TUMOUR-SUPPRESSIVE ACTIVITY OF THE GROWTH ARREST-SPECIFIC GENE, GAS1

A thesis submitted to the University of Adelaide as the requirement for the degree of Doctor of Philosophy

by

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SUMMARY

The growth arrest-specific gene, GAS1, codes for a membrane-associated protein which accumulates at the cell surface following growth arrest mediated by either serum starvation or density-dependent inhibition. Expression of both GAS1 mRNA and protein is rapidly down-regulated when cells are activated to enter S phase. Overexpression of the mouse gas-1 gene in normal and oncogene-transformed NIH3T3 cells blocks proliferation of actively-growing cells and inhibits entry by quiescent cells into S phase when activated by serum. The growth suppressing activity of the mouse gas-1 gene is mediated by a p53-dependent signalling pathway, but the transcriptional activation function of p53 is not required for gas-1-induced growth arrest. Since the expression of the GAS1 gene is tightly confined to the quiescent stage of the cell cycle and GAS1 has growth-inhibitory activity *in-vitro,* its expression either reflects requirements to sustain growth arrest, or may be involved in the control of differentiation.

Gene products with growth-inhibitory properties frequently act as tumour suppressor genes and the studies described in this thesis seek to establish a possible role for GAS1 as a candidate tumour suppressor gene whose function is to inhibit uncontrolled proliferation and to prevent the development of the malignant phenotype.

The first part of this thesis reports on the chromosomal localisation by *in-situ* hybridisation of the mouse and human GAS1 genes. The mapping of GAS1 to mouse Chromosome 13 at bands B3-C2 and, more particularly, to human chromosome 9 at bands q21.3-q22, places the human gene in a region of the genome which is highly significant as a site of a number of potential tumour suppressor genes. Genetic

alterations in this region of chromosome 9q have previously been implicated as early events in the progression of myeloid malignancy and bladder carcinoma.

The human GAS1 gene, when overexpressed in NIH3T3 cells, is able to block proliferation of actively growing cells. Cells overexpressing exogenous GAS1 display remarkable changes in phenotype that resemble growth arrested cells or cells in crisis. These results clearly demonstrate that the human GAS1 gene product is directly involved in mediating growth suppression in a similar manner to that previously observed with the mouse homologue.

To investigate whether GAS1 is able to suppress the growth of human tumours, GAS1-negative tumour cells were transfected with GAS1-expressing plasmids and the growth characteristics of stable transfectants were analysed. The A549 lung adenocarcinoma cell line contains wild type p53 and normal levels of the MDM2 oncogene product. When a constitutively-expressing GAS1 plasmid was transfected into A549 lung adenocarcinoma cells, no stable clones expressing GAS1 were isolated. When A549 cells were transfected with a dexamethasone-inducible GAS1 plasmid, expression of GAS1 inhibited growth *in vitro* and produced fewer slow-growing tumours in nude mice. GAS1 also inhibited proliferation of an HT1080 fibrosarcoma subline with wild-type (wt) p53 and normal levels of MDM2. In contrast, when the HT1080 subline, HTD114, was transfected with the constitutive GAS1 plasmid, there was no reduction in colony number. GAS1-transfectant clones had unaltered growth *in vitro*, and showed no difference in their ability to form tumours in nude mice. Although HTD114 cells contain wt p53, levels of MDM2 were elevated by 10-15 fold. The product of the MDM2 gene physically associates with wild type p53 protein and inhibits its transactivation function. Overexpression of MDM2 therefore provides an alternate mechanism by which p53 function is inhibited. The HT10806TGc5 subline with mutant

p53 and normal levels of MDM2 was also refractory to GAS1. These results indicate that GAS1 suppresses the growth and tumorigenicity of tumour cells in a p53 dependent manner and that p53 mutation and/or overexpression of MDM2 abrogates the GAS1-mediated growth-suppressing pathway.

To further define the role of MDM2 in the inhibition of GAS1-mediated growth suppression, the endogenous levels of MDM2 in the non-responsive HTD114 cells were downregulated using an antisense strategy. HTD114 cells were co-transfected with MDM2-antisense and GAS1 plasmid constructs. If overexpression of MDM2 in HTD114 cells inhibits the suppression of growth mediated by GAS1, then downregulation of the endogenous levels of MDM2 in these cells using the antisense construct, should increase the sensitivity of the co-transfectants to exogenous GAS1. Although there was a reduction in the levels of endogenous MDM2 mRNA, these cells remained refractory to overexpression of GAS1. However, these cells now expressed a previously unrecognised and novel MDM2 splice variant, which, by sequence analysis, was shown to contain most of the p53 binding domain and was devoid of the putative DNA binding motifs present in the carboxy end of the MDM2 gene. Based on these observations, the following model is proposed. The net effects of antisense MDM2 changes the balance in favour of free and functional p53 protein within the cell and that these cells now overcome the GAS1-mediated growth suppression by expressing the alternate splice variant of MDM2, thus maintaining the cells in a transformed state.

Taken together, the results presented in this thesis have established the growth-suppressive activity of the human GAS1 gene and provided the first direct evidence that GAS1 can inhibit the growth of tumours. The antiproliferative effects of GAS1 are mediated by a p53 dependent pathway. Functional inactivation of p53 by either p53 mutation and/or overexpression of the MDM2 oncogene product abrogates GAS1-

mediated growth-suppression. This study has therefore identified another downstream element, MDM2, that is involved in the cascade of events that lead to growth arrest mediated by GAS1. Further characterisation of the previously unrecognised alternate splice variant of MDM2 identified in HTD114 cells presents a challenge of defining its role and oncogenic potential.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in the University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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LIST OF ABBREVIATIONS

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
ATCC	American type culture collection
azaC	azacytidine
bp	base pairs
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
cDNA	complementary DNA
CHO	Chinese hamster ovary
cM	centimorgan
CMT1	charcot-Marie-Tooth disease type 1
CMV	cytomegalovirus
DCC	deleted in Colorectal Cancer
DEX	dexamethasone
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
FACC	fanconi's anaemia group C
FAP	familial adenomatous polyposis
FCS	foetal calf serum
FL	friend leukemia
G418	geneticin
GADD	growth arrest and DNA damage-inducible
GAS	growth arrest-specific
HDF	human diploid fibroblasts
HPV	human papilloma virus
hr	hour(s)
IFN	interferon
IL	interleukin
IR	ionising radiation

IU	international units
kb	kilobase
LOH	loss of heterozygosity
μg	microgram
min	minute(s)
μl	microlitre
MMS	methylmethanesulfonate
mRNA	messenger RNA
NBCCS	nevoid basal cell carcinoma
NF1	neurofibromatosis type 1
ng	nanogram
nt	nucleotide
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PMP22	peripheral myelin protein
RB	retinoblastoma
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription polymerase chain reaction
SSCP	single strand conformational polymorphism
TGF-β	transforming growth factor β
UTR	untranslated region
UV	ultra-violet
v/v	volume per volume
VSMC	vascular smooth muscle cells
w/v	weight per volume
wt	wild-type
WT1	wilms tumour

CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Control of mammalian cell growth and proliferation is mediated by a balance of a large number of genes that act in both a positive and negative manner. Any alteration of this balance can result in developmental anomalies or in tumour formation.

For a number of years, the major emphasis on the mechanisms of induction of growth from a quiescent state has been based on the assumption that growth control is positively regulated through the transcriptional activation of cellular genes. Many of the positive growth regulators, such as the products of the proto-oncogenes, have been well characterised and much is known about their function. Most proto-oncogenes encode for proteins that are involved in the cascade of events by which growth factors stimulate normal cell division. Many of these are growth factors themselves eg., c-*sis* or growth factor receptors eg., c-*erb,* c-*fms,* c-*kit*. Other genes encode for proteins that transmit the responses of growth factors eg., c-*src,* c-*ras*, and are thus involved in signal transduction pathways. Genes encoding transcription factors have also been identified, including c-*fos* and c-*jun*, which form the AP-1 transcription factor complex. The normal function of the AP-1 complex appears to be the regulation of DNA replication and cellular proliferation and its nuclear location suggest that it may be directly involved in gene expression (reviewed in Bishop, 1991).

The cell cycle possesses both negative as well as positive controlling elements. Over the last few years, negative control of cell growth has been increasingly investigated and proteins with antiproliferative properties have been identified. Genes preferentially expressed during growth arrest and which regulate the transition from the proliferative cell cycle to quiescence may act as tumour suppressors. Several classes of these genes are expected to have important roles in the transition, initiation and maintenance of quiescence. This review focuses on specific events occurring in

mammalian cells during quiescence, with emphasis on the role of growth inhibitory factors, tumour suppressor genes and other signals involved in the control of the cell cycle.

1.2 THE MAMMALIAN CELL CYCLE

In order for cells to proliferate, they must pass through an orderly series of processes known as the cell cycle. The initial observation that animal cells duplicate their DNA during a discrete interval, allowed the cell cycle to be divided into four phases. DNA is duplicated during a discrete phase (S phase) of interphase and chromosomes are segregated to each daughter cell during mitosis (M). These phases are separated from each other by G1 and G2 which are the gaps before S and M respectively. After completing mitosis, mammalian cells normally have the option to enter a new round of cell division or to leave the cell cycle and enter a separate and distinct resting state, usually referred to as quiescence or Go (Scher *et al.,* 1979; Baserga *et al.,* 1982; Pardee, 1989). Unlike terminally-differentiated cells, which have made a permanent exit from the cell cycle, cells in Go retain the ability to re-enter the proliferative cell cycle in response to extracellular factors (Fig 1.1). The decision to choose cell fate is mainly dependent on the concentration of the mitogens in the surrounding medium and the cell density. Once a cell has entered Go, it can remain in this state until the conditions are favourable and the cell resumes proliferation, for example in response to an increase level of mitogens. There has been considerable debate as to whether Go can be defined as a different state from G1 (Epifanova and Polunovsky, 1986). Go-arrested cells have an unduplicated DNA content, as do cycling cells in G1, but Go and G1 cells differ in many other properties (Baserga, 1985). Go cells decrease in size because their protein and RNA molecules are degraded and are not

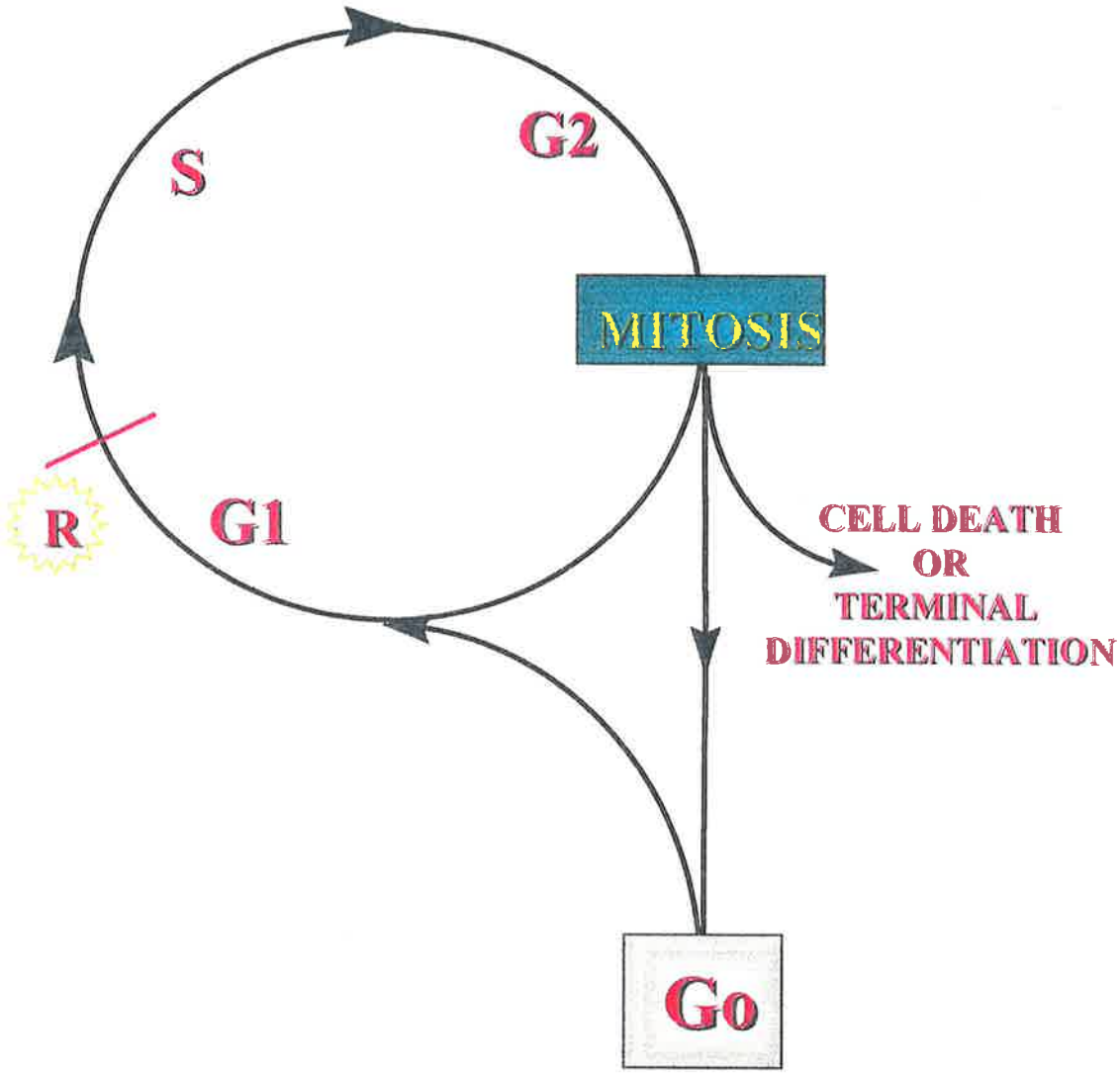


Figure 1.1 Schematic diagram of the cell cycle. After mitosis, cells enter the G1 phase in preparation for a second phase, the S phase in which DNA synthesis and replication occur. Following completion of DNA synthesis, the cells enter a second gap period (G2), in preparation for mitosis. After a certain number of divisions, some cells leave the cell cycle and become terminally differentiated or undergo cell death. Cells in the Go phase of the cell cycle are non-dividing cells, but unlike the terminally differentiated cells, Go cells retain the ability to re-enter the cell cycle when conditions are favorable for growth. The restriction (R) point in late G1 marks the point at which mammalian cells decide whether or not to initiate DNA synthesis or cease proliferation

rapidly resynthesised. Macromolecular synthesis is about one third as rapid in Go as in proliferating cells and enzymatic and other transmembrane transport activities are low in Go cells. Although it was initially argued that nondividing cells were merely arrested in G1, it became clear that upon mitogenic stimulation, quiescent cells do not progress through the cell cycle according to the normal schedule (Zetterberg and Larsson, 1991). Go cells require extra metabolism and therefore take longer to reach S phase than do G1 cells progressing to S from mitosis. In NIH3T3 mouse fibroblasts, which have often been used as a model, Go to S transition takes 12 hours, while in continuously growing NIH3T3 cells, the time between M and S is only 6 hours. Fibroblasts kept quiescent for prolonged periods enter a deeper Go state and become increasingly difficult to rescue (Rossini *et al.,* 1976). It is therefore clear that the progression from Go into S-phase is different from G1 to S-phase in cells that enter a new round of replication immediately after mitosis. This conclusion is strongly supported by the differences in gene expression in cells passing through G1 compared to Go cells which have been stimulated to enter the cell cycle (Pardee, 1989; Baserga *et al.,* 1982; Bravo, 1990; Lanahan *et al.,* 1992). Genes which are induced in mitogen stimulated cells or repressed in quiescent cells such as the early response genes *c-myc* and *c-fos,* are not regulated during cell cycle progression in proliferating cells (Hann *et al.,* 1985; Bravo *et al,* 1986). Other genes activated in response to growth arrest are repressed upon mitogenic stimulation and are not expressed in continuously growing cells. These growth arrest-specific genes or "GAS" genes are molecular markers for Go (Schneider *et al.,* 1988).

Conditions that induce cells to enter Go include serum deprivation (Bartholomew *et al.,* 1976; Lindgren and Westermark, 1976; Baserga, 1985; Pardee, 1987), high cell density (Robinson and Smith, 1976; Skehan and Friedman, 1976), limitation of some amino acids (Holley and Kiernan, 1974) or of other nutrients such as

phosphate, glucose, lipids, biotin and the presence of certain drugs. Serum is a complex mixture of substances and has been used routinely in cell culture medium to provide necessary growth factors. Limitation of some of these factors or certain amino acids in the medium have been reported to arrest cells in Go. Drugs such as streptovitacin A, caffeine, succinylated concanavalin A, glucocorticoids and interferons have all been reported to cause growth arrest (reviwed in Pardee *et al.,* 1978).

The mechanisms of cell density-dependent inhibition of growth is not well understood. Factors proposed include medium depletion due to competition, limitation of available growth surface, the formation of a diffusion boundary layer close to the cell surface, direct contact interactions, release of diffusible inhibitory factors by the cell and the amount of cell surface area available to the growth medium (Baserga, 1985). The existence of genes like *sdr* which is differentially expressed in serum-deprived and contact-inhibited quiescent cells, suggests that density-dependent growth arrest is not simply explained by local growth factor depletion in an overcrowded culture, but implies a more complex response. The difference is probably related to the relative growth restrictions imposed by cell to cell and cell to extracellular matrix interactions even in the presence of high levels of growth factors (Gustincich and Schneider, 1993).

Progression through the cell cycle may be viewed in terms of two principal regulatory cascades, the first governing transit from G1 to S, the second from G2 to M and the regulatory events involved in each transition have been described (Baserga, 1985; Cross *et al.,* 1989; Hartwell and Weinert, 1989; Pardee, 1989). Although both of these transitions represent critical decision points for cell cycle progression, the crucial control events for the regulation of growth seem to reside in the G1/S transition. There is considerable evidence for the existence of a restriction or commitment point in mid- to late G1 (Fig 1.1) which marks the point at which mammalian cells decide whether to

initiate DNA synthesis and undergo cell division or cease proliferation. For the purpose of this review, only the events that regulate the G1/S phase transition in eukaryotic cells will be considered.

The function and modulation of positively regulating genes such as *fos* and *myc*, have been important in our understanding of growth control processes governing the transition from Go to S phase and have been extensively analysed. However, the transition from the proliferative cell cycle to quiescence is far less understood at the molecular level. The presence of specific genes and their products which are actively associated with the process of growth arrest is demonstrated by a number of different observations. Somatic cell hybrid experiments with fusion of normal replicating cells with either quiescent or senescent cells have demonstrated that entry into S phase of the cell cycle can be strongly inhibited by the non replicating partner (Norwood *et al.,* 1974; Bunn and Tarrant, 1980). Similarly, fusion of normal and malignant cells suppresses of tumorigenicity (Harris *et al.,* 1969; Stanbridge *et al.,* 1981; Sager, 1985). The observation that mRNA species obtained from growth arrested cells can be microinjected into cycling cells to induce inhibition of proliferation (Lumpkin *et al.,* 1986; Pepperkok *et al.,* 1988a) and the differences in expression of mRNA and protein between cycling and non cycling cells (Williams and Penman, 1975), all provide evidence for the activity of growth-inhibitory gene products in growth arrested cells. A number of genes which are associated with growth arrest have been identified including the transforming growth factor β (TGF-β) family of genes (Barnard *et al.,* 1990; Pietenpol *et al.,* 1990b), interferons (IFN) (Pitha, 1990), interleukins (IL) (Sehgal, 1990), the growth arrest and DNA damage-inducible (GADD) genes (Fornace *et al.,* 1989) and other growth suppressive proteins such as prohibitin (Nuell *et al.,* 1991) and tumour suppressor gene products, such as retinoblastoma (RB), and P53.

One important set of checkpoint mechanisms that negatively regulate the cell cycle involves the inhibition of cyclin/cyclin dependent kinase (cyclin-CDK) complexes, which prevent some of the phosphorylation events essential for normal cell cycle progression. In mammalian cells, a number of these inhibitors have now been described and the activity of these causes growth arrest and inhibition of DNA synthesis (Harper *et al.,* 1993; Pines, 1994). There may also be a range of other novel strategies for regulating growth arrest, such as the finding that non-coding regions of messenger RNA can act as negative regulators of proliferation, differentiation and suppression of tumour formation (Rastinejad *et al.,* 1993a).

