THE ROLE OF TRANSFORMING GROWTH FACTORS TYPE BETA DURING MURINE EMBRYOGENESIS

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A thesis submitted for the degree of Doctor of Philosophy
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Contents

DECLARATION

I CERTIFY THAT THIS THESIS DOES NOT CONTAIN MATERIAL PREVIOUSLY PUBLISHED OR WRITTEN BY ANY OTHER PERSON, EXCEPT WHERE REFERRED TO IN THE TEXT AND THAT THE RESULTS IN THIS THESIS HAVE NOT BEEN SUBMITTED FOR ANY OTHER DEGREE OR DIPLOMA.

Fergus A. Millan

Acknowledgements

Many thanks to Rosemary Akhurst for giving me the opportunity to study for a Ph.D, and for providing excellent and patient supervision.

Thank you to Professor Mike Connor, and all the staff at the Duncan Guthrie Institute of Medical Genetics, for providing a very friendly and cooperative atmosphere in which to work.

I would like to thank all the members of the Developmental Genetics group (including Marie), those gone and those still here, for not only being great colleagues but also good companions. I would paricularly like to thank Liz for advice with all my photographic work, Marion for the M13 and sequencing stuff, Derek for providing living proof of my normality (compared to his!). Brian for teaching me all I know about tissue culture, and then asking me for advice! Caspar for playing with me!! and finally Frances for understanding someone who also likes Sci-Fi. Also thanks to everybody in the PGR lab for leaving me some apparatus to work with.

I would also like to thank my family for their love and support.

Finally, thank you, Deborah, for your love and understanding.

SUMMARY

The role of transforming growth factors type beta (TGF-β) was investigated during murine embryogenesis. There are three known mammalian TGF-β genes. The developmental pattern of expression of TGF-β1 RNA has previously been elucidated by *in situ* hybridisation and the localisation of TGF-β1 protein by immunohistochemistry. Based on this data it has been suggested that TGF-β1 acts in both a paracrine and autocrine role during development. The expression of both TGF-β2 and TGF-β3 RNA has been observed in murine embryos by Northern analysis. In order to understand the role of these two genes, individually, and in relation with each other, the developmental regulation of all three genes was investigated by in situ hybridisation using mouse specific probes on serial mouse embryo sections from 6.5 days *p.c.* to 16.5 days *p.c.*. A comparison was made of the data concerning the temporal and spatial expression patterns of TGF-β1, TGF-β2 and TGF-β3 RNA, and the role of each gene discussed. The pattern of expression of each of the TGF-β RNAs was compared with available protein localisation data.

TGF-β2 RNA was found to be expressed in the early embryo, where it was localised to the myocardium of the developing heart. This pattern of expression persisted, although it was regionally restricted to the myocardium overlying the presumptive heart valves. TGF-β1 RNA had previously been localised to the endothelial cells of the heart valves. It was suggested that TGF-β2 was providing the signal which induced the endothelial cells, overlying the myocardial tissue of the presumptive heart valve, to undergo a epithelial/mesenchymal transformation to form valve cushion tissue. TGF-β2 RNA was also expressed in prechondrogenic blastemae and growth zones of developing long bones. There was also widespread expression of TGF-β2 RNA in epithelial cells, including many which were undergoing differentiation. These included the nasal, retinal

and otic epithelium. TGF-β2 RNA was also transiently expressed in ventral horns of the neural chord at a time of motor neurone differentiation.

TGF-β3 RNA had a more restricted pattern of expression than TGF-β2. TGF-β3 RNA was expressed in intervertebral discs anlagen, cartilaginous capsules of the ear and nasal septum. TGF-β3 RNA was also expressed in epithelial tissue. The medial edge epithelium of the developing palate was an abundant source of TGF-β3 RNA both during and after fusion of the palates.

The three TGF-β genes were often expressed in the same developmental system, but rarely in the same cell or tissue. The lung develops as simple endodermal tubes which are induced to branch distally, the lung expands as rapid growth occurs at the terminal end buds and in the surrounding lung mesenchyme. The epithelial cells that line the respiratory tubes change in morphology at defined points, from a columnar to a simple cuboidal shape. The simple cuboidal epithelium eventually becomes more flattened in shape, forming the alveolar ducts. The change in epithelial morphology is marked by a change in expression from TGF-β3 RNA seen in the columnar epithelium to TGF-β2 RNA observed in the simple and flattened epithelium. TGF-β1 RNA is expressed in the mesenchyme of the lung, and TGF-β1 protein has been localised underneath the bronchiolar epithelium and at the clefts of the bronchioles. This data suggests there is close co-operation between the three TGF-β isoforms during morphogenetic events.

The role of the TGF-β genes remains unclear. The interactions of TGF-β isoforms with a variety of polypeptides in the ECM has been elucidated from *in vitro* analysis, this data, combined with RNA expression and protein localisation data, has not clarified the specific roles of the TGF-β isoforms. To determine a complete picture of the role of TGF-β isoforms during development, dominant-negative mutations were created and analysed *in vitro.*

The dimeric structure of mature TGF-β isoforms lends itself to the production of dominant-negative mutations which can bind to wild-type

monomers of TGF-β, resulting in the formation of heterodimers, which reduce biological activity of the selected TGF-β isoform.

Mutations of human TGF-β2 were generated which were sub-cloned into the eukaryotic expression vector πH3M and analysed *in vitro*. The first mutant was generated by a deletion of part of the human TGF-β2 cDNA sequence which encodes the signal peptide sequence at the N-terminal. The second mutation was generated by site-directed mutagenesis of the human TGF-β2 cDNA sequence which encodes the proteolytic cleavage site. The constructs were transiently transfected into COS-7 cells and the conditioned media assayed. The biological activity of the mutant constructs were compared to biological activity of constructs which contained the wild-type human TGF-β2 cDNA. A reduced level of biological activity of mutant proteins was detected by growth inhibition assay.

Co-transfections of wild-type TGF-β2 and the mutants were done at various ratios to determine if the mutant protein could inhibit wild-type activity. Growth inhibition assays showed that the mutants did not reduce wild-type levels of biological activity generated by the human TGF-β2 construct. The mutants were also co-transfected with simian TGF-$\beta1^{33}$ (a mutation which requires no acid activation but retains wild-type levels of biological activity). The mutants did not reduce the biological activity of the simian TGF-$\beta1^{33}$ protein.

Note: Some of the data presented in this thesis has been published elsewhere. Reprints are bound at the back.

Abbreviations

ATP	adenosine triphosphate
Ala	alanine
Asp	aspartic acid
Asn	asparigine
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol
cDNA	complimentary DNA
CIP	calf intestinal phosphatase
cm	centimetre
CMV	cytomegalovirus
CSF-1	colony stimulating factor 1
CTP	cytodine triphosphate
Cys	cysteine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytodine triphosphate
dpp	decapentaplegic
DEPC	diethylpyrocarbonate
dGTP	deoxyguanidine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotides
ddNTP	dideoxy nucleotides
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
DVR	decapentaplegic-Vg-related
ECM	extracellular matrix
EDTA	ethylenediaminetetraactic acid
EGF	epidermal growth factor
FGF	Fibroblast growth factor
g	gram
GIT	guanidium isothiocyanate
GTP	gaunidine triphosphate
Glu	glutamic acid

Gly	glycine
Gln	glutamine
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-etanesulphonic acid)
HIV	human immunodeficiency virus
His	histidine
Ile	isoleucine
kb	kilobase(s)
kDa	kiloDalton
kg	kilogram
L	litre
Lys	lysine
M	molar
Met	methionine
mg	milligram
ml	millilitre
mM	millimolar
MIS	Mullerian inhibiting substance
MOPS	3-[N-morpholino]propanesulphonic acid
mRNA	messenger RNA
ng	nanogram
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAI	Plasminogen activation inhibitor
PBS	phosphate buffered saline
p.c.	post coitum
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEG	polyethylene glycol (8000)
PFA	paraformaldehyde
pg	picogram
Phe	phenylalanine
Pro	proline
RA	retinoic acid
Rb	retinoblastoma
RNA	ribonucleic acid

rNTP	ribonucleotides
rpm	revolutions per minute
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Ser	serine
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TESPA	3-aminopropyltriethoxysilane
Tfb	transformation buffer
TGF-β	transforming growth factor type beta
TTP	thymidine triphosphate
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
UTP	uridine triphoshate
UT	untranslated
UV	ultraviolet
Val	valine
Vg	vegetal
Vgr	Vg-related
v/v	volume to volume
w/v	weight to volume
X-gal	5-bromo-4-chloro-3-inodyl-β-D-galactopyranoside

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Chapter 1

INTRODUCTION

1.1 Embryogenesis, pattern formation and cell fate.

The study of embryogenesis has been underway for a great many years. The first embryologists established fundamental questions which still remain to be answered. These questions include: How do differentiated cells arise and form tissues and organs? How do cells know the pattern to which they must conform? The emergence of molecular biological techniques has provided a variety of tools with which to disassemble the detailed events of embryogenesis and possibly provide a means to answer these questions.

Many genes have now been identified which have been shown to be active during embryogenesis. These include peptide growth factors, the homeobox-containing genes, pax genes and retinoic acid receptor genes. Homologues of these genes are found in a variety of species which diverged, in evolutionary terms, a very long time ago. This seems to indicate that whatever function these genes possess it must be very fundamental to the organism to ensure survival against evolutionary pressures. Melton (1991) suggested that a few types of genes are used similarly by diverse organisms to specify cell fates during development.

This project set out to examine the expression of two genes, which are members of the transforming growth factor type beta (TGF-β) superfamily, during embryogenesis. The relationship between these genes and

members of their immediate family, and the superfamily as a whole will be reviewed below.

1.2 Transforming growth factor superfamily

The transforming growth factor type β (TGF-β) superfamily consists of an extensive number of proteins that exist in a wide variety of species. These proteins are all related, the relationship based upon a number of common characterisics.

1. The mature regions of all members of the superfamily maintain a degree of homology at the amino acid level which ranges from 40% to 80%. The degree of homology that exists at the amino acid level, between the same mature protein isoforms, across species can be as high as 99%.

2. Each monomer of the superfamily exists as a mature protein region bound to a precursor, the latency associated peptide (LAP).

3. The removal of the precursor by proteolytic cleavage is necessary for activation of the mature region.

4. All members exists as homodimers.

There are five sub-groups within the TGF-β superfamily all of which are described in the following sections.

1.3 Transforming growth factors type β

DeLarco and Todaro (1978) reported the isolation of a novel growth factor from murine sarcoma virus-transformed mouse fibroblasts, which they termed "sarcoma growth factor" (SGF). They described the induction of a transformed phenotype in normal rat kidney (NRK) fibroblasts by SGF. This resulted in a loss of density-dependent growth inhibition, and acquisition of anchorage-independent growth, which led to colony formation in soft agar. The effect was immediately reversible upon removal

of the factor. SGF was shown to be distinct from epidermal growth factor (EGF), but still competed for the EGF receptor (Todaro *et al* 1980). Moses *et al* (1981) and Roberts *et al* (1982) later fractionated SGF into two types. Only one of these competed for the EGF receptor. The competing and non-competing factors were termed α and β respectively. These peptide growth factors were renamed "transforming growth factors" (Roberts *et al* 1980).

In 1985 Derynck and coworkers cloned the human cDNA for TGF-β, (now TGF-β1), and revealed its nature (Derynck *et al* 1985). The mature protein represents only a small carboxy terminal fraction, (112 amino acids) which is cleaved from a larger precursor. As expected for a secreted molecule, a short signal peptide sequence was present at the N-terminus of the precursor. As with all TGF-β-related molecules it exists as a dimer and is only activated upon cleavage from the precursor portion of the molecule, otherwise it remains inactive.

1.3.1 Structure of TGF-β genes and proteins.

There are three known vertebrate TGF-β genes all of which share common features in structure. The human TGF-β1 gene contains seven exons (Derynck *et al* 1987). The splice site junctions are conserved in bovine, porcine and murine TGF-β1 genes (Van Obberghen-Schilling *et al* 1987; Kondaiah *et al* 1989; Derynck *et al* 1987). The position of the intron/exon junctions are conserved in TGF-βs 2 and 3, with the exception of the first which differs by three nucleotides, suggesting that the various TGF-βs evolved from a common ancestor gene by duplication.

It is now becoming evident that TGF-β4, which was originally isolated from chicken DNA library and described by (Jakowlew *et al* 1988a), does not exist. Subsequent attempts, by many laboratories throughout the world, have failed to find the homologue in any other species. It was noted that the TGF-β4 gene was 2 nucleotides short of porcine TGF-β1. When these two nucleotides were taken into consideration the sequence was found to be identical to that of porcine TGF-β1 (David Burt Personal communication). This could be explained by a laboratory mix up, as both porcine TGF-β1 and chicken TGF-β4 were cloned in the same laboratory.

Further evidence was established by carrying out a variety of Southern blots which indicated that TGF-β4 picked up the same bands as TGF-β1, but that this was distinct from the pattern seen with either TGF-β2 or 3. Southern analysis carried out on DNA from a variety of species gave strength to the argument that chicken TGF-β4 was in fact porcine TGF-β1 (Marion Dickson personal communication). TGF-β5 has been isolated from *Xenopus*, (Kondaiah *et al* 1990), however little is known about this member of the family.

The mRNAs of the TGF-β genes contain approximately 1200 nucleotides of coding sequence, however the mRNA species do vary greatly in length which is accounted for by variations in 3' or 5' untranslated region (UT) length and also by alternative splicing. There is evidence for alternate splicing in TGF-β1 and β2. The alternatively spliced TGF-β1 mRNA transcripts do not generate novel proteins, but the alternatively spliced TGF-β2 mRNA does (Kondaiah *et al* 1989; Webb *et al* 1988). No alternatively spliced mRNAs have been identified for TGF-β3.

The three TGF-β proteins contain common features.

1. The translated protein can be divided into two regions. The latency associated peptide (LAP) makes up the first two thirds of the polypeptide. The last third of the protein is the mature region, which has to be cleaved from the LAP to become activated.

2. A short sequence of amino acid residues form a cleavage site which separates the precursor LAP region from the mature form.

3. The precursor sequence starts with a stretch of hydrophobic amino acids that correspond to a signal peptide sequence.

4. Only three cysteine residues are conserved in the precursor part of the protein whereas all nine cysteine residues in the mature region are conserved between the three TGF-β isoforms.

5. All the proteins contain potential sites for N-glycosylation, within the LAP, although these are at different locations only one being common to all.

6. Both TGF-β1 and β3 contain a potential integrin binding site which TGF-β2 lacks.

7. The mature domain of each TGF-β polypeptide is highly conserved and each is 112 amino acid residues long. As described, there is conservation of all nine cysteine residues and a perfectly matched C-terminal sequence.

Denhez *et al* (1989) suggested that the fact that both TGF-β1 and TGF-β3 have a potential integrin binding site, whereas TGF-β2 does not, could indicate that TGF-β1 and 3 are more closely related, in functional terms, than between either of them and TGF-β2. However, there is still greater overall homology between TGF-β2 and TGF-β3 and in most biological assays each isoform is interchangeable with only a variation in the degree of bioactivity.

1.3.2 Alternative splicing of TGF-β2 mRNA leads to an unique protein

The murine TGF-β2 gene can be transcribed to give five mRNA species which show different patterns of expression during development (Miller *et al* 1989). TGF-β2's five different transcript sizes identified by Northern analysis were 7.0, 6.0, 5.0, 4.0 and 3.5 Kb (Miller *et al* 1989) with the 6.0Kb species predominant. O'Reilly *et al* (1992) described the same number of transcripts for TGF-β2 but the sizes described were different, 5.8, 5.1, 4.0, 3.8 and 2.8Kb. Again the second largest transcript (5.1kb) was predominant. (The difference in size of transcripts described by these two groups probably reflects difference in the interpretation of Northern blots rather than any genuine difference in transcript size.) Four of the five aforementioned transcripts are generated by variation in polyadenylation length and give rise to identical proteins, however TGF-β2 is known to produce a transcript via alternative splicing, which results in a protein of different length (Webb *et al* 1988).

Simian and human cDNA clones of TGF-β2 revealed the existence of two types of TGF-β2 precursor. One would give rise to the expected 414 amino acid protein, the other would give rise to a 442 amino acid long protein, which were called TGF-β2:414 and TGF-β2:442 respectively. Webb *et al* (1988) described TGF-β2:442 as having an insertion of 84 bp at the *Asn*-116 codon of TGF-β2:414, which codes for an additional 28 amino acids. Comparison of the coding sequences is identical apart from this insertion. Webb and colleagues suggested that this could only arrive via alternative splicing rather than transcription from a new gene. Further analysis of the sequence around the splice-site junctions indicates that this short 84 bp fragment is indeed an exon in its own right. Whether TGF-β2:442 is differentially expressed was resolved when cells from human prostatic adenocarcinoma (PC-3) and a monkey cell line (BSC-40) were analysed. The 4.1, 5.1 and 6.5Kb mRNA species were found to be expressed in the PC-3 cell line whereas the BSC-40 line only expressed the 4.1 and 6.5 Kb mRNAs (de Martin *et al* 1987). The use of a probe based upon the short 84bp sequence showed that the 5.1Kb mRNA species encoded the larger precursor. TGF-β2:414 would then be encoded by either the 4.1 or 6.5 Kb mRNA species (Webb *et al* 1988). This suggested that there may be tissue specific expression of the exon containing the 84bp fragment. Recent data has indicated that there is no difference in expression pattern between the mRNA coding for TGF-β2:414 and TGF-β2:442 in human embryos (Marion Dickson personal communication).

TGF-β3 has been cloned from a variety of species including human, mouse, chicken and pig (ten Dijke *et al* 1988; Derynck *et al* 1988; Jakowlew *et al* 1988b). The mouse cDNA hybridises to a unique 3.5 Kb mRNA and is differentially expressed in various mouse organs and developmental stages (Denhez *et al* 1990; Miller *et al* 1989; Millan *et al* 1991; Schmid *et al* 1991; Fitzpatrick *et al* 1990; Gatherer *et al* 1990; Pelton *et al* 1990). At present no other transcript size of TGF-β3 mRNA has been reported although alternatively transcribed, or spliced species, cannot be ruled out. The mouse TGF-β3 coding region is 1230 nucleotides long and codes for a 410 amino acid protein very similar to its human counterpart (Derynck *et al* 1988).

1.3.3 TGF-β gene promoters

It is quite clear that TGF-β gene expression is regulated during development and *in vitro*. The variety of actions attributed to TGF-β isoforms have made it important to understand the mechanism by which gene expression is controlled, especially during embryogenesis when cell fates are being determined.

The three TGF-β gene promoters have all been isolated (Kim *et al* 1989; Lafyatis *et al* 1990; Noma *et al* 1991) (Figure 1). Despite high sequence homology between the three isoforms in the region of the mature proteins, there is no similarity in the promoter regions. However, there is striking homology between promoters of the same gene across species which implies that the evolutionary pressure on the sequences has been high. This is consistent with studies in the mouse and human, which demonstrate that the three TGF-β genes do have different patterns of expression, both spatially and temporally, during embryogenesis, but that these gene expression patterns are maintained across species (Pelton *et al* 1989; Gatherer *et al* 1990; Millan *et al* 1991; Schmid *et al* 1991).

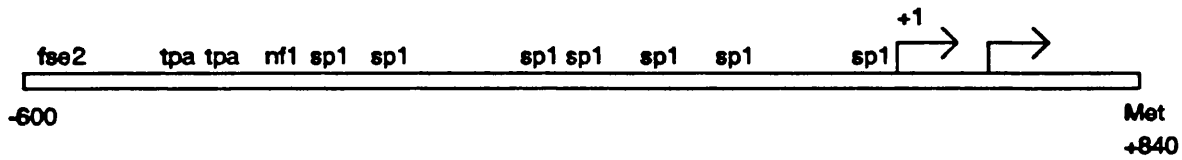
1.3.4 TGF-β1 promoter

Kim *et al* (1989) investigated the promoter activity of human TGF-β1. The analysis of the sequence upstream of the initiation site was carried out by generating chimaeric genes containing the bacterial reporter gene, chloramphenicol transferase (CAT), and variable regions of 5' flanking DNA. They were able to describe five distinct regulatory regions. These included a region having enhancer-like activity, two regions with negative regulatory activity and two different promoter regions. From analysis of CAT activity it is clear that the negative regulatory regions strongly repress the potential transcriptional activity. Kim and coworkers demonstrated that deletion of the enhancer region prevented the transcriptional activity of the upstream TGF-β1 promoter. They concluded that at least 453 bp upstream from the first transcription site was required for maximum expression of the hybrid gene. A number of consensus sequences were also found in the promoter region. These included several CCGCCC motifs in a repeat, some in reverse orientation. It is known that removal of such repeats from

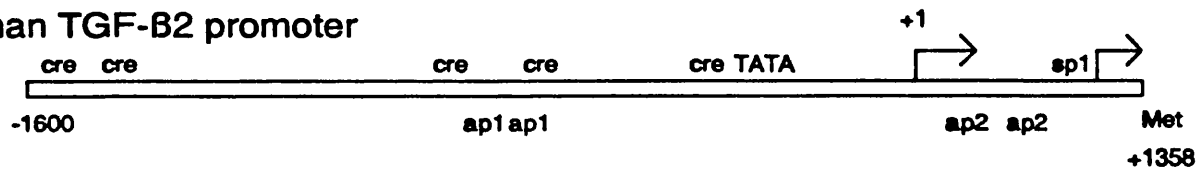
Figure 1. Comparison of the 5' untranslated regions of the three human TGF-ß genes. The major transcription factor binding sites are indicated, transcription starts from arrows above the sequence. The TGF-ß1 promoter lacks the conventional TATA box. In the TGF-ß2 promoter the AP-1 site appears not tobe functional; the existence of a second transcriptional start is suggested by indirect evidence regarding the size of various TGF-ß2 transcripts. The SP-1 sites in the TGF-ß1 and TGF-ß3 promoters and the CRE sites in the TGF-ß2 and 3 promoter have been demonstrated to be essential for transcription. Not drawn to scale.

Human TGF-ß gene promoters

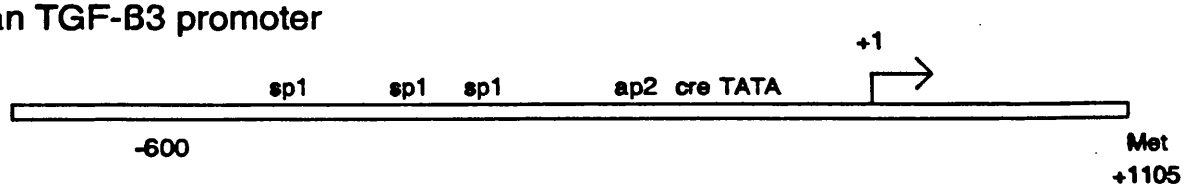
Human TGF-ß1 promoter



Human TGF-ß2 promoter



Human TGF-ß3 promoter



the SV40 early promoter has a detrimental effect on transcription, the motif is known to bind the SP-1 transcription factor (Fromm and Berg 1982). TGF-β1 promoter also contains a sequence which can bind NF-1, at sites –267 to –254, although in a reverse orientation to that described by (Nagata *et al* 1983). Akhurst *et al* (1988) have described the induction of TGF-β 1 by TPA. Two 7 base pair (bp) AP-1 sequences were found that are highly homologous to a common sequence found in the promoter region of several genes responsive to TPA. TGF-β1 does not possess the conventional TATA box.

1.3.5 TGF-β2 promoter

TGF-β2 protein is encoded by multiple mRNA transcripts (5.8, 5.1, 4.0, 3.8, and 2.8kb) that are expressed in a variety of cells. Some of the size differences are due to variation in length of the 5' untranslated region. The human TGF-β2 promoter region has recently been cloned independently by (Malipiero *et al* 1990; Noma *et al* 1991). Both reported sequences within the upstream region which are found in many promoters. They also reported some features unique to cytokine genes. Neither the promoter region of TGF-β1 nor TGF-β3 (described after this section) had any homology with the promoter region of TGF-β2. Noma *et al* determined the requirement for promotional activity by generating chimaeric constructs with the CAT gene. They showed that sequences capable of directing a high level of activity were contained up to –525 bp from the transcriptional start site. Several CRE like elements as well as AP-1, AP-2 and SP1 elements and a 50 nucleotide region characteristic of Z-DNA have also been identified (Noma *et al* 1991).

Multiple transcription initiation sites for TGF-β2 have also been identified (O'Reilly *et al* 1992). Transcription from one of these sites was dependent upon a CRE/ATF like element located 5' of the TATA box. Although the CRE/ATF like element deviated from the consensus sequence it was shown to be critical for promoter activity based upon point mutation analysis and the ability to form DNA-protein complex in a gel mobility shift assay. O'Reilly and coworkers also described different promotional activity in a variety of cell types, based on the use of sequences from –778 to –257 of the transcription start site. They argued that since the cells were from

different tissues then the DNA between –778 and –257 may contain tissue specific regulatory sequences.

1.3.6 TGF-β3 promoter

Lafyatis *et al* (1990) cloned the 5' untranslated region of human TGF-β3 in an attempt to define the structure of the promoter. They showed that a TATA box existed 21bp upstream of the putative transcription start site. 18 bp further upstream a consensus sequence for a CRE/ATF binding site was found. Immediately adjacent to this sequence, an AP 2 binding site was discovered. Three SP-1 sites were found at –358, –415 and –694 bp. The character of the 5' untranslated region of TGF-β3 was determined by the use of chimeric CAT constructs. This once again demonstrated that, as in TGF-β1 and TGF-β2, there were regions which could either up-regulate or down-regulate promoter activity (Lafyatis *et al* 1990). They demonstrated that the CRE modulated both basal and cAMP-inducible activity of the promoter. The fact that the CRE element was in close proximity to the TATA box was suggestive of the role of these two sites in the formation of a pre-initiation complex which had been previously described (Horikoshi *et al* 1988). The presence of the AP-2 site could be important due to the knowledge that retinoic acid increases expression of the AP-2 gene. This suggests that retinoic acid may play a role in regulating TGF-β3 during embryogenesis via the AP-2 site.

1.3.7 Latency of TGF-β

Most cells grown *in vitro* are known to secrete TGF-β in an inactive form (Delarco *et al* 1981; Moses *et al* 1981; Tucker *et al* 1983). It was demonstrated that treatment of conditioned media with acid (pH 3.6), followed by neutralisation, activated TGF-β as determined by anchorage-independent growth of NRK-49F cells (Pircher *et al* 1984). However it seems fairly unlikely that this is the favoured method of activation *in vivo*. Lyons *et al* (1988) pursued the mode of physiological activation further by investigating the effects of various proteases on latent TGF-β. By analysis of the effect of plasmin-treated conditioned media on anchorage-independent growth of NRK-49F cells, they were able to speculate that activation of latent TGF-β may take place by dissociation, or proteolytic

cleavage, from a precursor molecule or hypothetical TGF-β-binding protein (TGF-β-BP).

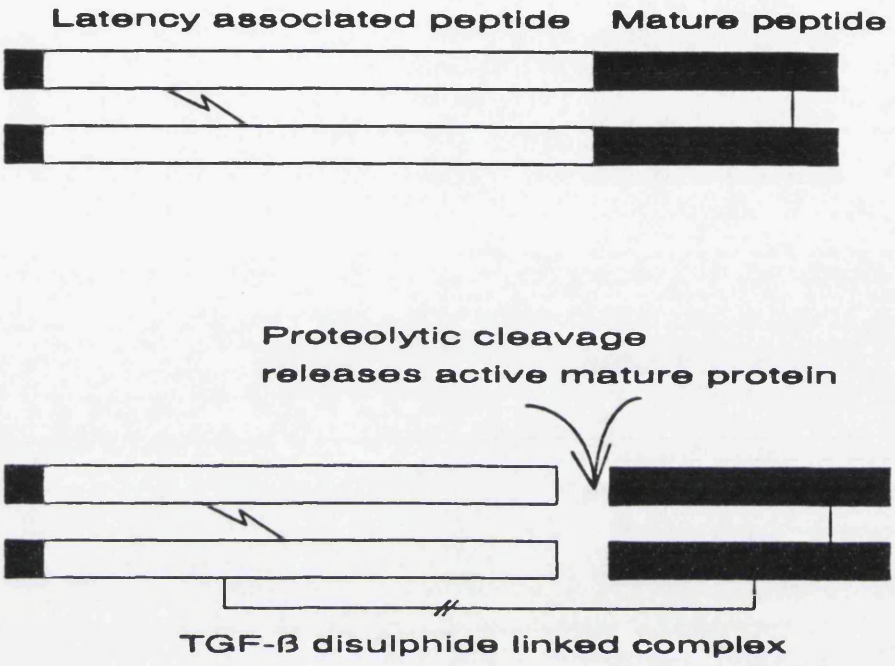
TGF-β1 is secreted from cells as a latent complex of high molecular weight. Since TGF-β1 is found universally *in vitro,* it is thought that activation of the latent complex may be a crucial step in the role of TGF-β1. Much of the work that has examined the latent complexes of the TGF-βs has revolved around TGF-β1. Although it is known that both TGF-β2 and TGF-β3 are produced in latent forms, and both can be activated, *in vitro*, in a similar manner described for TGF-β1. The structural characteristics of these forms remains unknown. The structure of both TGF-β2 and TGF-β3 proteins are fully characterised and do closely resemble that of TGF-β1, but it is possibly premature, to suggest that what we understand of the TGF-β1 latency complex applies in an identical manner in both TGF-β2 or β3 (Figure 2).

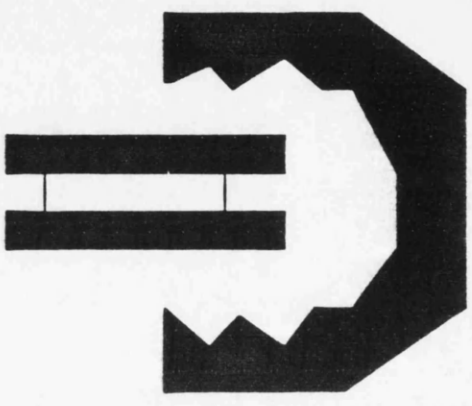
Miyazono *et al* (1991) analysed the large latent complex from mature platelets which consists of three components. The mature TGF-β1 is a covalently associated dimer, which is non-covalently associated with a disulphide-bonded dimer of the N-terminal remnant of the TGF-β1 precursor, known as the TGF-β1-latency associated peptide (LAP) (Gentry *et al* 1987). The third element of the large latent complex is the latent TGF-β1-binding protein (LTBP). It has been shown that LTBP is not a requirement for TGF-β latency, since transfection of Chinese hamster ovary cells with TGF-β1 precursor cDNA yielded a latent TGF-β1 molecule without LTBP. It is understood that the LAP is sufficient to confer latency on the TGF-β1 molecule. The complex which consists only of the LAP and TGF-β1 mature region is termed the "small latent complex" (Wakefield, 1989; Miyazono *et al* 1991).

Kanzaki *et al* (1990) cloned the cDNA coding for LTBP. Initially they purified LTBP from human platelets, and although attempts to sequence the N-terminal portion of the protein failed, indicating that the protein was blocked, sequence data was derived after the protein had been cleaved with proteases. Of 27 peptides sequenced, 22 showed similarity to EGF. They also found that several contained the consensus sequence for β-hydroxylation of asparagine/aspartic residues. These sequences are often

Figure 2. TGF-ß exists as a homodimer. The latency associated peptide is covalently bound to the mature peptide region. At the N-terminal end of the latency associated peptide is a signal sequence. The two monomers are bound to each other by disulphide bonds. The mature peptide is cleaved from the latency associated peptide at a cleavage recognition site. Inter-subunit disulphide bonds link the mature region to the latency associated peptide and prevent it's activation. The mature peptide of TGF-ß is not active until released from the latency associated peptide which can be done in vitro by low pH or heat. The latent TGF-ß binding protein is linked to the latency associated peptide. It's exact role is unclear as it is not required for latency. ß2-macroglobulin is thought to mop up active mature TGF-ß.

The TGF-ß polypeptide and its activation.





a2-macroglobulin mops up
active TGF-ß polypeptides

found in vitamin K-dependent proteins involved in blood coagulation. Although there are many proteins known to have hydroxylated asparagines, the exact nature of their function is still unclear, it is thought that they may play a role in Ca^{2+} binding. Kanzaki *et al* (1990) also demonstrated that LTBP does not bind nor inactivate mature TGF-β1. Incubation of LTBP with ^{125}I-labelled mature TGF-β1, followed by immunoprecipitation of the mixture with a TGF-β1 antibody, resulted in no observed binding by LTBP to the labelled mature TGF-β1. However, the labelled mature TGF-β1 was found to be cross-linked with the large latent complex. They suggested that this meant that in the large latent complex LTBP is located close to mature TGF-β1 but that LTBP in free form has no affinity for mature TGF-β1. They also suggested that if LTBP were to have a role in in keeping mature TGF-β1 latent it would be expected to interfere with the binding of mature TGF-β1 to cell surface receptors. Upon investigation, they were able to demonstrate that LTBP had no effect on the binding of labelled mature TGF-β1 to the cell surface receptors. This was compatible with their conclusion that LTBP does not bind and inactivate mature TGF-β1.

The role of carbohydrate stuctures in the latency of TGF-β have been assessed (Miyazono and Heldin, 1989; Sha *et al* 1989). Although mature TGF-β1 does not have any carbohydrate structures associated directly with it, both LAP and LTBP do. The LAP has three potential glycosylation sites, all of which are thought to be used (Miyazono and Heldin, 1989; Sha *et al* 1989). Treatment of LAP with endoglycosidase F leads to a dose-dependent activation of TGF-β1 (Miyazono and Heldin, 1989). Miyazono *et al* (1991) suggested that LTBP plays a critical role in the assembly and secretion of latent TGF-β1. Using pulse-chase experiments with labelled TGF-β1 they were able to follow the synthesis and secretion of the various forms of latent TGF-β1. They found that within 15 minutes of initiation of TGF-β1 synthesis, LTBP became associated with TGF-β1, and within 30 minutes this structure had been secreted. They also found that TGF-β1 which had not bound to LTBP was localised within the cell and was only secreted extremely slowly. Furthermore, the results of partial tryptic digestion suggested that this unattached TGF-β1 molecule contained inappropriate disulphide bonding.

O'Connor and Wakefield (1987) described a latent form of TGF-β1 which was in a complex with α_2-microglobulin found in serum. They proposed that this complex may be a method by which excess TGF-β1 is scavenged from the serum, possibly to restrict action of active TGF-β1.

Brunner *et al* (1989) demonstrated that by changing cysteine residues present in TGF-β1 it is possible to produce molecules which require no modifications to become active. By the use of site directed mutagenesis they changed two cysteine residues (Cys^{223} and Cys^{225}) to serine residues. When the construct containing these mutations was transfected into cells, only monomeric precursor proteins were expressed which released bioactive TGF-β1 that did not require acid activation. They suggested that dimerisation of the precursor pro-region may be necessary for latency.

1.4 TGF-β receptors

The first TGF-β receptor to be identified, by ligand-directed radioactive labelling, was a high molecular weight proteoglycan which was found in great abundance in many cell types (Massague and Like, 1985). Since then six receptors which bind TGF-βs have been described: types I and II (Laiho *et al* 1990; Frolik *et al* 1984; Cheifetz *et al* 1990; Lin *et al* 1992), type III (β-glycan) (Massague and Like, 1985; Lopez-Casillas *et al* 1991; Wang *et al* 1991), type IV (Cheifetz *et al* 1988a), and type V (O'Grady *et al* 1991).

The sixth and seventh TGF-β receptors have recently been identified (MacKay and Danielpour, 1991). They have been found on a variety of cell lines and have distinct molecular weights of 150kDa and 180 kDa. They have had no function attributed to them so far.

Receptors Type I,II and III have, been found in all cells examined, except for cells derived from retinoblastomas, which lack all three. The lack of receptors in these tumour cells is thought to play a specific role in the progression of this particular cancer (Kimchi *et al* 1988). Receptor type IV is limited to cells from a pituitary tumour cell line and is a special case in

itself as it is the only type of receptor which cross hybridises with other members of the TGF-β superfamily (e.g. activin). Receptor type V was purified from plasma membranes of bovine liver. The 400kDa receptor has been identified on cultured cells, including cells reported to lack the type III receptor (O'Grady *et al* 1991). It should maybe be noted that no cell line has been found which responds to TGF-β without having at least one type of TGF-β receptor present. Receptors Types I, II, and III are best characterised so far.

1.4.1 Type I receptor

Type 1 receptor is the smallest at 53 kD (Cheifetz *et al* 1990). The receptor binds TGF-β1 with a higher affinity than TGF-β2, whether TGF-β3 binds to this receptor is not known. About 8% of type I receptors have N-linked carbohydrates. This carbohydrate moiety is not required for integration of the receptor into the cell membrane nor is it required for ligand binding. However binding stability may be reduced if it is not present since the absolute labelling intensity of type I receptor is reduced in response to the inhibitor of co-translational glycosylation, tunimycin (Cheifetz and Massague, 1989). There are approximately 300 to 4000 type I receptor binding sites per cell on the cell surface which is in contrast to the type III receptor which has 10,000 sites per cell.

1.4.2 Type II receptor

Type II receptor is an 83kDa protein previously described due to its ability to bind radiolabelled TGF-β (Segarini *et al* 1989). The type II receptor was recently cloned (Lin *et al* 1992). Analysis of the cDNA predicted that the type II receptor would possess cysteine rich extracellular domain, a single hydrophobic transmembrane domain, and a cytoplasmic serine/threonine kinase domain. Lin and co-workers demonstrated that the serine/threonine kinase domain was functional, which implicates serine/threonine phosphorylation as an important mechanism of TGF-β receptor-mediated signalling. The type II receptor is related to two other serine/threonine kinase receptors. One is an activin receptor the other, daf-1, is found in *C. elegans*. Based on this fact Lin *et al* (1992) predicted

that the ligand for the daf-1 receptor will be a member of the TGF-β superfamily.

1.4.3 Type III receptor (β-glycan)

Receptor type III or β-glycan is an integral membrane proteoglycan, approximately 200 kD in size (Cheifetz *et al* 1988b). It is bound by either TGF-β1 or β2 with equal affinity. Type III also has the distinction of being able to bind a heterodimer composed of monomers of TGF-β1 and TGF-β2. Receptor type III was "expression cloned" very recently (Wang *et al* 1991). They determined that the gene coded for an 853 amino acid protein with a large N-terminal extracellular domain, containing at least one site for glycosaminoglycan addition, a single hydrophobic transmembrane portion and a 41 amino acid cytoplasmic tail with no obvious signalling motif. Wang *et al* (1991) demonstrated that overexpression of type III receptor in the L6 myoblast cell line, which lacks endogenous type III receptor, leads to an increase in amount of ligand bound, and cross-linked, to surface type II receptors. They suggested that this indicated the regulation of ligand binding ability or surface expression of type II receptor by type III receptor. The lack of an obvious signalling motif plus the control over type II receptor suggests that the type III receptor might function in an entirely different role than either type I or type II.

1.4.4 Type IV receptor and others

Type IV receptor was isolated from rat pituitary tumour and is unique amongst TGF-β receptors due to its ability to bind activin and inhibin as well as TGF-βs (Cheifetz *et al* 1988a). Type IV receptor was isolated from plasma membranes of bovine liver. The 400kDa non-proteoglycan receptor was also found on cells, *in vitro*, that had no type III receptor present. It also showed equal specificty for TGF-β1 and TGF-β2. The latest two receptors for the TGF-βs have been isloated from a variety of cell lines. The 150 and 180kDa glycoproteins were found to only bind TGF-β1 and did not bind TGF-β2 (MacKay and Danielpour, 1991). The role of these other receptors has not, as yet, been defined. MacKay and Danielpour suggested that the 150 nand 180 kDa receptors may be involved in

maintaining a pool of TGF-β1, or may be involved in the presentation of TGF-β1 to other receptors.

None of the receptors type I, II and III described above bind the latent form of TGF-βs, which is probably a mechanism by which binding is prevented from occuring in the golgi bodies during the production and excretion of the molecules (Fanger *et al* 1986). The affinity of TGF-β3 binding has not been determined, as thoroughly as for that of TGF-β1 and β2, for the receptors in many cell types but it is already known that TGF-β3 has a variety of effects on cells which probably reflects the binding affinity of TGF-β3 to these receptors, or other specific receptor(s), not yet identified.

1.4.5 Receptors modes of action

The multifarious effects of TGF-βs on cells is fairly well established (see section 1.6). However, it is still unclear as to how the TGF-β receptors mediate their response. It has been suggested that type I and type II receptors are involved in signal transduction (Laiho *et al* 1990). Laiho *et al* (1990) based their suggestion on the evidence gained from analysing two mutant mink lung epithelial cell lines (Mv1Lu). The first mutant had a normal profile of receptors type I, II and III but had lost the normal growth inhibitory response to TGF-βs (mutant S). The second class of mutants were also resistant to TGF-βs but they had selectively lost functional expression of receptor type I (mutant R). The lack of type I receptor and the phenotype of mutant R cells, in combination, led Laiho and co-workers to suggest that it was involved in trans-membrane signalling whereas the S mutants were suggestive of defects in some central component of the TGF-β transduction system. Laiho *et al* (1991) demonstrated the ability of the Mv1Lu cell mutants to be rescued by the formation of hybrids expressing the wild type receptors. Full responsiveness to TGF-β was produced as measured by inhibition of DNA synthesis, as well as stimulation of fibronectin and PAI-1 production. Their results suggested that R mutants may encode a normal type I receptor whose functional expression is rescued by the presence of a normal type II receptor provided by R cells in the hybrids. Their results provided evidence for an interaction between TGF-β receptor types I and II. They also demonstrated

that , in Mv1Lu cells, expression of both receptor types is required for mediation of biological responses to TGF-β1.

It has been proposed that cell surface proteoglycans may function as anchors for extracellular matrix molecules such as fibronectin and collagen, or as receptors for circulating molecules. This could implicate type III receptor as a port for activated TGF-βs thus removing it from circulation (Cheifetz *et al* 1988b). Induction of cell adhesion protein expression is an action of all TGF-β isoforms, and it has been suggested that this operates via the type III receptor based on the fact that this receptor binds to all TGF-βs equally well (Cheifetz *et al* 1988b).

Cheifetz *et al* (1990) showed that the same receptors and signalling pathways were utilised by TGF-β3, as by TGF-β2 and TGF-β1, at least in one cell line. Using one of the mutant Mv1Lu cell lines previously described, it was noted that, none of the three isoforms could inhibit the growth of the cells. They concluded that TGF-β3 did not have its own receptor/signalling pathway in this cell type. Although all three TGF-β isoforms inhibited growth in the parental Mv1Lu cell line, with similar concentrations producing a half maximal effect, there was still a 10 fold difference in receptor binding between the three isoforms, which could be physiologically important (Cheifetz *et al* 1990).

An important question, which arose from the investigations described above, is the apparent paradox observed in Mv1Lu cells. These cells are growth inhibited to an equal extent by each TGF-β, but it is clear that TGF-β2 binds less avidly than either TGF-β1 or TGF-β3 to the available receptors (Cheifetz *et al* 1990). How could TGF-β2 act via these receptors if its ability to bind them is much lower than that of either TGF-β1 or β3? This question was answered by identification of a subset of types I and II receptors which had high affinity for TGF-β2. The ability of TGF-β isoforms to compete with radioligands of TGF-β1 and TGF-β2 was examined. It was noted that although TGF-β3 and TGF-β1 competed successfully for binding with the type I receptor, at a concentration of 10pM, TGF-β2 minimally effected the binding of radiolabelled TGF-β1 to type I receptor, even when at high concentration (500pM). The same was true for the type II receptor, except that TGF-β3 had half the potency of TGF-β1. However TGF-β2 did

compete successfully against radiolabelled TGF-β2. Both TGF-β1 and β3 competed strongly for this apparent subset of high affinity receptors.

The combination of different TGF-βs binding a variety of receptors on a single cell multiplies the complexity of the effect of TGF-βs on the cell. It is therefore vital to elucidate the consequences to the cell after TGF-βs have bound to the receptor, and whether the combinations of receptor binding do affect the cell in a contrary way. Wakefield, (1987) demonstrated that binding of TGF-β to various cell types was not altered by heterologous agents which are known to effect the cellular response of TGF-β. These included EGF and PDGF, which are required for TGF-β to induce colony formation in soft agar. They demonstrated that EGF and PDGF caused a 20% and 0% reduction in binding, of TGF-β to receptors, respectively. Based on this evidence they suggested that modulation of TGF-β binding to its receptor may not be a primary control mechanism in TGF-β action.

1.5 Members of the TGF-β superfamily

There are five other gene sub-groups which are related to TGF-βs and each other, by dint of homology, at the amino acid sequence of the mature coding region.

1.5.1 Mullerian Inhibiting Substance

During male embryogenesis, the Mullerian duct, which would develop into the female reproductive tracts, regresses. Although testosterone, produced later in male sexual development, is responsible for the differentiation of the vas deferens, seminal vesicles and epididymus from the Wolffian duct, complete male sexual development is not possible without the regression of the Mullerian duct. The existence of a Mullerian inhibitory substance (MIS) was postulated to explain the phenomenon of Freemartin calves which are masculinised females of a heterosexual twin pair, whose Mullerian ducts have been obliterated (Lillie, 1916). It wasn't until over half a century later, that MIS was isolated and shown to be localised to the Sertoli cells of the testis (Josso, 1992; Blanchard and Josso, 1974) and was found to exist in the calf testis some considerable time after birth

(Donahoe *et al* 1977). MIS not only has a role in development but has been demonstrated to inhibit growth of tumours derived from tissues related to the Mullerian duct (Donahoe *et al* 1979; Fuller *et al* 1982).

MIS is thought to be a dimer of disulphide linked subunits with a total molecular weight of 140kDa (Budzik *et al* 1983). The dimer is made up of two monomeric units which differ slightly in molecular weight (70kDa and 74kDa). Cate *et al* (1986) suggested that the smaller polypeptide is a proteolytic product of the larger polypeptide, or that they undergo different forms of post-translational modification. Since the molecular weight of mature MIS protein is deduced to be 58kDa from the amino acid sequence it is obvious that MIS undergoes extreme post-translational modification.

A comparison of the bovine and human MIS protein shows a remarkable degree of homology (78%) with 108 of the last 112 amino acids being conserved. The conserved C-terminal region is a distinct domain with significant homology to the mature forms of the TGF-βs.

1.5.2 Decapentaplegic-Vgr related group

The decapentaplegic-Vgr related group (DVR) is an amalgamation of the bone morphogenetic proteins, the *Xenopus Vg* gene, and the mammalian homologue *Vgr-1*, plus the *drosophila* gene *dpp*. Previously these members of the TGF-β superfamily were considered distinct, recently (Lyons and Moses, 1990) has suggested grouping them together as they are more closely related to each other than to other members of the TGF-β superfamily.

1.5.2.1 Decapentaplegic gene

The *Drosophila* gene, decapentaplegic (*dpp*), cloned by Padgett *et al* (1987) is a member of the TGF-β superfamily. The C-terminal 110 amino acids of the *dpp* polypeptide show extensive similarity to the comparable regions of the vertebrate TGF-β family members. The *dpp* polypeptide contains seven of the nine cysteine residues conserved in the C-terminal region as well as three arginine-arginine dipeptides which are the potential site of proteolytic cleavage (Gelbart 1989). An analysis of amino acid

homology shows that mammalian BMP-2A and BMP 2B are very strong candidates for vertebrate analogues of *dpp* (Gelbart, 1989). It seems that the presence of a transforming growth factor-β-related-molecule, *dpp*, can specify the positional fate of cells through the regulation of homeotic genes in *Drosophila*. Panganiban *et al* (1990) demonstrated that *dpp* can regulate homeotic genes *lab* and *Scr*. Reuter *et al* (1990) also demonstrated that *dpp*, itself, is regulated by two homeotic genes, *Ubx* and *abd-A*.

Panganiban *et al* (1990) associated three responses during the development of the *Drosophila* midgut that involve homeotic gene expression.

The development of the midgut of *Drosophila* involves the migration and stretching of cells in two distinct germ layers to enclose the yolk. The double walled tube is then compartmentalised into four morphologically distinct chambers and four caeca. The four chambers of the midgut are created when three constrictions form at specific positions along the length of the migut. The caeca are formed as buds from the anterior end of the midgut (described in Panganiban *et al* 1990).

Four homeotic genes are expressed, in a non-overlapping manner, in the developing midgut *Scr*, *Antp, Ubx* and *abdA*. The absence of *Scr* results in abnormal caeca development. The lack of the other three homeotic genes, *Antp*, *Ubx*, and *abdA* results in no development of the first, second, and third constrictions respectively.

The expression of *dpp* in the visceral mesoderm of the developing midgut makes it an ideal candidate for the participation in the control of midgut development.

