Analysis of Multistage Mouse Skin Carcinogenesis by Retroviral Mediated Gene Transfer.

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Thesis submitted to the University of Glasgow

for the degree of Doctor of Philosophy.

The Beatson Institute for Cancer Research, Glasgow.

January, 1993.

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To Bob and Katie,

without their support and love this thesis would not have been possible.

Declaration:

The work in this thesis was performed by me, with the following exception. The cloning of the retroviral vector pZip LacZ SV(v-ras) was performed by Dr. Bernard Bailleuil at the Beatson Insitute for Cancer Research.

Uta Boeger-Brown

January, 1993.

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Abbreviations.

A	adenine
ada	adenosine deaminase
β-gal	β-galactosidase
B[a]P	benz[a]anthracene
bp	base pair
C	cytosine
cDNA	complementary deoxyribonucleic acid
cfu	colony forming units
Da	dalton
dhfr	dihydrofolate reductase
DMBA	7,12-dimethyl benz[a]anthracene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EtBr	ethidium bromide
FBS	foetal bovine serum
FCS	foetal calf serum
ffu	focus forming units
g	gravitational units
G	guanine
GDP	guanosine diphosphate
GTP	guanosine triphosphate
hprt	hypoxanthine posphoribosyl transferase
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride hydrate
kb	kilobase
kDa	kilo-Dalton
LTR	long terminal repeat
M	molar
min	minute(s)
µl	microlitre
ml	millilitre
mM	millimolar
MMP	matrix-degrading metalloproteinase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-nitroso-N'-methylurea
Mo-MuLV	Moloney murine leukemia virus
mRNA	messenger ribonucleic acid
neo	neomycin phosphotransferase
OD	optic density
PDGF	platelet-derived growth factor
PMSF	Phenylmethanylsulfonylfluorid
RNA	ribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease

RT	room temperature
SA-cfu	colony forming units in soft agar
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SV40	simian virus 40
T	thymine
TGF	transforming growth factor
tk	thymidine kinase
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl)methylamine
tRNA	transfer ribonucleic acid
v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside

Acknowledgements.

I would like to thank my supervisor, Dr. Allan Balmain, for his advice and encouragement throughout this project and during the writing of the thesis. In addition I am indebted to Dr. Ken Parkinson, Dr. Phil Burns, Dr. John Pitts, Dr. Keith Vass, Dr. Andrea Stoler and Mary Freshney for their advice and technical expertise. Thanks also to the various members of Research Groups R7 and R9, especially Sheena Haddow, Frances Fee, Mairi Clark, Phil Burns and Julie Burns, for their support and friendship. Liz Gordon deserves a special thank you for all those literature searches.

The biggest thank you by far goes to my husband, Bob Brown, and to my daughter, Katie, for being very patient, supportive, understanding and loving for such a long time.

I acknowledge the receipt of a research studentship from the Cancer Research Campaign.

Abstract.

In the present study I have explored the feasibility of using retroviral mediated gene transfer to study the role of given genes during chemically induced carcinogenesis of the mouse skin.

Retroviral vectors expressing parts of the murine c-Ha-*ras* gene as antisense RNA were compared to their sense counterparts for their ability to revert the transformed phenotype of cells containing a mutant Ha-*ras* gene. I have shown that one of the antisense retroviral constructs (pZN(X)RAS-1, containing exons 1 and 2 and 1.3 kb of the 5' untranslated region of murine c-Ha-*ras*) was effective in altering the transformed phenotype of NIH3T3 cells transformed by a Ha-*ras* gene containing a mutation at codon 61. Expression of this antisense Ha-*ras* retroviral construct altered the ratio of morphologically transformed to untransformed colonies compared to vector alone controls. Furthermore the ability to form colonies in soft agar of the Ha-*ras* transformed NIH3T3 cells was reduced by 68% after infection with pZN(X)RAS-1 compared to vector alone infectants. Infection of cells with the sense counterpart of pZN(X)RAS-1 did not reduce the ability of these cells to grow in soft agar. No significant reduction in soft agar cloning efficiency was observed with the other antisense or sense retroviral constructs.

Metalloproteinases have been implied to play a role in progression of tumours to an invasive and metastatic phenotype. The rat transin cDNA was introduced into murine epithelial cells. However no evidence was observed of transin expression influencing progression towards invasion or metastasis as assayed by a spontaneous metastasis assay following subcutaneous injection of cells in athymic nude mice.

The feasibility of histochemically tagging mouse keratinocytes through constitutive expression of a bacterial *lacZ* gene expressed within a retroviral vector was examined. Results suggested successful infection and expression of the *lacZ* gene in keratinocytes *in vitro* and *in vivo*. However a retroviral vector containing both the v-Ha-*ras* and *lacZ* genes failed to induce tumours in mouse skin. This appeared to be due to lack of efficient expression of the v-Ha-*ras* gene in murine epithelial cells.

The results presented in this study show the potential and limitations of retroviral vectors to study an *in vivo* tumour model system such as mouse skin carcinogenesis. The successful reversion of the transformed phenotype of cells *in vitro* using antisense Ha-*ras* expression may suggest this approach could be used to reverse or inhibit transformation in such tumour model systems.

Chapter 1

Cancer: A Multistep Process.

1. Cancer: A Multistep Process.

1.1. Genetic Events Involved in Neoplasia.

The hypothesis that genetic events are a major component of neoplasia is based on evidence accumulated over recent years involving studies on hereditary predisposition to cancer (HANSEN and CAVENEE, 1987; PONDER, 1990; HABER and HOUSMAN, 1991), studies on association of clonal karyotypic changes in cancers (HEIM et al., 1988) and the demonstration of the mutagenic capacity of most carcinogens. Direct evidence for causal genetic changes in cancer emerged with the identification and cloning of genes involved in neoplasia either in a dominant or recessive/dominant negative manner: oncogenes or tumour suppressor genes, respectively.

Oncogenes were first identified as part of oncogenic retroviruses (ELLERMANN and BAN, 1908; ROUS, 1911; GROSS, 1970), but are derived from normal cellular proto-oncogenes by mutations which activate the transforming activities of these genes. There are extensive examples of activation of proto-oncogenes by means other than retroviral transduction. Activation of proto-oncogenes involves either qualitative (by point mutations or larger scale events such as truncation and gene fusions as a result of chromosomal tanslocations) or quantitative changes (by elevated expression caused by a number of mechanisms including gene amplification, chromosome translocation, insertional mutagenesis and/or epigenetic events) (BISHOP, 1987; VARMUS, 1989). Most of the oncogenes isolated to date have been identified either by cellular transformation of mouse NIH 3T3 cells induced by DNA transfection (SHIH et al., 1979 and 1981; COOPER et al., 1980; KRONTIRIS and COOPER, 1981), by isolating new oncogenes located within amplified sequences present in tumour DNA (KINZLER et al. 1987) or adjacent to breakpoints of chromosomal translocations found in tumours (TSUJIMOTO et al., 1984 a and 1984b; TSUJIMOTO and CROCE, 1986; BISHOP, 1991). It has become clear in recent years, that the products of the very highly conserved proto-oncogenes are elements of a cellular signaling network whose functions range from external ligands and growth factors, through cytoplasmic protein kinases and GTP-binding proteins, to nuclear

transcription factors. (reviews for oncogenic function: EISENMAN, 1989; HUNTER, 1989 and 1991; McCORMICK, 1989; VARMUS, 1989; FORREST and CURRAN, 1992; PAWSON, 1992).

The existence of tumour suppressor genes has been postulated for a long time based on chromosomal deletions found *in vivo* in several inherited predispositions to cancer (KNUDSON, 1971), and on chromosome transfer and cell fusion experiments between normal and tumour cells *in vitro* where normal-tumour cell hybrids predominantly display a non-malignant phenotype (HARRIS, 1988; STANBRIDGE and CAVANEE, 1989). However, such genes have only recently been localized and cloned by using polymorphic DNA markers to search tumour cell genomes for repeated instances of loss of heterogenicity (LOH) and by gene mapping using linkage studies in families and chromosomal-rearrangement mutations in patients (SAGER, 1989; STANBRIDGE and CAVANEE, 1989; MARSHALL, 1991; MASSAGUE and WEINBERG, 1992; WHITE, 1992). Tumour suppressor genes have been hypothesized to act "recessively" at the cellular level (HERSKOWITZ, 1987), so that both copies of the allele must be inactivated or lost in order for the growth-suppressive function to be eliminated (KNUDSON, 1985).

1.2. The Multistep Nature of Carcinogenesis.

There is now compelling evidence that cancer is a multistep process as proposed by FOULDS (1954 and 1958) and KLEIN and KLEIN (1985). Evidence collected *in vitro* is based primarily on transformation of primary rodent fibroblasts. It has been demonstrated that there is a requirement for co-operation, in the classical sense, of two or more independent oncogenes (LAND et al., 1983a and 1983b) or, in a broader sense, the activation of one or more proto-oncogene(s) has to occur in conjunction with the loss of tumour suppressor genes. Evidence of co-operation is also found *in vivo* (HUNTER, 1991; BISHOP, 1991). So far relatively few tumours have been identified that contain two different activated oncogenes, whereas there are numerous examples of alterations of two or more tumour suppressor genes within one tumour (MARSHALL, 1991; FEARON and VOGELSTEIN, 1990).

Epidemiological studies also support the concept that cancer is a multistep process. Cancer development in humans shows a clear exponential

relationship of cancer incidence and age. Statistical analysis of age-incidence curves has suggested that 4-7 rate limiting steps are required for the development of carcinomas and 3-4 in leukaemias (KNUDSON, 1973; PETO et al., 1975; FARBER and CAMERON, 1980; DIX, 1989). Rate limiting steps are considered to reflect the minimum of events required, as non-rate limiting events will also contribute to the development of cancer.

Discrete morphological and histological stages have been identified in many cancers, again suggesting a stepwise progression towards malignancy. Molecular and karyotypic analysis of human cancers show multiple genetic events such as chromosomal translocations, gene amplification and point mutations in a single cancer of clonal origin which is accompanied by the successive emergence of more aneuploid subclones during tumour development (HEIM et al., 1988).

Animal model systems are invaluable resources for studies aimed at understanding the molecular mechanisms underlying carcinogenesis. Indeed, animal model systems helped to establish the now accepted concept of multistage tumorigenesis; comprising tumour initiation, promotion and progression (HECKER et al., 1982). The value of such animal model systems lies in the controllable and reproducible induction of specific tumour types by particular chemical and physical carcinogens; in contrast to the situation in most human tumours, for which the causative agents have only started to be identified (CAIRNS, 1981; MILLER, 1970; AMES, 1983; WILBOURN et al., 1986; AMES and SWIRSKY GOLD, 1990 and references therein). Carcinogen-induced animal tumour models therefore provide an ideal opportunity to investigate the molecular events associated with defined stages of carcinogenesis. Chemically-induced animal tumours like hepatocarcinomas in rats or mice (DRINKWATER, 1990), mammary tumours in rats (SUKUMAR, 1989 and 1990) and mouse skin carcinomas (BALMAIN and BROWN, 1988) are examples of the most studied experimental multistage carcinogenesis model systems. The activation of oncogenes in the various animal models of carcinogenesis has been summarized by GUERRERO and PELLICER (1987), BALMAIN and BROWN (1988) and SUKUMAR (1989, 1990).

1.3. Mouse Skin Carcinogenesis.

The two stage or initiation - promotion model of mouse skin carcinogenesis involves the single application of a subthreshold dose (i.e. one which will not induce tumour formation by itself) of a complete carcinogen or initiator followed by frequently repeated applications of a tumour promoter (SLAGA, 1983). The promotion stage can be subdivided into two further stages: conversion and propagation (BOUTWELL, 1964; FUERSTENBERGER et al., 1981; SLAGA et al., 1980). The mouse skin carcinogenesis model is of particular importance in the elucidation of genetic and/or epigenetic events associated with carcinogenesis involving epithelial tissues, keeping in mind that most human cancers are of epithelial origin (Cancer Statistics, Ca 1989). Molecular and cellular characterisation of the two stage model has now shown multiple events associated with tumorigenesis.

The initiation-promotion model of mouse skin carcinogenesis results in the appearance of benign papillomas within 6 to 20 weeks after the start of promotion by repeated treatments with a tumour promoter such as 12-O-tetradecanoylphorbol 13-acetate (TPA). Papillomas have a cauliflower-like structure consisting of several folds joined by one or a few stalks, linking them to the underlying skin. Each fold consists of epithelial projections covering vascular connective stalks. The basic stratified structure of the epidermal component is retained although tends to be thicker than normal. Approximately 5 to 10% of these papillomas will progress to malignant carcinomas. Macroscopically, carcinomas are firm expanding nodules which often ulcerate. They are characterized by a disorderly proliferation of epithelial cells and can be classified as grade 1 to 3, with grade 3 showing least differentiation, highest mitotic index and marked nuclear and cellular pleomorphism. The most differentiated tumours (G1) show extensive areas of keratinization and groups of terminally differentiated cells making up the so called "horny pearls" (KRUSZEWSKI et al., 1987).

1.3.1 Requirements for Chemical Carcinogenesis in Mouse Skin.

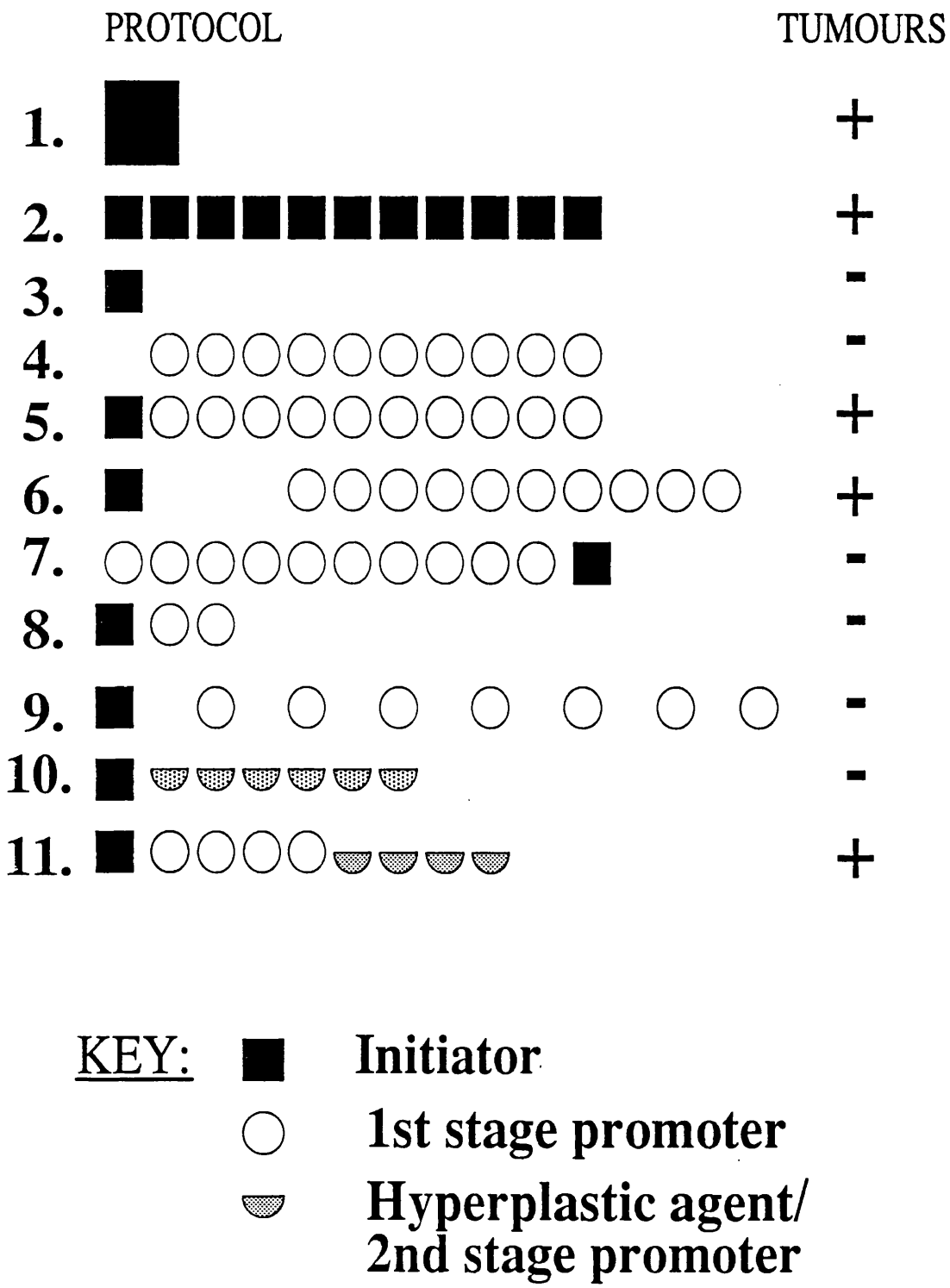
The requirements for tumour formation in mouse skin are summarized in Figure 1.1. Two basic protocols can be applied to induce mouse skin carcinogenesis:

a) complete carcinogenesis, which entails the application of either a single large dose or fractionated doses of a known carcinogen (protocols 1 and 2);

b) two-stage or initiation - promotion carcinogenesis using optimal doses of a carcinogen and tumour promoter (protocols 5, 6 and 11) (BURNS et al., 1984; SLAGA, 1984). The two protocols differ in the kinetics of appearance of benign papillomas and malignant carcinomas. The two-stage protocol has a relatively low conversion rate from benign papillomas to malignant carcinomas (only between 5 - 10% of the papillomas progress to carcinomas) (protocol 5). In contrast, complete carcinogenesis induces fewer papillomas (with a longer latency period) but has an increased efficiency to induce malignant carcinomas both in terms of incidence and shorted latency period compared to the two-stage protocol (HENNINGS et al., 1983).

Initiation requires only a single application of an initiating carcinogen and is irreversible, a delay between the initiating and promoting stage of up to 40 weeks does not alter the tumour incidence (protocol 6) (LOEHRKE et al., 1983; VAN DUUREN et al., 1975). Initiation by itself or promotion alone, however, does not result in the formation of tumours (protocol 3 and 4). Similarly, promotion prior to initiation (protocol 7), or promotion given only for a short period after initiation (protocol 8) or at increased intervals between individual promoter treatments (protocol 9) produce very few if any tumours. Tumour promotion can be subdivided into two stages: first stage or conversion and second stage or propagation (BOUTWELL, 1964; SLAGA et al., 1980; FUERSTENBERGER et al., 1981). Although all tumour promoters induce hyperplasia, not all hyperplastic agents can function as tumour promoters. However, the latter may complete promotion if the initiated mouse skin has been exposed to as little as one application of a tumour promoter (protocols 10 and 11). These observations led to the classification of stage 1 promoters (also named full or conversion promoters) and stage 2 promoters (propagation promoters). Wounding and TPA belong to the stage-1 promoters, whereas

Figure 1.1. Requirements for chemical carcinogenesis in mouse skin.



mezerein or 12-retinoylphorbol 13-acetate (RPA) function as stage-2 promoters (HENNINGS and BOUTWELL, 1970; ARGYRIS, 1989; MARKS et al., 1982).

For many years it has been accepted that around 90% of carcinomas arise directly from "identical site" promoter-independent papillomas (BURNS et al., 1978), which in turn were thought to arise from pre-existing promoter-dependent papillomas in a sequential manner (ALDAZ et al., 1988) This assumption has recently been questioned. REDDY et al. (1987), using a combination of photography, tumour mapping, and analysis of the X-linked polymorphic enzyme PGK, showed that 5 out of 18 carcinomas exhibited PGK phenotypes different from those detected at an earlier biopsy of a papilloma at the same site. The incidence of PGK phenotype change could be twice as high, as there is a 50% chance that a new tumour will have the same phenotype as the pre-existing lesion. Interestingly, the majority of carcinomas induced in this way do not appear to arise from visible papillomas (REDDY and FIALKOW, 1989). The simplest explanation for this is that while initiators efficiently induce mutations which can aid progression, these events are rarely induced or must occur spontaneously in initiated cells expanded by tumour promoter treatment (DRINKWATER, 1990). POTTER (1981) has suggested that the role of promotion is to increase the size of target cell population available for a second mutational event. The frequency of this event can be increased by applying mutagens to papillomas induced by an initiation-promotion protocol (HENNINGS et al., 1983). The mutations involved in this process have not been identified, but are likely to differ from those involved in initiation, since chemicals which are good initiators are not necessarily effective progression agents and vice versa (POTTER, 1981).

1.3.2. Molecular Events at Initiation.

The mutagenic capability of carcinogens (reviewed by SINGER and KUSMIEREK, 1982) together with the irreversibility of the generally phenotypically silent initiation event (LOEHRKE et al., 1983; VAN DUUREN et al., 1975; BOUTWELL et al., 1982) suggests that initiation involves a genetic event. Some of the target genes for initiation have been identified. Tumours induced in the skin, mammary gland liver and other

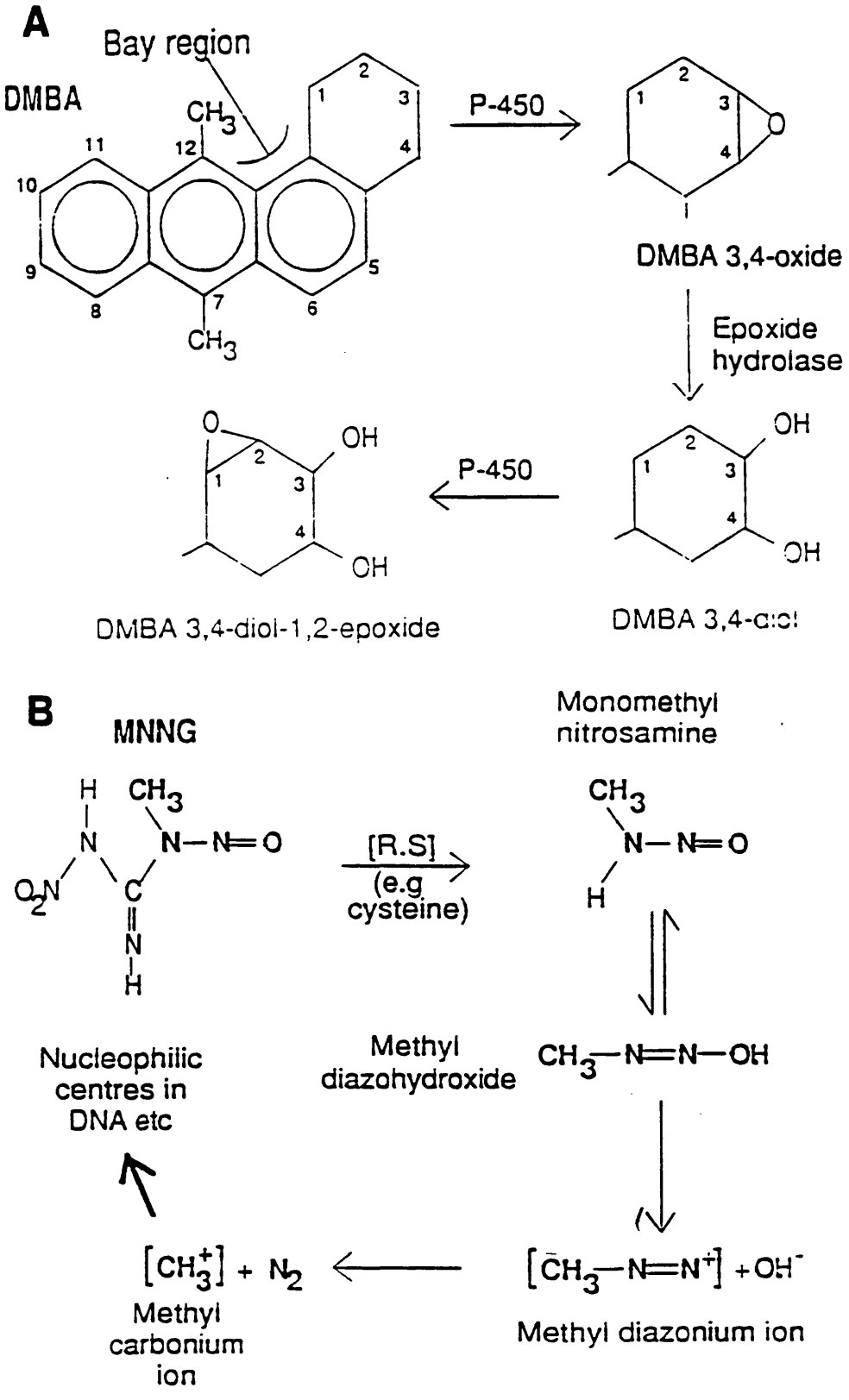
tissues in experimental animal model systems frequently contain activated *ras* oncogenes, capable of transforming NIH 3T3 cells after tumour DNA transfection (reviewed by BALMAIN and BROWN, 1988). Mutations in members of the *ras* gene family are also frequently found in human tumours (BOS, 1989; LEMOINE, 1990). Tumour suppressor genes have also been suggested as targets for chemical and physical carcinogens, especially in human cancers (HARRIS, 1991).

The two most commonly used mutagens in initiation of mouse skin carcinogenesis are 7,12-dimethyl benz(a)anthracene (DMBA) and N-methyl-N'-nitro-N-nitroso guanidine (MNNG). They belong to two distinct classes of chemical carcinogens: polycyclic aromatic hydrocarbons (PAH) and N-nitroso compounds, respectively.

PAHs do not interact with DNA directly but are metabolized and activated by P-450s (or monoamine oxidases) and an epoxide hydrolase to the ultimate carcinogen, the 'bay region' dihydrodiol epoxide (or diol epoxide). The chemical activation of PAHs has been elucidated mainly from studies on benz[a]pyrene (B[a]P) and its metabolites (reviewed by CONNEY, 1982). The metabolic activation of DMBA to its diol epoxide is shown in figure 1.2a. Both the *syn* and *anti* forms of the DMBA 'bay region' diol epoxide bind DNA (SAWICKI et al., 1983), but the carcinogenic properties of the chemical are largely due to the *syn* form. The *syn* form binds almost exclusively to deoxyadenosine (dA) residues in DNA, resulting in large dA adducts (CHENG et al., 1988). This predominant dA adduct formation is characteristic of DMBA and its metabolites, as other PAHs (including B[a]P and methylcholanthrene (MCA)) form major adducts with deoxyguanosine (dG) residues (JEFFREY, 1985). DMBA is about 30 fold more effective as an initiator than B[a]P (DIPPLE et al., 1983b).

Initiation with DMBA and promotion with phorbol esters induce papillomas and carcinomas with 90% showing activation by an A:T -> T:A transversion at the second base of codon 61 of the Ha-*ras* gene, generating a new XbaI restriction site (QUINTANILLA et al., 1986). The A->T mutation is consistent with the metabolism and DNA-binding characteristics of this carcinogen. PAHs, other than DMBA, induce a more heterogeneous pattern of mutations. B[a]P preferentially induces G:C -> T:A transversions

Figure 1.2. Mechanism of activation of DMBA and MNNG.



(WEINSTEIN et al., 1976), additional Ha-*ras* mutations have been reported (BALMAIN and BROWN, 1988). Only about 20% of MCA-induced mouse skin papillomas have the A:T -> T:A transversion at codon 61 of the Ha-*ras* gene, a further 20% contain G:C -> T:A transversion at codon 13. The codon 13 mutation is found in the majority of MCA-carcinomas (BROWN et al., 1990).

The N-nitroso compounds, including MNNG, are alkylating agents (SINGER and KUSMIEREK, 1982). The breakdown of these compounds to ultimate carcinogens occurs in the presence of a nucleophilic reagent (e.g. alkali or thiols in the cells) and does not require enzymatic catalysis. The breakdown of MNNG is illustrated in figure 1.2b. Although alkylation has been observed at all O and N positions in the deoxyribonucleotides (except the nitrogen attached to the sugar), the primary mutagenic lesion produced is the O6-methyl guanine adduct, resulting in G:C -> A:T transitions by mispairing with thymidine during DNA replication (TOORCHEN and TOPAL, 1983). Consistent with the mutational mechanism for MNNG, only G-> A lesions (at the middle base of codon 12) have been detected in the Ha-*ras* gene in mouse skin tumours initiated with MNNG (BROWN et al., 1990; BREMNER and BALMAIN, 1990). The simple methylating agent, MNU, induces G ->A mutations in mouse skin papillomas (BROWN et al., 1990) and in rat mammary carcinomas (ZARBL et al., 1985; SUKUMAR, 1989 and 1990). Interestingly, MNU-induced thymic lymphomas show a spectrum of N-*ras* gene alterations (C -> A or A -> T), in addition to the G -> A mutation, that are not always readily explicable with a simple mutational mechanism of methylating agents (see BALMAIN and BROWN, 1988).

1.3.3. Activation of *ras* Oncogenes: An Early or Late Event?

The discovery that the Ha-*ras* gene was activated not only in DMBA-induced carcinomas (BALMAIN and PRAGNELL, 1983) but also in benign papillomas (BALMAIN et al., 1984), suggested that the mutation of the gene is involved in the early stages of mouse skin carcinogenesis. QUINTANILLA et al. (1986) showed that the nature of the activating mutation in the Ha-*ras* gene reflected the chemical specificity of the initiating carcinogen. They found that over 90% of DMBA- initiated papillomas and carcinomas

contained an A:T -> T:A transversion in codon 61 of the Ha-*ras* gene, as predicted by the known metabolism and DNA-binding characteristics of the carcinogen both *in vitro* (CHENG et al., 1988) and *in vivo* (DIPPLE et al., 1983a). In MNU-induced rat mammary carcinogenesis, SUKUMAR et al. (1983) and ZARBL et al., (1985) also found a correlation between the type of Ha-*ras* gene mutation introduced and the carcinogen used. Further evidence that *ras* gene activation can occur at the initiation stage came from chemically induced hepatomas in B6C3F1 mice (WISEMAN et al., 1986). Cells from both liver tumours and hepatic foci, the precursors to hepatic neoplasms (PERAINO et al., 1983; SUKUMAR, 1989 and 1990), contain transforming, mutant c-Ha-*ras* genes following MNU treatment (BUCHMANN et al., 1989). It was demonstrated by using animal model systems, that the nature of the activating mutation in the *ras* gene accurately reflects the chemical specificity of the initiating carcinogen, but is independent from the tumour promoters used (QUINTANILLA et al., 1986; FUJIKI et al., 1989). Other examples are known, however, in which it is more difficult to correlate the mutagenic activity of the initiating carcinogen with the mutations detected in the resulting tumour (GUERRERO et al., 1985; GUERRERO and PELLICER, 1987; BALMAIN and BROWN, 1988).

The causal nature of mutations in the *ras* genes in initiating mouse skin carcinogenesis was demonstrated by BROWN et al. (1986). They found that chemical initiation with DMBA could be replaced by the application of either Harvey or BALB murine sarcoma virus (Ha-MSV, BALB-MSV), retroviruses containing the activated viral Ha-*ras* gene, to the mouse skin. As with chemical initiation, the presence of cells initiated by the stable integration of either retrovirus, was necessary but not sufficient for tumour formation to occur. Treatment with a phorbol ester was also required. Subsequently, ROOP et al. (1986) demonstrated, that keratinocytes expressing the viral Ha-*ras* gene (after infection by Ha-MSV) produce papillomas when grafted (combined with freshly isolated dermal fibroblasts) onto athymic nude mouse recipients. These experiments firmly established the correlation between initiation and Ha-*ras* gene activation in mouse skin carcinogenesis.

However, oncogene activation has also been shown to occur as a late event in experimental animal carcinogenesis systems, e.g. Ha-*ras* activation in

DMBA mediated progression of mammary hyperplastic outgrowths to mammary carcinomas in mice (KUMAR et al., 1990a). Activation of the N-*ras* gene in MNU-induced thymic lymphomas in mice (GUERRERO et l., 1985; GUERRERO and PELLICER, 1987) and the *neu* gene in schwannomas in rats exposed *in uteri* to MNU (SUKUMAR, 1989) have not yet been correlated to a particular stage in carcinogenesis.

1.3.4. Tumour Promotion.

Tumour promotion is an essential component of carcinogenesis in many *in vivo* systems (HECKER et al., 1982). Cells initiated by carcinogen treatment or virus application, do not develop into tumours in the absence of tumour promoting stimuli (VAN DUUREN, 1975; LOEHRKE et al., 1983; BROWN et al., 1986). A large variety of chemicals have tumour promoting activities. Phorbol esters are the strongest among these substances (SLAGA, 1983), whereas others have moderate (tobacco smoke condensate and benzoylperoxide) or weak tumour promoting activities (some long chain hydrocarbons). Several agents including asbestos, cigarette smoke, steroid hormones alcohol and dietary fat, have been identified as tumour promoters in human carcinogenesis based on epidemiological studies or animal model systems (PITOT, 1983; KODAMA and KODAMA, 1987; COHEN and ELLWEIN, 1990; WOODWARD and McMICHAEL, 1991).

Induced cell proliferation is a critical aspect of tumour promotion. Appropriate tumour promoters have mitogenic activity, inducing a hyperplastic response in epithelial tissues, such as skin, by increasing the mitotic activity in the basal cell layers and the number of suprabasal cell layers (RAICK et al., 1972). The induction of hyperplasia does not require a preceding initiation event, but appears to reflect the response of the normal cell population to tumour promotion. Similarly, initiated or preneoplastic cells are assumed to respond to tumour promotion by increasing their rate of cell proliferation (DRINKWATER, 1990 and references therein).

Hyperplasia is necessary, but on its own is not sufficient as a mechanistic explanation for tumour promotion. Other events such as clonal selection of initiated cells during tumour promotion have also been suggested to be important (YUSPA et al., 1982; PARKINSON, 1985). The selection

hypothesis has been based on the reported partial or complete resistance to TPA-induced terminal differentiation of initiated cells *in vitro* (YUSPA and MORGAN, 1981, YUSPA et al., 1983 and 1985). On the other hand TPA treatment induces terminal differentiation (and accelerates the rate of differentiation in committed cells) in the majority of epidermal basal cells *in vivo* (REINERS and SLAGA, 1983) and in normal keratinocytes *in vitro* (YUSPA et al., 1982; REINERS and SLAGA, 1983). The considerable loss of basal cells through accelerated differentiation of normal basal cells induced by the first TPA treatment might indirectly increase proliferation and lead to clonal expansion of initiated cells into the space left by suprabasally migrating normal keratinocytes (REINERS and SLAGA, 1983). Thus, enhanced proliferation and aberrant differentiation could operate synergistically during tumour promotion.

The target cells for initiation are unknown. The fact that initiation is irreversible suggests either epidermal stem cells or cells that have irreversibly dedifferentiated into stem cells as obvious target cell candidates. Furthermore, target cells for initiation have to be contained within a keratinocyte subpopulation which is resistant or at least less sensitive to the induction of terminal differentiation by TPA (YUSPA et al., 1981, 1982 and 1983b; REINERS and SLAGA, 1983; PARKINSON et al., 1983 and 1984). MORRIS et al. (1985) suggested that so called "label-retaining cells" (LRCs), located within a nuclear diameter of the central cell position of each of the numerous epidermal proliferative units (EPUs) of the mouse dorsal epidermis (for review see POTTEN, 1983) represents the epidermal stem cells. Groups of follicular LRC cells have been localized exclusively to the bulge area of the hair follicle (COTSARELIS et al., 1990). These LRC cells are relatively undifferentiated and extremely similar to the putative stem cells of the palm and sole epithelia (LAVKER and SUN, 1982 and 1983). The normally slow cycling LRC cells in the hair follicle bulge can be induced to proliferate in response to hyperproliferative stimuli such as TPA treatment. COTSARELIS et al. (1990) hypothesize that these follicular LRC cells not only represent the hair follicular stem cells but are also the pluripotent stem cells that can give rise to hair follicles, the sebaceous gland and the epidermis. A small percentage of the slow cycling population of mouse epidermal basal cells (2%)

and of infundibulum and outer root sheath cells (4-5%) have been shown to retain carcinogen for a long period of time and have been proposed to represent the target cells for initiation (MORRIS et al., 1986). There was no evidence of carcinogen retention in the matrix cells of the hair follicle nor the presence of LRC cells within that region which was previously thought to harbour hair follicle stem cells. Work by WEINBERG et al. (1991) demonstrated that interfollicular epidermal cells and some cells of the hair follicle (probably outer root sheath cells) can be transformed/initiated by a v-Ha-*ras* gene *in vitro* and following skin grafting onto nude mice result in benign papillomas, indistinguishable from each other. Morphologically distinct, so called "Dark Staining cells" (DSC), which are present in large numbers in embryonic epidermis but decrease throughout adulthood, have also been suggested to be some sort of primitive stem cells and possible target cells for initiation (KLEIN-SZANTO et al., 1980). DSC keratinocytes are visible in TPA-treated skin and are abundant in papillomas and carcinomas.

1.3.5. Genetic Events During Promotion.

There is now significant evidence in support of the involvement of aneuploidy in the development of papillomas on mouse skin. It is not known, as yet, whether the progressive aneuploidy observed during progression of mouse skin carcinogenesis is induced by TPA and/or if it occurs as a consequence of the presence of the activated Ha-*ras* gene. Karyotyping of solid tumours established a correlation between increasing aneuploidy, papilloma age and degree of dysplasia (CONTI et al., 1986; ALDAZ et al., 1987). Non-random sequential trisomy of chromosome 6 and 7 have been observed during the development of DMBA/TPA-papillomas (ALDAZ et al., 1989). Analysis of loss of heterozygosity using F1 hybrids of appropriate inbred mouse strains demonstrated a high incidence of imbalance between parental alleles on mouse chromosome 7 in carcinomas carrying activated Ha-*ras* genes (BREMNER and BALMAIN, 1990). Thus, activation of the Ha-*ras* gene, besides being the initiation event, can also influence the molecular nature of additional genetic changes occurring later in carcinogenesis.

TPA had been shown to induce aneuploidy in yeast (PARRY et al., 1981), mouse epidermal cells (DZARLIEVA and FUSENIG, 1982;

DZARLIEVA-PETRUSEVSKA and FUSENIG, 1985; PETRUSEVSKA et al., 1988) and human lymphocytes (EMERIT and CERRUTI, 1982). TPA causes severe numerical and structural chromosomal aberrations *in vitro*, such as tri- and quadradial chromatid interchanges, ring chromosomes, induction of gaps and translocations. In contrast, neither the second-stage tumour promoter RPA, nor non-promoting phorbol esters, like 4-O-MeTPA or 4-alpha-PDD, caused any substantial chromosomal alterations. The convertogenic activity of TPA as a first stage promoter, seems to correlate with cytogenic effects seen in primary keratinocyte cultures derived from mouse skin treated *in vivo* with TPA (PETRUSEVSKA et al., 1988).

1.3.6. Tumour Progression and Metastasis.

Malignant conversion is a result of the stepwise acquisition of more aggressive growth characteristics and more malignant behaviour (FOULDS, 1957). Clonal expansion of more malignant subclones within neoplasms is a major force in tumour progression (NICOLSON and ROSENBERG, 1987). The increased genetic instability of tumour cells compared to normal cells (NICOLSON, 1987) may be an important factor contributing to clonal evolution in neoplastic cell populations (NORWELL, 1976; 1986; 1989). Molecular events responsible for various malignant properties associated with tumour progression are discussed below.

Malignant conversion is phenotypically associated with invasion, metastasis and the progressive loss of tissue organization. Changes in the expression pattern of cytokeratins, differentiation markers in epithelial cells, are useful indicators of alterations in the hyperplastic and differentiation state of malignant tumours of the mouse skin (NELSON and SLAGA, 1982 and references therein). Corresponding to their state of differentiation, benign papillomas express cytokeratins specific to basal cells (K5 and K14), the differentiated suprabasal layer (K1 and K10) and hyperproliferative skin (K6 and K16). Repression of the suprabasal keratins K1 and K10 and the induction of K13 have been observed during the progression of papillomas to carcinomas (ALDAZ et al., 1988; ROOP et al., 1988; LAURIJSEN et al., 1989). In squamous cell carcinomas, there is a strong correlation between increasingly dedifferentiated phenotype and a dramatic reduction in

expression of differentiation specific cytokeratin K1 and K10, whereas the other cytokeratins seem to be unaffected (TOFTGARD et al., 1985; ROOP et al., 1988). Progression of squamous cell to spindle cell carcinoma is associated with the aberrant expression of the simple typeII cytokeratins K8 and K18 (endoA and endoB in mouse, respectively). Especially the expression of K8 correlated strongly with the state of epithelial cell differentiation, the levels of mutant Ha-ras p21 protein and the tumorigenic capabilities of the carcinoma cells (DIAZ-GUERRA et al., 1992).

Metastasis is a multistep process requiring successful completion of a series of complicated tumour-host interactions (reviewed in KERBEL, 1990; FIDLER and HART, 1982; SCHIRRMACHER, 1985; HILL, 1987; HART et al., 1989). In addition to growth control, an imbalanced regulation of motility and proteolysis appears to be required for invasion and metastasis (LIOTTA et al., 1991). Reviews have been written discussing the role of altered expression of oncogenes (LIOTTA, 1988; MUSCHEL and LIOTTA, 1988; GREENBERG et al., 1989), of cell-surface glycoproteins and oligosaccarides (NICOLSON, 1988; RAZ and LOTAN, 1987; DENNIS and LAFERTE, 1987), of specific adhesion molecules (TAYLOR-SHER et al., 1988) or of autocrine motility factors (LIOTTA and SCHIFFMANN, 1988) in metastatic processes. The contribution of basement membranes, extracellular matrix, and various proteolytic enzymes (LIOTTA et al., 1982; THORGEIRSSON et al., 1985; LIOTTA, 1986), and of growth factors (HERLYN et. al., 1989) to metastasis has also been reviewed extensively.

For many years, circumstantial evidence has been accumulating, suggesting that cellular and secreted proteinases play a central role in processes involved with invasion and metastatic tendencies of tumours. It has been suggested that other behavioural traits of metastatic cancer cells, such as a strong growth preference over their non-metastatic counterparts within the primary tumour, are probably also necessary, though not sufficient by itself, for expression of metastatic ability (reviewed KERBEL, 1990). Proteinases have been implicated in processing of mediators of the transformed state, such as autocrine growth factors, growth factor receptors, and angiogenic factors (LAIHO and KESKI-OJA, 1989), in induction of angiogenesis (LIOTTA et al., 1991), in evasion of the host immune system, as has been shown in the

case of the proteinase cathepsin L (McCOY et al., 1988; KANE and GOTTESMANN, 1990; GOTTESMANN, 1990) and in the degradation of basement membrane to allow invasion and metasatsis at later stages of tumour progression (LIOTTA and STETLER-STEVENSON, 1990 and references therein).

1.3.7. Molecular Events Associated with Malignant Progression in Mouse Skin Carcinogenesis.

Amplification of oncogenes appear to be a major mechanism for altered gene function, particular in the later stages of human tumour development (ALITALO and SCHWARB, 1986). A 2 to 20 fold amplification of the mutant Ha-*ras* has been observed in some mouse skin carcinomas (QUINTANILLA et al., 1986; BREMNER and BALMAIN, 1990; BUCHMANN et al., 1991). The reduction in the normal:mutant Ha-*ras* gene dosage seems to be the most significant difference between squamous cell and spindle cell carcinomas. It is still unclear, however, if the loss of the normal Ha-*ras* allele, the increased absolute expression of the mutant allele, or a combination of both represents the crucial feature of this progression event (BUCHMANN et al., 1991; BALMAIN et al., 1990).

Mutant *ras* genes have been implicated in the induction of the metastatic phenotype. Thus, evidence is accumulating that the Ha-*ras* gene may be involved at both, early and late stages of tumour progression. Other oncogenes have been tested for co-operativity with *ras* in the malignant conversion of mouse skin tumours. So far, only the introduction of v-*fos* or c-*fos* into murine papilloma cell lines resulted in the malignant conversion of these lines to squamous cell carcinomas. *Fos* activation, however, may not be commonly involved in the malignant progression of mouse skin tumours, as the resultant carcinomas lacked γ-glutamyl-transpeptidase activity, which is present in 90% of all chemically induced mouse skin carcinomas (CHIBA et al., 1986) and there is so far no evidence for *fos* gene activation in chemically induced tumours *in vivo*.

Functional alterations or loss of tumour suppressor genes are a major feature of progression in many human malignancies and seem to be also involved in experimental animal carcinogenesis model systems. The

progressive reduction in normal:mutant ratio of Ha-*ras* gene during tumour progression, resulting in the complete loss of the normal Ha-*ras* allele in spindle cell carcinomas (BUCHMANN et al., 1991), could represent such a loss of suppressor gene function in mouse skin carcinogenesis. It has been suggested that the normal Ha-*ras* allele, or a closely linked loci, could be the proposed tumour suppressor gene on chromosome 7 whose function seems to be specifically directed against the function of a mutant Ha-*ras* gene. The presence of a tumour suppressor gene on chromosome 7 was proposed as a number of loci on mouse chromosome 7, including the Ha-*ras* locus, are syntenic with a group of genes on the short arm of human chromosome 11, which in turn contains at least two putative tumour suppressor genes (SEARLE et al., 1989; BREMNER and BALMAIN, 1990). Inactivation of the mutant Ha-ras specific tumour suppressor gene, by epigenetic as well as genetic mechanisms, could be a major event during tumour progression. The trisomy of chromosome 7 detected in many carcinomas would correspond to an actual under-representation of this chromosome in the near-tetrapoid background of the progressing tumours (BREMNER and BALMAIN, 1990: FUSENIG et al., 1985). Loss of heterozygosity (LOH) for the p53 tumour suppressor loci on mouse chromosome 11, syntenic to the human p53-containing chromosome 17, was detected in 7 out of 40 carcinomas analysed, with the remaining chromosome having inactivating mutations in the p53 gene. LOH on chromosome 11 was not observed in 30 papillomas. Furthermore, carcinomas without LOH on chromosome 11 had mutations in both alleles of the p53 gene (BURNS et al., 1991; P.A. BURNS, personal communication). These results strongly suggest that complete loss of normal p53 function is associated with the malignant phenotype in mouse skin carcinogenesis. However, loss of normal p53 function does not appear to be directly responsible for further progression to undifferentiated spindle cell carcinoma, as loss of normal p53 function is found in well-differentiated squamous cell carcinomas (BURNS et al., 1991). Under-representation of mouse chromosome 4 in carcinomas has also been reported (C.J. KEMP, personal communication). Mouse chromosome 4 corresponds to the human chromosome 9 containing a putative tumour suppressor gene for epithelial tumours in man (GAILANI et al., 1992).

1.3.8. Transin/Stromelysin Expression and Tumour Progression.

The family of extracellular matrix-degrading metalloproteinases consists of three sub-groups characterized by the substrate preference of the various proteinases:

i. Interstitial collagenases, specifically degrade type I, II and III collagen found in the dermis and other connective tissues (TEMPELTON et al., 1990).

ii. Type IV collagenases or gelatinases with substrate specificity for basement membrane collagen (type IV collagen and gelatins) (FESSLER et al., 1984). The type IV collagenase, or 72kDA gelatinase, is also able to degrade type V and VII collagen. A positive correlation between type IV collagenase activity and tumour cell invasion has been demonstrated both *in vivo* and *in vitro*. The evidence for type IV collagenase playing a role in tumour invasion and metastasis has been extensively reviewed (LIOTTA et al., 1991; LIOTTA and STETLER-STEVENSON, 1990 and references therein).

iii. Stromelysins (WILHELM et al., 1987; MATRISIAN, 1992). The subclass stromelysin consists of three related gene products, stromelysin, stromelysin-2, and matrilysin, also called PUMP-1 (small putative metalloproteinase) or MMP7 (CHIN et al., 1985; WHITHAM et al., 1986; MULLER et al., 1988; WOESSNER and TAPLIN, 1988). Stromelysin proteinases degrade a wide variety of extracellular matrix components including proteoglycans and non-collagenous glycoproteins (e.g. laminin, fibronectin and gelatin), the non-collagenous domains of type IV collagen, as well as type III and V collagens (reviewed in McDONNELL and MATRISIAN, 1990 and references therein).

Transin mRNA was originally characterized as a mRNA whose levels are greatly increased following oncogenic transformation of rat embryo fibroblast cell lines (MATRISIAN et al., 1985). Additional transin-like genes have since been isolated: transin-2 (BREATHNACH et al., 1987) and matrix metalloproteinase 7 (MMP 7) (WOESSNER and TAPIN, 1988) or transin-3, also called matrilysin (QUANTIN et al., 1989; MATRISIAN, 1992). Subsequent studies and primary sequence comparisons have since revealed,

that the transin gene is the rat homolog of the extracellular matrix-degrading metalloproteinase (MMP) gene stromelysin described by CHIN et al., (1985) (WHITHAM et al., 1986; MULLER et al., 1988). Transin-2 and transin-3 genes code for the rat homologues of stromelysin-2 gene and PUMP-1 gene (putative metalloproteinase-1) (MULLER et al., 1988), respectively. The transin-like metalloproteinases have been grouped into the stromelysin subclass of MMPs according to their broad substrate specificity (reviewed in McDONNELL and MATRISIAN, 1990). The nomenclature for members of the stromelysin subclass is not consistent, since many of these enzymes have been given different names by different groups (Table 1.1 reproduced from MATRISIAN, 1992; reviewed in McDONNELL and MATRISIAN, 1990; MATRISIAN and BOWDEN, 1990; MATRISIAN, 1992). The regulation of expression of the transin/stromelysin genes, as well as the structure and proteolytic activation of metalloproteinases have been reviewed extensively (LIOTTA and STETLER-STEVENSON, 1990; MATRISIAN and BOWDEN, 1990; McDONNELL and MATRISIAN, 1990; MATRISIAN, 1992).

1.3.9. Transin/Stromelysin Expression During Chemical Carcinogenesis in Mouse Skin.

Analysis of tumours generated *in vivo* by the classical protocol of chemically induced mouse skin carcinogenesis (SLAGA, 1983), a single treatment of normal mouse skin with the initiating carcinogen DMBA followed by repeated treatments with the tumour promoter TPA, demonstrated a correlation between transin/stromelysin gene expression and malignant state of the tumours analysed. Only 6% of benign papillomas, arising after 15 weeks TPA treatment, had low levels of transin/stromelysin mRNA detectable (MATRISIAN and BOWDEN, 1990). However, squamous cell carcinomas (SCC), developing at around week 25 to 30 in 5-7% of DMBA/TPA treated animals, had an elevated incidence (73%) of high transin/stromelysin expression (MATRISIAN et al., 1986; MATRISIAN and BOWDEN, 1990). Similar observations were made in analysis of benign papillomas and SCC, induced by N-methyl-N-nitroso-N-nitroguanidine (MNNG)-initiation/TPA-promotion protocol. Transin/stromelysin expression was detected with much higher incidence in SCCs (80%) than in papillomas (25%) (MATRISIAN et

The stromelysin family of matrix degrading metolloproteinases (MMPs).

Group	Name	Members	Source	Substrate
1	Stromelysin	Stromelysin	Rabbit synovial fibroblasts	Proleoglycans
				Laminin
		Transin	Transformed rat fibroblasts	Fibronecton
		Proteoglycanose	Rabbit bone	III, IV, V, collagen
		Collagenase activator	Rabbit synovial fibroblasts	Gelatins
		MMP-3	Human rheumatoid synovial cells	
2	Stromelysin-2	Stromelysin-2	Human tumour cells	III, IV, V collagen
		Transin-2	Transformed rat fibroblasts	Fibronecton
		MMP-10		Gelatins
3	Matrilysin	Matrilysin (or transin-3)		Gelatins
		Pump-1	Human tumour cells	Fibronectons
		Small metallo proteinase of the uterus	Rat uterus	
		MMP-7		

Table 1.1.

The stromelysin family of matrix-degrading metalloproteinases (MMPs).

The stromelysin family of MMPs is subdivided into three groups: group 1 is represented by stromelysin, group 2 by stromelysin-2 and group 3 by matrilysin. The names given to purified enzymes or cloned cDNAs, their source, substrate specificity and classification within the family are indicated in this table. References are given in the text.

al., 1990). The correlation between transin/stromelysin expression and malignancy was even more striking in SCCs developing after repeated treatments with MNNG; 100% of these malignant tumours (5/5 SCC) showed very high levels of transin/stromelysin mRNA (OSTROWSKI et al., 1988; MATRISIAN and BOWDEN, 1990). These results correlated well with the observation that repeated MNNG treatment of mouse skin produces malignant tumours with a high probability of invading and metastasizing (PATSKAN et al., 1987). In none of the chemically induced SCCs, expressing transin/stromelysin at high levels, was any evidence found of amplification or rearrangement of the transin/stromelysin gene (MATRISIAN and BOWDEN, 1990). The induction of transin/stromelysin gene expression occurred solely at the transcriptional level. A very high percentage of mouse skin tumours initiated by ionizing radiation (JAFFE and BOWDEN., 1987), express transin/stromelysin (MATRISIAN and BOWDEN, 1990). The high percentage of transin/stromelysin expression found in benign tumours (near 100%) might be an indicator, that these tumours could be premalignant, as evidence suggested a high rate of conversion of radiation initiated papillomas to SCCs (JAFFE and BOWDEN. 1987). The fact that radiation initiated basal cell carcinomas (BCC) did not express transin/stromelysin, was consistent with the observation, that BCCs show little evidence of invasive behaviour (MATRISIAN and BOWDEN, 1990).