The first section of this literature review will consider some of these observations separately and in more detail. In addition, a set of genes termed growth arrest-specific (GAS) genes (Schneider *et al.,* 1988) may be examples of growth inhibitory genes and will form the second section of this review. It is possible that this group of genes represents a new class of growth-regulated genes. Their mutation could result in abnormal growth regulation and possibly tumour formation.

1.3 IDENTIFICATION OF GENE PRODUCTS ASSOCIATED WITH GROWTH ARREST

1.3.1 Antiproliferative mRNAS

Prior to the development of specific gene probes and as long ago as 1975, Williams and Penman, were the first to recognise the presence of antiproliferative mRNAs in quiescent cells. Cross-hybridisation of cDNA and mRNA classes from resting and growing mouse fibroblast cells has shown that the majority of mRNA sequences are the same in the two states. However cross hybridisation after the common sequences had been removed demonstrated that about 3% of the resting mRNA was not present in the growing state and *vice versa* (Williams and Penman, 1975). These differences suggested the presence of mRNAs that were related specifically to the quiescent state. Several investigators have shown that, when poly(A)-enriched RNA fractions from senescent or quiescent cells are microinjected into early passage diploid fibroblasts or HeLa cells, DNA synthesis is inhibited. However, no significant inhibition was observed when poly(A) RNA from young diploid fibroblast cells was microinjected (Lumpkin *et al.,* 1986). Growth inhibitory mRNAs were also identified by microinjection of poly(A) RNA from normal liver into human fibroblasts, resulting in inhibition of initiation of DNA synthesis. This inhibitory activity is absent or reduced in cells from regenerating liver (Lumpkin *et al.,* 1985). Human peripheral blood lymphocytes, which are normally quiescent, also contain mRNA which when microinjected into either normal or transformed cells, blocks the cells from entering S phase and delays DNA synthesis, whereas mRNA from mitogen-activated lymphocytes did not have this growth-inhibitory activity (Pepperkok *et al.,* 1988b). These studies

demonstrate the presence of antiproliferative genes which are active in the resting state of the cell cycle. These RNAs may code for factors that work antagonistically to the positive regulators of the cell cycle. The observation that antiproliferative mRNAs can inhibit the growth of transformed as well as normal cells, suggests that these mRNA species code for tumour suppressor gene products.

1.3.2 Interferons (IFN)

Interferons constitute a family of genes that can inhibit cell proliferation by blocking cells in the Go/G1 phase of the cell cycle. Interferons selectively inhibit the early synthesis of at least two platelet derived growth factor (PDGF) induced proteins and suppress the overall level of protein synthesis normally seen after PDGF addition (Lin *et al.,* 1986). Interferon (IFN) was shown to reduce *c-myc* mRNA levels by 75% in Daudi Burkitt Lymphoma cells, and this reduction was selective because neither the level of actin mRNA or the total cellular protein synthesis was affected. (Jonak and knight, 1984; Einat *et al.,* 1985a). Terminally differentiated cells that are in a resting phase of the cell cycle also produce IFNs (Einat *et al.,* 1985b). The endogenous IFN acts on the producer cells themselves as an autocrine growth inhibitor, by suppressing *c-myc* expression and thus appears to be involved in sustaining quiescence.

The interferon locus is on chromosome 9p22 in humans, which contain the genes encoding all known interferons α and a single gene encoding interferon β. This chromosomal region is frequently deleted in certain types of cancers including acute lymphocytic leukemia (ALL) and non-lymphoid neoplasias such as glioblastomas and melanomas (Cowan *et al.,* 1988;Diaz *et al.,* 1990). Inactivation of the INF genes by

chromosomal deletions, rearrangements, or other mechanisms of gene silencing may play a role in the aetiology of these cancers.

1.3.3 Transforming growth factor-β (TGF-β)

The transforming growth factor β (TGF-β) family consists of a group of closely related genes, including three highly homologous genes TGF-β1, TGF-β2 and TGF-β3, present in humans and other mammals, with additional related genes in other species (Sporn and Roberts, 1990). TGF-β was initially identified as a factor that could stimulate rodent fibroblasts to grow in soft agar (Assoian *et al.,* 1983). TGF-β1 is a mitogen for many mesenchymal cells and stimulates production of the extracellular matrix by these cells (Lyons and Moses, 1990). Since that time, TGF-β1 has emerged as a negative regulator of cell proliferation. It suppresses proliferation of normal and transformed cells of epithelial, endothelial and haematopoietic origin (Bascom *et al.,* 1989). The intracellular inhibitory effects produced by TGF-β1 are initiated following binding to complexes of high affinity TGF-β1 cell surface receptors type I, type II and type III (Saltis, 1996). Recent studies have suggested that a heteromeric complex of type I and type II receptors is required for TGF-β1 signal transduction. TGF-β1 initially binds to type II receptor which is a constitutively active serine/threonine kinase (Wrana *et al.,* 1994). Once bound to type II receptor, the function of the ligand is to recruit the type I receptor which, by itself, can not bind TGF-β1, forming a stable complex. The type I receptor is then phosphorylated at its serine/glycine rich domain (GS domain) by the constitutive kinase activity of the type II receptor. Thus receptor II is required for ligand binding, whereas receptor I is its substrate and a downstream component of signal transduction. Trans-phosphorylation of the type I receptor could activate its

kinase activity, or the phosphorylated residues could serve as substrate binding sites (Polyak, 1996). Although the mechanisms by which TGF-β1 mediates growth inhibition following binding to its receptor are poorly understood, there is evidence to suggest that TGF-β1-mediated inhibition of expression of growth-related genes such as *myc, KC,* and *JE* appears to play an important role. Unlike the other immediate early genes, expression of *c-myc* (a cellular proto-oncogene involved in positively regulating cell growth) remains elevated throughout G1 and during early S phase (Coffey *et al.,* 1988). Thus *c-myc* expression could be a target of TGF-β1 effects even in late G1. TGF-β1 was shown to downregulate the mRNA and protein levels of *c-myc* in mouse and human keratinocytes (Pietenpol *et al.,* 1990a; Munger *et al.,* 1992). Certain G1 cyclins and cyclin dependent kinases which are required for G1 to S phase transition may be targets for the negative signalling pathway induced by TGF-β1. Overexpression of *c-myc* in serum starved fibroblasts induces the expression of cyclin A and cyclin E, but has no effect in cycling cells (Jansen Durr *et al.,* 1993). Treatment of human keratinocytes with TGF-β1 in the early part of G1 results in a lack of transcriptional activation of both cyclin A and cyclin E in late G1 (Geng and Weinberg, 1993), suggesting that the down-regulation of *c-myc* is one way by which TGF-β1 blocks further cell cycle progression.

The involvement of the retinoblastoma protein, pRb, in the TGF-β1-mediated suppression of *c-myc* transcription has also been investigated and several lines of evidence suggest that the action of *c-myc* on proliferation may involve the cyclin-cyclin dependent kinase-retinoblastoma protein (cyclin-CDK-pRb) connection. This is discussed in more detail below (1.3.6).

TGF-β1 is a potent inhibitor of growth of epithelial cells, but is less effective in fibroblastic cell lines. Novel cDNAs have been isolated from growth arrested epithelial cells as well as from TGF-β1 inhibited cells. A cDNA clone was identified which was

down-regulated by TGF-β1 or by serum stimulation of quiescent cells. The protein encoded by this clone, TI-1 is a member of the tetraspan family of transmembrane glycoproteins which are expressed in haematopoietic and tumour cells. In actively growing cells, the expression of TI-1 is rapidly induced within 3 to 6 hours after addition of TGF-β1, corresponding to the intermediate time of induction of growth arrest in the same cells by TGF-β1. TI-1 is therefore a candidate for an epithelial GAS-like gene (Kallin *et al.,* 1991). TI-1 is preferentially expressed in bladder tissue, and recent evidence has shown that loss of TI-1 expression in bladder carcinoma is a frequent event, suggesting a possible involvement in the aetiology of bladder carcinoma (Finch *et al.,* submitted and Finch, personal communication).

1.3.4 Prohibitin

Prohibitin was originally identified as one of a set of cDNAs derived from mRNAs that were more highly expressed in normal than in regenerating liver (McClung, 1989). Prohibitin is an intracellular protein widely expressed in different tissues and highly conserved in evolution. Microinjection of prohibitin mRNA blocks entry into S phase of normal human diploid fibroblasts (HDF). Similarly, microinjection of an antisense oligonucleotide against prohibitin stimulates entry into S phase. Microinjection of prohibitin mRNA transcripts also inhibits human tumour cells from initiating DNA synthesis (Jupe *et al.,* 1995). These studies indicate that prohibitin is a negative regulator of cell growth (Nuell *et al.,* 1991). Sequence analysis identified prohibitin as the mammalian analogue of the *Cc* gene, a member of the dopa decarboxylase gene cluster of *Drosophila melanogaster* which is required for normal development. Loss of function in both alleles of the *Cc* gene, resulted in death during

larva-to-pupa formation(Eveleth and Marsh, 1986). Prohibitin could therefore be considered to be in a subset of tumour suppressor genes, since complete loss of this gene does not lead to increase cell proliferation and tumour formation, but rather leads to cell death. Studies with yeast cells have shown that overexpression of wild-type prohibitin causes a reduction in mean replicative lifespan, whereas prohibitin deletion mutants have an increase in their mean replicative life span (Franklin *et al.,* 1997). These results suggest a possible role for prohibitin in senescence.The prohibitin gene codes for a 30 kDa post-translationally modified protein, which is located primarily in the inner membrane of mitochondria and is involved in the regulation of Ca^{2+} efflux (McClung *et al.,* 1995; Dell'Orco *et al.,* 1996). It was proposed that prohibitin could control ATP production rates by regulating the three Ca^{2+}-dependent dehydrogenases (Unitt *et al.,* 1989). The antiproliferative effects of prohibitin mRNA transcripts when microinjected into a cell may therefore be due due to the lowering of ATP levels(Dell'Orco *et al.,* 1996). Prohibitin mRNA and protein levels increase from a basal level in Go to maximum at G1/S interface in both young and old cells with no significant differences in their levels in the two populations of cells (Liu *et al.,* 1994). However, postranslational modifications of the protein were noted and one form of the modified prohibitin, present in young cells was absent in senescent populations. It was therefore proposed that the functional activity of prohibitin may be regulated during the aging process in a way similar to the retinoblastoma protein, which is constitutively expressed throughout the cell cycle in both young and old cells, and its antiproliferative activity is inhibited by phosphorylation prior to the cells entry into S phase (Shigeoka and Yang, 1991). On this basis prohibitin, like RB may remain active in older cells if such post translational modifications are absent and alterations in prohibitins activity may lead to cellular immortalisation.

The human prohibitin gene family consists of one functional prohibitin gene on 17q21 and four processed puedogenes, each on a different chromosome (Sato *et al.,* 1993). Mutational analysis of a large number of human tumours suggested that, while somatic mutations in the prohibitin gene may be associated with sporadic breast cancer (5/120), this gene is not mutated in other types of human tumours tested such as ovarian, liver, and lung (Sato *et al.,* 1992).

1.3.5 Growth arrest and DNA damage-inducible (GADD) genes

Five growth arrest and DNA damage-inducible (GADD) genes were initially isolated on the basis of their induction by DNA-damaging agents, as well as by growth arrest in Chinese hamster ovary (CHO) cells (Fornace *et al.,* 1989). In particular *GADD* 45 and *GADD* 153 were found to be rapidly and coordinately induced by the DNA-damaging agent methylmethanesulfonate (MMS), that produces high levels of base changes in DNA. Every cell line tested including human, hamster, murine and rat showed this induction of GADD genes (Fornace *et al.,* 1992). Induction of these genes by growth arresting treatments other than DNA damage was slower, and not all treatments induced the GADD genes. Terminal differentiation of HL60 cells failed to induce expression of GADD genes. In addition, unrelated types of stress such as heat shock were also ineffective (Fornace *et al.,* 1992).The human GADD 45 gene was rapidly induced by ionising radiation (IR) in lymphoblasts and fibroblasts (Papathanasiou *et al.,* 1991), but this response was distinct from that observed after DNA damage induced by MMS. Only GADD 45 was strongly induced by IR at doses which produced relatively little DNA damage. However, the induction of GADD 45 by IR was absent in some tumour cell lines (Fornace *et al.,* 1992). The induction of GADD

45 was dependent on the presence of wild-type p53 and a conserved intronic p53 binding site was identified in the GADD 45 gene, supporting the role of p53 in the regulation of GADD 45 (Kastan *et al.,* 1992). However, the induction of GADD45 by MMS occurs in cells defective in p53 function, such as HeLa and HL60 (Fornace *et al.,* 1989), suggesting that other transcription factors which are independent of p53 may also regulate GADD 45 activity. GADD 45 binds to Proliferating Cell Nuclear Antigen (PCNA) *in vivo* after IR-induced DNA damage and stimulates DNA repair *in vitro* (Smith *et al.,* 1994). Evidence for a role of GADD 45 in the control of growth included the observation that overexpression of GADD 45 protein with a transfection expression vector significantly inhibited DNA synthesis. (Zhan et al 1994). Thus, induction of p53 after DNA damage results in inhibition of DNA replication followed by induction of DNA repair by the transcriptional activation of *GADD 45*. Cells with defective p53 allow DNA replication to proceed with damaged DNA which in turn could result in gene mutation and thus tumour formation (Hartwell and Kastan, 1994).

1.3.6 Cyclins, Cyclin-dependent kinases and their inhibitors

Cellular proliferation follows an orderly progression which is controlled by protein complexes composed of cyclins and cyclin dependent kinases (CDKs). Cyclins are a family of proteins which serve as the regulatory proteins for the CDKs and are differentially synthesised and degraded at specific points during the cell cycle (Murray, 1994; Sherr, 1994a). A network of inhibitors of the CDKs, the cyclin dependent kinase inhibitor (CDKI) family of proteins, regulate the activity of the cyclin complexes (Cordon-Cardon, 1995) and thus negatively regulate cell cycle progression.

In mammalian cells, progression through G1 is primarily controlled by CDK4 and CDK6, associated with one of three cyclin isoforms, D1, D2 and D3, and CDK2, associated with cyclin E. These complexes are activated following phosphorylation of the CDK subunit by a cyclin-activating kinase (CAK). The D-type cyclins are synthesised for as long as there is growth factor stimulation but are rapidly degraded when mitogens are withdrawn, regardless of the position of the cells in the cell cycle. Removal of growth factors while cells are in G1 results in failure of the cells to enter S phase. A major substrate of G1 CDKs is the retinoblastoma gene product pRb (Weinberg, 1995). The D-type cyclins bind directly to RB (Dowdy *et al.,* 1993; Ewen *et al.,* 1993), and in doing so, CDK4 or CDK6 are targeted to RB which is then phosphorylated (Matsushime *et al.,* 1992; Meyerson and Harlow, 1994). The RB protein inhibits cell cycle progression when a hypophosphorylated form of pRb binds to and represses the activity of transcriptional factors such as the E2F/DP1 complex (Lam and La Thangue, 1994). Phosphorylation of RB by CDKs physically releases E2F/DP1 from this negative constraint and allows the transcription of specific genes required for cell cycle progression (Nevins, 1991). Overexpression of cyclin D1 in G1, results in RB phosphorylation earlier in the cell cycle and progression through G1 is accelerated (Jiang *et al.,* 1993; Resnistzky *et al.,* 1994). However, microinjection of antibodies to cyclin D1 into normal fibroblasts during G1 phase prevents their entry into S phase, but injection near the G1-S transition has no effect, suggesting the critical function of cyclin D-dependent kinases is during mid to late G1 (Baldin *et al.,*1993; Quelle *et al.,* 1993).

CDK4 is a target for TGF-β1 mediated growth suppression. In Mv1Lu cells, treatment with TGF-β1 downregulates CDK4 protein without affecting mRNA levels, whereas constitutive expression of CDK4 renders these cells resistant to TGF-β1 inhibition (Ewen *et al.,* 1993). TGF-β1 causes G1 growth arrest by inhibiting the

CDK4-mediated phosphorylation of RB (Laiho *et al.,* 1990). No mutations have been reported in the CDK4 gene, but both CDK4 and CDK6 are overexpressed in some tumours (Khatib *et al.,* 1993), suggesting that some tumour cells may be insensitive to the growth-inhibitory effects of TGF-β1. TGF-β1 therefore acts through tumour suppressor gene products such as RB, to negatively regulate the transcription of growth-promoting genes such as *c-myc,* resulting in inhibition of cell proliferation. It has been suggested that *c-myc* regulates the expression of cyclin E, cyclin A and cyclin D (Jansenn Durr *et al.,* 1993). On this basis it is proposed that the TGF-β1–mediated down-regulation of *c-myc* is another mechanism by which CDK activity is inhibited and suggests that *c-myc* is regulated by a negative feedback. Since *c-myc* expression remains high throughout G1, abrogation of CDK activity by TGF-β1 is predicted not only to inhibit G1 progression but also to sustain cells in a growth-inhibited state by preventing further stimulation of late G1 events by *c-myc* (Alexandrow and Moses, 1995).

Negative regulation of cell cycle progression can occur via the upregulation of a family of proteins that bind to and inactivate CDKs (Alexandrow and Moses, 1995), known as the cyclin-dependent kinase inhibitors (CDKIs). These are subdivided into two groups on the basis of sequence homology. The first group is comprised of p27 and p21. In quiescent cells, p27 is normally complexed with cyclin D-CDK4 (Polyak *et al.,* 1994; Toyoshima and Hunter, 1994). Upon entry into the cell cycle, cyclin D-CDK4 complexes accumulate and their levels exceed those of p27, thus counteracting the inhibitory action of p27. As described above, treatment with TGF-β1 leads to a reduction in CDK4 synthesis and p27 is mobilised into complexes with cyclin E-CDK2, resulting in loss of activity of both kinases. The end result is the maintenance of RB in a hypophosphorylated form resulting in G1 arrest (Fig 1.2). The other member of this group of cyclin-dependent kinase inhibitors is p21 (also known as WAF1, CAP20 or

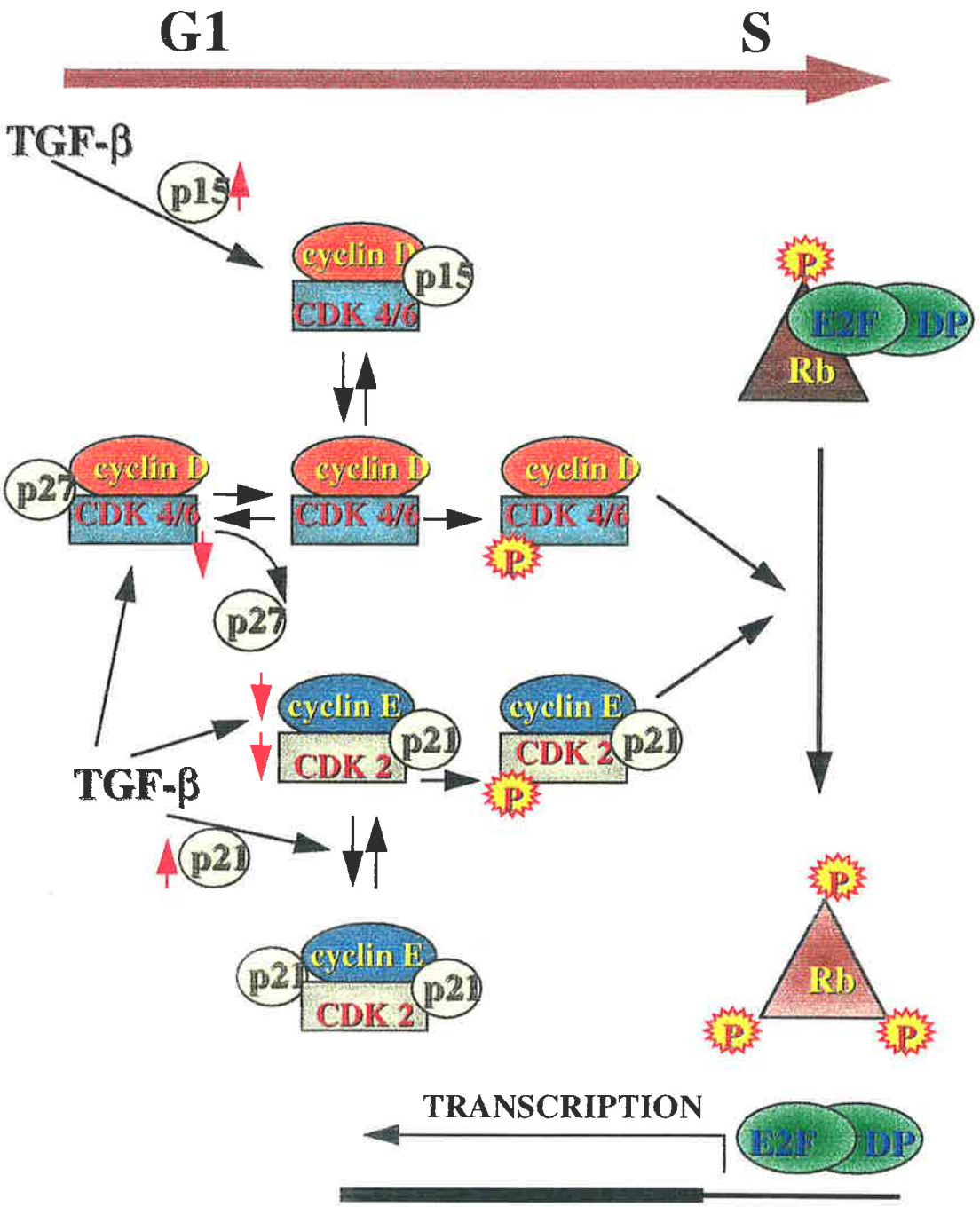


Figure 1.2 Control of the cell cycle by TGF-b. Shown is a model of the multiple actions of TGF-b on key components of the cell cycle machinery that ultimately leads to G1 arrest. Addition of TGF-b blocks the synthesis of CDK4/6, therefore in the absence of cyclin D/CDK4/6 the cells remain in quiescence. Exponentially growing cells have high levels of cyclin/CDK complexes part of which are bound to p27. Treatment with TGF-b induces the expression of p15 leading to its association with CDK4/6 and disruption of cyclin E/CDK4/6 complexes. The p27 is displaced and is now found complexed with cyclin E/CDK2. P21 is also upregulated by TGF-b leading to inactivation of cyclin E/CDK2. However, multiple molecules of p21 are required for this inactivation. The net effect is the inhibition of phosphorylation of the pRb protein which inhibits cell cycle progression.