Expression of both *dpp* and the homeobox gene *Ubx* is required for development of the midgut. These two genes positively regulate the expression of each other. In the absence of *Ubx*, *dpp* expression is reduced, also the level of expression of *dpp* is conditional on which *Ubx* allele is present and the lack of *dpp* causes a reduction in the level of *Ubx* expression. *dpp* polypeptide was noted to migrate from the visceral mesoderm across the endoderm and was thought to induce the expression

of the *lab* gene in the endoderm cells (Panganiban *et al* 1990; Reuter *et al* 1990).

Panganiban *et al* (1990) suggested that the significance of these regulatory relationships to morphogenesis by *dpp* is best indicated by the fact that mutations in *dpp* alter the same structures in the midgut of the *Drosophila* which are affected by mutations in two of the homeotic genes regulated by *dpp*. That *dpp* functions as a secreted molecule, similar to TGF-β, is illustrated by its negative regulation of a third homeotic gene, *Scr*, across germ layers. dpp^{shv} mutants, a series of mutations that affect 5' regulatory sequences that result in embryos with normal dorsal-ventral polarity but which cannot survive beyond larval stages, due to mutations in internal organs (originally described by (Segal and Gelbart, 1985) . The domain of the homeobox gene *Scr*'s expression is extended anteriorly to include those cells that normally express *dpp*. The ectopic expression of *Scr* in these cells may prevent them from evaginating as caeca. Alternatively, ectopic *Scr* expression by itself may not be sufficient to block development of the caeca but may serve as an indicator that the cell fate at the anterior end of the midgut is changed in the absence of *dpp*. The actual mechanism by which the suppression of *Scr* occurs still remains to be determined (Reuter *et al* 1990).

This example of a member of the TGF-β superfamily reciprocally interacting with a homeobox gene during development may be significant in terms of the general role played by TGF-βs in cell fate and patterning in vertebrate embryogenesis.

1.5.2.2 Bone Morphogenetic proteins

Many growth factors have been implicated in bone growth and repair. Fibroblast growth factor (FGF), TGF-β and platelet-derived growth factor (PDGF) have been found in bone, and have been shown to have multifarious effects on bone and cartilage cells *in vitro* . However none have been shown to induce cartilage or bone formation *in vivo*. Urist (1965) demonstrated that extracts from demineralised bone, if implanted into ectopic sites in rodents, could induce new bone to form. Mesenchymal cells invaded the implant and were observed to differentiate into cartilage-

forming cells. Bone cells then appeared at the site. These were responsible for the gradual removal of cartilage and the deposition of new bone in its place. The active component in the bone extract was identified as being of protein in nature and named bone morphogenetic protein (BMP). It was still unclear as to whether this effect could be attributed to just one protein or a combination of many, acting concordantly.

In 1988 Wozney and colleagues cloned four human BMP cDNAs (BMP 1, BMP 2A, BMP 2B and BMP 3) (Wozney *et al* 1988). It was shown that three of the BMP's (BMP 2A, 2B and 3) were related to members of the TGF-β superfamily of proteins based on their extensive homology at the protein level with the *dpp* gene product. Comparison of BMP 1 with other known sequences led Wozney *et al* (1988) to speculate that it was a novel unrelated protein and will therefore be discussed no further. Recently a further three BMPs have been isolated but as yet, like there immediate relatives, little is known about their expression patterns or role during embryogenesis.

Each of the BMP's tested *in vivo* induced cartilage formation. Interestingly neither TGF-β1 nor TGF-β2 were able to induce cartilage formation *in vivo.* Despite the fact that both were originally isolated as molecules which induce formation of cartilage specific molecules by fibroblasts *in vitro* (Seyedin *et al* 1986).

The expression pattern of BMP-2 RNA has been described (Lyons *et al* 1990). A number of sites of expression of this gene overlap with expression patterns described for other TGF-β superfamily expression patterns. The potential for coordinated interaction between various members of the TGF-β superfamily will be discussed later.

1.5.2.3 Vg1 and related proteins

Weeks and Melton (1987) reported that Vg1, a maternal mRNA isolated on the basis that it is localised to the vegetal hemisphere of frog eggs, encodes a member of the TGF-β superfamily. Lyons *et al* (1989) isolated the mammalian equivalent of Vg-1 and termed it Vgr-1 (for Vg-related).

A putative, though unproven, function of *Vg-1* in *Xenopus laevis* is a role in the induction of mesoderm. In an effort to explain how different cell types are produced from a fertilised egg it has been suggested that there are diffusible factors present which regulate gene expression in the very early embryo. It has previously been demonstrated that mesoderm, the third embryonic germ layer, arises by an inductive interaction between the prospective endoderm and ectoderm (Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971; Dale *et al* 1985; Gurdon, 1987). Although many growth factors have been shown to be effective in inducing mesoderm formation in *Xenopus* eggs, all were from external sources. However, this does point to the possibility that the natural inducer(s) of mesoderm induction share many properties with growth factors.

Weeks and Melton (1987) investigated the role of Vg-1 as an inducer of mesoderm for a variety of reasons. Firstly the RNA is synthesised during oogenesis, is inherited by eggs and persists in the cleaving embryo. After the blastula stage the RNA is generally degraded. Secondly, Vg-1 RNA is localised in a tight crescent at the vegetal end of mature oocytes. Therefore, because Vg-1 RNA is temporally and spatially limited to an area known to provide the mesoderm inducing signal, it appears to be a likely candidate for that special role. However there is still no functional evidence for this role. TGF-β1, in association with FGF, and TGF-β2 have both been found to be capable of inducing mesoderm (Rosa *et al* 1992; Kimelman and Kirschner, 1987).

1.5.3 Inhibins and Activins

The final members of the TGF-β superfamily that I will describe are the activins and inhibins. These proteins are gonadal hormones which act upon the mammalian pituitary to inhibit or stimulate the release of follicle stimulating hormone (FSH), respectively (Mason *et al* 1985; Ling *et al* 1986). Inhibin is a heterodimer of an α subunit with one of two possible β subunits (represented as $\alpha\beta_a$ and $\alpha\beta_b$). The two β chains are closely related to TGF-β at 46% and 38% homology. The α chain is also related but much more distantly. Activin molecules are heterodimers of the two β subunits of inhibin. Activin A ($\beta_a\beta_a$) is identical to erythroid differentiation factor described by (Murata *et al* 1988). Activin B ($\beta_b\beta_b$) has been shown to

be the mesoderm inducing factor (MIF) described by (Slack *et al* 1989), and has been isolated from the *Xenopus* XTC cell line (Smith, 1987; Smith, 1989a; Smith *et al* 1989b). Meunier *et al* (1988) reported the expression of inhibin α, β_a and β_b in a variety of tissues in the rat. These included; the placenta, pituitary and adrenal gland, bone marrow, kidney and brain. They observed that the expression of an inhibin subunit could vary by several-fold in a tissue-specific fashion. Meunier *et al* (1988) concluded that the dimers of inhibin possessed diverse functions and may act as growth and/or differentiation factors as well as hormones. Petraglia *et al* (1989) demonstrated that inhibin and activin modulated the release of gonadotropin-releasing hormone, human chorionic gonadotrophin and progesterone from cultured human placental cells. They also noted that TGF-β itself was unable to induce this response. The results described by Petraglia *et al* (1988) support the hypothesis that activins and inhibins play a role in regulating hormone release during pregnancy. Spencer *et al* (1990) described the dose dependent inhibition of fetal adrenal cells *in vitro* by activin A. They demonstrated that activin A inhibited epidermal growth factor-stimulated fetal zone cell proliferation, but did not alter basic fibroblast growth factor stimulated growth.

Of the TGF-β superfamily, the activins and inhibins, are the only other group to have had receptors isloated (Matthews and Vale, 1991; Nishimatsu *et al* 1992). The receptors identified were all serine/threonine kinase, which indicates a protein phosphorylation signalling pathway.

1.5.4 Recent additions to the TGF-β superfamily

Growth/differentiation factor (GDF-1) was recently described, having been isloated from an 8.5 day *p.c.* mouse cDNA library (Lee, 1991). Northern analysis has indicated that there are two transcripts, 1.4Kb and 3.0Kb, of this novel member of the TGF-β superfamily, which show distinct temporal and spatial patterns (Lee, 1991). The 3.0Kb transcript is exclusively expressed in the brain of embryonic mice. This expression continues into adulthood (Lee, 1991). Burt, (1992) determined that GDF-1 is a member of the BMP group of proteins, and is related to *Xenopus* Vg-1 protein. Another member of the BMP group is OP-1 which encodes an osteogenic protein (Ozkaynak *et al* 1990). This novel protein was isolated from

enriched bovine osteogenic protein preparations, due to its sequence homolgy to dpp and Vgr-1. Little else is know about this gene.

The newest member of the TGF-β superfamily, 60A, was isolated from *Drosophila* (Doctor *et al* 1992). This gene is also a member of the BMP family (Wharton *et al* 1991). The first detectable level of expression of 60A RNA, and localisation of its protein, was noted at the onset of gastrulation, mainly in the mesoderm of the extending germ band. 60A RNA and protein continue to be detected in cells of the developing foregut and hindgut (Doctor *et al* 1992).

1.6 Action of TGF-βs *in vitro* and *in vivo*

The effect of TGF-βs on cells *in vitro* has been extensively investigated (reviewed by (Lyons *et al* 1990; Moses *et al* 1990)). It should be borne in mind that much of the early work did not distinguish between TGF-β isoforms, therefore when describing data from these papers the phrase "TGF-β" could refer to one, two or all of the isoforms. Where possible the specific isoform will be mentioned and comparisons made.

A generalisation which could be made of these investigations would be that TGF-β acts on epithelial cells as a growth inhibitor, and on mesenchymal cells as a growth stimulator/inhibitor (Barnard *et al* 1989). In some systems it is known that the extent of the growth response of the cells to TGF-β can be determined by its concentration (Battegay *et al* 1990). However, the *in vivo* observations of TGF-β action tend not to support these findings.

Qualitative differences in TGF-β1 activity can be determined by quantitative differences in TGF-β1 concentration. For example TGF-β1 is thought illicit a chemotactic response *in vivo*. Cells migrate towards a local source of TGF-β1, once the cells have reached higher concentrations, TGF-β1 no longer elicits a chemotactic response and the cells become stationary. The cells can begin to be influenced by other factors to differentiate, as well as being influenced by the high concentration of TGF-β1 which inhibits their proliferation (Moses *et al* 1990).

1.6.1 Effect of TGF-β isoforms on the extracellular matrix

One important area of action of TGF-βs is on the extracellular matrix (ECM). TGF-β is known to galvanise the expression of fibronectin, various types of collagen and matrix proteoglycans in many cell types (Ignotz *et al* 1987; Keski-Oja *et al* 1988; Chen *et al* 1987; Overall *et al* 1989; Lund *et al* 1987). TGF-β initiated the incorporation of fibronectin and type I collagen into the ECM of pre-adipocytes (3T3-L1) via the increased expression of cell adhesion protein receptors (Ignotz *et al* 1987). Keski-Oja *et al* (1988) demonstrated that mRNA levels of type I plasminogen activator inhibitor (PAI-1) were elevated, in response to TGF-β1, in cell line A549, which is derived from a malignant human lung carcinoma, and human lung fibroblasts (WI-38). They suggested that TGF-β may be regulating the accumulation of ECM by regulating the synthesis of PAI-1. They also suggested that the ultimate goal of TGF-β control of PAI-1 may be in control of the conversion of plasminogen to plasmin which is a key enzyme in the activation of collagenases and other enzymes. Interestingly, plasmin was put forward as a candidate protein for the activation of latent TGF-β *in vivo* (Lyons *et al* 1988). This suggests that TGF-β, PAI-1 and plasmin could be involved in a negative feedback loop, which limits TGF-β production and ultimately ECM content. More recently it has been demonstrated that, *in vitro*, plasmin only activates approximately 1% of latent TGF-β (Brown *et al* 1990).

The increment in collagen and fibronectin protein levels, in the ECM, were shown to be due to an increase in mRNAs of these genes upon the application of TGF-β (Varga *et al* 1987; Keski Oja *et al* 1988; Ignotz *et al* 1987). Ignotz and Massague (1986) described the disruption of induction of fibronectin, pro-α-1 collagen and pro-α-2 collagen by actinomycin-D, suggesting an involvement of transcription. In the case of collagen the upregulation is dependent upon the presence of nuclear factor type 1 (NF-1) (Rossi *et al* 1988). The promoter region for collagen possesses a 6bp recognition site for NF-1. Others have demonstrated that TGF-β can stimulate the production in fibroblasts of type II collagen (Seyedin *et al* 1985), type III collagen (Varga *et al* 1987), tenascin (Pearson *et al* 1988) and thrombosponin (Penttinen *et al* 1988). An increase in fibronectin was

also demonstrated in keratinocytes (Wikner *et al* 1988). Edwards *et al* (1987) described the repression of induction of collagen and stromelysin but the super induction of tissue inhibitor of metalloproteinases (TIMP), by TGF-β in human fibroblasts. They suggested that the control of ECM deposition depended on the differential modulation of metalloproteinase and TIMP expression. Many other enzymes which are known to degrade components of the ECM also have their level of transcription negatively controlled by TGF-β. These include elastase (Redini *et al* 1988), transin/stromelysin (Machida *et al* 1988), and thiol proteases (Chiang and Nilsen-Hamilton, 1986). From the accumulated data it seems that TGF-β has three main effects upon the consistency of the ECM.

(a) TGF-β stimulates the production of molecules which are constitutive of the ECM.

(b) TGF-β inhibits the enzymes which degrade the molecules described in (a).

(c) TGF-β stimulates the factors which inhibit the degradative enzymes.

As stated earlier, most of the above experiments only mentioned TGF-β, and occasionally were more specific when they talked of the effect of TGF-β1. It is only in the last few years that attempts have been made to compare and contrast the effects of the three TGF-β isoforms. In the main, it seems that they all have similar actions upon the cells investigated, although the degree of action does vary. Graycar *et al* (1989) showed that TGF-β1 is more potent than TGF-β2, which in turn is more potent than TGF-β3, at inhibiting the proliferation of MCF-7 cells. However TGF-β3 is more potent than either TGF-β2 or β1 in inhibiting DNA synthesis of human and mouse keratinocytes (Graycar *et al* 1989).

1.6.2 Effect of TGF-β on muscle

The effect of TGF-β on muscle *in vitro* has been investigated and was found to be inhibitory to fusion of myoblasts and formation of multinucleated myotubes (Florini *et al* 1986; Olson *et al* 1986; Massague *et al* 1986; McCaffrey *et al* 1989; Lafyatis *et al* 1991). This was characterised by the inhibition of expression of the muscle specific genes, creatine

kinase and acetylcholine receptors (Olson *et al* 1986). The effect of TGF-β could be reversed by the removal of TGF-β from the culture conditions (Olson *et al* 1986; Massague *et al* 1986; McCaffrey *et al* 1989). McCaffrey *et al* (1989) demonstrated that heparin potentiated the action of TGF-β when applied to smooth muscle cells (SMC). The suggested that the mode of action of heparin was to release TGF-β from α2-microglobulin. The action of TGF-β3 was investigated by (Lafyatis *et al* 1991). They found that TGF-β3 was able to inhibit myoblast fusion in C_2C_{12} sketally derived cells. After fusion of C_2C_{12} cells, TGF-β3 mRNA expression was increased eightfold. The expression of TGF-β3 in myoblasts was dependent on regions of the TGF-β3 promoter being present at –301 to –47 and also the long 5' untranslated region. Lafyatis and coworkers speculated that the role of TGF-β3 in adult myoblasts may be related to the function of it's, electrochemically activated, contractile tissue.

1.6.3 Effect of TGF-β on bone

TGF-β is found in relatively large quantities in bones (>5mg protein/Kg tissue) (Roberts *et al* 1990a; Roberts and Sporn, 1990b). Centrella *et al* (1988) has suggested that TGF-β plays a central role in the formation and continuous remodelling of mineralised tissues. TGF-β is thought to act on mesenchymal precursor cells, chondrocytes, osteoblasts and osteoclasts, stimulate cell replication and collagen production in cultured fetal rat bone cells (Centrella *et al* 1988; Centrella and Canalis, 1987), and induce chondrogenesis of embryonic rat mesenchymal cells (Seyedin *et al* 1987). Carrington *et al* (1988) described a time-dependent accumulation of TGF-β protein when cartilage is being replaced by endochondral bone. The highest concentrations of TGF-β protein were present when the conversion from calcified cartilage to bone was taking place, and consequently when osteoblasts were present. The extraction and immunohistochemical analysis, by Carrington *et al* (1988), indicated that the TGF-β was tightly bound to mineralised matrix of calcified cartilage and bone. They suggested that the compartmentalisation of TGF-β in the mineral phase may be a mechanism for storing latent or processed protein.

1.6.4 Effect of TGF-β on endothelial cells

Modulation of the response of microvascular endothelial cells during angiogenesis has been observed to correlate with changes in the ECM (Madri *et al* 1988). It has been widely reported that TGF-β inhibits endothelial proliferation *in vitro* (Jennings *et al* 1988; Madri *et al* 1988; Takehara *et al* 1987; Muller *et al* 1987). TGF-β was also found to block angiogenesis in 2-D cell culture systems (Muller *et al* 1987; Madri *et al* 1988). Madri *et al* (1988) correlated this inhibition with an increase in cellular synthesis and deposition of fibronectin. Interestingly, they also found that application of TGF-β to endothelial cells growing in a 3-D collagen matrix gel provoked the formation of tube like structures which they suggested may be mimicking angiogenesis. This supported and confirmed the hypothesis that TGF-β is angiogenic (Yang and Moses 1990). Takehara *et al* 1987 described the inhibition of endothelial proliferation and ascribed this to the decrease in number of high affinity EGF receptors. They noted that EGF-induced expression of specific genes (c-myc, JE, KC) decreased, whereas the induction of c-fos gene expression, by EGF, was unaltered by application of TGF-β. Merwin *et al* (1991) have demonstrated that endothelial cells respond differently to TGF-β1 and TGF-β2 *in vitro*. They analysed the effect of these two isoforms of TGF-β on bovine aortic endothelial cells (BAECs), rat epididymal fat pad microvascular endothelium (RFCs) and bovine aortic smooth muscle cells (BASMCs). In 2-D cell system all three cell types were inhibited by TGF-β1. TGF-β2 fully inhibited BASMCs, partially inhibited RFCs and had no discernible effect upon BAECs. The variation in receptor types found on the surface of these cell types corresponded with the observed inhibition patterns. Merwin *et al* (1991) continued this line of investigation by comparing the vascular cell response of TGF-β3 with the other two isoforms. In general they found that application of TGF-β3 generated similar responses to that of TGF-β1.

1.6.5 Effect of TGF-β on epithelial cells

TGF-β suppresses the growth of most epithelial cells in culture including keratinocytes (Moses *et al* 1985; Shipley *et al* 1986; Coffey *et al* 1988); bronchial epithelial cells (Masui *et al* 1986) and tracheal epithelial cells

(Jetten *et al* 1986). However, there are exceptions to this rule. TGF-β has been found to stimulate human mesothelial cells (Gabrielson *et al* 1988). Growth and differentiation are closely linked in epithelial cells. Inhibition of proliferation by TGF-β is often accompanied by terminal differentiation of the cells. It has been demonstrated that upon application of TGF-β to bronchial epithelial cells in culture the appropriate specific markers of differentiation are found (Jetten *et al* 1986; Kurokowa *et al* 1987). Human keratinocytes have been shown to possess functional receptors to TGF-β and respond in the same manner as do other epithelial cells in that ECM molecules are increased and there is a reversible effect on growth (Wikner *et al* 1988). TGF-β has been shown to selectively reduce the expression of certain genes associated with cell proliferation, namely c-myc and KC (Coffey *et al* 1988). It is thought that the control of these genes is at the post-transcriptional level. Glick *et al* (1990) have described the inhibition of DNA synthesis associated with the differentiation of keratinocytes which is mediated through the production and autocrine action of TGF-β2.

1.6.6 Expression of TGF-βs during embryogenesis

Many cell lines derived from embryonic material have been investigated to determine their relationship to TGF-βs (Hill *et al* 1986; Anzano *et al* 1986; Robey *et al* 1987; Seyedin *et al* 1985). The response of cells to TGF-β is dependent upon the presence of receptors on those cells. Rizzino, (1987) demonstrated that there was a considerable increase in numbers of receptors on differentiated cells derived from embryonic stem cells which initially had few receptors themselves. Robey *et al* (1987) described the secretion of TGF-β by bovine cells derived from fetal osteoblasts and suggested that this could be evidence for autocrine action. Rizzino (1985) had previously provided evidence that, in an *in vitro* assay system, early embryonic cells are capable of both synthesising and secreting TGF-β like growth factors, implicating the production of these factors in the events of early development. Rappolee *et al* (1988) demonstrated the presence of TGF-β mRNA in pre-implantation mouse embryos by the use of reverse transcription-polymerase chain reaction techniques. They were also able to detect TGF-β antigens in blastocysts and noted that TGF-β1 only appeared after fertilisation.

The presence of TGF-β mRNA and protein in the early embryo prompted others to investigate later stages of embryogenesis. Initially these investigations took the form of Northern analysis, which demonstrated the presence of the three TGF-β mRNA isoforms in a variety of tissues and different developmental times (Denhez *et al* 1990; Miller *et al* 1989). Heine *et al* (1987) demonstrated the presence of TGF-β1 protein during murine embryogenesis by immunohistochemistry, using a polyclonal antibody. They described TGF-β expression which correlated with specific morphogenetic and histogenetic events. The localisation of TGF-β1 mRNA was described, using *in situ* hybridisation with a riboprobe derived from a template for TGF-β1 (Lehnert and Akhurst, 1988). They showed that TGF-β1 mRNA had a specific pattern of expression in late mouse embryos. The pattern of expression described, compared well to that for the TGF-β1 protein and led Lehnert and Akhurst to suggest that TGF-β1 acted in both a paracrine and autocrine manner. TGF-β1 RNA and protein wre localised in perichondral osteocytes and osteocytes, involved in intramembronous ossification, suggesting that the protein produced by the cells was used in an autocrine manner. In contrast to this data they noticed that in tissues which had both an epithelial and mesenchymal component the TGF-β1 protein was found in the mesenchymal areas underlying epithelia which was expressing RNA. Examples of tissues which were suggestive of a paracrine mode of action were the hair follicles, tooth bud, submandibular gland and fetal heart (Lehnert and Akhurst, 1988).

The expression of TGF-β mRNA and protein has also been investigated in earlier stages of embryos (Akhurst *et al* 1990b; Slager *et al* 1991). Slager *et al* (1991) described the use of an antibody to TGF-β2 to determine the expression in very early mouse embryos. They examined embryos from the four cell stage up to 7.4 days *post coitum*. Slager *et al* (1991) showed high levels of staining of TGF-β2 protein in the trophoectoderm of the blastocyst but that there was no staining in the inner cell mass. During postimplantation development the primitive and embryonic ectoderm was negative while the visceral endoderm was positive, as was the parietal endoderm.

1.6.7 Role of TGF-βs during development of the heart

Discrete clusters of cells in the sparse reticulum of mesenchyme, which form the precardiac mesoderm, migrate from their original bilateral paired regions on each side of the embrionic axis, antermedially up the cardiogenic crescent and come to rest, differentiating into the primitive heart tube. The heart of the 8.0 day *p.c.* mouse forms a twisted tube of which the wall is , in most places, one or two cells thick. The primitive ventricular division, along with the truncus arteriosus, forms a funnel-like structure. In the ventricle, endocardium forms attachments, in a number of discrete places, with the myocardium. Between these points of attachment, the myocardium extends into the lumen, forming convex surfaces, leaving relatively large spaces between the attachments to be occupied by cardiac jelly. In the truncus arteriosus the attachments are fewer in number, and more widely seperated, and the central lumen is small compared with the volume occupied by cardiac jelly. In the atrium the endocardium is more closely applied to the myocardium. At 9.0 days *p.c.* there is a constriction at the atrioventricular (av) channel, formed by the endocardium backed by cardiac jelly, which in turn is enclosed by a wider constriction of myocardium. In the ventricle a definite trabecular system has developed. As development proceeds the cardiac jelly at the av constriction is replaced by mesenchymal cells (Viragh and Challice, 1977; Viragh, 1973; Viragh and Challice, 1980; DeRuiter *et al* 1992).

Expression of TGF-βs are seen in the heart as early as 7.0 days *p.c.* in the cardiac mesoderm (Akhurst *et al* 1990b). By 8.5 days *p.c.* TGF-β1 is restricted to the endocardial cells, which by 9.5 days *p.c.* is localised in those endothelial cells which lie over the cardiac jelly at the atrioventricular junction. At the same time TGF-β2 is localised in the myocardium at the atrioventicular junction (Millan *et al* 1991). The process by which the endocardial cells transform into mesenchymal cells and migrate into the AV valve to replace the cardiac jelly has been studied extensively. It has been shown to involve an inductive event which emanates from the myocardium (Krug *et al* 1985; Mjaatvedt *et al* 1987). Potts and Runyan (1989) demonstrated that the epithelial-mesenchymal transformation could be

mediated by TGF-β and that application of antibodies to TGF-β which block this activity.

1.6.8 Interaction of TGF-β with other growth factors

TGF-βs interact with a variety of other growth factors as well as with each other. The interaction of TGF-βs with the various growth factors, as so often, depends upon the cell type. The specific synergistic interaction of TGF-β with EGF/TGFα in the stimulation of anchorage-independent growth of NRK49F cells is well documented (De Larco and Todaro, 1978). This effect is probably mediated by an increase in EGF receptor levels which TGF-β exposure brings about in these cells (Assoian *et al* 1984). However, TGF-β alone inhibits the anchorage-independent growth of NRK fibroblasts (Roberts *et al* 1985). This probably indicates that the rise in EGF receptors which was observed, is not the only mechanism by which NRK cells transform.

TGF-β acts antagonistically with other growth factors. Inhibition of mitogenic stimulation of endothelial cells by FGF was noted (Baird and Durkin, 1986). TGF-β also acts as an antagonist to interleukin-, and colony stimulating factor-, induced stimulation of cell division in haematopoietic and lymphopoietic precursor populations TGF-β acts with bFGF to control plasminogen activity (PA). FGF causes a pronounced increase in PA whereas TGF-β decreases PA. The control of PA depends entirely upon which of the growth factors is added to the cells first (Saksela *et al* 1987). TGF-β1 is known to act synergistically with FGF to induce mesoderm in *Xenopus* (Kimelman and Kirschner, 1987).

Many biological effects of TGF-β may be indirect and mediated via the induction of PDGF. Low concentrations of TGF-β, which is generally percieved as a growth inhibitor, can induce an autocrine PDGF-AA loop resulting in connective tissue cell proliferation. However, a necessary element of this autocrine PDGF loop, the PDGF receptor α subunit is reduced at higher concentrations of TGF-β, limiting the extent of this proliferative response (Battegay *et al* 1990).

TGF-β1, TGF-β2 and TGF-β3 have been found to be auto-regulated in murine fibroblasts and keratinocytes (Bascom *et al* 1989). Treatment of these cell types with TGF-β1 resulted in upregulation of TGF-β1 expression very early after induction. Increased expression of TGF-β1 was correlated with increased TGF-β protein. Neither TGF-β2 mRNA nor TGF-β3 mRNA levels were significantly increased, but both appeared to be down regulated some time after induction. In contrast TGF-β2 treatment of the same cells increased expression of all three TGF-βs but with different kinetics. Autoinduction of TGF-β2 occurred later than that seen with TGF-β1. Bascom *et al* (1989) demonstrated that TGF-β2 regulates TGF-β2 and TGF-β3 mRNA at the transcriptional level, but that the control exerted over TGF-β1 by TGF-β2 is at both the transcriptional and post-transcriptional level.

1.6.9 Interaction of TGF-β isoforms with other developmentally important proteins

The induction of mesoderm in early *Xenopus* embryos has become a model by which many developmental processes hope to be explained (Nieuwkoop, 1969). The formation of the basic body plan during early amphibian development is believed to result from a sequence of inductive interactions between different regions of the embryo (Slack, 1983; Smith *et al* 1989). Dale and Slack, (1987) demonstrated that an inducing agent from the vegetal region caused the formation of mesoderm. A portion of the vegetal region, the dorsoventral region, induces the formation of the Spemann organiser. A signal from the organiser then induces mesoderm to be dorsally specified. Those ventrally specified mesodermal regions give rise to different tissues such as mesenchyme and blood. Whereas dorsally specified mesoderm differentiates to muscle and notochord.

The primary axis of the egg is the animal-vegetal axis which is formed in early ôogenesis. A peptide growth factor Vg-1 (which is a maternally derived mRNA and a member of the TGF-β super family), is synthesised during this period of axis determination. Initially it is distributed uniformly, later it is found anchored in the presumptive endoderm of what will become the vegetal half of the ôocyte (Melton, 1991). At the early blastula stage,

vegetal endoderm induces mesoderm in overlying cells. The cells that will give rise to ectoderm, mesoderm and endoderm are roughly positioned in three layers, top to bottom. It was also demonstrated that FGF could act in a synergistic manner with a TGF-β-like molecule to induce mesoderm from isolated animal caps (Kimelman and Kirschner, 1987).

The induction of *xhox3*, a homeobox containing gene, is an early response to mesoderm induction, and the level of *xhox3* expression marks the antero-posterior character of the induced mesoderm (Ruiz i Altaba and Melton, 1989). The establishment of antero-posterior polarity requires a system of positional information that specifies different fates for mesodermal cells according to their position at the end of gastrulation. Ruiz i Altaba and Melton (1989) suggested that the system includes a diffusible graded signal to account for the observed graded axial deficiencies in embryos obtained after perturbation by ultraviolet irradiation, gastrulation arrest or *xhox3* over expression. They proposed that the graded signal is likely to be one or more peptide growth factors and that gradients of peptide growth factors could set positional information and polarity along the antero-posterior axis in the responding axial tissues by regulating the level of homeobox gene expression.

1.7 Molecular mechanisms of TGF-β action

Some insights into the mechanisms by which growth factors operate have been determined by investigating oncogenesis. Considerable progress has been made in the understanding of how genes and proteins act to promote normal cell growth, and the discovery that mutant forms of some genes, (oncogenes and tumour sppressor genes), participate in the formation of various types of tumours. However, it is only in the past few years that studies into the molecular elements that act to constrain cell growth have been investigated. Negative growth control depends on two types of components:

(a) Signals that pass from cell to cell, often carried by diffusible molecules.

(b) A receptor signal-transducing mechanism that enables a cell to recognize and respond appropriately to extracellular signals.

TGF-βs fill all the requirements of a molecule which can pass amongst cells, however little is known of how its signal is passed on within the cell nor what the signal is passed on to.

1.7.1 Cell cycle and retinoblastoma protein

The mechanism of signal transduction of the TGF-βs has been investigated with respect to a few of its known functions, in particular, growth inhibition by TGF-β1. Negative growth control by TGF-β has been linked to suppression of retinoblastoma protein phosphorylation (Laiho *et al* 1990; Pietenpol *et al* 1990). The retinoblastoma gene (Rb) encodes a product with presumptive growth-suppressive activity. This activity has been predicted on the basis of the phenotype of human familial retinoblastoma, a malignancy in which loss of function, by whatever means, of the two Rb alleles is associated with loss of cell growth control (Levine and Momand, 1990). TGF-β1 seems to exert its effect upon Rb anytime during G1 phase of the cell cycle, and that TGF-β1 prevents phosphorylation of Rb. Rb is known to exert a negative growth control over the cell when in an unphosphorylated state and can in fact arrest cell growth in G1. Phosphorylated Rb is required before the cell cycle can proceed. Kim *et al* (1991) have demonstrated that TGF-β1, *c-fos* and *c-myc* can be regulated by Rb via a retinoblastoma control region (RCE). Whether the regulation is positive or negative depends upon the cell type. Hamel *et al* (1988) suggested that Rb does not directly regulate *c-fos* but carries out its actions via another factor, possibly E2F, a cellular transcription factor. Also it has been suggested that with regards to *c-myc*, another element 5' of the promoter is essential for the effects of Rb on expression (Pietenpol *et al* 1991).

1.7.2 Signal transduction and G-proteins

It has been determined that retinoblastoma cells have lost types I, II and III receptors to TGF-β which are usually present in normal retinal cells (Kimchi *et al* 1988). However, the significance of the lack of receptors for

TGF-βs on retinoblastomas may not be great since there is no Rb gene product to be phosphorylated. It may be that the loss of receptors is a secondary event.

The next question to be answered concerning the molecular action of TGF-βs is by what mechanism is the cell informed of the binding of TGF-β to one of it's receptors. Unlike other growth factors (e.g. TGF α, EGF, PDGF, FGF, insulin and IGF-1) which act, at least in part, through stimulation of receptor tyrosine kinase activity and modulation of inositol phosphate/protein kinase C signalling pathway, the signalling transduction mechanisms of TGF-βs have not been fully elucidated (Murthy *et al* 1988). Recent investigations have demonstrated that a family of proteins known as guanine nucleotide-binding regulatory proteins (G-proteins) participate in transduction of polypeptide growth factors such as interleukin, and IGF-II. The mechanism by which TGF-βs operate has been investigated by the use of a variety of toxins which selectively inhibit the signal transduction pathways (Murthy *et al* 1988; Howe *et al* 1989). Murthy *et al* (1988) and Howe *et al* (1989) demonstrated that the substrate for the action of pertussis and cholera toxins (PT and CT) were a 41kDa and 45kDa proteins respectively. Both groups suggest that these two proteins correspond to α subunits of two G-proteins, G_i and G_s respectively. These particular G-proteins modulate the activity of adenylate cyclase. Further evidence that G-proteins are responsible for transducing the TGF-β signal comes from evidence which demonstrates that TGF-β1-stimulated proto-oncogene expression, as well as TGF-β-stimulated GTPase activity and mitogenicity, are inhibited by PT. Furthermore, Howe *et al* (1989), demonstrated that CT and PT had differential effects on the signal transducing pathway. CT was discovered to inhibit TGF-β1-stimulated monolayer cell growth, whereas PT was without effect.

1.8 Generation of mutants as a form of analysis of gene function

Despite the plethora of data concerning the role of TGF-βs *in vitro*, their exact mode of action *in vivo* remains unclear. The fact that the individual

members of the TGF-β family have an effect on cells, peculiar to that isoform and cell system, complicates the picture. One method of exploring the role of a gene is to knock out the function of that gene. This can be achieved by a variety of techniques.

(a) Use of antisense oligonucleotides which bind to the specific mRNA preventing translation.

(b) The generation of dominant-negative mutants which eliminate protein function.

(c) Finally, disruption or replacement of the gene of interest with another (usually a marker) gene by homologous recombination in embryonic stem cells.

1.8.1 Use of antisense oligonucleotides or RNA

The use of antisense oligonucleotides has been used quite extensively *in vitro*. Melton, (1985) injected, antisense oligonucleotide and sense globin mRNA into frog oocytes. He demonstrated that an RNA-RNA duplex was formed and inhibited translation. These experiments proved that it was possible to prevent translation of as much as 500 pg of globin mRNA or the equivalent of 1% of the total mRNA in an oocyte. The use of antisense oligonucleotides has also been explored with considerable success for a variety of genes including: HIV virus genes (Goodchild *et al* 1988), c-*myc* (Wickstrom *et al* 1988), c-*myb* (Anfossi *et al* 1989), bFGF (Becker *et al* 1989). Runyan and Markwald, (1990) demonstrated that an antisense oligonucleotide specific to TGF-β3, when injected into heart explants, inhibited the epithelial-mesenchymal transformation seen in atrio-ventricular regions of the heart. Neither antisense oligonucleotides to TGF-β1 nor TGF-β2 had any effect. Runyan *et al* (1990) suggested that TGF-β3 was providing the message required to induce this event. Kimelman *et al* (1988) demonstrated that Xenopus oocytes contained an antisense mRNA transcript from the bFGF gene which coded for a 25kd protein. However, they also showed that this antisense mRNA interacted with the sense mRNA and suggested it may be involved in regulating the stability of the bFGF mRNA.

1.8.2 Dominant-negative mutants

The use of dominant-negative mutants as a tool to knocking out protein function is potentially a very powerful weapon in the arsenal of the molecular biologist. There are two main approaches which can be utilised.

1. The use of mutated proteins which compete with wild-type proteins in the formation of naturally occurring dimers or oligomers. The mutated proteins then prevent correct functioning of the protein in question. The type of mutation can vary between subtle point mutations which change potential N-glycosylation sites, or cleavage sites or gross mutations such as the generation of proteins, containing major deletions, which still allow them to bind to the wild type but prevent normal processing.

2. The use of anti-sense RNA as a method to inhibit translation of sense mRNA thus eliminating the gene function. The use of anti-sense RNA of considearble length alleviates the potential problems of specificity.

Mercola *et al* (1990) demonstrated the use of mutant protein with respect to platelet derived growth factor (PDGF). Using site directed mutagenesis of a PDGF-A cDNA clone, they identified two domains which are required to generate stable, mitogenically inactive PDGF-AA homodimers. The first mutant was created by an alteration in the coding sequence of the tetrabasic cleavage site of the PDGF gene. The second mutant changed one of the cysteine residues, to a serine residue, found in mature region of the PDGF protein. The mutants were able to form dimers with wild type proteins and negate their function *in vitro* either by preventing correct processing or by upsetting the conformation of the protein. They also noted that only the cysteine mutant dimerized with PDGF-B supressing its expression. The suppression of wild type activity was specific for PDGF and not other secretory proteins. Dimers containing the cleavage site mutation, (the only one secreted in detectable amounts) did not compete with PDGF-AA for biological activity. Mercola *et al* (1990) suggested that this meant the mutant dimer was very unlikely to bind the PDGF receptor.

Amaya *et al* (1991) employed a dominant negative mutant FGF receptor to analyse the role of FGF in *Xenopus* mesoderm induction. By generating a

truncated form of the FGF receptor which lacked the tyrosine kinase domain, Amaya and colleagues demonstrated that explants containing this dominant negative mutant failed to induce mesoderm in response to stimulation with FGF. In whole embryos the mutant receptor caused specific defects in gastrulation and in posterior development. They also showed that overexpression of a wild type FGF receptor could reverse these developmental defects.

The use of dominant-negative mutants lends itself to the analysis of TGF-β isoforms as they are active when dimeric.

1.8.3 Homologous recombination

The generation of animals which have had the gene of interest replaced by another gene has become a very powerful tool to follow the gene function *in vivo*, examples of which are: *Hox 1.1* (Zimmer and Gruss, 1989), *Hox 1.5* (Chisaka and Capecchi, 1991), *Hox* 3.1 (Le Mouelic *et al* 1992), *Hox* 1.6 (Lufkin *et al* 1991). The gene of interest is replaced by homologous recombination with another gene (either neo^r or *lacZ*) in embryonic stem cells. Those stem cells which contain the recombinant can then be used to form chimeras. The use of *lacZ* allows the mutation to be followed *in situ* during embryogenesis. Following the fate of cells affected by the mutation is especially important when the disrupted gene is supposed to have a regionalizing role during embryogenesis. Furthermore the *lacZ* gene could also be used to monitor the level of activity of the disrupted gene promoter, taking into account that integration of the targeting vector has the potential to modify activity.

1.8.4 Tissue targeting

The main problem with the methods of gene inactivation described above is one of specificity in relation to tissue or cell targeting. Many of the genes which are investigated have extensive patterns of expression. This means that although you can specifically select a gene to knock out you cannot yet target only one cell or tissue type. All of the reported attempts at knocking out gene function have concentrated on deleting global expression of the gene concerned. This is particularly true when using

homologous recombination as a method for introducing your mutant gene. However the use of dominant-negative mutants and antisense mRNA for the introduction of mutants does lend itself to the potential for tissue targeting. The appropriate construct would have to be driven by a specific promoter and then introduced into the organism by well established transgenic techniques. However, although many promoters have been isolated they have not been characterised to an extent which would allow confident targetting of a specific tissue. It has been suggested that inducible promoters such as that which drives the metallothionein gene could be utelised (De *et al* 1989). Unfortunately the metallothionein gene product is known to be expressed in a variety of embryionic cells which make up the uterus and amnion (De *et al* 1989). Also it is not clear how well controlled transcription from such promoters is and the potential problem of "leaky" expression would have to be overcome. This is especially significant when utelising mutants which disrupt genes known to have very extensive patterns of expression such as the TGF-β genes.

1.9 Aims

1.9.1 A comparative study of TGF-β isoform expression in post implantation mouse embryos using *in situ* hybridisation

The aim of this project was to determine the pattern of expression of TGF-β2 mRNA and TGF-β3 mRNA during murine embryogenesis from early stages up to 18.5 days *post coitum.* Previously the pattern of expression for TGF-β1 protein and mRNA had been published (Heine *et al* 1987; Lehnert and Akhurst, 1988). Once the pattern of expression for these two genes was determined they could be compared and contrasted with that known for TGF-β1 mRNA and protein. They could also be compared with the pattern of expression for the two proteins when this data became available. The method of choice for detection of expression of these two genes was *in situ* hybridisation using mouse specific probes to mouse embryos ranging in age from 6.5 days *p.c.* to 18.5 days *p.c.*. *In situ*

hybridisation has a number of advantages compared to other techniques such as immunohistochemistry and Northern analysis.

Riboprobes for use in detecting RNA in mouse embryos can be very specific for the genes which you wish to detect. This can be of vital importance as many genes, especially growth factor genes, exist in large families which are closely related. Prudent choice of insert from which to make riboprobes and the ability to alter the stringency of the hybridisation conditions can almost guarantee the fidelity of the result. Also compared to Northern analysis, which can usually only provide a general picture of expression, *in situ* hybridisation allows the detection of RNA in single cells. Immunohistochemical analysis can often lend support to data generated via RNA analysis. However, a major problem with data generated via immunohistochemical techniques is the specificity of the antibodies employed. Particularly in the case of large families of genes where there has been a large degree of conservation of amino acids the likelihood of cross hybridisation is greatly increased. Evidence for this problem of epitope recognition comes from two antibodies for TGF-β1 which although raised against the same sequence of amino acids they detect the intracellular and extracellular form of TGF-β1 (Heine *et al* 1987).

1.9.2 Approaches to address the function of TGF-β2 *in vivo*

To investigate the role of TGF-β2 in various developmental events a dominant-negative mutational approach was undertaken.

There are many sites of the TGF-β2 sequence which are available for mutation and which could potentially alter function in a dominant-negative fashion. These included the signal peptide sequence, the N-glycosylation sites, the cleavage site, or any of the cysteine residues found in the mature protein.

The N-glycosylation sites (of which there are three in the precursor region of TGF-β2) of TGF-βs are thought to be important for proper secretion from the cell (Sha *et al* 1989). It had also been reported that TGF-β1 which has the N-glycosylated structures removed may not need to undergo the usual

activation steps (Allen and Boxhorn, 1989). If this is the case then this could generate the opposite result to that required. The presence of three N-glycosylation sites meant that there was a possibility of having to carry out three seperate site-directed mutagenic steps. For these reasons the generation of a mutant which possessed altered N-glycosylation sites was dismissed.

The cysteine residues offered a potental target which had proven succesful in creating a dominant-negative mutant of PDGF (Mercola *et al* 1990). However, the presence of nine cysteine residues and the length of time available made this option not feasible. It was also known that in at least two cases the alteration of cysteine residues in TGF-β1 had resulted in a fivefold increase in activity (Brunner *et al* 1989).

A further option was the use of the LAP alone to bind wild-type protein. It had been suggested that this inhibited the cleavage of the mature region thus creating the required effect. The beauty of this approach is the relative ease with which a gross deletion of the mature region can be achieved by use of restriction endonucleases. Another possibility was the use of antisense RNA generated from hTGF-β2 which had been inserted into the appropriate vector in the opposite orientation. Instead of relying upon antisense oligonucleotides a piece of antisense RNA ranging in size from 50 nucleotides to full length cDNA could be generated in an effort to block translation of the endogenous wild-type mRNA. Again this type of dominant-negative mutation was easy to generate by judicious cloning of the human TGF-β2 gene, and was very likely to be specific in its knockout effect.

The final two options were mutation of the cleavage site sequence. This had proved succesful in the generation of a dominant-negative PDGF mutant (Mercola *et al* 1990) and would only involve one site directed mutagenesis step. The second option was the removal of the signal peptide sequence. Both these options were investigated. Initial characterisation of the mutants and their effect upon normal TGF-β2 (and a TGF-$\beta1^{33}$ mutant provided by A Purchio, Oncogen) expression was carried out using an eukaryotic expression system. Conditioned media was collected and analysed by growth inhibition assays and Western blot.

It was expected that once one (or more) of the mutant TGF-β2 clones had been fully characterised, and shown to act in a dominant-negative fashion, it would be employed in the generation of transgenic mice under the control of an appropriate promoter. Aternatively it could be introduced into mice via homologous recombination.

Chapter 2

MATERIALS AND METHODS

2.1 Introduction

All materials used throughout this project were sterilised to prevent contamination or degradation of samples. Solutions used in DNA analysis were millipore-filtered (0.45μm) and autoclaved and plasticware for use with DNA was autoclaved. Gloves were worn at all times. Materials employed in procedures which involved the use of RNA were treated as follows:

(a) glassware was washed thoroughly, rinsed in Milli-Q water and baked for a minimum of four hours at 180°C.

(b) plasticware was submerged in Milli-Q water with freshly added 0.01% DEPC for a minimum of four hours and sterilised by autoclaving.

(c) solutions were millipore-filtered and treated with 0.01% DEPC (except any Tris based solutions and organic solvents) before being autoclaved.

All procedures involving bacterial cultures were carried out using aseptic technique. Tissue culture was also carried out aseptically in a flow hood see section tissue culture.

A list of the specialist chemicals and enzymes used during the project, along with a list of suppliers, can be found in appendix A.

2.2 Recombinant DNA methods

2.2.1 General

To generate specific gene constructs the DNA inserts of interest were subcloned into a variety of vectors, each of which had its own unique function and use. This was carried out essentially by the methods described by (Sambrook *et al* 1989) with minor modifications. All steps in the various methods employed are described below.

2.2.2 Preparation of phenol and phenol:chloroform

Phenol was equilibrated by the addition of equal volume of equilibration buffer (0.5M Tris-HCl pH 8.0). The phenol and equilibration buffer were mixed thoroughly and allowed to settle. The aqueous phase was discarded and this step repeated twice. The pH of the equilibrated phenol was checked with pH paper, and 1/10 volume of 0.1M Tris-HCl pH 8.0 and 0.1% hydroxyquiniline added. The phenol was stored at 4°C in a light tight bottle. RNA-phenol was prepared in the same manner as described above. However, the equilibration buffer used was 0.3M sodium acetate pH 5.5. The equilibrated RNA phenol was stored under 0.1M sodium acetate pH 5.5, at 4°C, in a light tight bottle.

Phenol:chloroform was made by adding an equal volume of chloroform:isoamyalcohol (24:1) to equilibrated phenol (pH 8.0). This was also stored at 4°C in a light tight bottle.

2.2.3 Ethanol precipitation of DNA and RNA

DNA and RNA was precipitated by the addition of either sodium acetate pH5.5 or ammonium acetate pH5.5 to final concentrations of 0.03M and 2M respectively plus the addition of 2 volumes 100% ethanol (3 volumes for RNA precipitation). The sample was incubated on dry ice for 20 minutes (-20°C overnight) and the nucleic acid pelleted by centrifugation at 14,000rpm for 15 minutes. The supernatant was removed and the pellet washed twice with 70% ethanol. The nucleic acid pellet was air dried and

resuspended in an appropriate volume of sterilised dH_2O (RNA was resuspended in DEPC treated dH_2O).

2.2.4 Preparation of competent bacterial cells

Competent DH5α and MC1016/p3 bacterial cells (see appendix) were prepared by the method described by (Hanahan, 1983). Essentially, the two strains were prepared the same way.

The bacterial cells were streaked out from a glycerol stock onto a TYM plate (2% Bacto-tryptone, 0.5% yeast extract, 0.1M NaCl, 10mM $MgSO_4$, 1.5% bacto-agar pH 7.0) and incubated for 12-16 hours at 37°C in an inverted position. A single colony was used to inoculate 20ml of TYM broth (2% bacto-tryptone, 0.5% yeast extract, 10mM $MgSO_4$, pH 7.0) and incubated for 12-16 hours at 37°C with vigorous shaking (225rpm). The whole culture was transferred to 100ml TYM broth and incubation continued until the OD_{600} reached 0.5. This culture was then used to inoculate 500ml of TYM broth (which had been pre-warmed to 37°C) and incubation continued, under the same conditions as before, until the OD_{600} reached 0.6. The culture was gently mixed on ice water to ensure rapid cooling. The bacterial cells were harvested by centrifugation at 4000rpm for 5 minutes at 4°C in sterile bottles. The supernatant was discarded and the pellet of bacterial cells gently resuspended in 100ml of ice-cold TfbI (30mM CH_3COOK, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% glycerol (pH 5.8)) The cells were incubated on ice for 5 minutes then pelleted by centrifugation at 4000rpm for 5 minutes at 4°C. The supernatant was again discarded and the cells resupended in 20ml of ice-cold TfbII (10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% glycerol (pH 6.5)). The cells were divided into 100µl aliqouts in pre-cooled cryostat tubes, using pre-cooled tips, and snap frozen in an ethanol/dry-ice mix. The cells were stored at –70°C until required. An aliquot was checked for efficiency of transformation. If the number of colony forming units fell below 10^7/ug supercoiled DNA, then the cells were discarded and fresh competent cells prepared.