OSTROWSKI et al. (1988) found an apparent decrease in the level of transin/stromelysin transcripts in metastatic lesions (1 lung metastasis and 2 lymph node metastases) when compared to the transcript levels found in the primary tumour, induced by repeated Benzo[a]pyrene (B[a]P) or MNNG treatment over a period of over 40 weeks, from the same animal. In one case (lung metastasis), no transin/stromelysin transcripts were detectable. They speculated that the level of transin/stromelysin expression could be downregulated in metastatic tumour cells, as its proteolytic activity might not be required for the process of establishing and maintenance of the metastatic colony at its new location (OSTROWSKI et al., 1988). Transin/stromelysin levels also correlated with the metastatic potential of a series of oncogene transformed rat embryo cells *in vitro* (POZZATTI et al., 1986). After transfection of the T24/EJ c-Ha-*ras* oncogene (PARADA et al., 1982), normal

rat embryo fibroblasts, as early as passage 3 to 4, exhibited great propensity to form metastatic lesions after intravenous injection into nude mice. Rat embryo fibroblasts transfected with cloned c-Ha-*ras* and adenovirus 2 E1a gene, however, gave rise to tumours, but no, or very few metastatic, lung nodules (POZZATTI et al., 1986). Unpublished results by POZZATTI (MATRISIAN and BOWDEN 1990), showed that 5/5 c-Ha-*ras* transformed cell lines had elevated levels of transin/stromelysin mRNA, whereas only 1/5 c-Ha-*ras*/E1a transformed cell lines expressed transin/stromelysin mRNA. These results were explained when VAN DAM et al. (1990) and OFFRINGA et al. (1990) showed that E1A protein suppresses the transcription of the collagenase gene by abolishing the transactivating function of AP-1 (Jun/Fos) which binds to the TRE element of the collagenase gene (AUBLE et al., 1991).

In summary, the induction of transin/stromelysin expression seemed to be a relatively late event in the process of tumour progression as demonstrated for ionizing radiation and chemically induced mouse skin carcinogenesis *in vivo* (MATRISIAN et al.,1986; MATRISIAN and BOWDEN, 1990) and for v-*ras*/v-*fos*-expressing keratinocytes upon grafting onto the backs of nude mice (GREENHALGH and YUSPA, 1989). There was a strong correlation between its expression and the invasive and metastatic potential of chemically induced mouse skin tumours, papilloma cell lines *in vitro* and human T24/EJ c-Ha-*ras* oncogene transformed, tumorigenic rat embryo fibroblasts (POZZATTI et al., 1986; KRIEG et al., 1988; GREENHALGH and YUSPA, 1989; MATRISIAN and BOWDEN, 1990). The observation that tumours with the greatest probability of becoming invasive and metastatic have the highest levels of transin/stromelysin (OSTROWSKI et. al., 1988; MATRISIAN and BOWDEN, 1990) leads to the speculation that the metalloproteinase transin/stromelysin might play a causal role in promoting invasion through the basement membrane.

1.4. The *Ras* Gene Family.

1.4.1. Structure and Function of the *ras* Proto-Oncogenes.

Three functional *ras* genes have been isolated from several mammalian species: Ha-*ras* 1, Ki-*ras* 2 and N-*ras*, alongside two

pseudogenes identified in rats and humans: Ha-*ras* 2 and Ki-*ras* 1 (BARBACID, 1987). The genes encode closely related proteins of 21 kDa, referred to as p21ras. By alternative splicing of its two fourth exon (IVA and IVB), the Ki-*ras* 2 gene can generate two forms of p21 (Ki-*ras* 2A and Ki-*ras* 2B), proteins of 188 amino acids and 189 amino acids, respectively, that differ in their C-terminal residues (McGRATH et al., 1983, CAPON et al., 1983). Whereas Ha-*ras* and Ki-*ras* have known retroviral homologues in the transforming genes of Harvey- and Kirsten murine sarcoma viruses (Ha-MSV and Ki-MSV), no such homologue has been identified for the N-*ras* gene (HALL et al., 1983; SHIMIZU et al., 1983). The p21ras proteins encoded by the various members of the *ras* gene family, have been shown to bind magnesium complexes of guanine nucleotides (GDP or GTP) (SCOLNICK et al., 1979; SHIH et al., 1980, TAMANOI et al., 1984; TEMELES et al., 1985; WITTINGHOFER and PAI, 1991), possess GTPase activity (GIBBS et al., 1984; McGRATH et al., 1984; SWEET et al., 1984; MANNE et al., 1985; TEMELES et al., 1985), and are associated with the plasma membrane (WILLINGHAM et al., 1980; WILLUMSEN et al., 1984). p21ras proteins show a significant sequence homology with G proteins, a group of signal-transducing proteins with intrinsic GTPase activity and an intracellular localisation on the inner surface of the plasma membrane similar to p21ras proteins (HURLEY et al., 1984; TANABE et al., 1985; LOCHRIE et al., 1985; ITOH et al., 1986). There is also a high degree of similarity, especially for the nucleotide binding site, between p21ras proteins and other GTP-binding proteins involved in polypeptide chain elongation, such as the bacterial EF-Tu factor (JURNAK, 1985; McCORMICK et al., 1985). The determination of the crystal structures of EF-Tu in its GDP-bound form (JURNAK et al., 1985; LA COUR et al., 1985) and the three dimensional structure of c-Ha-*ras* in both, its GDP-bound and GTP-bound forms (DE VOSS et al., 1988; PAI et al, 1989) confirmed the similarity at the nucleotide binding site of the two proteins. The three dimensional structure of p21ras proteins has been described in a number of reviews (WITTINGHOFER and PAI, 1991; SCHLICHTING et al., 1990; McCORMICK, 1989; BARBACID, 1987). These sequence and structure

similarities, especially to G proteins, and the properties of p21ras proteins suggest that they may function as molecular switches in signaling events of cell growth and differentiation. When bound to GTP p21ras proteins are in their active state ("on" switch); their GDP complexes comprise the inactive form ("off" switch) (BOURNE et al., 1991 and 1990; CHARDIN, 1991; KAZIRO et al., 1991).

The promoter regions of the cellular *ras* genes lack characteristic TATA boxes, but are highly GC rich (BARBACID, 1987), contain multiple potential GC-box SP1 protein binding sites (GGGCGG or its complement CCGCC) (YAMAMOTO and PERUCHO, 1988; ISHII et al., 1986; BROWN et al., 1988; NEADES et al., 1991; PLUMB et al., 1991) and a putative CCAAT box (BROWN et al., 1988) with two further potential CTF-1 binding sites (NEADES et al., 1991; JONES et al., 1987). The untranslated 5'-non-coding exon (E-1), located immediately downstream from the promoter region, contains multiple transcription start sites (BROWN et al., 1988; PLUMB et al., 1991). In the Ha-*ras* gene, the exact position and regulation of these multiple start sites are still disputed.

The amino acid sequence of the p21ras proteins are very similar and can be divided into four domains. The first 85 amino acids at the amino-terminus are identical in each member of the family, and the adjacent 80 amino acids show only a slight divergence with 85% identity. The third region between amino acids 165 and 185 is highly heterologous, but the sequence homology is restored in the last four amino acids. This fourth domain of four amino acids comprises a characteristic motif of Cys(186)-A-A-X-COOH ('A' representing any aliphatic amino acid; 'X' representing any amino acid) in all mammalian *ras* genes (BARBACID, 1987). The CAAX motif has an important signalling function in post-translational modification(s) of the p21ras proteins, necessary for their maturation into the membrane-bound forms (DER and COX, 1991). Three closely coupled post-translational modifications are required for efficient membrane binding of p21ras (HANCOCK et al., 1991): First, the addition of a prenoid derivate or a farnesly group to the cysteine of the CAAX sequence (HANCOCK et al., 1989; CASEY et al., 1989); second,

the removal of the AAX amino acids by proteolysis (GUTIERREZ et al., 1989) and third, the methyl-esterification of the now C-terminal cysteine residue (CLARKE et al., 1988; GUTIERREZ et al., 1989). The CAAX motif of p21 proteins must combine with a second "membrane association" signal, either a cysteine palmitoylation site (Ha-*ras*, N-*ras* and Ki-*ras* 4A) or a polybasic domain comprising six consecutive lysine residues (Ki-*ras*4B), contained within the hypervariable domain of the protein to target plasma membrane localization (HANCOCK et al., 1989; 1990 and 1991b). Post-translational modification of the p21ras proteins are essential for membrane localization and oncogenic activity (HANCOCK et al., 1989; WILLUMSEN et al., 1984). Recently it has been shown that irreversible myristylation of normal p21 ras activates the transforming properties of this protein (BUSS et al., 1989). Post-translational processing is also required for the interaction of the p21ras proteins with GDP dissociation stimulators (GDS) (MIZUNO et al., 1991).

Mammalian ras proteins have been implicated in a variety of biological events: p21ras proteins are capable of inducing proliferation and transformation in certain types of mammalian cells (fibroblasts, epithelial cells) (STACEY and KUNG, 1984; MULCAHY et al., 1985; YUSPA et al., 1985) and of inducing differentiation or growth arrest in others (rat pheochromocytoma (PC) 12 cells, Schwann cells) (NODA et al., 1985; BAR-SAGI and FERUMISCO, 1985). In contrast to normal p21ras, oncogenic p21ras proteins can induce terminal differentiation of PC12 cells. Microinjection of PC12 cells with antibodies against p21ras proteins inhibits neurite formation induced by NGF but not by cAMP (HAGAG et al., 1986). There is also evidence that in some cell types p21ras may promote transformation by inhibiting differentiation. The introduction of *ras* oncogenes into skeletal myoblasts (OLSEN et al., 1987) or mouse keratinocytes (YUSPA et al., 1983 and 1985) blocks the normal differentiation programme of these cell types (OLSEN et al., 1987). The high expression levels of p21ras in brain tissue (FURTH et al., 1987; CHESA et al., 1987) further supports a role for this protein in neural differentiation and offers an explanation for the lack of association

between *ras* gene activation and tumours of neuroectodermal origin (BOS, 1989). Detection of high levels of *ras* expression in both proliferating and terminally differentiated cell types (epithelial cells of the endocrine gland; neurons of the central nervous system) support the concept that *ras* genes can interact with diverse intracellular pathways involved in basic cellular proliferation and in certain specific functions of terminally differentiated cells.

1.4.2. Downstream Cellular Signalling Pathways Regulated by p21ras Proteins.

The elucidation of cellular signalling pathway(s) affected by normal or oncogenic activated p21ras proteins have made progress in recent years. One of the candidate intracellular pathways which mammalian p21ras proteins may influence is the phosphoinositide system, a system which regulates several processes including metabolism, secretion, neuronal activity and cell proliferation (BERRIDGE and IRVING, 1989). Evidence that p21ras may be the G protein involved in transducing a variety of signals from the cell surface to the enzyme phospholipase C (PLC) was inferred from the observation that p21ras-transformed cells contain elevated levels of inositol 1,4,5, triphosphate (IP3) (FLEISCHMAN et al., 1986; HANCOCK et al., 1988). The second messengers IP3 and diacylglycerol (DAG) are normally released following the PLC-catalysed hydrolysis of phosphatidyl inosito 4,5-biphosphate (PIP2). IP3 induces an increase in the level of intracellular Ca^{2+} (BERRIDGE and IRVINE, 1989), while DAG, in conjunction with Ca^{2+}, activates Protein kinase C (PKC) (NISHIZUKA, 1986 and 1988). It has been suggested that different p21ras proteins couple different receptors to PLC: the bombesin receptor via p21N-ras (WAKELAM et al., 1986) and the PDGF receptor via p21Ha-ras (MARSHALL, 1987). However, mutant p21ras has been observed to induce an increase in DAG in the absence of, or well above, any increase in inositol phosphates (LACAL et al., 1987a and 1987b; WOLFMANN and MACARA, 1987; SEUWEN et al., 1988; MORRIS et al., 1989). It has therefore been suggested that p21ras may mediate the breakdown of other phospholipids,

such as phosphatidylcholine (PC), phosphatidyl ethanolamine (LACAL et al., 1987a), or the substrates of phospholipase A2 (BAR-SAGI and FERAMISCO, 1986). However, downregulation of PKC by prior treatment with phorbol esters blocks ras-induced PC hydrolysis and elevation of DAG (PRICE et al., 1989), implying that DAG increase is downstream of PKC and that p21ras activates PKC by a novel mechanism.

More recently, activation of mitogen activated protein (MAP) kinases has been identified as an early consequence to p21ras deregulation following scrape loading quiescent cells with oncogenic p21ras (LEEVERS and MARSHALL, 1992). MAP kinases are constitutively activated in p21ras-transformed cell lines, suggesting that continued stimulation of these kinases is required for the transformed phenotype of the cells (LEEVERS and MARSHALL, 1992). Although initially described as the direct target for receptor linked tyrosine kinases (receptor for insulin, epidermal growth factor (EGF) and fibroblast growth factor (FGF)), the activation of the protein-serine MAP kinase is catalysed by a MAP kinase kinase which in turn is dependent on serine/threonine phosphorylation for its activity. There is evidence that the serine kinase product of the proto-oncogene c-*raf* is involved in activation of the MAP kinase kinase by directly phosphorylating the MAP kinase kinase (BRUDER et al., 1992; KYRIAKIS et al., 1992). Dominant negative Raf mutants inhibit p21ras-mediated signalling (BRUDER et al., 1992) and dominant negative p21ras mutants inhibit activation of c-*raf-1* by mitogens (KYRIAKIS et al., 1992). It has been shown *in vitro* that MAP kinases specifically modify by phosphorylation two serine residues within the transactivation domain of c-Jun, a component of the AP-1 transcription factor complex (PULVERER et al., 1991). Activation of c-Jun/AP-1 protein complex through phosphorylation seems to be necessary for deregulation of cell growth by p21ras proteins. However, the immediate downstream target for p21ras proteins is/are still elusive.

1.4.3. Oncogenic Activation of *ras* Genes.

The structural alterations responsible for the activation of *ras* genes are well documented (BARBACID, 1987; McCORMICK, 1989). The activating mutation of the c-Ha-*ras* gene of the T24/EJ bladder carcinoma cell line was the first to be determined. A single nucleotide substitution (G to T) at codon 12 results in an altered p21ras gene product containing a Val rather than a Gly residue at position 12 (TABIN et al., 1982; REDDY et al., 1982; TAPAROWSKY et al., 1982). Since then, codon 12, 13, and 61 mutations have been identified in activated *ras* oncogenes detected in naturally occurring tumours and in chemically induced tumours (GUERRERO and PELLICER, 1987, BARBACID, 1987; BALMAIN and BROWN, 1988; BOS, 1989; McCORMICK, 1989; SUKUMAR, 1989 and 1990; LEMOINE, 1990). In addition, an activating codon 59 mutation has been detected in viral Kirsten and Harvey *ras* genes (DHAR et al., 1982; TSUCHIDA et al., 1982), but never in an activated cellular *ras* gene. *In vitro* mutagenesis studies extended the list of activating mutations to include codons 63 (FASANO et al., 1984), 116 (WALTER et al., 1986), 117 (DER et al., 1988), 119 (SIJAL et al., 1986b) and 146 (SLOAN et al., 1990; ORITA et al., 1991). Except for codon 146 mutations identified in a range of naturally occurring human tumours including colon cancer, lung cancer and lymphoid malignancies (ORITA et al., 1991), these mutations have never been described in human tumours.

In general, *ras* gene activating mutations fall into two functional groups, those affecting codons 12, 13, 59, 61 and 63 residues have reduced intrinsic GTPase activity (GIBBS et al., 1984; McGRATH et al., 1984; SWEET et al., 1984; MANNE et al., 1985; TEMELESS et al., 1985) and are unable to respond to the stimulating effect of GTPase activating proteins (GAPs) (ADARI et al., 1988; CALES et al., 1988) while those at codons 116, 117, 119 and 146 have an increased GDP/GTP exchange rate (SIGAL et al., 1986a; WALTER et al., 1986).

Ras genes can also gain transforming properties by quantitative mechanisms. Overexpression of *ras*-proto-oncogenes as a result of linkage of normal *ras* genes to powerful enhancer elements such as

retroviral LTRs (CHANG et al., 1982) or obtained after integration of multiple copies of a normal human Ha-*ras*1 DNA clone (PULCIANI et al., 1985) result in the malignant transformation of NIH 3T3 cells. Amplification of *ras* has also been seen in a variety of human tumours, although the overall incidence of *ras* gene amplification in human neoplasia is estimated to be around 1% (PULCIANI et al., 1985; for reviews: BARBACID, 1987; BOS, 1989, 1990).

1.4.4. Ras Gene Activation in Human Tumorigenesis.

Oncogenic activation of members of the *ras* gene family plays an important role not only in mouse skin carcinogenesis and other animal model systems but is also implicated in human tumorigenesis, although the incidence of *ras* gene activation differs greatly in human tumours (LEMOINE, 1990; BOS, 1989). The highest incidences are found in adenocarcinomas of the exocrine pancreas, colorectal carcinomas, benign and malignant tumours of the thyroid gland and Acute Myeloid Leukemia (AML). Although lung carcinomas have a relatively low incidence of activating *ras* gene mutations, one histological type of lung carcinoma, adenocarcinomas have a high incidence of Ki-*ras* gene activation, there is also evidence that the mutational event could be a direct result of the presence and action of carcinogenic ingredient(s) of tobacco smoke (RODENHUIS et al., 1988). However, in several other types of tumours the incidence of *ras* gene activation is very low (less than 5%), these include carcinomas of the breast, cervix, ovaries and esophagus as well as glioblastoma, neuroblastoma, large cell lung cancer and chronic lymphocytic leukemia (LEMOINE, 1990; BOS, 1989; McCORMICK, 1989). The reason for the high incidence of *ras* mutations in certain tumours and their absence in others may relate to the tissue distribution of carcinogens and/or the sensitivity of individual tissues to *ras*-induced transformation. Studies using transgenic mice have demonstrated that the pancreas is particularly sensitive to *ras*-induced neoplasia whereas tumours arising in mammary (SINN et al., 1987) or lung tissue (SUDA et al., 1987) occur only after a long latency period.

A specificity regarding the activation of a particular member of the *ras* family is observed in certain tumours: the strong association between Ki-*ras* mutation and tumours of the colon, lung and pancreas (BOS, 1989; LEMOINE, 1990); highly significant incidence of activated N-*ras* genes in myeloid neoplasia and melanomas (BOS, 1989; LEMOINE, 1990; VAN'T VEER et al., 1989; ALBINO et al., 1989); Ha-*ras* gene mutations in rodent skin and mammary tumours and predominantly in mouse liver tumours (BALMAIN and BROWN, 1988; BALMAIN, 1990). This specificity is still not completely understood. The suggestion that *ras* genes have separate function may explain the specificity of activation of certain *ras* gene family members in certain tumours. However, the putative effector domains are identical in all three p21ras proteins (SIGAL et al., 1986a; WITTINGHOFER and PAI, 1991 and references therein) which speaks against the "separate function" theory and suggests the induction of the same effect in all cells upon activation of any one of the *ras* genes. However, the direct downstream effector for any of the *ras* genes has not yet been identified. On the other hand, the tissue specificity could reflect differential expression of the *ras* genes in different tissues. However, expression of all three *ras* genes is detected in most tissues, although at dissimilar levels (MULLER et al., 1983; LEON et al., 1987). Thymus and skin have high expression of N- and Ha-*ras*, respectively, consistent with the preferential activation of these genes in tumours derived from these tissues (GUERRERO and PELLICER, 1987; BALMAIN and BROWN, 1988).

1.5. Analysis of Carcinogenesis in the Mouse Skin Model.

1.5.1. Inhibition of Cell Transformation by Antisense *ras* RNA.

The Use of Artificial Antisense RNA in Regulation of Eukaryotic Gene Expression.

The identification of antisense RNA as a fine tuner of complex regulatory processes in prokaryotic systems (GREEN et al., 1986b; INOUYE 1988; TAKAYAMA and INOUYE, 1990; EGUCHI et al., 1991) lead to the

idea of developing methods where artificial antisense RNA genes could be used as specific inhibitors of gene expression in eukaryotes. The use of antisense RNA would make it possible to determine the function of genes by examining the consequences of the lack of expression of these genes and thus simulating dominant-negative "mutant" phenotypes.

Artificial antisense RNA genes are constructed by cloning the coding sequences of a gene in opposite orientation relative to the promoter and polyadenylation signal provided by the expression vector. The antisense RNA can be transcribed *in vitro* and microinjected into the cytoplasm of cells (ROSENBERG et al., 1985; CABERERA et al., 1987). Alternatively, the plasmid carrying the antisense gene can be transfected into cells and the antisense RNA is transcribed in the cell nucleus (IZANT and WEINTRAUB, 1985; TREVOR et al., 1987).

Experiments by IZANT and WEINTRAUB (1984) showed for the first time that the level of transient expression of an exogenous Herpes Simplex thymidine kinase (*tk*) gene could be significantly lowered when a mixture of plasmids containing cloned sense and antisense HSV-*tk* genes at a ratio of 1:100 (sense:antisense plasmids) was microinjected into mouse LTK$^-$ cells. They could also show, that the inhibition of antisense RNA is sequence specific, as antisense HSV-*tk* RNA could not repress the expression of the chicken tk gene and vice versa (IZANT and WEINTRAUB, 1985). An antisense HSV-*tk* RNA complementary to a sequence of 52 nucleotides from the 5' untranslated region of the target RNA showed the most effective inhibition; more effective than a long antisense RNA complementary to most of the *tk* coding region including the initiation codon (IZANT and WEINTRAUB, 1985). Transfection of LTK$^-$ cells with plasmids containing sense or antisense *tk* genes (at a ratio of 1:100) gave the same results as the microinjection experiments. Expressing the antisense HSV-*tk* gene from a glucocorticoid-inducible LTR from mouse mammary tumour virus (MMTV-LTR) showed a dose-dependent decrease in TK enzyme activity of up to 90% (IZANT and WEINTRAUB, 1985). KIM and WOLD (1985) achieved 80% to 90% inhibition of expression of the *tk* gene when expressing antisense *tk* RNA as part of a chimeric dihydrofolate reductase (DHFR) transcript. The increased levels of intracellular antisense *tk* RNA was a result of over-production of

DHFR message caused by selection of cells resistant to progressively higher levels of methotrexate. Transient inhibition of SV40 large T-antigen gene expression by an antisense RNA transcribed by RNA polymerase III was demonstrated in COS1 cells by JENNINGS and MOLLOY (1987). A 5 to 20 fold reduction in chloramphenicol acetyltransferase (CAT) activity was achieved by transient antisense inhibition of the bacterial CAT gene introduced into LTK^- cells (IZANT and WEINTRAUB, 1985).

Antisense RNAs were also used successfully to interfere with expression of a series of exogenous and endogenous genes including mouse *hprt* (STOUT et al., 1987), β-actin (IZANT and WEINTRAUB, 1985), globin (MELTON, 1985), *tk* gene (HARLAND and WEINTRAUB, 1985), and the murine Tissue Inhibitor of Metalloproteinases (TIMP) gene (KHOKHA et al., 1989).

Very elegant work has been done in *Dictyostelium discoidieum*, a eukaryotic cellular slime mold. Transfection of an antisense construct of the discoidin gene into *Dictyostelium* results in the repressed expression of three endogenous discoidin genes and leads to a >90% reduction in accumulated discoidin mRNA and protein. More importantly, however, the antisense transformants show a non-streaming phenotype similar to that of naturally occurring discoidin-minus mutants. Based on the detection of only low levels of endogenous discoidin mRNA by Northern analysis and the absence of detectable steady-state levels of antisense transcripts, sense and antisense RNA hybrids are believed to form in the nucleus and to be rapidly degraded (CROWLEY et al., 1985).

Transcription of a transfected antisense myosin heavy chain A (mhcA) gene leads to reduced accumulation of MHC A protein and subsequently to developmental abnormalities in *Dictyostelium* (slow growing, abnormally large and multinucleate phenotype). Phenotypic reversion can be achieved by increasing the amount of endogenous *mhc A* mRNA relative to the expression of the antisense RNA, as with accumulation of MHC A protein, *Dictyostelium* remains mononucleate and proceeds through development normally (KNECHT and LOOMIS, 1987).

Drosophila proved to be another valuable system where genes regulating development could be studied using antisense RNA. Wild-type

Drosophila injected at the syncytial blastoderm state with *in vitro* transcribed antisense *Kruppel* (*Kr*) RNA, complementary to a 2.3kb *Kr* cDNA fragment, developed into phenocopies of *Kr* mutants. A high frequency of lethal Kr phenocopies was observed. Phenocopy production is dose dependent and reflects the naturally found spectrum of *Kr* mutants. However, extreme Kr phenocopies indicative of complete $Kr^{(+)}$ inhibition are not produced. The ratio of 1000:1 (antisense to sense RNA) yielded about 50% of phenocopies, even the weakest response (4% phenocopies) required a greater than 50 fold excess of antisense RNA over endogenous *Kr* message (ROSENBERG et al., 1985).

Minimal to global *wingless* embryo phenocopy mutants are caused by injection into wild-type eggs of an antisense RNA complementary to a 3.0kb fragment of the w*ingless* cDNA of *Drosophila.* The *wingless* gene seems to be required for cooperation within discrete groups of cells during development. The wingless gene is the *Drosophila* homolog of the mouse *int-1* proto-oncogene (CABRERA et al., 1987).

The first successful whole-animal mouse model of antisense inhibition was demonstrated by KATSUKI et al. (1988) who generated transgenic mice carrying a 1.2 kb antisense myelin basic protein (MBP) cDNA sequence under its homologous promoter. Although the transgenic founder mice appeared phenotypically normal, 10 out of 21 transgenic offsprings from a cross between one of the founder males with a wild-type female mouse, converted to a mutant "shiverer" phenotype. The shiverer phenotype correlated with an observed reduction in MBP mRNA production, amount of MBP protein synthesized and degree of myelination. The mutant "shiverer" mouse harbours an autosomal recessive mutation in the MBP gene that results in its MBP deficiency and hypomyelination in the central nervous system (KATSUKI et al., 1988 and references therein). The production of an antisense RNA, complementary to exons 3-7 of the MBP gene, is presumed to be responsible for the reduced production of MBP protein in the mouse mutant "myelin-deficient" (*mld. shimid*). It was found that the *mld* mouse mutant had the MBP gene duplicated tandemly and that a large portion of the duplication is inverted upstream of the intact copy. The antisense RNA detected in *mld* mice corresponds to the inverted MBP segment (MIKOSHIBA et al., 1991).

A more detailed review of artificial antisense genes in eukaryotic systems has been written by TAKAYAMA and INOUYE (1990).

Application of Antisense RNA Inhibition in Studying Proto-Oncogene Function.

Antisense RNA targeted inhibition of gene expression has been used in the study of proto-oncogene function, as an alternative method to the limited use of specific antibodies directed against proto-oncogenes.

The protein kinase $pp60^{c\text{-}src}$ is thought to play an important function in polyoma virus (Py)-mediated transformation by interacting with the Py-encoded middle tumour antigen (MTAg). A 80-90% decrease in $pp60^{c\text{-}src}$ synthesis and protein kinase activity was observed in FR3T3 rat fibroblasts following the expression of antisense c-*src* RNA transcribed from a transfected plasmid containing the entire coding region of c-*src* in antisense orientation under the transcriptional control of the heavy-metal inducible mouse metallothioneine I promoter (AMINI et al., 1986). The expression of antisense c-*src* RNA did not result in phenotype revision of Py-transformed FR3T3 cells. However, an observed reduction in anchorage-independent growth, focus-formation and rate of tumour growth upon injection of antisense c-*src* RNA-expressing Py-transformed FR3T3 cells into syngenic rats was indicative of a less transformed cell type (AMINI et al., 1986).

In the human promyelocytic leukemia cell line HL60, antisense RNA expression from a stably integrated human anti-*myc* gene resulted in a 70% reduction of the steady-state levels of Myc protein. The antisense RNA hybridized with its complementary target transcripts in the nucleus and inhibited *myc* expression both, at the translational and transcriptional level. An enhancer-like 920bp fragment of the *myc* leader sequence is thought to be the primary transcriptional target region of the antisense RNA. Proliferation of HL60 cells decreased and an increased commitment of HL60 cells to monocytic differentiation as opposed to granulocytic differentiation was observed as a result of antisense RNA-mediated suppression of endogenous *myc* gene expression (YOKOYAMA and IMAMOTO, 1987).

A *raf*-expression dependent modulation in tumorigenicity and radiation-resistant phenotype of the human laryngeal squamous carcinoma

cells SQ-20B was demonstrated by KASID et al. (1989), implying an indirect role of *raf* in radiation response of tumour cells. Expression of full-length c-*raf*-1 antisense RNA following the transfection of SQ-20B cells with antisense *raf* sequences fused to the adenovirus 2 major late promoter, resulted in a greater than 10 fold reduction of the steady-state level of the endogenous c-*raf*-1 transcript. Anti-*raf* transfected cells showed a decreased tumour growth rate (compared to parental control cells) when inoculated into nude mice and appeared to have enhanced radiation sensitivity. SQ-20B cells expressing transfected sense-*raf* sequences had a significantly increased malignant potential and maintained a radiation-resistant phenotype compared to antisense *raf* RNA expressing SQ-20B cells. Results by KOLCH et al. (1991) demonstrated a requirement for c-*raf*-1 expression for proliferation of NIH 3T3 cells, normal and transformed by v-*raf*, v-Ki-*ras* and v-Ha-*ras*, as well as serum- or TPA-induced proliferation and DNA replication. Expression of antisense *raf* RNAs complementary to various portions of c-*raf*-1 cDNAs from the pMNC retroviral vector after transfection into v-*raf*-transformed NIH3T3 cells resulted in the partial or complete reversion of the transformed phenotype in approximately 50% of transfectants, the reversion correlated with loss of anchorage-independent growth. Morphological reversion of established v-Ras-transformed NIH3T3 cells was less efficient. However, transfection of a full-length antisense c-*raf*-1 construct at a four molar excess over a v-Ha-*ras* containing plasmid, could almost completely inhibit the initiation of p21ras transformation. Expression of dominant negative Raf-1 proteins, such as the kinase-defective Raf-1 mutant protein RRAF 301, confirmed the antisense *raf*-RNA results, but were at the same time more efficient in inhibiting serum- or TPA-induced NIH3T3-cell proliferation and v-Raf transformation (KOLCH et al., 1991). These results point to Raf-1 kinase functioning as an essential signal transducer downstream from serum growth factors receptors and p21ras but upstream of AP1 (KOLCH et al., 1991; WOODGETT, 1992 and references therein).

Antisense inhibition experiments provided additional information concerning function and location within the signalling pathways of the proto-oncogene c-*fos*. Following the DNA transfer of a MMTV-antisense-*fos-globin* hybrid construct into mouse 3T3 cells, steroid-induced production of antisense

fos-hybrid RNAs, complementary to the 5'-regions of the mouse or human c-*fos* gene, resulted in a significant reduction in colony formation and inhibited cell growth. In contrast, no such changes were observed following the induction of sense *fos*- hybrid RNA (HOLT et al., 1986). The use of the inducible MMTV promoter for antisense *fos* RNA production enabled the examination of the proto-oncogene for which constitutive suppression may otherwise render it refractory to analysis. Dexamethasone induction of 3T3 cells transformed with multicopies of MMTV-antisense-c-*fos* DNA prevented the usual large increase in c-*fos* mRNA and protein after platelet-derived-growth-factor (PDGF) stimulation. DNA replication was also greatly diminished in these cells. Thus, a large increase in c-*fos* expression seems to be required for PDGF-induced re-entry of quiescent cells into cell cycle (NISIKURA and MURRAY, 1987). Antisense c-*fos* RNA production in EJ-c-Ha-*ras* transformed NIH 3T3 mouse fibroblasts caused a marked reduction in the amount of c-Fos protein expressed after serum stimulation. Although antisense-c-*fos* RNA expressing EJ cells continued to over-express the EJ-*ras* oncogene and remained capable of proliferating *in vitro*, a partial reversion of the transformed phenotype occurred in form of restoration of contact inhibition of cell growth, inhibition of anchorage independent growth, reduction of tumorigenicity in nude mice and reversion to a more flat morphology (LEDWITH et al., 1990). These data provide evidence for the participation of c-Fos in p21ras-regulated signal transduction pathways (WOODGETT, 1992).

Mechanisms Involved in Gene Regulation by Artificial Antisense RNA.

As has been demonstrated in prokaryotic systems, artificial antisense RNAs in eukaryotic systems are likely to mediate their inhibitory effects on gene expression by annealing to the complementary region within the target mRNA. In eukaryotic systems, antisense RNA:target RNA duplex formation could occur in different cellular compartments, in the cytoplasm and in the cell nucleus.

The formation of antisense:sense globin RNA hybrids in the cytoplasm of *Xenopus* oocytes has been proven by MELTON (1985) after microinjection of *in vitro* synthesized RNAs into the oocyte cytoplasm. The duplexed RNA is

thought to inhibit gene expression by preventing initiation of translation (MELTON, 1985). WALDER and WALDER (1988) found that hybrid arrested translation using antisense oligodeoxynucleotides *in vitro* was predominantly due to cleavage of the mRNA by RNaseH at the site of the RNA:DNA heteroduplex, especially when antisense oligonucleotides were used that hybridize within the coding region of the mRNA or over the initiation codon. Hybridization of the oligodeoxynucleotides to the very 5' end of β-globin mRNA, also inhibited protein synthesis directly, with cleavage of the mRNA by RNaseH as the predominant pathway of hybrid arrest (WALDER and WALDER, 1988). SHUTTLEWORTH and COLMAN (1988) confirmed *in vivo* that antisense oligodeoxynucleotides appear to act specifically by directing RNaseH cleavage and destabilization of their complementary mRNA. RNaseH is required for DNA replication and is mainly a nuclear protein, but it also appears to be present in the cytoplasm, especially in *Xenopus* oocytes (WALDER and WALDER, 1988).

The formation of RNA:RNA duplexes in the nucleus was reported by KIM and WOLD (1985). The formation of antisense:sense RNA hybrids in the nucleus is thought to interfere with processing of the pre-mRNA and/or the export of the RNA into the cytoplasm. MUNROE (1988) demonstrated that antisense RNA inhibits splicing of pre-mRNA *in vitro*. Antisense RNAs complementary to >80 nucleotides downstream of the globin 3' splice site inhibited splicing at least as efficiently as those extending across the splice sites. Inhibition is mediated by factors which affect the annealing of antisense and sense RNAs. MUNROE (1988) could further identify an activity in HeLa nuclear extracts which promotes the rapid annealing of the complementary RNAs in an ATP-independent manner. RNA:RNA duplexes themselves are rapidly degraded by double strand specific ribonucleases such as RNaseIII.

The mode of action and the degree of inhibition of gene expression by artificial antisense RNAs in eukaryotic systems are dependent on a number of different factors:

i) Determination which part of the target mRNA is most susceptible to antisense RNA inhibition: It has been suggested that for effective inhibition of gene expression, antisense RNA should be complementary to the 5'-untranslated region of the target RNA, including the ribosomal binding site (LIEBHABER et al., 1984). Results by IZANT and WEINTRAUB (1985) confirmed the importance of the 5' untranslated region in antisense RNA-mediated inhibition of *tk* gene expression. However, KIM and WOLD (1985) using antisense constructs complementary to either the 3' or the 5' end of the HSV-*tk* gene, achieved the same degree of inhibition. Similarly, antisense transcripts complementary to only the 3'-terminal coding region of the *Xenopus* ribosomal protein L1 mRNA were found to repress translation as effectively as antisense RNAs spanning the entire L1 coding region (WORMINGTON, 1986). Antisense RNA complementary to large portions of the coding region of genes have also been described as efficient in inhibiting gene expression (COLEMAN et al., 1984; BEVILACQUA et al., 1988). The results listed above demonstrate, that the effectiveness of antisense inhibition is variable. There is also variability for preferred target regions in different genes for inhibition by antisense RNA. For any given gene the most effective sequence to be targeted needs to be determined empirically.

ii) Half-life of the target mRNA: The half-life of the target mRNA is a critical factor in antisense RNA mediated inhibition of gene expression. Antisense RNAs targeted against unstable mRNAs may not complete the duplex formation before the target mRNAs degrade. In prokaryotes, the expression of a gene producing an unstable mRNA can be targeted only at the transcriptional level, since protein synthesis can cease soon after the transcription of the gene is terminated. Antisense RNA class III-type regulation may also be possible in eukaryotic systems, if the mechanism for transcription termination in the eukaryotic cells are elucidated. Antisense RNAs complementary to relative stable mRNAs, on the other hand, can undoubtedly hybridize with the target RNAs. The expression of a gene producing a stable mRNA continues until the pool of the mRNA is degraded due to degradation of the RNA:RNA duplex by ribonucleases. The design of an antisense RNA with a 3' or 5' extra tail sequence containing a ribozyme activity has been suggested. Such a ribozyme may be able to specifically

digest, and hence inactivate, the target mRNA (INOUYE, 1988; TAKAYAMA and INOUYE, 1990).

iii) Stability of antisense RNA: Data concerning stability of antisense RNA are sketchy. IZANT and WEINTRAUB (1984) found that antisense *tk* lines contain a lower steady state concentration of antisense *tk* RNA than sense *tk* RNA. Antisense *tk* and antisense CAT RNAs injected into frog oocytes, however, were as stable as their sense counterparts (WEINTRAUB et al., 1985). Results by BEVILACQUA et al. (1988) show that the stability of guanosin-5'-triphospho-5'-guanosine (GpppG) capped β-glucuronidase antisense RNA is greatly enhanced as compared to the uncapped antisense RNA in the early stages of pre-implantation mouse embryo development. As a result of the increased stability of the antisense RNA the inhibitory effect of the antisense RNA is also increased. The incorporation of structural features, like stem-and-loop structures, into the design of antisense RNAs has been suggested to enhance their stability and resistance to cellular nucleases (INOUYE, 1988). As shown during the inhibition of *Xenopus* membrane skeleton protein 4.1 expression, the insertion of the antisense sequences into the coding region of a second gene, like CAT, may also lead to increased stability of the antisense RNA. Furthermore, the antisense transcript is expected to appear in both the nuclear and cytoplasmic compartments as a normal messenger ribonucleoprotein, thus being able to interfere with the processing and transport of the target mRNA as well as with its translation (GIEBELHAUS et al., 1988).

iv) Structure of sense and antisense RNAs: The negative regulation of ColEI plasmid regulation by RNAI in *E. coli* is a good example for the importance of the secondary structure. Mutations that interfere with the three stem-loop-secondary structure of RNAI decrease the inhibitory effects of the regulatory RNA (GREEN et al., 1986b). A high degree of secondary structure within the targeted region of the sense RNA can also prevent the inhibitory effect of antisense RNA (expression of *ompF* in *E. coli*) (GREEN et al., 1986b). In mouse pre-implantation embryos, the secondary structure of the β-glucuronidase antisense RNA does not seem to be important (BEVILACQUA et al., 1988). However, stable secondary structures within the complementary

region may prevent formation of the hybrid with the target mRNA (INOUYE, 1988).

v) Gene dosage effect of antisense RNA inhibition: Results by IZANT and WEINTRAUB (1985), KIM and WOLD (1985), ROSENBERG et al. (1985) and others clearly show that the degree of antisense RNA-specific inhibition is depending on the ratio of antisense:sense RNA. Generally, an excess of antisense over sense RNA is required for maximal inhibition; the ratio needed for inhibition of gene expression may vary from gene to gene. High expression of antisense RNAs can be obtained through the use of strong promoters, such as the SV40 promoter, cytomegalo virus (CMV) promoter and retroviral LTRs, or inducible promoters for the conditional inhibition of target gene expression, such as mouse metallothioneine promoter or MMTV-LTR. High levels of antisense RNA transcripts are achieved in cells resistant to selection by progressively higher levels of methotrexate when antisense RNA sequences are transcriptionally fused to the DHFR protein coding region, generating chimeric DHFR-antisense RNA (KIM and WOLD, 1985).

It has to be pointed out that a 100% inhibition of gene expression by antisense RNA has never been observed (ROSENBERG et al., 1985), but rather a reduction in expression level of the target gene, which in turn has important consequences depending on the phenotype examined.

Delivery of Antisense RNA: Use of Retroviral Vectors.

Two methods have been predominantly used to introduce antisense RNA into cells *in vitro*:

i) microinjection of *in vitro* synthesized RNA into the cytoplasm of cultured cells (MELTON, 1985; ROSENBERG et al., 1985; CABERERA et al., 1987),

ii) stable transfection of a target cell with a plasmid carrying an antisense gene; the target cell then expresses the antisense RNA (IZANT and WEINTRAUB, 1985; TREVOR et al., 1987).

The latter of these two possible approaches is the more feasible foe analysing large number of cells expressing antisense RNA and for approaches to gene inhibition *in vitro*. The degree of inhibition of a target gene is largely dependent on maintaining an excess of antisense RNA over the target mRNA

(IZANT and WEINTRAUB, 1985; ROSENBERG et al., 1985). The maintenance of an excess of antisense RNA can be achieved by microinjecting large amount of *in vitro* transcribed antisense RNA or by increasing the stability of micrionjected RNA. Increased antisense RNA in cells transfected by an antisense gene construct will depend on methods to optimize expression of the antisense gene. The stability of antisense RNA is increased when the antisense RNA is GpppG-capped at the 5'-end (BEVILACQUA et al., 1986), is transcribed as part of another gene into which coding regions the antisense sequences have been inserted (GIEBELHAUS et al., 1988) and by including stem loop structures into the design of antisense RNA sequences (INOUYE, 1988). Excess of antisense RNA can also be ensured by achieving high expression of the antisense RNA through the use of strong promoters such as SV40 early promoter, CMV promoter or retroviral long terminal repeat elements (LTR).

The use of retroviral mediated gene transfer to introduce antisense RNA into cells not only presents one of the most efficient ways of introduction of exogenous genes/DNA sequences into a much broader range of host cells (BERNSTEIN et al., 1985; VARMUS and BROWN, 1989), it also ensures high expression of the antisense RNA through the strong LTR promoter (WEISS, et al., 1985) and as a capped and polyadenylated RNA species (VARMUS and BROWN, 1989, see chapter 1.6). Furthermore, retroviral mediated gene transfer is to date the only possible DNA transfer technique to apply antisense RNA inhibition of gene expression in cells *in vivo*.

1.5.2. Cell Lineage Study in Mouse Skin and Mouse Skin Carcinogenesis.

Techniques Used in Cell Lineage Analysis.

The examination of cell lineage is an important step towards understanding the developmental events that specify the various cell types in the organism. Cell lineage analysis provides information about the numbers of cells and range of cell types that can be produced by stem or precursor cells at each stage of development. Knowledge of the cell lineage and of the potential of the stem and precursor cell is essential in order to understand the control of

cell proliferation and the selection of phenotypes. If the fate of individual cells, *in situ*, was known, the understanding of developmental mechanisms in higher eukaryotes, especially vertebrates, would be greatly enhanced. Similarly, considerable advances could be made in defining the role of activated oncogenes in tumorigenesis under conditions as they occur *in vivo*.

The determination of cell lineage requires a means to identify the descendants of single precursor cells. Single cell marking by microinjection of fluorescent lineage markers or enzyme tracers, such as fluorescently labelled dextran (GIMLICH and BRAUN, 1986) or horse radish peroxidase (WEISBLAT et al., 1978), respectively, have already been extensively applied to cell lineage analysis in a variety of vertebrate systems including analysis of embryonic development of *Zebrafish* (KIMMEL and WARGA, 1986; WARGA and KIMMEL, 1990), neural cell lineages in the frog retina (WETTS and FRASER, 1991 and references therein), and avian neural crest (BRONNER-FRASER and FRASER, 1991 and references therein). The microinjection approach is neither species nor tissue specific, allows the labelled cells to be identified in live animals, fixed whole-mounts or histological section, and permits the observation of cell movement *in situ* (KIMMEL and WARGA, 1986; WARGA and KIMMEL, 1990). However, the use of injectable tracers can be limited by possible inaccessibility of the target cell and dilution of the label by mitotic activity of the injected cell (PRICE, 1987; WETTS and FRASER, 1991).

In mammals, cell lineage and tumorigenesis studies have relied largely on the use of chimeric (reviewed in DAUARIN and McLAREN, 1984; PONDER et al., 1985; SCHMIDT et al., 1985) or transgenic animals (review: HANAHAN, 1986). Chimeras, constructed using multi-cellular grafts or mixed blastocysts, allow cell lineage studies in the earliest stages of embryonic development. They, however, do not allow definitive conclusions to be reached regarding the potential of individual cells (WINTON et al., 1988).

Later aspects of mammalian cell lineage such as the determination of familial relationships among cells within individual tissues, have been more difficult to study *in vivo*. Even in combination with the development and extensive use of the transgenic animal technique (HANAHAN, 1986),

limitations regarding their use in the analysis of multistage carcinogenesis persist. In transgenic mouse experiments, oncogene products are synthesized in all cells of a tissue to which expression of the gene has been targeted by the choice of a specific promoter (HANAHAN, 1986). The initiation of tumorigenesis *in vivo*, however, is very likely due to somatic mutations occurring in a single cell surrounded by normal tissue. In contrast, ubiquitous expression of oncogene products in transgenic tissues may not preserve cell-cell interactions that could play a critical role in early neoplasia, especially as it has been demonstrated that normal cells can inhibit the outgrowth of transformed cells (LAND et al., 1986; METHA et al., 1986; PITTS et al., 1987; YAMASAKI, 1990).

Cell lineage analysis in the small intestine of the mouse have been performed with F1 hybrid offspring of mouse strains which express variant isoforms of X chromosome-linked marker enzymes (PONDER et al., 1985) or differ in the expression pattern of marker genes (WINTON et al., 1988). Insight into the clonal organization of adult intestinal epithelium and the stem-cell organization in the mouse small intestine was gained by combining the F1 hybrid model with either the analysis of X chromosome-linked phosphoglycerate kinase (*pgk*) polymorphisms in $Pgk\text{-}1^{a}/Pgk\text{-}1^{b}$ F1 females, taking advantage of random X chromosome inactivation (PONDER et al., 1985), or the induction of a cellular marker by sporadic or ethylnitrsourea (ENU)-induced somatic mutation at the *Dlb-1* locus, which determines the tissue specific pattern of expression of the binding site for the lectin Dolichos biflorus agglutinin (DBA) (WINTON et al., 1988 and 1990).

Genetic Tagging of Cells with Retroviral Vectors Encoding Histochemical Markers.

For the study of lineage relationship of cells *in vivo* it is necessary to mark a cell such that its developmental capacity can be assayed. Marking cells with genetic markers has a number of advantages: the marker is heritable, need not damage a cell or interfere with normal development, and is always present and detectable. Cells can be marked using a variety of gene transfer techinques, such as DNA transfection for studies *in vitro* or microinjection of DNA into the mouse pronucleus to generate transgenic mice for

developmental studies *in vivo*. The limitation of the use of transgenic animals in analysis of multistage carcinogenesis has been already briefly outlined. In *Drosophila*, the development of P-element-mediated transformation (RUBIN and SPRADLING, 1982) permitted the stable introduction of cloned *Drosophila* genes and of fusion genes with the bacterial histochemical *lacZ* marker gene as single copy genes into the germline (LIS et al., 1983; LAWRENCE and MARTINEZ-ARIAS, 1985; HIROMI et al., 1986 and references therein).

In recent years, retroviruses have been applied more and more to the study of cell lineages. Retroviruses and the development of retroviral vector systems and their applications have been described earlier. The application of retroviruses in the study of cell lineage has been reviewed by PRICE (1987), CEPKO (1988), and SANES (1989). The possible horizontal spread of the virus, due to the ability of the infected cell to produce new retrovirus particles, constitutes a potential disadvantage as it can obscure any clonal analysis. However, development of replication deficient retroviral vectors provides the means of generating helper-free virus stocks and thus preventing the spread of virus to other cells. Retrovirus vectors have been used extensively in the hematopoietic system of mice and humans (DICK et al., 1986) not so much to prove the existence of a pluripotential haematopoietic stem cell (WU et al., 1986; ABRAMSON et al., 1979) but in demonstrating the relative ease with which it is possible to label such cells and follow their fate during development (DICK et al., 1986; PRICE, 1987 and references therein). Retroviruses have also been applied to the study of cell lineages in the preimplantation mouse embryo (reviewed in PRICE, 1987), although wild-type Mo-MuLV retrovirus does not express in cells of the preimplantation mouse embryo (JAENISCH et al., 1975) and infected clones are identified by Southern blot analysis. Retroviral sequences are introduced into a subpopulation of the embryonic cells by infecting early stage-embryos which are then introduced into pseudopregnant foster mothers (JAENISCH et al., 1975; RUBENSTEIN et al., 1986; SORIANO and JAENISCH, 1986; STEWART et al., 1987). Alternatively, embryonic carcinoma cells (STEWART et al., 1982; NICOLAS et al., 1985) or embryo-derived stem cells

can be infected in culture (EVANS et al., 1985; ROBERTSON et al., 1986) and then used to form chimeras.

Progeny of cells infected by a retroviral vector can not only be identified by the integration site of a retroviral provirus but also by the expression of a genetic marker present in the retroviral vector used for the genetic tagging of the original cell. The use of marker genes whose gene products are easily detectable by histochemical staining procedures offer an alternative to other detection methods, like Southern analysis and *in situ* hybridization. Furthermore, histochemical markers allow the study of small clones and are useful in identifying precisely which cells within a structure are part of a clone (SANES et al., 1986; PRICE et al., 1987; TURNER and CEPKO, 1987; TURNER et al., 1990; AUSTIN and CEPKO, 1990).

A widely used and versatile histochemical marker system is the bacterial *lacZ* gene coding for the β-galactosidase enzyme. Cells expressing the *lacZ* gene product in its active form are identifiable in cultured cells, in tissue sections, or whole mounts by a sensitive histochemical staining method using 4-Cl-5-Br-3-indolyl-β-galactosidase (X-Gal) as a substrate (LIS et al., 1983; HIROMI et al., 1986; SANES et al., 1986; DANNENBERG and SUGA, 1981). The *E.coli lacZ* marker gene, expressed by different retroviral vectors, has been used successfully in a number of different systems:

i) Lineage studies in the nervous system during pre- and postnatal development in the retina and the cerebral cortex of rat and mouse (PRICE et al., 1987; TURNER and CEPKO, 1987; SNYDER and CEPKO, 1990; AUSTIN and CEPKO, 1990; SNYDER et al., 1992; reviewed in PRICE, 1987 and 1991; CEPKO, 1988 and 1989). Histochemical identification of β-galactosidase expressing clones in the retina of adult rats which had been infected neonatally with the *lacZ* gene encoding retroviral vector BAG showed clearly that progenitor cells exist in the retina which can give rise to an array of cell types even very late in development (PRICE et al., 1987). Injection of the BAG virus into the cerebral vesicles of rat embryos *in utero* confirmed that in the mammalian central nervous system, however, separate neuronal, grey matter astrocyte and oligodendrocyte lineages are generated quite early in development. *In utero* infection with BAG virus also helped to identify early and more restricted, late precursor cells of these lineages in the

cerebral cortex (reviewed in PRICE, 1987; CEPKO, 1988 and 1989; PRICE et al., 1991). Following transplantation of BAG infected, *in vitro* immortalized clonal cerebellar cell lines into the developing mouse cerebellum, SNYDER et al. (1992) could identify progeny cells of transplanted cells by expression of the *lacZ* gene product and the identity of the viral integration site between the donor cell and the labelled cells. They could therefore show, that multipotent neural cell lines, derived from the cerebellar external germinal layer (EGL), can engraft and participate in the development of mouse cerebellum in a cyto-architecturally appropriate manner.

ii) Lineage study in post-implantation mouse embryos (SANES et. al., 1986). Clones of β-galactosidase positive cells were detected in a wide variety of tissues after injection of mid-gestation mouse embryos (E7 to E11) *in utero* with a *lacZ* encoding recombinant retrovirus. Injecting virus at different time points during embryo development and varying the expression time, resulted in more detailed analysis of cell lineage in visceral yolk sac, cranium and skin *in vivo*. SANES et al. (1986) showed that epidermal and peridermal cells originate from a common precursor, presumably located in the basal layer of the skin, and that hair forming precursors arise from epidermal cells by specialization as late as day E11 in mouse embryo development (reviewed in PRICE, 1987).

