Targeting gene-based therapeutics for canine osteoarthritis

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ABSTRACT

Osteoarthritis (OA) is a chronic, painful condition and is of major concern to both human and veterinary medicine. This degenerative joint disorder is characterised by pathological changes in articular cartilage, the underlying subchondral bone and synovial membrane. To date, treatment modalities are generally limited to 'symptom-modifying' therapies that only address joint pain. The primary aim of this project was to develop a targeted, 'structure-modifying' gene-based therapy for the future treatment of OA in the dog, with the prospect of developing an animal model for human disease.

To modify disease progression at the molecular level therapeutic genes can be introduced into arthritic joints to regulate those enzymes responsible for extra-cellular matrix (ECM) turnover. Since the matrix metalloproteinases (MMPs) play a central role in the development of OA, gene-based therapeutics should include mediators for controlling the synthesis and activity of these enzymes at the transcriptional and/or post-translational levels. Candidate proteins that may be of therapeutic benefit in treating OA include interleukin-1 receptor antagonist (IL-1Ra), soluble tumour necrosis factor receptors (sTNFR) and various tissue inhibitors of MMPs (TIMPs). The cDNA encoding the canine homologue of IL-1Ra was isolated using reverse transcription polymerase chain reaction (RT-PCR) and RNA harvested from canine peripheral blood mononuclear cells (PBMCs) as a template. Sequence analysis of the canine IL-1Ra demonstrated an open reading frame of 531 base pairs (bp) encoding a protein of 177 amino acids showing considerable sequence similarity with the homologous sequences published for other species. The canine-specific therapeutic proteins including IL-1Ra (19.5 kDa), TIMP-1 (22.8 kDa) and TIMP-2 9(24.3 kDa) were expressed using the *in vitro* transcription and translation techniques, however, sTNFRI (23.2 kDa) was unable to be expressed using this system.

Levels of therapeutic gene expression are strictly controlled at the transcriptional level by promoter regions of DNA. Candidate promoters that have potential for driving disease-specific expression in arthritic joints include those that are upregulated by pro-inflammatory cytokines and growth factors associated with the pathogenesis of OA. The canine homologues of MMP-9 and -13 promoters were cloned for use in a homologous species-specific targeted gene transfer study for canine disease. The 5′ untranslated regions (UTRs) were obtained by genome walking upstream of the canine MMP-9 and -13 translation start sites using genomic DNA (gDNA) as template. DNA fragments of 1894 and 1494 bp were isolated and on analysis demonstrated regions of sequence homology with the equivalent promoter sequences already determined for other species. In general, conserved regions correlated with numerous putative DNA binding motifs including Activator Protein-1 (AP-1) sites and a Nuclear Factor (NF)-κB binding domain. A consensus TATA-(like) box was identified in each case and shown to direct transcription initiation from specific positions upstream of the translation start site. The promoters were then characterised using various cell lines analysed for endogenous MMP-9 and -13 gene transcription using relative semi-quantitative reverse transcription PCR (RT-PCR). The canine MMP-9 and MMP-13 promoter fragments were sufficient to drive basal expression of a luciferase reporter gene in both Madin Darby canine kidney cells (MDCK) and primary rat cardiocytes. Basal activity of the MMP-13 promoter fragment (1494 bp) could be significantly enhanced by the treatment of transfected primary rat cardiocytes with interleukin-1 (IL-1β) and basic fibroblastic growth factor (bFGF), with some induction also observed with tumour necrosis factor (TNFα). However, no induction of this promoter was observed in the MDCK cells. In comparison the canine MMP-9 promoter fragment was enhanced by TNFα in the MDCK cells and bFGF in the rat cardiocytes. Five MMP-9 promoter deletion vectors were also analysed in MDCK cells and feline embryonic fibroblast (FEA) cells. The MMP-9 promoter deletion constructs were selectively enhanced by treatment of transfected MDCK cells with phorbol 12-myristate 13-acetate (PMA) suggesting that specific regions of the promoter were necessary for reporter gene expression. However, no effect was observed in the FEA cells.

The potential for undesirable systemic effects related to constitutive over-expression of certain therapeutic transgene products may be limited through the development of both 'disease and cell type specific' DNA targeting vectors that restricts therapeutic gene expression to diseased cell types of the joint through transcriptional regulation. To enable the incorporation of both disease and cell type specific promoter elements into one vector a novel dual-targeting vector system was designed. Two parameters were addressed during the development of this vector. Firstly, the promoters incorporated

into this system for driving gene expression required strategic manipulation to enhance promoter activity while maintaining both disease and cell type specificity. Secondly, the utilisation of Cre-lox technology was necessary to enable both disease and cell type targeting systems to be incorporated into a single vector system. Canine MMP-9 promoter luciferase reporter, pGL3/cMMP-9(1894) and deletion constructs, pGL3/cMMP-9(984), (628) and (534) were analysed in response to both pro-inflammatory cytokines, interleukin-1 (IL-1β) and tumour necrosis factor (TNFα) in human chondrosarcoma cells (SW1353). These deletion studies, in combination with mutagenesis analysis, guided the manipulation of the MMP-9 promoter with the incorporation of multiple NF-κB sites into the 5′ end to enhance activity while maintaining disease-specificity. The mouse collagen type XI (mColXI) promoter (1.2 kb) was also cloned from murine genomic DNA (gDNA) and analysed for cell type specific promoter activity in the chondrocyte-specific cells (SW1353) and undifferentiated chondroprogenitor cells (ATDC5). The mColXI promoter was manipulated with the incorporation of SOX9 enhancer sites into the vector to increase activity while maintaining cell type specificity. Attempts were then made to strategically sub-clone the canine MMP-9 and mouse ColXI promoters, manipulated with NF-κB and SOX9 sites respectively, into one plasmid vector containing the gene for 'Cre recombinase' and *lox*H sites to create a novel dual-targeting system. The aim of this vector system was to restrict therapeutic gene expression to diseased cell types of the osteoarthritic joint.

The introduction of DNA into target cells both *in vitro* and *in vivo* is a challenge in the development of efficient gene-based therapeutics. Although many chemical, biological and physical transfection delivery systems are routinely used, problems associated with cell type specificity, transfection efficiency and safety have encouraged the development of new transfer technologies such as particle-mediated bombardment using a gene gun. A relatively new physical approach for mammalian gene transfer is particle mediated bombardment using a gene gun where microscopic particles, coated with the gene of interest, are accelerated into cells by a motive force such as helium pressure discharge. Two reporter genes, β-Galactosidase (β-Gal) and Enhanced Green Fluorescent Protein (EGFP), were delivered to MDCK cells and rabbit synovial fibroblasts (HIG-82) respectively using gold particle (1.0 μm) bombardment to evaluate transfection efficiency at different helium pressures (100 and 150 psi). Transfection of both cell types was evident at these pressures despite some cell death. The *in vitro* delivery of gold particles into synovial tissue samples tissues taken from a freshly euthanatised dog at *post-mortem* examination (PME) was then analysed to evaluate the penetration depth of gold particles (1.0 μm) at helium pressures of 250 and 500 psi. Darkfield microscopy, used to evaluate histopathologic synovial specimens, demonstrated that particle-mediated bombardment could potentially be used to transfect the lining cells of the synovial membrane.

This thesis addresses some of the challenges in the development of gene-based therapeutics for canine OA. The aim of this study was to perform preliminary studies for the future development of a homologous gene transfer system for canine disease. The main focus of the project was to develop a targeted therapy using canine disease-specific promoters combined with a cell type specific promoter and Cre-lox technology to restrict therapeutic gene expression to diseased cell types of the osteoarthritic joint.

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DECLARATION

Apart from the help acknowledged, I declare that work described was carried out by me and is not that of any other person, and further has not been submitted in full or in part, for consideration for any other degree or qualification.

Sarah Elizabeth Campbell, August 2002

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Chapter I

General Introduction and review of the literature

1.1 ABSTRACT

Osteoarthritis (OA) is a degenerative joint disorder characterised by pathological changes in articular cartilage, the underlying subchondral bone and synovial membrane. Although the aetiology of OA is still largely unknown, recent advances in molecular biology have determined the pathogenic processes which result in the net loss of cartilage components. This common condition can be very painful and is often associated with a chronic persistent lameness, yet current treatments are still limited to 'symptom-modifying' therapies. This chapter reviews the pathogenesis of OA, with particular reference to the canine disorder, which is relevant to the development of 'structure-modifying' therapies. Considerable attention is paid to the newly emerging technologies of 'gene-based' therapeutics on which this thesis is based, with particular focus on the technical problems that must be overcome in order to provide appropriately regulated expression of therapeutic genes.

1.2 PATHOGENESIS OF OSTEOARTHRITIS

1.2.1 Definition and classification of osteoarthritis

Arthritis, the term used to define joint inflammation, is used in a broad sense to describe numerous disease entities affecting any tissues of the joint. Arthritis can be separated into two main categories, the degenerative and the inflammatory arthropathies, and further sub-divided to define specific disease types (Table 1-1). Although osteoarthritis (OA) is described as a degenerative condition, there is also varying degrees of inflammatory change within the synovium particularly evident in the canine species where OA is a much more inflammatory condition than in other species. Three types of canine OA are now recognised and have been described as primary (idiopathic), secondary and erosive (Table 1-2). Primary OA is relatively rare pertaining to specific breeds such as the Spaniel, Golden Retriever, Boxer and Samoyed. In comparison, OA secondary to other causes such as hip dysplasia, elbow dysplasia, osteochondrosis and cruciate disease is far more common (Bennett, 1999) and is particularly prevalent in the larger breeds of dog. Erosive OA is very rare and is most often seen in the canine hip joint where the OA, secondary to dysplasia, is complicated by destruction of the subchondral bone. However, the true definition of OA has yet to be resolved and many modern day rheumatologists regard it not so much as a disease, but as an end-point of joint failure. Thus whenever pathology affects a diarthrodial joint, the inevitable outcome will be OA. The idea that OA in man is restricted to one joint has recently been challenged by a new hypothesis. It is now considered that a generalised form of OA exists, directed by a primary metabolic disorder involving systemic factors in which many joints and other tissues are affected, remote from the focal (Aspden et al., 2001).

1.2.2 Prevalence of osteoarthritis

OA is a chronic, painful condition and is of major concern to both human and veterinary medicine, affecting one third of adults aged 25-74 years (Creamer and Hochberg, 1997) and 20% of the canine population aged over one year of age (Bennett, 1980). The incidence and prevalence of OA is closely associated with the ageing process and it has recently been hypothesised that *in vivo* replicative senescence may cause an age-related decline in the ability of chondrocytes to maintain articular cartilage, particularly through erosion of telomere length and senescence (Martin and Buckwalter, 2001). However, canine arthritis is often initiated early in life but clinical disease is more likely in the older patient since the disease is generally progressive and thus the pathology worsens with age.

Table 1-1 Classification of arthritis

Arthritis category	Type	Sub group
1) DEGENERATIVE	**a) Osteoarthritis** **b) Traumatic arthritis** **c) Haemophilic arthritis** **d) Neuropathic arthritis**	
2) INFLAMMATORY	**a) Infective**	Bacterial Discospondylitis Endocarditis and arthritis Borreliosis Bacterial L forms Tubercular Mycoplasmal Fungal Protozoal Viral
	b) Immune-based	<u>Erosive</u> Rheumatoid Periosteal proliferative polyarthritis Polyarthritis of Greyhounds Felty's syndrome <u>Non-erosive</u> Systemic lupus erythematosus Polyarthritis/polymyositis Polyarthritis/meningitis Arthritis of Japanese Akitas Amyloidosis of Chinese Shar Pei Polyarteritis nodosa Sjogren's syndrome Idiopathic Type I (no associations) Type II ('reactive') Type III (enteropathic) TypeIV (neoplastic) Drug-induced Vaccination 'reactions' Plasmacytic/lymphocytic gonitis
	c) Crystal-induced	Gout Pseudogout Hydroxyapatitie

Table 1-2 Categories of canine osteoarthritis

Osteoarthritis type	Aetiology	Characteristics	Breed predisposition
Primary (idiopathic)	No identifiable cause but possibly some inherent metabolic abnormality of the cartilage predisposing to early degeneration.	Multiple joints in young adult dogs	Dalmatian, Chow, Spaniel, Boxer, Golden Retriever, Samoyed
Secondary	2^{o} to identifiable joint diseases (e.g. hip dysplasia, cruciate disease, osteochondrosis), repeated and/or excessive trauma	One or many joints involved	Breed predisposition often associated with the underlying cause
Erosive	Generally of 2^{o} type	Destructive changes occur within the bone, mainly of the hip	No breed predisposition other than that associated with an underlying cause such as dysplasia

1.2.3 The molecular structure of cartilage

Cartilage tissue, lining the articular surface of joints, (Figure 1-1) consists of a small number of chondrocytes that produce and subsequently embed in an extra-cellular matrix (ECM) of water, proteoglycan and collagen (Figure 1-2). Proteoglycan monomers are large polyanions composed of polysaccharides, chondroitin sulphate (CS) and keratan sulphate (KS), bound by a core protein. Many of these proteoglycan sub-units are non-covalently bound to long filaments of hyaluronic acid forming a proteoglycan aggregate, also referred to as aggrecan, of highly negative charge (Hardingham and Fosang, 1992). Water molecules entrapped within this matrix containing negatively charged macromolecular complexes define the volume of the cartilage tissue, which in turn is prevented from unlimited expansion by the surrounding collagen network (Kuettner, 1992). Other molecules identified in the matrix such as decorin, fibromodulin and biglycan are currently under investigation and determination of their functions will enhance the understanding of cartilage metabolism (Knudson and Knudson, 2001). Collagen, the most abundant protein in the body and found throughout the ECM, consists of fibres made up of fibrils with each fibril containing recurring polypeptide sub-units. The amino acids forming these polypeptide chains produce a poly-L-proline triple helical structure that is unique to collagen. There are a number of different collagen types, varying slightly in their amino acid sequence and structure, but in articular cartilage type II collagen is the most important forming the fibrillar network with the minor types IX and XI each of which facilitate fibril interaction with proteoglycan molecules and regulate fibril size (Cremer et al., 1998).

Figure 1-1 Schematic drawing of a joint

Articular surfaces of the joint are covered by hyaline cartilage that is devoid of perichondrium. The capsule is formed by two parts, the external fibrous layer and the synovial layer (synovial membrane that lines the articular cavity except for the cartilaginous areas (Junqueira et al., 1992).

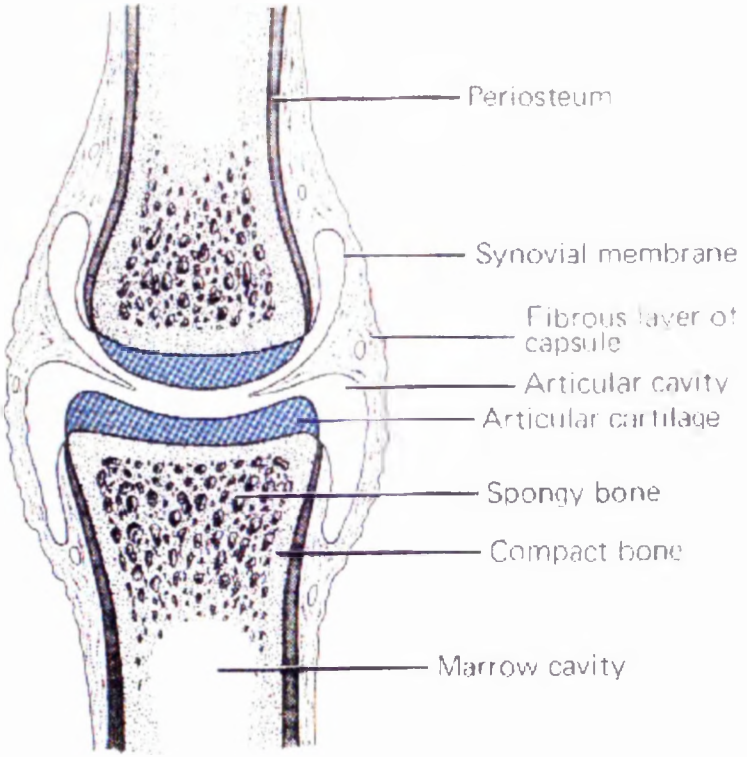
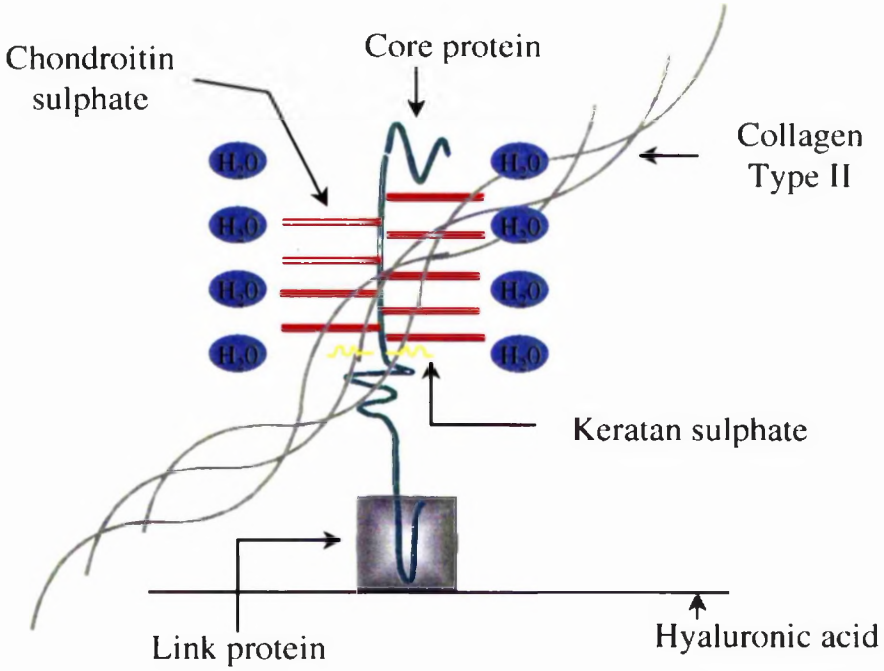


Figure 1-2 Molecular structure of articular cartilage

The molecular structure of healthy articular cartilage consists of chondrocytes that produce and subsequently embed in an extra-cellular matrix (ECM) of water, proteoglycan and collagen. The proteoglycan component consists of chondroitin sulphate (CS) and keratan sulphate (KS) molecules attached to a core protein; these in turn are bound to hyaluronic acid via link proteins. The negatively charged sulphate groups attract water molecules and the entire structure is prevented from unlimited expansion by the presence of collagen type II fibres.



1.2.4 Structural organisation of articular cartilage

Normal articular cartilage is organised into different layers each with a distinct structure and function. The first layer, referred to as the superficial tangential zone, is a thin region with high collagen to proteoglycan ratio that serves as the gliding surface for articulation. Collagen fibres are aligned in a parallel fashion to the cartilage surface while chondrocytes are elongated and quiescent. The adjacent layer, known as the transitional zone, is much thicker, occupying 40-60% of the cartilage depth where most of the cushioning and load distribution occurs. In this region the non-parallel collagen fibres are larger, proteoglycan levels are higher and chondrocytes more metabolically active. In the deep zone of the cartilage, collagen fibres are in a parallel alignment arranged perpendicular to the cartilage surface with chondrocytes arranged in columns. Immediately beneath this there is a transition from cartilage to subchondral bone where the collagen fibres extend into a zone of calcified cartilage; these fibres are thought to anchor the cartilage layer to bone. Within this organised structure the composition of the cartilage also varies with respect to the chondron, the chondrocyte and its surrounding pericellular matrix. For example the zone immediately surrounding the chondrocytes, the pericellular matrix, contains a high percentage of proteoglycan whereas the region adjacent to this, the territorial matrix, contains a lining of thin collagen fibrils forming a protective barrier to the chondrocyte (Ghivzzani et al., 2000).

1.2.5 Dynamic structure of the joint

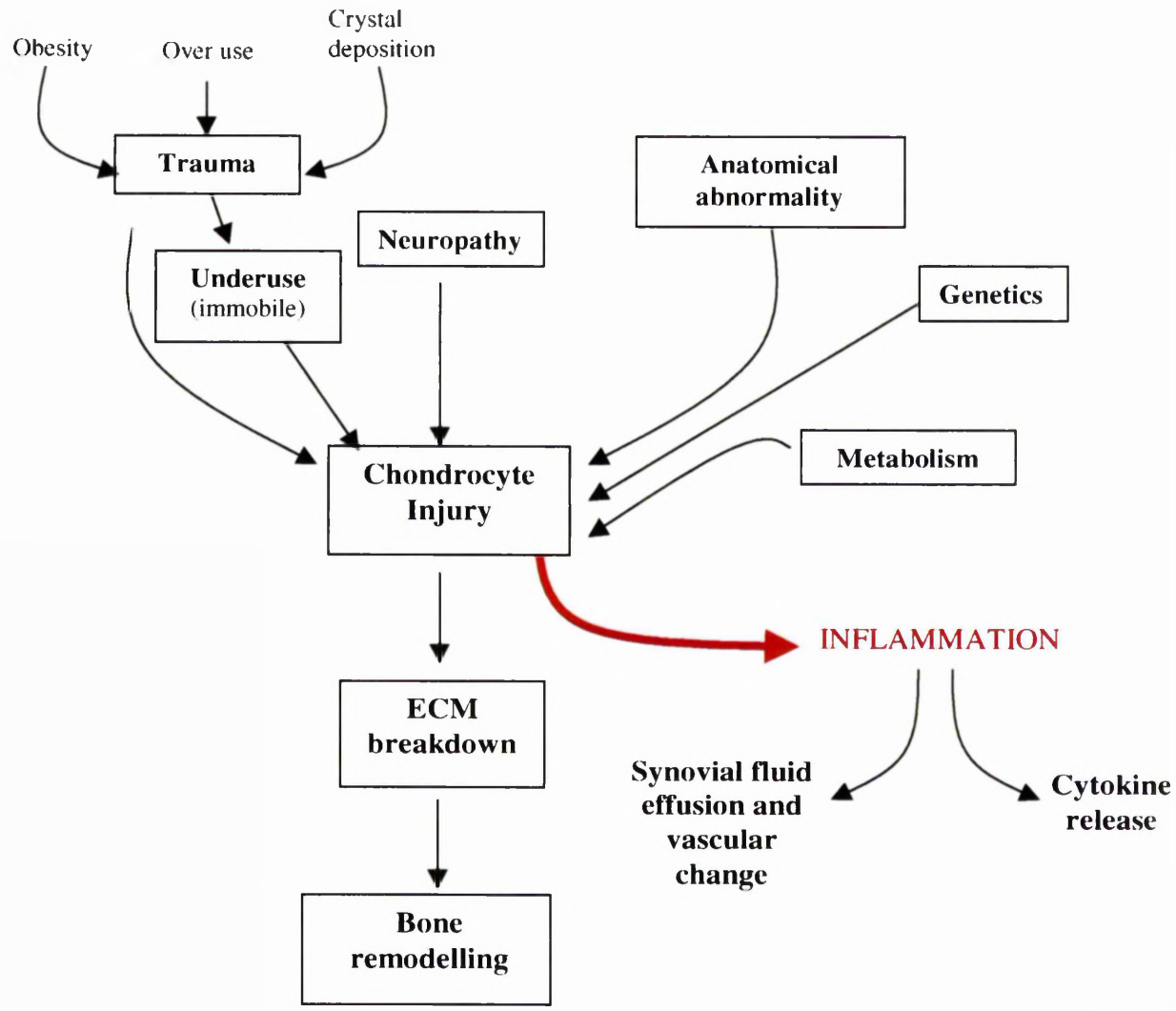
The unique mechanical property of articular cartilage relies not only on the structural properties of the proteoglycan and cartilage components but also on their specific three-dimensional arrangement relative to chondrocytes. This critical structural arrangement is mediated by cell adhesion receptors that enable cell/cell and cell/ECM interactions and intracellular plaques (peripheral membrane proteins). The latter provides a functional linkage between adhesion receptors and filaments of the cytoskeleton. These cell adhesion complexes are not static entities but are dynamic units that capture and integrate signals from the extra-cellular environment, regulated by biochemical events within cells. Thus the cell adhesion complex provides a two-way signalling between the cell and its external environment (Aplin et al., 1998). Type VI collagen immediately surrounding the cells may also assist in cellular-matrix interactions with other non-collagenous proteins such as tenascin and fibronectin (Aplin et al, 1998). It is therefore clear that the joint is a dynamic structure with the need for the continuous remodelling of the ECM to maintain health. Chondrocytes are responsible for producing the enzymes necessary for the balanced synthesis and degradation of the ECM. However, in OA the dynamics of this process are uncoupled with a change in this steady state situation, resulting in the net degradation of the ECM (Bullough and Cawston, 1994).

1.2.6 Aetiology of osteoarthritis

The cause of the uncoupling process in cartilage tissue turnover resulting in net degradation is uncertain but it is thought to be a combination of genetic, bio-mechanical and metabolic factors (Kraus, 1997) all resulting in a common final pathway of chondrocyte injury and ECM breakdown (Figure 1-3). It is becoming increasingly evident that abnormalities at the genetic level play an important role in both the initiation and maintenance of OA within an individual. However, this may also be complicated by the fact that many initiating causes of OA such as hip dysplasia and elbow dysfunction are also genetically influenced. A unique set of observations in Icelandic human families with a very high prevalence of hip OA suggests that there is a significant genetic contribution to OA (Ingvarsson et al, 2000). Although genetic susceptibility has not been evaluated in the dog it is thought that the variation in disease severity observed within a group of dogs suffering from the same initiating cause, strongly suggests genetic variation. Altered genes predisposing an individual to OA may not only be inherited but can also be acquired through mutation. Such mutations have been found to occur in genes encoding important structural components of cartilage including many of the collagen types (Kuivaniemi et al, 1997). A single base mutation in the gene encoding the human collagen type II gene has been shown to be responsible for the development of OA in man (Knowlton et al., 1990). Furthermore, changes in the type II collagen gene in transgenic mice altered the collagenous network softening the articular cartilage (Hyttinen et al, 2001) and caused degenerative changes in the articular cartilage similar to OA (Helminen et al., 1993). More commonly OA develops through bio-mechanical factors such as joint instability as a result of anatomical abnormalities (hip and elbow dysplasia and cruciate failure), following neuropathy or even as a direct result of repeated or severe joint trauma (Bennett and May, 1994; Vaughan, 1990). Metabolic factors also contribute to the pathological changes observed in primary OA (Oddis, 1996), particularly associated with the metabolic activity of the chondrocytes (Sulzbacher, 2000) influencing the turnover of collagens and proteoglycans (Fassbender, 1987). Whatever the cause, a similar process of degradation supervenes, with the only variation being the speed and severity of the disease process.

Figure 1-3 Aetiology of osteoarthritis

The cause of osteoarthritis (OA) is thought to be a combination of genetic, bio-mechanical and metabolic factors all resulting in a common final pathway of chondrocyte injury, extra-cellular matrix (ECM) breakdown and bone remodelling. Chondrocyte injury stimulates the inflammatory process with synovial effusion, vascular changes and the release of cytokines.



1.2.7 Pathological changes in osteoarthritis

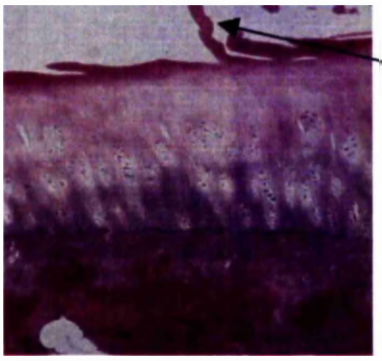
The first stage of OA is usually asymptomatic as the initial destruction of cartilage is balanced by an effective repair response mediated by growth factors, with the proliferation of chondrocytes and increased synthesis of the ECM. However, the biochemical changes that follow result in the net degradation of the ECM, partially resulting from the marked yet inadequate repair response failing to produce the normal balance of mature cartilage components. Instead the production of abnormal proteoglycan molecules within a disorganised collagen network results and is less able to counteract the osmotic pressure of the aggrecan, so influx of water occurs with associated swelling, fibrillation and cracking of the cartilage tissue (Figure 1-4a). This early of OA is characterised by an increased thickness of the articular cartilage and discolouration (Figure 1-4b). Only later does the cartilage slowly degrade progressing to a severely fibrillated and eroded tissue (Figure 1-4c), with the eventual disappearance of the full thickness surface of the joint (Figure 1-4d) (Dijkgraaf et al., 1995). The release of breakdown products from the degraded articular cartilage into the surrounding environment instigates an inflammatory process within the synovium that is characterised by hyperplasia and hypertrophy of the synovial lining. At the histological level there is a mononuclear cellular infiltrate (Figure 1-5) with the involvement of a complex network of cytokines even at an early stage of disease (Smith et al., 1997).

Although OA is commonly considered a disease primarily involving cartilage, studies support a role for considerable bone involvement within the subchondral region and articular margins. Whether these changes are primary or secondary to the disease process have yet to be determined and they are likely to vary with the stage of disease (Lajeunesse et al, 1999). It is thought that cytokines are intimately involved in these bony changes with synoviocytes secreting prostaglandins (PG) and matrix metalloproteinases (MMPs) leading to the resorption and destruction of the subchondral bone in some cases (Takahashi et al., 1998). Subchondral bone schlerosis however is commonly seen in OA and may be an early response to trauma in cases of OA secondary to repeated trauma, or a secondary response to cartilage loss in advanced OA. Osteophytes are found at the articular margins occurring as early as three days after initiation of the arthritic process (Gilbertson, 1975) (Figure 1-6). The presence of these may play a compensatory role by altering joint shape through a sophisticated remodelling process to allow the redistribution of forces providing some articular cartilage protection (Moskowitz, 1999) and altering the joint such that it copes better with the abnormal forces placed upon it.

Figure 1-4 Pathological changes in the canine osteoarthritic joint.

Pathological changes to joint tissue showing a) fibrillation, b) discoloured and thickened synovium, c) early cartilage erosion and d) late stage full thickness cartilage ulceration

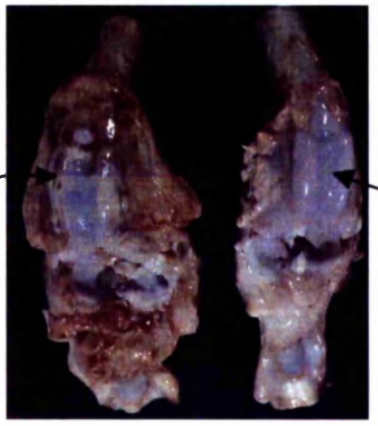
a)



Histological section showing fibrillation and cracking of articular cartilage

Magnification x400

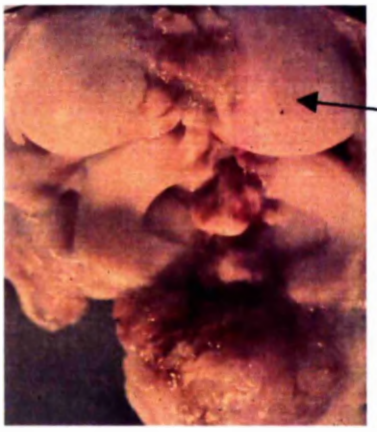
b)



Gross specimen of stifle joint showing discoloured and thickened synovium compared to the normal joint. Marginal osteophytes can also be seen

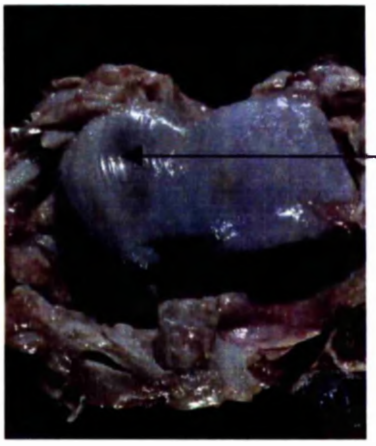
Normal joint

c)



Gross specimen of stifle joint showing cartilage erosion

d)



Gross specimen of the elbow joint. Disappearance of the full thickness articular cartilage with eburnation of the underlying subchondral bone

Figure 1-5: A histological section of synovial tissue taken from a canine osteoarthritic joint tissue.

A mononuclear cell infiltration provides evidence of synovitis.

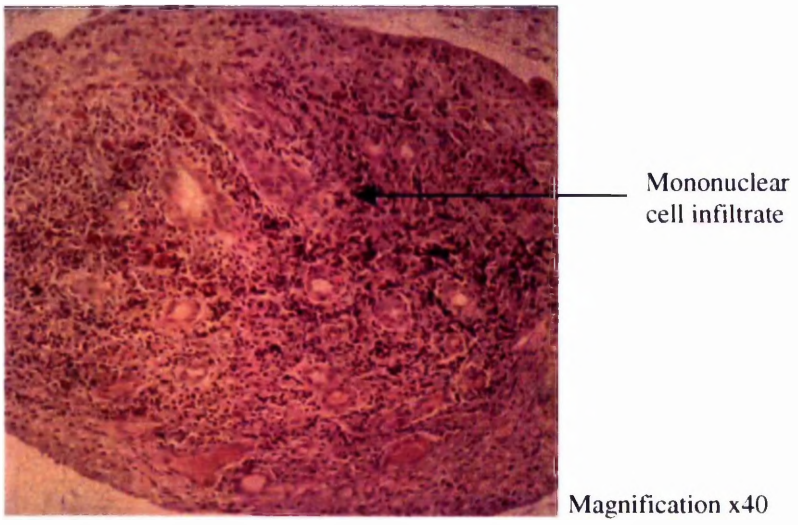
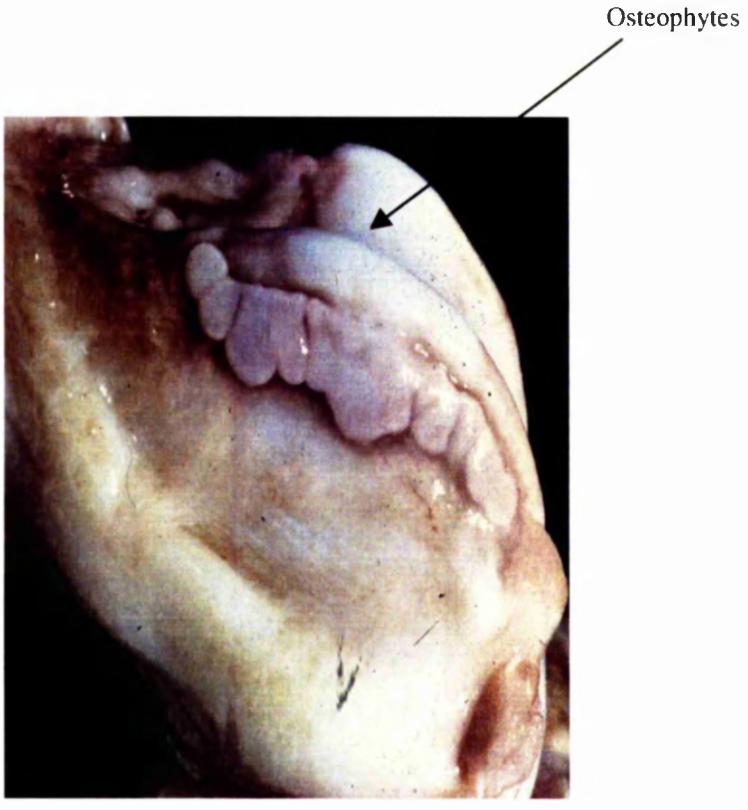


Figure 1-6: Development of osteophytes in canine osteoarthritis

Bony outgrowths along the edge of the trochlear groove of the stifle joint are evident on *post-mortem* examination (PME).

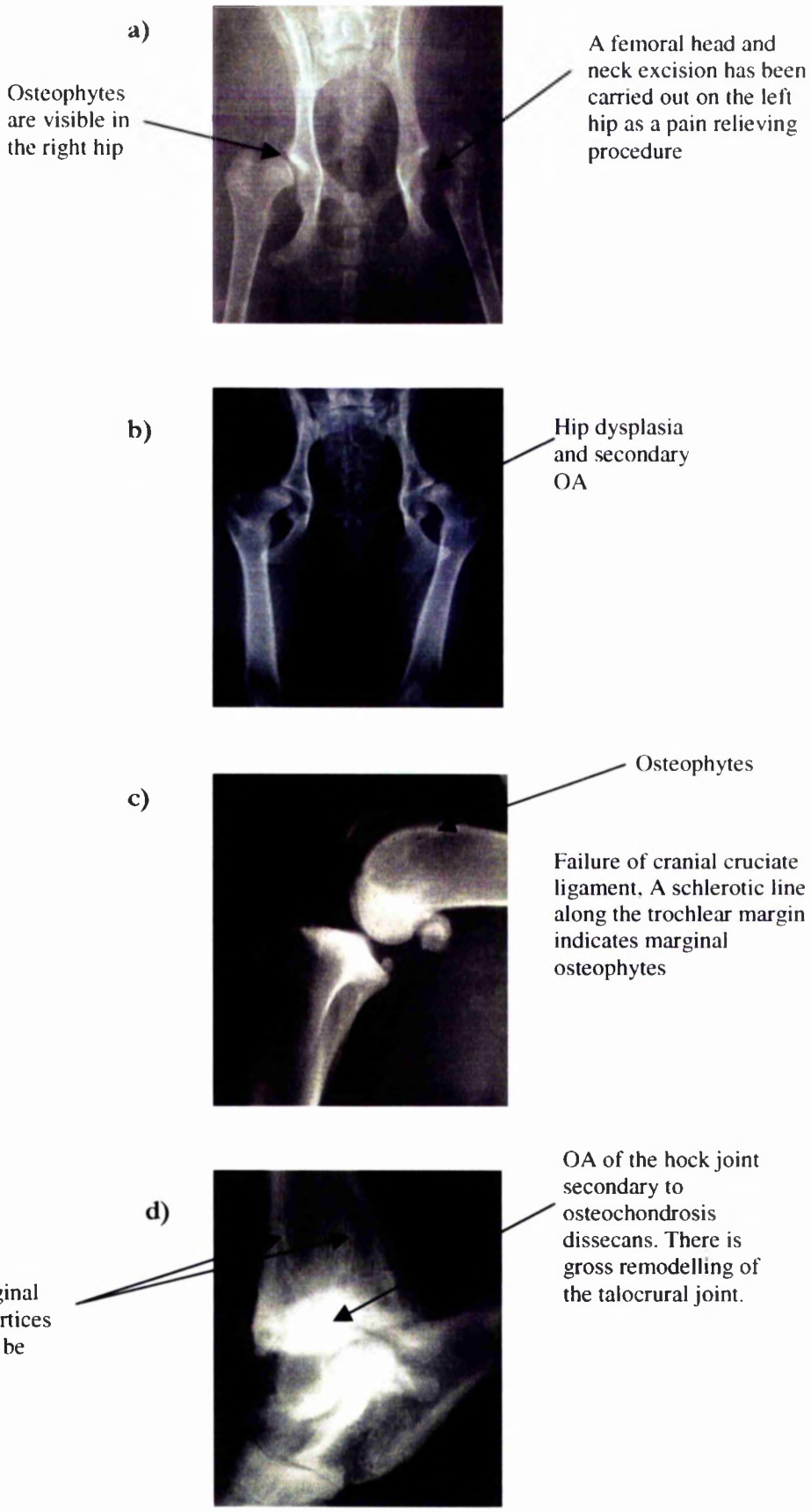


1.2.8 Diagnosis of osteoarthritis

Joint pain is usually the first clinical sign evident in canine patients suffering from OA. Usually this appears late in the disease when considerable joint destruction and remodelling have already occurred and is probably due to a combination of factors. It may simply be the stretching of nerve endings in the periosteum over osteophytes, microfractures in the subchondral bone, medullary hypertension due to blood flow distorted by thickened trabeculae, synovitis or joint instability. Reduced synovial hyaluronan and its destruction may also result in less efficient lubrication of the articular surfaces resulting in further inflammation and pain. The presence of pain can also be explained at the molecular level and has been associated with the presence of cytokines particularly interleukin-1 (IL-1) and tumour necrosis factor (TNF) (Takahashi et al., 1998; Emshoff et al., 2000). In the long term, chronic pain leads to sensitisation where the threshold of pain receptors is lowered and the nocioceptor unit not normally responsive becomes responsive. As a result stimulus not normally painful become painful (allodynia) with an exaggerated pain response to a given stimuli (hyperalgesia). Muscle pain is often another feature of OA and may be due to central sensitisation caused by the painful joint (Bajaj et al., 2001) or through inflammatory mediators diffusing from the joint to surrounding muscles with the added effect of altered muscle function to cope with the painful joint. Radiographical changes are evident much later in the diseased joint with areas of increased density in the subchondral bone, joint space narrowing (difficult to evaluate in the canine patient), osteophyte formation and bone remodelling (Figure 1-7a, b, c and d).

Since OA is frequently diagnosed late in the disease progression, numerous studies have attempted to identify diagnostic markers in synovial fluid and blood that may assist in the early identification of diseased individuals. This may not only assess the severity of disease but may also be used to monitor the effect of treatment (Rørvik and Grøndahl, 1995). Markers can be related to the mechanisms of cartilage degradation such as the MMP enzymes involved in joint tissue destruction (Lohmander et al., 1993) and the cytokines TNF, IL-6 (Venn et al., 1993) and IL-1 (Shinmei er al., 1990) that modulate ECM turnover. Components of cartilage and bone can also be used as markers of OA e.g. proteoglycans (Heinegard et al., 1985; Arican et al., 1994a), hyaluronan (Arican et al., 1994b), cartilage matrix glycoprotein (CMGP) (Fife and Brandt, 1989), cartilage oligometric matrix protein (COMP) (Lohmander et al., 1994; Saxne and Heinegard, 1992), cross-links markers of bone tumours such as pyridinloine and deoxypyridindine (Arican, 1995), bone sialoprotein (BSP) (Petersson et al., 1998), osteocalcin (Arican et al., 1996) and fibronectin (Lust et al, 1987). Antibodies to some of the collagens (Bari et al., 1989) and fragments of chondrocytes (Paroczai and Nemeth-Csoka., 1988) have also been found. To date it is reasonable to conclude that there is no reliable marker of OA that can be used for early diagnosis and/or disease monitoring.

Figure 1-7 Radiographical assessment of canine osteoarthritic joints

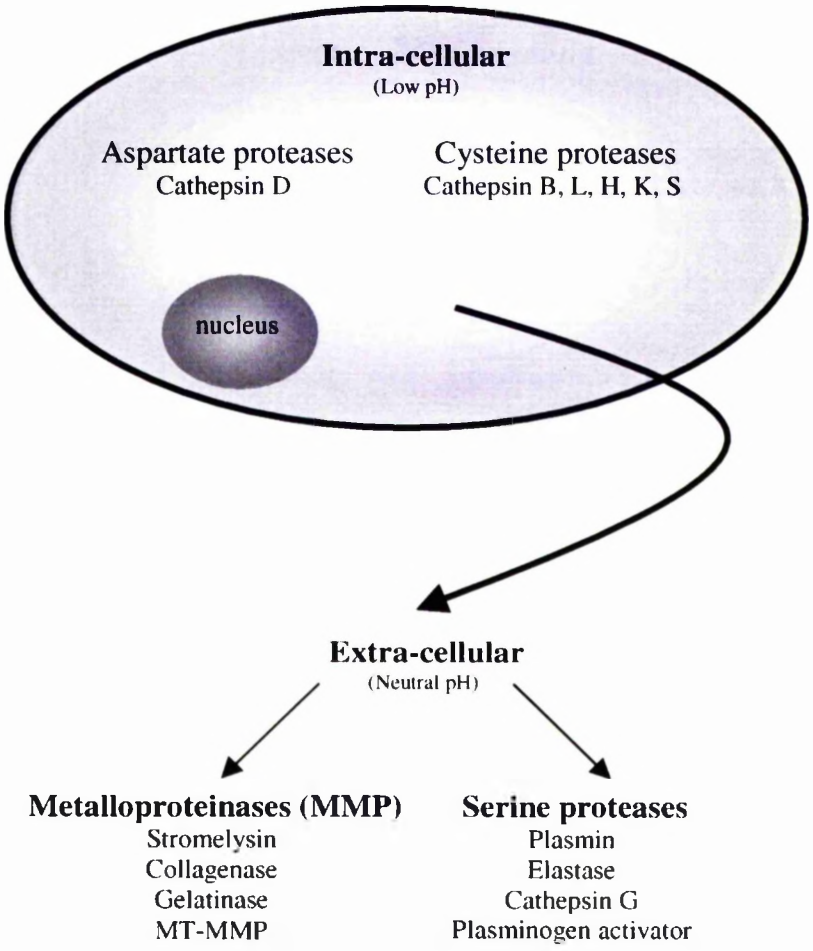


1.2.9 Enzyme-mediated degradation of the articular cartilage matrix

Although the pathogenesis of OA is complex, involving many different tissue types and biochemical pathways, it is the proteolytic enzymes involved in the destruction of the cartilage tissue that have been the focus of considerable research. The biochemical changes in the ECM can largely be attributed to the activity of proteolytic enzymes made within and secreted by a number of cell types within the joint. These enzymes can be divided into four main groups according to the chemical group responsible for hydrolysis (Figure 1-8). The aspartate and cysteine proteases (the cathepsins) act at a low pH within the cell while the MMPs and serine proteases function within a neutral extra-cellular location (Elliott and Cawston, 2001). All of these enzymes play a role in matrix remodelling but the MMPs produced by both the chondrocytes and synovial cells are particularly important and are primarily associated with the normal turnover of connective tissue (Matrisian, 1992) and in the pathological destruction of cartilage matrix (Martel-Pelletier, 1999b).

Figure 1-8 Enzyme-mediated degradation of articular cartilage matrix

The enzymes responsible for the degradation of the ECM can be segregated according to their intra- or extra-cellular location. The aspartate and cysteine proteases are responsible for the intra-cellular turnover at low pH while the MMPs and serine proteases are involved in the extra-cellular turnover at a neutral pH (Modified from Elliott and Cawston, 2001).



1.2.9.1 The matrix metalloproteinases

Over twenty different MMPs have been identified and classified into different groups according to their substrate specificity (Table 1-3), the collagenases (MMP-1, -8, -13, 18), gelatinases (MMP-2, -9), stromelysins (MMP-3, 10 –11,), the membrane type-MMP (MT-MMP-14, -15, -16, -17, -24, -25) and others such as Matrilysin (MMP-7), macrophage elastase (MMP-12) and MMP-19 (Elliot and Cawston, 2001; Nagase and Woessner, 1999). Many of these MMP's are elevated to varying degrees in osteoarthritic joint tissues including the collagenases MMP-1, 3, 13 (Bluteau et al., 2001) and MMP-8 (Chubinskaya et al., 1996), the gelatinases MMP-2 and 9 (Clegg et al., 1997; Coughlan et al., 1995) and MMP-7 (Ohta et al., 1998). The unique properties of the individual MMPs are distinguished by structural variations which provides substrate specificity (Matrisian, 1992). For example the carboxy-terminal domain has been reported to contribute to the specificity of the interstitial collagenases and stromelysins (Sanchez-Lopez et al., 1993). The gelatinases have an additional fibronectin-like gelatin-binding domain which contributes to the substrate specificity (Strongin et al., 1993) and the MT-MMP have been shown to be membrane associated via a transmembrane domain at the carboxy-terminal end of the molecule (Sato et al., 1994).

Despite their unique properties, the MMPs all belong to the same family sharing structural and functional domains (Figure 1-9). They vary slightly between different species but generally consist of regions encoding for a signal sequence, a prodomain, a zinc binding domain containing the catalytic site; a unique proline rich hinge region and a hemopexin-like carboxy-terminal domain. (Cawston, 1996). The MMPs are released from cells in a proenzyme state that requires activation (Figure 1-10). The latency of the MMPs is thought to be maintained by a bond between the unpaired cysteine residue in the prodomain and the zinc atom at the active site. Upon cleavage of this Cys-Zn bond, most of these enzymes undergo proteolysis, which irreversibly removes the propeptide domain; this mechanism of action is known as the 'cysteine switch' mechanism (Springman et al., 1990). This process of activation can be stimulated *in vitro* by physical (chaotrophic agents), chemical (HOCl, mercurials) and enzymatic (trypsin, plasmin) treatments that separate the cysteine residue from the zinc (Woessner, 1991). Many enzymes have since been implicated in the activation of the various MMPs and include the serine/cysteine dependent families such as the plasminogen activation / plasmin system and cathepsin B, stromelysin-1, collagenase-3, MT-MMP and gelatinase-A together forming an interactive web of regulation (Martel-Pelletier, 1999).

Table 1-3 Classification of the matrix metalloproteinases

Group	Name	Alternative names	Substrates
Collagenase	MMP-1	Interstitial collagenase	Collagens I, II, III, VII, VIII, X, gelatin, aggrecan, link protein,
	MMP-8	Neutrophil collagenase	Collagens I, II, III, V, VII, VIII, X, gelatin, aggrecan, fibronectin
	MMP-13	Collagenase-3	Collagens I, II, III, IV, gelatin, aggrecan
	MMP-18	Xenopus collagenase	
Gelatinases	MMP-2	Gelatinase A	Collagens IV, V, VII, X, XI, gelatin, aggrecan, link protein, elastin, fibronectin, laminin
	MMP-9	Gelatinase B	Collagens IV, V, VII, X, gelatin, aggrecan, link protein, elastin
Stromelysins	MMP-3	Stromelysin-1	Collagens III, IV, IX, X, gelatin, aggrecan, link protein, elastin, fibronectin, laminin, activates MMP-1, -8, -13
	MMP-10	Stromelysin-2	Collagens III, IV, V, gelatin, aggrecan, link protein, fibronectin, elastin, activates MMP-1, -7, -8, -9
	MMP-11	Stromelysin-3	
Membrane-type MMP	MMP-14	Membrane type-1 MMP	Collagens I, II, III, gelatin, aggrecan, fibronectin, activates MMP-2, -13
	MMP-15	MT2-MMP	Gelatin, fibronectin, activates MMP-2
	MMP-16	MT3-MMP	Activates MMP-2
	MMP-17	MT4-MMP	
	MMP-24	MT5-MMP	Activates MMP-2
	MMP-25	MT6-MMP	Gelatin
Others	MMP-7	Matrilysin	Collagens IV, X, gelatin, elastin, aggrecan, link protein, fibronectin, laminin, activates MMP-1
	MMP-12	Metalloelastase	Collagen IV, gelatin, fibronectin, laminin, vitronectin
	MMP-19		Aggrecan

Figure 1-9 Domain structure of the matrix metalloproteinases

The matrix metalloproteinas (MMP) family is represented schematically to demonstrate the relative size and conservation of sequences between members of this group. Each member of this family has at least four domains: i) the signal peptide sequence, ii) the propeptide domain that is lost during enzyme activation; iii) the catalytic domain that contains the conserved sequence VAAHEXGHXXGXXH in which the three histidine residues coordinate the active zinc site; iv) the carboxy-terminal domain that has homology to hemopexin and vitronectin, and contains a transmembrane domain in MT-MMP.

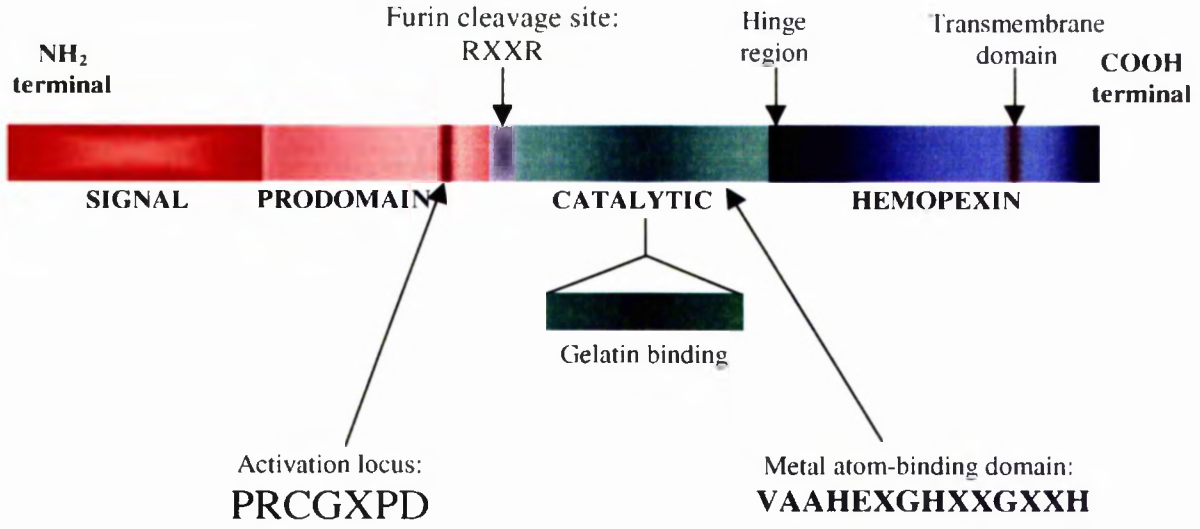
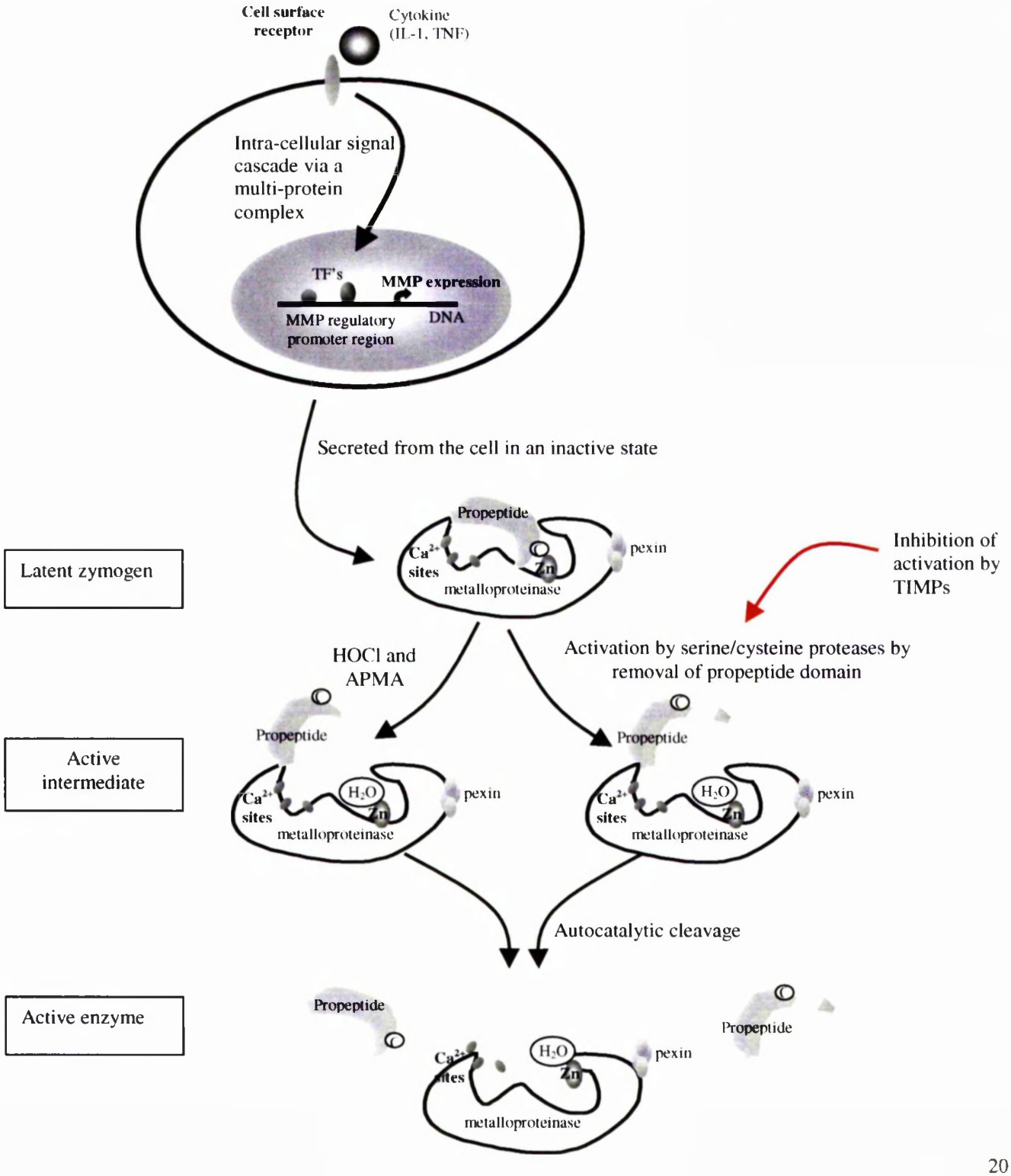


Figure 1-10 Synthesis and activation of the matrix metalloproteinases

The cysteine switch mechanism for activation of the matrix metalloproteinases has been proposed (Springman et al., 1990). Cysteine in the pro-enzyme domain contacts the zinc molecule to maintain latency of the enzyme. Physical agents such a sodium dodecyl sulfate (SDS) or chaotropic agents can unfold the structure to expose zinc. Reagents that react with sulfhydryl groups such as hypochlorous acid (HOCl) and organomercurials (aminophenylmercuric acetate, APMA) will inactivate the cysteine. Alternatively, proteolytic enzymes can initially cleave the pro-peptide, followed by autolytic cleavage by the activated MMP to remove the pro-peptide and confer permanent activity.

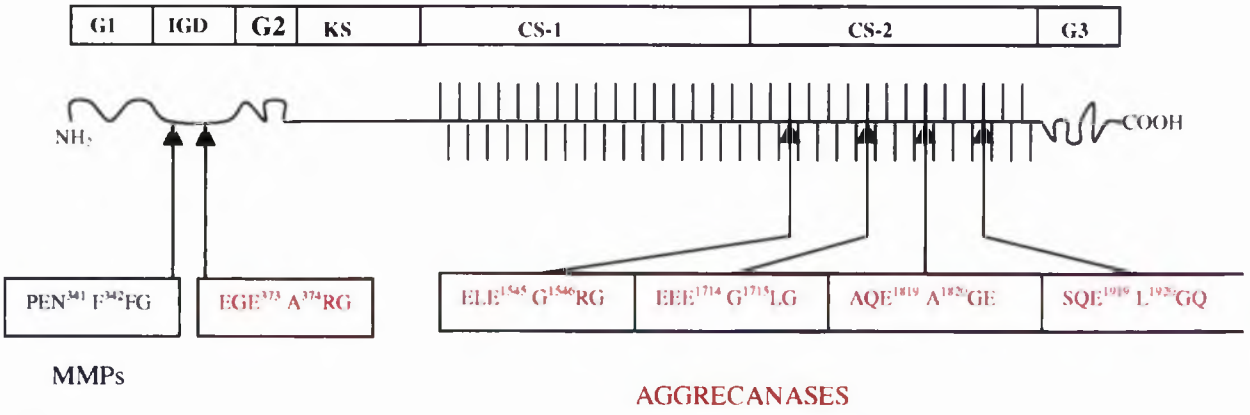


1.2.9.2 Aggrecanases

One of the central features of OA is the catabolism of the proteoglycan aggrecan component of cartilage tissue demonstrated by the increased loss of aggrecan metabolites from the cartilage matrix into the synovial fluid. Initial studies discovered two major sites of aggrecan proteolysis at the peptide bonds Asn^{341}-Phe^{342} ($DIPEN_{341}$-FFGVG) (Flannery et al., 1992) and Glu^{373}-Ala^{374} (Sandy et al., 1992) located within the highly conserved interglobular domain (IGD). Cleavage of the Asn^{341}-Phe^{342} bond can be achieved *in vitro* by the action of MMPs while proteolysis of the Glu^{373}-Ala^{374} and other specific Glu-Xaa peptide bonds has been associated with aggrecanse activity (Figure 1-11) (Caterson et al., 2000). The use of neoepitope antibodies specific for the newly generated N-terminal or C-terminal aggrecan sequences, cleaved by the MMP or aggrecanase, led to the purification and cloning of aggrecanase-1 and aggrecanase-2, two members of the 'A Disintegrin And Metalloproteinase with Thrombospondin motifs' (ADAMT) family (Abbaszade et al., 1999; Tortorella et al., 1999). Aggrecan fragments resulting from the *in situ* cleavage at the MMP-susceptible Asn^{341} APhe^{342} bond and aggrecanase-susceptible Glu^{373}-Ala^{374} bond have been detected in osteoarthritic articular cartilage (Lark et al., 1997). Furthermore studies have provided clear evidence that aggrecanase(s) are responsible for primary cleavage of the aggrecan IGD during cartilage degradation (Little et al., 1999).

Figure 1-11 Schematic representation of the domain organisation and structure of cartilage aggrecan

The cartilage aggrecan has been divided into domains represented by the globular (G) 1.2.3 domains, interglobular domain (IGD) keratan sulphate (KS) and chondroitin sulphate (CS)-2 regions. The cleavage sites for the matrix metalloproteinases (MMP) and aggrecanase enzymes have been identified on the aggrecan structure located in the IGD and CS-2 regions.



1.2.10 Regulation of the matrix metalloproteinase activity through the tissue inhibitors of metalloproteinase

Natural control mechanisms exist that regulate the process of MMP activation through the action of the tissue inhibitors of metalloproteinase (TIMPs) (Gomez et al., 1997). These inhibitors are responsible for maintaining the balanced physiological turnover of the ECM, through non-covalent binding of the active form of MMP in a 1:1 ratio. An imbalance of MMP over the inhibitor has been shown to contribute to cartilage degradation in OA (Woessner and Gunja-Smith., 1991; Martel-Pelletier et al., 1994). The N-terminal domain of the TIMP is inhibitory and the C-terminal domain confers binding specificity (Yu et al., 1996). So far four TIMPs have been identified, designated TIMP-1 (Murphy et al., 1977), TIMP-2 (Stetler-Stevenson et al., 1989), TIMP-3 (Silbiger et al., 1994) and TIMP-4 (Greene et al., 1996) each with unique biological properties (Table 1-4). Although the TIMPs can all function in MMP inhibition, differences are observed in their tissue distribution and transcriptional control. The TIMPs also have specific abilities to bind pro-MMPs with TIMP-1 and TIMP-3 able to bind pro-MMP-9 whereas TIMP-2, -3 and -4 can all form complexes with pro-MMP-2 (Elliott and Cawston, 2001; Brew et al., 2000). The TIMPs in turn are regulated by a series of cytokines and growth factors including transforming growth factor β (TGFβ), epidermal growth factor (EGF), platelet derived growth factor (PDGF), basic fibroblastic growth factor (bFGF), interleukin-1 (IL-1) and prostaglandin E_2 (PGE_2) (Borden and Heller, 1997). This strict regulation is essential since excessive production of TIMPs may have a detrimental effect on tissue degradation (Apparailly, 2001). A detailed discussion of TIMP-1 and TIMP-2 can be found in chapter III.

Table 1-4 Tissue inhibitors of metalloproteinase

Name	MMP inhibition	Mature protein size (kDa)	Glycosylated	Gene regulation	Tissue specific expression	Pro-enzyme binding
TIMP-1	All MMPs	20.2	yes	inducible	Bone and ovaries	MMP-9
TIMP-2	All MMPs	21.7	no	constitutive	Lung, ovaries, brain, testes, heart and placenta	MMP-2
TIMP-3	All MMPs	21.7	yes	inducible	Kidney, brain, lung, heart, and ovary	MMP-2 MMP-9
TIMP-4	MMP-1, 2, 3, 7, 9	22.6	no		Kidney, placenta, colon, testes, brain, heart, ovary and skeletal muscle	MMP-2

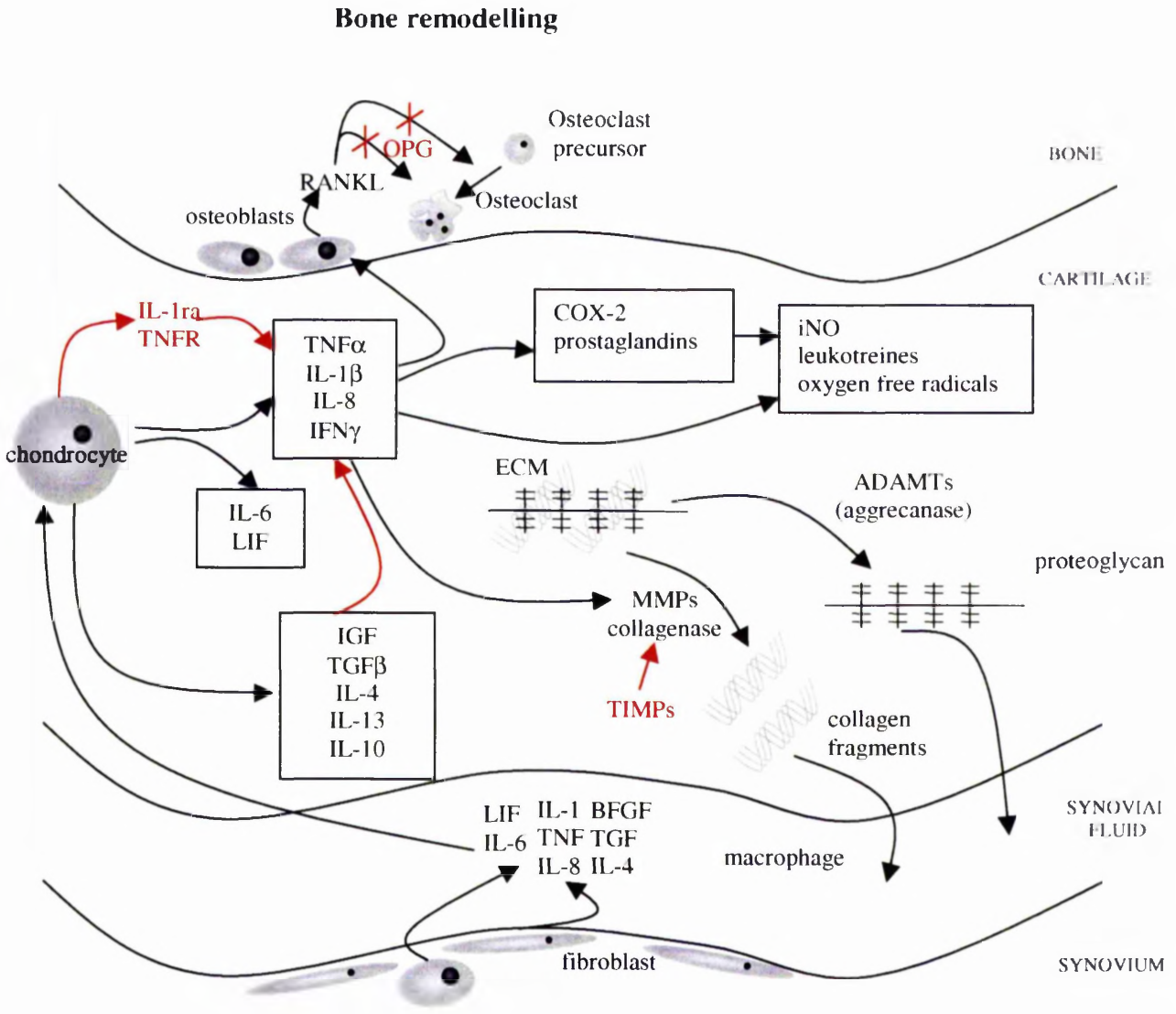
1.2.11 Regulation of the matrix metalloproteinases through cell signalling molecules

Although it is well established that multiple enzymes are responsible for the degradation of articular cartilage their gradual dys-regulation has yet to be fully defined. It is currently believed that articular cartilage integrity is maintained by the balance between a complex network of cytokine driven anabolic and catabolic processes with a disruption in this signalling network responsible for the pathological changes in OA. Signalling molecules are chemical, cellular messengers that are produced by all cells and referred to as cytokines and growth factors. Cytokines are released by cells in response to specific signals and influence the function of target cells by exerting a positive or negative effect on gene expression (Arend and Dayer, 1993). Many different signalling pathways are activated by cytokines each leading to the formation of multi-protein complexes and eventually to the binding of transcription factors to regulatory elements of DNA such as Activator protein-1 (AP-1) and Nuclear Factor-κB (NF-κB) sites within promoters (Adcock, 1997). The details of specific signalling pathways associated with OA are detailed in chapter V.

In general terms cytokines within the joint are primarily involved in the processes of synovial inflammation and cartilage degeneration where they are secreted from synovial cells following phagocytosis of degraded ECM (Ruggeri, 1996). The release of these cytokines stimulates synovial proliferation and activates the release of MMPs leading to the destruction of the ECM (Kacena et al., 2001). This continuous production of degraded ECM, its up-take by synovial cells and the subsequent production of cytokines forms a positive feedback mechanism, which leads to increased levels of joint destruction. Many different signalling molecules are implicated in the disease process but the pro-inflammatory cytokines IL-1 and TNF are particularly important (Martel-Pelletier, 1999a). However, these two cytokines form just a small part of a much more complex cell-signalling network that involves molecules such as interferon γ (IFNγ), TGFβ, leukaemia inhibitory factor (LIF), interleukin-1 receptor antagonist (IL-1Ra), leukotrienes, oxygen free radicals, PG and various other interleukins each with unique functions in enzyme regulation, acting independently and within the complex network. It is now clear that a cascade of cytokine activation occurs in OA with the potential for a functional hierarchy existing between them (Figure 1-12).

Figure 1-12: A simplified overview of the regulation of cartilage turnover.

A complex network of pro-inflammatory mediators enables specific interactions between cartilage, bone and synovium and results in the synthesis of active enzymes responsible for degradation of the extra-cellular matrix (ECM). This in turn is controlled through a series of anti-inflammatory and anabolic mediators.



1.2.11.1 Pro-inflammatory mediators

Both IL-1 and TNF have been intimately associated with the pathological changes in OA. Although these cytokines have overlapping and synergistic actions they have independent roles in cartilage destruction, with IL-1 thought to be responsible for cartilage degradation while TNF is thought to drive the inflammatory process (van den Berg et al, 1999a). However, it is evident that their roles are not exclusive but instead they have multiple functions dependent on the absolute levels and the presence of other ambient molecules. Previous studies suggested the importance of IL-1 as a mediator of joint damage in OA with high levels of IL-1α/β secreting macrophages detected in synovial tissue removed from osteoarthritic joints (Westacott et al., 1992) and increased expression of the IL-1 type I receptor identified on OA chondrocytes (Martel-Pelletier et al., 1992). It is now known that IL-1 is responsible for cartilage metabolism with its ability to suppress the synthesis of type II collagen while promoting the formation of type I collagen. IL-1 can also induce the production of enzymes involved in matrix degradation and suppress the ability of chondrocytes to synthesise new proteoglycan molecules (Pelletier et al., 1991). TNFα is also responsible for the degradation of articular cartilage albeit with a potency of approximately 10 fold lower than that of IL-1 (Campbell et al., 1990). The involvement of TNF in OA was implicated following the identification of TNFα production in synovial explants (Brennan et al., 1992) and synovial fluid samples (Hrycaj et al., 1995; Hopkins and Meager, 1988) from osteoarthritic joints. The density of TNFα receptors is also significantly higher in chondrocytes isolated from OA lesions as compared to chondrocytes from non-affected areas (Westacott et al., 1994). Synthesis of both IL-1 and TNF are up-regulated by a variety of cytokines including IL-17 which stimulates their production in macrophages (Jovanovic et al., 1998a).

Recent studies have revealed that these two principal pro-inflammatory cytokines IL-1β and TNFα may also be involved in the pathways responsible for the activation of osteoclast driven bone resorption (Lacey et al, 1998). They are thought to have an indirect effect through members of the TNF receptor-ligand super-family now known as the receptor activator of NK-κB ligand (RANKL) or osteoprotegerin ligand (OPGL). This ligand binds to the membrane-bound signalling receptor referred to as RANK leading to the resorption of bone (Suda et al., 1999; Li et al., 2000). Control of osteoclast resorption occurs through osteoprotegerin (OPG), an endogenous soluble antagonist of RANK ligand (Simonet et al, 1997). Although bone resorption is rare in OA, it is a feature of so-called erosive OA and the RANK ligand may be implicated in these cases.

Many different interleukins have been identified as important mediators of articular cartilage destruction. IL-6 is a multifunctional cytokine that has been identified in the canine osteoarthritic joint (Venn et al., 1993). Both IL-1β and TNFα can induce the production of IL-6 and its presence is required for IL-1-induced inhibition of proteoglycan synthesis in human articular cartilage (Nietfeld et al., 1990) suggesting its pro-inflammatory role in OA. However, IL-6 also induces the production of TIMPs rather than the MMPs and therefore had a dual function playing a part in the feedback mechanism and limiting enzyme damage (Lotz and Guerne, 1991). LIF has also been detected in synovial fluids from patients with OA and is secreted in the presence of IL-1β and

TNFα from cultured synoviocytes (Hamilton et al., 1993) and chondrocytes (Campbell et al., 1993). In the process of OA, LIF degrades human articular cartilage (Henrotin et al., 1994) and stimulates the loss of proteoglycans (Carroll and Bell, 1993) whilst synergising with interleukin-8 (IL-8) to potentiate the TNFα-PGE_2 effects on synovial fibroblasts (Alaaedine et al., 1999a; Martel-Pelletier 1999a). However, LIF also has an anti-inflammatory function similar to IL-6 and therefore has a dual role in the progression of OA (Westacott and Sharif, 1996). Oncostatin M (OSM), a member of the cytokine family that includes LIF, granulocyte colony-stimulating factor (GCSF) and IL-6 (Rose and Bruce, 1991), is another cytokine associated with the pathogenesis of arthritis. OSM combines with IL-1 to promote release of collagen fragments from bovine nasal cartilage in culture (Cawston et al., 1995) and increases the production of MMP-1 in human chondrocytes and synovial fibroblasts (Cawston et al., 1998).

Chemokines which are regulated by IL-1 and TNF function to recruit leukocytes to sites of inflammation and have also been implicated in the process of cartilage degradation in OA (Pulsatelli et al., 1999, Yuan et al., 2001, Alaaeddine et al., 2001). Chemokine subfamilies are distinguished according to the arrangement of the first two cysteines, which are separated by one amino acid (CXC) or are adjacent (CC). The CXC chemokines include IL-8 (CXCL8), and platelet factor 4 (CXCL4) and the CC chemokines include RANTES, macrophage inflammatory protein-1α (MIP-1α, CCL3), MIP-1β (CCL4) and the monocyte chemoattractant proteins (MCPs). Several of these chemokines including IL-8, MCP-1, MIP-1α and MIP-1β are over produced in arthritic joints (Taub and Oppenheim, 1993).

Reactive oxygen species (ROS) are yet another group of molecules thought to be involved in the degenerative changes associated with OA. ROS which include superoxide, hydrogen peroxide, the hydroxyl radical and nitric oxide (NO) are produced by chondrocytes, neutrophils and fibroblasts (Moulton, 1996). ROS affect the preservation of the cartilage matrix by mediating cytokine and growth factor stimulated intra-cellular signalling and concurrent gene activation (Lo and Cruz, 1995). Considerable research has focused on the involvement of NO in the pathogenesis of OA. NO is an inorganic, gaseous free radical synthesised from arginine in a reaction catalysed by NO synthase (NOS) from a variety of different cell types. This enzyme exists as isozymes, an inducible (iNOS) and constitutive (cNOS) form. The inducible form is up-regulated by IL-1β chondrocytes (Stadler et al., 1991) acting to suppress the synthesis of aggrecan (Taskiran et al., 1994) and type II collagen (Cao et al., 1997). NO has now been implicated as an important mediator of intra-articular pathophysiology (Evans et al., 1995) with increased concentrations of nitrites, the stable breakdown products of NO observed within the synovial fluid of osteoarthritic joints (Stefanovic-Racic et al., 1994; Farrell et al., 1992). Furthermore, explants of human cartilage released large amounts of nitrite when stimulated with IL-1β and TNFα (Murrell et al., 1996). The effects of NO in OA are likely to be exerted within cartilage, where NO promotes a number of catabolic effects on chondrocytes leading to ECM loss. These effects include i) inhibition of matrix synthesis (Stefanovic-Racic et al., 1995), ii) modulation of MMPs (Murrell et al., 1995), iii) increased susceptibility to other oxidants (Clancy et al., 1997) and iv) induction of apoptosis (Blanco et al.,

1995). It has been reported that inhibitors of inducible NO synthase (iNOS) have therapeutic effects on the progression of lesions in the experimental OA dog model (Pelletier et al., 1998).

1.2.11.2 Anti-inflammatory mediators

Activities of pro-inflammatory mediators are strictly regulated by a series of anti-inflammatory molecules. For example, the effects of IL-1α/β are controlled by the endogenous interleukin-1 receptor antagonist (IL-1Ra) functioning as a competitive inhibitor to the type I receptor (Arend, 1993) and shown to have chondroprotective effects in experimental OA (Caron et al., 1996). Soluble receptors that inhibit the binding of IL-1β have also been identified (Symons et al., 1991) and it has been suggested that IL-1RII, which binds IL-1β with high avidity, may function as an inhibitory decoy receptor (Colotta et al., 1994, Attur et al., 2000). In a similar manner, the effects of TNF can be controlled by the soluble receptors that bind and inactivate TNF (Mageed et al., 1998).

Modulators of cytokine activity also exist; these include IL-4, IL-13 and IL-10 which reverse the TNF induced release of PGE_2 by OA synovial fibroblasts (Alaaeddine, Di Battista, Pelletier, Kiansa, Cloutier, and Martel-Pelletier, 1999). Additional functions include the blocking of collagen release from IL-1/OSM stimulated cartilage tissue by IL-4 (Cawston et al., 1996) and IL-13 (Cleaver et al., 2001). IL-13 can also reduce the production of pro-inflammatory cytokines and MMPs, favouring the production of IL-1ra (Jovanovic et al, 1998b). Furthermore, IL-10 inhibits production of TNFα and IL-1 stimulated progression of established collagen-induced arthritis (Walmsley et al., 1996).

Numerous growth factors are also associated with the anabolic processes of cartilage matrix turnover. For example insulin-like growth factor-1 (IGF-1) is a potent mediator of cartilage proteoglycan synthesis even in the presence of pro-inflammatory cytokines (Tyler, 1989) and serum levels of IGF-1 are found to be low in OA (Denko et al., 1990). However, IGF-1 is important in both cartilage and bone turnover with the development of abnormal subchondral bone sclerosis (Martel-Pelletier et al., 1998) and osteophyte formation (Schouten et al., 1993). TGFβ is also a powerful mitogen for inducing cartilage and bone turnover while reducing inflammation and stimulating matrix synthesis (Wahl et al., 1989). TGFβ inhibits matrix degradation by stimulating protease inhibitor production, and blocking MMP release (Roberts and Sporn, 1990) and also promotes cartilage repair by stimulating production of collagen, fibronectin and inhibitors of plasminogen activators in the joint (Morales et al., 1988). TGFβ up-regulates the levels of TIMP within the joint, specifically by transcriptionally up-regulating the levels of TIMP-3 (Su et al., 1996). TGFβ also suppresses cartilage degradation, stimulates production of proteoglycans in the repair of articular cartilage (Glansbeek et al., 1998) and is thought to be involved in the negative feedback regulation of TNF induced collagenase activity (Shlopov et al., 2000). However, TGFβ has a dual role in OA since it can also induce IL-1β production (Wahl et al., 1993a) and excessive levels have been shown to induce OA after multiple intra-articular injections into murine joints (van Beuningen et al., 2000). All of these growth factors function together in a complex network, for example the insulin-like growth factor (IGF-1) and bone morphogenetic protein-2 (BMP-2) stimulate proteoglycan synthesis while transforming growth factor (TGFβ1) stimulates synthesis of

collagen (Smith et al., 2000) and fibroblast growth factor-2 (FGF-2) is important in the regeneration of articular cartilage (Otsuku et al., 1997).

OA is clearly a very complex pathological event. The initial inflammatory response stimulates a cascade of cytokine activation that enhances the production of proteolytic enzymes within the joint. Understanding the strict regulation of the biochemical pathways involved requires further research at the molecular level and holds the key to the development of new structure-modifying therapies.

1.3 THERAPIES FOR CANINE OSTEOARTHRITIS

The mainstay of treatment for OA in all species is drug based; however a variety of surgical treatments have been attempted in human and canine patients and include debridement, excision orthoplasts, lavage and mechanical penetration into the subchondral bone to promote cartilage resurfacing. Operative treatments to restore some of the functional capacity of affected joints by soft tissue releases and osteotomies are currently in use and even transplants using osteochondral, periosteal and perichondrial tissues are under development (Ghivizzani et al., 2000). Joint replacement surgery is frequently used in man to provide a pain free articulation, total hip replacement is now regularly performed in the dog, usually for the treatment of end-stage disease (Bennett and May, 1994).

1.3.1 Symptom modifying therapies

The main treatment for OA in canine patients relies on a combination of medical and physical modalities. In general the medical compounds available are limited to 'symptom-modifying' therapies that suppress one or more of the cardinal signs of inflammation with the principal value of pain relief. As such, the standard treatment for the canine patient includes anti-inflammatory drugs (Johnston and Budsberg, 1997) in combination with weight control and physiotherapy.

The non-steroidal anti-inflammatory drugs (NSAIDs) are indicated for use in a variety of painful musculoskeletal conditions, including OA. Almost all NSAIDs are weak carboxylic or enolic acids possessing analgesic, antipyretic, peripheral anti-inflammatory and anti-thrombotic qualities acting primarily through the inhibition of cyclooxygenase (COX). The COXs catalyse the first two steps in the synthesis of PG from arachidonic acid in a pathway that is clearly implicated in the pathogenesis of OA. A series of cartilage-explant studies demonstrated osteoarthritic cartilage released fifty times more PGs than normal cartilage which coincided with the up-regulation of chondrocyte COX-2 mRNA and protein (Amin et al., 1997). Furthermore, pro-inflammatory cytokines such as IL-1 have since been shown to be responsible for this increased production of PG in both chondrocytes and synovial cells (Knott et al., 1994).

There are at least two distinct isoforms of cyclooxygenase, COX-1 and COX-2. COX-1 is constitutively expressed in many tissues, where it plays a role in a range of physiological processes; COX-2 however, is an inducible form that is generally up-regulated in response to the inflammatory processes observed during disease progression. Although the NSAIDs are extremely efficacious drugs in the treatment of arthritis some of the original compounds developed were shown to have considerable side-effects within the joint causing further damage to the osteoarthritic cartilage (Huskisson et al., 1995) and to increase the risk of peptic ulcer disease and renal insufficiency. As such considerable research has been directed at developing selective COX-2 inhibitors that have anti-inflammatory properties without the adverse side-effects (Golden et al., 1999). The potency ratio for inhibition of COX-1: COX-2 can be determined and varies widely between the different NSAIDs, being high for Aspirin, Flunixin, Phenylbutazone and Piroxicam where the side-effects may be considerable and lower for Naproxen, Carprofen, Meloxicam and Nabumetone. However,

this depends on the *in vitro* and *in vivo* experimental systems used to determine these values. Although these new COX-2 inhibitors are reported to possess fewer side-effects than the non-selective agents is it now believed that COX-2 is important in the resolution of chronic inflammation, wound healing and angiogenesis. The selective COX-2 inhibitors therefore have the capability of reducing the resolution of chronic inflammation and for example delaying the healing of fractures (Simon et al., 2002). The NSAIDs can also reduce blood clotting, leading to excessive bleeding into the joint after injury. There is now increasing concern that blood within the joint can increase the risk of OA with early studies showing that blood elements, such as iron, are toxic to chondrocytes and can induce apoptosis in rabbits (Madhok et al., 1988). Certain NSAIDs have been shown to inhibit cytokines such as IL-1 and as such could theoretically have a structure modifying effect in addition to their symptom modifying effects (Herman et al., 1991).

Corticosteroids have also been used in the treatment of canine OA. Prednisolone, methylprednisolone and dexamethasone are compounds that are typically used and administered using standard protocols. Preparations are also available that contain combinations of NSAIDs, corticosteroids, opioid analgesics and non-opioid analgesics. The drugs included in each preparation may be additive in their toxic as well as therapeutic effects and therefore care must be taken when dispensing. A typical combination therapy used in the dog is Predleucotrophin (PLT) which is a combination of Cinchophen and Prednisolone.

The treatment regimen for canine OA not only relies on the use of the anti-inflammatory drugs but also on weight control to reduce the effects of weight-bearing on compromised joints. It has been demonstrated that a decrease in body fat is an important parameter for symptomatic pain relief in knee OA (Toda et al., 1998). Periods of regular controlled exercise is also indicated for osteoarthritic joints since daily moderate exercise augments glycosaminoglycan production and thickness of articular cartilage in the canine joint (Kiviranta et al., 1988).

1.3.2 Structure-modifying therapies

With an increasing awareness of the pathological processes occurring in the disease process it is now possible to develop 'structure-modifying' therapies. The drugs included in this category may be defined as agents that reverse, retard or stabilise the underlying pathology of OA, thereby providing symptomatic relief in the long-term by altering the progression of the disease. Many different compounds are thought to affect the turnover of the ECM components of the cartilage matrix, however their biological properties have not been fully determined and their long-term effects have yet to be established. Nevertheless almost all of these drugs do have 'symptom-modifying' effects and also have the potential to be 'structure-modifying'. Much evidence of a structure-modifying effect is based on *in vitro* tissue culture studies and these effects can not be directly translated to the whole animal.

1.3.2.1 Matrix supplements

1.3.2.1.1 Pentosan polysulphates

Sodium pentosan polysulphate (NaPPS), more commonly known as Cartrophen, is a semi-synthetic polymer with a molecular weight of 2000. This relatively new compound, available on the veterinary market, is delivered subcutaneously once a week over a period of one month. This compound and calcium pentosan polysulphate (CaPPS), an orally bio-available preparation (Innes et al., 2000), exhibit a wide range of pharmacological activities that are relevant to the metabolic pathways implicated in the pathobiology of OA. Polysulphated glycosaminoglycans (Adequan) is another simpler preparation which is used in the horse and dog, however there is no liscence for its use in the dog in the UK. The major effects of these drugs are i) preservation of cartilage integrity by supporting chondrocyte anabolic activities, including biosynthesis of aggrecan and downregulating proteases responsible for matrix catabolism, ii) reducing synovial inflammation. iii) normalising the ability to produce hyaluronan, iv) improving intra-articular blood flow and v) increasing the threshold for platelet activation (Ghosh, 1999). Although alleviation of clinical signs is quite evident, the effect only appears to be transient and the long-term effects of these drugs require further investigation.

1.3.2.1.2 Glucosamine, chondrotin sulphate and collagen preparations

Glucosamine hydrochloride is a supplement that facilitates the manufacture of hyaluronan (HA), collagen and PGs necessary for the restoration of the healthy articular cartilage and synovial fluid. When administered orally to the dog, a large percentage of the unionised compound passes into the systemic circulation within one hour (Setnikar et al., 1991). Since glucosamine hydrochloride is not plasma protein bound, free glucosamine is widely distributed with active up-take into the liver, kidneys and cartilage tissues, and in the latter it can be found 48 hours after administration (Setnikar et al., 1986). Recently, it has been demonstrated that orally ingested glucosamine can be incorporated into chondroitin sulphate of canine cartilage (Dodge et al., 2001). A well controlled clinical trial in man has shown that glucosamine has a structure-modifying effect in knee OA (Reginster et al., 2001). Glucosamine also inhibits IL-1 induced catabolism of aggrecan (Sandy et al., 1998), inhibits the release of leucocyte elastase (Kamel et al., 1991), and prevents the IL-1β mediated activation of human chondrocytes (Shikhman et al., 2001).

Chondroitin sulphate, an oral supplement extracted from shark, whale or beef cartilage, is used to reduce the indices of inflammation in OA synovial fluid, including reduction in collagenase activity, phospholipase A2 and n-acetyl-glucosaminidase, as well as increasing the HA concentration and synovial fluid viscosity (Ronca et al., 1998). Chondroitin sulphate is found in synovial fluid and cartilage within 24 hours of oral administration (Conte et al., 1995).

The term nutraceutical is often used to describe these oral supplements which are supplied individually or in combination; collagen preparations are often included as well. Additional nutraceuticals include minerals and vitamins such as zinc, vitamin C and the essential fatty acids.