CIP 1), which is structurally related to p27 and also preferentially binds to and inhibits a variety of cyclin-CDK complexes, including cyclin D-CDK4, cyclin E-CDK2, and cyclin A-CDK2. However, p21-mediated inhibition of activity of cyclin-CDK comples requires the binding of multiple p21 subunits (Zhang *et al.,* 1994). In addition, p21 associates with the proliferating cell nuclear antigen (PCNA) subunit which functions in both DNA replication and repair as a subunit of DNA polymerase δ (Waga *et al.,* 1994) and thus, acts as a direct inhibitor of DNA polymerase. The promoter of *p21* has a p53 binding site and transcription of *p21* is directly upregulated by wild-type but not mutant p53 (El-Deiry *et al.,* 1993). P21 acts therefore as a downstream effector of p53-induced G1 growth arrest which negatively regulates the cell cycle kinases required for G1-S transition. (Xiong *et al.,* 1993). Synthesis of p21 is increases when expression of p53 is induced after DNA damage caused by ionising radiation. Therefore, p53-mediated upregulation of p21 inhibits CDK activity by preventing the phosphorylation of RB and thus the release of E2F/DP1. On this basis, p21 serves to integrate the cell cycle control mediated by p53 and Rb. Recent studies demonstrated that the expression of exogenous p21 in human breast cancer brain, colon and lung cancer cell lines was sufficient to cause growth arrest (El-Deiry *et al.,* 1993; Sheikh *et al.,* 1995). The finding that many transformed cells express low levels of p21, suggests that this may be due to the absence of functional p53 within these cells (Li *et al.,* 1994). However, the induction of p21 in p53-negative cells also suggests that p21 can be regulated by other transcription factors independent of p53 activity (Michieli *et al.,* 1994). Like p27, p21 is also transcriptionally up-regulated upon TGF-β1 treatment, suggesting that TGF-β1 can act through multiple signalling pathways to induce growth arrest (Polyak *et al.,* 1994; Datto *et al.,* 1995).

The other CDK inhibitor subgroup includes p16, p15, and p18. The p16/INK4/MTS1 and p15/INK4/MTS2 genes are adjacent to each other and are located on chromosome 9p21, where they are found in tandem spanning a region of approximately 80 kb (Hannon and Beach, 1994). Both of these proteins bind and inhibit only CDK4 and CDK6 (Serrano *et al.,* 1993; Hannon and Beach, 1994), thereby inhibiting RB phosphorylation and thus cell cycle progression. P16 associates with CDK4 at the expense of cyclin D in RB-negative cells and in cells transformed by DNA tumour viruses (Parry *et al.,* 1994; Xiong *et al.,* 1993). RB negatively regulates the promoter of p16 (Li *et al.,* 1994) and p16 expression increases strongly towards the end of G1 after RB becomes phosphorylated (Tam *et al.,* 1994). Taken together, these observations suggest the operation of a negative feedback loop which begins with the association of cyclin D with CDK4/CDK6. This association triggers the phosphorylation of RB which in turn releases a transcription factor which induces p16 expression. p16 now binds the available CDK4/CDK6, displacing cyclin D which is now no longer protected by the association of its partner CDK4/CDK6 and undergoes degradation, and termination of its activities in late G1. In this way the participation of cyclin D is confined to a defined time just before the restriction point and ends at the G1/S phase transition.

The p16 locus is rearranged, deleted, or mutated in a majority of tumour cell lines (kamb *et al.,* 1994; Nobori *et al.,* 1994) suggesting that p16 may correspond to a tumour suppressor gene that maps to this region of chromosome 9 (MTS1), whose inactivation is implicated in a variety of sporadic carcinomas and familial melanomas. However, mutations of p16 were rarely found in primary tumours, suggesting that the mutations observed in the cell lines may be secondary events and loss of p16 may provide an additional growth advantage to some of these tumours (Cairns *et al.,* 1994;

Okamoto *et al.,* 1994). Methylation of the 5'CpG island of the p16 gene in normal and transformed human tissues correlates with gene silencing, suggesting an alternative mechanism by which p16 can be inactivated (Gonzalez-Zulueta *et al.,* 1995). At the level of protein sequence, p16 shares an 82% homology with p15, however their activities are regulated differently. Expression of p15 is induced almost 30-fold in human keratinocytes treated with TGF-β1, increasing the binding of p15 to CDK4 and CDK6 (Hannon and Beach, 1994), in a similar manner to that seen with p27 (Fig 1.2). At a later stage in G1, TGF-β1 action abolishes the expression of cyclin E and CDK2 (Geng and Weinberg, 1993). It was proposed that since TGF-β1 does not suppress CDK4 synthesis or alter p27 mRNA levels in human keratinocytes, then p15 may be the dominant effector of TGF-β1-mediated growth arrest (Sherr, 1994b).

1.4 TUMOUR SUPPRESSOR GENES

The existence of tumour suppressor genes has also given support to the hypothesis that neoplastic transformation involves alterations in genes whose products negatively regulate cell proliferation. Several lines of evidence including cell hybrids, familial cancer, and loss of heterozygosity in tumours supported their existence.

1.4.1 Cell hybrids

When highly malignant mouse cells were fused with normal embryo cells or mouse cells of low malignant potential, the resulting hybrid cells were suppressed in their ability to form tumours when injected into suitable hosts. When the hybrid clones were propagated in culture, tumorigenic segregants rapidly developed. As chromosomes in the hybrid cells were lost, the tumorigenicity of the hybrids increased to that of the parental cell with the high tumorigenic potential (Harris and Bramwell, 1987). Other studies with rodent and human interspecies hybrids (Miller and Miller, 1983; Sager, 1985; Stanbridge, 1988) confirmed these findings. These results demonstrated that the non-malignant cells contain one or more proteins capable of repressing the malignant phenotype. These "tumour suppressor"genes are often inactive or absent from the malignant cells

Human intraspecies hybrids were studied most extensively and provided the most penetrating insights into this phenomenon. Extensive studies by Stanbridge and his co-workers found that tumorigenicity in hybrids of HeLa cells and normal fibroblasts was associated with the loss of one copy of chromosome 11 and one copy of chromosome 14 (Stanbridge *et al.,* 1981). Similar evidence for human chromosome 11 were also reported by Kaelbling and Klinger. (1986), whereas chromosome 1 has been

implicated in the suppression of HT1080 fibrosarcoma by normal fibroblasts (Benedict *et al.,* 1984). HeLa and HT1080 cells produce nontumorigenic hybrids when fused with each other (Weissman and Stanbridge, 1983), suggesting that genetic complementation occurs between cells that carry tumour suppressor genes on different chromosomes. The results described above were confirmed by a more direct approach using the technique of microcell fusion, in which a single fibroblast chromosome 11 was introduced into HeLa cells and suppressed tumorigenicity of the recipient cells (Saxon *et al.,* 1986).

Cell hybrid studies, together with microcell transfer experiments, allowed the identification of the chromosomal location of some of these tumour suppressor genes, and demonstrated that different tumours have alterations in different suppressor genes (Stoler and Bouck, 1985; Shimizu *et al.,* 1990; Trent *et al.,* 1990;).

1.4.2 Familial cancers

The study of familial cancers provided the second clue which suggested the existence of tumour suppressor genes. These studies examined diseases such as retinoblastoma, Wilms tumour, neurofibromatosis and familial adenomatosis polyposis (FAP). Retinoblastoma represents the prototype of tumour suppressor genes identified in this way and will be discussed in more detail.

Retinoblastoma is a childhood cancer that occurs in familial and spontaneous forms. It was first proposed by Knudson, that retinoblastoma is caused by two mutational events (Knudson's two step hypothesis). In the familial form, the first step is a germ line mutation, in which the defective locus is present in all cells of the individual. This mutation predisposes the individual to retinoblastoma. A second step occurs in the somatic cells and leads to the inactivation of the wild type tumour

suppressor gene allele located on the homologous chromosome. Only after both tumour suppressor genes have been rendered inactive does retinoblastoma develop. In the spontaneous form, both mutations are somatic in origin (Knudson, 1971).

The retinoblastoma gene was initially mapped by cytogenetic analysis to band q14 of chromosome 13 (Friend *et al.,* 1986). The gene encompasses 180 kb of DNA that is transcribed into a 4.7 kb mRNA which encodes a 928 amino acid nuclear phosphoprotein (P105-RB). The ability of RB to bind to DNA suggests that it is important in regulating transcription of specific cellular genes involved in growth control or in DNA replication. The retinoblastoma protein is expressed not only in retinoblasts but also in most other tissues (Lee *et al.,* 1987). The neoplastic phenotype of retinoblastoma and osteosarcoma cells can be suppressed by the introduction of a cloned RB gene. In these cases complete loss of tumorigenic activity in nude mice has been observed (Huang *et al.,* 1988). Tumour-suppressive activity of a cloned RB gene was also shown in human prostate carcinoma cell lines (Bookstein *et al.,* 1990). RB mutations are not only restricted to retinoblastomas but also found in some osteosarcomas, soft tissue carcinomas, breast carcinomas and small cell lung carcinomas (Goodrich and Lee, 1990). The inactivation of RB has also been associated with a broad range of lymphoid neoplasms (Ginsberg *et al.,* 1991). These findings show that the inactivation of RB is associated with a broad range of tumours and loss of tumour suppressor function of RB influences the pathogenesis and/or progression of these tumours.

Although the function of the RB protein is not fully understood, it is believed to be regulated by differential levels of protein phosphorylation throughout the cell cycle. There are several lines of evidence which indicate that phosphorylation of RB causes the inactivation of its growth inhibitory functions. Firstly, the ability of some viral

oncoproteins to complex with the RB protein provided insights into the regulation of growth by phosphorylation of RB. The human adenovirus E1A, simian virus SV40 large T antigen and the human papilloma virus HPV E7 transforming viral oncoproteins form stable complexes with RB but only with the hypophosphorylated form of the protein, thus eliminating the suppressive potential of the hypophosphorylated form of RB. Secondly, it is the hypophosphorylated form of RB which is the active form and which binds and inhibits other factors required for cell cycle progression. Thirdly, conditions that favour RB phosphorylation also favour cell proliferation. The mechanisms of how RB phosphorylation controlls cell cycle progression and the importance of the cyclins, cyclin-dependent kinases and their inhibitors in these control mechanisms have been described elsewhere in this review (1.3.6).

The effector functions of the retinoblastoma protein are controlled primarily by the E2F transcription factor (Weinberg, 1995). As previously described (1.3.6) hypophosphorylated pRb binds to the E2F transcription factor, whereas phosphorylation of pRb causes the release of E2F from the complex, enabling E2F to activate a large number of genes such as *c-myc,* thymidine kinase (TK) dehydrofolate reductase (DHFR) and DNA polymerase α, which are required to prepare cells to enter S phase (Nevins *et al,* 1991,). Two other proteins, p107 and p130, that share structural similarities with the Rb protein, have also been identified. Both proteins were shown to inhibit E2F-mediated transcriptional activation and both can inhibit progression through G1 (Zhu *et al.,* 1993). The RB protein or the RB-related p107 and p130 proteins bind to promoters of genes as a complex with the E2F transcription factor at the E2F consensus sites. This finding suggested that RB may act at transcriptional promoters, using already bound E2F as the docking sites. RB can therefore actively repress transcription of promoters in which the E2F site is occupied (Qin *et al.,* 1995; Weinberg, 1995). The promoter of the

RB gene also contains an E2F recognition site and E2F regulates the expression of RB, suggesting a possible negative feedback loop for the regulation of pRb (Shan *et al.,* 1994). Other pRb-binding proteins have also been reported and they including Elf-1, MyoD, ATF-2 and c-abl proteins (Wang *et al.,* 1993), however the function of these are not fully understood.

The retinoblastoma protein exerts most of its effects in the first two thirds of the G1 phase of the cell cycle during which the commitment to undergo DNA synthesis and cell division or to enter quiescence is made. This decision is governed by a complex interplay of expression of both positive and negative regulators of growth. The main role of pRb is to act as a signal transducer and to control the expression of a large array of genes that mediate cell cycle progression.

1.4.3 Loss of Heterozygosity (LOH)

The method which is referred to as loss of heterozygosity (LOH), recognises losses of whole chromosomes or deletions of chromosomal regions when appropriate DNA markers are available. The closer that any DNA linked marker is to the putative locus, the higher will be the frequency with which loss of heterozygosity is scored in tumours. Many tumours show (LOH) at various chromosomal regions and some chromosomal deletions have been localised to regions containing putative tumour suppressor genes. Typically, such chromosomal deletions involve one of the two parental chromosome sets, or alleles, present in normal cells. In accordance with the hypothesis originally proposed by Knudson (1985), LOH usually unmasks a more subtle recessive mutation in the retained copy of a tumour suppressor gene. Two tumour

suppressor genes, p53 and the Deleted in Colorectal Cancer (DCC) genes are examples of such genes which were identified using this method and will be discussed below.

1.4.3.1 p53

Loss of heterozygosity of markers on the short arm of chromosome 17 occur at a high frequency in many tumours. These findings led to the identification of a putative tumour suppressor gene known as p53. Phosphoprotein p53 is a 53 kDa nuclear protein that is present in high amounts in transformed human and mouse cells (Crawford *et al.,* 1984). Originally it was demonstrated that p53 could cooperate with RAS in neoplastic transformation, and for a while p53 was thought to be an oncogene (Eliyahu *et al.,* 1984). However, it was later shown that the p53 clone used in those studies was mutated and not the wild type. In fact, it turned out that the wild type p53 was able to suppress the formation of transformed foci in these cells (Eliyahu *et al.,* 1989).

Like the retinoblastoma protein, a characteristic feature of p53 is its ability to form complexes with protein products of transforming viruses such as SV40 T-antigen (Linzer *et al.,* 1979), adenovirus E1B transforming protein (Sarnow *et al.,* 1982) as well as the E6 protein of the human papilloma virus 16 (Werness *et al.,* 1990). These similarities between Rb and p53 suggested that both proteins may be components of the same regulatory pathway.

The human p53 gene was localised by *in situ* hybridisation to the short arm of chromosome 17 at position 17p13.1 (Isobe *et al.,* 1986). Restriction fragment length polymorphism (RFLP) studies in human lung carcinoma, breast carcinoma, colorectal carcinoma and brain tumours have shown frequent loss of heterozygosity in this region of chromosome 17 (Mackay *et al.,* 1988; Baker *et al.,* 1989; James *et al.,* 1989;Yokota *et al.,*1989). Mutations within the p53 tumour suppressor gene have been detected in

more than 50% of all human tumours (Vogelstein, 1990; Hollstein *et al.,* 1991). Induction of expression of wild-type p53 in cell lines derived from human glioblastoma, prostate cancer and osteosarcoma has been shown to inhibit cell cycle progression of exponentially-growing cells. Moreover, expression of wild-type p53 in growth-arrested cells prevented these cells from progressing into S-phase following growth stimulation (Diller *et al.,* 1990; Mercer *et al.,* 1990). This inhibition was accompanied by selective down-regulation of the proliferating cell nuclear antigen gene (PCNA) which is a component of the DNA replication machinery of the cell (Mercer *et al.,* 1991). It was also demonstrated that p53 inhibited SV40 DNA replication by inhibiting the binding of the SV40 T-antigen to DNA polymerase α, which suggested that p53 can exert its suppressive activity by inhibiting cellular genes or gene products which are involved in DNA replication (Friedman *et al.,* 1990).

Although the precise role of p53 in the network of events that regulate cell proliferation are not fully understood, there is evidence to suggest that like pRb, p53 is another checkpoint regulator protein which mediates the progression of cells through the cell cycle. During the cell cycle, the levels of p53 increase from G1 to S phase and from G2 to M phase (Danova *et al.,* 1990). Microinjection of p53 -specific antibodies at the time of growth stimulation of quiescent cells, inhibits entry of these cells into S phase indicating a critical function of p53 in the Go/G1 to S transition (Mercer *et al.,* 1982; Mercer *et al.,* 1984). The subcellular localisation of p53 is dependent on growth conditions and can differ among cell types and tissues (Dippold *et al.,* 1981). In NIH3T3 cells p53 is cytoplasmic in G1, shifts into the nucleus at the G1/S transition and remains there until the completion of G2/M phase (Shaulsky *et al.,* 1990). P53 is hypophosphorylated in the Go/G1 phase of the cell cycle when compared to the S-

phase, suggesting that the hypophosphorylated form of p53 may exert the growth suppressive activity in G1 (Bischoff *et al.,* 1990).

One of the most important functions of p53 is its ability to cause G1 arrest in response to DNA damage. This is caused by an increase in p53 protein levels in the nucleus as well as an increase in transcriptional activity (Kastan *et al.,* 1991; Lu and Lane, 1993). It has been suggested that the DNA damage-induced accumulation of p53 may allow DNA-repair processes to occur by inhibiting proliferation (Lane, 1992). In the case of severe DNA damage, p53 initiates apoptosis preventing the cells from accumulating and replicating damaged DNA, thus preventing tumorigenesis. Cells lacking the p53 gene are completely resistant to DNA damage-induced apoptosis (Clarke *et al.,* 1993) In this context, p53 has been regarded as the " guardian of the genome" (Prives, 1993; Weinberg, 1991). The mechanisms by which p53 can arrest the cell cycle appear to be related to its ability to bind to DNA in a sequence-specific manner and trans-activate the expression of growth-inhibitory genes such as the genes encoding p21, the 21-kDa inhibitor of both cyclin-dependent kinases (CDK) and PCNA (El-Deiry *et al.,* 1993; Xiong *et al.,* 1993; Waga *et al.,* 1994). GADD 45, the DNA damage-inducible gene is another target gene which is positively regulated by p53 (Kastan *et al.,* 1991). In addition, p53 represses the activity of a variety of promoters, some of which correspond to genes including *c-fos* and *c-jun* whose expression is positively correlated with cell proliferation, or with increased malignancy (Ginsberg *et al.,* 1991; Mercer *et al.,* 1991; Chin *et al.,* 1992; Kley *et al.,* 1992). However, unlike the genes which are trans-activated by p53, these genes do not contain binding sites for p53 in their corresponding promoters. Because p53 suppresses transcription in a non-sequence-specific manner from a number of unrelated promoters, it is possible that it acts on some general transcription factor. One candidate is the TATA box binding

protein (TBP), which has been shown to bind p53. In fact the binding of TBP by p53 has been shown to repress transcription (Seto *et al.,* 1992). Other genes whose transcriptional activities are repressed by p53 include thymidine kinase, *c-myc* and DNA polymerase α (Lin *et al.,* 1992; Yuan *et al.,* 1993). These are the same genes which can be trans-activated by the E2F transcription factor, suggesting that p53 may interfere with E2F function in the normal cell cycle. One pathway by which p53 can mediate inhibition of E2F is via p21 and in which RB is the key component in this pathway (Weinberg, 1995). However, it has also been shown that p53 physically associates with E2F and thus can inhibit E2F transcriptional activity independently of RB (O'Connor,1995).