So far, there have been no reports of developmental abnormalities, which could have been attributed to high expression levels of β-galactosidase in mouse, rat or *Drosophila.*

1.6. Introduction to Retroviruses and Retroviral Vector Systems.

In order to have an efficient means of introducing genes into cells both *in vitro* and *in vivo* and to ensure efficient expression of such genes extensive use has been made in the present study of retroviral mediated gene transfer using retroviral vectors. These vectors can be manipulated *in vitro* to readily allow the insertion of DNA, such as antisense *ras* sequences, or given genes, such as the bacterial *lacZ* gene and the mammalian transin/stromelysin gene. Infectious virus can subsequently be derived which can transfer these DNA sequences both *in vitro* and *in vivo* with a higher efficiency than most

alternative DNA transfer methods. Sequences inserted into a retroviral vector are also expressed at relatively high levels. Since extensive use of retroviral vectors has been made in the present study, the uses and advantages of retroviral vectors will be reviewed in detail.

1.6.1. Retroviruses: A General Introduction.

The feature that distinguishes retroviruses from other viruses is the replication of a single-stranded RNA genome through a double-stranded DNA intermediate (VARMUS and SWANSTROM, 1985). Multiplication-competent retroviruses encode their virion associated proteins in three open reading frames (ORFs).

i) The *gag* ORF (group specific antigen) encodes non-glycosylated nucleocapsid proteins such as matrix protein (MA), found mainly between the nucleocapsid and the viral envelope; capsid protein (CA), the major structural component and dominant antigen of the viral capsid; nucleocapsid protein (NC), implicated in RNA packaging and dimer formation (LEIS and JENTOFT, 1983; MERIC and SPAHR, 1986; PRATS et al., 1988).

ii) The *pol* ORF (polymerase) encodes the reverse transcriptase (RT), containing an RNA- or DNA-directed DNA polymerase activity at its N'-terminal region and a C'-terminal RNaseH activity (BALTIMORE et al., 1970; TEMIN, 1985; JOHNSON et al., 1986, TANESE and GOFF, 1988). The integrase protein (IN), implicated in the integration mechanism of the viral DNA into the host cell genome, is encoded in the 3'-end of the *pol* coding region (DONEHOWER et al., 1984; SCHWARTZBERG et al., 1984; ALEXANDER et al., 1987).

iii) The *env* gene is translated from a spliced subgenomic mRNA and codes for two proteins: the highly glycosylated surface protein (SU) and the transmembrane protein (TM). The SU protein interacts with host cell receptors that mediate virus entry into the host cell. TM is thought to mediate the fusion of viral and host membranes during virus entry into the host cell.

iv) The position of the coding domain for the viral protease (PR), responsible for cleaving the primary translation products of the *gag-(prt)-pol*

region (SKALKA, 1989), differs in different viruses (VAN BEVEREN et al., 1985; YOSHINAKA et al., 1985).

All three polypeptide classes are synthesized as poly-proteins and are later cleaved into the final products. The template for the gag polyprotein is the full-length genomic RNA. The gag-pol fusion protein is the result of translational readthrough on the genomic template RNA, occurring in 4 to 20% of translational events, by nonsense suppression with (RSV, MMTV) or without frameshift (MoMLV) (YOSHINAKA et al., 1985, JACKS and VARMUS, 1985; JACKS et al., 1987).

1.6.2. Retroviral Life Cycle.

A schematic representation of the retrovirus life cycle is shown in figure 1.3. The life cycle begins with the entry of the extracellular virus particles into susceptible host cells. This process requires:

i) Attachment and adsorption mediated through the surface protein and through host-encoded specific transmembrane receptors (DALGLEISH et al., 1984; MADDON et al., 1986; ALBRITTON et al., 1989) which recognize eco- (exclusively murine), xeno- (exclusively non-murine), ampho- or polytropic (various species specific) strains of retroviruses (WEISS, 1982).

ii) Penetration of the enveloped particle by endocytosis involving fusion of the viral and cellular membranes (MIMS, 1986), resulting in a simpler intracytoplasmic nucleoprotein complex.

Subsequent early events in the life cycle include:

i) Synthesis of a linear double-stranded DNA copy of viral RNA within the viral nuclearprotein complex (FUETTERER and HOHN, 1987), leading to a DNA that is slightly larger than the viral RNA due to the generation of long terminal repeat sequences (LTR) at either end of the DNA (VARMUS and SWANSTROM, 1985; VARMUS and BROWN, 1989).

ii) migration of the DNA protein complex to the nucleus, where some of the DNA is covalently circularized and

iii) covalent integration of the viral DNA into the host chromosome, forming the provirus. During the integration process, 2bp are removed from the outer ends of each LTR and a short sequence of host DNA (4 to 6bp) is duplicated at the integration site to form a direct repeat that flanks the provirus

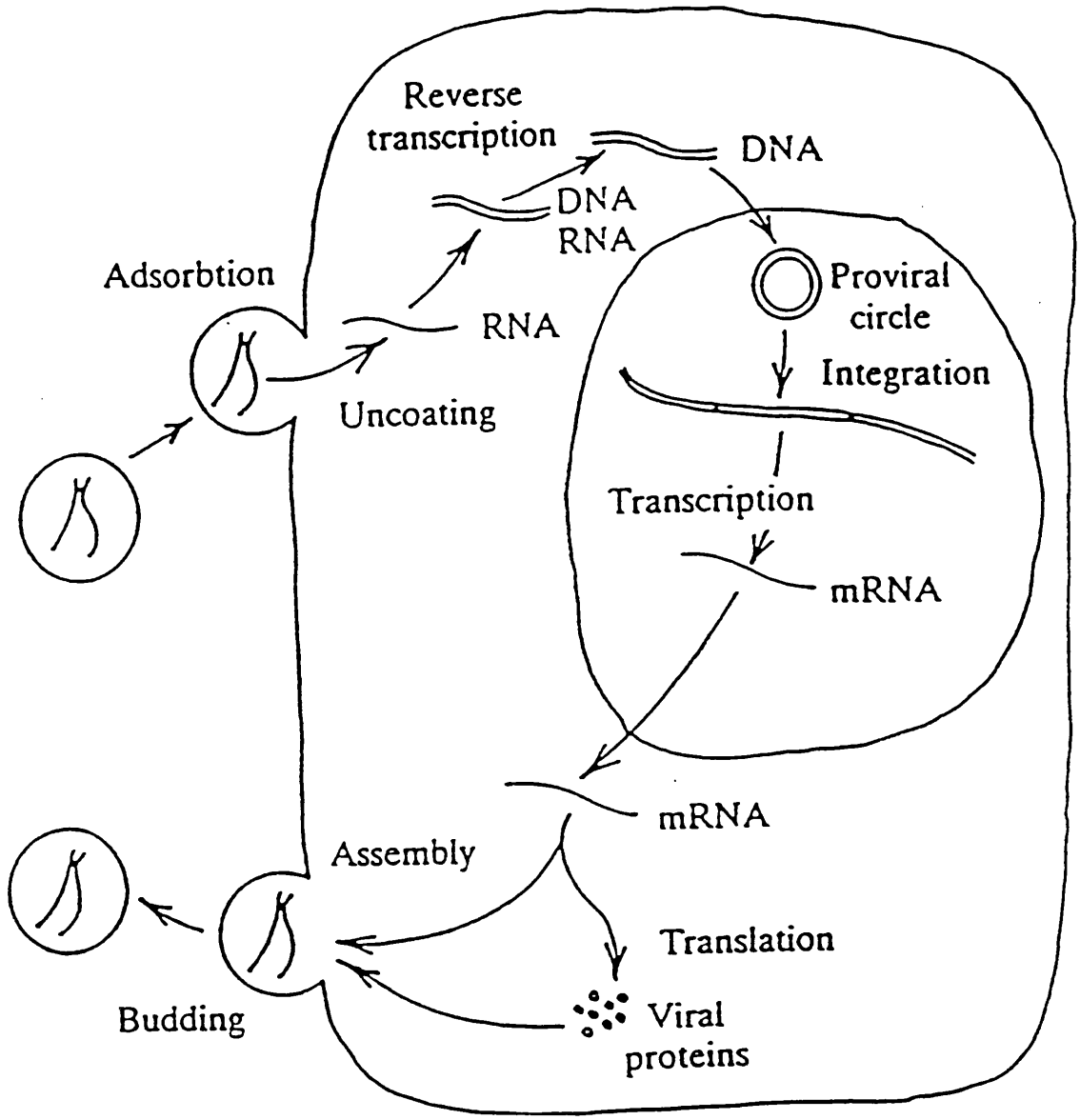


Figure 1.3:

The retroviral life cycle.

Figure 1.3., reproduced from PRICE (1987), is a schematic diagram of the life cycle of a typical wild-type retrovirus. The retroviral particle is adsorbed on to the cell plasma membrane by the binding of its envelope surface glycoproteins to a specific surface receptor. Following fusion, the retroviral genomic RNA passes into the cytoplasm, and is reverse transcribed into DNA, gains entry into the nucleus and as a proviral particle integrates randomly into the host cell chromosomal DNA. The integrated provirus acts as a typical chromosomal DNA in that it is inherited with both daughter cells whenever the host cell divides. The provirus is also transcribed and the retroviral genes are translated, using the cell's normal machinery. The assembly of a new retroviral particle completes the life cycle. The genomic retroviral RNA transcript comes together with the retroviral gene products and buds off to form a new free retroviral particle.

(PANGANIBAN, 1985; GOFF, 1990) Retroviral integration into the host genome is thought to occur essentially at random. However, a number of strongly preferred integration sites in the cellular genome seem to exist numbering perhaps 500 to 1000 (SHIH et al., 1988) although as yet no conserved cellular sequence features are apparent (SHIMOTONO and TEMIN, 1980; SHOEMAKER et al., 1981).

The retroviral LTR (conserved structure U3-R-U5) is between 300 to 1000bp long and is derived from a combination of unique sequences present at the 3'end (U3), the 5'end (U5) and a repeated sequence present at both ends (R) of the genomic RNA molecule. The U3 region contains control elements for retroviral transcription. Some LTRs have GC-rich boxes, proposed transcription factor SP1 binding sites (DYNAM and TIJAN, 1985; JONES et al., 1986) and enhancer elements that probably act as recognition signals for host-DNA-binding proteins that regulate transcriptional efficiency (GRUSS and KHOURY, 1983; YAMAMOTO, 1985; NABEL and BALTIMORE, 1987). Enhancer sequences have also been defined outside the U3 region in other parts of the retroviral genome such as the *gag* coding region (BROOME and GILBERT, 1985; ARRIGO et al., 1987; STOLZFUS et al., 1987). Specific sequences located at the termini of the LTRs are required in *cis* for correct integration (COLICELLI et al., 1985; PANGANIBAN, 1985; VARMUS and BROWN, 1989; GOFF, 1990). The integrated provirus has a defined structure: LTR-*gag-pol-env*-LTR and is a stable component of the host genome. The provirus generates two polyadenylated mRNAs: a full-length proviral transcript, initiated on the 5'-LTR and terminated on the 3'-LTR, containing a complete copy of the viral genome, and a spliced subgenomic RNA, essentially encoding env sequences (MULLIGAN, 1983). The full-length transcript can be used both for translation of *gag* and *gag-pol* products and for packaging into infectious viral particles (MULLIGAN, 1983; BERNSTEIN, 1985; GILBOA, 1986). Processing and translation of the viral RNA are followed by modification of the protein products, assembly of viral nucleoprotein cores and budding through the plasma membrane in places enriched with viral SU and TM glycoproteins to form extracellular virus particles (DICKSON et al., 1985; VARMUS and BROWN, 1989). Retroviruses rarely kill the cells they infect and the persistence of the infection

in both the origin target cell or its progeny, results in an amplification by transcription of a single provirus into many copies of viral RNA resulting in the release of thousands of virus particles per day during the process of virus growth.

1.6.3. Retroviral Vector-Mediated Gene Transfer.

Several methods have been developed to deliver recombinant DNA into cells in culture and to stably express transfected genes. The most widely used approach is DNA transfection by calcium phosphate co-precipitation (GRAHAM and VAN DER EB, 1973), However this method has several drawbacks:

i) It is inefficient, since on average a maximum of one in 10^4 to 10^6 fibroblast cells retain and express the integrated transfected sequences (PETERSON and McBRIDE, 1980; DEBENHAM et al., 1984). Additionally, only a small number of cultured cells are capable of being transfected at detectable frequencies.

ii) The transfected cell lines often contain multiple copies of the newly introduced sequences per cell (up to several hundred) integrated in tandem at a single chromosomal site (WIGLER et al., 1979; MULLIGAN, 1983). It is difficult to assess how many of these copies are expressed and non-mutated (LEBKOWSKI et al., 1984).

iii) The precise mechanism by which cells take up and retain the DNA is poorly understood (PERUCHO et al., 1980; LEBKOWSKI et al., 1984).

iv) Gene expression after DNA-mediated gene transfer tends to be relatively inefficient and unstable. Often constant selection is required to maintain the transfected DNA sequences (BERNSTEIN et al., 1985).

Other methods of DNA transfer such as protoplast fusion, lipofection and electroporation (POTTER et al., 1984) can be used with a wider range of recipient cells with a greater recovery rate compared to calcium phosphate mediated DNA transfer (TONEGUZZO and KEATING, 1986). However, the transfer efficiencies for these methods are also still often less than one in 10^3 cells, and many of the drawbacks discussed above still hold.

The first indication that retroviruses could be used as gene transfer vectors came from the extensive genetic and molecular studies of rapidly

transforming retroviruses whose genome contain sequences necessary for malignant transformation (viral oncogenes or v-onc), but are unrelated to the viral *gag*, *pol* and *env* coding regions (BISHOP and VARMUS, 1985), thus suggesting that retrovirus genomes are capable of accommodating a wide variety of different genetic sequences.

Many of the previously described unique features of retroviruses and their life cycle render these viruses uniquely suited for adaptation as gene transfer vectors *in vitro* and *in vivo*:

i) The infectious virus integrates stably into the host genome with high efficiency. The integrated proviruses are not specifically lost or inactivated at high frequency (MULLIGAN, 1983).

ii) The viral integration results in a defined and predictable proviral structure: cellular DNA-LTR-"transferred gene(s) of interest"-LTR-cellular DNA (MULLIGAN, 1983; GILBOA, 1986; GILBOA, 1990). The integrated provirus is exactly colinear with the unintegrated linear precursor, except for the terminal 2bp from each end of the precursor lost upon integration (VARMUS and BROWN, 1989; GOFF, 1990).

iii) The viral integration occurs at low copy number (often only one) per infected host cell (MULLIGAN, 1983).

iv) The expression of retroviral genes is very efficient due to the active transcriptional signals provided in the LTR (WEISS et al., 1985; VARMUS and BROWN, 1989).

v) The extended host range of retroviruses gives the possibility of gene transfer into a wide variety of species and cell types *in vitro* and *in vivo* (WEISS, 1982; STOCKING et al., 1986; CONE and MULLIGAN, 1984; KWOK et al., 1986).

vi) Retroviral infection is in general not toxic to the host cell and does not significantly interfere with the programme of cellular gene expression (WEISS et al., 1985).

vii) The retroviral genome has a flexible insert capacity for gene transfer (2 to 13kb) (BERNSTEIN et al., 1985), although the packaging efficiency declines beyond approximately 9 to 10kb (GELINAS and TEMIN, 1986). The lower limit for packageable size has not been determined,

however, RNA molecules as small as 3.0kb in size may be readily packaged (VARMUS and BROWN, 1989).

viii) Retroviral vector-mediated gene transfer can be obtained with relatively high efficiencies, with up to 1% of the cells being infected (BERNSTEIN et al., 1985).

1.6.4. Retroviral Vector-Mediated Gene Transfer Systems.

This overview of retroviral vector mediated gene transfer systems is divided into two parts: i) vector design and construction and ii) generation of infectious recombinant virus.

Vector Design and Construction:

Retrovirus vectors are generated by replacing varying amounts of the viral *gag*, *pol* and *env* genes with DNA sequences of interest. Using the efficient viral infection process, the inserted DNA sequences are transferred into the target cell as part of the viral genome. The basic retrovirus vector has to include the following essential sequences in *cis* for viral DNA synthesis, integration and expression of the recombinant provirus, as well as packaging of full length recombinant RNA into virus particles:

i) Sequences that form the LTR or are necessary for its synthesis: terminal repeat (R) necessary for DNA strand transfer during reverse transcription; two long unique sequences (U) between the primer binding site (PBS) for the minus-strand tRNA primer and R at the 5'-end (U5) and between the polypurine tract (PPT), which becomes the plus-strand primer, and R at the 3'-end (U3). U5 and U3 are duplicated during DNA synthesis to form the LTR (U3-R-U5) and contain signals for synthesis and processing of viral RNA.

ii) Short, usually imperfect inverted repeat sequences, so called attachment (att) sites, at the 3'-end of U5 and the 5'-end of U3 required for integration.

iii) Sequences that provide splice donor and splice acceptor sites for gene expression, although not strictly necessary for all types of vectors.

iv) So called "Psi" sequences (Ψ) necessary for the packaging of viral genomes into virions. These sequences are still poorly mapped but are found

adjacent to the 5'-LTR, residing in part downstream of the PBS (MANN and BALTIMORE, 1985; KATZ et al., 1986; BENDER et al., 1987).

A number of viraly encoded proteins, essential for the replication and propagation process, can be provided in *trans* by a replication-competent helper virus or by packaging-defective helper provirus present in specially designed packaging cell lines.

Retroviral Vector Types.

The first generation of retroviral vectors contained single selectable exogenous genes like thymidine kinase (tk) of Herpes Virus Simplex type I (SHIMOTOHNO and TEMIN, 1981) or cDNA of the human hypoxanthine phosphoribosyl transferase (HPRT) gene (MILLER et al., 1983) for gene transfer into tk- or hprt- recipient cells, respectively. The use of dominant selectable markers such as *neo* (G418 or neomycin resistance marker) or *gpt* (selection on mycophenolic acid) (COLBERE-GARAPIN et al., 1981; DAVIES and JIMENEZ, 1982; SOUTHERN and BERG, 1982; MULLIGAN and BERG, 1981) overcame the limitation of using drug resistant recipient cell lines. There are three basic types of retroviral vectors:

1) Double-expression vectors (DE vectors),
2) Vectors with internal promoters (VIP vectors),
3) Self-inactivating vectors (SIN vectors).

Double-Expression Vectors (DE Vectors).

DE vectors retain the identified viral splice donor and splice acceptor sequences, necessary to produce both full-length viral genomic transcripts and a spliced subgenomic RNA species required for *env* gene expression (MULLIGAN, 1983). Generally, two exogenous genes can be cloned into DE vectors, replacing the *gag/pol* and the *env* region. Thus the 5'-inserted exogenous gene is translated from the unspliced full-length transcript, and the 3'-inserted gene from the shorter, spliced RNA species (GILBOA, 1986). The efficiency of gene expression using DE vectors depends on a properly regulated splicing process. Although it is possible to express both genes from the full-length mRNA, efficient expression of the second gene decreases (PEABODY and BERG, 1986). The distinguishing feature of the DE vector is

that it not only provides the *cis* functions required for transmission of the exogenous genes into the target cells but also the *cis* functions necessary for their expression in the target cell.

The underlying assumption in the design of DE vectors is that the removal of the viral intron is regulated by sequences immediately surrounding the splice junctions (CEPKO et al., 1984). However, accumulating evidence suggests that sequences scattered throughout the viral intron play an essential role in modulating the frequency of RNA splicing (HWANG et al., 1984). Thus the absence of intron-contained sequences in DE vectors may be one explanation for their relatively poor performance when compared to other retroviral vector designs (GILBOA, 1986). More importantly, the use of DE vectors is limited to cells in which the viral regulatory promoter sequences are active. Both, host and viral factors are involved in regulating the correct proportion of full-length to sub-genomic RNAs. For example, in permissive avian cells, about 50% of the RSV RNA is spliced to form *env* and v-*src* mRNAs in about equal amounts. However, in mammalian cells, non-permissive for virus production, almost all viral RNA is spliced into v-*src* mRNA (VARMUS and SWANSTROM, 1985). One of the most widely used DE vectors is the pZIP NEO SV(X)1 vector designed by CEPKO et al. (1984) (figure 1.4a).

Vector with Internal Promoter (VIP Vector).

The VIP vector uses an internal promoter to control expression of one of the exogenous genes. In general, the transcription of the selectable marker gene is directed by the viral LTR. Gene expression in this type of vector does not rely on efficient splicing. A "minigene" consisting of a promoter linked to a cDNA of the gene of interest is inserted downstream of the selectable marker gene as shown in figure 1.4b (MILLER et al., 1984). The internal promoter can be ubiquitously active, tissue- and/or developmental specific, inducible or homologous to the inserted cDNA. However, internal promoter sequences present in the vector may negatively affect critical vector functions such as viral LTR activity and viral titer (EMERMAN and TEMIN, 1984a and 1984b; JOYNER and BERNSTEIN, 1983). The transcriptional enhancer element present in the viral LTR can also influence the expression of the inserted

Figure 1.4.:

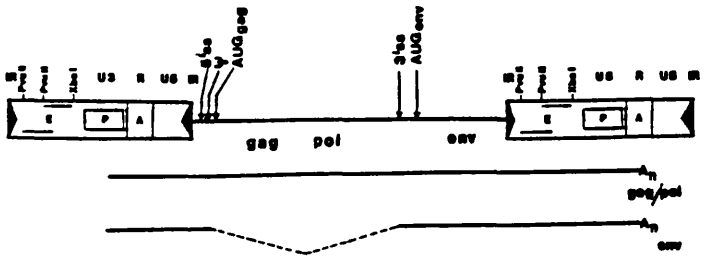
Three strategies of retroviral vector design.

Figure 1.4., derived from GILBOA (1986), illustrates the design of the three basic types of retroviral vector. Detailed description of the various vector designes is given in section 1.5.4.

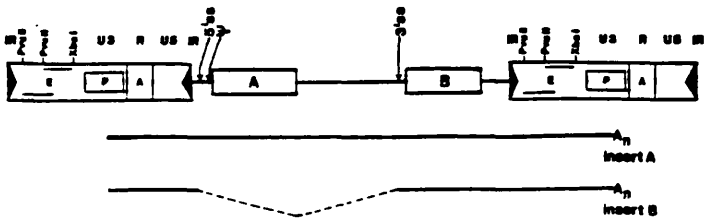
The schematical drawing of a retroviral provirus is shown in figure 1.4.A (top). The retroviral LTR (box) consists of the unique sequence present at the 3' end (U3), the unique sequence present at the 5' end (U5), and the repeated sequence present at both ends of the genomic RNA molecule (R). The short terminal inverted repeats (IR), enhancer (E), promoter (P), and the tract of polyadenylic acids [poly(A)] (A) of the 5' and 3' LTR are indicated. The coding domains for the virion structural proteins are indicated by *gag*, *pol*, and *env*. Normal retroviral genes are expressed from two RNA species: the *gag* and *pol* genes are expressed from an unspliced RNA form which is co-linear with the viral genome and the *env* gene is expressed from a spliced RNA form, produced by the removal of a long intron. The splice sites are indicated as 5'ss and 3'ss, respectively. The translation initiation codons for the *gag/pol* and the *env* transcripts are given as AUG_{gag} and AUG_{env}. Ψ indicated the location of the "Psi"-sequences, necessary for the packaging of the viral genomes into virions.

(A) Double expression vector

Provirus

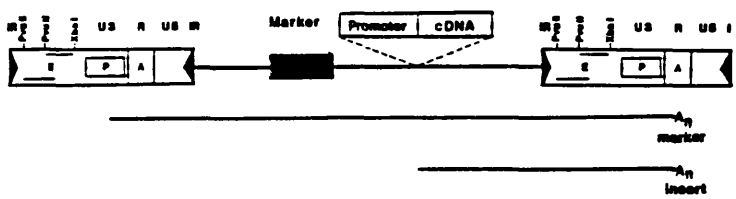


DE-AB

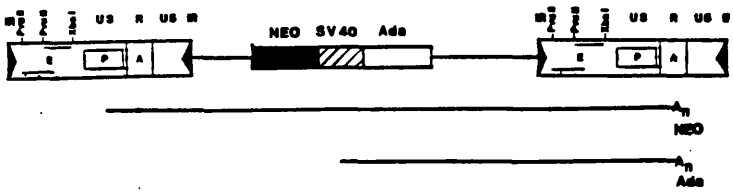


(B) Vectors with internal promoters

VIP

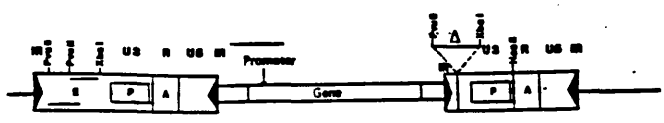


pLNSal



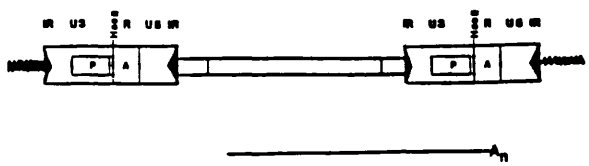
(C) Self-inactivating (SIN) vectors

Viral Vector



Infection

Provirus



minigene. To allow the study of sequences involved in control of tissue specific or inducible gene expression, it is possible to insert the minigene in the orientation opposite to the transcriptional direction of the viral LTR (EPISKOPOU et al., 1984; MILLER et al., 1984). However, viral enhancers could still influence some internal promoters irrespective of the orientation of the insert (YEE et al., 1987). An example of a VIP vector is the vector pLNSAL (figure 4.2b). The most widely used VIP vector is the high titer generating N2 vector. The N2 vector and its derivatives proved useful for the transfer of genes into lymphoid cells and bone marrow progenitor cells of various species. (Review: GILBOA, 1986 and 1990). LNL6, a safety modified derivative of N2 (BENDER et al., 1987), has been used in human gene therapy experiments (ANDERSON, 1992).

Self-Inactivating Vectors (SIN Vectors).

The enhancer present in the viral LTR can disturb the normal expression pattern of cellular genes, or more importantly cellular proto-oncogenes near which the retroviral provirus has integrated (NEEL et al., 1981). To circumvent the problems associated with abnormal gene expression induced by the presence of an integrated retroviral LTR, a third vector design was developed in the form of the SIN vector (figure 1.4c). SIN vectors contain a small deletion in the U3 region of the 3'-LTR, removing either the viral enhancer sequences (CONE et al., 1987) or the enhancer/promoter region (YEE et al., 1987). The original vector plasmid contains the deletion only in the 3'-U3 region, as full length transcripts originating from the 5'-U3 region are required to generate infectious virus upon transfection of the vector construct into packaging cells (GILBOA, 1986). During viral replication, the 3'-U3 deletion becomes duplicated and part of both proviral LTRs (WEISS et al., 1985; VARMUS and BROWN, 1989). Consequently, the integrated proviral DNA becomes transcriptionally inactive, but still enabling the expression of the exogenous minigene (GILBOA, 1986 and 1990). In addition, the absence of the viral enhancer will greatly reduce the possibility of activation of cellular oncogenes, an important condition for retroviral mediated gene transfer in human gene therapy (ANDERSON, 1984; 1992). However, SIN vectors produce infectious virus at very low titers.

1.6.5. Retroviral Vector Packaging Systems.

Infectious recombinant retrovirus can be recovered from cells which contain an integrated retrovirus vector construct after DNA-mediated gene transfer, as the transfected construct contains all functions required in *cis* for expression and encapsidation. The deleted viral coding sequences required in *trans* for generating infectious retroviral particles can be supplied by:

i) Replication competent helper retrovirus: The helper retrovirus is provided by co-transfection of the helper virus DNA together with the retroviral vector construct during DNA-mediated gene transfer into suitable recipient cells, or by superinfecting cells containing the integrated replication-defect retroviral vector construct with the helper virus. The infectious virus stock generated in this way contains both replication-defective and replication-competent virus. The procedure is outlined in detail in figure 1.5.

ii) Packaging cell lines: Specifically designed helper- or packaging-cell lines allow the production of helper-free virus stock form any retroviral vector construct. The generation of packaging cell lines involves the transfection of cells, mostly established mouse 3T3 fibroblasts, with the DNA of a helper virus, containing sequences required in *trans*, but having the Ψ-sequences, required for packaging of the retroviral RNA, deleted. Thus genomic RNA transcribed from the helper virus cannot be packaged into virions. Virus producer cell lines are established after the transfection of packaging cells with the retroviral vector construct following selection for expression of the selectable marker present within the vector itself or supplied on a separate plasmid by co-transfection. The most widely used selectable marker is the bacterial *neo* gene. Conditioned medium harvested from virus producer cells contains infectious viral particles which have only the full-length retroviral vector RNA packaged. The generation of infectious retroviral vector particles using helper cells is described schematically in figure 1.5.

Packaging-Cell Lines.

The most commonly used packaging cell line is the line Ψ2 generated by transfecting a plasmid containing a deletion mutant of Mo-MuLV virus into 3T3 mouse fibroblasts (MANN et al., 1983). The 351 bp deletion between the putative 5'-splice donor site and the AUG initiation codon of the *gag/pol* coding region, removing the Ψ packaging sequence. Virus stock generated by Ψ2 cells is mostly helper-virus-free, as reverse transcription activity is only seldom detectable. However, Ψ2 packaging cells do have a low reversion frequency, most likely due to *in vivo* recombination (MANN et al., 1983) or due to the presence of a small amount of contaminating wild-type helper virus plasmid at the time the Ψ2 packaging cells were generated (MILLER and BUTTIMORE, 1986) and thus can produce detectable amounts of replication competent helpervirus.

The problem of contaminating helper virus was overcome by generating the packaging cell line PA137 with the plasmid pAM containing a packaging-defective hybrid virus which carries a number of additional deletions and which has the 3'-LTR replaced by the Simian Virus SV40 polyadenylation signals (MILLER et al., 1986; MILLER and BUTTIMORE, 1986). Packaging of pAM-derived genomic RNA should be severely reduced, through the deletion of the packaging signal, and even if packaged, provirus formation in infected cells should be blocked both at the level of reverse transcription, through the deletion at the 3'-end of the virus removing the site for initiation of second-strand DNA synthesis and the 3'-R region required for translocation of the reverse transcriptase during first-strand DNA synthesis (VARMUS and SWANSTROM, 1985; VARMUS and BROWN, 1989), and at the level of provirus integration, through the deletion of the 5'-end of the 5'-LTR preventing formation of a functional integration signal (GOFF, 1992). The pAM plasmid contains still all the information necessary for the expression of the viral gene products but, at the same time, contains significantly less homology to sequences with which a retroviral vector can recombine to generate replication competent, packagable helper virus RNA (MILLER and BUTTIMORE, 1986). Safe packaging cell lines are also designed by using two retroviral clones containing overlapping deletions (WATANABE and TEMIN, 1982; MARKOWITZ et al., 1988).

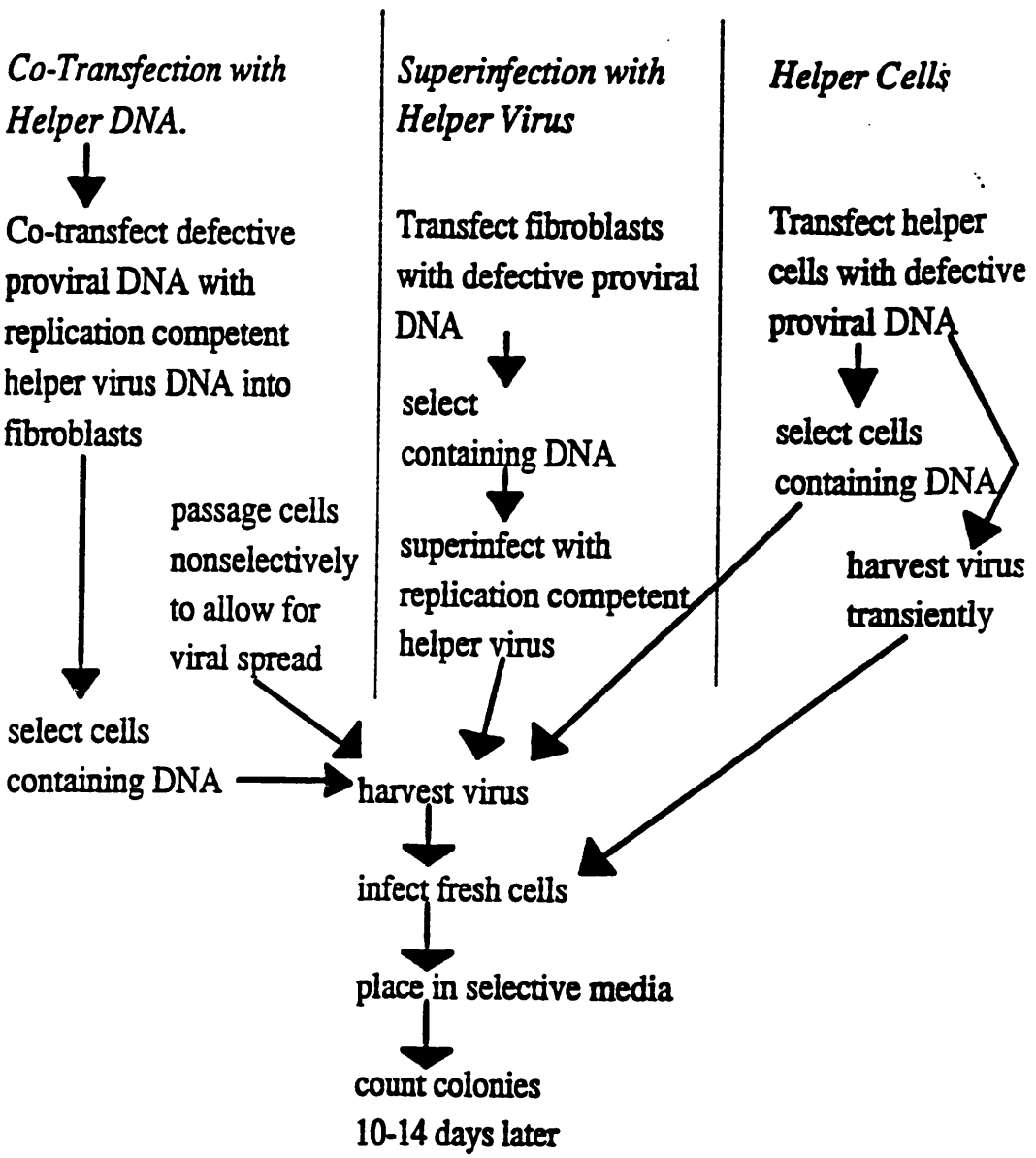


Figure 1.5:

Generation of infectious virus from cloned retroviral vector DNA.

Figure 1.5., reproduced from BERNSTEIN et al., 1985, outlines the major protocols for converting cloned retroviral vector DNA into infectious virus; see section 1.5.5. for detailed description.

Host Range of Recombinant Retroviruses.

The host range of retroviruses is primarily determined by the virus-encoded envelope glycoprotein. Retrovirus particles can only enter cells which express on their surface receptor molecules that are specific for their particular env protein allowing an env protein-receptor interaction (DALGLEISH et al., 1984; MADDON et al., 1986; ALBRITTON et al., 1989). The Mo-MuLV helper provirus present in Ψ2 cells expresses a viral envelope protein which recognizes only receptors present on mouse and closely related rodent cells (WEISS et al., 1985). The two most commonly used packaging cell lines with an extended, amphotropic host range are Psi Amp (amp: amphotropic) (CONE and MULLIGAN, 1984) and PA137 cells (MILLER and BUTTIMORE, 1986).

1.7. Aims of Thesis:

The mouse skin carcinogenesis model is of particular importance in the elucidation of genetic and/or epigenetic events associated with carcinogenesis involving epithelial tissues. Molecular and cellular characterization of the multistage model has now shown multiple events associated with the stages of initiation, promotion, progression and invasion. In this thesis, I have investigated the roles of given genes during chemically induced carcinogenesis of the mouse skin by inhibition of endogenous gene expression and by gene transfer.

Specific alterations of genes, either by introducing a gene into a cell or inhibiting expression of an endogenous gene, allows one to assess the role of expression of this gene in the phenotype of the cell. If these approaches can be extended to modulation of gene expression in progenitor cells of a tumour, then the role of this gene in tumorigenesis can be assessed. Transgenic mice, either expressing an exogenous gene or a knock-out mutation of a gene by insertional mutagenesis, have been invaluable in assessing gene function during development and tumorigenesis. However, the transgenic approach to study tumorigenesis has been restricted by the genetic alterations which allow development of viable animals. Furthermore, somatic mutations, rather than germ-line mutations, are more frequently observed in development of

tumours. Direct genetic alteration of somatic cells in an organism will avoid these problems. As will be discussed, the most efficient means of gene re-introduction uses infectious retroviral vectors which can infect cells both *in vitro* and *in vivo*.

In this thesis, I have explored the feasibility of using retroviral mediated gene transfer to study the roles of given genes during chemically induced carcinogenesis of the mouse skin. Although, several methods have been developed to deliver recombinant DNA into cells in culture and to stably express transfected genes, including DNA transfection by calcium phosphate co-precipitation (GRAHAM and VAN DER EB, 1973), lipofection and electroporation (POTTER et al., 1984), these methods have several drawbacks such as use *in vitro* only, limited range of recipient cell types and low transfer efficiencies. The identification and characterization of rapidly transforming retroviruses gave the first indication that retroviruses could be used as gene transfer vectors (BISHOP and VARMUS, 1985). Many of the unique features of retroviruses and their life cycle render these viruses uniquely suited for adaptation as gene transfer vectors *in vitro* and *in vivo*, including:

i) stable integration of the infectious virus as provirus into the host cell genome upon infection (MULLIGAN, 1983),

ii) defined and predictable structure of the provirus (MULLIGAN, 1983; GILBOA, 1986 and 1990),

iii) low copy number of integration (MULLIGAN, 1983),

iv) high efficiency of expression of the proviral genes (WEISS et al., 1985; VARMUS and BROWN, 1989),

v) extended host range *in vitro* and *in vivo* regarding both species and cell types (CONE and MULLIGAN, 1984; KWOK et al., 1986) and high efficiencies of gene transfer (BERNSTEIN et al., 1985),

vi) in general retroviral infection is not toxic to the host cell (WEISS et al., 1985),

vii) the retroviral genome has a flexible insert capacity for gene transfer (BERNSTEIN et al., 1985).

The design and construction of the different retrovirus vector systems used in retroviral mediated gene transfer as well as retroviral vector packaging systems have been described previously (chapter 1.6).

In this thesis three main studies were undertaken using retroviral vector mediated gene transfer:

i) The initiating event in tumours derived from mice initiated with the carcinogen 7,12-dimethyl benz[a]anthracene (DMBA) is a codon 61-mutation in the Ha-*ras* gene. Therefore, inhibition of Ha-*ras* gene expression in such tumours may lead to reversion of the transformed phenotype of the tumour cells. The first aim of my thesis was to explore the use of retroviral vectors to express an Ha-*ras* antisense RNA gene and infect cells transformed with mutated cellular Ha-*ras* gene. The feasibility of using such antisense Ha-*ras* retroviral vector systems to revert the transformed phenotype of the cells was analysed to assess the possibilities of using retroviral vectors for gene transfer of Ha-*ras* antisense RNA genes to suppress or revert transformation *in vivo*.

ii) Metalloproteinases have been implied to play a role in progression of tumours to an invasive and metastatic phenotype. The second aim of my thesis was to introduce the rat transin cDNA by retroviral mediated gene transfer into epithelial cells, which had been treated with DMBA *in vitro*, to examine if transin expression influenced progression of these cells towards invasion and metastasis as assessed by a spontaneous metastasis assay following subcutaneous injection of transin-retroviral vector infected cells into athymic nude mice.

iii) Retroviral vectors can be used to introduce histochemical marker genes, such as the β-galactosidase encoding bacterial *lacZ* gene, into mammalian cells. Infected cells, expressing active β-galactosidase, are easily identifiable by histochemical staining using the β-galactosidase specific substrate X-gal. The third aim of my thesis was to examine the feasibility of histochemical tagging mouse keratinocytes *in vivo* and *in vitro* through the constitutive expression of the lacZ marker gene following retroviral mediated gene transfer. Furthermore, the aim was to infect mouse skin cells *in vivo* with a retroviral vector containing both the v-Ha-*ras* and the *lacZ* gene. Successful infection should make it possible to follow v-*ras* retrovirally initiated cells through mouse skin carcinogenesis.

Chapter 2

Materials and Methods.

2. Materials and Methods.

2.1. Materials.

All chemicals used were of Analar grade and supplied by BDH Chemicals, Poole, Dorset unless otherwise stated.

All radioisotopes were obtained from Amersham International/ Amersham, Buckinghamshire.

Restriction endonucleases and enzymes employed in the manipulation of DNA or RNA were purchased mainly from Gibco/BRL (Europe), Paisley and Boehringer Mannheim, Lewes, East Sussex.

DNA and RNA size markers, were obtained from Gibco/BRL, Paisley.

Bacto-tryptone, Bacto-agar and yeast extract were obtained from DIFCO Laboratories, Surrey.

Serum, media and supplements for cell culture were obtained from Gibco/BRL, Paisley.

Guanidine thiocyanate was supplied by Fluorochem Ltd., Glossop, Derbyshire.

Phenol was obtained as a water-saturated liquid from Rathburn Chemicals Ltd., Walkerburn, Peebleshire.

Plastic-ware for cell culture was supplied by Nunc Intermed, Roskilde, Denmark and by Sterilin Ltd., Feltham, Middlesex.

NIH mice and nude mice were obtained from Marlan Olac Ltd., Bicester, Oxon.

2.2. General Methods.

2.2.1. Bacterial strains:

The bacterial strains used in this study were:

E.coli HB101, a hybrid of *E.coli* K-12 x *E.coli* B (BOYER and POULLAND-DUSSOIX, 1969);

E.coli DH5 (HANAHAN, 1985);

E.coli DH5α (FOCUS, 1986);

E.coli JM83 (VIEIRA and MESSING, 1982) and

E.coli JM101 (MESSING et al., 1981).

Strains in current use were maintained on Luria agar plates at 4°C. For long term storage, frozen cultures of each strain were prepared in nutrient broth containing 30% v/v glycerol and stored at -70°C.

2.2.2. Media and Antibiotics:

L-broth: 1% (w/v) tryptone,
0.5% (w/v) yeast extract,
1% (w/v) sodium chloride.
Ampicillin (100 μg/ml) (when required)
Kanomycin (50 μg/ml) (when required).

L-agar: L-broth containing 1.5% agar.

Top-agar for Blue-White Selection:
L-agar containing
100 μg/ml ampicillin;
250μg/ml X-gal.

2.2.3. Preparation of Competent Bacteria:

Bacteria were streaked on an L-broth agar plate and incubated overnight at 37°C. The next day a large colony was inoculated into 100ml of L-broth and grown at 37°C overnight with vigorous agitation. 2ml of this overnight culture were used to inoculate 100ml of L-broth (in a 500ml Erlenmayer flask) and grown at 37°C with agitation for about 4 hours. 5ml of this culture were inoculated into 500ml of L-broth and grown at 37°C with agitation until the desired optical density ($OD_{\lambda 650}$) of 0.2 was obtained

(around 1 to 1.5 hours). The bacterial culture was chilled in an ice-water bath for 5 min. The bacteria were transferred into cold sterile plastic bottles and spun down (Sorvall, 5000g, 10 min, 4°C). The medium was discarded and the bacterial pellet was resuspended gently, by pipetting up and down several times, in 100ml of ice-cold 100mM $MgCl_2$. The bacteria were spun down again (Sorvall, 4000g, 10 min, 4°C) and the pellet gently resuspended in 20ml of ice-cold 100mM $CaCl_2$. A further 180ml of ice-cold $CaCl_2$ were added, the suspension was mixed gently and left on ice for 20 min. Thereafter the bacteria were centrifuged as above and the pellet gently resuspended in 5ml (i.e. 1/100th of the original volume) of cold $CaCl_2$/glycerol (85% (v/v) 10mM $CaCl_2$ and 15% (v/v) glycerol). The competent bacteria were then aliquoted into sterile cold Eppendorf tubes and quick-frozen in liquid nitrogen. The aliquots were stored up to several months at -70°C. The bacteria prepared in this way are highly competent and transformation efficiencies obtained ranged from $5x10^6$ - $4x10^7$ colonies/µg plasmid DNA.

2.2.4. Transformation of Frozen Competent Bacteria:

The DNA solution of the ligation mixture (10µl volume, containing 50pg to 100ng plasmid DNA) was pipetted into a 10ml sterile polypropylene tube and left on ice. An aliquot of competent bacteria was thawed in an ice-water bath (10 min) and the bacteria were gently resuspended. With a chilled sterile Gilson tip 100µl of competent bacteria were pipetted onto the ligated plasmid DNA and then left for 20 to 30 min at 0°C. Then the bacteria were heat-shocked for 5 min at 37°C, thereafter 2ml of L-broth were added and the tubes were incubated at 37°C with agitation for 1 to 1.5 hours. Appropriate dilutions of the transformation mixture were plated on L-broth agar plates containing antibiotics and were incubated at 37°C overnight.

2.2.5. Plasmid Mini-Preps (HOLMES and QUIGLEY, 1981):

STET Buffer:

8% sucrose,
5% Triton X-100,
50mM EDTA,
50mM Tris-HCL pH 8.0.

TE-Buffer: 10mM Tris-HCL pH 7.5,
1mM EDTA.

Bacteria were transferred into 1.5ml Eppendorf tubes, resuspended in 400µl STET-buffer and mixed with 32µl of 10mg/ml lysozyme, dissolved in STET-buffer. Immediately after adding the lysozyme and mixing the bacteria suspension, the tubes were placed in a boiling water bath for 50 sec. After boiling, the bacterial suspension was spun down for 10 min in an Eppendorf centrifuge (13000g, 15 min, RT). After centrifugation the supernatant was taken and 400µl of cold isopropanol was then added, mixed and placed at -70°C for 5 min and centrifuged for 5 min in an Eppendorf centrifuge (13000g, RT). Following washes with 70% and 100% ethanol, the pellet was air dried for 5 to 10 min. After drying, the pellet was then resuspended in 200µl TE for pUC based plasmids (VIEIRA and MESSING, 1982) and in 50µl TE for pBR322/ColE1 based plasmids. Routinely 2 to 5µl were taken for analytical digests. Sub-cloning from the mini-prep DNA was carried out after isolation of the appropriate fragment from a low-melting agarose gel.

2.2.7. Large Scale Preparation of Plasmid DNA (HOLMES and QUIGELY, 1981, modified).

After growth in L-broth at 37°C, bacterial cells were harvested by centrifugation (Sorvall, 2500g, 5 min, 4°C). The medium was removed and the bacterial pellets were resuspended in 22ml STET. The bacterial suspension was transferred to a 100ml glass Erlenmayer flask Lysis was achieved by adding 2.6ml of a fresh lysozyme solution (10mg/ml in STET) and boiling the suspension over a gas flame. Alternatively, the bacterial suspensions could be lysed using a boiling water bath. The very viscous lysate was then transferred into Sorvall tubes and spun at 30000g for 30 min at 4°C. The supernatant was recovered into 50ml Falcon tubes and extracted once with 5ml of phenol/chloroform (1:1) and once with 5 ml chloroform/isoamylalcohol (20:1). The nucleic acids in the aqueous phase were the precipitated by adding 0.8 volume of isopropanol for one hour at -20°C. The precipitated material was pelleted by centrifugation. The pellet was washed with 70% and 100% ethanol, dried and resuspended in TE.

For CsCl preparations the DNA from above was then dissolved in 3.8ml TE and mixed with 4.3 g CsCl After the CsCl was dissolved, 0.5ml of 10mg/ml EtBr solution was added. This mixture was then transferred into Sorvall heat-seal centrifugation tubes which were topped up with 50% (w/w) CsCl, balanced, sealed and subsequently centrifuged (55000g, 48 hours, 20°C). The DNA band was collected. The EtBr was removed by adding three times 2ml of CsCl and H_2O-saturated isopropanol. The nucleic acid solution was diluted with 2 volumes TE and the DNA ethanol precipitated (0.1 volume 3M NaAc, 2.5 volumes absolute ethanol, -20°C, 1 hour) and the precipitate was spun down. The pellet was washed several times with 70% ethanol in order to remove precipitated CsCl, thereafter freeze-dried and resuspended in TE.

2.2.8. DNA Manipulation: General Techniques.

Disposable gloves were worn when DNA was handled. All buffers, reagents, glass and plastic ware was sterilized prior to use. Many of the techniques used are modified versions of those described in MANIATIS et al.,(1989).

Phenol Extraction.

DNA solutions were mixed with an equal volume of equilibrated phenol. The phenol phase and the aqueous phase were separated by centrifugation. The upper aqueous phase containing the DNA was removed and re-extracted with an equal volume of phenol/chloroform (1:1), followed by an extraction with an equal volume of chloroform/isoamylalcohol (25:1). The phenol was AR grade.

Ethanol Precipitation.

DNA was precipitated from solution by the addition of 1/10 volume of 3M sodium acetate (pH 6.5) or a 1/2 volume 7.5M ammonium acetate and 2.5 volumes of absolute ethanol. The precipitation mixture was chilled for at least 20 min at -20°C before the precipitated DNA was pelleted. The supernatant was discarded, the pellet washed in 70% ethanol and re-centrifugated. The DNA pellet was dried under vacuum and subsequently suspended as appropriate for further use. DNA in TE solution or in aqueous solutions were stored at -20°C.

2.2.9. Restriction Endonuclease Digestion of DNA.

Restriction Digestion:

All the restriction endonucleases used were obtained from Boehringer Mannheim, BRL or Biolabs. The digests were performed in sterile 1.5ml Eppendorf microcentrifuge tubes in the recommended restriction buffers according to the specifications provided by the suppliers. If no further information was available, restriction conditions were used according to MANIATIS et al. (1982).

Restriction buffers:

10xlow (10xLRB):

100mM Tris-HCl pH 7.5,
100mM $MgCl_2$,
10mM DTT.

10x medium (10xMRB):

100mM Tris-HCl pH 7.5,
100mM $MgCl_2$,
10mM DTT,
500mM NaCl.

10xhigh (10xHRB):

500mM Tris-HCl pH 7.5,
100mM $MgCl_2$,
10mM DTT,
1000mM NaCl.

10xCore (BRL):

500mM Tris-HCl pH 8.0,
100mM $MgCl_2$,
100mM NaCl.

2.2.10. Phosphatase Treatment.

To minimise recircularization of linear plasmid DNA, 5'-phosphates were removed from both ends of the linear DNA by phosphatase treatment.

TE Buffer: 10mM Tris-HCL pH 7.5,
1mM EDTA.

TEN Buffer: 40mM Tris-HCL pH 7.5,
1mM EDTA,
150mM NaCl.

Calf Intestinal Alkaline Phosphatase (CIP) (ULLRICH et al., 1977).

Calf Intestinal Alkaline Phosphatase (CIP): Molecular grade 1, Boehringer Mannheim.

10xCIP buffer: 500mM Tris-HCl, pH 9,0,
10mM $MgCl_2$,
1mM $ZnCl_2$,
10mM spermidine.

Prior to phosphatase treatment, the endonuclease digest of the vector DNA was checked by gel electrophoresis for completeness of the digest. The digested vector DNA was phenol extracted, precipitated with ethanol and resuspended in a minimum volume of 10mM Tris-HCl pH 8.0. All phosphatase reactions using CIP were carried out for 30 min at 37°C in 1xCIP buffer with a concentration of 0.1u CIP/pmol DNA ends. The reaction was stopped by phenol extraction. The phosphatase-treated DNA was recovered by ethanol precipitation and centrifugation. The DNA pellet was washed with 70% ethanol, dried and resuspended in TE to achieve DNA concentrations of 0.1µg/µl.

Bacterial Alkaline Phosphatase (BAP).

Prior to phosphatase treatment the endonuclease digest of the vector DNA was checked for completeness of digestion by gel electrophoresis. The desired amount of cut vector DNA was taken directly from the restriction digest mix. 1µl of BAP was added and the total volume adjusted to 100µl with TE. The reaction was carried out for 30 min at 37°C, followed by a further 30 min at 65°C. To stop the reaction, 1/10 volume (=10µl) 10xTEN was added and the enzyme BAP was inactivated by two successive phenol/chloroform (saturated in 1xTEN) extractions and a chloroform/isoamylalcohol (25:1) extraction. The phosphatase-treated DNA was recovered by ethanol precipitation. and centrifugation. The DNA pellet was washed with 70% ethanol, freeze dried and resuspended in TE to achieve DNA concentration of 0.1µg/µl DNA.

2.2.11. Ligation.

T4-DNA Ligase: New England Biolabs.

10xLigase Buffer: 660mM Tris-HCl, pH 7.5,
100mM $MgCl_2$,
100mM β-mercaptoethanol,
5mM ATP.