Zinc is a trace element involved in the enzymatic processes throughout the body, particularly implicated in growth plate function (Litchfield et al., 1998) and in the assembly of cartilage matrix proteins (Rosenberg et al., 1998). Vitamin C is a scavenger of ROS which are released by inflammatory cells and degrade the glucuronic acid component of hyaluronic acid (Jahn et al., 1999) and cartilage proteoglycan aggregates. Vitamin C is destroyed in preference to the components of the synovial fluid, thereby maintaining the visco-elasticity of the fluid. Essential fatty acids, including omega-3 (*n*-3) found in fish oils, have been shown to modulate the expression and activity of degradative and inflammatory factors that cause cartilage destruction during arthritis (Curtis et al., 2000). Furthermore, this omega-3 has been used to reduce inflammation in rheumatoid arthritis (RA) in human patients and lowered their requirements for NSAIDs (Belch et al., 1988). Diets are now available containing omega-3 for dogs but no studies have been performed to determine their clinical value.

Despite the widespread use of nutraceuticals in both human and veterinary medicine their application still remains controversial. The databases show a plethora of contradictory results taken from different clinical trials carried out world-wide and strictly controlled clinical trials need to be performed before their use can be fully justified.

1.3.2.1.3 Hyaluronan

Hyaluronan (HA) is widely distributed and an important component of the joint tissues where it plays an essential role in the formation of proteoglycan aggregates. Hyaluronidases are enzymes that degrade HA through the random cleavage of the β-N-acetyl-hexosamine-(1-4) glycosideic bonds. The low levels of HA associated with OA can be replaced by exogenous hyaluronan (sodium hyaluronate) which acts as a lubricant immediately post-injection and later functioning to reduce further destruction of cartilage and promote its repair (Niethard, 1999). HA also relieves pain and may provide damage-blocking effects associated with the ability of HA to block the penetration of fibronectin peptides mediating cartilage chondrolysis rather than by distinct effects on cartilage tissue itself (Menzel and Farr, 1998). HA is usually administered via an intra-articular injection where the joint compartment allows the introduced drugs to directly interact with its constituents. Chondroprotective drugs, such as hyaluronan, have been reported to favourably influence OA when administered intra-articularly (Graf et al., 1993).

1.3.2.2 Anti matrix metalloproteinase drugs

The antibiotic tetracycline and related compounds were the first major group of compounds identified to inhibit the MMPs. More specifically, doxycycline can reduce the severity of articular cartilage breakdown with structure modifying effects associated with reduction in the levels of MMPs in diseased articular cartilage extracts (Brandt, 1995). Initial attempts to develop synthetic MMP inhibitors used a zinc binding group attached to modified peptides that could bind to sub-sites around the catalytic zinc component of the MMP (Beckett et al., 1995). Since then, alternative inhibitors have been developed containing carboxylic acid, thiol and phosphorous ligands that co-

ordinate with the zinc atom in the active site to block the action of the MMP on its substrate. More recently, the determination of crystal structures for the MMPs has enabled the design of selective inhibitors with good oral bioavailability (Bottomley et al., 1998). Although these drugs are currently in clinical trials they are not in general use.

1.3.2.3 Inhibition of intra-cellular signalling mechanisms

A number of inhibitors are proposed to function via inhibition of intra-cellular signalling mechanisms. Esculetin (EST) is a coumarin derivative which was found to protect rabbit articular cartilage from proteoglycan loss in response to IL-1 (Watanbe et al., 1999) and inhibit cartilage resorption induced by IL-1/OSM (Elliott et al., 2001). Tenidap which belongs to the oxindole class has a dual action inhibiting cyclooxygenases and blocking the zymosan-induced expression of IL-1, TNF, IL-6 and lipopolysaccharide (LPS) in synovial membranes (Ounissi-Benkalha et al., 1996). Tendiap can also reduce the level of IL-1 receptors and collagenase expression in human arthritic synovial fibroblasts (Martel-Pelletier et al., 1996). Furthermore, *in vivo* administration of tenidap to experimental dogs markedly reduced progression of cartilage lesions, osteophyte formation and synovial inflammation (Fernandes et al., 1995) in addition to decreasing collagenase, stromelysin and gelatinase activity (Fernandes et al., 1997).

1.3.2.4 Recombinant protein technology and Immunotherapy

The development of 'structure-modifying' therapeutics in the treatment of arthritis has evolved with advances in molecular biology and recombinant protein technology. Over the past ten years research has identified certain species of RNA and proteins with promising chondroprotective and anti-inflammatory properties offering the prospect of disease modification for arthritis (Kang et al., 1997a). Recently the US Food and Drug Administration and regulatory bodies in the UK have approved a number of structure-modifying anti-rheumatic agents including infliximab, anakinra and etanercept (Fleischmann et al., 2002). Infliximab is a chimeric anti-tumour necrosis factorα monoclonal antibody used in combination with methotrexate for the treatment of RA. This monoclonal antibody reduces the inflammatory response and decreases MMP levels leading to a decreased joint destruction in patients with RA (Brennan et al., 1997). Anakinra (Kineret), a recombinant form of human IL-1Ra (Amgen) is used for the treatment of the signs and symptoms of RA either as a monotherapy or in combination with methotrexate. Significant improvement in both clinical signs and radiographical changes have been observed (Garces, 2001). Etanercept (Amgen) is a low affinity p75 soluble TNF receptor shown to down regulate MMP-1 and MMP-3 in RA (Catrina et al., 2002).

However, effective *in vivo* delivery of therapeutic RNA molecules and proteins is one of the major limitations of this therapy. Whether the therapeutic molecule is administered orally or parentally (subcutaneous, intraperitoneal, intramuscular or intravenous injection) its delivery to the joints relies upon the vascular perfusion of the synovium (Evans and Robbins, 1994). Since cartilage is avascular, drug delivery to this tissue relies on the inefficient passive diffusion from the

synovial capillaries into the joint cavity. The rapid clearance of proteins from the joint space due to their short intra-articular half-lives, means that even the use of direct intra-articular injections, the most promising form of delivery, has limited application. These limitations require the repeated administration of large protein concentrations in order to achieve stable therapeutic levels within joints. This results in the exposure of non-target tissues and undesirable side-effects, especially if systemic delivery is employed. In addition, most proteins of interest are excluded for serious consideration due to their large non-diffusible properties (Evans and Robbins, 1994). There is also the potential for an immune response even to chimeric and humanised monoclonal antibodies.

Most of these problems can now be circumvented by the delivery of genes encoding therapeutic RNA or protein molecules, the basis of gene therapy. By taking one step back along the molecular pathway the DNA encoding therapeutic molecules can be delivered to cells of diseased joints with many advantages (Kang et a., 1997a). Firstly, delivery of DNA into cells is not restricted by the size and diffusiblility of the molecule and therefore allows a broader range of potentially useful protein-based drugs to be investigated. Secondly, the need for repeated administration can be circumvented if the therapeutic gene is integrated into the genome or placed within a stable episomal vector. Thirdly, the ability to regulate gene expression in a tissue specific manner should reduce exposure of non-target organs and limit side-effects. However, the application of this powerful molecular technique must be thoroughly understood before being used in any clinical trials.

1.4 GENE THERAPY

Gene therapy is defined as the delivery of genes into the cells of an individual for therapeutic purposes and is one of the newest areas of molecular medicine. It was initially proposed in the late 1960's as a means of compensating for heritable genetic diseases (Wolff and Lederberg, 1994). It was thought that these diseases such as cystic fibrosis, phenylketonuria and β-thalassemia, the result of a single gene defect, could be resolved by the introduction of the wild-type gene into the patient (Anderson, 1994). However, it is now recognised that gene therapy can also be used in the treatment of acquired, multi-genic diseases such as cancer and arthritis with the transfer of appropriate genes as a type of drug delivery system. The potential for gene based therapeutics to become a leading therapy encouraged the Recombinant DNA Advisory Committee of the National Institute of Health to grant the first human gene transfer clinical trial in 1988. However, it wasn't until 1994 that permission was given for its application to the treatment of musculoskeletal disorders with the transfer of genes into joints affected by RA. Research into gene based therapy for arthritis in human medicine (Evans and Robbins, 1995) is now complemented by studies in domestic animals which not only provide ideal models for analysing the therapeutic benefits to human patients but also enables the development of new veterinary treatments (Argyle, 1999). Gene therapy is a powerful therapeutic tool with tremendous potential for not only controlling the progression of a disease, but through the genetic alteration of the germ-line DNA, it also offers the potential of permanently eliminating genetic diseases. As such there are numerous ethical concerns associated with this new technology including the spectre of eugenics (Latchman, 1994) and every step in its development must be handled with care. Safety assessment data must be obtained for *in vivo* clinical trials carefully evaluating every tissue for transgenic integration and for levels of foreign DNA and transgene expression. Although the idea of gene therapy is simple, the application of this new technology *in vivo* is much more complex with many technological problems that must be overcome before it can be accepted as a safe clinical practice.

1.4.1 Elements of gene therapy

1.4.1.1 Selection of appropriate therapeutic genes

The first step in developing a gene-based therapy is the selection of appropriate therapeutic genes, identified by understanding the disease pathogenesis. Two main pathologies afflict the joint affected by OA; one is inflammation, which occurs as a synovitis and the other is the breakdown of the ECM components of the articular cartilage. These two processes are ideal targets for gene therapy with the potential for arresting disease progression. However, repairing the damage already inflicted on the joint prior to therapy is also important, so there is also a need to introduce those genes that promote cartilage regeneration. A list of candidate proteins is shown in Table 1-5 with some of their effects on disease control. The stage and degree of the osteoarthritic process will also determine the selection of appropriate therapeutic genes, encoding for one or more anti-inflammatory, chondroprotective or chondroreparative proteins as determined for each individual.

The selection of intra-cellular or secreted therapeutic proteins is another important parameter and will be determined by the disease type. However, several problems are associated with the use of therapeutic gene products that remain intra-cellular since once synthesised they only benefit the transfected cell. Therefore to enable these proteins to be successful a large number of cells require to be transfected therefore limiting its use in the systemic and local *in vivo* delivery techniques. It is therefore likely that most therapeutic gene products will be secreted proteins that are compatible with both *ex vivo* and *in vivo* delivery systems.

Table 1-5 Potential therapeutic genes

Protein	Effects
IL-1Ra	Antagonise IL-1, Anti-inflammatory, chondroprotective
IL-1 soluble receptors	Antagonise IL-1, Anti-inflammatory, chondroprotective
TNF soluble receptors	Antagonise TNF, Anti-inflammatory, chondroprotective
IL-4	Anti-inflammatory, induces IL-1ra
IL-10	Anti-inflammatory, induces IL-1ra
γ-IFN	Inhibits MMP induction in chondrocytes
TGF-β	Antagonises responses of chondrocytes and synoviocytes to IL-1, Promotes cartilage matrix synthesis.
TIMPs	Inhibit MMPs, chondroprotective
Superoxide dismutase	Antagonises oxygen-derived free radicals
Soluble ICAM-1 CD44	Blocks cell to cell, cell to matrix interactions
IGF-1	Promotes cartilage matrix synthesis, Antagonises catabolic effect of IL-1
bFGF	Promotes cartilage matrix synthesis

Numerous cytokines have been identified as potential mediators of articular inflammation but IL-1 and TNF have been particularly implicated in the processes of ECM degradation stimulating cartilage breakdown whilst inhibiting repair (Arend and Dayer, 1990). Thus antagonists of these two cytokines hold particular promise as anti-arthritic agents (Evans et al., 1998). Agents that have successfully blocked the arthritic changes observed with IL-1 include IL-1Ra (Oligino et al., 1999) and soluble forms of the IL-1 receptors (Ghivizzani et al., 1998). Prevention of collagen-induced arthritis by the gene delivery of soluble TNFR receptors (sIL-1R) has also shown great promise as antagonists of TNF (Mageed et al., 1998). An alternative approach for disease control is to modulate the activity of IL-1 and TNR via other cytokines such as IL-4 (Joosten et al., 1999. Kim et al., 2000, 2001) and IL-10 (Fellowes et al., 2000, Lechman et al., 1999) both of which have demonstrated potential as effective treatments for collagen induced arthritis.

Degradation of articular cartilage involves the extra-cellular digestion of the matrix by proteinases (Cawston, 1996) and perhaps, oxygen-derived free radicals such as superoxide, hydroxyl radical or NO and its derivatives (Stefanovic-Racic et al., 1993). Antagonists of these mediators include TIMPs, plasminogen activator inhibitors (Hart and Fritzler, 1989) and inhibitors of cysteine proteinases. Free radical damage may be prevented by genes coding for enzymes such as superoxide dismutase and other anti-oxidant proteins. Increasing evidence has also implicated various types of cell adhesion molecules in the arthritic process such as CD18 (Jasin et al 1992), ICAM-1 (Iigo et al., 1991) and CD44 (Galea-Lauri et al., 1993). Genes encoding soluble forms of these molecules or other blocking proteins may be of future therapeutic use. Finally, cartilage repair through the increased synthesis of proteoglycans may be stimulated by growth factors such as IGF-1 (Mi et a.l., 2000), bFGF (Cuevas et al 1988) and TGFβ (Morales and Roberts, 1988).

1.4.1.2 Selection of appropriate gene organisation

Selected therapeutic genes can be expressed from a cloned complementary DNA (cDNA) or genomic DNA (gDNA) sequence. The cDNA sequence is an exact copy of the mRNA transcript and is directly transcribed and translated to a functional protein without the need for complex post-transcriptional processing such as splicing. cDNA is used for most standard applications due to size constraints of the current vector vehicles for transferring genes to cells, where most vectors have an upper gene size limit of 4-6 kb. In comparison gDNA not only contains intronic sequences spanning up to hundreds of kilobases presenting problems for gene delivery but also requires post-transcriptional modifications for effective protein expression. However, for future gene therapy, once vector technology has advanced, it may be beneficial to include intronic sequences and important 3′ and 5′ regulatory regions present in the gDNA for strictly regulating tissue-specific gene expression.

1.4.1.3 Selection of appropriate vector vehicles

Appropriate vehicles are required to enable the efficient delivery of therapeutic genes to cells of the joint. These vectors are responsible for transferring DNA to the nuclei of cells where the transcriptional machinery is responsible for gene expression. Most DNA vectors may remain episomal and gene expression from these is usually transient. Some vectors however, become integrated into the host chromosome which not only provides long-term expression but also ensures that the introduced gene will be transmitted to daughter cells on replication. Numerous vector systems have been developed for gene transfer and have been divided into viral and non-viral vectors (Oligino et al., 2000).

1.4.1.3.1 Viral vectors

Viruses are very efficient vectors for gene therapy since they take advantage of the natural ability of viruses to infect and deliver nucleic acids to specific cell types, whilst avoiding the host immune response. Viruses are made safe for use as gene transfer vectors by rendering them replication

incompetent through the inactivation of one or more genes essential for viral replication, which are replaced by the genes of interest (Robbins and Ghivizzani, 1998). Propagation of these deficient viruses is then performed in specialised cell lines providing the missing essential viral functions in *trans* (Krougliak and Graham, 1995). A number of viruses have been modified for vector application in gene transfer to joints as shown in Table 1-6.

Table 1-6 Viral vectors for gene therapy

Viral vector	Insert size (kb)	Viral genome state	Infection of non-dividing cells	Infection efficiency	Duration of transgene expression	Level of transgene expression
MLV	4-6	Integrated	No	low	Persistent	high
Lentivirus	4-6	Integrated	Yes	Moderate	Persistent	Moderate
AV	7.5	Episome	Yes	high	Transient	Very high
AAV	4	Integrated	Yes	Moderate	Persistent/ transient	Moderate
HSV	40	Episome	Yes	Very high	Transient	Very high

Retroviruses are RNA viruses that replicate through a double-stranded intermediate, integrating into the host cell chromosome for long-term viral RNA expression. Retrovirsuses are the most commonly used vector in *ex vivo* gene therapy clinical trials (Robbins et al., 1994) particularly the MMLV (Kim et al., 1998). This virus is non-pathogenic to humans and has no regions of homology with human retroviruses. The DNA sequence encodes for three major proteins for replication and packaging; structural proteins (gag), reverse transcriptase (pol) and envelope proteins (env). Construction of gene therapy vectors requires the replacement of these sequences with therapeutic genes followed by propagation in packaging cell lines that supply the gag, pol and env proteins. These therapeutic constructs are then capable of infecting cells and integrating into the genome but are unable to propagate. Retroviruses have been used for *in vivo* gene delivery to the synovium of the rabbit knee (Ghivizzani et al., 1997) and *ex vivo* delivery of IL-1ra to the synovium (Bandara et al., 1993). However, retroviral vectors have three limitations which has led to the development of other viral vectors. Firstly, the virus can only accept 8kb of foreign DNA, secondly the random insertion of DNA into the host genome has aroused the concern of mutagenesis and thirdly, their inability to transduce non-dividing cells, although their use in arthritis is possible since the synovium actively divides in OA.

Adeno-Associated Viruses (AAV) have the potential to circumvent some of the problems associated with retroviruses. AAVs belong to the Parvo virus family of single stranded small DNA viruses that require a helper virus such as Adeno Virus (AV) or Herpes Simplex Virus (HSV) for replication. Wild type virus is non-pathogenic, is not associated with any known disease and can infect a wide variety of dividing and non-dividing cells. Long-term expression is a possibility since

they are capable of integration into the host genome at a single site, although this feature is lost on generation of the recombinant virus. However, the main problem associated with the AAV vectors is the limitation on insert size (restricted to 4 kb) (Oligino et al., 2000).

Lentiviruses are a class of retroviruses that includes human immuno-deficiency virus (HIV), simian immunodeficiency virus (SIV), equine infectious anaemia (EIA) and caprine arthritis encephalitis virus (CAE). All lentiviruses are associated with a slow progressive destruction of the immune system and since wild type viruses are pathogenic these vectors are intrinsically more dangerous to use. Manipulation of these viral vectors is difficult since the production of additional accessory proteins through the alternative splicing of the primary transcript is necessary. However, lentiviruses can integrate into the genome of non-dividing cells and therefore show promise as a very useful vector system (Klimatcheva et al., 1999). The first successful use of lentiviruses as gene therapy vectors has been for *in vivo* gene delivery to the synovium (Gouze et al., 2002).

Adenoviruses (AV) have a double stranded DNA genome with inverted terminal repeats at either end; 47 serotypes exist but 2 and 5 are the two most commonly used for gene transfer and are excellent vehicles for gene delivery in a wide range of cell types (Bramson et al., 1995). The DNA of the adenovirus does not integrate into the host genome but remains as an episomal element with replication and packaging occurring in the nucleus. Several versions of replication-incompetent adenoviral vectors have been developed (Robbins and Ghivizzani, 1998) generally with the replacement of the early genes such as E1A and E1B genes with the transgene. AV have potential as gene therapy vectors since they can infect a wide range of dividing and non-dividing cells and are thus suited to *in vivo* gene delivery. They are also capable of producing high titre recombinant virus and can accept large amounts of additional DNA (approx 30 kb). These advantages have led to their wide-spread application (Evans et al., 2001) and examples used for orthopaedic application include the delivery of hIL-1Ra to synoviocytes (Roessler et al., 1995) and IGF-I to the synovium (Saxer et al., 2001).

Herpes Simplex Virus (HSV) contains a linear double-stranded DNA molecule (152 kb); this large genome has the potential to carry large amounts of foreign DNA in excess of 40 kb and has been used for the development of multigene vectors (Krisky et al., 1998). This virus can infect almost all known cell types including non-dividing cells both *in vitro* and *in vivo* but is difficult to make and only supports transient expression (Glorioso et al., 1997). However, replication-defective HSV vectors have been analysed for gene transfer *in vivo* (Marconi et al., 1996). Other viruses such as Simian Virus 40 (SV40), Polyoma Virus, Papilloma Virus and Picornavirus are being explored as potential vector systems for gene therapy (Evans and Robbins, 1995).

Although viral vector technology has already shown huge potential for gene delivery systems there is the concern that recombination of defective virus with viral sequences present in the host cell may generate replication-competent viruses that can propagate in the patient. Additionally there is the potential for an immune response to viral proteins. As a result considerable research has focused not only on refining these viral based vectors but also on the development of alternative non-viral methods.

Mammalian DNA expression plasmids are non-viral vectors that are widely used for standard gene cloning techniques and *in vitro* transfer studies. These synthetic vectors have the advantage of being non-immunogenic since they do not introduce strongly antigenic materials and they are easy to produce with a greater chemical stability. Plasmids however have two major disadvantages, they usually remain episomal, providing transient gene expression and are generally less efficient in the transfer of nucleic acid to cells when compared with viral vectors. The low transfection efficiency of naked DNA plasmids has been addressed with the development of a variety of chemical methods for introducing DNA into cells.

The transfer of DNA to mammalian cells have utilised liposomes as carriers which make stable complexes with DNA that are small enough to enter cells. Liposomes are grouped into two categories based on their mode of DNA entrapment. Since DNA is negatively charged it can be held with positively charged liposomes (cationic liposomes) to make a complex or it can be entrapped in the aqueous interior of the liposome (pH-sensitive liposomes) (Singhal and Huang, 1994). Cationic liposomes have been utilised to deliver DNA to articular chondrocytes (Madry and Trippel, 2000) despite the low transfection efficiency and transient expression. The use of conjugates between DNA, polylysine and specific antibodies or DNA conjugated to specific receptor binding proteins such as asialoglycoproteins as found on hepatocytes have also been developed (Perales et al, 1994). This DNA-ligand complex enables the DNA to be targeted to cells that express a cell-specific surface protein recognised by the antibody or protein. Plasmids can also be forced into cells by particle bombardment using a gene gun (Klein et al., 1987). A full description of particle bombardment is discussed in chapter VII. Briefly, particles of gold are coated with DNA and then accelerated at a high velocity using pressurised helium allowing the DNA to penetrate cells. In general plasmid vectors are designed for introducing DNA into cells in tissue culture and are therefore most applicable for *ex-vivo* gene delivery to cells.

Recent advances in technology have produced potential alternative vectors to plasmids for gene transfer studies. Mammalian artificial chromosomes (MAC) for example can now be constructed via two different methods (Grimes and Cook, 1998) and, although still in the early stages of development, have shown potential as gene transfer vectors for complementing genetic deficiencies (Mejia et al., 2001). This vector system offers the prospect of sustained therapeutic gene expression, important for the chronic condition of OA, as demonstrated by the stable expression of the hypoxanthine phosphoribosyltransferase locus (115 kb) for six months *in vitro* (Wade-Martins et al., 2000). The development of MACs began following the success of yeast artificial chromosomes (YACs) and bacterial and P1 artificial chromosomes (BACs) as large DNA cloning vectors (Monaco and Larin, 1994). Major efforts have aimed to define the vital chromosomal structures, alpha satellite and telomeric DNA, to enable these vectors to function as normal chromosomes and divide within the cell. MACs are fully functional autonomous vectors that do not integrate into the genome thus overcoming problems of gene silencing and oncogenesis from random integration into the host cell genome. Finally, the size of the vector enables the

incorporation of large pieces of genomic DNA allowing important up-stream and down-stream regulatory regions to be included for accurate transgene expression and providing the potential of introducing multiple therapeutic genes (Warburton, 1999). Efficient transfer of artificial chromosomal DNA into cells confronts the same problems associated with plasmid vectors, although there have been reports that a 60-Mb MAC was transferred into a hamster cell line using cationic lipids (de Jong et al., 2001). However, these problems are enhanced by the sheer size of the chromosome component and efforts to improve transfection efficiency will enable these vectors to become a realistic alternative to plasmid vectors.

1.4.1.4 Selection of an efficient gene transfer system

1.4.1.4.1 *In vivo* or *ex vivo* for gene delivery

There are currently two basic methods for gene transfer to joints referred to as the *in vivo* (direct) approach and the *ex vivo* (indirect) approach with selection based on the anatomy and physiology of the target tissues, the vectors employed and safety considerations. The direct delivery system involves the introduction of appropriate vectors into the joint cavity for transfer to target tissues of the joint. This technique is technically simpler but efficient delivery of vectors to target tissues such as the synovium is more difficult (Nita et al., 1996) and it may be difficult to regulate the efficiency of transduction and the level of gene expression. The indirect delivery system involves removal of joint cells from the patient followed by *in vitro* propagation and genetic modification before cells are screened for the therapeutic genes and re-introduced into the joint (Evans et al., 1992; Kang et al., 1997a). This is not only technically complex, expensive, time consuming and invasive but it is also currently very difficult for cells, especially chondrocytes, to re-establish themselves within the joint tissues. As an alternative to chondrocyte transduction, synovial cells are potentially easier to manipulate and can be used successfully in both the *in vivo* and *ex vivo* techniques for gene transfer to joints (Evans et al., 1999; Bandara et al., 1992). However, with the advent of new tissue engineering technologies the *ex vivo* technique may become a viable option with its advantages of regulating the efficiency of transduction and the level of gene expression.

1.4.1.4.2 Systemic or local gene delivery

Genes can be delivered locally to sites of disease, such as the joint space, or into the systemic circulation. Local delivery is used in preference to systemic delivery in order to restrict most of the gene expression to a small anatomical site and reduce undesirable side-effects. However, even using this technique it is likely that a small percentage of the DNA will enter the circulation through the inadvertent transduction of inflammatory cells; this has been demonstrated by the identification of a marker gene in the untreated contra-lateral joint (Evans et al., 1999). Systemic delivery may be desirable for RA where there is multiple joint involvement but side-effects are more likely.

1.4.1.5 Selection of appropriate methods for regulating gene expression

The efficient transfer of genes to target cells does not necessarily ensure successful treatment of disease. Genes must be expressed for specified lengths of time at appropriate therapeutic levels, as determined by the disease type. Although gene expression is regulated at many levels the control of gene expression for gene based therapeutics has generally focused on the level of gene transcription with the application of promoters (Whalen, 1994). Promoters are regulatory regions of DNA located in the 5′ untranslated region (UTR) of the gene; a full description of promoter function is described in chapter IV. Viral promoters are usually used in the development of gene therapy vectors and include the cytomegalovirus (CMV) and the SV40 both of which provide high levels of constitutive expression. However, gene expression from these promoters is often lost when cells stop dividing, possibly due to the methylation of cytosine residues, within the promoter, by cellular methylases (Hoeben et al., 1991; Challita, 1994). Eukaryotic promoters have been evaluated as alternatives to prolong gene expression and include those that drive constitutive expression of house-keeping genes within the cell such as the dihydrofolate reductase gene promoter (Scharfmann et al., 1991). However, the level of gene expression from eukaryotic promoters is generally far lower than that from viral promoters so hybrid promoters are under development to achieve high long-term expression. Eukaryotic gene promoters can be used for driving tissue-specific gene expression which is particularly useful for reducing expression of the transgene in non-target cell types. However, the context of the vector is important since there have been reports that promoter interference from viral vector promoters may affect activity and specificity of the tissue specific promoter (Miller and Vile, 1995).

In many diseases, high-level gene expression from constitutive promoters is acceptable since the therapeutic effect of the expressed proteins is well tolerated over a wide range. However, this is not true for all disease types in which sustained high levels may not only be unnecessary but may be detrimental to tissues. OA is an example of a disease that should be tightly regulated to avoid complications arising from the persistent overproduction of anti-inflammatory and chondroprotective molecules. The most efficient method for this is to use inducible promoters that regulate gene expression in response to the presence of exogenous or endogenous stimuli. Examples include those promoters that respond to exogenous stimuli such as the metallothionein promoter that responds to heavy metals (Mayo et al., 1982) and drug inducible promoters such as tetracycline (Ghersa et al., 1998) and rapamycin (Ye et al., 1999). Even radiotherapy has been used to stimulate promoter activity (Marples et al., 2002). Endogenously stimulated promoters can be used to harness pathological changes to gene expression and include promoters such as those for metalloproteinase, aggrecanase and hyaluronidase all of which are up-regulated by the inflammatory cytokines present in the disease state. Although research has focused on the involvement of promoters in regulating gene expression other elements of the transcriptional machinery are equally important such as enhancers and silencers which effect the relative activity of the promoter. Locus Control Regions (LCR) are also important since a considerable number of gene therapy approaches involve the expression of genes integrated into an unnatural position in

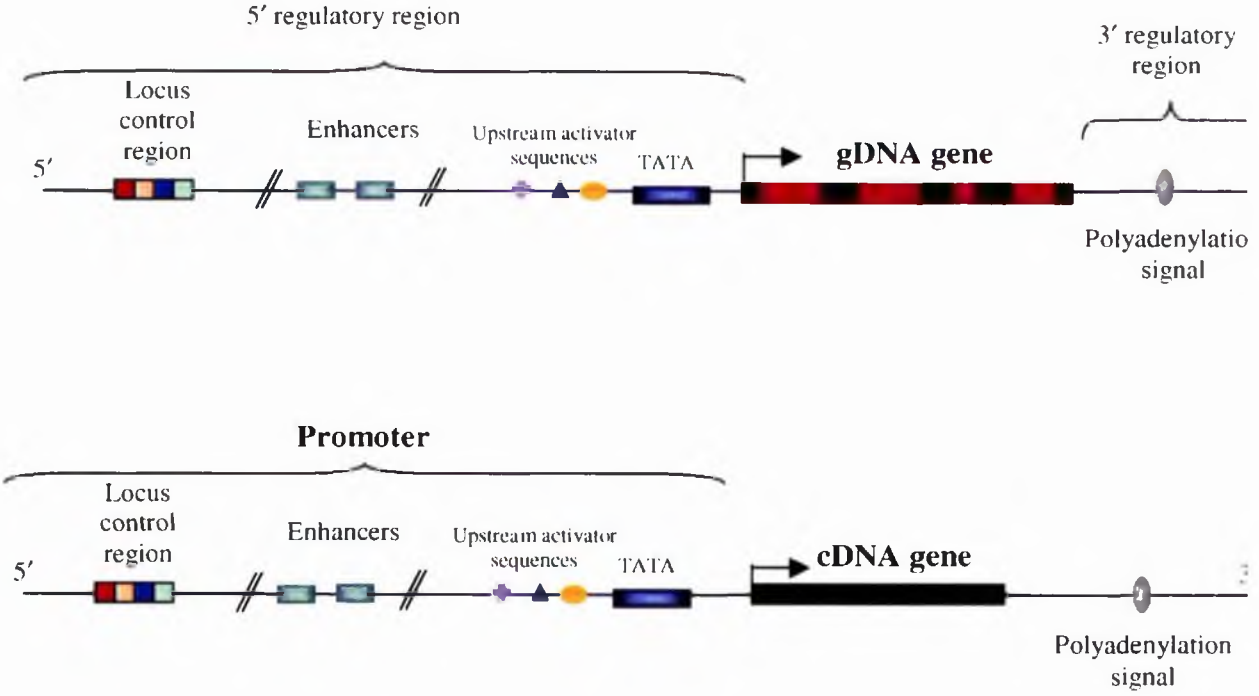
the host genome. Structural organisation of the chromatin is an important feature of gene regulation since minimal secondary structure associated with episomal locations may result in poor tissue specificity while integration into an area of highly complex secondary structure may result in gene silencing. Elements of the translational machinery are also important since the level of protein synthesis depends upon translational efficiency and the stability of the mRNA transcripts as discussed in chapter III. Ultimately the aim will be to control the expression of multiple therapeutic genes simultaneously from one vector thereby strictly regulating disease progression.

1.4.2 Construction of the gene therapy transcriptional unit

The design of a gene therapy vector system requires a 'transciptional unit' that consists of a region of protein-coding DNA (Jacob and Monod, 1961) and includes all associated regulatory regions referred to as promoters and enhancers based in the 3′ and 5′ UTRs. The expression of a gene for gene-based therapeutics requires the correct assembly of this transcriptional unit, as illustrated in Figure 1-13, to enable the *trans*-acting protein factors to specifically bind the regulatory regions of DNA and direct transcription. As discussed in detail in chapter III the transcriptional unit must include the gene sequence cloned either as cDNA (in frame) or gDNA with optimised translation start and stop sites terminating at the polyadenylation site present in the 3′ UTR. The 5′ UTR corresponding to the regulatory promoter must contain the core promoter elements, activation sequences, enhances and locus control elements to regulate gene transcription. The function of the 5′ regulatory region, is primarily responsible for the binding of appropriate transcription factors and the recruitment of RNA polymerase to the transcription initiation site. Gene regulation is discussed further in chapter IV. The transcriptional unit may then be modified with the manipulation of promoter sequences to target gene expression to diseased cell types of the canine osteoarthritic joint as attempted in chapter VI. Finally the gene therapy constructs must be easily delivered to the cells of the joint using *in vivo* or *ex vivo* techniques. The application of particle bombardment mediated gene delivery is described in chapter VII.

Figure 1-13 The gene therapy transcriptional unit

The 'transcriptional unit' consists of protein-coding DNA and includes all associated regulatory regions referred to as promoters and enhancers based in the 3′ and 5′ UTRs. The transcritpional unit must include the gene sequence cloned either as genomic DNA (containing black exons and red introns) or complementary DNA (containing only black exons) with optimised translation start and stop sites terminating at the polyadenylation site present in the 3′ UTR. The 5′ UTR corresponding to the regulatory promoter must contain the core promoter elements, activation sequences, enhances and locus control elements to regulate gene transcription. This region is primarily responsible for binding the appropriate transcription factors and recruitment of RNA polymerase to the transcription initiation site.



1.5 SUMMARY

As the details of the molecular mechanisms involved in the pathogenesis of OA are unveiled new 'structure-modifying' treatments such as gene therapy can evolve. Gene therapy is moving rapidly from the laboratory to the clinic with a number of clinical trials for orthopaedic disease already in progress. However current gene transfer studies have focused on the use of highly active constitutive promoters for driving high levels of gene expression in any cell type. This thesis identifies potential therapeutic genes and attempts to address the problems associated with non-specific gene expression with the development of a dual-promoter vector system for the transcriptional targeting of therapeutic genes to the diseased cells of the canine osteoarthritic joint.

Chapter II

Materials and Methods

2.1 MATERIALS

Materials in regular use, such as equipment, general reagents and solutions are detailed in this section.

2.1.1 Cell culture materials

2.1.1.1 Cell lines

MDCK cells were grown and maintained for use in transfection experiments by Mrs. Elizabeth Gault in the Molecular Therapeutics Research Group, University of Glasgow Veterinary School.
FEA cells were kindly supplied by Mr Matthew Golder, Feline Virus Unit, University of Glasgow.
Primary rat cardiocytes were kindly supplied by Dr. Arvind Sood, Department of Biochemistry and Molecular Biology, University of Glasgow.
APDC5 cells were kindly supplied by Dr. David Thomas, King's College London, Guy's Hospital.
SW1353 cells were supplied by The American Type Culture Collection (ATCC).
HTG-82 cells were supplied by The ATCC.

2.1.1.2 Plasticware

Tissue culture flasks, 6, 12, 24-well plates, Falcon conical centrifuge tubes (15 and 50ml) Pipettes (5, 10, 25, 50ml) were supplied by Greiner (Gloucestershire, UK). White, tissue culture treated ViewPlate™-96 were supplied by Packard (Berkshire, UK).

2.1.1.3 Solutions, media and supplements

All solutions and media for cell culture were supplied by Gibco BRL Life Technologies, unless otherwise stated.

2.1.1.3.1 Media

All media were supplied as sterile solutions and stored at 4°C

Dulbecco's MEM with Glutamax-I with sodium pyruvate, glucose and pyridoxine.
Dulbecco's MEM/ Nutrient mix F-12 (1:1) without L-Glutamine and with pyridoxine.
Dulbecco's MEM 25MM Hepes without sodium pyruvate, with glucose and pyridoxine.
Dulbecco's modified Eagle's medium with glucose and sodium bicarbonate, without L-Glutamine (Sigma).

Medium 199 with Earle's salts, L-glutamine and sodium bicarbonate (Sigma).

Nutrient Medium F-12(HAM) with L-Glutamine

2.1.1.3.2 Supplements

Foetal Calf Serum (FCS): virus and mycoplasma screened. FCS was heat inactivated at 56°C for 30 minutes, then stored in 50ml aliquots at -20°C.

Horse serum (HS): Heat inactivated at 56°C and stored at -20°C until use.

L-glutamine: supplied 200mM (100x) stock solution and was stored at -20°C in 5 ml aliquots.

Penicillin/streptomycin (P/S): supplied as a 100x stock solution of 10,000 units penicillin and 10,000 units streptomycin per ml and stored in 5 ml aliquots at -20°C.

Fungizione: supplied as a 100x stock solution and stored in 5 ml aliquots at -20°C.

Nystatin: supplied as a 100x stock solution and stored in 1 ml aliquots at -20°C.

Trypsin-EDTA: supplied as a 100x stock solution and stored in 5 ml aliquots at -20°C.

Sodium pyruvate: supplied as a 100mM stock solution and stored at 4°C.

Non Essential Amino Acids (NEAA): supplied as a 100x stock solution and stored at 4°C.

Ciprofloxacin: Anti-mycoplasma antibiotic added to tissue culture medium as necessary to a final concentration of 10mg/ml (Miles, Bayer Diagnostics)

Insulin: (bovine pancreas) lyophilized powder reconstituted in sterile water to a concentration of 10mg/ml and stored at -20°C (Sigma-Aldrich Company Ltd, Dorset, UK).

Human apo-transferrin: powder reconstituted in sterile water to a concentration of 10mg/ml and stored at -20°C (Sigma-Aldrich Company Ltd, Dorset, UK).

Sodium selenite: lyophilized powder reconstituted in sterile water (10^{-4}M), stored at -20°C (Sigma-Aldrich Company Ltd, Dorset, UK).

2.1.2 Radiochemicals

2.1.2.1 ^{35}S Methionine

EasyTag™ Methionine, L-[^{35}S], (specific activity of 43.5 TBq/mmol) was used in the *in vitro* transcription/translation protocol for labelling proteins. Supplied by PerkinElmer Life Sciences (NEN) and stored at 4°C.

2.1.3 General chemicals

Chemicals used were of analytic, ultrapure or molecular grade quality and were supplied by a range of biotech companies.

2.1.4 Complete kits

QIAquick® PCR Purification Kit (QIAGEN, UK)

QIAquick® Gel Extraction Kit (QIAGEN, UK)

QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK)

EndoFree® Plasmid Maxi Kit (QIAGEN, UK)

GeneRacer™ Kit (Invitrogen, UK)

Universal GenomeWalker Kit (Clontech, UK)

TOPO TA Cloning® (Invitrogen)

TNT® Quick Coupled Transcription/Translation Systems (Promega)

Dual-Luciferase® Reporter Assay System (Promega)

QuikChange™ Site-Directed Mutagenesis Kit (Stratgene)

ECL Western Botting Detection Reagents (Amersham Pharmacia Biotech)

DNA Sequencing Kit Big Dye™ Terminator Version 3.0 Cycle Sequencing Ready Reaction (ABI)

X-Gal Staining Assay Kit (Gene Therapy Systems)

2.1.5 Bacterial strains

2.1.5.1 E.coli One Shot® TOP10

Chemically Competent *E.coli* Cells (>1 x 10^9 cfu/μg) (Invitrogen). Genotype: F *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80 *lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

2.1.5.2 E.coli JM109

High Efficiency JM109 Competent Cells (>10^8 cfu/μg) (Promega). Genotype: *end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 (r_k^-, m_k^+) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F′, *tra*D36 *pro*AB *laq*I^qZΔM15]

2.1.5.3 XL1-Blue Supercompetent cells

(>1 x 10^9 cfu/μg) (Stratgene, The Netherlands). Genotype: *rec*A1 *end*A1 *gry*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F′, *pro*AB *laq*I^qZΔM15Tn 10 (Tet^r)]

2.1.6 DNA

Plasmid, molecular weight markers and oligonucleotide DNAs were stored at -20°C.

2.1.6.1 Plasmid vectors

2.1.6.1.1 pCR® 2.1-TOPO®

pCR®2.1-TOPO® (3900 bp) is supplied by Invitrogen and contains *lacZ*$^+$, Beta-lactamase gene (*amp*^{r}) and Kanamycin resistance (*Kan*^{r}) genes. T7 promoter and M13 forward and reverse primer binding sites are also present. This plasmid is designed for directly cloning PCR products with 3′ deoxyadenosine residues (A overhangs), generated by the non-template dependent activity of *Taq*

polymerase. The vector is supplied as linearised DNA with single 3′ deoxythymidine (T) residues and topoisomerase attached, allowing for efficient ligation of the target sequence to the vector.

2.1.6.1.2 pTarget™ Mammalian Expression Vector

pTarget™ Mammalian Expression Vector (5670 bp) is supplied by Promega and contains a T7 promoter, CMV immediate early promoter and enhancer sequences and SV40 late polyadenylation signal. A neomycin selectable marker (*Neo*^{R}), *lac*Zα, Beta-lactamase gene (*amp*^{r}) and T7 & pTarget primer binding sites are also present. A multiple cloning site (MCS) is available in addition to single 3′ deoxythymidine (T) for TA cloning.

2.1.6.1.3 pGL3-basic Vector

pGL3-basic Vector (4818 bp), supplied by Promega, lacks a eukaryotic promoter and enhancer sequences, allowing cloning of putative regulatory sequences into the MCS. The coding region for firefly (*Photinus pyralis*) luciferase gene (*luc*+) is followed by a SV40 poly(A) signal. This vector also contains Beta-lactamase gene (*amp*^{r}) and RV3, RV4 and GL2 primer binding sites.

2.1.6.1.4 pGL3-control Vector

pGL3-control Vector (5249 bp), supplied by Promega, contains a SV40 promoter, enhancer, late and up-stream poly(A) sequences resulting in strong expression of firefly (*Photinus pyralis*) luciferase gene (*luc*+) in many types of mammalian cells. Beta-lactamase gene (*amp*^{r}) and RV3, RV4 and GL2 primer binding sites are also present.

2.1.6.1.5 pRL-CMV Vector(f)

pRL-CMV Vector(f) (4079 bp), supplied by Promega, contains the CMV immediate-early enhancer/promoter region providing strong constitutive expression of Renilla (*Renilla reniformis*) [sea pansy] luciferase in a variety of cell types. The Beta-lactamase gene (*amp*^{r}) and SV40 late polyadenylation signal is also present.

2.1.6.1.6 pCAGGS-nlsCre

pCAGGS-nlsCre (5810 bp), supplied by Prof. Jun-ichi Miyazaki of Osaka University Medical Schoolin Japan, contains the CMV-immediate early/chicken β-Actin fusion promoter driving expression of nuclear localisation signal/Cre recombinase fusion protein. Polyadenylation signal and the Beta-lactamase gene (*amp*^{r}) are also present.

2.1.6.1.7 pUC18

pUC18 (Invitrogen): pBR322 based cloning vector contains the *lacZ*$^{+}$ and *amp*^{r} genes with a MCS and M13 forward and reverse primer annealing sites. This plasmid is supplied with TA cloning kit for use as positive control for verifying the transformation efficiency of competent bacteria at a concentration of 0.1 µg/ml.

2.1.6.2 Molecular Size Standards

All markers were supplied by GIBCOBRL Life Technologies and include 1 kb DNA Ladder (size range: 75- 12,216 bp), 1 Kb Plus DNA Ladder™ (size range: 100- 12.000 bp), 100 bp DNA Ladder (size range: 100-2072 bp), Low DNA Mass™ Ladder (size range: 100-2000 bp and 5-400 ng).

2.1.6.3 Oligonucleotide primers

Oligonucleotides for use in polymerase chain reactions (PCR) and cycle sequencing were synthesised by MWG Biotech and Sigma-Genosys. Primers were either reverse phase, desalted or Poly Acrylamide Gel electrophoresis (PAGE) purified and supplied as lyophilised DNA. Primers were reconstituted in dH_2O and stored at –20°C in 20 µl aliquots at 10µM.

2.1.7 Enzymes

All enzymes were stored at –20°C, being removed immediately before use.

2.1.7.1 *Restriction enzymes*

All restriction enzymes and their associated buffers were supplied by Promega, Kramel and Clontech.

Table 2-1 Restriction enzymes

Restriction enzyme	Restriction site	Buffer	Incubation Temp (°C)
*Eco*R 1	[G$^{\downarrow}$AATTC]	Buffer 6 (blue)	37
Stu 1	[AGG$^{\downarrow}$CCT]	Buffer B	37
Dra 1	[TTT$^{\downarrow}$AAA]	Buffer B	37
Pvu II	[CAG$^{\downarrow}$CTG]	Buffer B	37
*Eco*R V	[GAT$^{\downarrow}$ATC]	Multi-Core™	37
Xho 1	[C$^{\downarrow}$TCGAG]	Buffer 6 (blue)	37
Kpn 1	[GGTAC$^{\downarrow}$C]	Multi-Core™	37
Sma 1	[CCC$^{\downarrow}$GGG]	Multi-Core™	30
Hind II	[A$^{\downarrow}$AGCTT]	Buffer E	37
Xba 1	[T$^{\downarrow}$CTAGA]	Multi-Core™	37
Nsi 1	[ATGAA$^{\downarrow}$T]	Buffer D	37

2.1.7.2 T4 DNA Ligase

T4 DNA ligase was provided by Promega with ligation buffer (used at a final concentration of 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP) to catalyse the joining of two

strands of DNA between the 5′-phosphate and the 3′- hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration.

2.1.7.3 Taq DNA polymerase

Taq DNA polymerase was provided by QIAGEN and has 5' to 3' exonuclease activity and non-template dependant activity with the addition of 3′ deoxyadenosine (A) residues to PCR products for TA cloning.

2.1.7.4 Platinum Pfx DNA Polymerase

Platinum *Pfx* DNA polymerase was provided by Invitrogen and has proofreading 3′ to 5′ exonuclease activity with high fidelity. This enzyme is provided in an inactive form with a specifically bound antibody to provide an automatic 'hot-start' for PCR, enabling increased specificity, sensitivity, and yield. Full activity of the enzyme is re-gained on denaturation at 94°C. PCR Enhancer Solution is provided with this enzyme as a novel PCR co-solvent to facilitate efficient amplification of GC sequences by offering higher primer specificity, broader magnesium concentrations and annealing temperatures and improved thermo-stabalisation of DNA polymerases.

2.1.7.5 Advantage® Genomic Polymerase Mix

Advantage® Genomic Polymerase was provided by Clontech and provides efficient and accurate amplification of genomic DNA templates using a combination of the primary *Tth* DNA polymerase (thermostable DNA polymerase from *Thermus thermophilus*), a minor amount of a second DNA polymerase to provide 3′ to 5′ proof reading activity ($Vent_R$) and TthStart™ Antibody to provide automatic 'hot-start' PCR (Kellogg *et al.*, 1994). This antibody reduces or eliminates non-specific amplification products and primer-dimer artifacts created prior to the onset of thermal cycling. The antibody inhibits enzymatic activity during PCR reaction set-up at ambient temperatures. Polymerase activity is restored at the onset of thermo-cycling because the antibody is denatured at high temperatures. The loss of inhibition is complete and irreversible, so the polymerase regains full activity for PCR.

2.1.7.6 Ready-To-Go™ PCR beads (Amersham, Pharmacia)

Ready-To-Go™PCR beads (Amersham, Pharmacia) are designed as pre-mixed, pre-dispensed reactions for performing PCR amplifications. When brought to a final volume of 25 µl, each reaction contains *Taq* DNA Polymerase (1.5 units), 10 mM Tris-HCL, (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200µM of each dNTP and Bovine Serum Albumin (BSA).

2.1.7.7 Murine Moloney Virus Reverse Transcriptase Enzyme

Murine Moloney Leukaemia Virus Reverse Transcriptase (MMLV-RT) enzyme, provided by GIBCOBRL, uses single stranded RNA in the presence of a primer to synthesise a complementary

high temperatures. The loss of inhibition is complete and irreversible, so the polymerase regains full activity for PCR.

2.1.7.6 Ready-To-Go™ PCR beads (Amersham, Pharmacia)

Ready-To-Go™PCR beads (Amersham, Pharmacia) are designed as pre-mixed, pre-dispensed reactions for performing PCR amplifications. When brought to a final volume of 25 µl, each reaction contains *Taq* DNA Polymerase (1.5 units), 10 mM Tris-HCL, (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200µM of each dNTP and Bovine Serum Albumin (BSA).

2.1.7.7 Murine Moloney Virus Reverse Transcriptase Enzyme

Murine Moloney Leukaemia Virus Reverse Transcriptase (MMLV-RT) enzyme, provided by GIBCOBRL, uses single stranded RNA in the presence of a primer to synthesise a complementary DNA strand. This enzyme is isolated from *E.Coli* expressing a portion of the *pol* gene of the M-MLV on a plasmid.

2.1.7.8 Avian Myeloblastosis Virus Reverse Transcriptase (AML-RT)

Avian Myeloblastosis Virus Reverse Transcriptase functions in a similar manner to MMLV-RT.

2.1.7.9 RNaseOUT™ Recombinant Ribonuclease Inhibitor

RNaseOUT™ Recombinant Ribonuclease Inhibitor, provided by Invitrogen, is an acidic protein with a very high binding affinity for ribonucleases such as RNase A, B, C and is a non-competitive inhibitor.

2.1.7.10 Alkaline Phosphatase,

Calf Intestinal alkaline phosphatase provided by Promega catalyses the hydrolysis of 5′- phosphate groups from DNA.

2.1.7.11 DNase I: DNA-free™

DNase I provided by Ambion is a deoxyribonuclease I enzyme that cleaves double-stranded or single stranded DNA. Cleavage preferentially occurs adjacent to pyrimidine (C or T) residues, and the enzyme is therefore an endonuclease. Major products are 5'-phosphorylated di, tri and tetranucleotides. In the presence of magnesium ions, DNase I hydrolyzes each strand of duplex DNA independently, generating random cleavages. In the presence of manganese ions, the enzyme cleaves both strands of DNA at approximately the same site, producing blunt ends or fragments with 1-2 base overhangs. DNase I does not cleave RNA.

2.1.8 Protein SDS-PAGE standards

Wide Range (6.5 to 205 kDa) Color Markers molecular weight standard (Sigma).

2.1.9 Equipment

2.1.9.1 Major Equipment

Benchtop centrifuge: CPR Centrifuge (Beckman)

Microcentrifuges: Centrifuge 5402, 5415R and Minispin (eppendorf)

Water baths: Sub 36, and W6 (Grant)

Spectrophotometer: GeneQuant *pro* RNA/DNA calculator

Automatic Sequencing Apparatus: ABI 3100

Gel dryer: Model 583 Gel Dryer (Biorad)

Pipettes: Finnipipette Techpette (0.5-10, 5-40, 40-200, 200-1000 µl)

Ultraviolet trans-illuminator: T2201 (Sigma Chemical Company)

Gel documentation system: Uvi tec (Thistle Scientific)

Luminometer: Dynex MLX

Horizontal orbital shaker: 4628-1CE Labline Instruments inc (IL)

Incubator: B5042 (Heraeus)

Gel systems: Hoefer HE 33 Mini Horizontal Submarine Unit

Power packs: PAB 35-0.2 (Kikusui electronics corp)

Balance: Precisa 100A-300M (Precisa Balances Ltd, Bucks, UK)

Stirrer: Magnetic Stirrer Hotplate (Stuart Scientific, UK)

PCR Machines: GeneAmp PCR System 2400, 2700 and DNA Thermal Cycler 480 (Perkin Elmer)

2.1.9.2 Consumables

Syringe top filters (0.2µm pore size) were supplied by Nalgene™, (NY, USA) for sterilisation of ampicillin and tissue culture reagents.

Ependorf tubes: screw top 1.5ml and 0.5ml and 1.5ml flip top tubes were supplied by Thermo Life Sciences.

Pipette tips were supplied by Greiner.

Filter tip pipette tips (10, 100, 200, 1000µl) were supplied by Finnitip (Thermo Lab Systems)

Petri dishes were supplied by Sterilin (Staffs, UK)

Bijoux were supplied by Greiner

Universals were supplied by Greiner

Scalpel blades were supplied by Swan-Morton (Sheffield)

Parafilm was supplied by Sigma

2.1.10 Buffers, solutions and growth media

2.1.10.1 Water

Sterile water for procedures involving recombinant DNA, PCR etc was supplied by Baxter. Vivendi Water systems (USF ELGA) was used to supply water for preparation of general solutions and media.

2.1.10.2 Antibiotics

Ampicillin: Reconstituted with dH_2O to 10mg/ml, filter sterilised and stored in 1 ml aliquots at –20°C until use.

2.1.10.3 Buffers and solutions

Ammonium persulphate: 10% (w/v) stock solution in dH_2O

50 x TAE Buffer Solution: Tris base 484.5g, NaOAc 272.15 g, NaCl 116.8 g, Na_2EDTA 74.45 g. pH adjusted to 8.15 with glacial acetic acid and made up to 2 L volume.

TBE Buffer Solution: 0.09M Tris Borate, and 0.002M EDTA

Lysis Buffer: 25mM Tris HCL pH 8.0 with glacial acetic acid and made up to 2 L volume

1M Tris HCL: 121g Tris base, 800ml dH_2O. Adjusted to the desired pH with concentrated HCL and made up to 1L.

TE Buffer: 10mM Tris-HCL (pH 8.0), 1mM EDTA

1 x PBS: 140mM NaCl, 2.7mM KCl., 10mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3)

0.2M (2x) Phosphate Buffered Saline (PBS): 0.2M Na_2HPO_4, 0.2M NaH_2PO4, 1.8% w/v NaCl, pH 7.4

10x DNA Gel Loading Buffer: 20% w/v Ficoll 400, 0.1M Na_2EDTA, pH 8, 1.0% w/v sodium dodecyl sulphate, 0.25% bromophenol blue, 0.25% xylene cyanol. Stored at room temperature and used at a 1:10 dilution.

Ethidium bromide: made to a working dilution of 10mg/ml with dH_2O in a fume cupboard. Stored away from light.

X-Gal solution: (5-bromo-4-chloro-3-indole-b-galactoside) prepared as 50 mg/ml stock in dimethylformamide; stored at –20°C in the dark.

Transfast™ Transfection Reagent: (Promega) Supplied as dried lipid film that forms multi-lamellar vesicles upon hydration with nuclease-free water (400µl) and stored at –20°C.

DEPC water: 0.5ml of Diethylpyrocarbonate in 500ml water, overnight at room temperature in the fume cupboard and autoclaved.

Lysis mix: 0.32M sucrose, 10mM Tris, 5mM MgCl, 1% Triton X

Nuclei lysis mix: 10mM Tris, 0.4 NaCl, 2mM EDTA

TRIS-EDTA: (10/1 pH 7.4)

TRIS-EDTA: (10/0.1 pH7.5)

10 x SDS PAGE Electrode (Running) Buffer: tris base 60g, glycine 288g, SDS 20g. Made up to 2L by addition of dH_2O

Transfer Buffer: 1.93 g Tris, 9 g glycine. Made up to 1L by addition of dH_2O

10x Ponceau's Red Stain: 2 g Ponceau S, 30 g Trichloroacetic acid (TCA), 30 g Sulphopalicylic acid. Made up to 100 ml by addition of dH_2O

TBS-Tween: 150 mM NaCl (8.77 g). 50 mM Tris (6.06 g) and 0.05% Tween (0.5 ml). Made up to 1L by addition of dH_2O

Blocking Buffer: 1 g non-fat skimmed milk powder in 20 ml TBS-Tween

Antibody diluant: 0.2 g non-fat skimmed milk powder in 20 ml TBS-Tween.

SDS Protein sample loading buffer: 95% loading buffer (Biorad pre-made) and 5% 2-mercaptoethanol added immediately prior to use.

Gel Fixing solution for protein assays: Methanol (50 %), glacial acetic acid (10 %) and H_2O (40%)

Cell fixing solution for X-Gal assay: 4% w/v paraformaldehyde, 0.1M PBS, 0.12M sucrose, pH7.4. Dissolve sucrose (4.1g) in 50 ml of 0.2 M PBS pH 7.4, chill, heat 30 ml of dH_20 (55°C to 60°C) and add approximately 5 drops of 10 M NaOH. Dissolve 4g of paraformaldehyde and filter through a Whatman qualitative size filter paper circle into ice cold PBS sucrose. Adjust pH to 7.4 and make to 100 ml.

β-gal assay solution: potassium ferrocyanide (5mM), potassium ferricyanide (5mM), $MgCl_2$ (2mM), X-gal (1 mg/ml). The potassium ferrocyanide and potassium ferricyanide was prepared at 100 mM in PBS and could be stored at 4°C for up to a week. The x-gal was dissolved in dimethyl sulphoxide (DMSO) fresh when required. The x-gal was added to the assay solution prior to the assay.

2.1.10.4 Bacteriological Media

Media was sterilised by autoclaving at 121°C for 15 minutes, unless stated otherwise

LB Medium: 20g tryptone, 20g NaCl, 10g Yeast Extract to 2L with dH2O, pH adjusted to 7.0 with NaOH.

SOC Medium: 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.

2.2 METHODS

Common methods used throughout the thesis are described in this chapter, whilst techniques specific to one area are described in later chapters. Many of the methods described are based on standard techniques, which are detailed in Current Protocols in Molecular Biology Volumes 1 & 2 (Ausubel et al. 1994).

2.2.1 Growth and manipulation of mammalian cells

2.2.1.1 Basic technique

All procedures involving manipulation of mammalian cells were carried out using standard aseptic procedures. Where possible all procedures were performed in a laminar flow hood.

2.2.1.1.1 Cryopreservation of cells

In order to preserve stocks of cell lines for long term use, cells were stored over liquid nitrogen. Cells to be frozen were grown to mid-log phase (as described below) and removed into a sterile 50 ml centrifuge tube (using trypsin-EDTA as necessary). The cells were centrifuged at 1200g for 2 minutes, the supernatant discarded and cells re-suspended in freezing medium (appropriate culture medium supplemented with DMSO at 5%) to a concentration of approximately 2 x 10^6 cells/ml. The cell suspension was transferred in 1 ml aliquots, to labelled cryovials (NALGENE™) and brought to -70°C at a controlled rate (-1°C/min) using NALGENE™ Cryo 1°C Freezing Container (NALGENE, USA). The vials were then transferred to a liquid nitrogen freezer. Cell stocks were revived by rapid thawing in a 37°C water bath and subsequently used following standard techniques as described below.

2.2.1.1.2 Cell Counting

Cells were counted using a haemocytometer as follows. Cells were diluted ten fold in media and introduced to the haemocytometer chamber and counted under an inverted microscope with 4 x 10 objective. Cells lying on the top and right hand perimeter of each large (1 ml) square were included, those on the bottom and left hand perimeter were excluded. Cell concentrations (cells/ml) were calculated by multiplying the mean number of cells per large square by 10^4 and 10 to correct for the dilution factor. Where an estimation of live cell numbers was required 0.4% trypan blue (Sigma) was used to dilute the cell suspension and allowed to incubate for five minutes at room temperature prior to counting; dead cells take up the stain and therefore appear blue.

2.2.1.2 Cell lines

2.2.1.2.1 Madin Darby canine kidney (MDCK) cells

The MDCK cell line was derived from a kidney of an apparently normal female cocker spaniel in September 1958 by S.H. Madin and N.B. Darby. The cells grow as an adherent monolayer in

culture and were cultured in 20-30 ml Dulbecco's MEM with gluatamax-1 medium supplemented with 10% FCS and ciprofloxacin at 10µg/ml, 100 IU/ml penicillin and 100µg streptomycin and fungizone in 75 cm^2 tissue culture flasks at 37°C, 5% CO_2. Cultures were split, typically 1:9 every three and a half days when sub-confluent and were trypsinised and seeded into new flasks as described above.

2.2.1.2.2 Feline embryonic fibroblasts (FEA)

The FEA cell line is a fibroblast cell line derived from whole feline embryos (Jarrett et al., 1973). The cells grow as an adherent monolayer in culture and were cultured in 20-30 ml Dulbecco's MEM containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin and 100µg streptomycin in 75 cm^2 tissue culture flasks at 37°C. 5% CO_2. Cultures were split, typically 1:3 to 1:4 every three to four days, when sub-confluent. The medium was decanted from the cell monolayer; the cells washed twice with Hanks medium, then incubated in trypsin-EDTA (5ml) at 37°C for approximately five minutes. The detached cells were then washed in fresh medium and pelleted by centrifugation at 1200 rpm for 2 minutes, prior to re-suspending in fresh medium and seeding new tissue culture flasks.

2.2.1.2.3 Primary rat cardiocytes

The primary rat cardiocytes (Iwaki, Sukhatme et al., 1990), a gift from Dr. Arvind Sood, were provided pre-plated on 96 well plates. Cells were maintained in a mix of Dulbecco's modified Eagle's medium and medium 199 (at a ratio of 4:1) supplemented with 100units/ml of penicillin and streptomycin, 4% horse serum and 5% FCS at 37°C, 5% CO_2.

2.2.1.2.4 Human chondrosarcoma cells (SW1353)

The SW 1353 cell line was initiated by A. Leibovitz at the Scott and White Clinic, Texas in 1977 from a primary grade II chondrosarcoma of the right humerus obtained from a 72 year old female Caucasian. Stock cultures of cells were cultured in Dulbecco's MEM/F-12 supplemented with 10% foetal calf serum, 2mM Glutamine, 1x non-essential amino acids, 100 units/ml of penicillin and streptomycin, 20 U/ml nystatin in 75 cm^2 tissue culture flasks at 37°C, 5% CO_2. The cells grow as an adherent monolayer in culture and were split typically 1:4 when sub-confluent every 3.5 days and were trypsinised and seeded into new flasks as described above.

2.2.1.2.5 Mouse undifferentiated chondroprogenitor cells (ATDC5)

The ATDC5 cell line was isolated from a differentiating culture of murine AT805 teratocarcinoma expressing a fibroblastic cell phenotype (Atsumi et al., 1990). Cells were maintained in Dulbecco's MEM/F-12 supplemented with 5% FCS, 3x 10^8 M sodium selenite, 10µg/ml human apo-transferrin, 10µg/ml insulin in 75 cm^2 tissue culture flasks at 37°C, 5% CO_2. The cells grow as an adherent monolayer in culture and were split typically 1:9 when sub-confluent every 3.5 days and were trypsinised and seeded into new flasks as described above.

2.2.1.2.6 Rabbit synovial fibroblasts (HIG-82)

The HIG-82 synovial fibroblast cell line was derived from the intra-articular soft tissue from the knee joint of a young female rabbit (Georgescu et al., 1988). The cells grow as an adherent monolayer in culture and were cultured in 20-30 ml Ham's F-12 medium supplemented with 10% FCS and 100 IU/ml penicillin and 100µg streptomycin in 75 cm^2 tissue culture flasks at 37°C, 5% CO_2. Cultures were split, typically 1:4 every three and a half days, when sub-confluent and were trypsinised and seeded into new flasks as described above.

2.2.2 Recombinant DNA techniques

2.2.2.1 Storage and growth of bacteria

Plasmids were maintained in *E.coli* strains One Shot® TOP10 and JM109. To enable storage of these *E.coli* host strains and of transformants obtained during this work, glycerol stocks were prepared. The desired bacterial culture was streaked onto a 1.5% agar plate (1.5% agar in LB medium); in all cases where the bacterial stock contained a plasmid conferring ampicillin resistance the medium was supplemented with 50-100µg/ml ampicillin. The plate was incubated overnight at 37°C and the following day single colonies were picked using a pipette tip into 3-4 ml LB medium (supplemented with 50-100µg/ml ampicillin) in a sterile universal. The cultures were incubated at 37°C overnight in a horizontal orbital incubator (225 rpm). Confirmation that the overnight culture was derived from bacteria containing the desired recombinant plasmid was achieved by DNA extraction (see 2.2.2.2.1) and restriction digestion (see 2.2.2.4). Glycerol stocks (15%) were prepared by the addition of 90µl of glycerol (50%) to 210µl of culture and were stored at -20°C and -70°C. Bacterial stocks were revived for subsequent work by using a sterile pipette tip to scratch the surface of the stock, following which it was streaked onto an agar plate as outlined above.

2.2.2.2 Extraction and purification of plasmid DNA

Plasmid DNA was isolated using a modification of the alkali lysis technique (Birnboim and Doly, 1979).

2.2.2.2.1 Large Scale Plasmid Preparations

Large quantities of high quality endotoxin-free plasmid DNA, for sequencing purposes or transfections, were purified from 150ml of an exponentially growing overnight culture of recombinant bacteria using the EndoFree® Plasmid Maxi Kit (QIAGEN, UK). An overnight culture (3.5ml) of the desired transformant was grown and used to seed a 250 ml culture, which was then grown overnight at 37°C, with shaking. The remainder of the protocol was performed according to the manufacturer's instructions. DNA was stored at -20°C.

2.2.2.2.2 Small Scale Plasmid Preparations

Smaller quantities of high quality plasmid DNA, for molecular manipulation or sequencing purposes was purified from 4ml of an exponentially growing overnight culture of recombinant bacteria using the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK). The culture was spun at 3000 rpm for 10 minutes and the supernatant removed. DNA was purified from the cell pellet according to the manufacturer's instructions. In this procedure the cells were lysed to release the DNA constructs which were then separated by centrifugation and attached to a filter and washed with a buffer before being eluted in sterile water (50µl). DNA was stored at -20°C.

2.2.2.3 Determination of (ribo)nucleic acid concentration

2.2.2.3.1 Determination by spectrophotometry

(Ribo)nucleic acid samples were diluted 1:20 by addition of 5 µl of (ribo)nucleic acid to 95 µl of dH_2O. The optical density was measured at 260 nm and 280 nm, in comparison to a blank of dH_2O. An optical density (OD) reading of 1.0 at 260 nm corresponds to an approximate (ribo)nucleic acid concentration of 50 µg/ml for double stranded DNA, 40 µg/ml for RNA, or 33 µg/ml for single stranded oligonucleotides. The ratio of the OD readings at 260 nm and 280 nm (OD_{260}/OD_{280}) was used to estimate the purity of the (ribo)nucleic acid. Pure preparations of DNA and RNA have an OD_{260}/OD_{280} of 1.8 and 2.0, respectively; a lower value suggests possible protein or phenol contamination.

2.2.2.3.2 Estimation of double stranded DNA concentration via gel electrophoresis

In cases where there is insufficient sample to permit quantification via spectrophotometry or where it was desired to verify the purity of DNA fragments of a certain size the concentration of dsDNA was determined by running the sample on an agarose gel (see 2.2.2.5). The intensity of the fluorescence of the unknown DNA was compared to that of a known quantity of the appropriate size marker (Low DNA Mass™ Ladder) following staining with ethidium bromide and visualisation by UV transillumination.

2.2.2.4 Restriction endonuclease digestion

Typically, 1-2 µg of DNA were digested in a 20 µl reaction mix containing the appropriate buffer and 5 to 10 units of desired restriction enzyme. The reactions were incubated at the recommended temperature (typically 37°C) for a minimum of one hour. Where the isolation of restriction fragments was required, larger quantities of DNA, generally 5-10 µg, were digested, with the reaction volume and components being increased proportionally.

Recombinant plasmids contain a variety of restriction enzyme sites. One or more appropriate restriction enzymes can be incubated with DNA constructs at 37°C in the correct buffer, as determined by the manufacturer's recommendations, to cut at specific positions and produce

fragments of different sizes. The sizes of these fragments could be pre-determined and then viewed by gel electrophoresis.

2.2.2.5 Electrophoresis of DNA

DNA fragments of 0.1-10 kb were separated and identified by agarose gel electrophoresis using a Hoefer HE 33 Mini Submarine Electrophoresis Unit (Amersham Pharmacia Biotech, San Francisco, CA). Typically, 0.5 – 0.1 g of agarose was added to 50 ml of 0.5x TAE buffer, melted in the microwave (medium for 1.5 minutes), and mixed to produce a 1 to 2% gel. Once the gel mix had cooled to 55°C, 1.5µl of ethidium bromide (100mg/ml) was added and the gel poured into a gel support (100 x 65 mm) in its casting tray and an appropriate gel comb (twelve or twenty well) inserted. The gel was allowed to solidify before transferring to an electrophoresis tank; the gel was immersed 0.5x TAE buffer and the comb carefully removed. DNA samples were prepared by the addition of an appropriate volume of 10x gel loading buffer. Molecular size standard DNA was prepared similarly and the samples loaded into the wells using a micropipette. Gels were run at 100 volts for 20 to 30 minutes depending on the agarose percentage, then removed from the gel apparatus and visualised on a UV transilluminator (UVi tec, Thistle Scientific) and photographed using a Mitsubishi P91 photographic unit.

2.2.2.6 Purification of restriction enzyme fragments

Where purification of DNA fragments was required for construction of recombinant plasmids, DNA was purified from agarose gels using the QIAquick® Gel Extraction Kit (QIAGEN). Following electrophoresis, DNA fragments of interest were excised from agarose gels using a clean scalpel and the remainder of the procedure was performed according to the manufacturer's instructions.

2.2.2.7. Dephosphorylation

Vector DNA was linearised using an appropriate restriction enzyme(s). However, when cut with one enzyme, dephosphorylation steps were required to prevent re-circularisation of the vector. Both 5′ phosphate groups were hydrolysed with 0.05 units of calf intestinal alkaline phosphatase (CIAP, Promega) with 50 mM Tris-HCl (pH 9.3), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine in a total volume of 50 µl at 37°C for 2-3 hours.

2.2.2.8 Ligation of vector and insert DNA

2.2.2.8.1 Ligation of restriction digested DNA fragments

Fragments of DNA generated by restriction digestion were ligated into approximately 50-100 ng of vector using T4 DNA ligase (Promega) according to the manufacturer's instructions. Quantities of vector and insert DNA were calculated using a molar ratio in the range of 1:1 to 1:5 in the following equation:

$$\frac{\text{X ng vector x Y kb insert}}{\text{Z kb vector}} \times \text{insert : vector molar ratio} = \text{ng of insert required}$$

Vector and insert were mixed with ligation buffer (1x) and DNA ligase (1 unit), in a total volume of 10 µl. Ligation reactions were allowed to proceed at 16°C overnight.

2.2.2.8.2 Ligation using TA cloning methods

Ligations of DNA fragments generated by *Taq* Polymerase during PCR were carried out according to the manufacturer's instructions with the pCR2.1®TOPO TA Cloning Kit (Invitrogen). Briefly, freshly purified PCR products (15 ng) were directly cloned into the pCR®2.1-TOPO plasmid vector (0.5µl), incubated for 5 minutes at room temperature. Topoisomerase 1 is covalently bound to the ends of the linearised vector to facilitate the cloning procedure.

All ligations were stored at -20°C if not used immediately. A negative control ligation, omitting the DNA insert, was generally set up in parallel to the above, in order to check for 'background' when performing subsequent transformations.

2.2.2.9 Transformation of bacteria with plasmid DNA

In addition to the transformation with recombinant plasmid, the bacteria were also transformed with a control plasmid (as a positive control) and a ligation reaction from which the insert DNA had been omitted (as a negative control).

2.2.2.9.1 Transformation of TOP10 cells

Cells were thawed on wet ice, gently mixed and 25-50 µl of cells were transferred to chilled microcentrifuge tubes for each transformation as required. Ligation reaction (0.5-5µl) or control plasmid (pUC18) was added to cells (1-20 ng DNA), moving the pipette through the cells while dispensing in order to facilitate mixing. Cells were left on ice for 30 minutes, then heat shocked in a 42°C water bath for 30 seconds. After heat shocking, the cells were placed on ice for 2 minutes, 200 µl of SOC medium was added and the tubes were incubated at 37°C for 1 hour with shaking at 225 rpm. Variable volumes of cells were plated onto LB plates containing ampicillin (100 mg/ml) and X-Gal stock (35 µl) solution was spread on the plate one hour prior to use if blue-white colour selection was used and all plates incubated overnight at 37°C.

2.2.2.9.2 Transformation of JM109 cells

The transformation procedure for these cells was essentially as described for TOP10 cells with minor modifications. Cells were left on ice for 20 minutes before being heat shocked in a 42°C water bath for 45 seconds. A larger volume of SOC medium (450 µl) was necessary for cell recovery.

2.2.2.9.3 Transformation of XL1-Blue Supercompetent cells

The transformation procedure for these cells was essentially as described for TOP10 cells with minor modifications. Cells heat shocked in a water bath at 42°C for 45 seconds and SOC medium (500 µl) was added for cell recovery.

2.2.2.10 Screening of transformants for desired recombinant plasmids

Most plasmids used in this project conferred ampicillin resistance upon the host bacteria, allowing selection and maintenance of transformed bacteria with ampicillin supplemented media.

2.2.2.10.1 α- complementation

The pCR2.1®TOPO plasmids contain genes encoding the *lac*Zα fragment of the β-galactosidase and the *lac* promoter and are therefore capable of α-complementation with the φ fragment encoded by the *E.coli* host strains DH5α, JM109 and TOP10 One Shot™ giving active β-galactosidase. The incorporation of X-gal into LB agar plates allows the selection of transformants based on blue-white screening. Disruption of the *lac*Zα expression occurs with the cloning of fragments into the MCS of the vector, hence recombinants with plasmid containing insert DNA appear white whilst non-recombinants, expressing a functional β-galactosidase, appear blue.

2.2.2.10.2 Restriction analysis of small-scale plasmid preparations

Plasmid DNA, isolated as described in 2.2.2.2. was subjected to restriction digestion with the appropriate enzyme(s) (2.2.2.4), and the resulting products of digestion were run on an agarose gel. Bacteria with plasmids containing inserts of the desired size were stored as glycerol stocks as detailed in section 2.2.2.1.

2.2.2.10.3 PCR amplification of broth or small-scale plasmid preparations

A sample of bacterial culture (50 µl) was removed and heated at 95°C for 6 minutes and centrifuged at 12,000 rpm for 6 minutes. Aliquots of the supernatants (10µl) were then used as template in PCR reactions.

2.2.3 Preparation of total RNA

Clean, full-length RNA is essential as the starting material for molecular techniques, hence when isolating the RNA it was necessary to ensure that all the preparation material is free from ribonuclease (RNAse) activity. This is a very stable, active and ubiquitous enzyme that degrades RNA requiring no cofactors for function. Inhibition of this enzyme was instigated by wiping all equipment with RNase ERASE (ICN Biomedicals, Inc, Ohio) followed by a thorough rinse with DEPC treated water. All plastic-ware was treated by soaking overnight in DEPC treated water followed by autoclaving and drying. All solutions were prepared using DEPC treated water. Gloves were worn and changed frequently.

2.2.3.2 RNA extraction using RNAzol™ B solution

Various methods have been used for the isolation of undegraded RNA (Chirgwin et al., 1979; Cox, 1968). Progress in this field has made it possible to convert RNA isolation into a single-step procedure (Chomczynski and Sacchi 1987). The RNAzol™ B methods promote formation of complexes of RNA with guanidinium and water molecules and abolishes hydrophilic interactions of DNA and proteins. In effect, the DNA and proteins are efficiently removed from the aqueous phase with the RNA remaining in this phase during the sample extraction.

RNAzol™ B solution (AMS Biotechnology) (25ml) was added directly to the cell monolayer and the RNA solubilised by passing the lysate a few times through the pipette. Aliquots of the homogenate and chloroform in a 10:1 ratio were shaken for 15 seconds and then placed on ice for 5 minutes and centrifuged for 15 minutes at 2400 rpm and 4°C. At this point the homogenate formed two phases: the lower blue phenol-chloroform phase and the colourless upper aqueous phase; whereas DNA and proteins were in the interphase and organic phase. The upper aqueous phase was transferred to a clean ependorf tube containing an equal volume of isopropanol (750µl) and chilled at 4°C for 15 minutes. The samples were centrifuged again for 15 minutes at 13,000 rpm and 4°C, the supernatant discarded and the RNA pellet washed once with 75% ethanol by vortexing followed by a final centrifuge step for 8 minutes at 13,000 rpm and 4°C. The RNA samples were stored in 70% ethanol at -20°C until required. When required for further applications the RNA was centrifuged for 8 minutes at 13,000 rpm and 4°C, the supernatant removed and the RNA pellet dried at room temperature for 2 minutes (it is important not to let the RNA pellet dry completely as it will greatly decrease its solubility). The RNA was then re-suspended in 40 µl of DEPC water.

2.2.3.3 Assessment of RNA using agarose gel electrophoresis

RNA samples were assessed by gel electrophoresis on a 1% agarose TAE gel by comparing the bands to a 100 bp molecular weight standard (GIBCO BRL). The quality of the RNA samples were assessed by examining the integrity of the 18S and 28S ribosomal RNA bands which migrated through the gel at a rate corresponding to their predicted molecular weights.

2.2.3.4 DNase Treatment of RNA

Contaminating DNA was removed from RNA samples using DNA-free™ (Ambion) methods. Briefly, 0.1 volume of 10x DNase I (final concentration of: 10 mM Tris-HCl pH7.5, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$) and 2 units of DNase I were added directly to the RNA (35µl) samples, mixed and incubated at 37°C for variable lengths of time (generally > 1hr). The enzyme reaction was stopped by the addition of DNase Inactivation Reagent (5µl) for two minutes at room temperature. Samples were then centrifuged at 10,000g for one minute to pellet the Inactivation Reagent and the supernatant containing the pure RNA was then transferred to a clean ependorf tube.

2.2.4 First strand cDNA synthesis

The synthesis of complementary DNA (cDNA) requires the enzyme 'reverse transcriptase', initially identified as the enzyme involved in the formation of DNA-RNA hybrids in retrovirus replication. Reverse transcriptase synthesises a DNA strand complementary to an RNA template when provided with a primer that is base-paired to the RNA, containing a free 3′-OH group. Random primers, gene specific primers (GSP) or oligo-dT primers (the latter pairs with the poly-A sequence at the 3′ end of most eukaryotic mRNA molecules) can be used to instigate the synthesis of cDNA strands in the presence of the four dNTPs. The RNA-DNA hybrid is subsequently hydrolysed by raising the pH (unlike RNA, DNA is resistant to alkaline hydrolysis) or using a ribonuclease and the 3′- end of the newly-synthesised DNA strand forms a hairpin loop priming the synthesis of the opposite DNA strand. The hairpin loop is removed by digestion with S1 nuclease, which recognises unpaired nucleotides.

More specifically, total RNA samples in 9µl of DEPC water were denatured to remove any RNA secondary structure for 5 minutes at 65°C and then quenched on ice. First strand synthesis reactions were performed using the heat-treated RNA in a reaction mix containing reverse transcription buffer (1x), reverse transcriptase (units), DTT (mM), dNTPs (µM), RNAse inhibitor (units) and primers (random gene specific or oligo-dT) (µM). The reaction conditions vary slightly between protocols but always included incubation at 42°C for 60 minutes and were completed with incubation at 85-95°C for 5-15 minutes. A Perkin-Elmer (PE) thermal cycler 480 was used for the reaction. The reaction mix was stored at 4°C before immediate use or –20°C for long term storage.

MMLV reverse transcriptase and Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) are commonly used possessing RNA- and DNA-directed polymerase activity, and ribonuclease H activity specific to RNA in RNA-DNA hybrids

2.2.5 Amplification of DNA by the polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful technique for amplification of specific DNA sequences from a complex mixture of DNA. The procedure was developed by Mullis and co-workers in the mid 1980's (Mullis et al., 1986; Mullis and Faloona, 1987) enabling large amounts of a single copy gene to be generated from genomic (Saiki et al., 1985; 1986) or viral DNA (Kwok et al., 1987). The initial method used the Klenow fragment of DNA polymerase I, which had to be replenished during each cycle as it is readily denatured by the amplification conditions used. The substitution of the thermostable *Taq* polymerase, isolated from *Thermus aquaticus*, circumvented this problem and allowed the automation of thermal cycling (Saiki et al., 1988).

PCR enables the amplification of any unknown DNA sequence by the simultaneous extension of primer pairs, flanking the unknown sequence, each complimentary to opposite strands of DNA. The uses of PCR are many although it has been superseded by more conventional molecular biological methods in many areas, including sequencing (Innis et al., 1988), cloning (Scharf, 1990) and detection and analysis of RNA (Veres et al., 1987). An extensive overview of PCR and its

applications is available (Innis and Gelfand, 1990) but an overview of the procedure is given below, with more detail in the appropriate chapters.

2.2.5.1 Primer design

Primer design was aided by some basic guidelines (Innis and Gelfand, 1990). An optimal primer pair should hybridise efficiently to the sequence of interest with negligible hybridisation to other sequences present in the sample. The distance between primers is rather flexible, ranging up to 10 kbp, however the PCR reaction is considerably less efficient with distances > 3kbp. (Jeffreys et al., 1988). Smaller distances between primers lessen the ability to obtain much sequence information and to reamplify with nested internal oligonucleotides, if required. For any given pair, the annealing temperatures (Tm) and GC % were balanced.

For many applications, primers are designed to be exactly complementary to the template. For others, however, such as engineering for mutagenesis or new restriction endonuclease sites or for efforts to clone or detect gene homologues where sequence information is lacking, base-pair mismatches will be intentionally or unavoidably created. It is best to have mismatches, such as in a restriction endonuclease linker, at the 5′ end of the primer; the closer to the 3′ end of the primer, the more likely a mismatch will prevent extension.

The annealing portion of primers should generally be 18-30 nucleotides in length; it is unlikely that longer primers will help to increase specificity significantly. Additional sequences such as restriction enzyme sites, epitope tags or other desired motifs can be added to the 5′ end of the primer, and may effect specificity of primer binding at low temperatures on complex templates so these primers are successfully used when amplifying from a single template vector. Primers should be designed with a GC content similar to that of the template, avoiding unusual sequence distributions such as stretches of polypurines or polypyrimidines if possible. The formation of a secondary structure such as hairpin loops greatly effects efficiency of annealing.

Primer-dimers are a common artifact most frequently observed when small amounts of template are taken through many amplification cycles. They form when the 3′ end of one primer anneals to the 3′ end of the other primer and polymerase then extends each primer to the end of the other. Therefore, these can be avoided by using primers without complementary sequences, especially in the 3′ end (this can be minimised by optimising the $MgCl_2$ concentration).

2.2.5.2 Preparation of PCR reactions

As PCR is such a sensitive procedure it is essential to take stringent precautions to avoid PCR contamination from tube to tube or carry over of PCR products (Saiki et al., 1988). All PCR reactions were set up in a designated separate room, at the side of the main laboratory where PCR products were handled. A set of micropipettes was kept for the sole purpose of setting up PCR reactions. Filter tip pipette tips were used to decrease the risk of reaction components passing from one tube to the next. A bulk reaction mix was used in order to minimise the number of pipetting steps. Reaction components (including primers) were aliquoted prior to use and stored at –20°C.

2.2.5.3 Reaction conditions

The use of high quality reagents is essential for the success of PCR; to facilitate this a number of PCR amplification kits, each containing the necessary reagents, were used following the manufacturers' instructions. Typically reaction mixes were set up in either 50 µl or 25 µl volumes using thin walled ependorf tubes (0.5 ml or 0.2 ml respectively). Variable concentrations of primers (µM), dATPs, dCTP, dTTP and dGTP (mM), $MgCl_2$ (mmol/L), DNA polymerase (Units), 1x PCR Buffer containing Tris-HCl (mM). KCl (mM) and gelatin was mixed with an appropriate volume of DNA or cDNA template (ng). Thermal cycling was carried out using DNA thermal cyclers (Perkin Elmer). Two PCR machines were generally used, a Perkin Elmer (PE) 480 which required overlaying the reaction mix with mineral oil in 0.5 ml ependorf tubes, and a Perkin Elmer (PE) 2400 size for the 0.2 ml ependorf tubes requiring no mineral oil. Cycles varied between reactions but generally consisted of an initial denaturation of 94°C for five minutes, followed by 25-45 cycles of the following; denaturation at 94°C for one minute; annealing at 55-72°C for one minute; extension at 72°C for one minute; with a final extension step at 72°C for 4 – 30 minutes. Reaction products were visualised by agarose gel electrophoresis, generally using 5-10 µl of reaction product per well.

2.2.5.4 Purification and assessment of PCR products

Single PCR products were purified following the QIAquick®PCR purification kit protocol, (QIAGEN). Briefly, the DNA adhered to the filter within the column, separating it from all other components of the PCR reaction which were washed away with various buffer solutions. The PCR products were finally eluted in 30-50µl of sterile water, 4µl of which were assessed by gel electrophoresis on a 1% agarose TAE gel by comparing the bands created to a 100 bp molecular weight standard (GIBCO BRL)

2.2.6 DNA sequence analysis

2.2.6.1 Automated sequencing

Sanger dideoxy DNA sequencing is the most commonly used method for DNA sequencing, particularly in large-scale genomic sequencing (Sanger et al., 1977). A variation of automated DNA sequencing using dye-labelled terminators, in which the dyes are attached to the terminating dideoxynucleoside triphosphates (Prober et al., 1987) has been used in this project.

Table 2-2 Commonly used sequencing primers

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
M13 Reverse (M13F)	CAGGAAACAGCTATGAC	68.0 °C : 55.6 %
M13Forward (-20) (M13R)	GTAAAACGACGGCCAG	70.9 °C : 58.6 %
GL2	CTTTATGTTTTTGGCGTCTTCCA	57.1 °C : 39.1 %
RV3	CTAGCAAAATAGGCTGTCCC	57.3 °C : 50.0 %
RV4	GACGATAGTCATGCCCCGCGCCCACCGGAA	87.0 °C : 66.7 %

2.2.6.1.1 Sample preparation

During sample preparation, DNA fragments in a sample are chemically labelled with fluorescent dyes. The dyes facilitate the detection and identification of the DNA. Typically each DNA molecule is labelled with one dye molecule, but up to five dyes can be used to label the DNA sample. More specifically, PCR reactions were performed using plasmid DNA samples (200-500ng) in a total volume of 20µl containing 0.5µM of primers, 40mM Tris-HCl, 1mmol/L $MgCl_2$ and 4µl of Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism). Samples were prepared in the PE 2400 thermal cycler incorporating 25 cycles of amplification, each cycle consisting of a denaturation step 96°C for 10 seconds followed by an annealing temperature of 50°C for 5 seconds, and an elongation step of 60°C for four minutes. DNA was then purified by precipitation methods using 3M sodium acetate (Sigma) and ethanol (95%), room temperature for 15 minutes. Pelleted DNA (14,000 rpm for 20 minutes) was washed in ethanol (70%) and re-pelleted (14,000 rpm for 5 minutes) before all ethanol was removed and dried at 90°C for 1 minute. Template Suppression Reagent (25µl) was then added and the mixture heated to 95°C for 5 minutes and chilled before transfer to genetic analyser sample tubes.

2.2.6.1.2 Sample sequencing

Samples were finally loaded and run on the ABI PRISM® 3100 Genetic Analyzer (PE Applied Biosystems, UK) under standard sequencing conditions for generation of automated sequence data. The ABI PRISM® 3100 Genetic Analyzer is a multi-colour fluorescence-based DNA analysis system using the proven technology of capillary electrophoresis with 16 capillaries operating in parallel. The 3100 Genetic Analyzer is fully automated from sample loading to data analysis.

2.2.6.1.3 Sequence evaluation

The length of read is 750 bases at the 98.5% base calling accuracy with less than 2% ambiguity. The output is in the form of a chromas file. A series of different computational software were utilised for sequence analysis including 'Blast' search engine within the NBCI database, Genetics Computer Group (GCG) and ClustalW (http://www.ch.embnet.org/software/ClustalW.html), Transfac and Motif Search.

2.2.7 Transient transfection of mammalian cells

Cationic liposome mediated transfection involves the incubation of cationic lipid-containing liposomes and DNA to associate and compact the nucleic acid (Kabanov and Kabanov, 1995; Labat-Moleur et al., 1996), presumably from electrostatic interactions between the negatively charged nucleic acid and the positively charged head group of the synthetic lipid. The liposome/nucleic acid complex is dispersed over the culture, entering cells by the processes of endocytosis, or fusion with the plasma membrane via the lipid moieties of the liposome (Gao and Huang, 1995). The Trans™Fast Transfection Reagent (Promega) is comprised of the synthetic cationic lipid, (+)-N,N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide and the neutral lipid, DOPE, designed for high efficiency transfer of DNA into mammalian cells and was used in all cell types for this project.

Cells were seeded onto plates at various concentrations one day before the transfection experiment and incubated overnight at 37°C, 5% CO_2. For optimal assay conditions the cells should be approximately 80% confluent on the day of transfection. The TransFast™ Reagent was thawed, warmed to room temperature and mixed thoroughly by vortexing while the serum-free media was pre-warmed to 37°C. DNA to be transfected was re-suspended in TE buffer to a final concentration of 0.1-0.5 µg/µl and the DNA purity checked by determining the ratio of absorbency at 260nm (A_{260}) and 280nm (A_{280}) using a spectrophotometer. Transient transfections were carried out using a 1:1 ratio of Trans™Fast reagent with DNA according to the manufacturer's instructions. More specifically, total DNA (50-100ng) was added to pre-warmed serum free media (40µl) and thoroughly mixed before addition of TransFast™ Reagent (0.3µl) calculated per well using a 96-well plate. The DNA/transfection reagent samples were incubated for 10-15 minutes at room temperature to allow complex formation before the growth medium on the 96-well plates was aspirated and replaced with the transfection samples. The cells were incubated with the complexes for 1 hour at 37°C, 5% CO_2 before pre-warmed complete medium was added for cell recovery overnight at 37°C. Initially an optimisation procedure was performed, testing various amounts of DNA (0.25, 0.5, 0.75, 1.0 µg per well) at charge ratios (TransFast™ Reagent to DNA) of 1:1 and 2:1. For this initial optimisation, serum-free conditions with adherent cells in 24 well plates, using an exposure time of one hour was performed.