The activity of the p53 protein is also regulated by its association with a cellular oncogene product MDM2 (Momand *et al.,* 1992). The MDM2 oncogene was originally cloned from a spontaneously transformed Balb/c mouse cell line where it was found to be amplified and overexpressed (Fakharzadeh *et al.,* 1991). MDM2 protein forms a complex with p53 and blocks the ability of p53 to trans-activate target genes (Momand *et al.,* 1992; Oliner *et al.,* 1993). Overexpression of MDM2 inhibits both p53-mediated growth arrest and p53-mediated apoptosis (Haupt *et al.,* 1996). The first intron of the MDM2 gene itself contains a p53-responsive element, so increased levels of p53 result in increased levels of MDM2 protein (Wu *et al.,* 1993). In turn, the additional MDM2 binds to p53 and decreases its ability to stimulate the MDM2 gene. This provides an autoregulatory feedback loop (Fig 1.3) for the MDM2 gene which, in turn, regulates the activity of p53 (Picksley and Lane, 1993). When normal resting cells are stimulated to progress from Go to G1 phase of the cell cycle, there is an increase in the steady state levels of p53 protein until late G1 and prior to S phase (Reich and Levine, 1984). This is followed by a concomitant increase in transcriptional activation of MDM2 by p53 and

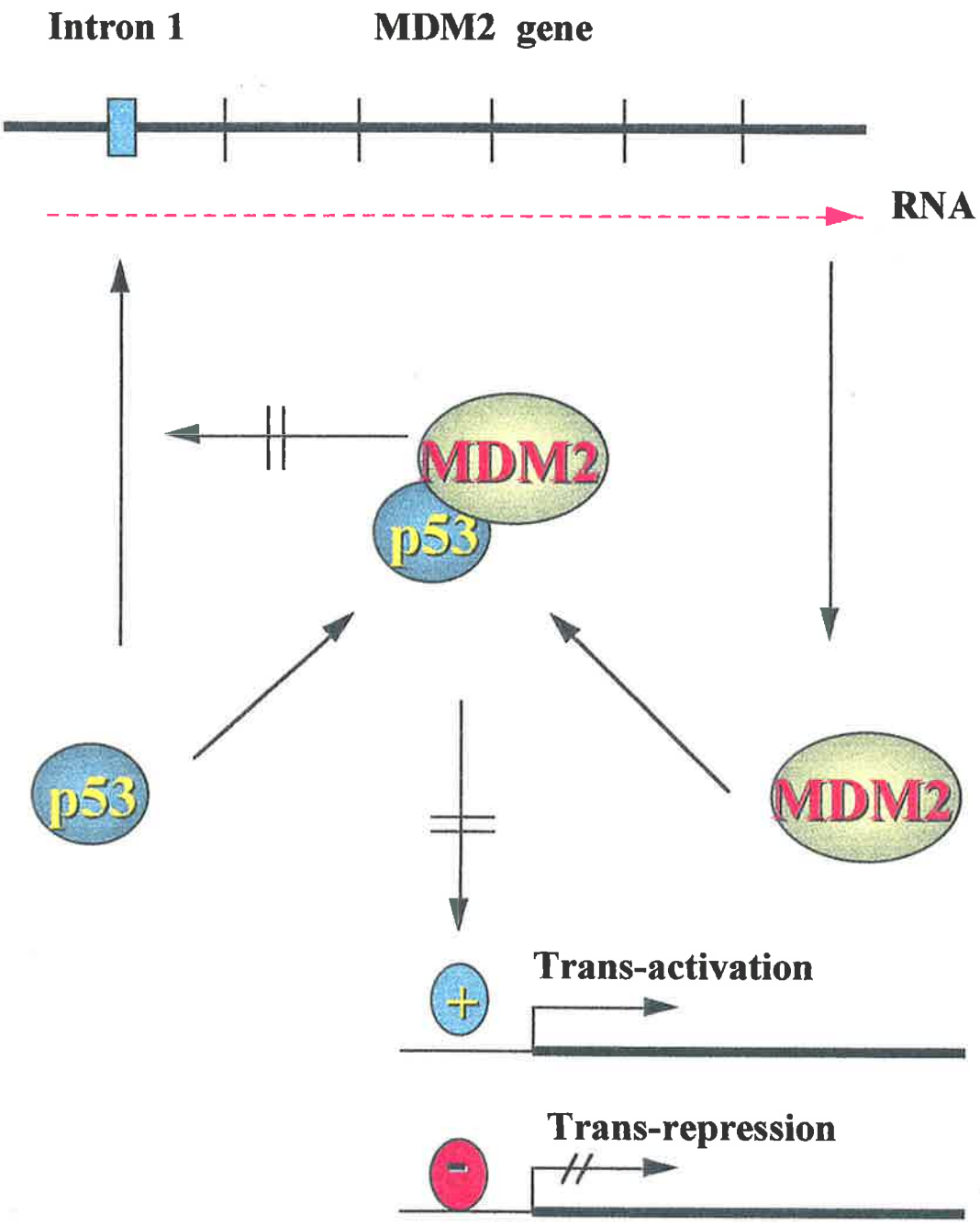


Figure 1.3 The p53-MDM2 autoregulatory loop. The p53 protein positively regulates MDM2 protein by activating *MDM2* gene expression. MDM2 on the other hand, inhibits the trans-activating and/or trans-repressing functions of p53 after forming a p53-MDM2 complex.

MDM2-p53 complexes are formed at about the same time in the cell cycle. The high levels of p53 protein would be expected to act as a check point block in late G1 (Kastan *et al.,* 1991), however the increased levels of MDM2 overcome this negative regulation of the cell cycle and permit entry into S phase. Thus the activity of p53 and the levels of MDM2 in a cell are kept in balance by this autoregulatory feedback loop. Similarly, in response to DNA damage, which leads to an increase in p53 (Kastan *et al.,* 1991), p53 activates the expression of the MDM2 gene (Wu *et al.,* 1993). The MDM2 protein binds to the trans-activation domain of p53, further inhibiting any activity of p53 as a transcription factor. Homozygous deletion of the MDM2 gene was found to be lethal to mouse embryos, but this lethality could be rescued in mice lacking both MDM2 and p53 (Montes de Oca Luna *et al.,* 1995; Jones *et al.,* 1995). These results suggested that the early lethality in the MDM2-null mice might arise as a result of the failure of MDM2 to inhibit p53-mediated suppression of cell cycling during the time of rapid cell division. The MDM2 product has therefore been implicated as a negative regulator of p53.

Tumours which contain elevated levels of MDM2 can lead to constitutive inhibition of p53 function. Amplification and/or overexpression of the MDM2 gene has been observed in more than 30% of soft tissue sarcomas and less often in other tumours. Tumours with elevated levels of MDM2 typically contain wild type p53 (Oliner *et al.,* 1992; Cordon-Cardo *et al.,* 1994), suggesting that MDM2 amplification may be an alternative mechanism to mutational inactivation of p53 in tumours. In addition, tumour cells overexpressing MDM2 can tolerate high levels of wild type p53 (Finlay 1993; Otto and Deppert, 1993).

One of the functions of the MDM2 protein is to bind and inactivate the functions of the p53 tumour suppressor gene. However, the MDM2 protein may have additional functions independent of p53. The primary amino acid sequence of the MDM2 protein

contains, in addition to an N-terminal p53 binding motif, several putative functional domains and motifs including zinc fingers, an acidic or highly negatively-charged region and nuclear localisation signals. It is likely that MDM2 can act as a transcription factor itself or in complex with other proteins (Wu *et al.,* 1993). Multiple-sized MDM2 proteins have been identified in both murine and human tumour cells (Olson *et al.,* 1993; Barak *et al.,* 1994). The identification of alternatively-spliced MDM2 transcripts with loss of p53-binding domain sequences, and the ability of these to transform cells, suggests an additional function for MDM2 which is independent of binding to p53 and inhibiting p53 activity (Sigalas *et al.,* 1996).

1.4.3.2 DCC

Allelic losses on the long arm of chromosome 18, centred around 18q21-18qter, are frequently seen in colorectal carcinomas where it occurs in more than 70% of the examined cases (Fearon *et al.,* 1990). Chromosome 18q losses have been reported in other malignancies including lung cancer (11%) (Yokota *et al.,* 1987), osteosarcoma (38%) (Toguchida *et al.,* 1988), renal cell carcinoma (33%) (Bergerheim *et al.,* 1989), gastric carcinoma (20%) (Motomura *et al.,* 1988) and in breast cancer (38%) (Devilee *et al.,* 1991). A gene sequence was cloned from this region of 18q21.1, the expression of which was absent or reduced in the majority of the cell lines or clinical samples of colon carcinomas relative to normal colonic mucosa. This gene falls within the common region of deletions on chromosome 18 found in a series of primary colon cancers and provides evidence that this gene might be a candidate tumour suppressor gene (Vogelstein *et al.,* 1988).

The human DCC gene is very large, spanning a 1350 kb genomic region. The cDNA of the DCC gene is encoded by at least 29 exons displaying alternative splicing

(Cho *et al.,* 1994). The DCC gene encodes a transmembrane protein with a single membrane spanning region separating the cytoplasmic and extracellular domains. The DCC protein bears sequence similarity to neural cell adhesion molecules and other related cell-surface glycoproteins. Many different classes of cell-surface glycoproteins, including integrins, cadherins and the immunoglobulin superfamily of cell adhesion molecules, are known to have critical roles in embryonic development, the differentiation of various cell types and the regulation of cell proliferation (Hynes, 1992; Takeichi, 1991; Edelman and Crossin, 1991). The normal function of the DCC protein has not yet been determined. It seems likely to be involved in cell-cell or cell-matrix interactions involving specific signalling which regulates cell proliferation and/or differentiation. However, specific ligands or receptors for DCC have not yet been identified.

The strongest evidence supporting DCC's role as a tumour suppressor gene came from studies showing suppression of tumorigenicity by reconstitution of DCC function in tumour cells lacking expression of DCC. Introduction of chromosome 18 into COKFu colon carcinoma cells resulted in suppression of growth in soft agar and suppression of tumorigenicity in nude mice (Tanaka *et al.,* 1991). In addition, expression of full length, but not truncated DCC, in transformed keratinocytes lacking DCC expression also suppressed tumorigenicity of these cells in nude mice (Klingelhutz *et al.,* 1995). More importantly, cells which reverted back to a transformed phenotype had lost the expression of DCC. Evidence supporting the involvement of DCC in cell adhesion came from experiments in which antisense DCC in Rat 1 fibroblasts resulted in neoplastic transformation (Narayanan *et al.*, 1992

1.5 NON-CODING RNAS AS TUMOUR SUPPRESSORS

Genetic complementation experiments revealed that untranslated regions of mRNA in the absence of coding sequences can also act in trans as regulators of growth and differentiation. In these experiments, a cDNA expression library was introduced into a nondifferentiating myogenic cell line, NMU2. Three cDNA sequences derived from the cytoskeletal genes troponin, tropomyosin, and α-cardiac actin were found to complement the mutant phenotype of the NMU2 cell line. The activity of these cDNAs was mapped to their 3' untranslated region (3'UTR). In both mutant and wild-type myogenic cell lines, expression of the 3'UTRs promoted the expression of muscle-specific genes. In fibroblasts, expression of the 3'UTR of tropomyosin cDNA markedly inhibited growth without inducing muscle gene expression (Rastinejad and Blau, 1993). The effects of the tropomyosin 3'UTR expression on the transformed state of NMU2 cells were further investigated and the results showed that expression of a 0.2 kb fragment of the tropomyosin 3'UTR was sufficient to inhibit anchorage-independent growth and tumorigenicity (Rastinejad *et al.,* 1993).

Untranslated RNAs may play a regulatory role in growth and development and have been associated with both congenital disease and malignancy. For example, myotonic dystrophy, an autosomal dominant degenerative disease of muscle tissue, results from a defect in the 3'UTR of a muscle specific kinase (Brook *et al.,* 1992) whereas, overexpression of the product of the H19 gene, a muscle transcript with no apparent open reading frame, leads to prenatal lethality in transgenic mice (Brannan *et al.,* 1990).

Several mechanisms of action of 3'UTRs have been proposed. Genes within 3'UTRs of other genes have previously been reported (Henikoff *et al.,* 1986), suggesting that the 3'UTRs may encode peptides initiated from cryptic start sites. It is also possible

that the 3'UTRs could act as an antisense RNA to block the function of other RNAs as is the case for fibroblast growth factor (Kimelman and Kirschner, 1989). The RNAs encoded by the 3'UTRs may also act as ribozymes (Latham and Cech, 1989). 3'UTRs may sequester and limit access of regulators of cell growth to their targets, or alternatively, the UTRs could suppress cell proliferation through a general disturbance of the translation machinery. Finally the RNAs may play a role in the regulation of progression through the cell cycle. The p53 protein binds covalently to the 5.8S RNA molecule, suggesting that RNAs may act as accessory factors to tumour suppressor proteins (Fontoura *et al.,* 1992). Similarly RNAs were shown to interact with cellular kinases involved in growth control (Koromilas *et al.,* 1992). The mechanisms by which untranslated RNAs can exert growth and tumour suppressive activity in trans are not clearly understood and remain to be elucidated. They nevertheless represent a novel strategy for the regulation of growth and differentiation.

1.6 GROWTH ARREST-SPECIFIC GENES (GAS)

In addition to the growth inhibitory genes described above, a novel set of genes have been identified, termed growth arrest-specific genes (GAS) and as such may also be involved in the negative regulation of the cell cycle and growth arrest. GAS genes as examples of cell-cycle regulating genes, may provide important new insights into the basic control of cell proliferation and growth arrest in both the normal and malignant cell. The GAS genes were first identified by subtraction hybridisation techniques on the basis of their preferential expression by cells in the Go or quiescent phase of the cell cycle (Schneider *et al.,* 1988). A cDNA library specific for the growth-arrested population was constructed using mRNA from serum-starved NIH3T3 mouse fibroblast

cells. The first cDNA strand was then hybridised with an excess amount of mRNA obtained from NIH3T3 cells that were actively growing in a nutrient rich medium. The arrest-specific cDNAs were isolated from the common sequences by a column chromatography technique. In this manner, six cDNA clones (Gas-1 to Gas-6) were isolated that detected mRNAs that were preferentially expressed when the cells were arrested in Go by serum starvation or by contact inhibition. The corresponding mRNAs were downregulated with different kinetics upon induction of growth with serum.

The GAS genes are a disparate group of genes which have no structural or functional similarities between them. The common link between these genes is their preferential expression in growth-arrested fibroblasts. They do not represent a family of related proteins having similar characteristics but may represent unrelated gene products involved in the cascade of events which regulate cellular proliferation.

1.6.1 Growth arrest-specific gene 1 (GAS1)

Unlike the other GAS genes, *GAS1* is controlled at the transcriptional level (Ciccarelli *et al.,* 1990; Del Sal *et al.,* 1992), while the remaining GAS genes are controlled postranscriptionally by events that modify the stability of their mRNAs. GAS1 encodes an integral membrane protein which accumulates at the cell surface following growth arrest and is rapidly down-regulated during growth. The GAS1 protein has two putative transmembrane domains flanking an extracellular region and bears no significant similarities to any other known proteins (Del Sal *et al.,* 1992). A consensus arginine-glycine-aspartic acid sequence proximal to the COOH-terminal transmembrane domain suggests that the GAS1 protein can associate with integrin-type receptors. GAS1 may therefore be involved in contact inhibition or in anchorage of the cells to the extracellular

matrix (Ruoslahti and Pierschbacher, 1987; Ruoslahti and Yamaguchi, 1991). An affinity-purified Gas-1 antibody preparation was used in both Western blot analysis and immunofluorescence studies to show that the Gas-1 protein was expressed on the cell surface of serum-starved, but not in exponentially-dividing NIH3T3 cells (Del Sal *et al.,* 1992). The expression of Gas-1 in several mouse tissues was analysed and shown to be abundant in mouse lung and heart and this may reflect functional requirement for the Gas-1 protein in these tissues (Schneider *et al.,* 1988). The expression of Gas-1 mRNA was inversely related to the level of expression of c-*myc*. As the expression of c-*myc* increased following the addition of serum to quiescent NIH3T3 cultures, the expression of Gas-1 mRNA was down-regulated (Del Sal *et al.,* 1992). Similarly, as cells entered Go after serum starvation the level of Gas-1 mRNA increased while the level of c-*myc* mRNA decreased. DNA synthesis was inhibited in both normal and transformed NIH3T3 cells when an increased level of ectopic expression of Gas-1 was artificially maintained while the endogenous level was down-regulated by serum stimulation. However, the induction of *c-fos* and *c-jun* proteins occured normally in Gas-1-overproducing cells, which suggested that the overexpression of Gas-1 does not interfere with the early response events in the signal transduction pathway (Del Sal *et al.,* 1992). These experiments suggested that a membrane protein such as the product of Gas-1 may suppress growth and its downregulation may be a prerequisite for cell cycle progression.

A human homologue to the mouse *Gas-1* was isolated by screening a normal human liver cDNA library with the mouse Gas-1 cDNA. Comparative analysis of mouse and human sequences demonstrated that the coding region of the human gene, GAS1, codes for a protein with 82% amino acid identity with the mouse Gas-1, maintaining the typical domains of an integral membrane protein (Del Sal *et al.,* 1994). Since cell cycle control elements are highly conserved between species, it is highly likely that the

products of the human GAS1 and mouse Gas-1 genes serve the same function. Like the mouse Gas-1, the expression of the human GAS1 gene was up-regulated in human fibroblasts when arrested by either serum deprivation or high cell density, while the level of GAS1 mRNA was significantly lower during exponential growth. Northern blot analysis of total RNA isolated from different human tissues has shown that GAS1 is expressed in several tissues including skeletal muscle, kidney, heart, and placenta. However, expression of GAS1 was restricted to fetal brain and expression in the adult brain was not detected, suggesting a functional significance of GAS1 in the developing brain and possibly in embryogenesis (Del Sal *et al.,* 1994).

Overexpression of GAS1, through microinjection experiments of cloned GAS1 in various malignant human cell lines has shown that, in the T24 bladder carcinoma and in the A549 lung adenocarcinoma cell lines, GAS1 was able to suppress cell growth, but this effect was not observed in the sarcoma derived cell line SAOS-2 or in the adenovirus type-5-transformed cell line 293 (Del Sal., *et al.,* 1994). The lack of response to GAS1 overexpression in the last two cell lines was similar to that previously noticed in the SV40-transformed NIH3T3 cell line when the mouse Gas-1 gene was used in similar microinjection experiments (Del Sal *et al.,* 1992). The growth-suppressive effects of GAS1 in these cell lines was correlated with the presence of functionally-active RB and/or p53. These observations suggested that tumour suppressor elements like Rb and/or P53 may mediate the growth-suppressive activity of both mouse and human GAS1 genes. A recent study demonstrated that overexpression of Gas-1 in NIH3T3 cells, blocks proliferation of these cells in a p53-dependent manner and that RB was not required. However, the N-terminal domain-dependent transactivating function of p53 was not involved in the Gas-1-induced growth arrest. It was therefore suggested that the other

transactivation-independent functions of p53, possibly related to regulation of apoptosis may mediate the Gas-1-induced growth arrest (Del Sal *et al.,* 1995).