2.2.5 Restriction endonuclease digests of dsDNA

An appropriate quantity of plasmid DNA, not exceeding 200μg/ml, was incubated with the restriction enzyme (under the conditions described by the supplier), at a concentration of at least 2units/ug DNA, in buffer (supplied with the restriction endonuclease). If necessary 0.4mg/ml BSA and 0.5mM spermidine were added to aid the reaction. An aliquot of the digest was analysed on an agarose gel containing ethidium bromide (1μg/ml). Gel loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol; 30% glycerol) was added to the aliquot before loading which allowed the migration of the DNA to be followed. An appropriate DNA size marker was run alongside the aliquot of restricted DNA. The percentage of agarose in the gel varied between 0.8% to 2% in 1xTBE buffer (0.045M Tris-borate, 0.001M EDTA pH 8.0). The gel was run at 100V in 1xTBE buffer and viewed under UV light.

2.2.6 Filling recessed 3' termini of DNA fragments using Klenow enzyme

DNA, in quantities from 1μg-10μg, was end-filled to generate "blunt ends". This procedure was carried out immediately after the completion of the restriction endonuclease digest using the Klenow fragment of *E.Coli* DNA polymerase I. If the restriction endonuclease digest had been carried out in a volume greater than 20μl, then the DNA was precipitated and resuspended in 10μl of dH_2O. dNTPs were added to the completed DNA digest to give a final concentration of 2mM followed by addition of 0.5 units/μl Klenow DNA polymerase. The mixture was incubated for 30 minutes at 37°C. The filled fragment was purified as described in section 2.2.12.

2.2.7 Phosphatasing linearised dsDNA

To minimise the number of vector self-ligations, especially if vector ends are compatible, the 5' terminal phosphate groups were removed. 1μg Linearised vector was phenol extracted and precipitated with ethanol. The DNA was resuspended in 1x calf intestinal phosphatase (CIP) buffer (100mM Tris HCl pH 8.3, 10mM $ZnCl_2$). 5 units of CIP were added and the

reaction incubated at 37°C for 30 minutes, followed by incubation at 68°C for 15 minutes to deactivate the enzyme. The reaction mix was subjected to phenol (pH 8.0) extraction, at least three times, to ensure complete removal of CIP, and finally precipitated with ethanol.

2.2.8 Ligation of DNA fragments into linearised vectors

Ligations were performed with insert DNA in a 9:1 (weight:weight) excess to vector DNA (150-500ng insert DNA/ligation in a total volume of 10μl). Vector and insert DNA were added to ligation buffer (50mM Tris-Cl pH 7.5, 100mM NaCl), and the mixture incubated at 65°C for 3 minutes followed by incubation on ice for 5 minutes. 10mM ATP (1μl) was added plus 5 units of T4 DNA ligase (1μl). The ligation was incubated at room temperature for 16 hours. A further 5 units of T4 DNA ligase was added and the reaction incubated for a further four hours. Half of the ligation mix was used to transform the appropriate competent bacterial cells.

2.2.9 Transfection of construct into bacterial cells

Transfection of competent bacterial cells with plasmid DNA was performed by essentially the same protocol for DH5α and MC1061/p3 bacterial strains. Ligation mixture (approximately 6μl) was added to 50μl of the appropriate competent cells and 25μl of TfbII. The transfection mix was incubated on ice for 30 minutes (45 minutes for MC1061/p3) followed by heat shock at 37°C for 2 minutes (5 minutes for MC1061/p3). The cells were incubated on ice for a further two minutes. 1ml of L-broth (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl pH 7.0, antibiotic free) was added to the cells and the culture incubated at 37°C for 60 minutes (90 minutes for MC1061/p3) at 225rpm. The transfected cells were plated out onto L-agar plates (L-broth containg 1.5% bacto-agar with the appropriate antibiotic(s) (ampicillin 50mg/ml, tetracycline 20mg/ml) and left to incubate at 37°C for 12-16 hours in an inverted position. Control transformations were carried out simultaneously. They consisted of competent cells with no DNA and supercoiled DNA of known concentration. After overnight incubation colonies were picked and the plasmid DNA extracted by the small scale preparation method described in section 2.2.10.

2.2.10 Small scale preparation of plasmid DNA

This method was based on that described by (Serghini *et al* 1989), but with minor modifications. A single bacterial colony was used to inoculate 5mls of L-broth (1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl) containing the appropriate antibiotic(s) and cultured overnight at 37°C at 225rpm. 1.5mls of the overnight culture was microcentrifuged at 10,000rpm for 3 minutes. The supernatant was discarded and the pellet of bacterial cells resuspended in 100µl Solution I (50mM glucose, 25mM Tris-HCl, 10mM EDTA pH 8.0). An equal volume of phenol:chloroform (pH 8.0) was added, mixed thoroughly, and microcentrifuged for five minutes at 10,000rpm. The aqueous phase was retained and incubated at 37°C for 30 minutes with 50ug RNAse A. The plasmid DNA was subjected to a further phenol:chloroform extraction and ethanol precipitated as previously described. The DNA pellet was resuspended in 50µl dH_2O at a concentration of 0.5ug-1ug/µl, which was sufficient to allow diagnostic restriction enzyme digests to be attempted.

2.2.11 Large scale preparation of plasmid DNA

Large scale preparation of plasmid DNA was carried out by the alkali-lysis method as described in (Sambrook *et al* 1989). Alternatively plasmid DNA was produced using Qiagen columns (Qiagen ™), described after this section. The DNA produced from these columns was suitable for dsDNA sequencing and eukaryotic cell transfection without further purification.

The bacteria containing the plasmid of interest were streaked onto an L-agar plate which contained the appropriate antibiotic(s). The plate was incubated overnight at 37°C and a single colony used to inoculate 5mls of L-broth containing the appropriate antibiotic(s). The inoculate was cultured overnight with shaking (225 rpm) at 37°C. The 5ml culture was used to inoculate 500mls of L-broth, containing the appropriate antibiotic, in a 2 litre flask. The large scale culture was incubated overnight with vigorous shaking (225rpm) at 37°C.

Bacterial cells were harvested by centrifugation at 4000rpm for 10 minutes at 4°C, in a Sorvall rotor. The supernatant was discarded and the pellet

gently resuspended in Solution I (50mM glucose, 25mM Tris-HCl, 10mM EDTA pH 8.0) (2ml/50ml culture). The cell suspension was incubated on ice for 5 minutes. Solution II (0.4M NaOH, 1% SDS made freshly) was added (4ml/50ml culture) and the mixture inverted gently until it cleared. The mixture was then incubated on ice for 5 minutes. Solution III (5M KCH_3COOH, 11.5% glacial acetic acid) (6ml/50ml culture) was added and the solution mixed gently until a heavy white protein precipitate formed. The solution was incubated on ice for 30 minutes and then centrifuged at 5000rpm for 10 minutes at 4°C to pellet the white precipitate (bacterial chromosomal DNA). At this point the two methods diverged. The crude DNA preparation was further purified either by the method of Sambrook *et al* (1989) or by Qiagen™ column.

(a) Alkali-lysis method (Sambrook et al 1989)

The supernatant, containing the plasmid, was filtered through sterile gauze. 0.6 volume of cold isopropanol was added to precipitate the plasmid DNA. The sample was mixed thoroughly and centrifuged at 5000rpm for 10 minutes at room temperature. The supernatant was drained off and the pellet of plasmid DNA washed in 70% ethanol and centrifuged (5000rpm) at room temperature. The pellet was resuspended in 1/50th volume TE pH 8.0 and incubated at 37°C for 30 minutes with 50ug/ml RNase A. The plasmid DNA was then subjected to a phenol:chloroform extraction and precipitated with ethanol. The pellet was resuspended in sterile water (500µl) and the concentration determined by spectrophotometric analysis.

(b) Large scale preparation using Qiagen™ columns.

The Qiagen™ column was equilibrated with 10ml QBT (750mM NaCl, 50mM MOPS, 15% ethanol ph 7.0, 0.15% Triton-X). The supernatant containing the plasmid DNA (from (a)) was added to the column and allowed to enter by gravity flow. 30ml buffer QC (1M NaCl, 50mM MOPS, 15% ethanol pH7.0) was added and allowed to pass through. The plasmid DNA was eluted with buffer QF (1.25M NaCl, 50mM MOPS, 15% ethanol pH 7.0) and the eluant mixed with 0.7 volumes of isopropanol to precipitate the plasmid DNA. The mixture was centrifuged at 5,000 rpm for 30 minutes

followed by one wash in 70% ethanol. The plasmid DNA pellet was resuspended in 500µl of dH_2O and the concentration determined. The DNA produced via this method could be used directly for double stranded sequencing or transfections into eukaryotic cells without undergoing further purification.

2.2.12 Extraction of DNA fragments from agarose gels

DNA to be purified was run on an 1% agarose gel containing 1µg/ml ethidium bromide. The gel was viewed under a long wavelength UV lamp in order to minimise damage to the DNA. The DNA fragment of interest was excised from the gel using a sterile scalpel blade and purified by centrifugation through siliconised glass wool at 6,000rpm for 10 minutes as described by Serghini *et al* (1989). Two 1.5ml eppendorfs had their caps removed. One had a hole punctured in the base and a plug of siliconised, sterile glass wool inserted. The first eppendorf was placed into the second eppendorf and the gel slice placed on top of the glass wool. The gel slice was spun through the glass wool at 6,000rpm for 10 minutes. The DNA fragment was precipitated with ethanol and resuspended in dH_2O.

2.2.13 Purification of DNA using PlasmidQuik™ columns

Small concentrations of dsDNA required for sequencing reactions which had not been prepared from the Qiagen column method were purified using PlasmidQuik™ columns (Stratagene). The DNA could either be purified directly from a 5ml culture or from already existing DNA. Cells from 1.5ml of the overnight culture were harvested by centrifugation at 10,000 rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 110µl lysis buffer (8% sucrose, 10mM Tris-HCl pH 8.0, 50mM EDTA, 0.5% Trito-X-100 plus RNace-IT cocktail). The sample was incubated at 100°C for 30 seconds and centrifuged at 10,000rpm for 5 minutes. The pellet was discarded and 110µl of loading buffer added (1M NaCl, 5mM Tris-HCl pH 8.0, 0.1mM EDTA pH 8.0). The sample was incubated at 100°C for 5 minutes and immediately placed on ice. Thereafter the sample was applied to the top of a Plasmidquik™ column containing resin and pushed through with pressure applied from a syringe. The DNA was precipitated from the eluate by addition of an equal volume

of isopropanol and washed in 70% ethanol twice. This usually yielded 5μg of DNA.

2.2.14 Generation of synthetic oligonucleotides

Synthetic oligonucleotides were designed based upon the sequence data for human TGF-β2 (M19154.Embl). The oligonucleotides (P1,P2 and P4) were manufactured by Enzymatics of Cambridge (England). Oligonucleotides P5 and P6 were manufactured on an Applied Biosystems DNA synthesiser™ (E. O'Hare) (Figure 3).

2.2.15 Polymerase chain reaction (PCR) methodology

100ng of plasmid DNA, containing the appropriate insert, was mixed with specific sense and antisense primers (50pmoles), 0.2mM dNTP's and 2 units TAQ polymerase (Cambio) plus PCR reaction buffer (50mM KCl, 15mM $MgCl_2$, 100mM Tris-HCl (pH 8.0)) in a total volume of 100μl. A layer of mineral oil was placed on top of the reaction to prevent evaporation. PCR was carried out using a DNA Thermocycler (Perkin Elmer Cetus). The amplification consisted of the following sequential steps: denaturation (95°C for 1 minute), annealing (43-52°C, 1 minute) and elongation (72°C, 2 minutes). The PCR was run for 30 cycles after which the reaction was incubated for 10 minutes at 72°C. 10μl of PCR product was analysed by agarose gel electrophoresis on a 1% gel. The layer of mineral oil was removed by extraction with an equal volume of chloroform:isoamylalcohol (24:1).

2.2.16 Site-directed mutagenesis by PCR

The generation of the cleavage site mutation was carried out by site directed mutagenesis using primer (P4) which contains three mismatches with the known sequence and the sense and antisense primers (P1 and P2 respectively) of human TGF-β2 cDNA by the method described by (Stappert *et al* 1992) (Figure 4).

Primers, P2 and P4, were annealed to single stranded DNA template, generated from an M13mp18 vector containing the human TGF-β2 cDNA (see section 2.4.4), in a volume of 10μl containing 0.1pmole template DNA,

Figure 3.

All sequences are written 5' to 3'.

Oligonucleotides P1, P2 and P3 were used for generating the cleavage site sequence mutant. P4 contains three mismatch bases from the original DNA sequence which disrupt three of the five codons which generate the five amino acid cleavage recognition site. P1, P2, P5 and P6 were also used to prime sequencing reactions. P5 will anneal x bp downstream of the cleavage site sequence. P6 anneals y bp downstream of the end of the signal peptide coding sequence.

Oligonucleotides for PCR, sequencing and SDM

P1

5' AAT TCA AGC AGG ATA CG

P2

5' AAC TCT AAG AGT AAG AAT GG

P4

5' CAA AGC AAG CTT CTT CTG CAG GTT GG

P5

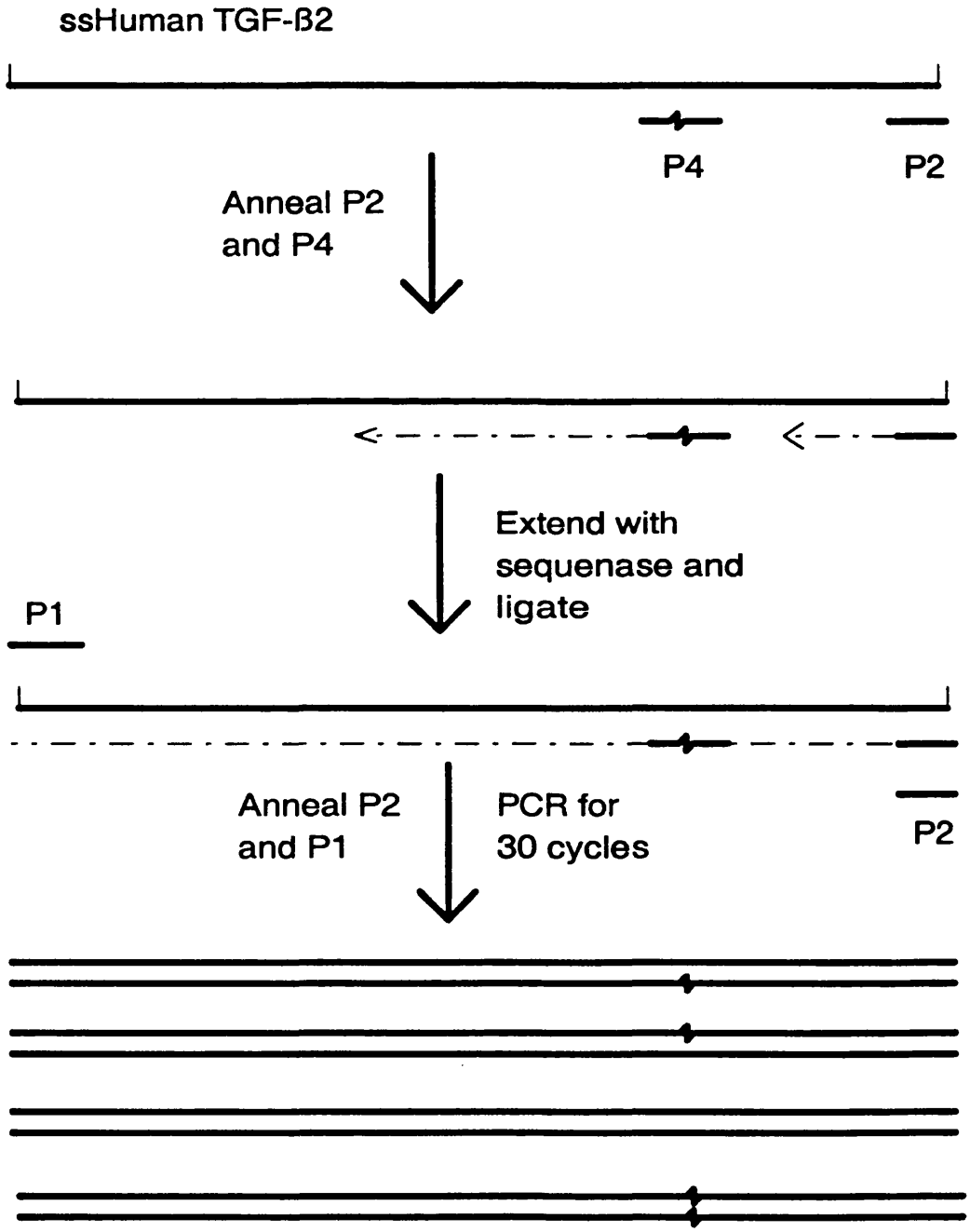
5' GTA AAG TGG ACG TAG G

P6

5' TCG CCT CGA TCC CTT G

Figure 4.
Site directed mutagenesis by PCR (See text for details). Single stranded human TGF-ß2 was generated and primers P2 and P4 annealed. Sequenase enzyme (USB) and T4 DNA ligase were added with dNTP's and a double stranded product formed with the mismatch P$ oligonucleotide incorporated into one of the strands. The heteroduplex is subjected to PCR using primers P1 and P2. The products are a mixture of four products. Two fragments contain one strand which has the mutated sequence in alternate strands. One fragment contains no mutated sequences. One fragment contains the mutated sequence in both strands. The PCR product which contains the latter fragment can be detected by restriction endinuclease digestion using HindIII and PstI which have had two new recognition sites generated by introduction of the mutated sequence. PCR products which contain products of interest were sub-cloned into piH3M

Site directed mutagenesis by PCR



10x excess of P2 and P4 (500pmoles each) and annealing buffer (20mM Tris-HCl pH 7.4, 2mM $MgCl_2$, 50mM NaCl). The mixture was heated to 65°C for 3 minutes and allowed to cool to room temperature for 30 minutes. Synthesis of the complementary strand was done in a volume of 20µl containing the same annealing mixture plus 2.5 units Sequenase™ (from the USB sequencing kit), 1 unit T4 DNA ligase (Pharmacia) and synthesis buffer (0.5mM each dNTP, 1mM ATP, 10mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 2mM DTT). The reaction was incubated at 37°C for 90 minutes and stopped by the addition of 10mM Tris-HCl pH 8.0 and 10mM EDTA. One fifth of the reaction was amplified by standard PCR using primers P1 and P2, as described above.

2.2.17 DNA sequencing

DNA sequencing by the dideoxy chain termination method (Sanger *et al* 1977) was performed upon double stranded (ds) DNA using Sequenase™ (USB). Prior to performing the sequencing reaction the dsDNA was treated in the following manner. Where the DNA had been produced with a Qiagen™ column it was not purified further. When, the DNA had been produced by the alkali-lysis method (see section 2.2.11), the sample was purified with a Plasmidquik™ purification kit (Stratagene) as described section 2.2.13.

A final concentration of 0.2M NaOH and 0.2mM EDTA (pH 8.0) was added to 5µg of purified dsDNA which was incubated at 37°C for 30 minutes. The reaction was neutralised by the addition of 1/10th volume 3M sodium acetate (pH 5.5). The samples were ethanol precipitated, washed at least twice with 70% ethanol, and the pellets of DNA resuspended in 7µl of dH_2O. The samples were treated exactly according to the protocol provided with the Sequenase Kit (USB). 2µl of Sequenase reaction buffer was added to the sample along with 1pM of the appropriate primer. The sample was incubated at 65°C for 2 minutes and allowed to cool to room temperature for 20 minutes. 1µl of 100mM DTT was added plus 2µl diluted labelling mix (diluted 1/5 in dH_2O), 1.0µCi of ^{35}S-dATP and 2µl of diluted Sequenase enzyme (diluted 1/15 in Sequenase enzyme dilution buffer). The samples were incubated at room temperature for 4 minutes. 3.5µl of each sample was mixed with 2.5 µl of each ddNTP, in tubes pre-warmed to

37°C, and incubated a further 3 minutes. 4μl of stop solution was added to each reaction. The samples were stored at 4°C until required. Prior to running the samples on a gel they were incubated at 80°C for 2 minutes and quenched on ice.

2.2.18 Preparation of sequencing gels

Sequencing gels containing 7.2% polyacrylamide were prepared by mixing 33g urea (Sigma), 13 mls 40% acrylamide (containing 2% bis-acrylamide (Sigma)), 0.75 mls 10% ammonium persulphate (BDH), 7.7 mls 10xTBE, 75μl TEMED (Sigma) and 33mls dH_2O and polymerised between glass plates 0.4mm apart. Gels were run at 34 Watts for a minimum of 2 hours, fixed in 10% methanol, 10% acetic acid for 15 minutes. The gel was dried for 45 minutes in a Biorad Gel Dryer at 80°C and exposed to X-ray film at room temperature overnight. The gels were read manually.

2.3 M13 recombinant DNA techniques

The M13 series of vectors are a very powerful tool in molecular biology because of the ease with which single stranded DNA can be produced from them. The analysis of M13 vectors was similar to that of other double stranded DNA vectors. dsDNA was sub-cloned into dsM13mp18 under the same conditions as described previously. Approximately 150ng DNA was used to transfect DH5αF' bacterial cells essentially by the same method described in subsection 2.2.9. However, after heat shock at 37°C for 2 minutes, 100μl of an overnight culture of either DH5α or TG1 cells and 3ml of top agar (0.5% NaCl, 0.7% Bacto-agar) was added and the mixture was plated immediately onto 2XNY agar plates (1% casein hydrolysate, 0.25% NaCl, 1.2% Bacto-agar) containing 20ug/ml *X-gal* and 25ug/ml IPTG. The plates were incubated overnight at 37°C in an inverted position. Blue plaques were indicative of the M13 vector religating with no insert present. Clear plaques, showing integration had occurred, were picked and used to inoculate 5ml of 2XNY broth (25% casein hydrolysate, 10% yeast extract, 2.5% NaCl). The inoculate was grown overnight at 37°C with vigorous shaking at 225rpm. Double stranded DNA was isolated by the small scale

plasmid preparation described in subsection 2.2.10. Single stranded DNA could be isolated as described in section 2.3.1.

2.3.1 Isolation of single stranded DNA from M13 recombinants

To retrieve single stranded DNA from an overnight preparation of M13 DNA, the supernatant produced when harvesting the bacterial cells was treated immediately. Alternatively the supernatant could be retained for later analysis although in order to maintain viability of the single stranded DNA the supernatant was incubated at 70°C for 10 minutes prior to storage at 4°C.

To 900µl of each supernatant, 250 µl PEG/NaCl (15% polyethylene glycol 8000, 2.5M NaCl) was added and the mixture incubated on ice for a minimum of 30 minutes. The supernatant mix was centrifuged for 5 minutes at 10,000rpm, at room temperature, after which the supernatant was discarded and the pellet washed with 300µl phenol buffer (0.3M NaCl, 0.1M Tris-HCl, 1mM Na_2EDTA pH 7.9). The pellet was resuspended in 150 µl of phenol buffer and subjected to a phenol/chloroform extraction followed by ethanol precipitation in 3 volumes of 100% ethanol. The pellet was finally resuspended in 20µl of dH_2O. The purity of the single stranded DNA was determined by gel electrophoresis and visualised by ethidium bromide staining. The single stranded DNA could then be used as a template to generate sequence data or for site directed mutagenesis.

2.4 Genes and vectors

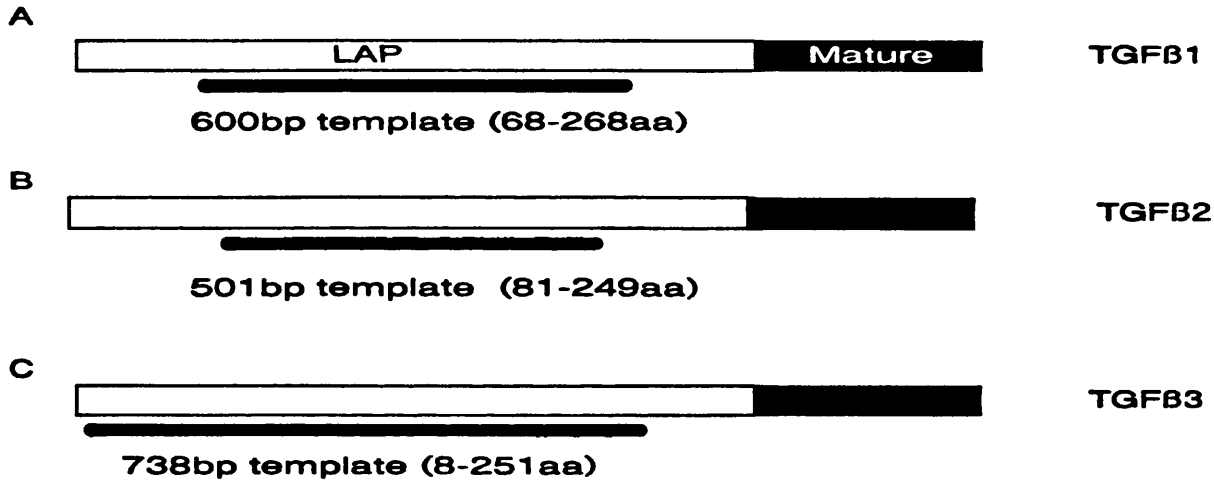
2.4.1 Constructs for generating TGF-β1, 2, and 3 specific riboprobes

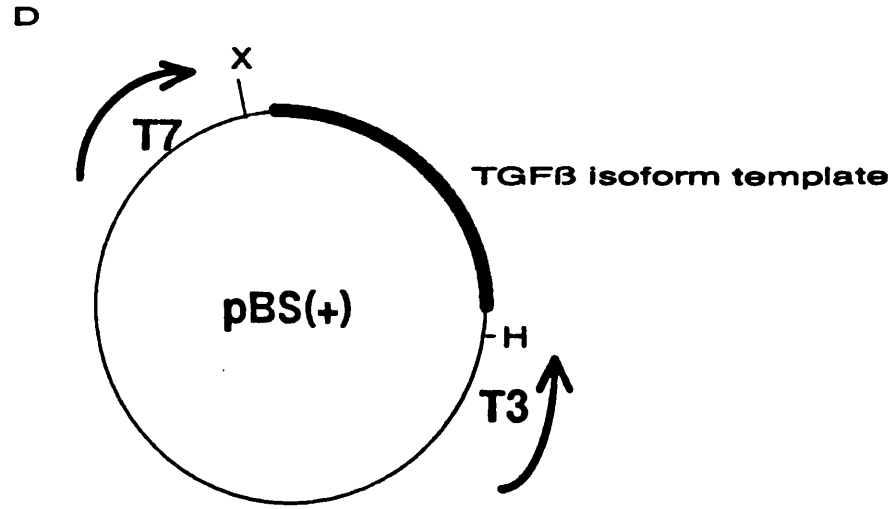
Murine TGF-β 2 and TGF-β3 cDNAs were supplied by F Dehnez and P Kondaiah. The murine TGF-β2 probe was a 501bp *PstI-SacI* fragment subcloned into Bluescribe in an antisense orientation with respect to the T7 promoter (by Margot McKerrell). The fragment coded for amino acids 81 to 249 of the TGF-β2 LAP. The TGF-β3 specific probe was generated from a

Figure 5.

Diagrammatic representation of the three mouse TGF-β isoforms. The solid line underneath each represents the template of DNA which was used to produce anti-sense RNA, and to show the region of protein which it encodes. (A) Mouse specific TGF-β1 template is 600bp and encodes amino acids 68-268 of the latency associated peptide (LAP) (Akhurst et al 1990). (B) Mouse specific TGF-β2 template is 501bp and encodes amino acids 81-249 of the LAP. (C) Mouse specific TGF-β3 template is 738bp and encodes amino acids 8-251 of the LAP. (D) Each of the templates was sub-cloned into transcription vector which has the bacterial promoters T3 and T7 either side of the multiple cloning site. The construct were linearised with respect to the promoter which would generate anti-sense RNA. X=XbaI, H=HindIII.

Specific TGF-ß isoform templates





738bp PCR fragment which had been sucloned into Bluescript KS II (by Paturu Kondaiah and Fabienne Denhez). The fragment coded for amino acids 8 to 251 of the TGF-β3 LAP and was orientated in Bluescript such that the T3 promoter generated antisense RNA. For *in situ* hybridisation analysis, a gene specific mouse TGF-β1 cDNA described by Akhurst *et al* (1990) was used. This consisted of a 600bp *KpnI – ApaI* fragment subcloned into Bluescribe (Stratagene) in an antisense orientation with respect to the T3 promoter. The fragment was derived from full length murine cDNA, (Derynck *et al* 1986), and corresponded to the region of the latency associated peptide (LAP) between amino acids 68 to 268. The three riboprobes shared between 35 to 47 % homology (See Figure 5).

2.4.2 Preparation of human TGF-β2 mutant

To investigate further, the role of TGF-β2 during embryogenesis, the generation of dominant-negative mutants was attempted as described in the Introduction. This involved the alteration of the coding sequence of human TGF-β2, and expression of the mutants proteins in an eukaryotic expression system. The analysis of the mutants generated required the use of a suitable vector and vector expression system. The vector chosen was πH3M which has a number of advantages compared to other vectors when expressing eukaryotic genes (see Figure 6).

Two constructs of πH3M containing simian TGF-β1 which had been mutated at cysteine residues 223/225, and cysteine residue 33 (Brunner *et al* 1989), were provided by Dr A F Purchio (Oncogen). These mutants had been shown to produce five times and normal levels of activity respectively. The πH3M-TGF-β1-(cys33) (TGF-$\beta1^{33}$) construct was used as a starting point for the generation of a series of further constructs that had the TGF-β1 cDNA replaced with human TGF-β2 cDNA (hTGF-β2) (provided by Dr G Bell). The πH3M-TGF-$\beta1^{33}$ construct was also used as a positive control. The hTGF-β2 cDNA was in a Bluescribe vector.

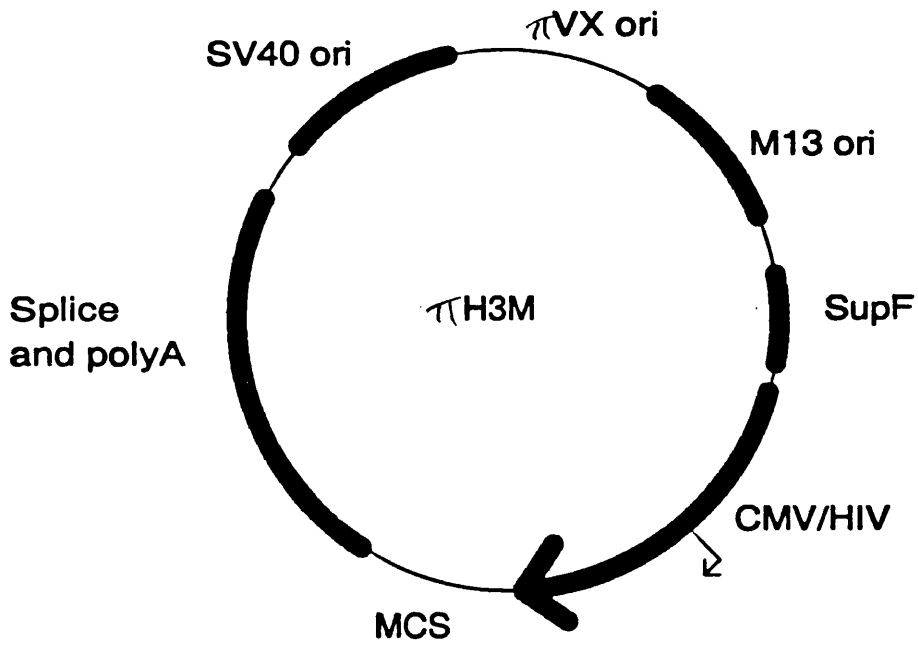
Firstly, the TGF-$\beta1^{33}$ was removed from the πH3M-TGF-$\beta1^{33}$ construct. The construct was digested with the restriction endonuclease *PstI*. The linearised construct had the recessed 3' termini filled in, by method described in section 2.2.6, to generate blunt ends. The construct was then

Figure 6

H3M SV40-based expression vector.

The direction of transcription from the eukaryotic promoter is indicated by the arrow. The vector is composed of seven segments. Segment 1 is the pBR322 origin of replication (pIVX). Segment 2 is the M13 origin of replication. Segment 3 is the supF gene (the selectable marker). Segment 4 is a chimeric cytomegalovirus/human immunodeficiency virus promoter. Segment 6 contains splice and polyadenylation signals. Segment 7 contains the SV40 origin of replication (M. Kriegler 1990). Not drawn to scale.

πH3M SV40-based expression vector



digested with *EcoRI* which isolated the vector from the TGF-β1[33] cDNA. This left the linearised πH3M vector with one blunt end and one *EcoRI* compatible end. The digested construct was run on a 1% agarose gel and the linearised vector excised as described in section 2.2.12.

The Bluescribe vector which contained a 2.3Kb fragment of hTGF-β2 was digested with the restriction enzymes *EcoRI* and *HhaI* which generated a 1.966Kb fragment. The 1.966Kb linearised fragment of hTGF-β2 had one EcoRI compatible end and one blunt end. This could be ligated into the πH3M vector in a directional sub-cloning step as described in section 2.2.8. The construct πH3M-hTGF-β2 was used as a starting point for the generation of the signal peptide sequence mutant (Figure 9 and 10).

2.4.3 Generation of signal-peptide sequence mutation

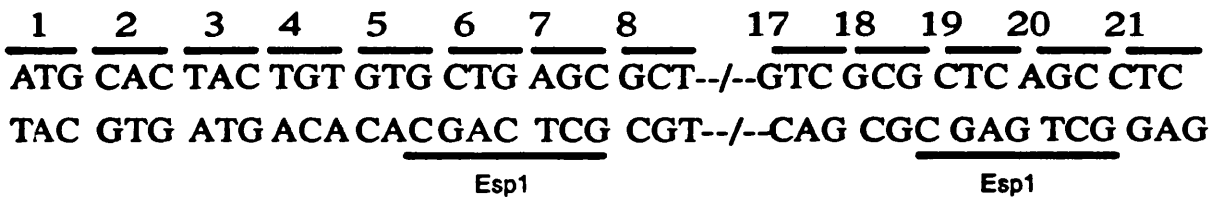
The signal peptide mutant was generated from the πH3M-hTGF-β2 construct by digestion with the restriction enzyme *EspI*. *EspI* cuts the hTGF-β2 gene at two sites, either side of the signal peptide coding sequence, and not at all in the vector. This resulted in the generation of two linearised DNA molecules (5.931Kb and 35bp). The digested construct was run on a 1% agarose gel and the 5.913Kb band removed from the gel and purified as described in section 2.2.12. The 35bp band, which contained most of the sequence which encodes the signal peptide was discarded.

The linearised construct, missing the 35bp of signal peptide coding sequence, could not be directly religated for two reasons. Firstly, this would have caused the sequence to jump out of frame (Fig 5). Secondly, it was not directly possible as the *EspI* restriction endonuclease recognition site is GC/TNAGC. The *EspI* sites were GC/TGAGC and GC/TCAGC respectively and were therefore did not produce compatible ends (Fig 7). The linearised construct was purified and the recessed termini end filled as described in section 2.2.6. The blunt ended construct was religated to produce the construct which coded for the signal peptide mutation (β2^{e}) (Figure 7).

Figure 7.
Diagram showing steps in the generation of the signal peptide mutation. (A) The first 21 amino acids make up the signal peptide sequence for hTGF-ß2. The solid lines under the DNA sequence are the locations of the Esp1 sites. (B) After restriction endonuclease digestion with Esp1 the two terminii are not compatible. (C) The two terminii are end-filled and can be ligated together. (D) The ligation of the two terminii result in the creation of a new amino acid residue x. The sequence has remained in frame with 5 of the original signal peptide residues still present.

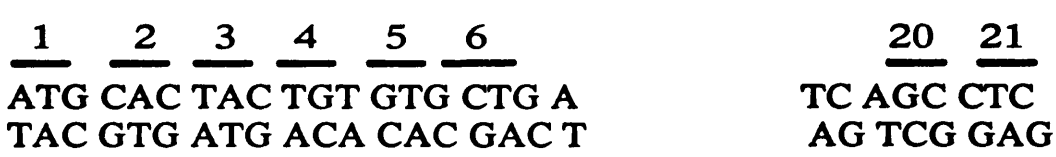
Generation of signal peptide mutation

(A)

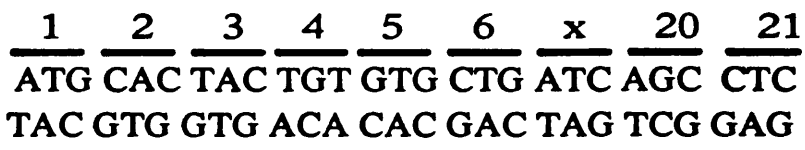


(B)

(C)



(D)



2.4.4 Generation of cleavage site sequence mutant

The generation of the second hTGF-β2 mutant involved the mutagenesis of the proteolytic cleavage site. This was attempted by PCR directed oligonucleotide mutagenesis (Stappert *et al* 1992) (Figure 4). Human TGF-β2 cDNA, isolated from the pBS construct described above, was subcloned into M13mp18 in such an orientation that single stranded DNA prepared from the M13 construct would be of the sense strand.

M13mp18 was digested with *EcoRI* and *HincII*. This generated a linearised M13mp18 vector with one *EcoRI* compatible end and one blunt end. The *EcoRI*/blunt fragment of hTGF-β2 generated for ligation into the πH3M vector (described above) was ligated into the M13mp18 vector. Single stranded DNA was generated from the M13-hTGF-β2 as described in section 2.3. Site directed mutagenesis was carried out on the ssDNA by PCR as described in section 2.2.16 (Fig 4 and 8). The mutation of the proteolytic cleavage site, by the introduction of 3 new base pairs, was confirmed by sequence analysis (Figure 9 and 10).

2.5 RNA analysis

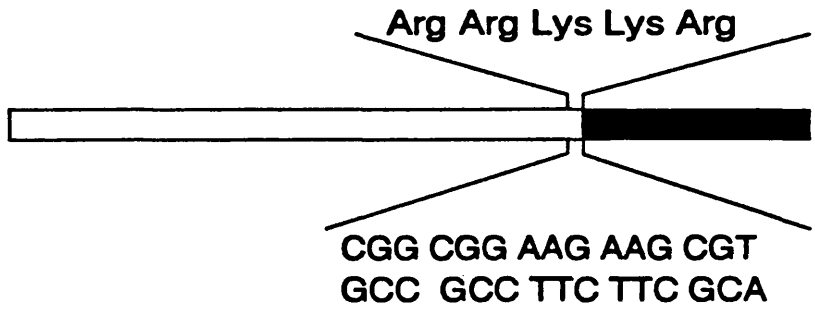
2.5.1 Extraction of cellular RNA

RNA was extracted from a variety of tissues and cultured cells by the method described by (Chomczynski and Sacchi, 1987). Mammalian cells were cultured in a 75cm^2 flask until confluent. The cells were washed twice in PBS (Life Technologies) and the flask drained. 1ml of solution D (4M Guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarkosyl, 0.1mM β-mercaptoethanol) was added and the cells scraped with a sterile "policeman". A further 2mls of solution D was added and the viscous mixture collected in a sterile 30ml polypropylene tube. The following reagents were added, 1/10th volume 0.2M sodium acetate pH 4.0, 1ml of unequilibrated phenol, 0.6ml chloroform:isoamylalcohol (49:1). The sample was mixed gently after the addition of each reagent and incubated on ice for 15 minutes. RNA was pelleted by centrifugation at 10,000rpm for 20

Figure 8

Generation of the cleavage site mutation. Diagram showing human TGF-ß2 polypeptide with normal cleavage site amino acid residues, and DNA sequence which encodes this region. P4 spans this coding region but contains three mismatch bases (*) in the codon which code for the three arginine residues. The result of the site-directed mutagenesis shows the introduction of the mismatches which results in the formation of two new restriction endonuclease sites for HindIII and PstI (solid lines above coding sequence). The altered amino acid sequence is shown below. The three basic argine residues have been replaced by two leucines and a glutamine residue.

Generation of cleavage site mutation.



Primer 4 GG TTG GAC GTC TTC TTC GAA CGA AAC

* * *

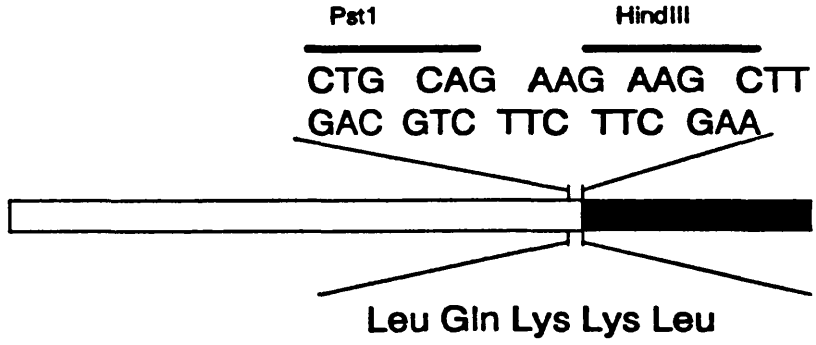


Figure 9.
Diagram showing monomers of (A) normal human TGF-ß2. (B) Human TGF-ß2e with deleted signal peptide sequence. (C) Human TGF-ß2c with altered cleavage site sequence. The length of the polypeptides is given in brackets. The human TGF-ß2e polypeptide lacks 23 amino acids which have been removed from the signal peptide sequence. The cleavage site mutant polypeptide will be the same size as normal human TGF-ß2 but the five amino acid cleavage recognition sequence has been altered by site-directed mutagenesis.

Polypeptides expected from mutant constructs.

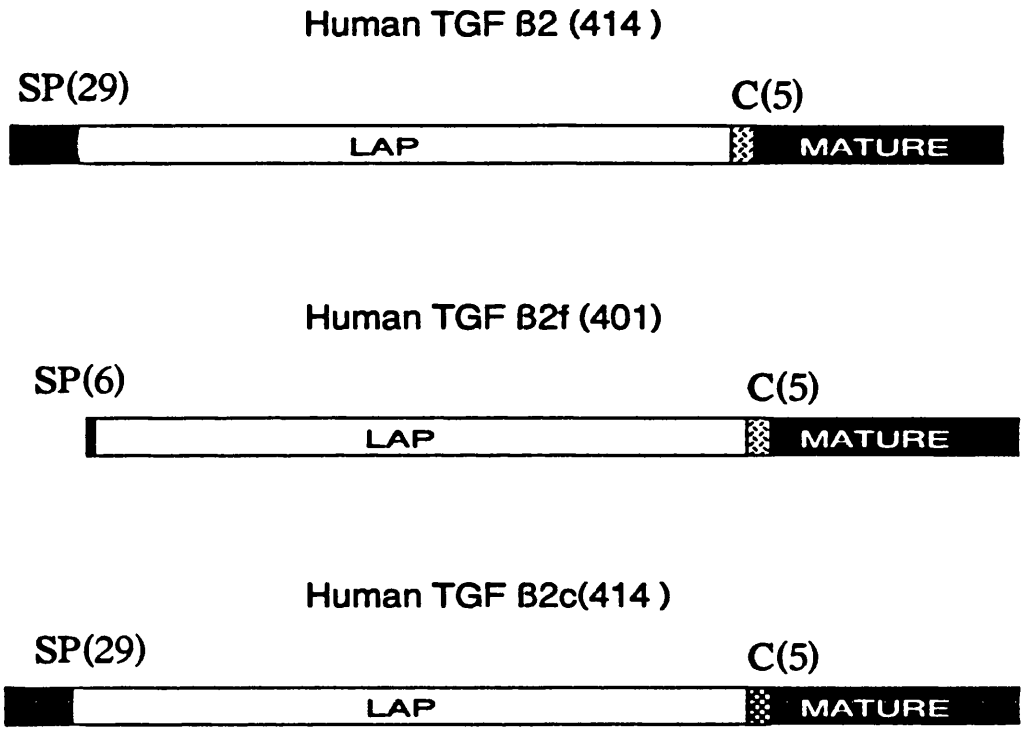
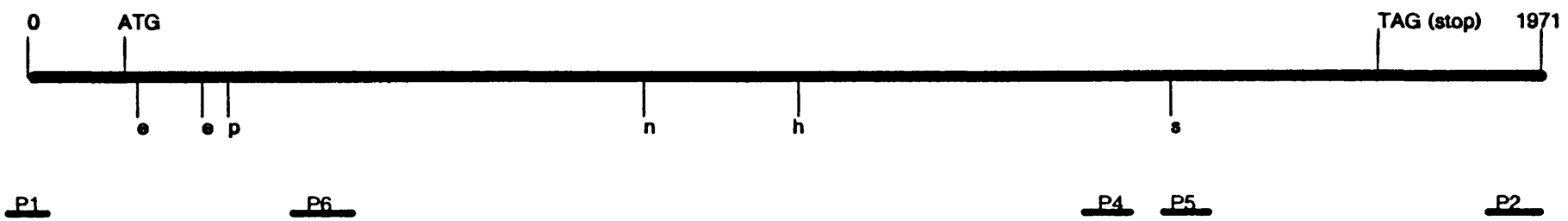


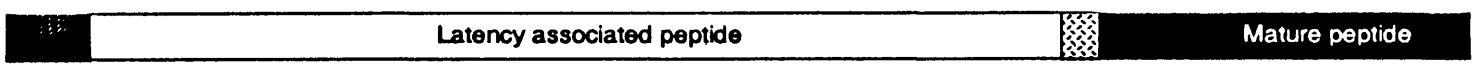
Figure 10

(A) Diagram of human TGF-B2 cDNA above (B) human TGF-B2 protein. The ATG initaition start site is indicated along with the in frame stop site. The primers used for sequencing, SDM and PCR are drawn below the cDNA in their approximate positions of homology. Not drawn to scale. e=EspI, p= PstI, N=NotI, H=HindIII, s=SphI restriction sites.

(A) cDNA Human TGF-β2



(B) Human TGF-β2 protein



Signal peptide

Cleavage site

minutes at 4°C in a Sorvall rotor. The aqueous phase was transferred to a fresh tube and 3ml of iso-propanol added. The solution was mixed and incubated at –20°C for 60 minutes. The RNA was pelleted at 10,000rpm for 20 minutes at 4°C and the supernatant discarded. The pellet was resuspended in 0.6ml Solution D and an equal volume of isopropanol was added. The RNA was precipitated by incubation at –20°C for 60 minutes followed by centrifugation at 10,000rpm for 10 minutes. The pellet of RNA was washed twice in 70% ethanol and resuspended in DEPC treated dH_2O. This method yielded about 200ug-300μg RNA/5x10^6 cells.

2.5.2 RNA gel electrophoresis

RNA was analysed on a 1.5% denaturing agarose gel containing, 5.2% formaldehyde, 1xMOPS buffer (0.2M MOPS sodium salt, 25mM Sodium acetate, 2.5mM EDTA sodium salt, pH 7.0). RNA samples (between 10-30μg RNA per lane at 1μg/μl) had an equal volume of 50% formamide, 5.2% formaldehyde, 1xMOPS buffer added, and incubated at 70°C for 10 minutes. The samples were quenched on ice and 1/6th volume loading dye added (20% Ficoll, 10mM EDTA, 0.25% xylene cyanol, 0.25% bromophenol blue). The gel was run in 1XMOPS, which was circulated to prevent depletion of ions at the anode. Ribosomal RNA or an RNA ladder (Life Technologies) was run alongside the samples as a size marker.

2.5.3 Transfer of RNA to a nitrocellulose membrane

For Northern analysis the samples were prepared and run as described above. When the gel had run a sufficient distance (as determined by migration of the loading dye) it was washed in Milli-Q water with frequent changes for 1 hour to remove formaldehyde. The marker lanes were removed from the main body of the gel and stained with ethidium bromide (50μg/ml) for 1 hour followed by destaining in Milli-Q water for up to 16 hours. The markers could then be viewed on a trans-illuminator and photographed alongside a scale.

The remainder of the gel was placed on two sheets of 3MM Whatmann paper which had been soaked in 10xSSC (1xSSC: 0.15M NaCl, 0.015M Na_3citrate) and were acting as a wick in a tray containing a reservoir of

10xSSC. A piece of nitrocellulose was cut to the size of the gel and was floated on Milli-Q dH_2O till wet and then submerged for 5 minutes. The wet filter was placed on top of the gel, and care taken to remove any air bubbles. Four pieces of dry 3MM Whatmann paper (cut to the same size as the gel) were placed on top of the filter followed by a stack of paper towels. A small weight (100g) was placed on top of the towels and the blot left for 16 hours. The filter was removed from the blot, rinsed in 2xSSC and baked at 80°C for two hours.