2.2.8 Genetic reporter systems

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Applications include the study of receptor activity, transcription factors and intracellular signalling in this study.

A dual reporter Assay system has been used in this project to improve experimental accuracy, by correcting for transfection efficiency. The term 'dual reporter' refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. In the Dual-Luciferase® Reporter (DLR™) Assay System, the experimental firefly (*Photinus pyralis*)

luciferase reporter (de Wet et al., 1985) is correlated with the effect of specific experimental conditions, while activity of the co-transfected control *Renilla* (*Renilla reniformis*) luciferase reporter (Lorenz et al., 1991) provides an internal control, which serves as a baseline response. Normalising the activity of the experimental reporter to the activity of the internal control minimises experimental variability caused by differences in cell viability or transfection efficiency. Other sources of variability, such as differences in pipetting volumes, cell lysis efficiency and assay efficiency can be effectively eliminated. Thus dual reporter assays often allow more reliable interpretation of the experimental data by reducing extraneous influences.

Both firefly and *Renilla* luciferase are immediately active upon mRNA translation, and as neither is naturally present in mammalian cells, their assays are not interfered with by endogenous enzymatic activities. As a dual-reporter system these enzymes also offer the advantage of having completely separate evolutionary histories and thus independent biochemistries, allowing their luminescent reactions to be distinguished.

DLR™ assays were performed 72 hours post transfection according to the manufacturer's protocol. More specifically, media was removed from the adherent cell mono-layer, washed once with PBS and lysed with Passive Lysis Buffer (PLB) (1x) for 15 minutes at room temperature. Using a Dynex, MLX luminometer, the activities of firefly and *Renilla* luciferase activities were measured sequentially from single samples (per well). The firefly luciferase reporter was measured first by adding Luciferase Assay Reagent II (LAR II) (50µl) to generate a 'glow-type' luminescent signal. After quantifying the firefly luminescence over 12 seconds, the Stop & Glo® Reagent (50µl) was then added to the same well to quench the first reaction (3 seconds) and then quantify the second 'glow-type' signal of *Renilla* luciferase activity which decayed slowly over the course of the 12 seconds measurement. Firefly and *Renilla* luciferases, because of their distinct evolutionary origins, have dissimilar enzyme structures and substrate requirements, enabling the discrimination between their respective bio-luminescent reactions. Firefly and *Renilla* luciferase values were obtained for each well and analysed using an Excel Spread- Sheet.

2.2.9 Analysis of recombinant proteins

The separation and analysis of proteins was facilitated by one-dimensional denaturing discontinuous gel electrophoresis (Laemmli, 1970). Proteins are denatured by boiling in the presence of SDS and 2-mercaptoethanol. The sample is then loaded onto a discontinuous gel consisting of a stacking buffer which concentrates the loaded protein sample into a narrow band and a separating gel which separates proteins on the basis of a molecular size, with smaller proteins migrating faster towards the anode. Ready Gel Precast Gels (Biorad, Herts) (8.6 cm x 6.8 cm x 1.0 mm) of specific resolving percentage gels (and 4 % stacking gel) were selected based on protein size and used with the Mini-Protean 3 Electrophoresis cell (Biorad, Herts) as recommended by the manufacturer. Both the wells and bottom of the gel were rinsed with 1x running buffer to remove all traces of the preservative solution and then placed on one side of the electrode core. The buffer dam was slotted into the other side and the complete assembly placed into the clamp unit before

being transferred to the electrophoresis tank. The top chamber and bottom of the tank were then filled with 1x running buffer. Protein samples (typically 5-30 µg of protein in 5-25 µl) were prepared by the addition of appropriate volume of protein sample loading buffer, followed by incubation at 100°C for 2-8 minutes. The samples were briefly centrifuged before being carefully loaded into the wells using pipette tips. A Wide Range (6.5 to 205 kDa) Color Markers molecular weight standard (10 µl) (Sigma) was loaded into both outer wells to allow the estimation of the size of sample proteins. Gels were electrophoresed at variable voltages until the bromophenol blue dye reached the lower part of the gel, the gel was removed from the cast and analysed further as determined by appropriate protein detection methods.

Chapter III

Cloning and expression of canine interleukin-1 receptor antagonist (IL-1Ra) cDNA

3.1 ABSTRACT

To modify disease progression at the molecular level 'structure-modifying' therapeutic genes can be introduced into arthritic joints to regulate those enzymes responsible for extra-cellular matrix (ECM) turnover. Since the matrix metalloproteinases (MMPs) play a central role in the development of osteoarthritis (OA), gene-based therapeutics should include mediators for controlling the synthesis and activity of these enzymes at the transcriptional and/or post-translational levels. Candidate proteins that may be of therapeutic benefit in treating OA include interleukin-1 receptor antagonist (IL-1Ra), soluble tumour necrosis factor receptors (sTNFR) and various tissue inhibitors of MMPs (TIMPs). All of these genes have been cloned and sequenced in a number of different species and considerable sequence homology has been demonstrated at both the nucleotide and amino acid levels. The canine homologues of these therapeutic genes were cloned for future use in a homologous gene transfer study for canine disease. This chapter describes the cloning and sequence analysis of the cDNA encoding the canine homologue of IL-1Ra using reverse transcription polymerase chain reaction (RT-PCR) and RNA harvested from canine peripheral blood mononuclear cells (PBMCs) as a template. Sequence analysis of the canine IL-1Ra gene demonstrated an open reading frame of 531 base pairs (bp) encoding a protein of 177 amino acids showing considerable sequence similarity with the homologous sequences published for other species. This chapter also describes the *in vitro* transcription and translation of candidate canine-specific therapeutic proteins including IL-1Ra, TIMP-1 and TIMP-2 of approximate molecular weights 19.5, 22.8 and 24.3 kilo Dalton (kDa) respectively; sTNFRI (23.2 kDa) was unable to be expressed using this system.

3.2 INTRODUCTION

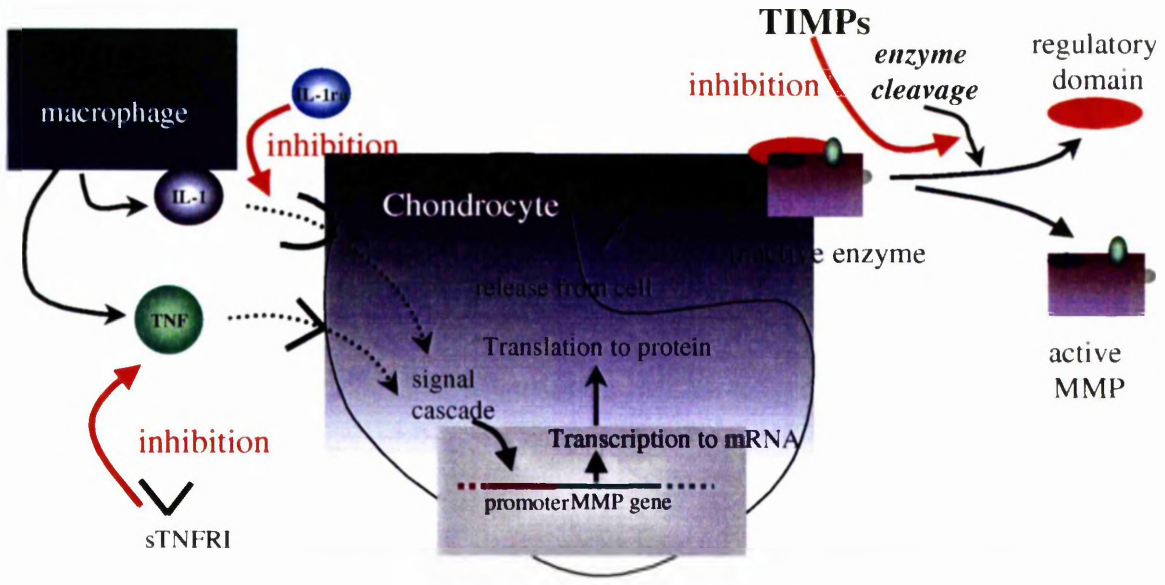
The pathogenesis of osteoarthritis (OA) involves two major components, inflammation of the synovial tissues and degeneration of the articular cartilage. Most standard therapies only address the inflammatory component since few drugs demonstrate real chondroprotective effects. However, the development of gene-based therapeutics promises to control both of these processes with the selection of appropriate therapeutic genes.

3.2.1 Candidate therapeutic proteins

Cytokines are multifunctional glycoproteins that participate in both immune and inflammatory responses (Kelso, 1998) and have been implicated in specific disease processes including cardiac failure (Sharma et al., 2000), cancer (Burke, 1999), diabetes mellitus (Falcone and Sarvetnick, 1999) and arthritis (Martel Pelletier et al., 1999b). In OA the regulation of matrix metalloproteinase (MMP) activity by cytokines and other cellular signalling molecules is an essential component of the pathogenesis, particularly associated with the processes of synovial inflammation and cartilage degeneration (Ruggeri et al., 1996). Within this cell-signalling network many potentially therapeutic compounds have already been identified to target disease progression at the transcriptional level including interleukin-1 receptor antagonist (IL-1Ra) (Caron et al., 1996) and soluble tumour necrosis factor receptor (sTNFR) (Mageed et al., 1998). However, gene-based therapeutics for OA should not only include mediators for controlling the MMPs at the transcriptional level but also at the post-translational level with the direct inhibition of active enzyme through the endogenous tissue inhibitors of MMPs (TIMPs) (Carmichael et al., 1989; Renggli-Zulliger et al., 1999) (Figure 3-1).

Figure 3-1 Candidate therapeutic proteins

Therapeutic proteins may be used to control the synthesis and activation of matrix metalloproteinases (MMPs) at the transcriptional level using interleukin-1 receptor antagonist (IL-1ra) which competitively inhibits the binding of IL-1 to its receptor and the soluble tumour necrosis factor type I receptor (sTNFRI) which binds and inhibits TNF, thus preventing receptor activation and MMP transcription. Therapeutic proteins can also be used to prevent the activation of pro-enzyme with the use of the tissue inhibitor of metalloproteinase (TIMPs) which function to prevent the removal of the regulatory domain of the MMP, thus inhibiting full activation of the MMPs.



3.2.1.1 Interleukin-1 and receptor antagonist

Interleukin-1 (IL-1) is a potent mediator of inflammation involved in regulating a wide variety of physiological and pathological events. This pro-inflammatory cytokine plays a crucial role in the pathogenesis of OA where it suppresses the synthesis of important structural components of the extracellular matrix (ECM) such the type II collagen and proteoglycans while promoting the synthesis of abnormal type I collagen. Furthermore, IL-1 enhances the levels of MMP gene transcription resulting in the degeneration of articular cartilage (Martel-Pelletier et al., 1999a; 1991).

To date the IL-1 family consists of a number of known genes encoding ligands including two agonists (IL-1α and IL-1β), an antagonist (IL-1Ra) and a rarely expressed transcript (IL1HY1) with 52% homology to IL-1Ra (Mulero et al., 1999). IL-1α, IL-1β and IL-1Ra share similar structural domains, with all three binding to the IL-1 receptor which exists in two forms, Type I (80 kDa) and Type II (68 kDa). Soluble forms of these receptors are produced by the cleavage of their extra-cellular domain and it is now known that the binding of the soluble portion of the receptor to the IL-1 ligands may function as inhibitors of this IL-1 family (Symons et al., 1991, 1995). Another mechanism for IL-1 inactivation is through the antagonistic behaviour of IL-1Ra which contains a signal peptide for extra-cellular secretion. Here, IL-1Ra functions as a competitive inhibitor by binding to the IL-1RI cell associated receptor without inducing the signalling cascade and biological response observed with IL-1 (Arend, 1991; Dinarello and Thompson, 1991).

Many substances including adherent IgG, cytokines, bacterial and viral components stimulate the production of IL-1Ra from cells which enabled the first purification of human IL-1Ra protein (18kDa) of 177 amino acids (22 kDa when glycosylated) (Hannum et al., 1990). Human IL-1Ra cDNA was cloned from a human monocyte library and the 18 kDa molecule, expressed in Escherichia coli (*E.coli*), demonstrated that the expected IL-1 induced PGE_2 production by fibroblasts was inhibited (Eisenberg et al., 1990). Further studies revealed that the recombinant IL-1Ra protein was capable of inhibiting IL-1 bioactivity on both T cells and endothelial cells *in vitro* and was also a potent inhibitor of IL-1 induced corticosterone production *in vivo* (Carter et al., 1990). Two structural variants of IL-1Ra have since been described and include secreted (sIL-1Ra) and intracellular (cIL-1Ra) forms. The sIL-1Ra (17 kDa) form is found in most cells capable of synthesising IL-1, except possibly endothelial cells and hepatocytes. The cIL-1Ra (18 kDa) form is generally found in skin where it remains in the cytoplasm of keratinocytes and other epithelial cells, monocytes and fibroblasts (Arend et al., 1998). These two isoforms of IL-1Ra are transcribed from the same gene through the use of alternative first exons to produce unique proteins (Butcher et al., 1994). The sIL-1Ra form (177 amino acids) contains a 25 amino acid leader sequence which is cleaved before being secreted as a variably glycosylated (152 amino acids) protein (Eisenberg, 1990). The cIL-1Ra form (159 amino acid) exists in two further forms Type 1 (Haskill et al., 1991) and Type 2 (Muzio et al., 1995) created by alternative splicing of cIL-1Ra. Yet another isoform of IL-1Ra (16 kDa) has also been described, binding to IL-1 receptors with less avidity than the other isoforms (Malyak et al., 1998). The biological significance of these isoforms remains unclear but

suggests a complex redundancy of protein function. In OA, the imbalance in the ratio of IL-1 and IL-1Ra suggested that the decreased production of IL-1Ra contributed to the destructive process rather than the up-regulation of IL-1 alone (Firestein et al., 1994). The suggestion of IL-1Ra as a therapeutic agent in OA has been reinforced by the use of intra-articular injections of IL-1Ra alone to reduce the pathological changes associated with osteoarthritis in the dog (Pelletier et al., 1997) and in the rabbit (Fernandes et al., 1999).

3.2.1.2 Tumour necrosis factor and receptors

TNF has also been implicated in the pathogenesis of OA (Martel-Pelletier, 1999a) as an important mediator of inflammation, tissue destruction and the immune response by inducing cell growth, proliferation and apoptosis (Edwards et al., 1994). TNFα is synthesised as an inactive precursor (proTNFα) requiring cleavage at the Ala76-Val77 bond for activation via TNFα converting enzyme (TACE), an adamalysin enzyme containing an MMP domain (Black et al., 1997) with a suggestion that TACE is up-regulated in OA-affected cartilage (Amin, 1999). Many cell types produce TNF and its biological effect is mediated by the binding to one of two structurally distinct receptors: type I (TNF-RI; p60 or p55) and type II (TNF-RII; p80 or p75). The extra-cellular domains of both receptors are shed from the cell surface by proteolytic cleavage by the MMPs and have the ability to bind TNFα and act as natural inhibitors. However since dissociation of TNFα from TNF-RI appears to be almost irreversible in comparison to the transient binding to TNF-RII it would appear that the former would act as a useful therapeutic target (Dinarello and Moldawer, 2000).

The control mechanism inhibiting the biological effects of TNF was first evaluated in the late eighties when two distinct TNF receptors were identified containing similar extra-cellular domains that bound with equal high affinity to TNF (Hohmann et al., 1989). It was then hypothesised that the soluble proteins, isolated from human urine and capable of specifically binding TNF, represented shed forms of cell surface cytokine receptor (Englemann et al., 1990). The cloning and structural analysis of the TNFR revealed a signal peptide followed by an extra-cellular domain, a trans-membrane region and an intra-cellular region (Schall et al., 1990). The prediction that the shedding of the extracellular domain of the TNFR might be used as a protective mechanism by cells was confirmed with the cloning of a 55 kDa TNF receptor. It was found that this domain bound stoichiometrically to TNF neutralising TNF bio-activity in different assay systems (Loetscher et al., 1991). In OA, imbalance in the ratio of TNF and its receptors, in particular the decreased production of sTNFR, may contribute to the destructive process rather than the up-regulation of TNF alone (Kammerman et al., 1996). The suggestion of sTNFR as a therapeutic agent in OA has been re-inforced by the delivery of sTNFR to SCID mice with B and T lymphocytes to prevent the development of arthritis (Mageed et al., 1998).

For the development of a homologous gene transfer study for canine OA the canine homologue for sTNFRI was selected. Canine sTNFRI has been cloned and sequenced to reveal an open reading frame of 633 bp encoding a predicted protein of 211 amino acid, corresponding to the soluble extra-cellular domain of the protein (Campbell et al., 2001b); submitted to Genbank Accession number:

AF013955. When compared with the sTNFRI sequences published for other species, the canine sequence showed considerable homology at the nucleotide level exhibiting 90, 84, 85, 80 and 80% similarity to feline (Accession number: U72344), human (Himmler et al., 1990), bovine (Lee et al., 1998), murine (Goodwin et al., 1991) and rat (Himmler et al., 1990) sequences respectively. The canine sTNFRI protein also demonstrated 89, 79, 71, 69 and 67% sequence similarity with the feline, human, bovine murine and rat sequences respectively at the amino acid level. The canine sTNFRI amino acid sequence alignment with other species (Campbell et al., 2001b) demonstrated 24 conserved cysteine residues thought to be important for ligand binding and two potential N-linked glycosylation sites. A signal peptide sequence of 21 amino acids starting at the first ATG codon for methionine is thought to exist with its cleavage necessary for maturation of the protein (Goodwin et al., 1991).

3.2.1.3 Tissue inhibitors of metalloproteinase

Control of MMP activity is not governed by cytokines alone but involves a system of complex protein interactions. One group of proteins particularly implicated in the regulation of the MMPs are the TIMPs (Brew et al., 2000), which are present in all connective tissues (Cawston, 1996). The TIMPs constitute a family of four polypeptide inhibitors that inhibit the action of MMPs at two levels, through the tight binding to the active forms of the MMPs (Murphy and Willenbrock, 1995) and through prevention or delay of MMP activation from the quiescent form (DeClerk et al., 1991). Human TIMP-1 was cloned (Docherty et al., 1985) and shown to be a 20 kDa variably glycosylated protein of 208 amino acids (Carmichael et al., 1986) and human TIMP-2 was later described as a 21 kDa non-glycosylated protein in its secreted form (Stetler-Stevenson et al., 1989). Mammalian TIMPs have two domains, at the N-terminal and C-terminals, each stabilised by three disulfide bonds (Williamson et al., 1990). The N-terminal domain is capable of forming a stable native molecule that has inhibitory activity against the different MMPs (Murphy et al., 1991). For example TIMP-1 was found to interact reversibly with MMP-1 and MMP-3 (Woessner, 1991) and MMP-9 (Goldberg et al., 1992), binding with high affinity as a major inhibitor of these enzymes. The C-terminal domain enables non-inhibitory complex formation with MMPs demonstrated by the binding of TIMP-2 to the pro-form of MMP-2 at a stabilisation site distinct from the active site (Kolkenbrock et al., 1991). Both TIMP-1 and -2 have been isolated from rheumatoid synovial fluids (Osthues et al., 1992) and disruption of the balance between the MMP and the TIMPs in favour of the MMP production has been implicated in the destruction of cartilage in OA (Dean et al., 1989). Furthermore, a decreased incidence of joint erosion in arthritic mice was demonstrated following systemic administration of TIMP-1 (Carmichael et al., 1989). Since then it has been found that both TIMP-1 and TIMP-2 block collagen degradation in IL-1 stimulated cartilage model systems (Ellis et al., 1994).

For the development of a homologous gene transfer study for canine OA the canine homologues for TIMP-1 and TIMP-2 were selected. The canine homologue for TIMP-1 was cloned and sequenced to reveal an open reading frame of 624 bp encoding a predicted protein of 207 amino

acids (23 kDa) (Zeiss et al., 1998); submitted to Genbank Accession number: AF077817. When compared with the TIMP-2 sequences published for other species the canine sequence was shown to share 80-85% nucleotide sequence identity and 66-83% identity in the deduced amino acid sequence with homologous mammalian human, bovine, sheep, pig, rabbit, mouse and rat sequences. The canine TIMP-1 amino acid sequence alignment with other species (Zeiss et al., 1998) demonstrated conserved regions including two potential glycosylation sites and 12 cysteine residues, forming six disulphide bonds, that divide the protein into two distinct domains. One domain binds the active metalloproteinase, and the other binds the latent, inactive form of the enzyme (Matrisian, 1990). A consensus sequence, VIRAK, found in all four TIMPs (Greene et al., 1996) is present at amino acid positions 41-46 in the canine promoter.

The canine homologue for TIMP-2 was also cloned and sequenced to reveal an open reading frame of 663 bp encoding a predicted protein of 220 amino acids (Campbell et al., 2001b); submitted to Genbank (Accession number: AF112115). When compared with the TIMP-2 sequences published for other species the canine sequence showed considerable homology at the nucleotide level exhibiting 92, 89, 89 91% similarity to human (Stetler-Stevenson et al., 1989), rat (Cook et al., 1994), mouse (Shimzu et al., 1992), bovine (Boone et al., 1990) sequences respectively. Furthermore, canine TIMP-2 protein demonstrated 96, 97, 96 and 93% sequence similarity with the human, rat, murine and bovine sequences respectively at the amino acid level. The canine TIMP-2 amino acid sequence alignment with other species (Campbell et al., 2001b) demonstrated conserved domains with 12 cysteine residues, forming six disulphide bonds (DeClerk et al., 1991). The N-terminal domain necessary for the MMP inhibitory activity is also conserved (DeClerk et al., 1993) with the VIRAK sequence present in this domain, thought to be of particular importance. A signal peptide of 26 amino acids is present starting at the ATG codon for methionine to the first conserved cysteine residue (Shimizu et al., 1992) and as with other published TIMP-2 sequences there are no predicted N-glycosylation sites within the canine coding sequence.

3.2.2 Construction of anti-arthritic therapeutic vectors

Candidate therapeutic genes can be evaluated using appropriate vector systems containing the necessary regulatory sequences for efficient gene expression. The therapeutic gene is cloned as a transcriptional unit containing the coding region of the gene as complementary DNA (cDNA) or genomic DNA (gDNA), and all necessary regulatory sequences in the immediate 5′ and 3′ untranslated regions (UTR) for efficient transcription and translation. However, the effect of the therapeutic protein is not only determined by the efficiency of transcription and translation but also on the steady state level of proteins.

3.2.2.1 The coding region of the gene

In eukaryotes most protein-coding regions of the DNA sequence do not read out co-linearly into messenger RNA (mRNA) but are composed of an alternating array of coding sequence, called exons separated by intervening pieces of non-protein coding DNA referred to as introns. The

removal of these intronic sequences from the initial RNA transcript and the joining of all exons in a head to tail arrangement occurs through a precise mechanism mediated by the splicing machinery in the nucleus to form mRNA (Weiner, 1993). Intronic sequences, present in gDNA, often contain functional enhancer elements necessary for efficient translation. It may therefore be desirable to use gDNA to express the gene of interest, however these intervening sequences can span up to hundreds of kilobases (kb) and present problems associated with the cloning and delivery systems using currently available mammalian expression vectors. As an alternative cDNA, which is a direct copy of the mRNA transcript, can be made *in vitro*, and used to directly transcribe and translate genes to a functional protein. The cDNAs encoding the therapeutic canine genes IL-1Ra, sTNFR1, TIMP-1 and TIMP-2 are discussed in this chapter.

3.2.2.2 The immediate 5′ untranslated region (UTR)

The 5′ UTR of the gene is important for the initiation of gene transcription since it is responsible for the binding of RNA polymerase to the initiation site enabling the addition of nucleotides in a 5′ to 3′ fashion to form the primary RNA transcript. The nucleotide at the 5′ end of the transcript is usually modified post-transcriptionally by the addition of a methylated guanine (G) residue linked by a 5′ to 5′ triphosphate-bridge. This feature, referred to as a 'Cap' structure, is essential for the efficient initiation of protein synthesis. The 5′ UTR is also important in translation initiation enabling the binding of the 40S ribosomal sub-unit to the region immediately up-stream to the translation start codon (AUG). This ribosomal component moves to the first AUG where the 60S ribosomal subunit joins to produce the first peptide bond. Translation initiation from the mRNA template is the rate limiting step in protein synthesis and is controlled by the context and position of the AUG with its surrounding secondary structure and length of 5′ UTR. The optimal context for initiation is 5′ GCC(A/G)CC**AUGG** 3′ where the **A** of the **AUG** codon is referred to as the +1 nucleotide. The most critical residues controlling the efficiency of initiation are purines at positions –3 and +4, usually an 'A' and 'G' respectively. The presence of multiple upstream initiation sites serves to suppress the initiation efficiency from the correct site. Position of the AUG codon is also extremely important since approximately 90% of vertebrate mRNAs are initiated from the first AUG codon without giving preference to an optimal context. Secondary structure of the RNA is also of great importance since the hairpin loop, 12-15 nucleotides downstream of the AUG, causes the 40S ribosomal subunit to temporarily stall and facilitate initiation. Secondary structure located in this position also serves to minimise 'leaky' initiation and permit translation from weak AUG codons. However, high degrees of secondary structure (hairpins) upstream of the AUG may have the opposite effect by preventing the translocation of the 40S ribosomal subunit to the AUG required for initiation; this serves as a useful control mechanism for translation. The length of the 5′ UTR is also a crucial factor in the efficiency of the translation process with a minimum of approximately 20 nucleotides required for initiation from an optimal AUG (Kozak, 1991). An optimal Kozak sequence (AXXATGG) was incorporated into the 5′ end of the cloned therapeutic cDNAs described in this chapter.

3.2.2.3 The immediate 3′ untranslated region

The 3′ UTR, defined as the mRNA sequence following the termination codon (TAA, TAG) is thought to play a role in mRNA transcript stability, providing a determinant of mRNA levels and also playing a role in protein synthesis through the control of translation (Jackson and Standart, 1990). The half-life of mRNA molecules determines the length of time that the template is available for protein synthesis and this can vary from several minutes for some cytokines to 100 hours for stable mRNAs (Peltz et al., 1991). However, the half-life may not always be constant and will change with the conditions of the cell. The rapid turnover of mRNA has been linked to cis-acting instability elements present within the RNA sequence. One of these instability elements is in the class of AU-rich elements (ARE) containing the pentameric motif AUUUA in a U-rich context sufficient for rapid mRNA degradation (Vakalopoulou et al., 1991). This rapid degradation process is important for transient induction of molecules and rapid cellular changes that occur in response to the environment providing the fine-tuning of protein levels within the cell.

The minimal, mammalian poly(A) signal, also located in the 3′ UTR, is composed of an AAUAAA motif 20-30 nucleotides in length and serves to control the termination and processing of the primary RNA transcript with the formation of the poly(A) tail on the mRNA transcript (Proudfoot, 2000). This homopolymeric stretch of adenine (A) residues is added to the primary transcript by a post-transcriptional nuclear processing mechanism, termed polyadenylation. The importance of the polyadenylation signal is in the formation of the poly(A) tract of the mRNA is to provide stability and translatability (Peltz et al., 1991). Lengthening of this poly(A) tract tends to control mRNA translation and increase its stability while shortening leads to degradation. An optimal mammalian poly(A) signal for the 3′ ends of the therapeutic mRNAs was provided by mammalian expression vector pTarget (Promega) as described in this chapter.

3.2.2.4 Therapeutic protein stability

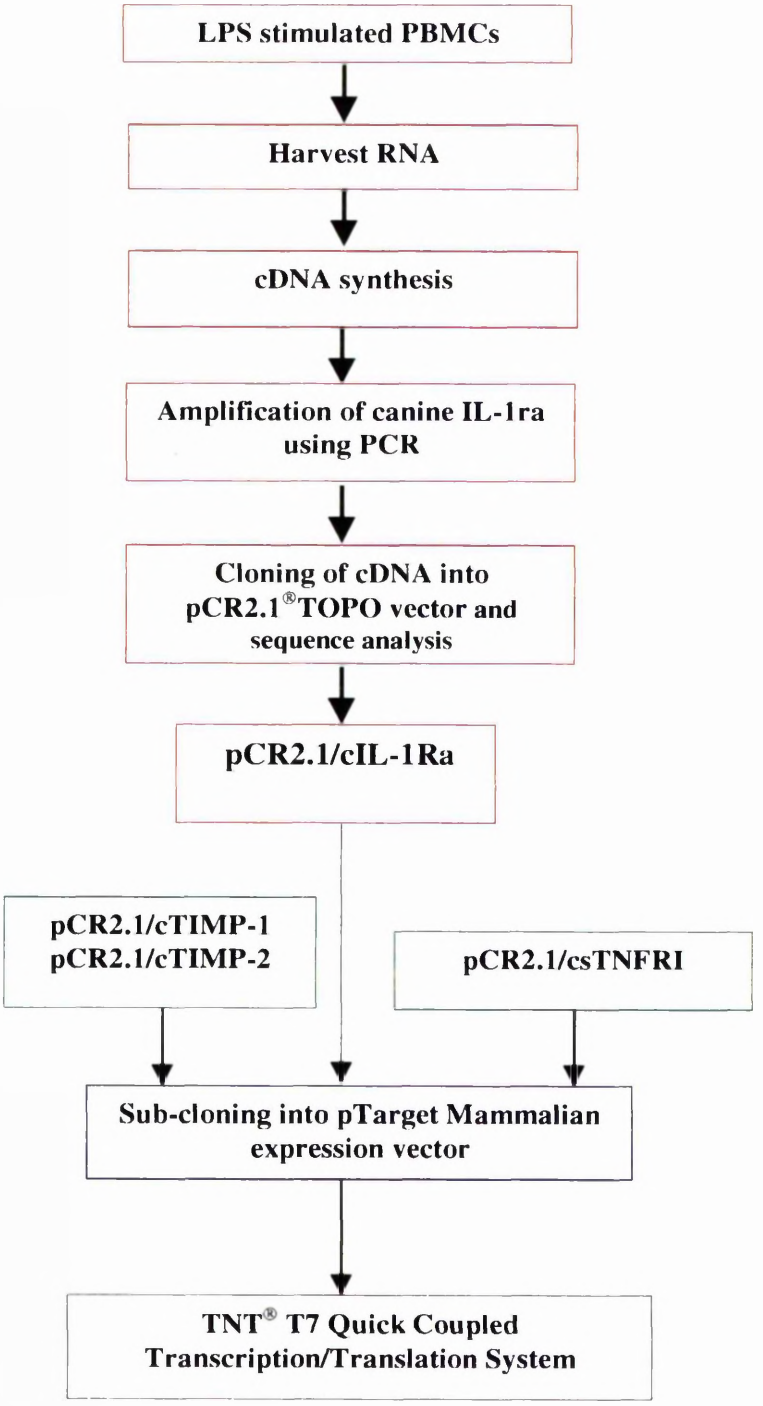
The effects of the therapeutic protein are not only determined by the efficiency of transcription and translation but also on the steady state level of proteins. The level of protein depends not only upon the rate at which mRNA is synthesised, processed, transported, translated and degraded but also the rate of protein turn-over. The half-life of a protein determines its steady state level with in the cell (Hargrove and Schmidt, 1989). Most proteins are stable, but those proteins that vary with time or in response to the physiological status of the cell are regulated by proteolysis (Varshavsky, 1992). Proteolysis occurs through cytosolic, ATP-dependent pathways that do or do not require ubiquitination, or by lysosomal pathways (Dice, 1990). Protein stability is more difficult to control and has not been considered in this chapter since the expression system used, *in vitro* transcription and translation, was independent of cellular function.

3.3 MATERIALS AND METHODS

Candidate canine-specific therapeutic genes, IL-1Ra, sTNFRI and TIMP-2 were cloned within our research group and a full description of cloning procedures can be found in appendix 1 (Campbell et al., 2001). The canine TIMP-1 construct was a generous gift from Dr. Noritake (Japan) (Noritake et al., 1999). Since the cloning procedures were similar for each gene this chapter only describes the cloning of canine IL-1Ra which was performed entirely by the author. Overviews of the experimental procedures employed in this chapter are illustrated in figure 3-2. Briefly, canine peripheral blood mononuclear cells (PBMC) were stimulated with lipopolysaccharide (LPS) and RNA isolated using RNAzol™B techniques. The canine IL-1Ra cDNA was isolated using reverse transcription polymerase chain reaction (RT-PCR) and primers based on sequence homology between the sequences published for different species. PCR products were cloned into pCR2.1®TOPO vector and sequenced. The canine IL-1Ra, sTNFRI, TIMP-1 and TIMP-2 cDNAs were sub-cloned into pTarget mammalian expression vector (Promega) and expressed using *in vitro* transcription translation protocols (Promega).

Figure 3-2 Overview of experimental procedures used to clone and express canine specific therapeutic cDNAs

The canine IL-1Ra cDNA was cloned using RT-PCR and the sequence analysed. The canine homologues of IL-1Ra, sTNFRI, TIMP-1 and TIMP-2 were sub-cloned into pTarget mammalian expression vector and expressed using *in vitro* transcription and translation techniques.



3.3.1 Preparation of RNA from canine peripheral blood mononuclear cells

3.3.1.1 Isolation of lipopolysaccharide stimulated canine peripheral blood mononuclear cells

Canine PBMCs, stimulated with LPS, were used as a substrate for the isolation of canine IL-1Ra by RT-PCR, based on the knowledge that LPS-activated mononuclear cells are a potential source of this antagonist (West et al., 1996). Canine whole blood (30ml) preparations were diluted with an equal volume of isotonic Alsevers solution (Scottish Antibody Production Unit) and 5µl aliquots were overlaid on Ficoll-Hypaque, Lymphoprep (gradient solution, Nycomed) before being centrifuged for 20 minutes at 2000 rpm, room temperature. The cells in the interface between the plasma and lymphoprep were removed, divided in two with additional Alsevers to create a final volume of 14 ml in each sample and then centrifuged (brake setting low) for 10 minutes at 1000 rpm, room temperature. The supernatants were discarded and the cells within the pellet slowly re-suspended in RPMI 1640 medium supplemented with L-glutamine, 10% foetal calf serum (FCS), Penstrep, 2-mercaptoethanol (2ME) and LPS (5µg ml^{-1}) at 37°C under 5% CO_2 in air for 24 hours. The total number of cells were then calculated with a ten fold dilution of the cell suspension in counting stain, viewed under the light microscope and the number of mononuclear cells counted.

3.3.1.2 Isolation of total RNA

Purified RNA was essential as the starting material for the cloning of the IL-1Ra gene, and thus when isolating the RNA it was necessary to ensure that all the preparation material was free of ribonuclease (RNAse) activity. RNAzol™ B solution (AMS Biotechnology) (25ml) was added directly to the flasks containing the adherent PBMCs for lysis. Aliquots (1.2ml) of the lysed cell solution were shaken for 15 seconds with chloroform (120µl) and RNA isolated as described in section 2.2.3. The RNA samples were re-suspended in DEPC water (40µl) and the quality and quantity assessed using spectrophotometry and agarose gel electrophoresis.

3.3.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The technique of RT-PCR was chosen to clone canine IL-1Ra since there was considerable sequence homology between the IL-1Ra sequence published for the different species, enabling the design of PCR primers based on conserved regions flanking the protein coding sequence. RT-PCR also offers a simpler and potentially faster approach than techniques involving the generation and screening of cDNA libraries. The ultimate aim of this project was to express the canine cytokine as protein using a mammalian expression vector system therefore it was only necessary to obtain the protein coding cDNA sequence.

3.3.2.1 First strand cDNA synthesis

The synthesis of first strand cDNA followed the basic principles as set out in section 2.2.4. More specifically, total RNA samples (2µg) in 9µl of DEPC water were heated for 5 minutes at 65°C and

then quenched on ice. Reactions were performed using the heat treated RNA in a 25µl reaction mix containing 5µl of reverse transcription buffer (5x), 200 units of Molony Murine Leukemia Virus reverse transcriptase (MMLV-RT) (GIBCO BRL), 10mM dTT, 250µM dNTPs, 25 units RNase inhibitor and 25µM random primers. The reaction was incubated at 37°C for 30 minutes followed by 42°C for 60 minutes and completed with 95°C for 5 minutes. A Perkin-Elmer (PE) thermal cycler 480 was used for the reaction.

3.3.2.2 PCR amplification

3.3.2.2.1 Primer design

Primers for PCR were designed as described in section 2.2.5.1, according to regions of sequence homology between different species and were synthesised, and supplied by MWG-Biotech (Table 3-1). The melting temperature (Tm) and guanine/cytosine (GC) content of each primer have been noted. These primers were expected to amplify a cDNA of approximately 550 nucleotides in size, containing the complete coding sequence of the canine IL-1Ra gene.

Table 3-1 Primer sequences for the PCR amplification of canine IL-1Ra cDNA

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
IL-1Raf	GCCTTGAATTCGAATTCATGGAAACCTGCAGGT	68.2°C : 45.5%
IL-1Rar	GGCCTCTAGATCTAGATTATTCCTTCTGGAAG	66.9°C : 43.8%

3.3.2.2.2 PCR conditions

PCR was performed as described in section 2.2.5. More specifically, canine cDNA samples (100 ng) were used as a template in a total volume of 50µl containing 0.4µM of both sense primer (IL-1Raf) and anti-sense primer (IL-1Rar), 0.2mM of dATPs, dCTP, dTTP and dGTP, 1.5 mmol/L $MgCl_2$ and 2 units of *Taq* DNA polymerase (QIAGEN, UK). Mineral oil was added to the surface of the mixture to prevent evaporation, before being placed in the PE 480 thermal cycler. Samples were subjected to an initial denaturation at 95°C for 5 minutes followed by 35 cycles of amplification, each cycle consisting of a denaturation step of 95°C for one minute and annealing temperature of 60°C for 1 minute, followed by an elongation step of 72°C for one minute. A final elongation step at 72°C for 30 minutes completed the reaction. *Taq* polymerase added a single deoxy-adenosine (A) to the 3' ends of duplex molecules to enable PCR products to be ligated efficiently with the linearised vector containing complementary single 3'deoxythymidine (T) residues. PCR products (8µl) were visualised by gel electrophoresis on a TAE agarose gel (1%) and compared with a 100 bp molecular weight standard. A negative control containing all PCR components (primers, dNTPs, PCR buffer and *Taq* polymerase) except template was included to

ensure that there was no contamination of the PCR reactions with extraneous DNA that might serve as a template for PCR amplification.

3.3.3. Cloning into pCR®2.1-TOPO

PCR products were cloned using methods described in section 2.2.2. Briefly, PCR products were purified following QIAquick®PCR purification kit protocol. and eluted in 50µl of sterile water, 4µl of which were assessed by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a low DNA Mass™ Ladder. Purified PCR products were directly ligated into the pCR®2.1-TOPO plasmid vector following the TOPO TA® cloning protocol. The ligation reactions (2µl) were then transformed into One Shot™ TOP10 competent cells and spread onto agar plates containing ampicillin and X-gal for incubation overnight at 37°C. White, ampicillin resistant, colonies were selected from the plates and cultured overnight in LB/ampicillin broth at 37°C and glycerol stocks of each culture were prepared for long term storage.

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep®PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. DNA samples were then assessed by spectrophotometry for quantification. Samples were screened for those containing the insert by restriction enzyme digestion. pCR2.1/cIL-1Ra constructs (500ng) were digested with *Eco*R1 restriction enzyme (7.5 units) and Buffer 6 in a total volume of 20µl at 37°C for 4 hours. Samples were analysed by agarose gel electrophoresis to identify the presence of inserts.

3.3.4 Sequence evaluation of constructs

Recombinant plasmids were sequenced and analysed as described in section 2.2.6. Briefly, recombinant plasmid samples (400 ng) were amplified using the Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism) with 0.5µM of both sense (M13R) and anti-sense (M13F) vector-based primers. The DNA was purified using ethanol precipitation methods before samples were loaded into the ABI 310 genetic analyser for generation of automated sequence data.

Sequences were stored and managed on a UNIX computer system using Genetics Computer Group (GCG) software (University of Wisconsin). 'Raw' sequence data was extracted from the Chromas files and edited using the 'SeqEd' program. The sequence was placed into the Blast search engine (NCBI database) to verify that the sequence was the canine homologue of IL-1Ra. Three independent sequences were then aligned using the 'pile-up' command to produce a consensus sequence for the canine IL-1Ra gene of 531 bp and the amino acid sequence was then predicted using the 'Translate' software on GCG. The sequence was finally submitted to the Genbank database.

3.3.5 Sub-cloning of therapeutic cDNAs into pTarget™ mammalian expression vectors

3.3.5.1 Primer design

Sense and anti-sense oligonucleotide primer pairs were designed according to the 5′ and 3′ ends of the canine IL-1Ra (cIL-1Raf and cIL-1Rar), sTNFRI (csTNFRIf and csTNFRIr), TIMP-1 (cTIMP-1f and cTIMP-1r) and TIMP-2 (cTIMP-2f and cTIMP-2r) gene sequences respectively (Table 3-2). A conserved kozak sequence (A**ATGG) was applied to all forward primers, flanking the start site, to provide optimal translation of the gene product. All primers were designed 'in frame' with a stop site (TAA) present in all reverse primers. Primers were synthesised and supplied by Sigma-Genosys.

Table 3-2 Primer sequences for the PCR amplification of canine IL-1Ra, sTNFRI, TIMP-1 and TIMP-2

Primer name	Oligonucleotide primer sequence 5′ to 3′	Tm (°C) & GC (%) content
cIL-1Raf	CCGGATATCATGGAAACCTGCAGGTGTCCT	78.0 °C : 53.3 %
cIL-1Rar	GGCTTATTCCTTCTGGAAGTAGAA	62.5 °C : 41.7 %
csTNFRIf	CCGGATATCATGGGCCTCCCCACCGTGCCT	85.5 °C : 66.7 %
csTNFRIr	GGCTTAGGTGGAGCCTGGGTCCTG	75.0 °C : 66.7 %
cTIMP-1f	CGGGTCGACTATGGCACCCTTTGCGCCCCTGGC	89.0 °C : 69.7 %
cTIMP-1r	CGGTTAGGCCATCCGGGGCCGCAGGGACTGCCA	91.4 °C : 72.7 %
cTIMP-2f	CGGGGTACCATGGGCGCCGCGGCCCGCAGCTT	94.8 °C : 78.1 %
cTIMP-2r	GGCTTATGGGTCCTCGATGTCGAGAAACTCCTG	79.1 °C : 54.5 %

3.3.5.2 PCR conditions

PCR reactions were performed using 100ng of canine pCR2.1/cIL-1Ra, (csTNFRI), (TIMP-1), (TIMP-2) samples as template in a total volume of 50µl containing 0.4µM of both sense primer and anti-sense primer pairs (Table 3-2), 0.3mM of dATPs, dCTP, dTTP and dGTP, 1mM $MgSO_4$ and 2 units of Platinum *Pfx* DNA Polymerase (GIBCOBRL UK). This high fidelity enzyme provided strict proof reading of the sequence to reduce the chance of introducing mutations. Using a PE 2400 thermal cycler, samples were subjected to an initial denaturation at 95°C for 5 minutes followed by 25 cycles of amplification, each cycle consisting of a denaturation step of 95°C for 30 seconds and annealing temperature of 60°C for 30 seconds, followed by an elongation step of 68°C for one minute.

3.3.5.3 Purification of PCR products and addition of dATPs

PCR products were analysed using TAE agarose gel (1.5%) electrophoresis and purified using the QIAquick®Gel Extraction kit (QIAGEN). Since Platinum *Pfx* DNA Polymerase can not add dATPs to the 3' ends of DNA, required for ligation to the complementary single dTTP residues found on the pTarget™ Mammalian Expression vector, an additional elongation step was then performed using *Taq* polymerase. As described earlier, *Taq* has a non template-dependent activity allowing the addition of a single deoxy-adenosine (dATP) to the 3' ends of duplex molecules. The purified DNA (35µl), in a total volume of 50µl containing 0.3mM of dATP, 1.5mM $MgCl_2$ and 1 unit *Taq* polymerase, was incubated at 72°C for 30 minutes. PCR products were purified following QIAquick®PCR purification kit protocol (QIAGEN) and eluted in 30µl of sterile water, 4µl of which were assessed by agarose gel electrophoresis.

3.3.5.4 Ligation and transformation canine therapeutic cDNAs

Freshly purified PCR products were directly ligated into the pTarget™ Mammalian Expression vector following the manufacturer's guidelines (Promega). The quantity of DNA for each ligation reaction was determined using the equation shown in section 2.2.2.8.1: where X is 60ng, Y is 0.6, Z is 5.67 and the molar ratio was 3. The ligation reactions were set up in a total volume of 10µl containing DNA as calculated above and 3 units of T4 DNA ligase and incubated at 16°C overnight. Subsequently, ligation reactions (1µl) were transformed into JM109 High efficiency Competent Cells (50µl). Transformed cells were spread onto agar plates containing ampicillin and incubated overnight at 37°C. Ampicillin resistant colonies containing the construct were selected from each of the plates and cultured overnight in LB/ampicillin broth at 37°C and glycerol stocks of each culture were prepared for long term storage.

3.3.5.5 Isolation and screening of recombinant plasmids

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep®PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50 µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. PCR product/pTarget vector constructs (500ng) were screened using *Eco*R1 restriction enzyme digestion as described in section 3.3.3.

3.3.5.6 Sequence evaluation of constructs

Recombinant plasmid samples (400 ng) were sequenced with 0.5µM of both sense (T7F) and anti-sense (pTargetR) vector-based primers (Table 3-3) using the ABI 310 genetic analyser as described earlier. Chromas files generated from the ABI 310 genetic analyser for each of the cDNAs were analysed using ClustalW software to align the correct predicted sequences with the sample sequences to check for the absence of mutations.

Table 3-3 Primers for sequencing pTarget/cDNAs

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
T7F:	TAATACGACTCACTATAGG	50.2 °C : 36.8 %
pTargetR:	AGATATGACCATGATTACGCCAAG	68.0 °C : 41.7 %

3.3.5.7 Plasmid vector maps

Using computer software, plasmid vector maps were drawn from the sequence data information incorporating all the important components of each vector including the cDNA sequence. CMV promoter, T7 promoter, polyadenylation signal and ampicillin resistance gene.

3.3.6 Expression of cDNAs

3.3.6.1 In vitro transcription/translation of cDNAs

The *in vitro* transcription/translation technique for expressing the canine cDNAs was chosen since it was a convenient, single tube reaction to verify that the cloned genes could be efficiently expressed from the mammalian expression vectors before employment in a tissue culture expression system. Using this technique the canine homologues of IL-1Ra, sTNFRI, TIMP-2 (Campbell et al., 2001b) and TIMP-1 (Zeiss et al., 1998) were expressed *in vitro* from the pTarget™ Mammalian Expression vectors using the TNT® T7 Quick Coupled Transcription/Translation System (Promega).

The TNT® Quick Coupled Transcription/Translation System enabled the *in vitro* eukaryotic transcription and translation of genes within circular plasmids, cloned down stream of a T7 RNA polymerase promoter. Using the radioisotope [^{35}S]methionine, the synthesised radioactively labelled proteins were then analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. To reduce the chance of RNase contamination, precautions were taken as outlined in section 2.2.3. More specifically, plasmid samples (500 ng) were mixed with TNT® Quick Master Mix (20 µl), [^{35}S]methionine (0.38MBq) to a final volume of 25 µl with nuclease free water and then incubated at 30°C for 90 minutes and stored at -20°C. The positive control plasmid used in this experiment was the Luciferase T7 Control DNA (4331 bp) for the expression of the firefly luciferase protein (61 kDa). A negative control was also employed containing no DNA to allow for the measurement of any background incorporation of labelled amino acids.

3.3.6.2 SDS-PAGE

Recombinant proteins were expressed and analysed using SDS-PAGE as described in section 2.2.9. A ready precast gel (8.6 cm x 6.8 cm x 1.0 mm) containing a 10 % resolving gel and a 4 % stacking gel with 10 wells was assembled in the Mini-Protean 3 Electrophoresis cell (Biorad, Herts). Protein samples (5 µl) were prepared by the addition of 20 µl of protein loading buffer, followed by

incubation at 100°C for 2 minutes and loaded onto the gel with a Wide Range (6.5 to 205 kDa) Color Markers molecular weight standard (10 µl) (Sigma). The gel was electrophoresed at 200 volts until the bromophenol blue dye reached the lower part of the gel and was then removed from the gel cast.

3.3.6.3 Protein Visualisation

The gel was fixed with 'fixing solution' for 30 minutes with slow agitation on an orbital shaker. The gel was then placed on a sheet of Whatman® 3MM filter paper, covered with plastic wrap and dried at 80°C for 3 hours under a vacuum using a gel drier. Once completely dry the gel was exposed to film for 20 hours at -80°C. The film was developed and then realigned with the gel to identify the positions of the molecular weight markers.

3.4 RESULTS

3.4.1 RNA quantity and quality

Full-length, total RNA was isolated from LPS stimulated canine PBMCs using RNAzol™B solution. Good quality RNA samples were obtained as determined by ultraviolet spectrophotometry (A260/A280 value of >1.6) and agarose gel electrophoresis assessment (Figure 3-3); the RNA migrated through an agarose gel (1%) revealing intact 18s and 28s ribosomal RNA components.

3.4.2 PCR amplification and cloning of the canine IL-1Ra cDNA

RNA samples (2μg) were subjected to first strand synthesis using MMLV-RT and random primers to produce canine cDNA. The cDNA samples were used as the template for the amplification of IL-1Ra gene using sense (IL-1Raf) and antisense (IL-1Rar) oligonucleotide primers and *Taq* DNA polymerase. The PCR products obtained migrated on a agarose gel (1%) to show a DNA fragment of approximately 550 bp (Figure 3-4). There were no bands present in the negative control reaction.

Subsequently the PCR products were cloned into pCR2.1®TOPO plasmid vectors and constructs were screened with *Eco*R1 restriction enzyme digestion for those containing the insert. *Eco*R1 restriction sites, present within the vector sequence, were positioned immediately upstream and downstream of the insert and released a 550 bp product on digestion. Plasmid clone numbers 2-4 were positive for a 550 bp product on restriction digestion and clone 1 contained an insert of a slightly larger size (Figure 3-5).

Figure 3-3 Assessment of RNA quality using TAE agarose gel electrophoresis.

The two distinct bands represent the undegraded sub-components (28s and 18s) of ribosomal RNA

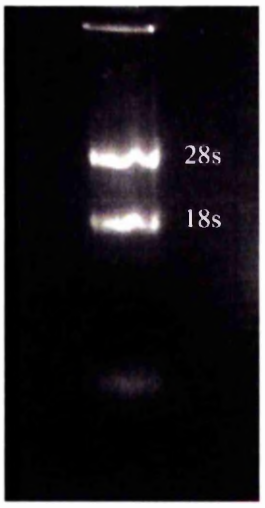


Figure 3-4 PCR amplification of IL-1Ra from canine cDNA.

A single PCR product was obtained which migrated on a agarose gel (1%) to show a DNA fragment of approximately 550 bp. No products were present in the negative control.

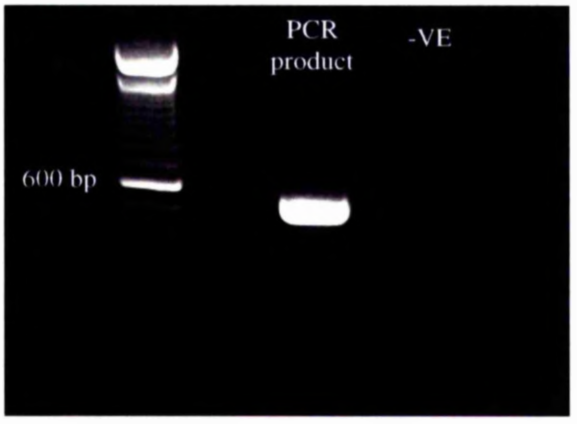
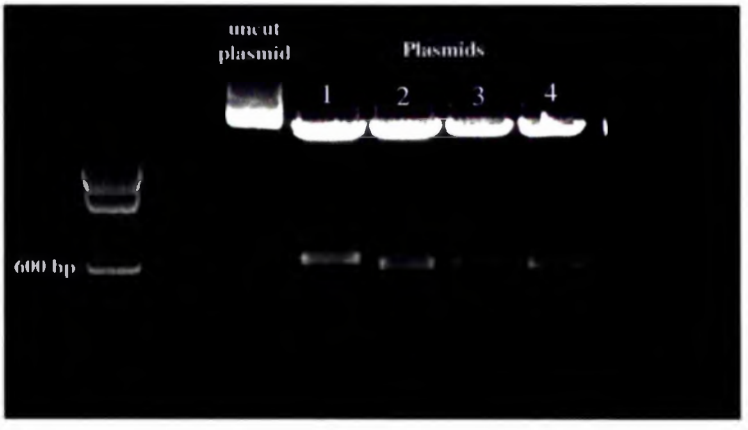


Figure 3-5 Restriction enzyme analysis of pCR2.1/cIL-1ra for positive clones

Plasmids 2, 3, 4 contain an insert of the correct size, approximately 550 bp, on *Eco*R I digestion



3.4.3 DNA sequence analysis of the canine IL-1Ra gene

Three independent PCR products independently cloned into pCR2.1®TOPO plasmid vectors and were selected and sequenced on an automated ABI310 genetic analyser using both forward (M13R) and reverse (M13F) vector-based primers. The three sequences were subjected to computational analysis.

3.4.3.1 Searching database with sequence

The nucleotide sequences determined from the genetic analyzer were assessed using the 'Blast' search engine within the NBCI database. The nucleotide sequence was compared to every gene sequence within the data bank to identify those genes with which this new sequence shared the greatest homology. The results revealed that the nucleotide sequences were comparable to the IL-1Ra sequence already cloned in other species, confirming that a unique canine IL-1Ra gene had been cloned.

3.4.3.2 Nucleotide and deduced amino acid sequence of canine IL-1Ra cDNA.

The sequence data obtained from the cloned individual PCR products were analysed using the GCG DNA sequence analysis software. The nucleotide sequences were aligned and examined for identical base pairs between sequences. Any anomalies were corrected by referring back to the appropriate chromas files until a consensus nucleotide sequence had been created. The canine IL-1Ra nucleotide sequence demonstrated a single open reading frame of 531 base pairs (Figure 3-6) encoding a protein of 177 amino acids (Figure 3-7) as determined by the GCG translation software.

3.4.3.3 Comparison of canine IL-1Ra nucleotide and protein sequences to other species.

Canine IL-1Ra was analysed in an alignment with the IL-1Ra nucleotide (Figure 3-8) and protein (Figure 3-9) sequences for the different species to identify regions of conserved sequence. The results showed that the canine sequence exhibited 85%, 84%, 85%, 88%, 83%, 88% similarity to human (Carter et al., 1990), murine (Zahedi et al., 1991), rabbit (Goto et al., 1992), equine (Kato et al., 1997), rat (Eisenberg et al., 1991) and porcine (Genbank L38849) sequences respectively (Table 3-4). A high level of sequence similarity was also evident at the protein level with canine IL-1Ra sequence demonstrating 79%, 77%, 76%, 79%, 75% and 83% amino acid sequence similarity with human, murine, rabbit, equine, rat and porcine sequences respectively (Table 3-4). The canine IL-1Ra amino acid sequence alignment demonstrated a conserved N-linked glycosylation site present at the amino acid position 109-111 and five cysteine residues at amino acid positions 25, 91, 94, 141 and 147.

Figure 3-6 The cDNA sequence of canine IL-1ra

The sequence has been deposited in Genbank DNA sequence database (Accession number: AF216526)

```
1    ATGGAAACCT GCAGGTGTCC TCTCAGCTAC CTAATCTCTT TCCTCCTTTT
51   CCTGTCCCAT TCAGAGACAG CCTGCCGTCC CTTGGGGAAG AGACCTTGCA
101  GGATGCAAGC CTTCAGAATC TGGGATGTTA ACCAGAAGAC CTTCTACCTG
151  AGGAATAACC AACTAGTCGC TGGATACTTG CAAGGATCAA ATACTAAATT
201  AGAAGAGAAG TTAGATGTGG TGCCCGTCGA GCCTCATGCC GTGTTCTTGG
251  GGATCCATGG GGGGAAGCTG TGCCTGGCCT GTGTCAAGTC TGGAGATGAG
301  ACCAGGCTCC AGCTGGAGGC CGTTAACATC ACTGACCTGA GTAAGAACAA
351  GGATCAAGAC AAGCGCTTTA CCTTCATCCT CTCAGACAGT GGCCCTACCA
401  CCAGCTTTGA GTCTGCTGCC TGCCCTGGCT GGTTCCTCTG CACAGCACTG
451  GAGGCCGACC GGCTTGTCAG CCTCACCAAC AGACCAGAAG AGGCCATGAT
501  GGTCACTAAG TTCTACTTCC AGAAGGAATA A
```

Figure 3-7 The deduced amino acid sequence of canine IL-1Ra

```
1   METCRCPLSY LISFLLFLSH SETACRPLGK RPCRMQAFRI WDVNQKTFYL
51  RNNQLVAGYL QGSNTKLEEK LDVVPVEPHA VFLGIHGGKL CLACVKSGDE
101  TRLQLEAVNI TDLSKNKDQD KRFTFILSDS GPTTSFESAA CPGWFLCTAL
151  EADRLVSLTN RPEEAMMVTK FYFQKE*
```

Figure 3-8 Alignment of the IL -1ra nucleotide sequences of different species

```
canine      ATGGAAACCTGCAGGTGTCCTCT-CAGCTACCTAATCTCTTTCCTCCTTTTCCT---GTC
mouse       ......AT....T..G.A-.C..A...TC..........C....T..CA....TCT..T
rat         ......AT.......G.A-.CT.A...TC..........C....T..CA....TCT..T
porcine     ......GTTA......A.-..G.G...T.........................---..T
human       ......AT......AG.C-.T.CG...TC........A..C.......C.....---..T
rabbit      ...AG.C...C....A.C-AC.CG...GC..........CC.......C.....---..T
equine      ......AT.C.....C..T..G.-...AC..........C..........TT---..T

canine      CCATTCAGAGACAGCCTGCCGTCCCTTGGGGAAGAGACCTTGCAGGATGCAAGCCTTCAG
mouse       T.........G..........C..T.CT.....A.....C....A..............
rat         T.G.......T....TG...AC..TGCT.....A.....C....A..............
porcine     ...C........T.......AC........A.............................
human       ............GAT......A....CT....GA.A.T.CA...A..............
rabbit      ......................C..T.CT.....A.................G........
equine      .T.C................AC..T.............C....A..............

canine      AATCTGGGATGTTAACCAGAAGACCTTCTACCTGAGGAATAACCAACTAGTCGCTGGATA
mouse       ..........AC..............T.........A..C.....G..CA.T.....G..
rat         ..........AC..............................C.....G..CA.T.....G..
porcine     ............C...........T.....................T....T........
human       ...............................T........C...........T........
rabbit      ................................T....A..C.................T..
equine      ................................A..................T........

canine      CTTGCAAGGATCAAATACTAAATTAGAAGAGAAGTTAGATGTGGTGCCCGTCGAGCCTCA
mouse       ...A......C......TC...C.......A...A....CA.......TA.T..C.T...
rat         ...A......C.......C...C.......A...A....CA.......TA.T..CTT..G
porcine     ..........C...........C.G.........A.............T..T........
human       ..........C.....GTC..T........A...A..........A...A.T........
rabbit      .........CC.....G.C...........A.G.A..............C.T........
equine      ........A................C.........A..............A.T......G.

canine      TGCCG-TGTTCTTGGGGATCCATGGGGGGAAGCTGTGCCTGGCCTGTGTCAAGTCTGGAG
mouse       .AGT.-...........C.....C.....C............T.T....C...........
rat         GAAT.-...........C.....C.....C............T.T................
porcine     .TTT.-.....C.............A................T................T.
human       ...TC-..........A........A......A........T................T.
rabbit      -..TCC.....C....C.....GA........T........T.T.....G........G.
equine      ...TC-.A...C....AC........A.................................T.

canine      ATGAGACCAGGCTCCAGCTGGAGGCCGTTAACATCACTGACCTGAGTAAGAACAAGGATC
mouse       ....T.T..A..............AA..............T.....C........A..AG
rat         ....C....A..............AG..............T....AC........A..AG
porcine     ......TG.A.......T....C..A...........A........A.......GC..G.
human       ..........A..............A.....................CG......GAA.G.
rabbit      ..A...TG.A......TT.........................G.C...........G.
equine      ......TT...T....AT.......A.....................C...........GG

canine      AAGACAAGCGCTTTACCTTCATCCTCTCAGACAGTGGCCCTACCACCAGCTTTGAGTCTG
mouse       ........................G...T..G.AA.....C.................A.
rat         ........................G...C..G.CA.................C..A..AC
porcine     .G..........C..........G...C..........C.................A.
human       .G...........CG.........G..............C........T..........
rabbit      .G...........C..........G...CA.T..C.............C...C.......
equine      .GA..........C..........G....A..........C...........C.......

canine      CTGCCTGCCCTGGCTGGTTCCTCTGCACAGCACTGGAGGCCGACCGGCTTGTCAGCCTCA
mouse       .......T..A..A..............A....A.....T.....T.C...G.......
rat         T......T..A..A..............A....A.....T..T.AT.CC..G.......
porcine     .C.....T.....C..............T.....A.....A....A..C...TG......
human       .C........C..T.................GA....A..T....A..CC..........
rabbit      .CT.......G........T........G..C........T....A..CC..........
equine      .C........................G..G.A......A.......CC..........

canine      CCAACAGACCAGAAGAGGCCATGATGGTCACTAAGTTCTACTTCCAGAAGG---AATAA
mouse       ......C...G......C..C.T..A.....G..............G.A.ACC....G
rat         ......C....A.....C..TGT.CA.....A..............G.A.ACC....G
porcine     ......CG..CA...CA...G.C.A......C..............C...ACC.G...
human       ....T.TG..T..C..A.G.G.C........C..A............G...ACG.G..G
rabbit      ......CG..C..C..CT....CG.......C..............G...ACC.G..G
equine      ......AG..CA.....T..T.C........C.........C.....G...ACC.G..G
```

Figure 3-9: Alignment of the IL-1ra amino acid sequences of different species

Alignment of different species: canine, murine, rat, porcine, human, rabbit and equine. Conserved cysteine residues are identified by an asterix (*) and the conserved N-linked glycosylation site is identified by an inverted arrow (^). The cleavage site, indicated by an arrow (↓) allows formation of the mature protein.

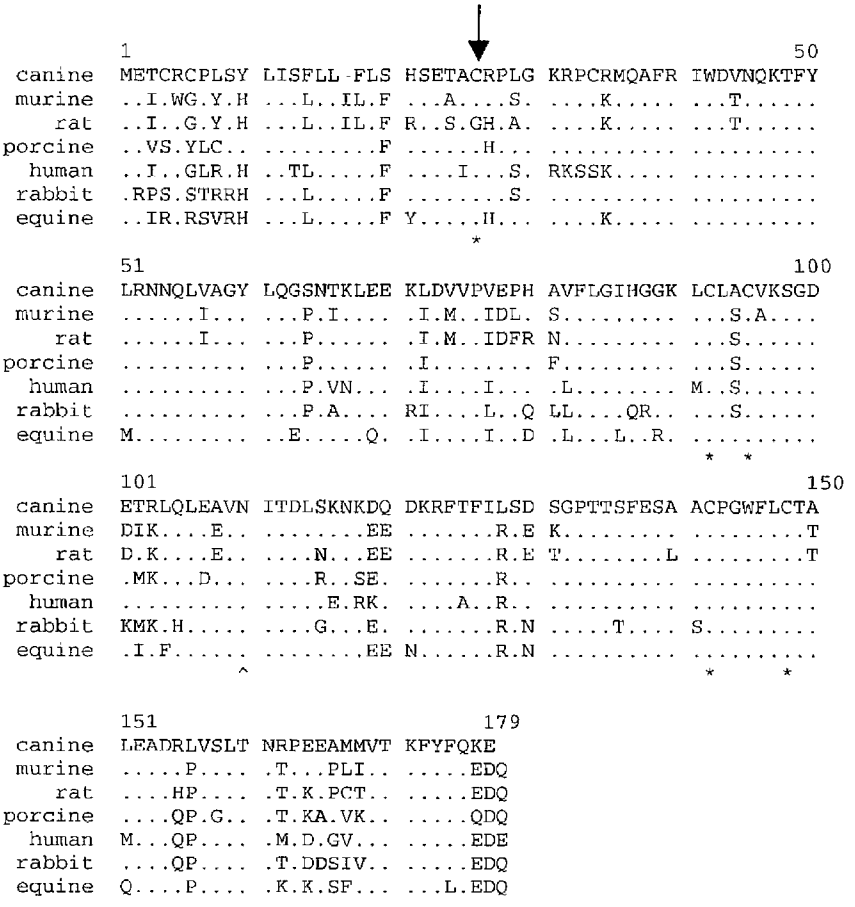


Table 3-4: The percentage of sequence homology between the canine IL-1ra nucleotide and amino acid sequences and other species

Species	Percentage of nucleotide homology to canine IL-1ra	Percentage of amino acid homology to canine IL-1ra
Equine	88	79
Pig	88	83
Human	85	79
Rabbit	85	76
Mouse	84	77
Rat	83	75

3.4.4 Sub-cloning of cDNAs into pTartget expression vector

PCR reactions were performed using canine pCR2.1/cIL-1Ra, (csTNFRI), (cTIMP-1), (cTIMP-2) samples as template and sequence-specific primer pairs with high fidelity Platinum *Pfx* DNA Polymerase. The PCR products obtained migrated on a TAE agarose gel (1.5%) to show DNA fragments of 550 bp, 600 bp, 620bp and 650 bp representing IL-1Ra, sTNFRI, TIMP-1, TIMP-2 respectively (Figure 3-10). Subsequently the PCR products were cloned into pTarget™ Mammalian Expression vector and screened with restriction enzymes for those containing the insert. One clone was selected from each and sequenced on an automated ABI310 genetic analyser using both forward (T7F) and reverse (pTarget) vector-based primers to confirm the sequence and ensure that no mutational errors had occurred during the cloning procedure. Vector maps for the canine cDNAs in pTarget mammalian expression vector (Promega) were drawn for pTarget/cIL-1Ra (Figure 3-11), pTarget/csTNFRI (Figure 3-12), pTarget/cTIMP-1 (Figure 3-13) and pTarget/cTIMP-2 (Figure 3-14).

3.4.5 Expression of cDNAs

The *in vitro* transcription/translation technique was used to express the cloned canine specific cDNAs from the pTarget mammalian expression vector. The radioactive isotope [^{35}S]methionine was used to visualise the proteins using autoradiography (Figure 3-15). The positive control showed a strong band at 61 kDa and there were no bands evident in the negative control. Bands of approximately 19.5 kDa, 22.8 kDa and 24.3 kDa were clearly visible for IL-1ra, TIMP-1 and TIMP-2 respectively providing evidence for their expression from the vector. No band was seen in the lane corresponding to the sTNFRI.

Figure 3-10 PCR amplification of the canine homologues of IL-1ra, sTNFRI, TIMP-1 and TIMP-2 for subcloning into the pTarget Mammalian expression vector.

The PCR products obtained migrated on a 1.5% TAE agarose gel to show a DNA fragments of 550 bp, 600 bp, 620bp, 650 bp representing IL-1Ra, sTNFRI, TIMP-1, TIPM-2 respectively

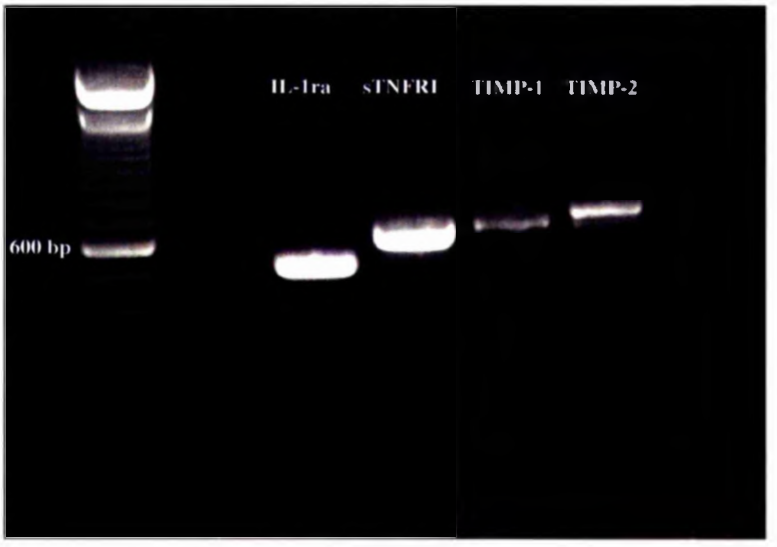


Figure 3-11 Vector map for pTARGET/cIL-1Ra

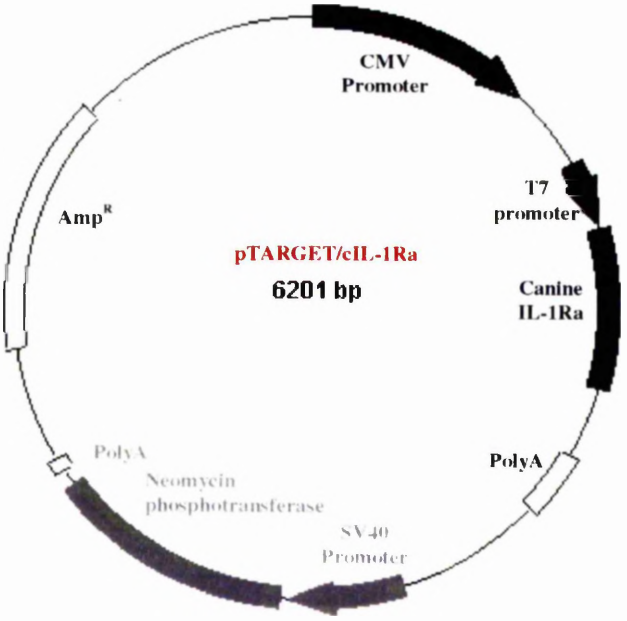


Figure 3-12 Vector map for pTARGET/csTNFRI

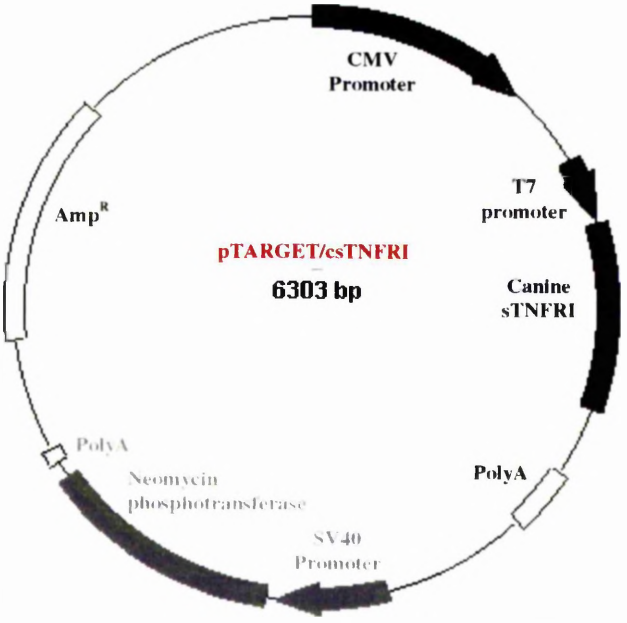


Figure 3-13 Vector maps for pTARGET/cTIMP-1

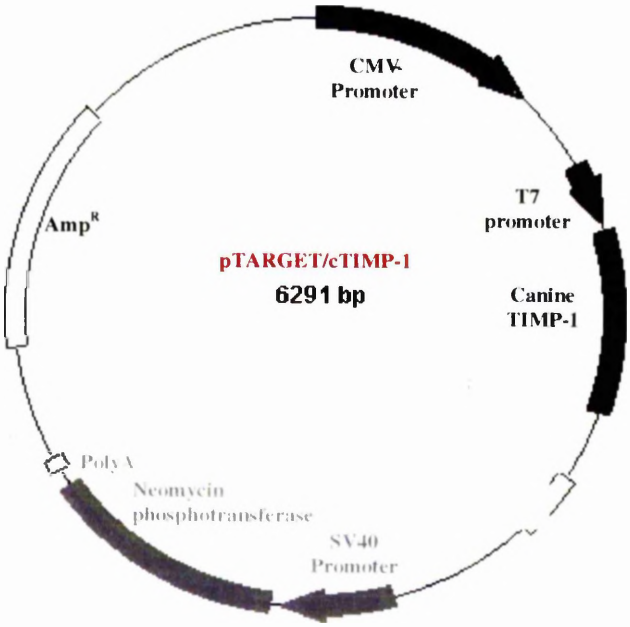


Figure 3-14 Vector maps for pTARGET/cTIMP-2

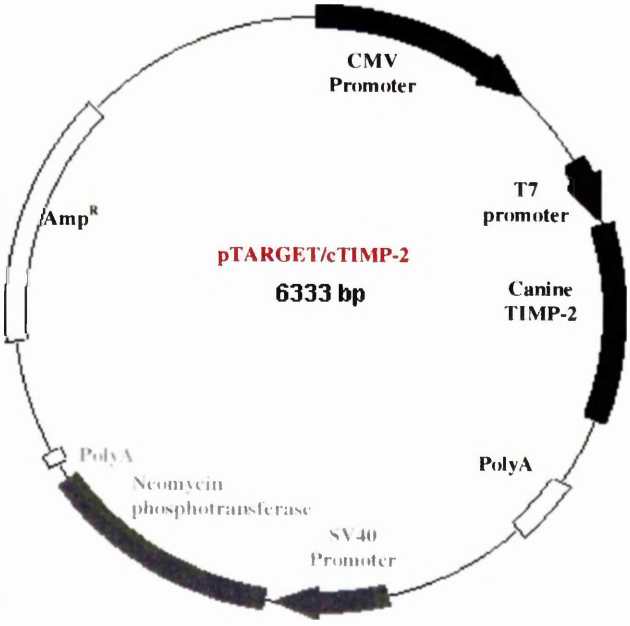
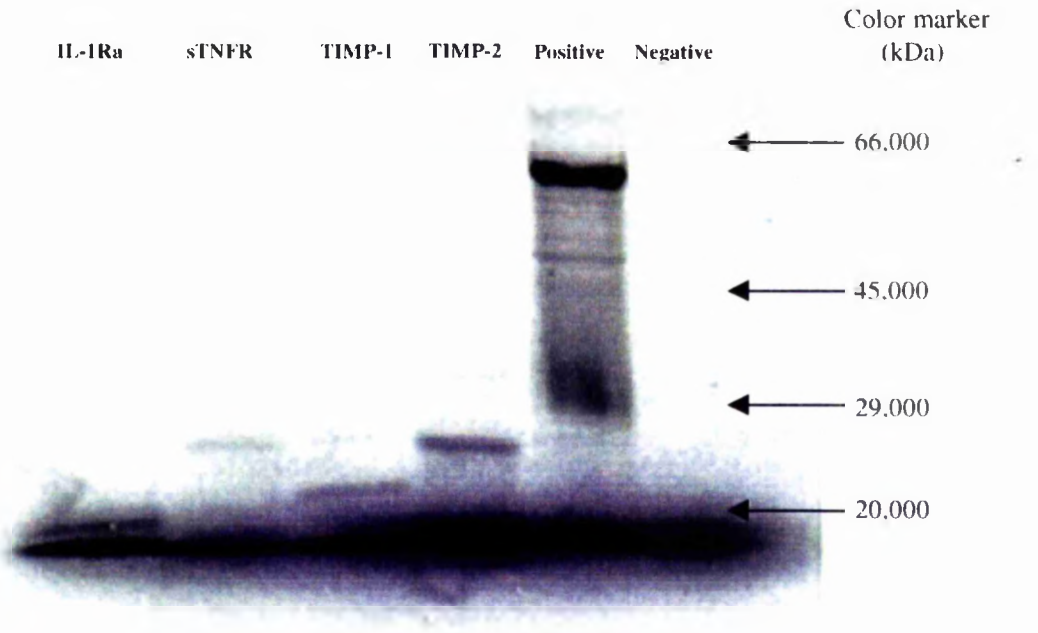


Figure 3-15 Expression of canine IL-1Ra, sTNFRI, TIMP-1 and TIMP-2 from pTarget mammalian expression vector.

The positive control showed a strong band at 61 kDa and there were no bands evident in the negative control showing low background levels. Bands of approximately 19.5 kDa, 22.8 kDa and 24.3 kDa were clearly visible for IL-1ra, TIMP-1 and TIMP-2 respectively providing evidence of their expression from the vector. No band was seen in the lane corresponding to the sTNFRI.



3.5 DISCUSSION

For the development of a homologous gene based therapy for canine OA, the canine homologue of IL-1ra was isolated, cloned and sequence analysed. Candidate canine specific proteins IL-1ra, sTNFRI, TIMP-1 and TIMP-2 were also analysed using *in vitro* transcription and translation techniques.

3.5.1 Prediction of canine IL-1Ra biological function based on sequence homology

The assumption that the cloned gene is the canine IL-1Ra equivalent is based on the high degree of sequence homology at both the nucleotide (Figure 3-8) and protein levels (Figure 3-9) with other species of IL-1Ra. The high level of nucleotide sequence conservation between the different species is not discussed in detail since it directly reflects the deduced amino acid sequence which is closely related to biological function.

The sequence alignments show many areas of homology including an N-linked glycosylation site present at amino acid positions 109-111 and five cysteine residues at positions 25, 91, 94, 141 and 147. The first cysteine residue at position 25 is thought to act as the cleavage site for the removal of the N-terminal 25 amino acid leader sequence via a leader peptidase to produce a single mature non-glycosylated protein of 152 amino acids (17kDa) (Eisenberg et al., 1990).

Extensive site-directed mutagenesis studies have been used to identify residues in IL-1Ra and IL-1 involved in the binding to IL-1RI. These studies revealed that the two IL-1α/β agonists contained two sites for IL-1RI binding whereas the IL-1Ra molecule only contained one. It was subsequently hypothesised that the presence of these two receptor binding sites was necessary for agonist activity (Evans et al., 1995a). Specific amino acids necessary for receptor activation once binding has occurred were subsequently identified by a series of amino acid substitution experiments where it was revealed that replacing the aspartate (D) residue at position 145 with lysine (K) conferred agonist activity to IL-1Ra. Furthermore, it was shown that the substitution of the β-pleated sheet structure (QGEESN) found within IL-1 at residues 48-53 into IL-1Ra sequence after position 51 or 53 resulted in a 3 to 4 fold increase in bioactivity (Greenfeder et al., 1995). The structural similarities of canine IL-1Ra with other species predict that the canine IL-1Ra will share the same basic biological functions. The high degree of homology within the sequences indicates the importance of this inhibitor among several mammalian species.

3.5.2 *In vitro* transcription/translation of the canine IL-1Ra, sTNFRI, TIMP-1 and TIMP-2

The *in vitro* transcription and translation of candidate therapeutic proteins was used to assess the expression levels from the pTarget mammalian expression vector system. Proteins of the predicted sizes for the non-glycosylated canine homologues of IL-1ra, TIMP-1, and TIMP-2 were evident on SDS PAGE analysis, although sTNFRI was not (Figure 3-15).

The predicted size for the canine non-glycosylated soluble IL-1ra protein was approximately 19.5kDa based on the pre-determined amino acid sequence. In comparison the human soluble IL-1Ra is translated with a leader sequence and promptly processed to a 17 kDa peptide, glycosylated, and secreted by cells as a 22 to 25 kDa species (Eisenberg et al., 1990). This protein is distinct from the intracellular form of IL-1Ra which is an 18 kDa peptide that lacks the leader sequence, is not glycosylated and remains within the intra-cellular space (Haskill et al., 1991). The predicted size for the canine non-glycosylated TIMP-1 is 22.8 kDa protein (Zeiss et al., 1998) which is larger than the human TIMP-1 described as a 20 kDa variably glycosylated protein of 184 amino acids (Carmichael et al., 1986). The predicted size for the canine non-glycosylated TIMP-2 was 24.3 kDa which again is larger than the human TIMP-2 protein which has been described as a 21 kDa non-glycosylated protein of 194 amino acids in its secreted form (Stetler-Stevenson et al., 1989).

The predicted size for the non-glycosylated sTNFRI molecule was 23.2 kDa; however expression of sTNFRI protein from the pTarget/sTNFR construct was not possible. This could be due to the presence of a strong hairpin secondary structure in the 5′ UTR of the plasmid sequence which can impair translation efficiency (Frances et al., 1992). There may also be an additional start codon (ATG), introduced into the 5′ end of the cloned region, that was not identified on sequence analysis. If the ribosome scans from the 5′ end of the RNA and begins translation at the first start site encountered, the generation of false sites may initiate translation prior to the desired start codon resulting in a frame shift of the open reading frame and the formation of a truncated protein. The sequence of the sTNFRI needs to be reanalysed before repeating the *in vitro* transcription translation procedure.

3.6 SUMMARY

This chapter describes the isolation and expression of the canine specific therapeutic gene IL-1Ra. Three additional candidate proteins were also analysed including sTNFR, TIMP-1 and TIMP-2. These therapeutic proteins were selected for incorporation into the gene-based therapy for canine OA based on the current understanding that they are intimately involved in the regulation of MMP activity in the joint where they play a critical role in tissue homeostasis.

Chapter IV

Cloning of canine matrix metalloproteinase -9 and -13 gene promoters

4.1 ABSTRACT

Levels of therapeutic gene expression are strictly controlled at the transcriptional level by regulatory regions of DNA termed promoters. Candidate promoters that have potential for use in driving disease-specific expression in arthritic joints include those that are up-regulated by pro-inflammatory cytokines and growth factors associated with the pathogenesis of osteoarthritis (OA). Since it is well documented that elevated levels of matrix metalloproteinases (MMPs) in the joint are primarily implicated in the degeneration of articular cartilage it was thought that MMP promoters would be useful in this targeting strategy. Many MMP genes and their promoters have been cloned and sequenced in a number of different species and considerable sequence homology has been demonstrated at both the nucleotide and amino acid levels. The canine homologues of MMP promoters were cloned for use in a homologous species-specific targeted gene transfer study for canine disease. This chapter describes the cloning and sequence analysis of the canine MMP-9 and MMP-13 gene promoters. The 5′ untranslated regions (UTRs) were obtained by genome walking upstream of the canine MMP-9 and -13 translation start sites using genomic DNA (gDNA) as template. DNA fragments of 1894 and 1494 bp were isolated and on analysis demonstrated regions of sequence homology with the equivalent promoter sequences already determined for other species. In general, conserved regions correlated with numerous putative DNA binding motifs including Activator Protein-1 (AP-1) sites and a Nuclear Factor (NF)-κB binding domain. A consensus TATA-(like) box was identified in each case and shown to direct transcription initiation from specific positions upstream of the translation start site.

4.2 INTRODUCTION

4.2.1 Targeting gene therapy

Successful gene therapy not only requires the identification of appropriate therapeutic genes but also on an accurate targeting system to limit therapeutic gene expression to specific cell types. Numerous gene delivery systems, both plasmid and viral, have been manipulated to improve targeting specificity at the cell surface and transcriptional levels. Targeting at the cell surface level can be achieved by manipulating the surface recognition components of viruses and liposomes and at the transcriptional level by incorporating transcriptional elements into plasmid or viral genomes such that the therapeutic gene is expressed only in certain cell types.

To target therapeutic gene expression at the level of the cell surface, retroviral vectors have been manipulated by replacing or altering the glycoprotein (Env) on the lipid envelope to recognise specific receptors on the host cell surface (Miller and Vile, 1995). Ligands, such as lactose, have also been coupled to the outside of retroviral particles allowing them to be recognised by asialoglycoproteins and internalised by hepatocytes (Neda et a., 1991). Adenoviral vectors have been targeted in a similar manner by covalently linking the fibre carbohydrate, responsible for viral binding and internalisation, to an asialoglycoprotein polylysine conjugate (Wu et al., 1994). Liposomes can also be targeted for *in vivo* use by displaying appropriate tropic and fusogenic such as transferrin used to increase specificity to bone marrow erythroblasts (Stavridis et al., 1987) and surfactant protein A for tropism to alveolar cells (Walther et al., 1993). Targeting of plasmid DNA can be achieved by coupling DNA to ligands with demonstrated cell or tissue affinity. For example, a polycation (e.g. polylysine) can be covalently linked to a ligand, to condense the plasmid DNA and expose the ligand on the cell surface (Michael and Curry, 1994) such as insulin (Rosenkranz et al., 1992), epidermal growth factor (EGF) (Chen et al., 1994) and lectins (Batra et al., 1994).

Gene therapy vectors can also be targeted at the genetic level by placing therapeutic gene expression under the control of regulatory promoter elements that possess binding sites specific for transcription factors required for controlling levels of gene expression. The transcriptional targeting approach for regulating therapeutic gene expression in response to the disease progression is discussed in this chapter.

4.2.2 Candidate disease-specific promoters

Mammalian matrix metalloproteinase (MMPs) constitute a family of proteolytic enzymes acting together to degrade the components of extra-cellular matrices thereby regulating connective tissue turnover and they have been implicated in many physiological processes including embryonic development and tissue remodelling (Matrisian, 1992). Furthermore, unregulated activity of MMPs have been associated with pathological processes such as tumour invasion (Nelson et al., 2000), heart failure (Spinale et al., 2000) and inflammatory processes such as arthritis (Martel Pelletier, 1999b). Of the MMPs, the gelatinase and collagenase groups have been intensively studied and associated with the development of arthritis. The gelatinase, MMP-9 and the collagenase, MMP-13

were therefore selected and the canine homologues of their promoters cloned and sequenced for driving disease specific expression of therapeutic genes.

4.2.2.1 Matrix metalloproteinase-9

The gelatinases are known to comprise two collagenases, MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) with substrate specificity for native type IV collagen, denatured collagen (gelatin), type V, VII, X, and XII collagens, elastin and vitronectin, but not type I collagen, proteoglycan or laminin (Huhtala et al., 1991). The MMP-2 gene is constitutively expressed from a wide range of cell types, in comparison the inducible MMP-9 gene is selectively expressed in neutrophils, monocytes and macrophages. Originally MMP-9 was identified as a product of polymorphonuclear leukocytes (Murphy et al., 1982) and pulmonary alveolar macrophages (Hibbs et al., 1987); yet it was not until the early 1990's that the sequence of the MMP-9 gene was determined (Huhtala et al., in 1990, 1991). The role of gelatinases in joint disease has been studied in man with elevated levels of MMP-9 demonstrated in human rheumatoid arthritis (RA) synovial fluid (Koolwikj et al., 1995). High levels of MM-9 have also been demonstrated in canine disease from both osteoarthritic (Coughlan et al., 1995) and rheumatoid synovial fluid samples (Coughlan et al., 1998) in addition to equine articular tissues (Clegg et al., 1997). More specifically, gelatinase B is intimately associated with the development of chronic synovitis (Grillet et al., 1997) and bone remodelling associated with the arthritic processes (Okada et al., 1995). The desire to determine the role of MMP-9, in both physiological and pathological states, has prompted the cloning and sequences analysis of the gene encoding this enzyme. As such the gene, along with the promoter in some cases, has been analysed in a number of different species including the human (Huhtala et al., 1991), rabbit (Fini et al., 1994), mouse (Masure et al., 1993) and rat (Eberhardt et al., 2000).

4.2.2.2 Matrix metalloproteinase-13

The collagenase group has been shown to comprise four different enzymes, MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase), MMP-13 (collagenase-3) (Cawston, 1996) and MMP-18 (Stowlow et al., 1996). The collagenases degrade fibrillar collagens cleaving all three α chains of Type I, II and III collagens at a single site, producing fragments approximately ¾ and ¼ the size of the original molecule, and in addition degrade type X collagen and serum amyloid A (Cawston, 1996). Human MMP-13 was first discovered in tissue samples taken from a breast carcinoma and found to be homologous to rat interstitial collagenase (MMP-1) (Frieje et al., 1994). However, there is only one rodent interstitial collagenase that has been described and this shares 86% homology with human MMP-13, but not with the human or rabbit MMP-1 (Schorpp et al., 1995). Elevated levels of MMP-13 have been found in a variety of connective tissues including osteoarthritic synovial tissue (Wernicke et al., 1996) and articular chondrocytes (Mitchell et al. 1996; Shlopov et al., 1997). Further evidence to support the role of MMP-13 in OA arose from studies showing that MMP-13 expression co-localised with type II collagen degradation in active OA lesions where it degraded type II collagen more rapidly than MMP-1 (Mitchell et al., 1996).