The expression of Gas-1 in NIH3T3 cells transfected *in vitro* with activated *H-ras* or *K-ras* oncogenes, was found to be significantly lower than untransfected cells. In addition, the down-regulation of Gas-1 mRNA by serum in these transformed cells was also less efficient than in the untransformed cells. Since transformed cells usually do not readily undergo growth arrest, the lower expression of Gas-1 in the transformed cells is consistent with its definition as a growth arrest-specific gene (Cairo.*et al.,* 1992). To assess whether the down-regulation of Gas-1 also occurs in transformed cells *in vivo*, the expression of Gas-1 in chemically-induced mouse tumours grown subcutaneously in syngenic mice was also investigated. The amount of Gas-1 mRNA was variable but not related to the proliferative activity of the tumours analysed. In general, the amount of Gas-1 mRNA in the tumours was notably higher than in normal lung or muscle, which were previously described as the tissues with the highest expression of this gene (Schneider *et al.,* 1988). These findings are not consistent with growth arrest-specific expression and differ from the *in vitro* results (Cairo *et al.,* 1992). In this context, it appears that Gas-1 may be insufficient to drive cells into quiescence. These findings are also contradictory of the findings of Del Sal (Del Sal *et al.,* 1992). These tumours could be expressing an abnormal Gas-1 RNA transcript and thus a non functional protein. Alternatively, it may be possible, that the tumour cell lines which express high levels of Gas-1 are defective in the down-stream elements like p53 normally required for Gas-1-mediated growth arrest. Further analysis of the down-stream elements in these tumours should clarify these findings.

1.6.2 Growth arrest-specific gene 2 (GAS2)

Expression of Gas-2 mRNA in mouse fibroblasts is abundant during growth arrest induced either by serum starvation or growth to confluence. Gas-2 mRNA is downregulated when growth-arrested cells are reintroduced back into the cell cycle by the addition of serum or by dilution (Schneider *et al.,* 1988). The protein product of the mouse Gas-2 cDNA has an apparent mass of 36 kDa and shows no similarities to any other known proteins. Western analysis of IMR90 human diploid fibroblasts also detected GAS2 as a band of 36 kDa suggesting that GAS2 has been well-conserved during evolution.

Expression of the Gas-2 gene is postranscriptionally regulated. Using specific antibodies, it was demonstrated that the protein product is also regulated at the postranslational level via a phosphorylation mechanism in which Gas-2 becomes highly phosphorylated after the addition of serum to growth arrested cells. Such a mechanism might therefore be more efficient in regulating its activity rather than regulation of its abundant level. The Gas-2 protein is a component of the microfilament system. Using double immunofluorescence analysis, Gas-2 was found to colocalize with actin filaments, its distribution being more abundant at the cell border and also along the stress fibrer in growth-arrested NIH3T3 cells. When growing cells were microinjected with purified Gas-2 protein, the pattern of distribution was found to be similar to that of endogenous Gas-2 in arrested cells (Brancolini *et al.,* 1992). In a series of single oncogene transformed NIH3T3 cells (*v-fos, v-myc, v-ras, v-src*), the expression of Gas-2 failed to increase in response to serum starvation, and the amount of Gas-2 protein in these cells was comparable to the amount detectable in growing cells. Cellular transformation results in the loss of stress fibre bundles of actin, with a concomitant alteration in contact inhibition and enhanced tumour-forming potential (Boschek *et al.,* 1981). Therefore Gas-

2 could be an actin-binding protein involved in the regulation of actin during different phases of cell growth. Abnormalities in this regulatory pathway could lead to cellular transformation and thus tumour formation (Brancolini *et al.,* 1992). Using antibodies specific to the carboxy or amino-terminal ends of the Gas-2 protein, it was demonstrated that, during the course of apoptosis, the carboxy-terminal end of Gas-2 is removed. In addition, when overexpressed in different cell types, only the artificially carboxy-terminal truncated forms of Gas-2 were able to trigger alterations in the microfilament system and in cell shape (Brancolini *et al.,* 1995). Such phenotypic alterations resemble changes in cell shape normally encountered during cell death by apoptosis (Arends and Wyllie, 1991; Martin *et al.,* 1994). Overexpression of wild type Gas-2 completely lacked such an effect, suggesting that the cleaved product was responsible for the apoptotic phenotype. The sequence from amino acid 278-280, Val-Asp-Gly, within the carboxy-terminal end of the Gas-2 gene, corresponds to a consensus site for the interleukin-1β-converting enzyme (ICE)-like protease, which is activated during apoptosis (Kumar, 1995). These findings provided evidence for a possible regulatory function of Gas-2 during apoptosis which is functionally associated with changes in cell architecture.

1.6.3 Growth arrest-specific gene 3 (GAS3)

The same striking regulation of GAS gene expression during growth arrest also hold true for Gas-3 which is specifically expressed in resting cells and down regulated by the addition of serum and/or density inhibition. Expression of Gas-3 mRNA was detected in various mouse tissues with highest abundance in lung and lower intestine (Schneider *et al.,* 1988). The full length Gas-3 cDNA clone has 1817 nucleotides and codes for an 144 amino-acid protein. *In vitro* translation experiments revealed an 18

kDa protein. The deduced protein sequence suggested the presence of three putative transmembrane domains with one N-glycosylation site. The Gas-3 product is a true integral membrane glycoprotein as it strongly associates with the detergent TX-114. Proteinase K protection experiments showed that it is fully protected by microsomes. A rat myelin protein SR13 has strong similarities with Gas-3 (Welcher *et al.,* 1991). Mouse Gas-3 and rat SR13 are 92% homologous at the nucleotide level, including the 3' untranslated region. SR13 is strongly expressed in sciatic nerve as a 1.8 kb transcript and a weak signal was detected in brain tissue. Immunohistochemical analysis has localised this protein to the fully-differentiated and quiescent schwann cells that make up the myelin sheath of the sciatic nerve. After nerve injury, the SR13 mRNA was rapidly downregulated, coinciding with the time of schwann cell proliferation. The human homologue to the *SR13/Gas-3,* now termed PMP22, maps to chromosome 17p11.2. Charcot-Marie-Tooth disease type 1A (CMT1, hereditary motor and sensory neuropathy type I) is associated with DNA duplication at this region. The clinical features of this disease are distal muscle wasting and weakness, areflexia and hollow feet, severely slowed nerve conduction and demyelination in nerve biopsies (Hoogendijk *et al.,* 1991). The peripheral myelin gene PMP22 is duplicated in this disease providing direct evidence for the involvement of PMP22 in its pathogenesis. Either the increased gene dosage or altered chromosomal environment of PMP22 caused by the duplication, could effect schwann cell proliferation resulting in a demyelinating polyneuropathy.

1.6.4 Growth arrest-specific gene 5 (GAS5)

The kinetics of induction after serum starvation and density inhibition of Gas-5 mRNA is similar to that of the other Gas genes (Ciccarelli *et al.,* 1990). Sequence analysis of the Gas-5 genomic clone has shown that the transcriptional unit is divided into 12 exons that span around 7 kb. The mature transcripts are derived from a complex splicing process which joins together 11 to 12 exons giving rise to two mRNA species. Both mRNAs are found in the cytoplasm of growth-arrested cells. The Gas-5 mRNAs are expressed in a growth regulated fashion in Friend leukemia (FL) and NIH3T3 cells, being almost undetectable in actively growing or density-arrested cells. Expression of the Gas-5 gene is under postranscriptional regulation in cycling cells, whereas in terminally differentiated cells this transcription is turned off. It appears that transcriptional regulation of Gas genes is effective only in those cells that retain the ability to re-enter the cell cycle during activation and are not irreversibly growth-arrested. No similarities with any other known proteins within the data bank have been detected to date and thus the biological function of the Gas-5 product remains unclear.

1.6.5 Growth arrest-specific gene 6 (GAS6)

Gas-6 is structurally similar to the bovine and human form of protein S in the coagulation system (Manfioletti *et al.,* 1993). The gene encodes a soluble protein belonging to the same family as protein S. When both murine and human cells reach confluency, large amounts of the Gas-6 protein are secreted, but in actively-growing cells only very small amounts of the protein are synthesised. Gas-6 is synthesised in many tissues although, protein S is predominantly synthesised in the liver. Gas-6 potentiates proliferation of vascular smooth muscle cells (VSMC) stimulated by Ca^{2+}-

mobilising growth factors, such as thrombin, and may be involved in thickening of the vascular wall accompanying atherosclerosis (Nakano *et al.,* 1995). Recent evidence suggested that Gas-6 is the ligand of the Sky receptor tyrosine kinase and is able to stimulate tyrosine phosphorylation of this receptor (Ohashi *et al.,* 1995). Receptor tyrosine kinases play a central role in signal transduction pathways leading to cell proliferation and differentiation (Ullrich and Schlessinger, 1990). Independently, two other research groups also reported that Gas-6 is the ligand for Axl, a receptor closely related to Sky, and is therefore a common ligand for both receptors (Varnum *et al.,* 1995; Stitt *et al.,* 1995).

1.7 AIMS OF THE THESIS

The functional role of the product of the GAS1 gene has not yet been established. The cell cycle-specific expression of GAS1 could reflect requirements to induce or sustain quiescence by blocking the pathway to cellular proliferation. The gene product of GAS1 may code for a repressor or suppressor protein that needs to be down regulated if a cell is to enter a state of proliferation. Abnormalities in the GAS1 gene such as deletions, translocations, and alterations in DNA methylation would allow a cell to escape from growth control restraints and possibly give rise to uncontrolled cellular proliferation and thus tumour formation.

The aim of this thesis was to determine if GAS1 is a tumour suppressor gene by addressing the following hypotheses:

1. The GAS1 gene is located at a chromosomal site that is abnormal or deleted in some tumours.

2. The normal function of the GAS1 gene is related to the control of growth and, when overexpressed in normal cells, is able to suppress proliferation.

3. Control of expression of the GAS1 gene is defective in some transformed cells and tumours.

4. When introduced into tumour cells which do not normally express GAS1, the product of the GAS1 gene will suppress proliferation *in vitro* and inhibit tumorigenicity in nude mice.

5. If expression of endogenous GAS1 is suppressed in normal cells by expression of an antisense GAS1 RNA, a transformed phenotype develops.

6. The GAS1 gene is mutated or deleted in tumours that show frequent cytogenetic abnormalities at the chromosomal location of GAS1.

In order to understand the mechanisms involved in the development of cancer, one must first know how the proliferation of normal cells is controlled. Tumours arise partly because the mechanisms which control cell cycle progression and proliferation are no longer effective. In this thesis, the function of GAS1 a cell cycle-regulating gene, will be investigated. These studies may provide evidence that GAS1 should be considered as a tumour suppressor gene, thereby identifying a further class of these genes and adding to the knowledge of the mechanisms of tumorigenesis. Identification of genes that have growth- and tumour-suppressive functions may also suggest new therapeutic interventions.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and reagents

The following chemicals and reagents were obtained from Sigma Chemical Co. Ltd., St Louis MO, USA.

Ammonium persulphate

5-Azacytidine

Antifoam A concentrate

Bovine serum albumin (BSA)

Bromophenol blue

Citric acid

Coomassie blue

Dextran sulphate

Diethyl pyrocarbonate (DEPC)

Dimethyl sulfoxide (DMSO)

Ethidium bromide

Ethylenediaminetetra-acetic acid (EDTA)

Formamide

Guanidine thiocyanate

N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)

3-[N'-morpholino]propanesulfonic acid (MOPS)

β-mercaptoethanol

Phenol

Phenylmethylsulphonyl fluoride (PMSF)

Tris[hydroxymethyl]aminomethane (Trizma base)

N,N,N',N'-tetramethyl-ethenediamine (TEMED)

Herring sperm DNA

Sodium acetate

Sodium dodecyl sulphate (SDS)

Sources of other important chemicals routinely used were as follows:

Acrylamide/bisacrylamide, 40% solutions, (19:1, 29:1), mixed bed resin, (Bio-Rad, Laboratories, California, USA). Agarose DNA grade, Low melt agarose, (Progen Industries, Queensland, Australia). Ampicillin, (Boehringer Mannheim, Germany). Formaldehyde, (Ajax Chemicals, Sydney Australia) Deoxyribonucleotide triphosphates, dNTP's (Promega Corporation, Maddison USA). Dexamethasone sodium phosphate inj. U.S.P. equivalent to dexamethasone phosphate, 4 mg/ml (David Bull)

All other chemicals and reagents used in this study were of molecular biology grade or analytical grade.

2.1.2 Enzymes

The enzymes used in this study were obtained from the following companies

Calf intestinal phosphatase	New England Biolabs, MA, USA
E, coli DNA polymerase I (Klenow fragment)	Bresatec, South Australia
Proteinase K	Boehringer Mannheim
Avian Myeloblastosis Virus Reverse transcriptase (AMV)	Promega, Corporation
Ribonuclease A	Sigma Chemical Co
RNAsin	Promega Corporation
T4 DNA ligase	Promega Corporation

Taq DNA polymerase	Promega Corporation
Trypsin-EDTA	Gibco BRL, Glen Waverly, Victoria, Australia

All restriction endonucleases in this study were obtained from New England Biolabs (Beverly, Massachusetts, USA) and Progen Industries (Queensland, Australia) unless specified

2.1.3 Radiochemicals

[α-^{32}P] dCTP, 3000 Ci/mmole	Bresatec

2.1.4 Kits

cDNA synthesis (AMV Reverse Transcriptase kit)	Promega, Maddison, USA
Cycle sequencing (Exo(-) *pfu* cyclist DNA sequencing kit)	Stratagene, La Jolla, CA,USA
Oligolabelling of DNA (Giga prime kit)	Bresatec
Plasmid isolation (Qiagen plasmid midi kit)	Qiagen Pty Ltd, Clifton Hill, Victoria, Australia
Purification of PCR products (Wizard PCR preps DNA purification system)	Promega
Single-Strand Conformational Polymorphism analysis, (SSCP-PCR control kit)	Stratagene

2.1.5 Buffers and solutions

Buffers and solutions routinely used in this study were as follows:

Formamide loading buffer (SSCP loading buffer)	95% deionised formamide, 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol
6 X DNA loading buffer	0.25% bromophenol blue, 40% (w/v) sucrose in H_2O
MOPS buffer (10X)	0.2 M MOPS free acid, 50 mM Na acetate, 10 mM EDTA
Phosphate buffered saline (PBS)	135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 20 mM KH_2PO_4
10 X PCR buffer (Magnesium free)	500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100
SSC	3 M NaCl, 0.3 M Na citrate, (pH 7.0)
STE	100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.
Protein Lysis buffer	10 mM Trizma base, 1% Triton X-100 0.1% SDS, 150 mM NaCl, (pH 7.6)
RNA lysis buffer	4 M guanidinium thiocyanate, 25 mM sodium citrate, 100 mM β-mercaptoethanol, 0.5% lauryl sarcosine, 0,1% antifoam A
RNA loading buffer	50% formamide, 6.2% formaldehyde 10% glycerol, 30 µg/ml ethidium-bromide, 1 x MOPS buffer.
SDS sample buffer (2X)	12 mM Trizma base, 6% SDS, 20% glycerol, 0.03% bromophenol blue (pH 6.8), 10% β-mercaptoethanol
Substrate buffer (colorimetric detection of proteins)	100 mM Trizma base, 100 mM NaCl, 100 mM $MgCl_2$, (add 45µl of NBT and 35 µl of X-phosphate per 10 ml just prior to use)

TAE	40 mM Tris-acetate, 1 mM EDTA (pH 8)
TBS	20 mM Trizma base, 500 mM NaCl, (pH 7.5)
TBE	90 mM Trizma base, 90 mM boric acid, 2.5 mM EDTA (pH 8.0).
TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA
Western transfer buffer	25 mM Trizma base, 192 mM glycine (pH 8.3)

All buffers were sterilised by autoclaving or where necessary by filtration through a 0.2μm filter (Schleicher and Schull, Germany)

2.1.6 Bacterial media

All liquid media were prepared using milliQ water and were sterilised by autoclaving.

The composition of the various media were as follows.

L-broth	1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl, (pH 7.0)
L-Agar	L-broth, 1.5% Bacto agar
L-Amp	L-broth, 1.5% Bacto agar, ampicillin (50 μg/ml)

2.1.7 Antibiotics

Ampicillin	Boehringer Mannheim
G418 sulphate (Geneticin, Neomycin)	Gibco (BRL), Vic, AustraliaPenicillin-
Streptomycin	ICN Biomedicals, California USA
Doxycycline hydrochloride	Sigma Chemical Co

2.1.8 Bacterial strains

The following E-coli strains were used as host for propagation of the various plasmids used in this study.

MC1061	*hsdR mcrB araD*139 Δ(*araABC-leu*)7679 Δ*lac*X74 *galU galK rpsL thi*
JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) F'[*tra D*36 *proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15]

2.1.9 Plasmid vectors

pBLUESCRIPT KS	Stratagene
pRc/CMV	Invitrogen, NV Leek, The Netherlands
pMAM neo	Clontech Laboratories, Inc, California, USA

2.1.10 Cloned DNA sequences

The following cloned DNA sequences used in this study were generous gifts from the following:

Mouse Gas-1 [PA-1] (nt 82-2252 of cDNA)	Dr. V. Sorrentino EMBL, Heidelberg, Germany
Human GAS1 [clone 181/HLEE] (3' fragment of human GAS1 cDNA)	Dr. V. Sorrentino EMBL, Heidelberg, Germany
Human GAS1 (6.5 kb *Xba*I genomic fragment in Bluescript)	Dr. R. de Martin EMBL, Heidelberg, Germany
MDM2 clone c14-2 (Human MDM2 cDNA)	Dr B Vogelstein, Johns Hopkins Oncology Research Laboratories Baltimore, USA (Oliner *et al.*, 1992)

2.1.11 Synthetic oligonucleotides

The following synthetic DNA primers were designed to amplify specific regions of the human GAS1 gene and were synthesised by the following centres:

Haematology Department of The Queen Elizabeth Hospital Adelaide:

HP1	5'-CGGGACCAGATCTCGACAGCTGTT-3'
HP2	5'-TCCTGCCCACTTGCATGAGTG-3'
HP5	5'-GAGTCACTGCATCTCGGCCCTCAT-3'
HP7	5'-GCAGCAGCGCCATCAGGCACAG-3'

Vector specific primers were synthesised by:

Bresatec:

5pMAMneo	5'-GGCTATCATCACAAGAGCGGAAC-3'

Operon Technologies:

5pRcCMV	5'-GAACCCACTGCTTAACTGGCTTAT-3'
3pRcCMV	5'-TGATCAGCGAGCTCTAGCATTTAG-3'

MDM2 specific primers

Gibco BRL:

MDM2 EXT1	5'-CTGGGGAGTCTTGAGGGACC-3'
MDM2 EXT2	5'-CAGGTTGTCTAAATTCCTAG-3'
MDM2 INT1	5'CGCGAAAACCCCGGGCAGGCAAATG TGCA-3'
MDM2 INT2	5'-CTCTTATAGACAGGTCAACTAG-3'

The following primers were used for the detection of mutations within the P53 gene and were designed by Mrs J Hardingham of the Department of Heamatology The Queen Elizabeth Hospital, Adelaide:

P53 Exon 5 sense	5'-TCTGTCTCCTTCCTCTTCCTACA-3'
P53 Exon 5 antisense	5'-AACCAGCCCTGTCGTCTCTC-3'
P53 Exon 6 sense	5'-GTCCCCAGGCCTCTGATTCC-3'
P53 Exon 6 antisense	5'-CCTCCTCCCAGAGACCCCCAGT-3'
P53 Exon 7 sense	5'-CCTCATCTTGGGCCTGTGTTATC-3'
P53 Exon 7 antisense	5' TGGCAAGTGGCTCCTGACCTGGA-3'
P53 Exon 8 sense	5'-CCTCTTGCTTCTCTTTTCCTATC-3'
P53 Exon 8 antisense	5'-CTGCTTGCTTACCTCGCTTAGT-3'

The following primers were used to amplify the ABO locus on chromosome 9 and are described in O'Keefe and Dobrovic, (1994):

ABO 1	5'-CGCATGGAGATGATCAGTGACTTC-3'
ABO 2	5'-GCTCGTAGGTGAAGGCCTCCC-3'

2.1.12 Tissue culture solutions

Dulbeccos minimal essential Medium (DMEM)	ICN Biomedicals, California, USA
Foetal calf serum	Gibco BRL
Glutamine	Flow Laboratories, Inc, NSW, Australia
Trypsin-EDTA	Gibco BRL

2.1.13 Cell lines

The sources of the following cell lines used throughout the course of this work are indicated:

A549 (Human bronchial adenocarcinoma)	American Tissue Culture Collection (ATCC)
HTD114 (*APRT*) (Human fibrosarcoma)	Dr Alex Dobrovic, Department of Haematology, The Queen Elizabeth Hospital
HT1080 p53WT (Human fibrosarcoma)	Dr R. Reddel, Children's Medical Research Institute, Sydney Westmead Australia
HT1080 6TGc5 (Human fibrosarcoma)	Professor E. Stanbridge, University of California, Irvine, USA
NIH3T3 (Mouse fibroblasts)	ATCC
Lewis Lung Carcinoma (mouse lung adenocarcinoma)	Dr. Lindsay Dent. Dept. of Microbiology, The University of Adelaide
B16 (mouse melanoma)	Dr Lindsay Dent
IMR90 (Human lung diploid fibroblasts)	Dr. Briony Forbes Department of Biochemistry, The University of Adelaide.
MRC-5 (Human lung diploid fibroblasts)	Flinders Medical Centre Department of Microbiology
SF1972 (human foreskin fibroblasts)	Dr. Briony Forbes Department of Biochemistry The University of Adelaide

2.1.14 Miscellaneous

Poly-prep chromatography columns (Separation of unincorporated nucleotides)	Bio-Rad
3MM chromatography paper	Whatman Ltd, Maidstone, UK
Nylon filters (GeneScreen*Plus*^{TM})	Dupont-NEN, Research products Boston, MA, USA
Positive land film, type 667	Polaroid, St Albans, UK
Sephadex G-50	Pharmacia Biotech, Hong Kong
X-Ray Hyperfilm MP	Amersham, International, Buckinghamshire, UK

2.2 GENERAL METHODS

The following methods were performed essentially as described in Sambrook *et al.* (1989); Growth, maintenance and preservation of bacteria, quantitation of DNA and RNA, autoradiography, agarose and polyacrylamide gel electrophoresis, DNA and RNA precipitations, phenol/chloroform extractions.