All ligations were carried out in 1xligation buffer at 15°C. Restricted plasmid vector DNA and inserts for ligation were mixed in a 1:3 molar proportion. Total DNA concentration was usually between 5 to 50µg/ml in a total volume 10 to 20µl. Restriction fragments having over-hanging sticky-ends, were ligated with 5 units of ligase (Biolabs unit definition) for 3 to 24 hours. Ligations involving cloning of flush-ended DNA fragments into a vector were carried out in two steps: i) overnight incubation with 250 units of ligase with a vector/insert ratio of approximately 1:3 respectively, and an overall DNA concentration of 100µg/ml. ii) followed by further 2 hours ligation after diluting the ligation reaction with 1xligation buffer to a DNA concentration of 20µg/ml.

2.2.12. Agarose Gel Electrophoresis (MANIATIS et al., 1989).

Electrophoresis Buffers:

10xTris-Borat Buffer (10xTBE):
900mM Tris-base,
889mM Boric-acid ,
5mM EDTA.

50xTris-Acetate Buffer (50xTAE):
2M Tris-base,
1M Acetic acid,
0.2M EDTA.

General DNA Loading Buffer (for all TBE and TAE gels):

20% Ficoll,
0.25% Bromphenol blue,
0.25% Xylene cyanol FF,
10mM Tris-HCl pH 7.5,
1mM EDTA.

Gel Electrophoresis:

Horizontal slap agarose gels (0.5 to 1.5% w/v) were prepared using BRL analytical agarose in 1xTAE or 0.5xTBE buffer. To allow staining of the DNA fragments during electrophoresis, ethidium bromide was added to the melted agarose to a final concentration of 0.1μg/ml. All DNA samples were mixed with 1/10 volume general DNA loading buffer. Electrophoresis was carried out with the gel completely submerged in appropriate electrophoresis buffer at 25V to 100V, until the dye front had migrated through at least 3/4 of the gel. DNA was visualised by transillumination with long wave UV light (λ_{260nm}). TBE gels can only separate up to 5μg of DNA per lane. TAE gels were used to separate up to 70μg of DNA per lane.

Low melting point agarose was dissolved in 1xTAE buffer. Electrophoresis was run at 40V, 4°C. The buffer was circulated using a peristaltic pump.

λ-DNA restricted with HindIII endonuclease (fragment sizes: 23.6kb, 9.46kb, 6.72kb, 4.34kb, 2.26kb, 1.98kb and 0.56kb) or with EcoRI endonuclease (fragment sizes: 21.7kb, 7.25kb, 5.83kb, 4.85kb and 3.48kb) were used as molecular weight markers.

2.2.13. Isolation of DNA Fragments from Agarose Gels.

Isolation of DNA Fragments from Low Melting Point Agarose Gels (MANIATIS et al., 1989).

Either Sigma type VII low gelling temperature agarose or BRL low melting point agarose 0.7% to 2.0% horizontal TAE gels (containing EtBr 0.1μg/ml) were used to separate and isolate DNA fragments ranging from 200bp to 20kb. The restriction digested DNA was loaded on an appropriate gel and ran in the cold-room (4°C) at 1-5V/cm with circulation of the buffer until fragments were separated. Generally the gels were run quickly (5V/cm) since

occasionally longer runs appeared to produce diffuse bands. DNA was then visualised with long-wave UV ($OD_{1300-360nm}$) and the desired band cut out with a scalpel. The agarose slice was diluted with TAE buffer to an approximate agarose concentration of 0.2% and heated at 70°C. When the agarose was melted the DNA was isolated from the agarose by several extractions: 2x phenol(equilibrated), 1x phenol/chloroform, each time the mixture was vortexed and centrifuged (2,500g, 5 min). Once the interphase was clear, the DNA was precipitated by the addition of ethanol (0.1vol 3M NaAc and 2vol ethanol, 5min in methanol/dry-ice). After centrifugation (5min Eppendorf centrifuge, 13000g) and washing with 70% ethanol the DNA was resuspended in TE. DNA extracted in this way could be: cut with restriction enzymes, ligated, nick-translated, kinased and cloned in a variety of vectors.

Recovery of DNA from Agarose Gels by Electroelution into Dialysis Bags (MANIATIS et al., 1982).

Electroelution:

Samples of restriction endonuclease digested DNA were electrophoresed in a 1.0% (w/v) agarose gel/0.5xTBE (containing EtBr 0.1μg/ml). The separated bands were visualised using a long-wave UV light (300 to 360nm) to minimize damage to the DNA. Gel slices containing the bands of interest were excised from the gel using a clean scalpel. The gel slice was placed inside a dialysis bag containing 1-2ml of 0.5xTBE buffer and the bag sealed with Medicel Mediclips. The bag was placed in a horizontal electrophoresis apparatus parallel to the platinum electrodes, just covered with 0.5x TBE buffer and electrophoresed for 1 to 2 hour at 100V. The current was reversed for 2 to 3 min at the end of the electroelution process to remove the DNA from the side of the dialysis tubing. The buffer containing the electroeluted DNA was pipetted from the bag and the gel slice and tubing were washed with 1 to 2ml 0.5xTBE buffer. Both the DNA sample and the wash were pooled and adjusted to 0.2M NaCl.

NACS Chromatography:

Buffer A: 0.2M NaCl,
10mM Tris-HCl, pH 7.2,
1mM EDTA.

Buffer B: 1M NaCl,
10mM Tris-HCl, pH 7.2,
1mM EDTA.

Buffer C: 2M NaCl,
10mM Tris-HCl, pH 7.2,
1mM EDTA.

The NACS PREPAC minicolumn (BRL) was hydrated with 3ml of buffer C. This was achieved by attaching the column to the barrel of a 1ml Gilson Pipetman, and the buffer was drawn up through the bottom of the column into the reservoir and then expelled by depressing the plunger. The resin was then equilibrated with 5ml buffer A in the same way as the column was hydrated. Up to 40µg of DNA loaded on to the top of the column and allowed to flow through by gravity flow to maximise binding of the DNA to the column. Thereafter, the DNA bound to the minicolumn was washed with 5ml buffer A. Restriction endonuclease fragments (greater than 1000bp) were eluated with 600µl of buffer C. Single-stranded oligonucleotides (less than 100 bases) were eluated with 600µl buffer B. The eluated DNA was preciptiated by adding 1/10 volume of 3M NaAc and 2 volumes of ethanol. The precipitation was carried out either at -20^{o}C overnight, or in a dry-ice/ethanol bath for 30 min. The DNA was recovered by centrifugation for 15 min in an Eppendorf centrifuge, dried for 15 min and resuspended in TE. DNA prepared in this way was stored at -20^{o}C.

2.2.14. Simultaneous Extraction of High Molecular Weight Genomic DNA and Total RNA from Cell Lines.

Handling of RNA:

An RNase-free environment was maintained for the RNA at all stages of its purification. The following guide-lines were observed at all times:

i) Never assume that anything in RNase free. Exception: sterile plastic ware, preferably individually wrapped, may be used without additional treatment.

ii) Dedicate laboratory glassware for use with RNA and mark it clearly.

iii) Autoclaving may not irreversibly inactivate RNases (RNase A and T1). Other RNases, especially if fungal in origin, may resist inactivation by autoclaving.

iv) At least one of the following methods was used to ensure that the reagents are RNase free. All water used for making solutions were treated with diethylpyrocarbonate (DEPC).

Phenol-SDS-Buffer Lysis (KRIEG et al., 1983):

SDS-Buffer (pH 7.0):

0.3M NaAc,
0.5% SDS,
5mM EDTA.

Phenol: 1 volume of phenol equilibrated with 1/2 volume of SDS-buffer and 0.1% Hydroxyquinolin.

Adherent cells were grown to just subconfluency the medium was removed and the cells washed twice in PBS. The cells were lysed *in situ* for 5min under agitation with 10ml equilibrated phenol and 10ml SDS-lysis-buffer. The lysate was transferred into a 50ml Falcon tube and gently shaken for additional 5min, before 10ml chloroform/isoamylalcohol (25:1) was added and the lysate-chloroform mixture shaken for a further 5min. The aqueous and organic phases were separated by centrifugation. The upper aqueous phase was collected and re-extracted with 10ml chloroform/isoamylalcohol (25:1) as described above. After centrifugation, the nucleic acids were precipitated out of the aqueous phase by addition of 2 volumes of absolute ethanol at -20°C for at least 1 hour. The ethanol precipitated nucleic acids were pelleted by centrifugation. The pellet was briefly dried and then resuspended in 2ml autoclaved DEPC-treated destilled water. The RNA was selectively precipitated out by addition of an equal volume of 4M LiCl at 4°C overnight. The RNA precipitate was pelleted in a Sorvall centrifuge (SS34 rotor, 15000g, 60min, 4°C). After the centrifugation, the pellet contained the precipitated RNA and the genomic DNA was present in the supernatant. RNA and DNA were from then on processed separately.

The RNA pellet was resuspended in sterile destilled DEPC-treated water. Any contaminating DNA was removed by DNAase I digestion, $MgCl_2$

was added to the RNA solution to the final concentration of 5mM, and 20µg/ml RNAase-free DNAase I (Boehringer Mannheim). After 20 to 30min incubation at RT, the DNAase I digestion was terminated by phenol extraction, chloroform/isoamylalcohol extraction followed by ethanol precipitation. The RNA pellet was washed twice with 70% ethanol, freeze dried and resuspended in up to 200µl sterile destilled DEPC-treated water. Total RNA was stored as ethanol-precipitated 20µg-aliquots at -70°C.

The genomic DNA was present in the supernatant recovered through centrifugation after the LiCl-precipitation of the RNA. The genomic DNA was precipitated at -20°C using 2 volumes of ethanol. After centrifugation the DNA pellet was washed twice with 70% ethanol, dried and resuspended in sterile destilled water or in sterile TE and stored at 4°C.

Guanidinium Thiocyanate Lysis (BALMAIN and PRAGNELL, 1983, modified):

Lysis-Buffer:

5M Guanidine thiocyanate,
50mM Tris-HCl, pH 7.0,
50mM EDTA,
5% β-mercaptoethanol,
2% N-Laurylsarcosine (sodium salt)
(added after lysis had occurred).

CsCl-Gradient:

5.7M CsCl,
50mM EDTA, pH 7.0,
1g/ml DEPC.

CsCl-gradient upper layer: Refraction index: 1.3925.

CsCl-gradient lower layer: Refraction index: 1.4025.

Nucleic Acid Extraction From Cultured Cell Lines:

Cultured adherent cells were grown to near confluency. The medium was removed, cells were washed twice in PBS at RT and then lysed in situ in 8 to 10ml lysis buffer (without 2.0% N-laurylsarcosine) for at least 30min. at room temperature. To isolate and separate the RNA and genomic DNA from

each other, the lysate was centrifugated through a CsCl/50mM EDTA step gradient. The gradient was prepared in pretreated 14ml polycarbonate Sorvall tubes (pretreatment: rinses in DEPC-treated destilled water followed by several rinses in autoclaved destilled water): 2ml CsCl/50mM EDTA (pH 7.0) (R.I. 1.3925) were underlaid with 2ml CsCl/50mM EDTA (pH 7.0) (R.I. 1.4025) using a gradient needle. The cell lysate was adjusted to 2.0% (v/v) N-laurylsarcosine, using a 20% stock solution, and carefully placed on top of the CsCl step gradient. The gradient was centrifugated at 25000g, 17°C for at least 36 hours. During centrifugation, the RNA was pelleted to the bottom of the centrifugation tube and the genomic DNA retained at the CsCl/CsCl interphase, whereas cellular proteins were retained in the guanidinium thiocyanate/CsCl interphase. After centrifugation the RNA and DNA were harvested and purified separately: The viscous CsCl fraction containing genomic DNA was transferred into a universal tube, the rest of the liquid discarded to leave the RNA pellet in the bottom of the centrifugation tube. The RNA pellet was resuspended in 200µl DEPC-treated, sterile water and the centrifugation tube was rinsed twice with 200µl DEPC-treated, sterile water. The resuspended RNA and the rinses were pooled.

The genomic DNA was precipitated in 3 volumes of cold 70% ethanol and spooled on to the tip of a pasteur pipette. After washes in cold 70% and cold 100% ethanol, the DNA was dried and then resuspended in 500µl TE/0.5% SDS. DNA associated proteins were removed by proteinase K treatment for 2 hours at 37°C: The DNA solution was adjusted to 150mM NaCl, 10 to 50mM EDTA and 100µg/ml proteinase K was added. The proteinase K stock solution had previously been predigested at 37°C for at least 30min. Two phenol/chloroform extractions (equal volume) and one chloroform/isoamylalcohol (25:1) inactivated and removed the proteinase K enzyme. The DNA was precipitated with 1/10 volume 3M NaAc and 3 volumes absolute ethanol (-20°C, 1hr), recovered by centrifugation, freeze-dried and resuspended in TE.

The RNA was precipitated with 1/10 volume 3M NaAc and 3 volumes absolute ethanol (-20°C, overnight), recovered by centrifugation, washed with 70% ethanol, freeze dried and resuspended in DEPC-treated, sterile water. The RNA was stored as ethanol-precipitated 20µg-aliquots at -70°C.

Nucleic acid concentrations were measured by determining the adsorption at wavelength λ:260nm in an spectrophotometer. The optic density of 1 (λ_{260nm}) is equivalent to a double stranded DNA concentration of 50µg/ml and equivalent to an RNA concentration of 40µg/ml, respectively (MANIATIS et al., 1982).

2.2.15. Northern Analysis: Blot and Hybridization (KRIEG et al., 1983).

Agarose Gel Electrophoresis for Northern Blot Analysis.

10X FAE Buffer (pH 7.0):

0.4M MOPS,
0.1M NaAc,
0.01M EDTA.

RNA Loading Buffer:

1x FAE buffer,
50% (v/v) Formamide,
2.2M Formaldehyde.

Gel Sample buffer:

50% (w/v) Glycerol,
1mM EDTA,
0.4% (w/v) Bromophenol Blue,
0.4% (w/v) Xylene Cyanol,
2 µg/ml EtBr.

Nitrocellulose Membrane: GeneScreen (NEN, Boston).

RNA samples (10 to 15 µg/lane) were mixed with 15µl RNA loading buffer and denatured at 65°C for 30 min. 5µl gel sample buffer were added to each RNA sample, briefly heated to 65°C prior to loading on 1.4% (w/v) agarose gel. The 1.4% (w/v) agarose gel was prepared by dissolving 2.8g of agar in 145ml of H_2O in a microwave oven. When cooled to 60°C, 35.8ml of formaldehyde (37%) and 20ml of 10x FAE buffer were added and the gel cast in the fume hood. Gel electrophoresis was run at 40V overnight, at 4°C in 1x FAE buffer (circulated). As the RNA was electrophoretic separated in the presence of EtBr, the RNA was made visible directly using a UV light source and a photograph was taken.

Northern Blot Transfer of RNA:

Denaturation Buffer:

50mM NaOH,
10mM NaCl.

Neutralisation Buffer:

0.1M Tris-HCl, pH 7.5.

Phosphate Buffer (20x):

0.5M Na_2HPO_4,
0.5M NaH_2PO_4.

The electrophoresed RNA samples were transferred on to GeneScreen membranes as follows: The RNA/formaldehyde gel was soaked in excess denaturation buffer for 30min at RT (on shaker) and the RNA subsequently neutralised in neutralisation buffer (30min, RT, shaker). The gel was equilibrated in 1x phosphate buffer (3x 20min, RT, shaker). The Northern blot was assembled as described in MANIATIS et al. (1982). The RNA transfer onto the nitrocellulose membrane (GeneScreen) was performed overnight in 1x phosphate buffer. After the transfer was completed, the membrane was rinsed in 1x phosphate buffer, dried on air and baked for 2 to 4 hrs at 80°C. The completeness of the RNA blot transfer was checked by examining the blotted gel under UV light for any remaining traces of RNA.

Hybridization:

Pre-Hybridization Buffer:

50% Formamide,
5x SSC,
2x Phosphate buffer,
5x Denhardt's,
100µg/ml salmon sperm DNA,
0.1% SDS,
10% Dextran sulphate.

Hybridization Buffer:

identical to Pre-Hybridization
buffer but with ^{32}P-labelled DNA
or RNA probe added.

Denhardt's Solution (100x):

2% (w/v) Ficoll-400,

2% (w/v) Polyvenylpyrolidone (PVP),

2% (w/v) Bovine Serum Albumin (BSA)

(Pentax fraction V).

Pre-Hybridization and Hybridization buffer were freshly prepared in a fumehood. Salmon Sperm DNA was denatured by boiling for 5min in water-bath and then leaving on ice for 5min before adding to the respective hybridization buffer. The SDS was always added last.

Pre-hybridization: Nitrocellulose membranes were pre-wetted in 1x phosphate buffer then hybridized in hybridization chambers in 20 to 25ml pre-hybridization solution for at least 3 hrs with agitation. Prehybridization was performed at 57°C, when ^{32}P-RNA-ribo probe was used, or at 50°C when probing with random primed ^{32}P-DNA probe.

Hybridization: Fresh hybridization buffer was prepared as described above. The pre-hybridization was stopped by exchanging pre-hybridization buffer. The ^{32}P-labelled probes were pretreated as follows: i) ^{32}P-RNA-ribo probe: the labelled RNA was denatured at 65°C for 5min and added straight into the freshly prepared hybridization buffer. ii) ^{32}P-random-primed DNA probe: 100µl 0.1xSSC/0.1%SDS was added to the DNA probe after labelling by random primed reactions. The labelled DNA probe was denatured in a boiling water-bath for 5 min, transferred back onto ice for 5 min and then added to the hybridization. Hybridization was performed overnight with agitation in a shaking water-bath at 57°C (^{32}P-RNA-Ribo Probe) or at 50°C (^{32}P- Random-Primed DNA Probe), respectively.

Washes:

Wash Buffer I:

2x SSC,

0.1% SDS.

Wash Buffer II:

0.1x SSC,

0.1% SDS.

After hybridization, the probed nitrocellulose membrane was rinsed twice in wash buffer I for 2 min at RT. Further washes took place in wash

buffer II at 65°C (routinely 2 times 20 min). The washed filters then were sealed in thin polythene bags and autoradiographed by exposing to X-ray film at -70°C.

2.2.16. Preparation of Radioactively-Labelled DNA Fragments (FEINBERG and VOGELSTEIN, 1983 and 1984).

Isolation of Plasmid Insert DNA for Use in Random Priming.

Electrophoresis of plasmid endonuclease restriction digests was carried out in 0.6 to 1,5% Sea-Plaque agarose/0.5x TBE gels. The size separated DNA fragments were visualised under UV light. Gel slices containing the bands of interest were excised from the gel using a clean scalpel. After adjusting the DNA concentration to approximately 3ng/ml, the DNA inserts were ready for labelling using the random priming method.

^{32}P-Random Priming Labelling Using Klenow Polymerase.

Oligo Labelling Buffer (OLB)):

50mM Tris-HCl pH 8.0,
5mM $MgCl_2$,
10mM β-mercaptoethanol,
4mM dATP,
4mM dGTP,
4mM dTTP,
0.2M HEPES pH 6.6,
110µg/ml mixed hexadeoxynucleotides,
0.4mg/ml BSA.

Double stranded DNA fragments were labelled using mixed hexadeoxyribonucleotide primers of random sequences as described by FEINBERG and VOGELSTEIN (1983 and 1984). A Sea-Plaque agarose suspension containing approximately 50 to 100ng of insert DNA was boiled for 7 min to denature the DNA. Then 50µl OLB-buffer, 5µl [α-^{32}P]dCTP (Amersham) and 1µl Klenow enzyme (5 u/µl, labelling grade) were added. The labelling mixture was mixed gently and incubated at RT for 3 hours or overnight. the unincorporated nucleotides were separated from the labelled probe by column chromatography using Biogel-A 1.5m Nick-columns

(Pharmacia) equilibrated with 1x TE/0.1% SDS according to manufacturers instructions.

2.2.17. Preparation of ^{32}P-Labelled RNA Transcripts (GREEN et al., 1983).

SP6/T7 RNA Transcription Kit: Boehringer, Mannheim.

T3 RNA Polymerase: Boehringer, Mannheim

The DNA template was first linearised with a restriction enzyme cutting downstream of the segment to be transcribed. Completeness of the restriction enzyme digestion was checked by gel electrophoresis on an overloaded agarose gel. If the digestion was deemed complete the DNA was extracted twice with phenol/chloroform, once with chloroform/isoamylalcohol (25:1) and ethanol precipitated. After centrifugation and two washes with 70% ethanol, the DNA was freeze-dried and resuspended in DEPC-treated H_2O to a final DNA concentration of 250ng/µl. Up to 1.0µg of linearised plasmid DNA were used in one transcription reaction. The transcription reaction was prepared as follows: 4µl DNA, 2µl 10x transcription buffer, 3µl cold ATP/UTP/GTP (1:1:1), 1µl RNAsin (20u/µl), 5µl DEPC-H_2O and 4µl ^{32}P-rCTP (Amersham) were pipetted together. The *in vitro* RNA transcription reaction was started by the addition of 1µl of the appropriate RNA polymerase, SP6, T7 or T3 RNA polymerase, depending on the linearized DNA template, and incubated for 90min at 37°C to 40°C. The transcription was stopped by adding of 20µl TNM buffer. The DNA template was removed by digestion with 1µl RNase-free-DNAase I for 15min at 37°C. Then 50µl DEPC-treated H_2O were added and the reaction mixture was phenol:chloroform extracted twice. Carrier tRNA was added (25µg) and the RNA probe was precipitated at -70°C with 0.5 volume 7.5M NH_4Ac and 3 volumes absolute ethanol. After centrifugation the RNA was resuspended in 50µl DEPC-treated H_2O and reprecipitated as above. The precipitate was centrifugated again, washed twice with 70% ethanol, air-dried and resuspended in 100µl DEPC-treated H_2O. ^{32}p-rCTP incorporation was checked by counting an aliquot in a scintillation counter. NH_4Ac/ethanol precipitation is very stringed and nucleotides are not precipitated; therefore the material recovered in the pellet could be counted directly. The unincorporated

32p-ribonucleotides were separated from the RNA probe by passing the RNA transcription reaction over a Nick-column (Pharmacia). The Nick column had previously been equilibrated with 1xTE/0.5%SDS (pH 7.5) according to manufacturers instructions.

2.2.18 Protein Extraction From Cell Lines.

Lysis Buffer:

1% Triton X100,
0.5% Sodium Deoxycholate,
0.1% SDS,
0.1M NaCl,
0.05M Tris-HCl, pH 7.4,
5mM $MgCl_2$,
3mM PMSF.

A $10cm_2$ dish of just subconfluent cells was washed three times with cold PBS. The cells were lysed on ice for around 15min in 1ml lysis buffer. Using a disposable cell scraper, the lysed cells were scraped off the dish and transferred into an eppendorf tube. The protein extract was cleared of cellular derbies by centrifugation at low speed for 15min at 4^oC. The precleared supernatant was removed, transferred into a new eppendorf tube and stored at -20^oC. The protein content of the extracts was measured as described by BRADFORD (1976).

2.2.19. Immunoprecipitation, Western Analysis and Immunodetection of Proteins.

Sample Preparation:

Samples of protein extracts, containing the same amount of protein and adjusted with lysis buffer to the same final volume of around .5 to 1.0ml, were cleared prior to Western analysis by incubation at 4^oC for 2 hours in the presence of 100ul 50% Protein A Sepharose coated with rabbit anti-rat IgG (IgG-PAS). IgG-PAS was removed from the protein samples by two successive low speed centrifugations. The cleared supernatant was transferred to a fresh eppendorf tube.

Immunoprecipitation:

Western Sample Buffer:

0.1M Dithiothreitol (DTT),
2% SDS,
25mM Tris, pH 6.8,
10% glycerol,
0.001% Bromophenol Blue.

The cleared lysates were incubated overnight at 4°C with Ha-ras specific momoclonal antibody YA6-172. Antigen-antibody complexes were precipitated with IgG-PAS (2 hours, 4°C). The precipitates were washed 5 times in 0.7ml cold lysis buffer (vortexing and spinning each time), resuspended in 50µl Western Sample buffer, denatured by boiling (about 3 to 5min), pelleted by centrifugation (5min, RT) and stored at -20°C.

Separation of Proteins by SDS-PAGE (LAEMMLI, 1970):

SDS-Polyacrylamide Gel (17.5%):

17.5ml 30% Acrylamide,
2.2ml 1% Bisacrylamide,
7.5ml 1.5M Tris, pH 8.7,
0.15ml 20% SDS,
2.5ml water,
0.1ml 10% Ammonium Persulphate,
10µl TEMED.

Stacking Gel (5%):

1.67ml 30% Acrylamide,
1.30ml 1% Bisacrylamide,
1.25ml 1M Tris, pH 6.8,
50µl 20% SDS,
4.4ml water,
0.1ml 10% Ammonium Persulphate,
10µl TEMED.

5xSDS/Electrophoreses Buffer:

15.1 g Tris base,
72 g glycine,
5 g SDS,
water to 1000ml.

Mini-Protean II gel tank system (Biorad).

Denaturing protein gels (17.5%) were cast in the caster with a 50ml syringe until gels were 5cm high, allowing 1.5cm for the stacking gel. Each gel was overlaid with 100ml H_2O-saturated isobutyl alcohol and were allowed to polymerize for around 1 hour. Isobutyl alcohol was removed and the gels were rinsed several times with 1xTris-Cl/SDS, pH 8.8. The stacking gel (5.0%) was added with a 10ml syringe, the combs were inserted and the gel was allowed to polymerize for around 1 hour. After the comb had been removed, the gel was placed into the gel tank, 1xSDS/electrophoreses buffer was added, and the protein samples loaded. The samples were electrophoresed at 10 to 25mA for 1 to 1.5 hours.

Electroblotting:

Anode Solution 1:

0.3M Tris,
20% methanol,
pH 10.4.

Anode Solution 2:

25mM Tris,
20% methanol,
pH 10.4.

Cathode Solution:

40mM 6-amino-n-hexanoic acid,
20% methanol,
pH 7.2.

Electroblotting was performed using a millipore semi-dry electroblotter. Six sheets of 3MM Whatman filter paper, soaked in anode solution 1, and three sheets of Whatman filter paper, soaked in anode solution 2, were placed on the anode plate. The nitrocellulose membrane, cut to size of protein SDS-PAGE gel and rinsed in H_2O, was layer on top of the filter paper,

followed by the protein gel itself. Next nine sheets of Whatmann filter paper, soaked in cathode solution were added. The transfer sandwich was covered with the cathode plate of the electroblotter. The transfer took place over 0.5 to 1 hour with 0.8mA per cm^2 (surface area of the gel).

Protein Detection by Chemiluminescence:

ECL chemiluminescence detection kit: Amersham.

Blotto:

5% Marvel,
0.5% NP40,
in PBS.

10xTBS:

116.9g NaCl,
24.22g Tris base,
6.7g EDTA,
make up to 2 litres at pH 7.6.

TBST:

1xTBS,
0.05% Tween 20.

Prior to probing, the membrane is blocked at RT for 1 to 2 hours in blotto. 3µl of the first antibody, pan ras NEI 704 (Dupont), was added in a total volume of 3ml blotto, incubation took place at RT overnight. The membrane was washes the following day for a total of 1.5 hours at RT in blotto, the blotto was changed every 15min. The second antibody, a anti-mouse IgG Horseradish peroxidase conjugate (Sigma), was diluted 1:5000 with blotto and incubated for 30min at RT. The membrane was washed for 1 hour in blotto and for 1 hour in TBST, both wash buffers were changed several times during the washing period. The membrane was then incubated with a chemiluminescence substrate (ECL kit from Amersham) as per manufacturers instructions and exposed to radiographic film.

2.3. Methods in Cell Culture.

2.3.1. Growth media and Buffers used in Tissue Culture:

Special Liquid Medium (SLM): Supplemented modified Eagles medium (SLM, GIBCO) containing 5% or 10% Foetal Calf Serum (FCS, GIBCO) and 2mM Glutamine.

1xDulbecco's modified Eagles Medium (DMEM, GIBCO): Supplemented to 5% or 10% Donor Calf Bovine Serum (GIBCO) and 2mM Glutamine.

2xDMEM (100ml):

20ml Dulbecco's MEM (10x) (GIBCO),
2ml glutamine (200mM),
20ml Serum (FBS or DCBS),
1ml penicilline (10000u/ml),
11.2ml sodium bicarbonate (7.5%),
2ml sodium pyruvate (100mM).

Temmin's Modified Dulbecco's Medium (TMDM) (100ml):

10ml Dulbecco's MEM (10x) (GIBCO),
0.1ml arginine/histidine (12.6% (w/v)/2.5% (w/v)),
5.6ml sodium bicarbonate (7.5%),
1ml glutamine (200mM),
10ml Foetal Bovine Serum,
0.5ml penicillin (10000u/ml),
1ml sodium pyruvate (100mM),
0.5ml sodium hydroxide (1M) (pH adjustment).

SF12 medium (100ml):

8.8ml SF12 medium (10x),
1.6ml essential amino acids for MEM (x50),
2ml glutamine (200mM),
2ml sodium bicarbonate (7.5%),
0.2ml penicillin (10000u/ml),
10ml Foetal Bovine Serum.

Dulbecco's Phosphate-Buffered Saline (PBS):

2.68mM KCl,

1.47mM KH_2PO_4,

0.137M NaCl,

8.06mM $Na_2HPO_4x7H_2O$.

Trypsin dilutant (PBS/EDTA): PBS buffer adjusted to 1mM EDTA.

Trypsin: 0.15% (w/v) trypsin in PBS/EDTA.

Hepes Buffered Saline (2x) (2xHBS):

280mM NaCl,

1.5mM Na_2HPO_4,

50mM Hepes, pH 7.4, to pH 7.12 with HCL.

HAT supplement (50x) (GIBCO):

8.8mg/l aminopterin,

680mg/l hypoxantine,

194mg/l thymidine.

2.3.2. Maintenance of Cell Lines:

Stocks of cell lines were kept subconfluent at all times. Cells were passaged once to twice per week by aspirating off the old media, washing the cells once in PBS and trypsinising in 2.5ml 0.125% trypsin per $75cm^2$ flask. Detached cells were diluted 1:10 to 1:30 in the appropriate growth medium depending on rate of cell growth and replated into new TC flasks containing the appropriate growth medium. Cells-containing TC flasks were transfered into 5% CO_2-37^oC-incubators.

2.3.3 Freezing of Cells:

Freezing medium:

11ml growth medium,

4ml DMSO,

5ml Serum (FBS or DCBS).

Cells to be frozen under liquid nitrogen were resuspended in 1/2 volume of growth medium and 1/2 volume of freshly prepared freezing medium was added dropwise to the cell suspension under agitation. The cell

suspension was aliquoted into 1ml NUNC cryotubes. The cryotubes were then transferred into polystyrol boxes and stored at -70°C for 12 hours, before being finally placed under liquid nitrogen.

2.3.4. Thawing cells:

A cryotube containing an aliquot of cells was taken out of the liquid nitrogen container and quickly thawed at 37°C. The cell suspension was added to 5 to 10ml of growth medium in a universal. The cells were pelleted at RT for 5min at 800 to 1000g. After the supernatant had been removed, the cell pellet was resuspended in the appropriate growth medium and replated into a new TC flask containing the appropriate growth medium.

2.3.5. Mycoplasma Test:

Fix-solution: methanol/acetic acid (3:1).

Cell to be tested for mycoplasma contamination were grown in a $25cm^2$ flask until they were semi to fully confluent. The medium was aspirated off and the cells were washed once with cold PBS. The fixation of the cells took place in 10ml fresh fixation solution for 10min on ice. After the fixative was removed, the cells were stained on ice for 10min in 20ml of cold PBS containing 1µl Hoechst stain (bisbenzimidazolefluorochrome, Hoechst 33258). The staining solution was taken off and the cells were rinsed under tap water. A piece of the size and shape of a micro-slide was cut out from the bottom of the $25cm^2$ flask. The cells on the plastic microslide were examined for mycoplasma DNA positive staining in the cell cytoplasma under a fluorescent microscope.

2.3.6. Stable Transfection by $CaPO_4$-DNA Co-precipitation Mediated Genetransfer (WIGLER et. al., 1978, modified).

Recipient Cell Lines: CT3 cells and Ψ2 cells

Growth medium: Temmin's Modified Dulbecco's Medium (TMDM).

2x HBS Buffer:

280mM NaCl,
1,5mM Na_2HPO_4,
50mM HEPES, pH 7.4,
adjusted to pH 7.12 with HCl.

Glycerol solution:

15% (v/v) glycerol/ 1xHBS,
sterile filtrated.

Recipient cells were plated in 15ml TMDM at a cell density of $5x10^5$ cells/$75cm^2$ flask one day prior to DNA transfection (day 0). On day 1, 4 hours prior to transfection, 10ml of fresh growth medium were added to the recipient cells. The DNA-$CaPO_4$ co-precipitate was prepared as follows: 100µl of 2,5M sterile $CaCl_2$ (Refraction index: 1.401) were added to 900µl of sterile water containing 40µg genomic carrier DNA and 5µg of plasmid DNA. Slowly, 1ml of 2x HBS was added dropwise while airbubbling (blowing air through cotton plugged pipet into solution). A precipitate was allowed to form at RT for 30 min. 4ml of growth medium were added to the slightly opague (but not clumpy) DNA-$CaPO_4$-precipitate, mixed and pipetted onto the cells. Usually the precipitate was left on the cells for 12 to 18 hours at 37°C. To increase the efficiency of the DNA-uptake by the recipient cells, on day 2 a glycerol shock was performed as follows: The growth medium was aspirated off, the cells were washed twice with serum-free medium and shocked for precisely 4min at RT with 4ml of 15% glycerol/HBS solution (v/v) per 75cm2 flask. The glycerol was removed, the cells were washed as above and subsequently fed with 20ml of fresh growth medium supplemented to 10% serum. After the glycerol shock, the transfected cells were incubated for further 48 hours at 37°C. On day 4 the cells were trypsinized, counted and replated in selective groth medium.

Foci-Selection:

Cells were replated into growth medium containing only 5% serum. The medium was changed routinely every 3 days and foci were scored after an incubation period of 2 to 3 weeks by Giemsa staining.

Giemsa staining:

For staining, growh medium was aspirated off the cells; cells were washed once with PBS and fixed with 10ml 100% methanol for 10min at RT. The methanol was removed and 10ml of Giemsa staining solution (Merck), diluted 1:10 with H_2O, were pipetted onto the cells. The cells were stained for 3min to 5min at RT. The Giemsa solution was poured off, cells were washed with deionised water and left to dry.

G418-Selection:

Normal growth medium (10% FCS/TMDMEM) containing 0.8mg/ml G418 was usedto select CT3 or Ψ2 cells sucessfully transfected with DNA of a *neo* gene containing plasmid DNA. The medium was changed once a week. Routinely $G418^r$-colonies were scored after 14 days incubation at 37^oC in 5% CO_2 by GIEMSA staining.

The concentration of G418 required to perform successful selection of transfected cells had been determined empirically for each cell line.

Gpt-Selection:

Selection medium: 1x DMEM medium containing:

10% fetal calf serum,
150µg/ml L-glutamine,
250µg/ml xanthine,
15µg/ml hypoxanthine (or 25µg/ml adenine),
10µg/ml thymidine,
2µg/ml aminopterine,
5µg/ml mycophenolic acid (sodium salt).

Ψ2 helper cells were continously propagated in the presence of 25µg/ml mycophenolic acid. The ecotropic retroviral packaging cell line Ψ2 is derived from the line NIH 3T3 by transfection with a replication deficient retroviral constuct containing the bacterial *gpt* marker gene MANN et al., 1983).

2.3.7. Retroviral Mediated Gene Transfer By Retrovirus Infection (MULLIGAN, 1983: MAGLI et al., 1987; LAKER et al., 1987).

Infectious Retrovirus Stock:

Stocks of infectious recombinant retrovirus were harvested from virus producer cell lines. Enough fresh medium was added to a semi-confluent flask of producer cell lines to just cover the cells. 24 hours later, the conditioned medium was harvested. The virus containing medium was taken off the cells, spun down (800g, 5min, RT), passed through a 2 to 4.5µm filter to remove contaminating cells. Aliquoted virus stock was used immediately in infection experiments or placed at -70°C for long term storage.

Retrovirus Infection:

Recipient cells were seeded out at a cell density of $5x10^5$ to $1x10^6$ cells/25cm^2 flask one day prior to infection (day 0). During infection, the recipient cells were exposed to 7 ml growth medium containing an aliquot of virus stock (usually 50µl) and 24µg/ml polybrene for 24 hours at 37°C (TOYOSHIMA and VOGT, 1969). Thereafter the cells were washed twice with PBS, fed with fresh medium and incubated for a further 48 hours at 37°C. The cells were then trypsinised, counted in the Coulter counter and replated at a cell density of $5x10^5$ cells per 10cm^2 petri dish in 10ml of selective medium. Most routinely G418-selection was used. A minimum of 4 dishes were used per experiment. After incubation of 1 week, the medium was changed (selective and non-selective). G418^r-colonies were single-cloned or pooled after 2 weeks of selection. The infection efficiency was calculated on the basis of the number of colonies obtained after selection. The replating efficiency of infected cells was determined by replating 200 cells into a 10cm^2 petri dish in 10ml non-selective medium.

Titration of Infectious Retrovirus Particles per Virus Stock:

CT3 cells were routinely used as recipient cells to determine retrovirus titers. CT3 cells, plated out at cell density of $5x10^5$ cells per 10cm^2 dish 24 hours prior to infection, were infected with 10µl to 100µl aliquots of virus stock in a total volume of 7ml growth medium containing 24µg/ml polybrene. After 48 hours expression time, the infected cells were trypsinized, counted

and replated in selective medium at cell densities of $1x10^3$, $1x10^4$ and $1x10^5$ cells per $10cm^2$ dish. Standard conditions for G418-selection and Giemsa staining were used. The number of $G418^r$-colonies was adjusted for re-plating efficiency, the fraction of infected cells plated out and the volume of virus supernatant used for infection to give the infection efficiency or titer as the number of $G418^r$-colony forming units per 1ml of infectious virus per 10^6 viable infected cells ($G418^r$-CFU/ml/10^6 viable cell).

2.3.8. Concentrating of Virus Stocks.

Centricon Microconcentrators (Amicon) with 10s molecular weight cut off point.

24-hour-conditioned medium harvested from four $175cm^2$ flasks of semi-confluent virus producer cells was used for concentration of infectious virus particles using centricon microconcentrators from Amicon. The virus supernatant was harvested form each flask, pooled and cleared of contaminating cells and cellular depirs by centrifugation (11000, 10min, RT). The clarified virus stock was passed through a 0.4µm sterile filter ansd stored on ice. The disposable centricon microconcentrators were assembled according to the manufactorer's instruction. 2ml of virus supernatant could be concentrated per microconcentrator per centrifugation run (Sorvall centrifuge, 7000g, 60min, 4^oC). The concentrated virus supernatant was retained in the upper compartment of the concentrator, wereas medium waste went through the filter into the lower, waste compartment. When the total of the conditioned medium had been passed through the microconcentrators, the concentrated virus supernatants retained in the upper compartment of each microconcentrator were pooled, aliquoted and stored for several months at -70^oC.

2.3.9. Test for Reverse Transcriptase Activity.

The reverse transcriptase test was used to detect and to quantitate the release of replication competent helper virus by virus producer cell lines. Disrupted retrovirus particles were incubated in the presence of poly rA template and ^{3}H-TTP nucleotides. When the virus stock contained replication competent hepler virus, synthesis of a poly ^{3}H-TTP product, wich is

precipitated by TCA, occurred. NIH 3T3 or CT3 cells were infected with recombinant virus stock harvested from producer cell lines. After two weeks of selection, G418-resistant colonies were pooled and propagated. Fresh medium was added to a sub-confluent flask of pooled retrovirus infectants. 24 hours later, the conditioned medium was harvested, clarified (11000g, 10min, RT) and filtered through 0.4μm sterile filter. The clarified medium was used directly in the reverse transcription test or could be frozen and stored at -70°C prior to testing for presence of replication competent helper virus.

Detection of Reverse Transcription Activity:

Reverse Transcriptase Buffers:

Disruption Buffer:

50μl 1M Tris/HCl, pH 8.0,
50μl 1M KCL,
200μl 0.1M DTT (stored at -20°C),
100μl 2% NP40,
600μl H_2O.

Reaction Mixes:

Reaction Mix A:

12.5μl 1M Tris/HCl, pH 8.0,
12.5μl 1M KCl,
25μl 0.02M MnCl (for FeLV or MoMuLV),
(25μl 0.02M $MgCl_2$ (for HIV)),
50μl Poly rA template (5.26u/ml),
25μl ^{3}H-TTP,
125μl H_20.

Reaction Mix B (control reaction):

as reaction mix A but with the following alterations:

50μl 0.01M Tris/HCl pH 8.1,
0.15M NaCl (instead of Poly rA).

Reaction Mix C (additional control):

as reaction mix A but with the following alterations:

50μl Poly dA instead of Poly rA.

Solutions TCA Precipitation and filtration:

10% TCA(v/v)/1% sodium-pyrophosphate(w/v)

5% TCA(v/v)/1% sodium-pyrophosphate(w/v)

5% TCA(v/v)

absolute ethanol

Virus particles were pelleted from the clarified medium supernatant by centrifugation at 35krpm for 60min at 4°C. The supernatant was carefully discarded, the centrifugation tubes were allowed to drain on a tissue for 1 to 2min and the neck of the tubes were wiped with a tissue to remove any traces of medium. 60.0µl of disruption buffer were added to the bottom of each tube and the virus pellet was scraped with the end of the pipette tip to resuspend the pellet (do not pipette up and down, as the samples will foam). For each virus sample to small falcon tubes (15ml) containing either reaction mix A (with poly rA template), reaction mix B (without template) or reaction mic C (with poly dA template) were prepared. 25µl of the virus samples in disruption buffer were added to each of the falcon tubes (change tips each time, danger of contamination). The virus sample and the reaction mixes were incubated for 1hour in a 37°C waterbath.

During the incubation period the millipore filter apparatus containing Whatman-GTC filters was set up. The filters had been pre-wetted in 10% (v/v) TCA/1% (w/v) sodium-pyrophosphate. At the end of the incubation period 20µl of yeast carrier RNA was added to each tube and the reaction was stopped with 10ml of ice-cold 10% (v/v) TCA/1% (w/v) sodium-pyrophosphate. The contence of each tube was poured onto a GFC filter and the solution was sucked through. Severall washes followed: once with 10ml of ice-cold 5% (v/v) TCA/1% (w/v) sodium-pyrophosphate, twice with 10ml of ice-cold 5% (v/v) TCA and once with 10ml of ice-cold absolute ethanol. The filters were then removed from the filter apparatus, dried on air and transfered into scintillation vials containing 5.0ml Ecosint scintillation fluid. To check wether the test had worked, the filters were counted for 1 min on tritium channel. The accurate incorporation of ^{3}H was determined by counting the filters twice for 10 min on a tritium channel.

2.3.10. X-Gal Staining.

X-Gal Staining of Cultured Cells (SANES et al., 1986).

Fixative:

2% Formaldehyde,
0.2% Glutaraldehyde,
in PBS.

X-Gal-stain:

5mM potassium ferricyanide,
5mM potassium ferrocyanide,
2mM MgCl2,
1mg/ml X-Gal (Boehringer),
in PBS.

X-Gal stock:

40mg/ml X-Gal disolved in DMF,
stored in the dark at +4^{o}C.

The medium was removed from the cells by aspiration and the cells were rinsed twice in PBS. 5ml to 10ml of fresh fixation solution was added to the cells. The fixative was removed after 5min fixation at on ice. The cells were washed twice in ice-cold PBS and finally overlayed with 5ml to 10ml of X-Gal stain. The cells were routinely stained overnight at 37^{o}C, although the first staining appeared after an incubation period of only 2 to 4 hours.

X-Gal Staining on Tissue Sections.

Whole Mount Staining (SANES et al., 1986).

Fixative: 1xPBS,
2% Formaldehyde,
0.5% Glutaraldehyde.

X-Gal Stain: 5mM potassium ferricyanide.
5mM potassium ferrocyanide,
2mM $MgCl_2$,
0.02% NP-40,
0.01% sodium deoxycholate,
1mg/ml X-gal in PBS.

To reveal β-galactosidase activity in whole mounts of BAG or ZipLacZSV9v-ras) infected mouse skins, the following procedure was used: Whole skins were fixed in fixative for 1hour at 4°C. The skins were rinsed in PBS and incubated in the X-Gal staining mixture for 12 to 18 hours at 30°C. Following staining, the tissues were rinsed in 3% DMSO/PBS and the stored in PBS at +4°C prior to paraffin enbedding.

Frozen Tissue Sections (PRICE et al., 1987):

Fixation solution:

0.1M PIPES, pH 6.9,
2% paraformaldehyde,
2mM $MgCl_2$,
1.25mM EDTA.

30% Sucrose solution:

1xPBS,
30% Sucrose,
2mM $MgCl_2$.

Permeabilization solution:

1xPBS,
2mM $MgCl_2$,
0.01% sodium deoxycholate,
0.02% NP-40.

X-Gal Staining solution:

1xPBS,
2mM $MgCl_2$,
0.01% sodium deoxycholate,
0.02% NP-40,
35mM potassium ferricyanide,
35mM potassium ferrocyanide,
1mg/ml X-gal.

Skin tissues were fixed for one hour at 4°C in the fixation solution. Tissues were then saturated in 30% sucrose/PBS/2mM $MgCl_2$ and quick-frozen on dry ice. Cryostate sections (5 to 20μm) were cut onto ploylysine-coated slides, refixed at 4°C in paraformaldehyde-fixative (4°C), and rinsed in

PBS containing 2mM $MgCl_2$. Following premeabilization for 10 min at 4°C in permeabilization solution, the sections were stained overnight at 30°C to 37°C in X-Gal staining solution. Slides were rinsed and mounted.

2.3.11. Soft Agar Cloning.

Replating in Soft Agar:

2xDMEM medium (100ml):

20ml Dulbecco's 10x medium (Gibco),
20ml FBS or DBS,
2ml Glutamine (200mM),
1ml Penicillin (10000u/ml),
11,2ml Sodium bicarbonate (7.5%),
2ml Sodium pyruvate (100mM),
adjust volume with sterile H_2O.

2xAgar:

1.2% agar in sterile water (underlay).
0.6% agar in sterile water (upper layer).

Preparation of the underlay agar: 2xDMEM medium was prepared as described above and equilibrated to 37°C. 1.2% agar were dissolved in 100ml sterile water by boiling for 2min in the microwave and transferred to a 57°C waterbath to prevent gelling. An equal volume of 1.2% agar was added to one volume of 2xDMEM medium, mixed and equilibrated to 37°C for at least 10min. Plastic disposable pipettes were used for all manipulations involving agar. 4ml of the underlay agar/DMEM mixture were used per $6cm^2$ bacterial petri dish. The underlay was allowed to gel and the so pre-prepared dishes could be stored overnight in sealed sterile bags at 4°C in the cold room.

Preparation of the upper layer: 0.6g agar were dissolved in 100ml sterile water by boiling for 2min in the microwave. The dissolved 0.6% agar was transferred to 57°C waterbath for at least 10min. To one volume of 2xDMEM medium equilibated to 37°C an equal volume of 0.6% agar was added, the now 0.3% agar/1xDMEM medium was placed bgack into the 37°C waterbath until used, but at least for 10min.

Preparation of cells: Cells were removed from the TC flask by trypsin treatment (2.5ml trypsin per $75cm^2$ flask) for a few minutes at 37°C and

transferred to an universal in the presence of 5ml to 10ml non-selective growth medium. The cell were pelleted by centrifugation (5min, 800g, RT), the supernatant removed and the cell pellet resuspended in 5ml to 10ml of non-selective growth medium. The cell number was determined by counting an 0.4ml aliquot in the Coulter counter. The cells were kept on ice until replating.

Replating in 0.3% soft agar: Pre-calculated volumes of the cell suspensions were added to the 0.3% soft agar/1xDMEM medium and gently mixed. A total of $1x10^4$, $2x10^3$, $1x10^3$ and $5x10^2$ cells were replated in 4ml of 0.3% soft agar onto the pre-prepared bacterial petri dishes equilibrated to room temperature (see preparation of underlay). Four dishes were replated per cell density. The upper layer soft agar containing the cells was allowed to set for about 30min, then the petri dishes were placed into an humid incubator (37°C, 5.0% CO2), the incubation period was between 2 to 4 weeks.

INT-staining of Viable Soft Agar Colonies. (BOLL et al., 1977).

INT-Stain: 2-(p-iodophenyl)-3-(p-nitriphenyl)-5-phenyl tetrazolium chloride hydrate (INT).

Preparation of INT-stock solution: 1mg/ml INT was dissolved in PBS using a boiling water bath or on heated stirrer. Undissolved particles were removed by sterile filtration (Filter: 0.22µm). The stock solution was stored at 4°C in the dark, not longer than one month.

After 2 to 4 weeks incubation at 37°C the soft agar colonies were stained with 0.6ml of 1mg/ml INT stain per $6cm^2$ plastic petri dish. The dishes were placed in a plastoc cake box with lid and the box was sealed with insolating tape. The dishes were gased with 100% CO_2 through a hole in the box or in the lid (about 20min). The hole was sealed with tape and the box was incubated overnight at 37°C. Viable cells take INT up, the metabolised product results in a red stain. Colonies greater 0.2mm were scored under a binocular dissection microscope.

Chapter 3

Inhibition of Cell Transformation by Antisense *Ras* RNA.

3. Inhibition of Cell Transformation by Antisense *ras* RNA.

3.1. Introduction.

The initiating event in skin tumours derived from mice initiated with DMBA is a codon 61-mutation in the c-Ha-*ras* gene. Therefore, inhibition of activated c-Ha-*ras* gene expression in such tumours may lead to reversion of the transformed phenotype of the tumour cells. Antisense RNA as a fine tuner of complex regulatory processes was first identified in prokaryotic systems (reviewed in EGUCHI et al., 1991). Artificial antisense genes have since been used in many eukaryotic systems facilitating the determination of function of genes by examining the consequences of the reduction or lack of expression of these genes (reviewed in TAKAYAMA and INOUYE, 1990). Antisense RNA targeted inhibition of gene expression has also been successfully applied to the study of proto-oncogene function and their participation in signal transduction pathways (AMINI et al., 1986; YOKOYAMA and IMAMOTO, 1987; KOLCH et al., 1991; NISIKURA and MURRAY, 1987; LEDWITH et al., 1990). The aim of this part of the present study was to explore the use of retroviral vectors expressing an c-Ha-*ras* antisense RNA gene in suppressing cell transformation of NIH 3T3 cells induced by a transfected codon 61-activated c-Ha-*ras* oncogene.

The first objective was to construct retroviral vectors expressing, as sense or antisense RNA, fragments of the 5'-region of the mouse c-Ha-*ras* gene. Three fragments spanning the whole of the genomic clone N1 (BROWN et al., 1988) were chosen for cloning into the retroviral shuttle vector pZip Neo SV(X)1 (CEPKO et al., 1984) in sense and antisense orientation. Fragment F1 contains 1.3kb of upstream sequences, coding exons E1 and E2, as well as intron I(1) and the first 112bp of intron I(2) (figure 3.2). Fragment F2 is comprised of 1.3kb upstream sequences, which contain 5' flanking sequences, the promoter region, untranslated exon E(-1), intron I(0), as well as the first 53bp of exon E1 (figure 3.2). Fragment F3 is made up by the remaining 3' 98bp of exon E1, intron I1, the entire exon E2 and the first 112bp of intron I2 (figure 3.2). Therefore, antisense RNA derived from fragment F1 was complementary to the 5'-untranslated region, translation initiation site and to parts of the coding region of the c-Ha-*ras* gene, whereas fragment F2

antisense RNA was mainly complementary to the 5'-untranslated region and fragment F3 antisense RNA to the coding region of the c-Ha-*ras* gene. Following the generation of producer cell lines for the sense and antisense c-Ha-*ras* retroviral ZN(X)RAS vectors, the second objective was to test ZN(X)RAS recombinant retroviruses for their ability to infect Ha-*ras*-transformed NIH 3T3 cells and to express the appropriate antisense c-Ha-*ras* RNA upon infection. Lastly, changes in cell phenotype of Ha-*ras* transformed NIH 3T3 cells, as a consequence of constitutive c-Ha-*ras* antisense RNA expression, were assessed by reversion of the transformed phenotype and by soft agar colony formation.

3.2. pZip Neo SV Retroviral Vectors.

The retroviral vectors pZip Neo SV(X)1 and pZip Neo SV(B)1 were designed by CEPKO et al. (1984) as murine retrovirus shuttle vector systems to introduce exogenous genes into a wide variety of mammalian cells or whole animals. (further references: BELMONT et al., 1986; RIJSEWIJK et al., 1986; CHANG et al., 1987; LEDLEY et al., 1987; YAMADA et al., 1987).