2.5.4 Random prime labelling of DNA

DNA inserts, to be used as probes, were labelled with $\alpha^{32}P$ dCTP using a random primer labelling kit (Boehringer Mannheim) following their protocol exactly. 200ng of double stranded DNA was resuspended in 7µl dH_2O, incubated for 5 minutes at 100°C and quenched on ice. 100mM (2µl) of each dNTP (except dCTP), plus $1/10^{th}$ volume reaction buffer, containing random hexanucleotides, 0.5µCi $\alpha^{32}P$ dCTP and 1 unit of Klenow was added to the DNA and the reaction incubated at 37°C for 30 minutes. The reaction was stopped by incubation at 65°C for 10 minutes. Unincorporated nucleotides were removed from the mixture by passing the sample through a Nick column (Pharmacia). The specific activity of the probe was determined and if this was below 10^8cpm/ug then it was discarded. The sample was kept on ice until required. Immediately prior to use the radioactive probe was incubated at 100°C for 10 minutes.

2.5.5 Northern hybridisation

The nitrocellulose filter, prepared by method above, was placed on 2xSET (0.15M NaCl, 0.03M Tris-HCl pH 8.0, 0.002M EDTA) until wet and then submerged for 5 minutes. The filter was placed in a hybridisation bottle, with 10mls of pre-hybridisation mix (50% formamide, 5xSET, 1xDenhardts (0.02% BSA (RNase free), 0.02% SDS, 0.02% Ficoll, 0.02% polyinypyrrolidine (all w/v)), 20mM phosphate buffer and 50ug/ml denatured and sheared salmon sperm DNA). The filter was incubated at 43°C for at least 4 hours. The probe was denatured and added to the pre-hybridisation mix. Incubation, at 43°C, continued for a further 16 hours.

The percentage of formamide could be altered depending on the stringency of hybridisation required.

2.5.6 Northern washes

Filters were washed twice at 65°C in 4xSET, 0.2% SDS for 15 minutes; twice at 65°C in 2xSET, 0.2% SDS for 30 minutes; twice at 65°C in 1xSET, 0.2% SDS for 30 minutes and finally once in 0.2xSET at 65°C for 30 minutes. The filter was exposed to Kodak X-Omat autoradiographic film.

2.6 In Situ hybridisation

2.6.1 Mouse husbandry

Embryos from 6.5 days *p.c.* to 18.5 days *p.c.* were generated from NIH males crossed with either Parkes females or NIH/Parkes F1 females. Noon on the day the copulation plug was found was considered as 0.5 day *p.c.*. The mice were kept under a 14 hour light to 10 hour dark cycle, the dark period from 1900 hours to 0500 hours.

2.6.2 Collection of embryos

Females from timed pregnancies were sacrificed by cervical dislocation. A ventral incision was made through the body wall and the uterus withdrawn from the body cavity. The decidua was cut from the uterus and placed in ice-cold, millipore-filtered PBS (130mM NaCl, 7mM Na_2HPO_4, 3mM $NaH_2PO_4.2H_2O$ pH 7.2). Embryos 10.5 days *p.c.* or older were dissected from the decidua and amnion. Embryos 9.5 days *p.c.* and younger were left in the decidua. All solutions were pre-cooled to 4°C. Embryonic material was fixed in filtered 4% paraformaldehyde (PFA) in PBS for 6 to 24 hours at 4°C with continuous rotation. The embryonic material was washed in PBS for 30 minutes; 0.85% saline for 30 minutes; ethanol:saline (0.425% saline : 50% ethanol) for 15 minutes; and 70% ethanol for 15 minutes. The 70% ethanol was replenished and the embryos could be stored at this stage for a number of weeks. The embryos were processed through a Histokinette automatic processor on a 24 hour cycle. The cycle

included: 100% ethanol; 5 washes in methanol; one wash in 100% ethanol; one wash in 50% ethanol:50% chloroform; two washes in chloroform; one wash in xylene and finally two washes in paraffin wax. The embryos were embedded in paraffin wax and stored at 4°C prior to use.

2.6.3 Preparation of slides and coverslips

Microscope slides for *in situ* hybridisation were washed at 65°C in a 10% Decon solution overnight, rinsed in hot running tap water for at least four hours followed by rinsing in Milli Q water. The slides were then baked for a minimum of four hours at 180°C, following which they were dipped in a 2% TESPA (Sigma) solution in acetone for 5 seconds followed by at four washes in 100% acetone and four washes in Milli-Q water. The slides were dried, in a dust free environment, at room temperature. Coverslips were treated in a similar manner although they were coated in dimethyldichlorosilane (BDH) solution instead of TESPA.

2.6.4 Cutting tissue sections

Embryos and tissues embedded in paraffin wax blocks were placed in a microtome and 5-7uM serial sections cut. These were floated onto water at 42°C. The sections were picked out with TESPA-coated microscope slides and left to dry at 42°C for at least four hours. The sections were then stored at 4°C, with desiccant, until required.

2.6.5 Radioactive riboprobe synthesis

To generate ^{35}S-labelled RNA probe, (riboprobe) to a high specific activity, Bluescript KSII or Bluescribe (+) vectors, containing the insert of choice, were linearised with respect to the orientation of the T3 and T7 promoters as described in section 2.4.1. 1ug of DNA template, in 1µl of dH_2O, was incubated at 37°C with the appropriate polymerase in a reaction buffer (0.04M Tris-HCl pH 8.0, 8mM $MgCl_2$, 1mM Spermidine, 0.025M NaCl.); plus the following 0.1mM DTT; 1mM of ATP, CTP, and GTP; 7.5uM UTP-S; 0.3mg/ml BSA (RNAse and DNAse free); 30 units RNA guard (Pharmacia) and 75uCi S^{35}UTP (dry)(Amersham) in a total volume of 20µl. The reaction was terminated by incubation with 0.15 units of DNAse 1 in 1xDNAse

buffer (0.1M NaOAc pH 5.0; 5mM $MgCl_2$; 10mM DTT (added fresh); 50 units RNA guard and finally 0.05mg Poly A). 50 µl of Orange G was added before the reaction mix was centrifuged through G50 sephadex column which had been equilibrated with column buffer (0.3M NaOAc pH 5.0; 10mM Tris-HCl pH 7.5;1mM EDTA pH 8.0; 0.1% SDS; 10mM DTT (added fresh)). The column was washed through with 2 volumes of column buffer and the eluants pooled. An equal volume of phenol pH 5.0 was added. The sample was thoroughly mixed and centrifuged at 10,000rpm for 5 minutes. The aqueous phase was retained and subjected to a chloroform:isoamylalcohol (24:1) extraction. Three volumes of 100% ethanol were added to precipitate the riboprobe. The riboprobe was pelleted by microcentrifugation for 15 minutes at 10,000g, washed twice in 70% ethanol and resuspended in 100µl ADB buffer (40 mM $NaHCO_3$ pH 10.2; 60 mM Na_2CO_3; 10 mM DTT). An aliquot was removed from the sample and kept on ice prior to analysis on a polyacrylamide gel (see Section 2.6.6). The remainder of the sample was digested by controlled alkaline digestion in ADB at 60°C. The length of time for the incubation of the riboprobe was calculated by the following formula (Cox *et al* 1984):

$$\text{Incubation time (minutes)} = (Lo - Li)/(0.11 \times Lo \times Li)$$

Lo = Original length of riboprobe (Kb)
Li = Required length of riboprobe (Kb)

After alkaline digestion was completed, 10µl 0.1M NaOAc pH 6.0, 10µl 0.5% sodium acetate, 5µl 0.05mg Poly A and 50 ul 1mg/ml Orange G was added to the reaction before the mixture was separated through a G50 sephadex column. The sample was washed through with two volumes column buffer and the eluants pooled. The sample was extracted with phenol (pH 5.0) and chloroform:isoamylalcohol (24:1) before ethanol precipitation. The pellet was resuspended in an appropriate volume of 50mM DTT such that a 10X stock was equal to 3×10^5 dpm/µl. The riboprobe was stored at –20°C for up to two weeks prior to use.

2.6.6 Polyacrylamide gel electrophoresis

Both full length and alkaline-digested samples of each riboprobe were analysed on a 6% polyacrylamide gel. Approximately 10^5 counts per minute (cpm) of each sample were loaded in each lane. The polyacrylamide gel solution contained (50% urea, 0.5X TBE, 6.5% polyacrylamide stock (acrylamide:bis-acrylamide, 19:1). Polymerisation was initiated by the addition of 1/100th volume of 10% ammonium persulphate plus 1/3000th volume of TEMED (N,N',N',N tetramethylethylenediamine). The solution was mixed thoroughly and quickly poured between two silanised glass plates separated by 1mm spacers. After the gel had set the wells were flushed out with running buffer (0.5X TBE) and the gel was pre-run at 300V for 30 minutes. The samples were taken up in loading buffer (98% Formamide, 1% Bromophenol blue, 1% Xylene Cyanol), incubated at 80°C for two minutes to denature the RNA, and quenched on ice for 2 minutes. The wells of the gel were flushed out again and the samples loaded. The gel was run at 300V for 1 hour, placed on 3MM Whatmann paper, covered in Clingfilm™ and finally exposed to autoradiographic film for 16 hours (See Figure 11).

2.6.7 Phosphorylation of DNA to generate radiolabelled markers for PAGE

Phosphorylation of DNA allows the replacement of the 5' hydroxyl group with a radioactive phosphate group supplied by τ^{32}P ATP (Amersham). The double stranded or single stranded DNA (1μg) was incubated in the presence of 10 units T4 polynucleotide kinase (Stratagene) with 0.01M ATP and react buffer (0.05M Tris-HCl pH7.6, 10mM $MgCl_2$, 5mM dithiothreitol, 0.1mM spermidine HCl, 0.1mM EDTA pH 8.0) for 45 minutes at 37°C followed by incubation at 65°C for 10 minutes to deactivate the enzyme.

2.6.8 Pretreatment of tissue sections

All treatments were performed in 250ml glass troughs using a 20 microscope slide carrier. The tissue sections were dewaxed twice in Histoclear™ for 10 minutes each, followed by rehydration in 100% ethanol

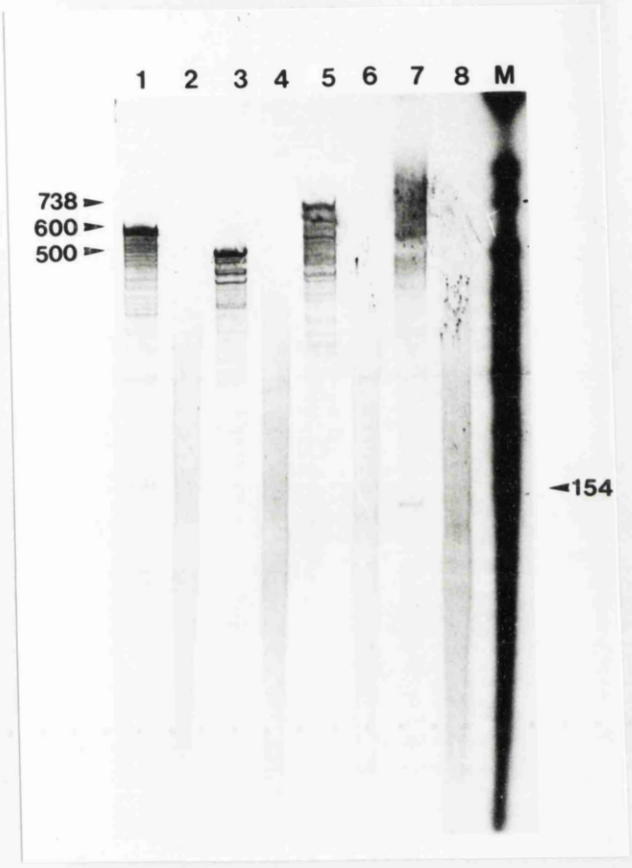
Figure 11.

PAGE gel electrophoresis of riboprobes.

Pre and post-alkaline digested riboprobe samples were run on a PAGE gel. The gel was exposed to autoradiographic film overnight. The sizes of the three mouse specific riboprobes are indicated at the side of the gel. The post alkaline digested riboprobes were determined to be on average ~100 nucleotides.

1. pre mTGF-β1
2. post mTGF-β1
3. pre mTGF-β2
4. post mTGF-β2
5. pre mTGF-β3
6. post mTGF-β3
7. pre hTGF-β2
8. post hTGF-β2

M. marker


1 2 3 4 5 6 7 8 M
738
600
500
154

twice for 2 minutes each, followed by immersion in an ethanol series (90%, 80%, 70%, 50% and 30% dilutions). The sections were equilibrated in 0.85% saline for 5 minutes, followed by PBS for 5 minutes. The sections were re-fixed in fresh 4% paraformaldehyde for 20 minutes at room temperature, followed by washes in PBS, twice, for 5 minutes each. The sections were incubated with proteinase K (40 ug/ml) in PKB buffer (50mM Tris-HCl pH 7.5; 5mM EDTA pH 8.0) for 7.5 minutes at room temperature followed by a brief wash in PBS before further fixation in 4% PFA for 5 minutes. The sections were acetylated in fresh 0.2% acetic anhydride, in 0.1M triethanolamine, for ten minutes at room temperature. After briefly washing the sections in PBS, followed by 0.85 % saline, they were dehydrated by immersion in an ethanol series for 2 minutes each (30%, 50%, 70%, 80%, 90%, 100%). The sections were left to air dry in a dust free environment prior to application of the radioactive riboprobe.

2.6.9 Hybridisation of riboprobe to tissue sections

The riboprobe was applied to the sections in a hybridisation mix containing: 60% formamide; 0.3M NaCl; 10mM Tris-HCl pH 8.0; 5mM EDTA; 10mM $NaPO_4$ pH 6.8; 10% dextran sulphate; 1xDenhardts; 0.5mg/ml yeast RNA; 0.5mg/ml Poly A RNA; 50mM DTT and 1x riboprobe ($3x10^4$ cpm/μl). The hybridisation mix was incubated at 80°C for 2 minutes and quenched on ice. 6μl per 11mm x 11mm coverslip of hybridisation mix was placed on the section. The coverslip was lowered gently on top and air bubbles removed. The slides were placed horizontally in a hybridisation box along with a tissue which had been soaked in 4xSSC, 50% formamide. The box was sealed and placed in an incubator at 52°C for 16 to 18 hours.

2.6.10 Post hybridisation washes

Microscope slides were placed vertically in a 20 microscope slide holder. The sections were then subjected to the following washes: 5x SSC,0.1% 2-mercaptoethanol for 15 minutes at 50°C; 2x SSC, 50% formamide, 1% 2-mercaptoethanol for 20 minutes at 65°C; four washes in RNase buffer (10mM Tris-HCl pH 8.0, 5mM EDTA, 0.5M NaCl) for ten minutes each at 37°C followed by a 30 minute incubation in RNase buffer plus 20ug/ml RNase A at 37°C; RNase buffer at 37°C for 15 minutes; 2x SSC, 50%

formamide, 1% 2-mercaptoethanol at 65°C for 20 minutes; 2x SSC 50°C, 15 minutes; 0.1x SSC at 50°C, for 15 minutes. The sections were dehydrated by immersion in a short ethanol series (50%, 70%, 100%) for 2 minutes each and allowed to air dry in a dust free atmosphere. The sections were dipped briefly in a gelatin:chromalum solution (0.1% gelatin: 0.01% chromic potassium sulphate) and allowed to dry overnight. This was a necessary step to allow the emulsion (Ilford K5) to adhere to the glass slide and section.

2.6.11 Autoradiography

Autoradiography was performed under dark room conditions using a Kodak 904 filter for illumination. Slides were dipped in a solution of Ilford K5 emulsion and glycerol (50% emulsion:1% glycerol) which had been prewarmed to, and maintained at, 45°C. The emulsion was allowed to dry for at least 2 hours in a light tight environment. The slides were sealed in light tight boxes with dessicant, and stored at 4°C prior to development. After the sections had been exposed for the appropriate length of time (3 days to 2 weeks) they were developed by agitation in 20% Phenisol (Ilford) for 2.5 minutes followed by 30 seconds in 1% acetic acid, 30 seconds in water and finally fixed for 5 minutes in 30% sodium thiosulphate. The sections were rinsed in cold running water for at least one hour before counter staining and mounting.

2.6.12 Staining and mounting

Sections were incubated for 5 to 30 seconds in Harris' Haematoxylin (Sigma), rinsed under cold running tap water for 30 seconds, followed by incubation in Scott's Tap water (20g $MgSO_4$, 20g $NaHCO_3$/litre of water) to provide an alkaline environment for the haemotoxylin stain. After a further rinse in cold running tap water the sections were dehydrated through an ethanol series (50%, 70% 100%, 100%, 2 minutes each). The sections were then incubated in two changes of Histoclear, 5 minutes each. Coverslips were mounted using Gurr's Neutral Mounting medium and left to dry overnight.

The sections were viewed with an Olympus BH2 microscope using brightfield and dark ground optics. Photographs were taken using Kodak Panatomic X, TMX 100 or PanF film, with a green filter plus LBD-2N filter for the brightfield views. Darkfield views were photographed without filters. No filters were used for high power (40X) brightfield views.

2.6.13 Alcian green staining

Sections were dewaxed prior to staining (see section 2.6.8), and incubated in 1% Alcian Green stain in 2% acetic acid for 5 minutes. This was followed by rinsing with dH_2O. The sections were transferred to a solution of 0.1% Nuclear Fast Red in 5% ammonium sulphate for 5 minutes, followed by a rinse in dH_2O. Finally the sections were incubated in 0.25% tartrazine, to which 2 drops of acetic acid /100ml had been added, and left for 30 minutes. The sections were dehydrated in a series of alcohols, cleared in Histoclear for 10 minutes and then mounted in Gurr's Neutral Mounting medium.

2.7 Tissue culture

2.7.1 Introduction

Cells were maintained at 37°C as subconfluent monolayers in $75cm^2$ tissue culture flasks (Nunc) in 10ml of Dulbecco's modified Eagles medium plus 10% foetal calf serum (FCS), 0.7% Na_2HCO_3, 10mM glutamine, 1% non-essential amino acids, 10mM NaOH (DMEM). All tissue culture media contained Gentamicin (0.1mg/ml). Each flask was flushed with 5% CO_2 and the cap screwed on tightly. Cultures were usually split every 3-4 days by washing cells twice in 10ml PBS (Life Technologies), followed by incubation in 2mls of Trypsin (Trypsin EDTA in Puck's saline) for 2 minutes at 37°C. Trypsin was deactivated by addition of 8mls of DMEM plus 10% FCS. Cell numbers were estimated using an "improved Neubauer" haemocytometer (BDH) and fresh culture medium was innoculated with $5\text{-}10x10^5$ cells/10mls.

2.7.2 Long term storage of eukaryotic cells

Cells were grown to 80% confluency and trypsinised as described above. Trypsin was deactivated by addition of DMEM plus 10% FCS and the cells centrifuged for 5 minutes at 1000rpm. The supernatant was aspirated off and the cells resuspended in 1ml of 10% dimethyl sulphoxide (DMSO) in DMEM plus 10% FCS. The cells were cooled by storage in Nunc cryostat tubes, wrapped in Bubblepak™, at –70°C for two days and then transferred to liquid nitrogen stores. Cells which had been frozen by this method were thawed quickly at 37°C and added to 9ml DMEM plus 10% FCS. The following day the media was aspirated off, the cells were washed in PBS and fresh media added.

COS-7 cells and CCL64 cells were maintained, as subconfluent monolayers, in DMEM plus 10% foetal calf serum (FCS) (Life Technologies). Cells were split every two to three days and seeded out into fresh DMEM at $1x10^6$ cells.

2.7.3 Transient transfection of COS-7 cells

COS-7 cells were transiently transfected by the DEAE-dextran method as described by Freshney (1987) with modifications. The COS-7 cells were seeded out in DMEM plus 10% FCS into $75cm^2$ flasks (Nunc) at a cell density of $1x10^6$ cells/flask and incubated overnight at 37°C. The following day the cells were checked to determine their confluency. If the cells were between 40-60% confluent they were suitable for transfection.

1 unit of construct πH3M-sTGF-$\beta1^{33}$ ($\beta1^{33}$) and πH3M-hTGF-β2 (β2) (10μg=1 unit), and 1, 2 and 4 units of constructs πH3M-TGF-$\beta2^e$ ($\beta2^e$), πH3M-hTGF-$\beta2^c$ ($\beta2^c$), and πH3M-hTGF-$\beta2^f$ ($\beta2^f$) were transfected independently into COS-7 cells (see section 2.4.2 for description of various constructs). The vector πH3M (π) was included in all transfections mixes to make the total DNA content equal to 50μg. Co-transfections of the two wild type constructs with each of the mutant constructs were carried out independently in ratios of 1:, 1:2 and 1:4 of wild-type construct to muutant construct.

A transfection mix containing 10μg DNA/ml DMEM plus 10% FCS, 100μM chloroquine, 0.04% DEAE dextran was prepared, and made up to 5ml with media. While the cells were washed twice with PBS the transfection mix was incubated at 55°C. The transfection mix was then added to the wells and incubated for 2.5 hours at 37°C. The transfection media was removed and 10% DMSO in PBS added to the cells and left for 2 minute at room temperature. The DMSO was aspirated off and 5ml DMEM plus 10% FCS added and the cells incubated for 48 hours.

2.7.4 Determination of efficiency of transfection

The construct pIRV (Beddington *et al* 1989) which contains six copies of the bacterial gene β-galactosidase driven by human actin gene promoter was used to determine the efficiency of the transfection procedure. 25μg pIRV was transfected into the COS-7 cells as described above. The cells were rinsed twice in PBS and fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 minutes at 4°C. The fix was aspirated from the cells which were washed twice with PBS. The reaction mix (1mg/ml X-Gal, 5mM potassium ferricyanide, 5mM potassium ferrocyanide and 2mM magnesium chloride in PBS) was added to the flask and the cells incubated for 24-48 hours at 37°C. The cells were examined under a light microscope and cells which stained blue counted. A flask of cells which had not been transfected with pIRV was examined simultaneously.

2.7.5 Collection of conditioned media

For the collection of conditioned media, transfected COS-7 cells, 48 hours post transfection, were incubated in serum-free DMEM after having been washed in four changes of PBS. The cells were left to condition the serum-free DMEM for 48 hours. The conditioned media was cleared by centrifugation at 1,000rpm for 10 minutes and a cocktail of protease inhibitors added (1μg/ml aprotonin, leupeptin and pepstatin A). The CM was immediately frozen at –20°C or acid dialysed against 0.2M acetic acid for 12 hours and lyophilised. Dialysis tubing was treated as described in (Sambrook *et al* 1989). The lyophilised sample was resuspended in water and used immediately.

2.7.6 Preparation of dialysis tubing

Dialysis tubing was cut into 15cm strips and boiled for 10 minutes in 2L of 2% $NaHCO_3$ and 1mM EDTA pH 8.0. The strips were rinsed thoroughly in dH_2O and boiled for a further 10 minutes in 1mM EDTA pH 8.0. The strips were allowed to cool and stored submerged under 1mM EDTA pH 8.0 4°C. The strips were rinsed thoroughly inside and out with dH_2O prior to use.

2.7.7 CCL64 inhibition assay

The CCL64 (Mv1Lu cells) assay for TGF-β biological activity was carried out according to the protocol of (Danielpour *et al* 1989). TGF-β inhibition of CCL64 epithelial cells was measured by assaying the tritiated thymidine incorporation in these cells over a period of time when incubated in conditioned media collected as described 2.7.5. A $75cm^2$ flask of subconfluent CCL64 cells were trypsinised and resuspended in DMEM plus 10% FCS. The cells were pelleted by centrifugation and media aspirated off. The cells were washed in assay buffer (DMEM plus 0.1% FCS and 10mM HEPES), and resuspended at 10^5 cells/ml in assay buffer. They were seeded into 24 well dishes at 0.5ml per well. The cells were returned to the incubator for one hour. The samples to be tested were added to the wells and the plates returned to the incubator for a further 22 hours. 1.0μCi ^3H (80 Ci/mmole) was added to each well and incubated for 2 hours at 37°C. The assay buffer was aspirated off and the cells washed twice in PBS and fixed in 1ml methanol:acetic acid (3:1 v/v) at room temperature for 1 hour. The fixed cells were washed twice with 80% methanol and finally trypsinised for one hour at room temperature. 0.5ml of 0.5% SDS was added to each well and the mix transferred to scintillation vials containing 10ml of Ecoscint A. All samples were analysed in triplicate. Samples were counted in a Texas Instruments scintillation counter.

2.7.8 Lysis of COS-7 cells growing in culture

Subconfluent cell cultures were trypsinised as described previously. The cells were harvested by centrifugation at 1,000rpm for 1 minute. The cells were resuspended in 100μl PBS and boiled for 10 minutes. The sample was sonicated to shear chromosomal DNA and finally centrifuged at

10,000rpm for 10 minutes at room temperature. The supernatant was retained and stored at –70°C until required.

2.8 Protein analysis

Western blot analysis was carried out in order to detect proteins produced from the various constructs either from the conditioned media or from cell lysates. A detection kit based on alkaline phosphatase staining from British Biotechnology (BDK-1) was used. The kit included rTGF-β1 as a control.

2.8.1 Preparation of Tris-glycine SDS-PAGE gels

SDS-PAGE gel was made in the same manner as described in section 84, but with minor differences. In order to seperate protein bands between 12-43kDa a 15% acrylamide gel was prepared. A gel mixture containing 15% acrylamide (29% acrylamide:1% bis-acrylamide w/v), 0.325M Tris-Cl pH8.8, 0.1% SDS. 0.1% ammonium persulphate and 0.01% TEMED were added and the gel mixture poured between glass plates. Butan-1-ol was layered on top of the acrylamide which was allowed to set. The butan-1-ol was removed and thoroughly rinsed away with dH_2O. A stacking gel mixture was prepared (5% acrylamide (29:1 acrylamide:bis-acrylamide), 120mM Tris-Cl pH 6.8, 0.1% SDS, 0.1% Ammonium persulphate and 0.1% TEMED), and poured on top of the acrylamide gel and allowed to set. The samples were prepared in 1XSDS gel loading buffer (50mM Tris-Cl pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Prior to loading the samples were incubated at 100°C for 3 minutes. Any wells which had no sample loaded, had 1XSDS gel loading buffer loaded. Appropriate molecular weight standards were run simultaneously (Sigma). The gel was run in Tris-glycine buffer (25mM Tris base, 250 mM Glycine, 0.1% SDS pH8.3), at 100V for 1 hour followed by 300V for a further 3 hours.

2.8.2 Transfer of proteins to nitrocellulose

The proteins were transferred to a nitrocellulose filter and probed with antibodies to TGF-β. The nitrocellulose was floated on a tray of Milli-Q H_2O and immersed for 5 minutes. Six pieces of Whatmann paper were soaked in Transfer buffer (TBS)(39mM glycine, 48mM Tris, 0.037% SDS, 20% methanol). The gel was placed in a sandwich between Whatmann paper and with the nitrocellulose next to the gel. The whole sandwich was placed in a Bio-Rad electro blot tank and a current passed through the gel (200mA for 2 hours). The filter was dried at room temperature for 1 hour and stored at 4°C.

Prior to detection of the proteins of interest, the molecular weight ladder can be detected with Ponceau S stain (Sigma). The dried filter was placed onto Milli-Q H_2O and submerged for 5 minutes. The wet filter was transferred to a tray containing a working solution of Ponceau S stain (1:9 Ponceau S: Milli-Q H_2O) and incubated for 5-10 minutes with gentle agitation. When the bands of the molecular weight ladder bacame visible the stain was discarded and the filter washed with several changes of Milli-Q H_2O. The positions of the proteins were marked as the Ponceau S stain will wash off during the rest of the detection process.

2.8.3 Detection of TGF-βs on nitrocellulose filters with specific antibodies

The detection of TGF-β2 was carried out with a kit supplied by British Biotechnology using a chicken polyclonal antibody which detects both TGF-β1 and TGF-β2. The protocol provided was also used with some minor modifications. The stock solution of TGF-β antibody (10mg/ml) was diluted in blocking buffer (1/1000) (TBS, 1% EIA grade BSA, 0.05% Tween-20 EIA grade)(TBS= 500mM NaCl, 20mM Tris-HCl pH 7.2). The membrane was placed on Milli-Q H_2O for a few minutes then incubated in blocking buffer for 2 hours at room temperature. The blocked nitrocellulose filter was incubated with the diluted antibody for 2 hours at room temperature in a heat sealed plastic bag. The antibody dilution was removed and the filter washed three times in TBS containing 0.05% Tween-20 for 5 minutes. Affinity purified, biotin-conjugated anti-rabbit IgG

was diluted in blocking bufer (1:1000). The filter was then incubated with the diluted anti-rabbit IgG second antibody for 1 hour at room temperature. The filter was washed as before. Diluted alkaline phosphatase-conjugated avidin in blocking buffer (1:500) was incubated with the filter for 1 hour at room temperature. The washes were repeated. Finally, the colour solution was prepared by adding 66µl of colour reagent A (50mg/ml p-nitro blue tetrazolium chloride in 70% N.N dimethyl formamide) to 10ml of AP buffer (100mM Tris-HCl pH 9.5, 100mM NaCl, 5mM $MgCl_2$). 33µl of colour reagent B (50mg/ml 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt in N.N-dimethyl formamide) was added and mixed. The filter was incubated with the colour solution for several hours. After suitable colour development, the colour reaction was removed and the filter rinsed with ddH_2O. The filter was photographed and the membrane dried and stored in the dark indefinitely.

Chapter 3

RESULTS

3.1 Introduction

At the start of this project it was known that each of the TGF-β isoforms was expressed during mammalian embryogenesis. The majority of this data was based upon expression analysis by *in situ* hybridisation and Northern blot analysis. Additionally, there was a large body of data available on TGF-β1 expression in murine embryogenesis gathered by *in situ* hybridisation and immunohistochemistry. Very little data existed on the detailed developmental expression of TGF-β2 or TGF-β3 and what there was was based entirely upon Northern blot analysis (Denhez *et al* 1990; Miller *et al* 1989). To contribute more comprehensive data on the temporal and spatial patterns of expression of these two, relatively new, members of the TGF-β gene family, global *in situ* hybridisation studies were performed on serial sections of murine embryos, from gestational age 6.5 days up to 16.5 days using mouse gene probes which were specific for TGF-β2 and TGF-β3. Dissected skin from 18.5 days *p.c.* embryos were also examined. No expression of either gene was detected in the embryo prior to 8.5 days *p.c.*. However both were expressed in extraembryonic material from 6.5 days *p.c.* to at least 9.5 days *p.c.* (described in section 3.2.9)

One of the first major hurdles to overcome when carrying out *in situ* hybridisation using genes from a conserved family is to minimise the amount of cross-reactivity between the riboprobes and other members of the gene family. The riboprobe templates were chosen from the region of the cDNA which encodes the LAP, which has considerably less homology between the isoforms than does the DNA sequence encoding the mature

region. The riboprobes were between 35 to 47% homologous which, under the conditions of the *in situ* hybridisation employed, ensured that there was no cross-reactivity. The TGF-β1-specific riboprobe utilised by Akhurst *et al* (1990) provided a positive control whilst a sense TGF-β2 riboprobe was used as a negative control. No specific autoradiographic signal was observed at any time, when using the sense riboprobe, during these experiments (Figure 1 D, H, P. Figure 2 C. Figure 9 C, F, I. Figure 10 C. Figure 11 I). To confirm the specificity of the three templates they were used to detect total RNA on a Northen blot (Fig 12). The TGF-β1 specific template detected a band of 2.5Kb. The TGF-β2 detected at least two bands at 4.0 and 5.0Kb, there may have been other bands hidden by the ribosomal RNA. The specific TGF-β3 probe picked up the expected 3.5Kb transcript (Figure 12).

3.2 Expression of TGF-β isoforms during murine embryogenesis

During this stage of the investigation at least three embryos from each stage described were studied. 18.5 days *p.c.* embryos had their skin removed and investigated.

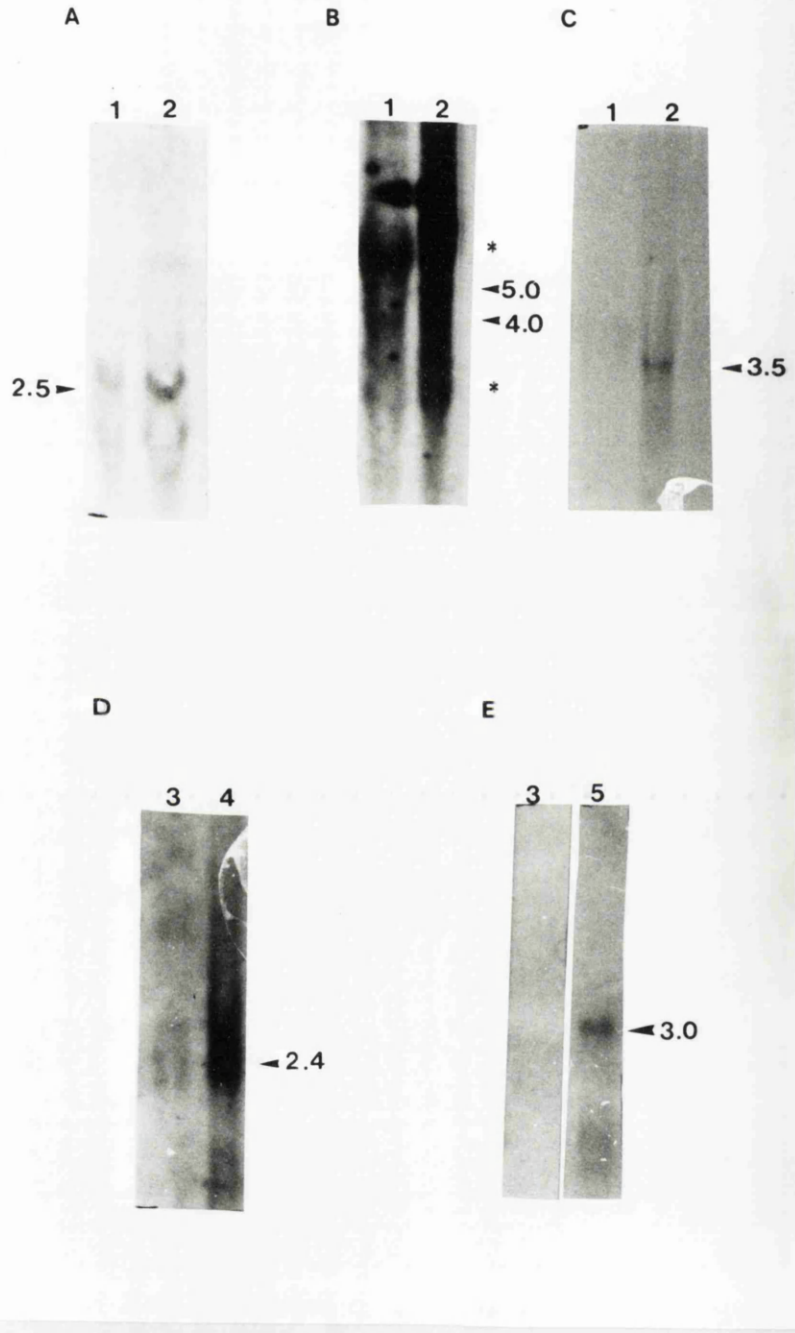
3.2.1 Expression during and osteogenesis and chondrogenesis

There are two main routes to bone formation. Intramembranous ossification is the conversion of primitive connective tissue into bone, whereas replacement of cartilage by bone is known as endochondral ossification. Cartilage in the embryo arises from condensations of mesenchymal cells which form a precartilage blastema in those parts of the embryo where cartilaginous elements later develop. In general, the formation of bone by endochondral ossification can be considered as a model for both types of bone formation. Mesenchymal cells condense to form pre-chondrogenic blastema, around which a perichondrium is formed, with inner chondrogenic and outer fibrous layers. Chondroblasts give rise to chondrocytes and mature cartilage is deposited. The outer fibrous

Figure 12.

Northern analysis to show specificty of riboprobes and to determine RNA expression by COS-transfected cells.

(A, B, C) 15μg of total mouse RNA from 16.5 day p.c. liver (1) and 14.5 day p.c. mouse RNA (2) was probed with (A) mTGF-β1, or (B) mTGF-β2, or (C) mTGF-β3. The mTGF-β1 probe recognised a 2.5Kb band in both the liver and whole mouse samples. mTGF-β2 probe hybridised to bands of 4.0 and 5.0Kb in the whole mouse RNA sample. The asteroks indicate positions of the rRNA which masked any other bands present. The mTGF-β3 probe detected the expected 3.5 Kb band in whole mouse RNA but detected no RNA in the liver. (D,E) 15μg of RNA extracted from COS-7 cells which had been untreated (1) or transfected with either $\beta1^{33}$ construct (4) or β2 construct (5) were subjected to Northern analysis. (D) Northern probed with hTGF-β1. A 2.4Kb band was detected in the COS cells transfected with $\beta1^{33}$. (E) Northern probed with hTGF-β2. A band was detected in the RNA transfected with β2 at 3.0Kb.


A
B
C
1
2
2.5
5.0
4.0
3.5
D
E
3
4
5
2.4
3.0

perichondrial layer becomes vascularised and forms periosteum, while the inner perichondrial cells become osteogenic. Osteoblasts differentiate from mesenchymal cells, and deposit matrix that is subsequently mineralised. Bone is initially laid down as a network of immature trabeculae, the primary spongiosa; the primary spongiosa is replaced by secondary bone, or removed to form bone marrow, or converted into primary cortical bone by the filling of spaces between the trabeculae (Hamilton *et al* 1978)).

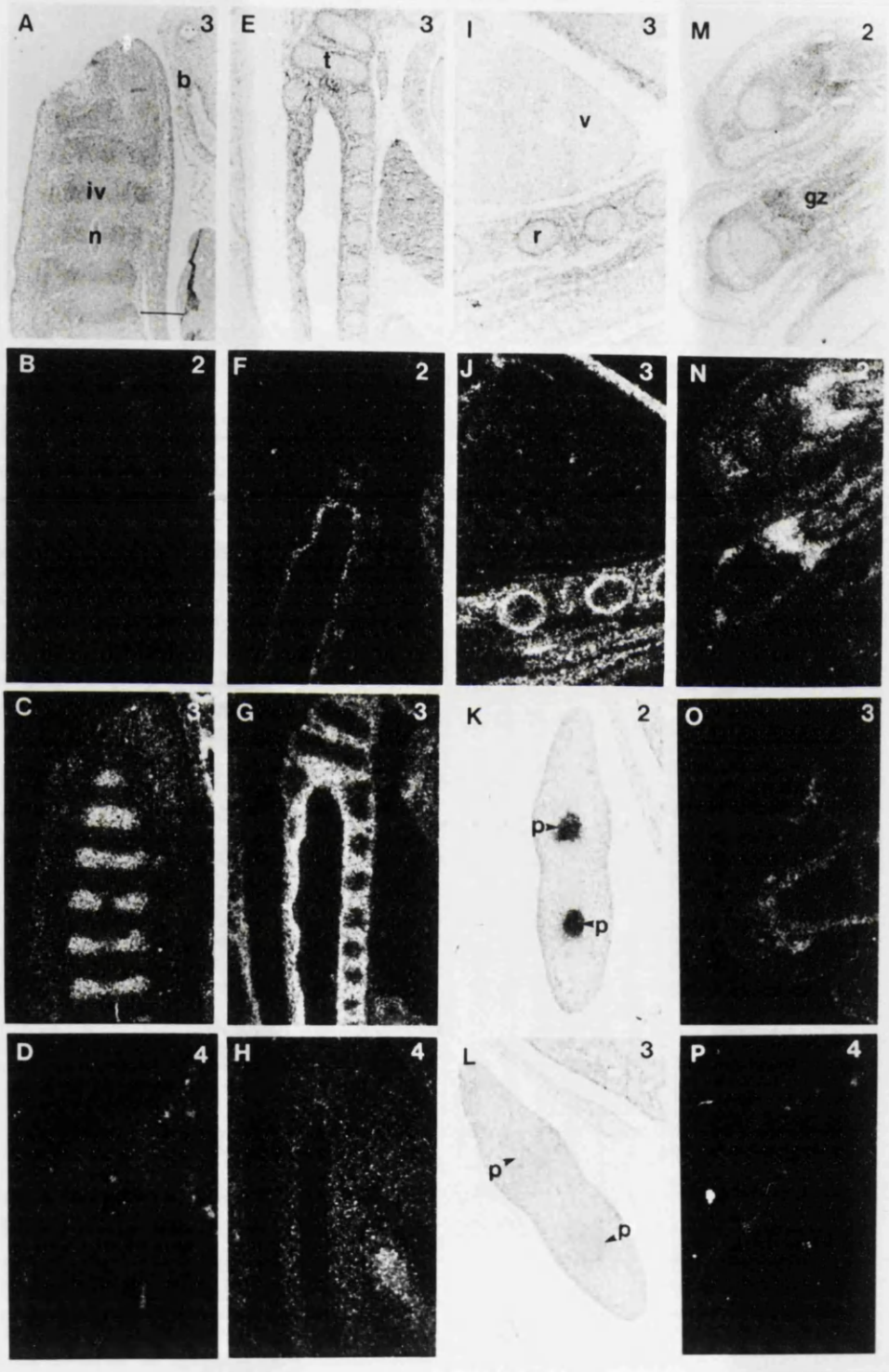
In previous studies (Lehnert and Akhurst, 1988), TGF-β1 RNA expression was observed in areas of ossification, including osteoblasts and osteoclasts. Neither TGF-β2 RNA nor TGF-β3 RNA were seen at high levels within these cell types. The earliest abundant expression of any TGF-β isoform was that of TGF-β3 RNA, seen at 10.5 days *p.c.* in the intervertebral disc anlagen (Figure 13 A,C.). This expression pattern of TGF β3 RNA persisted up to at least 16.5 days *p.c.*. TGF-β3 RNA was also present, in abundance, in mesenchyme within, and adjacent to, the perichondria of non-ossifying cartilage. This included tracheal cartilage (Figure 13. E,G), nasal septum (Figure 19. C, D), the otic capsule (Fig 5 E) and sclerotic coat. Transcripts of TGF-β3 RNA were also seen in the perichondria of cartilage models of the vertebrae, ribs and the presumptive long bones and digits (Figure 13, I, J and O). The TGF-β3 riboprobe also hybridised to the perichondria of jaw bone and Meckel's cartilage. Hypertrophic chondrocytes no longer express this gene.

TGF-β2 RNA was seen in the pre-chondrogenic blastemae of the limb buds at 12.5 days *p.c.*, before there is any morphological distinction between these cells and the surrounding mesenchyme (Figure 13 K). Adjacent sections containing pre-chondrogenic blastemae were analysed to determine if there was any difference between the cells which made up the blastema and the surrounding mesenchyme. The stain, alcian green, picks up cells expressing proteo-lycans which would be expected in the pre-chondrogenic blastemae but not surrounding mesenchyme if they have begun to differentiate. However, it is quite clear that the pre-chondrogenic blastemae are apparently identical to their neighbouring cells (Figure 14 A). TGF-β3 RNA was not detected in the pre-chondrogenic blastemae or elsewhere in the developing limb until much later when the bones were being laid down (Figure 13,L). As development of the long bones proceeds

Figure 13

Expression of TGF β isoforms during chondrogenesis and osteogenesis.

Bright field sections hybridised with either TGF β2 (K, M) or TGF β3 (A, E, I, L); Dark field of sections hybridised with TGF β2 (B, F, N) or TGF β3 (C, G, J, O) or control probe (D, H, P). (A, B, C, D) serial sections through tip of 14.5 day p.c. embryo tail. (A) light field of section hybridised with antisense TGF β3 riboprobe. (C) corresponding dark field section to (A) showing hybridisation of TGF β3 riboprobe to intervertebral discs. TGF β2 RNA was not expressed in the IV discs (B). (E, F, G, H) serial sections of trachea of 16.5 day p.c. embryo. (E and G) light field and corresponding darkfield image of section hybridised with antisense TGF β3 probe shows expression of TGF β3 RNA in the mesenchyme surrounding the tracheal cartilage but no expression in tracheal epithelium. (F) parallel section to (E) showing hybridisation of TGF β2 riboprobe to mesenchyme directly underlying tracheal epithelium. Also signal from cartilage of tracheal discs. (I, J) Light and dark field showing hybridisation of TGF β3 to the perichondria of ribs, also expression in the diaphragm and slight expression in mesothelia of heart of 14.5 day p.c. embryo. (K and L) Light fields of parallel sections of 12.5 day p.c. embryo showing intense hybridisation of TGF β2 riboprobe to prechondrogenic blastemas. (L) No expression was seen with TGF β3 riboprobe to these regions. (M, N, O, P) serial section of 16.5 day p.c. forelimb. (M, N) Light and corresponding darkfield demonstrating hybridisation of TGF β2 probe to growth zone of digits and also tendons. The fingers are bent over giving the impression that the growth zone is more central than it really is. (O) parallel section probed with TGF β3 showing expression in the perichondrium. (D, H, P) dark fields of sections hybridised with sense TGF β2 probe demonstrating no hybridisation. Numbers in top right of photographs correspond to which riboprobe was hybridised to section: 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. b=blood vessel, n= notochord, iv=intervertebral discs, t=tracheal cartilage, v=ventricle, r=rib, p=prechondrogenic blastema, gz=growth zone. Scale bars represents 200μm.


A 3
b
iv
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E 3
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I 3
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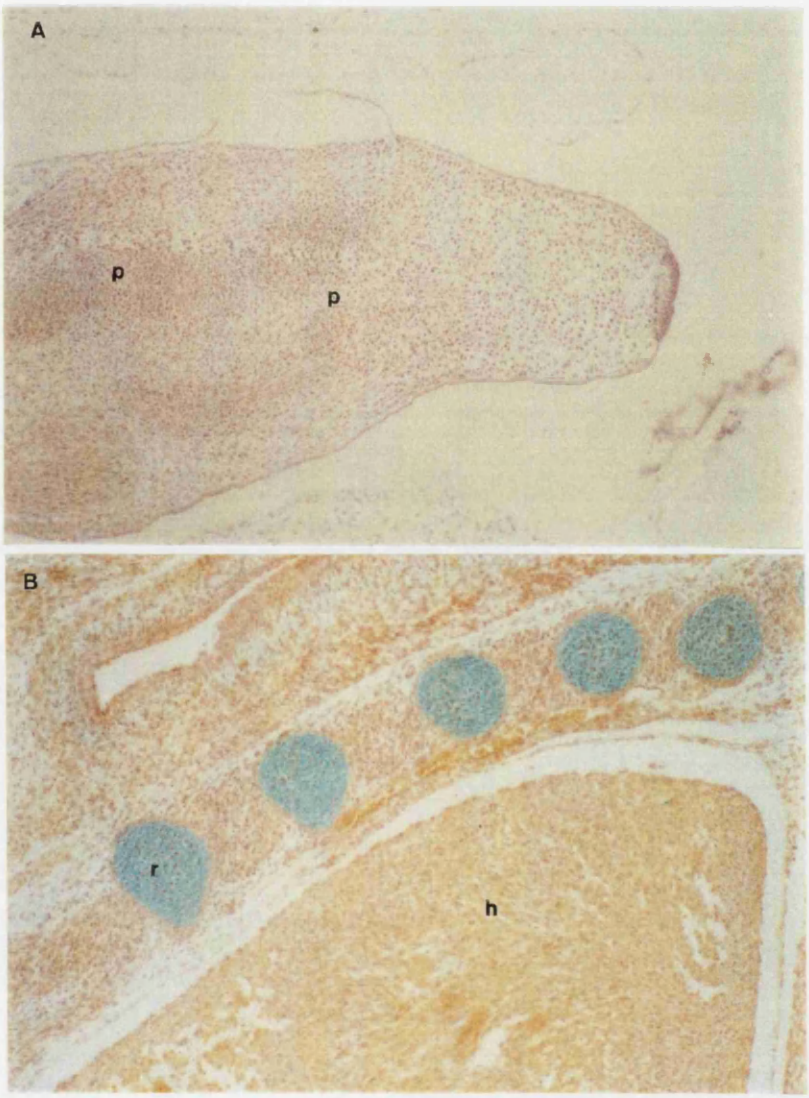
M 2
gz
B 2
F 2
J 3
N
C 3
G 3
K 2
p
p
O 3
D 4
H 4
L 3
p
p
P 4

Figure 14.

Alcian green staining of prechondrogenic blastemae.

(A) Transverse section through limb bud of 12.5 day p.c. embryo stained with Alcian green. No green staining is visible at the prechondrogenic blastemae (p).
(B) Sagittal section through a 14.5 day p.c. embryo to show staining of rib cartilage (r), with alcian green. h=heart. Scale bar represents 200μm.


A
p
p
B
r
h

TGF-β2 RNA becomes restricted to the proliferating chondroblast zone of the growth plate at the termini of the long bones and digits (Figure 13, M, N). No expression of TGF-β2 RNA is seen in areas of intramembranous or endochondral ossification.