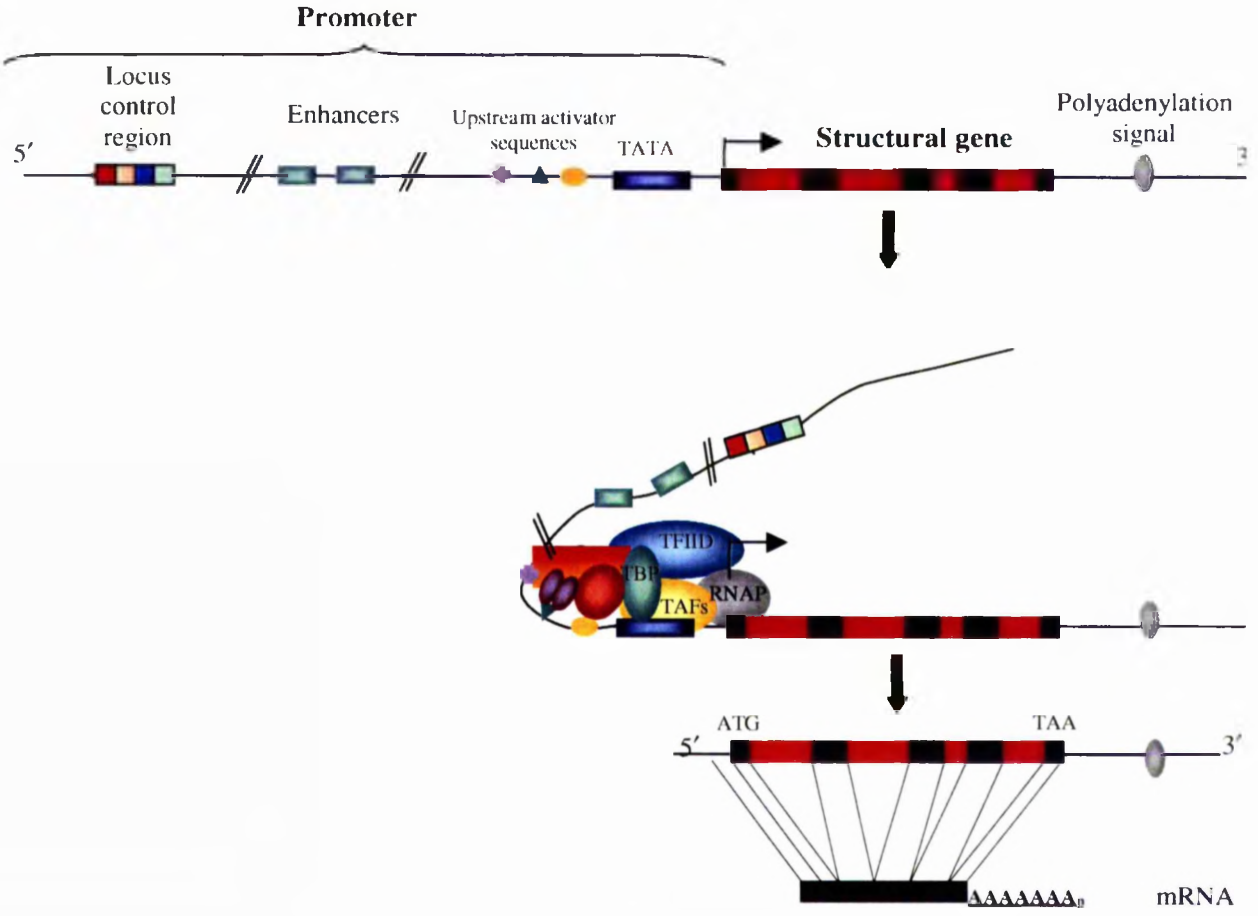
Additionally, inhibition of MMP-13 in cartilage explant models greatly reduced type II collagen destruction while its over-expression in transgenic mice caused cartilage destruction strongly resembling OA (Neuhold et al., 2001). The desire to determine the role of MMP-13 in both physiological and pathological states has prompted the cloning and sequence analysis of the gene encoding this enzyme. As such the genes, along with the promoter in some cases, has been analysed in a number of different species including human (Tardif, Pelletier et al., 1997) and rabbit (Vincenti, Coon et al., 1998). These gene sequences also show considerable sequence similarity to the collagenase-1 (interstitial collagenase) promoter region identified in the mouse (Schorpp, Mattei et al., 1995) and rat (Rajakumar and Quinn, 1996).

4.2.3 Construction of anti-arthritic therapeutic vectors

Disease-inducible promoters are evaluated using appropriate vector systems containing all the necessary regulatory sequences for efficient gene expression. The promoter region of a gene refers to the DNA sequence immediately 5′ to the translation start site containing those elements required for controlling the rate of gene transcription initiation. The DNA sequence of the promoter has no coding function, but contains a series of sequence motifs, ranging from a few to up to tens of nucleotides in length, that serve as sites for the interaction with *trans*-acting protein components of the transcriptional machinery. The locus control region (LCR) also serves to regulate genes positioned in the down-stream location. (Figure 4-1).

Figure 4-1 Transcription

The transcriptional unit in genomic DNA consists of the structural gene and the flanking extra-genic DNA containing the promoter. The position marked (⌐→) corresponds to the nucleotide position at which initiation of RNA transcription of the gene begins. this defines the start of the structural gene. The promoter is further dissected into component parts. the core promoter. upstream activator sequences (UAS). enhancers and locus control region (LCR). The binding of TBP and TAFs to the TATA box and formation of the TFIID complex results in the recruitment of RNA polymerase to the transcription initiation site. The folding of the DNA in this region allows transcription factors bound to up-stream DNA motifs to participate in promoter activation and gene transcription. In most cases the primary RNA transcript begins at a sequence which serves a non-coding function in the final mRNA product. The translation start codon (ATG: methionine) is found down stream of the transcription initiation site. in the first exon. The protein coding sequence of the RNA transcript terminates at the stop codon (TAA. TAG or TGA) and then continues into the 3′ region incorporating the polyadenylation signal (AATAAA) which signals the termination of the transcript and the addition of polyA residues. Splicing of the primary RNA transcript joins the different exons in a precise head to tail fashion followed by capping of the 5′ end giving rise to the final mRNA transcript.



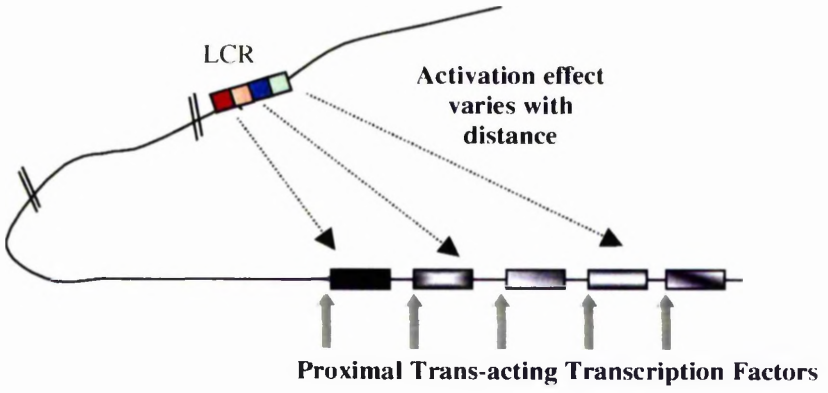
4.2.3.1 5′ Untranslated region

The promoter/regulatory region of the gene comprises four important components functioning together to recruit RNA polymerase to the transcription initiation site. These include the core promoter, adjacent up-stream activator sequences (UAS), enhancers and a locus control region (LCR) (Whalen, 1994). The core promoter can be defined as the 30 or 40 nucleotides immediately 5′ to the transcription initiation site. Within this short region most promoters usually contain a TATA box sequence motif $TAT(^{A}/_{T})(^{A}/_{T})(^{A}/_{T})(^{A}/_{T})$, one of the minimal elements necessary for transcriptional initiation providing low levels of transcription. Upstream activator sequences comprise a collection of regulatory elements found within several hundred nucleotides upstream of the core promoter. These motifs appear to play a major role in stimulating transcription by interaction with the core promoter elements. The enhancer region forms part of the complex array of regulatory elements, usually spanning several kb and functioning bi-directionally. They are also located throughout the coding region, within intronic sequence, and can also be found in the 3′ UTR.

The LCR is composed of several types of activator elements. Although the precise role of this region still needs to be defined, it has a major effect on gene expression. A series of *in vitro* and transgenic mouse studies of the human β-globin gene have shown that the LCR appears to confer position and copy number independent expression of the genes placed under its control suggesting that the LCR-promoter-gene complex functions as an independent regulatory unit. The LCR is considered an area of chromatin containing DNA sequence motifs accessible to numerous GATA-1, NF-E2 (Andrews et al., 1993) and SP1 proteins already known for their importance in promoter regions. However, these sequence motifs may not necessarily be involved in the binding of these proteins but instead have roles unique to the LCR. It has also been demonstrated that if a foreign promoter is experimentally introduced closer to the LCR than the endogenous gene that it regulates then the introduced transgene is activated at the expense of the endogenous one (Kim et al., 1992). The mechanism of this action is still not elucidated and requires further research; two current theories of action are shown in Figure 4-2.

Figure 4-2 Mechanism of action of the LCR

Two theories have been offered for the mechanism of action of the LCR using the human β-globin gene as an example. The first is the polar effect on expression on the different genes during development. It is thought that in this model the activation effect of the LCR is proportional to the distance from the individual promoters. The second model is that the differential expression of genes is due to competition between the different genes based on the promoter components. A final explanation may be a combination of both theories.



4.2.3.2 Transcription factors

The final component of a functional promoter is the presence of transacting factors (protein transcription factors); representing the transcriptional machinery that binds to specific DNA sequences within the 5′ regulatory region (Whalen, 1994). Several classes of these proteins exist which can be differentiated based on their protein structure which enables specific interaction with DNA or other proteins to form multimers (Harrison, 1991). Examples of protein domains that participate directly in the binding to DNA include the homeodomain proteins containing helix-turn-helix motifs and the Zinc finger containing proteins. Examples of protein domains that participate directly in the binding to other proteins and form multimers through a dimerisation motif are known as leucine zippers and include transcription factors c-Fos and c-Jun (Alber, 1992); the adjacent basic region allows the dimer to bind DNA. Dimerisation is an important feature of transcription factor formation especially where heterodimers can be formed since there is the potential for a combinatorial increase in the possible regulatory elements formed. An example of this is the AP-1 transcription factor complex containing both c-Jun and c-Fos proteins. The dimerisation process also applies to all proteins containing the helix-loop-helix (HLH) motif responsible for protein-protein interactions and includes c-myc and MyoD.

4.2.3.3 Gene transcription

The binding of transcription factors to the promoter regions of DNA is necessary for the process of transcription with the recruitment of RNA polymerase to the transcription initiation sites. Basal levels of transcription initiation involve the binding of specific proteins to the core promoter. A protein called TBP (TATA-binding protein) binds to the TATA box sequence within this region (Nikolov et al., 1992), in addition to other proteins called TAFs (TBP-Associated Factors) (Greenblatt, 1992). A protein complex is then built up around the TBP (referred to as TFIID) providing multiple points of interaction with factors binding to other regulatory elements capable of interacting with the TAFs of the core promoter protein-DNA complex (Gill and Tijan, 1992). The arrangement of these transcription factors at this site helps to position the RNA polymerase molecule in the vicinity of the start site so that accurate initiation can take place (Roeder, 1991). The basal level of gene transcription varies between promoters due to specific combinations of transcription factors TFIIB, TFIIE, TFIIF and TFIIE involved in the transcription initiation complex (Parvin et al., 1992). However, these levels can be increased with the involvement of the upstream activation sequences (UAS). Although the multiple UAS for the binding of transcription factors appear to lie within a linear sequence, the precise 3-dimensional arrangement is somewhat different and provides an important contribution to the level and specificity of transcriptional activity driven from the core promoter. It is now widely known that the interaction of the transcription factors with TBP and TAF complex invokes specific bending of the DNA to bring all necessary factors into contact; the actual linear sequence of the UAS may not be a crucial determinant of promoter function.

4.3 MATERIALS AND METHODS

Overviews of the experimental procedures employed in this chapter are illustrated in figure 4-3. Briefly, the canine MMP-9 and MMP-13 promoters were cloned from genomic DNA isolated from peripheral mononuclear cells (PBMCs) using GenomeWalking™ techniques (Clontech) (Figure 4-4) and primers designed on the 5′ end of the cDNA sequences encoding the canine MMP-9 and MMP-13 genes. Two walks were performed for each promoter and all PCR products generated were cloned into pCR2.1®TOPO and sequenced. A consensus sequence was generated for each promoter from three independent PCRs and the sequences were finally submitted to the Genbank database. Transcription initiation sites were determined using GeneRacer™ techniques (Invitrogen) (Figure 4-5) within both the endogenous MMP-9 and MMP-13 gene sequences and the cloned promoter sequence within pGL3/cMMP-9(1894), pGL3/cMMP-13(1494) constructs.

Figure 4-3 Overview of the experimental procedure used to clone canine MMP-9 & -13 promoters

The canine MMP-9 and MMP-13 promoters were cloned from gDNA using GenomeWalking™ techniques (Clontech) and the subsequent sequences analysed. The transcription initiation sites were determined using GeneRacer™ techniques (Invitrogen) for both the endogenous MMP-9 and MMP-13 genes and the cloned promoters in pGL3/cMMP-9(1894). pGL3/cMMP-13(1494).

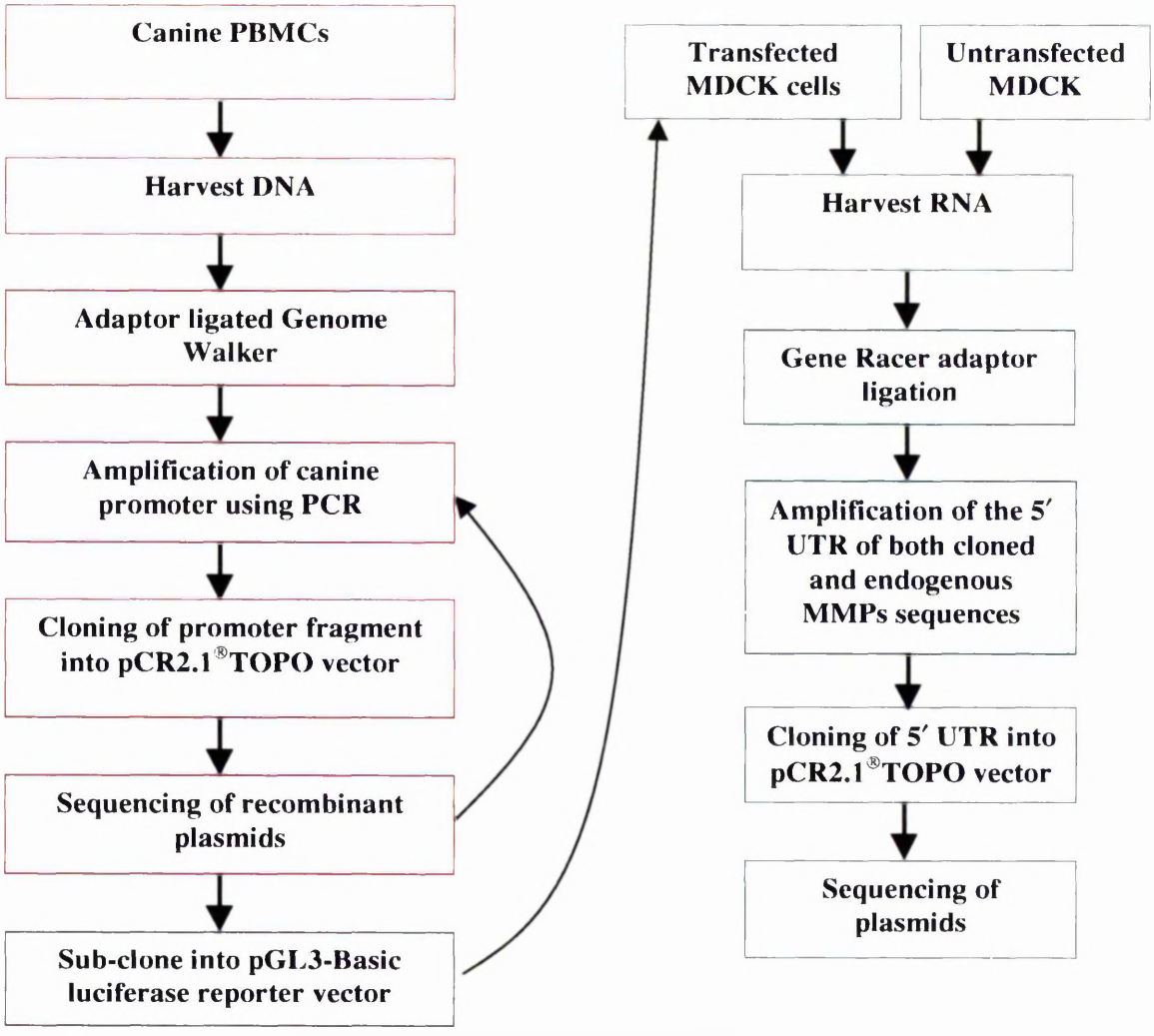


Figure 4-4: Summary of Genome Walking

Canine genomic DNA (gDNA) was first digested with four different restriction enzymes. Genome Walker adaptors were then ligated to 5′ ends of the cut fragments to form four libraries. These libraries were then used as template for each Genome™Walk consisting of two PCR amplifications using sense adaptor primers and anti-sense gene specific primers (GSP).

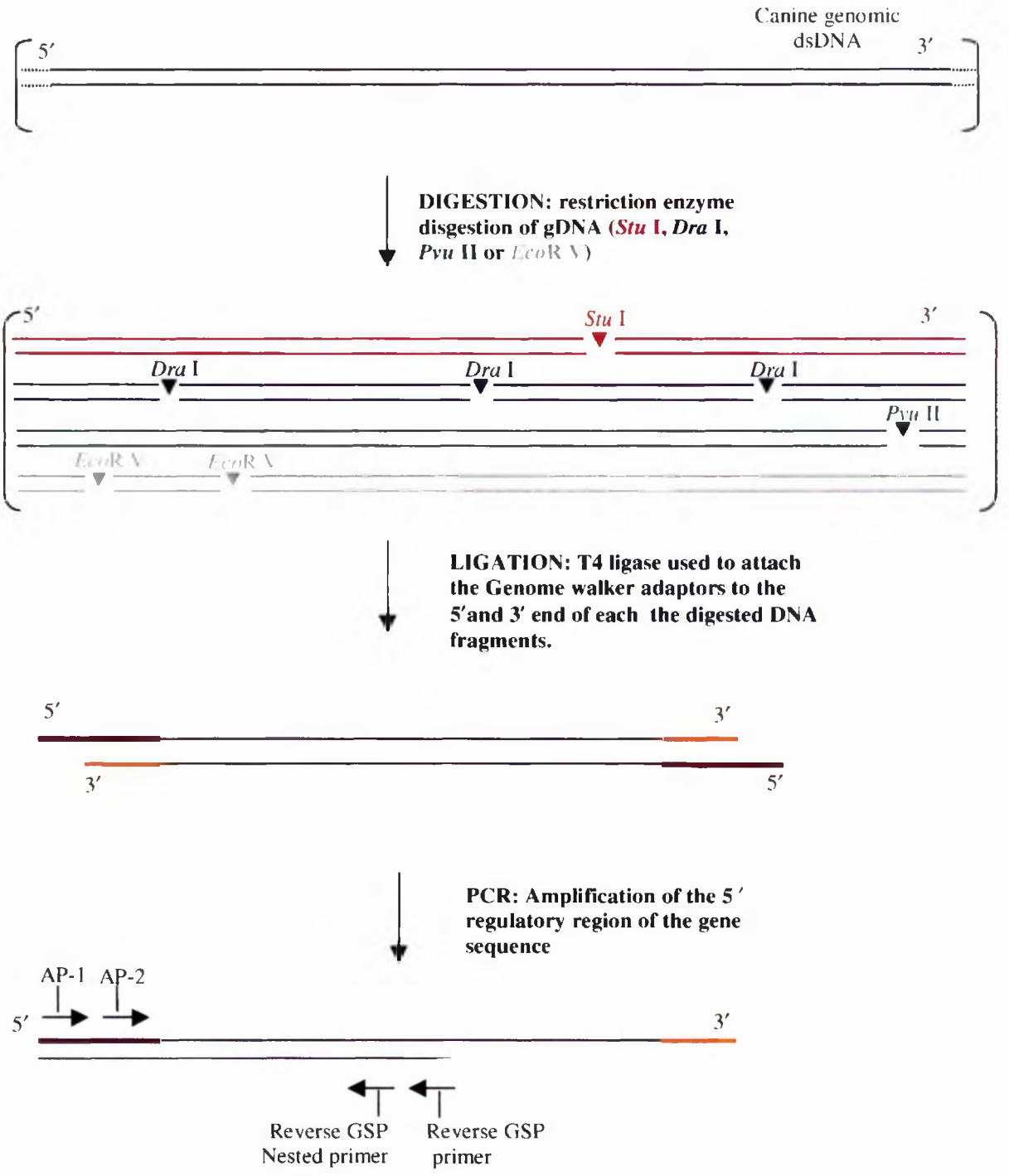
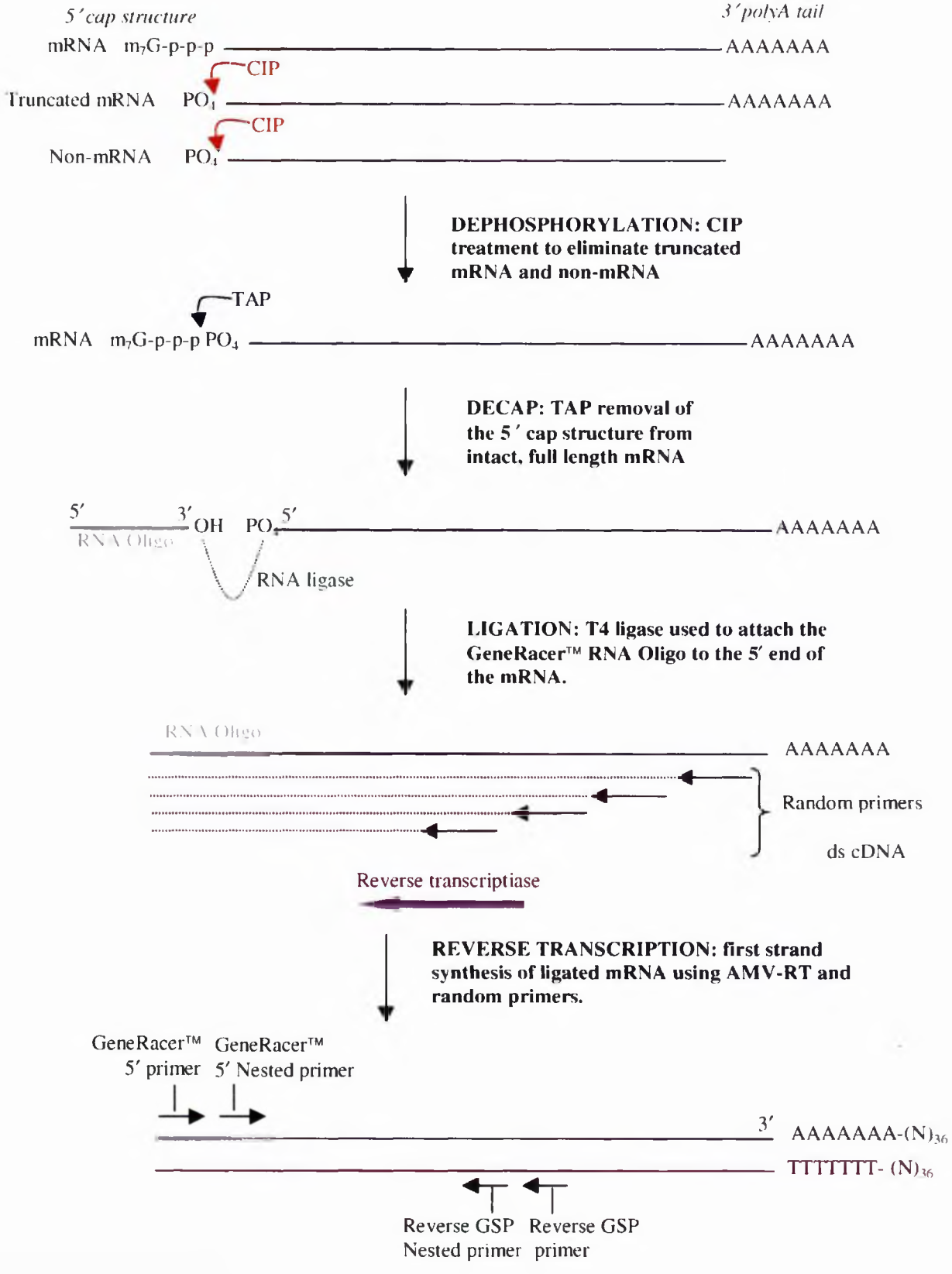


Figure 4-5: Summary of GeneRacer™ protocol

The isolated RNA was dephosphorylated and decapped before ligating to the GeneRacer™ RNA Oligo. Reverse transcription was then performed using random primers followed by PCR using reverse primers based on the 5′ ends of the MMP-9, MMP-13 or luciferase cDNA sequences.



4.3.1 Amplification of canine MMP-9 & -13 gene promoters, 5′ UTRs using GenomeWalker™ techniques

The GenomeWalker™ technique (Clontech) provides access to a novel method for walking up-stream, towards promoters, in gDNA from known cDNA sequences (Siebert et al., 1995) and is a simpler and potentially faster approach than those protocols involving the generation and screening of gDNA libraries. In summary, canine gDNA was digested with four different restriction enzymes and GenomeWalker Adaptors were then ligated to 5′ ends of the cut fragments to form four libraries. These libraries were then used as templates for each genome walk consisting of two PCR amplifications using sense adaptor primers and anti-sense gene specific primers (Figure 4-4). The gene specific primer (GSP) sequences were based on the 5′ end of the canine MMP-9 and MMP-13 cDNAs available in the Genbank database. PCR products were cloned and sequenced allowing the design of new gene specific primers for the second genome walk.

4.3.1.1 Primer design

Two primers per cDNA sequence were designed for each nested PCR as described in section 2.2.5.1 and were synthesised and supplied by MWG-Biotech (Table 4-1). Each primer of 25-28 nucleotides in length required 40-60% GC content to ensure that primers would effectively anneal to the template at the recommended annealing and extension temperature of 67°C. Primer sequences were designed to prevent the folding and formation of intra-molecular hydrogen bonds and particular attention was paid to reducing the GC content at the 3′ end of the gene specific primers to prevent their binding to the 3′ end of the adaptor primers.

4.3.1.1.1 MMP-9 promoter specific primers

Using these criteria, two anti-sense oligonucleotide primers (MMP-9GSP1R and MMP-9GSP2R) were designed according to the 5′ region the MMP-9 cDNA sequence. On completion of the first genome walk it was possible to design two more anti-sense oligonucleotide primers (MMP-9GSP3R and MMP-9GSP4R) for the second genome walk along the MMP-9 promoter sequence (Figure 4-12).

4.3.1.1.2 MMP-13 promoter specific primers

Two anti-sense oligonucleotide primers (MMP-13GSP1R and MMP-13GSP2R) were also designed according to the 5′ region the MMP-13 cDNA sequence. On completion of the first genome walk it was possible once again to design two more anti-sense oligonucleotide primers (MMP-13GSP3R and MMP-13GSP4R) for the second genome walk along the MMP-13 promoter sequence (Figure 4-14).

4.3.1.1.3 GenomeWalker Adaptor primers

Forward primers, specific to the Adaptor sequence attached to the 5′ end of the gDNA fragments, were supplied with the kit and are referred to as Adaptor primer-1 (AP1) and nested Adaptor Primer-2 (AP2) (see 4.3.1.4)

Table 4-1 Primer sequences for genome walking

Primer name	Oligonucleotide primer sequence 5′to 3′	Tm (°C) & GC (%) content
MMP-9GSP1R	TGGAAAGACCACAACGGTGGGCTTGTG	68.0 °C : 55.6 %
MMP-9GSP2R	ATAGAGCAGCAGCCCAGCACCAGGAACAC	70.9 °C : 58.6 %
MMP-9GSP3R	AGGGAGAGAGTTAAGGCTACAGGACTC	66.5 °C : 51.9 %
MMP-9GSP4R	TAAGTGGTCAGCCTAAGGGCAAGGGAT	66.5 °C : 51.9 %
MMP-13GSP1R	CTGCAAGCTGGAAGTCTTCCTCAGACA	66.5 °C : 51.9 %
MMP-13GSP2R	AGCACTGAGTCCAGCTCAAGAAGAGGA	66.5 °C : 51.9 %
MMP-13GSP3R	CCCTCTGTAGAAAAGCAGCTACTTTTACTC	65.4 °C : 43.3 %
MMP-13GSP4R	CAAGAGTATCAAGATGATGCTCTCACTTCC	65.4 °C : 43.3 %
AP1	GTAATACGACTCACTATAGGGC	58.4 °C : 45.4 %
AP2	ACTATAGGGCACGCGTGGT	58.8 °C : 57.9 %

4.3.1.2 Preparation of canine genomic DNA from peripheral blood mononuclear cells

Any canine cell line could be used for isolating canine gDNA for use as a template in the isolation of the promoter sequences, since gDNA is identical in every cell, so PBMCs were selected. Canine whole blood samples (5 ml) were mixed with lysis mix (45 ml) and incubated on ice for 10 mins. The mixture was then centrifuged at 2800g for 10 mins and 4°C and the pellet re-suspended in nuclei lysis mix (3 ml). After addition of 10% SDS (200µl) and 75µl of proteinase K (10mg/ml) the mixture was incubated at 55°C for 2 hours. Two extractions with 3ml phenol/chloroform (50:50) were performed, each centrifuged for 10 mins at 2800g and room temperature. The upper aqueous phase was then transferred to a fresh tube and the DNA precipitated by adding 3 volumes of 100% ethanol. The DNA was then re-suspended in 250µl of TE Buffer and the quantity and quality of gDNA was assessed using UV spectrophotometry and agarose gel electrophoresis.

4.3.1.3 Digestion and purification of canine genomic DNA

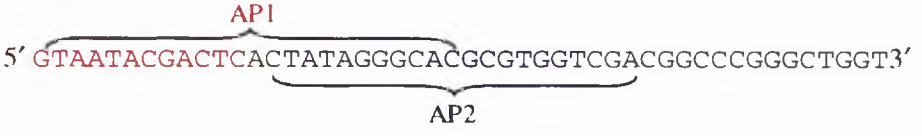
Four blunt ended digestions of canine gDNA (2.5µg) were performed, each in a total volume of 100µl with the appropriate restriction enzyme *Stu* I, *Dra* I, *Pvu* II or *Eco*R V (80 units) and restriction enzyme buffer (1x) combination (Table 2.1). Reactions were incubated at 37°C overnight before samples (5µl) were analysed for complete digestion by TAE agarose gel (1%) electrophoresis. For the purification of DNA, phenol (95µl) was added to each reaction, vortexed and flash spun to separate the aqueous and organic phases. The upper aqueous layer from each tube

was mixed with an equal volume of chloroform added, vortexed and briefly spun to separate the aqueous and organic phases. This time the DNA in the upper layer was precipitated with 2 volumes of ethanol (95%), 1/10 volume 3M NaOAc (pH 4.5) and 20µg of glycogen. After vortexing and centrifugation at 15,000rpm for 10 minutes, supernatants were decanted and the pellet washed in 100µl ethanol (80%). After a final centrifuge at 15,000 rpm for 5 minutes and supernatant discarded the pellet was air-dried and dissolved in 20µl of Tris-EDTA (10/0.1, pH 7.5). The quality of gDNA was again assessed using TAE agarose gel analysis.

4.3.1.4 Ligation of digested canine gDNA to GenomeWalker Adaptors

The digested, purified DNA (4µl) was ligated to the GenomeWalker Adaptors (25µM) in a total volume of 8 µl with T4 DNA ligase (4 units) and ligation Buffer (1x) at 16°C overnight. The reactions were incubated at 70°C for 5 minutes before dilution to a total volume of 80 µl with Tris-EDTA (10/1 pH 7.4).

GenomeWalker Adaptor sequence



4.3.1.5 PCR-based DNA Walking in GenomicWalker Libraries

The four different libraries were subjected to two rounds of PCR (primary and secondary) using a PE 2400 thermal cycler. For the first genome walk, the primary round of amplification used the adaptor-ligated gDNA as template with 0.2µM of sense primer 'Adaptor Primer 1' (AP1) and anti-sense 'Gene Specific Primers' (MMP-9GSP1R or MMP-13GSP1R) (Table 4-1), *Tth* PCR Reaction Buffer (40 mM Tris-HCl, 15 mM KOAc, 0.02% Triton X-100), dNTPs (0.2mM), $Mg(OAc)_2$ (1.1mM) and Advantage Genomic Polymerase Mix (1x) (5 units of *Tth* DNA polymerase, 0.5µg of TthStart Antibody, 1% glycerol, 0.2 mM Tris-HCl pH 7.5 and 4.6 mM KCl) (see 2.1.7.5) in a total volume of 50µl. Primary PCR conditions were 94°C for 2s, 70°C for 3 mins for 7 cycles, and 94°C for 2s, 65°C for 3 mins for 32 cycles, then 65°C for 4 mins. Primary PCR products were diluted 50 fold and 1µl was used as a template for secondary, nested PCR with 0.2µM of sense primer 'Adaptor Primer 2' (AP2) and anti-sense 'Gene Specific Primers' (MMP-9GSP2R or MMP-13GSP2R) (Table 4-1) in a reaction mix as described for primary PCR. Secondary PCR conditions were 94°C for 2s, 70°C for 3 mins for 5 cycles, and 94°C for 2s, 65°C for 3 mins for 20 cycles, then 65°C for 10 mins.

The second genome walk used anti-sense 'Gene Specific Primers' (MMP-9GSP3R and MMP-13GSP3R) in the primary round of PCR and nested primers (MMP-9GSP4R and MMP-13GSP4R) in the secondary round of PCR. PCR conditions were altered slightly with the annealing temperature reduced from 70 to 69°C and 65 to 64°C in secondary PCR. A negative control containing all PCR components (primers, dNTPs, PCR buffer and Advantage Genomic Polymerase

Mix) except template was performed to ensure no contamination of the PCR reactions with extraneous DNA that might serve as a template for PCR amplification

4.3.2 Cloning of canine MMP-9 and MMP-13 promoter fragments

PCR products were cloned using methods described in section 2.2.2.

4.3.2.1. Purification, ligation and transformation

PCR products were purified following QIAquick®Gel Extraction Kit (QIAGEN, UK) and eluted in 50µl of sterile water, 4µl of which were assessed by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a 1 kb molecular weight standard. Purified PCR products were directly ligated into the pCR®2.1-TOPO plasmid vector following the TOPO TA® cloning protocol. The ligation reactions (2µl) were then transformed into One Shot™ TOP10 competent cells and spread onto agar plates containing ampicillin and X-gal for incubation overnight at 37°C. White, ampicillin resistant, colonies were selected from the plates and cultured overnight in LB/ampicillin broth at 37°C and glycerol stocks of each culture were prepared for long-term storage.

4.3.2.2 Isolation and screening of recombinant plasmids

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. Constructs were screened for inserts using PCR techniques in a total volume of 50µl containing 0.4µM of both sense primer (AP2) and appropriate anti-sense primer (MMP-9GSP2R, MMP-9GSP4R, MMP-13GSP2R and MMP-13GSP4R), 0.2mM of dATPs, dCTP, dTTP and dGTP, 1.5 mmol/L $MgCl_2$ and 2 units of *Taq* DNA polymerase (QIAGEN, UK). Using a PE 480 thermal cycler, samples were subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of amplification with each cycle consisting of a denaturation step of 95°C for one minute and annealing temperature of 58°C for 1 minute, followed by an elongation step of 72°C for one minute. A final elongation step at 72°C for 30 minutes completed the reaction. PCR products (8µl) were visualised by gel electrophoresis on a TAE agarose gel (1%) to visualise those constructs containing the insert.

4.3.3 Sequence evaluation of constructs

Recombinant plasmids were sequenced and analysed as described in section 2.2.6. Briefly, plasmid samples were prepared for sequencing with PCR reactions using plasmid DNA samples (400 ng) and 0.5µM of both sense (M13F) and anti-sense (M13R) vector-based primers and the Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism). Samples were amplified, and the DNA purified by precipitation methods before samples were loaded into the ABI 310 genetic analyser for generation of automated sequence data.

Sequence data was stored and managed on a UNIX computer system using Genetics Computer Group (GCG) software (University of Wisconsin). 'Raw' sequence data was extracted from the Chromas files generated from the ABI 310 genetic analyser and edited using the 'SeqEd' program. The Blast search engine (NCBI database) was used to verify that the sequences were the canine homologues MMP-9 and MMP-13 promoter sequences.

4.3.4 Isolation and sequence analysis of the canine MMP-9 (1894 bp) and MMP-13 (1494 bp) promoter fragments

4.3.4.1 Primer design

Primers pairs were designed for the amplification of the canine MMP-9 and MMP-13 promoter fragments as described in section 2.2.5.1 and were synthesised and supplied by MWG-Biotech (Table 4-2). Each primer was 30 nucleotides in length but the GC content and therefore Tm varied between the primers, as the 3′ region of DNA over which the reverse primers could be designed was limited. However, the design of forward primers at the 3′ region was more flexible and attempts were made to match Tm of primer pairs as close as possible.

4.3.4.1.1 MMP-9 promoter specific primers

Using these criteria, two oligonucleotide primers, cMMP-9F and cMMP-9R, were designed according to the 5′ and 3′ regions of the MMP-9 promoter sequence respectively to amplify the 1894 bp promoter fragment (Figure 4-12).

4.3.4.1.2 MMP-13 promoter specific primers

Two oligonucleotide primers, cMMP-13F and cMMP-13R, were also designed according to the 5′ and 3′ regions the MMP-13 promoter sequence respectively to amplify the 1494 bp promoter fragment (Figure 4-14).

Table 4-2 Primers to amplify the canine MMP-9 and -13 promoter sequences

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
cMMP-9F	GGTCTGGGTGACTCCAAAGCCAATGCTCAT	69.5 °C : 53.3 %
cMMP-9R	GGTGAGGGTAGTGGTGTGTCTAGCTACTAG	69.5 °C : 53.3 %
cMMP-13F	GCGTTGCTGGTTGAGCCCAATTCTAATCTC	68.1 °C : 50.0 %
cMMP-13R	CTTGGTGGTTGTGCCTGGAGCCTGCTGTCT	72.2 °C : 60.0 %

4.3.4.2 Amplification and cloning of canine MMP-9 and MMP-13 promoter fragments

PCR was performed as described in section 2.2.5 to amplify the canine MMP-9 and -13 promoter sequences from gDNA. More specifically, canine gDNA (150ng) samples were amplified in a total

volume of 50µl containing 0.4µM of both sense primers (cMMP-9F and cMMP-13F) and anti-sense primers (cMM-9R and cMMP-13R), 0.2mM of dNTPs, 1.5mmol/L $MgCl_2$, and 2 units of *Taq* polymerase (QIAGEN, UK) (see 2.1.7.3). Using a PE 2400, thermal cycler samples were subjected to an initial denaturation at 95°C for 5 minutes followed by 35 cycles of amplification, each cycle consisting of a denaturation step of 95°C for one minute and annealing temperature of 60°C for 1 minute, followed by an elongation step of 72°C for two minutes. A final elongation step at 72°C for 30 minutes completed the reaction. PCR products were analysed by electrophoresis on a agarose gel (1.5%) and cloned into pCR®2.1-TOPO vector (Invitrogen) as described for the genome walking promoter fragments. Clones were screened for insert using PCR reagents and conditions as described for their original amplification.

4.3.4.3 Sequence evaluation of constructs containing whole MMP-9 and MMP-13 promoter fragments

4.3.4.3.1 Primer design for sequencing

Primers were designed for sequencing the internal region of the canine MMP-9 and MMP-13 promoter fragments. Each primer was varied in length and GC content. All primers were synthesised and supplied by MWG-Biotech (Table 4-3). Two oligonucleotide primers (MMP-9SEQF and MMP-9SEQ2F) were designed according to the internal sequence of the canine MMP-9 promoter sequence (Figure 4-12). One oligonucleotide primer (MMP-13SEQF) was designed according to the internal region the canine MMP-13 promoter sequence (Figure 4-14).

Table 4-3 Primers for the internal sequencing of the canine MMP-9 and –13 promoter sequences

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
MMP-9SEQF	CCAATAATGGCCATGGTCGTT	57.9 °C : 47.6 %
MMP-9SEQ2F	TATTATTCATTCATGAGAATC	48.1 °C : 23.8 %
MMP-13SEQF	GTCCTTGTCTTCTTGTGTTCCGGACCT	66.5 °C : 51.9 %

4.3.4.3.2 Sample preparation and data analysis

Recombinant plasmid samples were prepared for sequencing as described previously with PCR reactions using plasmid DNA samples (400 ng) and the Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism). A series of different primers (0.5µM) were used to cover the entire length of each promoter fragment. These included both vector-based primers M13F and M13R (see Table 2-2) and sequence based primers cMMP-9F, cMMP-9R, cMMP-9GSP3R, cMMP-9GSP4R, MMP-9SEQF, and MMP-9SEQ2F for canine MMP-9 promoter sequence and MMP-13F, MMP-13R, MMP-13GSP3R, MMP-13GSP4R and MMP-13SEQF for canine MMP-13 promoter sequence (Tables 4-1, 4-2 and 4-3). Samples were amplified, and the DNA purified by precipitation

methods before samples were loaded into the ABI 310 genetic analyser for generation of automated sequence data.

Sequence data was stored and managed on a UNIX computer system using Genetics Computer Group (GCG) software. 'Raw' sequence data was extracted from the Chromas files and edited using the 'SeqEd' program. Three independent sequence derived from three different PCR products were aligned using the 'pile-up' command to produce a consensus sequence for the canine MMP-9 and MMP-13 promoter sequences. The promoter sequences were then further analysed for DNA binding motifs using the web-based 'Sequence Motif Search' (http://motif.genome.ad.jp/) for searching nucleic acid sequence motifs. The sequences were finally submitted to the Genbank sequence database.

4.3.4.3.3 Alignment of the canine sequence with homologous sequences published for other species

The canine sequence was compared to those sequences already published for others species using ClustalW (http://www.ch.embnet.org/software/ClustalW.html). This software enables the easy manipulation and alignment of regions of homologous (DNA binding motifs) between sequences rather than finding the best-fit over the whole promoter sequence. For this application the chromas files were converted to 'FASTA' format and saved as 'Word' documents where they were manipulated into the correct format. All conserved regions were identified.

4.3.5 Determination of transcription initiation sites using GeneRacer™ techniques

The Rapid Amplification of cDNA ends (RACE) technique is a simple PCR-based approach for the identification of transcription initiation sites. The Gene™Racer Kit (Invitrogen) is based on RNA ligase-mediated (RLM-RACE) and oligo-capping RACE methods. This results in the selective ligation of the RNA oligonucleotide (GeneRacer™ RNA Oligo) to the 5′ ends of the decapped mRNA using T4 RNA ligase (Maruyama and Sugano, 1994; Volloch et al., 1994; Schaefer, 1995). In summary, RNA samples were first dephosphorylated and decapped before GeneRacer™ RNA Oligo adaptors were ligated to the 5′ ends mRNA molecules. Reverse transcription was then performed using random primers and the cDNA generated used as template for each PCR reaction consisting of two PCR amplifications using sense Gene™Racer primers and anti-sense gene specific primers (see Figure 4-4). All PCR products isolated were then cloned and sequenced to identify the position of transcription initiation.

This protocol is based on RT-PCR to amplify the 5′ end of cDNA generated from the endogenous expression of the canine MMP-9 and MMP-13 genes in the canine MDCK cell line. However, to identify the transcription initiation sites within the cloned MMP-9 and MMP-13 promoter sequences the pGL3/cMMP-9(1894) and pGL3/cMMP-13(1894) luciferase reporter constructs (as described in chapter V) were utilised. The constructs were transfected into the MDCK cells

(although any cell line could be used) with the PCR reverse primers based on the luciferase reporter gene.

4.3.5.1 Primer design

Two primers per gene sequence were designed for nested PCR as described in section 2.2.5.1 and were synthesised and supplied by SigmaGenosys (Table 4-4). Each primer of 23-28 nucleotides in length required 50-70% GC content to obtain a high annealing temperature (70°C) for improving the binding specificity within the PCR. Low GC contents at the 3′ end of primer sequences minimised extension by DNA polymerase at non-target sites. Additionally, no self-complementary sequences within the primer itself or to the kit-based primers prevented primer dimer formation. All gene specific primers were located approximately 400 bp downstream of the translation start site to optimise the PCR.

4.3.5.1.1 MMP-9 gene specific primers

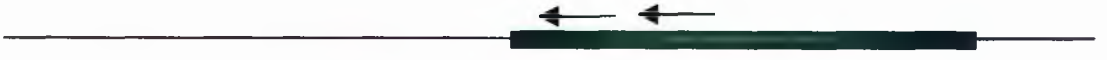
Using these criteria, two anti-sense oligonucleotide primers (MMP-9TISR and MMP-9TISnestedR) were designed according to regions within the canine MMP-9 cDNA sequence to identify the transcription initiation site within the endogenous promoter (Figure 4-13).

4.3.5.1.2 MMP-13 gene specific primers

Two anti-sense oligonucleotide primers (MMP-13TISR and MMP-13TISnestedR) were also designed according to regions within the MMP-13 cDNA sequence to identify the transcription initiation site within the endogenous promoter (Figure 4-15).

4.3.5.1.3 Firefly luciferase gene specific primers

Two anti-sense oligonucleotide primers (FFLR and FFLnestedR) were designed according to regions within the firefly luciferase cDNA sequence present in the pGL3-Basic reporter vector to identify the transcription initiation site within the luciferase plasmid vectors, pGL3/cMMP-9(1984) and pGL3/cMMP-13(1494).



Luciferase reporter gene

4.3.5.1.4 GeneRacer™ primers

Forward primers specific to the GeneRacer™ RNA Oligo sequence ligated to the 5′ end of the cDNA fragments were supplied with the kit and are referred to as GeneRacer™ 5′ Primer and GeneRacer™ 5′ Nested Primer (see 4.3.5.8).

Table-4-4 Primer sequences for RACE techniques

Primer name	Primer sequence (5′ to 3′)	TM (°C) & GC (%) content
MMP-9TISR	GTCGTCGAAGTGGGCGTCTCCCTGAAT	78.1 °C : 59.3 %
MMP-9TISnestedR	TTCTTCCCATCGAAGGGATACCCATCT	65.0 °C : 48.1 %
MMP-13TISR	CCCAGGAGGAAAAGCATGAGCCAGAAGA	76.8 °C : 53.6 %
MMP-13TISnestedR	GGACCACTTGAGAGTTCGGGGGAAAAC	74.8 °C : 55.6 %
FFLR	CGCGGGCGCAACTGCAACTCCGATAAA	69.5 °C : 59.3 %
FFLnestedR	TTCATAGCTTCTGCCAACCGAACGGA	64.8 °C : 50.0 %
GeneRacer™ 5′ Primer	CGACTGGAGCACGAGGACACTGA	74.0 °C : 60.9 %
GeneRacer™ 5′ Nested Primer	GGACACTGACATGGACTGAAGGAGTA	79.1 °C : 50.0 %

4.3.5.2 Cell line and transfections

Madin Darby Canine Kidney (MDCK) cells, maintained in DMEM with gluatamax-I medium described in section 2.2.1.2.1, were seeded onto 6 well plates at a concentration of 6 x 10^4 cells/ml. The pGL3/cMMP-9(1894) and pGL3/cMMP-13(1494) luciferase reporter vectors (construction described chapter V) were transiently transfected into cells using the cationic liposome mediated transfection system, TransFast™ Transfection Reagent (see 2.2.7). Briefly, transient transfections were carried out using DNA (2 µg per well) in a 1:1 ratio with the TransFast™ Reagent, in a total volume of 1 ml of serum free media per well. After one hour of incubation at 37°C, cells were left to recover overnight in supplemented media and 48 hours later all cells were harvested.

4.3.5.3 Isolation of total RNA

To enable the amplification of the 5′ UTR of the MMP-9 and MMP-13 promoters, purified full-length RNA was essential as the template and therefore all preparation material was free from ribonuclease activity. The media was removed from the 6 well plates and RNAzol™ B solution (500µl/well) was added directly to the wells containing the adherent MDCK cells for lysis. Aliquots (1ml) of the lysed cell solution were shaken for 15 seconds with chloroform (100µl) and then the RNA isolated as described in section 2.2.3. The RNA samples were stored in 70% ethanol at -20°C until required.

4.3.5.4 DNase Treatment and removal of reagents

The RNA was resuspended in 40µl of DEPC treated water and quality analysed by spectrophotometry and agarose gel electrophoresis. Contaminating DNA was removed using DNA-free™ (Ambion) methods (2.2.3.4). Briefly, 0.1 volume of 10x DNase I and 2 units of DNase I were added directly to the RNA (35µl) samples, mixed and incubated at 37°C for two hours. The enzyme was inactivated by the addition of DNase Inactivation Reagent, which was later removed, and the supernatant containing the pure RNA was stored at –20°C.

4.3.5.5 Dephosphorylation of RNA

Phosphatase treatment of total RNA removed the 5′ phosphates to eliminate truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo. Briefly, in a total volume of 10µl, total RNA (1µg) was incubated with 10 units of Calf Intestinal Phosphatase (CIP), CIP buffer (1x) and RNASEOUT™ (40 units) for 1 hour at 50°C.

4.3.5.6 Purification of RNA

RNA was then purified using the following precipitation protocol. DEPC water (90µl) was added to the reaction with phenol:chloroform (100µl), vortexed for 30 seconds before centrifugation for 5 minutes at 14,000 rpm. The aqueous (top) phase was transferred to a new tube and the RNA precipitated with 2µl mussel glycogen (10mg/ml), 10µl 3M sodium acetate (pH 5.2) and 220µl ethanol (95%). After briefly vortexing, the RNA was rapidly frozen on dry ice for 10 minutes. The RNA was then pelleted at 14,000 rpm for 20 minutes at 4°C and the supernatant carefully removed. 500µl of ethanol (75%) was then added, mixed and centrifuged at 14,000 rpm for 2 minutes at 4°C, the supernatant was once again removed and the pellet air dried for 2 minutes before re-suspension in 7µl of DEPC water.

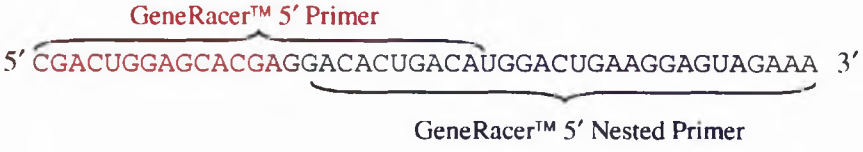
4.3.5.7 Removal of the mRNA cap structure

Pyrophosphatase treatment of dephosphorylated RNA removed the 5′ cap structure from intact, full-length mRNA. This treatment left a 5′ phosphate required for ligation to the GeneRacer™ RNA Oligo. Briefly, in a total volume of 10µl, dephosphorylated RNA (1µg) was incubated with 0.5 units of tobacco acid pyrophosphatase (TAP), TAP buffer (1x) and RNASEOUT™ (40 units) for 1 hour at 37°C. RNA was precipitated as described in 4.3.5.6 and re-suspended in 7µl of DEPC water.

4.3.5.8 Ligation of the GeneRacer™ RNA Oligonucleotide to the decapped mRNA

Ligation of the GeneRacer™ RNA Oligos' to the 5′ ends of the dephosphorylated, decapped mRNA was performed using T4 ligase. The GeneRacer™ RNA provided a known priming site for the GeneRacer™ PCR primers after transcription of the mRNA to cDNA.

GeneRacer™ RNA Oligo sequence:



Briefly, dephosphorylated, decapped mRNA (7µl) was added to the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25µg), mixed and heated to 65°C for 5 minutes to relax the RNA secondary structure. The RNA/oligo mixture was then chilled on ice before ligation in a total

volume of 10µl incubated with 5 units of T4 RNA ligase, Ligase Buffer (1x), 1mM ATP and RNASEOUT™ (40 units) for 1 hour at 37°C. RNA was precipitated as described in 4.3.5.6 and re-suspended in 10µl of DEPC water.

4.3.5.9 First strand cDNA synthesis

The synthesis of cDNA followed the basic principles as set out in section 2.2.4. Reverse transcription of the ligated mRNA used reverse transcriptase and random primers to create RACE-ready cDNA with known priming sites at the 5′ ends. Briefly, random primers (1µl) were added to RNA samples (10µl), heated for 5 minutes at 65°C to remove any RNA secondary structure and then quenched on ice for two minutes. First strand synthesis reactions were performed using the heat treated RNA in a 20µl reaction mix containing 5 units of Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), Reverse Transcription (RT) Buffer (1x), 5mm dNTPs and RNASEOUT™ (40 units). The reaction was incubated at 42°C for 1 hour followed by 85°C for 15 minutes to inactivate AMV-RT. A PE 480 thermal cycler was used for the reaction.

4.3.5.10 PCR amplification

Amplification of the 5′ ends used a reverse gene-specific primer and forward GeneRacer™ 5′ Primer homologous to the GeneRacer™ RNA Oligo. Only mRNA with the GeneRacer™ RNA Oligo ligated to the 5′ end completely reverse-transcribed would be amplified during PCR. Primary rounds of PCR were performed using Ready·To·Go™ PCR Beads (Amersham) and a PE 2400 thermal cycler. Canine cDNA samples (2µl) were mixed in a total volume of 25µl with 0.4µM of both sense primer (GeneRacer™ 5′ Primer) and gene specific anti-sense primers (MP9TISR, MP13TISR) (Table 4-4), 0.2mM of dNTPs, 50mM KCl, 1.5 mM $MgCl_2$ and 1.5 units of *Taq* DNA polymerase. Reaction conditions as shown in table 4-5 employs touch down PCR (Don et al., 1991; Roux, 1995) that exploits the high annealing temperatures of the primers used in this protocol to selectively amplify the gene specific cDNAs tagged with the GeneRacer™ RNA Oligonucleotide. By starting at a high annealing temperature only gene-specific or GeneRacer™-tagged cDNAs are amplified, allowing the desired product to accumulate. Decreasing the annealing temperature through the remaining PCR cycles permits efficient, exponential amplification of the tagged, gene specific template.

Table 4-5 PCR conditions for RACE protocols

Temperature	Time	Cycles
94°C	2 minutes	1
94 °C	30 seconds	
70 °C	3 minutes	5
94 °C	30 seconds	
68 °C	30 seconds	5
72 °C	3 minutes	
94 °C	30 seconds	
68 °C	30 seconds	45
72 °C	3 minutes	
72 °C	10 minutes	1

A second round of nested PCR was then performed, again using Ready·To·Go™ PCR Beads (Amersham) and a PE 2400 thermal cycler. Primary PCR samples (0.5 µl) were mixed in a total volume of 25µl with 0.4µM of both sense (GeneRacer™ 5′ Nested Primer) and nested gene specific anti-sense primers (MP9TISnestedR, MP13TISnestedR) (Table 4-4), 0.2mM of dNTPs, 50mM KCl, 1.5 mM $MgCl_2$ and 1.5 units of *Taq* DNA polymerase. Samples were subjected to an initial denaturation at 94°C for 2 minutes followed by 25 cycles of amplification, each cycle consisting of a denaturation step of 94°C for 30 seconds and annealing temperature of 68°C for 30 seconds, followed by an elongation step of 72°C for 1 minute. A final elongation step at 72°C for 10 minutes completed the reaction.

4.3.5.11 Cloning into pCR®2.1-TOPO vector

PCR products were cloned using methods described in section 2.2.2. Briefly, PCR products were purified using the QIAquick®Gel Extraction kit (QIAGEN) and purified products were directly cloned into the pCR®2.1-TOPO plasmid vector (3.9kb) following TOPO TA® cloning protocol. DNA constructs (500ng) were screened using PCR techniques as described in the secondary/nested PCR reaction (see 4.3.5.10) for those clones containing an insert.

4.3.5.12 Sequence evaluation of the transcription initiation sites

Recombinant plasmids were sequenced and analysed as described in section 2.2.6. Briefly, plasmid DNA samples were prepared for sequencing with PCR reactions using plasmid DNA samples (400 ng) and vector-based primers M13F (0.5µM) on the ABI 310 genetic analyser.

Ten positive clones for each initiation site were sequenced and the Chromas files were analysed to identify the transcriptional start sites relating to both the endogenous and vector-based promoter sequences for each MMP promoter. The transcriptional start sites were then mapped on the promoter sequence as shown in Figure 4-16 and 4-17 for MMP-9 and MMP-13 respectively.

4.4 RESULTS

4.4.1 Isolation of the canine MMP-9 and MMP-13 promoter sequences using GenomeWalking™ techniques .

4.4.1.1 DNA quantity and quality

gDNA was isolated from canine PBMCs and the quality of samples assessed by ultraviolet spectrophotometry (A260/A280 value of >1.6) and agarose gel electrophoresis (Figure 4-6). The gDNA migrated through the agarose gel (1%) consistent with high molecular weight molecules. The gDNA was completely digested with four restriction enzymes and analysed using agarose gel (1%) electrophoresis (Figure 4-7). The digested gDNA migrated through the gel over a wide range of molecular weights demonstrated by the long smears.

4.4.1.2 PCR Amplification of the promoter fragments.

Digested canine gDNA samples were used as the template for the amplification of the canine MMP promoter regions using anti-sense oligonucleotide primers (Table 4-1) and Advantage®Genomic Polymerase Mix. In all cases the negative control showed no contamination.

4.4.1.2.1 MMP-9 promoter genome walk

The first genome walk amplified a PCR product that migrated on an agarose gel (1.5%) to show a single DNA fragment of approximately 600 bp within the *Stu* I library (Figure 4-8); no bands were observed in the other lanes. The second genome walk amplified two PCR products that migrated on an agarose gel (1.5%) to show DNA fragments of approximately 1 kb and 1.5 kb for the *Dra* I and *Pvu* II libraries respectively (Figure 4-9). Again no bands were visible in any of the other libraries.

4.4.1.2.2 MMP-13 promoter genome walk

The first genome walk amplified a PCR product that migrated on a agarose gel (1.5%) to show a single DNA fragment of approximately 550 bp within the *Stu* I library (Figure 4-10). The second genome walk amplified a PCR product that migrated on a agarose gel (1.5%) to show a DNA fragment of approximately 1.1 for the *Dra* I library (Figure 4-11).

Figure 4-6 Purified genomic DNA samples

The genomic DNA samples migrated through an agarose gel showing good quality, high molecular weight DNA. The absence of DNA smears suggested undegraded DNA

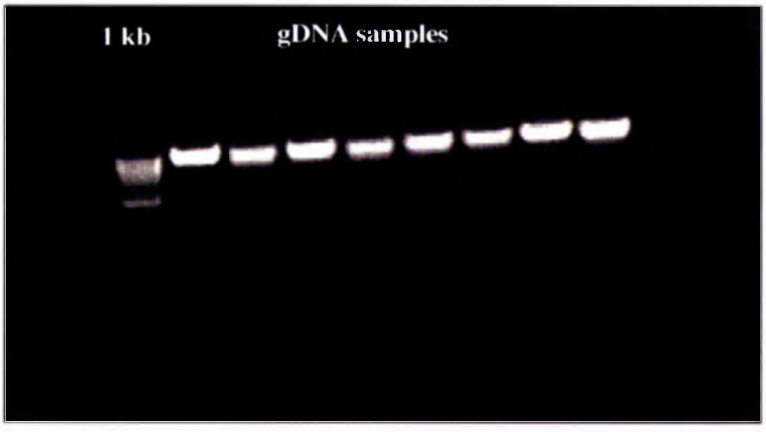


Figure 4-7 Digested genomic DNA samples pre (a) and post (b) purification

Genomic DNA samples were digested with four different restriction enzymes (EcoRV, Pvu II, Dra I and Stu I) and analysed for complete digestion, by agarose gel electrophoresis. The DNA migrated through the gel with a range of molecular weights shown by the long smears on the agarose gel; all samples have been completely digested.

Figure 4-8 First genome walk for the MMP-9 promoter sequence

The first genome walk amplified a PCR product that migrated on an agarose gel to show a single DNA fragment of approximately 600 bp within the *Stu* I library, no bands were observed in the other lanes.

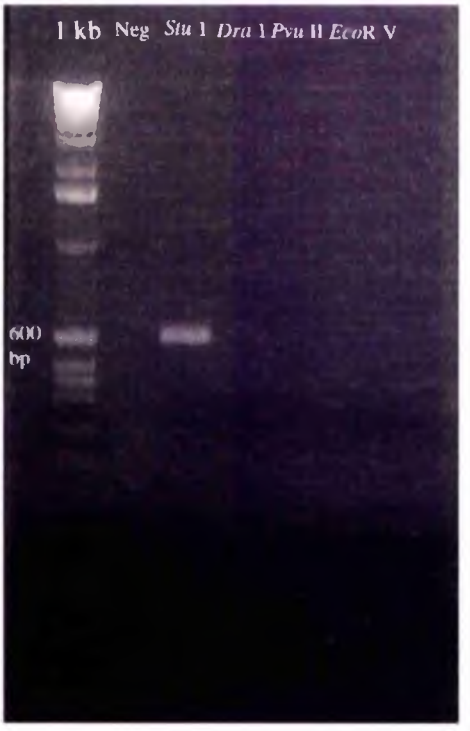


Figure 4-9 Second genome walk for the MMP-9 promoter sequence

The second genome walk amplified two PCR products that migrated on an agarose gel to show DNA fragments of approximately 1 kb and 1.5 kb for the *Dra* I and *Pvu* II libraries respectively; no bands were visible in the other libraries.

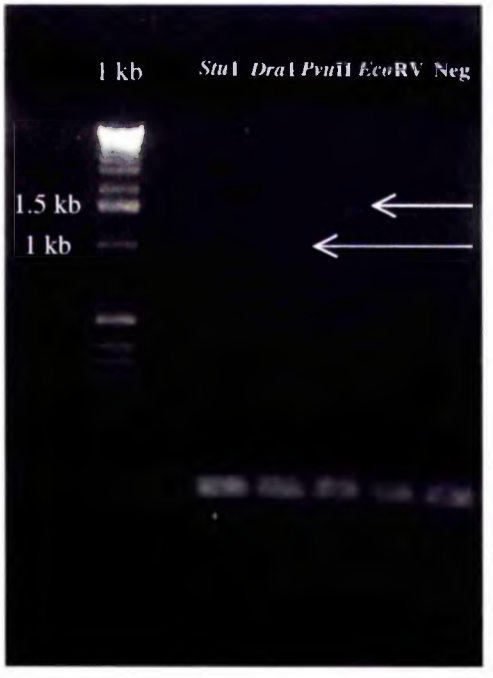
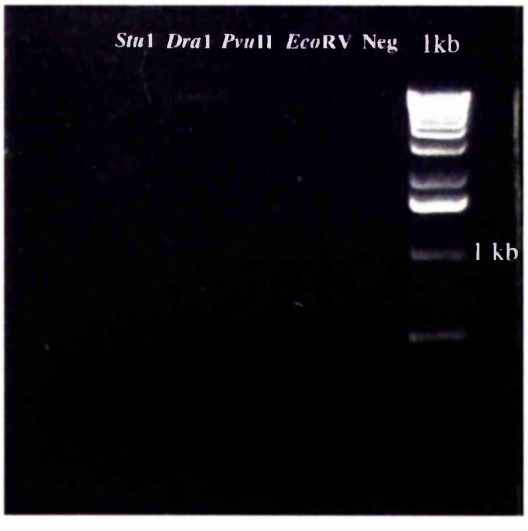


Figure 4-10 First genome walk for MMP-13 promoter

The first genome walk amplified a PCR product that migrated on a 1.5% agarose gel to show a single DNA fragment of approximately 550 bp within the *Stu* I library

Figure 4-11 Second genome walk for MMP-13 promoter

The second genome walk amplified a PCR product that migrated on a 1.5% agarose gel to show a DNA fragment of approximately 1.1 kb from the *Dra* I library, no bands were visible in the other lanes.



4.4.1.3 Cloning and screening for positive clones containing the canine MMP promoters

All PCR products were cloned into pCR2.1®TOPO plasmid vectors and screened by PCR using M13R and cMMP-9R or cMMP-13R primers to determine those constructs containing inserts.

4.4.2 DNA sequence evaluation of the canine MMP promoters

Three independent PCR products, cloned into pCR®2.1-TOPO vector, were selected and sequenced on an automated ABI310 genetic analyser using both forward (M13F) and reverse (M13R) vector-based primers. The three sequences were subjected to computational analysis.

4.4.2.1. Blast Search of NCBI database with sequences

The nucleotide sequences determined from the genetic analyzer were assessed using the 'Blast' search engine within the NBCI database. The nucleotide sequences were compared to every gene sequence within the data bank to identify those genes with which these new sequences shared the greatest homology. The results revealed that the nucleotide sequences were comparable to the MMP-9 and MMP-13 promoter sequences already cloned in other species. This was enough evidence to confirm that a unique region within the canine MMP-9 and MMP-13 promoter sequences had been cloned.

4.4.2.2 Sequence data analysis

The sequence data obtained from the individually cloned PCR products were analysed using GCG DNA sequence analysis software. The nucleotide sequences were aligned and examined for identical base pairs between sequences with any anomalies corrected by referring back to the appropriate chromas files until a consensus nucleotide sequence had been created. The 1894 bp canine MMP-9 nucleotide sequence and 1494 bp canine MMP-13 promoter sequences are shown in Figures 4-12 and 4-14 respectively. All primers used in the cloning of these fragments are highlighted on the sequences in colour.

4.4.2.3 Identification of putative DNA binding domains

The canine MMP-9 and MMP-13 promoter sequences were analysed using a web-based program (Transfac, Motif search) to identify putative DNA binding domains. The two promoter sequences were then aligned with the homologous sequences published for other species, using ClustalW, to identify conserved regions of DNA that corresponded to the identified binding motifs as shown in Figures 4-16 and 4-17. It was hypothesised that the conserved regions would correspond to important regulatory regions of the DNA.

4.4.2.3.1 Canine MMP-9 promoter alignment

The nucleotide sequence of the 1894 bp canine MMP-9 promoter region (Accession number: AF280420) was aligned with the sequences, where available in Genbank, for other species

including rabbit (L36050), human (M68343), mouse (X72794) and rat (AF148065) (Figure 4-16). The numbering system corresponds to the canine sequence with position number -1 immediately up-stream of the translation start site (ATG) indicated by a large arrow. The DNA binding motifs within the canine MMP-9 promoter sequence are highlighted in bold type and labelled throughout including a TATA-like box, GC boxes/SP1 sites, AP-1, AP-2, SRY, Lyf-1, PEA3, NFκB domain, and GATA sites. The d(CA) repeat sequence indicated by capital letters is conserved in all of the species except the canine sequence.

4.4.2.3.2 Canine MMP-13 promoter alignment

The nucleotide sequence of the 1494 bp canine MMP-13 promoter region (Accession number: AF384859) was aligned with the sequences, where available in Genbank, for other species including rabbit (AF059201), human (U52692), mouse (X82000) and rat (U53605) (Figure 4-17). The numbering system again corresponds to the canine sequence with position number -1 immediately up-stream of the translation start site (ATG) indicated by a large arrow. The DNA binding motifs within the canine MMP-13 promoter sequence are highlighted in bold type and labelled throughout including a TATA box, AP-1, SRY, Lyf-1, PEA3, NFκB-like, Cbfa, AML, FREAK-7, Nkx2.5, Nfat, Hsf-2, HN3B, STATX, Oct-1 and GATA sites.

4.4.3 Transcription initiation sites

The GeneRacer™ protocol enabled the full-length 5′ ends of cDNA to be isolated using known cDNA sequence from expressed sequence tags. The protocol ensured the amplification of only full-length transcripts via elimination of truncated messages from the amplification process. This enabled the identification of the 5′ untranslated regions of genes leading to the study of heterogeneous transcriptional start sites.

4.4.3.1 Analysis of RNA quantity and quality

Full-length, total RNA was isolated from MDCK cells using RNAzol™B solution. Good quality RNA samples were obtained as determined by ultraviolet spectrophotometry (A260/A280 value of >1.6) and agarose gel electrophoresis. The RNA migrated through the agarose gel (1%) demonstrating intact 18s and 28s ribosomal RNA components.

4.4.3.2 PCR amplification and cloning of the MMP transcription initiation sites

RNA samples (2μg) were subjected to first strand synthesis using AVM-RT and random primers to produce canine cDNA. The cDNA samples were used as the template for the amplification of the transcription initiation sites using oligonucleotide primers (Table 4-4) and *Taq* DNA polymerase. The PCR products obtained migrated on agarose gel (1.5%) revealing DNA fragments of approximately 400bp. Since the position of the transcriptional start site was unknown the exact size of the PCR product could not be predicted. PCR products were cloned into pCR2.1®TOPO plasmid vectors and positive constructs, screened by PCR, were sequenced.

4.4.3.3 DNA sequence analysis of the canine transcription initiation sites and comparison of to other species.

Ten independent clones for each promoter sequence, both cloned and endogenous, were selected for automated sequencing with reverse (M13F) vector-based primers and analysed by directly observing the chromas file sequences. The transcription initiation sites were marked on the promoter sequence and compared to the position in other species.

4.4.3.3.1 Canine MMP-9 transcriptional start sites

The transcriptional start site(s) for the canine MMP-9 promoter region were analysed from twenty independent clones generated using the RACE technique from both the plasmid vector and endogenous promoters (Figure 4-16). The multiple positions of transcription initiation within the canine promoter sequence are shown at positions -17, -21, -22, -24, -25, -40, -55 bp for the plasmid vector [pGL3/cMMP-9(1894)] (>) and positions -17, -21, -25 and –40 bp for the endogenous (*) sequences relative to ATG translation start codon. The equivalent regions of transcribed sequence in the other species have been identified and underlined.

4.4.3.3.2 Canine MMP-13 transcriptional start sites

The transcriptional start site(s) for the canine MMP-13 promoter region were also analysed from twenty independent clones generated using the RACE technique from both the plasmid vector and endogenous promoters (Figure 4-17). The region of transcription initiation from the endogenous canine promoter sequence spans from –25 to –30 bp and the single site within the plasmid vector [pGL3/cMMP-13(1494)] (*) is located-28 bp relative to ATG translation start codon. The equivalent regions of transcribed sequence in the other species have been identified and underlined.

Figure 4-12: Nucleotide sequence of canine MMP-9 promoter (AF280420).

Positions of primer sequences have been identified in colour.

```
   1  GGTCTGGGTG ACTCCAAAGC CAATGCTCAT TAATTCGTGA ATTCTAGAGC   cMMP-9F
  51  TTCAATTTTC CTATAAGAGA AGTATCTTCA CCTCACAGAT TCATTTATTA
 101  GATAAGCATA GAAAGTTCCC AGCAGGAGTG CCTGGGTGAC TCGGTTAAGC
 151  ATCTGACTTG GGTTCAGGTC CTGGGATGGA GTCTGCATCA GGTCCATGCT
 201  CAGTTAGGAG TCTGCTTGTC CCTCTGCCTC TCCCTCTGCC CCTCCCCCTG
 251  TTCATGCTCT CCCTCTCTCT CAGATAAATA AATATTTTTT AAAAAGATTT
 301  TATTTATTCA TTCATGAGAA TCAGAGAGAG AGAGGGAGAG AGAGAGAGAG   MMP-9SEQ2F
 351  AGAGAGAGAG AGGCAGAGAC ACAGGTAGAG GGAGAAGCAG GCTCCATGCA
 401  GGGAGCCCAA TGTCGGACTT GATTGGATCC GGGTCTCCGG GATCAGGCTC
 451  TGGGCTGAAG GCGGTGCTAA ACTGCTGAGC CACCCAGGCT GCCCAATAAA
 501  ATTTTTTAGA AAAAAGAAGA AGAAAAAGAA AGTGCCTGGC ACATAGTGAG
 551  CCCTTTAAAT CCATATTCTT GTTTTTTTTT TTTAAGATTT TATTTATTTA
 601  TTTGAGAGAG AGAGAACACA AGCAGGGGGA GCGGCAGGCA GAGGGAGAAA
 651  CAGGCTGCTC ATGGAGCAGG GAGCCTGATA CAGGGCTGTA TCGCAGGACC
 701  CCGGGATCAA GATCTGAGCT GAAGGCAGAT GCTTAACTGA GCAACCCAGG
 751  CACCCCACTT CTTGGGTTGT TATGTTGCCT TTCCCAAGTG ATGCCTCCAG
 801  GAAGGCAAAC ACAGACCCTT CCTTAAAGGG GCTGGTGGGG AAGAGAGAAG
 851  GGAGGGACCC ATACTGGGGA AACTTTGGGG CTGAAGCTCC GTGCTCAGTG
 901  CAGAAGGGGC TGGAGAACTG AAAGCCACCT ACCCCTCAAC GCCATCATCC
 951  GCCCTGCCCC GTTTTGCAGT TGAAGAGTCC CAAACCTAGA GAGATAAGAA
1001  GGCATTTGTT GTAAGTCACA CGGGGCAGAT TGGAGTCCAA GTCCCCAATC
1051  TCCTGATCCC AAGTGGAATC CCTGTCTTGA CCTGTAATTA AAAGCGACCA   MMP-9SEQF
1101  TGGCCATTAT TGGGGGACTT GCGACGTGCA CTGCGGTAAG CATTTTCTGT
1151  GTTTGATCTC ATTTTGTCCT CACATCAATT TAGGGACAAA GGGCCCTTTT
1201  TTTACAGTTG AGGAAGCAAA TTCAGAGTGA GTGGAGGACA TCTGCCTGGA
1251  GCCCTGGAGA AGAGTGAAGC CTGTCTGCTG GTTTCCAGAT GCCCTGCACT
1301  TCCCCCTTTA CTCCCCTTGG AAGACAGGGC TTGCTGCGCT GGAATTCCCC
1351  AATCCCTGCC TGAGGGAGCA CACTCCTTCC GCCTTGAGGT GGGGTAGGGG
1401  GAGGAAGCTG ATGAGCAAGG CTTCCAGGGA GGGGAAAAGA GGATGGAGCC
1451  CAGCAGTGTG GAAGGGGGAT TGCTGGGCTC AGGAAGAGGC CTGGGGAAAC
1501  TTCCATCCCT TGCCCTTAGG CTGACCACTT AGGGCCTTTG GGTGGTGAGC   MMP-9GSP4R
1551  TCAGGGAGTC CTGTAGCCTT AACTCTCTCC CTTGCTTTCA TTCAGTTCCC   MMP-9GSP3R
1601  ACAAGCTCTG CAGTTTGCAA AACCCAACCC TTCCCCTATG GGCCTGTGGT
1651  TTCCTGTGGG TCGGGGGTCC TGCCTGACTC GGCAGTGGGA ACTGTGGGCA
1701  GGGGGTAGAG GAGGACGTGG TGTAAGCCCT TTCTTTGCTT TCTCATGCTG
1751  GGGCCGCCCC CCTCCCCAAC ACACCCCATC CCCTGAGTCA GCCCCTACTT
1801  GCCAGGGAGG GGCGGGGTTA CTGATTCAGT GAACAGTGCC TTCTTAAACC
1851  CCTCACAGGG TCAGCTAGTA GCTAGACACA CCACTACCCT CACCATGAGC   cMMP-9R
1901  CCCAGGCAGC CCCTGGTCCT GGTGTTCCTG GTGCTGGGCT GCTGCTCTGC   MMP-9GSP2R
1951  AGCTCCCAGA CCACACAAGC CCACCGTTGT GGTCTTTCCA GGAGACCTGA   MMP-9GSP1R
2001  GACT
```

Figure 4-13: Nucleotide sequence of canine MMP-9 partial cDNA (AF169244).

Positions of primer sequences have been identified in colour.

```
   1  ATGAGCCCCA GGCAGCCCCT GGTCCTGGTG TTCCTGGTGC TGGGCTGCTG
  51  CTCTGCAGCT CCCAGACCAC ACAAGCCCAC CGTTGTGGTC TTTCCAGGAG
 101  ACCTGAGAAC TAATCTCACT GACAAGCAGC TGGCAGAGGA ATATCTGTTT
 151  CGCTATGGCT ACACTCAAGT GGCCGAGCTG AGCGACGACA AGCAGTCCCT
 201  GAGTCGCGGG CTGCGGCTTC TCCAGAGGCG CCTGGCTCTG CCTGAGACTG
 251  GAGAGCTGGA CAAAACCACC CTGGAGGCCA TGCGGGCCCC GCGCTGCGGC
 301  GTCCCGGACC TGGGCAAATT CCAGACCTTT GAGGGCGACC TCAAGTGGCA
 351  CCACAACGAC ATCACTTACT GGATACAAAA CTACTCGGAA GACTTGCCCC
 401  GCGACGTGAT CGACGACGCC TTTGCCCGAG CCTTCGCGGT CTGGAGCGCG
 451  GTGACACCGC TCACCTTCAC TCGCGTGTAC GGCCCCGAAG CCGACATCAT
 501  CATTCAGTTT GGTGTTAGGG AGCACGGAGA TGGGTATCCC TTCGATGGGA   MMP-9TISnested R
 551  AGAACGGGCT TCTGGCTCAC GCCTTTCCTC CCGGCCCGGG CATTCAGGGA   MMP-9TISR
 601  GACGCCCACT TCGACGACGA GGAGTTATGG ACTCTGGGCA AGGGCGTCGT
 651  GGTTCCGACC CACTTCGGAA ACGCAGATGG CGCCCCCTGC CACTTCCCCT
```

Figure 4-14: Nucleotide sequence of canine MMP-13 promoter (AF384859)
Positions of primer sequences have been identified in colour.

```
   1  GCGTTGCTGG TTGAGCCCAA TTCTAATCTC AAACTCATTG GAAATATTTA   cMMP-13R
  51  TTGTAAGGAT CTGTTTTTGG ACAAGGTGTA ATAATTGTCC TCCGCCGATA
 101  GCTTACATTT CATAAGCTTG TCTGGTTCCG TCTGAGGCTC TGGCCAACTC
 151  AGGTGACAGC ACGTCACTCA GATGTGGAAT GCCCCCTCCC TACCCACCCT
 201  AACCTGTGCT CCACTCCTGT CCTTGTCTTC TTGTGTTCCG GACCTGAGCT   MMP-13SEQF
 251  GCTCTCTGAT TCCTGATCAC TTAAATTAAC TTCATTTTCA GTCATTCAAT
 301  CTGAAGATCA TGACTTGTAT TTTCTATCCA TTTACATCTA TGCTGGACCA
 351  AAGATGGCAT TCTGATTTCC TGGTACTAAA TGTGGGATGT TCTGTAACAG
 401  ACTGGACGCC CCTCGTGGAT GATTTTCCTG AGTTTTTGGA ACTCAGGTAC
 451  ATCTACTATT CAAACTTCTT AAAAGTCAAA ATTAAGAAAC TTGAAGTTGG
 501  CCAAGAGCTT ACCTAGACCC TAGTTTTCCT GGTACTTCCA GGGGTGTGGG
 551  AACAAAGCAG CTCCATTTTT GAGAAAATTT CTGAGAATTA ATGGGGTAGA
 601  AGCCATACTG GATGGTGGTT AAAAAGATGA AGTCTTAAGT CTACTGGCTT
 651  GGAGTCAAAT GCTGACAGTA TCATTTATTA ACTTTTGACC TTTATTAGAG
 701  GTTAGTATTT AATTTCTCTG TGCTCAGATT ATTCTTATGT CAAATGAAAA
 751  CAAGCCTTCC AGTAGCACCT CCTGCTTAGG CTACTGTACA GATTTAACGA
 801  GATCATGCCT TTTAAGCCTA GTAATCCCCG GGTATGTGCT AGCCATTATA
 851  TTGTATTCCA TATAGGCTCC TACCCCTTCC TGAACCTGTG GGGAATCCAT
 901  GGAGGATTCC ATGTGTATCG AGAGTCACAC CAGGGCCCTG TGGGTGTGCC
 951  CTAGTATCTG AGCCTCCAAT CTCGGGCTTG CCCCAATTGT TTGGCCCTCA
1001  GGTACTCATG TCTCCTTCTG TAATCGCTCT AGGGAAAATG ATGTTCAGAA
1051  GTCACCAGGT TCTAGATATT CTAGAAGCAG GACTAAGTAT CTCTTTAAGG   MMP-13GSP4
1101  AAGTGAGAGC ATCATCTTGA TACTCTTGTC TGAAAAGAGT AAAAGTAGCT
1151  GCTTTTCTAC AGAGGGAAAA ATATTTTTGC CCAGTGAAGT GAAATATTAC   MMP-13GSP3
1201  TACTCTCTGC TTCTTCCCAC AGTATCCATA AATATGCTGA GGCTGTTTAT
1251  TTTGCCAGAT GGTTTTTGAG ACCCCGCTGA AATGAGAGAT GCCCTCATTT
1301  TATATTTCCC TCAAATTCTA CCACAAACCA CACTTTCTTG GGAGGAAAAA
1351  AAAAAAGTCG CCATGTAAGC ATGTTTACCT TCAATAGACT AGGAAGCGGG
1401  AACTTGTCCA TAAAGGATGA GTCACCACTG CAGGTCTATA AAAGTAAAGG
1451  TGCTTGTCTG GGAGAGACAG CAGGCTCCAG GCACAACCAC CAAGATGCTC   cMMP-13R
1501  CCAGGCATCC TGGCTGCCTT CCTCTTCTTG AGCTGGACTC AGTGCTGGTC   MMP-13GSP2
1551  CCTGCCCCTT CCCAGCGATG GTGATGATGA TCTGTCTGAG GAAGACTTCC   MMP-13GSP1
1601  AGCTTGCAG AGCGCTACCT GAAATCCTAC TACTATCCCC TGAATCCTGCT
1651  GGAA
```

Figure 4-15: Nucleotide sequence of canine MMP-13 partial cDNA (AF201729).
Positions of primer sequences have been identified in colour.

```
   1  ATGCTCCCAG GCATCCTGGC TGCCTTCCTC TTCTTGAGCT GGACTCAGTG
  51  CTGGTCCCTG CCCCTTCCCA GCGATGGTGA TGATGATCTG TCTGAGGAAG
 101  ACTTCCAGCT TGCAGAGCGC TACCTGAAAT CCTACTACTA TCCCCTGAAT
 151  CCTGCTGGAA TCCTGAAGAA GTCTGCAGCA GGCTCAGTGG CTGACAGGCT
 201  TCGAGAAATG CAGTCCTTCT TCGGCTTAGA GGTCACTGGC AAACTTGATG
 251  ATAATACCTT GGACATCATG AAGAAACCAA GATGTGGGGT CCCTGATGTG
 301  GGCGAGTACA ATGTTTTCCC CCGAACTCTC AAGTGGTCCA AAACGAACCT   MMP-13TISnestedR
 351  AACCTACAGG ATTGTGAACT ATACCCCTGA TCTGACTCAT TCTGAAGTTG
 401  AAAAGGCATT CAAAAAAGCT TTCAAAGTTT GGTCAGATGT GACACCTCTG
 451  AATTTTACCA GACTTCATGA CGGCACTGCA GATATCATGA TCTCTTTTGG
 501  AACTAAAGAG CATGGGGACT TCTATCCATT TGATGGACCT TCTGGTCTTC   MMP-13TISR
 551  TGGCTCATGC TTTTCCTCCT GGGCCAAATT ATGGAGGAGA TGCCCATTTT
 601  GATGATGATG AAACTTGGAC AAGTAGTTCC AAAGGCTACA ACTTGTTCCT
 651  TGTCGCTGCC CATGAGTTCG GCCACTCCTT AGGTCTTGAC CACTCCAAGG
```

Figure 4-16 Alignment of canine MMP-9 promoter sequence with other species

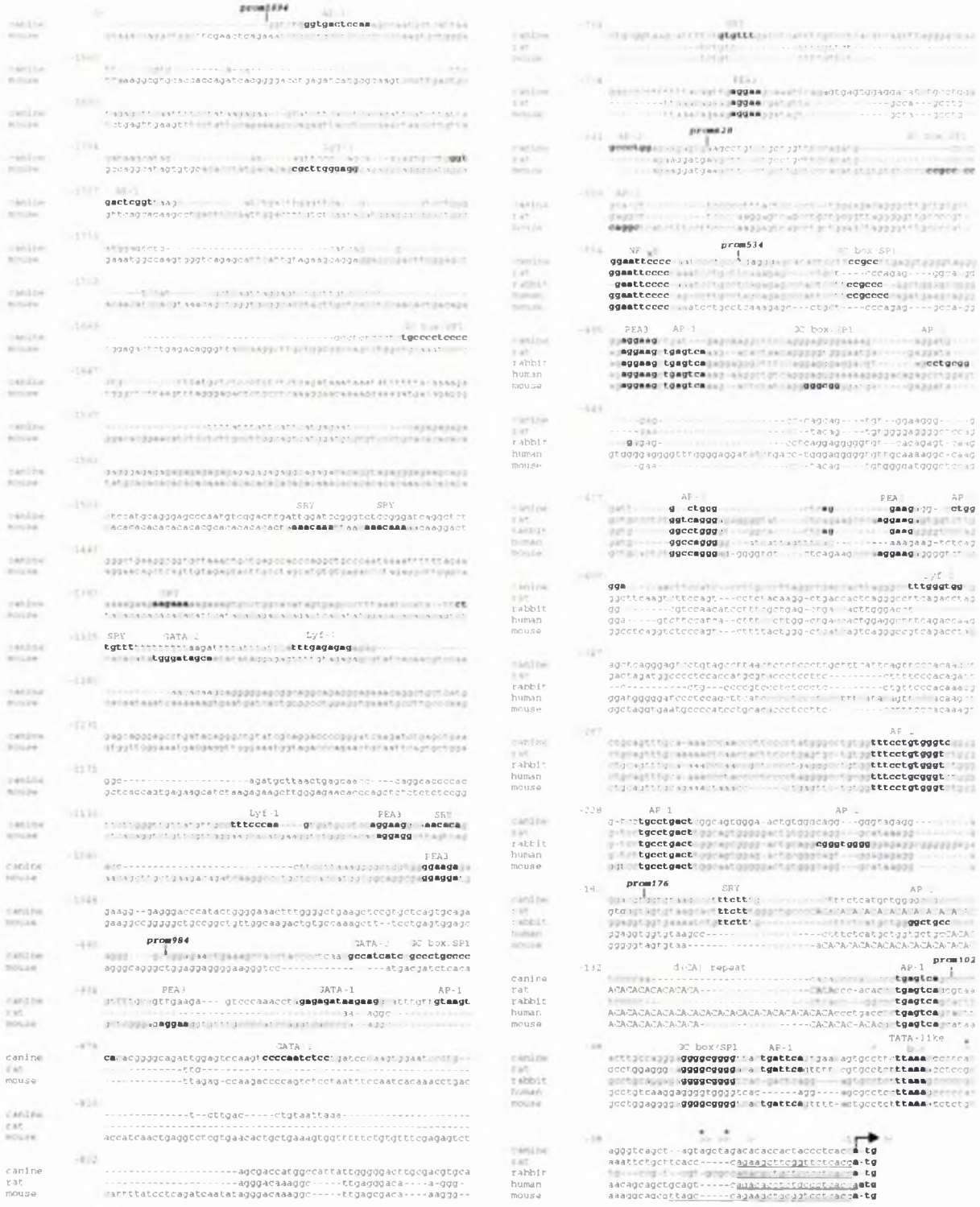
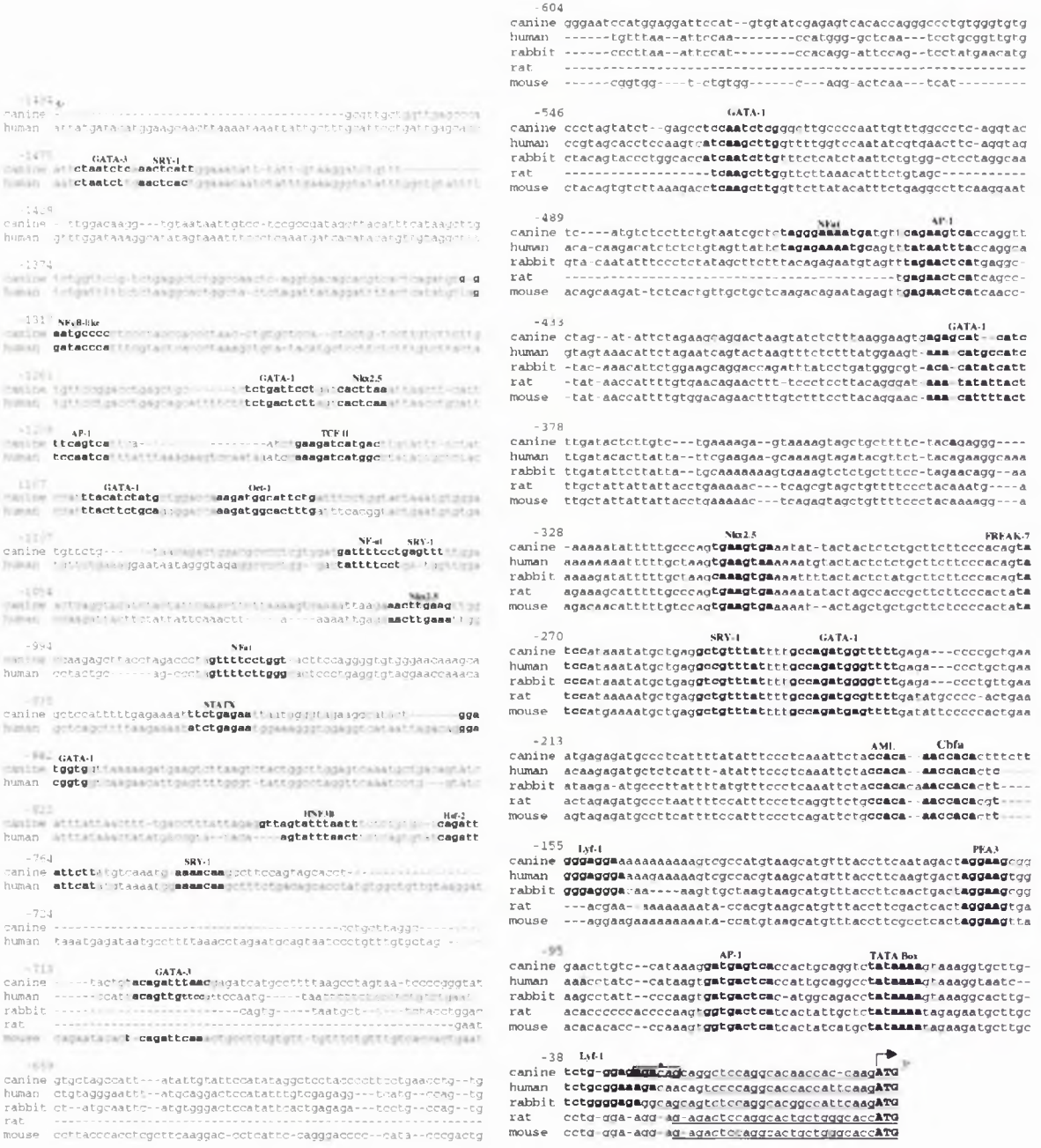


Figure 4-17 Alignment of canine MMP-13 promoter sequence with other species



4.5 DISCUSSION

4.5.1 Analysis of the 5′ regulatory region of canine MMP-9 and MMP-13

4.5.1.1 Analysis of the 5′ regulatory region of canine MMP-9

Comparison of the MMP-9 promoter sequence between the different species including human (Huhtala et al., 1991), rabbit (Fini et al., 1994), mouse (Masure et al., 1993) and rat (Eberhardt et al., 2000) shows deviation from a common ancestral promoter and species specificity. However, several conserved sequence motifs throughout the promoter have been identified which correlate with potential sites for the binding of transcription regulatory factors as determined by computational analysis of the canine DNA sequence (1894 bp) using 'MOTIF' (http://motif.genome.ad.jp/) suggesting functional importance (Figure 4-16).