The basic vectors consist of:

i) pBR322 sequences necessary for the propagation of the vector DNA in *E.coli*,

ii) control units derived from a cloned Moloney murine leukemia provirus (Mo-MuLV) (HOFFMAN et al., 1982) which are required in *cis* for retroviral gene expression (viral LTRs), packaging of retroviral genomic RNAs (Ψ sequence) (MANN et al., 1983), and sequences involved in generating 5' and 3' splicing signals required for the processing of the subgenomic *env* RNA. The retroviral *gag/pol* and *env* coding regions had been removed to allow the insertion of genes or DNA fragments of interest, using two single cutter cloning sites, XhoI and BamHI. A cassette containing the *neomycin*^{r}-marker gene from transposon Tn5 (*neo*-gene) (COLBERE-GARAPIN et al., 1981; DAVIES and JIMENEZ, 1982; SOUTHERN and BERG, 1982) and the origins of replication of plasmid pBR322 and SV40 virus, is inserted into either one of the single cloning sites, leaving only one cloning site for the insertion of the exogenous sequences: pZip Neo SV(X)1 has the "neo-cassette" inserted in the XhoI site whereas the "neo-cassette" is

inserted in the BamHI site in the pZip Neo SV(B)1 vector (figure 3.1). The sequences present in the cassette allow selection of mammalian cells harbouring either Zip Neo SV(X) or SV(B) provirus (*neo*^{r}-marker), the rapid recovery of free or integrated proviral genes as bacterial clones (pBR322 origin of replication) and the propagation of the pZip Neo constructs as extrachromosomal plasmids in high copy number in Cos or CV-1 cells (SV40 origin of replication). (MELLON et al., 1981; GLUZMAN, 1981).

The pZip Neo vectors are double expression vectors (chapter 4). The expression of any exogenous gene, inserted into the 3'-Xho-I cloning site, depends on efficient splicing. Therefore, the pZip Neo SV(X)1 retroviral vector was chosen for *in vitro* gene transfer of genomic c-Ha-*ras* fragments inserted into the 5'-BamHI cloning site since its expression is then not as dependent on efficient splicing. Using the pZip Neo SV(X)1 vector, any neo^r-colonies isolated after retrovirus mediated gene transfer are more likely to co-express the gene inserted into the BamHI cloning site and the *neo* gene. In the basic vectors, there is a 1:1 ratio of genomic to subgenomic message (CEPKO et al., 1984). However, it is possible that some inserted sequences may inhibit splicing and so reduce the efficiency of expression of the 3' gene. For instance, insertion of either *c-myc* or of murine granulocyte/macrophage-colony stimulating factor (GM-CSF) into the vectors resulted in a decrease of up to 50% of the subgenomic message in infected cells (LANG et al., 1985; CORY et al., 1987).

3.3. Results.

3.3.1. Cloning of Antisense c-Ha-*ras* Retroviral Vectors.

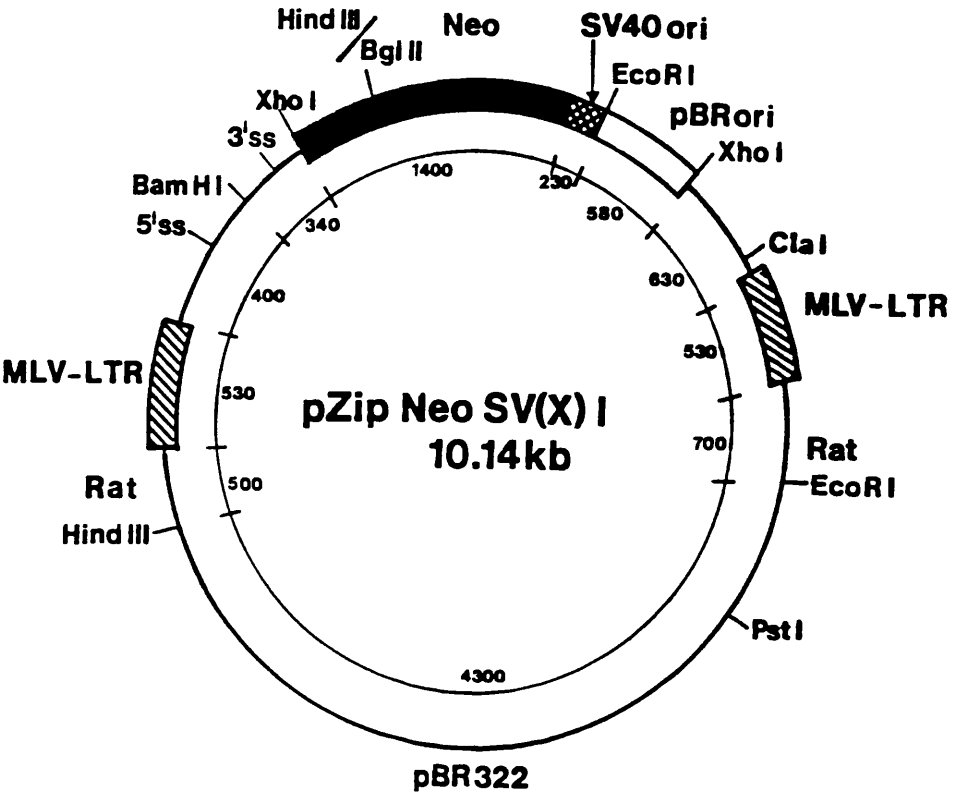
Three fragments spanning the genomic clone N1 of the mouse cellular Ha-*ras* proto-oncogene (BROWN et al., 1988) were chosen to be inserted in both orientations, "sense" and "antisense", into the single BamHI site of the pZip Neo SV(X)1 retroviral vector (CEPKO et al., 1984).

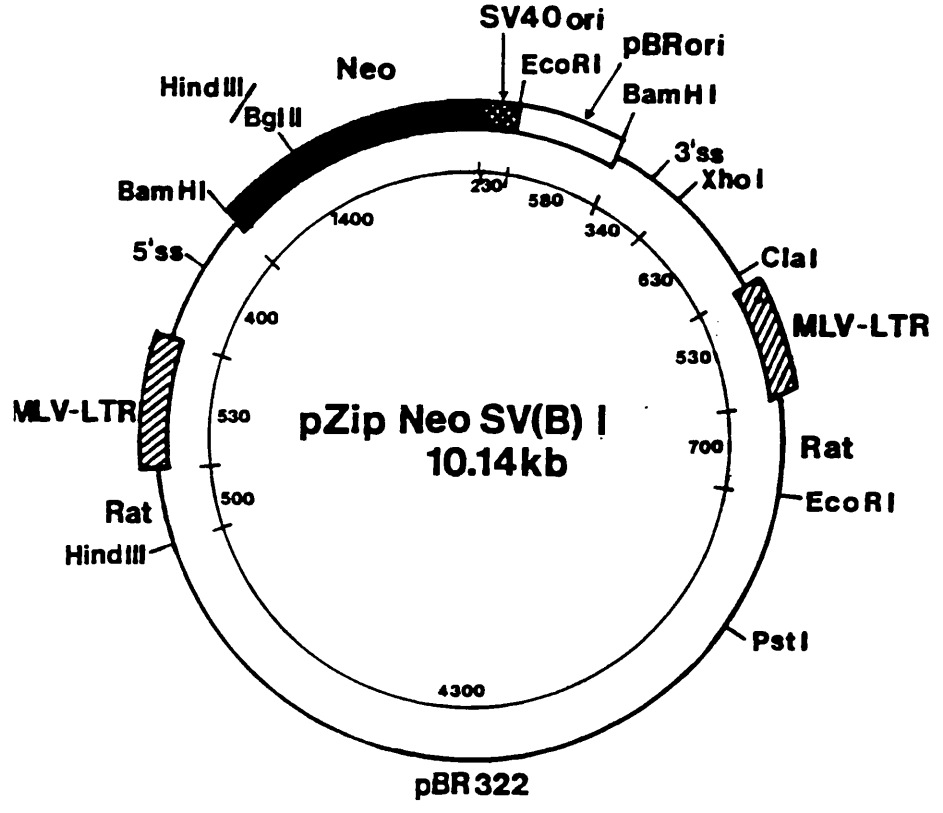
The clone N1 of the c-Ha-*ras* gene contains 1.3kb of upstream sequences, the coding exons E1 and E2, as well as intron I(1) and the first 112bp of intron I(2). The upstream sequences are composed of 5'-flanking sequences, the promoter region, the untranslated exon E(-1) and intron I(0). 3'-

Figure 3.1.

pZip Neo SV Retroviral Shuttle Vectors.

The pZip Neo SV retroviral shuttle vectors were cloned by CEPKO et al. (1984). The Mo-MuLV LTRs (diagonal boxes), and other *cis* regulatory functions required for retroviral RNA packaging and splicing (5'ss and 3'ss), as well as rat genomic sequences flanking the LTRs were derived from the Mo-MuLV provirus clone pZip. The fragment containing the *neo* gene (filled box) was isolated from Transposon Tn5 (SOUTHERN and BERG, 1982). The origins of replication of simian virus SV40 (crossed box) and of plasmid pBR322 (open box) allow the propagation of the shuttle vector as extrachromosomal plasmids in Cos cells and the rapid recovery of free or integrated proviral genes as bacterial clones. Recognition sites for restriction enzymes Hind III, Bam HI, Bgl II, Eco RI, ClaI and PstI are indicated. The numbers reflect the size of various fragments in bp. Detailed description of the cloning strategy is given in CEPKO et al., 1984).


Hind III
Bgl II
Neo
SV40 ori
EcoR I
pBR ori
Xho I
3'ss
Xho I
Bam H I
5'ss
Cla I
MLV-LTR
MLV-LTR
Rat
EcoR I
Hind III
Rat
Pst I
pZip Neo SV(X) I
10.14kb
pBR322
1400
230
580
340
630
400
530
530
700
500
4300


SV40 ori
pBR ori
Neo
EcoR I
Hind III
Bgl II
Bam H I
3'ss
Xho I
Bam H I
Cla I
5'ss
MLV-LTR
MLV-LTR
Rat
EcoR I
Rat
Hind III
Pst I
pZip Neo SV(B) I
10.14kb
pBR322
230
580
1400
340
630
400
530
530
700
500
4300

sequences including coding exons E(3) and E(4) are absent from the genomic c-Ha-*ras* clone N1 (figure 3.2). The 5'-region of the mouse c-Ha-*ras* gene has been characterized extensively by BROWN et al. (1988), PLUMB et al. (1991) and NEADES et al. (1991).

The three c-Ha-*ras* fragments used were:

i) fragment F1, the 1982bp long PstI-PstI fragment spanning the whole of the genomic clone N1,

ii) fragment F2, the PstI-HindIII fragment of 1384bp in length containing the 5'-flanking sequences, the promoter region, untranslated exon E(-1), intron I(0) and the first 53bp of exon E(1),

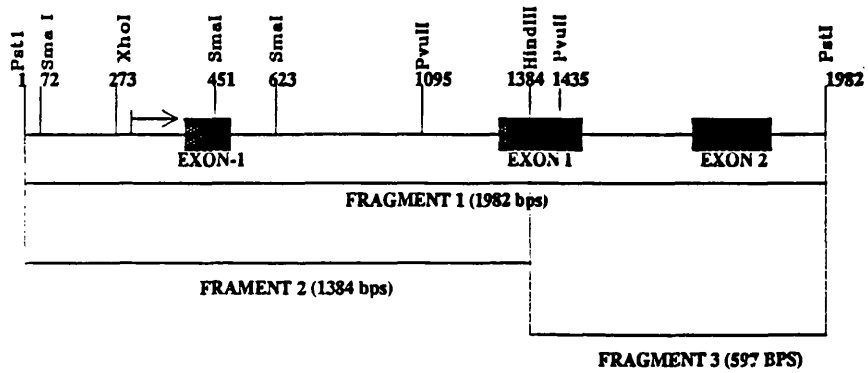
iii) fragment F3, the HindIII-PstI fragment, 597bp long, comprising of the remaining 3'-98bp of exon E(1), intron I(1), the whole of exon E(2) and the first 112bp of intron I(2).

It was necessary to subclone the c-Ha-*ras* fragments into the plasmid pIC20R (MARSH et al., 1984), generating three intermediate plasmids pIC-RASF1, pIC-RASF2 and pIC-RASF3, from which the genomic Ha-*ras* fragments could be re-isolated with BamHI compatible ends by BamHI-BglII double restriction enzyme digest. The re-isolated fragments F1', F2' and F3', now slightly larger due to the presence of additional polylinker sequences acquired during the subcloning into pIC20R (F1': 2.0kb, F2': 1.4kb, F3': 0.6kb), were inserted in both orientations into the single BamHI cloning site of the pZip Neo SV(X)1 vector. The "sense" orientation is defined by the orientation of the transcriptional active 5'-Mo-MuLV-LTR of the retroviral vector and the direction of the transcription initiated at this LTR. The "antisense" retroviral constructs, pZN(X)RAS-1, -2 and -3, contain the c-Ha-*ras* inserts in the opposite orientation to the transcriptional direction defined by the 5'-Mo MuLV-LTR. The retroviral constructs have been named as follows:

pZN(X)RAS+1/-1: c-Ha-*ras* fragment F1' cloned into pZip Neo SV(X)1 vector in "sense" (+1) or "antisense" (-1) orientation.

pZN(X)RAS+2/-2: c-Ha-*ras* fragment F2' cloned into pZip Neo SV(X)1 vector in "sense" (+2) or "antisense" (-2) orientation.

pZN(X)RAS+3/-3: c-Ha-*ras* fragment F3' cloned into pZip Neo SV(X)1 vector in "sense" (+3) or "antisense" (-3) orientation.



EXON -1: 116 bps (362-478)

EXON 1: 351 bps (1131-1482)

EXON 2: 178 bps (1692-1870)

⟶ : major transcription start sites.

Figure 3.2.

Schematic map of the 5' c-Ha-*ras* region used for antisense retroviral vectors.

Figure 3.2. shows a schematic diagram of the genomic clone N1 containing the 5'-region of the mouse c-Ha-*ras* gene. The untranslated exon E(-1) and the coding exons E1 and E2 (chequered boxes) as well as the major transcriptional start sites (arrow) are indicated as described in BROWN et al. (1988) and PLUMB et al. (1991). Genomic fragment F1 (PstI-PstI fragment, 1982bp) encompasses the whole of clone N1, whereas fragments F2 (PstI-HindIII fragment, 1384bp) and fragment F3 (HindIII-PstI fragment, 597bp) are subfragments thereof. Further details, see text.

Figure 3.3 summarizes in a flow diagram the cloning steps required to construct the recombinant sense and antisense pZN(X)RAS retroviral vectors. Plasmid maps of the antisense retroviral constructs are shown in figure 3.4. Maps for the sense retroviral constructs are not included in this figure, as they differ from their antisense counterparts only in the orientation of the c-Ha-*ras* fragment inserts.

3.3.2. Generation of ZN(X)RAS Virus Producing Cell Pools and Infection of CT3 Fibroblasts.

Virus producing cell pools were generated by stable transfection of ZN(X)RAS retroviral vector DNA into Ψ2 packaging cells (MANN et al., 1983) using calcium phosphate co-precipitation (WIGLER et al., 1978). Table 3.1 lists the transfection efficiencies using the different retroviral vector DNAs averaged over three independent transfection experiments. Significant variations in transfection efficiencies were observed, which could partly be explained by the size of the transfected retroviral plasmids. The presence of c-Ha-*ras* fragment F1' and F2' in sense orientation within the recombinant retroviral vector constructs had a negative effect on transfection efficiencies. It seemed likely for both fragments that "sense" expression reduced the efficiency of the *neo* gene expression, thus leading to fewer $G418^r$-colonies and poorer transfection efficiencies. The expression of fragments F1' or F2' inserted in antisense orientation did not have any reducing influence on *neo* gene expression and transfection efficiencies. No such orientation-dependent influence on transfection efficiency and *neo* gene expression was observed for the c-Ha-*ras* fragment F3' when inserted into pZip Neo SV(X)1 vector (table 3.1). A virus producer pool for the pZN(X)RAS+1 construct could only be established after repeated transfection experiments using Ψ2 cells continuously selected in mycophenolic acid for the expression of the *gpt* marker present on the packaging-defect helper virus construct used to generate the packaging cell line (MANN et al., 1983). LANG et al. (1985) and CORY et al. (1987) also observed a reduction in the ratio of the levels of subgenomic RNA to genomic RNA, possibly caused by inefficient splicing, after the insertion of a GM-CSF cDNA or a c-*myc* cDNA into the pZip Neo SV(X)1 vector, respectively. Insertion of the c-*myc* cDNA in "antisense" orientation

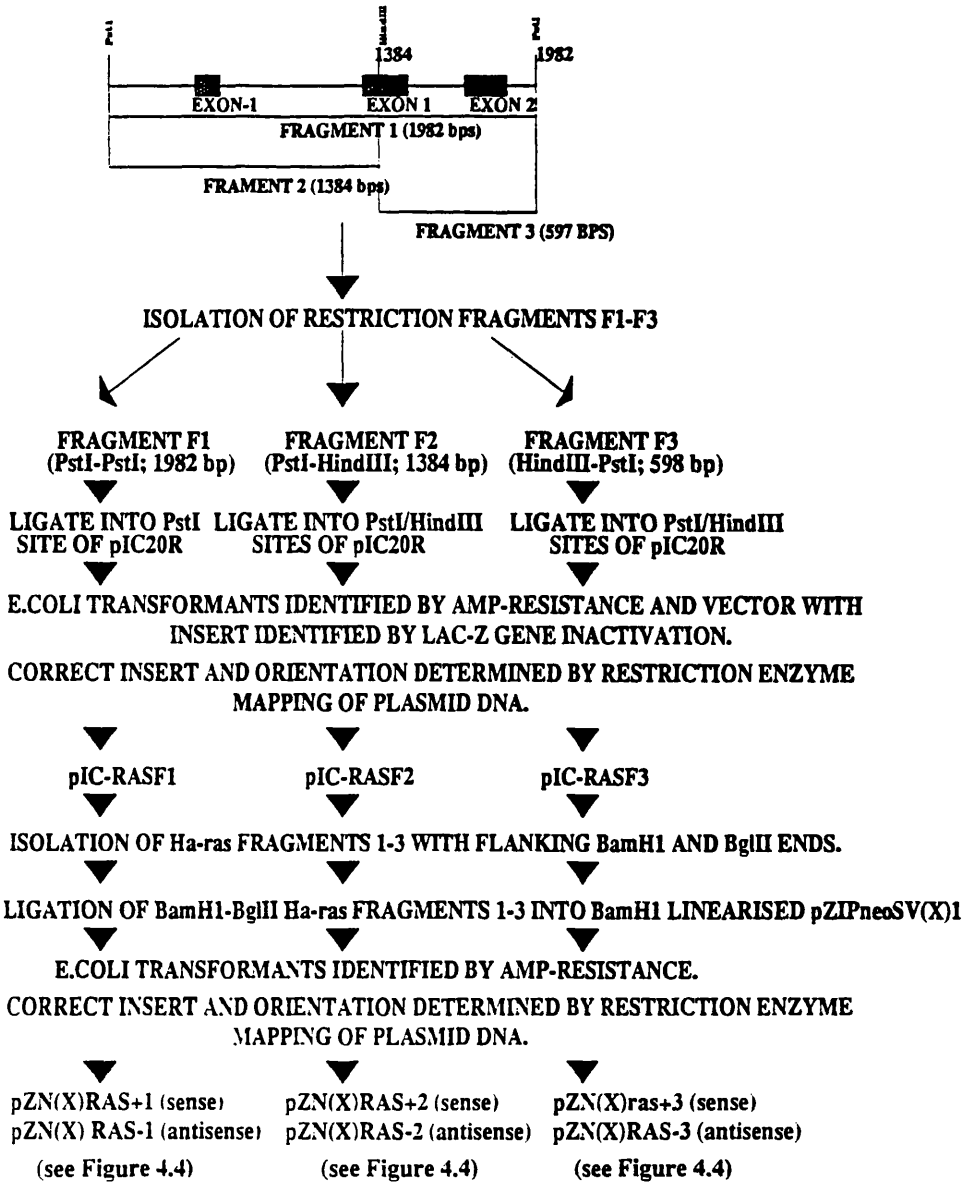


Figure 3.3.

Flow diagramm of the cloning of the sense and antisense pZN(X)RAS retroviral vectors.

The flow diagramm summarizes the cloning steps required in the construction of the sense (+) and antisense (-) pZN(X)RAS retroviral vectors.

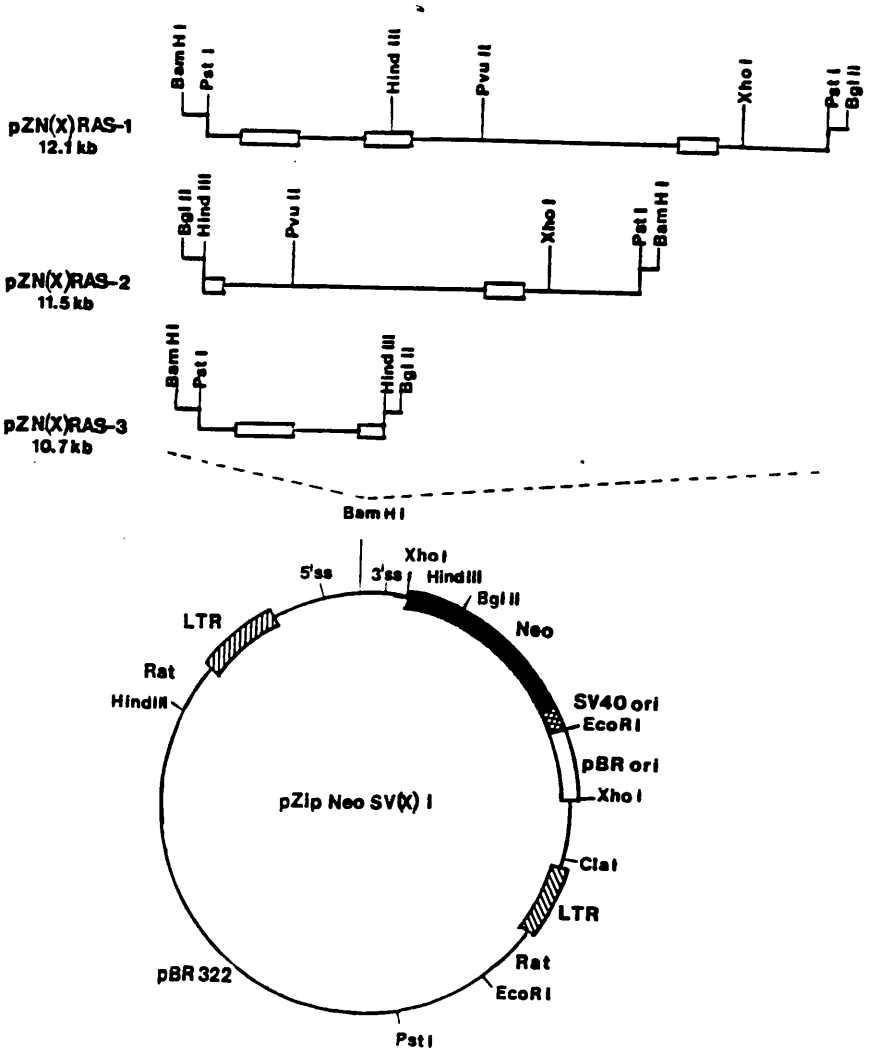


Figure 3.4.

Antisense pZN(X)RAS retroviral vector constructs.

Antisense pZN(X)RAS retroviral vectors are derived from the pZip Neo SV(X)1 retroviral vector (CEPKO et al., 1984) by inserting genomic c-Ha-*ras* gene fragments F1', F2' or F3' (figure 3.2), respectively, in the opposite orientation to the transcriptional direction defined by the 5' MoMuLV-LTR, into the Bam HI cloning site of the pZip Neo SV(X)1 vector (figure 3.1.). Recognition sites for several restriction enzymes are indicated and the sizes of the retroviral vector constructs pZN(X)RAS-1, pZN(X)RAS-2 and pZN(X)RAS-3 are given in kb.

Table 3.1.

Transfection efficiencies of Ψ2 cells transfected with ZN(X)RAS retroviral vector plasmid DNA.

(a): pZN(X)RAS are pZIP NEO SV(X)1-derived retroviral vectors containing genomic c-Ha-*ras* sequences in sense (+) or antisense orientation (-).

(b): Virus producing cell pools were generated by stable transfection of ZN(X)RAS retroviral vector DNA into Ψ2 packaging cells (MANN et al., 1983) using calcium phosphate co-precipitation (WIGLER et al., 1978). 5μg of each retroviral plasmid to be transfected was added to sheared genomic carrier DNA (human white blood cell DNA) and precipitated onto Ψ2 cells seeded out 24 hours previously at a cell density of $1x10^6$ cells per 75 cm^2 flask. Medium was replaced after 18 hours. After a further 48 hours, the transfected Ψ2 cells were trypsinized, counted and seeded at a density of $5x10^5$ cells per 10 cm^2 plate in the presence of 800μg/ml G418. After a selection period of two weeks, pools of virus producer cells were generated. As controls for the transfection procedure, negative (carrier DNA only) and positive controls (pAG60, a non-retroviral plasmid conferring G418-resistance; pZIP NEO SV(X)1, the parental retroviral vector) had been included. Transfection efficiency is the number of G418-resistant colonies observed per 10^6 viable cells replated into G418 selection after calcium phosphate transfection of Ψ2 cells with 5μg of plasmid DNA and 20μg carrier human white blood cell DNA.

(c): Due to initial difficulties in transfecting pZN(X)RAS+1 plasmid DNA, the results shown in brackets were from a later experiment using Ψ2 cells continuously selected in mycophenolic acid.

Transfection efficiencies of ψ2 cells transfected with ZN(X)RAS retroviral vector plasmid DNA.

Transfected retroviral vector plasmid DNA [a]	Transfection Efficiency [b] $G418^r$ - cfu/5μg DNA/10^6 viable cells
pAG60	8.3×10^3
pZip Neo SV(X)1	1.1×10^3
pZN(X) RAS + 1	0 (1.5×10^1) [c]
pZN(X) RAS - 1	1.6×10^2
pZN(X) RAS + 2	2.7×10^1
pZN(X) RAS - 2	1.8×10^2
pZN(X) RAS + 3	2.6×10^2
pZN(X) RAS - 3	6.3×10^2

into the same retroviral vector did not alter the expression of the *neo*-gene (CORY et al., 1987).

The virus titer released from each pool was assayed by determining the number of G418-resistant CT3 colony forming units following infection of CT3 cells. The number of $G418^r$-colonies was adjusted for re-plating efficiency, the fraction of infected cells plated out and the volume of virus supernatant used for infection to give the infection efficiency or titer as the number of $G418^r$-CT3 colony forming units per ml of virus supernatant per 10^6 viable infected cells ($G418^r$-CFU/ml/10^6 viable cells). Table 3.2 shows the titer of 7 virus producing pools averaged over up to three separate infection experiments. All viruses were able to efficiently infect CT3 cells to give G418-resistant colonies. Infectious virus particles were released by the virus producer cells at titers between $3.0x10^5$ to $1.6x10^6$ $G418^r$-cfu/ml/10^6 viable CT3 cells (table 3.2). These titers compare favourably with values published by CEPKO et al. (1984). The number of infectious virus particles released by pools of producer cells tends to be on average lower than the viral titer that can be achieved by clonal lines of producer cells (data not shown).

3.3.3. Northern Analysis of Antisense ZN(X)RAS Virus Infectants.

The ZN(X)RAS producing cell pools listed in tables 5.1 and 5.2 were analyzed for the expression of the exogenous c-Ha-*ras* sequences and *neo*-gene, both encoded in the ZN(X)RAS retroviral vector constructs. In Northern analysis of total RNA isolated from these transfectants, hybridization of an *in vitro* transcribed "antisense" *neo*-riboprobe (pBSneo was a gift from N. KEITH) detected a *neo*-specific transcript in Ψ2 cells transfected with the *neo*-gene-containing ZN(X)RAS retroviral constructs (figure 3.5). The detected messenger RNA species was consistent in size with being the spliced retroviral subgenomic RNA of about 4.2kb in length. A further, larger sized transcript was detected in each lane of RNA from ZN(X)RAS transfected Ψ2 cells. The larger RNAs differed in size but again the respective size was consistent with being the full-length retroviral genomic RNAs of the ZN(X)RAS proviruses present in the transfectants. The observed size variations corresponded with the differences in size of the c-Ha-*ras* fragments F1' to F3' which were inserted into the BamHI site of the basic pZip Neo

Efficiency of infection of CT3 and SEP14 cells by recombinant sense and antisense *ras* retroviral vectors.

Recombinant retroviral vectors (a)	Infection efficiency $G418^r$-cfu/ml/10^6 viable cells (b)	
	CT3 cells	SEP14 cells
Zip Neo SV(X)1	2.3×10^6	1.5×10^6
ZN(X) RAS+1	1.5×10^6	9.2×10^5
ZN(X) RAS-1	3.0×10^5	2.8×10^5
ZN(X) RAS+2	5.6×10^5	6.7×10^4
ZN(X) RAS-2	1.6×10^6	2.5×10^5
ZN(X) RAS+3	4.7×10^5	1.5×10^5
ZN(X) RAS-3	7.8×10^5	1.8×10^5

Table 3.2.

Efficiency of infection of CT3 and SEP14 cells by recombinant sense and antisense *ras* containing retroviral vectors.

(a): ZN(X)RAS are ZIP NEO SV(X)1-derived retriviral vectors containing genomic c-Ha-*ras* sequences in sense (+) or antisense orientation (-).

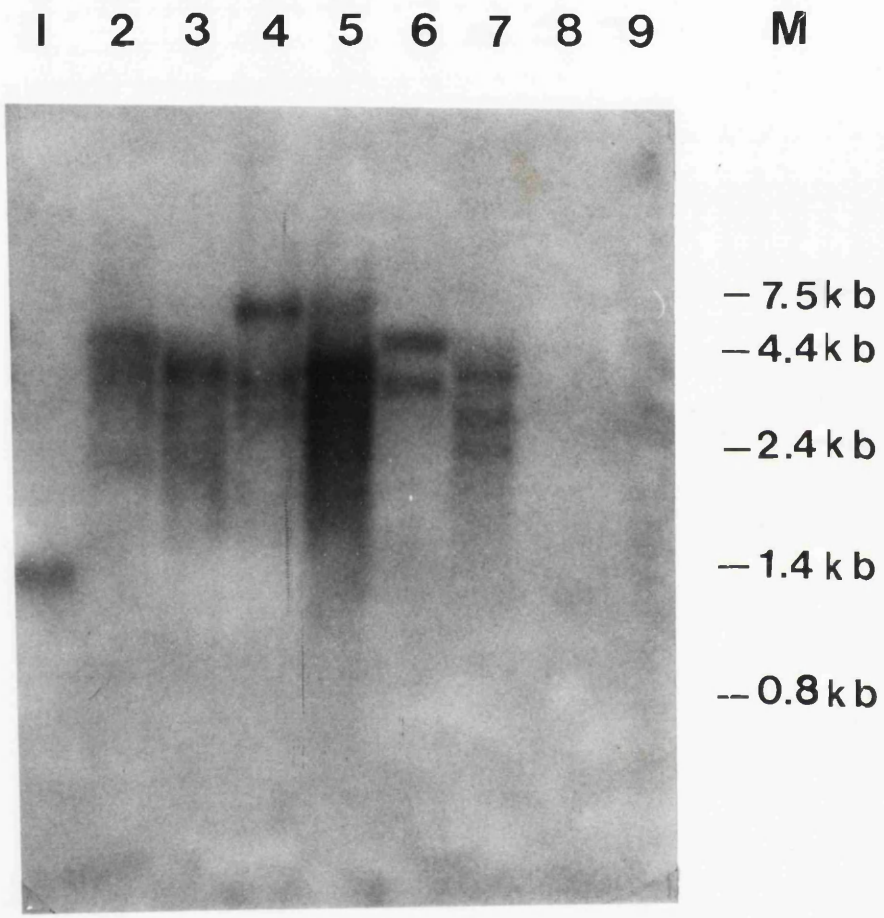
(b); Virus-producing Ψ2 pools were plated out at subconfluent cell density ($5x10^5$ cells per 25 cm^2 TC flask) in 5ml of fresh, non-selective media. After 24 hours the media was removed and used immediately for virus assay. A 200µl aliquot of virus supernatant, in a total volume of 7ml containing 24µg/ml polybrene, was added to CT3 fibroblast cells seeded out 24 hours previously at $5x10^5$ cells per F25 flask. Cells were washed with PBS and 5-7ml of fresh media was added 24 hours after infection. After an expression period of 48 hours, the CT3 cells were trypsinized, counted and seeded at $1x10^5$, $1x10^4$ and $1x10^3$ cells per $10cm^2$ plate in the presence of 0.8mg/ml G418. The plates were incubated for two weeks, with a medium change after the first week, and the frequency of $G418^r$-colony formation was calculated. Infection efficiency is the number of G418-resistant colonies observed per 10^6 viable cells replated into G418 selection after infection of CT3 or SEP14 cells with 200µl of viral supernatant from Ψ2 cells producing the recombinant retrovirus shown.

Figure 3.5.

Northern analysis of Ψ2 cells transfected with pZN(X)RAS retroviral vectors.

10μg of total cellular RNA, isolated from Ψ2 cells transfected with sense (+) or antisense (-) pZN(X)RAS retroviral vector plasmid DNA, were electrophoresed, Northern blotted and hybridized with the antisense *neo* riboprobe, transcribed *in vitro* by T3 RNA polymerase from SstI-linearized pBS NEO plasmid DNA. The plasmid pBS NEO was cloned by inserting a 1kb BglII-SmaI fragment of the neo^r gene from plasmid p61cl-24 into BamHI-HincII linearized bluescribe vector in sense orientation to the T7 promoter (N. KEITH Ph.D. Thesis, 1988).

Lane 1: total RNA form Ψ2 cells transfected with pAG60 plasmid DNA. Lane 2: total RNA from Ψ2 cells transfected with pZN(X)RAS-3 plasmid DNA (Ψ2RAS-3 producer cells). Lane 3: total RNA from Ψ2 cells transfected with pZN(X)RAS+3 plasmid DNA (Ψ2RAS+3 producer cells). Lane 4: total RNA from Ψ2 cells transfected with pZN(X)RAS-2 plasmid DNA (Ψ2RAS-2 producer cells). Lane 5: total RNA from Ψ2 cells transfected with pZN(X)RAS+2 plasmid DNA (Ψ2RAS+2 producer cells). Lane 6: total RNA from Ψ2 cells transfected with pZN(X)RAS-1 plasmid DNA (Ψ2RAS-1 producer cells). Lane 7: total RNA from Ψ2 cells transfected with pZip Neo SV(X)1 plasmid DNA (Ψ2SV(X)1 producer cells). Lane 8: total RNA from untransfected SEP14 cells. Lane 9: total RNA from untransfected Ψ2 cells. Lane M: RNA size marker (Gibco/BRL) *in vitro* transcribed in the presence of ^{32}P-rUTP. The RNA size marker indicated the migartion distance of 9.5kb, 7.5kb, 4.4kb, 2.4kb, 1.4kb and 0.8kb RNA molecules.


1 2 3 4 5 6 7 8 9 M
7.5kb
4.4kb
2.4kb
1.4kb
0.8kb

SV(X)1 retroviral vector to generate the ZN(X)RAS retoviral constructs (figures 5.3 and 5.4). These results confirm the expression of the ZN(X)RAS retroviral vector construct encoded *neo*-gene via the subgenomic RNA species and are consistant with expression of the 5'-inserted genomic c-Ha-*ras* sequences via the full-length RNA species of the transcriptional active ZN(X)RAS proviral constructs after transfection into Ψ2 recipient cells: a spliced subgenomic RNA of around 4.2kb in length and full-length genomic RNAs of varying sizes depending on the size of the Ha-*ras* fragment present in the ZN(X)RAS retroviral constructs (figure 3.4). The *neo*-gene is transcribed into the spliced message and the genomic c-Ha-*ras* fragments are transcribed as part of the full-length RNA species.

Non-specific effects of c-Ha-*ras* antisense RNA detecting riboprobes, hybridizing presumably to ribosomal RNA, made the analysis of antisense c-Ha-*ras* RNA expression from the ZN(X)RAS proviruses more difficult (Figures 5.6a and 5.6b). Nevertheless, riboprobes designed to detect antisense c-Ha-*ras* F2' and F3' expression were used successfully to demonstrate antisense RNA expression of ZN(X)RAS-2 and ZN(X)RAS-3 retroviral constructs present in the Ψ2 transfectants. The riboprobe constructs pBS19rasF2 (F2) and pBS19rasF3 (F3) were cloned by inserting c-Ha-*ras* fragments F2 and F3 into the bluescribe vector pBS19 (Stratagene), which had previously been linearized in its pUC19 polylinker region by a PstI/HindIII double restriction digest. The F2-riboprobe, *in vitro* transcribed from the HindIII-linearized pBS19rasF2 plasmid by T7 RNA polymerase, hybridized to a transcript of the expected size of about 5.5kb only in the lane which contained total RNA from ZN(X)RAS-2 transfectants (figure 3.6a). Similarly, F3-riboprobe, *in vitro* transcribed by the T3 RNA polymerase from the PstI-linearized pBS19rasF3 plasmid, detected a transcript of around 4.7kb in RNA isolated from ZN(X)RAS-3 retroviral construct containing Ψ2 transfectants, representing c-Ha-*ras* fragment F3' antisense RNA expression in ZN(X)RAS-3 transfectants (figure 3.6b). The F2- and F3-riboprobes did not hybridize to mRNA transcripts in lanes containing total RNA from ZN(X)RAS+2 and ZN(X)RAS+3 transfectants. No ZN(X)RAS specific retroviral gene expression was detected with either of the riboprobes in uninfected Ψ2 and SEP14 cells (figure 3.6). Attempts were made to analyze ZN(X)RAS-1

Figure 3.6.

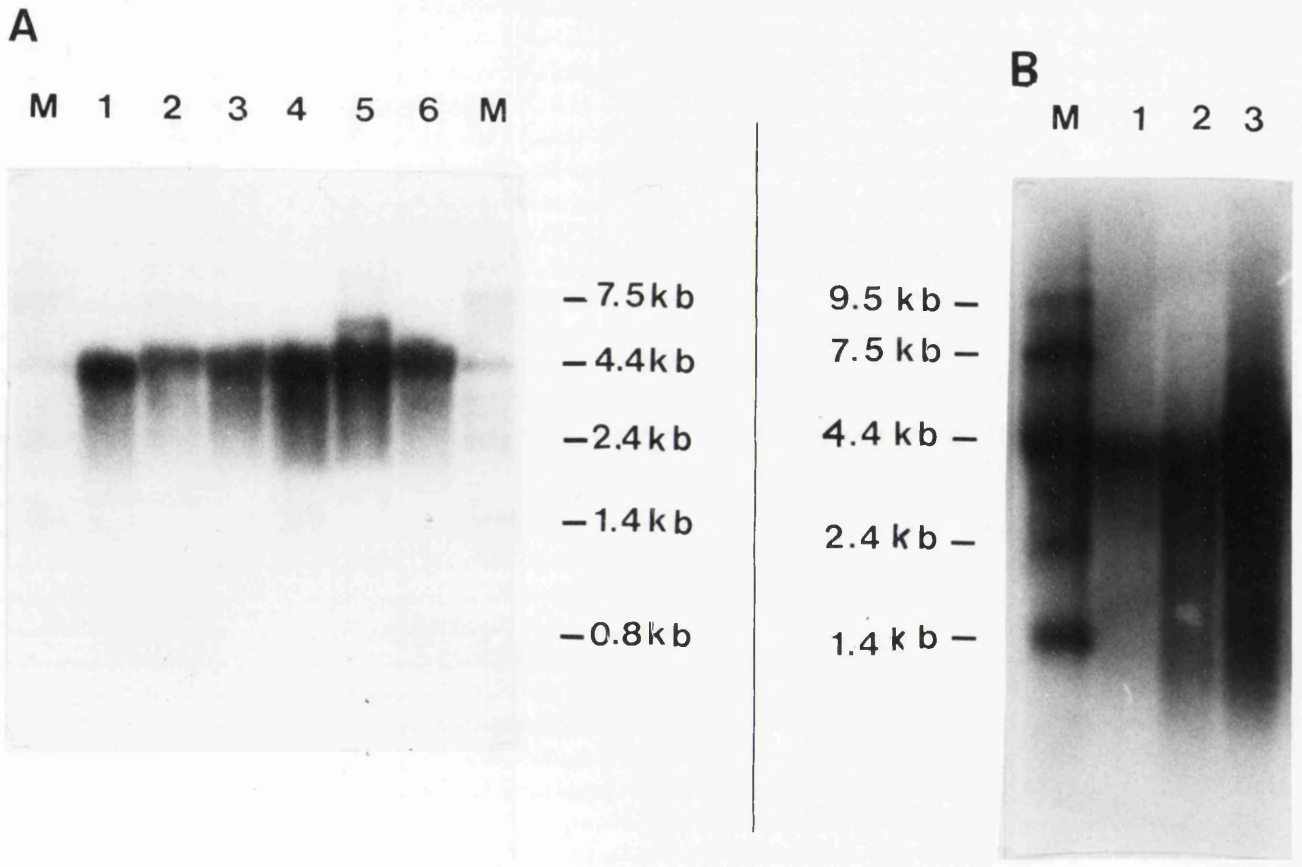
Northern analysis of Ψ2 cells transfected with pZN(X)RAS retroviral vector plasmid DNA for antisense c-Ha-*ras* RNA expression.

15μg of total RNA isolated from sense (+) and antisense (-) ZN(X)RAS transfected Ψ2 cells were electrophoresed, Northern blotted and hybridized with *in vitro* transcribed sense F2-riboprobe (figure 3.6a) or sense F3-riboprobe (figure 3.6b). The riboprobes have been descibed in the text.

Figure 3.6a: Lane M: *in vitro* ^{32}P-rUTP labelled RNA size marker (see legend figure 5.5). Lane 1: total RNA isolated from untransfected CT3 cells. Lane 2: total RNA isolated from untransfected Ψ2 cells. Lane 3: total RNA isolated from Ψ2SV(X)1 producer cells (figure 3.5). Lane 4: total RNA isolated from Ψ2RAS+2 producer cells (figure 3.5). Lane 5: total RNA isolated from Ψ2RAS-2 producer cells (figure 3.5). Lane 6: total RNA isolated from untransfected SEP14 cells.

Figure 3.6b: Lane M: *in vitro* ^{32}P-rUTP labelled RNA size marker (see legend figure 5.5). Lane 1: total RNA isolated from untransfected Ψ2 cells. Lane 2: total RNA isolated from Ψ2RAS+3 produced cells (figure 3.5). Lane 3: total RNA isolated from Ψ2RAS-3 producer cells (figure 3.5).

The sense F2-riboprobe detected a transcript of the size expected for full-length ZN(X)RAS-2 RNA (5.5kb) in lanes containing total RNA isolated from pZN(X)RAS-2 transfected Ψ2 cells (figure 3.6a, lane 5). No signal was detected in pZN(X)RAS+2 transfected Y2 cells and in RNA isolated from untransfected Ψ2, CT3 or SEP14 cells (figure 3.6a). In figure 3.6b, the sense F3-riboprobe detected a transcript of the size expected for full-length (ZN(X)RAS-3 RNA (4.7kb) in lanes containing total RNA isolated from Ψ2RAS-3 producer cells (lane 3), no such transcript was detected in total RNA from Y2RAS+3 producer cells (lane 2) or from untransfected Ψ2 cells (lane 1). Hybridization to transcripts of about 4.4kb in size, detected each lane (figures 3.6a and b), repesented non-specific hybridization of the F2 and F3-riboprobes to presumably ribosomal RNA.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retroviral vector transfectants but due to non-specific hybridization, antisense specific transcripts could not be clearly identified (see also later discussion on possible rapid turn-over of c-Ha-*ras* fragment F1'-antisense RNA.).

Northern analysis was performed on total RNA isolated from ZN(X)RAS infected SEP14 and CT3 cells and uninfected parental SEP14 and CT3 cells. The F2- and F3-riboprobes were used for hybridization, their specificity for detection of c-Ha-*ras* antisense RNA transcripts had been demonstrated during RNA analysis on ZN(X)RAS transfectants figure 3.6a and 5.6b).

Non-specific effects of both riboprobes hybridizing presumably to ribosomal RNA was again observed (figure 3.7a and b). However, using the F2-riboprobe, a transcript was detected in lanes containing RNA from ZN(X)RAS-2 infected SEP14 and CT3 cells. The transcript was consistent in size with being the full-length genomic RNA transcribed from the ZN(X)RAS-2 recombinant provirus. No transcript was detected in RNAs isolated from uninfected SEP14 and CT3 cells nor cells infected with the ZN(X)RAS+2 virus (figure 3.7a). Corresponding results were obtained for ZN(X)RAS-3 infected SEP14 and CT3 cells. The F3-riboprobe hybridized to a transcript of the size expected for full-length genomic ZN(X)RAS-3 retroviral vector RNA in lanes containing RNA from ZN(X)RAS-3 infected SEP14 and CT3 cells and Ψ2 cells transfected with the ZN(X)RAS-3 retroviral construct. No hybridization signal was detected in lanes containing total RNA isolated from either ZN(X)RAS+3-infectants or the ZN(X)RAS+3 transfected Ψ2 cells, nor the parental Ψ2, SEP14 and CT3 cells (figure 3.7b).

Northern analysis on RNAs from ZN(X)RAS-1 infected SEP14 cells had been performed repeatedly. However, at no time was a c-Ha-*ras* F1'-antisense RNA specific transcript detected. Figure 3.8 shows that even under conditions where c-Ha-*ras* F2'-antisense RNA expression in ZN(X)RAS-2 infectants was readily detectable, using the F2-riboprobe, the predicted larger-sized c-Ha-*ras* F1'-antisense RNA transcript of the ZN(X)RAS-1 provirus was not detected. It could be speculated that the failure to detect expression of a 6.7kb c-Ha-*ras* F1' antisense RNA was due to the binding of the antisense transcript to its target, the cellular c-Ha-*ras* mRNA, resulting in an RNA:RNA duplex, which is rapidly degraded by double-strand specific ribonucleases like

Figure 3.7.

Northern analysis of SEP14 and CT3 cells infected with sense and antisense ZN(X)RAS retroviral vectors.

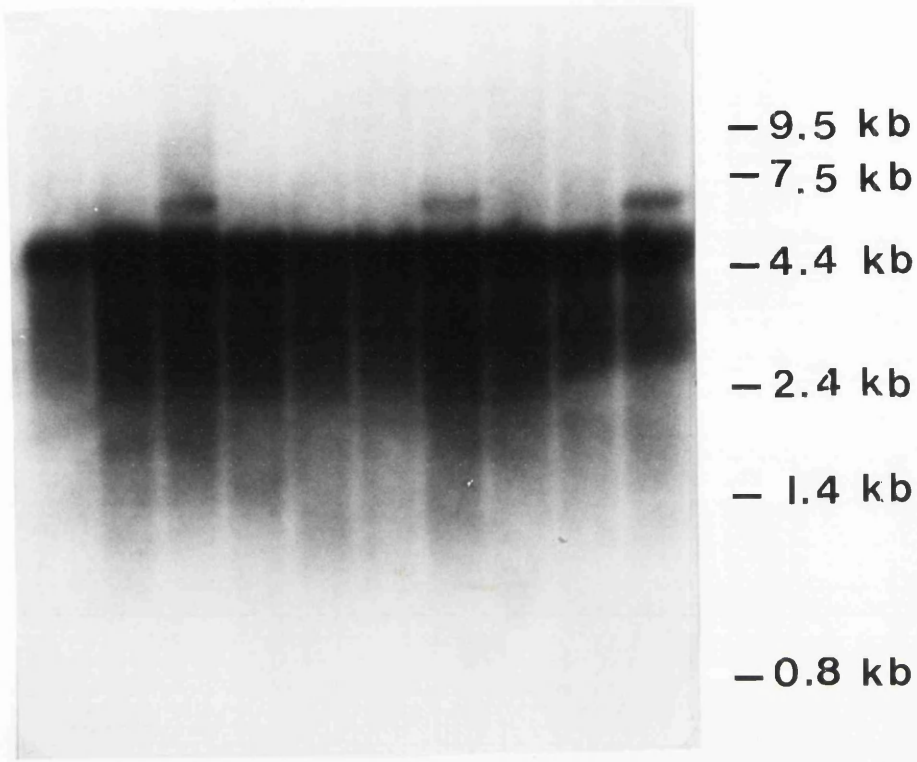
15μg of total RNA, isolated from SEP14 and CT3 cells infected with sense(+) or antisense (-) ZN(X)RAS retoviral vectors, were electrophoresed, Northern blotted and hybridized with *in vitro* transcribed sense F2-riboprobe (figure 3.7.a) or sense F3-riboprobe (figure 3.7b).

Figure 3.7a: Lane 1: total RNA isolated from untransfected Ψ2 cells. Lane 2:
total RNA isolated from Ψ2RAS+2 producer cells (figure 3.5). Lane 3: total RNA isolated from Ψ2RAS-2 producer cells (figure 3.5). Lane 4: total RNA isolated from uninfected CT3 cells. Lanes 5 and 6: total RNA isolated from two separate pools of CT3 cells infected with ZN(X)RAS+2 retroviral vector. Lane 7: total RNA isolated from CT3 cells infected with ZN(X)RAS-2 retroviral vector. Lane 8: total RNA isolated from uninfected SEP14 cells. Lane 9: total RNA isolated from SEP14 cells infected with ZN(X)RAS+2 retroviral vector. Lane 10: total RNA isolated from SEP14 cells infected with ZN(X)RAS-2 retroviral vector. M: ^{32}P-rUTP labelled RNA size marker.

Figure 3.7b: Lane 1: total RNA isolated from untransfected Ψ2 cells. Lane 2: total RNA isolated from Ψ2RAS+3 producer cells (figure 3.5). Lane 3: total RNA isolated from Ψ2RAS-3 producer cells (figure 3.5). Lane 4: total RNA isolated from uninfected CT3 cells. Lane 5: total RNA isolated from CT3 cells infected with ZN(X)RAS+3 retroviral vector. Lane 6: total RNA isolated from CT3 cells infected with ZN(X)RAS-3 retroviral vector. Lane 7: total RNA isolated from uninfected SEP14 cells. Lane 8: total RNA isolated from SEP14 cells infected with ZN(X)RAS+3 retroviral vector. Lane 9: total RNA isolated from SEP14 cells infected with ZN(X)RAS-3 virus. Lane M: ^{32}P-rUTP labelled RNA size marker.

In figure 3.7a, hybridization with the sense F2-riboprobe detected a transcript of the size expected for full-length ZN(X)RAS-2 RNA (5.5kb) in lanes containing total RNA from Ψ2RAS-2 producer cells (lane 3) and from CT3 and SEP 14 cells infected with ZN(X)RAS-2 retroviral vector (lanes 7 and 10).No such transcript was deteceted in lanes containing RNA from Ψ2RAS+2 producer cells (lane 2), uninfected SEP14 and CT3 cells (lanes 8 and 4), or ZN(X)RAS+2 infected SEP14 and CT3 cells (lanes 9, 5, and 6). In figure 3.7b, hybridization with the sense F3-riboprobe detected a transcript of the size expected for full-length ZN(X)RAS-3 RNA (4.7kb) in lanes containing total RNA from Ψ2RAS-3 producer cells (lane 3), and from CT3 and SEP14 cells infected with ZN(X)RAS-3 retroviral vector (lanes 6 and 9). No such transcript was detected in lanes containing RNA from Ψ2RAS+3 producer cells (lane 2), uninfected SEP14 and CT3 cells (lanes 7 and 4), or ZN(X)RAS+3 infected SEP14 or CT3 cells (lanes 8 and 5). Hybridization to transcripts of about 4.4 kb in size, detected in each lane, represented non-specific hybridization of the riboprobes F2 and F3 presumably to ribosomal RNA.