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA, the Council of the University of Adelaide and biohazards committee of The Queen Elizabeth Hospital.

Handling of animals and procedures were carried out at the animal house facilities of the Queen Elizabeth Hospital in accordance and approval from the Animal Ethics Committees of The University of Adelaide and the Queen Elizabeth Hospital.

2.2.1 Plasmid DNA preparation

Plasmid minipreps were essentially prepared as described by Birnboim and Doly (1979). Briefly, single colonies were grown overnight in 5 ml of L-broth containing 50 μg/ml of ampicillin. Cells (2 ml) were pelleted by centrifugation at 12,000 g for 1 min and the pellet was resuspended in 100 μl of solution I containing 50 mM glucose, 25 mM Tris-HCl (PH 8.0) and 10 mM EDTA. The suspension was incubated at room temperature for 5 min. Freshly prepared 0.2 M NaOH, 1% (w/v) SDS added to each tube (200 μl), mixed by inversion and incubated on ice for 5 min followed by the addition of 150 μl of an ice cold solution of 3 M sodium acetate, pH 5.5. The solution was incubated on ice for a further 5 min. The cellular debris and bacterial DNA was pelleted by centrifugation at 12,000g for 5 min and the supernatant extracted with an equal volume of phenol/chloroform, followed by precipitation in 2 volumes of ethanol. The DNA pellet was washed in 70% ethanol, vacuum-dried and resuspended in 30 μl of water or TE buffer. (RNase A was added to digestions of the miniprep plasmid DNA to a final concentration of 200 μg/ml).

For large-scale plasmid preparation, recombinant clones, were grown overnight in 50 ml of L-broth supplemented with 50 μg/ml of ampicillin. The cells were pelleted by centrifugation at 4,000 rpm for 30 min at 4°C and plasmids isolated using the Qiagen Plasmid Midi kit (Qiagen) according to the instructions supplied by the manufacturer.

2.2.2 Restriction enzyme digestions

For analytical digests, 0.5-1 μg of plasmid DNA was incubated with 5-10 units of each of the appropriate restriction enzyme(s) for a minimum of 1 hr in the buffer

conditions specified by the manufacturer. Restriction fragments were electrophoresed on 1% mini-agarose gels in TAE buffer. In preparative digests, 5-10 μg of DNA was used in a total volume of 30-100 μl and the desired DNA fragments were isolated as described below.

2.2.3 Preparation of DNA restriction fragments

The plasmid DNA was digested with the appropriate restriction enzyme(s) as described above (2.2.2). Fragments were isolated from horizontal 0.8%-1.5% low melting point agarose gels depending on the size of the DNA restriction fragment. Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide, and the appropriate fragment(s) excised from the gel using a sterile scalpel blade. Gel slices were placed at 70°C until melted and for DNA fragments greater than 3000 bp in length, the melted agarose was mixed with 3 volumes of TE buffer, and extracted with an equal volume of phenol chloroform.The plasmid DNA was then precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. For purification of restriction fragments smaller that 3000 bp in length, the Wizard (PCR preps) DNA purification kit (Promega) was used according to the instructions supplied by the manufacturer.

2.2.4 Preparation of cloning vectors

Vector DNA for use in ligation reactions was linearised with the appropriate restriction enzyme(s) and the complete digestion of DNA confirmed on agarose gels. To prevent self-ligation of the vector, 5' terminal phosphate groups were removed by an initial incubation in 10 units of calf intestinal phosphatase, CIP (New England Biolabs), in a total volume of 100 μl and in the buffer supplied by the manufacturer. The reaction

was incubated at 37°C for 30 min, followed by a further addition of 10 units of CIP and a further incubation for 45 min at 55°C. The ligated vector was then purified by either phenol/chloroform extractions followed by DNA precipitation or using the Wizard PCR prep DNA purification kit (Promega). The amount of vector DNA recovered was estimated by agarose gel electrophoresis stain with ethidium bromide.

2.2.5 Ligation of DNA fragments into plasmid vectors

The DNA insert and the appropriate plasmid vector (100 ng) were combined in molar ratios of 1:1, 1:3, and 3:1, in a 20 µl reaction volume with 15 units of T4 DNA ligase (Promega) and in the buffer supplied by the manufacturer. The reactions were routinely incubated overnight at 4°C. A control ligation of vector without insert was usually set up and included in the subsequent transformations to determine background levels of uncut or recircularised vector. The pGEM-T Easy vector system (Promega) was used for the direct cloning of PCR products according to the supplied protocol.

2.2.6 Preparation of competent bacteria

A single colony of the *E. coli* host strain JM109 or MC1061 was inoculated into 10 ml of L-broth and the culture incubated overnight at 37°C with continuous shaking. One ml of the overnight culture was then added to 50 ml of L- broth in the presence of the appropriate antibiotic and the incubation continued at 37°C with shaking until the culture reached an absorbence at 600 nm of 0.4-0.6. The cells were pelleted by centrifugation at 4,000 g for 5 min at 4°C, resuspended in 20 ml of an ice-cold solution of 100 mM $MgCl_2$ and incubated on ice for 30 min. Following centrifugation, the cells were resuspended in 2.5 ml of 100 mM $CaCl_2$ and left on ice for at least 1 hr. The cells

were mixed with an equal volume of 80% glycerol, and aliquots were then stored in cryovials (Nunc) at -80°C.

2.2.7 Transformation of *E. coli.*

Plasmid vectors (20-50 ng) or DNA ligation reactions corresponding to half the volume of the ligation mixture were mixed with 200 µl of competent bacteria (2.2.6) and incubated on ice for 30 min. The cells were then heat shocked at 42°C for 2 to 5 min, placed in 1 ml of L-broth and incubated at 37°C with shaking for 30 min. Cells were pelleted and resuspended in 100 µl of L-broth and plated using a sterile spreader.onto L-agar containing 50 µg/ml of ampicillin The agar plates were routinely incubated overnight at 37°C .

2.2.8 Southern transfer

DNA (10 µg) digested with the appropriate restriction endonucleases was mixed with 6 x DNA loading buffer and electrophoresed overnight at 42 volts on 1% agarose gels and in 1 x TAE running buffer. Following staining with ethidium bromide, the DNA was visualised with UV light and photographed onto Polaroid 667 film. DNA was transferred to GeneScreen*Plus*™ (Dupont, NEN) nylon membrane using an optimised protocol for the downward alkaline transfer method essentially as described in Koetsier *et al.* (1993). To acid nick the DNA, the gel was soaked in 0.25 M HCl for 15 min with gentle shaking and then immersed in transfer solution (0.4 M NaOH) for 15 min with gentle shaking. The nylon membrane was cut to the size of the gel, briefly placed in distilled H_2O and then in transfer solution for 5 min. The downward transfer of the DNA was carried out using a stack of paper towels onto which three Whatman 3MM filters cut to the size of the gel were positioned on top. The top filter was soaked in

transfer solution and the nylon membrane was placed on top followed by the gel. Three Whatman 3MM filters were soaked in transfer solution and placed on top of the gel followed by a stack of ROAR wipers (Kimberly-Clark Australia) that were also soaked in transfer solution. Transfer was allowed to take place for 3 hr. Following transfer, the filter was placed in 2 x SSC buffer for 5 min, air dried and UV cross linked using the Stratalinker UV Crosslinker 1800 (Stratagene). Filters were then prehybridised and hybridised as described in section 2.2.10.

2.2.9 Labelling of DNA with ^{32}P

The Gigaprime kit (Bresatec) was used for the random-labelling of plasmids or isolated inserts. DNA (100-200 ng) was labelled with ^{32}P-dCTP according to the manufacturer's recommended protocol. The labelling reaction was incubated at 37°C for 15 min and the labelled probe was purified from unincorporated nucleotides through a Sephadex G-50 column, prepared as follows.

Sephadex G-50 (2.5 g) was suspended in 60 ml of distilled H_20 and allowed to swell overnight at room temperature or alternatively for at least 2 hr at 65°C. A poly-Prep chromatography column (Biorad) was filled with 2.0 ml of Sephadex solution and then washed with 200 µl of 2 mM β-mercaptoethanol and 10 µl of 10 mg/ml herring sperm DNA. The column was centrifuged for 6 min at 400 rpm to pack the Sephadex and to remove excess liquid. The oligolabelling reaction was diluted with 200 µl of 2 mM β-mercaptoethanol and the column centrifuged at 800 rpm for 8 min. The recovered probe was then used in subsequent hybridisation reactions of Southerns and Northerns.

2.2.10 Prehybridisation, hybridisation and washing of filters

Prehybridisation was carried out in a solution consisting of 50% deionised formamide, 1% SDS, 1 M NaCl, 10% Dextran sulphate and 400 μg/ml of sheared and denatured herring sperm DNA. Incubation was carried out at 42°C for at least 2 hr in a plastic bag submersed in a water bath or in hybridisation bottles in the micro 4 mini rotary incubator (Hybaid). After prehybridisation, the purified ^{32}P-labelled probe was denatured by heating to 100°C for 5 min, snap-cooled on ice and added directly to the bag or bottle containing the prehybridisation solution. Hybridisation was then allowed to take place overnight at 42°C. Filters were initially washed at 2 x SSC at room temperature for 5 min with constant agitation and then twice in 2 x SSC, 1% SDS for 30 min with a gradual increase in temperature from room temperature to 65°C. Signal to background noise ratio was periodically assessed using a radiation mini-monitor series 900 A final high-stringency wash in 0.1 x SSC, 0.1% SDS at 65°C was generally performed to reduce background to signal ratio. The membrane was wrapped in cling film, placed in a cassette with intensifying screens and X-Ray film (Hyperfilm MP, Amersham), and exposed at -80°C for varying times. The film was developed using an automatic developer (Kodak Australia) at the Radiology Department of The Queen Elizabeth Hospital

2.2.11 Re-use of filters

For Southern blots, the bound probe was stripped from the membrane by an initial wash in 0.4 M NaOH for 30 min at 42°C followed by a second wash in 0.5 M Tris-HCl, (PH 7.5), 0.1 x SSC, 0.1% SDS for 30 min at 42°C. The filter was then briefly

washed in 2 x SSC for 5 min at room temperature, air dried and stored sealed in plastic for subsequent hybridisations.

For Northern blots, the bound probe was stripped from the membrane by 3 sequential washes of 15 min each, in a boiling solution of 0.1 x SSC, 0.1% SDS, finally followed by a single 5 min wash in 2 x SSC at room temperature. This procedure led to satisfactory re-hybridisation of the same blot at least 4 times.

2.2.12 RNA preparation

Total RNA was isolated from tissue or cultured cells as previously described (Chomczynski and Sachi, 1987). Cultured cells, fresh tissue or tissue that had previously been frozen in liquid nitrogen was placed in RNA lysis solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 100 mM β-mercaptoethanol, 0.5% lauryl sarcosine and 0.1% antifoam A. Tissues were homogenised on ice in a glass teflon homogeniser. Lysates were placed into polypropylene tubes and the following reagents added per ml of RNA lysis buffer: 0.1 ml of 2 M sodium acetate, PH 4.0, 1.0 ml of phenol saturated with DEPC treated water and 2.0 ml of chloroform. The mixture was vortexed for 10 seconds and placed on ice for 15 min. Samples were then centrifuged at 4,000 rpm for 15 min at 4°C to separate the RNA, which remained in the aqueous phase, from the DNA and protein contained in the interface and the phenol layer. The aqueous phase was transferred to a fresh tube, an equal volume of ice-cold isopropanol added and the solution incubated at -20°C for at least 1 hr. The RNA was pelleted by centrifugation at 4,000 rpm for 30 min at 4°C, the pellet resuspended in 0.5 ml of RNA lysis buffer and transferred to 1.5 ml microcentrifuge tubes. Samples were centrifuged for 5 min at 12,000 g at 4°C the supernatant removed into a fresh tube and 2

volumes of ice cold absolute ethanol added and placed at -20°C for at least 1 hr to precipitate the RNA. The sample was centrifugation for 5 min at 12,000 g at 4°C and the RNA pellet washed in 70% ethanol, dried and dissolved in DEPC-H_2O. The integrity and yield of the RNA was checked by electrophoresis through a formaldehyde agarose gel.

2.2.13 Northern blot analysis

Total RNA (10-20 μg) was mixed with 2 x RNA loading buffer containing 30 μg/ml ethidium bromide, denatured at 68°C for 5 min and immediately electrophoresed through 1% agarose / 16.6% formaldehyde gels in 1 x MOPS running buffer. After electrophoresis the RNA was transferred to GeneScreen*Plus*™ nylon membrane using 10 x SSC buffer as the transfer solution and a downward transfer performed as previously described for Southern analysis (2.2.8). The RNA on the filters was visualised under UV and photographed to confirmed equal loading. Prehybridisation, hybridisation and washing conditions were as previously described (2.2 10).

2.2.14 Purification of oligonucleotides

Oligonucleotides from the Department of Haematology/Oncology The Queen Elizabeth Hospital, were synthesised and purified as follows:

Oligonucleotides were synthesised on 0.2 μM columns (Pharmacia-LKB) in a Pharmacia-LKB Gene Assembler Plus DNA synthesiser according to the manufacturer's instructions. Excess synthesis reagents were initially removed from the column by centrifugation in 1.5 ml Sarsdedt tubes for 1 min at 1,000 g. The oligonucleotides were cleaved from their support by placing the column in a 1.5 ml tube

containing ice cold 28% NH_4OH and incubating overnight at 37°C. The ammonia solution was then removed and oligonucleotides were concentrated by drying in a vacuum centrifuge for approximately 2 hr. The lyophilised oligonucleotides were resuspended in 1 ml of TE buffer and the concentration determined by spectrophotometry at 260nm (A_{260} 1 = 33 μg/ml). Oligonucleotides not synthesised at The Queen Elizabeth Hospital were supplied as fully-deprotected and desalted lyophilised stocks from the various companies listed in section 2.2.11 of this chapter.

2.2.15 Polymerase chain reaction (PCR)

Unless otherwise stated, all PCR reactions were carried out as follows. Target DNA (50-100 ng), was placed in a 0.5 ml reaction tube in a total volume of 50 μl containing 100 ng of each of the oligonucleotide primers, 0.8 mM dNTP's, 2.5 mM of $MgCl_2$ in the PCR reaction buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C, and 1% Triton X-100. Each PCR reaction contained 1 unit of Taq DNA polymerase (Promega) and 5-10% (v/v) dimethyl sulfoxide was used in the reaction mixes where indicated. The reaction was overlaid with one drop of mineral oil (Sigma) to prevent evaporation. The DNA was then denatured by heating the reaction to 94°C for 5 min, followed by annealing at the designated temperature, dependent on the specific pair of primers being used, and elongation at 72°C for the appropriate time, dependent on the length of the PCR product expected. In general, 35-40 cycles were performed and annealing temperatures for amplifying GAS1 varied between 60°C and 65°C. Appropriate positive and negative buffer-only controls were always included in each set of reactions. All PCR reactions were carried out in a water-cooled thermal cycler (ARN Electronics, Belair, South Australia). PCR products were analysed by electrophoresis on agarose gels containing ethidium bromide.

2.2.16 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated as previously described in section 2.2.12 and first-strand cDNA synthesis carried out using an RT-PCR kit (Promega) according to the manufacturer's instructions Briefly, 2 μg of total RNA was reverse transcribed into single-stranded cDNA at 42°C for 1 hr in a 20 μl reaction containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100, $MgCl_2$, 1 mM each dNTP, 1 μg of either oligo$(dT)_{15}$ (Promega) or 100 ng of a specific GAS1 down-stream primer, 10 units RNAsin (Promega) and 30 units of AMV reverse transcriptase (Promega). The resulting cDNA was either stored at -20°C or 5-10 μl of the reaction used in subsequen PCR as described in 2.2.15.

2.2.17 Removal of contaminating DNA prior to reverse-transcription.

The GAS1 gene contains no introns, and for RT-PCR analysis of expression of GAS1 mRNA, it was necessary to remove contaminating DNA from RNA preparations prior to reverse transcription. Twenty units of RNAase-free DNAase (Boehringer) was added to the cDNA reaction mix without AMV transcriptase or primer and incubated at 37°C for 30 min. The DNAase was then denatured by heating the reaction to 95°C for 5 min and an aliquot removed to be used in PCR reactions to assess the efficiency of the DNase step. AMV reverse transcriptase and primer was added to the remainder of the reaction and cDNA synthesis performed as described above.

2.2.18 Preparation of protein from cell extracts

Adherent cells grown in 75 cm^2 culture flasks were initially washed with PBS buffer. Protein lysis buffer (500 µl) containing 10 mM Trizma base, 1% Triton X-100, 1% SDS and 150 mM NaCl, (pH 7.6) was directly added to the flask and left at room temperature until cells detached. Lysates were then removed and stored at -80°C for later analysis. Prior to electrophoresis, an aliquot of cells in lysis buffer were mixed with an equal volume of 2 x SDS loading buffer containing 12 mM Trizma base, 6% SDS, 20% glycerol, 0.03% bromophenol blue (pH 6.8) and freshly added 10% β-mercaptoethanol. Immediately before loading, the samples were denatured for 10 min in boiling water, followed by centrifugation at 12,000 g for 15 min. Aliquots loaded onto SDS polyacrylamide gels were withdrawn from the top of the tube to avoid collecting any insoluble material from the bottom. Protein standads were also loaded on each gel with out denaturation and electrophoresis was carried out as described below in section 2.2.19.

2.219 SDS-polyacrylamide gel electrophoresis and Western blot analysis

SDS-polyacrylamide (36:1, acrylamide : bis acrylamide) gel electrophoresis of cell extracts was carried out using the Bio-Rad Mini Protean II Dual Slab Cell system according to the manufacturer's instructions. Duplicate gels were run using the Rainbow coloured molecular weight markers (Amersham) as indicators of the size of the expected protein. Sufficient separation of the MDM2 protein was obtained using a 4.0% stacking gel and 10% separating gel. Electrophoresis was carried at 200 volts and until the indicator dye had eluted from the bottom of the gel.

Following disassembly, one gel was placed in 0.25% Coomassie Blue stain for 30 min with gentle agitation and subsequently destained as required in 45% methanol, 10% acetic acid. The duplicate gel was transferred to PVDF transfer membrane (Bio-Rad) using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell according to the manufacturer's instructions. Electrotransfer was performed at 70 V; 350-750 mA for 2 hr with cooling and circulating transfer buffer.