More specifically, a CAAT motif was absent in all cases but a TATA-like box was consistently present, found in the canine sequence at position –51 to –47. Six AP-1 sites, the recognition sequence for members of the c-Fos and c-Jun families of transcription factors, were also identified within the canine sequence at various positions (–73 to –67, -111 to -105, -224 to -216, -877 to –884, -1760 to -1750 and –1888 to –1878). The highly conserved AP-1 site (-111 to –105) found throughout the species, has been shown to be necessary for basal and induced promoter activity in human tumour cells (Sato and Seiki, 1993) with mutation abolishing all promoter activity (Gum et al., 1997). Another important binding domain is the NF-κB site found at position –554 to –545. This again is highly conserved throughout the species and acts synergistically with other motifs, in particular the AP-1 site (Yokoo and Kitamura, 1996), to participate in MMP-9 expression (Sato and Seike, 1993). Numerous GC boxes for the binding of transcription factor SP1 are found at positions –86 to –78, -516 to –512, -944 to –935, -1658 to-1648. The most distal site is conserved and present in most of the species. Another important element, the polyoma enhancer activator-3 (PEA3) motif that is recognised by the products of the Ets-1 and Ets-2 protooncogenes, was also found in the 5′-flanking sequence and is known to function synergistically with the AP-1 sites in collagenase promoters (Gutman and Wasylyk, 1990). The microsatellite segment of alternating CA residues d(CA) sequence, identified in the rat, human and mouse promoter, is absent in both the canine and rabbit sequences. The importance of this motif has yet to be determined since conflicting studies show both the requirement of d(CA) for upregulating expression (Shimajiri et al., 1999) while others show no function at all (Sato and Seiki, 1993). The canine sequence also contains AP-2, SRY, GATA-1, -2 and Lyf-1 sequences but the functional role of each regulatory element and their combined effect remains to be determined.

4.5.1.2 Analysis of the 5′ regulatory region of canine MMP-13

The canine MMP-13 promoter sequence was also aligned and compared to the sequences already published for the different species showing regions of sequence similarity to the human (Tardif,

Pelletier et al., 1997) and rabbit (Vincenti, Coon et al., 1998) collagenase-3 (MMP-13) promoters. The canine MMP-13 promoter also showed considerable sequence similarity to the collagenase-1 (interstitial collagenase) promoter region identified in the mouse (Schorpp, Mattei et al., 1995) and rat (Rajakumar and Quinn, 1996). Potential sites for the binding of transcription regulatory factors were determined by computational analysis of the canine DNA sequence (1494 bp) using 'MOTIF' software. These sites were again located in regions of strong sequence conservation and thought to have functional importance (Figure 4-17).

More specifically, a consensus TATA box (–58 to –52) was consistently present throughout the species in addition to three AP-1 sites (–79 to –71, –449 to –441 and –1208 to –1201), the recognition sequence for members of the c-Fos and c-Jun families of transcription factors. Another important binding domain, the NF-κB-like site, found at position –1319 to –1310 is also present in the human counterpart and is likely to act synergistically with other motifs, in particular the AP-1 site, to participate in MMP-13 expression (Adcock, 1997). Many other conserved regions of the promoter have been shown to correspond to specific transcription factor binding sites. These include a Cbfa element (–169 to –163) considered to play a role in bone remodelling (Jimenez, Balbin et al., 1999) and chondrocyte maturation (Enomoto et al., 2000). The Cbfa site also corresponds to the Runx-2 site that was shown to confer IL-1 responsiveness by p38 MAPK and JNK pathways (Mengshol et al., 2001). The PEA3 site at position (–104 to –99) is believed to interact with neighbouring AP-1 site or others more distally (Benbow and Brinckerhoff, 1997). The multiple cardiac specific Nkx2.5 sites (–310 to –303, –1008 to –999 and –1227 to –1221) have been proposed to co-operate with GATA factors in cardiac specific gene expression in the developing heart (Molkentin, Antos et al., 2000). The canine sequence also contains Lyf-1, AML, NFat GATA, SRY-1, FREAK-7, TCF II, Oct-1, STATX, Hsf-2 and HNF3B motifs but the functional role of each regulatory element and their combined effect remains to be determined.

4.5.2 Analysis of the MMP-9 and MMP-13 transcription initiation sites

4.5.2.1 Determination of the MMP-9 transcriptional start site

Sequence analysis of transcription initiation sites for both the plasmid vector and endogenous promoter revealed multiple transcription initiation sites (Figure 1-16). Transcription initiation sites were identified at positions -17, -21, -22, -24, -25, -40, -55 bp relative to ATG translation start codon for the cloned promoter while expression from the endogenous promoter appeared to be more restricted over three sites –21, -22, -40 bp. However, the regions corresponding to transcription initiation throughout the species have been shown to originate from single sites at positions –19, -19, -19 and –24 bp upstream of the start site for translation in the rat (Eberhardt et al., 2000), rabbit (Fini et al., 1994), human (Huhtala et al., 1991) and mouse (Masure et al., 1993) sequences respectively.

There is an obvious difference between the transcription start sites between the endogenous and cloned promoter sequences that may be explained by the limitations of the technique used. If more

clones were sequenced then the transcriptional start sites may have been identified over similar regions. However, if the differences are real it may be explained by the presence of secondary and tertiary DNA structure within the endogenous genomic sequence unlike the cloned DNA sequence positioned in the exposed environment of the plasmid vector. There is also a variation between the multiple canine start sites and the single site published for each of the species. This may be due to the increased sensitivity of the Gene™Racer technique over the S1 nuclease protocol used by the other authors.

4.5.2.2 Determination of the MMP-13 transcriptional start site

The transcriptional start site(s) for the canine MMP-13 promoter region started from one defined position within the vector, 27 bp up-stream of the translation start site (*), in comparison to the endogenous promoter in which transcription initiation spanned an area of 6 bp (shown within box in figure 4-17). The regions corresponding to transcription initiation throughout the species have been underlined in Figure 4-17 and shown to originate from sites at positions 25, 22, 28, 25 bp upstream of the start site for translation in the rat (Rajakumar and Quinn, 1996), human (Tardif, Pelletier, Dupuis, Hambor, and Martel-Pelletier, 1997), rabbit (Vincenti, Coon, Mengshol, Yocum, Mitchell, and Brinckerhoff, 1998) and mouse (Schorpp, Mattei, Herr, Gack, Schaper, Angel, and P., 1995) promoter sequences respectively. The same theories apply to the canine MMP-13 as described for the differences between the MMP-9 sequence for the different species. However, the MMP-13 transcription initiation spanned a much smaller region of the promoter in comparison to the MMP-9 promoter which may be explained by the presence of a truly conserved TATA-box unlike the TATA-like box of the MMP-9 promoter.

4.6 SUMMARY

This chapter describes the isolation and sequence analysis of the canine specific MMP-9 and MMP-13 gene promoters and the identification of transcription initiation sites within these sequences. These canine promoters were selected for incorporation into the gene-based therapy for canine OA based on the understanding that collagenases and gelatinases are important enzymes associated with degradation of articular cartilage in the joint with increased levels of both enzymes demonstrated in OA. Understanding the unique ways in which each MMP gene is regulated through promoter sequence analysis is a crucial step in determining the role of these enzymes in the pathogenesis at the molecular level and their use in new targeted structure-modifying therapies.

Chapter V

Characterisation of canine matrix metalloproteinase -9 and -13 gene promoters

5.1 ABSTRACT

The identification of conserved DNA binding sequences within the canine matrix metalloproteinase (MMP)-9 and -13 gene promoters sequences, corresponding to potential pro-inflammatory induction, does not necessarily correlate with disease-inducible promoter activity. To this end it was necessary to characterise both the basal and pro-inflammatory induced activity of these promoter regions using *in vitro* tissue culture techniques, in order to determine their potential as promoters for driving disease-specific expression of therapeutic genes. This chapter describes the characterisation of the canine MMP-9 and -13 gene promoters using various cell lines analysed for endogenous MMP-9 and -13 gene transcription using relative semi-quantitative reverse transcription PCR (RT-PCR). The canine MMP-9 and MMP-13 promoter fragments were sufficient to drive basal expression of a luciferase reporter gene in both Madin Darby canine kidney cells (MDCK) and primary rat cardiocytes. Basal activity of the MMP-13 promoter fragment (1494 bp) could be significantly enhanced by the treatment of transfected primary rat cardiocytes with interleukin-1 (IL-1β) and basic fibroblastic growth factor (bFGF), with some induction also observed with tumour necrosis factor (TNFα). However, no induction of this promoter was observed in the MDCK cells. In comparison the canine MMP-9 promoter fragment was enhanced by TNFα in the MDCK cells and bFGF in the rat cardiocytes. This chapter also describes the construction of five MMP-9 promoter deletion vectors, analysed in MDCK cells and feline embryonic fibroblast (FEA) cells. The MMP-9 promoter deletion constructs were selectively enhanced by treatment of transfected MDCK cells with phorbol 12-myristate 13-acetate (PMA) suggesting that specific regions of the promoter were necessary for reporter gene expression. However, no effect was observed in the FEA cells.

5.2 INTRODUCTION

5.2.1 Regulation of matrix metalloproteinase gene transcription

It is well documented that increased levels of matrix metalloproteinase (MMP) gene expression and its activity is closely associated with disease progression in osteoarthritis (OA) (Martel-Pelletier, 1999b). Determining the pathways involved in the regulation of MMPs at the transcriptional, translational and activation levels is a prerequisite for the fundamental understanding of their roles in both normal and pathological states. This chapter will focus on level of transcriptional control following the identification of potential DNA regulatory elements, such the Nuclear factor (NF)-κB and Activator Protein-1 (AP-1) sites within the MMP-9 and MM-13 promoter sequences, known to be responsive to pro-inflammatory mediators including interleukin-1 (IL-1), tumour necrosis factor (TNF,) and basic fibroblastic growth factor (bFGF) (Bond et al., 1998, 1999; Benbow and Brinkerhoff, 1997).

5.2.1.1 Regulation of MMP-9 (Gelatinase B) transcription

Basal levels of MMP-9 expression is usually low or absent in most normal cell types but can be upregulated *in vitro* by various cytokines, growth factors and other inducible agents (Borden and Heller, 1997). Inflammatory cytokines such as TNF induces MMP-9 expression (Sato and Seiki, 1993) while IL-1 increases the levels of MMP-9 in human osteoarthritic cartilage (Mohtai et al., 1993). MMP-9 activity is also enhanced by growth factors such as transforming growth factor (TGFβ) (Wahl et al., 1993b) and epidermal growth factor (EGF) often associated with cytokines such as IL-1 (Lyons et al., 1993), while platelet derived growth factor (PDGF) also functions synergistically with IL-1 (Fabunmi et al., 1996). The levels of MMP-9 gene expression can also be increased by phorbol myristate acetate (PMA) which mimics the action of growth factors and cytokines (Huhtala et al., 1991).

The gelatinase B promoter requires the NF-κB element for induction by inflammatory cytokines. NF-κB is a pleiotropic transcription factor that binds to the NF-κB element and is involved in the expression of various viral and cellular genes (Lenardo and Baltimore, 1989). It was first discovered as one of the B-cell proteins interacting with the immunoglobulin κ light chain gene enhancer (Sen and Baltimore, 1986). Later it was purified from human B lymphocytes and shown to contain 51 kDa and 68 kDa proteins (p51 and p68) of which p51 bound to the DNA (Kawakami et al., 1988). NF-κB is either constitutively active, as in monocytes and macrophages (Griffin et al., 1989) or present in an inactive cytosolic complex together with an inhibitory protein called IκB (Baeuerle and Baltimore, 1988; Liou and Baltimore, 1993). Inactive NF-κB is activated by stimulation of cells with a variety of agents, such as PMA and lipopolysaccharide (LPS) or cytokines TNF and IL-1 (Meyer et al., 1991). It is the phosphorylation of IκB that leads to its dissociation and release of NF-κB *in vitro* (Gosh and Baltimore, 1990).

The AP-1 site is another DNA binding element that plays a pivotal role in the regulation of MMP-9 expression by growth factors and cytokines. Early studies suggested this site was a major player in MMP transcriptional activation with a transient rapid increase in AP-1 proteins prior to transcription. However, further studies have indicated that the AP-1 site does not function as the sole regulator of MMP gene expression but depends on interaction with several other *cis*-acting sequences located throughout the promoter sequence (Benbow and Brinckerhoff, 1997). The MMP-9 sequence contains a perfect consensus for this sequence (TGAGTCA) (Sato and Seiki, 1993) which binds to dimers of the c-Fos and c-Jun families of transcription factors (White and Brinckerhoff, 1995). However, this site is not sufficient for stimulation by PMA but requires co-operation with other upstream binding sites. MMP-9 is also expressed in a large number of malignancies and *in vitro* tumour cell over-expression studies have revealed the importance of the NF-κB and AP-1 sites in the development of the metastatic phenotype (Himelstein et al., 1997).

5.2.1.2 Regulation of MMP-13 (Collagenase-3) transcription

Collagenase-3 gene expression is also inducible in chondrocytes, regulated by a number of different agents including growth factors and cytokines depending on the physiologic sate of the cell (Tardif et al., 1999). The two major inflammatory cytokines IL-1 and TNF modulate MMP-13 expression supporting their roles in the pathogenesis of OA (Reboul et al., 1996). The MMP-13 promoter sequence contains a proximal AP-1 site and although it does not contain a perfect consensus it can still bind c-Fos and c-Jun factors to modulate gene transcription (Tardif et al., 1997). This AP-1 site is thought to play a pivotal role in the regulation of MMP-13 expression, in particular through the activity of IL-1 (Mengshol et al., 2000, 2001) and through the effects of IL-6 (Solis-Herruzo et al., 1999). The collagenase-3 gene promoter also has a requirement for the NF-κB element for induction by inflammatory cytokines. Both IL-1 and TNFα induction of MMP-13 gene expression in chondrocytes requires both the transcription factor NF-κB and AP-1 binding sites (Mengshol et al., 2000; Johansson et al., 2000). The transcriptional regulation of MMP-13 is complex and involves many transcription factors. For example the tissue specific expression of MMP-13 requires the presence of the AP-1 site interacting with the tissue-specific transcription factor Runx-2 binding domain for tissue specific regulation in chondrocytes (Mengshol et al., 2001).

5.2.2 Pro-inflammatory signalling pathways

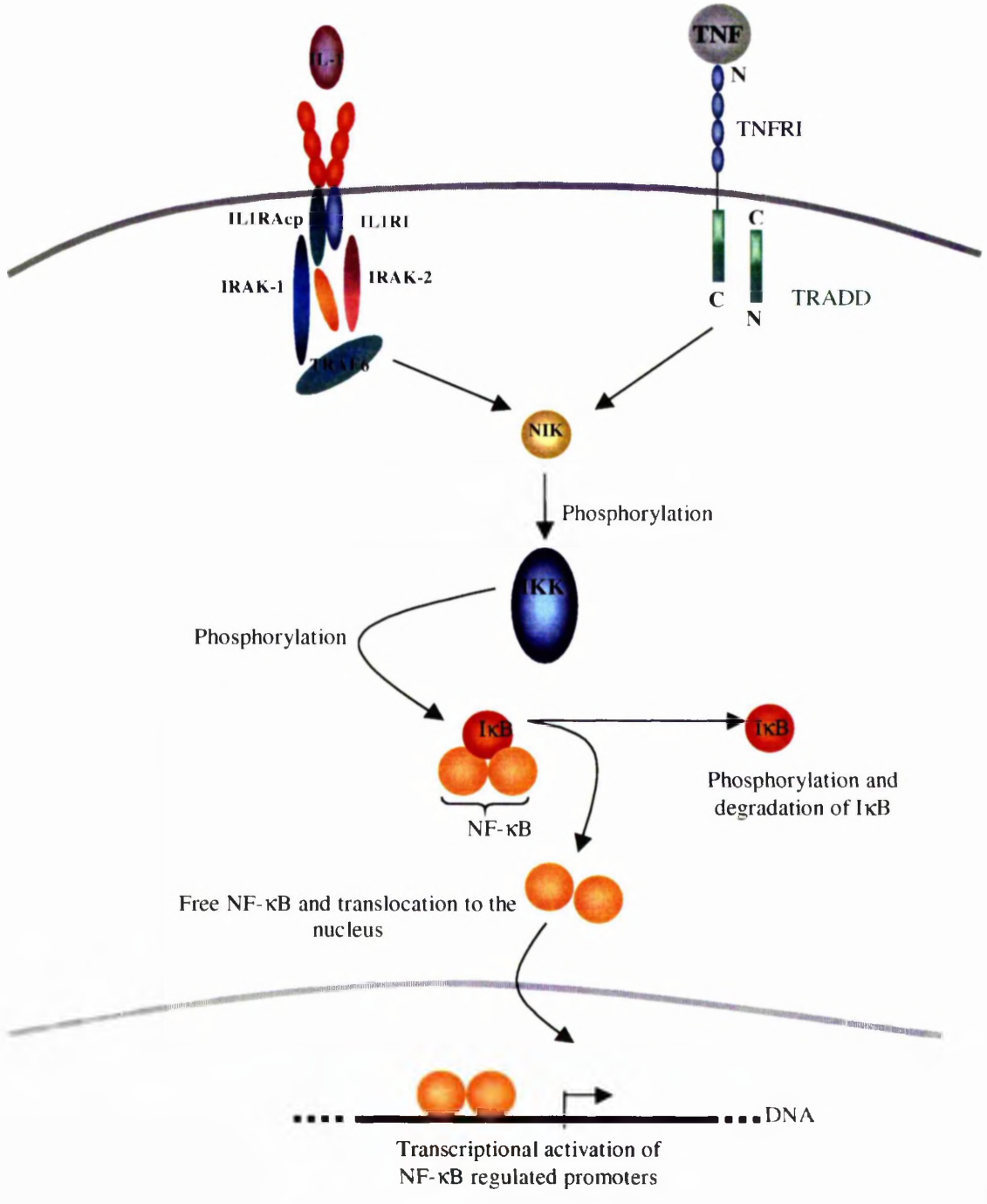
The pro-inflammatory cytokines and growth factors modulate gene expression through several complex signalling pathways each functioning independently and as part of a much larger integrated cascade. The details of these signalling cascades are complex and although numerous pathways have been described, many still remain to be determined. A brief description of the signalling pathways activated by the agents IL-1, TNF, bFGF and PMA is described.

5.2.2.1 Nuclear Factor (NF)-κB signalling pathway

Although IL-1 and TNF bind to distinct cellular receptors they both potently induce the transcription of a multitude of genes through the activation of the transcription factor NF-κB (Baeuerle, 1998; Baeuerle and Henkal, 1994) through slightly different pathways (Figure 5-1). In the TNF-induced signaling pathway the cytoplasmic segment of the TNF receptor type I (TNFRI), containing an eighty amino acid 'death domain', directly interacts with the death domain of TRADD, a 34 kDa protein, (Hsu et al., 1995) to result in NF-κB signalling. IL-1 however, activates NF-κB via a multiprotein complex involving IL1RI/IRAK-2, IL-1RacP/IRAK-1 and MyD88 leading to the recruitment of Traf6 (O'Neill and Greene, 1998). The signalling pathways of both IL-1 and TNF converge with the stimulation of the NF-κB inducing kinase (NIK) which phosphorylates and activates another group of kinases known as the IκB kinases (IKKs). The IKK then phosphorylates IkB, the cytoplasmic inhibitor of NF-κB, for ubiquitination and proteosome-mediated degradation. The NF-κB then translocates to the nucleus where it increases transcription of inflammatory genes. However, IL-1 can also activate NF-κB through IKK-independent pathways.

Figure 5-1 Nuclear Factor (NF)-κB signalling pathway

The TNF-induced signalling pathway leads to the cytoplasmic segment of the TNF receptor type I (TNFRI), directly interacting with TRADD to result in NF-κB signalling. The IL-1-induced signalling pathway however, activates NF-κB via a multiprotein complex involving IL1RI/IRAK-2, IL-1RacP/IRAK-1 and MyD88 leading to the recruitment of Traf6. These two signalling pathways converge with the stimulation of the NF-κB inducing kinase (NIK) which phosphorylates and activates another group of kinases termed the IκB kinases (IKKs). The IKK then phosphorylate IkB, the cytoplasmic inhibitor of NF-κB for ubiquitination and proteosome-mediated degradation. The NF-κB then translocates to the nucleus to up-regulate transcription of inflammatory genes.



5.2.2.2 Mitogen-activated protein kinase (MAPK) pathway

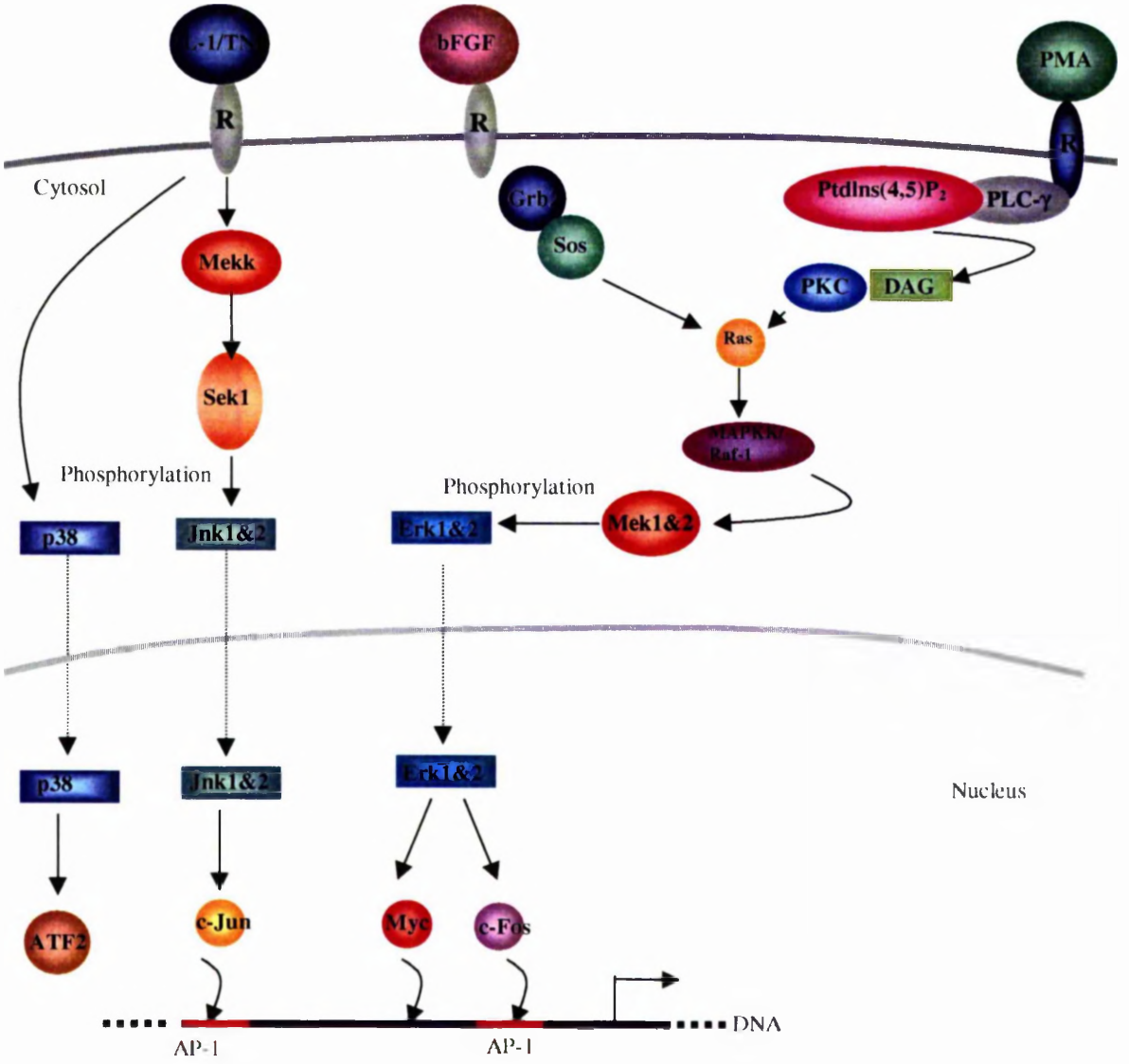
Concomitant with the activation of the NF-κB pathway, IL-1 and TNF induce a group of serine and threonine kinases known as the mitogen-activated protein kinases (MAPK). Once active, the MAPK travel to the nucleus where they phosphorylate and activate specific transcription factors involved in inflammatory and growth responses. Three main subgroups of MAPK have been described (Figure 5-2). The extra-cellular stimulus-regulated kinases (ERK) become active in response to cytokines, phorbol esters such as PMA and growth factors such as bFGF and activate the AP-1 family member c-Jun (Pulverer et al., 1991; Frost et al., 1994). The stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNK) are primarily activated in response to cytokines (IL-1 and TNF) or stresses such as UV radiation, hyperosmolarity and hypoxia (Minden and Karin, 1997). The third group of MAPK consists of the p38 or reactive kinases, which are similar to the SAPK/JNK with respect to the extracellular stimuli that activate them and the transcription factors they target (Raingeaud et al., 1995). In general all of the MAPKs become catalytically active when phosphorylayed by MAPK kinases (MAPKK) which are in turn activated through phosphorylation by MAPKK kinases. The SAPK/JNK and ERK kinases can phosphorylate and activate c-Jun directly and p38 activates ATF2 which mediates transcription of the c-Jun gene. This suggests that all of the MAPK cascades can contribute to AP-1 dependant transcription (Barchowsky et al., 2000).

More specifically, the MAP kinase (p42 and p44) or Erk1 and 2 (extra-cellular signal-regulated kinase) cellular signalling pathway is activated by a variety of mitogens including phorbol esters (PMA) (Rossomando et al., 1989) and growth factors such as bFGF (Berrou et al., 1996). This pathway consisting of Ras, Raf, Mek, MAP kinase and Rsk spans from the plasma membrane to the nucleus and serves to transduce mitotic signals downstream from the membrane receptor tyrosine kinases. This pathway requires the transient phosphorylation of Erk (42 kDa protein) on tyrosine (Y187) and threonine (T185) residues for full activity (Blumer and Johnson, 1994) which is mediated by a MAP kinase kinase (MAPKK or MEK). The functional significance of the MAP kinase pathway is through the phosphorylation of intra-cellular protein kinases and activation of transcription factors (Marshall, 1995).

The stress-activated MAPKs require phosphorylation on tyrosyl and threonyl residues for activation (Blumer and Johnson, 1994). p54 MAPKs (stress-activated kinase: SAPK-1 or c-Jun N-terminal kinase: JNK) (Kyriakis et al., 1994) and p38 MAPK (ASPK-2, reactivating kinase, cytokine suppressive binding protein) are only weakly activated by mitogens, but are strongly activated by cell stresses, bacterial LPS, IL-1 and TNFα (Lee et al., 1994). P54 MAPK/JNK strongly phosphorylates the *trans*-activation domains of the c-Jun (Pulverer et al., 1991) playing a role in the activation of AP-1-dependent genes. IL-1 activated MAPK/JNK pathways affect numerous regulatory processes, including the synthesis and activity of the AP-1 transcription factor composed of c-Fos/c-Jun heterodimers and c-Jun/Jun homodimers (Karin et al., 1997).

Figure 5-2 Mitogen activated protein kinase pathway (MAPK)

IL-1 and TNF induce a group of serine and threonine kinases known as the mitogen-activated protein kinases (MAPK) through receptor (R) binding. Once active, the MAPK travel to the nucleus where they phosphorylate and activate specific transcription factors involved in inflammatory and growth responses. Three main subgroups of MAPK have been described. The extracellular stimulus-regulated kinases (ERK) become active in response to cytokines, phorbol esters such as PMA and growth factors. The stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNK) are primarily activated in response to cytokines (IL-1 and TNF) or stresses such as UV radiation, hyperosmolarity and hypoxia . The third group of MAPK consists of the p38 or reactive kinases, which are similar to the SAPK/JNK with respect to the extracellular stimuli that activate them and the transcription factors they target.

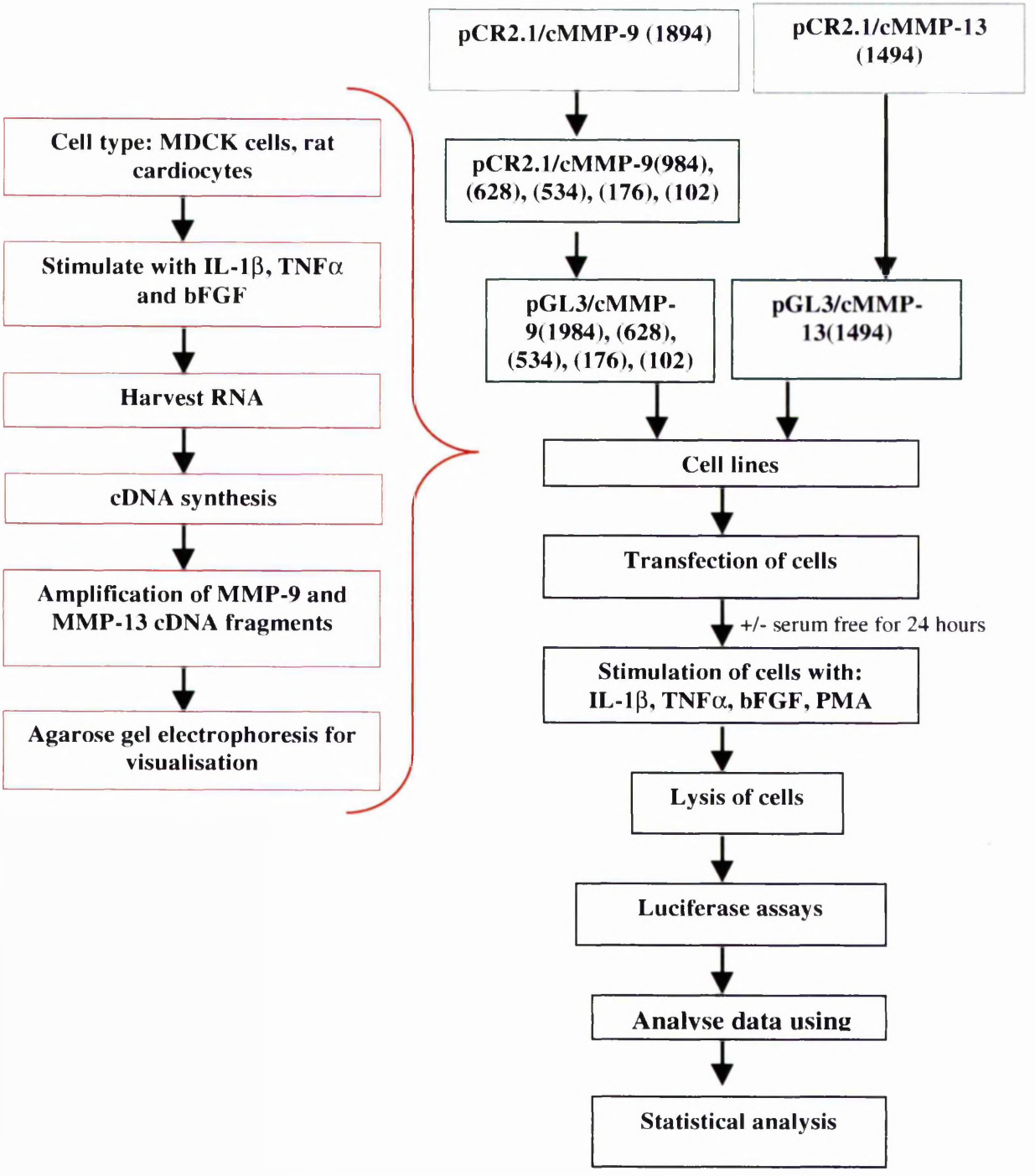


5.3 MATERIALS AND METHODS

Overviews of the experimental procedures employed in this chapter are illustrated in figure 5-3 Briefly, the canine MMP-13 and MMP-19 promoters, plus the MMP-9 deletions, were sub-cloned into pGL3-Basic luciferase reporter vector (Promega) using restriction enzyme digestion (*Kpn* 1 and *Eco*RV/*Sma*1) and ligation techniques. Madin Darby canine kidney (MDCK) cells and primary rat cardiocytes were analysed for basal and induced (IL-1β, TNFα and bFGF) endogenous levels of MMP-9 and MMP-13 gene transcription using reverse transcription (RT)-PCR to be used for analysing the cloned promoter fragments. The cloned canine MMP-9 and MMP-13 promoter fragments, pGL3/cMMP-9(1894) and pGL3/cMMP-13(1894) were transfected into the two cells lines, stimulated with IL-1β, TNFα and bFGF and activities determined using Dual-Luciferase®Reporter Assays (Promega). The cloned canine MMP-9 promoter deletion constructs pGL3/cMMP-9(1894), (984), (628), (534), (176) and (102) were transfected into the MDCK cells and feline embryonic fibroblasts (FEA), stimulated with PMA and activities determined using similar Dual-Luciferase®Reporter Assays (Promega).

Figure 5-3 Overview of the experimental procedures used to characterise the canine MMP-9 and MMP-13 promoter fragments

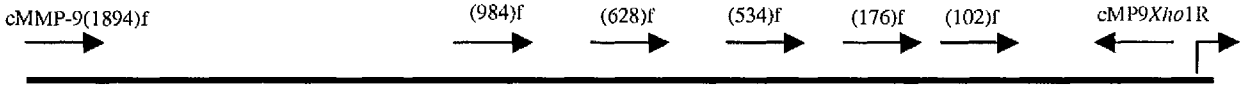
The canine MMP-13 and MMP-19 promoters (plus MMP-9 deletions) were sub-cloned into pGL3-Basic luciferase reporter vector. Appropriate cell lines were then analysed for endogenous transcription of MMP-9 and MMP-13 using RT-PCR. Finally activity of the cloned MMP-9 and MMP-13 promoter fragments were analysed using Dual-Luciferase® Reporter Assays



5.3.1 Cloning of MMP-9 promoter deletion constructs into pCR®2.1-TOPO

5.3.1.1 Primer design

For the PCR-based cloning of the MMP-9 promoter deletions into pCR®2.1-TOPO vector, six forward (sense) primers and one reverse anti-sense primer were designed as described in section 2.2.5.1 and supplied by MWG-Biotech (Table 5-1). The forward primers were based on specific regions of the canine MMP-9 promoter sequence, guided by motif location as described in figure 4-16. Each primer of 25-28 nucleotides in length required 40-60% GC content and also contained *Xho*1 restriction enzyme sites for the sub-cloning into the luciferase reporter vector, pGL3-Basic (Promega). Using these criteria, sense oligonucleotide primers (cMMP-9(1894)f, cMMP-9(984)f, cMMP-9(628)f, cMMP-9(534)f, cMMP-9(176)f, cMMP-9(102)f) and anti-sense oligonucleotide primer (cMP9*Xho*1R) were designed according to the canine MMP-9 promoter sequence (Figure 5-4).



Canine MMP-9 promoter

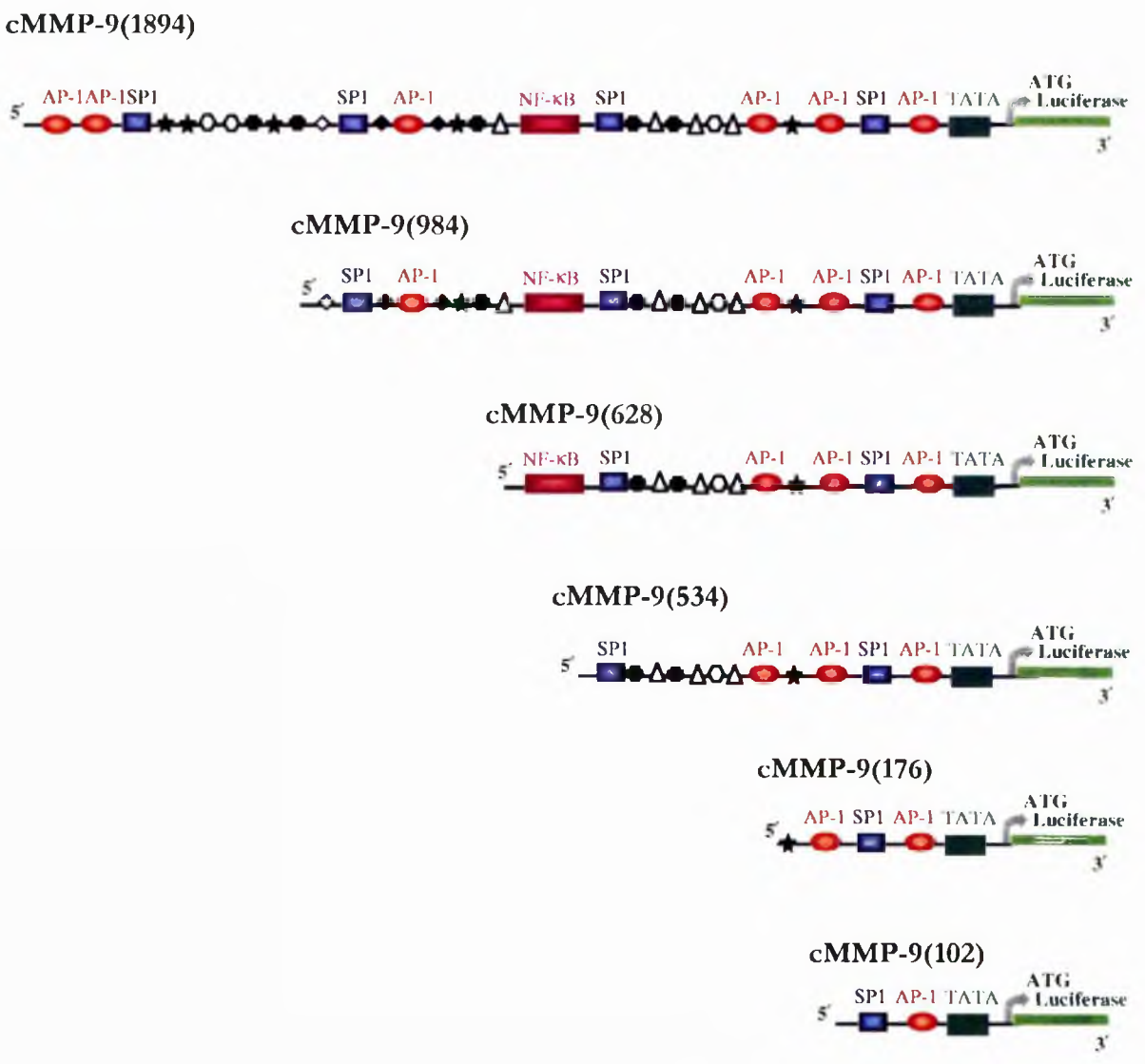
Table 5-1. Primer sequences for the PCR amplification of the canine MMP-9 promoter deletion constructs

Primer Identification	Oligonucleotide primer sequence (5′-3′)	Tm (°C) & GC (%) content
cMMP-9(1894)f	GGCTCTCGAGCTCGAGTCTGGGTGACTCCAAA	73.3 °C : 59.4 %
cMMP-9(984)f	CGGCTCGAGCTCGAGTGGAGAACTGAAAGCCACCT	75.0 °C : 60.0 %
cMMP-9(628)f	CGGCTCGAGCTCGAGAAGCCTGTCTGCTGGTTT	74.5 °C : 60.6 %
cMMP-9(534)f	CGGTTCTCGAGCTCGAGTGAGGGAGCACACTCCTT	75.0 °C : 60.0 %
cMMP-9(176)f	CGGCTCGAGCTCGAGGGTGTAAGCCCTTTCTTTGC	75.0 °C : 60.0 %
cMMP-9(102)f	CGGTTTTCTCGAGCTCGAGCCCTACTTGCCAGGGA	75.0 °C : 60.0 %
cMP9*Xho*1R	GGCTTTCTCGAGCTCGAGGGTGAGGGTAGTGGTGT	75.0 °C : 60.0 %

Figure 5-4 MMP-9 promoter deletion fragments

Illustration of the MMP-9 promoter deletion constructs with sequential portions of the 5′ region of the promoter removed to eliminate cytokine responsive elements. Constructs have been named cMMP-9(1894), cMMP-9(984), cMMP-9(628), cMMP-9(534), cMMP-9(176), cMMP-9(102) with each number corresponding to the size of the promoter deletion construct in base pairs.

SRY.★: AP-2.Δ: LYF-1. O: PEA3.●: GATA-1.◆: GATA-2. ◇



5.3.1.2 PCR amplification of the canine MMP-9 promoter deletions

PCR was performed as described in section 2.2.5 using pCR®2.1/cMMP-9 vector samples (100 ng) as template in a final volume of 50µl containing 0.4µm of both forward primer (cMMP-9(1894)f, cMMP-9(984)f, cMMP-9(628)f, cMMP-9(534)f, cMMP-9(176)f and cMMP-9(102)f) and reverse primer (MP9*Xho*1R) pairs, 0.2mM of dNTPs, 1.5Mm $MgCl_2$ and 2 units of *Taq* DNA polymerase (QIAGEN). Using a PE 480 thermal cycler samples were covered in mineral oil and subjected to an initial denaturation at 95°C for 5 minutes followed by 35 cycles of amplification, each cycle consisting of a denaturation step of 95°C for 1 min and an annealing temperature of 70°C for one minute followed by an elongation step of 72°C for one minute. The final elongation step of 72°C for 10 minutes completed the reaction. PCR products (8 µl) were visualised by agarose gel electrophoresis (1.5%) gel by comparing the bands created to a 100 bp molecular weight standard.

5.3.1.3 Cloning into pCR2.1® TOPO vector

PCR products were cloned using methods described in section 2.2.2. Briefly, PCR products were purified following QIAquick® Gel Extraction Kit (QIAGEN, UK) and eluted in 50µl of sterile water 4µl of which were assessed by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a 1 kb molecular weight standard. Purified PCR products were directly ligated into the pCR®2.1-TOPO plasmid vector following the TOPO TA® cloning protocol. The ligation reaction (2µl) was then transformed into One Shot™ TOP10 competent cells and spread onto agar plates containing ampicillin and X-gal for incubation overnight at 37°C. White, ampicillin resistant, colonies were selected from the plates and cultured overnight in LB/ampicillin broth at 37°C and glycerol stocks of each culture were prepared for long-term storage.

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. Samples were screened for those containing the insert by restriction enzyme digestion. Briefly, each pCR2.1/cMMP construct (500ng) was digested with *Xho*1 restriction enzyme (7.5 units) and Buffer 6 in a total volume of 20µl at 37°C for 4 hours. Samples were analysed by agarose gel electrophoresis to identify the presence of inserts.

5.3.2 Sub-cloning of the entire MMP-9 & MMP-13 promoter fragments and MMP-9 promoter deletions into pGL3-Basic luciferase reporter vector

Originally it was intended that the *Xho*1 restriction sites would be used to sub-clone the promoter deletion fragments into the pGL3-Basic luciferase reporter vector. However, after multiple unsuccessful attempts, an uni-directional sticky/blunt ended ligation reaction was successfully used instead. This involved ligating the *Kpn* I and *Sma* I restriction enzymes sites located in the multiple cloning site (MCS) of the pGL3-Basic reporter vector with the *Kpn* I and *Eco*R V restriction enzymes sites positioned either side of the promoter fragments cloned into the pCR®2.1-TOPO

vector. To enable this procedure it was necessary to ensure that promoter sequences did not contain *Kpn* I or *Eco*R V restriction enzyme sites within their sequences.

5.3.2.1 Restriction enzyme digestion of vectors

All pCR®2.1/cMMP vector constructs, including the entire canine MMP-9, its deletions and the MMP-13 promoter, were digested with *Kpn* I and *Eco*R V restriction enzymes. The luciferase reporter vector, pGL3-Basic (Promega) was digested with *Kpn* I and *Sma* I restriction enzymes. More specifically, the pCR®2.1/cMMP vectors (5µg) were digested for four hours with *Kpn* I (5 units) and *Eco*R V (5 units) in MULTI-CORE™ (1x) buffer at 37°C. The recipient pGL3-Basic luciferase reporter vector (5µg) was digested with *Kpn* I (5 units) and *Sma* I (5 units) at 37°C and 30°C for 2 hours with each enzyme respectively.

5.3.2.2. Purification, ligation and transformation

Digested DNA samples (linearised pGL3-Basic and released cMMP promoter fragments) were purified following QIAquick®Gel Extraction Kit (QIAGEN, UK) and eluted in 30µl of sterile water. The DNA was quantified (4µl) by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a Low DNA Mass™ Ladder (4µl).

The quantity of vector (pGL3-Basic) and insert (promoter fragment) for each ligation reaction was calculated according to the equation shown in 2.2.2.8.1. For each calculation the vector mass (X) was 50 ng, the insert size (Y) were approximately 2, 1, 0.5, 0.6, 0.5, 0.15 and 0.1 kb for the MMP-9 promoter and its deletions (in descending size) and 1.5 kb for MMP-13 promoter. The molar ratio of insert to vector used was a value of three. The cut ends of the inserts were then ligated to complementary ends of the cut pGL3-Basic vector using T4 DNA ligase (Promega) over night at 16°C. The ligations (20ng) were transformed into 25µl of JM109 competent cells, heat shocked for 45 seconds at 42°C and then grown overnight at 37 °C on LB/ampicillin agarose plates. Six colonies were selected from each plate and transferred to LB broth for overnight culture at 37°C.

5.3.2.3 Isolation and screening of recombinant plasmids

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep®PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. pGL3/cMMP-9 constructs were screened with *Xho* I restriction enzyme as described in section 5.3.1.3. The clones containing the pGL3/cMMP-13 constructs did not contain flanking *Xho* 1 restriction enzyme sites incorporated into the primer sequences and could not be screened in the same manner as the MMP-9 constructs. In addition, the *Kpn* I and *Eco*R V restriction enzyme reaction used in the vector construction, could not be used in the screening process either since the blunt ended ligation of the *Eco*R V and *Sma* I sites abolished the use of either site for this purpose. Instead a PCR-based screening protocol was applied using reagents and conditions similar to those

described earlier for the amplification of the MMP-13 promoter as described in 4.3.4. Since all the promoter inserts were directionally cloned it was not necessary to screen for correct orientation.

5.3.2.4 Sequence evaluation

Recombinant plasmids were sequenced and analysed as described in section 2.2.6. Briefly, plasmid samples were prepared for sequencing with PCR reactions using plasmid DNA samples (400 ng) and the Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism) with 0.5μM of both sense (RV3) and anti-sense (GL2) vector-based primers. Samples were amplified, and the DNA purified by precipitation methods before samples were loaded into the ABI 310 genetic analyser for generation of automated sequence data. Sequence files were downloaded from the chromas file, saved as Word documents using the appropriate format, and then lined up with the relevant correct sequence for the MMP-9 and MMP-13 sequences using ClustalW to check for the absence of mutational errors.

5.3.2.5 Plasmid vector maps

Using computer software, plasmid vector maps were drawn from the sequence data information incorporating all important components of each vector including the luciferase cDNA sequence, relevant canine MMP promoter sequence, polyadenylation signal and ampicillin resistance gene.

5.3.3 Analysis of endogenous MMP-9 and MMP-13 gene transcription using relative semi-quantitative RT-PCR

Semi-quantitative reverse transcription (RT)-PCR has previously been used to detect and analyse cytokine mRNA levels in various cell lines and tissues (Walker, 1998). This system was therefore adapted to measure the relative levels of endogenous MMP-9 and MMP-13 transcription from different cell lines to assess their suitability in the characterisation of the MMP-9 and MMP-13 promoter fragments. RT-PCR is a sensitive technique that can detect minimal levels of gene transcription, due to the amplification step, unlike standard Northern blot protocols. Additionally, the results generated from the RT-PCR directly reflected levels of gene transcription rather than the absolute levels of protein expression as determined by Western blot analysis. The latter technique could not be used since canine specific antibodies to both MMP-9 and MMP-13 were unavailable.

Both basal and induced levels of endogenous MMP-9 and MMP-13 gene transcription were analysed in the MDCK cells and primary rat cardiocytes using RT-PCR. However, it was not possible to assess the FEA cells since the feline sequence for MMP-9 cDNA was not available in the database, for primer design.

5.3.3.1 Cell lines and reagents

MDCK cells were maintained in Dulbecco's MEM with gluatamax-1 medium supplemented with 10% foetal calf serum (FCS) as described in section 2.2.1.2.1. Primary rat cardiocytes, a gift from Dr. Arvind Sood, were cultured in a mix of Dulbecco's modified Eagle's medium and medium 199

(at a ratio of 4:1) supplemented with 4% horse serum and 5% FCS as described in section 2.2.1.2.3. MDCK cells and rat primary cardiocytes were seeded onto 6 well plates at a concentration of 6x 10^4 and 5x 10^5 cells/ml respectively and allowed to grow to confluence over 24 hours at 37°C and 5% CO_2.

5.3.3.2 Stimulation of cells

MDCK cells and primary rat cardiocytes used in the comparison of full-length MMP-9 and MMP-13 transcripts were serum-starved for 24 hours prior to stimulation with IL-1β (10 ng/ml), TNFα (10 ng/ml) and bFGF (20 ng/ml) for a further 24 hours. MDCK cells used in the characterisation of the MMP-9 promoter deletion constructs were not serum starved but were immediately exposed to Phorbol Myristate Acetate (PMA) (5^{-9}M) for the 24 hours prior to harvesting cells.

5.3.3.3 Isolation of total RNA

Total RNA was harvested from both untreated (basal) and treated (PMA, IL-1β, TNFα and bFGF) cells using RNAzol™ B solution (AMS Biotechnology) (500µl/well) added directly to the wells containing the adherent MDCK cells and rat cardiocytes for lysis. Aliquots (1 ml) of the lysed cell solution were shaken for 15 seconds with chloroform (100µl) and RNA extracted as described in section 2.2.3. The RNA samples were re-suspended in 40µl DEPC water and the quality and quantity assessed using spectrophotometry and agarose gel electrophoresis. Care was taken when estimating the RNA concentrations to enable equal amounts of RNA (2µg) to be used for each reaction in semi-quantitative RT-PCR. Steps were also taken to ensure no contamination of genomic DNA by treating all RNA preparations with DNase 1 (DNA-free™, Ambion) as described in section 2.2.3.4.

5.3.3.4 First strand cDNA synthesis

The synthesis of first strand cDNA followed the basic principles as set out in section 2.2.4. More specifically, total RNA samples (2µg) in 9µl of DEPC water were heated for 5 minutes at 65°C and then quenched on ice. Reactions were performed using the heat treated RNA in a 25µl reaction mix containing 5µl of reverse transcription buffer (5x), 200 units of Molony Murine Leukemia Virus reverse transcriptase (MMLV RT) (GIBCO BRL), 10mM dTT, 250µM dNTPs, 25 units RNAse inhibitor and 25µM random primers. The reaction was incubated at 37°C for 30 minutes followed by 42°C for 60 minutes and completed with 95°C for 5 minutes. A PE 480 thermal cycler was used for the reaction. The cDNA concentration of each sample was determined using UV spectrophotometry.

5.3.3.5 Primer design

PCR primers were designed as described in section 2.2.5.1 and were synthesised and supplied by Sigma-Genosys (Table 5-2). In order to amplify portions of the canine MMP-9 & MMP-13 cDNA sequences for both canine and rat species it was necessary to design oligonucleotide primer pairs

spanning a 350 bp region of each sequence. Both sense and anti-sense species-specific primers pairs were designed within the MMP-9 cDNA sequence for canine (cMMP9f and cMMP9r) and rat (ratMMP-9f and ratMMP-9r). Similarly, Primers were designed within the MMP-13 cDNA sequence for canine (cMMP13f and cMMP13r) and rat (ratMMP-13f and ratMMP-13r).

The constitutively expressed cyclophilin gene, encoding a periplasmic protein involved in protein folding (Andreeva et al., 1999) was used as an internal control for the canine (AF243140) and rat (NM_017101) species. Species specific primers were subsequently designed within the cyclophilin cDNA sequence for the canine (cCyclophilinf and cCyclophilinf) and rat (ratCyclophilinf and ratCyclophilinr) to amplify a region of 265 bp from the cDNA templates. The position of the control primers, based on the cDNA sequence to span an intron, enabled the identification of contaminating gDNA as a 450 bp fragment. Primer pairs (27 bp) were matched as closely as possible for Tm (70°C) and GC (40-60%) content.

Table 5-2 Primer sequences for RT-PCR 9(MMP-9, MMP-13, cyclophilin)

Primer Identification	Oligonucleotide primer sequence (5′-3′)	Tm (°C) & GC (%) content
cCyclophilinf	CGTGCTCTGAGTACTGGAGAGAAGGGA	72.1 °C : 55.6 %
cCyclophilinf	CCACTCAGTCTTGGCGGTGCAGATGAA	77.7 °C : 55.6 %
cMMP9f	GCTGGACAAAACCACCCTGGAGGCCAT	79.1 °C : 59.3 %
cMMP9r	GTCGTCGAAGTGGGCGTCTCCCTGAAT	78.1 °C : 59.3 %
cMMP13f	CTCTTCTTGAGCTGGACTCAGTGCTGG	72.8 °C : 55.6 %
cMMP13r	GGACCACTTGAGAGTTCGGGGGAAAAC	74.8 °C : 55.6 %
ratCyclophilinf	GTGCTCTGAGCACTGGGGAGAAAGGAT	74.4 °C : 55.6 %
ratCyclophilinr	CCACTCAGTCTTGGCAGTGCAGATAAA	71.6 °C : 48.1 %
ratMMP-9f	AACCTTCGAAGGCGACCTCAAGTGGCA	78.2 °C : 55.6 %
ratMMP-9r	CGTCCCTCGAAGGTGAAGGGAAAGTGA	76.3 °C : 55.6 %
ratMMP-13f	CCCTCGAACACTCAAATGGTCCCAAAC	74.7 °C : 51.9 %
ratMMP-13r	AGTGGCCAAGCTCATGGGCAGCAACAA	79.9 °C : 55.6 %

5.3.3.6 PCR amplification of MMP regions using PCR

PCR was performed as described in section 2.2.5 using Ready-To-Go™ PCR beads (25 µl) (Amersham, Pharmacia) with cDNA samples (1µg) as template and 0.4µM of species specific primer pairs for MMP-9 or MMP-13 each with 0.1µM of species specific cyclophilin primers as an internal control (Table 5-2). Samples were subjected to an initial denaturation at 95°C for 5 min

followed by a variable number of cycles for amplification, each cycle consisting of a denaturation step of 95 °C for 1 min, an annealing temperature of 67°C followed by an elongation step of 72 °C for 1 min. A final elongation step of 72°C for 10 min completed the reaction.

Only PCR samples taken from the exponential phase of the reactions (basal and treated) could be directly compared to semi-quantify the levels of transcription. This was determined by removing samples at multiple (3 to 5) intervals 5 cycles apart and comparing the density of the PCR products. All PCR products were analysed using agarose gel (1.5 %) electrophoresis. PCR products (8µl) were analysed by TAE agarose gel (1%) electrophoresis by comparing the bands created to a 100 bp molecular weight standard. Care was taken to load equal volumes of each PCR sample into wells on the agarose to enable accurate comparison between samples. A negative control containing the PCR bead with all components (dNTPs, PCR buffer and *Taq* polymerase) and primer pairs without template was included to check that there was no contamination of the PCR reactions with extraneous DNA that might serve as a template for PCR amplification.

5.3.4 Luciferase reporter assays to analyse the activity of the cloned canine MMP-9 and MMP-13 promoter fragments

The pGL3-Basic luciferase reporter vector was chosen to analyse the canine MMP-9 and MMP-13 promoters since luciferase reporters offer an extremely sensitive system for measuring the activity of regulatory regions of DNA such as promoters. A variety of luciferase-based vectors are available enabling strictly controlled experiments including a negative control (promoter-less vector, pGL3-Basic), positive control (SV40 luciferase, pGL3-Control) and a *Renilla* luciferase vector (pRL-CMV) as an internal standardised control. This Dual-Luciferase®Reporter assay system has the unique ability to assay both the fire-fly and *Renilla* luciferase activities sequentially in the same well to control for both cell number and transfection efficiency. A modern luminometer housing a 96-well plate format and dual injector system was available for use enabling multiple, fast and accurate assays to be performed.

5.3.4.1 Transfection of cells with luciferase reporter constructs

Maintenance of MDCK cells and primary rat cardiocytes is described in section 5.3.3.1. FEA cells were maintained in Dulbecco's MEM supplemented with 10% FCS as described in section 2.2.1.2.2. White, tissue culture treated ViewPlate™-96 (Packard) were seeded with MDCK, FEA3 and primary rat cardiocytes at concentrations of $6x10^4$ cells/ml, $5x10^4$ cells/ml and $5x10^5$cells/ml respectively and incubated overnight at 37°C, 5% CO_2. Cells should be approximately 70-80% confluent on the day of transfection for optimal assay conditions. Transient transfections were carried out using the cationic liposome mediated methods as described in section 2.2.7 with TransFast™ Reagent (Promega) used at a 1:1 ratio with DNA according to the manufacturer's instructions. More specifically, total DNA (50-100ng) was added to pre-warmed serum free media (40µl) and thoroughly mixed before addition of TransFast™ Reagent (0.3µl). The DNA/transfection reagent samples were incubated for 10-15 minutes at room temperature to allow

complex formation before the growth medium on the 96-well plates was aspirated and replaced with the transfection samples. The cells were incubated with the complexes for 1 hour at 37°C. 5% CO_2 before pre-warmed complete medium was added for cell recovery overnight at 37°C.

5.3.4.2 Stimulation of cells

To induce transcription from the MMP promoter constructs, transfected cells were treated with different stimulants for the 24 hours prior to luciferase assays. MDCK cells and primary rat cardiocytes used in the comparison of full-length MMP-9 and MMP-13 transcripts were serum-starved for 24 hours prior to stimulation with IL-1β (10 ng/ml), TNFα (10 ng/ml) and bFGF (20 ng/ml) (R&D Systems) for a further 24 hours. MDCK cells used in the characterisation of the MMP-9 promoter deletion constructs were not serum starved but were immediately exposed to PMA (5^{-9}M) (Sigma) for the 24 hours prior to harvesting cells.

5.3.4.3 Luciferase assays

Dual-Luciferase®Reporter assays were performed as described in section 2.2.8. Briefly, 72 hours post transfection the media was removed from the adherent cells, washed once with Phoshate Buffered Saline (PBS) and lysed with Passive Lysis Buffer (PLB) (1x) for 15 minutes at room temperature. Using a Dynex, MLX luminometer and injector system, freshly prepared Luciferase Assay Buffer II (LAR II) (50µl) and Stop & Glo® Reagent (50µl) were dispensed into each well with an interval of 30 seconds, each taking a luciferase count for 10 seconds. Both firefly and *Renilla* luciferase values were attained for each well and all luciferase values were analysed using an excel spread sheet and a statistical analysis performed using the Mann-Whitney test.

5.4.1 Cloning of the canine MMP-13 and MMP-9 promoter fragments and deletion constructs into the pGL3-Basic luciferase reporter vector

The regions of the canine MMP-9 promoter, required for the deletion constructs, were successfully amplified from the PCR2.1®TOPO templates using the primers described in Table 5-1. All six canine MMP-9 promoter fragments (1984), (894), (628), (534), (176), (102) and canine MMP-13 promoter (1494) were then sub-cloned into the pGL3-Basic luciferase reporter vector using the *kpn* 1 (sticky) and *EcoRV*/*Sma* 1 (blunt) restriction enzyme sites. The bacterial clones containing the MMP-9 promoter deletions were screened using the *Xho*I restriction sites and the clones containing the MMP-13 promoter were screened using PCR techniques.

One clone from each promoter construct was selected and sequenced on an automated ABI310 genetic analyser using both forward (RV3) and reverse (GL2) vector based primers to confirm the sequences and ensure that no mutational errors had occurred during the cloning procedure.

Vector maps for the canine MMP promoter sequences in pGL3-Basic luciferase reporter vector were drawn for pGL3/cMMP-9(1894) (Figure 5-5), pGL3/cMMP-9(984) (Figure 5-6), pGL3/cMMP-9(628) (Figure 5-7), pGL3MMP-9/c(534) (Figure 5-8), pGL3/cMMP-9(176) (Figure 5-9), pGL3/cMMP-9(102) (Figure 5-10) and pGL3/cMMP-13(1494) (Figure 5-11).

Figure 5-5 Vector map for pGL3/cMMP-9(1894)

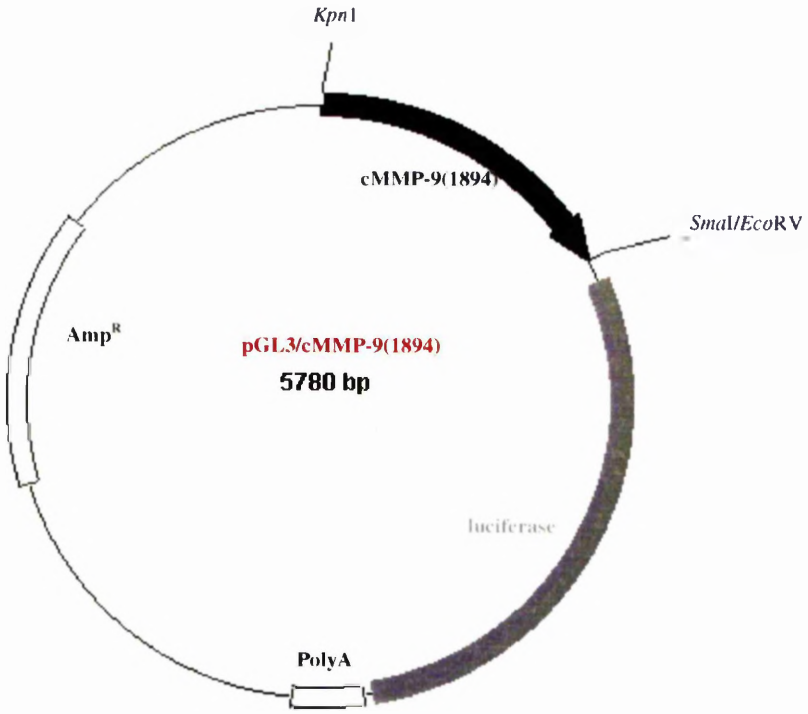


Figure 5-6 Vector maps for pGL3/cMMP-9(984)

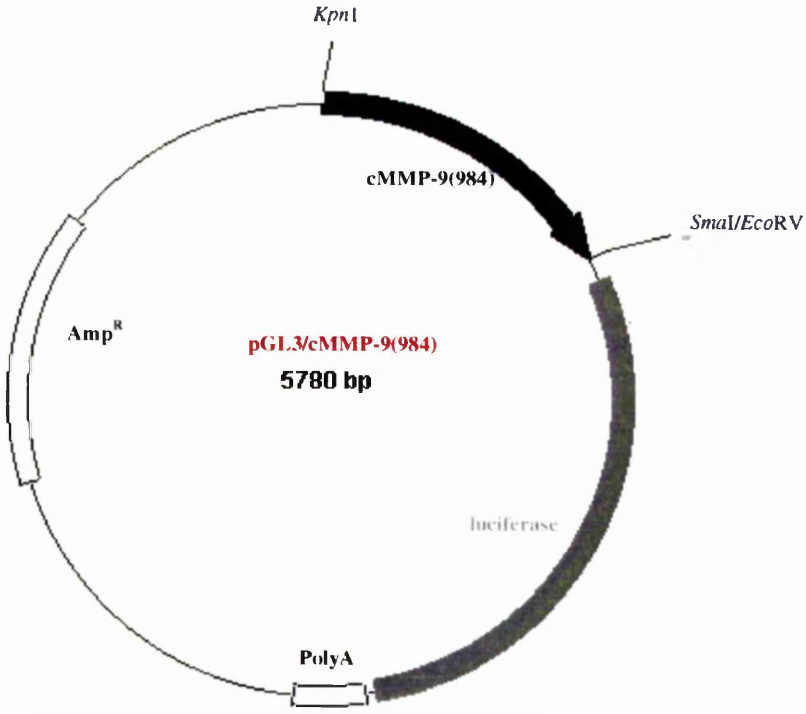


Figure 5-7 Vector map for pGL3/cMMP-9(628)

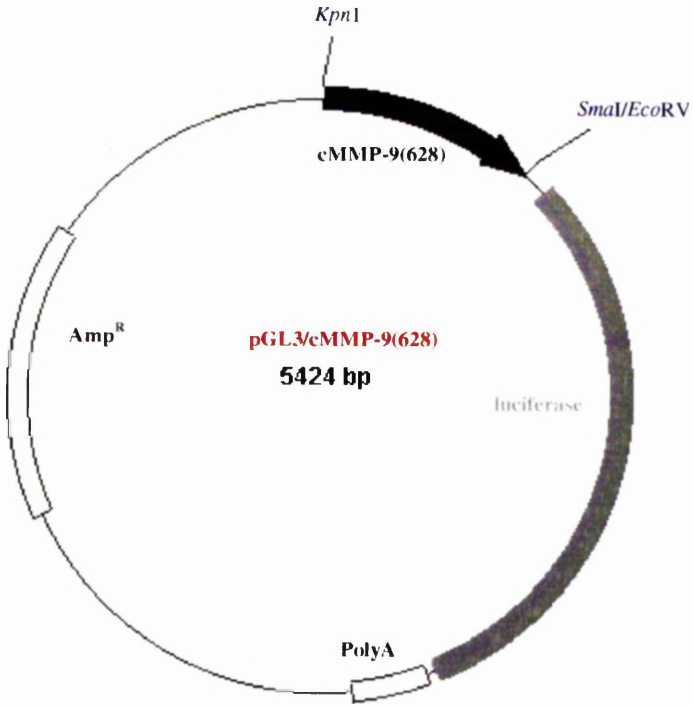


Figure 5-8 Vector map for pGL3/cMMP-9(534)

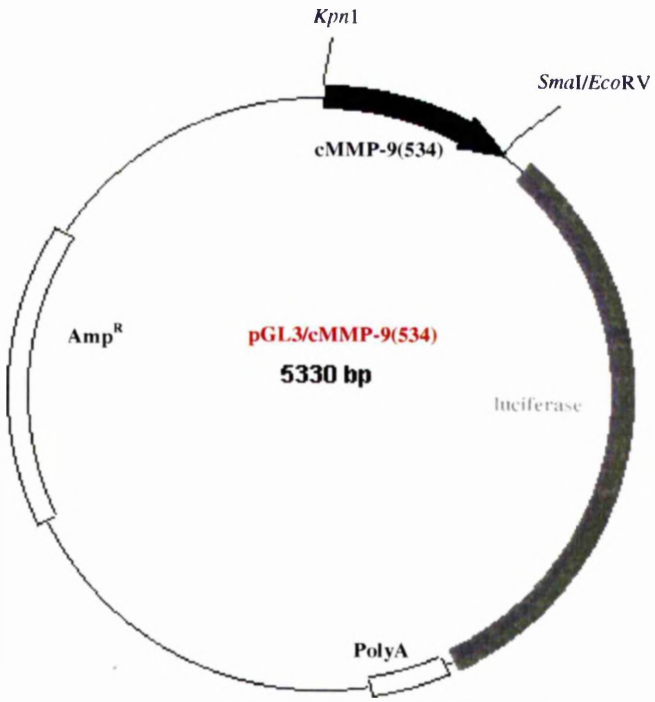


Figure 5-9 Vector map for pGL3/cMMP-9(176)

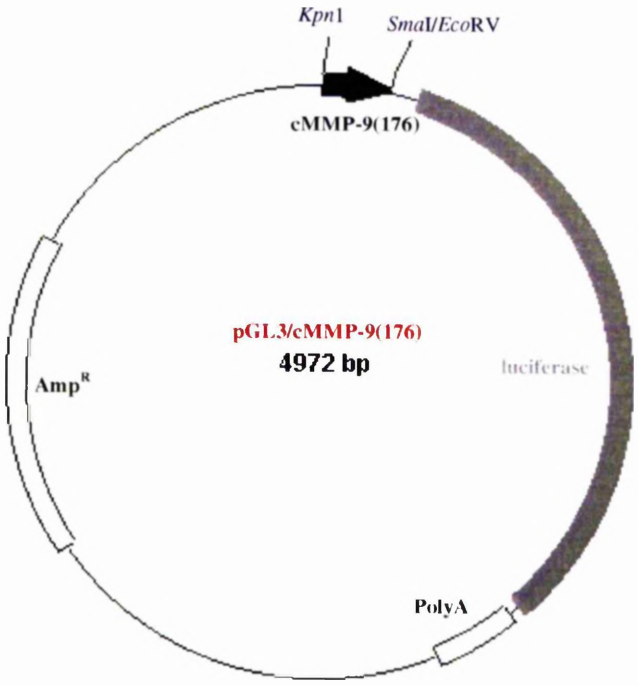


Figure 5-10 Vector map for pGL3/cMMP-9(102)

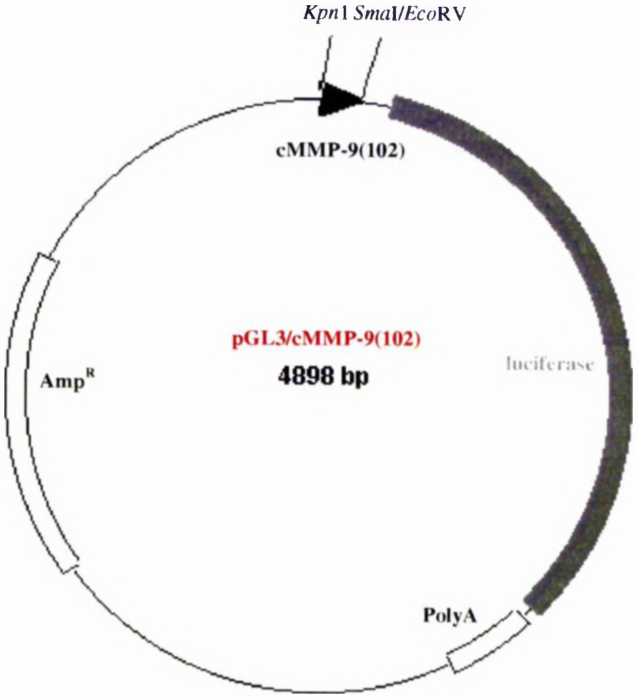
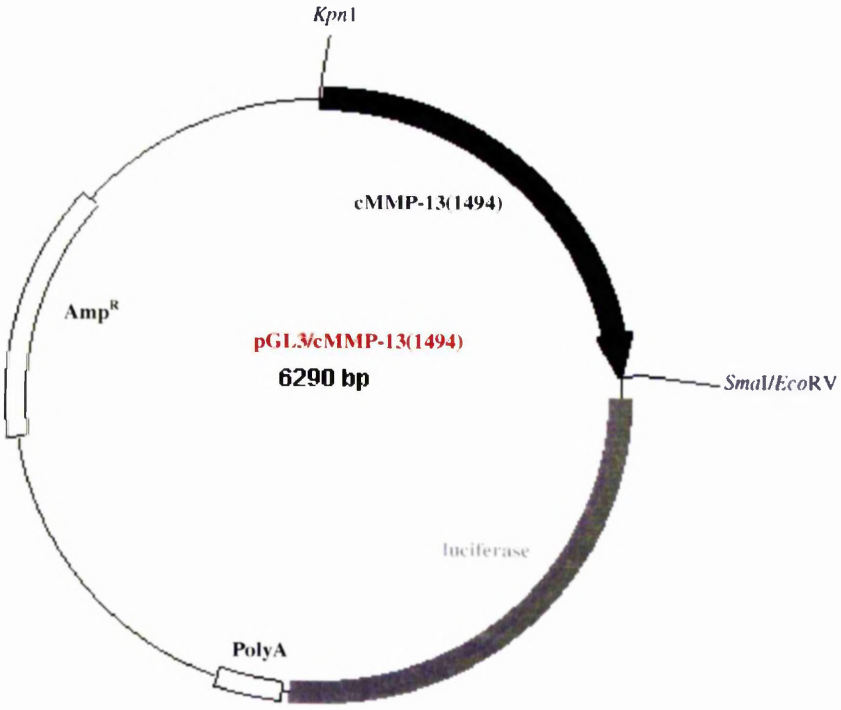


Figure 5-11 Vector map for pGL3/cMMP-13(1494)



5.4.2 Characterisation of the cloned canine MMP-9(1894) and MMP-13(1494) promoter fragments in MDCK and primary rat cardiocytes

The aim of this first study was to evaluate and compare the basal and induced (IL-1β, TNFα and bFGF) activities of the largest cloned canine MMP-9 promoter fragment (1894 bp) to the MMP-13 promoter (1494 bp); to examine their potential for driving disease-specific gene expression.

5.4.2.1 Analysis of the endogenous basal and induced MMP-13 and MMP-9 gene transcription levels in MDCK cells and primary rat cardiocytes

To analyse the endogenous levels of MMP-13 and MMP-9 transcription in the cell lines to be used for the transfection of the canine MMP-13 and MMP-9 promoter fragments, relative semi-quantitative RT-PCR was performed (Figure 5-12, 13, 14, 15). A portion of each gene was amplified from cDNA samples prepared from untreated (basal) and treated (IL-1β, TNFα and bFGF) cells using species-specific primer pairs. A portion of the cyclophilin gene was also amplified (255 bp) as an internal control using primers designed to span an intron and control for the presence of contaminating gDNA (absence of a 474 bp PCR product) (Table 5-2). Samples were removed at three intervals, 5 cycles apart, to determine the exponential phase of amplification and enable the selection of one cycle number for each PCR for the semi-quantification of gene transcription levels. Basal transcription of the endogenous MMP-13 gene was evident in both the MDCK cells (Figure 5-12) and rat cardiocytes (Figure 5-13) but only the rat primary cardiocytes could be induced with IL-1β, TNFα and bFGF. In comparison basal levels of the canine MMP-9 promoter could be only be enhanced by TNFα in MDCK cells (Figure 5-14), while in the primary rat cardiocytes the endogenous MMP-9 promoter activity was clearly up-regulated with IL-1β and to a lesser degree with TNFα and bFGF (Figure 5-15).

5.4.2.2 Analysis of cloned canine pGL3/MMP-9(1984) and pGL3/MMP-13(1494) promoter constructs using Dual® Luciferase Reporter assays

All Dual®Luciferase assays were conducted in triplicate for statistical significance and to ensure reproducibility, all transfections were carried out three times. To account for differences in transfection efficiency the cells were co-transfected with *Renilla* luciferase vector (Promega) and the firefly luciferase values were adjusted accordingly. *Renilla* was expressed and shown to be active in cell types used. Representative *Renilla* values for the rat cardiocytes and MDCK cells were 159 and 1882 respectively. The corrected luciferase activity of each construct represents the mean +/-SEM (n=3). The canine MMP-9 and MMP-13 luciferase reporter constructs, together with the promoter-less luciferase vector, pGL3-Basic vector as the negative control, were transiently transfected into MDCK and primary rat cardiocytes. Basal luciferase activity for each promoter was compared to treated samples (IL-1β, TNFα and bFGF).

Basal activity of the cloned canine MMP-13 promoter fragment was present in the MDCK cells but could not be induced by any of the cytokines (Figure 5-16). However, the cloned canine MMP-

13 promoter fragment could be significantly enhanced with IL-1β and bFGF. and to a lesser degree TNF, in the rat primary cardiac cells (Figure 5-17). Basal activity of the cloned canine MMP-9 promoter fragment was significantly increased with TNFα (Figure 5-18). In comparison, basal levels of activity of the in primary rat cardiocytes could be significantly increased by bFGF (Figure 5-19).

Figure 5-12 Basal and induced (IL-1, TNF and bFGF) levels of endogenous MMP-13 transcription in MDCK cells

A portion (365 bp) of the canine MMP-13 gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from MDCK cells. During PCR amplification samples were removed within the exponential phase of the reaction (35 cycles) revealing that basal levels of MMP-13 transcription were not significantly up-regulated by pro-inflammatory cytokines IL-1β. TNFα or bFGF.

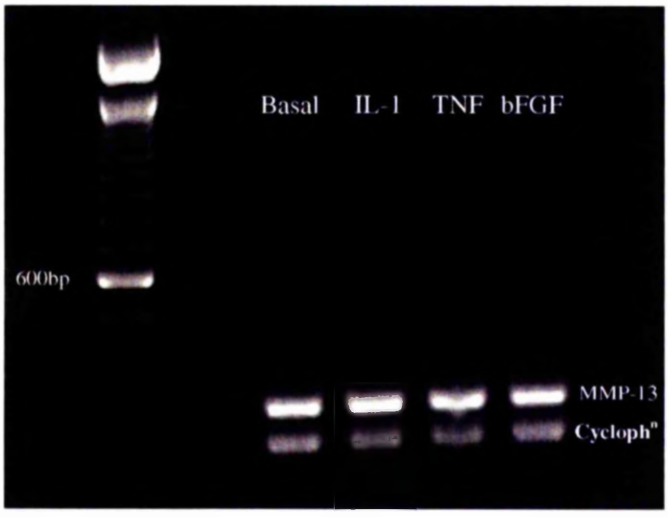


Figure 5-13: Basal and induced (IL-1, TNF and bFGF) levels of endogenous MMP-13 transcription in primary rat cardiocytes

A portion (365 bp) of the rat MMP-13 gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from primary rat cardiocytes. During PCR amplification samples were removed within the exponential phase of the reaction (45 cycles) revealing that basal levels of MMP-13 transcription were up-regulated by pro-inflammatory cytokines IL-1β. TNFα or bFGF.

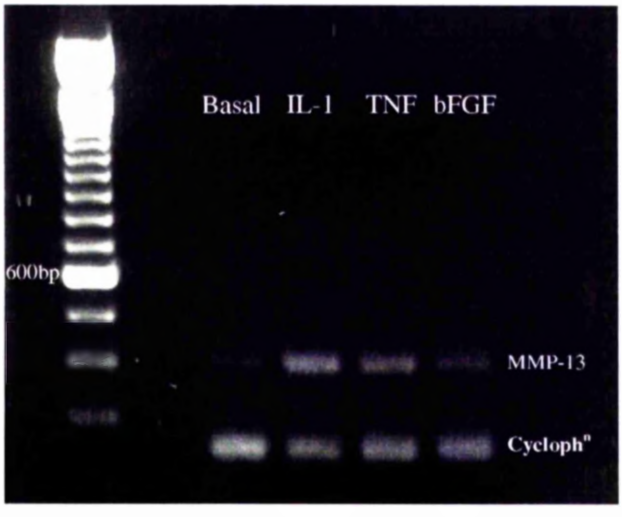


Figure 5-14 Basal and induced (IL-1, TNF and bFGF) levels of endogenous MMP-9 transcription in MDCK cells

A portion (364 bp) of the canine MMP-9 gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from MDCK cells. During PCR amplification samples were removed within the exponential phase of the reaction (40 cycles) revealing that basal levels of MMP-9 transcription were significantly up-regulated by the pro-inflammatory cytokine TNFα.

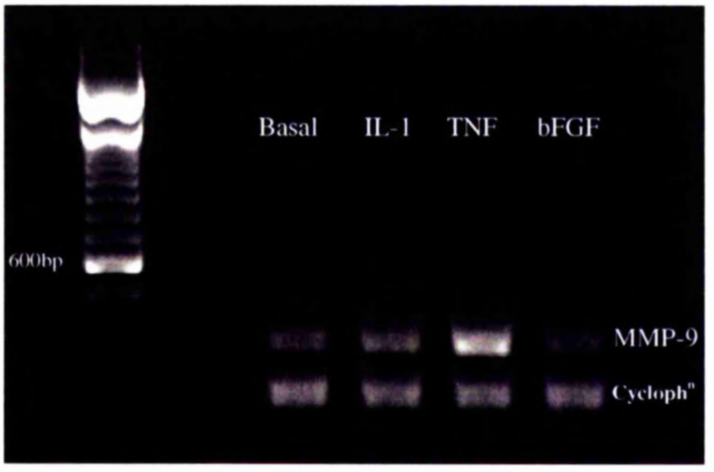


Figure 5-15 Basal and induced (IL-1, TNF and bFGF) levels of endogenous MMP-9 transcription in primary rat cardiocytes

A portion (365 bp) of the rat MMP-9 gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from primary rat cardiocytes. During PCR amplification samples were removed within the exponential phase of the reaction (45 cycles) revealing that basal levels of MMP-9 transcription were significantly up-regulated by the pro-inflammatory cytokine IL-1β. while some increase was also observed with TNFα and bFGF

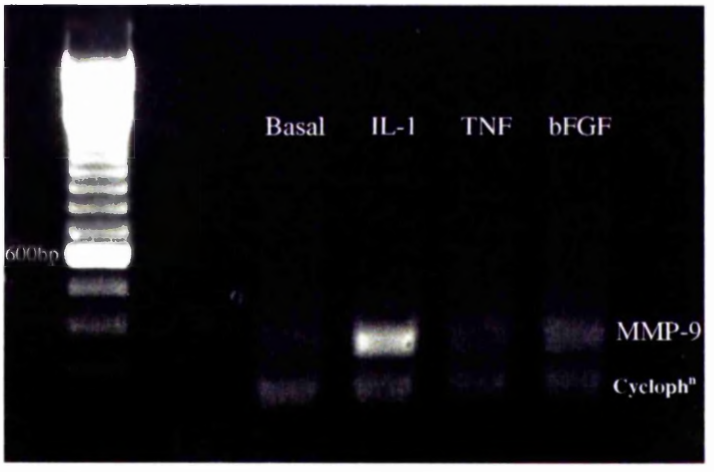


Figure 5-16: pGL3/cMMP-13(1494) promoter construct activity in MDCK cells

Dual® Luciferase Reporter assays were used to determine the activity of the cloned canine MMP-13(1494) promoter fragment in MDCK cells. Basal activity of cloned canine MMP-13 promoter fragment was present in the MDCK cells but could not be enhanced by any of the cytokines.

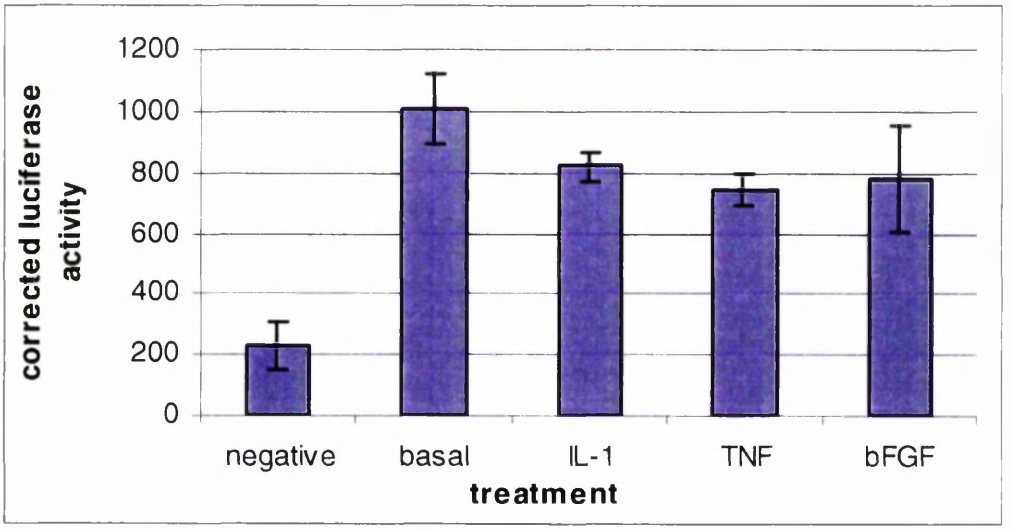


Figure 5-17 pGL3/cMMP-13(1494) promoter activity in primary rat cardiocytes

Dual® Luciferase Reporter assays were used to determine the activity of the cloned canine MMP-13(1494) promoter fragment in primary rat cardiocytes. Basal activity of cloned canine MMP-13 promoter fragment was evident in the primary rat cardiocytes. Statistical analysis showed that the basal activity could be significantly enhanced by IL-1β and bFGF (*P<0.05 Mann-Whitney test).