A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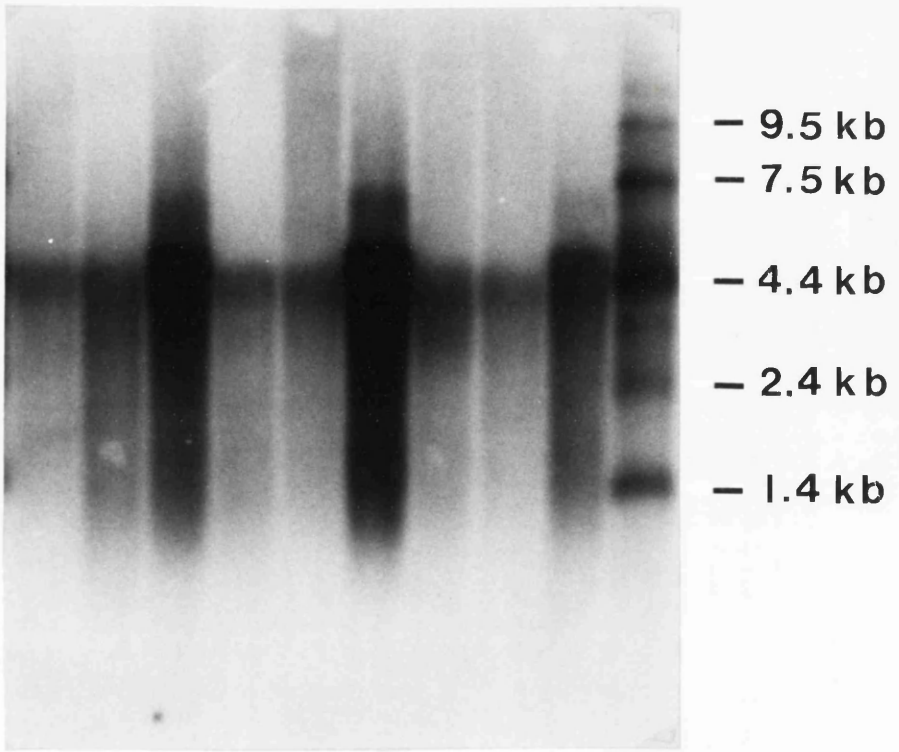
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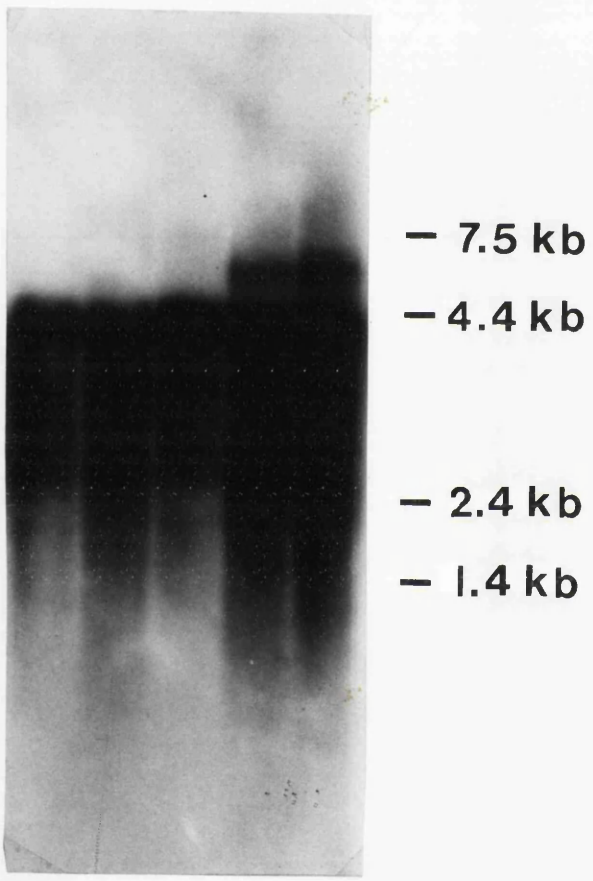
Figure 3.8.

Northern analysis of SEP14 cells infected with ZN(X)RAS-1 retroviral vector.

15μg of total RNA isolated from SEP14 cells infected with antisense (-) and sense (+) ZN(X)RAS retroviral vectors were electrophoresed, Northern blotted and hybridized with *in vitro* transcribed sense F2-riboprobe. Lane 1: total RNA isolated from uninfected SEP14 cells. Lane 2: total RNA isolated from SEP14 cells infected with ZN(X)RAS-1 retroviral vector. Lane 3: total RNA isolated from SEP14 cells infected with ZN(X)RAS+2 retroviral vector. Lanes 4 and 5: total RNAs isolated from two independent pools of SEP14 cells infected with ZN(X)RAS-2 retroviral vector. M: ^{32}P-rUTP labelled RNA size marker.

Under conditions where c-Ha-*ras* F2'-antisense specific transcripts were readily detected by hybridization with *in vitro* transcribed sense F2-riboprobe in total RNA isolated from ZN(X)RAS-2 infected SEP14 cells (lanes 4 and 5), no c-Ha-*ras* F1'-antisense transcript was detected in lane 2 containing total RNA from ZN(X)RAS-1 infected SEP14 cells. Hybridization to transcripts of about 4.4kb, detected in each lane, represented non-specific hybridisation of the F2-riboprobe to presumable ribosomal RNA.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RNaseIII (chapter 5.1.4.). Figure 3.5 has shown that a larger, full-length genomic RNA transcript can be detected in Ψ2 cells transfected with the ZN(X)RAS-1 retroviral vector construct, consistent with c-Ha-*ras* F1'-antisense RNA being expressed from the transfected retroviral construct. As will be described later, it is also interesting to note, that the ZN(X)RAS-1 virus, when infected into SEP14 cells, has given the most significant reversals of transformation characteristics.

In summary, retroviral constructs were made containing genomic fragments of the murine c-Ha-*ras* gene in sense and antisense orientation. These retroviral vectors were able to infect cells with high efficiency *in vitro* (table 3.2). Antisense RNA expression of F2' and F3' were shown (figures 5.6, 5.7 and 5.8). Although antisense F1' RNA appeared to be expressed as part of the full-length viral transcript of ZN(X)RAS-1 virus, a transcript corresponding to antisense F1' mRNA was not observed in steady-state level.

3.4. Inhibition of Cell Transformation by Genomic c-Ha-*ras* Antisense RNA.

Two cell lines, the non-transformed cell line CT3 and the highly transformed cell line SEP14, were used to assay possible effects of constitutive expression of c-Ha-*ras* antisense RNA, under the control of MoMuLV-LTR, on the expression levels of cellular Ha-ras proto-oncogene and/or its oncogenic form activated by a codon 61 mutation. Changes in cellular phenotype and anchorage independent growth properties were assayed.

The cell line CT3 had been obtained from G.M. COOPER's laboratory (COPELAND et al., 1979). The CT3 cell line is one of several lines derived from mouse NIH 3T3 fibroblasts with unlimited life-span. The cell line NIH 3T3 was established from clones of primary NIH mouse embryo fibroblasts which survived crisis while being passaged frequently for a long period of time at low cell density ($3x10^5$ cells replated every 3 days) (JAINCHILL et al, 1969).

Highly transformed SEP14 cells were derived from an NIH 3T3 focus following transfection of DNA isolated from a DMBA/TPA initiated and promoted mouse skin papilloma containing a codon 61-activated c-Ha-*ras*

oncogene. SEP14 cells have a highly transformed phenotype, produce large amounts of mutant p21 Ha-ras protein and are highly tumorigenic in NIH 3T3 mice (tumours develop within 8 days after subcutaneous injection) (QUINTANILLA et al., 1986).

An additional cell line, SEP11, was included in the c-Ha-*ras* antisense RNA study. The clonal line SEP11, generated following the same protocol as for SEP14, had a less transformed phenotype than SEP14, which was reflected in reduced tumorigenicity in NIH mice and the lower amounts of mutant p21 Ha-ras protein detectable. Unfortunately, for reasons not understood, SEP11 cells had lost their transformation properties as became clear following careful characterization *in vivo* (tumorigenicity in NIH mice) and *in vitro* (focus formation). After putting the cells through renewed foci selection (5% FBS; confluency for 2 to 3 weeks; medium changes every 3 to 4 days), a more transformed appearing SEP11 sub-line could be established. However, tumorigenicity tests with this SEP11 subline in nude mice were negative (data not presented). Therefore, efforts to re-establish the transformed phenotype of SEP11 failed and results from SEP11 cells were left out of the data presented.

3.4.1. Effects of c-Ha-*ras* Antisense RNA Expression on Transformation Phenotype.

SEP14 cells were infected with the c-Ha-*ras* antisense retroviral vectors to assess possible effects of the expression of the antisense RNA on the morphology of the transformed SEP14 cells. As controls, retroviral vectors expressing the c-Ha-*ras* fragments in sense were included in the infection experiments. Additionally, the quality of the virus stocks was tested by infecting CT3 cells in parallel to allow the determination of the viral titer of each stock at the time of infection of SEP14 cells. The infection efficiencies are summarized in table 3.3. The infection efficiency values obtained for SEP14 cells were between 20% to 60% lower than those calculated for CT3 cells, showing that SEP14 are less readily infectable than CT3 cells.

$G418^r$-SEP14 colonies obtained after G418 selection following infection of SEP14 cells with recombinant sense or antisense ZN(X)RAS retroviruses, displayed various degrees of transformation. Within a single $10cm^2$ plate, colonies with a broad range of phenotypic morphology, from

Ratio of morphologically untransformed to transformed colonies of SEP14 cells after infection with sense and antisense *ras* retroviral vectors.

Recombinant retroviral vectors (a)	Percentage viability (b)	U/T ratio (c)	Percentage c U/T ratio (d)
uninfected	13.3	4.8 (266)	0
ZN(X) RAS-1	6.0	1.7 (135)	-64.5
ZN(X) RAS+2	20.5	3.9 (107)	-18.8
ZN(X) RAS-2	15.5	4.0 (134)	-16.7
ZN(X) RAS+3	14.0	4.5 (171)	-6.3
ZN(X) RAS-3	18.0	4.3 (386)	-10.4

Table 3.3.

Ratio of morphologicallly untransformed to transformed colonies of SEP14 cells after infection with sense and antisense *ras* containing retroviral vectors.

(a): ZN(X)RAS are ZIP NEO SV(X)1-derived retroviral vectors containing genomic c-Ha-*ras* sequences in sense (+) or antisense orientation (-).

(b): The cell viability of cells was tested by replating 200 cells per $10cm^2$ plate into non-selective growth medium. The number of colonies observed 10 to 14 days after replating is expressed as percentage of number of cells replated.

(c): SEP14 cells infected with ZN(X)RAS viruses had been replated into G418 selection 48 hours after infection at cell densities of 10^5 or 104 cell per $10cm^2$ plate. Uninfected SEP14 cells were replated into non-selecting growth medium at cell densities of 200 to 500 cells per $10cm^2$ plate. The number of colonies and their morphology was scored 10 to 14 days after replating. The ratio of morphologically untransformed to transformed colonies observed after infection of SEP14 cells with sense and antisense *ras* retroviral vectors or replating of uninfected SEP14 cells at low cell density. Numbers in brackets are the total number of colonies scored.

(d): Percentage change in untransformed to transformed ratio compared to uninfected SEP14 cells.

highly transformed to flat, almost revertant-like phenotype, were found. The abundance of phenotypically untransformed (U) to transformed (T) colonies was expressed as the U/T ratio. Uninfected SEP14 cells, plated at low cell density in non-selective medium, formed colonies within 10 to 14 days. These SEP14 colonies were not homogeneous in their degree of transformation but showed also a wide spectrum of transformed phenotypes (table 3.3). Even recloning of phenotypically transformed SEP14 colonies did not give uniformly transformed colonies in subsequent colony formation experiments. Uninfected SEP14 colonies had a U/T ratio of 4.8 (untransformed colonies to every transformed colony), a value which was used as the reference against which any changes of the U/T ratio observed in ZN(X)RAS retrovirus infected SEP14 colonies were evaluated. Results are summarized in table 3.3.

No significant alterations in the U/T ratio was observed in SEP14 colonies derived after infection with ZN(X)RAS+3 (U/T ratio: 4.5) or ZN(X)RAS-3 (U/T ratio: 4.3) when compared either to each other or to the U/T value of uninfected SEP14 colonies (U/T ratio: 4.8) (table 3.3).

A slight difference in the distribution of flat to transformed colonies was observed in SEP14 cells infected with ZN(X)RAS+2 (U/T ratio: 3.9) and ZN(X)RAS-2 virus (U/T ratio: 4.0) in relation to uninfected SEP14 cells (U/T ratio: 4.8). The changes in the U/T ratios could not be attributed to the expression of the antisense RNA, as infection with either sense or antisense recombinant retrovirus resulted in the same degree of reduction of the U/T value (table 3.3).

There was, however, a striking difference in the U/T ratio between SEP14 infected with ZN(X)RAS-1 virus and uninfected cells. ZN(X)RAS-1 infected, G418^r-SEP14 cells had a U/T value of 1.7, representing a 64.5% reduction compared to the U/T ratio of 4.8 of uninfected SEP14 cells (table 3.3). Unfortunately, cell pools producing ZN(X)RAS+1 infectious virus had not yet been successfully established at the time these experiments were performed, as it proved extremely difficult to generate a ZN(X)RAS+1 virus producing cell pool by transfection of Ψ2 cells (table 3.1). Thus, a direct comparison of alterations of U/T ratios as an effect of the expression of genomic c-Ha-*ras* fragment F1 as sense or antisense RNA was not possible. Subsequent experiments of cell transformation used soft-agar cloning of

SEP14 cells infected with sense and antisense ZN(X)RAS1 virus. Nevertheless, the observed 64.5% reduction in the U/T value seemed too significant to be explained solely by the presence of the Zip Neo SV(X)1 derived provirus. Furthermore, G418^r-SEP14 colonies containing sense or antisense ZN(X)RAS2 or ZN(X)RAS3 proviruses did not show nearly the same extent of U/T ratio reduction. As all c-Ha-*ras* sense and antisense ZN(X)RAS proviruses share the same basic retroviral vector, it seemed unlikely that the Zip Neo SV(X)1 vector sequences should have caused the dramatic reduction in the U/T value observed in ZN(X)RAS-1 infected G418^r-SEP14 cells. However, the scoring of phenotypically transformed or untransformed colonies was highly subjective. Therefore, soft agar cloning was used in subsequent experiments to assess possible reductions in cell transformation by changes in anchorage independent growth properties.

3.4.2. Effects of c-Ha-*ras* Antisense RNA Expression on Soft Agar Cloning Ability.

One of the definitions of cellular transformation is the ability of transformed cells to grow in an anchorage-independent manner in semi-solid medium. Normal fibroblasts do not proliferate when suspended in a gel or semi solid growth medium, such as soft agar, as fibroblastic cell division requires attachment to a solid surface. Fully transformed fibroblasts, however, grow anchorage-independently and readily form colonies under semi solid growth conditions (SHIH et al., 1979, 1981; COOPER et al., 1980; KRONTIRIS and COOPER, 1981).

Several independent pools of SEP14 and CT3 cells, generated after individual infections with either of the sense or antisense ZN(X)RAS recombinant retroviral vectors followed by G418-selection, were replated in semi-solid growth medium (0.3% agar). Changes in the efficiency of colony formation were assessed after a 4-week-incubation period and selective staining of viable colonies with 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (1.0mg/ml in PBS) (BOL et al., 1977). INT staining is specific for viable cells, as only these are able to metabolize INT following uptake into the cell. The metabolic intermediate of INT is a red stain, causing viable colonies to appear red (BOL et al., 1977). Red colonies

were scored and used to calculate soft agar cloning efficiency of the different SEP14 and CT3 cell pools. The results of three soft agar cloning experiments with ZN(X)RAS-infected SEP14 pools (experiments 1 to 3) and two experiments with ZN(X)RAS-infected CT3 pools (experiments 2 and 3) are listed in table 3.4. Typical values regarding virus titer and infection efficiencies obtained after infection and G418-selection of SEP14 cells of the recombinant ZN(X)RAS virus stocks used were described earlier (table 3.2). ZN(X)RAS-infected SEP14 and CT3 pools were replated into semi-solid medium in parallel (experiments 2 and 3). Soft agar cloning efficiencies achieved by uninfected SEP14 or CT3 cells were used as the standard against which soft agar colony formation efficiencies of ZN(X)RAS-infected cell pools were assessed.

In experiments 1 and 2, the most prominent reduction in soft agar cloning efficiency was seen with $G418^r$-pools of ZN(X)RAS-1 infected SEP14 cells. The observed reductions were -67.3% and -54.9%, respectively (table 3.4). Reductions to a lesser extent of colony formation in soft agar by -39.0% and -30.5% were observed in ZN(X)RAS-2 infected SEP14 cells. No conclusions on changes in anchorage independent growth could be drawn with ZN(X)RAS-3 infected SEP14 cells, as these repeatedly failed to grow in 10% FBS-containing growth medium in culture and thus could not be assayed in soft agar. The batch of FBS used was changed during the course of the study and the failure of ZN(X)RAS3 infected SEP14 cells to grow presumably was due to differences in the growth factors present in the FBS.

The dramatic reduction in colony formation of ZN(X)RAS-1-infected SEP14 cells in semi-solid medium, seen in experiments 1 and 2 (table 3.4), could only indirectly be attributed to the expression of c-Ha-*ras* fragment F1' antisense RNA. The appropriate control of ZN(X)RAS+1-infected SEP14 cells was not available, as a ZN(X)RAS+1-producer cell line had still not been successfully established. Any effects of the presence of ZN(X)RAS-1 provirus in SEP14 cells on colony formation in soft agar were compared to the soft agar cloning efficiency of uninfected SEP14 cells. However, the reduction in soft agar cloning efficiencies of -67.3% and -54.9% appeared too significant to be attributed solely to the presence of the Zip Neo SV(X) basic retroviral vector. This was confirmed in experiment 3, where the ZN(X)RAS+1 control

Table 3.4.

Soft agar cloning efficiency of SEP14 cells infected with sense or antisense *ras* containing retroviral vectors.

(a): The designated number of each experimental series (see text).

(b): The cell line infected with sense or antisense *ras* retroviral vector.

(c): ZN(X)RAS are ZIP NEO SV(X)1-derived retroviral vectors containing genomic c-Ha-*ras* sequences in sense (+) or antisense orientation (-).

(d): Cell viability was tested by replating 200 cells per $10cm^2$ plate into non-selective growth medium. The number of colonies observed 10 to 14 days after plating is expressed as percentage of number of cells replated.

(e): ZN(X)RAS-infected and uninfected SEP14 cells were replated into 0.3% soft agar at cell densities of 10^4, 10^3 or $5x10^2$ cells per $6cm^2$ plate. Soft agar plates were incubated for up to three weeks (5% CO_2, 37°C, humid atmosphere) before viable colonies were stained with INT and scored. The number of viable colonies observed growing in soft agar expressed as percentage of number of cells replated. One standart error of the mean is shown. Number in brackets are the number of experiments.

(f): The percentage change in soft agar cloning efficiency compared to uninfected SEP14 cells.

Soft agar cloning efficiency of SEP14 cells infected with sense or antisens *ras* retroviral vectors.

Experimental series [a]	Cell Line [b]	Recombinant retroviral vector [c]	Percentage viability [d]	Percentage soft agar cloning efficiency [e]	Percentage change in soft agar cloning efficiency [f]
1.	SEP14	none	33.8	11.26±1.29(16)	0
	SEP14	ZN(X)RAS-1	25.8	3.68±0.94(25)	-67.3
	SEP14	ZN(X)RAS+2	24.6	6.63±1.00(33)	-41.1
	SEP14	ZN(X)RAS-2	16.3	6.83±1.65(33)	-39.0
	SEP14	ZN(X)RAS+3	36.0	13.82±0.60(7)	+22.7
2.	SEP14	none	35.2	0.82±0.14(12)	0
	CT3		62.5	0 (12)	
	SEP14	ZN(X)RAS-1	33.3	0.37±0.15(12)	-54.9
	CT3		30.9	0 (12)	
	SEP14	ZN(X)RAS+2	23.3	0.81±0.12(12)	-1.2
	CT3		36.0	0 (12)	
	SEP14	ZN(X)RAS-2	27.3	0.57±0.26(7)	-30.5
	CT3		30.5	0 (12)	
3.	SEP14	none	13.3	0.55±0.01(7)	0
	CT3		46.3	0 (12)	
	SEP14	ZN(X)RAS+1	13.0	0.96±0.22(7)	+74.5
	CT3		48.3	0 (12)	
	SEP14	ZN(X)RAS-1	12.5	0.1±0.03(8)	-81.8
	CT3		43.3	0 (12)	

was included. The soft agar cloning efficiencies of uninfected SEP14 cells were directly compared to the cloning efficiencies obtained for ZN(X)RAS-1 and ZN(X)RAS+1 infected SEP14 cells. ZN(X)RAS+1-infected SEP14 cells (experiment 3, table 3.4) showed an increase in colony formation of +74.5%. Equally, ZN(X)RAS-1-infected SEP14 cells had a reduced cloning efficiency in soft agar by -81,8% (experiment 3, table 3.4). The ZN(X)RAS+1 and ZN(X)RAS-1 proviruses differed only in the orientation of the c-Ha-*ras* fragment F1 insert, which in turn determined the production of sense or antisense RNA. It seemed therefore plausible to conclude, that the expression of the c-Ha-*ras* fragment F1 as antisense RNA resulted in the dramatic reduction of the growth in soft agar and consequently transformed phenotype. Reduced cloning efficiencies in soft agar were also observed with ZN(X)RAS-2-infected SEP14 cells. The reductions in colony formation of -39.0% (experiment 1, table 3.4) and -30.5% (experiment 2, table 3.4) were consistent but not as dramatic as with ZN(X)RAS-1-infected SEP14 cells. The presence of sense ZN(X)RAS recombinant proviruses in infected SEP14 cells resulted in increasing, rather then reducing, soft agar cloning efficiencies (table 3.4). CT3 cells infected in parallel to SEP14 cells with the same ZN(X)RAS recombinant viruses failed to grow in soft agar as did uninfected CT3 cells (experiments 2 and 3, table 3.4). As CT3 cells have an untransformed cellular phenotype, the failure of these cells to form colonies in semi-solid medium was expected (SHIH et al., 1979, 1981; COOPER et al., 1980; KRONTIRIS and COOPER, 1981).

The results of experiment 3, together with the more circumstantial evidence provided by experiments 1 and 2, demonstrated clearly that the reduction in cloning efficiency observed for ZN(X)RAS-1 and ZN(X)RAS-2-infected SEP14 cells could not solely be attributed to the presence of the Zip Neo SV(X) backbone of the recombinant proviruses but must be an effect of c-Ha-*ras* antisense RNA expression. Antisense RNA expressed from ZN(X)RAS1-1 provirus was the most effective in reducing cell transformation, both in soft agar cloning assays and the ratio of untransformed and transformed colonies. The results from the cell transformation assays are summarised in table 3.5.

Summary of observed changes in transformed phonotype observed on SEP14 cell after infection with sense and antisense *ras* retroviral vectors.

Recombinant retroviral vector[a]	Percentage change in U/T ratio[b]	Percentage change in soft agar cloning efficiency[c]
none	0	0
ZN(X) RAS+1	not done	+74.5
ZN(X) RAS-1	-64.5	-68.0
ZN(X) RAS+2	-18.8	-21.0
ZN(X) RAS-2	-16.7	-32.0
ZN(X) RAS+3	-6.3	+22.7
ZN(X) RAS-3	-10.4	not done

Table 3.5.

Summary of observed changes in transformed phenotype observed on SEP14 cells after infection with sense and antisense *ras* containing retroviral vectors.

(a): ZN(X)RAS are ZIP NEO SV(X)1-derived retroviral vectors containing genomic c-Ha-*ras* sequences in sense (+) or antisense (-) orientation.

(b): Percentage change in untransformed to transformed ratio compared to uninfected SEP14 cells.

(c): The number of viable colonies observed growing in soft agar expressed as percentage of number of cells replated. the values given are the mean of the values listed in table 3.4.

Experiments to assess changes in the RNA levels from the endogenous c-Ha-*ras* gene and/or from the transfected codon 61-activated Ha-*ras* oncogene in ZN(X)RAS-1 infected SEP14 cells were not undertaken due to time limitations. A correlation between the observed changes in transformation of ZN(X)RAS-1 infectants with a reduction in the target RNA could be expected. Analysis of the negative effects of the presence of antisense RNA on Ha-*ras* gene expression at the protein level are more important still.

3.5. Summary.

pZip Neo SV(X)1-based retroviral vectors had been constructed which constitutively expressed antisense RNA from genomic c-Ha-*ras* sequences. Northern analysis of ZN(X)RAS transfected Ψ2 cells clearly demonstrated the synthesis of a spliced, subgenomic RNA and full-length, genomic retroviral RNA from each ZN(X)RAS retroviral constructs upon transfection. In Ψ2 transfectants, the expression of antisense RNA from the genomic c-Ha-*ras* fragments F2' and F3' present in the ZN(X)RAS-2 and ZN(X)RAS-3 constructs, respectively, had been shown by hybridization with *in vitro* transcribed "sense" riboprobes. No antisense transcripts were detected in RNA from parental Ψ2 cells or those transfected with the ZN(X)RAS+2 and ZN(X)RAS+3 constructs. ZN(X)RAS-2 and ZN(X)RAS-3 encoded antisense c-Ha-*ras* RNA expression was also detected upon infection of SEP14 and CT3 cells with the corresponding recombinant viruses. At no time was it possible to demonstrate F1'-specific antisense RNA expression from the ZN(X)RAS-1 provirus. The ZN(X)RAS-1 encoded antisense RNA was expected to be as stable as antisense RNA transcribed from ZN(X)RAS-2 and ZN(X)RAS-3 proviruses (GIEBELHAUS et al., 1988). The failure to detect antisense RNA expression in ZN(X)RAS-1 infected SEP14 and CT3 cells could be due to rapid duplex formation between the antisense RNA and its target, the endogenous cellular c-Ha-*ras* mRNA, followed by rapid degradation of the RNA duplex through double-strand specific ribonucleases (CROWLEY et al., 1985; WALDER and WALDER, 1988).

3.6. Discussion.

3.6.1. Northern Analysis of ZN(X)RAS Antisense Virus Infected SEP14 Cells.

The mechanisms by which antisense RNA alters expression of the target gene have been variously attributed to effects in transcription (KRYSTAL et al., 1988), nuclear processing (MUNROE, 1988), nuclear transport, translation (MELTON, 1985) and mRNA stability (KINLEMAN and KIRSCHNER, 1989) (reviewed by VAN DER KROL et al., 1988). The most significant reversion of cell transformation has been observed in ZN(X)RAS-1-infected SEP14 cells. Repeated efforts to demonstrate F1'-antisense RNA expression in SEP14 and CT3 cells infected with ZN(X)RAS-1 virus failed, even under conditions where F2'-antisense RNA expression in ZN(X)RAS-2 infected SEP14 and CT3 cells was readily detectable. Upon infection, transcription of the antisense ZN(X)RAS proviruses should result in post-transcriptionally modified, thus stable c-Ha-*ras* antisense RNA. All three antisense RNAs are expressed as part of the full-length genomic retroviral RNA (chapter 4) and should therefore be post-transcriptionally modified by 5'-capping and 3'-polyadanylation. 5'-capping of antisense RNA has been shown to lead to increased RNA stability and to a greater degree of inhibition of target gene expression (BEVILACQUA et al., 1988; GIEBELHAUS et al., 1988). The failure to detect F1'-antisense RNA expression in ZN(X)RAS-1 infected SEP14 cells is therefore unlikely to be the result of RNA instability. It is possible that hybridization of the antisense RNA to endogenous c-Ha-*ras* mRNA could have lead to the rapid degradation of RNA:RNA duplexes by double-strand specific ribonucleases and therefore the inability to detect antisense transcript (COWLEY et al., 1985; WALDER and WALDER, 1988; SHUTTLEWORTH and COLEMAN, 1988). The ability of the ZN(X)RAS-1 retroviral construct to direct antisense c-Ha-ras F1' RNA transcription had been demonstrated with ZN(X)RAS-1-transfected Ψ2 cells. Nevertheless, the performance of nuclear runoff analysis would be necessary to confirm c-Ha-*ras* F1' antisense RNA synthesis in SEP14 infectants.

It seems likely that c-Ha-*ras* F1'-antisense RNA will be involved in RNA:RNA duplex formation in the nucleus. Duplex formation in the

cytoplasm is also possible. As part of the full-length genomic retroviral RNA, the c-Ha-*ras* F1'-antisense RNA, as other eukaryotic pre-mRNAs, will be processed in the nucleus and transported into the cytoplasm. Thus c-Ha-*ras* antisense RNA is expected to be able to interfere with the processing and transport of the target mRNA in the nucleus as well as with its translation in the cytoplasm (GIEBELHAUS et al., 1988). Duplex formation between artificial antisense RNAs and their target mRNAs, in the cytoplasm and in the nucleus, had been demonstrated by MELTON (1985) and KIM and WOLD (1985), respectively. RNA:RNA duplex formation in the nucleus inhibits gene expression by preventing processing of the pre-mRNA (MUNROE, 1988) and export of the spliced product into the cytoplasm, whereas duplex formation in the cytoplasm is thought to led to inhibition of gene expression by preventing the initiation of translation of the target mRNA (VAN DER KROL et al., 1988). RNA:RNA duplex formation between F1'-antisense RNA and the endogenous c-Ha-*ras* mRNA had not been analysed due to time limitations. However, electron microscopy and RNase protection techniques as described by KIM and WOLD (1985), MELTON (1985) and KRYSTAL et al. (1990) can be used to detect and confirm RNA:RNA duplex formation.

3.6.2. Effects of Genomic c-Ha-*ras* Antisense RNA Expression on Transformation Phenotype.

Expression of c-Ha-*ras* antisense RNA using ZN(X)RAS antisense retroviral vectors causes reversion of the transformed phenotype of NIH 3T3 cells transformed by c-Ha-*ras* gene activated through codon 61 mutation (SEP14).

The cell line SEP14, although originally isolated as a highly transformed cell line, displays wide range of degrees of transformation. An unexpectedly high ratio of untransformed to transformed colonies (U/T ratio) was observed in SEP14 colony formation assays. Infection of SEP14 cells with various c-Ha-*ras* antisense ZN(X)RAS retroviral vectors and subsequent expression of the antisense RNAs as part of the full length retroviral RNA was hoped to lead to changes in the U/T ratio. Antisense RNA expression of genomic c-Ha-*ras* fragments F2 and F3 led to small reductions in the U/T ratios (-16.7% for ZN(X)RAS-2; -10.4% for ZN(X)RAS-3). However, the

reductions could not be solely attributed to c-Ha-*ras* antisense RNA expression, as sense RNA expression of c-Ha-*ras* fragments F2 and F3 led to equivalent reductions in the U/T values (-18.8% for ZN(X)RAS+2; -6.3% for ZN(X)RAS+3). However, a significant U/T ratio reduction of -64.5% was observed in pools of ZN(X)RAS-1 infected SEP14 cells. This reduction is most likely due to expression of the antisense RNA of c-Ha-*ras* fragment F1, rather than the expression of Zip Neo SV(X)1 retroviral sequences per se (table 3.3). Generally, c-Ha-*ras* antisense RNA expression was hoped to lead to an at least partial inhibition of the expression of codon 61-activated c-Ha-*ras* oncogene and/or the normal proto-oncogene in SEP14 cells infected with the antisense *ras* retroviral vector. Thus, the number of untransformed or less transformed colonies would have been expected to increase resulting in a subsequent increase of the U/T value. However, c-Ha-*ras* fragment F1 antisense RNA might also interfere with the viability of flat, morphologically untransformed appearing SEP14 cells by inhibiting or reducing the expression of the cellular Ha-*ras* oncogene and/or proto-oncogene, subsequently blocking the signalling pathways through which Ha-*ras* exerts its functions. Inhibition of c-Ha-*ras* oncogene or proto-oncogene expression in turn could decrease the viability of untransformed cells, thus causing the unexpected but observed reduction in the number of untransformed $G418^r$-colonies of ZN(X)RAS-1 infected SEP14 cells. A low average viability of 6% had been observed for ZN(X)RAS-1 infected SEP14 cells, whereas the average replating viability of uninfected SEP14 cells (13.3%) and SEP14 cells infected with ZN(X)RAS2 or ZN(X)RAS3 retroviral vectors was significantly higher (ZN(X)RAS+2 infected cells: 20.5%; ZN(X)RAS-2 infected cells: 15.5%; ZN(X)RAS+3 infected cells: 14.0%; ZN(X)RAS-3 infected cells: 18.0%) (table 3.3).

In summary, there is some evidence suggesting that antisense RNA expression of c-Ha-*ras* fragment F1 results in morphological changes of transformed SEP14 cells most likely due to the inhibition of c-Ha-*ras* oncogene and/or proto-oncogene expression. The use of the U/T ratio in assessing effects of c-Ha-ras antisense RNA expression on cellular morphology depended significantly on the stability of the transformed (and untransformed) phenotype of SEP14 cells. However, even clonal derived transformed SEP14 infectant lines did not stably maintain a transformed

phenotype. Very high incidences of phenotypic reversions from transformed to untransformed cellular morphology ranging between 33.0% to 62.3% were detected. It seemed highly unlikely that the observed reversion was caused through the outgrowth of contaminating untransformed cells, present at the time of colony cloning, as the viability of untransformed cells was generally greatly reduced compared to the viability of transformed cells (not shown). Also, untransformed CT3 cells infected with the sense and antisense ZN(X)RAS retroviral vectors had not been tested for changes in viability. It would have been interesting to know, if antisense RNA expression from the ZN(X)RAS-1 retroviral vector would have caused a similar reduction in replating viability of infected CT3 cells as seen in ZN(X)RAS-1 infected SEP14 cells.

Scoring untransformed versus transformed colonies, as a criterion to assess any effects of c-Ha-*ras* antisense RNA expression, has obvious disadvantages:

i) Scoring of phenotypically transformed or untransformed colonies was highly subjective. Although in most cases there was a clear difference between the U and T colonies after staining with GIEMSA. Generally, transformed colonies stained more intensely with GIEMSA stain than untransformed colonies, as a colony of transformed cells, growing contact uninhibited in several layers, contained a larger number of cells. The less intense stained untransformed colonies were made up of fewer cells growing in a monolayer of contact inhibited cells. It had been attempted to use Computer aided colony analysis to solve the question of subjectivity involved in scoring colonies. Computer aided colony analysis allowed the definition and storage of parameters, like colony size and intensity of staining, resulting, in principle, in consistent analysis of data from single experiments or whole experimental series. However, it soon became clear at the time, that the quality of the computer hardware and software available within the institute was not sufficient to allow computer aided analysis to be used routinely and reliably.

ii) Furthermore, the instability of the transformed phenotype of SEP14 cells made it more difficult to evaluate any effects of c-Ha-*ras* antisense RNA expression upon reduction of transformation. It had been observed that

transformed cell lines generated by transfection were very unstable, unless the expression of these transforming DNA fragments were under constant selection. One example being the transfectant cell line SEP11 (chapter 5.3.). Phenotypic reversion of human Ha-*ras* oncogene-transformed NIH 3T3 cells (SHIH and WEINBERG, 1982) was also reported by SALMONS et al. (1986), although the phenotypic reversion was due to the loss of all human Ha-*ras* sequences.

3.6.3. Effects of Genomic c-Ha-*ras* Antisense RNA Expression on Soft Agar Cloning Ability.

SEP14 cells infected with ZN(X)RAS-1 recombinant virus showed decreased colony formation in soft agar in relation to the cloning efficiency value obtained for uninfected SEP14 cells. However, the overall reductions in cloning efficiency varied from 81.8% (experiment 3, table 3.4) to 54.9% (experiment 2, table 3.4). Although the extent of the reduction in anchorage-independent growth clearly depended on the recombinant antisense ZN(X)RAS provirus present, variations were observed. Several factors could influence the degree of variations observed.

i) Cell pools were tested for changes in anchorage-independent growth: The cell pools tested for anchorage-independent growth were made by pooling individual colonies present on one petri dish after G418 selection. Within one cell pool, the expression levels of recombinant ZN(X)RAS proviruses were likely to vary from one clonal colony to another. Factors such as site of proviral integration, mutation frequency and methylation pattern can influence the expression levels of proviruses (VARMUS and BROWN, 1989.). Although, each cell pool contained at least 20 individual colonies, the number might not have been high enough to average out the differences in expression levels of the recombinant provirus from one cell pool to another.

ii) Expression levels of the transfected codon 61-activated c-Ha-*ras* oncogene: In addition to the variation in the levels of expression of the recombinant provirus encoding c-Ha-*ras* antisense RNAs, the expression levels of the transfected codon 61-activated c-Ha-*ras* oncogene might also differ between individual SEP14 cells, probably reflected in the relative phenotypic instability of SEP14 cells. A partial or complete represion of

activated c-Ha-*ras* gene expression could contribute partly to the reduced ability of SEP14 cells to grow in soft agar. This effect does clearly not account for the full extent of the reductions in soft agar cloning efficiencies observed, as an obvious relationship between the extent of the reductions and the presence of individual antisense ZN(X)RAS retroviral vectors exists (table 3.4).

iii) Experimental variability due to the quality of reagents used: Independently from the cell lines and the retroviral constructs, other, experimental factors can effect the colony formation in soft agar. An important factor was the quality of the agar itself. During the course of the soft agar cloning experiments, agar from two different manufacturers was used (due to delivery problems experienced by the normal supplier). Agar from DIFCO was used for the first soft agar cloning experiment (experiment 1, table 3.4) and successful colony formation was obtained. When NOBEL agar was used instead, cell pools previously successfully tested for colony formation in DIFCO agar, failed to give rise to any colonies (data not shown). Enough DIFCO agar was obtained within the institute to enable all soft agar cloning experiments to be performed with agar from the same manufacturer, however from two different batches. The low soft agar cloning efficiencies seen in experiments 2 and 3 were most likely due to the difference in the quality of the agar from the two batches. In experiment 1, uninfected SEP14 control cells had a soft agar cloning efficiency of 11.26%, wereas in experiments 2 and 3, the cloning efficiencies had dropped dramatically to 0.82 % and 0.55% (table 3.4). In each of the three experiments, the SEP14 cells used as controls were from the same frozen stock, had been kept in culture for the same time and undergone the same number of passages. Furthermore, no significant variations in the viability of the SEP14 cells were detected at the time of replating into semi-solid medium. The drop in soft agar cloning efficiencies from 11.26% (experiment 1) to around 0.7% (experiments 2 and 3) seemed therefore most likely due to the differences in the quality of agar used for the three experiments.

The presence of sense ZN(X)RAS retroviral vectors in SEP14 cells did overall not result in a reduction of soft agar cloning efficiency, with the exception of ZN(X)RAS+2-infected SEP14 pool in experiment 1 (table 3.4).

No significant alteration in soft agar cloning efficiency was seen with ZN(X)RAS+2-infected SEP14 cells in experiment 2. The discrepency between the changes in soft agar cloning efficiencies two independent ZN(X)RAS+2-infected pools of SEP14 cells is not completely clear. However, taking the general tendency of sense ZN(X)RAS vectors not to reduce colony formation, the discrepancy seemed most likely due to experimental variations, not least influenced by factors such as quality of serum and agar used during the experiment. ZN(X)RAS+3-infected SEP14 cells (experiment 1) displayed a 22.7% increased ability to grow in semi-solid medium. The most dramatic increase of soft agar colony formation by +74.5% was seen with ZN(X)RAS+1 infected SEP14 cells (experiment 3; table 3.4). Although, the presence of sense ZN(X)RAS vectors in SEP14 cells had at best an overall stimulating effect, or at least no effect, on growth in soft agar, an increase in colony formation was not seen with pools of CT3 cells infected by the same recombinant sense ZN(X)RAS vectors. CT3 cells were not able to grow in soft agar, due to their untransformed phenotype (SHIH et al., 1979, 1981; COOPER et al., 1980; KRONTIRIS and COOPER, 1981). The failure of sense ZN(X)RAS vectors to stimulate infected CT3 cells to grow in soft agar is probably due to the lack of c-Ha-*ras* exons 3 and 4 in these retroviral constructs, preventing membrane association of any translated protein product (WILLUMSEN et al., 1984; HANCOCK et al., 1989; HANCOCK et al., 1991). Association with the plasma membrane is a prerequisite for any transforming activity of p21 ras proteins (WILLUMSEN et al., 1984; DER and COX, 1991; HANCOCK et al., 1991).

The exact mechanism by which sense ZN(X)RAS retroviral constructs increase growth in soft agar of infected SEP14 cells had not been examined. It seemed unlikely that protein products would be made from retroviral transcripts containing the genomic c-Ha-*ras* fragments F2 and F3, as F2 codes for only 4 amino acids and there is no translation initiation codon in fragments F3. However, any protein product translated from c-Ha-*ras* fragment F1'sense RNA could compete for positive regulators of the GTP-ase activity of normal NIH 3T3 endogenous p21 Ha-ras proteins, further reducing the levels of regulators already deminished due to their binding to the highly expressed codon 61-activated p21Ha-ras protein (VOGEL et al., 1988; QUINTANILLA

et al., 1986). Ha-*ras* fragment F1 codes for three regions of the p21ras protein identified by three dimensional structure analysis as required for the interaction between p21ras and p120GAP (GIDEON et al., 1992). The proposed competition of ZN(X)RAS+1-encoded protein with normal p21ras proteins for regulators, such as the ubiquitous, cytoplasmic p120GAP, could depleat the levels of regulators of endogenous p21ras activity below a critical threshold required for the switching of active p21 to the inactive GDP-bound form, thus mimicking, to a small degree, an overexpression of normal p21 Ha-*ras* protein. The suggested reduction in regulator levels would work in inverse analogy to findings published by ZHANG et al. (1990), who showed that overexpression of p120GAP in NIH 3T3 cells can prevent and reverse the transformation induced by overexpression of normal p21ras proteins. This competition effect is not be sufficient, however, to induce CT3 cells to change to a transformed phenotype, as even 100 fold over-expression of normal c-Ha-ras protein is not as effective in inducing transformation as the presence of oncogenic activated p21 Ha-ras. It also has to be pointed out however, that Ha-ras proteins which have the majority of the amino acids delected which are implicated in GDP/GTP binding, as a protein product derived from fragment F1 would be, are extremely unstable (C. MARSHALL, pers. communication).

It is not completely clear why fragment F1' of genomic mouse c-Ha-*ras* clone N1 is the most effective fragment for antisense RNA induced reversion of cell transformation as measured by reduction in soft agar colony formation (table 3.5). The genomic PstI-fragment F1 encompasses the first and second coding exons of the mouse gene, intron I1 and the first 112bp of intron I2, together with 1.3kb of upstream sequence, including the short GC rich promoter region and the untranslated exon E-1. In soft agar cloning experiments, ZN(X)RAS-2 infected SEP14 cells had a reduced ability to form colonies (-30.5% to -39.0%), however, not to the same extent as SEP14 cells infected with ZN(X)RAS-1 virus (up to -81.8%). Recent results of deletion analysis experiments (J.B. TELLIEZ, pers. communication) identify within intron I(0) of the cellular mouse Ha-*ras* gene three elements which are important in regulating c-Ha-*ras* transcription. The regulatory elements consist of a transcriptional enhancer and two elements 3' to the enhancer which negatively and positively regulated the activity of this intron I(0) enhancer.

Therefore, antisense RNA complementary to intron I(0) sequences, as transcribed from ZN(X)RAS-1 and ZN(X)RAS-2 retroviral vectors, might be expected to result in a reduction of c-Ha-*ras* gene expression. As observed in ZN(X)RAS-2 infected SEP14 cells, 5'-untranslated sequences of the mouse c-Ha-*ras* gene, while effective, is not be sufficient for extensive inhibition of c-Ha-*ras* gene expression by antisense RNA. It has previously been shown that antisense RNA directed against the translation initiation site is particularly effective in reducing expression of the target gene (INUOYE et al., 1988; EGUCHI et al., 1991). The translation initiation site for p21 synthesis is about 12bp 5' to the HindIII site in exon E1 of the mouse c-Ha-*ras* gene. therefore, only antisense RNA transcribed from fragment F1 will have extensive regions of complementarity surrounding the endogenous translation initiation site of the c-Ha-*ras* gene. The 3' complementarity of antisense RNA derived from fragment F2 extends only over 12bp 3' the translation initiation site and duplex formation in the 3'-region of overlap may therefore be more unstable compared to F1'-antisense RNA. Only antisense RNA transcribed from fragment F1' has extensive regions of complementarity to regulatory elements within the 5'-untranslated region and to sequences surrounding the endogenous translation initiation site, thus supporting that antisense RNA derived from ZN(X)RAS-1 retroviral vector most effective in reducing cell transformation of c-Ha-ras transformed NIH 3T3 cells.

Antisense RNA expression in ZN(X)RAS-3 infected SEP14 cells resulted in -8.35% reversion of cell phenotype compared to -64.5% reversion for ZN(X)RAS-1 infected SEP14 cells. F3'-antisense RNA is not complementary to the translation initiation region of the c-Ha-*ras* gene which might explain the low percentage reduction in soft agar cloning ability observed with ZN(X)RAS-3 infected SEP14 cells. Also, flat revertants of SEP14 cells containing ZN(X)RAS-3 provirus showed reduced viability in normal tissue culture conditions (6.3%) compared to flat revertant SEP14 cells containing ZN(X)RAS-1 provirus (8.5%). LINGELBACH and DOBBERSTEIN (1988) identified in HeLa cell extracts an RNA:RNA unwinding activity associated with the translating ribosome which led to the unrestricted ability of the ribosome to translate mRNA efficiently even when extensive RNA:RNA duplexes were present within the coding region of the

mRNA. Extensive RNA:RNA duplex formation within the coding region of the target mRNA did not reduce the efficiency with which translation was initiated. Under the assumption that in the case of ZN(X)RAS-3 infected cells, the majority of antisense RNA:RNA duplex formation ocurres in the cytoplasm, the observation by LINGELBACH and DOBBERSTEIN (1988) could explane the reduced activity of F3' antisense RNA. Only antisense RNA expression derived from fragment F1, combining both 5'-untranslated sequences and coding sequences, leads to the dramatic changes in cell transformation as seen with ZN(X)RAS-1 infected SEP14 cells.

3.7. Future Prospects.

Results presented in this chapter demonstrate, that constitutive c-Ha-*ras* antisense RNA expression following retrovirus mediated gene transfer, can lead to reversion of cell transformation of SEP14 cells, a transformed NIH 3T3 cell line derived after transfection of total cellular DNA isolated from a DMBA/TPA induced mouse skin papilloma containing a codon 61-activated Ha-*ras* oncogene.

There are several matters arising from the study described here which require further investigations. First: although antisense RNA expression from ZN(X)RAS-2 and ZN(X)RAS-3 vectors has been demonstrated following infection into SEP14 cells, however, the synthesis of antisense RNA in ZN(X)RAS-1 infected SEP14 cells remains to be confirmed by nuclear run-off experiments. Second: it is important to investigate changes in expression of the endogenous c-Ha-*ras* gene and the transfected c-Ha-*ras* oncogene, in order to correlate these with the degree of cell transformation reversion. The levels of RNA transcribed should be analysed by Northern blotting or cDNA-PCR; changes in p21 protein levels by immunoprecipitation and Western blotting. Based on the reductions of cell transformation observed in ZN(X)RAS-1 infected SEP14 cells, reduced mRNA and protein levels of both, the endogenous proto-oncogene and the transfected c-Ha-*ras* oncogene are expected., even though the antisense RNA is not complementary to the mutated codon 61. Analysis of p21 Ha-ras and activated p21 Ha-ras protein levels would contribute to the understanding of their roles cell viability and in maintaining cell transformation. Third: complete repression of both c-Ha-*ras*

genes present in SEP14 cells would be required to assess any possible redundancy amongst the members of the *ras* gene family. This would require i) testing and subcloning of additional c-Ha-ras gene fragments for their suitability in antisense RNA inhibition of gene expression and ii) increasing the expression of antisense RNA. Using a 2.0kb Ki-*ras* genomic DNA segment carrying second and third exon together with flanking sequences for antisense RNA expression, MUKHOPADHYAY et al. (1991) obtained a 95% reduction in p21 Ki-ras protein synthesis resulting in reduced tumorigenicity in *nu/nu* mice. No increased expression of endogenous N-*ras* and the Ha-*ras* genes was observed in these tumours, suggesting that a functional redundancy amongst the mammalian p21ras proteins seems unlikely (MUKHOPADHYAY et al., 1991). The importance of gene dosage in antisense RNA inhibition had been demonstrated by IZANT and WEINTRAUB (1985), KIM and WOLD (1985) and ROSENBERG et al. (1985). Retroviral vectors containing DHFR protein coding sequences are available for cloning of antisense RNA genes (WILLIAMS et al., 1986). Upon infection the antisense RNA gene-DHFR recombinant proviruses can be amplified by selection of cells in progressively higher levels of methotrexate, leading to the over-production of DHFR message and antisense RNA. Fourth: The results on changes in cell transformation of SEP14 cells induced by the expression of antisense c-Ha-*ras* RNA were clearly encouraging. Although analysis of endogenous c-Ha-*ras* mRNA and p21 protein levels had not been performed due to time limitations, the data presented justifies the introduction of the ZN(X)RAS antisense viruses into *in vitro* derived cell lines of DMBA/TPA-induced mouse skin papillomas and carcinomas. BREMNER and BALMAIN (1990) and BUCHMANN et al (1991) have shown that during progression of mouse skin tumours quantitative increases in mutant c-Ha-*ras* gene copy number relative to the normal c-Ha-*ras* gene occur. The results presented in this chapter suggest that inhibition of mutant c-Ha-*ras* gene expression could be a useful approach to examining the role of mutant c-Ha-*ras* in the maintenance of transformation and during progression. Retroviral vectors expressing antisense c-Ha-*ras* RNA could be used to infect cell lines representing different stages of tumour progression to examine if phenotypic reversal of progression is observed *in vitro*.

Chapter 4

Involvement of the Transin-1/Stromelysin-1 Metalloproteinase in Tumour Invasion and Metastasis.

4. Involvement of the Transin-1/Stromelysin-1 Proteinase in Tumour Invasion and Metastasis.

4.1. Introduction.

Ras gene activation are often found as an initiating event in chemically induced carcinogenesis using animal model systems *in vivo* (reviews: BALMAIN and BROWN, 1988; SUKUMAR, 1989 and 1990; DRINKWATER, 1990) and the causal nature of the *ras* gene mutations in initiating carcinogenesis has been confirmed by introducing activated retroviral v-*ras* genes into mouse skin *in vivo* (BROWN et al., 1986; ROOP et al., 1986) or by expressing human T24/EJ mutant *c-Ha-ras* in transgenic animals (BAILLEUL et al., 1990). However, the expression of the mutated c-Ha-*ras* gene found in benign lesions of the DMBA treated mouse skin (BALMAIN et al., 1984) is not sufficient to induce progression towards a more complete tumorigenic phenotype (QUINTANILLA et al., 1991). Therefore, additional factors and events are likely to play an important role(s) in tumour progression. Members of the metalloproteinase family have been specifically implicated in the multistep process of metastasis formation by mediating invasion of transformed cells through the basement membrane. In particular, the expression of the transin-1/stromelysi-1 gene during chemical carcinogenesis in mouse skin has been studied extensively. Originally identified by means of its highly induced mRNA in rat embryo fibroblast cell lines upon oncogenic transformation, the transin-1/stromelysin-1 gene showed a strong correlation between its expression and the invasive and metastatic potential of chemically induced mouse skin tumours (reviewed in MATRISIAN and BOWDEN, 1990). This observation lead to the speculation that the transin-1/stromelysin-1 metalloproteinase might play a causal role in promoting invasion through the basement membrane. The experiments described below are part of an effort to establish an *in vivo* system which could be used to assess the role and the effect of expression of transin-1/stromelysin-1 gene in mediating the metastatic phenotype.

The cell lines PDV (FUSENIG et al., 1978 and 1982), C5 and AT5 (DIAZ-GUERRA et al., 1992; QUINTANILLA et al., 1991) could be looked upon as representing *in vitro* discrete stages characterized in chemically

induced mouse skin carcinogenesis *in vivo*. All three cell lines are derived from primary epidermal cell cultures after *in vitro* treatment with DMBA. They differ from each other by the presence or absence of an activated Ha-*ras* gene, the level at which the Ha-*ras* oncogene is expressed and the degree of tumorigenicity in nude mice (table 4.2). All three cell lines are non-metastatic upon subcutaneous injection into nude mice. This indicates that the expression of mutated Ha-*ras* gene, even at high levels, is by itself not sufficient to convert the immortalized phenotype of these cell lines to a full neoplastic phenotype (QUINTANILLA et al., 1991). Transferring the rat transin/stromelysin cDNA into these cell lines via retroviral mediated gene transfer was hoped to give a better insight into the role of metalloproteinases in cell invasiveness, the process of metastasis formation and the requirement for the presence of an activated Ha-*ras* gene and the level of its expression in metastatic processes. Any changes in cell behaviour due to the N2 retroviral vector itself will be evaluated with the help of the control retroviral construct pLNSal containing the human adenosine deaminase (*ada*) gene in place of the transin cDNA. Changes in tumorigenic and metastatic phenotype will be assessed using the spontaneous metastasis assay (MUSCHEL and LIOTTA, 1988) *in vivo*. It had been reported that the calcium phosphate transfection procedure might in itself produce independent changes in the transfected cell which can contribute to alter the metastatic behaviour of some transfected cells (KERBEL et al., 1987). Thus retroviral mediated gene transfer was chosen to avoid effects of calcium phosphate and the incorporation of unwanted carrier DNA into the recipient cell (GREENBERG et al., 1989).