TGF-β3 RNA is seen in a number of areas of condensing mesenchyme which were part of the developing neurocranium. These areas included the optic and otic capsules as well as the nasal cartilage (Figures 18 and 19).

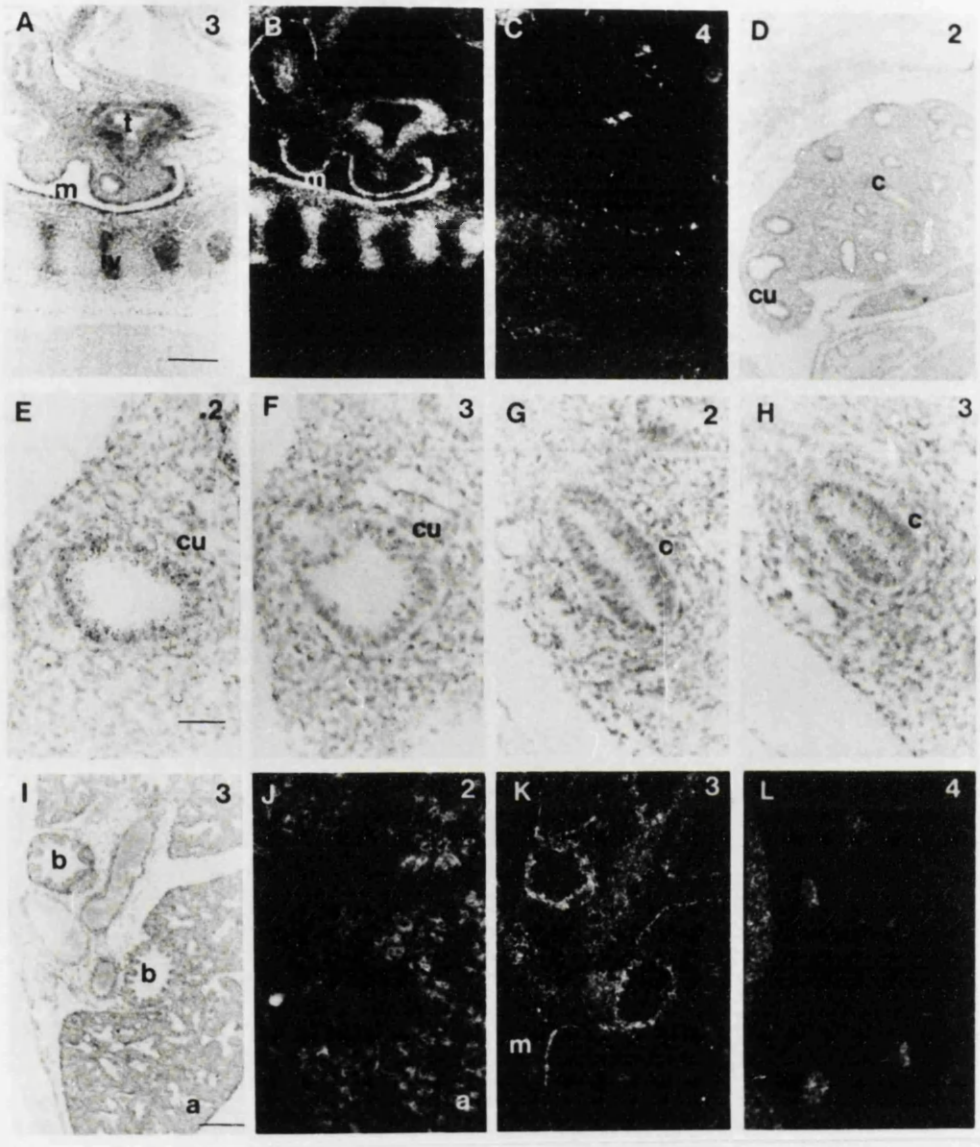
3.2.2 Expression in the Pulmonary system

The lungs are derivatives of the digestive tube and are formed from two endodermal outgrowths, which invade the splanchnic mesoderm at about 11.5 days *p.c.*. These simple endodermal tubes are then induced to branch distally, and the lung expands as rapid growth occurs at the terminal end buds and in the surrounding mesenchyme. The epithelial cells which line the respiratory tubes change in morphology at defined points. In the trachea the lining is made up of columnar epithelial cells, this type of epithelial cell is also seen in the bronchii and bronchioles. However in the more distal branches of the respiratory tract there is a change to a simple cuboidal epithelial. Furthermore, the fully differentiated alveolar epithelial cells (which begin to appear at about 16.5 days *p.c.*) are flattened in shape. These morphological rearrangements correspond chronologically to events in pulmonary epithelial cell differentiation such that primitive columnar epithelial cells transform/differentiate into simple cuboidal phenotype (Joyce-Brady and Brody, 1990). Expression of TGF-β isoforms in the pulmonary epithelial cells depends upon the stage and type of epithelial cell. At no stage of the development of the lung were either TGF-β2 RNA nor TGF-β3 RNA expressed in the mesenchyme of the lung.

TGF-β3 RNA is seen submucosally in the trachea and proximal bronchii at 12.5 days *p.c.* (Figure 15 A,B). Simultaneously, intense expression was seen in the immature columnar epithelial cells of the growing bronchioles (Figure 15 A,B) and in the mesothelium which surrounds the lung at this stage (Figure 15 A, B). TGF-β3 RNA is also expressed in the mesothelial cells which are opposite the lung buds on the dorsal wall adjacent to the lung bud (Figure 15 A, B). As lung growth proceeds and the distal

Figure 15.

Expression in the developing lung. Bright field of section hybridised with either TGF β2 (E, G) or TGF β3 (A, D, F, H, I). Dark field sections hybridised with TGF β2 (J) or TGF β3 (B, K) or control probe (C and L). (A and B) Sagital section through 12.5 day p.c. lung buds showing expression of TGF β3 RNA in bronchiolar epithelium and underlying tracheal epithelium. Expression also seen in mesothelium of lung buds and IV discs. (C) parallel section hybridised with sense TGF β2 probe showing no hybridisation. (D) Transverse section through 14.5 days p.c. lung probed with TGF β3. (E and F) High power of terminal end buds showing expression of TGF β2 in simple cuboidal epithelium (E). No silver grains are seen over epithelium of parallel section probed with TGF β3 (F). (G and H) High power of bronchioles showing no expression of TGF β2 in columnar epithelium (G). Parallel section probed with TGF β3 demonstrating specific expression in columnar epithelium of bronchioles (H). (I, J, K, L) Sagital sections of 16.5 day p.c. lung. Expression of TGF β2 is in all terminal end sacs, but no expression around bronchi (J). TGF β3 is limited to the mesothelia of the lung and the mesenchyme underlying bronchi epithelium (I, K). (L) Parallel section to (I) probed with sense TGF β2. 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. t=trachea, m=mesothelium, iv=intervertebral discs, c=columnar epithelium, cu=cuboidal epithelium, b=bronchi, a=developing alveoli. Scale bars represents 200μm (A, B, C, D, I, J, K, L) or 50μm (E, F, G, H).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branches of the respiratory tract differentiate to a cuboidal epithelium TGF-β3 RNA expression remains restricted to the columnar epithelial cells of the proximal respiratory tract (Figure 15 F,H). No TGF-β3 RNA expression was seen in simple cuboidal epithelia of the terminal end buds (Figure 15 F K). By 16.5 days *p.c.*, when differentiation of most bronchiolar epithelium has occurred and alveolar epithelium is widespread, expression of TGF-β3 RNA in the lung is negligible (Figure 15 K), being restricted to the mesenchyme surrounding the epithelial cells of the bronchi. At this stage TGF-β3 RNA is still expressed in the mesothelium.

TGF-β2 RNA expression is wholly restricted to the more distal cuboidal epithelia of the terminal end buds at 12.5 days p.c up to 14.5 days *p.c.* (Figure 15 E,G). By 16.5 days *p.c.*, as the lung is nearly fully differentiated to alveoli, there is widespread expression of TGF-β2 RNA in the flattened squamous epithelial cells (Figure 15 J). By 16.5 days *p.c.*, neither TGF-β2 RNA nor TGF-β3 RNA is detected in the epithelial cells which line the trachea or the bronchi (Figure 15 F and Figure 15 K).

3.2.3 Expression in the heart

Organogenesis begins at about 7.5 days *p.c.* in the mouse embryo. Between the ectoderm of the head process and the related endoderm appears the notochord. As the head process extends anteriorly, the underlying endoderm is carried with it. This will constitute the lining of the foregut. Directly beneath the foregut, loose mesenchymal cells are found. At this stage of development these cells form the heart primordia. As development proceeds the loose mesenchymal cells between the splanchnic mesoderm and the floor of the foregut form the endocardium. This is at first a straight tube which will, over the next 48-72 hours, twist and coil, due to the limited space available to growth in the pericardial cavity, to form the various chambers of the heart. Prior to the formation of the heart tube, which occurs at about 8.0 days *p.c.*, TGF-β2 RNA is present in the cells of the splanchnipleuric mesoderm arranged at the ventral side of the primitive foregut. However it is at the next stage that the expression pattern of TGF-β2 becomes more interesting. At about 8.0 days *p.c.* the heart begins to turn and twist back upon itself. This is caused by unequal growth down one side of the heart tube but also because of the

Figure 16.

Expression of TGF βs in the heart from 8.5 days p.c. to 9.5 days p.c.. Bright field of sections hybridised with TGF β2 (A, D). Dark field sections hybridised with either TGF β2 (B, E) or TGF β1 (C, F). (A, B, C) Transverse sections through an 8.5 day p.c. embryo. Expression of TGF-β2 is found in all the ventricular and outflow tract myocardium but not in the atrial myocardium. Intense expression is also noted in the body wall and beside the foregut (A, B). TGF-β1 is restricted to the cells which make up the endothelial inner tube of the heart. (D, E, F) TRansverse sections through 9.5 day p.c. embryo. The expression of TGF-β2 appears more intense at this time and is limited to the portion of the ventricular myocardium where the valves will form. Note the lack of expression of TGF-β2 in the myocardium of the heart coming from the outflow tract (m) (D,E). Intense expression is still observed in the region of the foregut (D, E). TGF-β1 is expressed in the endothelial cells but is beginning to be limited to those endothelial cells which lie over the regions where heart valves will form (F). 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. a=primitive atrium, e=endothelium, j=cardiac jelly, o= outflow tract, m=myocardium. Scale bars represents 200μm

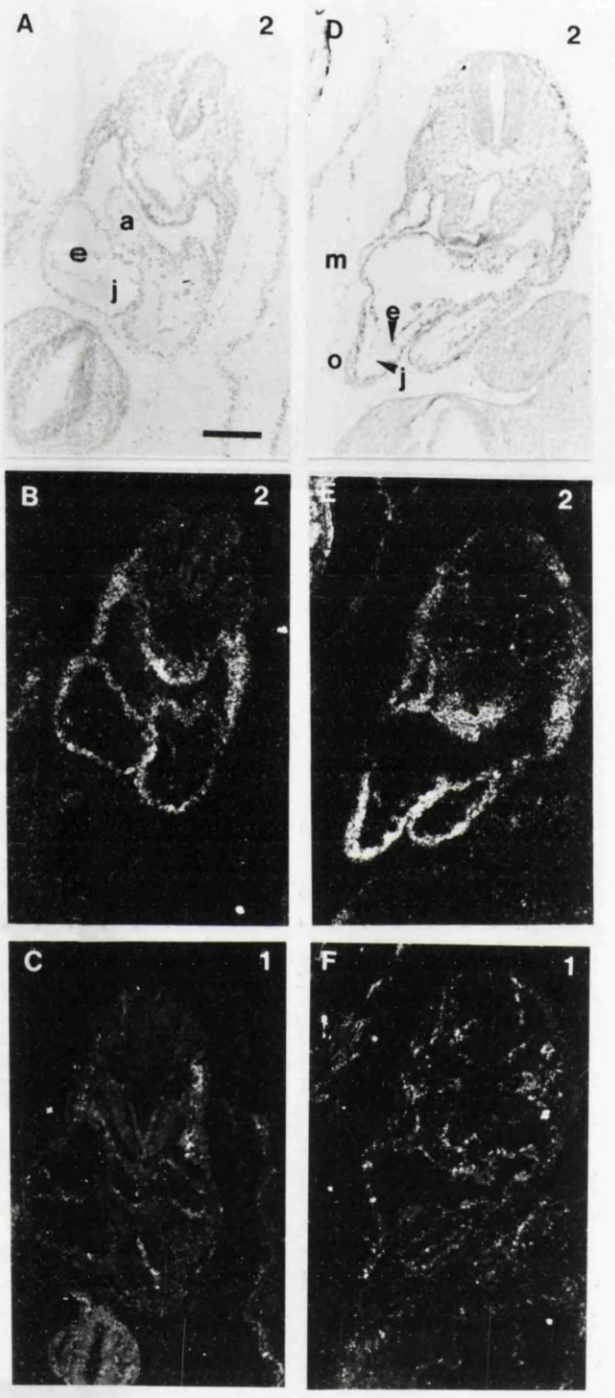
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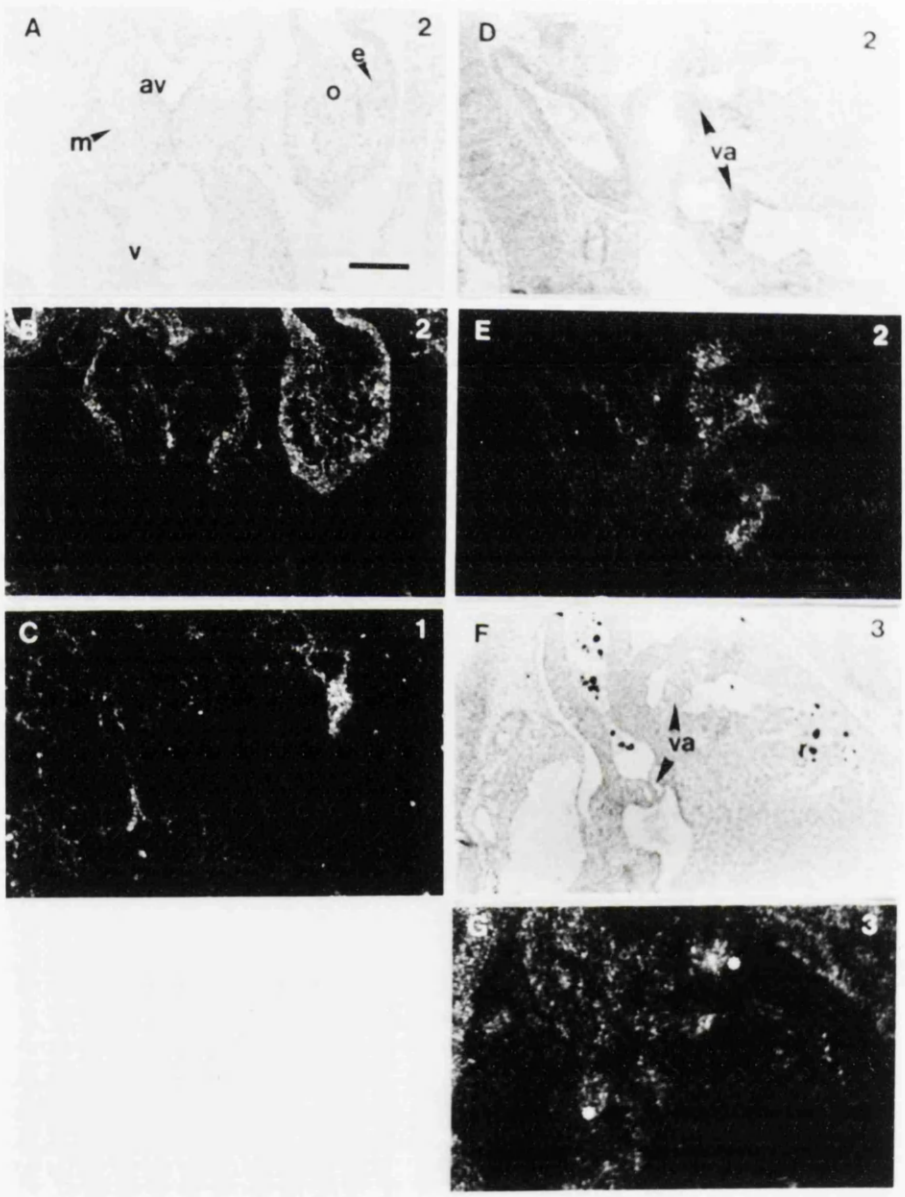
Figure 17.

Expression of TGF βs in heart at 10.5 days p.c. and 14.5 days p.c. Bright field of sections hybridised with TGF β2 (A, D) or TGF-β3 (F); Dark field of sections hybridised with TGF β2 (B, E), or TGF β3 (G) or TGF β1 (C). (A, B) sagittal section through atrioventricular region and outflow tract of 10.5 day p.c. heart. (A, B) Specialised myocardium overlying AV valve region expressing TGF β2 RNA. Note also slight expression in cushion tissue but no expression in myocardium of ventricle. TGF β2 also expressed in the myocardium and cushion tissue of outflow tract (B). (C) corresponding parallel section probed with TGF β1 demonstrating RNA expression in endothelial cells of AV region and outflow tract. (D, E, F, G) Sagittal section through 14.5 day p.c. heart. Expression of TGF β2 is limited to the cells of the cushion tissue at the valves (D, E). TGF β3 RNA is expressed in small condensations below valves (*) (F, G). 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. m=specialised myocardial cells, av=atrioventricular valve, v=ventricle, o=outflow tract, va=valves. Scale bars represents 200μm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limited space in the coelomic cavity. As the heart begins to form the various chambers associated with a normal adult heart, a number of constrictions begin to appear. These are the primitive valves which regulate the flow of blood through the heart. The endocardial cells become more intimate with the atrial myocardium, while in the ventricle they form attachments with the myocardial wall. At the junction between the atrium and ventricle the atrioventricular valve anlage forms. At the same time TGF-β1 becomes restricted to the endocardial cells which cover the primitive AV valve (Akhurst *et al* 1990, Lehnert and Akhurst 1988). In this study, no expression of either TGF-β2 RNA or TGF-β3 RNA was seen in the heart earlier than 8.5 days *p.c.*. At 8.5 days *p.c.* the first expression of TGF-β2 RNA was seen in the myocardium of the heart, the ventral endoderm of the foregut, and the mesenchyme which corresponds to splanchnic mesoderm derivatives (Fig 16 A, B). Later, at 9.5 days *p.c.* the expression in the foregut region is localised to the dorsal mesocardium (Fig 16 E). The early expression of TGF-β2 RNA appears to be associated with all myocardial tissue (Fig 16 A, B). However by 9.5 days *p.c.* the pattern of TGF-β2 RNA expression is restricted to that myocardium associated with the presumptive heart valve regions (Fig 16 D, E). TGF-β2 RNA is also seen in the myocardium of the bulbous arteriosus from day 8.5 *p.c.* up to 10.5 days *p.c.* (Figure 16 and 17). At 8.5 days *p.c.* TGF-β1 RNA is seen in the endothelial cells of the heart particularly in the region of the cushion tissue as described by (Akhurst *et al* 1990) which was confirmed by this investigation (Figure 15 C, F). This pattern of expression persists until after birth (Akhurst *et al* 1990). During this developmental stage TGF-β3 RNA is not expressed in any region of the heart.

At about 10.5 days *p.c.* in the region of the atrio-ventricular canal the dorsal atrial myocardial wall splits into two cell layers. The inner specialised cell layer adjacent to the dorsal endocardial cushion (which is about 2 to 3 cells thick) is continuous with the ventricular trabecular system. The outer cell layer underneath the epicardium (1 to 2 cells thick), which is degenerate and necrotic, is in continuity with the outer ventricular wall (DeRuiter *et al* 1992). By 10.5 days *p.c.* the expression pattern of TGF-β2 RNA is restricted entirely to the specialised cell layer, which forms a band of myocardium facing the dorsal endocardial cushion surrounding

the atrioventricular canal. TGF-β2 RNA is also expressed in the myocardium of the outflow tract (Figure 17 A, B). Expression of this gene is not seen in the atrial nor the ventricular myocardium *per se*, although slight expression is seen in the cushion tissue. TGF-β1 RNA is localised to the endothelial cells overlying the cushion tissue at this developmental stage (Fig 17, C). TGF-β2 RNA was not observed in the endothelial or epicardial cells in any part of the heart. At 12.5 days p.c the expression of TGF-β2 RNA has diminished considerably and by 14.5 days p.c the only area of expression was in the mesenchyme of the atrioventricular valve itself (Figure 17 D, E). At 14.5 day *p.c.* TGF-β3 RNA was observed to be expressed for the one and only time in the heart in a small localised spot just below the atrioventricular valve (Figure 17 F, G). By 16.5 days *p.c.* expression of TGF-β2 RNA and TGF-β3 RNA could be detected in the mesenchyme of the heart valves and TGF-β3 RNA in the wall of the sinus venosus. No expression of either RNA could be detected in the adult heart (data not shown).

TGF-β3 RNA was expressed in the tunica media and tunica intima of blood vessels at 14.5 days *p.c.* (Figure 13 C).

3.2.4 Expression of TGF-β genes in epithelial cells

All three TGF-β isoforms are expressed in epithelial cells during embryogenesis. In section 3.2.2, expression of TGF-β2 RNA and TGF-β3 RNA in epithelial cells of the pulmonary system, and the expression of TGF-β1 RNA in endothelial cells has already been reported.

3.2.5 Expression in sensory epithelia

Neither TGF-β1 RNA nor TGF-β3 RNA were seen to be expressed at any time in the sensory components of the developing olfactory, visual or auditory apparatus. TGF-β3 RNA was localised in areas of chondrification within the otic capsule and nasal septum (see section 3.2.1). Hybridisation with the TGF-β2 probe was seen in specific epithelial structures of all three of these organs.

3.2.5.1 Otic epithelia

The inner ear begins to develop from 8.0 days *p.c.* when the otic invagination is seen on the surface of the head, lateral to the myelencephalon. At 8.5 days *p.c.* the vesicle is completely formed and goes on to deepen and close over. At 11 days *p.c.* the endolymphatic cavities elongate mesio-ventrally to form the cochlear duct. The dorsal part of the otocyst consists largely of simple epithelium, the nuclei of the ventral part are stratified in two or three layers. The ganglion of the eigth acoustic nerve lies medio-rostral to the thicker, ventral part of the otocyst (Sher, 1971). Twelve hours later the otic vesicles are surrounded by mesenchyme with both the utricle and saccule parts visible. At 12 days *p.c.* the semi-circular canals begin to form from the utricle and are lined with epithelium (Rugh, 1990). By 14 days *p.c.* all three semi-circular canals have formed. Their walls are made up of simple cuboidal epithelium except on their convex sides, where there appears to be pseudostratified cuboidal epithelium with two or three levels of nuclei (Sher, 1971).

Prior to 10.5 days *p.c.* no TGF-β2 RNA was localised to the otic vesicle or the surrounding mesenchyme. At 10.5 days *p.c.* the first expression of TGF-β2 RNA was observed in a restricted pattern to one side of the otic vesicle adjacent to acoustic ganglion (VIII) (Fig 18, A, B). As development progresses TGF-β2 RNA was localised to the thickened epithelia on the convex side of the semi-circular canals from 12.5 days *p.c.*. This pattern of expression persists until at least 16.5 days *p.c.* (Figure 18 A,B and D). No TGF-β2 RNA expression was seen in the simple cuboidal epithelium opposite (Fig 18 F). TGF-β3 RNA was localised to the auditory capsule as described in section 3.2.1 (Figure 18 E). The continuation of the development of the inner ear was not followed beyond 16.5 days *p.c.*.

3.2.5.2 Nasal epithelia

The olfactory apparatus develops as two ectodermal thickenings above the stomatodaeum below and lateral to the forebrain. By proliferation of the surrounding mesoderm these placodes become depressed to form the olfactory pits. Further growth causes the epithelium of each nasal placode to come to lie in the medial and lateral walls of the nasal cavity. Expression

of TGF-β2 RNA is seen in the pseudostratified epithelium of the olfactory organ at 14.5 days *p.c.* and persists up to 16.5 days *p.c.* (Figure 19 A, B). The epithelium at this stage is quite thick, up to as many as 6 cells in depth and it is clear that the TGF-β2 RNA is localised to that part of the epithelia adjacent to the nasal cavity (Figure 19 E). TGF-β2 RNA also hybridises to Jacobson's organ, which is destined to become the glandular and sensitive olfactory epithelia (Fig 19 A, B). TGF-β3 mRNA expression is limited to the cartilaginous capsule which surrounds the nasal passages (Figure 19 C, D). This pattern of expression of TGF-β3 RNA is first seen at 14.5 days *p.c.* and persists up to at least 16.5 days *p.c.*.

3.2.5.3 Optic epithelial

The eye begins to develop by invagination of the optic vesicle from the wall of the forebrain at 8.0 days *p.c.*. As development proceeds the optic cup forms, when the embryonic wall invaginates into the optic vesicle. The invaginating embryonic wall forms the lens vesicle which by day 11 will have formed a completely circular vesicle which is partially engulfed by the optic cup. The optic cup is bilayered. The layer immediately adjacent the optic vesicle is destined to become the pigmented layer of retina, while the outer layer will become the sensory layer. The formation of the optic vesicle generates an optic stalk from which the optic nerve will develop (Rugh 1990). As the eye continues to develop the lens becomes seperate from the epidermis. Lens fibres are observed at about 13 days *p.c.* and nerve fibres appear in the optic stalk. By 13.5 days other structure associated with a adult eye begin to form. These include the iris, and various ocular muscles. By 14 days the lens epithelium, adjacent to the anterior chamber is cellular and as the lens enlarges the epithelium thins. At about this stage the eyelids fuse to cover the eye before birth. At 15 days *p.c.* the retina can be defined into two regions, the inner and outer nuclear layers. The inner nuclear layer contains the neurones which recieve signals from the rods, found in the outer nuclear layer, before transmitting that signal via the optic nerve to the brain. The epidermis begins to differentiate into the cornea. By 17 days the iris is well established and the seperation between the retinal and lenticular zones of the eye cup, the ora serrata, can be distinguished. The retina is undergoing

Figure 18.

Expression in sensory epithelia: the ear. Bright field of sections hybridised with TGF β2 (A, C, F) and dark field of sections hybridised with either TGF β2 (B, D) or TGF β3 (E). (A, B) sagittal section through otic vesicle of 10.5 days p.c. embryo. TGF β2 RNA is expressed in a localised region of the otic epithelia adjacent to acoustic ganglion VIII. (C, D, E) Serial sagittal sections through a 16.5 day p.c. embryo. TGF β2 is localised to the pseudostratified sensory epithelium rather than the simple cuboidal epithelium opposite (C,D). TGF β3 RNA is not expressed in any otic structure but is present in the mesenchymal condensations which form the otic capsule (E). (F) High power of (C). 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. o=otic vesicle, g=ganglion, s=pseudostratified epithelium, c=simple cuboidal epithelium, p=peri-otic capsule. Scale bars represents 200μm (A, B, C, D, E) or 50μm (F)

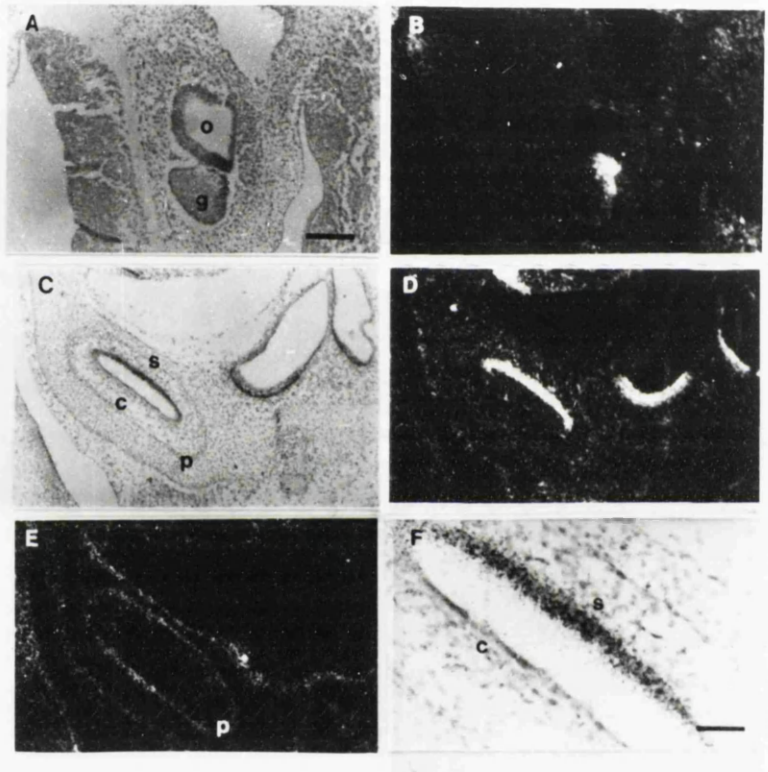
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Figure 19.

Expression in sensory epithelia: the nose. Bright fields of sections hybridised with TGF β2 probe (A, E) or TGF β3 (C). Dark field of sections hybridised with TGF β2 (B) or TGF β3 (D). (A, B, C, D, E) transverse sections through 14.5 day p.c mouse head. TGF β2 RNA is expressed in the nasal epithelium adjacent to the cavity. It is also expressed in Jacobson's organ (A, B). (E) High power of (A) demonstrating expression of TGF-β2 in the epithelium nearest the nasal cavity. TGF β3 is expressed in the mesenchymal condensations which will become cartilage and also in the muscle of the tongue (C, D). 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. o=olfactory epithelium, j=Jacobson's organ, t=tongue. Scale bars represents 200μm (A, B, C, D) or 50μm (E)

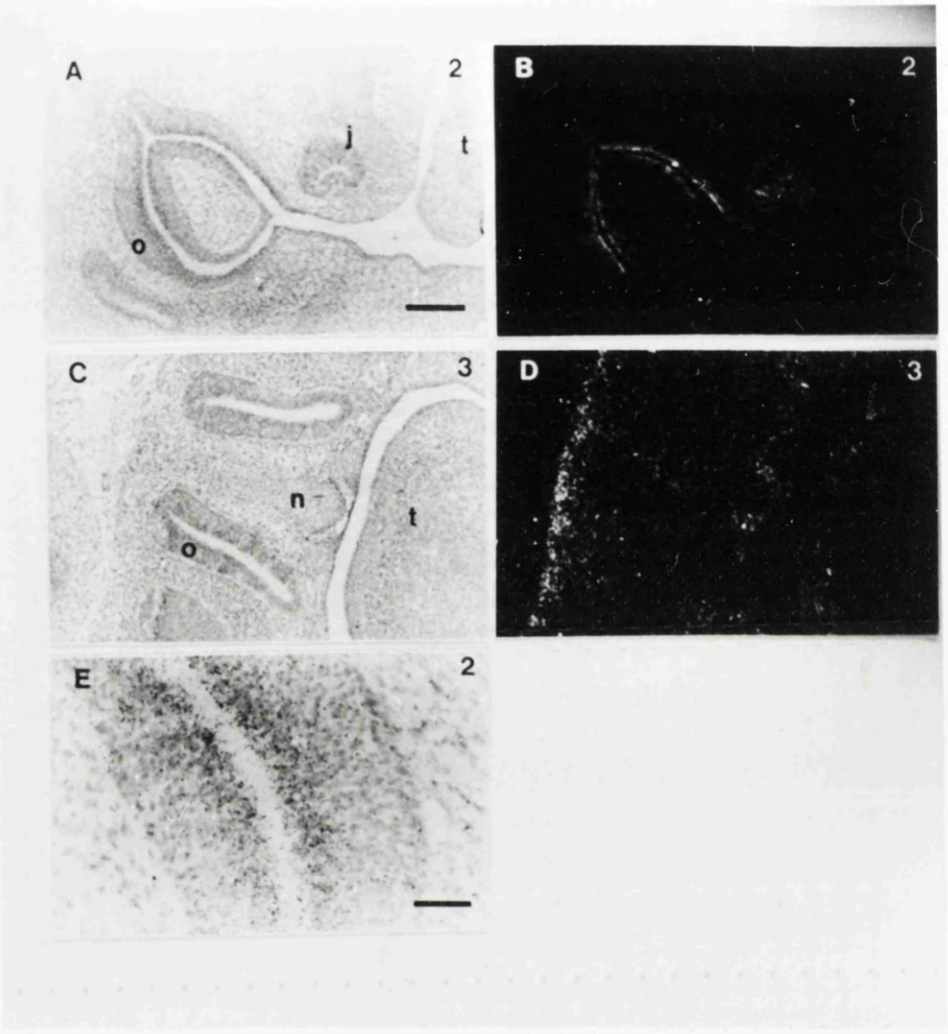
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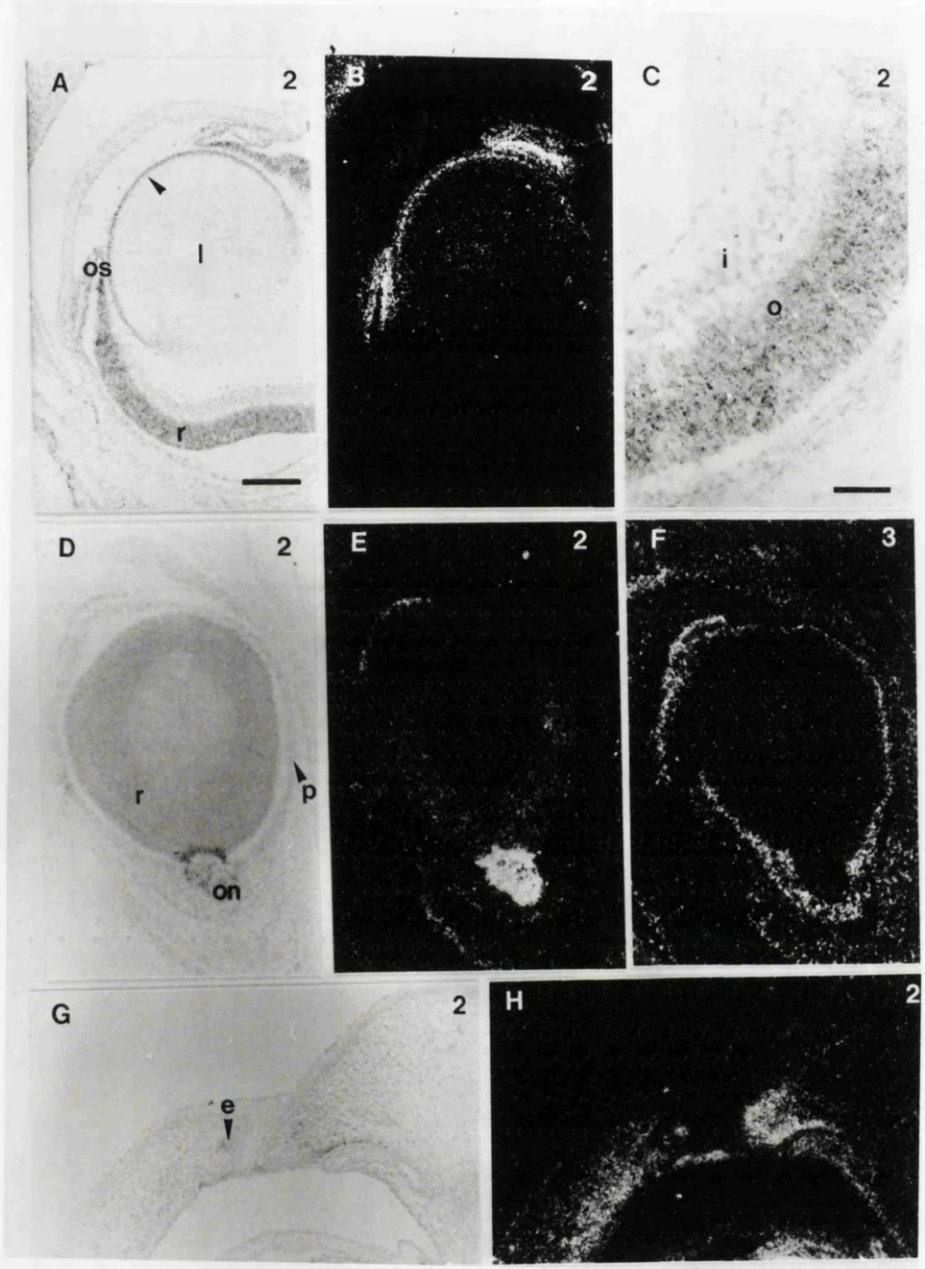
Figure 20.

Expression in sensory epithelia: the eye. Bright field (A, C, D, G) and dark field (B, E, H) of sections hybridised with TGF β2. (A, B, C, D, E, F, G, H) Sagittal sections through eye of 16.5 day p.c. embryo. TGF β2 RNA is expressed in the anterior side of the lens epithelium, and in the ora serata (A, B). (C) High power of (A) showing hybridisation of TGF β2 to the outer layer of the neural retina. (D, E, F) A more sagittal section through the eye to that of (A) demonstrating TGF-β2 in the neural retina and also very intense expression in the region of the eye where the neurones from the neural retina leave through the optic nerve. (F) Adjacent section hybridised with TGF-β3 riboprobe showing no expression in the pigmented layer of the eye. No expression in the optic nerve was seen with this riboprobe demonstrating the specificity of TGF-β2 expression. TGF β2 RNA was also expressed in the mesenchyme of the eyelides behind the eyelid furrow. There was also expression of RNA in the epithelia of the eyelid furrow adjacent to the eye (G, H). 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. os= ora serata, l=lens, r=retina, i=inner retina, o=outer neuronal retina, p=pigmented epithelium, on=optic nerve, e=eyelash follicle. Scale bars represents 200μm (A, B, D, E, F, G, H) or 50μm (C)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further differentiation, although it has no cones. By 18 days the eye has completed development (Rugh 1990)

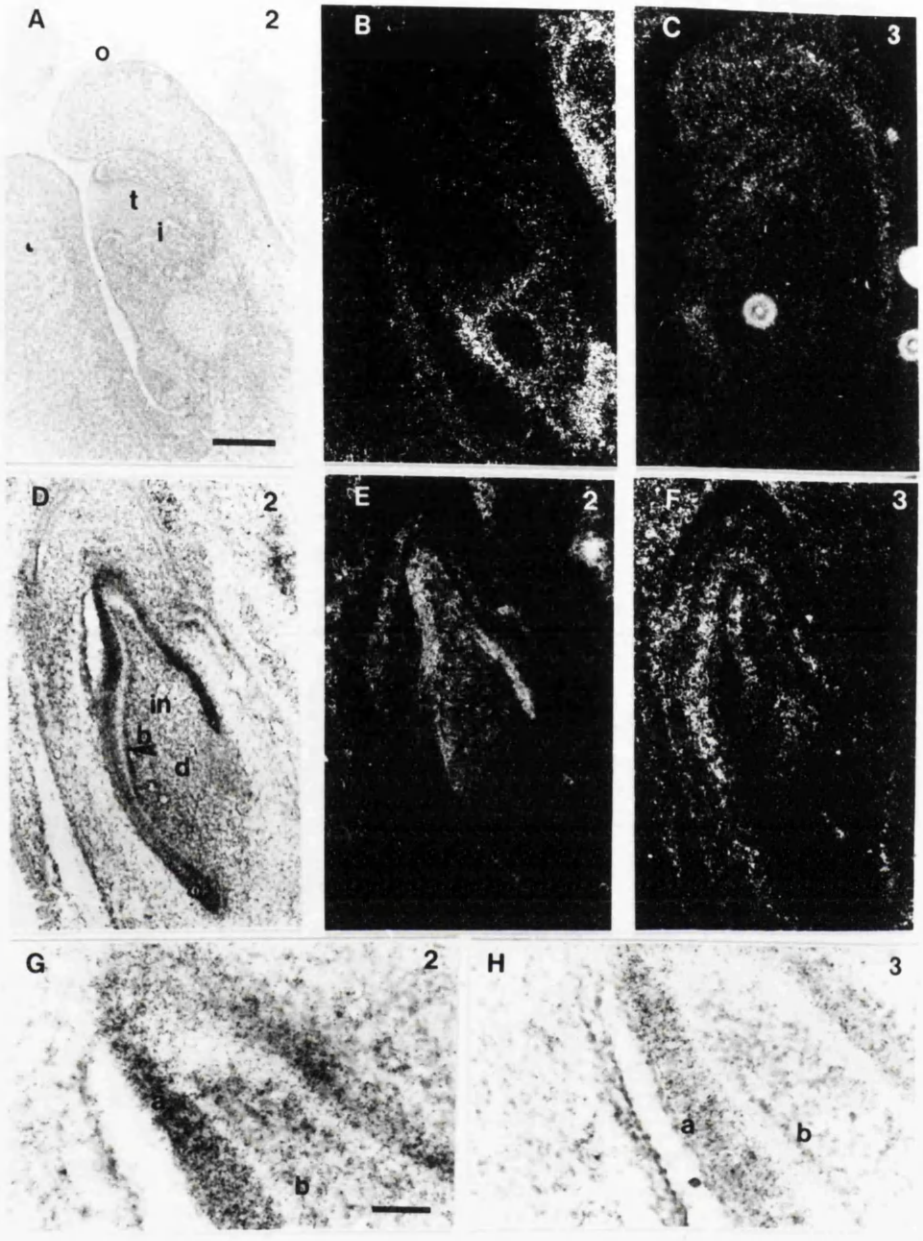
The earliest expression of TGF-β2 RNA is seen at 10.5 days p.c around the retina of the optic cup. Expresson of TGF-β2 RNA continues to be seen in this region but it is not until 16.5 days *p.c.* that it can be localised to the outer retinal layer of neural cells (Fig 20 C). Expression of TGF-β2 RNA is also seen over the optic nerve at 12.5 days *p.c.* and an intense hybridisation signal was observed at the point where all the nerves of the retina leave the eye (Fig 20 D, E). At 16.5 days *p.c.* TGF-β2 RNA was also seen in the ora serata and in the lens epithelium. The expression in the lens epithelium was restricted to the anterior portion of the lens itself and also limited to the inner cells of the epithelium (Fig 20 A, B). TGF-β2 RNA was also seen in the mesenchyme of the lower and upper eyelids at 16.5 days *p.c.* (Fig 20, G, H). TGF-β2 RNA was also expressed in the innermost layer of cells at the region of the fused epithelium at 16.5 days *p.c.* but not in the eyelid furrow *per se* (Fig 20 G, H). Note that TGF-β2 RNA was detected in the condensation of cells which are the developing follicles of eyelashes of the mouse (Figure 20 G, H). There is also expression in the cells along the innermost side of the eyelids which are destined to become the conjuctiva (Fig 20, G, H). TGF-β3 RNA was expressed in the sclera, a tough mesenchymatous and partially cartilaginous coat, outside of the choroid coat of the eye, at 16.5 days *p.c.* and in the pigmented epithelium of the eye at 16.5 days *p.c.* (Fig 20 F).

3.2.6 Expression of TGF-βs in developing teeth

Tooth development begins at about 12 gestation days and continues beyond birth. The tooth buds of the first molars and incisors appear at the same time. The first indication of teeth is the appearance of thickening and elongation of the dental lamina within the epithelial lining of the oral cavity, the growth of which parallels the growth of the jaw. The dental lamina is an invagination and ingrowth of the stratified oral epithelium and consists mainly of cuboidal cells with a basal layer made up of low columnar cells. A day later the first tooth buds (destined to become molars, other teeth such as inscisors follow later) show invagination in the dental lamina. This invagination will be filled with mesenchymal cells which becomes the

Figure 21.

Expression of TGF β isoforms in the developing teeth. Bright field (A, D, G) and dark field (B, E) of sections hybridised with TGF β2. Brightfield (H) and dark field (C, F) of sections hybridised with TGF β3. (A, B, C) Serial sagittal section through lower jaw of 14.5 day p.c. mouse embryo, with a tooth bud. TGF-β2 riboprobe hybridised to the mesenchyme surrounding the tooth bud but was not expressed within the stuctures of the tooth bud at this stage (A, B). TGF-β3 was only expressed in a line of muscle at the lower part of the jaw and not in the tooth bud or surrounding area (C). By 16.5 days p.c. both TGF-β2 and TGF-β3 are expressed in the tooth bud. (D, E, F) Sagittal sections through incisor of 16.5 day p.c. embryo. TGF β2 RNA is expressed in the ameloblasts and odontblasts as wells as dental papilla (D, E). TGF β3 RNA is localised to the ameloblasts but not the odontoblasts. Expression is also seen in the dental papilla but not as extensive as TGF β2 (F). There is also extensive expression of both genes around the tooth buds at this time. (G and H) are high power views of the incisor hybridised with either TGF-β2 (G) or TGF-β3 (H). Expression of TGF-β2 is very intense in the ameloblasts and there is also expression in the odontoblasts. TGF-β3 is also expressed in the ameloblasts and although difficult to compare in situs quantitatively the expression of TGF-β2 appears to be greater than TGF-β3. Neither gene is expressed in the single layer of epithelial cells which overly the ameloblasts. 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. t=tooth bud, i and in=incisor, d=dental papilla, b=odontoblasts, a=ameloblasts. Scale bars represents 200μm (A, B, C, D, E, F) or 50μm (G, H)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dental papilla. The stellate reticulum appears between the central cells of the tooth bud epithelia which defines the inner and outer enamel epithelium. The inner enamel epithelium will differentiate to ameloblasts which secrete the enamel cap of the tooth. By 16 days *p.c.* the enamel epithelium cells are continuing to proliferate which increases the volume of the dental papilla. The inner enamel epithelium is one cell thick while the outer epithelium is a single layer of columnar cells overlying a thick layer of squamous cells. At this stage the dental sac which surrounds the primordial tooth consists of several layers of flattened cells. As development proceeds at 17 days *p.c.*, bone is laid down around the tooth, and tooth buds of the second set of molars appears. Development of the teeth continues up to 5 days after birth for the first teeth that appeared but up to 35 days *post partum* for the last teeth to develop.

No expression of any TGF-β isoform was detected in the early tooth buds at 14.5 days *p.c.* or earlier (Fig 21 A,B). Both TGF-β2 RNA and TGF-β3 RNA were expressed in epithelial and mesenchymal components of developing teeth, but not until after 14.5 days *p.c.*. Prior to this stage only TGF-β1 RNA was detected in the tooth buds, which had been previously reported by Lehnert and Akhurst (1988), and which was confirmed by this study.

Both TGF-β2 RNA and TGF-β3 RNA were expressed in mesenchyme between the tooth bud and the epithelium of the oral cavity. The expression of TGF-β2 RNA was more defined, an intense band of expression visible directly underneath the oral epithelium. The expression of TGF-β3 RNA was not limited to the mesenchymal cells underlying the oral epithelium but appeared to have a low level of expression in all the cells surrounding the tooth bud (Figure 21 A, B). There is also a disparity of expression patterns between the TGF-β isoforms. TGF-β1, unlike the other two, was not expressed in the dental papilla in later stages of tooth development. There was some over lap of expression with both TGF-β1 and TGF-β2 expressed in the inner enamel epithelium. Also TGF-β2 showed similar expression to that described for TGF-β1 in the cervical loops of the dental epithelium.

At 16.5 days *p.c.* the expression of TGF-β2 RNA appeared to be more extensive and intense than TGF-β3 RNA, although it is difficult to compare quantitatively the intensity of expression of different probes (Fig 21).

Both TGF-β2 RNA and TGF-β3 RNA were expressed in the ameloblasts (Fig 21 D, E, F, G, H). Neither gene was expressed in the outer enamel although there was intense expression of TGF-β2 in the ameloblasts at 16.5 days *p.c.* (Fig 21 G) there was no expression of TGF-β2 RNA (or TGF-β3 RNA) in the stratum intermedium, which are the single layer of epithelial cells overlying the ameloblasts (Fig 21 G, H). Both were also localised in the dental papilla, although with apparently different patterns of expression (Fig 21 E, F). TGF-β2 RNA was localised in a majority of the cells which make up the dental papilla with a gradient of expression becoming more intense towards the anterior portion of the tooth, this could reflect cell density. The expression of TGF-β3 RNA was not as widespread as compared to TGF-β2 RNA. TGF-β3 RNA expression was localised more centrally in the dental papilla (Fig 21 F). The expression of TGF-β3 RNA in this region did seem to coincide with cell density. The immediate anterior portion of the dental papilla was negative for TGF-β3 RNA.