MDM2 protein was detected by probing with a mouse monoclonal IgG1 antibody (SMP14) specific for the epitope corresponding to amino acids 154-167 of MDM2 of human origin (Santa Cruz Biotechnology). The filter was initially rinsed in PBS and non-specific binding of proteins blocked by incubating for 1 hr in 15 mg/ml BSA in 1 x TBS and 0.02% sodium azide. The primary MDM2 antibody was added to fresh blocking solution at a 1:300 dilution and incubation continued for a further hour at room temperature. The filter was then washed 3 times for 5 min each in PBS buffer. The secondary antibody (alkaline phosphatase conjugated anti-mouse antibody) was diluted (1:5000) in fresh blocking solution and incubated with the filter for an additional hour at room temperature before being washed as above. The filter was then equilibrated for 5 min in substrate buffer containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 100 mM 100 mM $MgCl_2$ and the colorimetric reaction was developed after incubation with NBT (0.3 mg/ml) and BCIP (0.2 mg/ml) in the same buffer as described by Bers and Garfin (1985). When bands were sufficiently intense, the reaction was terminated with 20 mM EDTA.

2.2.20 Maintenance of cultured cell lines

All culture cell lines were routinely grown in Dulbecco's Modified Eagles medium (DMEM), pH 7.4, supplemented with 10% fetal calf serum, 20 mM HEPES, 2 mM glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin in 25 cm^2 or 75 cm^2 flasks (Nunc) at 37°C in an atmosphere of 5% CO_2. Rapidly dividing cells were subcultured by 1:10 dilutions into fresh media, and slower dividing cells by 1:4 dilutions. Prior to subculturing of suspension cells, the cells were pelleted and washed in phosphate buffered saline (PBS). To subculture or harvest adherent cells, the culture media was removed and the cells washed in sterile phosphate buffered saline (PBS) before the addition of 1.0 ml of 0.05% trypsin / 0.53 mM EDTA solution.for 25 cm^2 flasks or 3.0 ml for 75 cm^2 flasks. The cells were left at room temperature until they began to detach from the flask. and 5 ml of culture media was added and the flask washed to remove any remaining cells.

2.2.21 Transfection of adherent cells with expression vectors

DNA transfections were performed with either vector DNA or recombinant plasmids using the calcium phosphate precipitation method (Wigler *et al.,* 1977). Cells were seeded at a density of 1 x 10^6 per 25 cm^2 flask, and allowed to settle overnight prior to transfection. Approximately 2 hr before transfection, the medium was replaced with 12 ml of fresh medium. In a 10 ml sterile tube, 1.25 ml of 2 x HeBS (pH 7.1: The correct pH is critical for the transfection to be successful) was mixed with 10 μg of plasmid DNA and 20 μg of inert carrier DNA of either herring sperm DNA or genomic DNA from the cell line to be transfected. The solution was aerated by pipetting air through a 1 ml plastic pipette with an autopipettor and 1.25 ml of $CaCl_2$ was added dropwise and the solution was allow to stand for 30-40 min at room temperature for the

precipitate to form. The DNA mixture was then added dropwise while gently rocking the flask. The flasks were then incubated for 24 hr at 37°C, the precipitate removed by replacing the culture medium. The cells were allowed to recover for a further 24 hr at 37°C before the medium was replaced with fresh medium containing 400-800 μg/ml of G418 (Geneticin) depending on the cell line. Cells were maintained in the presence of G418 until individual colonies appeared usually two weeks. G418 resistant colonies were counted, individual clones picked manually with a 200 μl Gilson tip and propagated in the continual presence of G418 for subsequent analysis.

2.2.21 Sequencing

Automatic sequencing of PCR products and cloned DNA fragments was performed at Flinders Medical Centre, Bedford Park, South Australia on an ABI model 373A automatic sequencer (Applied Biosystems). The sequence was read by the software supplied with the sequencer (version 2.0.1S). The sequence was also read manually and the bases that were called "N" by the program were often resolved manually.

Manual sequencing of PCR products and recombinant plasmids was also carried out using the Stratagenes Cyclist Exo⁻*pfu* DNA sequencing kit with α–32P-dATP according to the supplied protocol. PCR products were sequenced without further purification whereas recombinant plasmids were purified using the Qiagen midi plasmid purification kit (Promega).

2.2.22 Sequencing gels

Sequencing reaction products were resolved on 6% polyacrylamide gels containing 7 M urea in 1 x TBE buffer. A 40% stock solution of acrylamide (19:1; acrylamide : *bis*-acrylamide), was diluted in 1 x TBE buffer, to which 420 µl of 10% ammonium persulphate and 70 µl of TEMED (Sigma) were added. Electrophoresis was carried out at 55 watts for 2-3 hr, and the gel was soaked in 5% acetic acid, 15% methanol for 15 min. Gels were then transferred to Whatman 3MM paper and vacuum dried for 45-90 min at 80°C. The dried gels were exposed overnight at room temperature to Hyperfilm-MP (Amersham) and developed using an automatic developer. Sequence data was read manually and analysed using DNA analysis software (see section 2.2 24)

2.2.23 Single strand conformational polymorphism analysis (SSCP)

For the analysis of p53 mutations, 50 ng of genomic DNA was added in PCR mix containing 1 x PCR buffer, 2.5 mM $MgCl_2$, 0.8 mM of each of the dNTPs ,1.0 units of Taq DNA polymerase (Promega), 1.5 µCi [^{32}P] dCTP (3000 Ci/mmol), and 100 ng of specific primers around exons 5, 6, 7 and 8 of the p53 gene (section 2.1.11). The final volume of the PCR reaction was 50 µl. Samples were initially denatured at 94°C for 5min and were then subjected to 35 cycles of denaturation at 94°C for 1 min, followed by primer annealing at 60°C for 1 min and extension at 72°C for 1 min. Prior to electrophoresis on non-denaturing gels, samples were run on agarose gels to confirm the presence of a single PCR product. The PCR reaction mixture (5.0 µl) was diluted with 5.0 µl of formamide loading buffer containing 95% deionised formamide, 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples were heated at 95°C for 5 min, chilled on ice and 2.0 µl loaded onto sequencing size non

denaturing polyacrylamide gels (6%) in 1 x TBE buffer containing 5-10% glycerol. SSCP gels were prepared essentially as previously described in section 2.2.22 with the omission of urea but the addition of glycerol. Gels were prerun at room temperature for 30 min at 30 watts prior to loading the samples. After loading, gels were run at 45 watts for 4-5 hrs at room temperature, transferred to 3MM Whatman paper, dried and autoradiographed as previously described (2.2.22).

2.2.24 Computer programmes

Compilation and analysis of DNA sequence data was performed using the following Macintosh molecular biology software: DNA Strider version 1.0 and Gene Jockey. Data base searches were done by screening the most recent updates of the following databases: GeneBank, EMBL and SWISS-PROT. The collection of programs from the ANGIS group were used for database searches, in particular FASTA.

Chromatographs were produced from printed output using a Biorad scanner. Word processing and typesetting were performed on an IBM compatible personal computer using word version 6.0 (Microsoft Corporation). Diagrams and graphs were produced using MacDraw II version 1.1 and Graph pad Prism version 2.0 software.

CHAPTER 3

CHROMOSOMAL LOCALISATION OF THE MOUSE AND HUMAN GAS1 GENES

3.1 INTRODUCTION

A variety of strategies are now available for the localisation of cloned DNA sequences within the human genome. Hybridisation of a gene probe to a panel of DNA isolated from somatic cell hybrids containing different chromosomes is one approach in which genes can be included or excluded from the whole chromosome. For regional localisation within a particular segment of the chromosome, cloned sequences can be hybridised to DNA from cell lines carrying deletions or rearrangements of that chromosome and thereby mapped within or outside the deleted or rearranged region. In this way, the smallest region of overlap which gives rise to a positive hybridisation signal can then be determined. However, these strategies require extensive cytogenetic characterisation of the cell lines being used and a precise definition of the structural abnormalities present. *In situ* hybridisation provides a direct approach to regional mapping. Cloned DNA sequences can be directly hybridised to fixed chromosome preparations on glass slides. After autoradiography, a significant excess of silver grains can be scored within one region of a particular chromosome. This procedure will also indicate whether any other sites of hybridisation are present with in the genome suggesting the presence of multiple genes or pseudogenes.

Mapping of cloned genes and random DNA sequences within the human genome by a variety of strategies had been useful for a number of different purposes over the years. Mapping of genes to specific chromosomes has been useful to compile a genetic map of chromosomes and to associate the mapped gene to other genes. More importantly, mapping has been useful for the analysis of the association of the gene with rearrangement of the chromosomal material at its locus. In some cases, this situation is reversed in that the identification of rearrangements may point to the existence of the

gene. Retinoblastoma is the prototype of the tumour suppressor genes and provides the best example of a gene identified in this way. (reviewed in Goodrich and Lee, 1993). Molecular analysis of the cloned retinoblastoma gene, has identified numerous mutations or deletions of the gene in human tumours. However, before such an analysis was possible, the retinoblastoma locus was mapped to a specific chromosome. A number of retinoblastoma patients, with a cytogenetically visible deletion in the region of band q14 of chromosome 13 were identified and it was inferred that the retinoblastoma gene lay in this region. (Balaban *et al.,* 1981; Orye *et al.,* 1974). Narrowing the region containing the RB1 gene made it feasible to clone the gene and study the mutations at the molecular level. The Wilms tumour gene, WT1 (Gessler *et al.,* 1990), p53 (Nigro *et al.,* 1989), DCC (Deleted in Colon Cancer, Fearon and Vogelstein., 1990) and the NF1 gene (Collins *et al.,* 1989), involved in neurofibromatosis type 1, are among other examples of tumour suppressor genes that have been identified and cloned on the basis of their association with recurrent and specific chromosomal deletions.

The chromosomal site of *GAS1* may have important implications for its function, as the location of putative tumour suppressor genes is often the site of mutations and deletions in human cancers. On this basis, the aim of this study was to identify the chromosomal site of the Gas-1 gene in both the mouse and human genomes.

Initially, only a murine Gas-1 cDNA clone was available, hence the first part of this chapter reports the mapping by in-situ hybridisation of *Gas-1* to mouse chromosome 13 bands B1.3-C2 (Webb *et al.,* 1992). Subsequently, a human homologue of the mouse Gas-1 gene was cloned by screening a human cDNA library with the mouse *Gas-1* cDNA probe (Del Sal *et al.,* 1994). The human GAS1 gene has similar growth arrest-specific patterns of expression as the mouse homologue and comparative

analysis of mouse and human cDNA sequences demonstrated a strong sequence homology. The coding region of the human GAS1 gene results in a protein with 82% amino acid identity with the mouse Gas-1, maintaining the typical domains of an integral membrane protein (Del Sal *et al.*, 1994). Since cell cycle control elements are highly conserved between species, it is highly likely that the products of the human GAS1 and mouse Gas-1 genes serve the same function.

The second part of this chapter reports on the localisation of the human GAS1 gene to chromosome arm 9q at bands q21.3-q22 (Evdokiou *et al.*, 1993). The implications of the importance of this localisation are also discussed.

3.2 MATERIALS AND METHODS

3.2.1 DNA Probes for *in situ* hybridisation

A 1.3 kb fragment of the mouse Gas-1 cDNA (PA-1), cloned at the *Eco*RI sites in the plasmid Bluescript was provided by Dr. C Schneider, Centro Internationale per L'Ingegneria Genetica e Biotechnologia, Trieste. The cDNA fragment was labelled by nick translation with tritiated bases to a specific activity of 5.9×10^7 cpm/μg.

A Bluescript plasmid containing part of the human GAS1 cDNA (clone 181/HLEE, was cut with *Eco*RI and *Xho*I. This yielded inserts of 400 and 800 bp fragments which were tritium-labelled to specific activities of 4.8×10^6 and 6.7×10^6 cpm/μg respectively.

3.2.2 Labelling of cDNA probes with ^{3}H-dNTP's by nick translation

Labelling of both the murine and human GAS1 cDNA probes was carried out using a nick translation kit (code N5500) and tritiated dATP, dCTP and dTTP (Amersham). Each of the labelled nucleotide triphosphate, (25 μCi), ([1',2',2,8-^{3}H]dATP, [1',2',5-^{3}H]dCTP, [*methyl*-1',2'-^{3}H]dTTP were evaporated in a vacuum desiccator and the following were added to the dried tube: 300 ng of the cDNA probe, H_2O to 20.7 μl, 1.8 μl cold dGTP and 2.5 μl enzyme mix (DNase/DNA pol I) in a total volume of 25 μl. The reaction mixture was incubated at 15°C for 2 hr then 15 units of DNA polymerase I was added and the reaction allowed to continue for a further 30 min at 15°C.

A Sephadex G-50 column was prepared by packing the resin in a siliconised glass pipette plugged with sterile non-absorbent cotton wool. Prior to the addition of the reaction mixture, the column was washed three times with $T_{10}E_1$ (10 mM Tris, 1 mM EDTA pH 7.6). The reaction mixture was then layered onto the column, and fresh $T_{10}E_1$ was added. Fractions were collected in sterile 1.5 ml centrifuge tubes. The volumes of the fractions were as follows: tube 1 (300 μl), tubes 2-12 (100 μl), tubes 13-17 (300 μl) and tube 18 (600 μl). Two μl of each fraction was mixed with 500 μl of H_2O and 5 ml of scintillant containing Triton and counted in a Beckman LS 2800 scintillation counter. The fractions containing the labelled probe were determined, pooled and divided into 3 aliquots each containing 100 ng of labelled probe. The labelled probe was ethanol-precipitated with 1/10 vol 3 M Na acetate pH 5.2 and 2.75 vol of ice cold ethanol and stored at -20 °C.

The following formula was used to determine the fraction of tritium incorporated and the specific activity of the probes.

$$\text{Specific activity (cpm/}\mu\text{g)} = \frac{\text{Total microcurie x fraction incorporated x } 2.22 \times 10^6}{\text{mass of probe in } \mu\text{g}}$$

using:

i) $$\text{Fraction Incorporated} = \frac{\text{Total counts in probe}}{\text{Total counts in probe plus unincorporated bases}}$$

ii) 1 microcurie = 2.22×10^6 dpm)

3.2.3 Chromosome preparation from mouse splenic lymphocytes

Spleens from Balb/c mice were dissected out under aseptic conditions, minced with a scalpel blade and cells passed through a sieve into RPMI 1640 culture medium, supplemented with 10% foetal calf serum, 0.85 gm/L of sodium bicarbonate, 1 μg/ml of Gentamycin, 0.02 M HEPES and 3.0 μg/ml of Concanavalin A (Sigma). Cells were incubated for 2-3 days in 5% CO_2 at 37 °C. To label the chromosomes, 300-500 μg/ml of 5-bromodeoxyuredine (5-Brdu, Sigma) was added to the cultures and incubated overnight. Cells were then washed twice in PBS buffer and once in medium, resuspended in 10 ml of culture medium containing 10^{-5} M Thymidine, transferred to culture flasks and incubated for a further 4.5 hr. In order to destroy the metaphase spindle, colchicine (Fluka, 10 μg per 1.0 ml of culture) was added and the cells were incubated for a further 15-30 min at 37°C. Cells were resuspended in a hypotonic solution of 0.075 M KCl prewarmed to 37°C and left for 10 min at 37°C before they were fixed with 2.0 ml of 3 parts methanol : 1 part glacial acetic acid. Cells were spread onto clean glass microscope slides, dried and stored desiccated at -20°C.

3.2.4 Chromosome preparations from human peripheral blood lymphocyte cultures

RPMI 1640 with sodium bicarbonate was supplemented with 20% foetal calf serum, 20 mM glutamine, 8 mg/ml Gentamycin, 2% v/v of phytohaemaglutin (PHA, Gibco, 10ml), and 0.1% v/v of 1000 units/ml of sodium heparin. Freshly taken peripheral blood was mixed with RPMI 1640 culture medium in a ratio of 0.25 parts : 4.5 parts respectively. Cells were incubated for 72 hr at 37 ^{o}C in 5% CO_2. On the fourth day, 5-bromodeoxyuridine at a final concentration of 200 μg/ml was added to the cultures and incubated for a further 17 hr. Cells were harvested by centrifugation, washed twice in PBS buffer at 37^{o}C followed by a single wash in RPMI 1640 medium before resuspending the cells in fresh medium containing 10^{-5} M Thymidine. Cultures were incubated for a further 6-7 hr, colchicine added to a final concentration of 0.5 μg/ml and incubated for a further 10 min Cells were treated with hypotonic solution of 0.075 M KCl at 37^{o}C for 10 min and subsequently fixed with 2.0 ml of 3 parts methanol : 1 part glacial acetic acid. Cells were spread onto clean slides, dried and stored desiccated at -20^{o}C.

3.2.5 *In situ* hybridisation

3.2.5.1 Treatment of slides with RNase

Slides were treated with RNase A at 100 μg/ml in 2 x SSC (using stock RNase A, boiled for 10 min and cooled slowly to remove contaminating DNAase) and incubated for 1 hr at 37^{o}C. Slides were then rinsed in 4 changes of 2 x SSC, 2 min each at room temperature, followed by dehydration in 35, 70, 95, 100% ethanol.

3.2.5.2 Acetylation

To reduce charge-induced background, the slides were acetylated (Pardue *et al.*, 1985). Slides were placed in 500 ml of 0.1 M triethanolamine-HCl, pH 8.0 and 2.5 ml of acetic anhydride was added drop by drop while stirring continuously. Slides were allowed to stand without stirring for 10 min and then rinsed in 4 changes of 2 x SSC followed by dehydration in an ethanol series (3.2.5.1) and air dried.

3.2.5.3 Denaturation and hybridisation

Chromosomal DNA was denatured by immersing slides preheated to 70°C in 70% deionised formamide / 2 x SSC pH 7.0, at 70°C for 2 min with frequent agitation. Slides were placed in ice-cold 70% ethanol for 1 min, rinsed through an ethanol series of approximately 1 min each in 80, 95, and 100% ethanol and air dried.

The labelled cDNA probes prepared in section 3.2.2 were centrifuged for 15 min to pellet the DNA, the pellet dried under vacuum, and the labelled DNA resuspended in H_2O to give a final concentration of 1 ng/µl.

The probe mixture was prepared in the following proportions: 5 parts of deionised formamide with 20% dextran sulphate (freshly prepared), 2 parts of 10 x SSCP (10 x SSCP: 1.2 M NaCl, 0.15 Na citrate, 0.2 M NaPO4, pH 6.0), 2 parts of labelled probe to give a final concentration of 200 ng/ml, and 1 part of salmon sperm DNA. The probe mix was denatured by heating at 70°C for 10 min and then rapidly cooled in an ice/water mixture.

Each slide was covered with 50 µl of the hybridisation mixture, covered with a 24 x 50 mm coverslip which was wiped clean with 70% ethanol, sealed with rubber cement and incubated in a humid environment at 42°C for a maximum of 16 hr. The

coverslip was then removed and slides were briefly rinsed in 50% deionised formamide in 2 x SSC to remove the hybridisation mixture immediately prior to stringency washes. Slides were initially rinsed with 3 changes of 50% deionised formamide / 2 x SSC pH 7.0 for 3 min each at 44°C followed by 5 changes in 2 x SSC at the same temperature. All rinses were carried out with spasmotic agitation. The slides were finally dried through an ethanol series of, 35, 70, 95 and 100% ethanol as previously described.

3.2.5.4 Autoradiography and staining

Ilford L4 emulsion was prepared under a safe light by melting bulk emulsion threats in a 1:1 ratio with 1% glycerol/H_2O prewarmed to 46°C. Slides were dipped back to back into the diluted emulsion for 15 seconds, removed and the ends blotted to remove excess emulsion. The slides were placed on a cooled rack to set the emulsion and then placed in a light-tight drying box to dry in an air flow for at least 30 min while the emulsion hardened. They slides were exposed in small light-tight boxes with drying agent and stored at 4°C to protect the latent images from fading.

TheSlides were allowed to warm to room temperature for about 1 hr before they were dipped for 5 min without agitation in Kodak D19 developer diluted 1:1 with H_20 at 20°C. The slides were removed and placed in 4% developer for a few seconds to rinse out the bulk of the developer and then fixed for 5 min in film-strength Ilford Hypam fixer with frequent agitation. Finally, the slides were rinsed in 5 changes of H_2O of 2 min each. The slides were not allowed to dry before staining.

For GBG banding, the slides were sensitised for 30 min at room temperature in Hoescht 33258 diluted to 10 μg/ml in 2 x SSC and then rinsed twice in 2 x SSC. The slides were then placed on a flat surface covered with a thin film of 2 x SSC and exposed for 1 hr at a distance of 10 cm to long wavelength UV light (Blacklite-blue, 15

watt, 350 nm). The slides were stained for 20 min in Giemsa stain diluted to 8-15% in phosphate buffer pH 6.8 and glass coverslips were fixed on top of the slides with depex before being analysed.