4.2. Results.

4.2.1. Retrovirus Mediated Gene Transfer of Rat Transin/Stromelysin cDNA into epithelial cells *in vitro*.

Full-length c-DNA of the rat transin/stromelysin gene was introduced into three different epithelial cell lines, PDV, C5 and AT5, and the mouse CT3 fibroblast cell line through infection with the LNTR2 recombinant retroviral vector. The characteristics of the different cell lines are summarized in table 4.2.

Recipient cell lines for retroviral mediated gene transfer of the rat transin cDNA.

Cell line	Cell type	Initiation	Promotion	Ha *ras* gene alteration	Relative tumorigenicity (e)
C5 (a)	epithelial	DMBA	TPA (2.5months)	wild type	-
AT5 (b)	epithelial	DMBA	TPA (2.5months)	codon 12	+/-
PDV (c)	epithelial	DMBA	TPA (1month)	codon 61	+
CT3 (d)	fibroblasts	none	none	wild type	-

Table 4.2.

Recipient cell lines for retroviral mediated gene transfer of the rat transin cDNA.

(a) The epithelial C5N cell line had been obtained by single cell cloning of epithelial MCA 3D cells (QUINTANILLA et al., 1991). The cell line MCA 3D is derived from mouse primary epithelial cell culture after initiation with DMBA *in vitro* and selection by calcium switch (KULEZ-MARTIN et al., 1983).

(b) The epithelial cell line AT5 was derived by stable transfection of epithelial MCA 3D cells with the plasmid pAGT1 containing the human EJ-T24 mutant Ha-*ras* gene and the *neo*^{r} marker gene (DIAZ-GUERRA et al., 1992).

(c) The cell line PDV is a rare transformant isolated after treatment of epidermal cell cultures from newborn mice with DMBA. PDV cells have three copies of mouse chromosome 7 (FUSENIG et al., 1983) on which the c-Ha-*ras* gene is located. One of these alleles has an A to T transversion mutation in codon 61 as seen *in vivo* in mouse skin papillomas and carcinomas initiated by DMBA treatment (QUINTANILLA et al., 1991).

(d) The CT3 fibroblast cell line is derived from immortalized NIH 3T3 cells (COPELAND et al., 1979).

(e) The relative tumorigenicity indicates the incidence of tumour induction upon subcutaneous injection into nude mice.

G. M. COOPER's CT3 cell line is one of several clones derived from NIH 3T3 mouse fibroblasts (COPELAND et al., 1979; JAINCHILL et al., 1969). CT3 cells are non-tumorigenic in nude mouse assay. The CT3 cell line is included in the experiments described below as a non-epithelial, non-initiated cell control.

The PDV cell line was established as a consequence of a rare transformation event after *in vitro* DMBA treatment of primary epithelial cell cultures from newborn mice. PDV cells show an increased proliferation rate and reach higher cell densities in culture than their normal counterparts. (FUSENIG et al., 1978 and 1982). The cell line is tumorigenic in nude mice with a latency period of around 3 weeks but rarely produce tumours when injected into syngeneic C57Bl/6 mice (DIAZ-GUERRA et al., 1992). Subcutaneous cell injection induces SCCs with varying degrees of keratinization (FUSENIG et al., 1978 and 1982). Karyotypic analysis of PDV cells has indicated the presence of three copies of chromosome 7 which harbour the mouse c-Ha-*ras* locus (FUSENIG et al., 1985; KOZAK et al., 1983), however, only one of the three Ha-*ras* alleles has the DMBA specific mutation at codon 61 (QUINTANILLA et al., 1991), as found in a high proportion of mouse skin tumours initiated with DMBA (BALMAIN and PRAGNELL, 1983; BALMAIN et al., 1984; QUINTANILLA et al., 1986 and 1991; BIZUB et al., 1986; STRICKLAND et al., 1986; FUSENIG et al., 1985; KOZAK et al., 1983).

The cell line MCA 3D was established from newborn Balb/C mouse epidermal keratinocytes following treatment with the carcinogen DMBA *in vitro* (KULESZ-MARTIN et al., 1983). C5, derived by single cell cloning from MCA 3D cells, express the normal p21 Ha-ras protein, are non-tumorigenic in nude mice and show a resistance to terminal differentiation induced by high Ca^{2+} concentration (QUINTANILLA et al., 1991). The AT5 cell line, derived by stable transfection of the plasmid pAGT containing the human T24/EJ Ha-*ras* oncogene with the codon 12 mutation isolated from the human bladder carcinoma cell line T24/EJ (DER et al., 1982; QUINTANILLA et al., 1991). The cell line has been classified as weakly tumorigenic with a latency period of approximately three months (DIAZ-GUERRA et. al., 1992).

4.2.2. *In vitro* infection of epithelial cells by recombinant retroviral vector LNTR2 containing the rat transin cDNA.

The rat transin cDNA expressing retroviral vector, pLNTR2, and a control vector containing the cDNA of the human *ada* gene, pLNSal (PALMER et al., 1987), were a gift from R. BREATHNACH. The retroviral vector pLNTR2 was derived from the vector pLNSal by exchanging the StuI-BglII fragment of the *ada* cDNA with an compatible insert containing full length rat transin cDNA (R. BREATHNACH, personal communication.). The LNTR2 and the LNSal retroviral vector are based on the N2 vector (GILBOA, 1987). The high titer producing N2 vector retained besides the 5'- M-MuLV LTR, the *gag* AUG initiation codon and the first 418 bp of the *gag* coding region to which the coding sequences of the selectable *neo*-marker were fused out of frame (GILBOA, 1987). The *neo* gene was expressed from a spliced RNA generated by the activation of a cryptic 3'-splice site in the *gag* coding sequences upstream of the *neo* gene (ARMENTANO et al., 1987). In the constructs pLNTR2 and pLNSal, the rat transin and human *ada* cDNAs, respectively, are under transcriptional control of an internal SV40 promoter. Plasmid maps of the pLNTR2 and pLNSal constructs are shown in figure 4.2. Clonal cell lines producing ecotropic LNTR2 virus (ΨLNTR3 and ΨLNTR5) and ecotropic LNSal virus (ΨLNSal1 and ΨLNSal2) had been kindly provided by Dr. R. BREATHNACH. The producer lines generated between 1.2 to $1.5x10^6$ infectious virus particles per ml supernatant (table 4.3). Using the reverse transcriptase assay, helper virus production was detected for cell lines ΨLNTR5 and ΨLNSal2 (data not presented). The cell lines ΨLNTR3 and ΨLNSal1 were assumed to also release helper virus, however, they had not been tested for helpervirus production.

During pilot experiments, CT3, PDV, MCA 3D and C5 cells had been successfully infected with ecotropic $G418^r$-conferring Zip Neo SV(X)1 virus (data not shown). Virus-containing medium from producer cell lines ΨLNTR3, ΨLNTR5 and ΨLNSal1 was used to infect CT3, PDV, C5 and AT5 cells. The infection efficiencies for LNTR2 and LNSal viruses, based on the number of $G418^r$-colonies observed after selection, are summarized in table 4.3. Efficiency values could not be determined for retroviral vector infections

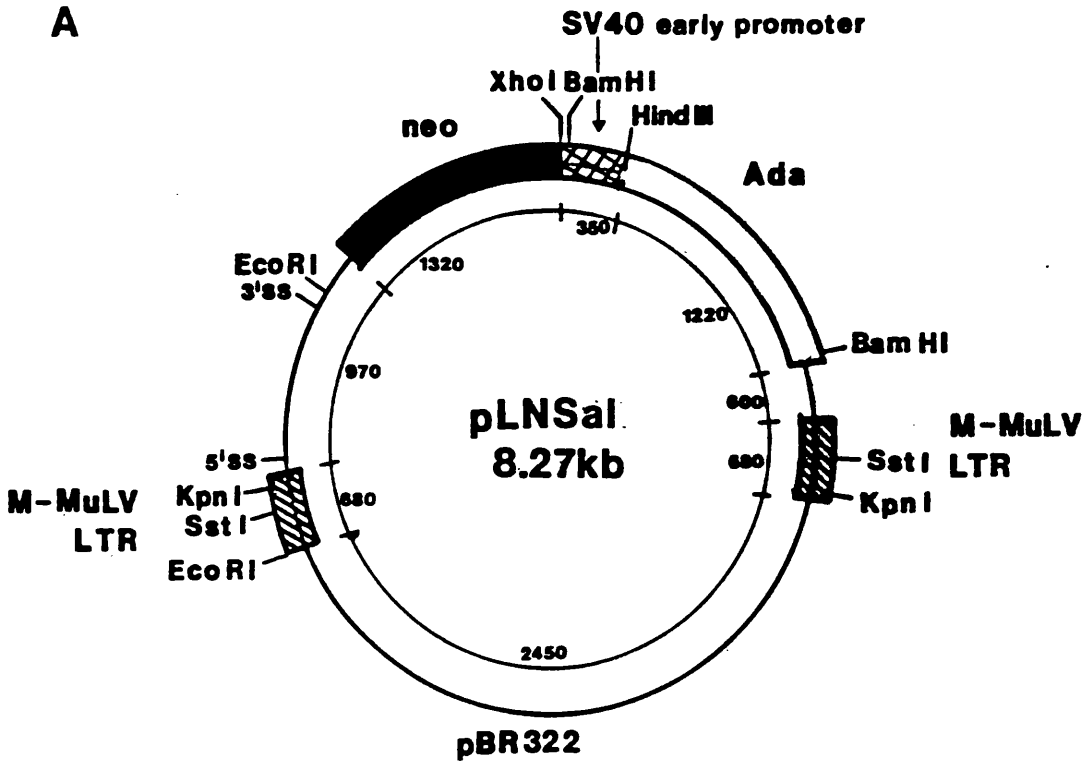
Figure 4.2.

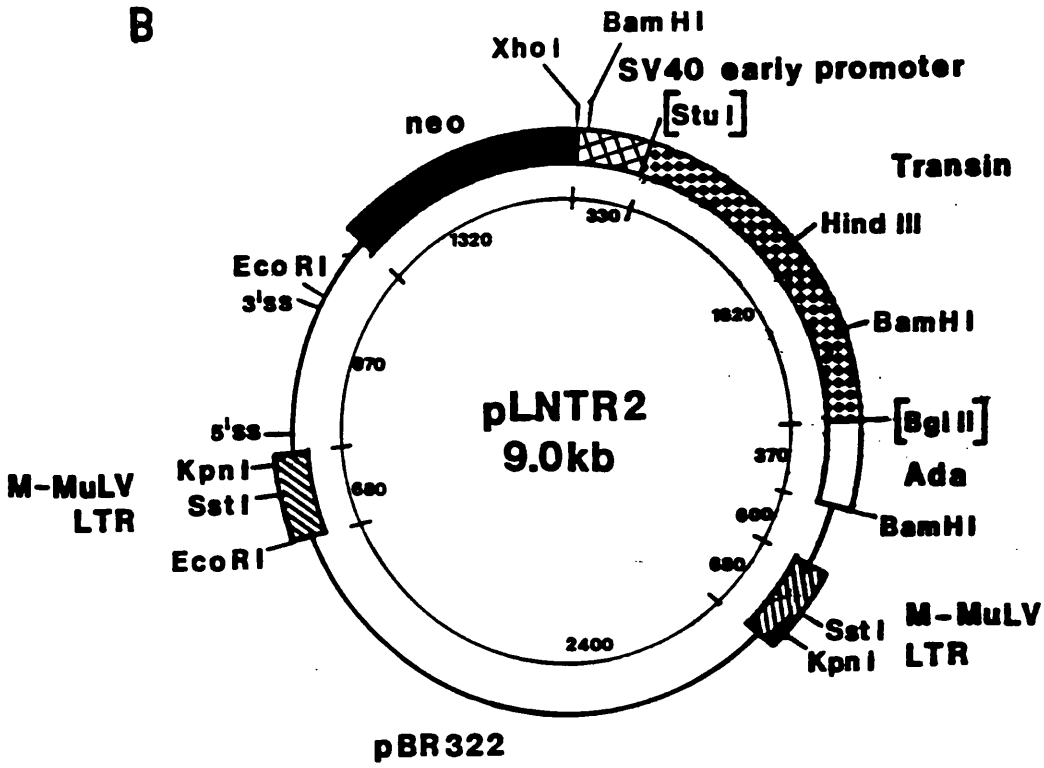
pLNSal and pLNTR2 retroviral vectors.

Figure 4.2a: The 8.27kb pLNSal retroviral vector has been derived from the N2 retroviral vector by inserting a human adenosine deaminase (ada) cDNA (open box) under the transcriptional control of the SV40 early region enhancer/promoter (hatched box) downstream of the *neo*^{r} gene (filled box). The Mo-MuLV-LTRs (diagonal boxes) and splicing signals (5'ss, 3'ss) are indicated. pLNSal contains around 400bp of the *gag* coding region of wild type Mo-MuLV. pBR322 sequences (line) allow propagation of the retroviral vector in *E. coli.* (PALMER et al., 1987 and references therein).

Figure 4.2b: The 9.0kb pLNTR2 retroviral vector is derived fromthe pLNSAl retroviral vector by replacing the StuI-BglII fragment of the human ada cDNA (open box) with a full-length rat transin-1/stromelysin-1 cDNA (chequered box). 370bp of the 3' end of the human ada cDNA remain in the construct (R> BREATHNACH, pers. communication).

Recognition sites for endonucleases EcoRI, SstI, KpnI, XhoI, and BamHI are indicated.


A
SV40 early promoter
XhoI BamHI
HindIII
neo
Ada
EcoRI
3'SS
1320
350
1220
Bam HI
870
pLNSal
8.27kb
600
680
M-MuLV
LTR
SstI
KpnI
5'SS
M-MuLV
LTR
KpnI
SstI
EcoRI
680
2450
pBR322


B
BamHI
XhoI
SV40 early promoter
[StuI]
neo
Transin
330
Hind III
1320
EcoRI
3'SS
1620
BamHI
870
pLNTR2
9.0kb
[BglII]
5'SS
370
M-MuLV
LTR
KpnI
SstI
Ada
680
600
BamHI
EcoRI
680
M-MuLV
LTR
SstI
KpnI
2400
pBR322

Efficiency of infection of epithelial cells by recombinant transin retroviral vector LNTR.

Recombinant retroviral vector (a)	Infection Efficiency $G418^V$ -cfu/ml/10^6 viable cells (b)			
	CT3	PDV	C5	AT5 (c)
None	0	$1.1x10^1$	0	($1.0x10^6$)
LNSaL	$1.5x10^6$	$4.6x10^2$	$4.0x10^4$	($1.0x10^6$)
LNTR2	$1.2x10^6$	$2.0x10^3$	$9.4x10^4$	($2.2x10^6$)

Table4.3.

Efficiency of infection of epithelial cells by recombinant transin retroviral vector LNTR2.

(a) The retroviral vectors LNSal and LNTR2 are derived from the N2 retroviral vector and express human adenosine deaminase (ada) cDNA or rat transin cDNA, respectively, under the transcriptional control of the internal SV40 early promoter (see figure4.2.).

(b) The infection efficiency is the number of G418 resistant colonies observed per 10^6 viable cells replated into G418 selection after infection of recipient cells (CT3, PDV, C5) with 200µl of viral supernatant from clonal Ψ2LNTR3 or Ψ2LNSal2 producer cell lines.

(c) The efficiency of infection of MCA 3D AT5 cells by LNTR2 and LNSal retroviral vectors could not be determined, as AT5 cells were already G418 resistant . AT5 cells were infected three times within a 24-hour period with infectious supernatant containing LNTR2 and LNSal retroviral vectors.

of AT5 cells, as these cells were already G418 resistant due to the *neo*-marker gene, present on the pAGT plasmid used to establish the cell line. Therefore, to ensure efficient infection, AT5 cells were infected three times within 24 hours with infectious LNTR2 or LNSal recombinant virus. In general, the efficiency in infecting epithelial cell lines was significantly reduced compared to mouse fibroblasts (table 4.3: CT3: 100%; PDV: 0.17%; C5: 7.9%). MORGAN et al. (1987) and FRIEDMANN (1989) reported similar findings upon infection of epithelial cells *in vitro* by retroviral vectors.

4.2.3. In Vivo Assay for Metastatic Phenotype.

$G418^r$-pools of LNTR2 and LNSal infected cells from each cell line were tested for changes in their invasive or metastatic phenotype caused by expression of the transin gene or *ada* gene, respectively, upon retroviral mediated gene transfer. (The spontaneous metastasis assay was used to assess changes in tumorigenicity or metastatic behaviour of infected cells.)

The ability of tumorigenic cells to form metastases can be assayed *in vivo*. Young nude mice (<8 weeks old) are routinely used in metastasis assays *in vivo* to reduce immunological barriers (HANNA and FIDLER, 1981; POLLACK and FIDLER, 1982). The two assays most widely used, are the spontaneous metastasis assay and the experimental metastasis assay (MUSCHEL and LIOTTA, 1988). In the experimental metastasis assay, cells are injected intravenously. The resultant lung nodules indicate the extent of metastatic potential. This assay is based upon metastasis formation involving the exodus of cells from the circulation via the blood stream into distant sites and the formation of secondary tumours at those sites. It gives high reproducible numbers for quantitative comparisons. In the spontaneous metastasis assay, however, cells are injected subcutaneously, a tumour forms at the site of injection, and later the animal is autopsied and evaluated for metastasis on distant sites. In a variation of the assay, removal of the primary tumour after its formation will increase the time after which the animal can be evaluated. The results of both metastasis assays do correlate often, but not always. For a review of assays for metastasis, see NICOLSON and POSTE (1983). The spontaneous metastasis assay was chosen for evaluation of metastatic phenotype of LNTR2 and LNSal infected epidermal cells, as the

experimental metastasis assays duplicate only the later steps involved in metastasis.

G418^r-pools of LNTR2 or LNSal infected CT3, PDV, C5 and AT5 cells were injected subcutaneously into 8 week old female nude mice. Controls of uninfected parental cells were also included. The animals were observed for tumour formation for several months. All tumours had reached a minimum size of 1 cm in diameter before the animals were sacrificed. An autopsy was performed at the time of sacrifice to evaluate metastasis formation at distant sites. The results of the spontaneous metastasis assay are summarized in table 4.4.

PDV cells induced tumours in nude mice at high frequency (6/8 tumours/injection site) with a latency of 6 weeks. Similar results were published by DIAZ-GUERRA et al. (1992) who reported PDV cells to be tumorigenic in nude mice (3/3 tumours/injection site) with a latency period of 3 weeks. No significant differences in tumorigenicity, both in frequency and latency periods, were detected in LNTR2 or LNSal infected PDV cells compared to the uninfected control cell (LNTR2: 3/4 tumours/injection site, 5 weeks latency; LNSal: 3/4 tumours/injection site, 7 weeks latency). Clearly, the presence of the LNTR2 or LNSal vectors in PDV cells had no effect on the tumorigenic phenotype of the PDV cells. None of the PDV cells, LNTR2 or LNSal infected or uninfected, gave rise to metastatic tumours upon subcutaneous injection (table 4.4). This suggested that either the introduction of the transin gene into PDV cells was in itself not sufficient to induce progression towards a more invasive and/or metastatic phenotype and that additional changes were required (e.g. higher expression levels of the mutated c-Ha-*ras* gene (MUSCHEL and LIOTTA, 1988)) or that the transin gene was not expressed or the protein was not activated following secretion.

CT3 cells are non-transformed and should as such be non-tumorigenic in nude mice. However, low frequency tumour formation was detected following subcutaneous injection of uninfected CT3 cells into nude mice (1/8 tumours/ injection site, 3 months latency). LNTR2 and LNSal infected CT3 cells showed a further increase in tumour formation (LNTR2: 3/8 tumours/injection site; LNSal: 3/8 tumours/injection site), with the same latency periods. CT3 cells were routinely used in 3T3 transfection assays,

Spontaneous metastasis formation of LNTR2 infected epithelial cells.

Cell Line (a)	Recombinant retroviral vector (b)	No of primary tumours (c)	No of secondary tumours (d)	Observation period (e)
CT3	none	1/8	0	78
CT3	LNSal	3/8	0	80
CT3	LNTR2	3/8	0	80
PDV	none	6/8	0	44
PDV	LNSal	3/4	0	36
PDV	LNTR2	3/4	0	44
C5	none	0/8	0	78
C5	LNSal	2/6	0	98
C5	LNTR2	4/16	0	98
AT5	none	7/8	0	71
AT5	LNSal	5/8	0	71
AT5	LNTR2	10/16	1 Lymphnode tumour	71

Table 4.4.

Spontaneous metastasis formation of LNTR2 infected epithelial cells.

(a) Recipient cell line for infection with LNTR2 and LNSal retroviral vectors.

(b) LNSal and LNTR2 are N2-derived retroviral vectors containing human adenosine deaminase cDNA or rat transin cDNA, respectively, under the transcriptional control of the internal SV40 early promoter (see figure 4.2.).

(c) The number of tumours observed per number of injection sites. 8-week old female nude mice were used for subcutaneous injection of infected cells. Each animal was injected twice and received between 2.5 to $5x10^6$ cells per site of injection.

(d) The number of secondary tumours observed. Animals were sacrificed once the primary tumours had reached an average size of 10mm in diameter. Autopsy was performed on each animal to score for the formation of secondary tumours.

(e) Numbers of days between date of subcutaneous injection of cells and the date of sacrifice of the animal.

designed to detect transforming activities present in genomic donor DNAs (KRONTIRIS et al., 1981; LANE et al., 1981). However, CT3 cells, like other 3T3 mouse fibroblast cell lines, could only be propagated for a limited number of passages under sub-confluent conditions, otherwise the incidence of transformed phenotype within the CT3 cell population increased dramatically. The CT3 cells used in the infection and injection experiments were of low passage number to ensure a non-transformed phenotype. Therefore, there is no obvious explanation for the tumorigenic phenotype of the parental CT3 cells. The observation, that both LNTR2-infected and LNSal-infected CT3 cells had an increased tumorigenic phenotype compared to the uninfected control, suggested, that the retroviral vector N2, rather than the inserted rat transin and human *ada* cDNA sequences, caused the increased tumorigenic phenotype. No evidence of secondary tumour formation was found in any of the CT3 pools tested in the spontaneous metastasis assay (table 4.4).

The cell line C5 was non-tumorigenic in nude mice throughout the 3 month observation period (0/8 tumours/injection sites), as was the parental cell line MCA 3D (0/6 tumours/injection sites). $G418^r$-LNTR-transfectants of C5 cells were moderately tumorigenic in nude mice with a long latency period of 3 to 4 months (4/16 tumours/injection site). LNSal infectants induced tumours with a higher incidence (2/6 tumours/injection site), the latency period, however, remained the same as by LNTR2-infected $G418^r$-C5 cells. These results again implicated the N2 retroviral vector background in the tumour formation process rather than either the rat transin or the human *ada* cDNA inserts (table 4.4).

In comparison to the cell lines MCA 3D and C5, AT5 cells were highly tumorigenic in nude mice (7/8 tumours/injection site). The incidence of tumour formation following subcutaneous injection into nude mice was higher than that reported by DIAZ-GUERRA et al. (1992), who reported an incidence of 1/3 tumours/injection site. The latency period was also shortened from 90 days (DIAZ-GUERRA et al., 1992) to around 70 days. Introduction of either LNTR2 or LNSal virus and subsequent G418-selection *in vitro* seemed to reduce the tumorigenic phenotype of AT5 cells, without affecting the latency period of around 70 days. The incidence of tumour induction for LNSal-infected AT5 cells and LNTR2-infected AT5 cells were 5/8 tumours/injection

site and 10/16 tumours/injection sites, respectively. A lymph node tumour was detected in one nude mouse (table 4.5).

4.3. Discussion.

Overall there was no indication, that retroviral mediated gene transfer of rat transin/stromelysin cDNA into *in vitro* DMBA-initiated epithelial cell lines induced progression of phenotype of the infected cells, neither towards increased tumorigenicity nor to the induction of metastasis formation (table 4.4). The retroviral vector pLNTR2 was derived from pLNSal (PALMER et al., 1987), an N2-based retroviral vector which contained a human ada cDNA and which had been used successfully to infect *in vitro* human skin fibroblasts from a patient with ADA deficiency. In the experiments described above, epithelial cells infected by LNSal virus had been included to control for retroviral vector-induced changes in tumorigenic and metastatic phenotype. Upon subcutaneous injection into female nude mice, CT3 cells infected by LNTR2 or LNSal virus were slightly more tumorigenic than uninfected CT3 cells (table 4.4). No changes in tumorigenicity were observed for subcutaneously injected LNTR2- or LNSal-infected PDV cells and uninfected parental PDV cells (table 4.4). The non-tumorigenic cell line C5 became moderately tumorigenic after infection with LNTR2 or LNSal (table 4.4), whereas LNTR2- or LNSal-infected AT5 cells showed a reduction in tumorigenicity upon subcutaneous injection (table 4.4). No metastasis formation was observed in any of LNTR2- or LNSal-infected epithelial cells nor in CT3 fibroblasts with the exception of one lymph node tumour identified on a nude mouse injected with LNTR2-infected AT5 cells. The animal was in relative poor health at time of sacrifice and removal of the tumour. Each time an animal was sacrificed, an autopsy had been performed. With the above mentioned exception, no secondary tumours were found in the animals. However, severe liver damage was observed in many animals, this could have been due to infection by mouse hepatitis virus. The poor health of the animals could have contributed to the increased tumorigenic phenotype of AT5 cells in this experiment. DIAZ-GUERRA et al. (1992) reported a less tumorigenic phenotype for AT5 cells upon subcutaneous injection into nude mice. The development of tumours in nude mice upon subcutaneous injection of CT3

cells was unexpected. CT3 cells infected with LNTR2 or LNSal recombinant retrovirus showed increased tumorigenicity compared to uninfected CT3 cells. The poor health status of the animals could have been responsible for the unexcepted tumour formation along side with the occurrence of spontaneous transformation of CT3 cell during propagation in tissue culture prior to injection into nude mice.

In summary, upon subcutaneous injection into nude mice, LNSal-infected CT3, PDV, C5 and AT5 cells showed the same altered tumorigenic phenotype then equivalent LNTR2-infectants. These results suggested, therefore, that the retrovirus vector per se, rather than the expression of either the ada cDNA or the transin/stromelysin cDNA, seemed to induce the few observed changes in the respective tumorigenic phenotypes of the infected cells. Since no biological effects were observed, it was suggested not to further pursue the role of transin/stromelysin in metastasis, although a more detailed analysis of transin/stromelysin expression would be required to demonstrate that transin/stromelysin did not confer a metastatic phenotype. studies by BREATHNACH and colleagues have shown that the pLNTR2 retroviral vector does express transin/stromelysin, however, expression studies of transin/stromelysin activity were not straight forward.

At the same time when the above described experiments were performed, S. YUSPA and T. BOWDEN and their co-workers also attempted to induce progression towards an invasive and metastatic phenotype by introducing transin/stromelysin cDNA into epithelial cells *in vitro*. Stable transfection of full-length transin/stromelysin cDNA into cells of chemically induced mouse skin papilloma derived cell lines also failed to result in an invasive phenotype of the transfectants (L.MATRISIAN, personal communication). It seemed likely therefore, that both, experiments performed by YUSPA and BOWDEN and by myself were unsuccessful in inducing metastatic phenotypes because the protein products translated from transin/stromelysin cDNAs were probably enzymatically inactive. At the time these experiments were performed, it was not appreciated that metalloproteinases required proteolytic activation after secretion. There are many stages in the biosynthesis of transin/stromelysin and other metalloproteinases where regulation of expression and activity occurs *in vivo*

(MATRISIAN and BOWDEN, 1990; LIOTTA and STETLER-STEVENSON, 1990; LIOTTA et al., 1991; MATRISIAN, 1992). Furthermore, by introducing a constitutively active form of matrilysin or transin-3 (table 4.1) into human stomach and colon cancers derived cell lines, it has been shown that matrilysin activity is necessary but on its own not sufficient to induce of tumour invasiveness. An extensive correlation had been demonstrated previously between matrilysin gene expression and activity in human gastric tumours and primary prostate adenocarcinomas and their invasive phenotype (McDONNEL and MATRISIAN, 1990; SIADAT PAJOUH et al., 1991; MATRISIAN, 1992).

Recent observations made in the groups of D. STEHELIN and L. MATRISIAN seem to requires an adjustment of the traditional concept of invasion, where matrix-degrading proteinases are expressed within the tumour cells themselves and localized secretion of latent forms of the degrading proteins by the tumour cells, followed by activation of the proteins, results in localized digestion of the matrix immediately underlying the invasive tumour (reviewed in MATRISIAN and BOWDEN, 1990; LIOTTA and STETLER-STEVENSON, 1990; LIOTTA et al., 1991; MATRISIAN, 1992). Recent results by D.STEHELIN and co-workers showed a transient and localized induction in c-*ets*-1 RNA expression, followed by a localized induction of collagenase gene expression in the endothelia during the invasive process of angiogenesis in developing embryos (WERNERT et al., 1992). Moreover, c-*ets*-1 gene expression was also induced in endothelial cells which were in immediate neighbourhood to vascularizing tumours. *In situ* hybridization could detect no signs of c-*ets*-1 or collagenase gene expression in the actual tumour cells themselves at any stage during tumour progression (D. STEHELIN, pers. communication). Similar results were obtained after *in situ* hybridization of sections through invasive human ovarian tumours which had been grafted onto the stroma of nude mice. Mouse c-*ets*-1 mRNA was readily detected in fibrocytes of the mouse stroma immediately surrounded by the invasive human ovarian tumour, which itself proved negative for c-*ets*-1 gene expression. In some cases, co-expression of c-*ets*-1 and *collagenase* mRNA occurred in the mouse stroma cells. It had been suggested that the invasive human ovarian tumour releases factors into the remnants of the tumour-

surrounded mouse stroma which induce c-*ets*-1 gene expression, leading in turn to the induction of expression of metalloproteinase genes. Induction of metalloproteinase genes in the stroma cells will result in the stroma "dissolving" itself and thus allows the invasive tumour to grow (D. STEHELIN, pers. communication).

High levels of *transin/stromelysin* gene expression were detected in 80% of invasive mouse skin carcinomas, induced by DMBA-TPA induction and promotion, and in 100% of invasive mouse skin carcinomas, induced by repeated MNNG treatments, but not necessarily in metastasis derived from these tumours (MATRISIAN et al., 1986; OSTROWSKI et al., 1988; MATRISIAN and BOWDEN, 1990). Recent *in situ* hybridization experiments, however, showed that transin/stromelysin mRNA is synthesized in the stromal cells immediately surrounding or located within the mouse skin lesions rather than in the papilloma or carcinoma cells themselves. Upon progression to undifferentiated spindle cell carcinomas, transin/stromelysin expression can also be detected in the undifferentiated, fibroblastic-appearing carcinoma cells (L. MATRISIAN, pers. communication). Considering these very recent results by L.MATRISIAN et al.(unpublished results), it seemed likely, that retroviral mediated gene transfer of the rat transin/stromelysin cDNA into epithelial cells representing the more benign stages of mouse skin carcinogenesis was the wrong choice. Introduction of the rat transin/stromelysin cDNA into carcinoma or spindle cell carcinoma cell lines, however, might have been more appropriate for the induction of invasiveness and metastasis formation. Interestingly, preliminary results by P. DAUBERSIES seemed to indicate a shift in AP-1 protein complex concentrations during progression of chemically induced mouse skin lesions. High levels of AP-1 protein complex have been found in benign papillomas, whereas spindle cell carcinomas seem to contain very little AP-1. A more detailed knowledge about the distribution and abundance of the transcription factors AP-1 and c-Ets-1 during tumour progression might help to understand the tight regulation of transin/stromelysin gene expression at the various stages of tumour development and metastasis.

Chapter 5

The Use of Retroviral Vectors in Cell Lineage Study in Mouse Skin and Mouse Skin Carcinogenesis.

5. The Use of Retroviral Vectors in Cell Lineage Study in Mouse Skin and Mouse Skin Carcinogenesis.

5.1. Introduction.

Knowledge of the cell lineage and of the potential of stem cells or precursor cells is essential to further the understanding of control of cell proliferation. In tumorigenesis studies specifically, single cell marking techniques could assist in defining the role of activated oncogenes during tumour development under conditions as they occur *in vivo*. The use of microinjection of fluorescent cell lineage markers or enzyme tracers, although successfully used in other systems (KIMMEL and WARGA, 1986; WARGA and KIMMEL, 1990) seemed to be inappropriate in cell lineage studies in mouse skin carcinogenesis, as the target cells for initiation are as yet unidentified. Even the generation and use of transgenic animals (HANAHAN, 1986) has limitations regarding their use in the analysis of multistage mouse skin carcinogenesis. The initiation of tumorigenesis *in vivo* is due to somatic mutations in the c-Ha-*ras* gene occurring in a single cell surrounded by normal tissue. In transgenic mice, however, the oncogene product is synthesized in all cells of a tissue to which the expression of the gene has been targeted through the choice of the promoter directing expressing of the transgene. In recent years, retroviruses have been increasingly applied to the study of cell lineage (reviewed in PRICE, 1987). The relative ease with which it was possible to tag a pluripotential haematopoietic stem cell in man and mouse has been demonstrated by DICK et al. (1986). progeny of cells infected by a retroviral vector can not only be identified by the integration site of the retroviral provirus but also by the expression of a genetic marker gene present in the retroviral vector used for the genetic tagging of the original cell. The most widely used genetic marker whose gene product is easily detectable by histochemical staining procedures is the bacterial *lacZ* gene coding for the β-galactosidase enzyme. The successful use of *LacZ*-encoding retroviral vectors has been reviewed in PRICE (1987 and 1991) and CEPKO (1988 and 1989). So far, no developmental abnormalities attributed to high expression of the *lacZ* gene have been reported.

The aim of this part of the study was to examine the feasibility of using retroviral vectors expressing β-galactosidase encoded by the bacterial *lacZ* gene

to label epithelial cells *in vivo* in mouse skin. Initial experiments examined the titer of the virus and expression of β-galactosidase in murine epithelial cells *in vitro*. Conditions for detecting the expression of β-galactosidase by histochemical techniques were established in cells *in vitro* and in tissue sections. The ability of a β-galactosidase expressing retroviral vector, pBAG, to infect and label mouse skin cells *in vivo* was examined with the objective of attempting to identify skin stem cells and differentiation compartments. Retroviral vectors containing the v-*ras* gene and the β-galactosidase marker gene were also examined for their ability to co-express both genes. The possibility of using ras/β-gal retroviral vectors to initiate papilloma formation in mouse skin *in vivo* was also explored, with the hope of using such an approach to identify histochemically v-*ras* initiated cells in mouse skin *in situ*.

5. Results.

5.2. Cell Lineage Study in Normal Mouse Skin.

5.2.1. BAG Virus Infection of Epithelial Cells *In Vitro*.

The retroviral vector, pBAG, encodes two marker genes: the *neo* gene encoding the neomycin phosphotransferase for G418 selection (COLBERE-GARAPIN et al., 1981; DAVIES and JIMENEZ, 1982) and the *E. coli* β-galactosidase (β-gal)-encoding *lacZ* gene (figure 5.1). This retroviral vector system has been used to study cell lineages in the developing vertebrate nervous system, both *in vivo* and *in vitro* (PRICE, et al., 1987). The ecotropic BAG virus producer clone G4 12.2-Y7 was kindly supplied by J. PRICE. BAG virus from G4 12.2-Y7 was tested on CT3 cells for the ability of infected cells to simultaneously express the *lacZ* and *neo* genes. G4 12.2-Y7 released BAG virus at a titre of 4.3×10^5 G418-resistant colony forming units per ml of viral supernatant per 10^6 viable CT3 cells infected ($G418^r$-cfu/ml/10^6 viable CT3 cells) or 4.0×10^5 $\beta\text{-gal}^+$-$G418^r$-cfu/ml/10^6 viable CT3 cells, respectively (table 5.1). This compares favourably with published results of 10^5 $G418^r$-cfu/ml (PRICE et al., 1987).

BAG virus was also tested for the ability to infect epithelial C5N cells *in vitro* (table 5.1). The C5N cell line is a subclone of the non-tumorigenic epithelial

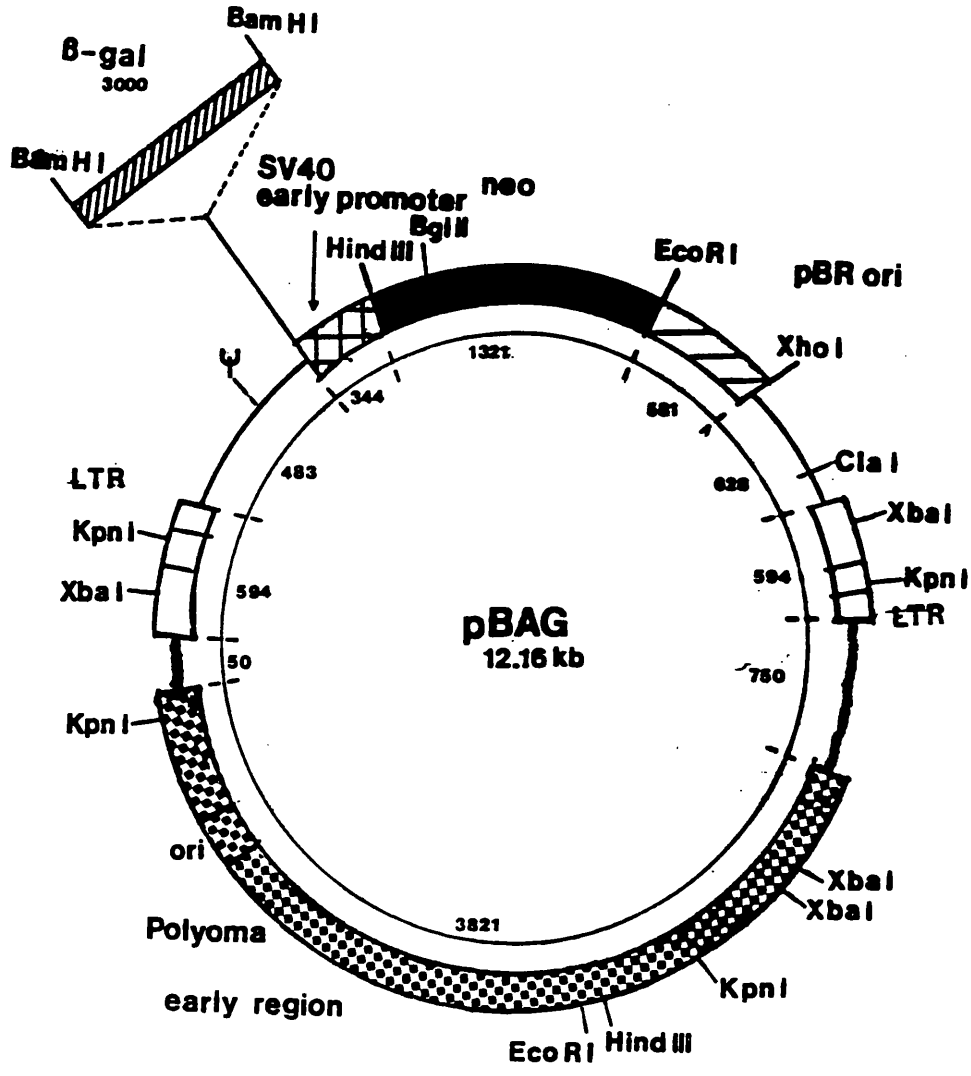


Figure 5.1

Retroviral Vector BAG.

The retroviral vector BAG was cloned by PRICE et al. (1987). The 5'Mo-MuLV-LTR (open box) provided the promoter for the *E.coli lacZ* gene (3.0kb BamHI-fragment, narrow diagonal box). The ATG translation initiation codon for translation of the retroviral *lacZ* message was supplied by modified Mo-MuLV gag sequences. The SV40 early promoter (hatched box) and the neo^r-gene (filled box) were isolated from plasmid pSV2neo (SOUTHERN and BERG, 1982); the pBR322 origin of replication (broad diagonal box) was derived from the pZip Neo SV(X)1 retroviral vector (CEPKO et al., 1984), and the remaining 3' Mo-MuLV sequences were wild-type. The polyoma early region (BamHI-HindIII fragment, chequered box) was ligated into the BAG plasmid outside the viral LTRs (open box) and rat chromosomal sequences (wavy line). See PRICE et al. (1987) for detailed description of the cloning of the pBAG retroviral vectors and for references. Recognition sites for a number of restriction endonucleases are indicated in the figure.

Efficiency of infection of CT3 and C5N cells by BAG retroviral vector.

Retroviral vector (a)	Infection Efficiency CT3		Infection Efficiency C5N	
	$G418^{r}$ (b)	$\beta\text{-gal}^{+}/G418^{r}$ (c)	$G418^{r}$ (b)	$\beta\text{-gal}^{+}/G418^{r}$ (c)
Zip Neo SV(x)1	1.7×10^{5} (593) (d)	-	4.4×10^{4} (30.4) (d)	-
BAG	4.3×10^{5} (6) (54.4) (d)	4.0×10^{5} (6)	1.4×10^{5} (5) (30.6) (d)	9.0×10^{4} (5)

Table 5.1

Efficiency of infection of CT3 and C5N cells by BAG retroviral vector.

(a) Description of retroviral vectors Zip Neo SV(X)1 (CEPKO et al., 1984) and BAG (PRICE et al., 1987) see text.

(b) Infection efficiency is the number of G418-resistant colonies observed per 10^{6} viable cells replated in G418 selection after infection of CT3 or C5N cells with 100 to 200µl of infectious supernatant harvested from Ψ2 cells producing infectious recombinant Zip Neo SV(X)1 or BAG retroviral vectors. Infected CT3 and C5N cells were selected in the presence of 0.8mg/ml or 0.2mg/ml G418, respectively.

(c) Infection efficiency is the number of β-galactosidase positive G418-resistant colonies observed under (b). Colonies were scored positive for β-galactosidase activity when stained blue within 5 to 24 hours after overlaying the colonies with X-Gal containing staining solution (SANES et al., 1986).

(d) The percentage viability of pooled colonies after infection by Zip Neo SV(X)1 or BAG and G418 selection.

cell line MCA 3D (KULESZ-MARTIN et al., 1983). By determining the number of G418 resistant colonies and the number of G418-resistant colonies that stained positive for β-galactosidase expression, the relative effectiveness of expression of the two marker genes, *lacZ* and *neo*, were assessed in epithelial C5N cell infectants. C5N were readily infectable by ecotropic BAG virus, however, the infection efficiency of C5N cells was reduced compared to CT3 cells (table 5.1). Although, the two mammalian promoters, SV40 early promoter and Mo-MuLV LTR, allowed the expression of their respective marker genes quite efficiently in both cell lines, the reduction in viral titre and number of G418-resistant colonies expressing *lacZ* suggests that the overall efficiency of both promoters appears to be reduced in C5N cells.

The possibility that lacZ gene expression could adversely effect cell viability, cell morphology or tumorigenicity of cells was also explored. Infection of CT3 cells or C5N cells by BAG or ZIP NEO SV(X)1 retroviral vector virus (PRICE et al., 1987; CEPKO et al., 1984) caused no reduction in viability of the cells (table 5.1). There were no obvious differences in cell morphology detectable between β-gal-expressing CT3 cells and uninfected parental cells. However, the intensity of the X-gal staining was heterogeneous within colonies. The heterogeneity of the staining might reflect a heterogeneity in *lacZ* gene expression or β-gal activity. PRICE et. al. (1987) had previously reported that mosaic expression of the *lacZ* gene could occur occasionally even in clones of infected fibroblasts grown *in vitro*.

The CarcB cell line was derived from a mouse squamous cell carcinoma induced by DMBA/TPA treatment (QUINTANILLA et al., 1986; DIAZ-GUERRA et al., 1992). After infection of CarcB cells with BAG virus, G418-resistant cells were injected subcutaneously into adult NIH or nude mice ($2.5x10^6$ cells/injection point). Within 3 days of the injection, rapidly growing tumours started to develop at each site of injection. No difference in tumorigenicity could be detected between $G418^r/\beta\text{–gal}^+$ CarcB cells and the parental CarcB cell line. X-gal staining showed, that the tumours contained a large proportion of cells expressing high amounts of active β-galactosidase protein (D. MORGAN, personal communication).

5.2.2. BAG Virus Infection of Mouse Skin *In Vivo*.

Concentrated BAG virus stock was used for *in vivo* infection of epithelial cells in normal mouse skin. Two days prior to BAG virus application, the fur from the back of two months old NIH female mice was removed, by shaving and treating with depilatory agent. The back skin of each animal was treated with a single dose of TPA 24 hours prior to BAG virus application (BROWN et al., 1986). BAG virus was applied either by injection of virus into the epidermis of the skin or by scarification of the back skin in the presence of BAG virus. A control group of animals had their back skin scarified in the presence of PBS or remained untreated for the duration of the experiment with the exception of the single TPA treatment.

BAG-infected animals and PBS-control animals were sacrificed at day 6 post virus application. Samples of the treated skin were taken together with control samples of untreated skin derived from the same animals. The skin samples were fixed (PRICE et. al., 1987), stained at 37°C overnight in X-gal staining solution (SANES et al., 1986), taken through a dehydration procedure and embedded in paraffin.

Examination of 10µm sections of skin taken from BAG infected animals revealed β-galactosidase-specific staining in the suprabasal layer of the epidermis as well as in and around hair follicles (figure 5.2a and b). Whereas in negative control skins, scarified in the presence of PBS, no β-galactosidase positive staining was seen within the hair follicles (figure 5.2c). Strong background staining was seen, however, over a wide range of endothelial cells (figure 5.2b and c). High background of β-galactosidase-positive staining had previously been reported by SANES et. al. (1986), who attributed the background staining to the presence of endogenous galactosidase enzymes. Furthermore, characteristic blue staining (red stain in dark field microscopy) was not always solely localized within the cytoplasm of β-galactosidase positive cells, but could also be found extensively within the extracellular spaces in the skin sections (figure 5.2b and c). This seemed to suggest that β-galactosidase might diffuse easily. The "leakage" of β-galactosidase-positive staining was probably due to insufficient fixation of the skin specimen prior to staining or possible rupture of β-galactosidase expressing cells during staining, dehydration and/or paraffin embedding.

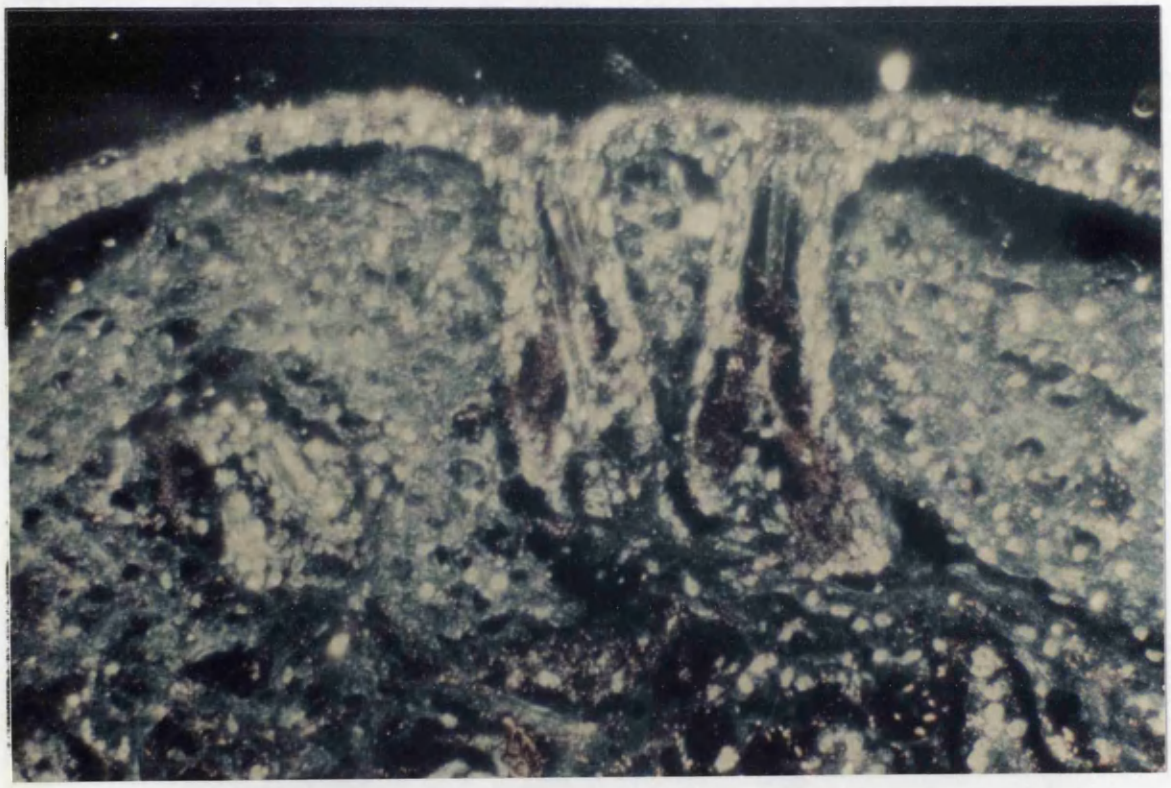
The results of these experiments showed that it was possible to successfully infect epithelial cells *in vivo* by applying concentrated BAG virus

Figure 5.2.

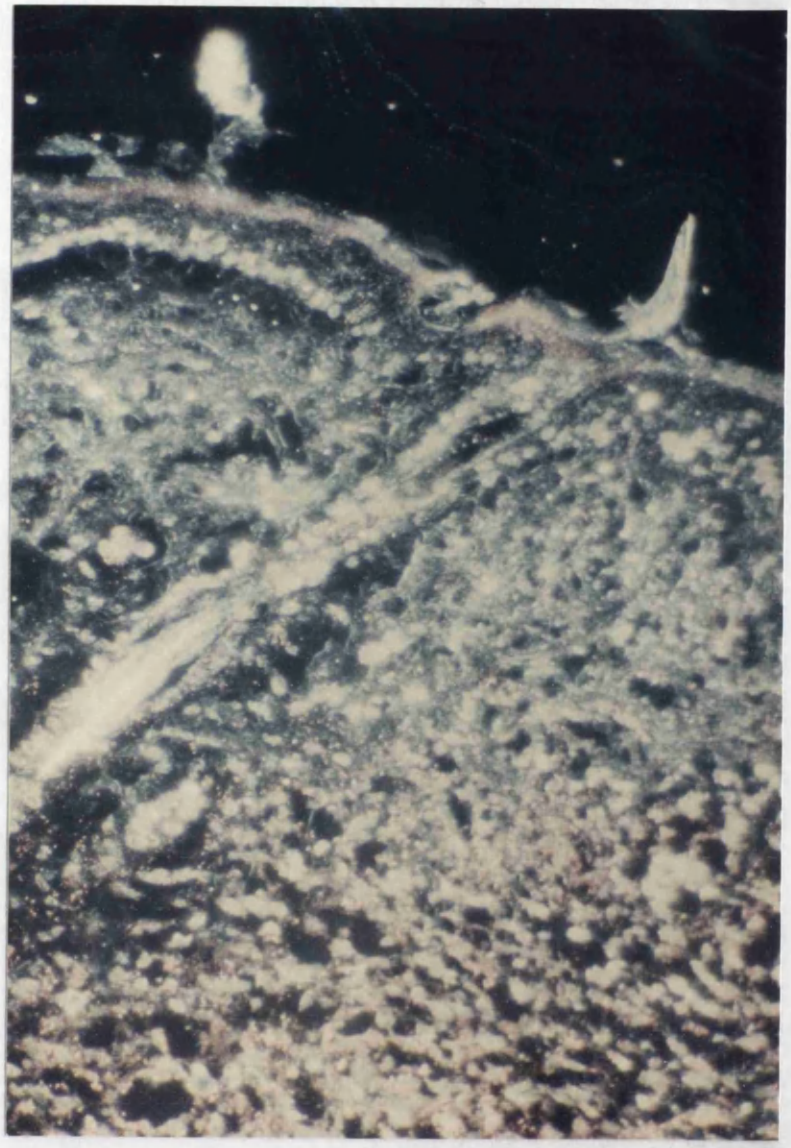
Histochemical analysis of mouse skin.

Six days post virus application, samples of mouse skin were taken from animals scarified in the presence of infectious BAG retroviral vector or phosphate buffered saline (PBS). The skin samples were fixed at 4°C in paraformaldehyde fixative (PRICE et al., 1987), stained overnight at 37°C in X-Gal staining solution (SANES et al., 1986), dehydrated and enbedded in paraffin. Dark field microscopy of 10μm skin sections taken from skins of BAG-infected animals revealed β-galactosidase positive staining (red) in the suprabasal layer of the epidermis and in hair follicles (figure 5.2a and b), however, not in hair follicles of PBS-infected control skins (figure 5.2c). Strong background staining was seen in sections of both BAG-infected and PBS-infected skins, especially over endothelial cells (figure 5.2a. and c.).