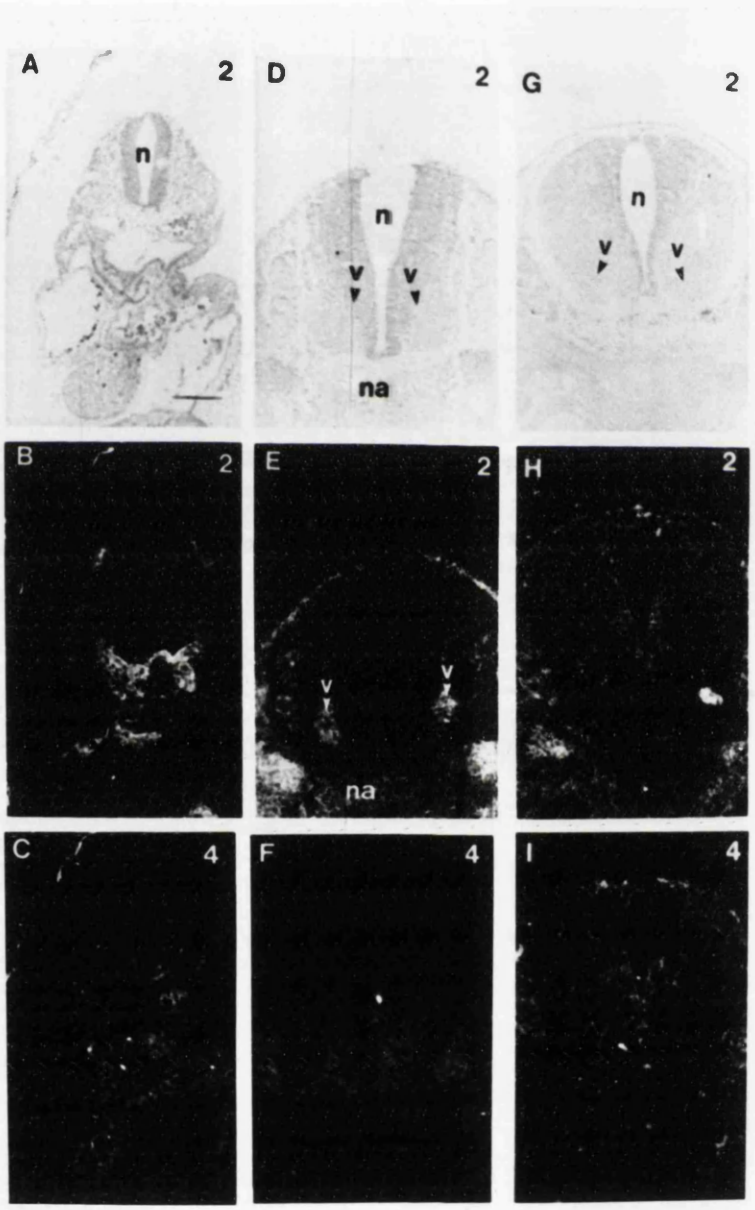
3.2.7 Expression of TGF-βs in neural tissue

The central nervous system is one of the first systems to develop and to differentiate and one of the last to be completed during embryogenesis. The spinal cord begins to appear at about 8.5 days *p.c.*. At 10 days *p.c.* the neural groove is closed in limited regions, forming a large neural canal with a thick layer of surrounding neuroblasts. Neural crests appear simultaneously as the neural tube closes (Kaufman, 1992). By 11.0 days *p.c.* the spinal cord has thickened and the ventral horns, spinal ganglia and spinal nerves become visible. Sclerotome mesenchyme is distributed around the cord and encloses the notochord which is located ventrally. This will degenerate as a cartilaginous centrum develops around it.

In this study expression of TGF-β2 RNA and TGF-β3 RNA appeared to be very limited in neural tissue of the embryonic mouse. At 10.5 days *p.c.* TGF-β2 probe showed hybridisation to the forebrain in the telenchephalon (Figure 23 A, B). Expression in the brain appeared to be very limited, the

Figure 22.

Expression of TGF β2 in the ventral horns. Bright field (A, D, G) and dark field sections (B, E, F) hybridised with TGF β2. Dark field of sections hybridised with sense TGF β2 probe (C, F, I). 9.5 (A, B, C) 11.5 (D, E, F) and 12.5 (G, H, I) day p.c. embryos transversely sectioned. TGF β2 RNA is expressed at only 11.5 days p.c. in the ventral horns of the spinal cord (D, E). Expression of TGF β2 is not seen prior (A, B) or post (G, H) this developmental stage. 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. n=neural canal, v= ventral horns, na= neural arch. Scale bars represents 200μm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n
D
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na
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n
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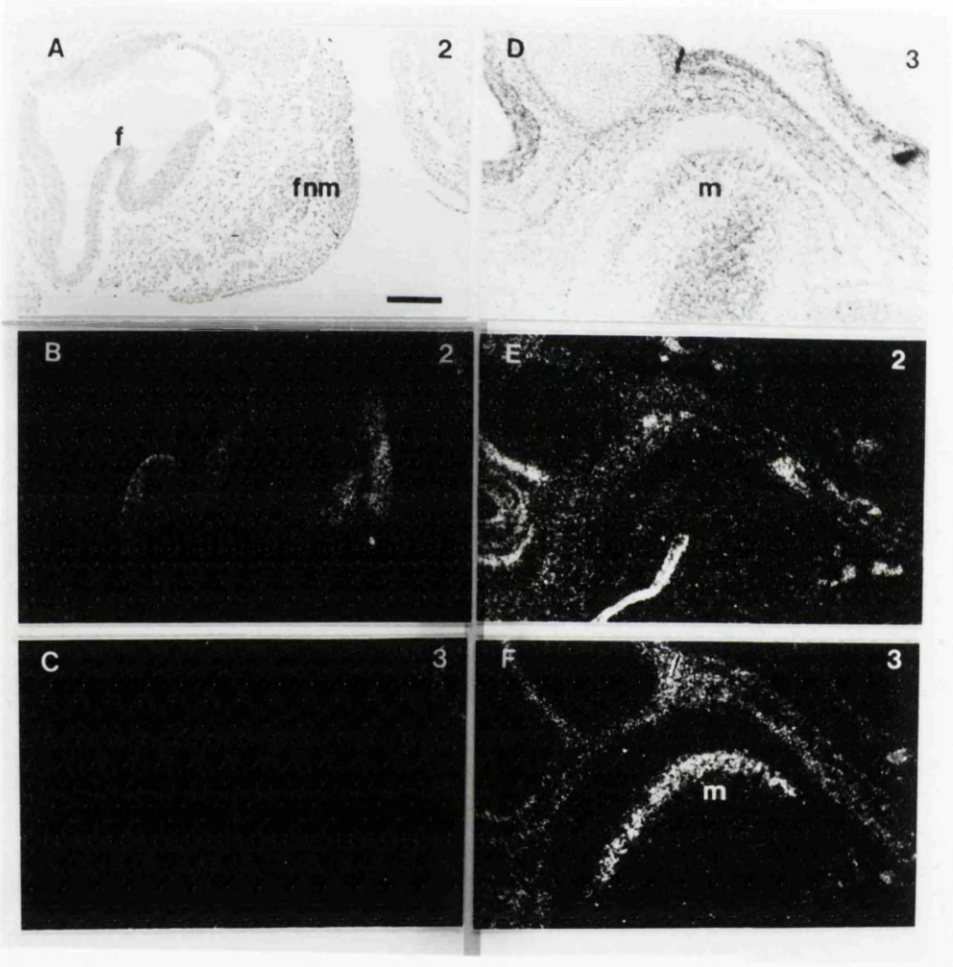
v
na
H
2
C
4
F
4
I
4

Figure 23.

Expression of TGF β isoforms in other neural tissue. Bright field (A) and dark field (B, E) of sections hybridised with TGF β2 riboprobe. Light field (D) and dark field (C, F) of sections hybridised with TGF β3 probe. (A, B, C) 10.5 day p.c. embryo sectioned sagittally showing expression of TGF β2 RNA in the fronto-nasal mesenchyme and developing brain (A, B). At this stage of development expression of TGF-β3 is limited to the intervertebral discs. Sagittal section through brain of 16.5 day p.c. embryo showing hybridisation of TGF β3 probe to the mitral cells of the developing olfactory lobe (D, F). TGF-β2 is not expressed in the brain at this stage of development. 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. f=forebrain, fnm=frontonasal mesenchyme, m=mitral cell layer. Scale bars represents 200μm


A
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3
m

only other time TGF-β2 RNA being observed was at 16.5 days *p.c.* in the mesencephalon (data not shown). TGF-β2 RNA is transiently expressed at comparatively low levels in the ventral horns of the spinal cord at about 11.0 days *p.c.* (Figure 22 D,E). The transcripts are not seen prior to 10.5 days *p.c.* or after 11.5 days *p.c.* (Figure 22 A,B, G,H). No expression of TGF-β2 RNA was seen in any other structure of the spinal cord or related nervous tissue. TGF-β3 RNA is not expressed in any part of the spinal cord. TGF-β3 RNA is only found to be expressed at later stages of development. At 14.5 days *p.c.* transcripts of TGF-β3 RNA were seen in the mitral cells of the developing olfactory lobes. This expression persists up to at least 16.5 days *p.c.* (Figure 23 D, F). Apart from this expression of TGF-β2 RNA and TGF-β3 RNA in the regions described, the presence of TGF-β2 RNA during the epithelial differentiation of various sense organs was the only other potential site of TGF-β RNA isoforms expression in the nervous system.

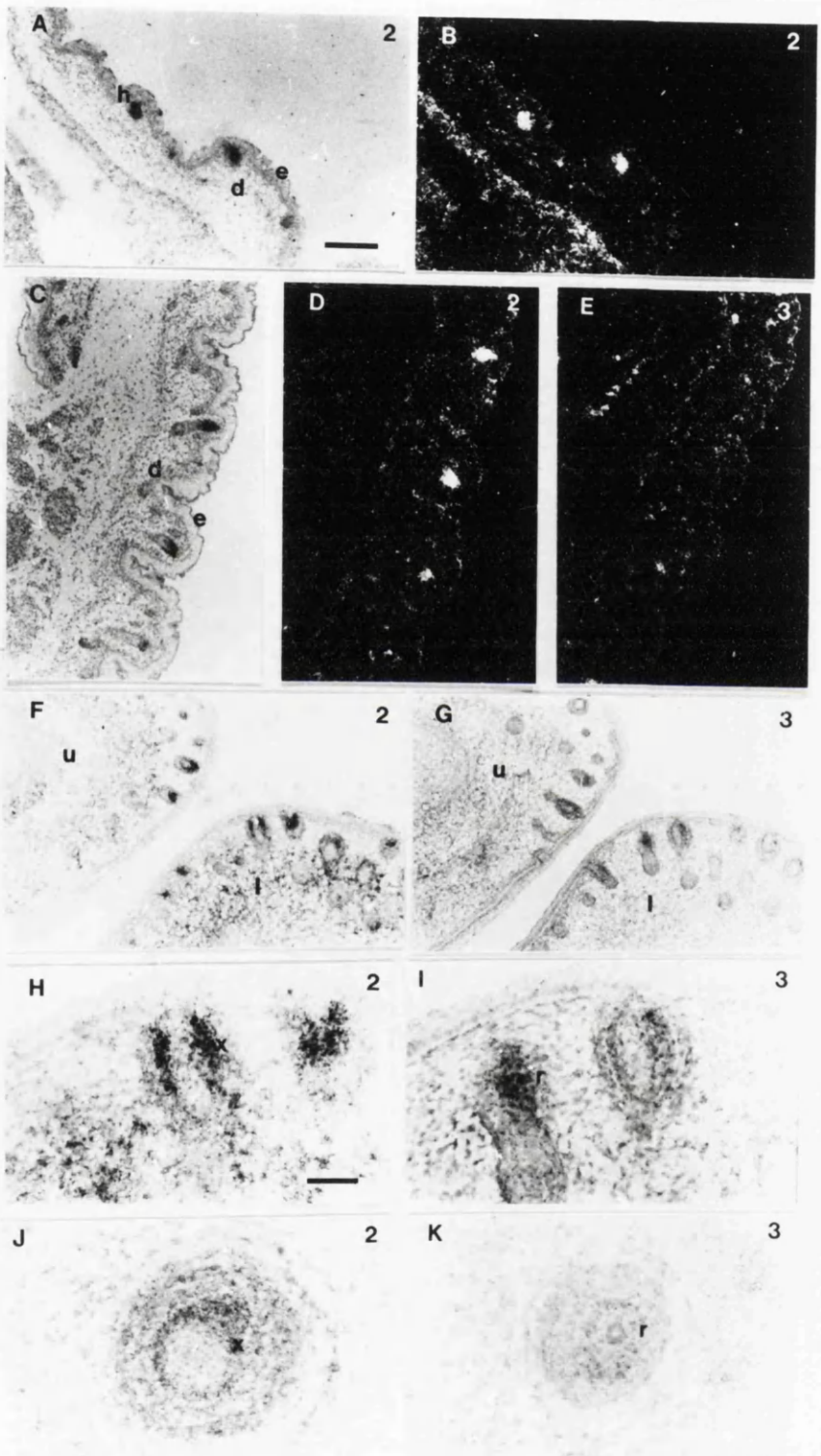
3.2.8 Expression of TGF-β isoforms in epidermis and whisker follicles

Although the embryo has an ectodermal covering from an early stage of embryogenesis, it is not until about 14 days *p.c.* that differentiation of the epidermis begins. At this time congregations of cells, hair follicle primordia, appear in the single layer of ectoderm. At 15 days *p.c.* a second skin layer, the stratum germanitum arises, and between it and the first layer a basal layer develops. In between these two events the vibrissae papilla have invaginated. Prior to 14.5 *p.c.* days no expression of either TGF-β2 RNA nor TGF-β3 RNA was seen on the surface of the embryo. At 14.5 days *p.c.* the TGF-β2 riboprobe was seen to hybridise to the congregation of cells which are the forerunners of the hair follicles. At this stage TGF-β3 RNA is not localised in either the epidermis nor the primordia of the hair follicles.

At 14.5 days *p.c.* expression of TGF-β2 RNA is restricted to the hair follicle primordia which persist up to 16.5 days *p.c.* (Fig 24 A, B). By 18.5 days *p.c.* the skin is almost fully developed. The hair follicles have differentiated rapidly and are abundant. By this stage the hair bulb, core and inner and outer sheaths all project deeply into the dermis. Again TGF-β2 RNA was

Figure 24.

Expression of TGF β isoforms in the skin and vibrissae. Light fields (A, C, F, H, J) and dark fields (B and D) of sections probed with TGF β2. Light fields (G, I and K) and dark field (E) of sections hybridised with TGF β3. (A, B) Sagittal sections through 16.5 day p.c. skin. No hybridisation is seen in the ectoderm by either TGF β2 or TGF β3 probes (A,B). TGF β2 probe hybridises to the condensation of cells which will develop into hair follicles. (C, D, E) sections through skin of 18.5 day p.c. embryo. Neither TGF-β2 nor TGF-β3 is expressed at levels of detection in the dermis or epidermis. TGF-β2 is expressed in the hair follicles (C, D). There is slight expression of TGF-β3 RNA in the hair follicle at this stage (E). (F,G) Sagittal section through snout of 16.5 day p.c. embryo showing expression of TGF β2 and TGF β3 in the hair follicles. There is still no expression in the skin. The expression of TGF-β2 is more intense in the hair follicles than that of TGF-β3. (H, I) High power of hair follicles demonstrating expression of TGF-β2 in the outer root sheath (H). TGF-β3 RNA also appears to be expressed in the outer root sheath (I). The hair follicle in (I) is from the adjacent section to that in (H) and is giving the impression that TGF-β3 is expressed in the inner root sheath. (J, K) High power transverse sections through whisker follicles at 16.5 days p.c. hybridised with TGF-β2 (J) and TGF-β3 (K). TGF-β2 RNA is expressed in Henle's layer and outer root sheath (J). (K) deeper transverse section than in (J), TGF-β3 RNA is expressed in the outer root sheath. Sagittal section through skin of 18.5 day p.c. embryo. Neither TGF β2 nor TGF β3 is expressed at a high level in the epidermis or dermis. Both are expressed in the condensation of cells which are developing hair follicles and in the sub basal mucosa. (J, K, L, M) Sagittal section through head of 16.5 day p.c. embryo which has cut vibrissae in transverse sections. TGF β2 RNA is expressed in the inner and outer epithelium of the hair follicle (J, K). (L) high power of (J). (M) high power of vibrissae showing hybridisation of TGF β3 probe to inner epithelium of whisker follicle. 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. h=hair follicle, d= dermis, e= epidermis, u=upper jaw, l=lower jaw, x and r =outer root sheath. Scale bars represents 200μm (A, B, C, D, E, F, G) or 50μm (H, I, J, K)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only found in the hair follicles with no expression seen in the dermis or epidermis (Fig 24 C, D). TGF-β3 RNA was not expressed at this stage of skin development but was expressed, although to a lesser extent in the hair follicles (Fig 24 E). The expression of both genes in the hair follicles appeared to be associated within different cell types. TGF-β2 RNA was restricted to the outer hair sheath while TGF-β3 RNA was localised to the cells which would form the mesenchymal sheath (Fig 24 F, G, H, I).

The expression of TGF-β2 and β3 RNA in the whisker follicles was noted as early as 14.5 days *p.c.* when the vibrissae have invaginated. TGF-β2 RNA and TGF-β3 RNA were expressed in the whisker follicles at 16.5 days *p.c.* (Fig 24 J, K). TGF-β2 RNA was expressed in the inner or outer root sheaths at this stage in development (Fig 24 J). TGF-β3 RNA was restricted to the inner root sheath (Fig 24 K). Expression of TGF-β2 RNA in the mesenchyme of the snout was restricted around the vibrissae. No expression of TGF-β2 RNA was observed immediately adjacent to the skin.

3.2.9 Extraembryonic expression of TGF-β isoforms

All three TGF-βs are expressed in extraembryonic membranes although all have a distinct pattern of expression. The expression of the TGF-βs was only followed up to 9.5 days *p.c.* as after this time the embryo was dissected from the decidua.

The earliest embryos investigated were 6.5 days *p.c.* No expression of either TGF-β2 or β3 RNA was seen in any embryonic stucture at 6.5 or 7.5 days *p.c.*. However, there was expression of both genes in extraembryonic membranes. This expression continued up to 9.5 days *p.c.*. At 10.5 days *p.c.*, and after, the embryo was dissected from the decidua, which was discarded.

At 6.5 days *p.c.* TGF-β2 RNA was RNA was present in the decidua capsularis although the expression was not seen at the surface of the decidua (Fig 25, A, B). TGF-β3 RNA was also seen in the decidua capsularis but the expression pattern was in direct contrast to that observed for TGF-β2 RNA, and was possibly located in the uterine

Figure 25.

Expression of TGF β isoforms in extraembryonic membranes. Bright field (A, D, G) and dark field (B, E, H) of sections hybridised to TGF β2 probe or TGF β3 (C, F, I). (A, B, C) section through 6.5 day p.c. decidua. TGF β2 is expressed in a wide band of cells around the outside of the decidua (A, B). TGF β3 is expressed in a tighter band around the edge of the decidua (C). (D, E, F) Section through 8.5 day p.c. decidua. TGF β2 is intensely expressed in the uterine epithelium (D, E). TGF β3 probe hybridised to the decidua. No expression of TGF-β3 was seen in the uterine epithelium (F). (G, H, I) Section through decidua at 9.5 days p.c. TGF β2 is intensely expressed in the giant cells of the decidua (I, J). TGF β3 is limited to a tight band of cells on the outer limits of the decidua (K). 1=TGF-β1, 2=TGF-β2, 3=TGF-β3 and 4=sense riboprobe. d=decidua, e=uterine epithelium, m=mesometrial decidua. Scale bars represents 200μm

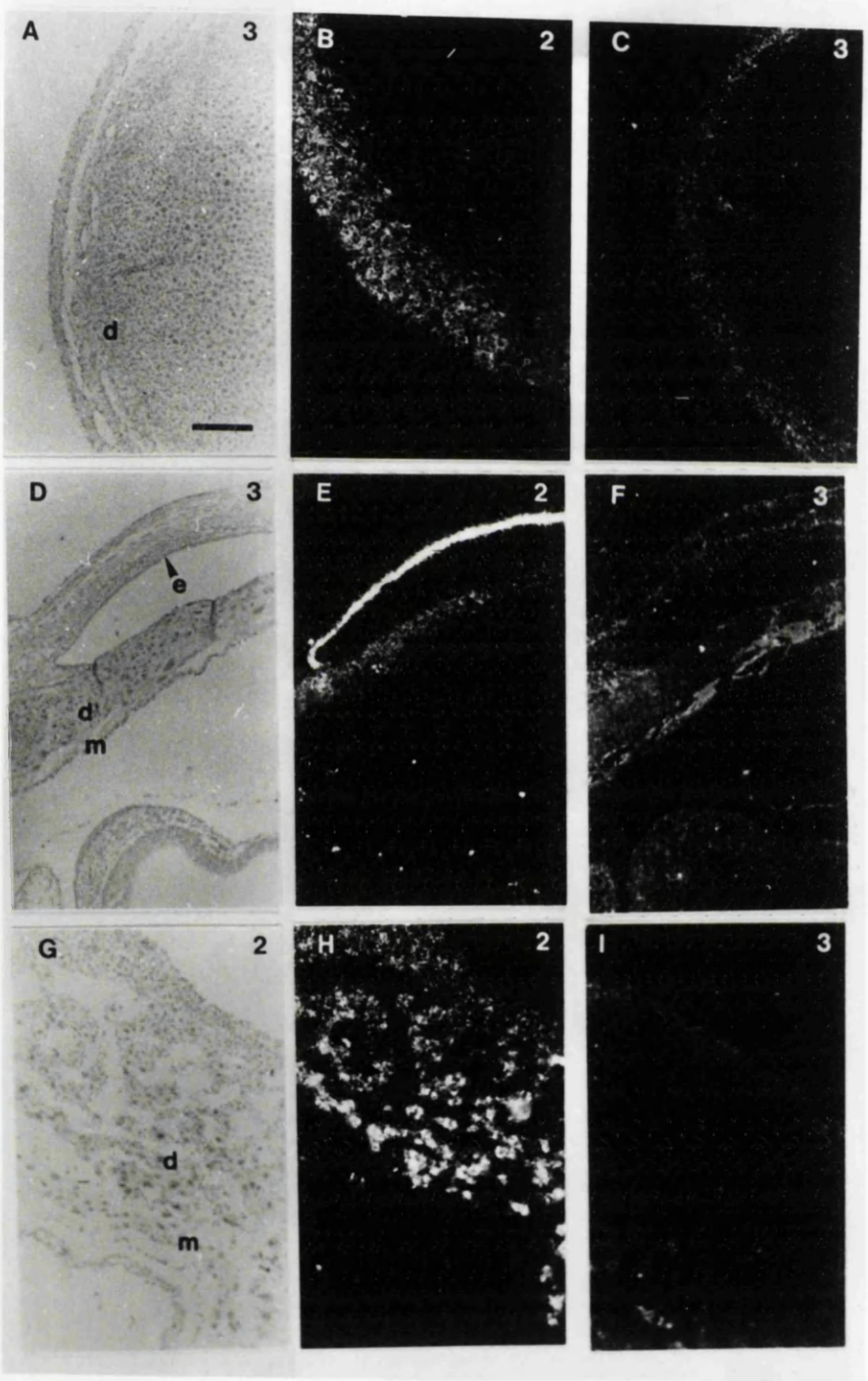
A
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TABLE 1

Summary of TGF-β RNA expression during embryogenesis

+ denotes that RNA is abundant at some stage between 6.5 days *p.c.* and birth, though expression may be transient. –, is below the detection level of *in situ* hybridisation.

* Data from Lehnert and Akhurst (1988) and Akhurst et al (1990)

1. Data taken from (Sandberg *et al* 1988a; Sandberg *et al* 1988b), (Wilcox and Derynck, 1988), (Lehnert and Akhurst, 1988), and (Akhurst *et al* 1990b; Akhurst *et al* 1990a).

2. Data taken from (Fitzpatrick *et al* 1990; Pelton *et al* 1990)

3. Data taken from (Pelton *et al* 1989)

c=cuboidal, s=squamous, iv=intervertebral disc anlagen

Cell type	β1*	β2	β3
Haematopoietic tissue	+	–	–
Endothelia	+	–	–
Thyroid	+	–	–
Parathyroid	+	–	–
Thymus	+	–	–
Kidney	–	–	–
Intestine	–	–	–
Stomach	–	–	–
Epithelia			
Whisker follicles	+	+	+
Salivary gland	+	+	–
Tooth bud	+	+	+
Secondary palate	+	–	+
Bronchiole epithelium	–	+(s)	+(c)
Otic epithelium	–	+	–
Olfactory epithelium	–	+	–
Lens epithelium	–	+	–
Neural retina	–	+	–
Hyperplastic nodules	–	+[1]	–
Suprabasal keratinocytes	–	+[2]	–
Cartilage and bone			
Precartilaginous blastema-		+(limb)	+(iv)
Growth zone of long bone	–	+	–
Perichondria	+	–	+
Hypertrophic cartilage	–	–	–
Osteoblasts,osteoclasts	+	–	–
Cardiac tissue			
Pre-valvular endothelium	+	–	–
Pre-valvular myocardium	–	+	–
Neuronal tissue			
Ventral spinal cord	–	+	–
Ventral forebrain	–	+	–
Olfactory lobe	–	–	+
Extraembryonic membranes	+	+	+
Muscle	–	+	+
Mesothelia	–	–	+

longitudinal muscle (Fig 25, C, F). TGF-β2 mRNA was also present, in considerable quantities, in the uterine epithelium at this stage of development (Fig 25, D, E). The expression of TGF-β2 RNA and TGF-β3 RNA at 8.5 days *p.c.* was seen in identical patterns to that described for 6.5 days *p.c.*. However the level of TGF-β2 RNA expression seemed to have increased (Fig 25 G, H), whilst the level of TGF-β3 expression had dropped (Fig 25 I). TGF-β1 RNA appeared to have an overall low level of expression in the extra-embryonic structures (data not shown).

3.3 Generation and analysis of mutant TGF-β2 constructs

A functional approach to investigate the potential role(s) of TGF-β isoforms during embryogenesis was undertaken. Mutations of one isoform were generated with the objective of producing a dominant-negative mutant. The mutant proteins produced were analysed, *in vitro,* to determine their potential biological activities. Human TGF-β2 (hTGF-β2) was chosen as a starting point for the generation of two mutants.

Human TGF-β2 is 414 amino acids long. There are two constitutive parts to the polypeptide, these are the latency-associated peptide (LAP) which constitutes the first 302 amino acids, followed directly by 112 amino acids which make up the mature TGF-β2 protein (see figure 11). Two mutant hTGF-β2 genes were made:

(a) In the first mutant, 36bp of the region coding for the signal peptide sequence was removed from the cDNA.

(b) The second mutant hTGF-β2 was generated by targeted mutagenesis of cDNA sequence encoding the amino acids which are recognised as the proteolytic cleavage site sequence of hTGF-β2.
A detailed description of the strategy for mutant generation is given in the previous chapter and figure 10. Prior to analysing their biological effects, the mutants were sequenced across the areas that had been altered to confirm the expected sequence change and to ensure the maintainance of the correct reading frame.

3.3.1 Generation of the signal peptide sequence mutation of human TGF-β2

The signal peptide sequence of human TGF-β2 is encoded by the first fifty seven nucleotides (19 amino acids) including the initiation codon. Thirty six base pairs of this sequence were removed to disrupt the amino acid sequence, but leaving the sequence in the correct reading frame. This resulted in the removal of twelve of the nineteen amino acids which make up the signal sequence (see figure 7). Of the original nineteen amino acids the first five codons were left intact as was the last codon. Between these original codons one new codon was generated by the ligation of the two blunt ends. This codon (ATC) translates to isoleucine. These changes maintained the correct reading frame, and if translated should generate a shorter TGF-β2 polypeptide of 401 amino acids. The lack of the signal peptide sequence would hopefully prevent the secretion of the mutant TGF-β molecule.

The generation of the signal peptide sequence mutant was facilitated by the fact that the restriction endonuclease *EspI* (with a heptanucleotide recognition sequence) was found to cut hTGF-β2 at two sites. These were at positions 225 and 265 of the cDNA. *EspI* does not cut the vector πH3M. The construct πH3M-TGF-β2 (6.070Kb) was digested with *EspI* which generated two linear pieces of DNA (6.031Kb and 39bp). The digested construct was analysed to check for complete digestion and run on a 1% gel to seperate the two fragments. The larger fragment was excised from the gel and purified. The *EspI* restriction endonuclease can in some cases generate compatible "sticky ends." In this case this was not so and the termini had to be end-filled to create blunt ends. Even if the digest had produced compatible ends they would have had to be end filled in order to maintain the correct reading frame (see fig 7). Once the termini were end filled they were ligated to generate the first mutant (called $\beta 2^{e}$). The ligation mix was transfected into competent MC1061/P3 bacterial cells. DNA was extracted from colonies by the small scale isolation method. To determine which of the colonies contained the mutated construct, with the expected 39bp deleted, DNA was digested with two enzymes, *EcoRI* and *PstI*. *EcoRI* cut the construct at the immediate junction between the vector and the

cDNA insert at position 0. *PstI* cut the construct at position 248 in the hTGF-β2 cDNA. A double digest of the mutant construct with these two restriction endonucleases should generate two bands; a 5.8Kb and a 209bp. This was compared to πH3M-hTGF-β2 construct which had also been digested with these two enzymes which would generate bands of; 5.8Kb and 248bp. The digested samples were run on a 2-3% agarose gel and the DNA visualised by staining with ethidium bromide.

To check the restriction endonuclease digestion, end filling and ligation had generated, precisely, the expected changes, the mutant construct was sequenced over the area of interest. Sequence data was generated using oligonucleotide primer P6 which was 41 nucleotides upstream of the end of the coding region for the signal peptide sequence (see figure 10).

The first time that this cloning procedure was completed, the generated mutant, $\beta 2^{e}$, did not produce the expected sequence. This was confirmed by sequence analysis of the alternate DNA strand using primer P1 as the sequencing template. P1 anneals to the sequence 200bp upstream of the region which encodes the signal peptide sequence.

It was determined that one of the nucleotides from the 5' terminal end of the coding sequence, produced by restriction endonuclease digestion with *Esp I* had been removed (see Fig 8). This was thought to have occurred during the reaction to endfill the recessed termini prior to ligation. It is known that the Klenow fragment, which is utilised for the end filling step, does have 3' exonuclease activity, especially if the concentration of dNTPs is too low. The removal of this single nucleotide resulted in the sequence moving out of frame after the sixth codon (see Fig 8). A stop codon was present in this reading frame 24 codons further upstream, which would result in a polypeptide of 32 amino acids being generated from the original ATG site.

This mutant was not discarded immediately, as an ATG site, normally 28 amino acids beyond the original initiation site, could possibly provide an alternative startpoint for initiation of translation. A protein produced from this initiation site would be 28 amino acids short of the wild type TGF-β2 at 386 amino acids long. If this protein was produced then it would lack the

whole signal peptide sequence, and could have unusual biological activity. This mutant was designated as $\beta 2^e$.

The signal peptide mutation was generated again in order to produce the original defined mutant and was called $\beta 2^f$. This mutant was also sequenced to confirm the maintainance of the reading frame.

3.3.2 Generation of cleavage site mutation of hTGF-β2

The second mutant hTGF-β2 molecule, ($\beta 2^c$,) was produced by altering the sequence which coded for the proteolytic cleavage site. The proteolytic cleavage site is required to allow the mature TGF-β polypetide to escape from the LAP and become activeted.

The hTGF-β2 polypeptide cleavage site is a five amino acid recognition sequence (Arg-Arg-Lys-Lys-Arg) at position 298aa to 302aa. The mature hTGF-β2 protein is cleaved from the latency-associated peptide directly after the last arginine residue in the above sequence. The cleavage site mutant was generated by altering the DNA sequence from which the cleavage site is encoded. This resulted in a change in three of the five amino acids which make up the cleavage site sequence, and simultaneously introducing two new restriction endonuclease recognition sequences into the cDNA.

This was achieved by using a 26 base long oligonucleotide (P4) (Fig 3), which contained three mismatches compared to the wild type sequence, in a PCR-site directed mutagenesis (PCR-SDM) reaction (described in detail in section sdm). The incorporation of this mismatch oligonucleotide into hTGF-β2 cDNA would alter three codons in the cleavage site sequence. This would result in the replacement of the three arginine residues with two leucines and a glutamine residue respectively to give a new sequence of amino acids (Leu-Gln-Lys-Lys-Leu). The new sequence would not be recognised as a site for proteolytic cleavage and would therefore prevent the cleavage of the mature TGF-β2 molecule from the LAP.

3.3.2.1 Identification of PCR products and clones which carried the mutant sequence

The mismatch oligonucleotide, P4, was also designed to generate new restriction sites which could be used to quickly analyse clones. The two new restriction sites were a *PstI* site introduced at position 1155, of the cDNA, and an *HindIII* site at position 1169 of the cDNA. The PCR-SDM products were produced as described in Materials and Methods. Amongst the PCR products there were, potentially, a variety of double stranded products. These were:

(a) wild-type products

(b) heteroduplexes (either strand contains mutation)

(c) products which contained the mutation in both DNA strands (Fig 4).

The introduction of the restriction endonuclease recognition sites for PstI and HindIII, meant that a PCR-SDM product containing these two new sites would, after digestion with these restriction endonucleases, generate different fragments of DNA that were compared to wild-type hTGF-β2 which had been subjected to normal PCR (Fig 26 B).

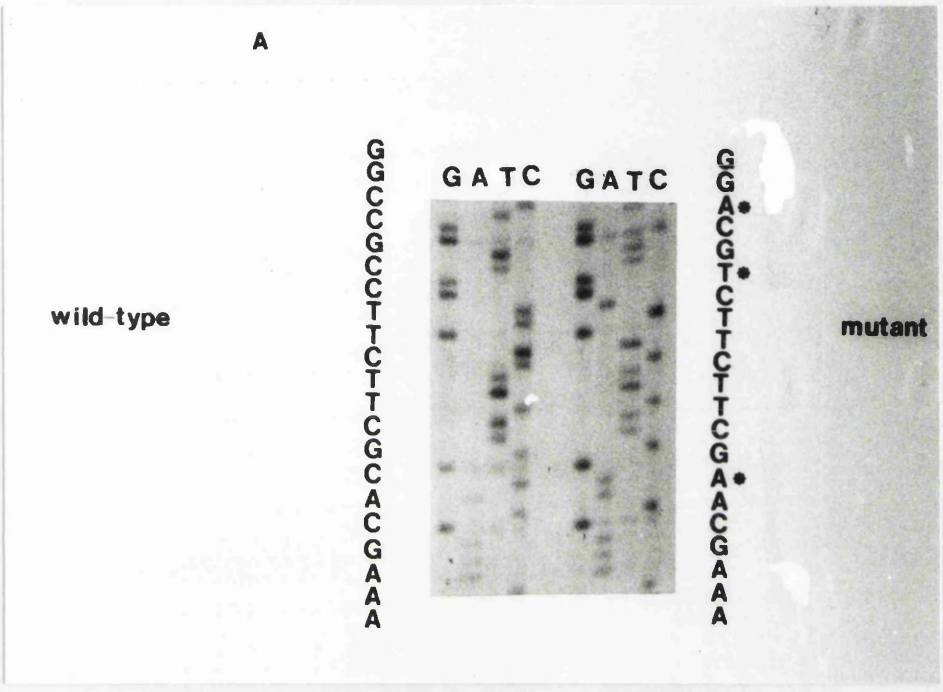
The PCR-SDM product which contained both mutated strands was sub-cloned into the πH3M vector after appropriate digestion with restriction endonucleases.

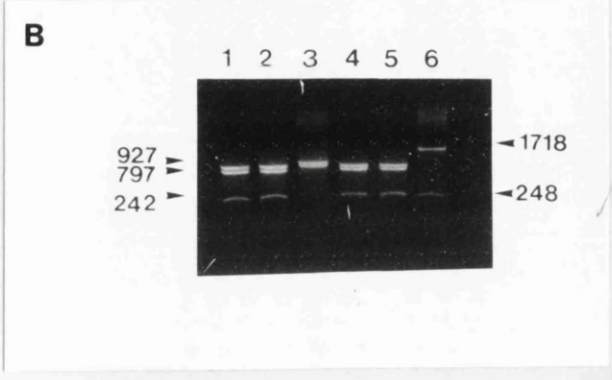
The PCR-SDM product was digested with *NotI* and *SphI* which have recognition sequences at positions 730 and 1300 of the hTGF-β2 cDNA respectively (Fig 10). This double digest generated an 872bp fragment that contained the potential mutant cleavage site (position 1155-1169 of hTGF-β2 cDNA). The πH3M-hTGF-β2 construct was also digested with *NotI* and *SphI* and the comparable 872bp fragment containing the wild-type cleavage site coding sequence removed (see Fig 10). The 872bp fragment of cDNA generated from the PCR-SDM was ligated into the linearised πH3M-hTGF-β2 construct.

Figure 26.

Sequence analysis of mutant construct generated by SDM-PCR.

(A) Sequence across wild-type and mutant strand using primer P5. The asteriks beside the bases on the mutant side indicate the changed nucleotides. (B) The wild type and mutant constucts were subjected to PCR using primers P1 and P2. The PCR products were digested with *Hind III* and *Pst I*. The wild type samples gave the expected fragments of DNA 1039 and 927 bp, when digested with *Hind III* and 1718 and 248 when digested with *Pst I* (3 and 6). Samples 1 and 2 were two products of the SDM-PCR which were suspected to have the mismatched base pairs introduced. When digested with *Hind III* and *Pst I* they generated DNA fragments of (*Hind III*) 927, 797, and 242bp; and (*Pst I*) 907, 811 and 248bp. This indicated that the correct base pair mutations had been introduced.


A
wild-type
GGCCGGCCTTCTTCGCACGAAA
GATC GATC
GGACGTCTTCTTCGAACGAAA
mutant


B
1 2 3 4 5 6
927
797
242
1718
248

The construct was transfected into MC1061/p3 and the clones analysed. 24 colonies were picked and subjected to small scale DNA preparation. The plasmid DNA was analysed by digestion with restiction endonucleases *PstI* and *HindIII*. Of the 24 colonies picked 12 were found to contain these two new restriction sites and therefore the mutated cleavage site sequence.

Conformation that the base pair changes had occurred was achieved by sequence analysis of this region with primer P5 (See Fig 26).

3.4 Analysis of the biological activity of polypeptides generated from the mutant and wild type constructs

3.4.1 Optimization of COS-7 cell transfection and collection of conditioned media

The mutant constructs were transfected into COS-7 cells as described in materials and methods. To confirm that the predicted proteins could be expressed from the mutant genes, conditioned media was collected and treated and assayed as described in materials and methods.

Each transfection was assayed for efficiency using a vector which contained a bacterial reporter gene. COS-7 cells were transfected with pIRV (2.5units), a construct containing the bacterial gene β-galactosidase driven by actin promoters (Beddington *et al* 1989). The transfection of this construct into COS-7 cells provided an indication of the efficiency of transfection. After conditioned media had been collected from cells which had been transfected with pIRV they were fixed and stained. Approximately 80% of cells were stained blue after a 24-36 hour incubation. Cells which had not been transfected with any DNA, but had undergone the complete transfection protocol, were also fixed and stained. No staining was observed.

The three mutant hTGF-β2 constructs, $\beta2^{e}$, $\beta2^{c}$, $\beta2^{f}$, as well as $\beta1^{33}$, β2 and the πH3M vector were transfected into COS-7 cells.

$\beta1^{33}$ was generated by Brunner *et al* (1989). They described the mutation of simian cDNA by SDM, causing amino acid 33 in the LAP to change from a cysteine to a serine residue. This resulted in normal levels of biological activity. However, $\beta1^{33}$ does not require acid activation, unlike other TGF-β polypeptides. For the purpose of these experiments it was considered to give wild-type activity.

One flask of cells underwent the transfection protocol but without the presence of any DNA, to act as a negative control. Media was conditioned for 24, 48 and 72 hours. Conditioned media collected after 48 hours was determined to have the highest level of TGF-β expression from the constructs. At 72 hours cell death was quite extensive and the cells looked very poorly. The level of TGF-β expression at this time was equivalent to that seen at 24 hours. All biological activity from the various constructs described below was from conditioned media collected at 48 hours. The potential instability of TGF-β polypeptides in the conditioned media was investigated by incubating rTGF-β1 in media collected from COS cells transfected with no DNA. The conditioned media was treated as normal and assayed. No loss in biological activity of the rTGF-β1 was detected. The effect of protease inhibitors on TGF-β polypeptides and CCL64 assay was determined. Conditioned media from COS cells transfected with $\beta1^{33}$, had either all three protease inhibitors added (see Materials and Methods), or no protease inhibitors added and the media assayed. The protease inhibitors did not effect the biological activity of $\beta1^{33}$. The three protease inhibitors were also added seperately to CCL64 cells during a biological assay. The presence of protease inhibitors did not influence the incorporation of 3H-thymidine by the CCL64 cells. Conditioned media, which was stored at 4°C or at room temperature, did show considerable loss of biological activity. For this reason conditioned media was either stored frozen or dialysed immediately after collection. The dialysed conditioned media was also stored frozen in aliquots and lyophilised when required.

3.4.2 Western blot analysis of TGF-β

Conditioned media from various transfections were dialysed, lyophilised and resuspended in sample buffer. Detection of (10-200μl conditioned media) TGF-β was carried out as described in Materials and Methods. The antibody detected 5ng rTGF-β1 which had been run simultaneously. No $\beta1^{33}$, β2, $\beta2^{e}$, $\beta2^{c}$ or $\beta2^{f}$ protein was detected. The limit of detection with this antibody is 5ng (British Biotechnology).

3.4.3 Growth inhibition of CCL64 cells by rTGF-β1

Dilutions of rTGF-β1 were freshly prepared in assay media and analysed each time a CCL64 assay was performed. A representative titration curve is shown in figure 27. Maximum inhibition was observed at a rTGF-β1 concentration of 285pg/ml with 50% inhibition at between 5-15pg/ml. The inhibition by rTGF-β1 (and conditioned medias) was comparable to the level of inhibition described by Danielpour *et al* (1989). They described 50% inhibition occuring at a concentration of between 12.5-17.5pg/ml of rTGF-β1. Maximum inhibition was observed at 250pg/ml TGF-β1 by (Danielpour *et al* 1989) (Figure 27).

The inhibition of CCL64 cells by rTGF-β1 could be blocked by pre-incubation of the rTGF-β1 with an anti-TGF-β neutralising antibody (British Biotechnology). CCL64 cells which were treated with the antibody-blocked rTGF-β1 protein (200pg/ml) showed 96% incorporation of ^{3}H-thymidine compared to less than 3% incorporation if the rTGF-β1 protein had not been blocked. This demonstrated that TGF-β was responsible for the growth inhibitory effects on CCL64 cells. Comparable results were seen with conditioned media from the constructs $\beta1^{33}$ and β2, which had been pre-incubated with anti-TGF-β neutralising antibody (Figure 28).

3.4.4 Biological activity of sTGF-$\beta1^{33}$ and hTGF-β2

Conditioned media from COS-7 cells which had been transfected with the constructs $\beta1^{33}$, β2, pIRV, πH3M alone and also conditioned media from cells which had been through the transfection protocol but with no DNA present in the transfection mix were assayed for biological activity. The

biological activity determined from 4 independent transfections of the constructs using the equivalent of 10μl of conditioned media in each assay is presented in Table 2. The biological assay was carried out at least twice for each transfection (Figure 29).

The figures generated for $\beta1^{33}$ and β2 compared to the results generated using rTGF-β1 suggest that the two wild type constructs are producing 280pg/ml and 140pg/ml respectively. Conditioned media from COS cells transfected with the control constructs or no DNA also showed some biological activity. The average incorporation of 3H-thymidine by CCL64 cells treated with 200μl conditioned media was 46% (±4%). This suggests that the COS cells are producing approximately 35pg/ml. The absolute concentration of recombinant TGF-β133 and TGF-β2 is approximately 245pg/ml and 105pg/ml (Table 3). This does not compare well with the reported production of 60-90ng/ml from the $\beta1^{33}$ construct by Brunner *et al* (1989) (Table 3).

3.4.5 Biological assay of conditioned media from COS-7 cells transfected with mutant constructs

The three mutant constructs had the proteins they generated assayed for biological activity exactly as described above (Table 2). Figure 29 shows the average biological activity of 10μl of conditioned media collected from COS-7 cells transfected with 4 units (40μg) of each individual mutant. $\beta2^{e}$ showed 83.0% (±40.6%) growth as determined by ^{3}H-thymidine incorporation into CCL64 cells. $\beta2^{c}$ showed 66.0% (±31.3%) incorporation and $\beta2^{f}$ showed 63% (±31.2%) incorporation. Biological activity of conditioned media collected from COS-7 cells which had been transfected with 1 unit of each mutant all showed >90% incorporation (Fig 30).

3.4.6 Biological assay of conditioned media from COS-7 cells co-transfected with hTGF-β2 and mutant constructs

Co-transfections, into COS-7 cells, were carried out such that the wild type:mutant molar ratios were 1:1, 1:2 and 1:4. Transfections and collection of conditioned media was carried out in an identical manner as described above. 10μl of conditioned media was assayed against CCL64

Figure 27.

Standard curve of percentage 3H-thymidine incorporation by CCL64 cells against known concentrations of rTGF-β1.

Known concentrations of rTGF-β1 were assayed and a standard curve plotted. The half maximal incorporation was between 5-15pg/ml. Total inhibition of CCL64 growth was achieved at a concentration of about 285pg/ml.

Percentage ^{3}H-thymidine incorporation against concentration of TGF-B1

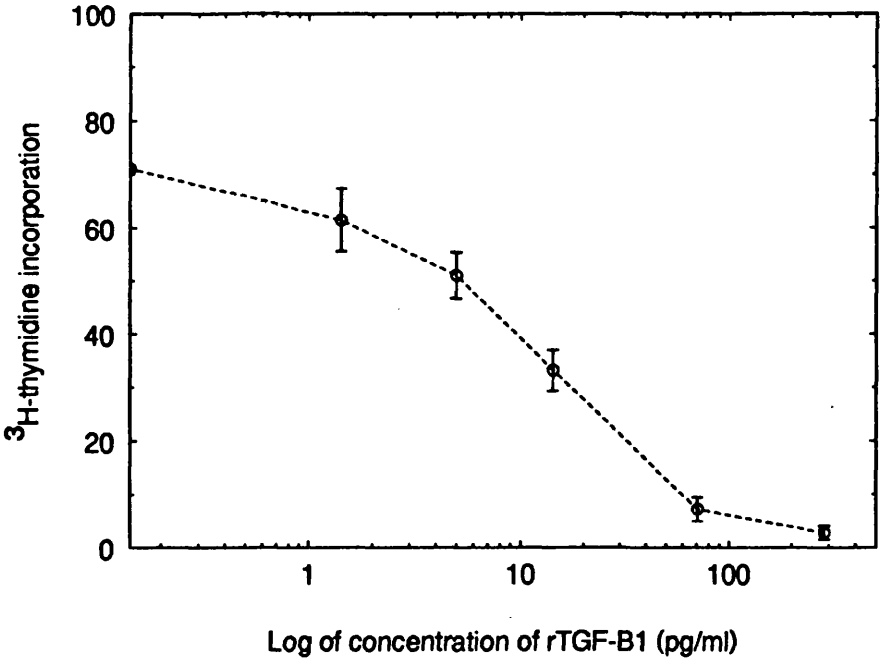


Figure 28.

Standard curve of 3H-thymidine incorporation by CCL64 cells assayed against conditioned media from COS-7 cells transfected with $\beta1^{33}$ and $\beta2$ constructs.

Different volumes of conditioned media from COS-7 cells which had been transfected with 1 unit (10μg) of $\beta1^{33}$ and β2 constructs were assayed for biological activity. The concentration of each was determined in relation to the standard curve generated using rTGF-β1. The half maximal incorporation of 3H-thymidine was achieved at a concentration of 12pg/ml and 15pg/ml for $\beta1^{33}$ and β2 respectively. This is within the range expected as determined for the rTGF-β1. Total inhibition was observed at 200pg/ml for both construct. The dip in the curve between 15 and 100pg/ml may refelect errors in the assay.

Percentage ^{3}H-thymidine incorporation against concentration of TGF-B

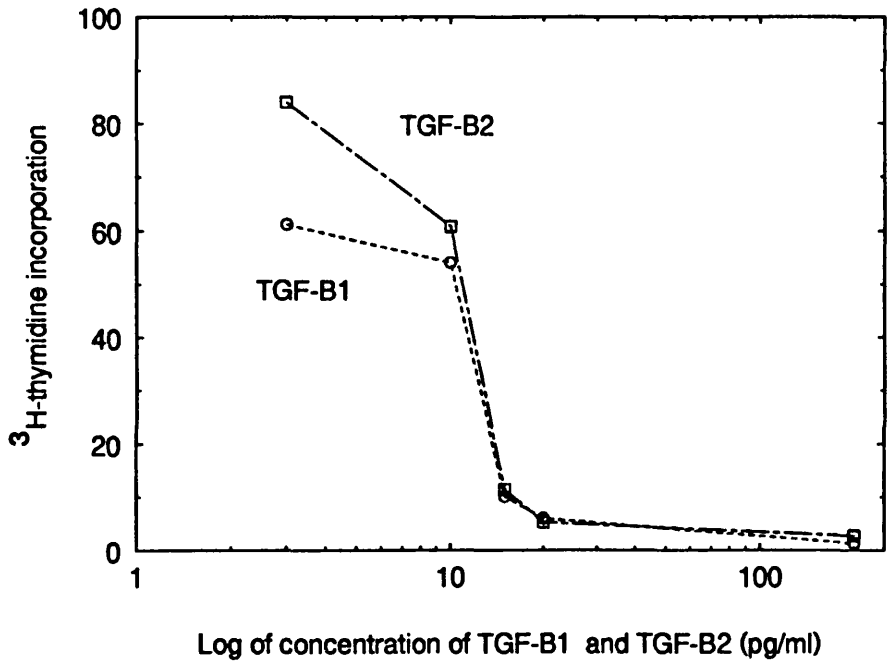


Figure 29.