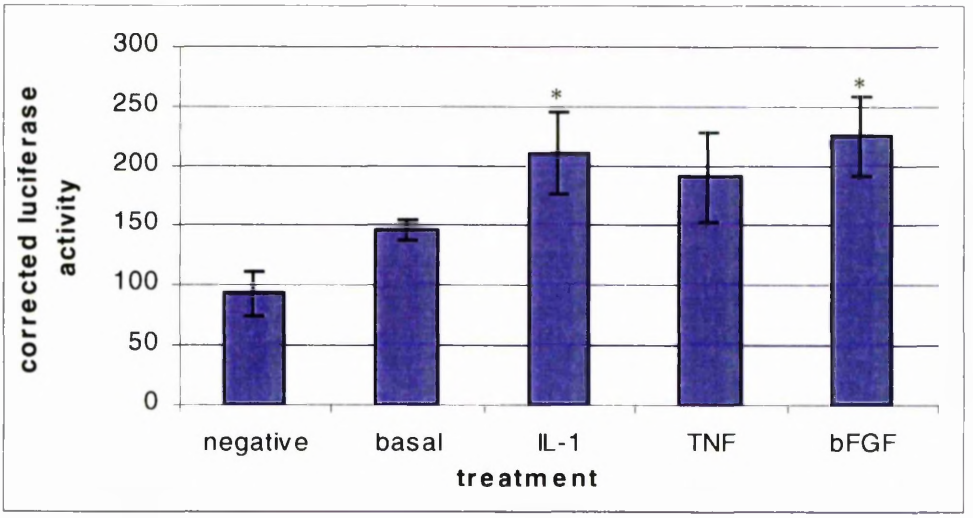


Figure 5-18 pGL3/cMMP-9(1894) promoter construct activity in MDCK cells

Dual®Luciferase Reporter assays were used to determine the activity of the cloned canine MMP-19(1494) promoter fragment in MDCK cells. Basal activity of cloned canine MMP-9 promoter fragment was evident in the MDCK and statistical analysis showed that the basal activity could be significantly enhanced by TNFα (*P<0.05 Mann-Whitney test).

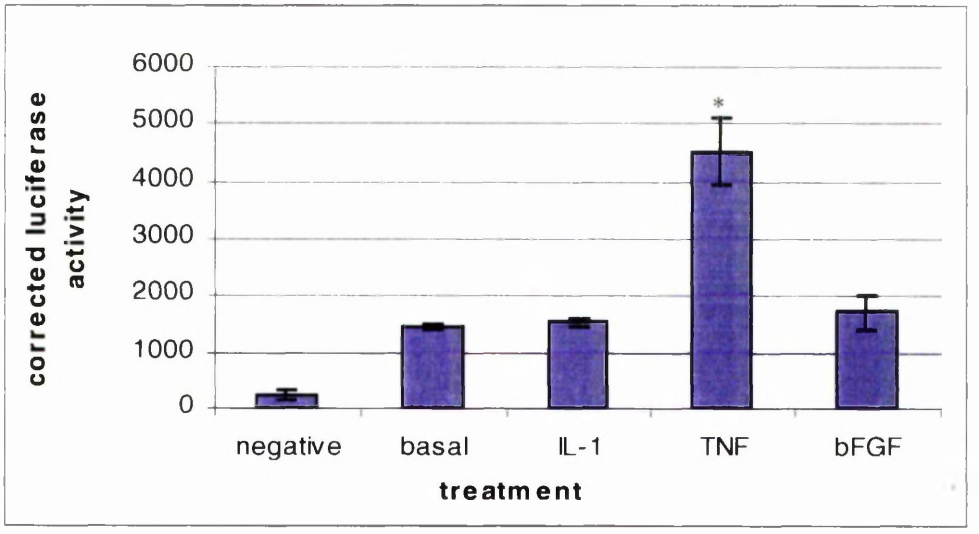
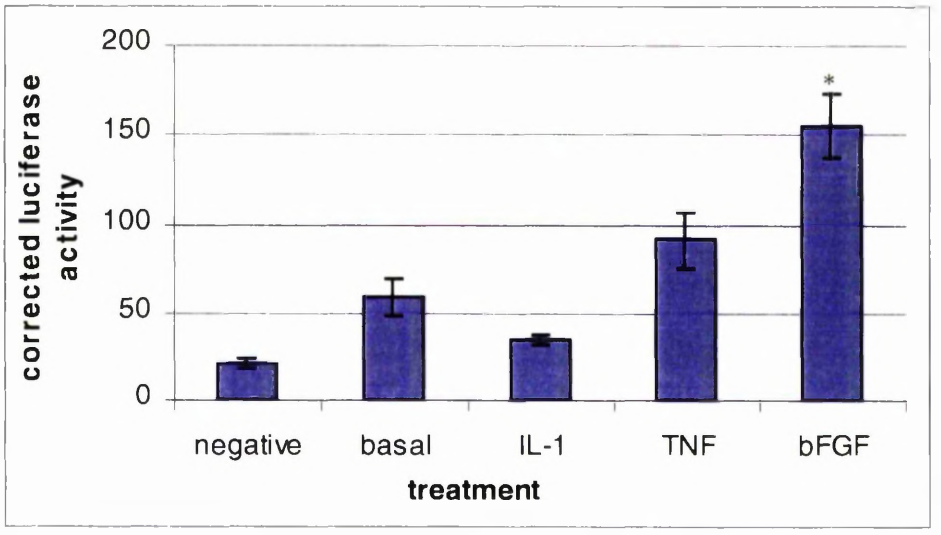


Figure 5-19 pGL3/cMMP-9(1894) promoter construct activity in primary rat cardiocytes

Dual®Luciferase Reporter assays were used to determine the activity of the cloned canine MMP-19(1494) promoter fragment in primary rat cardiocytes. Basal activity of cloned canine MMP-9 promoter fragment was evident and statistical analysis showed that the basal activity could be significantly enhanced by bFGF (*P<0.05 Mann-Whitney test).



5.4.3 Identification of PMA responsive elements in the canine MMP-9 promoter sequence

The aim of the second study was to evaluate and compare the basal and induced activities of the cloned canine MMP-9 promoter deletion constructs pGL3/cMMP-9(1894), (984), (628), (534), (176), and (102). These studies aimed to identify regions of the MMP-9 promoter that may be responsive to PMA, mimicking the effects of cytokines and growth factors, in the disease-state.

5.4.3.1 Endogenous expression of canine MMP-9 in MDCK cells

The endogenous and PMA induced levels of canine MMP-9 gene transcription in MDCK cells was investigated using relative semi-quantitative RT-PCR (Figure 5-20). A portion of the canine MMP-9 gene (364 bp) was amplified from cDNA samples prepared from basal and PMA treated MDCK cells. Samples removed at 5 cycle intervals from 15 to 35 cycles showed that the exponential/linear phase of the MMP-9 gene amplification occurred within this range and could be used to semi-quantify the levels of MMP-9 expression in these cells. Basal expression of endogenous MMP-9 gene was evident in MDCK cells and treatment with PMA was shown to induce expression of this gene. A portion of the canine cyclophilin gene was also amplified (255 bp) as an internal control. The primers designed for PCR spanned an intron to control for the presence of contaminating genomic DNA (absence of a 474 bp PCR product).

The endogenous levels of feline MMP-9 transcription could not be analysed in the FEA cells since the sequence for the feline MMP-9 cDNA was not available in the Genbank sequence database for primer design.

5.4.3.2 Analysis of cloned canine full-length MMP-9 promoter fragments and deletions using luciferase reporters

Dual-Luciferase®Reporter Assays were conducted as described in section 5.4.2.2. *Renilla* was expressed and shown to be active in cell types used. Representative *Renilla* values for the MDCK cells and FEA cells were 1882 and 824 respectively. The six canine pGL3/MMP-9 luciferase reporter constructs (Figure 5-5, 6, 7, 8, 9, 10) containing motifs as illustrated in Figure 5-4 were transiently transfected into both the canine MDCK cells (Figure 5-21) and FEA cells (Figure 5-22). In these experiments the promoter-less luciferase vector, pGL3-Basic vector was used as the negative control and the pGL3-Control, containing the SV40 promoter and enhancer sequences was used as a positive control vector

In the MDCK cells basal expression of the largest promoter fragment pGL3/cMMP-9(1894) was found to be high, approximately one third of the value of the positive control. Comparison of the basal luciferase expression levels between the various promoter deletions revealed a trend of maximal expression with pGL3/cMMP-9(628) containing the NF-κB motif. This trend was observed in three independent experiments but the difference between the promoter constructs was

not significant. The promoter activity was not completely lost with the smallest promoter fragment pGL3/cMMP-9(102). Basal levels of expression from the four largest promoter constructs pGL3/cMMP-9(1894), (984), (628), (534) could be significantly enhanced by the addition of PMA. Comparison of the PMA luciferase expression levels between the various promoter deletions revealed a trend of maximal expression again with the pGL3/cMMP-9(628) construct. This trend was observed in three independent experiments but this time the difference between the promoter constructs was significant. Activity of the constructs pGL3/cMMP-9(1894), (984), (628), (534) were significantly higher than the smallest construct pGL3/cMMP-9(102). In direct contrast there appeared to be very low levels of MMP-9 expression in FEA cells where basal expression of the MMP-9 promoter was shown to be approximately one sixth of the value of the SV40 driven luciferase expression. The level of basal expression could not be enhanced with PMA in these cells.

Figure 5-20 Basal and PMA induced levels of endogenous MMP-9 transcription in MDCK cells

A portion (364 bp) of the canine MMP-9 gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from MDCK cells. During PCR amplification samples were removed within the exponential phase of the reaction (15 to 35 cycles) revealing that basal levels of MMP-9 transcription were significantly up-regulated by the pro-inflammatory mediator PMA.

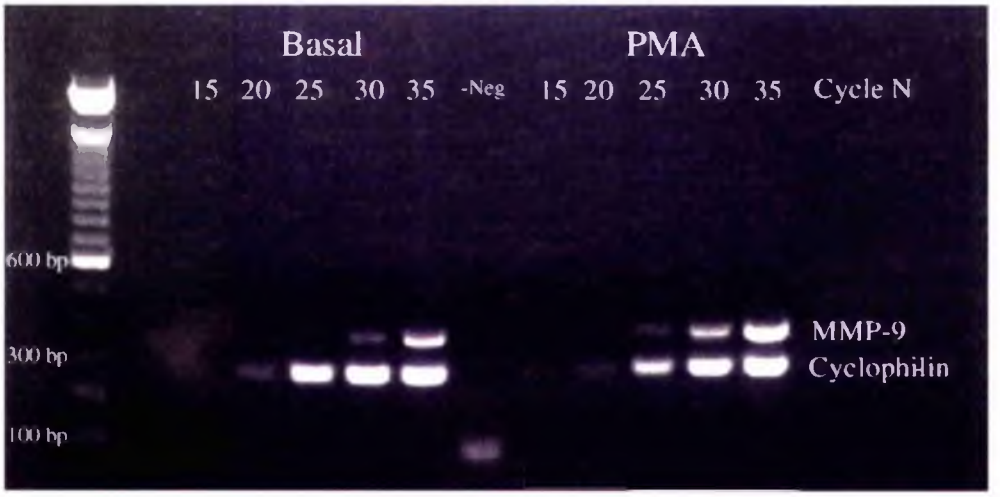


Figure 5-21 pGL3/cMMP-9 promoter deletion construct activity in MDCK cells

Dual®Luciferase Reporter assays were used to determine the activity of the cloned canine MMP-9 promoter deletion constructs in MDCK cells. Basal activity of the cloned canine MMP-9 promoter fragments were evident in the MDCK cells and statistical analysis showed that the basal activity could be significantly enhanced by PMA in the four largest constructs (*P<0.05 Mann-Whitney test). Comparison of the promoter deletion constructs using the Kruskal-Wallis test showed that there was no significant difference in promoter activity between the basal promoter deletions but that the PMA stimulated constructs were significantly different (P< 0.05). A follow-up comparison revealed that the PMA stimulated pGL3/MMP-9(102) was significantly lower than the four largest promoter constructs.

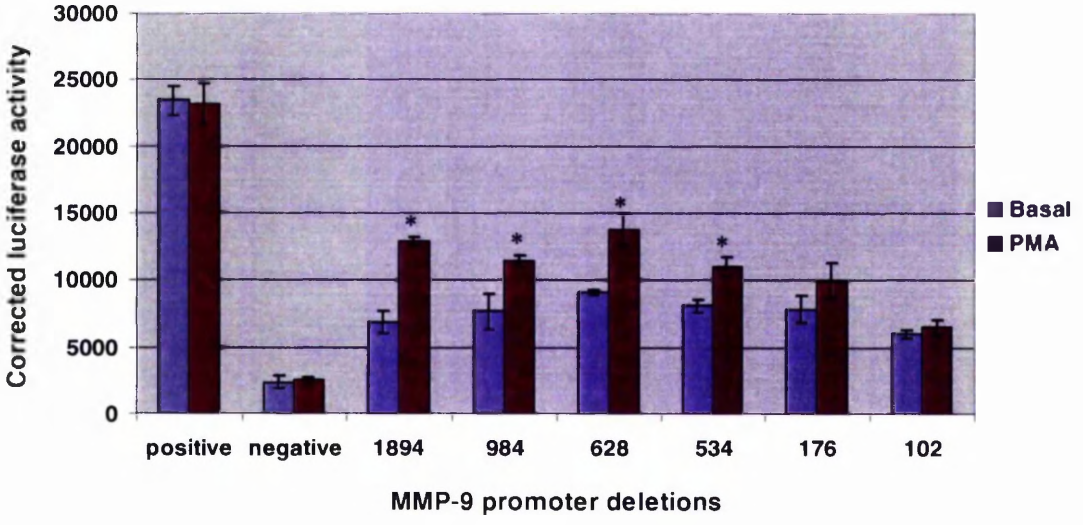
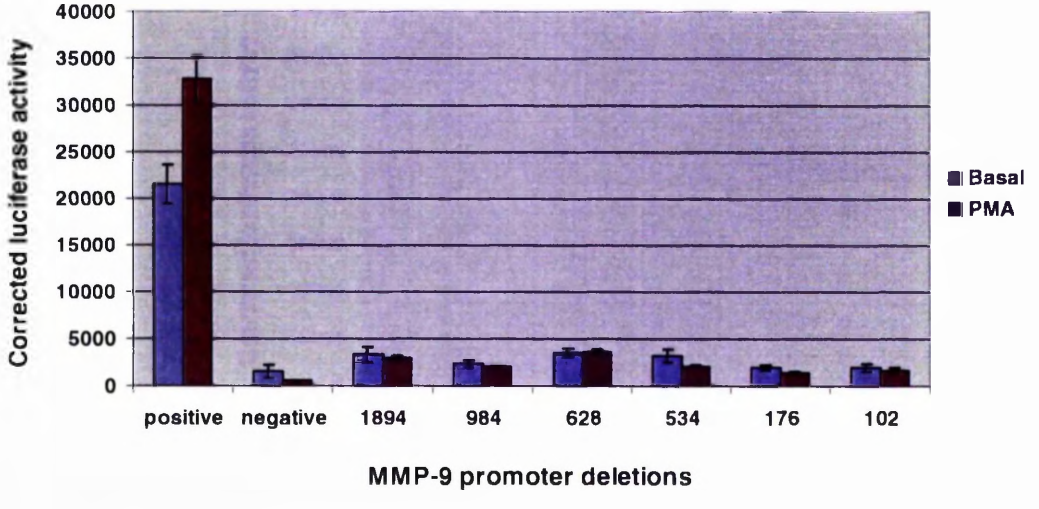


Figure 5-22 pGL3/cMMP-9 promoter deletion construct activity in FEA cells

Dual®Luciferase Reporter assays were used to determine the activity of the cloned canine MMP-9 promoter deletion constructs in FEA cells. Basal activity of the cloned canine MMP-9 promoter fragment was low in FEA cells and could not be enhanced by PMA.



5.5 DISCUSSION

5.5.1 Characterisation of the cloned canine pGL3/cMMP-9(1894) and MMP-13(1494) promoter fragments in MDCK and primary rat cardiocytes

To analyse the endogenous levels of MMP-9 and MMP-13 transcription in the cell lines to be used for the transfection of the canine pGL3/cMMP-9(1894) and pGL3/cMMP-13(1494) promoter constructs relative semi-quantitaive RT-PCR was performed. The two constructs were transiently transfected into the MDCK cells and primary rat cardiocytes where basal luciferase activity for each promoter was compared to treated samples (IL-1β, TNFα and bFGF) along with a promoter-less luciferase vector as a negative control.

5.5.1.1 Assessment of basal and induced (IL-1β, TNFα and bFGF) endogenous transcription levels of MMP-9 and MMP-13 genes in MDCK cells and rat cardiocytes.

An immortalised rabbit synovial cell line (HIG-82) was analysed first for use in the transfection experiments; however, the high levels of basal transcription could not be enhanced by any of the stimulants used (data not shown). These results are contradictory to studies showing that cultured rheumatoid synovial fibroblasts responded to PMA, IL-1, TNFα, TGFβ and IL-6 with increased collagenase-3 mRNA (Moore et al., 2000). The difference in these results may be explained by the phenotypic changes that have occurred during development of arthritis where the diseased cells are more likely to express the receptors for these cytokines and growth factors than the HIG-82 cells. However, MMP-13 expression can be stimulated with PMA, IL-1β and TNFα in isolated primary rabbit synovial fibroblasts (Vincenti et al., 1998).

Endogenous levels of MMP gene expression were subsequently evaluated in MDCK cells and primary rat cardiocytes using RT-PCR techniques. Basal expression of the endogenous MMP-13 gene was evident in both cell types but only the rat primary cardiocytes could be induced with IL-1β, TNFα and bFGF (Figure 5-12, 5-13). These results are in concordance with a study showing MMP-13 up-regulation with these cytokines in adult rat cardiac fibroblasts (Siwik, Chang et al., 2000). In comparison basal and enhanced expression of the canine MMP-9 promoter was evident in both cell types (Figure 5-14, 5-15). In the MDCK cells only TNF was able to increase activity contradicting a previous study which demonstrated increased expression of MMP-9 in a primary rat mesangial cell line by IL-1 (Yokoo and Kitamura, 1996). This suggests that phenotypic changes may occur during immortalisation of cells and it is possible that the MDCK cells either fail to express IL-1 receptors or do not contain the necessary machinery for IL-1 induction. In the primary rat cardiocytes the endogenous MMP-9 promoter activity was clearly upregulated with IL-1β and to a lesser degree with TNFα and bFGF.

The results of endogenous activity of the MMP-13 and MM-9 promoters suggested that the cloned canine MMP-13 & 9 promoter fragments could be analysed in the primary rat cardiocytes.

5.5.1.2 Comparison of basal and induced (IL-1β, TNFα and bFGF) activity of pGL3/MMP-9 MMP-13 promoter constructs

Activity of the canine pGL3/cMMP-13(1494) promoter construct was similar to the endogenous MMP-13 transcriptional activity in both cell lines. Basal activity present in the MDCK cells was non-responsive to any of the cytokines (IL-1β, TNFα and bFGF) (Figure 5-16), confirming that the cloned canine MMP-13 promoter could not be characterised in this cell line. The results from the rat primary cardiac cells revealed that basal expression of the cloned canine MMP-13 promoter fragment could be significantly enhanced with IL-1β and bFGF, and to a lesser degree TNF (Figure 5-17). However, it is thought that the level of induction seen with the cytokines may be enhanced by the stable transfection of the promoter constructs (Menghol et al., 2001).

Activity of the cloned canine MMP-9 promoter fragment was also similar to the endogenous MMP-9 promoter activity in the MDCK cells showing significant induction with TNFα (Figure 5-18); this suggests that the portion of the cloned canine MMP-9 promoter was functional and responsive to this cytokine. However, the luciferase results from the MMP-9 promoter in primary rat cardiocytes varied slightly from the endogenous data showing that although TNF could be used to increase activity only bFGF showed significant induction (Figure 5-19). Contradictory to the endogenous data, IL-1β showed no up-regulation at all suggesting that IL-1 responsive promoter elements may lie out-with the cloned fragment necessary for this cell type. The potential for synergistic activation of each cytokine with bFGF was also examined but no synergism was evident (data not shown) contradicting evidence for combined IL-1 and bFGF activation seen in rabbit dermal fibroblasts (Bond, Baker et al., 1999).

It is clear that selection of cell type is important when analysing the activity of cloned promoter fragments. These data suggest that the region of the cloned canine MMP-13 promoter described in this chapter is sufficient to drive basal expression in both cell types and contains promoter elements responsive to IL-1β, TNFα and bFGF signalling pathways as observed in the primary rat cardiocytes. However, the response of the fragments was not as large as endogenous levels and therefore likely to be missing some enhancer elements. The canine MMP-9 promoter fragment is also able to drive basal expression but only appears to contain the necessary binding motifs for TNF and bFGF signalling pathways in these cell types.

5.5.2 Analysis of canine MMP-9 promoter for the identification of PMA responsive elements using deletion constructs

To analyse the endogenous levels of MMP-9 transcription in the cell lines to be used for the transfection of the canine pGL3/cMMP-9(1894), (984), (628), (534), (176) and (102) promoter deletion constructs relative semi-quantitative RT-PCR was performed. The six constructs were transiently transfected into the MDCK cells and FEA cells where basal luciferase activity for each promoter was compared to PMA treated samples along with pGL3-Basic, a promoter-less luciferase vector as a negative control, and pGL3-Control a SV40 promoter driving luciferase gene expression.

5.5.2.1 Assessment of basal and PMA induced endogenous transcription levels of MMP-9 in MDCK and FEA3 cells.

Basal and PMA induced levels of endogenous MMP-9 gene transcription in MDCK cells is shown in Figure 5-20. Basal and PMA induced transcription was evident over cycle range 25 to 35 showing that samples could be compared over this exponential phase. Basal levels of transcription were up-regulated by PMA supporting previous documentation that PMA could be both upregulated and suppressed dependant on cell type (Hanemaaijer et al., 1993). The results of the basal and PMA induced endogenous MM-9 transcription suggested that the cloned canine MMP-9 promoter deletion fragments could be analysed in the MDCK cells using PMA.

5.5.2.2 Characterisation of the canine MMP-9 promoter using deletion analysis

In MDCK cells basal expression of the largest promoter construct pGL3/cMMP-9(1894) was found to be high (Figure 5-13) confirming studies showing MMP-9 is normally expressed in cells specific to the kidney (Yokoo and Kitamura, 1996). Comparison of the basal luciferase expression levels between the various promoter deletions revealed a trend of maximal expression with the pGL3/cMMP-9(628) construct containing the NF-κB motif indicating that it may serve to enhance transcription. This trend was observed in three independent experiments but the difference between the promoter constructs was not significant. The promoter activity was not completely lost with the removal of the conserved AP-1 site at position (-111 to 105) as described for the human promoter (Sato and Seiki, 1993). Since the canine sequence contains an additional AP-1 site (-73 to –67) not found in the human counterpart we speculate this extra AP-1 site may perhaps be sufficient to drive basal expression. Similar results have been reported in the rat promoter with mutation of the conserved AP-1 (-111 to –105) in the rat sequence only reducing basal expression by twenty-five percent (Eberhardt et al., 2000). Up-stream repressor sites have been identified in the mouse MMP-9 promoter which may function to regulate basal levels of promoter activity in a tissue specific manner (Roach et al., 1998) but this region of the canine promoter has not been analysed. Basal levels of expression from the four largest promoter constructs pGL3/cMMP-9(1894), (984), (628), (534) could be significantly enhanced by the addition of PMA suggesting the presence of inducible regions in all constructs. Comparison of the PMA luciferase expression levels between the various

promoter deletions revealed a trend of maximal expression again with the pGL3/cMMP-9(628) construct. This trend was observed in three independent experiments but this time the difference between the promoter constructs was significant. The constructs pGL3/cMMP-9(1894), (984), (628), (534) were significantly higher than the smallest construct pGL3/cMMP-9(102) suggesting that the latter construct does not contain any inducible regions.

In direct contrast there was very low levels of MMP-9 gene transcription in the FEA cells (Figure 5-22). This could be due to a cell-type specificity since species-specificity could be ruled out by studies showing activity of human promoter in rabbit cells (He, 1996). Cell-type specific expression was thought to reside in the DNA sequence found upstream of -2722 bp in the mouse promoter (Munaut et al., 1999), and since only the 1894 bp of canine promoter DNA had been cloned it was not possible to assess cell type specificity in this study. The level of basal expression could not be enhanced with PMA in these cells.

5.5.3 Relevance of MMP promoters to gene-based therapy

The canine specific MMP-9 and MMP-13 promoters were selected to drive expression of therapeutic genes in preference to commonly utilised promoters such as the cytomegalovirus (CMV) promoter which provides high levels of constitutive gene expression. It was thought that the MMP promoters would provide regulated levels of gene expression, in response to pro-inflammatory cytokines unlike viral promoters. However, although the low levels of basal expression observed could be up-regulated to some extent with the pro-inflammatory cytokines it is debatable whether these induced levels of transgenes will provide therapeutic levels of gene expression, equivalent to those levels observed with viral promoters. As such it is likely that these promoters will require manipulation to enhance activity in disease state while maintaining low levels of basal expression, by the incorporation of enhancer elements into the promoter sequences.

5.6 SUMMARY

This chapter describes the characterisation of the canine specific MMP-9 (1894 bp) and MMP-13 (1494 bp) gene promoters in MDCK cells and primary rat cardiocytes in response to pro-inflammatory mediators IL-1, TNF and bFGF. A series of promoter deletion constructs, based on the canine MMP-9 promoter sequence pGL3/cMMP-9 (1894), (984), (628), (534), (176) and (102), were analysed in MDCK and FEA cells in response to PMA to identify inducible elements within the promoter sequence. The canine MMP-9 and MMP-13 promoters were analysed to investigate the unique ways in which each MMP gene is regulated through pro-inflammatory mediators during the disease process and to enable their use in targeted structure-modifying therapies.

Chapter VI

Targeting therapeutic gene expression to the diseased cell types of the canine osteoarthritic joint

6.1 ABSTRACT

The potential for undesirable systemic effects related to constitutive over-expression of certain therapeutic transgene products may be limited through the development of both 'disease and cell type specific' DNA targeting vectors that restricts therapeutic gene expression to diseased cell types of the joint through transcriptional regulation. To enable the incorporation of both disease and cell type specific promoter elements into one vector a novel dual-targeting vector system was designed. Two parameters were addressed during the development of this vector. Firstly, the promoters incorporated into this system for driving gene expression required strategic manipulation to enhance promoter activity while maintaining both disease and cell type specificity. Secondly, the utilisation of Cre-lox technology was necessary to enable both disease and cell type targeting systems to be incorporated into a single vector system. This chapter describes the analysis of the canine MMP-9 promoter luciferase reporter, pGL3/cMMP-9(1894) and deletion constructs, pGL3/cMMP-9(984), (628) and (534) in response to both pro-inflammatory cytokines, interleukin-1 (IL-1β) and tumour necrosis factor (TNFα) in human chondrosarcoma cells (SW1353). These deletion studies, in combination with mutagenesis analysis, guided the manipulation of the MMP-9 promoter with the incorporation of multiple NF-κB sites into the 5′ end to enhance activity while maintaining disease-specificity. This chapter also describes the cloning of the mouse collagen type XI (mColXI) promoter (1.2 kb) from murine genomic DNA (gDNA) and analysis of cell type specific promoter activity in the chondrocyte-specific cells (SW1353) and undifferentiated chondroprogenitor cells (ATDC5). The mColXI promoter was manipulated with the incorporation of SOX9 enhancer sites into the vector to increase activity while maintaining cell type specificity. This chapter finally describes attempts to strategically sub-clone the canine MMP-9 and mouse ColXI promoters, manipulated with NF-κB and SOX9 sites respectively, into one plasmid vector containing the gene for 'Cre recombinase' and *lox*H sites to create a novel dual-targeting system.

6.2 INTRODUCTION

Developing gene-based therapy for osteoarthritis (OA) in both human and veterinary patients represents an exciting challenge. The detailed understanding of disease pathogenesis has already enabled the introduction of 'structure-modifying' therapeutic genes into arthritic joints to control disease progression at the molecular level by inhibiting the enzymes responsible for cartilage degradation while enhancing tissue repair. However, despite considerable advances in molecular biology several technical problems still exist, which must be solved before gene therapy can be considered for use in clinical practice. In the development of a gene-based therapy for OA appropriate therapeutic genes and vector vehicles must be selected and methods devised to efficiently deliver these constructs to cells of the joint where sustained therapeutic levels of gene expression can be provided. However, it is also necessary to minimise undesirable side-effects associated with most therapies by accurately targeting therapeutic gene expression to diseased joint cell of the osteoarthritic joint such as chondrocytes and synovial cells. To this end, regulatory promoter elements can be modulated to enhance therapeutic levels of gene expression whilst maintaining both disease and cell type specificity.

6.2.1 Transcriptional regulation of transgenes

The ultimate success of any gene-based therapy strategy will therefore rely on the efficiency and duration of transcription, directed by promoter DNA sequences and associated regulatory elements, to provide adequate levels of transgene expression. Manipulation of these regulatory sequences may enable the rate of transcription initiation, crucial in the achievement of modulated therapeutic levels of transgene expression, to be obtained *in vivo*. The modular nature of the promoter region allows a considerable degree of flexibility when deciding on the design of the promoter/enhancer elements to be used to control gene expression using artificial transcriptional units. For example enhancers isolated from one regulatory region can be incorporated into a promoter with whom they are not necessarily associated to produce an optimised, manipulated promoter for the regulated control of therapeutic gene expression. Many different methods of targeting gene expression with promoter manipulation have been investigated.

6.2.1.1 Manipulation of promoters for targeting gene therapy

Some eukaryotic promoters are ideally suited for gene therapy since they combine strong transcriptional activity with a high degree of specificity; this is true for the melanocyte-specific tyrosinase promoter (Siders et al., 1996). However, often the application of highly specific promoters results in inefficient levels of transcriptional activity. A typical example is the von Willebrand factor (vWF) promoter which is highly specific for endothelial cells but is a poor activator of transcription (Ferreira et al., 1993). Subsequently, different methods for enhancing the transcriptional activity of eukaryotic promoters while maintaining their specificity have been developed with the incorporation of transcriptional control elements into promoter regions. One system has described the use of 'self-enhancing promoters' which incorporates a positive feedback

loop provided by a chimeric transcription factor consisting of the strong herpes simplex virus VP16 transcriptional activation domain fused to the DNA-binding domain of LexA (Nettlebeck et al., 1998). Disease-specific components have also been incorporated into promoter regulatory systems to enhance transcription in response to the disease-state. For example, therapeutic gene expression can be driven in response to a disease-induced hypoxic environment with the inclusion of a hypoxia-resonsive element (HRE) into the promoter which interacts with the transcriptional complex hypoxia-inducible factor-1 (HIF-1) (Dachs et al., 1997). Other studies have utilised concurrent treatments to enhance promoter activity such as the radiation-inducible promoter region of the Egr-1 gene which can specifically direct expression of TNFα in tumour tissues in response to radiation treatment (Hallahan et al., 1995). Furthermore, it is may also be desirable to switch gene expression on or off with the use of regulatable promoters. One of the most elegant promoter systems in use today is the tetracycline-switch mechanism (Gossen et al., 1995) which involves the E.Coli tetracycline (tc) repressor fused with the C-terminal domain of eukaryotic transcriptional activator VP16. This tetracycline-regulatable transcription factor (tTA) specifically trans-activates artificial minimal promoters carrying multiple tc operator sites (tc-Op). Adding tc to the system reversibly inhibits binding of tTA to promoter DNA and blocks gene expression. This system has been used in a number of systems including transgenic mice (Ghersa et al., 1998).

6.2.1.2 Tissue specific promoters for targeting gene therapy

The construction of vectors enabling tissue-specific gene expression, is one of the current challenges in the field of gene therapy now thought possible by placing therapeutic genes under the control of regulatory promoters that possess binding sites for tissue specific factors. However, correctly regulated expression may not only require promoter regions but also the distant 5′ and 3′ elements that influence tissue specific promoter activity (Miller and Vile, 1995). Tissue-specific regulatory elements have already been used to target gene expression to certain cell types. For example, using the transgenic mouse model for muscular dystrophy, the creatine kinase promoter has been used to restrict dystrophin cDNA expression to skeletal and cardiac muscles to correct the clinical signs of disease without deleterious side-effects (Cox et al., 1993). The promoter of the Ig heavy chain has also been used to direct tissue-specific expression of the diphtheria toxin A (DT-A) gene in lymphoid cells (Maxwell et al., 1991). However, vector context is an important parameter when designing tissue-specific targeting systems. Although tissue specific promoters frequently retain their specificity in the context of retroviral vectors (Hatzoglou et al., 1990) this is not always the case, and the design of the viral vectors may have significant effects on cell type specificity due to promoter interference (Vile et al., 1994).

This cell-type specific targeting system not only applies to the treatment of systemic diseases but also local gene therapy, whose efficacy and safety can be improved by the use of cell-type specific promoters keeping the expression of therapeutic genes in non-target cells to a minimum. This tissue-specific promoter system can easily be applied to the gene therapy for OA by incorporating promoter elements that are specifically activated in cell types specific to the joint

such as chondrocytes or synovial cells. For example the collagen types II, IX and XI are expressed in chondrocytes (Mendler et al., 1989) and may be used to drive cell type specific gene expression in joints. The discovery of synovial-specific promoter elements such as hyaluronan (although the expression of this gene is not entirely tissue specific) will enable the development of targeting systems to accompany the advanced delivery systems already established for this cell type.

6.2.1.2.1 Chondrocyte specific gene expression

Collagens are expressed in a tissue-specific manner with specific types unique to the articular cartilage. The type XI collagen molecules co-assembles with the major type II collagen, to form cartilage fibrils, whereas type IX collagen is associated with the surface of the fibrils (Mendler et al., 1989). The type XI collagen molecule regulates the diameter of cartilage collagen fibrils (Mendler et al., 1989) as illustrated by a null mutation in the α1(XI) gene leading to abnormally thick collagen fibrils in chondrodysplastic mice (Li et al., 1995). This observation suggests that the fidelity of the spatio-temporal expression of type XI collagen is crucial for the development and maintenance of the normal structure of cartilage. The type XI collagen molecule is composed of three distinct sub-units, α1(XI), α2(XI), and α3(XI), (Morris and Bachinger 1987) each with unique patterns of expression. The α1(XI) and α3(XI) are found in a variety of non-cartilaginous tissues (Yoshioka et al., 1995) whereas the expression of the collagen α2(XI) gene appears to be more restricted. Although low levels of alternatively spliced variants of the α2(XI) transcripts are found in non-cartilaginous tissues the major transcripts are pre-dominantly found in cartilage tissue (Tsumaki and Kitamura, 1995). The 5′ flanking region of the mouse α2(XI) collagen gene (Col11a2) was cloned and analysed to reveal that the first 742 base pairs of the promoter, immediately up-stream of the transcriptional start site, contained information for gene expression specific to the cartilage of long bones and ribs (Tsumaki et al., 1996). *Cis* regulatory regions of this promoter, necessary for cartilage specific expression, were then identified and shown to consist of an enhancer site (24 bp) located within the 500 and 530 base pairs region of the promoter (Tsumaki et al., 1998). This sequence was sufficient to bind nuclear proteins selectively expressed in chondrocytes (Lefebvre et al., 1996) which are now specifically referred to as the SOX9 proteins. The SOX9 protein contains a high mobility group (HMG)-type DNA binding domain and a trans-activation domain that has been mapped to the C terminus (Sudbeck et al., 1996). It is expressed abundantly in chondrocytes and in chondroprogenitor cells (Ng et al., 1997) and can activate the chondrocyte-specific enhancer in non-chondrocytic cells in transient transfection experiments (Lefebvre et al., 1997). This suggests that SOX9 proteins play an important role in chondrogenesis perhaps by activating collagen type XI gene expression. Other HMG-like sites are present in the enhancer site which participate in the formation of a large chondrocyte-specific protein complex that includes SOX9 and other proteins which appear to belong to the HMG domain protein family (Zhou et al., 1998). These enhancer sites are also present in the other sub-units of the collagen Type IX gene sequence, such as α1(IX) (Bridgewater et al., 1998).

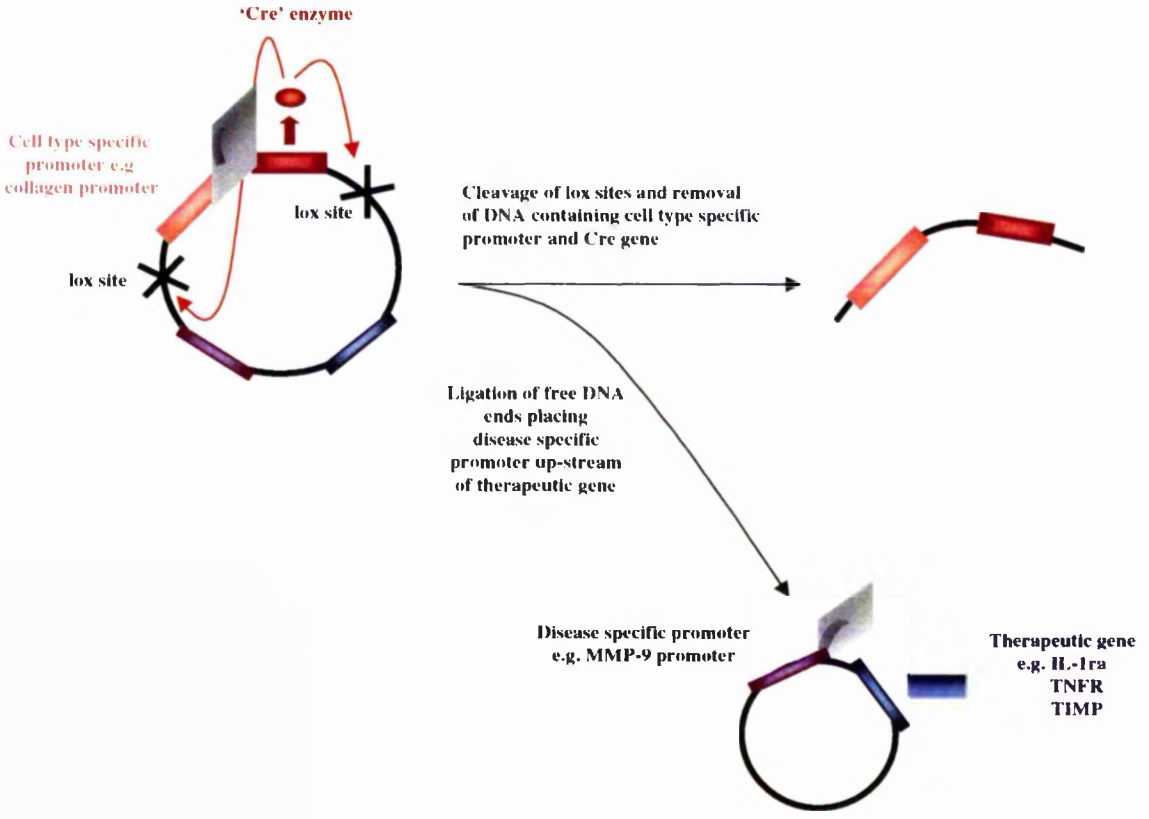
6.2.1.3 A dual-promoter system for targeting gene therapy

Novel methods for targeting therapeutic gene delivery are now possible with the application of Cre-lox technology. The recombinase Cre protein (38 kDa) of bacteriophage P1 (Sterberg et al., 1978) is a member of the family of site-specific recombinases and integrases that catalyse inter- and intra-molecular DNA rearrangements. Cre recombinase catalyses the excision of DNA located between flanking *lox* sites, leaving one site behind. *Lox* sites are 34 base pair sequences, with two 13 base pair inverted repeats separated by an 8 base pair space region (Sauer and Henderson, 1990; 1989; 1988). This technology has become widely used since its first application in mammalian cells and is now the universal reagent for genome tailoring (Nagy, 2000) with applications in the *in vitro* manipulation of DNA without the use of restriction enzymes (Buchholz and Bishop, 2001). Methods of regulating Cre gene expression by linking the gene to a number of inducible promoters has already been described (Kuhn et al., 1995). For example the combination of cell-type specific and temporal controls has enabled the expression of a chimeric protein, consisting of a fusion between Cre and mutated steroid ligand-binding domain of the receptor, under the control of tissue specific promoters. The chimeric protein becomes active upon interaction with the synthetic ligand tamoxifen (Schwenk et al., 1998). A temporal, spatial and cell-type-specific control of Cre-mediated DNA recombination has also been developed using the tetracycline inducibility in transgenic mice (Utomo et al., 1999). This self-containing Cre/lox site-specific recombination system has also been described to target telomerase positive/p53-negative tumour cells for bladder cancer (Pan and Koeneman, 1998). Furthermore, a double infection system has also been described for targeted treatment of hepatic carcinoma using a regulator recombinant adenovirus producing site specific recombinase Cre expression under the control of the hepatocarcinoma-specific α-feto-protein (AFP) promoter in combination with the suicide gene thymidine kinase (Sato et al., 1998). Self deleting retroviral vectors using Cre gene expression have also been developed to overcome problems encountered with recombination into the host cell genome (Russ et al., 1996).

Using Cre-lox technology the potential for undesirable systemic effects related to constitutive over-expression of certain therapeutic transgene products may be limited through the development of a 'disease and cell type specific' DNA targeting vector that restricts transgene expression to diseased chondrocytes (Figure 6-1). It was hypothesised that transgene expression could be tightly controlled at the transcriptional level through the proper assembly of the appropriate *cis*-acting regulatory regions of DNA. Since metalloproteinase (MMP) promoters are upregulated during the arthritic disease process, these regulatory sequences could be utilised to direct disease-specific transgene expression while simultaneously avoiding undesirable expression in healthy tissues. A second level of targeting could be incorporated into the system using chondrocyte specific promoters and enhancers, such as those for the collagen type XI gene, to limit expression of therapeutic genes to cell types specific for the diarthrodial joint.

Figure 6-1 Targeting therapeutic gene expression to diseased cell types of the joint

This dual targeting vector system utilises a cell type specific promoter to drive expression of a gene encoding the P1 bacteriophage recombinase enzyme referred to as 'Cre'. This enzyme is capable of cutting DNA at specific lox sites before recombining the two free ends of DNA. This results in the removal of the DNA encoding the cell type specific promoter and Cre gene bringing the disease specific promoter up-stream of the therapeutic gene.



6.3 MATERIALS AND METHODS

Overviews of the experimental procedures employed in this chapter are illustrated in figure 6-2, 6-3 and 6-4. The construction of pGL3/cMMP-9(1984) and deletions pGL3/cMMP-9(984), (628) and (534) are described in detail in chapter V. The human chondrosarcoma cell line (SW1353) was analysed for basal and induced (IL-1β and TNFα) levels of endogenous MMP-9 gene transcription using reverse transcription (RT)-PCR. The canine MMP-9 promoter deletions pGL3/cMMP-9(1894), (984), (628) and (534) were transiently transfected into SW1353 cells and basal and induced (IL-1β and TNFα) activities were determined using Dual-Luciferase®Reporter Assays. In a similar fashion the mutated, pGL3/cMMP-9(628)(NF-κBmut[a]), and manipulated pGL3/cMMP-9(5NF-κB), (3NF-κB) and (1NF-κB) constructs were analysed using the same assay system.

The human chondrosarcoma cell line (SW1353) and undifferentiated chondrosarcoma cell line (ATDC5) were analysed for basal and induced (IL-1 and TNF) levels of endogenous collagen type XI gene transcription using RT-PCR. The mouse collagen type XI promoter and intron 1 regions were amplified by PCR and into cloned pCR2.1®TOPO and then sub-cloned into pGL3-Basic luciferase reporter vector. Activity of the promoter was analysed in the two cell lines using Dual-Luciferase®Reporter Assays. The mouse collagen type XI promoter and intron 1 sequences were manipulated with the addition of SOX9 sites and activities determined using the same assay system.

Once the individual disease and cell type specific targeting elements had been evaluated, attempts were to made to construct the dual-targeting vector in four steps. The mouse collagen type XI promoter was amplified by PCR incorporating a *lox*H site into the 5′ end of the sequence. This was sub-cloned into pGL3-Basic (Vector A). The Cre gene plus polyadenylation site was cut from the pCAGGS-nls Cre vector and sub-cloned into vector A to create vector B. The canine MMP-9 promoter, containing three NF-κB sites in the 5′ end of the sequence, was cut from the pGL3/cMMP-9(1894)(3NF-κB) vector and sub-cloned into vector B to form vector C. The mouse collagen type XI intronic region containing three flanking SOX9 sites was amplified, incorporating a *lox*H site in the 3′ end of the sequence, and efforts were made to sub-clone this into vector C to make vector D.

The final step in the construction of the dual targeting vector was not possible due to technical problems. Evidence for the endogenous expression of Cre during the transformation procedure was sought using Western blot protocols.

Figure 6-2 Overview of experimental procedures used to manipulate and analyse the canine MMP-9 promoter

The human chondrosarcoma cell line (SW1353) was analysed for basal and induced (IL-1β and TNFα) levels of endogenous MMP-9 gene transcription using RT-PCR. The activities of MMP-9 promoter deletions pGL3/cMMP-9(1894), (984), (628) and (534) were analysed in the SW1353 cells using Dual-Luciferase® Reporter assays. The mutated, pGL3/cMMP-9(628)(NF-κBmut^{n}), and manipulated, pGL3/cMMP-9(5NF-κB), (3NF-κB) and (1NF-κB), constructs were analysed using the same assay system.

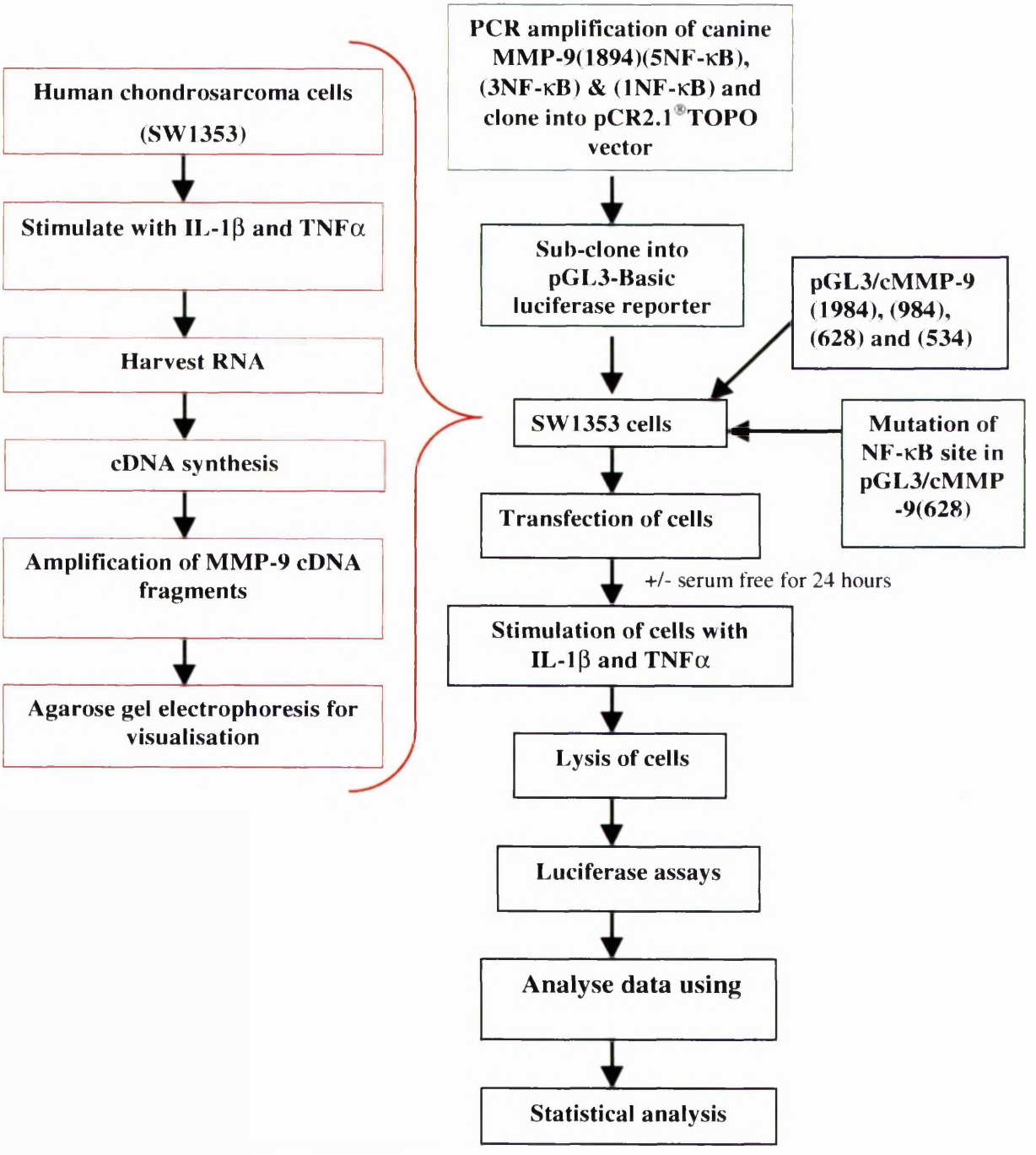


Figure 6-3 Overview of the experimental procedures used to clone and analyse the mouse collagen type XI promoter

The human chondrosarcoma cell line (SW1353) and undifferentiated chondrosarcoma cell line (ATDC5) were analysed for basal and induced (IL-1β and TNFα) levels of endogenous collagen type XI gene transcription using RT-PCR. The mouse collagen type XI promoter and intron 1 were amplified by PCR and cloned into pCR2.1®TOPO before sub-cloning into pGL3-Basic luciferase reporter vector. Activity of the promoter was analysed in the two cell lines using Dual-Luciferase®Reporter Assays. The mouse collagen type XI promoter and intronic sequences were manipulated with the addition of SOX9 sites and activities determined using the same assay system.

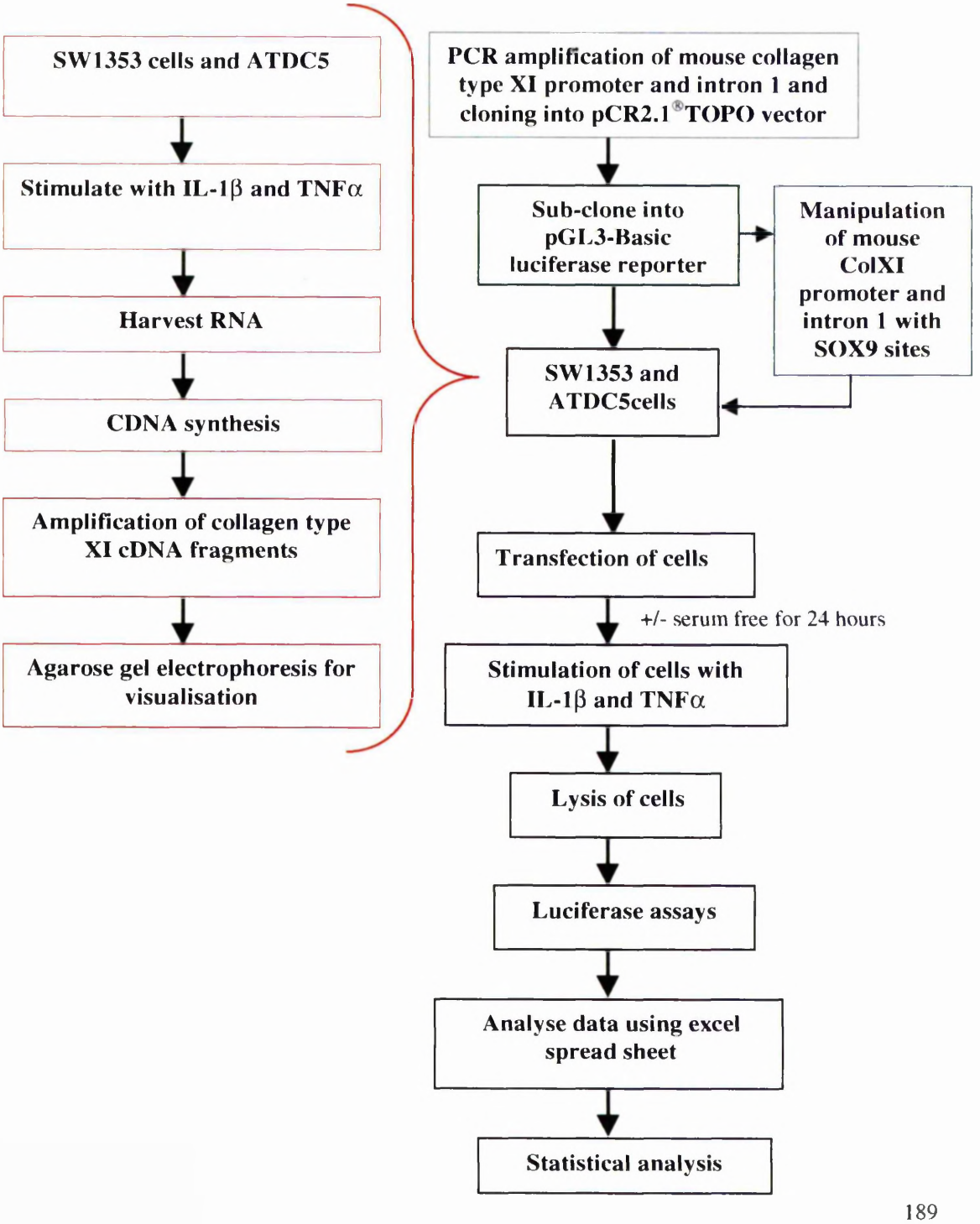
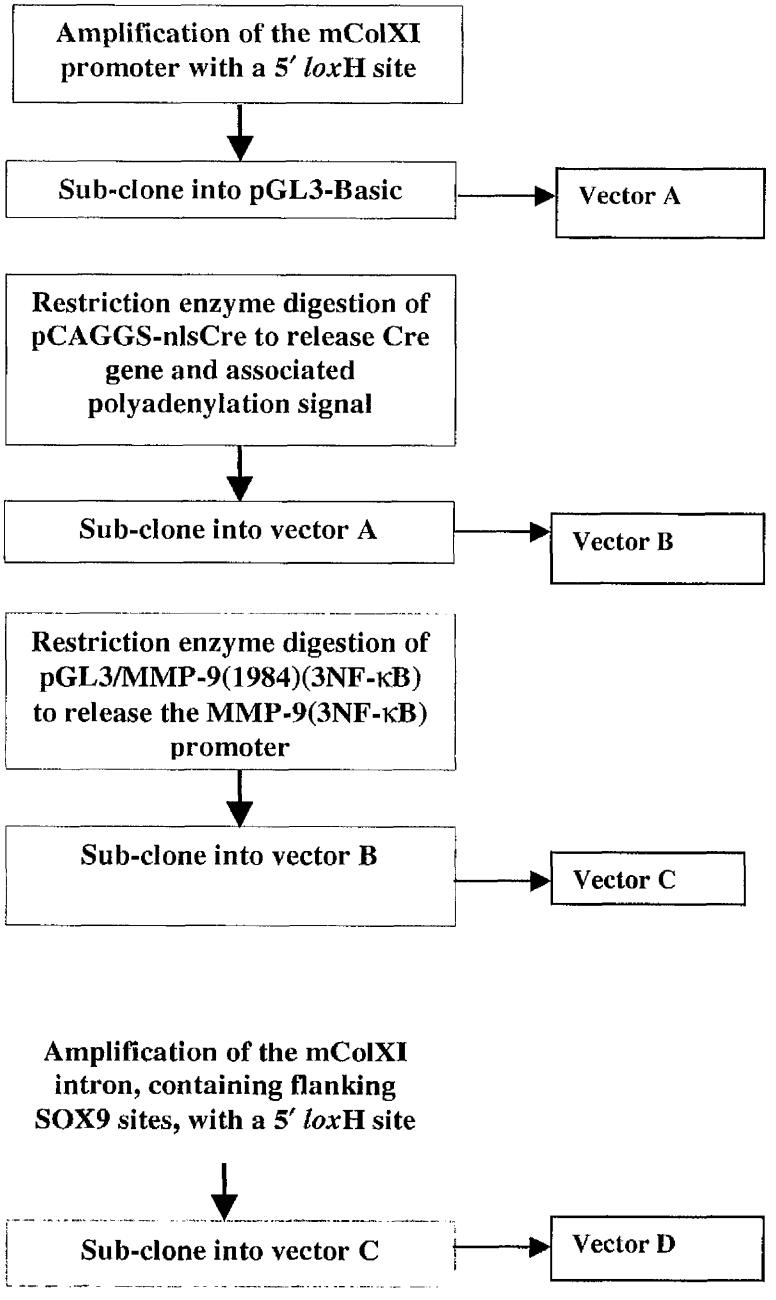


Figure 6-4 Overview of the experimental procedures used to clone the dual-targeting vector

Construction of the dual-targeting vector was attempted in four steps. The mouse collagen type XI promoter was amplified by PCR incorporating a *lox*H site into the 5′ end of the sequence and sub-cloned into pGL3-Basic (Vector A). The Cre gene containing a downstream polyadenylation site was cut from the pCAGGS-nls Cre vector and sub-cloned into vector A to create vector B. The canine MMP-9 promoter, containing three NF-κB sites in the 5′ end of the sequence, was cut from the pGL3/cMMP-9(1894)(3NF-κB) vector and sub-cloned into vector B to form vector C. The mouse collagen type XI intronic region containing three flanking SOX9 sites was amplified, incorporating a *lox*H site in the 3′ end of the sequence, and efforts were made to sub-clone this into vector C to make vector D.



6.3.1 Cloning of all promoters into pCR2.1®TOPO vector

The cloning procedures used in this chapter were simplified, as described in chapter V, by the initial cloning of PCR generated inserts (containing the appropriately modified sequence) into the pCR2.1®TOPO vector before sub-cloning into the pGL3-Basic luciferase reporter vector. This intermediate cloning step, via the pCR2.1®TOPO vector, had a number of advantages. The 'T-A' cloning system is highly efficient and allows the newly generated PCR fragments to be quickly cloned and their sequence analysed before sub-cloning into the luciferase reporter vector. Restriction enzyme digestion of the insert from the pCR2.1®TOPO vector is more efficient than the direct digestion of PCR products (containing flanking restriction enzyme sites) since a larger area of DNA is available to support the enzyme. Restriction digestion of the fragment could then be positively controlled, since the digested insert is visibly separated from the vector backbone on agarose gel analysis. The pCR2.1®TOPO vector containing the PCR products can easily be grown in bacterial cells to repeat the sub-cloning procedure if necessary; this is preferable to repeating the PCR which is often variable and may introduce sequence mutations.

6.3.1.1 Primer design

Forward (sense) primers and reverse (anti-sense) primers were designed, as described in section 2.2.5.1 and supplied by Sigma-Genosys (Table 6-1), for the PCR-based cloning of the canine MMP-9 promoter with additional NF-κB sites, the mouse collagen type XI promoter +/- SOX9 sites and mouse collagen type XI intron +/- SOX9 sites into pCR®2.1-TOPO.

6.3.1.1.1 Canine MMP-9 promoter manipulation with NF-κB sites

Forward primers were designed to incorporate five, three and one NF-κB sites (GGAATTCCC) into the 5′ end of the MMP-9 promoter sequence (5NF-κBf, 3NF-κBf, 1NF-κBf). These primers, used for the PCR-based cloning of these constructs, contained *Xho* 1 (CTCGAG) restriction enzyme sites incorporated into the forward primers to facilitate screening for insert orientation.

6.3.1.1.2 Mouse collagen type XI promoter (+/- manipulation with SOX9 sites)

Primers pairs based on specific regions of the mouse collagen type XI gene sequence (Genbank Number: D84066), guided by motif location, were designed for the PCR-based cloning of the collagen promoter region into pCR®2.1-TOPO. Sense (mColXIpromf) and anti-sense (mColXIpromr) primers were designed to amplify a region of the promoter (1178 bp) immediately 5′ to the translation start site. To manipulate this promoter with the addition of chondrocyte specific enhancers, SOX9 sites (CTCGAAAG), into the 5′ end of the promoter, two further sense oligonucleotide primers were designed containing three and five chondrocyte specific enhancers. SOX9 sites, referred to as 3SOX9promf and 5SOX9promf respectively.

6.3.1.1.3 Mouse collagen type XI intron 1 (+/- manipulation with SOX9 sites)

Primer pairs were also based on the first intronic region of the collagen type XI gene sequence (Genbank Number: D84066) to enable the PCR-based cloning of intron 1 into pCR®2.1-TOPO. Sense primer (mColXIIntronf) and anti-sense primer (mColXIIntronr) were designed to amplify most of intron 1 (2427bp) between exon 1 and exon 2. To manipulate the mouse collagen type XI intron 1 region, sense (3Sox9intronf) and anti-sense (3Sox9intronr) oligonucleotide primers were designed to amplify a smaller region of the intron (242 bp) both containing three chondrocyte specific SOX9 enhancer sites to flank a region of intron 1.

Illustration of primer position on the mouse collagen type XI gene

Table 6-1 Primer sequences for manipulating the canine MMP-9 sequence with NF-κB sites and the cloning and manipulation of the mouse ColXI promoter and intron with SOX9 sites

Primer Identification	Oligonucleotide primer sequence (5′-3′)	Tm (°C) & GC (%) content
5NF-κBf	GGCCTCGAGGGAATTCCCGGAATTCCCGGAATTCCCGGAATTCCCGGAATTCCCGGTTCTGGGTGACTCCAACGCCAATGCTCAT	98.1 °C : 57.1 %
3NF-κBf	GGCCTCGAGGGAATTCCCGGAATTCCCGGAATTCCCGGTCTGGGTGACTCCAAAGCCAATGCTCAT	95.6 °C : 57.6 %
1NF-κBf	GGCCTCGAGGGAATTCCCGGTCTGGGTGACTCCAAAGCCAATGCTCAT	91.1 °C : 58.3 %
mColXIpromf	ACCCTGACATGTTCCCTGGATGCTGCCACG	83.0 °C : 60.0 %
mColXIpromr	GGCTCAGAATGCCAGATCCCAGGCAGTCCT	80.9 °C : 60.0 %
3Sox9promf	GGCCTCGAGCTCGAAAGCTCGAAAGCTCGAAAGACCCTGACATGTTCCCTGGAT	88.5 °C : 52.9 %
5Sox9promf	GGCCTCGAGCTCGAAAGCTCGAAAGCTCGAAAGCTCGAAAGCTCGAAAGACCCTGACATGTTCCCTGGAT	90.9 °C : 50.8 %
mColXIIntronf	GGTGTCAGTCAGTTGGAGGTTGTAGCATCT	72.9 °C : 50.0 %
mColXIIntronr	GGCAGGGTTAGTTAGGAGTGAGAACATGTC	71.3 °C : 50.0 %
3SOX9intronf	GGCGGATCCGCTAGCCTCAAAGCTCAAGCTCAAAGGTCTCTGTGAGTCTCTGTGTCTC	89.4 °C : 54.2 %
3SOX9intronr	GGCGGATCCGCTAGCCTTTGAGCTTTGAGCTTTGAGACCATCTCCACAGCAGAGAACTC	90.2 °C : 54.2 %

6.3.1.2 PCR amplification

6.3.1.2.1 MMP-9 manipulation with multiple NF-κB sites

Manipulation of the canine MMP-9 promoter with multiple NF-κB sites was performed by PCR using pCR®2.1/cMMP-9 vector samples (100 ng) as template and Ready-To-Go™ PCR beads (Amersham, Pharmacia) in a total volume of 25µl containing 0.4 µM of sense (5NF-κBf, 3NF-κBf, 1NF-κBf) (Table 6-1) and anti-sense (cMMP-9r) (Table 4-2) primer pairs. Samples were subjected to an initial denaturation at 95°C for 5 mins followed by 30 cycles of amplification, each consisting of a denaturation step of 95 °C for 1 min, an annealing temperature of 66 °C for 1 min and an elongation step of 72 °C for 2 mins. A final extension step of 72 °C for 30 mins completed the reaction.

6.3.1.2.2 Mouse collagen type XI promoter amplification and manipulation

Using Ready-To-Go™ PCR beads (Amersham, Pharmacia) in a total volume of 25µl the mouse collagen type XI promoter (mColXI) and manipulated mColXI(3SOX9), (5SOX9) promoters were amplified from mouse gDNA (150ng) and pCR2.1/mColXI vector samples (100 ng) respectively with 0.4µM of both sense (mColXIpromf, 3SOX9promf, 5SOX9promf) and anti-sense (mColXIpromr) primer pairs (Table 6-1). Samples were subjected to an initial denaturation at 95°C for 5 mins followed by 30 cycles of amplification, each consisting of a denaturation step of 95 °C for 1 min, an annealing temperature of 60 °C for 1 min and an elongation step of 72 °C for 2 mins. A final extension step of 72 °C for 30 mins completed the reaction.

6.3.1.2.3 Mouse collagen type XI intronic amplification and manipulation

Using Ready-To-Go™ PCR beads (Amersham, Pharmacia) in a total volume of 25µl the mouse collagen type XI intron 1 and manipulated [3SOX9(intron)3SOX9] intronic regions were amplified from mouse gDNA (150ng) and pCR®2.1/mCol(XI)intron vector samples (100 ng) respectively with 0.4µM of both sense (ColXIIntronf and 3SOX9intronf) and anti-sense (ColXIIntronr and 3SOX9intronr) primer pairs (Table 6-1). Samples were subjected to an initial denaturation at 95°C for 5 mins followed by 30 cycles of amplification, each consisting of a denaturation step of 95 °C for 1 min, an annealing temperature of 60 °C for 1 min and an elongation step of 72 °C for 1 min. A final extension step of 72 °C for 30 mins completed the reaction. PCR products (8 µl) were visualised by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a 100 bp molecular weight standard.

6.3.1.3 Cloning into pCR®2.1-TOPO vector

PCR products were cloned using methods described in section 2.2.2. Briefly, PCR products were purified using the QIAquick®Gel Extraction Kit (QIAGEN, UK) and eluted in 50µl of sterile water,

4µl of which were assessed by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a 1 kb molecular weight standard. Purified PCR products were directly ligated into the pCR®2.1-TOPO plasmid vector following the TOPO TA® cloning protocol. The ligation reaction (2µl) was then transformed into One Shot™ TOP10 competent cells and spread onto agar plates containing ampicillin and X-gal for incubation overnight at 37°C. White, ampicillin resistant, colonies were selected from the plates and cultured overnight in LB/ampicillin broth at 37°C and glycerol stocks of each culture were prepared for long-term storage.

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation using the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. All constructs were screened using PCR conditions as described above but replacing one of the primers with a vector primer (either M13R or M13F) to determine orientation.

6.3.1.4 Sequence evaluation

Recombinant plasmids were sequenced and analysed as described in section 2.2.6. Briefly, Plasmid samples were prepared for sequencing with PCR reactions using plasmid DNA samples (400 ng) and the Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism) with 0.5µM of both sense (M13R) and anti-sense (M13F) vector-based primers. Samples were amplified, and the DNA purified by precipitation methods before samples were loaded into the ABI 310 genetic analyser for generation of automated sequence data. Sequence files were downloaded from the chromas file, saved as Word documents using the appropriate format, and then lined up with the relevant correct sequence for the MMP-9 and MMP-13 sequences using ClustalW to check for the absence of mutational errors.

6.3.2 Sub-cloning canine manipulated MMP-9 promoter & mouse collagen type XI promoter/intronic fragments into pGL3-Basic luciferase reporter vectors

6.3.2.1 Restriction enzyme digestion

6.3.2.1.1 Canine MMP-9(multiple NF-κB sites) and mouse collagen type XI promoter +/- (multiple SOX9 sites)

The uni-directional sticky/blunt ended ligation reaction described in chapter V was successfully used to sub-clone all promoter fragments into pGL3-Basic luciferase reporter vectors. This involved ligating the *Kpn* I and *Sma* I restriction enzyme sites located in the multiple cloning site (MCS) of the pGL3-Basic reporter vector with the *Kpn* I and *Eco*R V restriction enzyme sites positioned either side of the promoter fragments cloned into pCR®2.1-TOPO. To enable this procedure it was necessary to ensure that promoter sequences did not contain *Kpn* I and *Eco*R V

restriction enzyme sequences. More specifically, the promoter/pCR®2.1-TOPO vectors (5µg) were digested for four hours with *Kpn* I (5 units) and *Eco*R V (5 units) in MULTI-CORE™ (1x) buffer at 37°C. The recipient pGL3-Basic luciferase reporter vector (5µg) was digested with *Kpn* I (5 units) and *Sma* I (5 units) at 37°C and 30°C for 2 hours with each enzyme respectively (see 2.1.7.1).

6.3.2.1.1 Mouse collagen type XI [3SOX9(intron)3SOX9] sites

A non-directional sticky ended ligation reaction was successfully used to sub-clone the intronic fragment containing flanking SOX9 sites into the down-stream enhancer site of the pGL3-Basic luciferase reporter vector. This involved ligating the *Bam*H 1 restriction enzyme site located in the enhancer region of the pGL3-Basic reporter vector with the *Bam*H1 restriction enzyme sites positioned either side of the intronic fragment cloned into pCR®2.1-TOPO. To enable this procedure it was necessary to ensure that intronic sequences did not contain *Bam*H1 restriction enzymes sites. More specifically, the pCR®2.1/[3SOX9(intron)3SOX9] (5µg) and recipient pGL3-Basic luciferase reporter vector (5µg) were digested for 2 hours with *Bam*H1 (5 units) in MULTI-CORE™ (1x) buffer at 37°C (2.1.7.1). The pGL3-basic vector was dephosphorylated for 2 hours with CIAP as described in section 2.2.2.7.

6.3.2.2. Purification, ligation and transformation

Digested DNA samples, linearised pGL3-Basic and released promoter/intronic fragments, were purified using QIAquick®Gel Extraction Kit (QIAGEN, UK) and eluted in 30µl of sterile water. The DNA was quantified (4µl) by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a Low DNA Mass™ Ladder (4µl). The quantity of vector (pGL3-Basic) and insert (promoter/intronic fragment) for each ligation reaction was calculated according to the equation shown in 2.2.2.8.1. For each calculation the vector mass (X) was 50 ng, the insert size (Y) was approximately 2 kb for the manipulated MMP-9 promoter, 1.2 kb for the collagen type XI promoter and 0.5 kb for intronic regions. The molar ratio of insert to vector used was a value of three. The cut ends of the inserts were then ligated to complementary ends of the cut pGL3-Basic vector using T4 DNA ligase over night at 16°C. The ligations (20ng) were transformed into 25µl of JM109 competent cells, heat shocked for 45 seconds at 42°C and then grown overnight at 37 °C on LB/ampicillin agarose plates. Six colonies were selected from each plate and transferred to LB broth for overnight culture at 37°C.

6.3.2.3 Isolation and screening of recombinant plasmids

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation using the QIAprep®PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. All constructs were screened using PCR techniques as described for their original amplification. However, although there was no need to screen for orientation of the promoter constructs since

they were directionally cloned into pGL3-Basic but, the intronic clones were not and therefore the reverse primer was replaced with the vector primer (RV4) to determine orientation using PCR techniques.

6.3.2.4 Plasmid vector maps

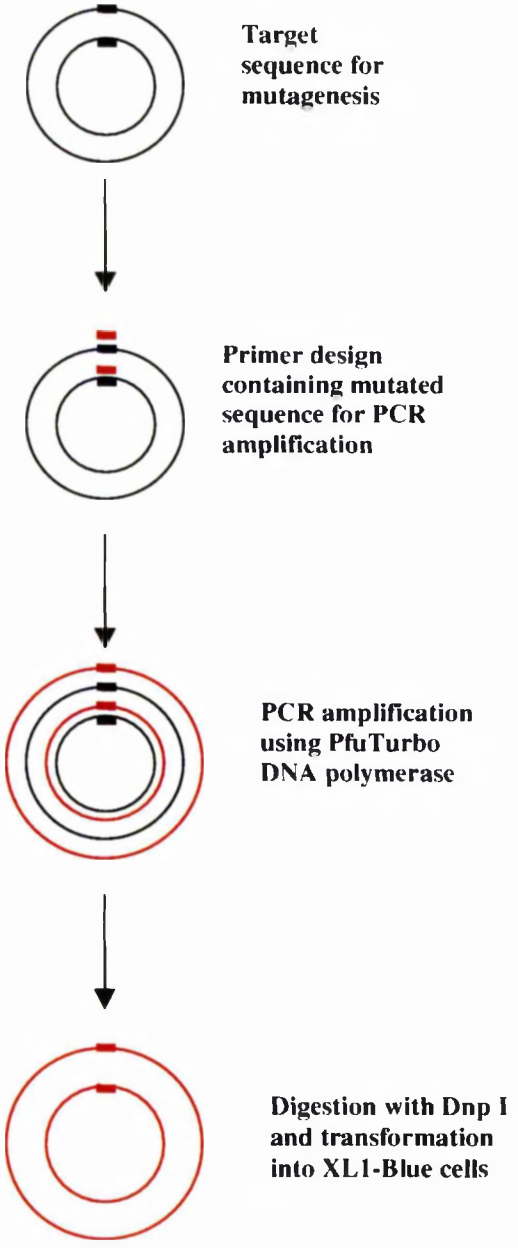
Using computer software plasmid vector maps were drawn from the sequence data information incorporating all important components of each vector including the luciferase cDNA sequence, relevant canine MMP or mouse collagen type XI promoter/ intronic sequences with and without modifications, polyadenylation signal and ampicillin resistance gene.

6.3.3 Mutagensis

In vitro site-directed mutagensis using the QuikChange™ site-directed mutagenesis protocol (Stratgene) was applied for the site-specific mutation in supercoiled double-stranded DNA (dsDNA) vectors. This technique was chosen to mutate the NF-κB site from the pGL3/cMMP-9(628) construct as it is a rapid technique generating mutants with 80% efficiency and eliminating the need for sub-cloning into specialised vectors. This four step procedure (Figure 6-5) utilised *PfuTurbo*® DNA Polymerase II and a thermal cycler replicating both plasmid strands with high fidelity without displacing the two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers each complementary to opposite strands of the vector were extended during temperature cycling by *PfuTurbo* DNA polymerase generating mutated plasmids containing staggered nicks. Following temperature cycling, the product was treated with *Dpn* I, an endonuclease (target sequence: 5′- G_m6ATC-3′) that is specific for methylated and hemi-methylated DNA and used to digest the parental DNA template thereby selecting for mutation-containing synthesised DNA (Nelson and McClelland, 1992). DNA isolated from almost all *E.coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA containing the desired mutation was then transformed into XL1-Blue supercompetent cells.

Figure 6-5 Simplified diagram of mutagenesis

The *in vitro* QuikChange™ site-directed mutagenesis protocol (Stratgene) is a simple four step procedure used for the site-specific mutation in supercoiled double-stranded DNA (dsDNA) vectors. The mutation sequence was identified and two synthetic oligonucleotide primers containing the desired mutation were designed. During PCR cycling the primers annealed to the vector sequence and the nonstrand-displacing action of *PfuTurbo*® DNA Polymerase II extended and incorporated the mutagenic primers into new nicked circular DNA strands. The methylated, non-mutated parental DNA was then digested with *Dpn* I, an endonuclease. The newly mutated vectors were then transformed into XL1-Blue supercompetent cells that repaired the nicks in the mutated plasmid.



6.3.3.1 Primer design

Both primers contained the desired mutation annealing to the same sequence on opposite strands of the plasmid. The primers were between 25 and 45 bp in length with the desired mutation in the centre of the primer with approximately 10 to 15 bases of correct sequence on both sides. The primers contained a minimum GC content of 40% and terminating in one or more G or C bases. The Tm value was greater than 78°C using equation (where *N* is the primer length in bases and %GC and % mismatch are whole numbers): Tm = 81.5 + 0.41(%GC) - 675/*N*- % mismatch. Primers were synthesised and PAGE purified by Sigma Genosys.

Conserved NF-kB sequence: GGAATTCCCC

Mutated NF-kB sequence: TTAATTCCAA

Table 6-2 Primer sequences for NF-κB mutagenesis

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
NF-kBmut^{n}f	GGGCTTGCTGCGCTTTAATTCCAAAATCCCTGCCTGAGGG	86.2 °C : 55.0 %
NF-kBmut^{n}r	CCCTCAGGCAGGGATTTTGGAATTAAAGCGCAGCAAGCCC	86.2 °C : 55.0 %

6.3.3.2 PCR amplification of mutated vectors

PCR reactions were performed using 5-50 ng pGL3/cMMP-9(628) vector as template in a total volume of 50µl containing both 125 ng of sense (NFkBmut^{n}f) and anti-sense (NFkBmut^{n}r) primers, 1 µl of dNTP mix, 2 mM KCl, 2 mM $(NH_4)_2SO_4$, 4 mM Tris-HCL (pH 8.8), 0.4 mM $MgCl_2$, 1% Triton® X-100, 1 mg/ml nuclease-free bovine serum albumin (BSA) and 2.5 units *PfuTurbo*® DNA polymerase. Using a PE 2400 thermal cycler, samples were subjected to an initial denaturation at 95°C for 30 seconds, followed by 18 cycles of amplification, each cycle consisting of a denaturation step of 95°C for 30 seconds and annealing temperature of 55°C for 1 minute, followed by an elongation step of 68°C for fourteen minutes. The nonstrand-displacing action of *PfuTurbo* polymerase extended and incorporated the mutagenic primers resulting in a nicked circular DNA vector.

6.3.3.3 Endonuclease Digestion

All methylated, non-mutated parental dsDNA templates were digested with *Dpn* I (10 units) added directly to the reaction mix and incubated at 37°C for one hour.

6.3.3.4 Transformation and plasmid preparation

Dpn I- treated circular nicked dsDNA (1 µl) was transformed into XL1-Blue super-competent cells (2.2.2.9.3) (50 µl) to repair the nicks in the mutated plasmid. The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation using the

QIAprep®PCR Spin Miniprep Kit (QIAGEN. UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification.

6.3.3.5 Sequence evaluation

Recombinant plasmids were sequenced and analysed as described in section 2.2.6 using plasmid samples (400 ng) and 0.5µM of both sense (RV3) and anti-sense (GL2) vector-based primers. Automated sequence generated from the ABI 310 genetic analyser were downloaded as chromas files and saved as Word documents. The canine MMP-9 sequence was checked to ensure correct mutation of the NF-κB site.

6.3.3.6 Plasmid vector maps

Using computer software, a plasmid vector map was drawn from the sequence data information incorporating all important components of each vector including the luciferase cDNA sequence, mutated canine MMP-9 promoter sequence, polyadenylation signal and ampicillin resistance gene.

6.3.4 Relative semi-quantitative RT-PCR to analyse endogenous transcription of MMP-9 and collagen type XI genes in different cell lines

To assess the suitability of different cell lines in the characterisation of the MMP-9 and collagen type XI gene promoter fragments relative semi-quantitative RT-PCR was once again performed. Both basal and induced (IL-1β and TNFα) endogenous levels of the MMP-9 and collagen type XI gene transcription were analysed in the human chondrosarcoma cells (SW1353) and undiffereniated chondroprogenitor cells (ATDC5). Care was taken when using relative, semi-quantitative RT-PCR to compare levels of gene transcription between cell lines since numerous reports have shown that the expression of internal controls such as β-actin and GAPDH can be affected by experimental treatments, stage of development and cell type (Spanakis et al., 1993).

6.3.4.1 Cell lines and reagents

Human chondrosarcoma cells (SW1353 cells) were maintained in Dulbecco's MEM/F-12 medium supplemented with 10% foetal calf serum (FCS) as described in section 2.2.1.2.4. Undifferentiated chondroprogenitor cells (ATDC5) were cultured in a mix of Dulbecco's MEM/F-12 medium supplemented with 5% FCS as described in section 2.2.1.2.5. SW1353 cells and ATDC5 cells were seeded onto 6 well plates at a concentration of $5.5x\ 10^4$ and $5x\ 10^4$ cells/ml respectively and allowed to grow to confluence over 24 hours. SW1353 cells and ATDC5 cells used in all experiments were serum-starved for 24 hours prior to stimulation with IL-1β (10 ng/ml) and TNFα (10 ng/ml) for a further 24 hours prior to harvesting.

6.3.4.2 Isolation of purified total RNA

Total RNA was harvested from both untreated (basal) and treated (IL-1β and TNFα) cells using RNAzol™ B solution (AMS Biotechnology) (500µl/well) which was added directly to the wells containing the adherent SW1353 cells and ATDC5 cells for lysis. Aliquots (1 ml) of the lysed cell solution were shaken for 15 seconds with chloroform (100µl) and RNA extracted as described in section 2.2.3. The RNA samples were re-suspended in 40µl DEPC water and the quality and quantity assessed using spectrophotometry and agarose gel electrophoresis. To remove contaminating genomic DNA from the RNA preparation the RNA was treated with DNA-free™ (Ambion) as described in section 2.2.3.4.

6.3.4.3 First strand cDNA synthesis

The synthesis of first strand cDNA followed the basic principles as set out in section 2.2.4. More specifically, total RNA samples (2µg) in 9µl of DEPC water were heated for 5 minutes at 65°C and then quenched on ice. First strand synthesis reactions were performed using the heat treated RNA in a 25µl reaction mix containing 5µl of reverse transcription buffer (5x), 200 units of Molony Murine Leukaemia Virus reverse transcriptase (MMLV-RT) (GIBCO BRL), 10mM dTT, 250µM dNTPs, 25 units RNAse inhibitor and 25µM random primers. Using a PE 480 thermal cycler the reaction was incubated at 37°C for 30 minutes followed by 42°C for 60 minutes and completed with 95°C for 5 minutes.

6.3.4.4 Primer design

In order to amplify approximately 350 bp regions of the human and mouse MMP-9 and collagen type XI cDNAs it was necessary to design oligonucleotide primer pairs for each gene. PCR primers were designed as described in section 2.2.5.1 and were synthesised and supplied by Sigma-Genosys (Table 6-3). Both sense and anti-sense species-specific primer pairs were designed based on the human (hMMP-9f and hMMP-9r) and mouse (mMMP-9f and mMMP-9r) MMP-9 gene sequences. Primer pairs were also designed based on the human (hColXIf and hColXIr) and mouse (mColXIf and mColXIr) collagen type XI gene sequences. The constitutively expressed cyclophilin gene was used as an internal control for the human (NM_021130) and mouse (XM_125205) species. Species specific primers were subsequently designed based on the human (hcyclophilinf and hcyclophilinf) and mouse (mcyclophilinf and mcyclophilinr) genes to amplify a region of 265bp from cDNA template. The position of the control primers were again based on the cDNA sequence to span an intron, to enable the identification of contaminating genomic DNA as a 450 bp fragment. Primer pairs (27 bp) were matched as closely as possible for Tm (70°C) and GC (40-60%) content.

Table 6-3 Primer sequences for RT-PCR (MMP-9, ColXI and cyclophilin)

Primer name	Oligonucleotide primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
hMMP-9f	GCTGGATAGCGCCACGCTGAAGGCCAT	81.2 °C : 63.0 %
hMMP-9r	ATCGTCGAAATGGGCGTCTCCCTGAAT	77.0 °C : 51.9 %
mMMP-9f	CTGGACAGCCAGACACTAAAGGCCATT	73.0 °C : 51.9 %
mMMP-9r	GTCGTCGAAATGGGCATCTCCCTGAAC	76.7 °C : 55.6 %
hColXIf	ATCTGTCCAGCTGATGTGGCCTACCGA	76.2 °C : 55.6 %
hColXIr	TCTTCATCCAGAATACGGGCACCAAAG	73.4 °C : 48.1 %
mColXIf	GTCTGTCCGGGTGATGTGGCTTACCGT	77.0 °C : 59.3 %
mColXIr	TCATCGTCGAGGATGTGGGCACCAAAG	78.9 °C : 55.6 %
hcyclophilinf	CGTGCTCTGAGCACTGGAGAGAAAGGA	74.9 °C : 55.6 %
hcyclophilinr	CCACTCAGTCTTGGCAGTGCAGATGAA	74.7 °C : 51.9 %
mcyclophilinf	CGAGCTCTGAGCACTGGAGAGAAAGGA	74.1 °C : 55.6 %
mcyclophilinr	CCATTCAGTCTTGGCAGTGCAGATAAA	71.2 °C : 44.4 %

6.3.4.5 PCR amplification of MMP-9 and collagen cDNA fragments using PCR

PCR reactions were performed using Ready-To-Go™ PCR beads (25 µl) (Amersham. Pharmacia) with cDNA samples (1µg) as template and 0.4µM of species specific primer pairs for MMP-9 or collagen type XI each with 0.1µM of species specific cyclophilin primers as an internal control (Table 6-3). Samples were subjected to an initial denaturation at 95°C for 5 min followed by a variable number of cycles for amplification, each cycle consisting of a denaturation step of 95 °C for 1 min, an annealing temperature of 67°C followed by an elongation step of 72 °C for 1 min. A final elongation step of 72°C for 10 min completed the reaction. Samples were removed at multiple (3 to 5) intervals 5 cycles apart to determine the exponential phase of the reaction where basal and treated samples could be semi-quantified and compared. A negative control containing a PCR bead with all components (dNTPs, PCR buffer and *Taq* polymerase) and primer pairs without template was performed to check for contamination of the PCR reactions with extraneous DNA that might serve as a template for PCR amplification. PCR products (8µl) were analysed by TAE agarose gel (1%) electrophoresis by comparing the bands created to a 100 bp molecular weight standard.

6.3.5 Luciferase reporter assays to analyse the basal and induced activity of the manipulated canine MMP-9 promoter fragments and the mouse collagen Type XI promoter/intronic fragments

The Dual-Luciferase® Reporter assays as discussed in section 2.2.8 were chosen to analyse the cloned canine MMP-9 and mouse collagen type XI promoters for reasons as described in chapter V.

6.3.5.1 Transfection of cells

SW1353 and ATDC5 cells were maintained as described in section 6.3.4.1. White, tissue culture treated ViewPlate™-96 (Packard) were seeded with SW1353 cells, and ATDC5 cells at concentrations of $5.5x10^4$ cells/ml and $5x10^4$ cells/ml respectively and incubated overnight at 37°C in 5% CO_2. Cells should be approximately 70-80% confluent on the day of transfection for optimal assay conditions. Transient transfections were carried out using TransFast™ Reagent (Promega) at a 1:1 ratio with DNA according to the manufacturer's instructions as described in section 2.2.7. More specifically, total DNA (50-100ng) was added to pre-warmed serum free media (40µl) and thoroughly mixed before addition of TransFast™ Reagent (0.3µl). The DNA/transfection reagent samples were incubated for 10-15 minutes at room temperature to allow complex formation before the growth medium on the 96-well plates was aspirated and replaced with the transfection samples. The cells were incubated with the complexes for 1 hour at 37°C in 5% CO_2 before pre-warmed complete medium was added for cell recovery overnight at 37°C.

6.3.5.2 Stimulation of cells

To induce transcription from the promoter constructs, transfected cells were treated with different stimulants for the 24 hours prior to luciferase assays. SW1353 cells and ATDC5 cells were both serum-starved for 24 hours prior to stimulation with IL-1β (10 ng/ml), and TNFα (10 ng/ml) (R&D Systems) for a further 24 hours prior to harvesting the cells.

6.3.5.3 Luciferase assays

Dual-Luciferase®Reporter assays were performed 72 hours post transfection, according to the manufacturer's protocol (Promega). More specifically, media was removed from the adherent cells, washed once with PBS and lysed with Passive Lysis Buffer (PLB) (1x) for 15 minutes at room temperature. Using a Dynex, MLX luminometer and injector system, freshly prepared Luciferase Assay Buffer II (LAR II) (50µl) and Stop & Glo® Reagent (50µl) were dispensed into each well with an interval of 30 seconds, each taking a luciferase count for 10 seconds. Both firefly and *Renilla* luciferase values were obtained for each well and all luciferase values were analysed using an excel spread sheet.

6.3.6 Preparation of components required for the dual-targeting vector construction

The strategy for sub-cloning all components of the dual-targeting vector into the pGL3-Basic vector was designed to be as simple as possible using restriction enzyme digestion where possible from tested plasmids. New components, including the *lox*H sites and restriction enzyme sites, were incorporated into primer sequences for sub-cloning into the final vector by PCR methods. However, The dual-targeting CRE-lox vector construction was not completed due to technical problems in the final stages of the cloning procedure.

6.3.6.1 The incorporation of loxH sites into the dual targeting vector

The sequence of the *lox*P site consists of 34 base pairs containing a 13 bp inverted repeat, separated by an 8 bp spacer region. The inverted repeat (underlined) may form a stem and loop structure which may reduce expression of the gene of interest. A variation of the *lox*P (*loxH*) was created by Invitrogen to eliminate the formation of stem and loop structure and potentially improve transcription. Mutated bases are shown in bold face.