A



B



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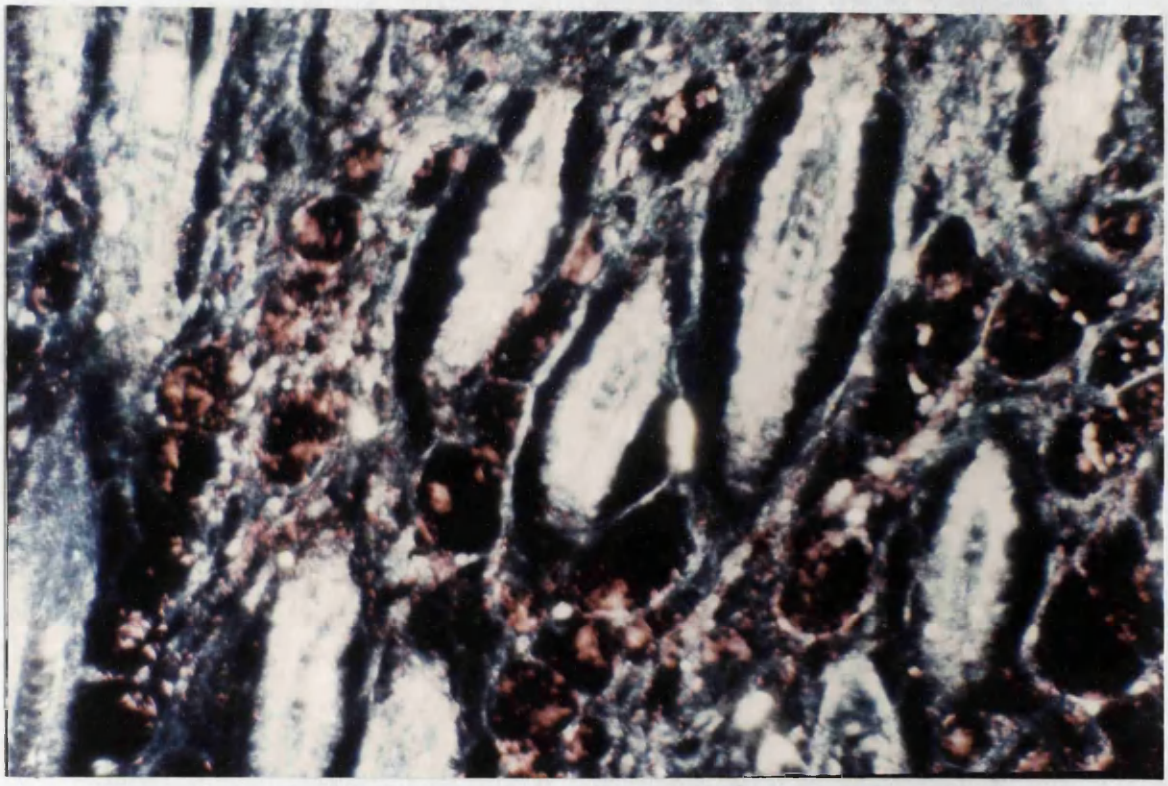

onto mouse skin by scarification and injection. The β-galactosidase-specific staining seemed to be concentrated at or near the base of hair follicles within the mouse skin. Several groups of cells within the epidermis have been suggested to represent epithelial stem cells, some of which are thought to be localized in the bulge area of the hair follicle (COTSRAELIS et al., 1990). As yet, no candidate stem cells has been histochemically identified *in situ*. Infection of mouse skin *in vivo* with the BAG or related recombinant retroviruses, would provide an excellent experimental system to identify epithelial stem cells and to analyse the genealogical relationship of mouse skin epithelial cells *in vivo*. The presence and expression of histochemical markers, such as the *lacZ* gene, would enable the clear identification of progenitor cells at any stage of the normal differentiation pathway and in mouse skin development.

5.3. Cell Lineage Study in Ha-*ras* Virus Initiated Mouse Skin Carcinogenesis.

Results by BROWN et. al. (1986) showed that activated v-*ras* genes were able to replace chemical carcinogens in the initiation of mouse skin carcinogenesis. Harvey and BALB murine sarcoma virus was directly applied to the mouse skin by scarification and subsequent treatments with the tumour promoter TPA induced benign papillomas, some of which progressed to invasive carcinomas. Therefore, upon infection of mouse skin with recombinant retroviral vector virus coding for the v-Ha-*ras* gene (DHAR et al., 1982) and the *lacZ* histochemical marker gene, it should be possible to follow virally initiated cells through promotion to the development of benign papillomas and perhaps ultimately through progression to the development of invasive carcinomas. The successful application of genetic cell marking with a v-Ha-*ras* and *lacZ* gene encoding retrovirus could provide information about the developmental capacity, localization and involvement of (virally) initiated cells in multistage mouse skin carcinogenesis.

5.3.1. Cloning of pZip LacZ SV(v-ras) Retroviral Vector.

The retroviral vector pZip LacZ SV(v-ras) (figure 5.3) was designed to contain two genes of interest, v-Ha-*ras* isolated from a cloned Harvey murine sarcoma virus (DHAR et al., 1982) and the *E. coli lacZ* histochemical marker

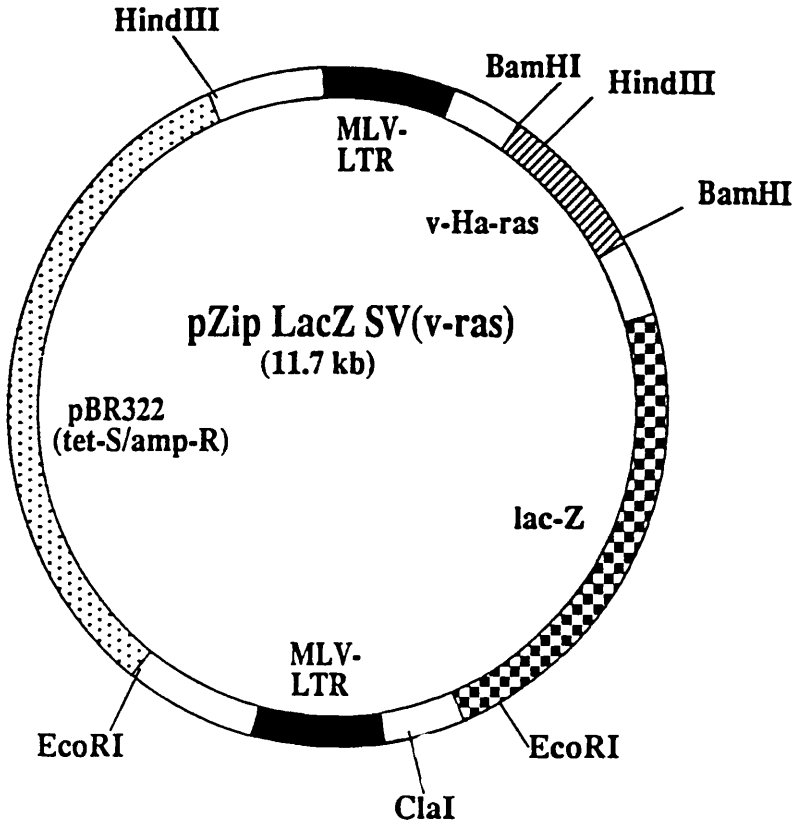

Figure 5.3

The pZip LacZ SV(v-ras) retroviral vector.

The retroviral vector pZip LacZ SV(v-ras) was cloned by B. BAILLEUL (unpublished results) by replacing the neo- cassette (chapter 5.2.1) in the XhoI cloning site of the pZip Neo SV(v-ras) retroviral vector (DOTTO et al., 1985) with the *lacZ* gene encoded in the SalI fragment (chequered box) isolated from plasmid pGH101 (HERMAN et al., 1986). The v-Ha-*ras* gene is represented by the striped box, retroviral Mo-MuLV-LTR sequences by filled boxes, and the pBR322 plasmid sequences by the dotted box. Recognition sites for restriction endonucleases ClaI, EcoRI, BamHI and HindIII are indicated.

gene ligated to an "in frame" AUG codon (HERMAN et. al., 1986). The retroviral vector pZip LacZ SV(v-ras) was cloned by B.BAILLEUL (unpublished results) by replacing the XhoI *neo*-cassette from the retroviral vector pZip Neo SV(v-ras) (DOTTO et. al., 1985) through a SalI-SalI fragment encoding the *lacZ* gene from plasmid pGH101 (HERMAN et. al., 1986), in which the *lacZ* gene was ligated to an "in frame" AUG codon (HERMAN et. al., 1986). The expression of both exogenous genes, the *neo* and the *lacZ* gene, is under transcriptional control of the Mo-MuLV-LTR in the pZip LacZ SV(v-ras) vector (figure 5.3). The expression of the *lacZ* gene in particular is greatly dependant on the efficiency of splicing taking place in the cells infected by Zip LacZ SV(v-ras) virus (HWANG et al., 1984; MILLER and TEMIN, 1986; PEABODY and BERG, 1986; GILBOA, 1986). pZip LacZ SV(v-ras) encodes no drug resistance marker gene, that can be used to select for infectants.

5.3.2. Generation of Zip LacZ SV(v-ras) Virus Producer Cell Pools and Infection of CT3 Fibroblasts.

Infectious Zip LacZ SV(v-ras) virus producer cells were generated by stable co-transfection of pZip LacZ SV(v-ras) and pAG60 plasmid DNA into the ecotropic Ψ2 cells (MANN et al., 1983) by calcium phosphate co-precipitation (WIGLER et al., 1978). The plasmid pAG60 (kindly donated by J. LANG) contains the dominant selectable *neo* gene (COLBERE-GARAPIN et. al., 1981). Co-transfecting the pAG60 plasmid with pZip LacZ SV(v-ras) allowed G418 selection in order to identify successfully transfected Ψ2 cells. Transfected Ψ2 cells were either selected for G418-resistance (selecting for the *neo* gene) or for their ability to form foci on a monolayer of untransformed Ψ2 cells (selecting for the transforming v-*ras* gene function).

Upon calcium-phosphate mediated plasmid DNA transfection pZip LacZ SV(v-ras) retroviral vector DNA induced transformed foci in Ψ2 cells. The majority of pZip LacZ SV(v-ras)-transfected Ψ2 foci were also positive for β-galactosidase expression. Therefore, Zip LacZ SV(v-ras) driven v-Ha-*ras* and *lacZ* gene expression had been demonstrated. Controls had been included to test for overall transfection efficiency and to provide a positive control for G418 selection (pAG60); plasmids pIC-TK-ras (J. LANG, unpublished results) and pZip Neo SV(v-ras) (DOTTO et. al., 1985), both containing v-Ha-*ras* gene, were

used as positive controls in focus forming assay (SHIH et al., 1979 and 1981; COOPER et al., 1980; KRONTIRIS and COOPER, 1981; PERUCHO et al., 1981) with untransfected Ψ2 cells giving very little or no background of spontaneous foci; pBAG DNA (PRICE et. al., 1987) was included as the positive control for X-gal specific staining (data not shown).

Three pools of G418-resistant Ψ2 cells, selected after co-transfection of pZip LacZ SV(v-ras) and pAG60 plasmid DNA, were tested for Zip LacZ SV(v-ras) virus production as described earlier (table 5.2. transfection). Zip LacZ SV(v-ras) virus does not encode a drug-selectable marker gene. Therefore, CT3 cells infected by the recombinant retroviral vector virus were selected in focus forming assays to test for v-Ha-*ras* transforming activity through the loss of contact inhibited growth. The virus titres given were based on the number of foci positive for β-galactosidase expression. Although all three pools tested released infectious Zip LacZ SV(v-ras) virus, the actual virus titre varied from $6.0x10^3$ to $8.5x10^5$ β-gal$^+$-focus forming units per ml virus supernatant per 10^6 viable cells (β-gal$^+$-ffu/ml/10^6 viable cells) (table 5.2. transfection). Zip LacZ SV(v-ras) producer pool 1, releasing the highest amount of infectious virus, was used as the Zip LacZ SV(v-ras) producer cell line for all subsequent experiments. Upon infection of CT3 cells, the incidence of β-gal$^+$-foci to β-gal$^-$-foci demonstrated high coincidence of v-Ha-*ras* expression and β-galactosidase activity. More than 96% of v-Ha-*ras*-induced foci stained blue after incubation of the cells with the β-galactosidase substrate X-Gal, while monolayers of morphologically normal CT3 cells remained unstained. However, since the aim was to attempt to infect epithelial cells *in vivo*, it was important to explore methods to increase the titre of virus released by the producer cell line. The importance of virus titre in retroviral mediated gene transfer had previously been demonstrated by BROWN et al (1986) and THOMPSON et al (1989).

5.3.3. Attempts to Increase Production of Infectious Recombinant Virus.

Several experimental designs were followed up aiming to increase the titre of the Zip LacZ SV(v-ras) producer cells:

A) Zip LacZ SV(v-ras) Virus Rescue By Superinfection With a Wild-Type Helper Virus.

Table 5.2

Comparison of virus titres of v-ras/lacZ retroviral vectors from producer lines generated by different methods.

(a) Zip LacZ SV(v-ras) virus producer cells were generated by stable transfection with 20μg of pZip LacZ SV(v-ras) plasmid DNA and 5μg of pAG60 plasmid DNA in the presence of 40μg of human white blood cell carrier DNA into Ψ2 cells (MANN et al., 1983) by calcium phosphate co-precipitation (WIGLER et al., 1978). Three independent producer pools were established by pooling G418-resistant transfected Ψ2 colonies.

(b) CT3 cells derived from a clonal β-gal$^+$-focus established following infection of CT3 cells with infectious Zip LacZ SV(v-ras) retroviral vector were superinfected with 100μl of wild-type Mo-MuLV helper virus. The technique of rescuing replication-incompetent retroviral vector, integrated into the host cell genome, by superinfection of host cells with replication-competent helper virus is outlined in figure 1.5 and is described in chapter 1.5.6)

(c) In a variation to the shuttle technique described by MILLER et al (1986), amphotropic packaging cells PA137 (MILLER et al., 1985) were infected with infectious ecotropic Zip LacZ SV(v-ras) retroviral vector. 48 hours after the infection, supernatant containing infectious amphotropic retroviral vector was harvested and used to infect fresh ecotropic Ψ2 cells. Infected Ψ2 cells were selected for focus formation in growth medium supplemented with 5% serum.

(d) Two clonal packaging cell lines releasing infectious Zip ras/β-gal retroviral vector (THOMPSON et al., 1989) had been obtained from H. LAND. The Zip ras/β-gal producer cell lines had been established by stable co-transfection of Ψ2 cells with pZip ras/β-gal and pSV2neo (SOUTHERN and BERG, 1982) plasmid DNA followed by G418 selection. The two cell lines (Ψ2 ras/β-gal 5 and Ψ2 ras/β-gal 9) had been characterized by THOMPSON et al. (1989) as releasing infectious retroviral vector at high titre.

(e) The viral titre is calculated form the number of focus forming units (ffu) observed per ml of supernatant per 10^6 cells replated into growth medium supplemented with 5% serum.

(f) The viral titre is calculated from the number of soft agar colony forming units (SA-cfu) observed per ml of supernatant per 10^6 cells replated into semi solid growth conditions (0.3% soft agar).

Comparison of virus titres in v-ras/LacZ retroviral vectors on produce lines generated by different methods.

Method to generate producer cell line	Virus titre on CT3 cells	
	ffu/ml[e]	SA-cfu/ml[f]
Transfection[a]	1) $0.8\text{-}2.6\times10^{6}$ 2) 2.2×10^{4} 3) 6×10^{3}	1) 1.2×10^{5} 2) not done 3) not done
Helper virus rescue[b]	not done	3.7×10^{5}
Shuttle infection[c]	1) 5.3×10^{5} 2) 6.6×10^{5} 3) 5.7×10^{5}	not done
Transfection[d] (Thompson *et al*,1989)	1) 1.2×10^{5} 2) 1.1×10^{5}	not done not done

The rescue of Zip LacZ SV(v-ras) virus from infected but non-producing CT3 cells by superinfection with replication-competent Mo-MuLV helper virus will lead to virus stock containing replication-defective Zip LacZ SV(v-ras) virus and helper virus. The presence of replication-competent helper virus in the infectious virus stock will allow horizontal spread, due to infected cells being able to release infectious virus. This should lead to enlarged clusters of infected cells, of non-clonal origin, facilitating the identification of β-gal$^+$-cells *in vivo*. Horizontal virus spread could be advantageous in Zip LacZ SV(v-ras) initiated mouse skin carcinogenesis for the following reasons:

i) Virus stock containing Zip LacZ SV(v-ras) virus and replication competent Mo-MuMLV helper virus would be similar to the Ha-MSV virus stock used by BROWN et al. (1986) in viral initiation of mouse skin carcinogenesis. The presence of helper virus could, however, alter the clonality of any arising tumour.

ii) Results by THOMPSON et al. (1989) suggested that epithelial cells might be less infectable by Mo-MuLV derived retroviral vector virus. Using infectious virus stock that also contains replication-competent helper virus, would perhaps increase the chance of infecting epithelial cells by horizontal virus spread after the initial virus application.

The technique of superinfection is outlined in figure 1.5. After infection with Zip LacZ SV(v-ras) virus, CT3 cells cloned from a β-gal$^+$-focus were superinfected with Moloney Murine Leukemia virus (Mo-MuLV). The results of infection of CT3 cells with helper rescued Zip LacZ SV(v-ras) virus stock are shown in table 5.2 (helper virus rescue). The overall virus titre was 4.1×10^5 soft agar-colony forming units per ml infectious supernatant (SA-cfu/ml). Soft agar cloning was used to assess transformation of infected CT3 cells by v-Ha-*ras* to avoid horizontal virus spread. The vast majority of soft agar colonies (90%) were also positive for β-galactosidase activity, resulting in a Mo-MuLV rescued Zip LacZ SV(v-ras) virus titre of 3.7×10^5 SA-cfu/ml. Only 10% of the soft agar colonies (0.4×10^5 SA-cfu/ml) did not express the β-galactosidase enzyme in its active form, as they failed to convert the X-Gal substrate.

The titre of the Mo-MuLV-rescued Zip LacZ SV(v-ras) virus stock was less than 4 fold higher than helper-free virus stock. Nevertheless, the Zip LacZ

SV(v-ras) producer cell line 2, generated through rescue with Mo-MuLV helper virus, was also used in subsequent experiments infecting mouse skin.

B) Shuttle of Retroviral Vectors using Packaging Cell Lines with Different Host Ranges.

The shuttle technique for creation of cell lines secreting helper free-retroviral vectors was first described by MILLER et. al. (1986). This technique involved the direct infection of retrovirus packaging cells of one (e.g. ecotropic) host range with the viral vector generated by another packaging cell line displaying a different host range (e.g. amphotropic). MILLER et. al. (1986) suggested that the titre of recombinant virus produced from infected packaging cell lines would be on average higher than the recombinant virus titre produced when the provirus had been introduced into the packaging cell lines by transfection with the plasmid DNA.

In a variation to the shuttle protocol described by MILLER et. al. (1986), amphotropic packaging cells PA137 (MILLER et. al., 1985) were transiently infected with ecotropic Zip LacZ SV(v-ras) virus released from Zip LacZ SV(v-ras) producer line 1. Virus secreted from infected PA137 cells was harvested and used to infect fresh cells of the ecotropic packaging cell line Ψ2 (MANN et. al., 1983). Ψ2 cells infected with amphotropic Zip LacZ SV(v-ras) virus were selected for focus formation. A total of 11 individual foci were established as clonal cell lines. Only three out of the 11 cell lines examined were clearly strongly positive for β-galactosidase activity. Each of the three focus derived cell lines were tested for Zip LacZ SV(v-ras) virus production by infecting CT3 cells followed by selection for focus formation (table 5.2. shuttle infection). The percentage of β-gal$^+$-ffu were between 96% to 90% of the total number of ffu. However, upon passage of the "shuttled" producer cell lines, the ability to form colonies in soft agar and the percentage of colonies staining positive for β-galactosidase activity declined. None of the three producer lines obtained after shuttling of recombinant Zip LacZ SV(v-ras) virus showed a significantly increased virus titre compared to the Zip lacZ SV(v-ras) producer line 1 generated by transfection of Ψ2 cells. However, it can be seen that there was less clonal variation in the titre of virus released. Therefore, the "shuttle technique" did not help to establish producer cell lines releasing recombinant Zip LacZ

SV(v-ras)-retrovirus vector at high titre. More important still, "shuttled" producer lines did not seem to display a stable β-gal$^+$/v-Ha-ras$^+$-phenotype. Neither of the three "shuttled" producer cell lines were used in any further experiments.

C) Virus Titre of Zip ras/-gal Producer Cell Lines.

Two clonal packaging cell lines releasing recombinant Zip ras/β-gal virus (THOMPSON et. al., 1989) had been obtained from H. LAND. The retrovirus vector Zip ras/β-gal, cloned by THOMPSON et. al. (1989), was identical to the Zip LacZ SV(v-ras) vector, both recombinant retroviral vectors transduced the activated viral Ha-*ras* gene and the *E.coli lacZ* gene. Producer cell lines Ψ2 ras/β-gal 5 and 9 were tested for virus production at the same time as Zip LacZ SV(v-ras) producer lines. Table 5.2 gives the result of the direct virus titre comparison of the different Zip LacZ SV(v-ras) virus and Zip ras/β-gal virus producer cell lines, respectively. The results show that neither of the two Zip v-ras/β-gal producer lines obtained from H.LAND produced the recombinant v-Ha-*ras* and *lacZ* gene expressing retroviral vector with higher titre than eitherthe producer lines for Zip LacZ SV(v-ras) or Zip LacZ SV(v-ras)/Mo-MuLV (1.1 to 1.2x10^5 β-gal$^+$-ffu/ml; table 5.2. transfection by THOMPSON et al., 1989). On the contrary, the viral titre determined for Zip LacZ SV(v-ras) producer cell line 1 was slightly higher than the titre of the other v-Ha-ras/β-gal expressing viruses. The incidence with which both genes were expressed simultaneously in the same infected cell were broadly similar: 85% of the Zip LacZ SV(v-ras) induced CT3 foci were also β-gal positive, Zip ras/β-gal-CT3 foci were β-gal positive in 73% (Ψ2 ras/β-gal 9) and 96% (Ψ2 ras/β-gal 5) of the cases.

D) Concentration of Viral Supernatants.

As it had so far not been possible to clone a Zip LacZ SV(X)1-virus producer line releasing the recombinant virus at a higher viral titre, freshly harvested viral supernatants were concentrated by centrifugation through Amicon micro-concentraters prior to use or short-term storage at -70^{o}C. All concentrated virus stocks were titred on CT3 cells. On average, concentration of viral supernatant led to a 5 to 15 fold increase in virus titre (data not shown).

In summary, none of the experiments described above have been successful in generating a new Zip LacZ SV(v-ras) producer cell line with a

higher virus titre. The superinfection of Zip LacZ SV(v-ras) infected CT3 cells with replication-competent Mo-MuLV helper virus was successful in rescuing the retroviral vector. However, a direct comparison of Zip LacZ SV(v-ras) viral titres, determined on the basis of β-gal^{+}-CT3 foci formation, revealed that producer cell lines 1 and 2 released recombinant Zip LacZ SV(v-ras) virus with a similar titre (table 5.2. helper virus rescue). Clonal Zip LacZ SV(v-ras) producer cell lines generated by the shuttle technique described by MILLER et al. (1986) also failed to release recombinant virus at higher titres (table 5.2. shuttle infection). Therefore, no further attempts were undertaken to generate producer cell lines which would release recombinant Zip LacZ SV(v-ras) virus at a higher titre. Clonal Zip ras/β-gal producer cell lines (THOMPSON et al., 1989), did not have a higher titre than the Zip LacZ SV(v-ras) producer line 1 (table 5.2. transfection by THOMPSON et al., 1989). The concentration of viral particles in supernatants harvested from virus producer lines through Amicon micro-concentrators led to an average 5 to 15 fold increase in virus titre. Subsequently, concentrated stocks of the ras/β-gal retroviral vectors were used when infecting mouse skin *in vivo*.

5.4. LacZ SV(v-ras) Retroviral Vector Mediated Gene Transfer *In Vivo*.

5.4.1. Nude Mice Tumorigenicity Test.

It had been demonstrated that the activated v-Ha-*ras* gene, cloned from the Harvey murine sarcoma virus (DHAR et. al., 1982), inserted into the recombinant Zip LacZ SV(v-ras) retroviral vector was capable of inducing transformation in CT3 fibroblasts *in vitro* (table 5.2). Zip LacZ SV(v-ras) infected CT3 cells also efficiently induced tumours in nude mice after subcutaneous injection of cells (10/10 possible tumours in Zip LacZ SV(v-ras) infected CT3 cells, 0/10 from uninfected CT3 cells). Frozen sections through these tumour samples were tested for the presence of β-galactosidase activity, using a modified histochemical staining protocol (J. PRICE, personal communication). The treated sections showed intensive blue staining in all tumour cells, whereas no traces of staining could be detected in the adjacent skin tissue (figure 5.4). These results confirmed, that the viral Ha-*ras* gene and the bacterial *lacZ* gene encoded in the recombinant Zip LacZ SV(v-ras) vector, were functionally expressed *in vivo*. Tumorigenicity tests were also carried out on packaging cell lines producing either one of the ras/β-gal recombinant retrovirus stocks, Zip LacZ SV(v-ras), Zip LacZ SV(v-ras)/Mo-MuLV and Zip ras/β-gal (THOMPSON et. al., 1989). The presence of the Mo-MuLV helper-virus in Zip LacZ SV(v-ras) virus stock (producer cell line 2) resulted in a 100% incidence of tumour induction in nude mice when injected subcutaneously. In comparison, tumour formation upon subcutaneous injection of helper virus-free Zip LacZ SV(v-ras) producer cells also occurred at every injection point, but was delayed by two days. The rate of tumour formation induced by subcutaneous injection of Zip ras/β-gal producer cells (THOMPSON et. al., 1989) was reduced further, regarding both tumour incidence and latency period. The altered tumorigenic behaviour of the Zip ras/β-gal producer cells (Ψ2 ras/β-gal 9) could perhaps be explained by a less stable β-gal$^+$/v-Ha-ras$^+$ phenotype. The periodical screening for the expression of the activated viral Ha-*ras* gene (focus formation and/or soft agar colony formation assay) and the bacterial *lacZ* gene (testing for β-galactosidase activity by staining with the substrate X-gal) did not indicate any loss of expression of either of the two genes.

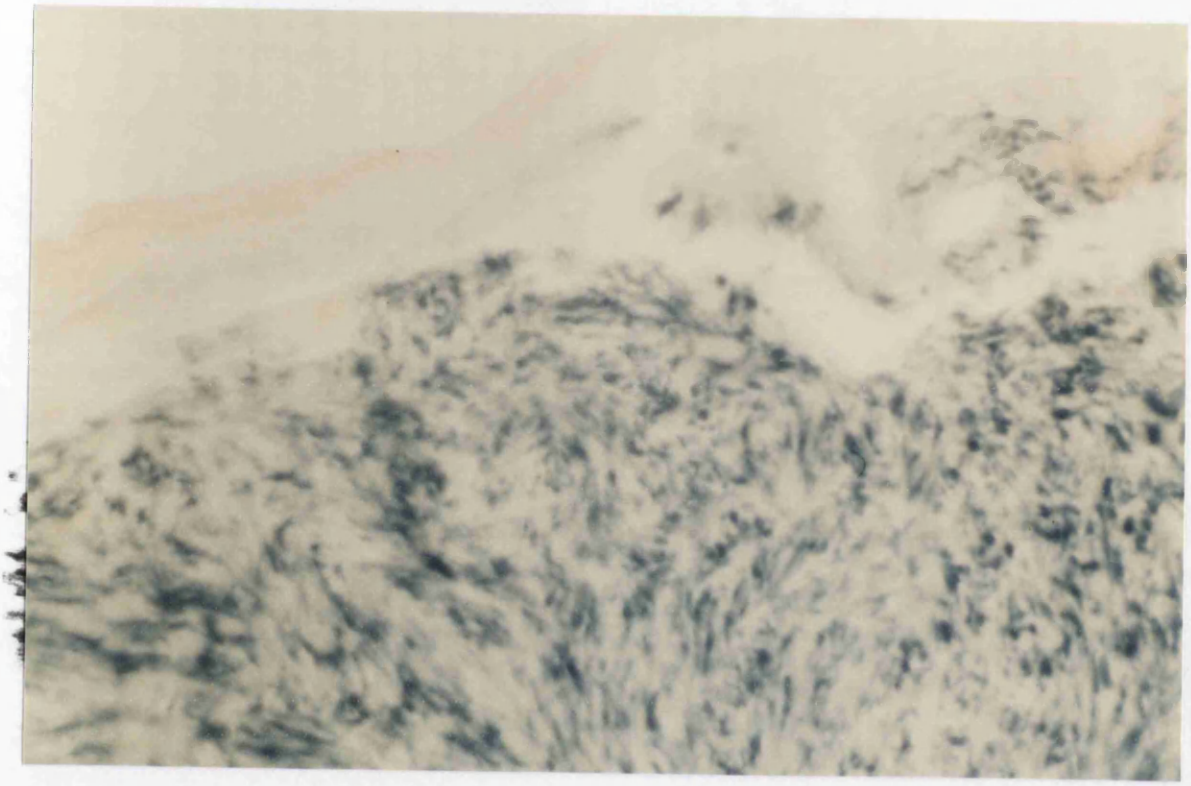

Figure 5.5.

Immunoprecipitatio of p21 Ha-ras protein of ras/β-gal infected C5N cells.

Following a single TPA treatment (table 5.4. legend), protein lysates of uninfected C5N cells (lane1), C5N cells infected with Zip Neo SV(X)1 (lane 2), BAG (lane 3), or Zip LacZ SV(v-ras) retroviral vector in the absence or presence of Mo-MuLV helper virus (lanes 4 and 5, respectively), or with wild type BALB-MSV (lane6) were immuniprecipitated with YA6-172 monoclonal antibody. Antigen-antibody complexes were precipitated with protein A-sepharose coated with rabbit anti-rat IgG and run on a denaturing polyacrylamide gel (17.5%) as described in QUINTANILLA et al., 1986. The gel was electroblotted and p21 Ha-ras proteins were detected by chemiluminescence using an ECL Chemiluminescence detection kit (Amersham).

A p21 v-Ha-ras protein specific band was detected only in lane 6 containing protein from BALB-MSV infected C5N cells. A signal corresponding to cellular p21 c-Ha-ras protein was detected in all lanes.

The Zip LacZ SV(v-ras) producer cell lines 1 and 2, as well as Ψ2 ras/β-gal 9 (THOMPSON et al., 1989) released principally the same infectious recombinant retroviral vector. Any differences in the ability to initiate viral multistage carcinogenesis in mouse skin would be most likely due to the titres with which the recombinant viruses were generated by their respective producer cells (BROWN et al., 1986). A higher titre would lead to a higher chance of infecting epithelial cells *in vivo*. If the time delay observed of tumour induction by subcutaneously injected Zip ras/β–gal producer cells (Ψ2 ras/β-gal 9) was reflected in a reduced titre for the released Zip ras/β-gal virus, then a reduced frequency of viral initiation of multistage mouse skin carcinogenesis would be expected. The Zip SV(v-ras)/Mo-MuLV virus stock was anticipated to be the one most likely to succeed in initiating virally induced multistage carcinogenesis processes in mouse skin. The presence of the replication competent wild-type Mo-MuLV helper-virus would make horizontal spread of the Zip lacZ SV(v-ras) virus possible, thus increasing the chances of infecting epithelial stem cells within the mouse skin (BROWN et al., 1986).

5.4.2. Virus Initiated Mouse Skin Carcinogenesis.

Female NIH mice aged between 8 to 12 weeks were used in virus initiation experiments *in vivo*. The mice had been obtained from the breeding colony within the Beatson Institute or from a commercial supplier. The mice were shaved at least two days prior to virus application. All mice were treated with depilatory agent and with a single dose of TPA (200µl of 10^{-4}M TPA in acetone) 24 hours prior to virus application, although pretreatment with TPA was not essential for tumour formation (BROWN et al., 1986). Approximately 100µl of viral supernatant containing 80 µg/ml polybrene were applied to the back skin of fully anaesthetised mice by scarification. TPA treatment was started 7 days after virus application and thereafter given twice weekly over a period of 17 weeks. Control groups were treated twice weekly with 200µl acetone alone.

Table 5.3 lists the experimental groups as defined by initiating virus used and TPA treatment and summarizes the respective number of tumours observed. In experimental groups 1 and 3, virally initiated mice treated with acetone alone did not develop skin tumours throughout the observation period of 18 weeks. The lack of tumour development in Zip LacZ SV(v-ras) or Zip LacZ SV(v-ras)/Mo-

Infection of mouse skin with recombinant v-ras/β-gal retroviral vectors.

Experimental group [a]	Number of mice	Retroviral construct	Virus titre [b] β-gal$^+$-ffu/ml/10^6 viable cells	Promoter treatment [c]	Total No. papillomas Total No. of mice
1	9	ZipLacZ SV(v-ras)	$2.6x10^5$	-	0/0
2	9	BAG	$3.7x10^5$	-	0/0
3	9	ZipLacZ SV(v-ras)	$3.5x10^5$	-	0/0
4	33	ZipLacZ SV(v-ras)	$2.6x10^5$	TPA	0/33
5	18	BAG	$3.7x10^5$	TPA	0/18
6	33	ZipLacZ SV(v-ras)	$3.5x10^5$	TPA	1/33 [d]
7	12	Zipras/β-gal	$2.1x10^5$	TPA	1/12 [e]
8	6	BALB-MSV	$>8.6x10^6$	TPA	18/6 [f]

Table 5.3

Infection of mouse skin with recombinant v-ras/β-gal retroviral vectors *in vivo*.

(a) Experimental groups: see text.

(b) The viral titre is the number of β-gal$^+$-focus forming units observed per ml of supernatant per 10^6 viable cells replated into growth medium supplemented with 5% serum. β-galactosidase activity in foci was demonstrated by X-Gal staining (SANES et al., 1986).

(c) Dash indicated treatment twice weekly with 200µl acetone for 17 weeks (experimental groups 1 to 3). TPA treatment was given by applying twice weekly 200µl 10^{-4}M TPA/acetone onto the back of mice for 17 weeks. Promoter treatment was stopped after 17 weeks. However, mice were maintained for further 4 weeks without any treatment.

(d) A small papilloma (<2mm in diameter) developed by week 15 of promoter treatment. This papilloma persisted after TPA treatment had been stopped, but showed no β-galactosidase activity in frozen sections after X-Gal staining.

(e) A small, promoter-dependent papilloma (<2mm in diameter) developed by week 15 of TPA treatment, but regressed as soon as promoter treatment was stopped.

(f) Several BALB MSV-initiated papillomas had developed after 4 to 6 weeks of TPA treatment. By 11 weeks of promoter treatment, the average size of the papillomas was between 5 and 10mm in diameter.

MLV initiated mice was in agreement with results published by BROWN et. al. (1986) who had previously demonstrated, that initiation of epithelial cells *in vivo* by application of Ha-MSV or BALB-MSV retrovirus without subsequent tumour promotion was not sufficient to complete the carcinogenesis process.

No skin tumours were observed on 9 animals, infected with the BAG virus and promoted with acetone for 17 weeks during an observation period of up to 17.5 months (table 5.3. experimental group 2). Initiation with BAG virus and promotion through twice weekly TPA treatments over a period of 17 weeks also failed to induce any tumour development (table 5.3. experimental group 5). BAG-viral initiation followed by TPA promotion was not expected to give rise to tumour formation, as the initiating BAG virus did not contain an activated v-Ha-*ras* gene. TPA treatment alone, without previous viral or chemical initiation, did not lead to papilloma formation, as had been shown previously (BROWN et al., 1986). Mice initiated with the Balb-MSV virus (Balb-MSV virus titre: $>8.6x10^6$ ffu/ml/viable cells) and followed by twice-weekly TPA treatment over a period of 11 weeks, developed papillomas by 4 to 6 weeks (table 5.3. experimental group 8). A total of 18 papillomas were counted on the 6 BALB-MSV-initiated NIH mice. The majority of these papillomas reached a size of 5 to 10 mm in diameter (table 5.3). No carcinomas had been observed. The observation period of 11 weeks had been too short to expect any malignant conversion of BALB-MSV-initiated papillomas to carcinomas. The expected latency period for malignant conversion of HaMSV-initiated papillomas had been determined as 3 to 5 months (BROWN et al. (1986).

Initiation of mice with the helper-free Zip LacZ SV(v-ras) virus stock in combination with twice weekly TPA treatments over 17 weeks, did not give rise to any papillomas on any of the 33 mice (table 5.3. experimental group 4). The virus titre of the Zip LacZ SV(v-ras) virus stocks used was between $2.6x10^5$ and $1.0x10^6$ β-gal$^+$-ffu/ml/viable cells and was thus comparable to the viral titres of the recombinant retroviral vectors Zip LacZ SV(v-ras)/Mo-MLV (viral titre: $3.5x10^5$ β-gal$^+$-ffu/ml/viable cells) and the Zip ras/β-gal (viral titre: $2.1x10^5$ β-gal$^+$-ffu/ml/viable cells).

In total 33 mice were initiated with Zip LacZ SV(v-ras)/Mo-MuLV helper virus stock (Zip LacZ SV(v-ras)/Mo-MuLV virus titre: $3.5x10^5$ β-gal$^+$-ffu/ml/viable cells) and TPA treated twice weekly for 17 weeks. Only one of the

33 animals developed one small papilloma by week 15. The papilloma remained small, only 2mm in diameter by week 20, but did persist for 4 weeks after TPA treatment had been stopped at week 17 (table 5.3. experimental group 6). However, histochemical staining of frozen sections derived from this papilloma failed to detect expression of the β-galactosidase gene (data not shown).

One animal developed one small papilloma (2mm in diameter) by week 15 from experimental group 7, initiated by Zip ras/β-gal virus (Zip ras/β-gal virus titre: $2.1x10^5$ β-gal$^+$-ffu/ml/viable cells) and promoted by twice weekly TPA applications over a period of 17 weeks (table 5.3). The papilloma proved to be tumour promoter dependent, as it started to regress as soon as TPA treatment had been stopped. By week 22 the papilloma was no longer detectable. BROWN et al. (1986) also reported regression of HaMSV-initiated papillomas after about 12 weeks of promoter treatment although these papillomas had previously reached a considerably larger size.

In total 78 mice had been initiated with recombinant ras/β-gal retroviral vectors and promoted with TPA. But only 2 of these mice went on to develop one papilloma each after a relatively long latency period of 15 weeks. One of the two papillomas proved to be TPA dependent, as it regressed completely after the promoter treatment had been stopped (table 5.3), whereas the other failed to express the active form of β-galactosidase as shown by histochemical staining. The total number of tumours observed in the experimental groups described above compared poorly with findings by BROWN et al. (1986) who counted 12 papillomas on 4 papilloma-bearing mice out of a group of 9 NIH mice initiated with Balb-MSV virus (virus titre: 0.2 to $5x10^5$ ffu/ml/viable cells). The viral titres of the three different recombinant ras/β-gal virus stocks used in the *in vivo* initiation were between $2.1x10^5$ to $3.5x10^5$ β-gal$^+$-ffu/ml/viable cells, thus being similar to the virus titre of the Balb-MSV virus stock used by BROWN et al. (1986). It seemed unlikely, therefore, that the low tumour incidence was solely due to lack of successful initiation as a possible consequence of applying too few infectious virus particles. A different explanation, based on the relative sensitivity of NIH mice to virus-initiated papilloma formation, would suggest that NIH mice breeding strains could develop differences with regards to sensitivity to virus-initiated papilloma formation over a period of 4 years. This hypothesis was dismissed, however, by the result of papilloma formation observed with Balb-

MSV virus-initiated NIH mice (table 5.3). Therefore, the most likely explanation for the low tumour incidence had to lie within the identical recombinant retroviral vectors Zip LacZ SV(v-ras) virus and Zip ras/β-gal virus themselves. The transforming activity of the v-Ha-*ras* gene present in both retroviral constructs in epithelial cells was re-examined in more detail *in vitro*, as the lack of reduced transforming activity of the retroviral vector encoded v-Ha-*ras* gene in epithelial cells would be the most probable explanation for the failure of the recombinant v-Ha-ras/β-gal retroviral vectors to induce papilloma formation in mouse skin *in vivo*.

5.5. Transforming Activity of Recombinant v-Ha-ras/β-gal Vectors *In Vitro*.

Previous experiments demonstrated clearly that the promoter region within the Mo-MuLV LTR functioned efficiently in directing the expression of the *neo*^{r}-marker gene and the bacterial *lacZ* gene in epithelial C5N cells (table 5.1). Recombinant v-Ha-*ras*/β-gal retroviral vectors had also been shown to induce transformation in infected CT3 cells, as demonstrated through focus-forming assay (table 5.2), soft agar cloning assay (table 5.2) and tumorigenicity assay in nude mice. However, transformation of C5N cells induced by the expression of the v-Ha-*ras* gene under the transcriptional control of the Mo-MuLV LTR promoter had not been previously examined. As the *in vivo* experiments described above demonstrated a failure of the recombinant v-Ha-*ras*/β-gal vectors to induce papillomas in mouse skin, the question had to be addressed whether the recombinant vectors Zip LacZ SV(v-ras) and/or Zip ras/β-gal (THOMPSON et al., 1989) could induce transformation in epithelial C5N cells *in vitro*.

Two approaches were used to assess transformation of C5N cells *in vitro*. C5N cells infected with either of the recombinant v-Ha-*ras*/β-gal retroviral vectors were analysed with respect to their serum requirement for growth and response to TPA treatment.

A) The requirement of serum for cell growth of C5N cells infected with Zip LacZ SV(v-ras) or Zip ras/β-gal (THOMPSON et. al., 1989) was tested in different serum concentrations (5% and 2%). Untransformed C5N cells had been reported to be extremely serum-dependent (A. STOLER, personal communication). However, attempts to show differential growth in low serum

conditions of v-Ha-*ras* transformed C5N cells failed. No significant differences were detected regarding the ability to grow in SF12 medium containing 5% or 2% serum. Neither of the infecting recombinant v-Ha-*ras*/β-gal viruses induced any changes regarding serum requirements for growth. Furthermore, C5N cells infected by control viruses such as Zip Neo SV(X)1 or BAG, neither of which contained a transforming v-Ha-*ras* gene, or by the transforming BALB-MSV sarcoma virus (H9) showed near identical growth characteristics to C5N cells infected with either of the three recombinant Ha-*ras*/β-gal virus stocks (data not shown).

B) The second approach used to assess v-Ha-*ras*/β-gal retroviral vector induced transformation of C5N cells *in vitro* was based on work by PARKINSON et al. (1983), KULESZ-MARTRIN et al. (1980), YUSPA and MORGAN (1981), and YUSPA et al. (1982).

PARKINSON et al. (1983) described a subpopulation of normal cultured human keratinocytes which was insensitive to TPA-induced loss of cloning efficiency and suggested that the resistant cells were not sufficiently committed to terminal differentiation. They reported further, that the number of cells resistant to TPA-induced terminal differentiation were greater in transformed lines, as these possessed a reduced competence to trigger terminal differentiation *in vitro*. YUSPA and colleagues suggested that mouse epidermal cells which had been initiated *in vivo* (YUSPA and MORGAN, 1981) or *in vitro* (KULESZ-MARTIN et al., 1980) showed a reduced capacity to terminally differentiate in culture and would further, as a response to repeated treatments with the tumour promoters like TPA, expand at the expense of the normal cells. Similarly, neoplastic mouse keratinocytes had been shown to be much less sensitive to growth inhibition by phorbol esters than their normal counterparts maintained in the same culture conditions (FUSENIG et al., 1979).

Due to time limitations, only the response of Zip LacZ SV(v-ras) or Zip ras/βgal-infected C5N cells to a single treatment with the tumour promoter TPA *in vitro* was analysed. Colony-formation was taken as the parameter to assess the commitment to terminal differentiation to a single TPA treatment (Table 5.4). The response of C5N cells to the single TPA treatment was expressed as the percentage change in colony-formation of infected and parental C5N cells after TPA treatment compared to their non-treated counterparts (table 5.4). C5N cells

Transformation of C5N cells following infection with ZipLacZ SV(v-ras) retroviral vectors.

Retroviral vector	TPA (a)	Percentage colony formation (b)	Percentage change in colony formation (c)
ZipNeo SV(x)1	-	29 (3)	0
	+	15 (4)	-48
ZipLacZ SV(v-ras)	-	23 (3)	0
	+	15 (4)	-34
ZipLacZ SV(v-ras) +Mo-MuLV	-	12 (2)	0
	+	9 (4)	-25

Table 5.4

Transformation of C5N cells following infection with Zip LacZ SV(v-ras) retroviral vector.

Following a single TPA treatment, Zip LacZ SV(v-ras)-infected C5N cells were assessed for their commitment to terminal differentiation by colony formation assay. Loss of terminal differentiation can be regarded as a parameter of transformation in epithelial cells (PARKINSON et al., 1983). 48 hours after infection C5N cells had been split into two groups, one group was subsequently treated with TPA (0.1ng/ml in SF12 growth medium) for 24 hours, whereas the second group of infected C5N cells was replated into normal SF12 growth medium. TPA-treated and untreated C5N cells were then replated at a cell density of 10^3 cells per 10 cm^2 plate to test for colony-formation. The response of C5N cells to the single TPA treatment was expressed as the percentage change in colony formation of infected and parental C5N cells after TPA treatment compared to their non-treated counterparts.

(a) C5N cells were treated for 24 hours with 0.1ng/ml TPA in growth medium (+) or remained untreated (-).

(b) Percentage of colony formation is the percentage number of colonies observed after replating 103 cells in normal growth medium. the numbers in brackets is the number of experiments.

(c) The percentage change in colony formation is the percentage of changes seen in colony formation between C5N cells.

infected with the parental Zip Neo SV(X)1 virus showed a 48% reduction in colony formation after TPA treatment. Following TPA treatment, a 35% reduced colony formation was observed with Zip LacZ SV(v-ras)-infected C5N cells and a 25% reduction with Zip LacZ SV(v-ras)/Mo-MuLV virus-infected C5N cells compared to their counterparts which had not been treated with TPA. In C5N cells infected with wild type BALB-MSV sarcoma virus, TPA treatment resulted in colony-formation which was reduced by 22% compared to non-treated cells. PARKINSON et al. (1983) suggested that more transformed epithelial cells would be more resistant to TPA induced terminal differentiation. on this basis, the present results would suggest that C5N cells infected with BALB-MSV virus are more transformed than C5N cells infected with Zip LacZ SV(v-ras)/Mo-MuLV, which in turn are more transformed than Zip LacZ SV(v-ras) infected C5N cells and the least transformed are C5N cells infected with Zip Neo SV(X)1 which does not contain a v-Ha-*ras* gene. These results correlate with the *in vivo* initiation experiments described in the previous section (table 5.3). It has be to pointed out, however, that these observations are based on small numbers. A more extensive analysis would be required to allow more definite conclusions.

It had not been possible to select C5N cells successfully infected by either the Zip LacZ SV(v-ras) virus or the wild type BALB-MSV virus prior to TPA treatment, as neither of the viruses used encoded a positive selection marker. Selection prior to TPA treatment would allow a more direct comparison of the viability data based on the same number of infected cells used in each experiment and would thus help in assessing the response of transformed cells to TPA treatment.

C5N cells had not been previously characterized regarding the response to a single or repeated treatments of TPA nor had the concentration of TPA been optimized for C5N cells. A comparison of the infection efficiencies achieved by Zip Neo SV(X)1 and BAG virus in C5N cells, which had been treated with TPA 48 hours after infection and in untreated cells allowed an assessment of the extent of cell differentiation and cell death induced by the single treatment with TPA. C5N cells infected with Zip Neo SV(X)1 virus followed by TPA treatment showed a dramatic 75%-reduction of infection efficiency ($3.9x10^4$ $G418^r$-cfu/ml/10^6 viable cells) compared to Zip Neo SV(X)1-infected, nontreated C5N cells ($1.6x10^6$ $G418^r$-cfu/ml/10^6 viable cells). A 92%-reduction of infection

efficiency was seen by BAG-infected, TPA-treated C5N cells ($1.1x10^4$ β-gal$^+$/G418^r-cfu/ml/10^6 viable cells) compared to BAG-infected, nontreated C5N cells ($1.3x10^5$ β-gal$^+$/G418^r-cfu/ml/10^6 viable cells). However, at the time no alternative *in vitro* epithelial cell system had been characterized regarding response to TPA treatment to be used as a test system for v-Ha-*ras* induced transformation of epithelial cells upon infection with recombinant Zip LacZ (v-ras) retroviral vectors *in vitro*.

A further explanation why v-Ha-*ras*/β-gal recombinant retroviruses failed to initiate papillomas upon TPA treatment of the infected mouse skin could be that the v-Ha-ras p21 protein was not produced to a high enough level to allow transformation *in vivo* to occur. It was therefore important to measure the levels of v-Ha-ras p21 protein in C5N cells infected with either the recombinant v-Ha-*ras*/β-gal retrovirus vectors or the wild type Balb-MSV virus. The p21 Ha-ras specific monoclonal antibody YA6-172 was used to immunoprecipitate the cellular normal and the viral Ha-ras p21 proteins (QUINTANILLA et al., 1986). The normal and the viral form of the p21 Ha-ras proteins can be easily distinguished from each other through changes in migration properties on polyacrylamide gels. The viral Ha-*ras* gene of BALB-MSV virus differs from its cellular counterpart in that it has mutations at codons 12 and 59 that lead to amino acid changes in the p21 protein product (DHAR et al., 1982), resulting in a slower mobility in polyacrylamide gels than the cellular p21 Ha-ras protein (ULSH and SHIH, 1984). Following SDS-polyacrylamide gel electrophoresis and western analysis of the immunoprecipitates of parental C5N cells and of cells infected by either recombinant v-Ha-*ras*/β-gal retroviral vectors or by wild type Balb-MSV virus, a p21 v-Ha-ras specific protein band was only detected in BALB-MSV infected C5N cells. No viral specific band were detected in immunoprecipitates of C5N cells infected by ZIP NEO SV(X), BAG, or Zip LacZ SV(v-ras) in the presence or absence of Mo-MuLV helper virus. Whereas a protein band corresponding to cellular p21 Ha-ras was detected in all immunoprecipitates (figure 5.5).

5.6. Conclusions.

The recombinant Zip LacZ SV(v-ras) retroviral vector on its own or with the presence of Mo-MuLV helper virus was able to transform CT3 fibroblast

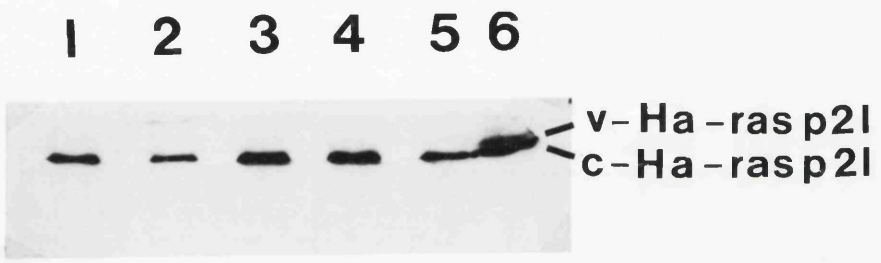

Figure 5.4.

Histochemical staining of nude mouse tumours induced by subcutaneous injection of Zip LacZ SV(v-ras) infected CT3 cells.

Tumours developed within 8 to 10 days after subcutaneous injection of Zip LacZ SV(v-ras) infected CT3 cells into nude mice ($2.5x10^6$ cells per injection site). Tumours were fixed for 1 hour at 4oC in paraformaldehyde fixative, saturated in 30% sucrose/PBS/2mM $MgCl_2$ and quick frozen on dry ice/ethanol. 10μm sections were taken, refixed at 4^oC in paraformaldehyde fixative, rinsed in PBS/2mM $MgCl_2$ at 4^oC, permeabilized for 10 min at 4^oC and stained overnight at 30^oC in X-Gal staining solution.

Intensive blue staining, indicative of β-galactosidase activity, was seen in cells of the tumour mass but not in the adjacent skin.

cells following infection *in vitro*. The transformed phenotype of Zip LacZ SV(v-ras) infected CT3 cells was confirmed by focus formation, growth in soft agar and by induction of tumour formation in nude mice following subcutaneous injection. However, the Zip LacZ SV(v-ras) retroviral vector was not able to initiate mouse skin papilloma formation *in vivo*. Infection of epithelial C5N cells by the same recombinant retroviral vectors did not show any evidence for transformation *in vitro*. In addition, western analysis of C5N cells infected by Zip LacZ SV(v-ras) vector showed no expression of p21 v-Ha-ras protein. While wild type BALB-MSV virus was able to express p21 v-Ha-ras protein in infected C5N cells *in vitro* and initiate mouse skin papilloma formation *in vivo*. This suggests that the failure of Zip LacZ SV(v-ras) retroviral vector to initiate papilloma formation *in vivo* may be due to the lack of v-Ha-*ras* expression in epithelial cells.

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