Biological activity of conditioned media from COS-7 cells transfected with wild-type and mutant constructs.

Histogram showing percentage 3H-thymidine incorporation by CCL64 cells when assayed with 10μl of conditioned media from various transfections.

1. $\beta1^{33}$ (1 unit)
2. β2 (1 unit)
3. $\beta2^{e}$ (1 unit)
4. $\beta2^{c}$ (1 unit)
5. $\beta2^{f}$ (1 unit)
6. pIRV (2.5 units)
7. xH3M (5 units)
8. $\beta2^{e}$ (4 units)
9. $\beta2^{c}$ (4 units)
10. $\beta2^{f}$ (4 units)

Biological activity of wild-type, mutant and control constructs

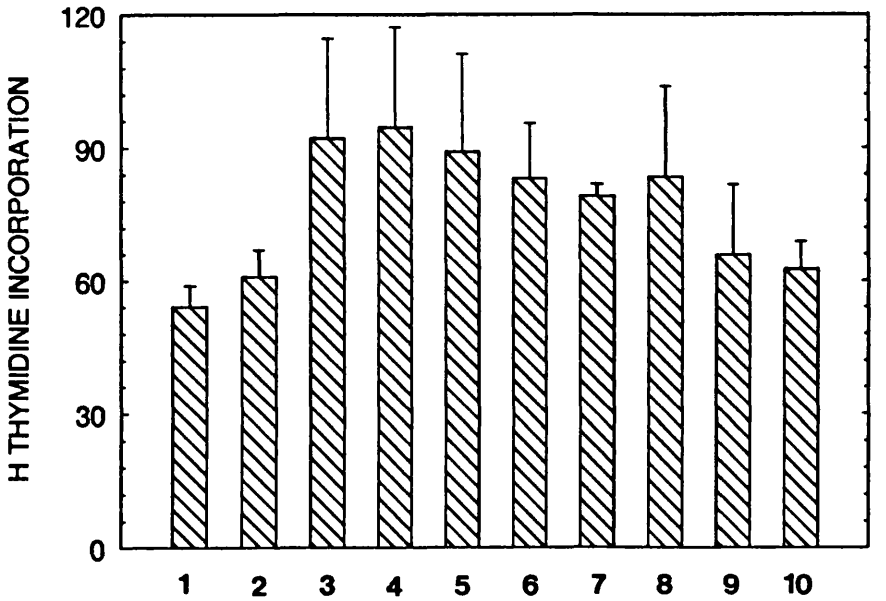


Figure 30.

Biological activity of conditioned media from COS-7 cells co-transfected with β2 and three mutant constructs.

1. β2 (1 unit)

2. β2 (1 unit)/$\beta2^{e}$ (1 unit)
3. β2 (1 unit)/$\beta2^{c}$ (1 unit)
4. β2 (1 unit)/$\beta2^{f}$ (1 unit)

5. β2 (1 unit)/$\beta2^{e}$ (2 unit)
6. β2 (1 unit)/$\beta2^{c}$ (2 unit)
7. β2 (1 unit)/$\beta2^{f}$ (2 unit)

8. β2 (1 unit)/$\beta2^{e}$ (4 unit)
9. β2 (1 unit)/$\beta2^{c}$ (4 unit)
10. β2 (1 unit)/$\beta2^{f}$ (4 unit)

Biological activity of conditioned media from co-transfected cells

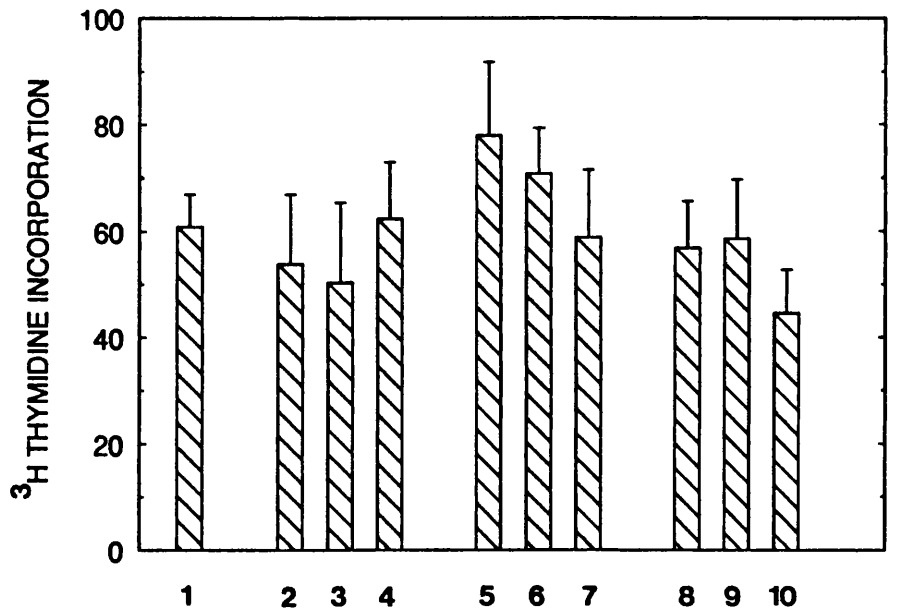


Figure 32.

Biological activity of conditioned media from COS-7 cells co-transfected with $\beta 1^{33}$ and $\beta 2^{e}$ or $\beta 2^{c}$.

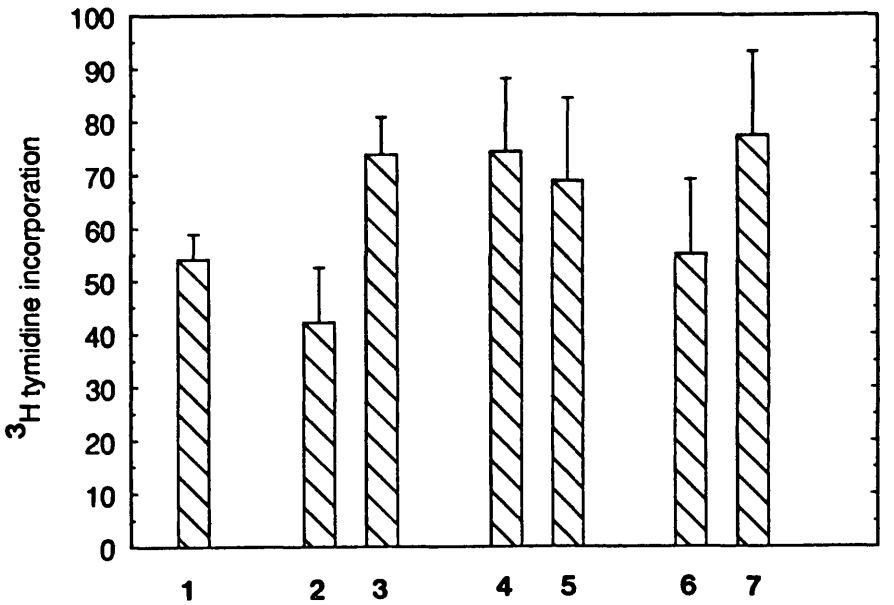
1. $\beta 1^{33}$ (1 unit)

2. $\beta 1^{33}$ (1 unit)/$\beta 2^{e}$ (1 unit)
3. $\beta 1^{33}$ (1 unit)/$\beta 2^{c}$ (1 unit)

4. $\beta 1^{33}$ (1 unit)/$\beta 2^{e}$ (2 unit)
5. $\beta 1^{33}$ (1 unit)/$\beta 2^{c}$ (2 unit)

6. $\beta 1^{33}$ (1 unit)/$\beta 2^{e}$ (4 unit)
7. $\beta 1^{33}$ (1 unit)/$\beta 2^{c}$ (4 unit)

Biological activity of conditioned media from co-transfected cells



cells. The average percent inhibition of ^{3}H-thymidine was determined for at least two independent transfections which were assayed twice, each time in triplicate (Table 2).

Figure 29 shows a the average incorporation of ^{3}H-thymidine incorporation into CCL64 cells treated with conditioned media from the various co-transfections and also the average incorporation assayed for expression of hTGF-β2 (61.0%). The average percentage incorporation of 3H-thymidine was between 50 and 62% at a 1:1 ratio of wild-type:mutant construct, between 58 and 77% when in a 1:2 ratio, wild-type:mutant and between 44 and 58% when wild-type:mutant ratio was 1:4 (Table 2 and Figure 31).

3.4.7 Co-transfection of mutant constructs with sTGF-$\beta1^{33}$ construct

In order to determine whether the TGF-β2 mutants would form heterodimers with TGF-β1, the mutant constructs were co-transfected with the $\beta1^{33}$ construct in the same ratios described above. The conditioned media was also treated and assayed in an identical manner as described above. The $\beta1^{33}$ construct was not transfected with the mutant construct $\beta2^{f}$.

The incorporation of 3H-thymidine by CCL64 cells was 62%, 74% and 55% wher.. assaying conditioned media from COS cells transfected with $\beta1^{33}$ and $\beta2^{e}$ constructs in a 1:1, 1:2 and 1:4 ratio respectively. COS cells transfected with β133 and β2c in a 1:1, 1:2 and 1:4 ratio produced 73%, 68% and 77.3% incorporation of 3H-thymidine resepectively (Table 2 and Figure 32)

TABLE 2 Percentage of 3H-thymidine incorporation by CCL64 cells assayed with conditioned media from COS-7 cells transfected with wild-type and mutant constructs.

Four independent transfections were completed and 10μl of conditioned media which had been treated as described in Materials and methods were tested for biological activity in a CCL64 assay. Incorporation of 3H-thymidine by CCL64 cells was determined as a percentage of conditioned media from COS-7 cells which had been through the transfection procedure but with no DNA in the transfection mix. u=unit

TRANSFECTION	% 3H-thymidine incorporation									
	T1		T2		T3		T4		x	SD
β1[33] (1 unit)	62	47	37	67	57	50	59	—	**54.1**	**9.4**
β2 (1 unit)	72	52	53	63	47	87	54	58	**60.6**	**12.2**
β2[e] (4 units)	80	151	8	65	82	126	59	96	**83.3**	**40.6**
β2[c] (4 units)	83	109	7	48	49	104	54	72	**65.8**	**31.3**
β2[f] (4 units)	—	—	41	57	37	115	—	—	**62.5**	**31.2**
Co-transfection (1unit:1unit)										
β2/β2[e]	25	59	47	15	76	63	42	103	**53.8**	**26.3**
β2/β2[c]	16	39	35	32	33	106	45	96	**50.3**	**30.4**
β2/β2[f]	—	—	79	48	35	87	—	—	**62.3**	**21.4**
β1/β2[e]	56	7	—	—	—	—	57	49	**42.2**	**20.6**
β1/β2[c]	56	89	—	—	—	—	64	86	**73.8**	**14.1**
β1/β2[f]	—	—	—	—	—	—	—	—	——	——
Co-transfection (1 unit:2 units)										
β2/β2[e]	101	82	31	87	36	114	79	93	**77.9**	**27.7**
β2/β2[c]	70	34	70	72	68	102	71	79	**70.8**	**17.3**
β2/β2[f]	—	—	41	50	42	102	—	—	**58.8**	**25.2**
β1/β2[e]	99	28	—	—	—	—	82	88	**74.3**	**27.4**
β1/β2[c]	95	16	—	—	—	—	84	80	**68.8**	**31.0**
β1/β2[f]	—	—	—	—	—	—	—	—	——	——
Co-transfection (1 unit:4 units)										
β2/β2[e]	67	51	—	—	26	80	49	68	**56.8**	**17.4**
β2/β2[c]	63	50	—	—	—	—	30	91	**58.5**	**22.1**
β2/β2[f]	—	—	—	—	28	61	—	—	**44.5**	**16.5**
β1/β2[e]	73	7	—	—	—	—	63	77	**55.0**	**28.2**
β1/β2[c]	109	40	—	—	—	—	52	108	**77.3**	**31.5**
β1/β2[f]	—	—	—	—	—	—	—	—		
pIRV (2.5u)	70	125	90	79	64	104	—	45	**82.4**	**24.6**
πH3M (5u)	74	102	83	50	88	79	—	77	**79.0**	**14.6**

TABLE 3

Estimation of concentration of protein produced by transfected COS-7 cells

The approximate amount of TGF-β polypeptide being produced from the COS cells after transfection with β133 and β2, was determined by assaying 200μl of conditioned media. The concentrations of both β133 and β2 polypeptide was estimated using the standard curve generated by known concentrations of rTGF-β1. A basal level of TGF-β polypeptide production by COS cells was produced by averaging the biological activity of 200μl of conditioned media from control samples. The basal concentration of TGF-β production by COS cells was subtracted from the estimated concentrations of β133 and β2 transfections to give a final concentration of β133 and β2 polypeptide.

Samples	% incorporation of 3H-thymidine	+ blocking antibody % incorporation of 3H thymidine	concentration TGF-β
β133(200μl)	13%	96%	280pg/ml
β2(200μl)	27%	97%	140pg/ml
controls(200ul)	46%	—%	35pg/ml

3.5 Statistical analysis of biological activities

3.5.1 Student's t test on difference between mean of two samples

To test the null hypothesis that there is no difference between the means of two samples, Student's t test was applied to the results (see Appendix B for statistical equations).

The means of the ^{3}H-thymidine incorporation of the conditioned media from co-transfections were compared against the mean generated by the analysis of the conditioned media from cells transfected with the appropriate wild-type construct.

The values of t are presented in Table 4. The majority of the results gave $0.1<p<0.5$ or $p\geq0.5$ indicating that the null hypothesis is correct. Only one co-transfection, $\beta1^{33}/\beta2^{c}$ at a 1:1 ratio gave $0.1<p<0.05$ which is still not significant (Table 4).

TABLE 4
Values of t and their associated probabilities

The difference between the means of biological activity produced from the co-transfections of wild-type and mutants was compared to the means of the transfections of wild-type constructs alone, using Student's "t-test" to examine the null hypothesis that: There is no difference between the means. The values of t and their associated probabilities indicate that the null hypothesis is correct and there is no difference between the means.
d of f = degrees of freedom

	t	d of f	probability
(1:1)			
$\beta 2/\beta 2^{e}$	0.61	14	$p<0.5$
$\beta 2/\beta 2^{c}$	0.79	14	$0.5<p<0.1$
$\beta 2/\beta 2^{f}$	0.12	10	$p<0.5$
$\beta 1^{33}/\beta 2^{e}$	1.055	9	$0.5<p<0.1$
$\beta 1^{33}/\beta 2^{c}$	2.218	9	$0.1<p<0.05$
(1:2)			
$\beta 2/\beta 2^{e}$	1.395	14	$0.5<p<0.1$
$\beta 2/\beta 2^{c}$	1.160	14	$0.5<p<0.1$
$\beta 2/\beta 2^{f}$	0.154	10	$p<0.5$
$\beta 1^{33}/\beta 2^{e}$	1.421	3	$0.5<p<0.1$
$\beta 1^{33}/\beta 2^{c}$	0.930	3	$0.5<p<0.1$
(1:4)			
$\beta 2/\beta 2^{e}$	0.449	13	$p<0.5$
$\beta 2/\beta 2^{c}$	0.190	10	$p<0.5$
$\beta 2/\beta 2^{f}$	1.034	8	$0.5<p<0.1$
$\beta 1^{33}/\beta 2^{e}$	0.062	3	$p<0.5$
$\beta 1^{33}/\beta 2^{c}$	1.450	3	$0.5<p<0.1$

Chapter 4

DISCUSSION

4.1 Introduction

This thesis presents data concerning the expression of three members of the transforming growth factor type beta (TGF-β) family, TGF-β1, TGF-β2 and TGF-β3, during murine embryogenesis. TGF-β1 expression has previously been reported extensively in both mouse (Lehnert and Akhurst 1988, Akhurst *et al* 1990a, b) and human (Gatherer *et al* 1990) but investigation of the expression of this gene along with the other two family members provides an opportunity to compare their respective expression patterns.

These genes are members of the TGF-β family of genes which is itself just one group of a larger, TGF-β super-family of over 20 related genes. The various members of the super-family are expressed during embryogenesis and are thought to be vital for normal development. It has previously been demonstrated that both mouse TGF-β2 and TGF-β3 mRNA are expressed during embryogenesis, as determined by Northern analysis, and that there was variable expression during gestation. In order to determine the exact spatial and temporal localisation of these two genes, *in situ* hybridisation studies were performed on serial mouse sections from 6.5 days *p.c.* to 16.5 days *p.c.*.

The latter part of the thesis presents data which begins to assess the biological function of TGF-β2 by the generation of mutants and their investigation *in vitro*.

4.1.1 *In situ* hybridisation: Limitations of the current study and discrepancies in the literature

There are a number of points which have to be considered when carrying out any technique which involves nucleic acid hybridisation. These considerations are multiplied when examining stages of development.

1. When working with genes which are members of large gene families it is vital to minimise the extent of cross-hybridisation of probes to homologues within the gene family. The problem of cross-hybridisation was easily overcome by prudent choice of template from which to generate riboprobes. In the case of the TGF-β family this usually means that the template is from the sequence of the gene which codes for the latency associated peptide (LAP) or, from the 5' or 3' untranslated regions.

2. Although embryo age was calculated from the day the plug was found, variation in embryonic age within the litter will occur. Also the embryo's actual developmental age can appear either more or less advanced than expected. This is particularly true of outbred strains which develop at a faster rate than inbred strains. Investigation of developmental stages at 24 hour intervals can result in morphogenetic events being missed. The only method to ensure complete coverage of any particular developmental stage is to harvest embryos at close time intervals.

3. The interpretation of RNA localisation studies often makes an assumption that RNA transcripts are translated into a biologically active protein at the site of manufacture. This is probably a simplification of the process, especially when considering TGF-β genes. It is well established that the TGF-βs are under considerable post-translational control (Miyazono *et al* 1991; Gentry *et al* 1987) and that TGF-β isoforms are secreted in an inactive form, and only activated when removed from the cell or tissue of origin.

Data produced from early investigations, via the *in situ* hybridisation technique, used full length TGF-β probes or probes based on homologous genes from a different species (Lehnert and Akhurst, 1988; Pelton *et al* 1989). Lehnert and Akhurst (1988), although having used a full length

mouse cDNA TGF-β1 as a template on mouse material, commented that under the stringent conditions they employed for the hybridisation and washes, there was no difference between the results they obtained initially with the full length TGF-β1 riboprobe, and data generated from a template which was based on the latency-associated peptide region and therefore specific (Akhurst *et al* 1990b). Their results were confirmed during this investigation which used the same specific TGF-β1 template as a positive control (See materials and methods). Pelton *et al* (1989) described the temporal and spatial localisation of TGF-β2 during murine embryogenesis. Throughout their investigation they utilised a 750bp of the human TGF-β2 cDNA, which encodes a region of the LAP, as a template. Due to the expected difference in sequence homology between man and mouse the stringency under which they carried out the *in situ* hybridisation was lower than that described in this study or by (Lehnert and Akhurst, 1988; Gatherer *et al* 1990; Fitzpatrick *et al* 1990; Schmid *et al* 1991), and therefore more likely to lead to non-specific hybridisations of the human TGF-β2 riboprobe to the mouse TGF-β gene family. The mouse TGF-β2 homologue which was subsequently cloned was found to be 85% homologous in the region chosen by Pelton and coworkers as a template. Previously Lehnert and Akhurst (1988) described a different pattern of TGF-β1 expression when they reduced the stringency of their hybridisation conditions, observing a signal in the ventral horns and dorsal root ganglia. Data presented in this thesis shows that this expression was, probably, due to TGF-β2. However, the less stringent conditions employed in that experiment were still higher than those employed by Pelton *et al* (1989).

One possible explanation for the differences in expression patterns observed by different groups is due to mouse strain. Fowlis *et al* (1992) described a novel finding in that some mouse strains show a different response to TPA induction of TGF-β1. Previously a strong and rapid induction of TGF-β1 mRNA and protein had been observed when treating an inbred colony of NIH-Beatson mice (Akhurst *et al* 1988). Fowlis *et al* (1992) examined outbred and inbred mice of a fresh stock (NIH-Olac) and did not observe the the same strong induction of TGF-β1 mRNA which had previously been described. They suggested that their results demonstrated that elevated TGF-β1 protein production is a consistent response to TPA

treatment, but that the mechanism by which this is achieved can vary between strains. This may go some way to explaining the discrepencies between the work presented in this thesis and data published by Pelton *et al* (1989). However, this cannot be the complete explanation, as it is argued that the TGF-β genes are of fundamental importance in development, so much so, that there is conservation of them throughout a variety of species. To then suggest that mouse strain differences can produce different localisation patterns is maybe pushing that explanation too far.

4.1.2 Reported expression patterns of TGF-β2 and TGF-β3 RNA in the literature

Pelton *et al* (1989) investigated the temporal and spatial patterns of expression of TGF-β2 RNA in mouse embryos from 10.5 days *p.c.* to 3 days post partum. They described the expression of TGF-β2 RNA in a variety of tissues including bone, cartilage, tendon, gut, blood vessels, skin and foetal placenta. They noted that the expression of TGF-β2 RNA in these tissues was generally mesenchymal. FitzPatrick *et al* (1990) and Pelton *et al* (1990) simultaneously published the localisation of the three murine TGF-β gene transcripts in the developing palate. Both groups reported essentially identical patterns of expression of the three TGF-β genes although there were some minor discrepencies. Both described intense expression of TGF-β3 in the medial edge epithelium both before and after palatal shelf fusion. TGF-β1 RNA was also localised to the medial edge epithelium but only after shelf elevation. TGF-β2 RNA was described by FitzPatrick *et al* (1990) as localised in the palate mesenchyme underlying the medial edge epithelium when the shelves were elevated and after fusion. Pelton *et al* (1990) described a similar pattern of expression but also suggested that there was localisation of TGF-β2 RNA transcripts in the medial edge epithelium. Gatherer *et al* (1990) described the localisation of all three human TGF-β gene transcripts in human embryos from 32 to 57 days *p.c.*. The pattern of expression for the three murine TGF-β RNAs were described by Schmid *et al* (1991) and were identical to that reported here, in this thesis, and by Gatherer *et al* (1990). Neither of these reports described patterns of TGF-β2 RNA expression

previously described by Pelton *et al* (1989). However, the reported pattern of expression of TGF-β1, 2 and 3 RNA by both Gatherer *et al* (1990) and Schmid *et al* (1991) were identical.

A comparison of data produced from this study, which employed specific mouse cDNAs as templates, and that reported by Pelton *et al* (1989) demonstrates a clear difference in the temporal and spatial pattern encountered. The results generated from this investigation have been confirmed independently by others who have also used specific mouse probes under comparable stringent hybridisation conditions (Schmid *et al* 1991). Furthermore the temporal and spatial pattern of human TGF-β isoforms has been examined in comparable gestational age human fetuses, using specific human TGF-β probes under identical stringent conditions as employed in this investigation (Gatherer *et al* 1990), and have described identical patterns of expression to that described in this thesis and by Schmid *et al* (1991). More recently both Vaahtokari *et al* (1991) and Manova *et al* (1992) have also described data concerning expression of TGF-β RNAs which is in agreement with data presented in this thesis. A final point which indicates that the data presented here represents specific hybridisation is the fact that each gene does have a unique pattern of expression both temporally and spatially with very few areas of overlap.

To further minimise the chance that the *in situ* hybridisation might give spurious results each stage of development described was investigated at least three times. At all times a mouse specific TGF-β1 template was used as a positive control, which produced the expected pattern of expression previously described by Lehnert and Akhurst (1988). As a negative control a full length sense human TGF-β2 template was employed. At no time of the developmental stages investigated was there any indication of specific hybridisation using this probe.

4.1.3 Antibodies to TGF-β2 and TGF-β3

The TGF-β2 and TGF-β3 antibodies described were raised against different epitopes of the isoforms (Pelton *et al* 1991, Flanders *et al* 1990 and van den Eijnden-van Raaij *et al* 1990). Pelton and coworkers made

antibodies to TGF-β2 and TGF-β3 against amino acids 4-19 and 9-20 of the LAP, respectively. Flanders and coworkers raised antibodies to TGF-β2 against amino acids 50-75 of the mature protein, and two antibodies to TGF-β3 raised against amino acids 50-60 of the mature region and 81-100 of the LAP. The TGF-β2 antibody employed by Slager *et al* (1991) had been raised against the first 29 amino acids of the mature TGF-β2 protein and was previously described by van den Eijnden-van Raaij *et al* (1990).

Pelton *et al* (1991) described the expression of the TGF-β2 polypeptide in cartilage, including chondrocytes; bone, pulp and odontoblasts of teeth, muscle, kidney, adrenal cortex, basement membrane of the gut and inner ear, in the meninges and extensive expression in the skin. The expression of TGF-β3 protein, described by Pelton and coworkers was different to that described for TGF-β2 protein although there were areas of overlap. These included cartilage, bone, muscle, gut and skin. Areas of unique TGF-β3 protein expression included periosteum of bone, ameloblasts, basal epithelia of the bronchi, endothelium of blood vessels, and the liver capsule. Flanders *et al* (1991) described the localisation of TGF-β2 and TGF-β3 protein in the developing nervous system. They described the localisation of TGF-β2 and TGF-β3 protein to neuronal perikarya and axons as well as radial glial cells. In the central nervous system, they commented that staining for the two isoforms was most prominent in zones where neuronal differentiation occurred and less intense in zones of active proliferation. They also described strong staining in many nerve fibres of the peripheral nervous system. The antibody to TGF-β2 described by Van den Eijnden-van Raaij *et al* (1990) was employed in a study of heart development (Dickson,M,C. *et al* 1992 submitted).

Protein localisation studies of TGF-β isoforms, as with most other proteins, has limitations concerning the interpretation of the data produced. The first limitation is the specificity of the antibody. As antibodies described by Pelton *et al* (1991), Flanders *et al* (1991) and Van den Eijnden-van Raaij *et al* (1990), were raised against synthetic peptides chosen from only a portion of the whole protein, there is no guarantee that this polypeptide is an epitope on the surface of the protein in vivo. Recently the three-dimensional structure of mature human TGF-β2 has been elucidated (Schlunegger and Grutter, 1992). A comparison of their data and the

antibodies raised against portions of the mature TGF-β2 by Flanders *et al* (1991) and Van den Eijnden-van Raaij (1990) demonstrates the difficulty encountered when trying to produce antibodies. Of the first 29 amino acids, of the mature TGF-β2 region, used by Van den Eijnden-van Raaij (1990) to generate antibodies, only 5 are exposed and 10 buried within the three dimensional structure of the protein. The synthetic peptide described by Flanders and coworkers has only one amino acid exposed and 14 buried. The specificity of the antibodies raised against the synthetic peptides described are usually determined by Western blot analysis. However, the structure of the protein on such gels has been altered by the conditions and probably does not reflect the structure which will be encountered *in vivo*.

Furthermore, the detection of TGF-β isoforms by antibodies is limited by the fact that it is not known whether the protein detected is in a latent, active or spent form. Also, the localisation to a particular cell type indicates nothing concerning the relation of the cell to the protein. That is; is the cell a producer or a responder to the protein? The questions raised by the localisation of the protein can only be resolved by the interpretation of that data alongside RNA localisation data and knowledge of receptor distribution.

4.2 Expression of TGF-β isoforms during murine embryogenesis

Although much of the data presented in this thesis is analysed without reference to comparable data concerning the localisation of the specific proteins. This was beyond the scope of the study as the antibodies to TGF-β2 and TGF-β3 protein were unavailable. Recently, however other workers have reported data concerning TGF-β2 and TGF-β3 protein localisations in specific cells and organs (Slager *et al* 1991, Pelton *et al* 1991, Flanders *et al* 1992), and where possible, the expression patterns of RNA and protein will be compared and the role of the gene considered.

4.2.1 The role of TGF-β isoforms during chondrification and ossification

In vitro TGF-βs are potent inducers of chondrogenesis in appropriately responsive mesenchyme (Seyedin *et al* 1987; Kulyk *et al* 1989). Kulyk *et al* (1989) demonstrated that exposure of cells cultured from chick limbs to TGF-β1 and TGF-β2 for as little as two hours was enough to stimulate chondrogenesis. They observed an increase in Alcian blue positive cartilage matrix plus an increase in glycosaminoglycans. They also noted an increase in the steady state levels of cartilage-characteristic type II collagen. Previously TGF-β1 and TGF-β2 had been termed cartilage inducing factors A and B respectively (Seyedin *et al* 1985). Both had been shown to induce fibroblastic, mesenchymal cells to synthesize cartilage matrix proteins *in vitro* (Seyedin *et al* 1987).

Lyons *et al* (1989) proposed a simple model to explain the role of the TGF-β family of genes in determining the cartilage model of bone formation, from condensing mesenchyme to hypertrophic cartilage. Their model suggested that BMP2-A and TGF-β2 was involved in the formation of the precartilaginous blastema and induced the differentiation of mesenchyme into actively proliferating chondroblasts and chondrocytes. They suggested that this would in turn induce TGF-β2 in the more terminally differentiated cells in an autocrine and paracrine manner. This would ensure the coordinate progression through the cell lineage to hypertrophic cartilage where Vgr-1 gene is expressed possibly promoting differentiation of these cells in an autocrine manner, as well as stimulating chondrocytes in a paracrine way. However, this model seems to over complicate the process and some of its data is not consistent with that produced in this thesis and by others (Gatherer *et al* 1990; Schmid *et al* 1991). No evidence of TGF-β2 RNA expression in chondroblasts was seen during the course of this thesis, nor was it observed in these cells by Gatherer *et al* 1991 or Schmid *et al* (1991).

To describe the process of bone formation in more detail, the developing long bones provide a useful model. In the developing limb, the formation of the skeleton involves a sequence of events which includes the accumulation of the extracellular glycoproteins including fibronectin, the

formation of clusters of mesenchymal cells (precartilaginous blastema) and the cytodifferentiation of cartilage at these sites of condensation (Rugh 1990). Leonard *et al* (1991) demonstrated, by analysis of the mechanism of enhancement of chondrogenesis in chick wing bud mesenchyme *in vitro,* that exogenous TGF-β1 could cause the accumulation of ECM and raised levels of fibronectin mRNA. This coincided with an acceleration and an increase in the extent of precartilaginous blastema (PCB) formation determined microscopically. They also detected endogenous TGF-β like activity in limb mesenchyme. This correlates with the time of expression of TGF-β2 RNA observed in the PCBs of embryos seen during this investigation and others (Gatherer *et al* 1990, Schmid *et al* 1991). It therefore seems likely that the endogenous TGF-β like molecule found in chick limbs is the chicken TGF-β2 homologue (Leonard *et al* 1991). These data combined, suggest that TGF-β2 is a major factor in the histogenesis of the cartilage model. In later stages of long bone development, TGF-β2 was limited to the growth zone with TGF-β3 mRNA present in the perichondrium. The expression of TGF-β2 in the growth zones is probably related to its involvment in the continued proliferation of chondroblasts in that area. The expression of TGF-β1 mRNA and protein in the development of the long bone has previously been described (Heine *et al* 1987; Sandberg *et al* 1988a; Lehnert and Akhurst, 1988; Gatherer *et al* 1990). TGF-β1 RNA was localised in osteogenic cells, osteoblasts, osteocytes and osteoclasts, of the zone of ossification (Lehnert and Akhurst 1988, Sanberg *et al* 1988, Gatherer *et al* 1990). TGF-β1 protein was present in calcifying parts of long bones (Heine *et al* 1987).

The expression of TGF-β3 mRNA in the perichondrium of developing ribs is similar to that described for TGF-β1 by Lehnert and Akhurst (1988). However, there is a dissimilarity in that TGF-β1 is expressed on the innermost chondrogenic cell layer of the perichondrium (Lehnert 1989) whereas TGF-β3 mRNA is found in the outermost fibrous layer. The cells expressing TGF-β1 may have a similar function to that seen *in vitro* systems of chondrogenesis, where TGF-β1 stimulates chondrogenic differentiation of fibroblasts. The role of TGF-β3 in the perichondrium is more of a problem as it seems unlikely that it would be required to perform the same function as TGF-β1, and, if they were acting synergistically, it

might seem reasonable for them to be expressed in the same cell layer unless they were operating in a paracrine manner. Data concerning protein localisation presented by (Pelton *et al* 1991) did show that an antibody specific for TGF-β3 was staining in the perichondrium. Although Pelton and coworkers did not specify which cell type, the fact that it was not present in the hypertrophic cartilage may suggest that it is operating in an autocrine manner. The action of TGF-β3 in a paracrine manner upon the innermost chondrogenic layer of the perichondrium cannot be discounted. Immunohistochemical data, also from Pelton and coworkers, showed TGF-β1 protein present in the perichondrium as well as in mature cartilage, as previously described by Heine *et al* (1987), suggesting that TGF-β1 may act in a paracrine fashion, for example to inhibit proliferation and induce ECM production by mature chondrocytes. Both these functions have been observed *in vitro* (Rosen *et al* 1988; O Keefe *et al* 1988). Thorp *et al* (1992) described the localisation of TGF-β1, 2 and 3 protein in 3 week old chicken long bones using specific antibodies raised against human TGF-β isoforms. They observed staining in chondrocytes recently derived from the perichondrium.

If TGF-β3 has an alternative function to that of TGF-β1 it may be that it promotes vascularisation of the fibrous layer of the perichondrium which occurs prior to the formation of the periosteum. This conforms with *in vitro* analysis which demonstrated that TGF-β can induce angiogenesis (Yang and Moses 1990). Alternatively TGF-β3 could be cooperating with TGF-β1 to maintain the perichondrium at this stage.

TGF-β3 mRNA was also observed in the perichondria of cartilage capsules of the ear and nose and also tracheal cartilage. TGF-β3 RNA is also present in the condensations of the optic capsule however this, unlike other cartilage models, will not under chondrogenesis. It apppears only to be associated with precartilage condensations. Neither TGF-β1 nor TGF-β2 were observed in these condensations. The expression of TGF-β3 in the condensations which are destined to become cartilage capsules of the ear and nose, but also in condensations around the eye which does not form cartilage (Hamilton *et al* 1978) seems to suggest that TGF-β3 may be necessary, but not sufficient, for cartilage formation. What role TGF-β3 is playing in these condensations is unclear. It seems that TGF-β3 is

expressed in many fibrous capsules, e.g. mesothelium, perichondrium, pericardium. This might suggest that TGF-β3 is inducing specialised fibrous connective tissue elements necessary for structral integrity.

TGF-β3 RNA was also detected in the prechondrogenic blastemas which will ultimately form the intervertebral discs. The gene *Pax1*, a member of the paired box family, is also expressed in this area and is thought to be playing a role in the formation of segmented structures of the mouse embryo (Deutsch *et al* 1988). The timing of expression of both these genes in the intervertebral discs anlagen correlates well although it appears that *Pax1* gene transcripts may appear slightly before TGF-β3 gene transcripts. There is a fundamental difference between the expression pattern of *Pax1* and murine homeobox genes. The *Hox* gene 1.3 is region specific for thoracic prevertebrate at 12 days *p.c.*(Dony and Gruss, 1987) . It has been speculated that *Hox* genes specify positional information whereas *Pax1* gene is expressed, independent of position, and may mediate a process required for the entire axis. This process may involve the initiation of TGF-β3 transcription in the region of the intervertebral discs.

4.2.2 The role of TGF-β isoforms in pulmonary system

Both TGF-β2 and TGF-β3 mRNA were expressed in the developing pulmonary system. Both were restricted to the epithelial component of the lung but with distinct patterns of expression which reflected a change in epithelial morphology which is part of the developmental process of lung formation. Lehnert and Akhurst (1988) have previously reported the expression of TGF-β1 RNA in the lung, however the pattern of expression was limited to the mesenchyme, and was at no time seen in any epithelial cells of the pulmonary system.

Although a lung bud is first seen at 9.5 days *p.c.* in the mouse embryo, no expression of any TGF-β isoform was detected until 3 days later. It is from this point that the primordial system will develop with columnar epithelia lining the primitive tubes of the lung. During this stage only TGF-β3 RNA is expressed in the epithelial component of the lung. Not until later, at about 14 days, will there be a demarcation in the lining of the lung tubes, when there is a change in morphology from columnar epithelia in the proximal

lung tubes to the cuboidal epithelia in the distal lung tubes. The expression of TGF-β2 RNA correlates with the change in epithelial morphology, and is expressed only in the simple cuboidal epithelium, while TGF-β3 is still expressed in the columnar epithelia of the bronchioles. TGF-β2 expression remains in the epithelia of the distal portion of the lung even as they become more flattened in shape as the alveoli are formed. The correlation of expression of TGF-β2 and TGF-β3 RNA with the change in epithelial morphology leads to the speculation that these two genes are in some way responsible for the differentiation to a simple cuboidal epithelium in the distal part of the lung. However, the expression of TGF-β3 RNA cannot induce a differentiation to a columnar epithelial cell in the lung tubes, as the columnar morphology appears before TGF-β3 RNA expression. As the tubular system continues to divide there comes a point when there is a change in epithelial cell morphology, from columnar to simple cuboidal. The simple cuboidal cells are found at the ends of the lung tubes at the point where they terminate. TGF-β3 RNA was not expressed in any of these cells. This means that TGF-β3 RNA transcription is switched off as TGF-β2 RNA transcription is switched on. During no stage investigated was TGF-β2 or TGF-β3 RNA localised in the same epithelial cell type of the lung. However, it was very difficult to distinguish the exact expression pattern at the points where epithelial morphology changed.

The continual growth of the lung and branching of the tubes to form the alveoli, ultimately leads to an almost total epithelial expression of TGF-β2 RNA towards the end of gestation. At this stage there has once again been a change in epithelial morphology with the simple cuboidal epithelium becoming flattened. This flattening has no effect upon TGF-β2 RNA expression.

As described above, all three TGF-β isoforms are expressed in developing lung, each with a unique pattern and each presumably with a unique role. The RNA expression pattern of the TGF-β isoforms in the lung was identical to that described by Gatherer *et al* (1990) when they investigated human embryogenesis, except that they described the localisation of TGF-β2 RNA to the epithelium of bronchioles, but only at the terminal portion. The stage of human lung development when this was seen was probably equivalent to a 14.5 day *p.c.* mouse and could reflect the time

when there is the change in epithelial morphology. This might suggest that TGF-β2 RNA expression takes over from TGF-β3 in the terminal portion of the bronchiolar epithelium before there is any morphological change to the simple cuboidal epithelium.

The role of TGF-β2 and TGF-β3 at this time could be associated with the epithelial change at about 14 days *p.c.*. Pelton *et al* (1991) observed that the three TGF-β proteins had identical patterns of localisation in the embryonic lung. They did describe slight expression of TGF-β3 (and TGF-β1) in epithelium of 17.5 day bronchioles, but in general, staining for TGF-β isoforms was seen mainly just below the respiratory epithelium. Pelton and coworkers did not distinguish between the epithelial cell types. It is known that all three TGF-β isoforms inhibit growth of lung epithelial cells. In fact the inhibition of a mink lung epithelial cell line, (CCL64), forms the basis of a standard biological assay for TGF-β isoforms (Danielpour *et al* 1989). However, the fact that the expression of TGF-β2 RNA and TGF-β3 RNA are quite specific to distinct epithelial cell types would imply that these isoforms are differentially utilised by different epithelial cells. Masui *et al* (1986) demonstrated that TGF-β was responsible for inducing normal human bronchial epithelial (NHBE) cells to undergo squamous differentiation. It seems that the switch from TGF-β3 expression in the columnar epithelial cells of the bronchioles to TGF-β2 expression is coincident with this change in epithelial cell morphology and could be implicated in controlling this event. The question as to whether it is the change to TGF-β2 expression that causes a change in cell morphology or the change in cell morphology that causes a change in TGF-β isoform expression is very interesting. Since the application of serum containing TGF-β can induce NHBE cells to differentiate to a more squamous morphology, Masui *et al* (1986), it would seem that it was the TGF-βs which brought about the change.

TGF-β1 protein expression has been investigated during lung development (Heine *et al* 1990). They demonstrated that TGF-β1 protein was present at the epithelial-mesenchymal interfaces of stalks and clefts and suggested that TGF-β1 was having a direct effect upon lung branching. Heine *et al* (1990) also described the localisation of collagen III, fibronectin, collagen I and glycosaminoglycan I using immunohistochemistry on parallel sections.

They found that these four ECM proteins co-localised with the expression of TGF-β1 protein in the clefts of the tubes and in a sheath around the bronchioles. The closer localisation of TGF-β1 protein in smooth muscle cells under the bronchiolar epithelium at later stages was thought to be stabilising the epithelia above through an intermediate such as tenascin. Tenascin is also expressed in smooth muscle cells underlying bronchiolar epithelia in chicken lung, (Crossin *et al* 1986) and is known to be induced by TGF-β1 (Pearson *et al* 1988). Heine *et al* (1990) also pointed out that there was an enhanced expression of TGF-β1 protein at about 13 days *p.c.* a time when there is massive vascularisation and innervation of the lung.

Epithelial-mesenchymal interactions are required for normal lung morphogenesis. A variety of ECM proteins are found in the area of the interactions, these include types I, III and IV collagen, laminin, heparan sulphate, proteoglycans, fibronectin, chondroitin sulphate and enactin (Timpl and Martin, 1982; Hay, 1981). Blum *et al* (1987), demonstrated that the role of one ECM protein, laminin, was intimately involved in epithelial/mesenchymal interactions. Laminin is known to regulate cell differentiation *in vitro*, in part by influencing gene expression (Blum *et al* 1987). They suggested that epithelial cell interactions with laminin might result in the expression of specific genes. That these genes, which are influenced by laminin, may be the TGF-βs could be investigated by examining the effect of blocking antibodies to laminin on TGF-β expression.

4.2.3 The role of TGF-β isoforms in heart development

The embryonic heart is an extremely complex organ which develops very rapidly thus presenting a problem in a study such as this when embryos of limited stages were investigated. From a period starting at about 7.5 days *p.c.* up to 9.0 days *p.c.* the heart undergoes an amazing evolution, from a simple two layered tube, to an elaborate structure. The results described in this thesis can only be considered a crude analysis of TGF-β isoform expression during this complicated morphogenetic event.

The expression of TGF-β2 RNA in the heart, described in this thesis, is transient and specific. The low level expression of TGF-β2 RNA localised

in most myocardial cells at early stages of heart development changes to a more elevated expression pattern in a specific subset of myocardial cells which underly the region of the heart at the region which will become the atrio-ventricular (AV) and outflow tract cushion tissue, which later contribute to septation and valve formation. TGF-β2 is not the only growth factor which has been found to be expressed in the heart, or indeed in this specific pattern. The expression of these other factors will be discussed later.

The first cells of the embryo to contain detectable amounts of TGF-β1 RNA are cardiac precursor cells, at 7.0 days *p.c.*, which arise from the splachnic mesoderm overlying the pharyngeal endoderm (Akhurst *et al* 1990a). As the early heart develops into a myocardial tube with an endocardial lining, separated by "cardiac jelly," the expression of TGF-β1 is restricted to the endocardial cells.

As development proceeds there is an invasion of the cardiac jelly at the AV valve by mesenchymal cells which are known to arise from the endocardial "epithelial" cells, as demonstrated in chicken heart cultures (Krug *et al* 1985). Krug *et al* (1985) also showed that the signal which is required to induce the epithelial/mesenchymal transformation, of the endocardial cells, arises from the myocardium, specifically from the same myocardium region that has been shown to express TGF-β2 RNA.

A considerable amount of work has been carried out on chick hearts. Krug *et al* (1985) demonstrated that a signal emanating from the myocardium at the AV valve causes the adjacent endothelial cells to transform and migrate towards the myocardium thus forming the AV cushion tissue. In an effort to determine whether one of the TGF-β isoforms was providing the signal, (Potts and Runyan, 1989) applied blocking antibodies to disaggregated chick heart, *in vitro*. The application of antibodies to the TGF-βs blocked the epithelial/mesenchymal transformation implicating one or more of the TGF-βs as the signal molecule. They continued their investigations using modified specific antisense oligonucleotides in an attempt to knock out the individual TGF-β isoforms to determine which was providing the signal (Potts *et al* 1991). The oligonucleotides were modified in the sense that they had phosphoramadite linkages at their 5' and 3' ends which minimises

nucleolytic degradation. They discovered that a modified antisense oligonucleotide to TGF-β3 inhibited normal cell invasion by 84% *in vitro* but failed to inhibit normal epithelial activation in the same *in vitro* heart cultures. Other modified oligonucleotides specific for the other two TGF-βs failed to inhibit the cell invasion. This result indicated that the epithelial/mesenchymal transformation, and subsequent invasion of the AV region, is a two step process, and that TGF-β3 is required for the mesenchymal cells to invade.

The results of Potts et al (1991), are at odds with the data described in this thesis in that no evidence of TGF-β3 was found in the heart at these stages of development. There are a variety of reasons why this may be so. Firstly, the level of TGF-β3 in the mouse hearts may be low, such that it is beyond the limit of detection by *in situ* hybridisation. If the TGF-β3 RNA level is low there may, still, be very efficient translation. This question will be easily resolved by the use of specific TGF-β3 antibodies when they become available. The antibody can be used in two different ways, either as a blocking antibody in a similar experiment as described by Potts and Runyan, or in immunohistochemistry. The second possibility is that heart development in mouse and chicken, although proceeding in a similar fashion, is controlled by different genes of the same family. This arguement must be wrong if the TGF-βs are supposed to be conserved in a variety of species due to their fundamental importance during embryogenesis. The third possibility is that the data is not valid. The interpretation of a positive result in this system involves counting the number of cells which have invaded the collagen gel-matrix upon which the chick heart disaggregates have been cultured. The number of cells required to have "invaded" to provide a positive result was very low.

Other members of the TGF-β superfamily are also expressed in the heart at about these stages of development. Gatherer *et al* (1990) described the expression of TGF-β3 RNA in the mesenchyme of the human AV valve cushion tissue at a low level. At no time during this stage of development were transcripts of TGF-β3 RNA detected during this investigation, in mouse, although later TGF-β3 RNA was detected in a slight condensation underneath the AV valve. Vgr-1 has also been found in the mesenchymal cells of the AV cushion tissue, (Jones *et al* 1991), and activin B has been

cloned from 10 day *p.c.* mouse heart although the spatial localisation of this peptide has not been determined. Lyons *et al* (1990) described the expression of the bone morphogenetic protein, BMP-2, in the myocardium but not the endocardium of the AV canal at 9.5 days *p.c.*. This persisted until at least 14.5 days *p.c.*. By contrast BMP-4 appears first in the myocardial layer of the AV canal at about 9.0 days *p.c.* and by 10.0 day *p.c.* is restricted to the ventricular outflow tract (Jones *et al* 1991). Jones *et al* (1991) suggested that BMP-2 and BMP-4 cooperate during early development to influence the differentiation of the AV cushions but that later in development BMP-4 alone mediates some process unique to the outflow tract. However, it is simplistic to assume that it is just BMP-2 and BMP-4 which are cooperating when there are at least three other TGF-β superfamily members known to be present at the same developmental stages and with similar expression patterns.

A comparison of the various TGF-β superfamily members patterns of expression might provide an insight into how they interact. TGF-β1 is unique in that it alone is expressed in the endothelial cells. BMP-4, BMP-2 and TGF-β2 RNA are all expressed in the myocardial cells, at 9.5 days *p.c.*, in the AV region and the outflow tract. Vgr-1 and TGF-β2 are also expressed at low levels in the AV cushion tissue at 10.5 days *p.c.*. There is no evidence, as yet, of interaction of TGF-β superfamily members with each other. Although there is evidence that TGF-β genes can induce members of their own group. The expression of TGF-β2 RNA and BMP-2 RNA is also seen in prechondrogenic blastemas. The fact that both genes are expressed at similar times and similar locations might indicate that these two genes cooperate in developmental functions. How these two genes might cooperate is unknown, but both the formation of myocardium around the heart valves and formation of prechondrogenic blastemas involve the movement of cells.

Recently a mouse antibody to TGF-β2 has been used to detect the protein during heart development (Dickson *et al* submitted). Dickson and colleagues described the localisation of protein in the whole myocardium at 9.0 days *p.c.* with the broad distribution more obvious at later stages of embryogenesis. This pattern of protein expression does not conform with what might have been expected if TGF-β2 was involved in providing the

signal for endothelial cells to transform at the AV region. Dickson and colleagues determined that the protein staining occured some 12-24 hours after RNA transcript localisation. They suggested that translational control of TGF-β2 protein production might regulate some aspects of cardiomyogenesis, or the growth factor might play an important role in maintainance of the differentiated function of the cardiomyocyte. Nevertheless, they did not rule out the possibility that TGF-β2 was providing the signal which caused the epithelial/mesenchymal transformation.