*Lox*P site:

ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T

*Lox*H site:

ATT ACC TCA TAT AGC ATA CAT TAT ACG AAG TTA T

6.3.6.2 Primer design for cloning into pCR® 2.1-TOPO vector

PCR primers were designed as described in section 2.2.5.1 and were synthesised and supplied by Sigma-Genosys (Table 6-4). Two primer pairs were designed for the PCR-based cloning of intermediate products into pCR®2.1-TOPO for further sub-cloning into the dual-targeting vector. This enabled the incorporation of *lox*H sites and restriction enzyme sites, not found within pGL3-Basic vector multiple cloning site (MCS), into the vector system.

6.3.6.2.1 Mouse collagen type XI gene promoter plus *lox*H site:

One primer pair was based on specific regions of the mouse collagen type XI gene promoter sequence. Sense (mColXI*lox*Hf) and anti-sense (mColXI*lox*Hr) primers designed were designed to amplify a region of the promoter (1178 bp) immediately 5′ to the translation start site. A *lox*H site was incorporated into the sense primer in addition to a *Bgl* II (AGATCT) and a *Nsi* 1 (ATGCAT) to facilitate the sub-cloning steps into pGL3-Basic. The anti-sense primer contained a *Bgl* II and a *Xba*1 (TCTAGT) for the same reasons.

6.3.6.2.2 Mouse collagen type XI intron/sox9 sites plus *lox*H site

The second primer pair was based on the manipulated [3SOX9(intron)3SOX9] sequence designed from the mouse collagen type XI intron 1 sequence. Sense (mIntron*lox*Hf) and anti-sense (mIntron*lox*Hr) primers were designed to amplify a region of the intron (242 bp) with three flanking SOX9 sites (CTCAAAG). A *lox*H site was also incorporated into the anti-sense primer in addition to a *Hind* III (AAGCTT) to facilitate the sub-cloning steps into pGL3-Basic. The anti-sense primer also contained a *Hind* III site.

Table 6-4 Primer seqences for the cloning of the dual-targeting vector

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
mColXI*lox*Hf	GGCAGATCTATGCATATTACCTCATATAGCATACATTATACGAAGTTATACCCTGACATGTTCCCTGGAT	81.9 °C : 54.8 %
mColXI*lox*Hr	GGCAGATCTTCTAGTGGCTCAGAATGCCAGATCCCAGGCAGT	84.3 °C : 38.6 %
mIntron*lox*Hf	GGCAAGCTTCTCAAAGCTCAAAGCTCAAAGGTCTCTAGTCTCTGTGTC	84.7 °C : 49.0 %
mIntron*lox*Hr	GGCAAGCTTATAACTTCGTATAATGTATGCTATATGAGGTAATCTTTGAGCTTTGAGCTTTGAGACCATCTCCACAGCAGAGAAC	85.3 °C : 80.6 %

6.3.6.3 PCR amplification

6.3.6.3.1 Mouse collagen type XI promoter/*lox*H

PCR amplification of the mouse collagen type XI promoter with the *lox*H site was performed using pCR®2.1/mColXI vector samples (100 ng) as template and Ready-To-Go™ PCR beads (Amersham. Pharmacia) in a total volume of 25µl containing 0.4 µM of sense (mColXI*lox*Hf) and anti-sense (mColXI*lox*Hr) primer pairs (Table 6-4). Samples were subjected to an initial denaturation at 95°C for 5 mins followed by 30 cycles of amplification. each consisting of a denaturation step of 95 °C for 30 secs. an annealing temperature of 60 °C for 30 secs and an elongation step of 72 °C for 2 mins. A final extension step of 72 °C for 10 mins completed the reaction.

6.3.6.3.2 Mouse collagen type XI intron/SOX9/*lox*H

PCR amplification of the mouse collagen type XI manipulated intron with the *lox*H site was performed using pCR2.1/mColXI[3SOX9(intron)3SOX9] vector samples (100 ng) as template and Ready-To-Go™ PCR beads (Amersham. Pharmacia) in a total volume of 25µl containing 0.4 µM of sense (mIntronloxHf) and anti-sense (mIntronloxHr) primer pairs (Table 6-4). Samples were subjected to an initial denaturation at 95°C for 5 mins followed by 30 cycles of amplification. each consisting of a denaturation step of 95 °C for 30 secs. an annealing temperature of 68 °C for 30 secs and an elongation step of 72 °C for 1 min. A final extension step of 72 °C for 10 mins completed the reaction. PCR products (8 µl) were visualised by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a 100 bp molecular weight standard.

6.3.6.4 Cloning of PCR products into pCR®2.1-TOPO vectors

PCR products were cloned using methods described in section 2.2.2. Briefly, PCR products were purified following QIAquick®Gel Extraction Kit (QIAGEN, UK) and eluted in 50µl of sterile water, 4µl of which were assessed by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created with a 1 kb molecular weight standard. Purified PCR products were directly ligated into the pCR®2.1-TOPO plasmid vector following the TOPO TA® cloning protocol. The ligation reaction (2µl) was then transformed into One Shot™ TOP10 competent cells and spread onto agar plates containing ampicillin and X-gal for incubation overnight at 37°C. White, ampicillin resistant, colonies were selected from the plates and cultured overnight in LB/ampicillin broth at 37°C and glycerol stocks of each culture were prepared for long-term storage. The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. All constructs were screened using PCR conditions as described above but substituting one of the primers with a vector primer (either M13R or M13F) to determine orientation.

6.3.6.5 Sequence evaluation

Recombinant plasmids (400 ng) were sequenced as described in section 2.2.6 using 0.5µM of both sense (M13R) and anti-sense (M13F) vector-based primers. Sequences generated from the ABI 310 genetic analyser were downloaded as chromas files and saved as Word documents. Sequences were aligned with the relevant correct sequence for the mouse collagen type XI gene sequence using ClustalW to check for the absence of mutational errors.

6.3.7 Construction of dual targeting vector

6.3.7.1 Restriction enzyme (RE) digestion

See figure 6-4 for a flow chart of the cloning strategy.

6.3.7.1.1 RE digestion for the construction of vector (A): pGL3/mColXI*lox*II

The non-directional sticky ended ligation reaction was successfully used to sub-clone the mouse collagen type XI promoter containing the 5′ *lox*H site into the MCS of pGL3-Basic luciferase reporter vector. This involved ligating the cut *Bgl* II restriction enzyme sites located in the MCS of the pGL3-Basic reporter vector with complementary *Bgl* II restriction enzymes sites positioned either side of the mouse collagen type XI promoter fragment cloned into pCR®2.1-TOPO. To enable this procedure it was necessary to ensure that promoter sequences did not contain *Bgl* II restriction enzyme sites. More specifically, the pCR®2.1/mColXI*lox*H vector (5µg) was digested for two hours with *Bgl* II (5 units) in MULTI-CORE™ (1x) buffer at 37°C to release the insert. The recipient pGL3-Basic luciferase reporter vector (5µg) was also digested with *Bgl* II (5 units) at

37°C for 2 hours (see 2.1.7.1) but was also dephosphorylated for 2 hours with CIAP as described in section 2.2.2.7.

6.3.7.1.2 RE digesion for the construction of vector B: pGL3/mColXI*lox*H-Cre

A uni-directional sticky ended ligation reaction was then performed to sub-clone the Cre gene together with its nuclear localisation signal (nls) and polyadenylation signal (PolyA) into vector A: pGL3/mColXI*lox*H. This involved ligating the *Xba* I and *Hind* III restriction enzymes sites located in the MCS immediately downstream of the collagen promoter into the pGL3/mColXI*lox*H vector with the *Xba* I and *Hind* III restriction enzymes sites positioned either side of the nls-Cre-PolyA sequence cloned into pCAGGS-nlsCre vector (kindly provided by Dr. Nagy, Colorado). To enable this procedure it was necessary to ensure that the nls-Cre-PolyA sequence did not contain *Xba* I or *Hind* III restriction enzymes sites. More specifically, the pCAGGS-nlsCre vector (5µg) was digested for two hours with *Xba* I (5 units) and *Hind* III (5 units) in MULTI-CORE™ (1x) buffer at 37°C to release the insert. The recipient pGL3/mColXI*lox*H (5µg) was first partially digested with *Xba* 1 (0.5 units) since a second *Xba* I was present in the backbone of the pGL3 vector sequence. The mixed population of full-length linearised DNA fragments was separated from both the uncut and completely digested vectors by agarose gel electrophoresis and gel extraction. To separate the vectors digested within the MCS from those cut within the back bone of the vector, and to produce the second sticky end for ligation, complete digestion with *Hind* III (5 units) (2.1.7.1) was performed. The digestion with *Hind* III provided a sticky end for cloning at the end of the linearised strand of DNA in the correctly linearised vector (in MCS), while those previously digested in the backbone were cut into two fragments. Agarose gel electrophoresis and gel extraction was then used to separate the full-length *Xba* I and *Hind* III digested vector for further sub-cloning.

6.3.7.1.3.RE digestion for the construction of vector C: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB)

A uni-directional sticky ended ligation reaction was then performed to sub-clone the canine MMP-9(1894) promoter manipulated with three NF-κB sites into vector B: pGL3/mColXI*lox*H-Cre. This involved ligating the *kpn* 1 and *Nsi* 1 restriction enzymes sites located in the MCS immediately upstream of the collagen type XI promoter within the pGL3/mColXI*lox*H-Cre vector with the *kpn* 1 and *Nsi* 1 restriction enzymes sites positioned either side of the pCR2.1/cMMP-9(3NF-κB) vector. To enable this procedure it was necessary to ensure that the MMP-9(3NF-κB) sequence did not contain *kpn* 1 or *Nsi* restriction enzymes sites. More specifically, the pCR2.1/cMMP-9(3NF-κB) vector (5µg) and pGL3/mColXI*lox*H-Cre vector(5µg) were both digested for two hours with *kpn* 1 (5 units) in MULTI-CORE™ (1x) buffer at 37°C followed by *Nsi* 1 (5 units) in buffer D (1x) at 37°C to release the insert and linearise the vector respectively.

6.3.7.1.4 RE digestion for construction of vector D: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB)[SOX9(intron)SOX9]

A non-directional sticky ended ligation reaction was attempted to sub-clone the mouse collagen type XI intron, manipulated with six SOX9 sites, containing the second *lox*H site into the MCS of vector C: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB). This involved ligating the cut *Hind* III restriction enzyme sites located in the MCS of the vector C, downstream of the Cre gene, with the complementary *Hind* III restriction enzymes sites positioned either side of the mouse collagen type XI intron containing six SOX9 sites in pCR®2.1-TOPO. To enable this procedure it was necessary to ensure that the intronic sequence did not contain *Hind* III restriction enzymes sites. More specifically, the pCR®2.1/mColXI[3sox9(intron)3sox9] vector (5µg) was digested for two hours with *Hind* III (5 units) in MULTI-CORE™ (1x) buffer at 37°C to release the insert. The recipient pGL3/mColXIloxH-Cre-cMMP-9(3NFκB) vector (5µg) was also digested with *Hind* III (5 units) at 37°C for 2 hours (see 2.1.7.1) but was also dephosphorylated for 2 hours with CIAP as described in section 2.2.2.7.

6.3.7.2 Purification, ligation and transformation

All digested DNA products were cloned using methods described in section 2.2.2. Briefly, digested DNA samples (linearised vectors and DNA inserts) were purified following QIAquick® Gel Extraction Kit (QIAGEN, UK) and eluted in 30µl of sterile water. The DNA was quantified (4µl) by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created with a Low DNA Mass™ Ladder (4µl). The quantities of vectors and inserts for each ligation reaction were calculated according to the equation shown in 2.2.2.8. For each calculation the vector mass (X) was 50 ng, the insert size (Y) was approximately 1.2 for the collagen type XI promoter, 1.6 for the nls-Cre-PolyA, 2 for the manipulated cMMP-9 promoter, and 0.5 for the intronic region. The molar ratio of insert to vector used was generally a value of three. The cut ends of the inserts were then ligated to complementary ends of the cut vector using T4 DNA ligase (Roche) over night at 16°C. The ligations (20ng) were transformed into 25µl of TOP10 competent cells, heat shocked for 30 seconds at 42°C and then grown overnight at 37 °C on LB/ampicillin agarose plates. Six colonies were selected from each plate and transferred to LB broth for overnight culture at 37°C.

6.3.7.3 Isolation and screening of recombinant plasmids

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep®PCR Spin Miniprep Kit (QIAGEN, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. Constructs were either screened using PCR techniques as described for their original amplification (using vector-based primers where possible) or by restriction enzyme digestion. Vector A was screened by PCR using primers RV3 and ColXI*lox*Hr, Vector B was screened by restriction

digestion using *Nsi* I and *Hind* III restriction enzymes. Vector C was screened by PCR using primers 3NF-κBf and cMMP-9r. Vector D was screened by restriction digestion using *Hind* III.

6.3.7.4 Sequence evaluation

At each stage positive samples were selected and sequenced as described in section 2.2.6 to confirm that all sequences were correct with no mutational errors. Plasmid samples (400 ng) were amplified using 0.5μM of both sense both sense (RV3, mColXIpromseqf, mIntron*lox*Hf) and anti-sense (GL2, Creseqr and mIntron*lox*Hr) primers (Table 6-5) and sequenced on the ABI 310 genetic analyser. Sequence files were downloaded from the chromas file, saved as Word documents using the appropriate format, and then lined up with the relevant correct sequence for the canine MMP-9 promoter, mouse collagen type XI gene and Cre gene sequences using ClustalW to check for the absence mutational errors.

Table 6-5 Primers for sequencing the dual targeting vector

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
mcolXIpromseqf	GGGTCCTGGTTCCGAGGCTCAGCTGGCTT	82.3 °C : 65.5 %
Creseqr	GGGCATATGTTGCCAAACTCTAAACCA	71.3 °C : 44.4 %

6.3.7.5 Plasmid vector maps

Using computer software, plasmid vector maps were drawn from the sequence data information incorporating all important components of each vector including the luciferase cDNA sequence, the relevant canine MMP promoter sequence or mouse collagen type XI promoter and intronic sequences, Cre gene polyadenylation signals and ampicillin resistance gene.

6.3.8 Western blot analysis of Cre expression during the construction of the dual targeting vector

To evaluate why the cloning of vector D was not possible, investigation of Cre gene expression using Western blot procedures was carried out. The TOP10 E.coli competent cells (Invitrogen) used for the transformation procedure were analysed for endogenous Cre gene expression and cells transformed with vector C were also analysed for Cre gene expression from the plasmid sequence.

6.3.8.1 Sample preparation

Vector C: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB) (20ng) and pGL3-Basic (20ng) (as a negative control) were transformed into 25μl of TOP10 competent cells, heat shocked for 30 seconds at 42°C and then grown overnight at 37 °C on LB/ampicillin agarose plates. Untransformed bacterial cells were also plated on LB agarose plates. One colony was selected from each plate and transferred to

LB broth +/- ampicillin for overnight culture at 37°C. TOP10 bacterial cells (100µl) were removed from each broth and spun at 13,000 rpm for 10 minutes to pellet the cells.

6.3.8.2 SDS-PAGE

A ready pre-cast gel (8.6 cm x 6.8 cm x 1.0 mm), comprising a 10 % resolving and 4 % stacking gel, with ten wells was assembled in the Mini-Protean 3 Electrophoresis cell (Biorad, Herts) as described in section 2.2.9. Cell pellets were resuspended in 40 µl of protein loading buffer, followed by incubation at 100°C for 8 minutes and loaded onto the gel with a Wide Range (6.5 to 205 kDa) Color Markers molecular weight standard (10 µl) (Sigma). The gel was electrophoresed at 200 volts until the bromophenol blue dye reached the lower part of the gel and was then removed from the gel cast.

6.3.8.3 Blotting and blocking the membrane

Two pieces of filter paper (3MM Watmann paper) and one piece of nitrocellulose membrane (Biorad Transblot) were cut to size and soaked in Transfer buffer with two sponges for 30 minutes at room temperature. On completion of electrophoresis, the gel was carefully removed and placed directly on the membrane and then sandwiched between the two pieces of filter paper and sponges. The blotting assembly was then placed in the transfer tank, filled with transfer buffer and run at 200V for one hour. Marker positions were highlighted on the gel in pencil before staining with Ponceau's red staining for 10 minutes. After de-staining three times with distilled water, bands were visualised. The membrane was blocked overnight in 5% non-fat milk in TBS-Tween at 4°C. The membrane was rinsed with TBS-Tween (2x) and then washed three times for 10 minutes each.

6.3.8.4 Antibody binding and detection using ECL™ techniques

The primary polyclonal antibody against Cre recombinase (crude rabbit antiserum) (Covance, CA) was diluted 1:1000 in 1% milk/TBS-Tween. The membrane from 6.3.8.3 was incubated in 20ml of the diluted antibody for one hour at room temperature with gentle rocking and then washed three times in 2x TBS-Tween.

Binding of the Cre antibody was evaluated using the Enhanced Chemoluminescence (ECL™) Western kit. This is a sensitive, rapid system for protein blotting where an antibody system coupled to horse radish peroxidase (HRP) catalyses the conversion of the ECL substrate, luminol into a light signal (maximum wavelength emmision at 430nm) that can be detected by autoradiography. The secondary antibody anti-rabbit HRP conjugate was diluted 1:3000 with 1% milk/TBS-Tween and 20ml was added to the membrane and incubated at room temperature for one hour. The membrane was washed three times in 2x TBS-Tween before being completely removed. Equal volumes (15 ml) of ECL reagent #1 was mixed with reagent #2 and placed onto the membrane. The membrane was transferred to a piece of clear plastic sheet (Saran Wrap), taped to a cassette and exposed to film for 3 seconds.

6.4 RESULTS

6.4.1 Cloning of the canine MMP-9 promoter deletions, NF-κB mutation and multiple NF-κB sites into the pGL3-Basic luciferase reporter vector

The successful cloning of the four canine MMP-9 deletion constructs: pGL3/cMMP-9(1894), (984), (628) and (534) is described in chapter V. The pGL3/cMMP-9(628)(NFκBmut^{n}) construct was made using the QuikChange™ site-directed mutagenesis protocol (Stratgene). The conserved NF-κB site within the canine MMP-9 promoter sequence was successfully mutated using primers shown in Table 6-2. The bacterial clones containing the mutated MMP-9 promoter sequence were confirmed by sequence analysis using both forward (RV3) and reverse (GL2) vector based primers. Multiple NF-κB sites were added to the 5′ end of the canine MMP-9 promoter using PCR techniques and primers described in Table 6-3 containing one, three and five NF-κB sites and were immediately cloned into pCR2.1®TOPO. All three manipulated MMP-9 promoters were then sub-cloned into the pGL3-Basic luciferase reporter vector using the *kpn* 1 (sticky) and *EcoRV*/*Sma* 1 (blunt) restriction enzyme sites. The bacterial clones containing the manipulated MMP-9 promoters were screened using the described PCR techniques for their amplification and were sequenced with both forward (RV3) and reverse (GL2) vector based primers.

6.4.1.1 Vector maps

Vector maps for the canine MMP promoter sequences in pGL3-Basic luciferase reporter vector (Promega) are shown for pGL3/cMMP-9(1894) (Figure 5-4), pGL3/cMMP-9(984) (Figure 5-5), pGL3/cMMP-9(628) (Figure 5-6), pGL3/cMMP-9(534) (Figure 5-7). A vector map for the mutated canine MMP promoter construct pGL3/cMMP-9(628)(NF-κBmut^{n}) is shown in Figure 6-6. Vector maps for the manipulated canine MMP promoter sequences in pGL3-Basic luciferase reporter vector are shown accordingly: pGL3/cMMP-9(5NFκB) (Figure 6-7), pGL3/cMMP-9(3NFκB) (Figure 6-8), pGL3/cMMP-9(1NFκB) (Figure 6-9).

Figure 6-6 Vector map for pGL3/cMMP-9(628)(NF-κBmut^n)

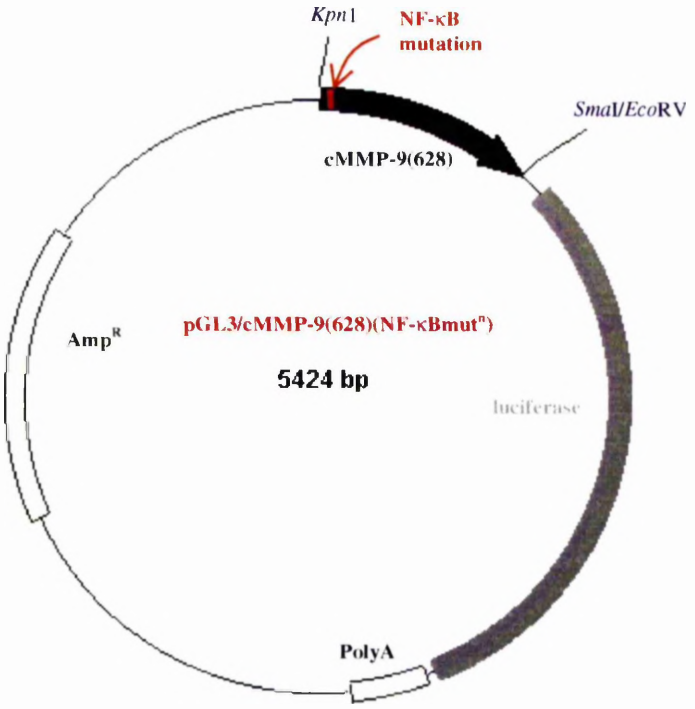


Figure 6-7 Vector map for pGL3/cMMP9(1894)(5NF-κB)

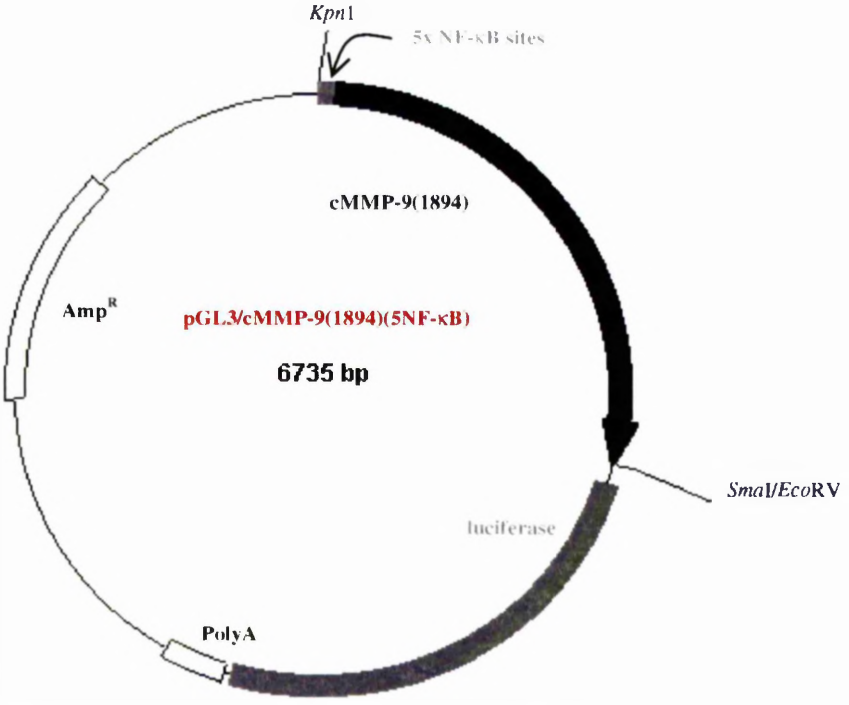


Figure 6-8 Vector map for pGL3/cMMP-9(1894)(3NF-κB)

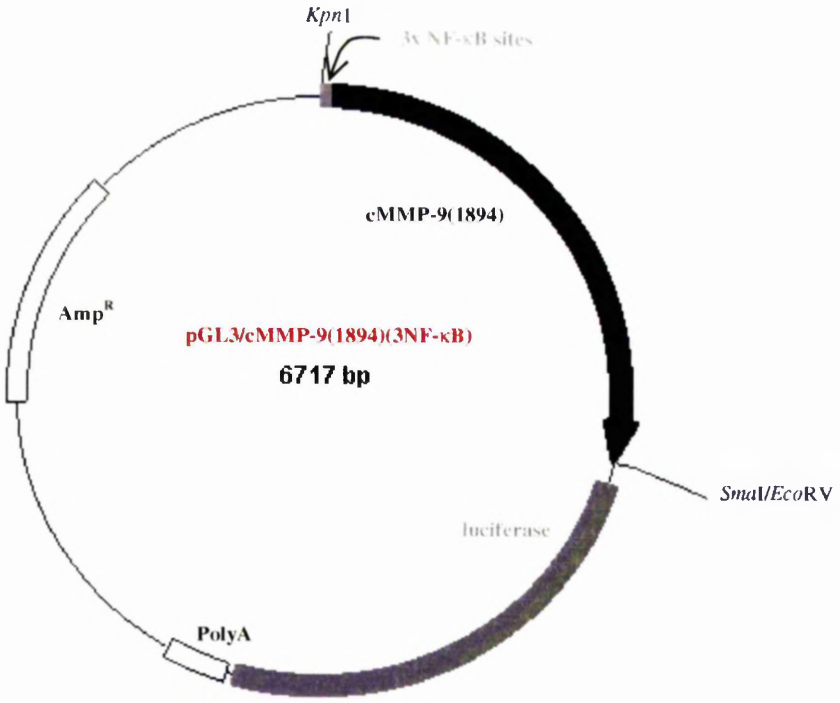
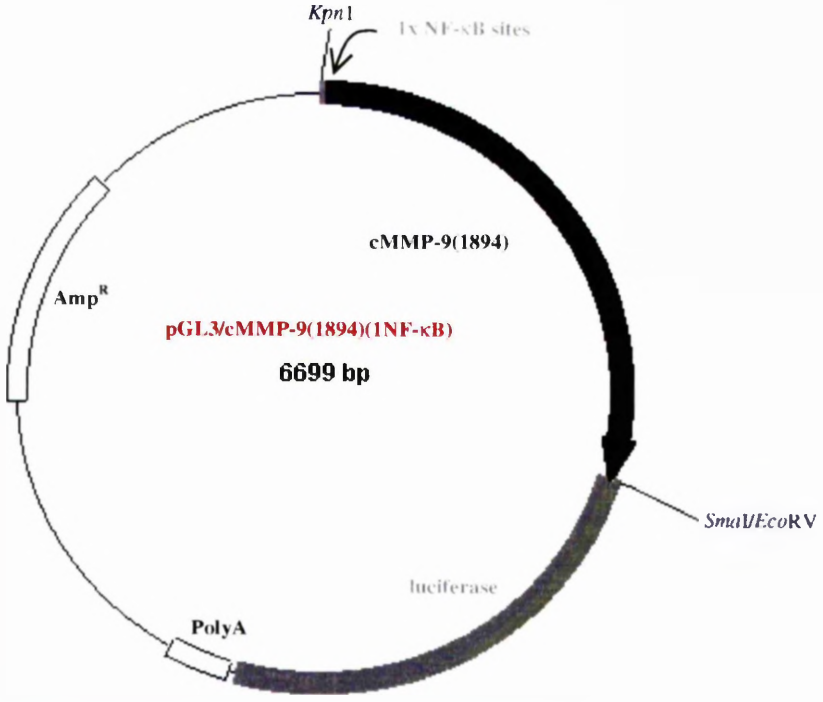


Figure 6-9 Vector map for pGL3/cMMP-9(1894)(1NF-κB)



6.4.2 Characterisation of the canine pGL3/cMMP-9 promoter deletions, NF-κB mutation and multiple NF-κB sites in human chondrosarcoma cells (SW1353)

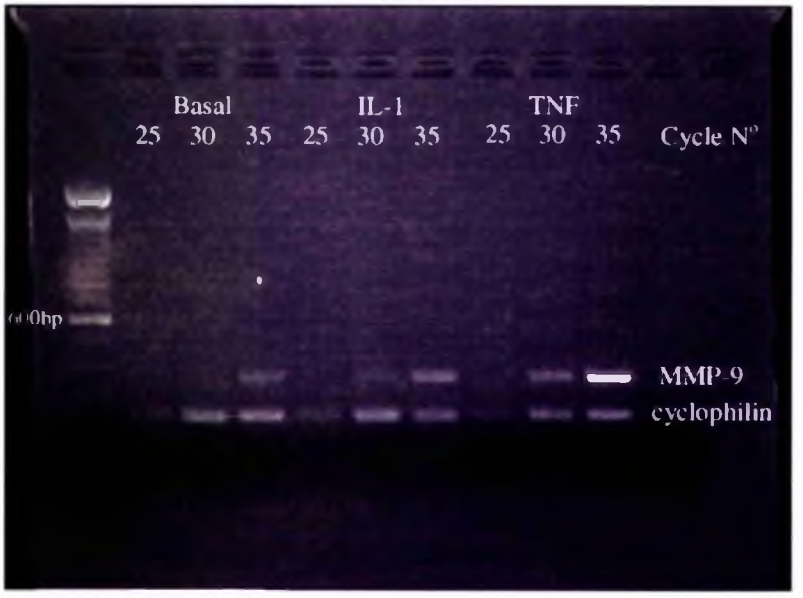
The aim of this first study was to evaluate and compare the basal and induced (IL-1β and TNFα) activities of the four largest cloned canine MMP-9 promoter deletion constructs to identify regions containing cytokine responsive elements. Cytokine responsive element(s) were then to be mutated from the promoter sequence to confirm their involvement in cytokine induction. Multiple copies of these element were then to be introduced into the MMP-9 promoter sequence to enhance activity in response to cytokines while maintaining low basal activity.

6.4.2.1 Analysis of endogenous basal and induced transcription of MMP-9 genes in SW1353 cells

To analyse the basal and cytokine induced (IL-1β and TNFα) levels of MMP-9 gene transcription in the human chondrosarcoma cell line (SW1353) to be used for the transfections of all of the canine MMP-9 promoter constructs relative semi-quantitative RT-PCR was performed. A portion of the gene was amplified from cDNA samples prepared from untreated (basal) and treated (IL-1β and TNFα) cells using species-specific primer pairs (Table 6-2). Samples removed at three intervals, 25, 30 and 35 cycles to enable the semi-quantification of MMP-9 gene transcription over the exponential phase of the PCR. A portion of the human cyclophilin gene was also amplified (255 bp), using species-specific primer pairs, as an internal control. The primers were designed to span an intron and control for the presence of contaminating genomic DNA (absence of a 474 bp PCR product). Basal transcription of the endogenous MMP-9 gene was evident in the human chondrosarcoma cells and could be upregulated by cytokines, IL-1β and TNFα (Figure 6-10). Subsequently this cell line was used to analyse all of the cloned MMP-9 promoter constructs.

Figure 6-10 Endogenous levels of MMP-9 transcription in SW1353 cells using relative semi quantitative RT-PCR

A portion (364 bp) of the human MMP-9 gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from human chondrosarcoma cells (SW1353). During PCR amplification, samples were removed within the exponential phase of the reaction (25 to 35 cycles) revealing that basal levels of MMP-9 transcription were significantly up-regulated by the pro-inflammatory cytokines IL-1β and TNFα.



6.4.2.2 Analysis of all cloned canine MMP-9 promoter constructs using Dual-Luciferase® Reporter assays

All Dual®Luciferase assays were conducted in triplicate for statistical significance and to ensure reproducibility, and all transfections were carried out three times. To account for differences in transfection efficiency the cells were co-transfected with *Renilla* luciferase vector (Promega) and the firefly luciferase values were adjusted accordingly. *Renilla* was expressed and shown to be active the cell type used. Representative *Renilla* values for SW1353 cells was 242. The corrected luciferase activity of each construct represents the mean +/-SEM (n=3). The canine MMP-9 deletion reporter constructs, together with the promoter-less luciferase vector, pGL3-Basic vector as the negative control, were transiently transfected into human chondrosarcoma cells (SW1353). Basal luciferase activities for each promoter were compared to treated samples (IL-1β and TNFα). The Mann-Whitney statistical analysis was performed on all assays.

6.4.2.2.1 Analysis of canine pGL3/cMMP-9(1984), (984), (628), (534) luciferase reporter constucts

Basal activities of all pGL3/cMMP-9 deletion constructs were evident in the SW1353 cell line. All constructs MMP-9(1894), (984), (628) and (534) could be significantly induced by TNFα to varying degrees (*P<0.05). Although some induction was also observed with IL-1β the results were not significant. The general trend of promoter activity showed that smallest construct (534) had significantly less activity than the three largest constructs MMP-9(1894), (984) and (628) at both basal and induced (IL-1βand TNFα) levels (•P<0.05) (Figure 6-11).

6.4.2.2.2 Analysis of mutated canine pGL3/cMMP-9(628)(NF-κBmut") luciferase reporter constuct

Basal activity of pGL3/cMMP-9(628)(NFκBmut") construct was evident in the SW1353 cell line which could be significantly enhanced by TNFα (*P<0.05), and although induction with IL-1β was evident this again was not significant. Basal and induced (TNFα and IL-1β) activity of the mutated construct was significantly lower than the MMP-9(628) promoter construct (ΔP<0.05). Basal and induced (TNFα) was also significantly lower than the MMP-9(534) construct (P<0.05) (Figure 6-11).

6.4.2.2.3 Analysis of manipulated canine pGL3/cMMP-9(1894)(5NF-κB), (3NF-κB) and (1NF-κB) luciferase reporter constucts

The canine MMP-9 promoter constructs, manipulated with additional NF-κB sites, were directly compared with the unmanipulated MMP-9(1894) promoter construct (Figure 6-12). The general trend of promoter activity showed that the addition of five NF-κB sites increased basal and cytokine-induced activity the most, followed by the addition of three three NF-κB sites while the addition of one alone had little effect. More specifically, basal activities of the canine MMP-9

promoter constructs cMMP-9(1894). (5NFκB) and (3NFκB) were significantly enhanced by TNFα (*P<0.05 Mann Whitney test). In comparison, the MMP-9(1984) and (3NF-κB) were significantly enhanced by IL-1β (•P<0.05). The addition of five NF-κB sites significantly enhanced basal and induced (TNFα and IL-1β) promoter activity (ΔP<0.05) in comparison to the unmanipulated cMMP-9(1894) promoter construct. The addition of three NF-κB sites only significantly enhanced TNFα induced activity (P<0.05) compared to the unmanipulated MMP-9(1894) promoter construct. The addition of one NF-κB site had no effect on increasing promoter activity at the basal or induced levels.

Figure 6-11 Deletion and mutational analysis of the canine MMP-9 promoter.

Dual-Luciferase® Reporter assays were used to determine the activity of the cloned canine MMP-9 promoter deletion MMP-9(1894), (984), (628), (534) and mutation (MMP-9NF-κBmut^{n}) constructs in the human chondrosarcoma (SW1353) cells. Basal activities of the canine MMP-9 promoter constructs were significantly enhanced by TNFα (*P<0.05 Mann-Whitney test), but not IL-1β. Basal and induced (TNFα and IL-1β) activities of the MMP-9(534) construct were significantly lower than the other MMP-9 promoter deletion constructs (•P<0.05). Basal and induced (TNFα and IL-1β) activities of the MMP-9(NFκBmut^{n}) construct were significantly lower than the other MMP-9(638) promoter (ΔP<0.05). Basal and induced (TNFα) activities of the MMP-9(mut^{n}) construct were also significantly lower than the other MMP-9(534) promoter (P<0.05).

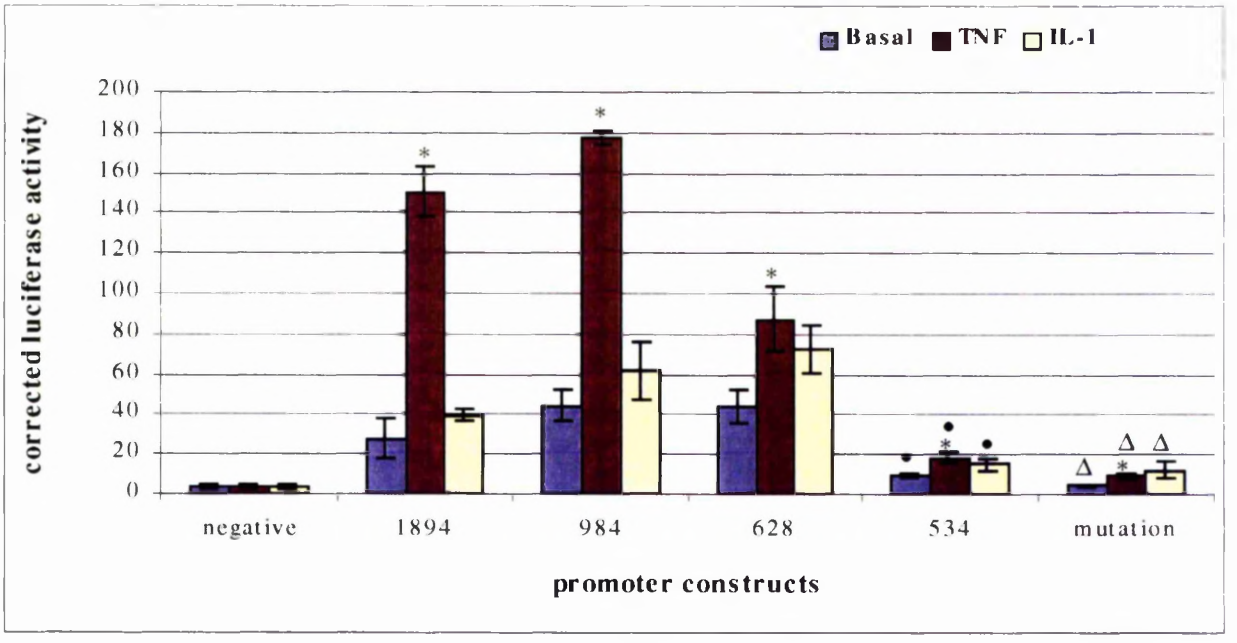
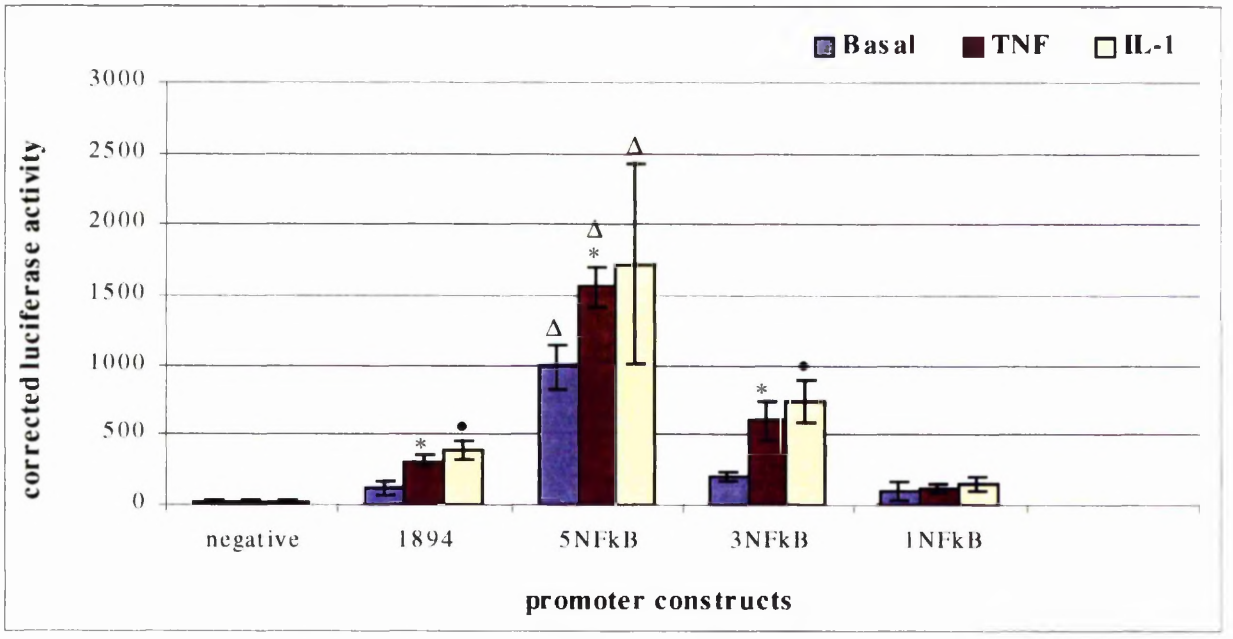


Figure 6-12 Manipulation of the canine MMP-9 promoter fragment with multiple NF-κB sites

Dual-Luciferase® Reporter assays were used to determine the activity of the cloned canine MMP-9 promoter, MMP-9(1894) and manipulated MMP-9(5NF-κB) (3NF-κB) (1NF-κB) constructs in the human chondrosarcoma (SW1353) cells. Basal activities of the canine MMP-9 promoter constructs MMP-9(1894), (5NF-κB) and (3NF-κB) were significantly enhanced by TNFα (*P<0.05 Mann Whitney test). In comparison, the MMP-9(1984) and (3NF-κB) were significantly enhanced by IL-1β (•P<0.05). The addition of five NF-κB sites significantly enhanced basal and induced (TNFα and IL-1β) promoter activity (ΔP<0.05) over the unmanipulated MMP-9(1894) promoter construct. The addition of three NF-κB sites only significantly enhanced TNFα induced activity (P<0.05) over the unmanipulated MMP-9(1894) promoter construct. The addition of one NF-κB site had no effect on increasing promoter activity at the basal or induced levels.



6.4.3 Cloning of the mouse collagen Type XI promoter, 3&5SOX9 promoters, intron/SOX9 into the pGL3-Basic luciferase reporter vector

The mouse collagen type XI promoter was successfully amplified by PCR using mouse genomic DNA as template and primers described in Table 6-4. The promoter was cloned into pCR2.1®TOPO before being sub-cloned into the pGL3-Basic luciferase reporter vector using the *kpn* 1 (sticky) and *EcoRV/Sma* 1 (blunt) restriction enzyme sites. The mouse collagen type XI promoter was successfully amplified with three and five SOX9 sites into the 5′ end of the promoter using the pCR2.1/mColXI construct as template and primers described in Table 6-4. The promoter was cloned into pCR2.1®TOPO before being sub-cloned into the pGL3-Basic luciferase reporter vector using the *kpn* 1 (sticky) and *EcoRV/Sma* 1 (blunt) restriction enzyme sites. The mouse collagen type XI intron was successfully amplified with three SOX9 sites flanking the sequence using the pCR2.1/mColXIintron construct as template and primers described in Table 6-4. The promoter was cloned into pCR2.1®TOPO before being sub-cloned into the pGL3-Basic luciferase reporter vector using the *Hind* III (sticky) restriction enzyme sites. All bacterial clones containing the promoter and intronic sequences were screened using the described PCR techniques, for their amplification.

6.4.3.1 Vector maps

Vector maps for the mouse collagen type XI promoter +/- SOX9 sites and intron +SOX9 sites in pGL3-Basic are shown for pGL3/mColXI (Figure 6-13) pGL3/mColXI(3SOX9) (Figure 6-14) pG3/mColXI(5SOX9) (Figure 6-15) and pGL3/mColXI[3SOX9(intron)3SOX9] (Figure 6-16).

Figure 6-13 Vector map for pGL3/mColXI

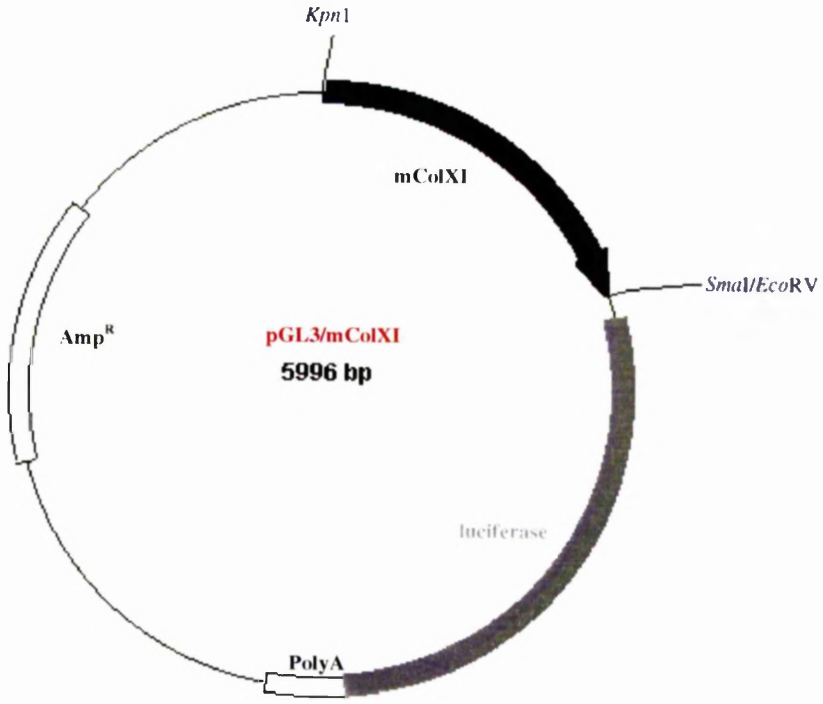


Figure 6-14 Vector map for pGL3/mColXI(3SOX9)

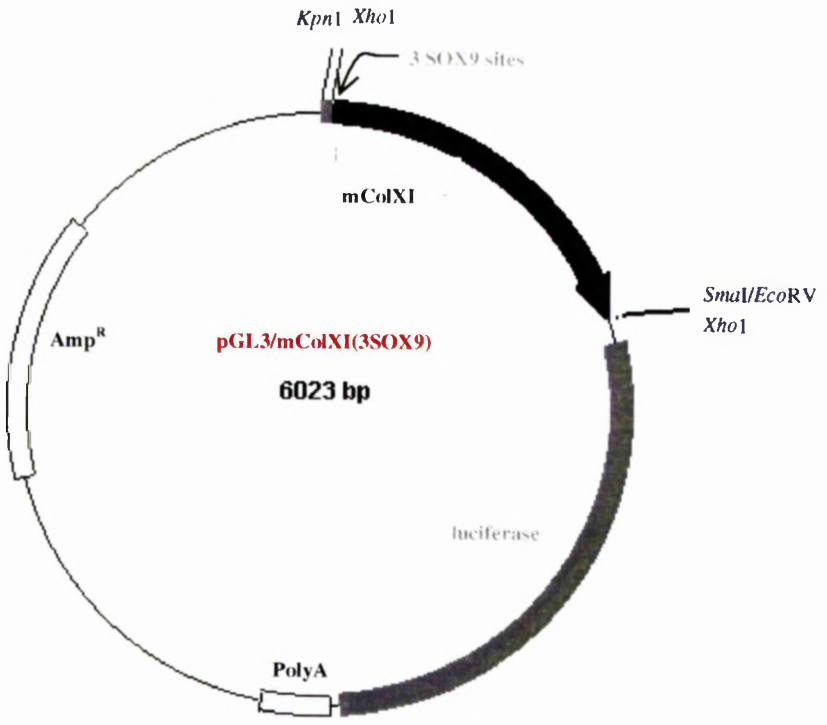


Figure 6-15 Vector map for pGL3/mColXI(5SOX9)

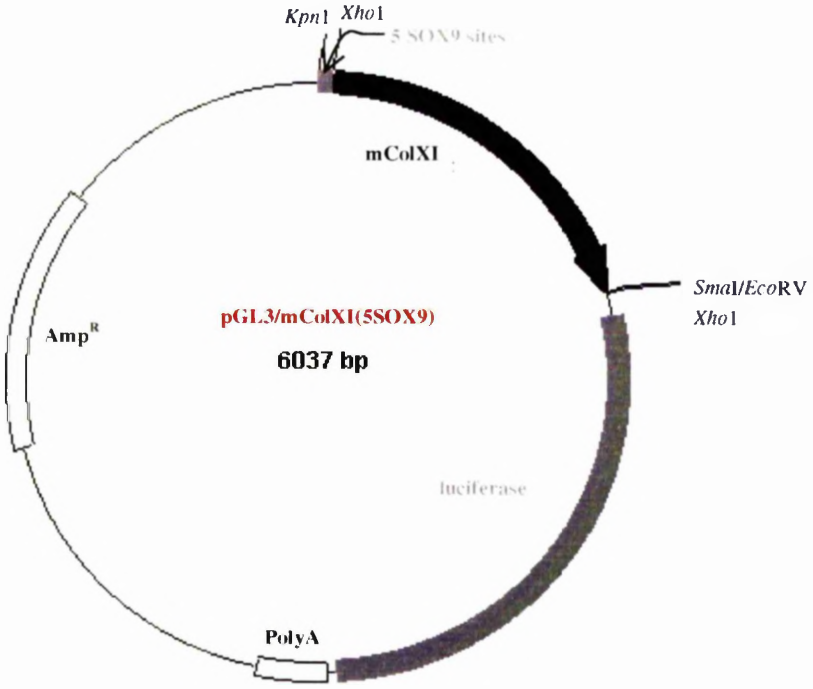
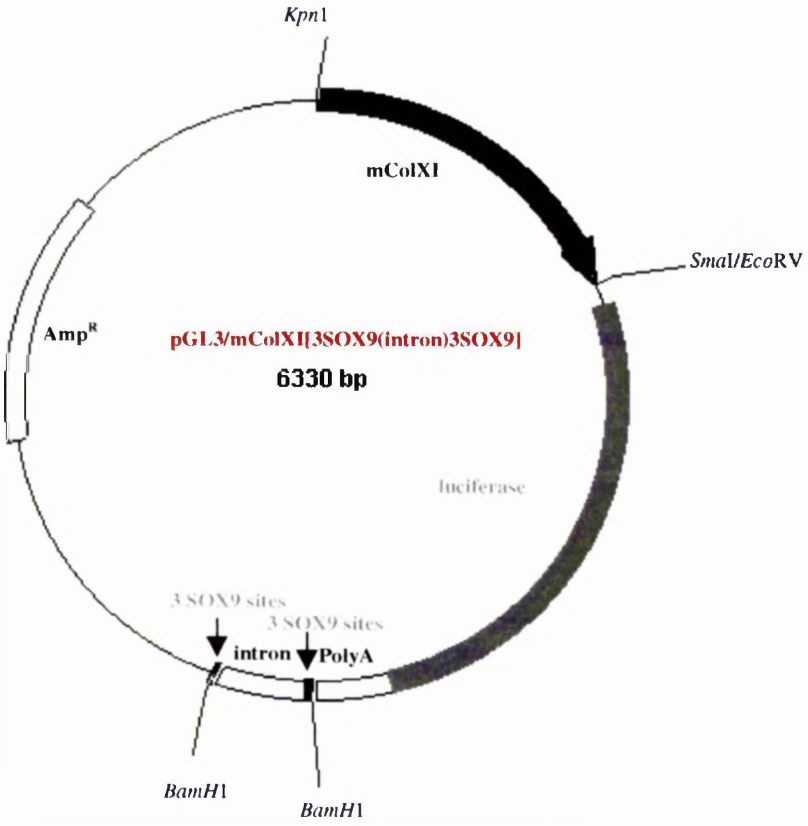


Figure 6-16 Vector map for pGL3/mColXI[3SOX9(intron)3SOX9]



6.4.4 Characterisation of mouse collagen type XI promoter and intronic constructs manipulated with SOX9 sites in SW1353 and ATDC5 cells

6.4.4.1 Analysis of endogenous transcription of collagen type XI genes in SW1353 cells and ATDC5 cells

To analyse endogenous activity of collagen type XI transcription in the SW1353 and ATDC5 cell lines to be used for the transfection of the mouse collagen type XI promoter fragment, relative semi-quantitative RT-PCR was once again performed. A portion of each gene was amplified from cDNA samples prepared from untreated (basal) and treated (IL-1β and TNFα) cells using species-specific primer pairs (Table 6-3). Samples were removed at three intervals, 3 cycles apart, to enable the semi-quantification of collagen type XI gene transcription over the exponential phase of the PCR. A portion of the cyclophilin gene was also amplified (255 bp) using species specific primers as an internal control. The primers were designed to span an intron and control for the presence of contaminating gDNA (absence of a 474 bp PCR product). Basal levels of endogenous transcription were present in both the SW1353 cells (Figure 6-17) and the ATDC5 cells (Figure 6-18) neither of which could be up-regulated by IL-1 and TNF. These cell lines could therefore be used to analyse the mouse collagen type XI promoter fragments.

Figure 6-17 Endogenous levels of collagen type XI (mColXI) transcription in SW1353 cells using relative semi-quantitative RT-PCR.

A portion (395 bp) of the human collagen type XI gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from human chondrosarcoma cells (SW1353). During PCR amplification, samples were removed within the exponential phase of the reaction (30 to 40 cycles) revealing that basal levels of collagen type XI transcription were present which were not up-regulated by the pro-inflammatory cytokines IL-1β and TNFα.

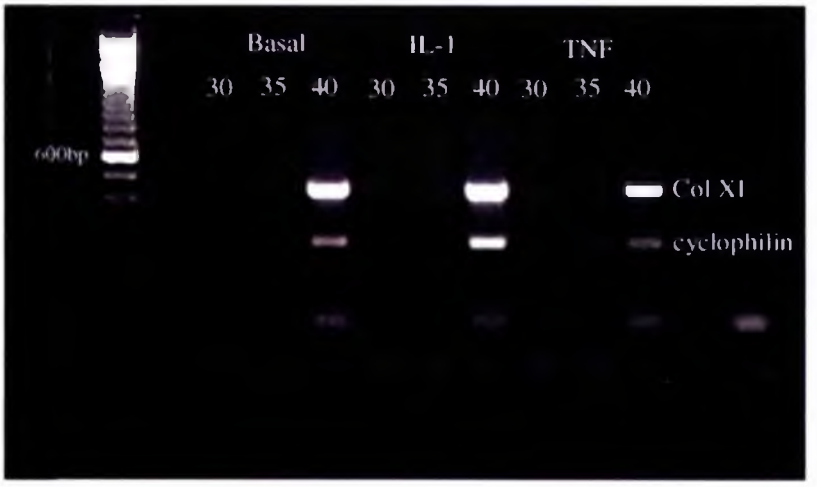
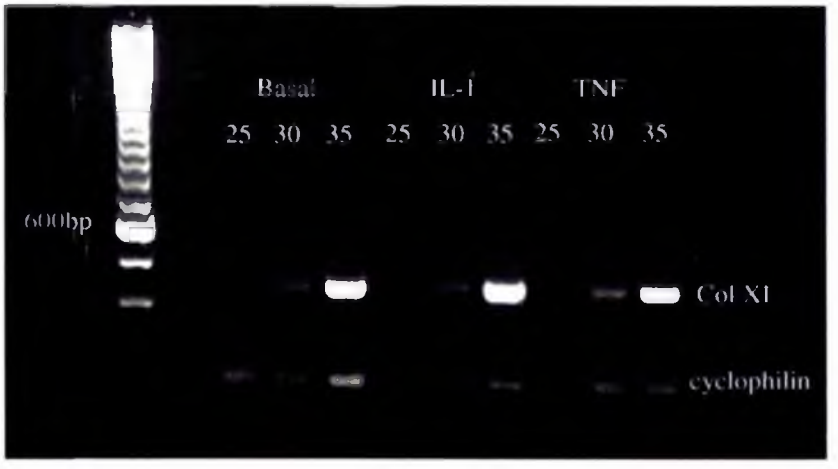


Figure 6-18 Endogenous levels of collagen type XI (Col XI) transcription in ATDC5 cells using relative semi-quantitative RT-PCR.

A portion (395 bp) of the mouse collagen type XI gene (upper band) was amplified from cDNA samples prepared from total RNA isolated from undifferentiated chondrosarcoma cells (ATDC5). During PCR amplification samples were removed within the exponential phase of the reaction (25-35 cycles) revealing that basal levels of collagen type XI transcription were present which were not up-regulated by the pro-inflammatory cytokines IL-1β and TNFα.



6.4.4.2 Analysis of mouse collagen type XI promoter and intronic sequences manipulated with SOX9 sites using Dual-Luciferase® Reporter Assays

Dual-Luciferase®Reporter Assays were conducted in triplicate as described for the various MMP-9 promoter constructs using the *Renilla* luciferase vector as the internal control. *Renilla* was expressed and shown to be active in cell types used. Representative *Renilla* values for the SW1353 and ATDC5 cells were 322 and 225 respectively. The different mouse collagen type XI promoter +/- intron luciferase reporter constructs were transiently transfected into human chondrosarcoma cells (SW1353) and undifferentiated chondrosarcoma cells (ATDC5). Basal luciferase activities of these constructs were directly compared along with a promoter-less luciferase vector (pGL3-Basic) as a negative control. The Mann-Whitney statistical test was used to analyse the results.

6.4.4.2.1 Mouse collagen type XI promoter +/- (3SOX9) and (5SOX9) in pGL3 luciferase reporter vector

Activities of the cloned mouse collagen type XI promoter and manipulated (3SOX9) and (5SOX9) promoter constructs were significantly higher than the negative control in both the SW1353 (*P<0.05) and ATDC5 (•P<0.05) cell lines. However, the activities of the mouse collagen type XI promoter and the two manipulated promoter constructs mColXI(3SOX9) and (5SOX9) were significantly higher in the SW1353 cells than the ATDC5 cells (ΔP<0.05). Nevertheless, the addition of these SOX9 sites did not enhance activity in either cell line (Figure 6-19).

6.4.4.2.2 Mouse collagen type XI promoter and (3SOX9)intron(3SOX9) in pGL3 luciferase reporter vector

Activities of cloned mouse collagen type XI promoter with and without the additional intronic sequence flanked by three SOX9 sites were significantly higher than the negative control in both the SW1353 (*P<0.05) and ATDC5 (•P<0.05) cells. Activity of the promoter +/- the intronic sequence was also significantly higher in the SW1353 cells than the ATDC5 cells (ΔP<0.05). Furthermore, the addition of the intronic sequence containing flanking SOX9 sites significantly enhanced promoter activity in the SW1353 cells line (P<0.05) (Figure 6-20).

Figure 6-19 Analysis of the mouse collagen type XI promoter +/- 3 and 5 SOX9 sites.

Dual-Luciferase® Reporter Assays were used to determine the activity of the cloned mouse collagen type XI promoter with additional three and five SOX9 sites in the human chondrosarcoma cells (SW1353) and undifferentiated chondroprogenitor cells (ATDC5). Activities of cloned mouse collagen type XI promoter and manipulated promoters (3SOX9; 5SOX9) were significantly higher than the negative control in both the SW1353 (*P<0.05) and ATDC5 (•P<0.05) cells (P<0.05 using the Mann-Whitney test). Activity of the three promoter constructs was also significantly higher in the SW1353 cells than the ATDC5 cells (ΔP<0.05); however the addition of the three and five SOX9 sites did not significantly enhance activity in either cell line.

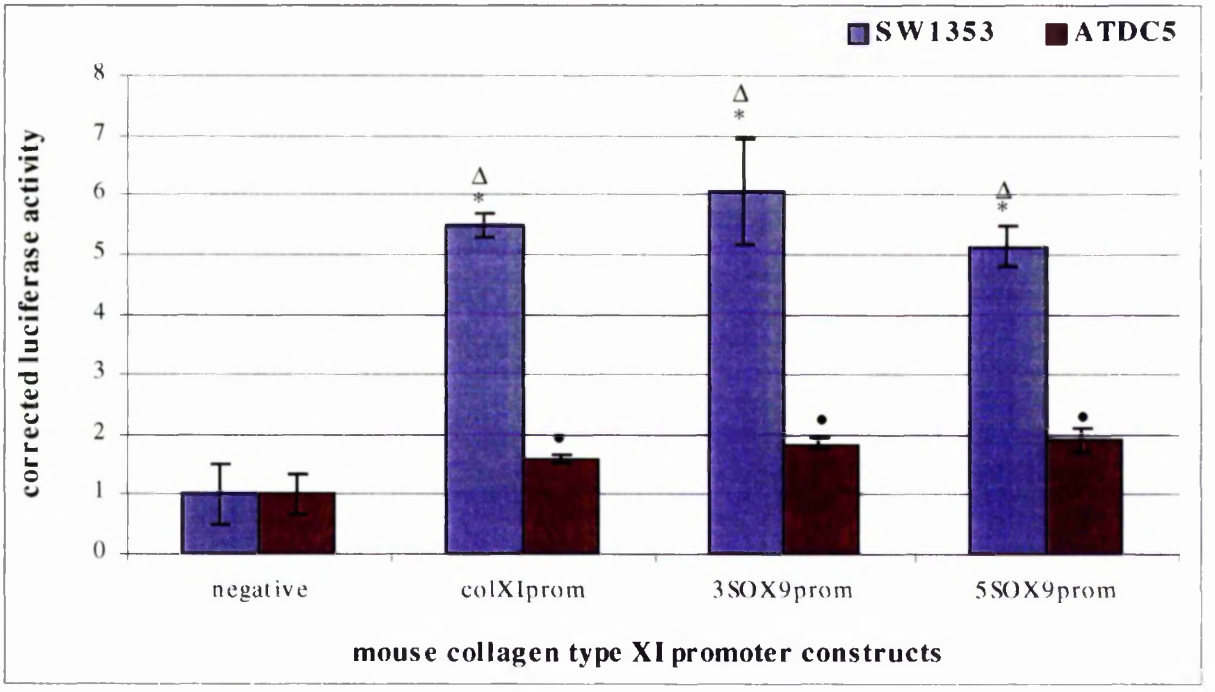
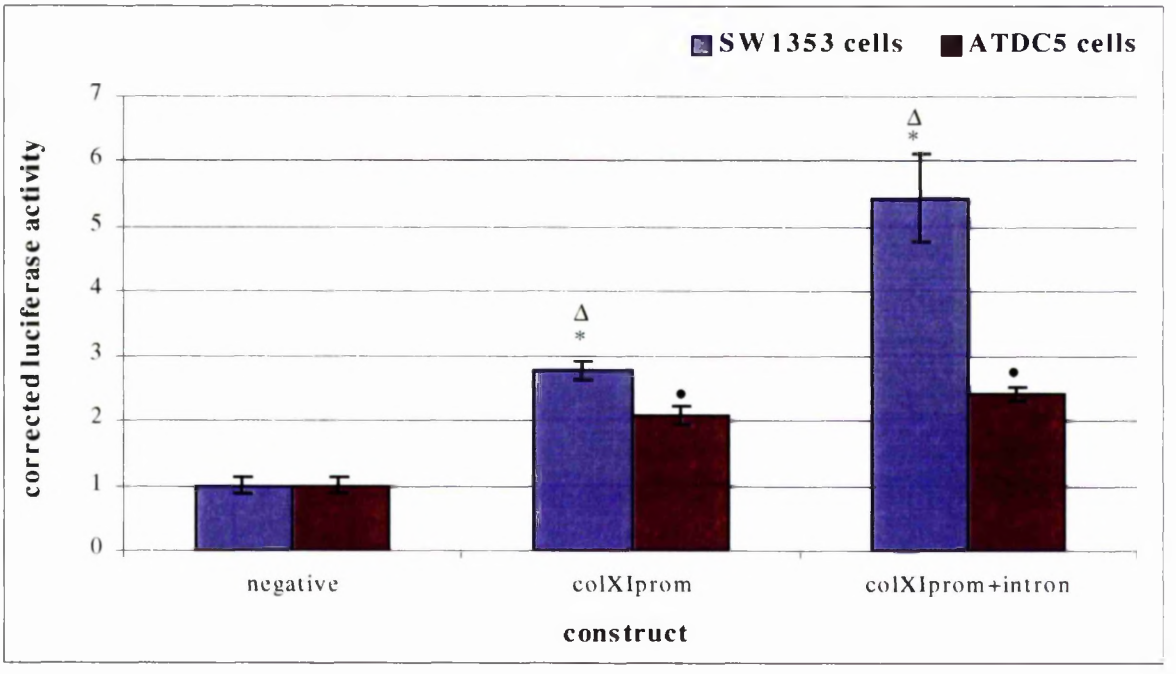


Figure 6-20 Analysis of the mouse collagen type XI promoter with intronic SOX9 sites. Dual-Luciferase® Reporter Assays were used to determine the activity of the cloned mouse collagen type XI promoter with and without the additional intronic sequence containing three flanking SOX9 sites in the human chondrosarcoma cells (SW1353) and undifferentiated chondroprogenitor cells (ATDC5). Activities of cloned mouse collagen type XI promoter with and without the additional intronic sequence were significantly higher than the negative control in both the SW1353 (*P<0.05 using the Mann-Whitney test) and ATDC5 (•P<0.05) cells. Activity of these two constructs was also significantly higher in the SW1353 cells than the ATDC5 cells (ΔP<0.05). Furthermore, the addition of the intronic sequence containing flanking SOX9 sites significantly enhanced promoter activity in the SW1353 cells line (P<0.05).



6.4.5 Cloning of the dual targeting vector

Unfortunately, the construction of the dual targeting vector was not possible due to technical problems. The first three vectors A, B and C were all successfully constructed, confirmed by sequence analysis using a combination of vector-based and internal sequence-based primers (Table 6-2,3,4). Vector A was a high copy number plasmid, but after the insertion of the second and subsequent DNA fragments Vectors B and C were converted to low copy number plasmids.

Problems arose during the construction of Vector D. Both Vector C and the final 400 bp insert were successfully digested with *Hind* III, visible on agarose gel analysis. On transformation into TOP 10 bacterial cells, several hundred colonies were produced, twenty of which were selected and grown overnight at 37°C. The DNA was isolated and the quantity determined by UV spectrophotometry which revealed that a percentage of these clones had reverted to a high copy number plasmid. Low copy number plasmids were selected and screened for insert with *Hind* III, irrespective of orientation. Positive clones were sent for sequencing and were all found to contain the final insert in inverse orientation. It was thought that the presence of Cre protein, in the bacterial cells used for the transformation step, could be responsible for the recombination of Vector D on insertion of the final insert containing the second *lox*H site in the correct orientation.

Vector maps for the four intermediate plasmids produced in the construction of the dual targeting vector can be found accordingly: Vector A: pGL3/mColXI*lox*H (Figure 6-21), Vector B: pGL3/mColXI*lox*H-Cre (Figure 6-22), Vector C: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB) (Figure 6-23), Vector D: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB) (Figure 6-24)

Figure 6-21: Vector map for Vector A construct: pGL3/mColXI*lox*H

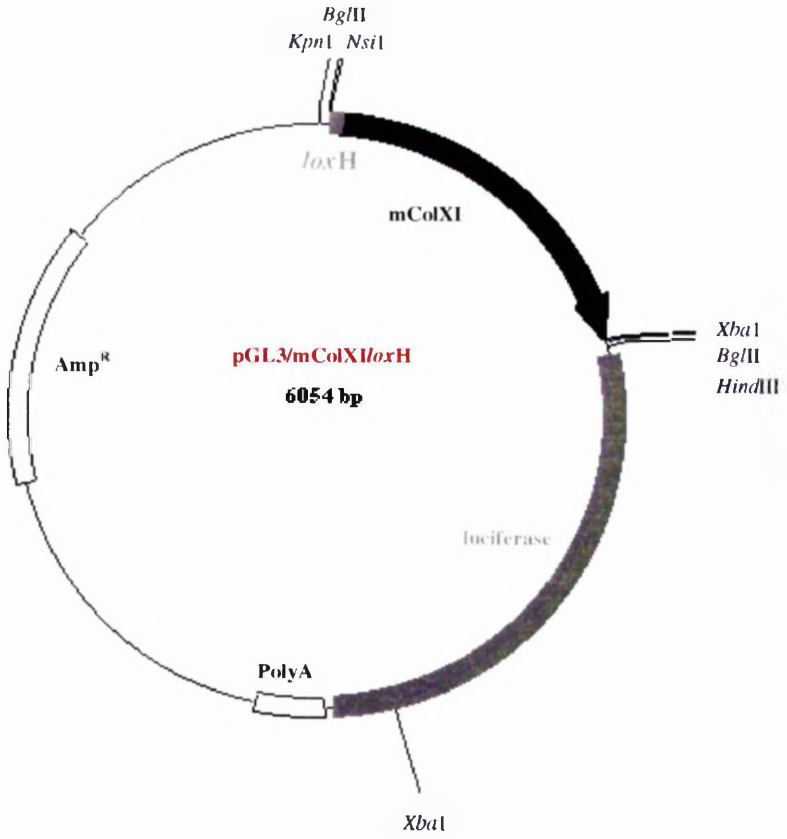


Figure 6-22: Vector map for Vector B construct: pGL3/mColXI*lox*H-Cre

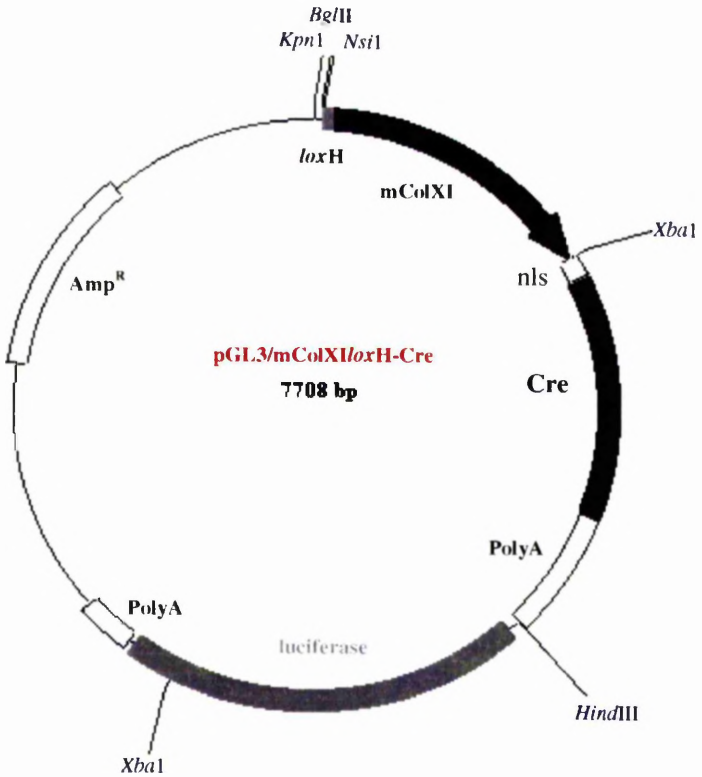


Figure 6-23 Vector map for Vector C construct: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB)

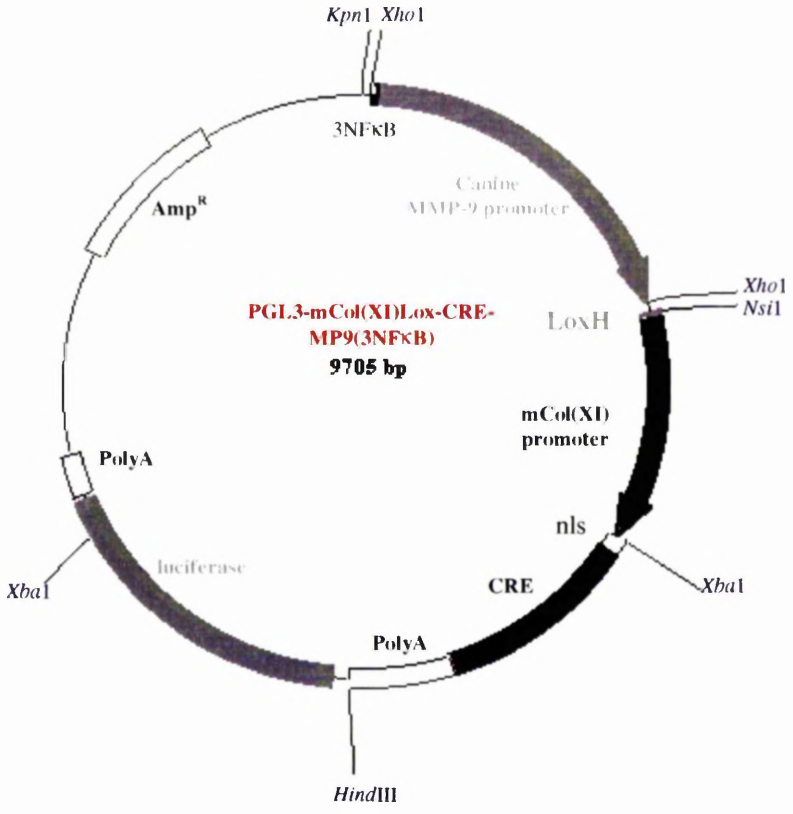
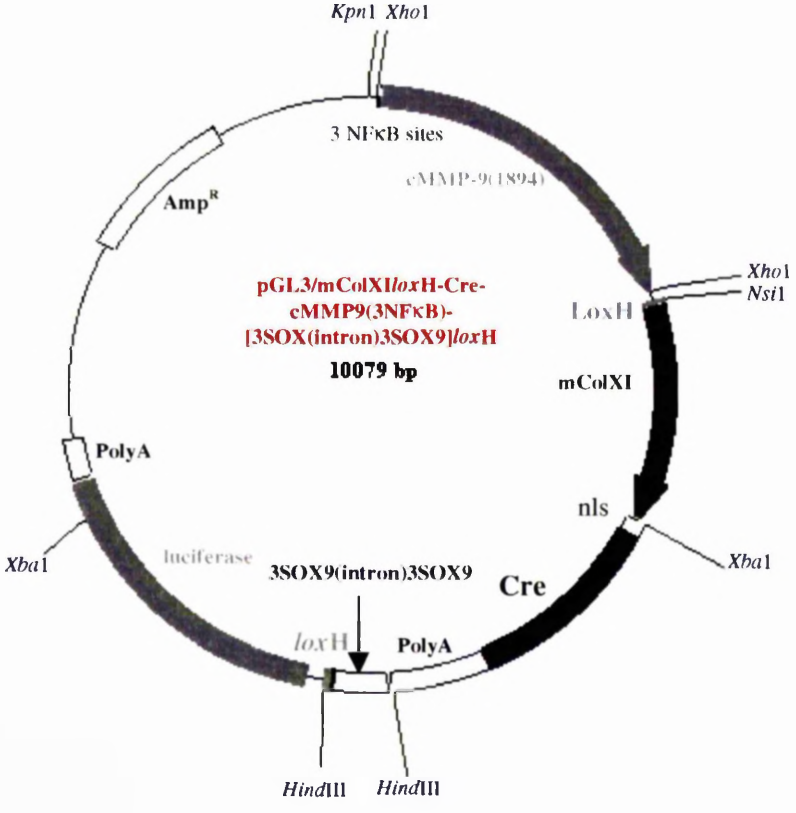


Figure 6-24 Vector map for Vector D: pGL3/mColXI*lox*H-Cre-cMMP-9(3NFκB)-[3SOX9(intron)3SOX9]

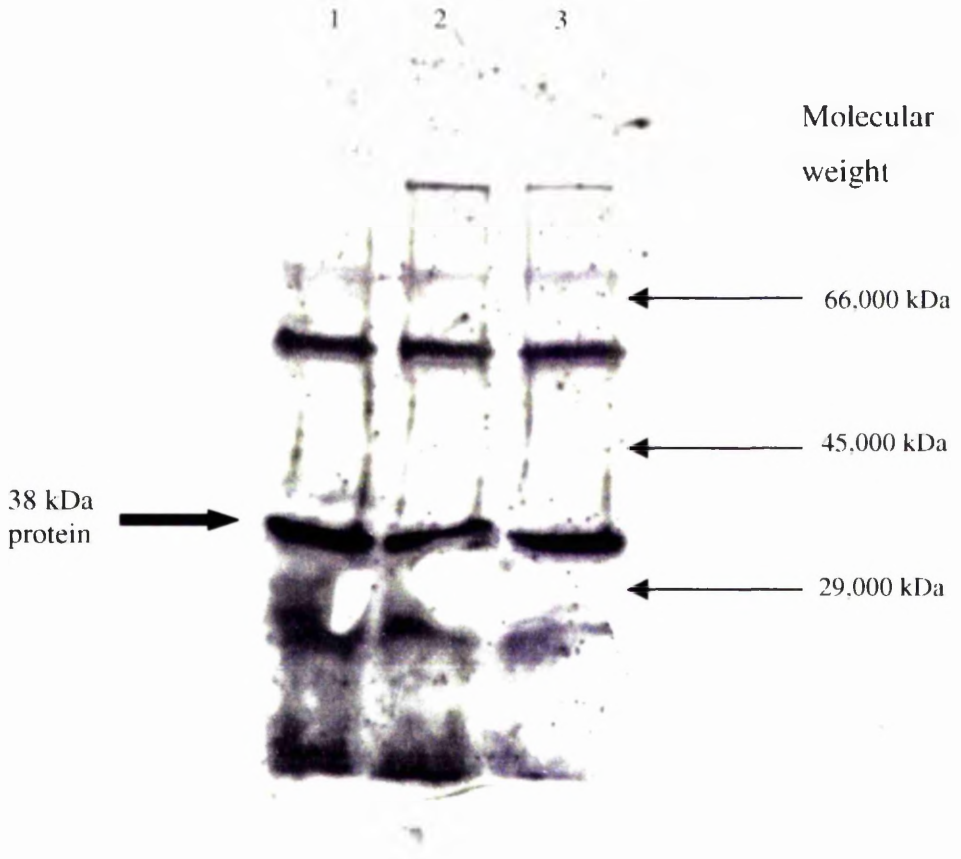


6.4.6 Western blot analysis of Cre recombination during construction

It was thought that the presence of Cre protein in the bacterial cells used for the transformation step could be responsible for the recombination of Vector D on correct insertion of the final insert containing the second *lox*H site. A Western blot was subsequently performed to investigate both endogenous or plasmid mediated (under the control of the mouse collagen type XI promoter) Cre gene expression. Three samples were analysed for Cre protein, TOP10 bacterial cells alone, TOP10 bacterial cells transfected with the promoterless pGL3-Basic vector and TOP10 bacterial cells transfected with Vector C. All three samples, analysed for Cre gene expression, showed a 38 kDa band corresponding to the predicted size of the Cre protein (Figure 6-25).

Figure 6-25 Western blot analysis of the Cre gene expression in the TOP10 bacterial cells

Western blot analysis of 1) TOP10 E.Coli bacterial cells, 2) pGL3-Basic in TOP10 cells and 3) Vector 3 in TOP10 bacterial cells. A band of 38 kDa corresponding to the Cre protein was found in all three lanes suggesting that the Cre gene is expressed endogenously in the TOP10 bacterial cells



6.5 DISCUSSION

6.5.1 Can the canine MMP-9 promoter be manipulated to enhance activity while maintaining disease-specificity?

6.5.1.1 Analysis of endogenous basal and induced transcription of MMP-9 genes in SW1353 cells

Basal and cytokine stimulated endogenous levels of MMP-9 transcription were examined in the human chondrosarcoma cell line (SW1353) using relative semi-quantitative RT-PCR to investigate the potential for analysing the numerous canine MMP-9 promoter/luciferase constructs in this cell line. The SW1353 cell line was selected since it serves as an appropriate model for primary chondrocytes in OA (Vincenti and Brinckerhoff et al., 2001). The levels of MMP-9 transcription were upregulated by inflammatory cytokines IL-1β and TNFα (Figure 6-10) which complements previous studies showing MMP-9 up-regulated by IL-1β, PMA (Cowell et al., 1998) and TNFα (Hattori et al., 2001) in human chondrosarcoma cell lines.

6.5.1.2 Analysis of all cloned canine MMP-9 promoter constructs using luciferase reporters

The MMP-9 promoter deletion constructs were transiently transfected into the SW1353 cells in an attempt to identify promoter elements involved in both basal and pro-inflammatory (IL-1β and TNFα) induced gene transcription. All of the MMP-9 promoter deletion constructs cMMP-9(1894), (984), (628), (534) were shown to have basal activity that could be significantly enhanced by the addition of TNFα, and some induction was observed with IL-1β (Figure 6-11). This suggested that the distal AP-1 sites (-73 to –67; -111 to 105), and other promoter elements adjacent to the TATA box, were sufficient for upregulating gene expression in response to both cytokines. This result complemented findings described in chapter V, where it was demonstrated that basal activity of these canine MMP-9 deletion constructs could be significantly enhanced by PMA. However, the actual levels of promoter activity were much greater in the three largest constructs pGL3/cMMP-9(1984), (984) (628) in comparison to the smallest vector pGL3/cMMP-9(534), suggesting that additional cytokine-responsive elements, located up-stream of position 534 bp, may co-operate with the distal promoter elements. This may be explained by the presence of a conserved NF-κB DNA binding domain (-554 to –545) that is able to cross-couple with the AP-1 site acting synergistically to increase promoter activity in response to pro-inflammatory cytokines (Adcock, 1997). To investigate this further the NF-κB binding site was mutated from the pGL3/cMMP-9(628) construct.

The MMP-9 promoter construct containing the mutated NF-κB site (cMMP-9/NF-κBmut[n]) was also transfected into SW1353 cells and shown to have similar levels of activity to the pGL3/cMMP-9(534) reporter construct (Figure 6-11). Again basal levels of activity could be significantly

enhanced by TNFα with some increase in response to IL-1β suggesting that the NFκB was not essential for cytokine induction and that other elements were also important. However, actual levels of promoter activity were greatly reduced suggesting that the NF-κB site was probably important for co-operating with the AP-1 and other sites for both TNF and IL-1β induction. These results are similar to other studies showing the importance of the NF-κB site in the mouse MMP-9 promoter (He, 1996), and that the human and rabbit MMP-9 promoters both requiring the synergistic action of NF-κB and AP-1 sites for cytokine induction with TNF and IL-1 and growth factors (Bond et al., 1998). Furthermore, site-directed mutagenesis of the N-FκB site has been shown to decrease the TNFα induced activity of the human E-Selectin promoter (Schindler and Baichwal, 1994) and IL-1-induced activity of the human MCP-1 (Martin et al., 1997) and COX-2 (Allport et al., 2000) promoters.

Further to suggestions that multiple NF-κB sites within the inducible nitric oxide synthase gene may co-operate to confer inducibility to both IL-1β and TNFα (Taylor et al., 1998), it was decided to incorporate multiple (5, 3 and 1) NF-κB sites into the 5′ end of the promoter to enhance the response to cytokines. Activities of the manipulated MMP-9 promoter constructs, pGL3/cMMP-9(5NF-κB), (3NF-κB) and (1NF-κB), were analysed using the SW1353 cells (Figure 6-12). The addition of five NF-κB sites significantly increased both basal and cytokine (IL-1β and TNFα) induced promoter activity suggesting that disease-specificty had been lost. The addition of one NF-κB site did not increase promoter activity at the basal or induced levels, in fact promoter activity appeared to have decreased, the reasons for this are not apparent and further studies need to be performed. The addition of three NF-κB sites appeared to be optimal with a significant increase in promoter activity in response to TNFα and IL-1β while basal levels remained unaffected, indicating that disease specificity had been maintained. The differences observed in promoter activity observed after the addition of five, three and one NF-κB sites into the 5′ end of the MMP-9 promoter is difficult to explain. One would think that the effect of adding NF-κB sites into a promoter sequence would be similar irrespective of number, but there are obviously complex transcriptional changes occurring that are closely associated with the number of these additional sites. It may be explained by the presence of the AP-1 site (-1888 to –1877) located 6 bp downstream of the 5′ end of the MMP-9 promoter. The binding of the NF-κB transcription factor to one extra NF-κB site may interfere with the binding of transcription factors to this proximal AP-1 site required for promoter activity. However, the addition of three and five extra NFκB sites may enable the NFκB transcription factor to bind further up-stream without interfering with the AP-1 binding. In addition to this, there may be conformational changes in the DNA that are dependent on the number and type of motifs present within the sequence which not only alters the efficiency of the transcription factor binding but may also directly influence other sites present in the adjacent sequence. This may enhance transcription in the case of five and three additional sites or repress activity in the presence of just one site. Finally, there is an obvious discrepancy in the IL-1 response between experiments; this may be explained by the slight variations between batches of IL-1β

bought from the manufacturer and the stability of the recombinant protein after a number of freeze thaw cycles.

6.5.2 Can the mouse collagen type XI promoter be manipulated to enhance activity while maintaining cell-type specificity?

6.5.2.1 Analysis of endogenous transcription of collagen type XI genes in SW1353 cells and ATDC5 cells

Endogenous levels of collagen type XI gene transcription in the two cell lines, SW1353 and ATDC5, were analysed using relative semi-quantitative RT-PCR. These cell lines were chosen since chondrosarcoma cells had previously been shown to the express the collagen type XI gene whilst the undifferentiated ATDC5 cells have not (Liu et al., 2000).

Basal levels of endogenous transcription were evident in the differentiated SW1353 cells (Figure 6-17) as expected. However, the level of basal transcription could not be enhanced by the addition of TNFα or IL-1β. In contrast, studies have shown that TNFα can up-regulate collagen gene expression such as the collagen type II in chondrosarcoma cells (Hattori et al., 2001), while other groups have reported a TNF-induced decrease in the synthesis of the components of the ECM (Enomoto et al., 1990). More importantly, it was clear that the undifferentiated cells (ATDC5) were also capable of collagen type XI transcription, contradicting reports that collagen type XI was not expressed in this cell line (Liu et al., 2000). Since these authors had not analysed the endogenous expression of the gene in the ATDC5 cells but had shown insignificant levels of collagen XI promoter gene expression from a transfected construct it was decided to continue with this cell line using the cloned collagen type XI promoter constructs. It was hoped that there would be a large enough difference in the degree of gene expression between the SW1353 and ATDC5 cell lines to enable the cell type specific analysis of the mouse collagen type XI promoter fragments.

6.5.2.2 Analysis of all cloned mouse collagen type XI promoter fragments using luciferase reporters in SW1353 cells and ATDC5 cells

The mouse collagen type XI promoter construct was transiently transfected into the SW1353 and ATDC5 cells in an attempt to show cell-type specificity. Basal levels of promoter activity were normalised with the negative control for each cell line and then directly compared (Figure 6-19). Activity of the collagen type XI promoter construct was evident in both SW1353 and ATDC5 cell types, which is in concordance with the endogenous data, however the promoter had significantly more activity in the human SW1353 cells. This suggested that the collagen type XI promoter contained cell-type specific binding elements within its sequence enabling higher levels of promoter activity in the differentiated chondrocytes. These results are in agreement with a series of transfection studies demonstrating that the collagen type XI promoter had comparable activity in similar cell lines (Liu et al., 2000). However, DNA sequences containing minimal promoter

elements may serve as a promoter if placed upstream of a gene containing all the necessary elements required for transcription. In gDNA, cell type specificity is not only determined by the primary structure of a gene, such as the promoter and enhancer sequences, but also by the secondary and tertiary structural elements in the region of that gene. The 3-dimensional folded arrangement of the DNA in combination with the presence of histone acetylation and DNA methylation (Ng and Bird, 1999) maintains cell-type specificity of a promoter by regulating the binding of transcription factors and thus to the recruitment of RNA polymerase to the transcription initiation site. As such it is very difficult to attain a high level of cell type specific regulation when evaluating the level of gene transcription from a promoter positioned in a plasmid vector which contains minimal secondary and tertiary structure. In this situation, the promoter under analysis is easily accessible to the transcriptional machinery of the cell and therefore will not necessarily reflect the action of the promoter *in vivo*.

The size of the promoter fragment under analysis is also important for cell type specificity. In this study a relatively small portion of the mouse collagen type XI promoter (1.2 kb) was analysed. Although studies have shown this region to contain cartilage specific elements (Tsumaki et al., 1998), such elements are often located hundreds of kilo bases upstream of the transcription start site. For example an up-stream repressor site in the mouse MMP-9 promoter has been identified and shown to regulate basal levels of promoter activity in a tissue specific manner (Roach et al., 1998). However, until vector technology advances for example with the development of mammalian artificial chromosomes which can deliver the entire genomic fragment containing the promoter and other elements, it is necessary to try and design the best cell-type targeting vector possible within the constraints of available DNA plasmid vectors. To this end, the collagen type XI promoter sequence was manipulated with chondrocyte specific enhancers (SOX9 sites), in an attempt to incorporate enhanced promoter activity and maintain cell type specificity.

The first attempt to modify the collagen type XI promoter involved the introduction of three and five SOX9 sites into the 5′ end of the promoter sequence as described for the manipulation of the canine MMP-9 promoter with NF-κB sites. The two constructs pGL3/mColXI(3SOX9) and (5SOX9) were transiently transfected into the SW1353 and ATDC5 cell lines as described before and compared to the unmanipulated pGL3/mCOLXI construct (Figure 6-19). Although activities of the two manipulated promoter constructs were again significantly higher in the SW1353 cells compared to the ATDC5 cells the addition of SOX9 sites did not enhance activity in either cell line (Figure 6-19). This contradicted evidence that SOX9 proteins bind to the collagen type XI gene to up-regulate promoter activity in chondrocytes (Lefebvre et al., 1997). However, further studies to investigate the role of SOX9 in cartilage specific expression identified enhancer sites in the first intron of the collagen type XI gene (Liu et al., 2000). This suggested that SOX9 sites downstream of the transcription start site were important for enhancing chondrocyte specific expression of the gene. As a result it was decided to clone a 300 bp region of the mouse collagen type XI intron 1, containing two SOX9 sites within its sequence, and flanking it with a further six SOX9 sites. The manipulated intron was then cloned down-stream of the luciferase reporter gene into the

pGL3/mCOLXI vector luciferase reporter construct. The modified vector, pGL3/mColXI[3SOX9(intron)3SOX9], was transiently transfected into the SW1353 and ATDC5 cell lines and compared to the unmanipulated mColXI construct (Figure 6-20). Activity of the modified construct was again significantly higher in the SW1353 cells compared to the ATDC5 cells. However, this time the incorporation of the intronic sequence, containing flanking SOX9 sites, into the promoter sequence significantly enhanced the mouse collagen type XI promoter activity in the SW1353 cell line while maintaining low levels in the ATDC5 cells. This is in agreement with studies showing that SOX9 expression is considerably higher in chondrosarcoma cells than undifferentiated ATDC5 cells (Morishita et al., 2001).