4.2.4 The role of TGF-β isoforms in epithelia

The results presented in this thesis show that both TGF-β2 and TGF-β3 RNA were found in a variety of epithelial cells during embryogenesis. The expression of TGF-β1 RNA in epithelial cells has previously been reported (Lehnert and Akhurst 1988). This was in contrast to the accumulation of TGF-β1 polypeptide in the mesenchyme underlying the epithelia (Heine *et al* 1987). This phenomenon was observed in tissue undergoing morphogenetic activity such as the whisker follicle, toothbud, submandibular gland and heart (Lehnert and Akhurst 1988). A comparison of the RNA expression of the three TGF-β isoforms showed that TGF-β2 was found in the widest variety of epithelia.

Potent growth inhibition by TGF-β on epithelial cells, *in vitro,* has been well established (Graycar *et al* 1989). Some have been induced to differentiate in the presence of TGF-β (Reiss and Sartorelli, 1987; Moses *et al* 1990; Gehris and Greene, 1992). It is important to know whether epithelially-derived TGF-βs are involved in autocrine regulation of growth and/or differentiation, or in paracrine interactions with the underlying mesenchyme. Lehnert and Akhurst (1988) proposed that TGF-β1 acted in both a paracrine and autocrine mode. They based this hypothesis on a comparison of RNA expression they found and TGF-β1 protein immunohistochemistry data from Heine and coworkers (Heine *et al* 1987). Lehnert and Akhurst found that TGF-β1 RNA was expressed in epithelial cells overlying mesenchyme which had previously been shown to contain TGF-β1 protein. They argued that TGF-β1 protein was being produced in the epithelial cells and subsequently secreted in to the underlying

mesenchyme where it would control the ECM and the mesenchymal cells themselves in morphogenetic interactions.

Unlike TGF-β1 or TGF-β3, TGF-β2 RNA expression was not limited to morphogenetically active tissue, but was also found in epithelial cells of established structures that are in the process of differentiation. These include epithelia of the sense organs, alveolar epithelia (discussed in section 4.2.2) and the hyperplastic nodules of palatal epithelium (Fitzpatrick *et al* 1990).

Pelton *et al* (1991) has described the localisation of TGF-β1, 2 and 3 proteins in murine embryos from gestational age 12.5 to 18.5 days. They described the localisation of TGF-β2 protein in the epithelia of the inner ear. However, in contrast to the expression of TGF-β2 RNA in the pseudostratified epithelium (described in this thesis and by Pelton *et al* (1990), and Schmid *et al* (1991)), immunostaining demonstrated that TGF-β2 protein was localised to the basement membrane below the simple epithelium, opposite the pseudostratified epithelium. Pelton *et al* (1990) did describe the localisation of TGF-β3 protein in the pseudostratified epithelium, although nobody has described RNA for TGF-β3 in any otic structures. The intensity of staining described by Pelton and coworkers for TGF-β3 protein seems to match the intensity of expression of TGF-β2 RNA in the otic epithelium, this might suggest that the antibody for TGF-β3 is in fact detecting TGF-β2. The localisation of TGF-β2 RNA in sensory epithelia suggests that it may be playing a role in the innervation of those epithelial cells. The expression of TGF-β2 RNA in the neural retina seems to back up this arguement.

The investigation of the development of the palate with respect to the expression of TGF-β isoforms has been carried out by FitzPatrick *et al* (1990) and Pelton *et al* (1991) . Although this thesis did not attempt such an in depth study of palate formation as described by either of these two authors and their coworkers, the observations from this study matched those described. TGF-β3 RNA is expressed early in the development of the palate in the medial edge epithelium (MEE) of the palatal shelves. This pattern persists until the palatal shelves have fused. Once the palatal shelves have fused the MEE is lost and the expression of TGF-β3 is no

longer seen. TGF-β1 RNA is also seen in the palatal shelve MEE, but only after the shelves have elevated and TGF-β2 RNA is expressed in the mesenchyme underlying the MEE both prior to fusion and for some time after (FitzPatrick *et al* 1990). The only difference in expression patterns seen by Pelton *et al* (1991) when compared with FitzPatrick *et al* (1990) is the presence of TGF-β2 RNA transcripts in the MEE seen by Pelton and colleagues. This minor discrepency could be due to the differentiative status of the MEE cells. FitzPatrick *et al* (1992) described the localisation of TGF-β2 RNA in MEE of mice which had been treated with retinoic acid. FitzPatrick and coworkers suggested that both TGF-β1 and TGF-β3 could be acting on the underlying mesenchyme. Sharpe *et al* (1988) claimed that TGF-β is inhibitory to palatal mesenchyme growth, although this is at odds with other reports of the effect of TGF-βs on mesenchymal cells. The induction of a variety of ECM proteins by TGF-βs has already been noted. FitzPatrick *et al* (1990) noted that the high level of TGF-β3 expression in the MEE occurred 24 hours prior to shelf elevation. They proposed that a possible function of the intense expression of TGF-β3 at this point was to induce chondroitin/dermatan proteoglycans which are thought to be important in causing the palatal shelfs to rise by mechanical means (Brinkley and Morris-Wiman, 1987). The difference in expression pattern of TGF-β2, compared to that of the other isoforms led FitzPatrick and coworkers to suggest that it was possibly involved in the maintainance of the overlying MEE.

4.2.5 Expression of TGF-β isoforms during tooth development

The expression pattern described in this thesis for TGF-β2 and TGF-β3 during tooth development, was similar to that described by Pelton *et al* (1990). TGF-β1 RNA has previously been localised to the epithelial bud and in the condensed mesenchyme (Vaahtokari *et al* 1991). This group also described intense TGF-β1 RNA expression in the morphologically active cervical loops of the dental epithelium. Later at about 16.5 days *p.c.* Vaahtokari and coworkers described expression of TGF-β1 RNA in the inner enamel epithelium where it subsequently dissappeared.

A number of other genes are expressed in a variety of cell types during tooth morphogenesis. Both tenascin, fibronectin and collagen are developmentally regulated during tooth morphogenesis (Thesleff *et al* 1979; Thesleff *et al* 1987). Tenascin was expressed in dental mesenchymal cells throughout tooth development. This expression was downregulated at one point, the cap stage, during which teeth undergo massive morphogenetic changes. Tenascin was also found in the basal membrane at the time of odontoblast differentiation (Thesleff *et al* 1987). Fibronectin has also been localised to dental mesenchyme, as well as basement membranes but not to epithelial tissues (Thesleff 1979).

Tooth development is not only a complicated event morphologically, but also in relation to expression of the TGF-β isoforms and ECM proteins. The distribution of all three TGF-β isoforms changes during tooth development. It is clear that none of the TGF-βs are involved in the initial events of tooth bud formation since no RNA or protein for any of the isoforms has been localised to dental lamina which elongates and thickens before invaginating to form tooth buds.

Vaahtokari *et al* (1991) described the regulation of TGF-β1 expression in epithelial cells by mesenchyme. They demonstrated that when bud-stage epithelium was cultured with bud-stage mesenchyme TGF-β1 RNA was detected. However, if the bud-stage epithelium was cultured with jaw mesenchyme then no expression of TGF-β1 RNA was seen. They also demonstrated that dental mesenchyme induced TGF-β1 RNA expression in oral epithelium where normally it is not found. They concluded that the bud stage mesenchyme regulates expression of epithelial TGF-β1.

Hox7.1, a homeobox gene, has been found to be expressed in dental mesenchyme some time before any of the TGF-β isoforms are detected (Mackenzie *et al* 1992). Vaahtokari *et al* (1991) argued that since it has been shown that a member of the TGF-β superfamily can be regulated by homeotic genes, *ie dpp* (Reuter *et al* 1990), then it could be possible that *Hox7.1* is regulating expression of some or all the TGF-βs.

4.2.6 Neuronal expression of TGF-β isoforms

TGF-β isoforms affects on a variety of nerve cells *in vitro.* These include mitogenic activity with respect to Schwann cells (Ridley *et al* 1989), and a response by neuronal accessory cells to TGF-β treatment (Eccleston *et al* 1989). In astroglial cells application of TGF-β delays the peak of DNA synthesis induced by serum (Toru-Delbauffe *et al* 1990). Martinou *et al* (1990) reported that TGF-β1 can increase the survival of embryonic spinal cord neurons *in vitro.* Rogers *et al* (1992) demonstrated that TGF-β alters differentiation of avian neural crest cells having an effect on morphology, proliferation, fibronectin expression and melanogenesis.

The data presented in this thesis represents the first global RNA localisation of TGF-β RNA isoforms in the developing nervous system. No TGF-β isoform was detected in the developing nervous system prior to 10.5 days *p.c.*. Both TGF-β2 and TGF-β3 RNAs were expressed in neuronal cells at later stages in development but in temporally and spatially unique patterns. The expression of TGF-β3 at 14.5-16.5 days *p.c.* in the mitral cell layer, described in this thesis, was also observed by Pelton *et al* (1990), however this was the only neuronal expression pattern of TGF-β3 which was observed. Pelton *et al* (1990) also described expression of TGF-β3 RNA in developing olfactory bulbs at 14.5 days *p.c.*. They described the expression of TGF-β3 RNA along the entire periphery of the olfactory bulb at later stages of development. They suggested that expression of TGF-β3 in the developing olfactory bulbs, at a time when complex cell interactions between the the olfactory bulb and innervating afferent axons, may be playing a role in proliferation and/or differentiation of neuronal or glial cells within the mitral cell layer. Pelton *et al* (1990) also described the localisation of TGF-β3 transcripts in the meningeal layers surrounding the brain and in the spinal cord. At no time was TGF-β3 RNA detected in the olfactory bulbs, or in the meningeal layers surrounding either the brain or the spinal cord.

The first detection of any TGF-β isoform in the developing nervous system was TGF-β2 in the forebrain at 10.5 days *p.c.*. TGF-β2 RNA was detected in the ventral horns of the spinal cord but with a very short temporal window of expression at 11.5 days *p.c.*. This compared well to expression

detected in the spinal cord of an approximately similar age human embryo described by Gatherer *et al* (1990). This transient appearance of TGF-β2 RNA in the ventral horns does correlate with a period of motor neuron differentiation.

Flanders *et al* (1991) described the localisation of TGF-β protein isoforms in the developing mouse nervous system. They examined embryos from 12 days *p.c.* to 18 days *p.c.*. They reported a more extensive localisation for both TGF-β2 and TGF-β3 which does not reflect the level of RNA transcripts described in this thesis nor the levels reported by Pelton *et al* (1990) and Schmid *et al* (1991). They also noted that the pattern of localisation for both TGF-β2 and TGF-β3 were so similar that only one set of results was shown. This begs the question as to whether the two antibodies were detecting the same species of protein. The localisation of protein in areas described by Flanders *et al* (1991), not found to express RNA from either gene may be due to very low transcript levels being very effectively translated, thus producing discordant levels of RNA and protein. Certainly the *in situ* hybridisation technique would not be able to distinguish low levels of expression from background. Also the choice of stages investigated by Flanders and coworkers probably meant that they would not have seen expression of TGF-β2 protein in the ventral horns and therefore did not report such. This even allows for the time taken from transcription to translation (estimated by Fowlis *et al* (1991) to be in the region of 12-24 hours) plus the half-life of the protein. However, they did suggest that both TGF-β2 and TGF-β3 might have a role in regulation of neuronal migration and differentiation, which correlates well with the interpretation of the RNA expression data presented in this thesis.

4.2.7 TGF-β isoforms and their role in skin development

TGF-β2 RNA and TGF-β3 RNA transcripts were expressed at a low level in the dermis of the developing skin. Both genes transcripts were also seen in whisker follicles and TGF-β2 RNA was localised to the primordia of hair follicles and in more mature hair follicles at later stages of development. Both TGF-β2 and TGF-β3 transcripts were observed in the sub-dermal mucosa. Previously, Pelton *et al* (1989) described the localisation of TGF-β2 in both epidermis and dermis from 13.5 days *p.c.* at high levels. By 15.5

days *p.c.* Pelton *et al* (1989) found that the TGF-β2 RNA was localised to the dermis and not seen in the epidermis or hair follicles. At 18.5 days *p.c.* the expression of TGF-β2 RNA was restricted to the suprabasal layer of the epidermis and was now found in the hair follicles. Apart from the hair follicle expression at 18.5 days *p.c.* this pattern of intense expression does not compare with that described in this thesis. Pelton *et al* (1991) have also described the protein localisation of TGF-β2 and TGF-β3 in the skin at similar stages of development. They found that TGF-β3 RNA was present in the epidermis and hair follicles at later stages of development, whereas TGF-β2 protein seemed to be ubiquitously expressed in all components of the skin. However, Pelton *et al* (1991) did comment that the highest level of staining of TGF-β2 antibody was in the developing hair follicles which correlates with the higher levels of expression of TGF-β2 RNA presented in this thesis.

Glick *et al* (1989) found that TGF-β2 could be induced by application of retinoic acid to keratinocytes in culture and in intact epidermis. This supports the evidence that embryonic skin has the potential to express TGF-β2. The proliferation of both mouse and human keratinocytes is known to be potently, and reversibly, inhibited by TGF-β (Shipley *et al* 1986; Coffey *et al* 1988). Choi and Fuchs, (1990) described plietropic effects of both TGF-β and RA on keratinocytes. They found that TGF-βs acted on mitotically active basal cells to retard cell proliferation. Although stopping the cell cycle is a prerequisite for terminal differentiation, TGF-βs inhibited normal keratinisation and promoted the type of differentiation normally seen in wound healing and epidermal hyperproliferation (Choi and Fuchs, 1990; Mansbridge *et al* 1989). Also, treatment of epidermis with phorbol esters, which induces differentiation of cultured keratinocytes and hyperplasia, *in vivo*, is known to increase levels of TGF-β1 RNA (Akhurst *et al* 1988). Neither TGF-β2 nor TGF-β3 RNA levels are raised in the skin on treatment with phorbol esters (Fowlis *et al* 1992).

The vibrissae and hair follicles, which begin to appear at about 13 and 14 days *p.c.* respectively, express RNA from all three isoforms (this thesis, and Lehnert and Akhurst 1988). The vibrissae arise by epithelial thickenings which invade the primitive epidermis. Lehnert and Akhurst (1988) described the localisation of TGF-β1 RNA, initially in the basal

keratinocytes of the invaginating whisker follicle and later in the outer root sheaths of the more advanced whisker follicle. TGF-β1 protein was localised in the adjacent mesenchyme (Heine *et al* 1987).

Tenascin, an ECM protein which is known to be induced by TGF-β1 (Pearson *et al* 1988), is also expressed in the mesenchyme underlying the epithelia of the developing vibrissae. This would suggest that the epithelially synthesised TGF-β1 is directly inducing tenascin accumulation in the mesenchyme around the developing vibrissae. However, the exact role of tenascin in the development of this structure remains unknown.

The expression of the TGF-β isoforms in the hair follicles seems to be similar to that of the vibrissae. Early on in their development both TGF-β2 and TGF-β3 are expressed to a similar extent in the primordial condensations of mesenchymal cells that will become the hair follicles.

4.2.8 Role of TGF-β isoforms in extra-embryonic material

The expression of both TGF-β2 RNA and TGF-β3 RNA in the extra-embryonic material suggests that they may play a role in the proliferation and maintainance of decidual structures. The expression of TGF-β2 RNA in the uterine epithelium was first observed at 8.5 days *p.c.*. However, it is not, limited to this one cell type as it also localises to the myometrium. This is similar to the pattern of expression seen by Manova et al (1992). They suggested that factors such as TGFβ2, which have mesoderm-inducing capability, are expressed only within the decidua before and during primary period of mesoderm formation. The pattern of expression, described for TGF-β2 RNA, was also observed for colony stimulating factor 1 (CSF-1) Arceci et al (1989). They suggested that CSF-1 was not only regulating the proliferation of macrophages but controlling the formation and differentiation of decidual tissue. These genes are not the only ones expressed in pre- and post-implantation deciduas suggesting many lelvels of control over the maintainance and differentiation of decidual structures.

4.2.9 Interaction of TGF-β isoforms with other molecules

The TGF-βs are known to regulate and be regulated by a wide variety of molecules ranging from other peptide growth factors, homeobox genes, transcription factors, phorbol esters, retinoids and each other.

The induction of TGF-β2 by RA in cultured keratinocytes has been described above (Glick *et al* 1989). Glick *et al* (1991) have also described the regulation of TGF-β expression by retinoic acid (RA) in vitamin A deficient rats. They showed that systemic RA induces expression of all TGF-β isoforms in the epidermis, and of TGF-β2 and TGF-β3 in the respiratory epithelium as well as in the intestinal mucosa and lamina propria. In contrast they also showed that retinoid deficiency results in increased expression of all isoforms in the vaginal epithelium, and subsequent addition of RA decreased expression. Vaginal epithelium is one of the few epithelial cell types which is stimulated by TGF-β *in vivo* (Takahashi *et al* 1989). They noted that TGF-β2 was the most sensitive to tissue retinoid levels, but the response by all three isoforms was transient and expression returned to original levels after a few days. Glick *et al* (1989) indicated that the control of TGF-β2 expression by RA was at the post-transcriptional level. The data of (Glick *et al* 1990) suggest that systemic levels of retinoids maintain epithelial homeostasis in part through the local regulation of TGF-β expression in responsive epithelia. Previously they had shown that TGF-β2 which had been induced by RA is in a biologically active form. This suggests that the induction of TGF-β isoforms is an important element of the regulation of epithelial differentiation by retinoids *in vivo*, not only in adult tissues but in embryological tissues. This is supported by the observation that TGF-β2 is induced by RA in embryonal carcinoma and stem cells (Mummery *et al* 1990). A number of investigations of the developmental expression of the RA receptors RNAs (RAR), have found that RAR-beta RNA expression is closely parallel to that of TGF-β2 in the developing limb (Dolle *et al* 1989) and that RAR-gamma RNA is coordinately expressed with TGF-β3 in the developing spinal column (Ruberte *et al* 1990). Both RAR-gamma and RAR-beta are also expressed in the frontonasal mesenchyme at a similar time described in this thesis and by Gatherer (1991). The cellular retinol binding protein

(CRBP) has been localised in the ventral horns at the same stage as TGF-β2 RNA in this thesis (Maden *et al* 1989). It is a well established fact that application of exogenous RA to developing embryos can produce severe cranio-facial abnormalities (Webster *et al* (1986). This would seem to suggest that RA is acting upon endogenously expressed TGF-βs, perturbing the expression and causing the changes. However, FitzPatrick *et al* (1992) have demonstrated that the pattern of expression of RNA of the three TGF-β isoforms is unaltered, at least with respect to palate formation, after treatment with RA except for TGF-β2 which was expressed in the medial edge epithelium. It is possible that RA is regulating the TGF-βs as suggested by Glick *et al* (1991). At the same time, very high levels of TGF-β2 RNA, compared to non-treated mice, were observed in the cuboidal epithelium of the developing lung (D. FitzPatrick unpublished observations). This might suggest that there is already a level of expression in the MEE of TGF-β2 RNA, which is normally beyond the level of detection by *in situ* hybridisation, or possibly the control of TGF-β2 expression by RA is dependent upon other factors.

4.3 Generation of mutants as a form of analysis of biological function

As has been described above, the TGF-β genes are expressed in developmentally specific patterns. All appear to be involved in morphogenetic events, interacting with the ECM and other proteins to direct growth, either by inhibition, promotion or differentiation. However, the mass of data produced by expression studies and *in vitro* analysis does not answer the basic question: What is the role of TGF-β isoforms *in vivo*? *In vitro* analysis can only provide so much information which is generated in an artificial environment which may never be found naturally. Herskowitz, (1987) described a method by which the role of genes could be investigated. He suggested that by the manipulation of cloned genes to create the generation of dominant-negative mutations which, when overexpressed, disrupt the activity of the wild-type gene, might provide an answer to the question above.

Other strategies available for the disruption of gene function include directly knocking the gene out. The advantage to this method is that the entire gene function is obliterated. Recently this method has been employed to disrupt mouse homeobox genes (Zimmer and Gruss, 1989; Chisaka and Capecchi, 1991; Le Mouelic *et al* 1992; Lufkin *et al* 1991). As might be expected most of the mice carrying these mutations have not survived, probably due to the fundemental importance to development of the homeobox genes. However, there is an exception, the *Hox 3.1*$^{-/-}$ null mutation generated by (Le Mouelic *et al* 1992) has survived to sexual maturity and produced viable offspring. The survival of these null-mutant mice is probably indicative of the ability of other *Hox* family members to take over the role of the missing gene. This may not be a direct action of the Hox genes but possibly an indirect response to the lack of the missing genes signal and a change in the gradient of expression normally observed with *Hox* genes. It is unlikely that this method of gene disruption would be applicable to the TGF-β family of genes. There are also many areas of morphogenesis where the TGF-β genes are possibly interacting directly or indirectly. Whether the other TGF-β genes could replace the function of the lost gene, without disrupting the smooth developmental progression is doubtful. However, the example of leukaemia inhibitory factor (LIF) which is thought to be fundemental to mouse embryo implantation, can be knocked out without changing the viability of the mouse, may indicate that TGF-βs are not necessary either (Stewart et al 1992). If, however, the TGF-β mutated gene, or mutational event, could be targeted to a specific tissue at a specific time then this would provide a more useful model for examining the role(s) of TGF-β isoforms. Recently, Hasty *et al* (1992) have described a "hit and run" approach to mutation of *Hox* genes. This method allows the selection of very specific mutations in genes which may circumvent the problem associated with previous homologous recombination techniques and allow the TGF-β genes to be targeted in a more subtle fashion.

The use of antisense RNA as a method of disrupting gene function has also been successfully attempted in vitro (Anfossi *et al* 1989; Becker *et al* 1989; Goodchild *et al* 1988; Wickstrom *et al* 1988). These authors all utilised short antisense oligonucleotides to knock out gene function, though

there is no known limit on the size of the antisense RNA which could be used. This might be particularly significant as the requirement for specificity, especially when dealing with genes like the TGF-βs which share high sequence homology, is important. Whether an antisense RNA would be able to bind and control expression of the targeted gene is not clear. It has been demonstrated that Xenopus oocytes contain naturally occurring antisense transcripts of bFGF (Kimelman *et al* 1988). Although the antisense RNA produced a protein it was also shown to interact with the sense RNA, possibly regulating the expression of the normal gene product. If the cell has the ability to cope with antisense RNA molecules and allows them to interact with sense RNA then this approach seems to offer considerable scope for the generation of dominant-negative mutations. Cockayne *et al* (1991) have employed this approach to knock out the gene, granulocyte-macrophage stimulating factor (GM-CSF). They generated stable transfected cell lines containing a portion of the GM-CSF coding sequence, in the reverse orientation, driven by a B19 parvovirus promoter. They were able to show a reduction to 10% of normal levels of GM-CSF production. However, Cockayne *et al* (1991) could not prove conclusively that the reduced level of GM-CSF was due to the formation of RNA duplexes between the endogenous antisense RNA and their sense RNA, due to clonal heterogeneity observed in the transfected cell populations.

The generation of mutants which compete with wild-type gene products also offer a method by which to knock out a specific gene function (Meeks-Wagner and Hartwell, 1986). The over-expression of the mutant gene product might compete with the wild-type gene product at a variety of levels, depending on the function of that particular protein. In the case of TGF-βs there exists a potential variety of sites which could be mutated. These include the areas of the protein which bind to the numerous promoters and other active sites. This method of creating a dominant-negative mutant is complicated by the fact that no active sites on the TGF-β isoforms have as yet been identified. Recently, an analysis of regions of mature TGF-β polypeptide, by generation of TGF-β1-2-1 and assessing the chimaeras biological activity, has been reported (Qian *et al* 1992). This sort of analysis will provide information useful for determining areas to mutate.

The approach, to generating a dominant-negative mutation, in this thesis concentrated on the genration of a mutant TGF-β protein which would have the potential to bind to endogenous wild-type TGF-β protein forming a heterodimer. The lack of a particular structure in the mutant protein would result in the heterodimer being unable to function at normal levels and therefore reduce the bioactivity of TGF-β. The TGF-β protein contains a number of sites which are suitable for the introduction of subtle mutations. These include the signal peptide sequence, the N-glycosylation sites, the proteolytic cleavage site and the cysteine residues. Gross mutations including the complete removal of the sequence of DNA which encodes either the LAP or the mature region are also possible targets for analysis.

Recently, site-directed mutagenesis of PDGF-A cDNA to disrupt the proteolytic cleavage site and substitution of a cysteine residue for a serine residue resulted in the succesful generation of stable dominant-negative mutations, described by Mercola *et al* (1990). Mercola *et al* (1990) demonstrated the ability of both types of mutation to inhibit wild-type PDGF-A activity but only the protein carrying the Cys to Ser change had the ability to disrupt the activity of PDGF-B. Brunner *et al* (1989) described mutagenesis of a simian TGF-β1 cDNA to change cysteine residues to serine residues in the translated protein. They chose which cysteine residues to mutate based upon the expected function in the formation of the 3-D structure of the protein. One cysteine residue was involved in inter-subunit disulphide bonds between the LAP and mature region at amino acid position 33. The other cysteine residues were involved in disulphide bonding within the mature peptide at amino acid positions 223/225. They generated two mutant TGF-β1 cDNAs and subcloned them into the πH3M vector and transiently transfected COS cells. They demonstrated that the position 33 had normal biological activity but did not require acid activation. The other mutation, at position 223/225, did require acid activation but produced the equivalent of five times normal biological activity. These mutant TGF-β1 proteins were not generated for their potential dominant-negative activity. Instead they have provided valuable insights into how the interaction of disulphide bonds between the LAP and the mature region act in TGF-β proteins. However, it might be possible to take the mutated cDNA which has five times normal activity and use that

as the basis for generating a dominant-negative mutation which might compete with wild-type TGF-β proteins.

4.3.1 Which TGF-β isoform to mutate and where to mutate it

In this thesis, human TGF-β2 was chosen as a starting cDNA for the attempt to create dominant-negative mutants. The reasons for this choice were twofold. Firstly, from the *in situ* hybridisation analysis, our main interest had been the function of TGF-β2. The expression pattern of mouse TGF-β2, included many epithelial sites of expression and in particular expression in the myocardium of the heart at a time of valve formation. From the data presented in this thesis, it had been speculated that TGF-β2 was the molecule that induced an epithelial/mesenchymal transformation at the region of the presumptive heart valve and regulates epithelial differentiation. It was hoped that the generation of a dominant-negative mutant for TGF-β2 could be used, eventually, to specifically knock out wild-type TGF-β2 at that particular developmental stage. Secondly, at the initiation of the project this was the only cDNA which was immediately available which possessed both 5' and 3' untranslated regions.

Two mutations of human TGF-β2 were generated. These were (a) deletion of the signal peptide sequence which makes up the first 19 amino acids and (b) ablation of the cleavage site sequence by site directed mutagenesis. These sites were chosen for mutation rather than either the N-glycosylation sites or the other cysteine residues for a variety of reasons. Firstly, the N-glycosylation sites were discounted as it had been reported that prevention of glycosylation of the LAP by endoglycosidase F had resulted in the activation of the TGF-β protein (Miyazono and Heldin 1989). Miyazono and Heldin (1989) suggested that removal of carbohydrate structures might be a method of activation of the TGF-β protein. Sha *et al* (1989) investigated further the role of glycosylation on the activity of TGF-β protein. They described the prevention of secretion, and increase in cellular levels of the LAP, when cells expressing TGF-β1 were treated with tunimyacin, which blocked the glycosylation steps. However, when they treated the cells with swainsonine, which inhibits correct glycoprotein processing, the abnormal TGF-β1 proteins containing the altered

carbohydrates were secreted readily by the cells. They suggested that oligosaccharide addition, and remodelling, are important for secretory exit of TGF-β1, but play no role in the specific proteolysis to activate TGF-β1. Sha *et al* (1989) did not investigate the biological activity of media from the cell line expressing TGF-β1, but relied solely on detection of radio-labelled protein extracts or Western blots. They did note that the levels of LAP and mature protein were at a comparable ratio to each other in the cell lysates, which they suggested meant that no active mature protein was released from the cell. However, since most TGF-β antibodies can only detect a minimum of 1-5ng of TGF-β protein, this analysis does not indicate the potential inactivivty, especially when only picogram quantities of TGF-β are required for biological activity.

The cysteine residues also offer a target for generation of mutations by site directed mutagenesis. However, the number of potential target sites available, combined with the lack of knowledge concerning their specific role, made the cysteine residues an unappetising target. Brunner *et al* (1989) had already shown that alteration of some of these cysteine residues to serine reidues had resulted in activation of the protein. Although this had been expected since it was known that the particular residues they altered were involved in inter-disulphide bonding (see above).

4.3.2 Biological activity of homodimers and heterodimers of mutated human TGF-β2

The two wild-type constructs, $\beta1^{33}$ and β2, were estimated to be producing 215pg/ml and 105pg/ml respectively. This does not compare with the production of 60-90ng/ml reported by Brunner *et al* (1989) when using the exact same $\beta1^{33}$ construct. The only difference between the method employed in this thesis and that employed by Brunner *et al* (1989) was in the transfection protocol. Although almost identical, Brunner *et al* (1989) transfected their constructs into the COS cells in NuSerum (In this thesis the transfection was carried out in DMEM + 10%FCS). Nuserum, is normally used for the growth of primary cultures and was not considered ideal for stable cell line growth. The method of Brunner *et al* (1989) was

attempted, but during and after cell transfection, cell death was considerable with over 80% of cells lost. This technique was not pursued further, and although the production of TGF-β polypeptides by the method, described in this thesis, was lower than expected, it was producing quantities of TGF-β which were detectable in a biological assay.

The transfection efficiency was determined to be in the expected range of 50-90% by analysing COS cells which had been transfected with the construct pIRV (Beddington *et al* 1989). The problem with lack of production does not seem to be poor transfection efficiency, although this can only be judged by comparison with the efficiency of the pIRV transfection. If the expected number of DNA molecules are getting into the cells then maybe there is a breakdown in the replication, transcription or translation steps.

Another possibility is the sensitivity of COS-7 cells to TGF-β. Previous reports which involved the use of the πH3M vector utilised COS-1 cells, although Brunner *et al* (1989) did not specify which type of COS cell they used. Both these cells are derived from the same source and have been transformed with an SV40 virus which lacks an origin of replication. Both are reported to be able to support vectors such as πH3M. The continuous culture of cells can lead to changes in phenotype and the COS-7 cells may have developed sensitivity to TGF-β. If this was the case then the COS cells might go through a selection procedure killing off many of the cells. Cell death was not extensive after 24 or 48 hours although by 72 hours it was beginning to increase, although this was, probably, as much to do with the fact that the cells were cultured in serum-free medium.

The πH3M vector is designed specifically to give high copy number in COS cells, which have been transformed by an origin-defective mutant of SV40. The cells support the growth of recombinant SV40 viruses. As the πH3M vector has been designed with an SV40 origin of replication, there is a massive replication of the πH3M vector to between 10,000 and 100,000 copies per cell (Kaufman, 1990). Since the constructs were generated from a πH3M-TGF-β1[33] construct described and provided by Brunner *et al* (1989) which had been proven to generate nanogram quantities of protein, then it would be unlikely that there was a mistake in the vector resulting in

either defective replication or transcription. This is based on the assumption that this is indeed the correct construct as determined by restriction endonuclease digests. The lack of high copy number in the cell lines was not tested at the time although this is possible by re-extracting the construct from the cells and possibly estimating the concentration, of plasmid DNA, compared with what was initially transfected. However, this would not tell you if the lack of copy number was due to defective replication caused by a defect in the cell line or a defect in the construct.

The transfections were carried out at least twice and the biological activity assayed twice for each transfection, each time in triplicate. This was carried out by measuring growth of the epithelial cell line CCL64, in a standard biological assay, after they had been incubated with conditioned media from COS cells which had undergone transfection with the various constructs, either individually or as co-transfections.

The mutant constructs $\beta2^c$ (cleavage site mutant) and $\beta2^f$ (in frame signal peptide mutant) had a reduced biological activity compared to β2, although it was not completely lost. This suggests that these two mutants were not inactivating the effect of TGF-β2 polypeptide, and it was being produced, cleaved and secreted normally. This was borne out by the analysis of a comparison of these mutants co-transfected in the ratios described with wild-type human TGF-β2. All the mean percentages of ^{3}H-thymidine incorporation were below the mean produced by hTGF-β2. This probably reflects the increase in number of copies of vector, whether mutant or wild type being introduced into the COS cells. When the means were compared by Student's t-test, $p<0.1$ which is not significant and the null hypothesis is not rejected.

Mutant $\beta2^e$, which contained the frame-shift mutation produced a mean of incorporation at 83% after 4 units of construct had been transfected. Comparison with rTGF-β1 standard curve suggested that this mutant was producing between 0-100pg/ml. The pIRV construct which was employed to test the efficieny of transfection also provided a mean of 82% which might reflect the basal level of activity of COS cells after transfection estimated at 35pg/ml. Co-transfection of this mutant with wild-type TGF-β2

did not significantly alter the mean level of incorporation of ^{3}H-thymidine, observed with β2, in the CCL64 cells.

To test the specificity of the mutant β2 constructs they were co-transfected with $\beta1^{33}$ in the same ratios as described above. Means of ^{3}H-thymidine incorporation of the various co-transfections were compared to the mean generated from $\beta1^{33}$ construct transfected alone. This was also done by Student's t-test. The value of p was determined to be: $p<0.5$ or $0.5<p<0.1$ for most of the co-transfections which is not significant. However, the cotransfection of $\beta2^{c}$ in a 1:1 ratio with $\beta1^{33}$, (mean 74% ±14.1%) did produce a value of p between 0.1 and 0.05. This level of significance probably does not mean rejecting the null hypothesis that there is no difference between the means.

4.3.3 Mutations introduced into human TGF-β2 do not alter biological activity.

Two of the three hTGF-β2 mutations generated in the latter half of this thesis do not produce a slight reduction of biological activity. The other mutation, $\beta2^{e}$, does not appear to generate detectable levels of activity and might either not be stable or be translated.

The likelihood that $\beta2^{e}$ does not translate to a protein is quite high. The most likely reason for this is the requirement of the construct to generate a protein from a secondary ATG codon some 87bp downstream of the original ATG. The lack of biological activity ascertained from this construct probably reflects the inability of the translational machinery to recognise anything but the original ATG initiation site. If this is the case, then the polypeptide generated from this site enters a frame shift at position 5 and hits a stop codon at position 24. The fate of this short polypeptide is unknown.

The reason for the other two mutants not behaving in a dominant-negative manner obviously stems from the fact that the both produce some level of activity.

The $\beta 2^f$ mutant which lacks the DNA sequence encoding the signal peptide demonstrates that this structure is not required for TGF-β2 to follow the normal secretory pathways, and that it can find it's own way out of the cell, whether by passive means or by attachment to other polypeptides involved in a secretory pathway. This would account for the lack of inhibition of activity of wild-type forms of TGF-β.

What is the signal peptide for, if it is not required for secretion from the cell? Secreted proteins are generally synthesised with an N-terminal signal sequence 15-25 residues long, which somehow initiates the export process. Several functions have been associated within this short peptide. These include mediation of interaction between ribosome and the signal recognition peptide which arrests translation until an occupied site on the membrane is found (Perlman and Halvorson, 1983). It is also thought that it may influence the release of the polypeptide from the ribosome. It also contains the information required to remove it from the rest of the polypeptide (Von Heijne, 1984). It is quite possible that it is used to secrete TGF-β2 across organelle membranes within the cell itself. This would suggest that TGF-β2 functions in an intracrine manner, which is supported by data from the in situ hybridisation analysis, (presented in this thesis and Gatherer *et al* 1990, Schmid *et al* 1991) which described the localisation of TGF-β2 RNA in many epithelial cells which were undergoing differentiation. This does not preclude TGF-β2 operating in a paracrine manner. Whether this is the case for TGF-β1 and TGF-β3 could be ascertained by deletion of their signal peptide sequences. Cell lines which are known to respond to TGF-β2 in an autocrine manner could be transfected with the $\beta 2^f$ mutant to establish whether this suggestion is true. It is unknown which organelle TGF-β2 acts upon in the cell, although recently it has been reported that TGF-β1 is localised in the mitochondria of rat and mouse cardiomyocytes (Heine *et al* 1991).

The levels of activity observed with the mutant $\beta 2^c$ and it's lack of effect, when co-transfected with β2 and $\beta 1^{33}$, on biological activity was disappointing. Complete biological inactivity was not observed. Mercola *et al* (1990) and Lopez *et al* (1992) have both described the generation of dominant-negative mutations based on alteration of cleavage recognition site in PDGF-A and TGF-β1 respectively.

Six mutations were generated by Lopez *et al* (1992) designed to specifically knock out TGF-β1. Three of the mutations were generated by disruption of the N-glycosylation sites, at amino acid positions 82, 136 and both (82-136). This was achieved by replacing the asparagine residues with glutamine residues. A further two mutants were generated by deletion of a small charged peptide sequence (residues 42-63 of the LAP, which is found in all three TGF-β isoforms). The final mutation involved the alteration of the tetrabasic proteolytic cleavage site which would prevent release of the mature protein from the LAP. The mutant TGF-β1 genes were expressed in human 293S cells, known to secrete very little TGF-β.

Lopez *et al* (1992) demonstrated that conditioned media from transfected cell lines was assessed for presence of TGF-β1 by immunoprecipitation. The two mutations which carried the single altered N-glycosylation site showed reduced levels of TGF-β in the conditioned media. However, the mutant TGF-β1 which contained both disrupted N-glycosylation sites showed no secreted TGF-β1, and was apparently more succesful than the constructs carrying the individual N-glycosylation mutations at preventing correct processing. The most succesful mutation described by Lopez *et al* (1992) was the construct which had had residues 42-63 of the LAP removed. They also described succesful inhibition of secretion of TGF-β1 generated from the construct which had the proteolytic cleavage site altered.

Lopez *et al* (1992) continued by investigating the specificity of action of the dominant-negative mutations. They described co-transfections of the mutants with constructs carrying wild-type TGF-β1, 2, and 3 and showed that the mutations would inhibit secretion of TGF-β2 and TGF-β3 but that they would preferably form dimers with TGF-β1. The mutant constructs described by Lopez *et al* (1992) did not interfere with secretion of protein from other members of the TGF-β superfamily or unrelated polypeptides.

However, Lopez *et al* (1992) did not describe biological activity of the various constructs on their own, or from co-transfections with wild-type constructs. Even if this mutant only prevented secretion of 99% of the wild-type polypeptide the remaining 1% may still retain enough biological activity to elicit a normal response. Mercola *et al* (1990) demonstrated

reduced levels of PDGF-A by both biological response assays and immunoprecipitation. The effect of the mutant construct was dependent upon its concentration against the concentration of the wild-type PDGF construct. A ratio of 4:1 in favour of the mutant construct almost obliterating the biological activity of the wild-type PDGF-A polypeptide.

Why these mutations, described by Lopez *et al* (1992) and Mercola *et al* (1989), operated in the expected manner and the mutations generated during this project did not, may be due to two reasons. Firstly, the mutation of the cleavage site in PDGF polypeptide resulted in the change from four basic amino acids to one. Lopez *et al* (1992) described the complete alteration of the TGF-β1 cleavage site leaving no basic residues. The $\beta 2^{c}$ mutant generated in this project left two adjacent basic residues intact, although the other three were changed (Arg-Arg-**Lys-Lys**-Arg to Leu-Gln-**Lys-Lys**-Leu). It is known that specific proteases can cut the protein when only one basic residue is present, but usually they recognise a specific peptide sequence (Schulz and Schirmer 1985). This suggests that the two basic Lys residues provided enough of a recognition site for proteolytic cleavage. However, it is clear that at least 4 times the amount of construct had to be transfected into the COS cells, to produce a comparable level of biological activity to that described for COS cells which had been transfected with only 1 unit of β2. Secondly, it is possible that proteolytic cleavage was occurring at an alternative site. If this was the case then an alternative size of mature polypeptide would be produced which could possibly be detected by Western analysis. Unfortunately Western analysis was precluded in this thesis because of the low levels of production of TGF-β proteins, (estimated concentrations were based on biological assays of rTGF-β1), and the requirement for a minimum of 5ng of protein for antibody detection.

4.4 Conclusions and perspectives

One of the most interesting pieces of information to emerge from the expression studies of the TGF-β isoforms presented here and elsewhere is the fact that each has a unique pattern of expression both temporally and

spatially (Lehnert and Akhurst 1988, Pelton *et al* 1989, FitzPatrick *et al* 1990, Gatherer *et al* 1990, Pelton *et al* 1990, Schmid *et al* 1991, Millan *et al* 1991). There are occasional areas of overlap, such as whisker follicles, tooth buds, but in general the RNA of each isoforms has a specific and unique pattern in which it is expressed. It brings to the fore the question of why is there a requirement for such identical mature proteins to have these unique patterns of expression? One answer is that each TGF-β isoform has a unique function, which only that isoform can perform, *in vivo*. This is not necessarily borne out by the accumulation of data concerning the biological activities of the three isoforms. In most biological systems they are interchangeable, but there are exceptions, for instance only TGF-β2 can promote mesoderm induction in *Xenopus* (Rosa *et al* 1992). A more probable explanation might be that the three genes have essentially identical functions *in vivo* (as expected from *in vitro* data), but that the diversity of the promoter regions play a vital role in expression of the three genes. To complicate the situation further, the interaction of the isoforms with receptors has to be considered. As has been described, there have been seven receptor types found which interact with TGF-β. The specificity for each of the receptors with respect to the TGF-β isoforms has not been fully characterised, but the fact that most cell types, with notable exceptions, seem to express at least one receptor indicates another level of control of TGF-β activity. An obvious next step in the understanding of the role of TGF-β genes in development might be the elucidation of the expression patterns of the various receptor genes.

One of the most obvious problems when attempting to interpret RNA localisations studies, is that of whether the TGF-β protein is active at the site of synthesis, or is it excreted from the cell and activated elsewhere. This problem has been answered to a certain extent by the RNA and protein data generated concerning TGF-β1 (Heine *et al* 1987, Lehnert and Akhurst 1988). A comparison of the protein and RNA expression patterns indicated that TGF-β1 could act in a paracrine or autocrine manner. Whether the same can be said of TGF-β2 and TGF-β3 has been partially resolved by comparison of protein localisation data generated recently (Pelton *et al* 1991, Flanders *et al* 1991, Akhurst *et al* 1992). The localisation of protein for both these genes occurred, like TGF-β1, either in

cells where RNA had been observed, or in adjacent cells. This suggests that both TGF-β2 and TGF-β3, like TGF-β1, are undergoing extensive post-transcriptional control and operating in a paracrine or autocrine manner.

There is abundant evidence in the literature of the control exerted upon the ECM and its constituent parts by TGF-βs. Not only are they known to upregulate a variety of proteins they are also known to inhibit the production of proteases. An interesting and important question is what regulates the TGF-βs? How do the TGF-β genes know when to switch on, and just as importantly, off? There is considerable evidence accumulating that TGF-β genes, like many other developmentally important genes, respond to morphogens. So far only one natural morphogen, retinoic acid, has been identified in mammals (Tickle *et al* 1982). This has been shown to regulate the expression of TGF-βs *in vitro*, especially TGF-β2 (Glick *et al* 1991). It may be a possibility that the TGF-βs are regulated by other morphogens which are yet to be discovered. Recently TGF-β2 has been shown to be activated by the retinoblastoma (pRB) gene product through the transcription factor ATF-2 (Manova *et al* 1992). A full investigation into the transcriptional control of the TGF-βs can only provide insights as to how cellular mechanisms utilise TGF-β.

The role of TGF-β isoforms has begun to be investigated more directly with the attempts to generate mutations which can increase the level of expression, act in a dominant-negative manner or have different pieces of genes spliced into each other to determine the active sites of the protein (Brunner *et al* 1989a; Lopez-Casillas *et al* 1991; Qian *et al* 1992). The attempts, presented in this thesis, to generate dominant-negative mutations have pointed out the pitfalls in attempting such a course of action. The deletion of signal peptide from TGF-β2 apparently has no effect on secretion from the cell. What can this suggest as to the function of the signal peptide sequence of TGF-β2, and will its function be the same as that of the signal peptide sequence of TGF-β1 or TGF-β3. The mutation of the cleavage sequence, had no discernible effect on the biological activity of wild-type polypeptides. There did appear to be limited inhibition of proteolysis at the mutated site, but not enough to prevent activation in vitro.

The investigation into the generation of mutations to analyse the role of TGF-β isoforms will eventually move into the realms of transgenic technology. However, before this should be embarked upon two important areas cannot be overlooked. These are control of expression in the cell or tissue of study and, specificity of action.

Appendix A

Source of chemicals and enzymes

A.1 Chemicals

agarose	Sigma
ampicillin	Sigma
bacto Agar	Difco
bacto Tryptone	Difco
bovine Serum Albumin (BSA)	Sigma
BSA, DNase free	Gibco BRL
BSA, DNAse and RNAse free	Pharmacia
chloroform	BDH
Decon 90	Decon Labs
α-^{32}P deoxycytodine triphosphate	Amersham
deoxyribonucleotides	Pharmacia
dextran Sulphate	Pharmacia
diethylpyrocarbonate (DEPC)	Sigma
dithiothreitol	Sigma
Dulbecco's modified Eagle's medium	Gibco BRL
EDTA	BDH
eosin	BDH
ethanol	University store
ethidium bromide	Sigma
foetal Calf serum	NBL

ficoll	Pharmacia
formamide	Fluka
formaldehyde	BDH
τ-^{32}P deoxyadenosine triphosphate	Amersham
GeneClean Kit	B i o
101	
glutamine	Gibco
BRL	
guanidium thiocyante	Fluka
Histoclear	National
	Diagnostics
Harris' haemotoxylin	Sigma
hydroxyquinoline	Sigma
IPTG	Sigma
isoamylalcohol	Sigma
K5 nuclear track emulsion	Ilford
MOPS sodium salts	Sigma
Panatomic X film	Kodak
Pan F film	Kodak
paraformaldehyde	Sigma
phosphate buffered saline (PBS)	Gibco
BRL	
PBS without Ca^{2+} or Mg^{2+}	Gibco
BRL	
phenol (Water saturated)	Rathburn
	Chemicals
phenisol developer	Ilford
polyvinylpyrolidine	Sigma
proteinase K	Sigma
random prime DNA labelling kit	Boehringer
	Mannheim
Repelcote (2% Silane solution)	BDH
ribonucleotides	Pharmacia
ribosomal RNA	Boehringer
	Mannheim

RNA ladder	Gibco BRL
salmon sperm DNA	Sigma
7X detergent	Sterilin
Sephadex G50 (medium)	Sigma
spermidine	Sigma
tetracycline	Sigma
tetramethylethylenediamine (TEMED)	BDH
TESPA	Sigma
^{3}H thymidine	Amersham
TGF β antibody detection kit	British Biotec
triethanolamine	Sigma
tris base	Sigma
α-^{35}S uridine triphosphate	Amersham
UTP-S	NEN
yeast extract	Difco
X-gal	NBL

A.2 Enzymes

Alkaline Phosphatase	Boehringer Mannheim
DNAse I (RNAse free)	Pharmacia
Klenow fragment (DNA polmerase I)	NBL
Restriction endonucleases	NBL, Pharmacia, Stratagene, BCL
RNA guard	Pharmacia
RNAse A	Sigma
T7 RNA polymerase	Gibco BRL
T3 RNA polymerase	Gibco BRL

T4 DNA ligase	Pharmacia
T4 polynucleotide kinase	Stratagene
Protease inhibitors	Sigma

All other common laboratory chemicals not mentioned above were supplied by BDH (Analar grade).

Appendix B

Statistical equations for Student's t test

Σx_1^2 = sum of the squares in observation in sample 1
Σx_2^2 = sum of the squares in observation in sample 2
$(\Sigma x_1)^2$ = square of the total of the observations in sample 1
$(\Sigma x_2)^2$ = square of the total of the observations in sample 2

sum of the squares of the difference from their res[pective means:

$$\Sigma(x_1-x_1)^2= \Sigma x_1^2-(\Sigma x_1)^2/n_1$$

$$\Sigma(x_2-x_2)_2= \Sigma x_2^2-(\Sigma x_2)^2/n_2$$

The variance for the two samples combined is:

$$(\Sigma(x_1-x_1)^2 - \Sigma(x_2-x_2)_2)/ (n_1-1)+(n_2-1) = SD^2$$

The divisors n_1-1 and n_2-1 represent degrees of freedom.

The standard error of the difference between the means is:

$$\text{SE diff}=\sqrt{SD^2/n_1+SD^2/n_2}$$

When the difference between the means is divivded by this standard error the result is t.

Thus $$t=x_1-x_2/\sqrt{SD^2/n_1+SD^2/n_2}$$

The probability for a given value of t can be looked up in statistical tables, with $(n_1-1)+(n_2-1)$ degrees of freedom.

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