6.5.3 Can a dual-targeting vector system be developed to restrict therapeutic gene expression to diseased cell types of the canine osteoarthritic joint?

Once the disease and cell-type specific transcriptional control systems had been evaluated separately, attempts were made to incorporate both components into one system using Cre-lox technology for the development of a dual-targeting vector.

6.5.3.1 Construction of the dual targeting vector

Technical problems were associated with the construction of the final vector (Vector D). The clones selected from the final ligation step contained a mixture of high and low copy number plasmids. The low copy number plasmids were screened with *Hind* III for the presence of the insert and then sequenced; however all constructs contained the final insert in reverse orientation. This suggested that on correct orientation of the insert, the DNA sequence would encode a toxic gene. This was not likely since the final insert containing the intronic sequence flanked by SOX9 enhancer sites had already been successfully cloned into the pGL3/mColXI construct. It was therefore suggested that on ligation of the insert into the vector, in the correct orientation, the presence of Cre recombinase protein within the bacterial cells used for the transformation step, may be responsible for recombining Vector D. This would result in the removal of the collagen promoter and Cre gene from the vector with the joining of the two *loxH* sites (leaving one site behind) to produce the high copy number plasmid corresponding to the unique vector pGL3MMP-9(3NF-κB)*lox*H. A selection of the high copy number plasmids was therefore sequenced from both the 5′ and 3′ ends of the region using vector primers (RV3 and GL2). The forward primer (RV3) confirmed that the new construct contained the manipulated MMP-9(3NF-κB) promoter fragment. However, the reverse primer (GL2) would not generate any sequence data suggesting that unpredictable recombination events may have occurred between constructs and possibly with the gDNA. After several attempts at sequencing this region it was decided to simply confirm the presence of Cre protein in the cells used for transformation, using Western blot analysis. However, it was not known whether the

endogenous expression of Cre protein or expression from the vector under construction was responsible for the recombination event.

6.5.3.2 Western blot analysis of Cre recombination during construction

Western blot analysis for Cre gene expression was subsequently investigated in TOP10 bacterial cells alone and cells transfected with Vector C and pGL3-Basic. A band corresponding to a protein of 38 kDa was observed in all samples suggesting the endogenous expression of the Cre gene in the TOP10 cells was responsible for the problems associated with the cloning procedure. A web-based search led to the discovery of a cell line (*PIR1, Invitrogen) with gDNA containing a mutation in the sequence encoding the Cre gene, for use in the Echo™ Cloning System (Invitrogen). The genotype of the cells used for this procedure has the cysteine at position 510 in the Cre gene mutated to prevent the formation of functional Cre enzyme. However, this Western blot can not rule out expression of the Cre gene from the plasmid vector which may also occur.

6.5.4 Future direction for the dual targeting vector

To enable the construction of the dual targeting vector new technology needs to be developed. If endogenous Cre gene expression is responsible for the recombination event then the use of the Cre knockout (PIR′) cells will enable the final construction of the vector. However, if the Cre gene is also expressed from the plasmid, complex cloning techniques will be required. One method would be to develop a cell-free cloning system. To date, there is no efficient method for amplifying DNA without the use of bacterial cells although a combination of the Site Directed Mutagenesis kit (Stratgene) and the Amplitaq (Amersham) offers a potentially unique method. Two complementary primers (with 5′ phosphate modification) can be designed, based on the overlapping sequence between the insert and vector to eliminate all incorrectly orientated inserts. PCR can then be performed using a high fidelity *Pfx* polymerase and the ligation sample as a template to selectively amplify correct vectors. The linearised DNA can then be ligated to form circular vectors to be used as template in the Amplitaq rolling circle protocol. Since this results in linearised concatamers of the vector sequence the DNA must then be digested using a single cutting restriction enzyme, ensuring that the recognition sequence is located in an unimportant region of the vector. The vector must then be dephosphorylated (to prevent the concatameric DNA reforming) and finally ligated with small filler fragments also digested with the same restriction enzyme. Although this technique may be theoretically possible, it was attempted and found to be technically impossible.

An easier alternative may be to re-design the vector incorporating regions of DNA that prevent Cre expression from the vector in bacterial cells without hindering expression in mammalian cells. One way in which this can be done is to clone the binding region for LacI protein (seven base pairs) into the immediate 5′ region of the Cre recombinase gene sequence. On transformation into bacterial cells, which specifically express high levels of the LacI protein from a plasmid vector, the

* PIR1:F' Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA (ΔMlu I)::pir-116

LacI protein will bind to its recognition site and prevent the recruited RNA polymerase from reaching the 5′ end of the Cre gene thus blocking transcription. However, this system must 100% efficient at blocking Cre transcription since the expression of a single molecule of Cre the enzyme will prevent cloning.

Even if the problems associated with the cloning of the dual targeting vector are solved there are still several concerns with the ultimate application of such a vector system *in vivo*. Cre recombinase is a bacterial protein and although on expression in mammalian cells it will be directed to the nucleus small protein fragments will be expressed in the major histocompatibility complex (MHC) of the cell. As such the expression of this protein will be immunogenic and therefore abrogate all potentially therapeutic properties of the delivered therapeutic gene. However, this problem may ultimately be circumvented with the identification and application of mammalian species-specific recombination systems. For example mature B lymphocytes are able to specifically alter their Ig isotype expression in response to extra-cellular stimuli through a regulated recombination process called isotype switch recombination. An endonuclease, EndoSR (32 kDa) has been shown to preferentially cleave at highly repetitive, G-rich switch regions containing variable numbers of pentameric motifs TGGGN and TGAGC located directly upstream of the immunoglobulin heavy-chain constant region genes (Lyon et al., 1996; Lyon and Aguilera, 1997). However, further research is necessary to characterise these mammalian enzymes before they can be applied to such a targeting system.

6.6 SUMMARY

This chapter describes the modification of the canine MMP-9 promoter with multiple NF-κB sites to enhance activity while maintaining disease specificity. This was guided by a series of promoter deletion constructs, pGL3/MMP-9(984), (628), (534), and mutagenesis using human chondrosarcoma cells (SW1353). A second level of cell type specific targeting was investigated using the mouse collagen type XI promoter to restrict gene expression to chondrocytes. Activity of this promoter was enhanced while maintaining cell type specificity by the incorporation of intronic sequence containing flanking SOX9 enhancer sites. Finally, attempts were made to incorporate the two promoter systems into one vector utilising Cre-*lox* technology. Both disease and cell type specific promoters can be utilised individually to target therapeutic gene expression to diseased cell types of the joint. However, incorporating the two promoter systems into one vector is not possible at present but may be a possibility in the future with advances in molecular biology.

Chapter VII

Delivery of genes to synovial cells using particle-mediated bombardment transfection techniques

7.1 ABSTRACT

The introduction of DNA into target cells both *in vitro* and *in vivo* is a challenge in the development of efficient gene-based therapeutics. Therefore many chemical, biological and physical transfection techniques methods have been developed. Although these delivery systems are routinely used for the study of gene regulation and function, problems associated with cell type specificity, transfection efficiency and safety have encouraged the development of new transfer technologies. A relatively new physical approach for mammalian gene transfer is particle mediated bombardment using a gene gun where microscopic particles, coated with the gene of interest, are accelerated into cells by a motive force such as helium pressure discharge. This chapter evaluates the use of the gene gun (BioRad) for the *in vitro* delivery of DNA to cells in tissue culture. Two reporter genes, β-Galactosidase (β-Gal) and Enhanced Green Fluorescent Protein (EGFP), were delivered to Madin-Darby canine kidney (MDCK) cells and rabbit synovial fibroblasts (HIG-82) respectively using gold particle (1.0 μm) bombardment to evaluate transfection efficiency at different helium pressures (100 and 150 psi). Transfection of both cell types was evident at these pressures despite some cell death. This chapter also describes the *in vitro* delivery of gold particles into synovial tissue samples tissues taken from a freshly euthanatised dog at *post-mortem* examination (PME) to evaluate the penetration depth of gold particles (1.0 μm) at helium pressures of 250 and 500 psi. Darkfield microscopy, used to evaluate histopathologic synovial specimens, demonstrated that particle-mediated bombardment could potentially be used to transfect the lining cells of the synovial membrane.

7.2 INTRODUCTION

7.2.1 Gene delivery systems

Different methods of gene delivery are currently being used to transfer and express therapeutic DNA in many types of mammalian cells. These include agents that deliver DNA by chemical means and include calcium phosphate (Wigler et al., 1977), DEAE dextran (Ishikawa and Homcy, 1992), polylysine conjugates (Wu and Wu, 1987; Wagner et al., 1990), polybrene-dimethyl sulfoxide (Kawai and Nishizawa, 1984) and liposmes (Strauss et al., 1996). Another method uses biological agents such as viral vectors to deliver DNA (Robbins and Ghivizzani, 1998) and finally there are agents that deliver DNA by physical means including microinjection (Capecchi, 1980), electroporation (Lurquin et al., 1997) and particle bombardment (Yang et al., 1990).

7.2.1.1 Liposome-mediated gene delivery

The liposome-mediated gene delivery system has been of particular use in the efficient transfer of genes *in vitro*, and depending on the cell type, can result in higher transfection rates than calcium phosphate or DAEA-dextran (Felgner et al., 1987). Cationic liposomes form complexes with negatively charged molecules. Since DNA is highly negatively charged it can be held within the positively charged liposomes, to make a compact and stable complex, small enough for cellular entry (Kabanov and Kabanov 1995; Labat-Moleur et al., 1996). The liposome-DNA complex has been used for both transient and stable transfections of various cell lines. These include T lymphoblastoid and macrophage cell lines in suspension culture (Dorman and Yong, 1989), primary cultures of rat pituitary cells (Maurer, 1989), pancreatic islet cells (Welsh et al., 1990;) fibroblasts and hepatocytes (Legendre and Szoka, 1992). Liposomes have also been used to deliver RNA (Malone et al., 1989) and proteins to cells (Debs et al., 1990). However, the significant toxicities observed in various cells has limited its use as a general carrier for DNA *in vivo* (Felgner et al., 1987).

7.2.1.2 Viral vector mediated gene delivery

Viral vectors, which often give a high level of transduction have received a considerable amount of interest for gene-based therapeutics. Details of this biological delivery system are described in chapter I.

7.2.1.2 Particle bombardment mediated gene delivery

Particle bombardment has many technical advantages over standard gene transfer methods including the delivery of a high concentration of large DNA molecules such as cosmids or MACs to individual cells (Yang, 1992). Also, since DNA-coated micro-projectiles can penetrate multiple cell layers of a target tissue, particle bombardment may enhance the simultaneous use of other gene transfer methods. The creation of millions of microscopic paths through the tissue will enhance the deeper penetration of a transducing virus or lipofectin methods used concurrently (Yang et al.,

1994). In addition, since the gene gun uses inert gold particles to physically carry the DNA into the cell, the culture is not contaminated with bioactive substances such as calcium phosphate ($CaPO_4$), lipids or resins. Furthermore, particle mediated gene transfer is essentially instantaneous.

Particle bombardment (Biolistic transfection) is a physical method of cell transformation in which high density, sub-cellular-sized particles are accelerated to high velocity to carry DNA into cells. This gene delivery system does not depend on specific ligand receptors and/or biochemical features of the structural components present on the surface of cells and can be applied in a variety of biological systems for both *in vitro* and *in vivo* transfection of recombinant genes. Biolistic transfection methods were originally developed to introduce genetic material into plants, whose walls presented a physical barrier to conventional techniques (Klein et al., 1987). However, they have since been used to transfect bacteria (Smith et al., 1992), yeast (Johnston et al., 1988), fungi (Armaleo et al 1990) and are now available for many mammalian experimental systems.

7.2.1.2.1 *In vitro and ex vivo* gene transfer

Particle bombardment is suited for the introduction of recombinant genes into many cell types, but is particularly useful for those that are difficult to transfect by conventional means. Particle bombardment has been successfully used to transfect primary cultures of macrophages (Burkholder et al., 1993), dorsal root ganglion cells (Usachev et al., 2000) and foetal rat brain cells (Jiao et al 1993). Particle bombardment has also been successfully used to transfect organotypic tissue explants such as the cerebellum (Arnold et al., 1997), hippocampus and hypothalamus (Thomas et al., 1998), mammary ductal organoids (Yang et al., 1990), liver and kidney tissues (Zelenin et al., 1991). However, there is limited information concerning the use of the gene gun for *in vitro* delivery to the tissue types, specific for the joint.

7.2.1.2.2 *In vivo* and *in situ* gene transfer

Particle bombardment has also been used successfully for *in situ* and *in vivo* gene transfer in a variety of mammalian species (Williams et al., 1991). In vertebrates, the accessible epidermal cells of the skin are the most obvious targets for particle bombardment with *in vivo* experimental systems targeting this tissue type for vaccination studies (Tang et al., 1992; Fynan et al., 1993; Eisenbraun et al., 1993). In addition to skin, muscle and internal organs such as the liver, pancreas, spleen, kidney and central nervous system (CNS) have also be targeted *in vivo* after appropriate surgical exposure (Yang et al., 1990, Williams et al., 1991; Cheng et al., 1993; Zhang and Selzer, 2001). Particle bombardment has also been used to transfer genes encoding IL-6, TNFα, IFNγ, IL-2 and IL-12 into neoplastic tissues for the regression of tumours in mice (Sun et al., 1995; Rakhmilevich et al., 1996). Reporter genes, β-galactosidase and luciferase, and granulocyte macrophage-colony stimulating factor have also been successfully delivered to the oral mucosa and epidermis of dogs (Keller et al., 1996). However, there is limited literature reporting the use of the gene gun for *in vivo* delivery to tissue types of the joint.

7.2.1.2.3 Mechanical and functional parameters of particle bombardment

In all systems of particle bombardment microscopic particles are coated with the gene of interest and accelerated by a motive force to penetrate cells and deliver the DNA coated particles (Sanford, 1988). The motive force can be generated by a high-voltage electrical discharge (McCabe et al., 1988) or helium pressure discharge (Williams et al., 1991). In either of these systems the parameters for efficient gene delivery can be modified to enhance expression in specific tissues or cell types (Yang et al., 1994). The first important parameter is the particle acceleration rate that is varied by the discharge voltage or pressure to give optimal penetration of monolayer cells or multi-layered tissues and organs. A low particle acceleration rate is generally sufficient to transfect samples in tissue culture (Yang et al., 1990); a higher acceleration rate is usually required for optimal particle bombardment of somatic tissues *in vivo* (Cheng et al., 1993). The material used for this microprojectile system is another very important parameter. Gold has been selected for mammalian gene transfer due to its inert and non-toxic nature; its high density permits greater momentum and deeper penetration. A range of gold particles is commercially available for particle bombardment ranging from 0.6 to 15 μm diameter. The 1 μm particles are suitable for most cell culture studies where 1-3 μm sized particles are generally used for *in vivo* somatic tissue delivery. The larger beads can be used when much deeper penetration is required but greater destruction is observed which, thus limits their use (Yang et al., 1994). The gold particle loading rate also has to be adjusted to optimise cell transfection efficiency which will vary between cell types. However, cells bombarded with an optimised concentration of particles generally results in the sealing of cell membranes in ten minutes. Greater than 90% of adherent tissue culture cells are viable after this period, when optimised concentrations are used; whereas a higher gold particle density causes extensive cell and tissue damage (Jiao et al., 1993).

7.2.1.2.4 Limitations of particle bombardment

As an emerging technology, the gene gun method of gene transfer has certain technical limitations. Unlike integrating viral vectors, *in vivo* gene transfer to most somatic tissues using particle bombardment results in only transient expression lasting a few days to two weeks this probably results from the lack of stable integration into the bombarded target cells. However, strategies for long-term transgene expression may include the repeated bombardment of tissues to provide a continuous therapeutic level of transgene expression or combining particle bombardment with artificial chromosome technology to maintain stable expression levels for adequate periods of time (Yang et al., 1994). Another current limitation of particle bombardment for *in vivo* use is the low level of transgene expression despite deep tissue penetration. This is thought to be related to the high levels of cell damage observed in bombarded tissues (Yang et al., 1994). However, modification of particle forms, acceleration hardware designs and the optimisation of bombardment parameters are continually under revision to reduce this problem. Safety is also a key issue when using this technique and the fate of the gold particles will have to be thoroughly addressed before particle bombardment is used in clinical practice.

7.2.2 Therapeutic gene delivery to cell types of the joint

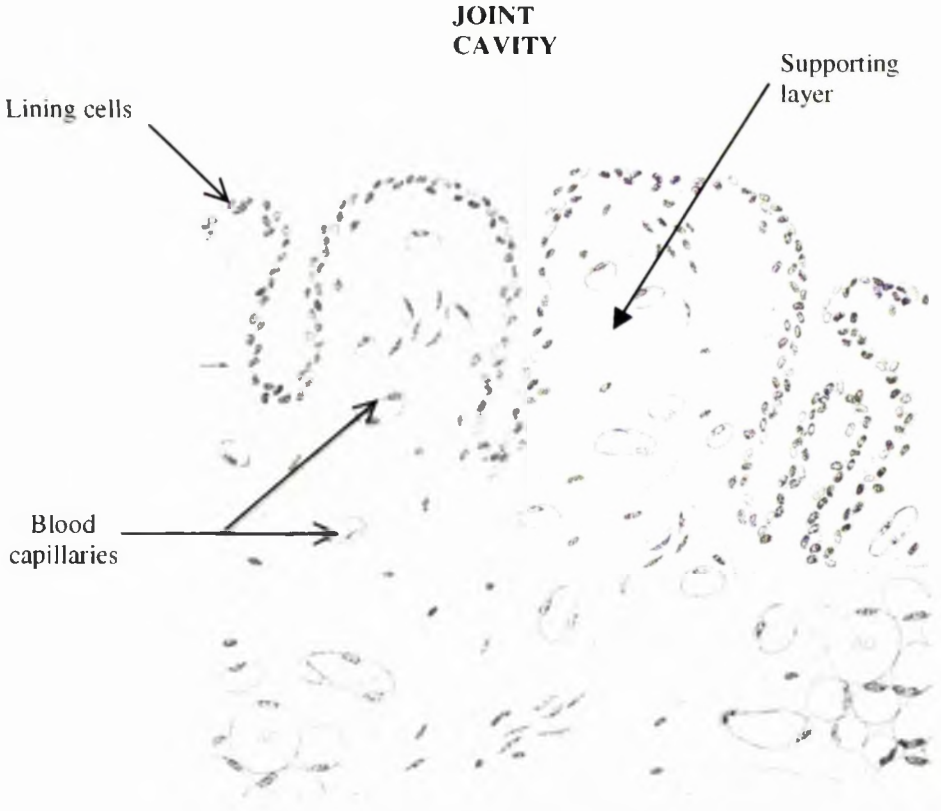
Delivery of therapeutic genes to tissue-types specific for the joint using intravenous, intramuscular and oral routes of delivery is not only inefficient but results in the undesirable systemic exposure to the therapeutic agents. An intra-articular approach however, provides higher levels of therapeutic gene transfection in target cells within the localised joint compartment without exposing other tissues and is therefore the technique generally used for gene transfer to the joint.

Hyaline cartilage is the tissue primarily involved in the osteoarthritic processes and would be the obvious tissue to target in gene based therapeutics. However, hyaline cartilage consists of a dense extra-cellular matrix (ECM) containing type II collagen and proteoglycan which may be responsible for the low transfection efficiency of chondrocytes. Furthermore, despite the presence of a few immature chondrocytes at the periphery of hyaline cartilage most are located deep in the tissue appearing in groups where they are difficult to access for efficient transfection using *in vivo* methods of gene delivery. However, one study has demonstrated that the IL-1Ra gene can be transduced directly *in vivo* using non-viral plasmid technology for delivery to chondrocytes to reduce progression of experimental osteoarthritis (Fernandes et al., 1999). Although articular chondrocytes can be transfected in tissue culture using adenoviral vectors (Brower-Toland et al., 2001) there are significant difficulties in using these cells to re-surface hyaline cartilage (Brittberg et al., 1994) which currently excludes this for *ex-vivo* gene delivery technique.

Synovial tissue however can be easily targeted for transfection using both *in vivo* (direct) and *in vitro* (indirect) delivery systems. The synovium is particularly applicable in the direct approach since the synovium lacks a basement membrane and therefore presents no physical or chemical barrier between itself and the joint space. Any type of particle introduced into the joint is quickly captured by the synovium which consists of loose a connective tissue into which particles may penetrate freely (Figure 7-1). The synovium lines all the non-cartilaginous internal surfaces of the joint and therefore possesses a huge surface area for gene transfer. Viral vectors, in particular retroviruses, have been used for this approach even though they require cell division for infection and integration. The normally quiescent synovial lining is induced by inflammation in OA and shows hyperplasia, encouraging transduction by viruses *in situ*. This has been demonstrated with the transduction of synovial cells *in vivo* by the intra-articular injection of retroviral vectors containing the human IL-1Ra to reduce the progression of experimental OA in the dog (Pelletier et al., 1997). The indirect approach is also possible since the type B synovial fibroblasts readily divide in culture and can be easily transduced before re-introduction into the joint. Intra-articular injection of suspensions of these synoviocytes should result in the attachment and maintenance of transplanted cells in the host synovial lining since synovial fibroblasts appear to possess specific homing receptors that help restrict them to the synovial lining (Galea-lauri et al., 1991). The *ex vivo* transfer of human IL-1Ra genes has been shown to suppress collagen-induced arthritis in mice (Bakker et al, 1997) and this approach has been recently used in a human clinical trial (Evans et al., 1996).

Figure 7-1 Histological structure of the synovial membrane

The synovial layer is arranged in folds that occasionally penetrate into the interior of the articular cavity. The internal surface of the synovial membrane is lined by a layer of squamous or cuboidal cells with a layer of loose or dense connective tissue underneath. The lining cells of the synovial membrane are separated from each other by a small amount of connective tissue ground substance. The synovium originates from the mesenchye and thus there is no basal lamina between the lining cells and the underlying connective tissue and the entire tissue is rich in blood capillaries with variable amounts of adipose tissue (AD). The supporting layer may consist of areolar, fibrous and adipose connective tissue or a mixture of all three.

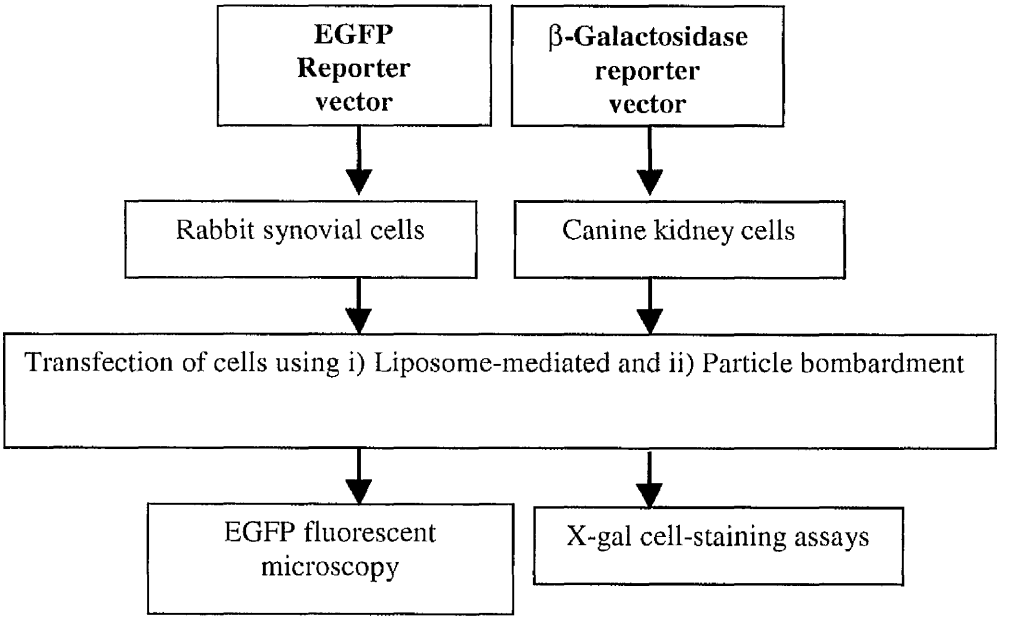


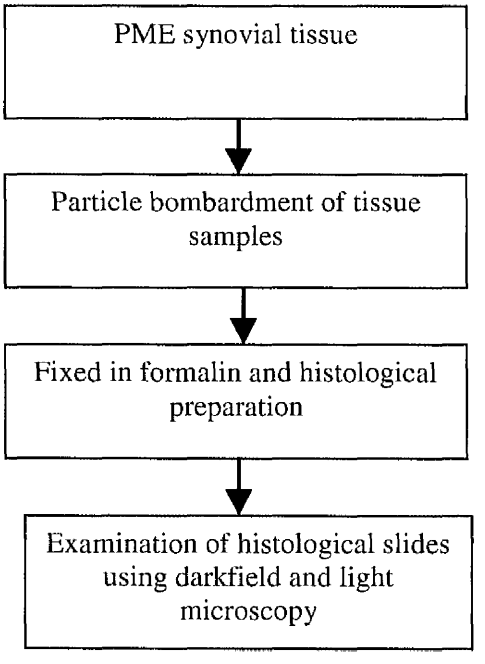
7.3 MATERIALS AND METHODS

Overviews of the experimental procedures employed in this chapter are illustrated in figure 7-2. Particle mediated transfection, using the gene gun (BioRad) apparatus, was evaluated for gene transfer and gold penetration into the synovial tissues of the joint. Briefly, *in vitro* tissue culture studies were performed to analyse the transfection ability of the gene gun. Vectors containing reporter genes, Enhanced Green Fluorescent protein (EGFP) and β-galactosidase (β-Gal), were transiently transfected into rabbit synovial (HIG-82) and Madin Darby canine kidney (MDCK) cells respectively using cationic liposome and gene gun methods. Fluorescence microscopy and X-gal staining assays were used to visualise transfected cells. Particle mediated bombardment was then evaluated for the penetration depth of gold particles into explant synovial tissues taken from a 10 year old dog at *post-mortem* examination (PME). Samples were fixed in formalin, stained with hematoxylin and eosin (H/E) and visualised using light microscopy to identify synovial tissue structure. Dark field microscopy was than used to identify the position of gold particles in the lining cells of the synovium.

Figure 7-2 Overview of experimental procedures for particle-mediated bombardment transfection of cells

The Enhanced Green Fluorescent protein (EGFP) and β-galactosidase (β-Gal) reporter vectors were transiently transfected into rabbit synovial (Hig-82) and Madin Darby canie kidney (MDCK) cells respectively using cationic liposome and gene gun methods. Fluorescence microscopy and X-gal staining assays were used to determine transfection efficiency. Particle bombardment of gold particles into synovial tissues was performed to determine penetration efficiency analysed by histopathological techniques.





7.3.1 Preparation of components for gene gun system

7.3.1.1 Preparation of cartridges

Bullets used in this study were made of Tefzel tubing coated with gold particles (1.0 μm) carrying plasmid DNA encoding Enhanced Green Fluorescent Protein (EGFP, Clontech) or β-galactosidase (pCMVβ, Clontech) and made following the manufacturer's instructions. Ethanol (100%), from a previously unopened bottle, was first divided into 10 ml aliquots. A fresh aliquot was opened each time cartridges were prepared since opened bottles of ethanol absorb water and may result in the production of poor quality cartridges during the drying step with streaking, clumping and uneven coating of micro-carriers over the inner surface of the Gold-Coat (Tefzel) tubing.

7.3.1.1.1 Precipitation of DNA onto micocarriers (gold particles)

A fresh stock solution of Polyvinylpyrrolidine (PVP), in ethanol (20 mg/ml) was prepared as an adhesive during the cartridge preparation. This PVP stock solution was diluted with ethanol to 0.1 mg/ml (3.5 ml of this dilute solution was required for each 30″ length of Gold-Coat tubing in the tubing Prep Station). The following procedure refers to a Micro-carrier Loading Quantity (MLQ) of 0.5 and a DNA Loading Ratio (DLR) of 2. Gold micro-carriers (25 mg of 0.1μm) were mixed with 100μl spermidine (0.05M), briefly vortexed, then sonicated for 3-5 seconds using an ultrasonic cleaner to break up gold clumps. DNA (50 μg) samples were added and the contents mixed thoroughly by vortexing for 5 seconds. While vortexing at a moderate rate on a variable speed vortexer, 100 μl of $CaCl_2$ was added drop-wise to the mixture to allow the DNA to associate with the gold micro-carriers. The mixture was incubated at room temperature for 10 minutes and flash spun to pellet the precipitate enabling the transparent supernatant to be discarded. The pellet was then re-suspended in the remaining supernatant by briefly vortexing and then washed three times with fresh 100% ethanol (1 ml), flash spinning between each wash and discarding all supernatants. After the final ethanol wash, the pellet was re-suspended in 200 μl of PVP/ethanol solution (0.1 mg/ml) and transferred to a polyproplene centrifuge tube (15 ml). The microfuge tube was washed with 200 μl of PVP/ethanol solution (0.1 mg/ml) and added to the same centrifuge tube to produce a DNA/micro-carrier solution corresponding to a MLQ of 0.5.

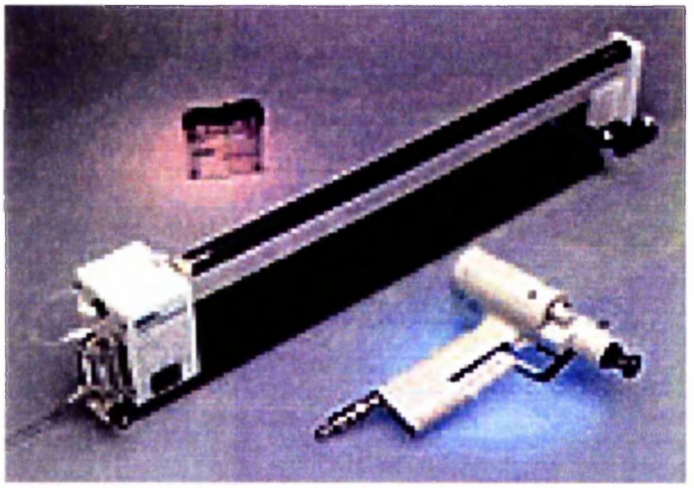
7.3.1.1.2 Loading the DNA/Micro-carrier suspension into Gold-Coat Tubing

The Tubing Prep Station, as shown in figure 7-3, was connected to the nitrogen tank and the Gold-Coat tubing was completely dried by purging with nitrogen (0.3-0.4 LPM) for at least 15 minutes. Once ready to prepare the cartridges, the tubing was removed from the Prep Station and the flow of nitrogen stopped. A 30″ length of dried Tefzel tubing was cut and one end inserted into the adaptor tubing fitted to the 10cc syringe. The micro-carrier suspension was briefly vortexed, sonicated and inverted several times to achieve an even suspension of gold. The micro-carrier suspension was immediately and quickly drawn into the Tefzel tubing for approximately 22-24″ after which the

tubing was withdrawn from the suspension while continuing to draw in air to leave some space at each end; care was taken to ensure that no bubbles were drawn into the tubing. The Gold-Coat tubing was immediately brought to a horizontal position and loaded into the Tubing Prep Station. The micro-carriers were allowed to settle for 3-5 minutes before the ethanol was removed using the 10 cc syringe at a rate of 0.5 to 1.0″/sec (taking approximately 40 seconds). A delay in the removal of the ethanol may cause the gold to adhere too strongly to the tubing and coat unevenly, however, it has been shown that a reduction in PVP concentration does not overcome this problem (O'Brien et al., 2001). The syringe was then detached from the tubing which was immediately rotated 180° to allow the gold to begin coating the inside surface of the tubing for 3-4 seconds. The Tubing prep Station was automatically rotated to allow the gold to evenly coat the inside of the tube for 25 seconds; nitrogen was then passed through the tube at 0.35-0.4 LPM for 3 to 5 minutes to dry the inside of the tubing while it continued to rotate. Once complete the tubing was removed from the Tubing Prep Station and examined to ensure that the micro-carriers were evenly distributed over the length of the tubing. Using scissors, sparsely coated regions were discarded and the remaining usable tubing was cut into cartridge sized portions (0.5″) using the Tubing Cutter. All cartridges were stored in a tightly sealed container, wrapped in parafilm and stored at 4°C.

Figure 7-3 The Tubing Prep Station

The Tubing Prep Station was connected to the nitrogen tank and the Gold-Coat tubing was completely dried by purging with nitrogen. The tubing was then removed from the Prep Station and the flow of nitrogen stopped before the tubing was loaded with the gold/DNA preparation and then reloaded into the Tubing Prep Station. The ethanol was removed and the tubing prep apparatus immediately rotated 180° before being allowed to automatically rotate with nitrogen passing through the tube.



7.3.1.2 Preparation of gene gun

The BioRad Gene Gun, as illustrated in figure 7-4 and 7-5, uses a pulse of helium fired through the apparatus to strip gold microcarriers coated with DNA from the inner surface of a cartridge constructed of Gold-Coat tubing. The accelerator channel fits against the cartridge carrying the gold/DNA microparticles with a tight seal produced by two 'O' rings. This tight seal directs the flow of helium down the channel towards the outer barrel. The expanding gas stream propels the particles forwards down the accelerator channel to the coned shaped outer barrel at the distal end. As the helium leaves this coned surface it follows the contour thereby dispersing the gold/DNA particles in a continuing outward motion that defines the extent of the target area. The standard configuration of the cone is optimised to project particles over a relatively wide area and the spacer that is attached distal to the cone barrel determines the radius of this area. This spacer also defines the minimum distance between the cone barrel and the target tissue. There is a decreased likelihood that gold/DNA particles will penetrate the target if the distance from the barrel to the tissue is too great (Wellmann et al., 1999), but if the distance is to short there will be an increased risk of tissue damage from the pressure wave. The performance of the Gene Gun in terms of target area, tissue penetration and tissue damage is determined by the balance between gas pressure, the area of dispersion over the target, and the proximity of the outer barrel to the tissue. The major feature that affects these factors is the profile of the accelerator channel, which causes the microcarriers to spread out from their original 2mm diameter of up to 1.5 cm at the target site (Yoshida et al., 1997).

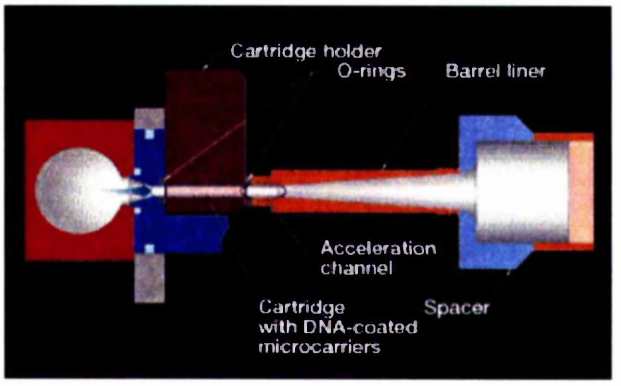
To activate the Helios Gene Gun an empty cartridge holder was inserted into the barrel liner, before connection to the helium source where the flow of helium into the gun was adjusted to the required setting (100-500 psi). A 70 µm nylon mesh was placed directly over the barrel to reduce cellular damage. Hearing and eye protection was then worn for the remainder of the procedure. The gene gun device was pointed away from any by-standers and the trigger depressed 2-3 times to discharge the device and equilibrate the pressure. The cartridge holder was then removed and replaced with a fully loaded cartridge. Six well plates were used for the cell culture as the diameter of the wells enabled the gene gun's plastic spacer to fit inside the well and reduce the distance between the end barrel and the cells/tissue samples.

Figure 7-4 The gene gun

The gene gun (BioRad) is considered a convenient, hand-held device that provides rapid and direct gene transfer into a range of targets both *in vitro* and *in vivo*. The unit uses an adjustable low-pressure helium pulse to sweep DNA-coated gold microcarriers from the inner wall of a small plastic cartridge directly into the target. Up to twelve DNA-coated gold cartridges can be loaded into the holder, which is inserted into the device and fired.

Figure 7-5 Mechanics of the gene gun

The BioRad gene gun produces a helium pulse to sweep the DNA-coated gold micro-carriers from the inside wall of the sample cartridge. The micro-carriers accelerate for maximum penetration as they move through the barrel, while the helium pulse diffuses outward. The accelerator channel fits against the cartridge with a tight seal produced by two 'O' rings. This tight seal directs the flow of helium down the channel towards the outer barrel and out of the coned surface. The standard configuration of the cone is optimised to project particles over a relatively wide area and the spacer that is attached distal to the cone barrel determines the radius of this area. The spacer maintains the optimal target distance for most applications and vents the helium gas away from the target to minimise cell surface impact.



7.3.2 Delivery of reporter plasmid vectors to cells in tissue culture using particle bombardment and liposome-mediated techniques

7.3.2.1 Cell lines and reagents

MDCK cells were maintained in Dulbecco's MEM with gluatamax-1 medium supplemented with 10% foetal calf serum (FCS) as described in section 2.2.1.2.1 Rabbit synovial fibroblasts (HIG-82) were maintained in Hams F-12 medium supplemented with 10% FCS as described in section 2.2.2.1.6. Both MDCK and HIG-82 cells were seeded onto 6 well plates at concentrations of $6x10^4$ cells/ml and incubated overnight at 37°C, 5% CO_2.

7.3.2.2 Reporter plasmid vectors

7.3.2.2.1 Beta-Galactosidase(β-Gal) reporter plasmid

The pCMVβ-Galactosidase DNA plasmid (Clontech) is a mammalian reporter vector designed to express beta-galactosidase in mammalian cells driven by the human cytomegalovirus immediate early gene promoter (MacGregor and Caskey, 1989). pCMVβ contains a polyadenylation signal from SV40 and the full length E.coli beta-galactosidase gene with eukaryotic translation initiation signals (MacGregor et al., 1987).

7.3.2.2.2 Enhanced Green Fluorescent Protein reporter plasmid (EGFP)

The Enhanced Green Fluorescent Protein (EGFP) mammalian reporter vector (Clontech) contains a unique EGFP variant which has a chromophore that is 35 times brighter than wild-type GFP (wt GFP). The EGFP vector encodes the GFPmut1 variant, consisting of the double substitution of the Phe-64 to Leucine and Serine 65 to Threonine. (Cormak et al., 1996). The EGFP has a single strong red-shifted excitation peak at 488 nm in filter sets used in fluorescence microscopy. This vector contains the kanamycin resistance gene.

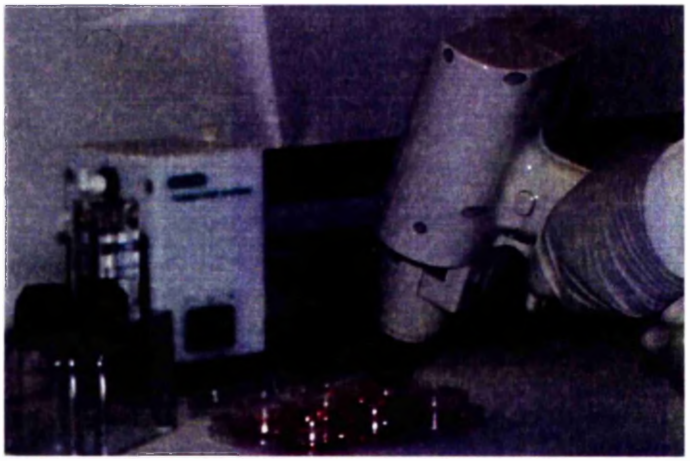
7.3.2.3 Transient transfection of tissue culture monolayer cells with reporter constructs

7.3.2.3.1 Particle bombardment method of transfection

The CMVβ-Galactosidase (Clontech) and Enhanced Green Fluorescent protein (EGFP) (Clontech) reporter plasmid vectors were transiently transfected into cells using the Helios Gene Gun system (Figure 7-6). After aspiration of the media, the gene gun was held perpendicular to the tissue culture plates with the plastic spacer held as close to the target as possible. The gene gun was then discharged at different pressures (100 and 150 psi) once (or twice at low pressures) before 5 ml of media was added to each well and the tissue culture plates were returned to the incubator for cell recovery at 37°C and 5% CO_2.

Figure 7-6: Transfection using the gene gun apparatus

After aspiration of the media, the gene gun was held perpendicular to tissue culture plates with the plastic spacer held as close to the target as possible. The gene gun was then discharged once, or twice at low helium pressures, before 5 ml of media was added to each well and the tissue culture plates returned to the incubator for cell recovery at 37°C and 5% CO_2.



7.3.2.3.2 Liposome-mediated transfection

The CMVβ-Galactosidase (Clontech) and the Enhanced Green Fluorescent protein (EGFP) (Clontech) reporter plasmid vectors were transiently transfected into cells using the cationic liposome mediated transfection system. TransFast™ Transfection Reagent (see 2.2.7). Briefly, transient transfections were carried out using DNA (2 μg per well) in a 1:1 ratio with the TransFast™ Reagent, in a total volume of 1 ml of serum free media per well. After one hour of incubation at 37°C cells were allowed to recover overnight in supplemented media and 48 hours later all cells were harvested.

7.3.2.4 Reporter Assays

7.3.2.4.1 X-gal staining assay

The X-Gal Staining Assay was used to detect *in situ* levels of β-galactosidase activity from cells transfected with plasmids expressing *lacZ*. *LacZ* is a bacterial gene used as a reporter construct in eukaryotic transfection experiments because the gene product, β-galactosidase is very stable, resistant to proteolysis in cellular lysates, and its activity is easily assayed. The X-gal staining system provides a rapid and simple method to analyse cells transfected with β-galactosidase-expressing plasmid. The β-galactosidase enzyme catalyses the hydrolysis of X-gal (5-bromo-3-indole-b-d-galactopyranoside), a β-galactoside. Following fixation and incubation with the X-gal substrate, transfected cells appeared blue and could be easily visualised by light microscopy.

More specifically, forty-eight hours post transfection the media was aspirated from the cells and washed in PBS (0.1M, pH. 7.4) before the cell fixing solution was added for 15 minutes at room temperature. The cells were then washed three times with PBS (0.1 M), each for five minutes, before being permeabilised for five minutes with 0.1% v/v Triton/PBS. The cells were washed for a second time in PBS (0.1 M) as before, the X-Gal assay solution was added to each well (just enough to cover the cells) and then incubated at 37°C for three hours. The cell monolayer was observed under a light microscope and blue cells observed. An empty plasmid vector was used as a negative control for determining the level of background activity caused by endogenous β-galactosidase activity. Photographs were taken for a permanent record of the results and then digitalised using Coolscan apparatus (Nikon)

7.3.2.4.2 Enhanced Green Fluorescent Protein (EGFP) Assay

Green fluorescent protein (GFP) was discovered as a companion protein to aequorin, a chemiluminescent protein from *Aequorea* jellyfish (Shimomura et al., 1962), the emission spectrum of which peaked at 508 nm (Johnson et al., 1962). However, the emission spectrum of purified aequorin was blue and peaked near 470 nm, the approximate value of one of the excitation peaks of GFP. These data implied that the GFP converted the blue light emitted from aequorin into green fluorescence in intact cells. The GFP chromophore was later identified (Shimomura et al., 1979)

and the GFP gene cloned (Prasher et al., 1992) and subsequently shown to retain its fluorescent properties when expressed in other organisms (Chalfie 1994).

Briefly, forty-eight hours post transfection, the cells (in media) were observed under fluorescent microscopy for GFP expression. The EGFP fluorescence was excited and collected through 480+/-10 and 540 +/- nm bandpass filters on a fluorescent microscope (Olympus 1x 70). Images were captured using Image Pro-plus software (assisted by Lynn Stevenson).

7.3.3 Delivery of gold particles to tissue samples taken at post mortem examination using particle bombardment

7.3.3.1 Sample preparation

A Labrador-cross dog, ten years of age, with no history of OA was humanely euthanatised for clinical reasons. At *post-mortem* examination (PME), both stifle joints appeared grossly normal and were dissected to remove synovium from both joints. Sections (approximately 0.5 cm^3) were immediately trimmed and placed into wells of a six-well tissue culture plate.

7.3.3.2 Particle bombardment of tissues

The plastic spacer of the gene gun was held as close to the target tissue as possible and the gene gun discharged at 250 and 500 psi. Samples were immediately placed in formalin (10%) for 24 hours for fixation and then sent for histopathological H/E staining. Untransfected cells were used as negative controls. Samples were analysed for gold particle penetration into tissue samples using darkfield microscopy (Zeiss A xloskop 2). This particular method of microscopy allowed the gold particles to reflect incident light and so become visible as bright granules (De Waele and Beesley, 1989). The same part of the section was observed in light microscopy and all images were digitally recorded using Zeiss KS 300 3-0 software.

7.4.1 Comparison of liposome-mediated and particle-bombardment-mediated transfection of monolayer cells in tissue culture

7.4.1.1 The β-galactosidase assay

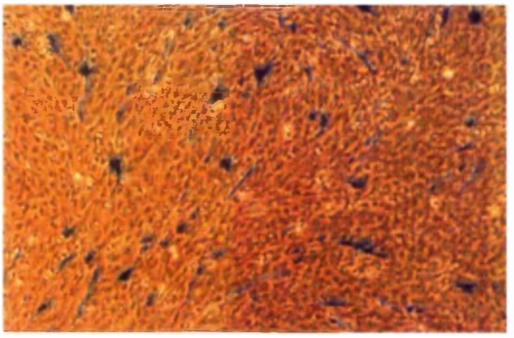
MDCK cells were transfected with the reporter vectors containing the β-galactosidase gene using both liposome-mediated and particle bombardment methods and then analysed using the X-gal staining assay to observe for the presence of blue-stained cells under light microscopy. The liposome-mediated method of transfection resulted in efficient transfection of cells, evenly distributed throughout the well, with little cell death (Figure 7-7). The particle bombardment method of transfection also resulted in the transfection of cells with both 100 psi (Figure 7-8a) and 150 psi (Figure 7-8b). In both particle-mediated deliveries the transfection distribution was uneven, determined by the position of the gene gun barrel during bombardment. There was some cell death in the centre where the main focus of the gene gun barrel had been positioned, providing the greatest helium pressure. Surrounding the small area of cell death a ring of efficiently transfected cells was evident. The higher the pressure, the greater the cell death but the larger the area of transfected cells. Untransfected cells did not stain blue with the X-gal stain (Figure 7-9).

7.4.1.1 The EGFP assay

Rabbit synovial fibroblast cells (HIG-82) were transfected with the reporter vector containing the EGFP gene using both liposome-mediated and particle bombardment methods then analysed using fluorescent microscopy to observe the presence of green fluorescent cells. The liposome-mediated method of transfection resulted in efficient transfection of cells, evenly distributed throughout the well, with little cell death (Figure 7-10). The particle bombardment method of transfection also resulted in the transfection of cells with both 100 psi (Figure 7-11a) and 150 psi (Figure 7-11b). Again, at both pressures the distribution of transfection was uneven, determined by the position of the gene gun barrel during bombardment. There was considerably more cell death observed with the rabbit fibroblasts as compared to the MDCK cells. Again, the cell death was present in the centre consistent with the main focus of the gene gun barrel. Surrounding the small area of cell death was a ring of efficiently transfected cells seen by a large number of fluorescent cells. The higher the pressure, the greater the cell death but the larger the area of transfected cells. Untransfected cells were not fluorescent (data not shown).

Figure 7-7: Liposome-mediated method of gene delivery

MDCK cells were transfected with the β-galactosidase reporter gene using liposome-mediated transfection methods. Light microscopy of X-gal stained cells revealed that the transfected cells were evenly distributed throughout the well, with little cell death

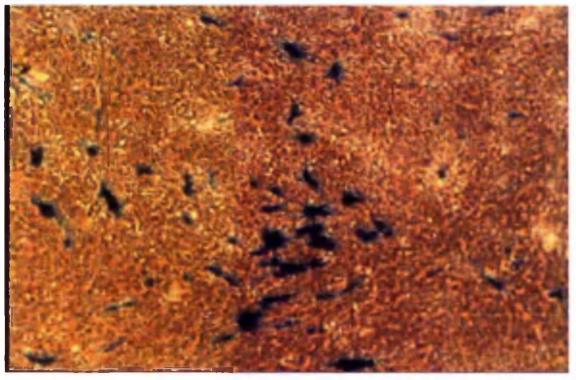


Magnification x10

Figure 7-8: Particle bombardment-mediated method of gene delivery

MDCK cells were transfected with the β-galactosidase reporter gene using a particle bombardment method of transfection at helium pressures of a) 100 and b) 150 psi. Light microscopy of X-gal stained cells revealed that transfection of cells was possible, however their distribution was uneven, with some evidence of cell death.

a)



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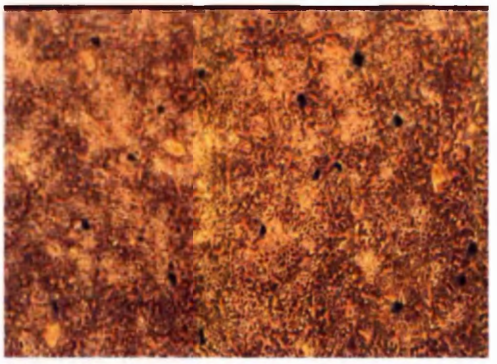
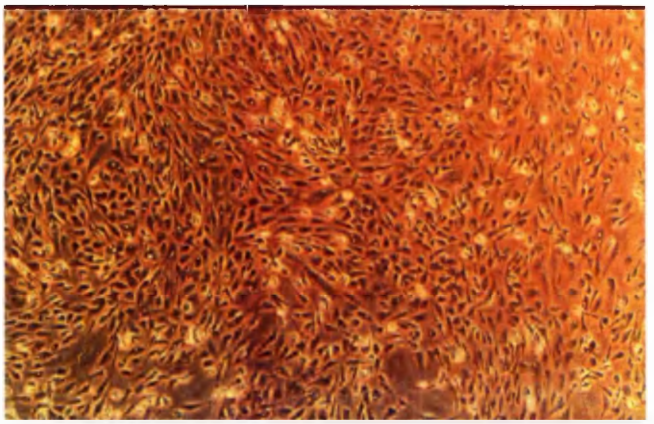
b)



Magnification x10

Figure 7-9: Untransfected MDCK cells

There was no evidence of blue cell staining in the untransfected samples



Magnification x10

Figure 7-10: Liposome-mediated gene delivery

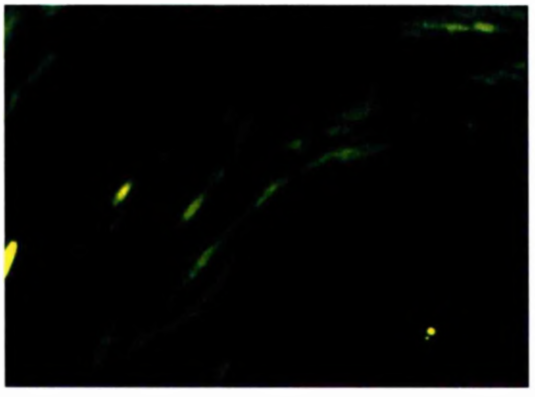
Rabbit synovial fibroblasts (HIG-82 cells) were transfected with the EGFP reporter vector using liposome-mediated delivery and analysed using fluorescent microscopy to observe the presence of green fluorescent cells. The liposome-mediated method of transfection resulted in efficient transfection of cells, evenly distributed throughout the well, with little cell death.

Magnification x20

Figure 7-11: Particle bombardment-mediated gene delivery

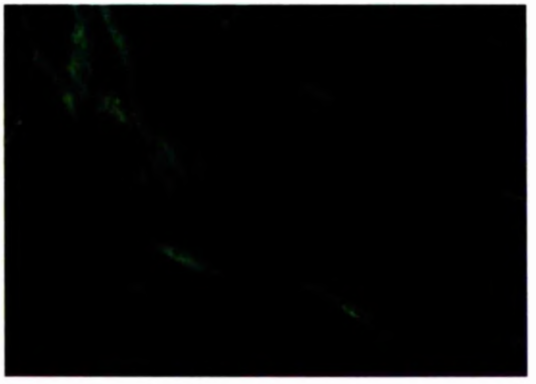
Rabbit synovial fibroblasts (HIG-82 cells) were transfected with the EGFP reporter vector using the particle bombardment method of transfection at helium pressures of a) 100 psi and b) 150 psi, and analysed using fluorescent microscopy. The particle-bombardment method of transfection resulted in transfection of cells although unevenly distributed throughout the well, with some cell death.

a)



Magnification x20

b)



Magnification x20

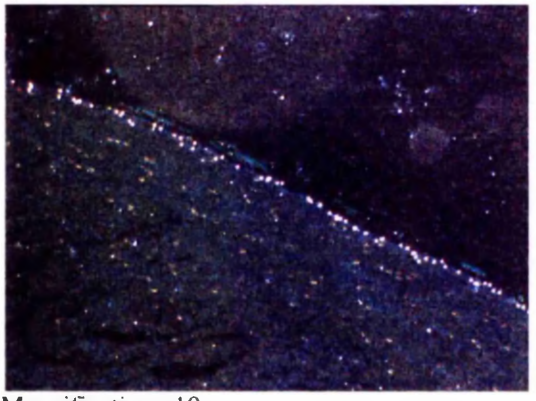
7.4.2 Gold particle delivery to cell types of the canine joint

The efficiency of gold particle bombardment was assessed using the Gene Gun system (BioRad) for penetration into canine synovial tissue dissected from the stifle joint. Using six-well tissue culture plates the plastic spacer of the gene gun was held as close to the target tissue as possible and the gene gun discharged at 250 and 500 psi before samples were immediately fixed in formalin and sent for histopathological H/E staining. Using darkfield microscopy samples were analysed for gold particle penetration into the tissue samples, the gold particles were easily visible through their ability to reflect light. All gold particles were distinguished from dust contaminants by their uniformly circular shape.

H/E stained synovial tissue samples were observed by light microscopy and divided into sub-types referred to as fibrous (Figures 7-12b, 13b, 14b), fibroareolar (Figure 7-15b, 16b) and adipose (Figure 7-17b) synovium. The same samples were then analysed by dark-field microscopy to identify the position of gold particles within the tissue structure. Gold particles were found to penetrate the lining cells of the fibrous synovium at helium pressures of 500 psi (Figure 7-12a) and 250 psi (Figure 7-13b). Gold particles could also penetrate the fibroareolar synovium at a helium pressure 250 psi (Figure 7-15a). Finally, gold particles could easily penetrate the adipose synovium at a pressure of 250 psi (Figure 7-17a). Untransfected tissues did not contain any gold particles (Figure 7-14a, 16a).

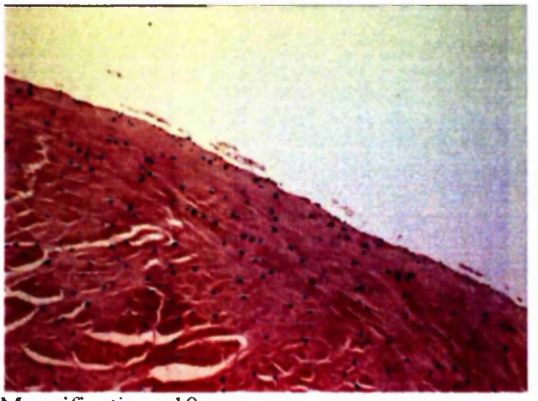
Figure 7-12: Gold particle penetration of canine fibrous synovium with particle bombardment pressures of 500 psi observed using a) darkfield and b) light microscopy (H&E)

a)



Magnifiaction x10

b)



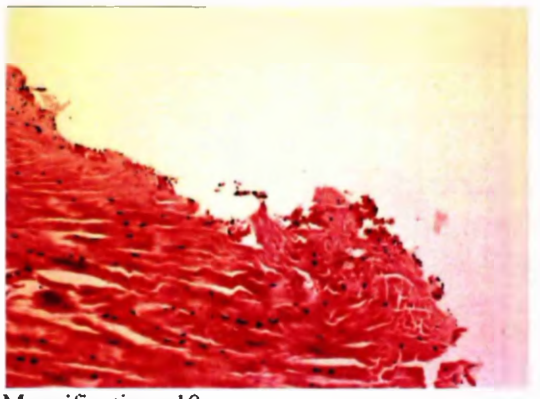
Magnification x10

Figure 7-13: Gold particle penetration of canine fibrous synovium tissue with particle bombardment pressure of 250 psi observed using a) darkfield and b) light microscopy (H&E)

a)

Magnification x10

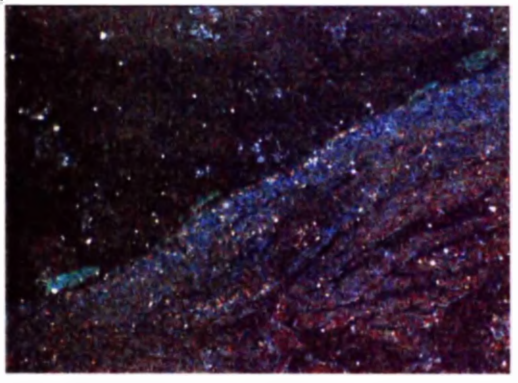
b)



Magnification x10

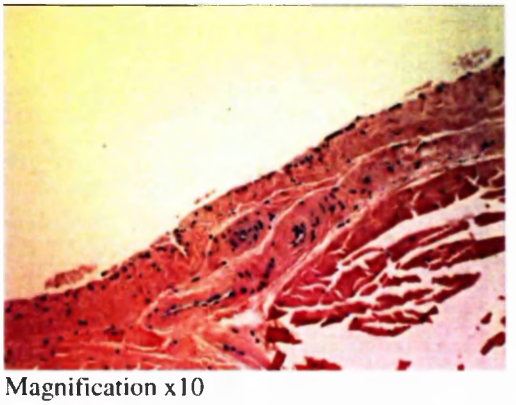
Figure 7-14: Untransfected canine fibrous synovium observed using a) darkfield and b) light microscopy (H&E)

a)



Magnification x10

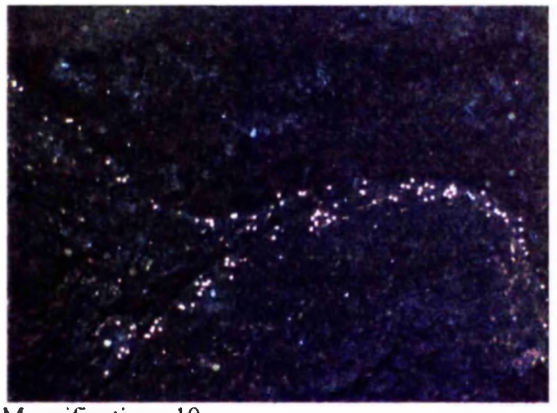
b)



Magnification x10

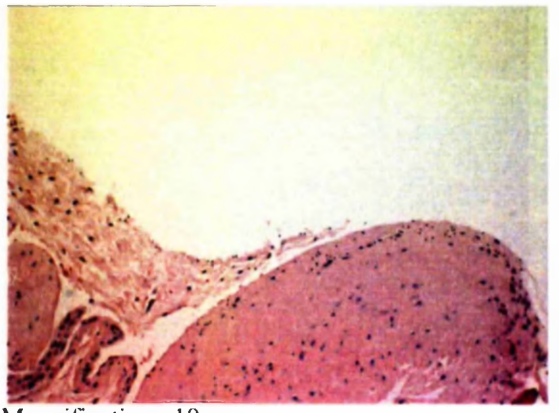
Figure 7-15: Gold particle penetration of canine fibroareolar synovium with particle bombardment pressure of 250 psi observed using a) darkfield and b) light microscopy (H&E)

a)



Magnification x10

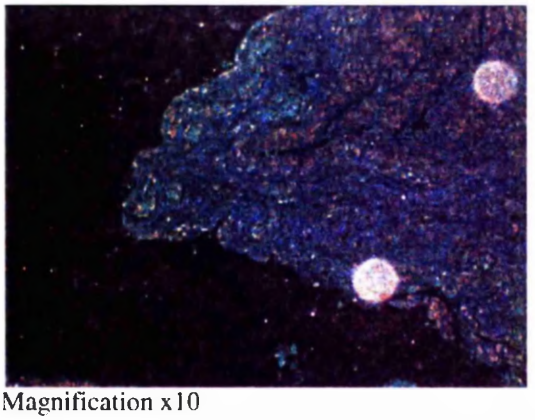
b)



Magnification x10

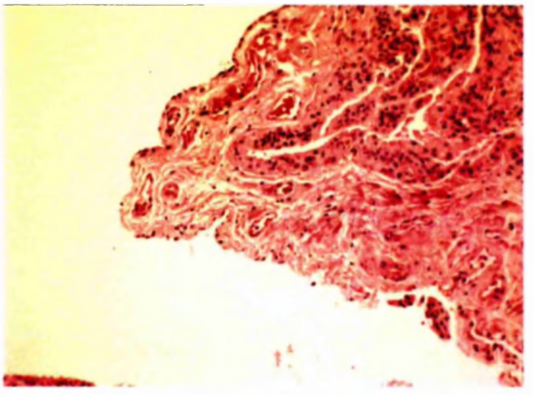
Figure 7-16: Untransfected canine fibroareolar synovium observed using a) darkfield and b) light microscopy (H&E)

a)



Magnification x10

b)



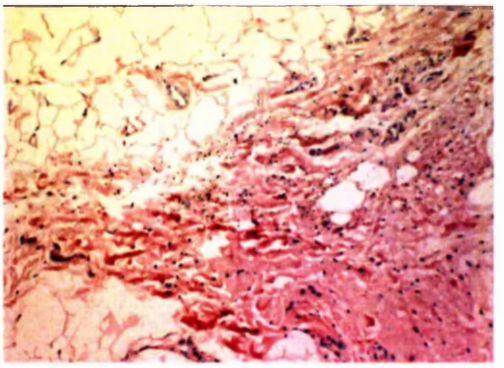
Magnification x10

Figure 7-17: Gold particle penetration of canine adipose synovial tissue at particle bombardment pressure of 250 psi observed using a) darkfield and b) light microscopy (H&E)

a)

Magnification x10

b)



Magnification x10

7.5 DISCUSSION

The BioRad Gene Gun is considered a convenient and effective means of transfecting a wide range of tissue and cell types (Pecorino et al., 1992), particularly for DNA-based immunisation (Fynan, 1993) and has now been adopted for cancer therapy (Rakhmilevich et al., 1996; Sun et al., 1995; Mahvi et al., 1996). This chapter assesses the potential application of the gene gun in the delivery of therapeutic genes to the synovial tissues of the joint for arthritis gene therapy.

7.5.1 Liposome-mediated and particle bombardment-mediated methods of gene transfection into monolayer cells in tissue culture

Particle-mediated gene transfer represents a useful approach for the transfection of cells in tissue culture systems. First of all it was necessary to establish that the gene gun could be used to successfully deliver reporter constructs to different cells lines in tissue culture with the cationic liposome-mediated method used as a positive control. Confluent cultures of MDCK and rabbit synovial cells (HIG-82) were bombarded with the gene gun apparatus at different pressures (100 and 150 psi) with the spacer against the cell monolayer (i.e target 40 mm from the distal cone of the accelerator channel). Successful transfection was identified by the expression of β-galactosidase and EGFP reporter genes in MDCK cells and rabbit synovial fibroblasts respectively.

When used at 100 psi the gene gun produced diffuse areas of transfected cells in the MDCK cells (Figure 7-8a) and rabbit synovial cells (Figures 7-11a) with little cell death. This is consistent with experiments using 100 psi to transfect neurons in tissue culture (Wellman et al., 1999) and a primary culture of rat neonatal cardiomyocytes (Eizema et al., 2000). However, contradictory studies have shown that pressures of 100 psi and below may not result in efficient delivery of genes to Hela or HEK293 cells (O'Brien et al., 2001). The difference in these results may be related to the preparation of the gold/DNA tubing, since very adherent gold particles, due to a high PVP concentration, would require a higher pressure to displace them from the cartridge. It may also be due to using a double firing process adopted to remove all micro-carriers from the inner tube and maximise transfection efficiency (Gan et al., 2000), although it could just be due to cell-type specificity. When used at 150 psi, the gene gun produced areas of transfected cells in both MDCK (Figure 7-8b) and rabbit synovial cells (Figure 7-11b) at the periphery of the well which is consistent with experiments using 150 psi to transfect COS cells (Yoshida et al., 1997). Contradictory studies showed that monocyte-derived dendritic cells could not be transfected with a reporter gene at 150 psi (Smith et al., 2001) or HeLa and HEK cells (O'Brien et al., 2001). Despite the reasonable transfection rates there was considerable cell death in the centre of the well, attributed to the gas impact and heavy gold particle loading. A similar 'dead zone' has been reported with pressures of 175 psi that could destroy HeLa and HEK293 cells in the centre of a cover slip (O'Brien et al., 2001; Yoshida et al., 1997). A pattern of transfection was evident with a radial increase in transfection frequency with increasing distance from the centre (Wellman et al., 1999). This peaked and then tended to fall away again as the distance increased from the target

centre. At even higher pressures cell transfection is still possible with considerable cell viability as shown with the transfection of islet cells *in vitro* at a pressure of 250 psi (Guo et al., 1997).

Variability in cell death was observed between the two cell types, with the rabbit synovial cells more susceptible to cell death than the MDCK cells. This may simply be due to an ability of the MDCK cells to firmly adhere to the matrix provided by the tissue culture base unlike the synovial cells which are more easily displaced on cell splitting (authors observations). Variation in cell death between different cell types has previously been described when comparing the particle-mediated delivery of reporter genes to rat dorsal root ganglion and hippocampal neurons in primary culture. Few toxic effects were observed with the peripheral neurons but this delivery system was detrimental to the integrity of the synaptic network in cells isolated form the CNS (Usachev et al, 2000). It has subsequently been suggested that the pressure of the helium pulse will affect cell survival because higher pressures cause shock-waves across cultured cells, detaching them from the matrix (O'Brien et al. 2001).

The standard configuration of the Gene Gun is known to induce appreciable cell death when used at the higher gas pressures required for effective transfection (Yoshida et al., 1997; Zang and Selzer, 2001). Therefore modifications to this Gene Gun system are necessary to reduce the potential for cell damage. The use of a 70 μm mesh between the Gene Gun and target is essential for effective transfection with the use of a finer mesh (3μm pore size) to cover the end of the accelerator channel recommended to further reduce tissue damage (Gan et al., 2000). Another method to reduce cell death is to limit the amount of gold micro-carriers per shot (Eizema et al., 2000).

7.5.2 Gold particle delivery to cell types of the canine joint

Particle-mediated gene transfer also represents a useful approach for the transfection of organotypic/explant cultures as shown by several groups with the successful transfection of neuronal cells in short-term slice-explant cultures of the cerebellum and cortex (Arnold et al, 1994; Lo et al., 1994). However, the efficiency of particle bombardment has not been assessed in explant tissues specific to the joint. First of all it is necessary to establish the penetration power of the gold particles using the gene gun system for delivery to the synovial tisues of the joint before gene transfection studies using explants can be performed. To this end gold particle bombardment was performed on canine synovial tissue dissected from the stifle joint at PME. Two different helium pressures were analysed (250 and 500 psi) for efficiency in projecting gold particles through the tissue samples. Gold particles were easily visible using darkfield microscopy with their ability to reflect light and distinguished by their uniformly circular shape.

Analysis of the bombarded synovial samples revealed that particle bombardment could be used to deliver gold particles to the lining cells of the fibrous, fibroareolar and adipose synovium at a helium pressure of 250 psi. This is consistent with studies demonstrating that the hand-held Gene Gun is optimised to transfect superficial tissues or dispersed cells in culture, propelling microparticles superficially over a wide area (O'Brien et al., 2001). This also corresponds to data showing that the particle bombardment-mediated delivery has been useful for the *in situ*

transfection of external cells in mammalian skin (Johnston and Tang, 1993). However, gold particle penetration did not extend beyond the outer lining which is consistent with previous reports suggesting that deeper penetration into tissues is limited (O'Brien et al., 2001). Nevertheless, one report has demonstrated penetration of some 30 to 50 cells layers in various tissues types (Yang et al., 1995). The discrepancy in these results is probably due to the difference in composition and structure between different tissue samples. However modifications to this system are already being developed to enable the accelerator channel to achieve a more compact, focused shot of gold particles and increased penetration depth (O' Brien et al., 2001). It is true that the lining layer is the most relevant part of the synovium for introducing genes and thus the pattern of penetration recorded here would be satisfactory.

7.5.3 Optimisation of transfection parameters

The success of the particle-mediated bombardment transfection technique depends on the optimisation of several variables, most of which balance cell death, as observed in the cell monolayer studies against tissue penetration as shown in the tissue explant study. One of the most important parameters is the velocity of the particles which in turn is dependent on the helium pressure, the distance of the particle source from the target tissue and the particle size. The pressure of the helium gas must be finely balanced between the high pressures that result in excessive cellular death and the low pressures that restrict penetration of microcarriers to the outer layers of the tissue. The helium pressure regulator used in these experiments did not permit accurate control of pressures at intervals smaller than 25 psi; therefore a more accurate regulator may be required for future studies in order to refine the pressure conditions. The distance of the particle source from the target tissue is also important since particle acceleration is dependent on atmospheric effects. To reduce this slowing effect, the gene gun spacer was kept as close to the target as possible. However, modification of spacer attachments are also possible with shortening of the stem to reduce the particle flight distance (Eizema et al., 2000). Particle size is also an important variable (Tanner et al., 1997) with gold particles available in a range of sizes from 0.6 to 1.6 μm (supplied with Gene Gun Kit, BioRad). Small particles are more susceptible to the slowing effects of the atmosphere, whereas larger particles may inflict greater cellular injury during bombardment. In the present study, 1.0 μm gold particles were used but optimisation with other sizes will be necessary for future studies.

However, the ultimate levels of transfection efficiency are determined by many additional factors including the concentration of particles delivered to the tissue. The more particles that enter a tissue, the greater the chance that cells will express the transgene, but smaller the chance that cells will survive membrane damage (Klein et al., 1987). In fact, in several mammalian cell lines, increasing the concentration of gold particles to more than 1 mg per cartridge load reduced transfection efficiency (Heiser et al., 1994). DNA concentration is another important consideration and several studies have shown that increasing the concentration of DNA coating the gold particles results in higher expression levels of the transfected gene (Burkholder et al., 1993, Thompson et al., 1993).

Contamination of DNA samples with protein and higher DNA loading concentrations are believed to enhance clumping which results in the uneven coating of the inner surface of the tubing. This and the coating state of the DNA on the gold micro-particles results in uneven particle penetration, increased tissue damage and variable transfection efficiencies. Another problem is the uneven distribution of microcarriers in the well, since the area of the gun barrel is smaller than the area of the well; however a special delivery device has now been developed in order to evenly bombard all cells present in the well (Eizema et al., 2000).

Many of these factors are difficult to standardise and are responsible for considerable variability in biolistic transfection (Biewenga et al., 1997). As a result it is difficult to reliably reproduce particle-mediated bombardment transfection between cultures. To adjust for this variability a standard must be used when attempting to quantify biolistic transfections. One quantitative method that has been used is based on evidence that when two DNA constructs are added to the gold particles, both constructs are virtually always expressed in the same cell (Arnold et al., 1994; Arnold and Heintz, 1997). Therefore two plasmids can be co-transfected, and the plasmid under evaluation can be corrected by the second internal control. This observation that several DNA constructs mixed with gold particles are expressed in the same cell has been adopted for multi-gene based therapies for cancer. The simultaneous delivery of IL-12, pro-IL-18 and IL-1β converting enzyme (ICE) using the gene gun has been shown to significantly augment anti-tumour effects (Oshikawa et al., 1999). The co-expression of two plasmids can be accomplished by simply mixing the plasmids, rather than constructing a vector encoding both genes (Usachev et al., 2000). Many of these parameters need to optimised before the gene gun apparatus can be used to efficiently deliver genes to the lining cells of the synovium for gene-based therapeutics.

7.6 SUMMARY

This chapter describes the preliminary evaluation of particle mediated bombardment for the delivery of therapeutic genes into synovial cells for future use in therapeutic gene delivery systems to the joint. Rabbit synovial fibroblasts and MDCK cells were successfully transfected with reporter genes, EGFP and β-Gal, respectively in tissue culture using this delivery system. Synovial tissue samples, collected at PME were also bombarded with gold particles to demonstrate that the gene gun could be used to penetrate the lining cells of the synovium. Particle bombardment-mediated gene delivery is a simple, fast and versatile technique for gene transfer and may be applicable for gene therapy research in the delivery of candidate therapeutic genes to somatic tissues. This technique has been shown to be effective under various experimental conditions within *ex vivo*, *in vivo* and *in vitro* mammalian gene transfer systems. With future modification and optimisation, this evolving technology may serve as a suitable approach for gene transfer in osteoarthritic disease.

Chapter VIII

General Discussion

8.1 ABSTRACT

Osteoarthritis (OA) is a chronic, painful condition and is of major concern to both human and veterinary medicine. To date, treatment modalities in both species are generally limited to 'symptom-modifying' therapies that only address joint pain. The primary aim of this project was to develop a targeted, 'structure-modifying' gene based therapy for the future treatment of OA in the dog, with the prospect of developing an animal model for human disease. Candidate canine-specific therapeutic genes, based on their ability to regulate the levels of matrix metalloproteinase (MMP) activity within arthritic joints, were cloned and expressed. Pro-inflammatory cytokine-inducible MMP promoters were subsequently cloned and characterised to evaluate their potential for driving disease-specific expression of therapeutic genes in joints. Targeting therapeutic gene expression to diseased cell types of the osteoarthritic joint was attempted with the development of a novel dual-targeting vector incorporating both disease-inducible and cell-type specific regulatory elements, with both components modulated to enhance activity whilst maintaining specificity. Finally, particle-mediated bombardment methods of synovial tissue transfection were evaluated as a method of gene delivery to the joint.

8.2 DISCUSSION

8.2.1 Work described in this thesis

Recent research has identified several proteins that have promising chondroprotective and anti-inflammatory properties which offer the prospect of establishing 'structure-modifying' therapies. However, recombinant proteins have limited half-lives *in vivo*: thus, it is difficult to deliver and maintain therapeutic concentrations of these agents for sustained intervals. By delivering cDNAs that encode these proteins to the diseased joints and providing for their expression in a relevant manner, it may now be possible to devise an effective treatment strategy for arthritic disease. The design of a gene delivery strategy for osteoarthritis (OA) should include mediators for controlling the synthesis and activity of the matrix metalloproteinases (MMPs) at either or both the transcriptional and post-translational levels using therapeutic inhibitory proteins. This is the first step in controlling the disease at the molecular level using gene therapy by inhibiting the enzymes responsible for cartilage degradation while enhancing tissue repair. Subsequently canine specific cDNAs for IL-1Ra, sTNFRI, TIMP-2 (Campbell et al., 2001b) and TIMP-1 (Zeiss et al., 1998) have been isolated for gene-based therapeutics for canine OA.

Developing an effective yet safe gene based therapy for OA in both human and veterinary patients is an exciting challenge, yet several technical problems must be overcome before it can be considered for use in clinical practice. Methods can be devised to regulate therapeutic gene expression, at the transcriptional level, in a disease-specific manner. Since MMPs are up-regulated during the arthritic disease process it is hypothesised that these regulatory sequences could be utilised to direct disease-specific transgene expression while simultaneously avoiding undesirable expression in healthy tissues. The canine MMP-9 promoter (Campbell et al., 2001a) and canine MMP-13 promoter (Campbell et al., 2002) were cloned and characterised for driving disease specific therapeutic gene expression. Using computer-based analysis, inducible elements were identified in both promoter sequences corresponding to the conserved regions found in sequence alignments with the promoters published for other species. Both promoters were characterised in various cell lines using a luciferase reporter gene to determine promoter activity in response to pro-inflammatory mediators, Interleukin-1 (IL-1β), tumour necrosis factor (TNFα) an basic fibroblastic factor (bFGF). A series of promoter deletion contructs were also made, based on the canine MMP-9 promoter sequence, to evaluate phorbol myristate acetate (PMA)-responsive elements within the promoter sequence.

The potential for undesirable systemic effects related to constitutive over-expression of certain therapeutic transgene products may be limited through the development of both 'disease and cell type specific' DNA targeting vectors that restricts transgene expression to diseased cell types of the joint. Therefore, in addition to the development of MMP promoters for driving disease-specific expression, it was necessary to investigate a second level of targeting using cartilage specific promoters and enhancers, such as those for the collagen type XI gene to limit expression of therapeutic genes to chondrocytes. It was thought that these two targeting elements could be

combined into one plasmid vector utilising 'Cre Recombinase' technology, an enzyme system which affects DNA recombination between *lox* recognition sequences to develop a dual targeting vector.

First of all it was necessary to modulate the two components of the dual targeting vector to enhance activity while maintaining specificity. It was demonstrated that the canine MMP-9 promoter could be manipulated to increase activity while maintaining disease specificity through the introduction of multiple NF-kB sites at the 5′ end, guided by a series of promoter deletion studies and mutational analysis. The mouse cell-type specific collagen type XI promoter, had selectively higher activity in the chondrosarcoma cell line (SW1353) compared to the undifferentiated chondroprogenitor cell line (ATDC5). However, this promoter activity could be modulated to enhance activity while maintaining cell type specificity with the incorporation of a region of the collagen type XI intron 1 sequence, containing three flanking SOX9 enhancer sites in the vector. Attempts were finally made to combine these two modulated promoter sequences into one plasmid vector containing the gene for Cre Recombinase and *loxH* sites to restrict therapeutic gene expression to diseased chondrocytes in tissue culture. Although the analysis of the individual components of the targeting system was successful, the incorporation of each of these components into one vector, to demonstrate that a simple recombination-based system can produce a functional targeting vector, was not technically possible. Nevertheless, considerable experience and knowledge was gained during the construction steps and it is now apparent that further studies are required to develop this technology for *in vivo* application. It is possible that this specific system may not have provided sufficient levels of transgene expression *in vivo* and the potential for an immunogenic response to the bacterial Cre protein may have prohibited its use. Therefore, before novel targeting vectors such as this are delivered and analysed *in vivo*, methodical steps need to be taken to evaluate the overall feasibility and efficacy of using gene therapy to treat a canine model of OA.

Methods must also be devised to efficiently deliver therapeutic genes to cells of the joint. Synovial cells are the most promising cell-type for targeting gene-based therapeutics in OA since they can be directly transfected *in vivo*, due to their location lining the joint cavity but can also be used for *ex vivo* gene delivery methods (Bandara et al., 1992). Furthermore, synovial cells are more abundant than chondrocytes and line the joint space for efficient secretion of soluble therapeutic gene products into the joint fluid. Synovial cells were therefore selected to analyse particle-mediated methods of gene delivery. Two reporter genes β-galactosidase (β-Gal) and enhnaced green fluorescent protein (EGFP) were successfully transfected into monolayer cells in tissue culture using the gene gun apparatus. Synovial tissue samples, collected at *post-mortem* examination PME, were also successfully bombarded with gold particles to the lining cells of the synovial tissue.

8.2.2 Future studies

Future studies need to build on this *in vitro* work directed towards the development of an *in vivo* homologous gene-based therapy for canine OA, as a model for treating human disease. The long-term goal is to establish a method for delivering species-specific therapeutic cDNAs directly to the synovial lining of joints, whose subsequent expression is regulated by the amplitude of disease.

Fundamental tissue-explant studies should first be performed to characterise and quantify the expression and chondroprotective capacity of canine-specific therapeutic genes for *in vivo* application. Then viral vectors, such as recombinant Adeno Associated Virus (AAV) vectors incorporating the disease inducible promoters, should be generated and evaluated for levels of *in vivo* gene expression in the joints of standard laboratory animal models such as the rat. AAV mediated, intra-articular gene transfer of cDNAs encoding homologous therapeutic proteins can finally be evaluated for feasibility and therapeutic efficacy using an *in vivo* homologous canine study such as the experimental Pond-Nuki canine model of OA (Pond and Nuki, 1973).

Developing gene based therapies using the canine model of OA not only has veterinary importance but also represents an ideal *in vivo* model of human disease from both a bio-mechanical and biological perspective. More specifically, section of the cranial cruciate ligament (CCL) in the canine Pond-Nuki Model of OA mimics the naturally occurring failure of the CCL (Pond and Nuki, 1973) the single most common cause of OA in the dog. A recent report has revealed that many cases of so-called primary OA of the human knee are in fact secondary to failure of the anterior cruciate ligament (ACL) (Wada et al., 1996) reinforcing the relevance of the dog model to the human patient. Alternative animal models, such as the rat, have evolutionary diverged from the human species and therefore have several disadvantages. Not only are the effects of locomotion and weight bearing on the joint different, but there is also a disparity in collagenase gene expression within articular cartilage. Rodents have two rather than the three functional collagenases so far identified in the human and canine species (Cawston et al., 1996). The size of the canine joint compared to other smaller laboratory species is also of distinct benefit allowing easy sampling and gene delivery.

8.2.2.1 Details of proposed future research project

8.2.2.1.1 Expression and biological characterisation of the canine specific therapeutic proteins using a tissue culture explant system

A biological assay for the evaluation of the canine therapeutic proteins can be developed using an *in vitro* tissue culture explant system where portions of bovine nasal septum cartilage tissue are co-cultured with adherent rabbit synovial fibroblasts (HIG-82 cells). Constitutive expression (CMV promoter) of the respective therapeutic gene (IL-1ra, TNFR, TIMP-1, -2), or gene combination from simple mammalian expression plasmids can then be analysed after stable transfection into the synovial cell monolayer and incubation with inflammatory cytokines (IL-1, TNF). The relative chondroprotective capacity of the secreted soluble therapeutic proteins can then be measured by

their ability to inhibit the cytokine-induced changes within the cartilage explants by analysing the harvested supernatants and cartilage tissue samples for established markers of OA.

8.2.2.1.2 Local and systemic assessment of inducible disease specific promoters *in vivo* using Adeno Associated Viral (AAV) vectors in rats.

Activity of the inducible disease specific promoters (MMP-9, MMP-13), which have already been assessed *in vitro* can be evaluated *in vivo* using viral vector technology. The MMP-9 and MMP-13 disease-specific promoters can be inserted into recombinant AAV vectors using the cDNA for human interleukin-1 receptor antagonist (hIL-1Ra) as a surrogate reporter gene. The AAV constructs can be analysed in rats using the adjuvant-induced arthritis system to determine the levels of intra-articular hIL-1ra expression in response to the disease-state. The tissues of the knee joints can be assayed for hIL-1Ra concentrations using a commercially available ELISA kit, that does not cross-react with the rat homologue of IL-1Ra, and the bio-distribution of the hIL-1Ra transgene product and vector can be evaluated in selected tissues using a combination of ELISA and RT-PCR techniques.

8.2.2.1.3 *In vivo* study using the canine model of osteoarthritis

The previous studies will determine the appropriate combination of canine therapeutic cDNA(s) and MMP promoter for use in gene transfer/efficacy studies in the canine model of OA. A recombinant AAV vector containing this cDNA/promoter combination can then be generated and characterised for functional expression and titre, and the biological effects of therapeutic gene expression at the gross and microscopic level in the canine model can then be determined. Dogs should be divided into three groups, one group to include untreated naive animals, the other two groups to undergo surgical section of the cranial cruciate ligament for induction of OA. Following an appropriate interval, one group of diseased animals should be treated with an empty AAV vector and the other group with the therapeutic AAV vector via intra-articular injection. Clinical evaluation and radiographic analysis of joints should occur on a daily and weekly basis, respectively. Joint biopsies and synovial samples can be taken at intervals for evaluation at the microscopic and molecular level. More specifically, cartilage and synovial tissue samples can be evaluated for differential protein expression using proteomic techniques (Pandey and Mann, 2000). At PME tissue samples can be taken from selected tissues and studied at the gross and microscopic levels for pathological changes and for the bio-distribution of the IL-1Ra transgene product using RT-PCR.

8.3 SUMMARY

These studies are essential for the development of a canine-specific animal model to analyse a homologous gene transfer system for OA. Once the biological effects of the most effective therapeutic protein (IL-1ra, sTNFRI, TIMP-1, TIMP-2) combinations have been shown and the efficacy of disease-specific (MMP-9 & -13) promoter constructs demonstrated *in vivo,* the first *in vivo* targeted homologous gene transfer study using the cruciate transection experimental canine model of OA can be performed. These data will enable the implemention of a Phase II clinical trial in dogs which is an essential step to take in the promotion of gene-based therapeutics for the human disease.

GLOSSARY

A	Adenosine
AAV	Adeno associated virus
ADAM	A Disintegrin and metalloproteinase Thrombospondin motif
AMV-RT	Avian myeloblastosis virus RT
AP-1	Activator protein-1
APMA	Aminophenylmercuric acetate
ATDC5	Undifferentiated chondroprogenitor cells
ATCC	American Type Tissue Culture
AV	Adeno virus
bFGF	Basic Fibroblastic growth factor
β-Gal	β-Galactosidase
bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine
CDNA	Complementary DNA
cIL-1Ra	Cytoplasmic IL-1Ra
COX	Cyclooxygenase
CMV	Cytomegalovirus
CNS	Central nervous sysstem
CS	Chondroitin sulphate
DEPC	Diethylpyrocarbonate
dH_2O	distilled water
DLR	DNA loading ratio
ECM	Extra-cellular matrix
E.Coli	Escherichia coli
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbant assay
ERK	Extra-cellular stimulus-regulated kinases
EST	Esculetin
FEA	Feline embryonic fibroblasts
FGF-2	Fibroblast growth factor-2
FCS	Foetal calf serum
G	Guanine
G/C	Guanine/Cytosine ratio
GCG	Genetics Computer Group
GCSF	Granulocyte colony stimulating factor
gDNA	Genomic DNA
HA	Hyaluronan
H/E	Hematoxylin and eosin dye
HIG-82	Rabbit synovial fibroblasts
HMG	High mobility group
HOCl	Hypochlorous acid
HSV	Herpes simplex virus
IFNγ	Interferon γ
IGD	Interglobular domain
IGF-1	Insulin-like growth factor-1
IκB	Inhibitory κB
IKK	IκB
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
JNK	c-Jun N-terminal kinase
Kb	Kilobase(s)
KDa	Kilodalton
KS	Keratan sulphate
LCR	Locus control region
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide

MAC	Mammalian artificial chromosome
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MBq	Mega Beqerels
MCS	Multiple cloning site
MDCK	Madin Darby canine kidney cells
MEK	MAPK kinase
MLQ	Micro-carrier loading quantity
MMLV	Molony murine leukaemia virus
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MT-MMP	Membrane Type MMP
mut^n	Mutation
NF-κB	Nuclear factor-κB
NIK	NF-κB inducing kinase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OD	Optical density
OPGL	Osteoprotegerin ligand
OSM	Oncostatin M
PAGE	Poly acrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE 480, 2400	Perkin-Elmer 490, 2400
PDGF	Platelet derived growth factor
PG	Prostaglandin
PKC	Protein kinase C
PLC	Phosholipase C
PMA	Phorbol myristate acetate
PME	*Post-mortem* examination
PVP	Polyvinylpyrrolidine
R	Receptor
RA	Rheumatoid arthritis
RACE	Rapid amplification of cDNA ends
RANKL	Receptor activator of NF-κB ligand
RE	Restriction enzyme
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
SAPK	Stress-activated protein kinases
sIL-1R	Soluble IL-1 receptors
sIL-1Ra	Soluble IL-1Ra
sTNRI	Soluble TNFRI
SV 40	Simian virus-40
SW1353	Human chondrosarcoma cells
TACE	TNFα converting enzyme
TAFs	TBP-Associated Factors
TBP	TATA-binding protein
TF	Transcription factor
TGF	Transforming growth factor
TIMPs	Tissue inhibitor of MMP
Tm	Temperature of primer melting point
TNF	Tumour necrosis factor
UAS	Upstream activator sequence
UTR	Untranslated region

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