Analysis and scoring of the slides was carried out entirely by Dr. Webb who had no previous knowledge of the location of GAS1 in the mouse or human genomes.

3.2.6 Somatic Cell Hybrid DNA Panel

DNA from human/rodent somatic cell hybrid cultures with a reduced number of human chromosomes (Table 3.1) was used to verify the chromosomal location of *GAS1* to human chromosome 9. The hybrids, designated GM/NA, were obtained from the Human Genetic Mutant Repository (Camden, NJ) and kindly provided by Dr David Callen of the Women's and Children's Hospital, Adelaide, South Australia.

3.2.7 Polymerase Chain Reaction

The primers for *GAS1* were designed to amplify a 138 bp sequence from the cDNA region of the human GAS1 gene and correspond to residues 182-206, and 296-320, respectively of the published cDNA sequence (Del Sal *et al.,* 1994). The oligonucleotides were both 24 bases long with the following sequences:

5HP1

5'-CGGGACCAGATCTCGACAGCTGTT-3'

3HP2

5'-TCCTGCCCACTTCTTGCATGAGTG-3'

ABO primers were also synthesised to amplify a 181 bp sequence flanking a portion of the *ABO* gene (O'Keefe and Dobrovic, 1994).

ABO 1

5'-CGCATGGAGATGATCAGTGACTTC-3'

ABO 2

5'-GCTCGTAGGTGAAGGCCTCCC-3'

PCR (Saiki *et al.,* 1985) was performed with 50 ng of hybrid DNA and 12.5 pmol of each oligonucleotide primer, 0.2mM each of dATP, dGTP, dCTP, and dTTP and 0.5U *Taq* DNA polymerase (Boehringer Mannheim) in the presence of the buffer supplied by the manufacturer, in a total volume of 50 µl. Amplification was obtained by an initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of heating to 94^{o}C for 1 min, 67^{o}C for 1 min and 72^{o}C for 1 min in a thermal cycler (A.R.N. Electronics, Belair, South Australia). A 15 µl sample of the product of each PCR reaction was analysed by electrophoresis through a 2% agarose gel containing ethidium bromide.

3.3 RESULTS

3.3.1 Localisation of *Gas-1* to mouse chromosome 13

Since the specific activity of the murine Gas-1 probe described in section 3.2.1 was low and following an unsuccessful exposure for 7 days, the slides were exposed to Ilford L-4 emulsion for 3 months. Individual grains were scored onto standard idiograms of G-banded mouse chromosomes (Nesbitt and Francke., 1973).

In approximately 100 cells, 50% of the grains were located over Chromosome 13 (Fig 3.1) and the background grains on the other chromosomes showed no significant subsidiary peaks (Fig 3.2). Of the 101 grains over Chromosome 13, 86 were in the four tall peaks over bands B and C. The two tallest peaks, containing 62 grains, were located over the sub-bands 13B1.3 and 13C1-C2. This region constitutes the most precise localisation of *Gas-1*.

An independent study by Colombo *et al.* (1992), has shown linkage of *Gas-1* to the following markers on Chromosome 13: *Xmv-13* (xenotropic murine leukemia virus 13), no recombination; DNA segment *D13Pas2*, approximately 8% recombinants and *Dhfr* (dihydrofolate reductase), approximately 17% recombinants. These data hav been incorporated into the map of mouse Chromosome 13 of Justice and Stephenson (1991), which shows *Gas-1* at 45 map units and physically aligned with band C1, which is the central band of the three to which the Gas-1 gene has been localised above. From all these sources of data, it seems likely that *Gas-1* is in band 13C1 in the mouse. This assignment contradicts that of Colombo *et al.* (1989) who assigned *Gas-1* to Chromosome 12 on the basis of the pattern of inheritance of an *Ava*I restriction fragment length polymorphism. However, *Ava*I is a methylation-sensitive enzyme and the contradiction between the localisation to Chromosome 13 and that of Colombo *et al.* (1989) may be related to different banding patterns associated with differentially-methylated DNA from the inbred strains used, resulting in an error of interpretation of the data.

Figure 3.1 Metaphase chromosome spread from a single G-banded murine cell, showing localisation of grains to chromosome 13 at band B2 (arrowed).

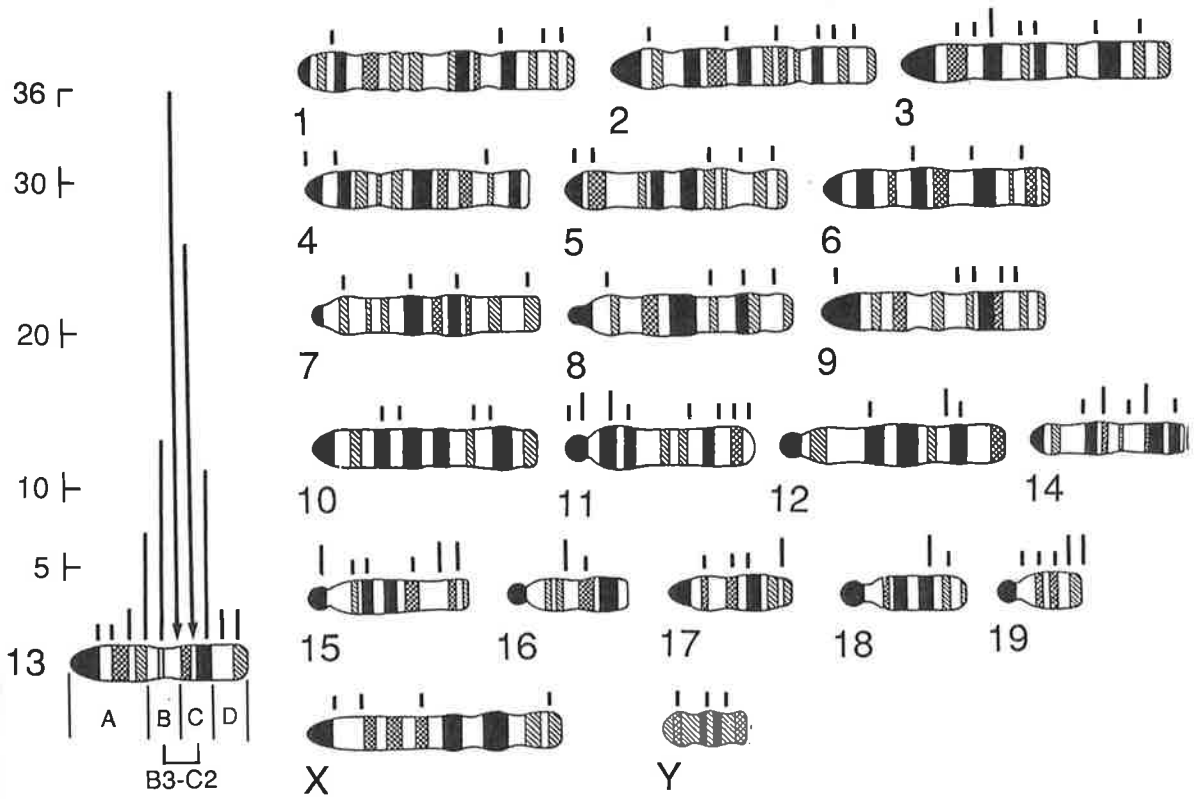


Figure 3.2 Localisation of grains over the chromosomes in approximately 100 metaphase spreads following hybridisation with the tritiated *Gas-1* cDNA probe. Band B2 of chromosome 13 is shown as a double line (Evans *et al.*, 1989). The three bands under the two tallest peaks of grains (arrowed) are marked as the most precise localisation of *Gas-1* (from Webb *et al.*, 1992).

3.3.2 The human GAS1 gene does not map to human chromosome 5

The localisation of Gas-1 to murine Chromosome 13 has considerable significance when the synteny and order of homologous human genes are considered. Mouse Chromosome 13 contains 8 loci within a 30-cM segment that are conserved in human chromosome arm 5q, mainly in 5q11-q14 (Justice and Stephenson, 1991). *Gas-1* is close to the IL-9 gene in the mouse, one of the loci in the segment conserved on human Chromosome 5. On this basis, it was predicted that the human homologue of Gas-1 should map to Chromosome arm 5q. This region of the genome is highly significant as a site of a number of potential tumour suppressor and growth factor genes (Wasmuth *et al.,* 1988) and is altered or deleted in a number of human tumours. Chromosome arm 5q is the site of the familial adenomatous polyposis (FAP) gene, which predisposes to colon cancer (Bodmer *et al.,* 1987). This region is mutated or deleted in colon cancer (Solomon *et al.,* 1987), myelodisplastic syndromes (LeBeau *et al.,* 1986) and acute myeloid leukemia (AML, LeBeau et al,. 1987). Therefore, if the Gas-1 gene is located on chromosome arm 5q, loss of Gas-1 by mutation or deletion may be important in the development of colon cancer and AML.

DNA was isolated from a human/hamster hybrid cell line containing a single human chromosome 5 designated GMNA-10114 * (Table 3.1) and analysed by Southern blotting for the presence of GAS1 sequences specific to the human genome. DNA was digested with the restriction enzymes *Eco*RI or *Hin*dIII, electrophoresed through a 1% agarose gel, and the DNA transferred to Genescreen Plus nylon membranes (2.2.8). The resulting Southern blot was hybridised initially with the mouse *Gas-1* probe, PA-1 (Fig 3.3A) and, subsequently with a human GAS1 probe, GAS1-181[HLEE] (Fig 3.3B). Both probes failed to detect human-specific GAS1 sequences in

Table 3.1. **Human/Rodent Somatic Cell Hybrid DNA Panel**

Hybrid DNA Repository Number	**Human chromosome Content**
GMNA 07299	1,X
GMNA 108268	2
GMNA 10253	3
GMNA 10115	4
GMNA 10114 *	5
GMNA 10629	6
GMNA 10791	7
GMNA 101568	8
GMNA 10611	9
GMNA 109268	10
GMNA 10927A	11
GMNA 10868	12
GMNA 10898	13
GMNA 10479	14
GMNA 11418	15
GMNA 10567	16
GMNA 10498	17
GMNA 11010	18
GMNA 10449	19
GMNA 10478	20, 4
GMNA 10323	21
GMNA 10888	22
GMNA 063188	X
GMNA 06317	Y
GMNA 1M291	Human chromosomes only
GMNA 05862	Mouse chromosomes only
GMNA 10658	Hamster chromosomes only

* Hybrid DNA used in Southen analysis to exclude localisation of the human GAS1 gene to chromosome 5 (3.3.2 and Fig 3.3).

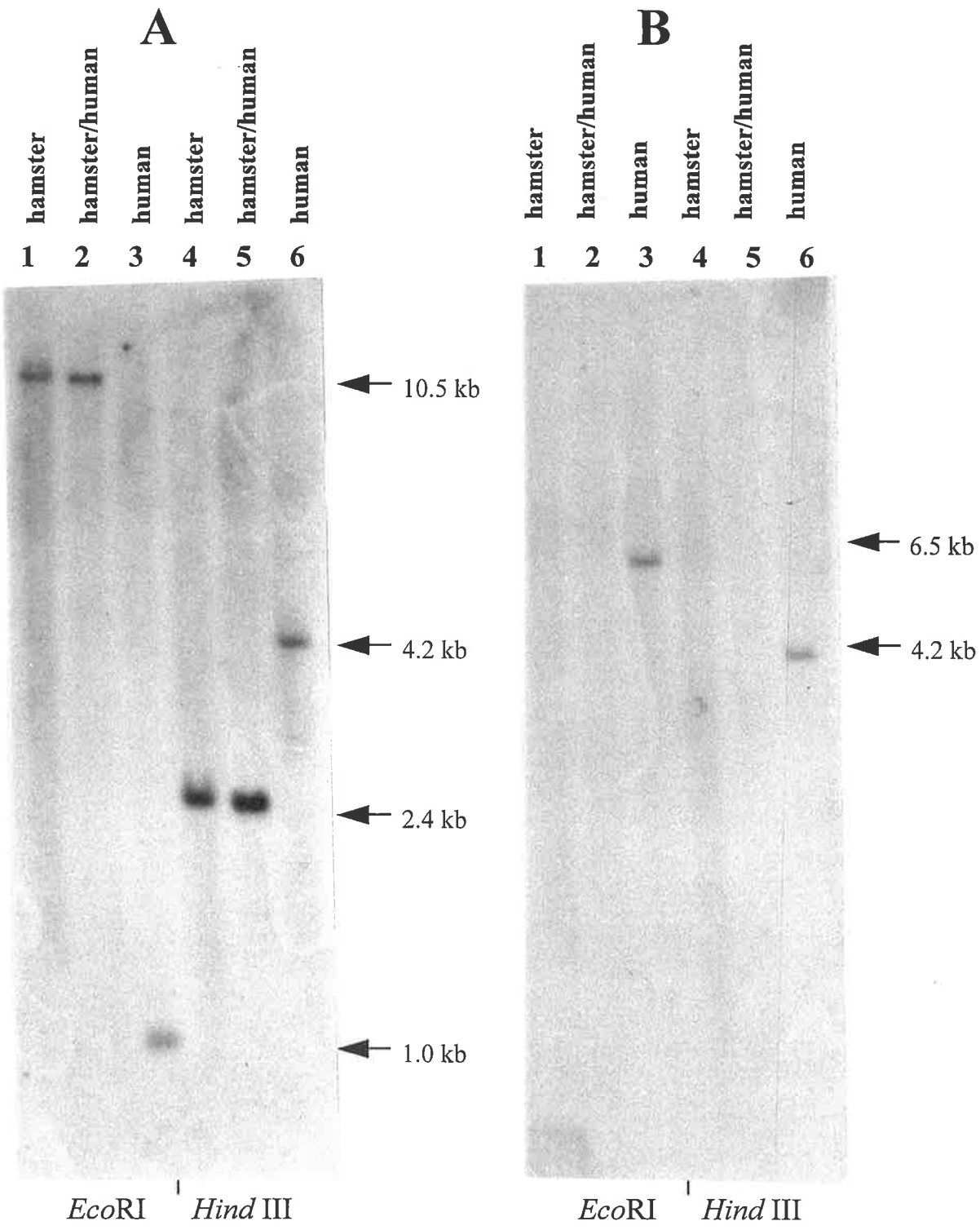


Figure 3.3 Absence of human *GAS1* sequences in a hamster/human hybrid cell line containing a single human chromosome 5. *(A)* Southern analysis using a mouse Gas-1 cDNA probe (PA-1). *(B)* The same filter hybridised with a human GAS1 cDNA probe 181[HLEE]. Hamster genomic DNA, (lanes 1 and 4), hamster/human hybrid cell line GMNA 10114, (lanes 2 and 5) and human genomic DNA (lanes 3 and 6). DNA was digested with either *Eco*RI (lanes 1-3) or *Hin*dIII (lanes 4-6).

the hamster/human hybrid. (Note the absence of the 1.0 kb *Eco*RI or the 4.2 kb *Hin*dIII fragments from the human/hamster hybrid tracks (Fig 3.3A). Similarly,when the same blot is hybridised with the mouse *Gas-1* probe, the 6.5 kb *Eco*RI or the 4.2 kb *Hin*dIII human-specific bands were not detected in the hamster/human hybrid (Fig 3.3B). These results clearly demonstrate that the human GAS1 gene does not map to human chromosome 5.

3.3.3 Localisation of the human GAS1 gene to human chromosome 9q21.3-q22

The two human probes labelled with tritium as described in sections 3.2.1 and 3.2.2, were hybridised *in situ* to the chromosomes of two normal human subjects, male and female, using standard techniques described above. After trial exposures of 21 and 42 days, the remaining slides were exposed for 102 days. This unusually long exposure was necessary due to the low activities of the probes. The data presented here is from the slides exposed for 102 days. These slides were acetylated (3.2.5.2) to control charge-induced background. Grains were scored onto a 550 band idiogram of the human chromosomes (ISCN, 1985).

Both probes were localised by *in situ* hybridisation to the long arm of chromosome 9 (9q), (Fig 3.4). Retrospective analysis of the slides exposed to emulsion for 42 days also showed localisation to 9q.

The 800bp fragment probe produced 33% of grains over 9q out of a total of 376 grains on all chromosomes. The distribution of silver grains in both the male and female subjects were very similar and are shown pooled (Fig. 3.5). Of the grains over 9q, 77 (62%) were in the three tall peaks over bands 9q21.3-q22, and this chromosomal segment is regarded as the probable location of *GAS1*. A very prominent peak of 40 grains,

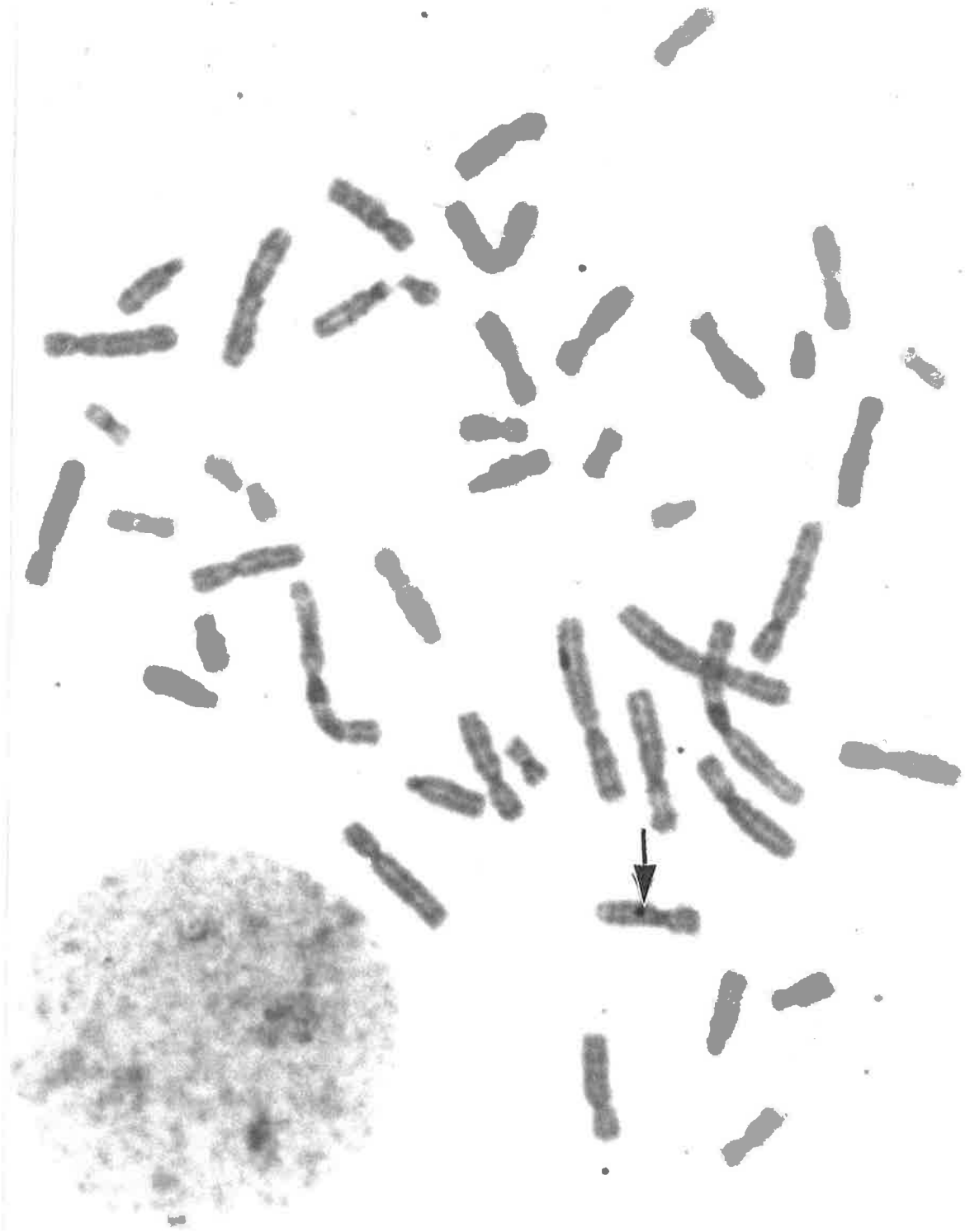


Figure 3.4 Metaphase chromosome spread from a single human cell, showing localisation of a grain to chromosome 9 at band q21.3-q22 (arrowed).

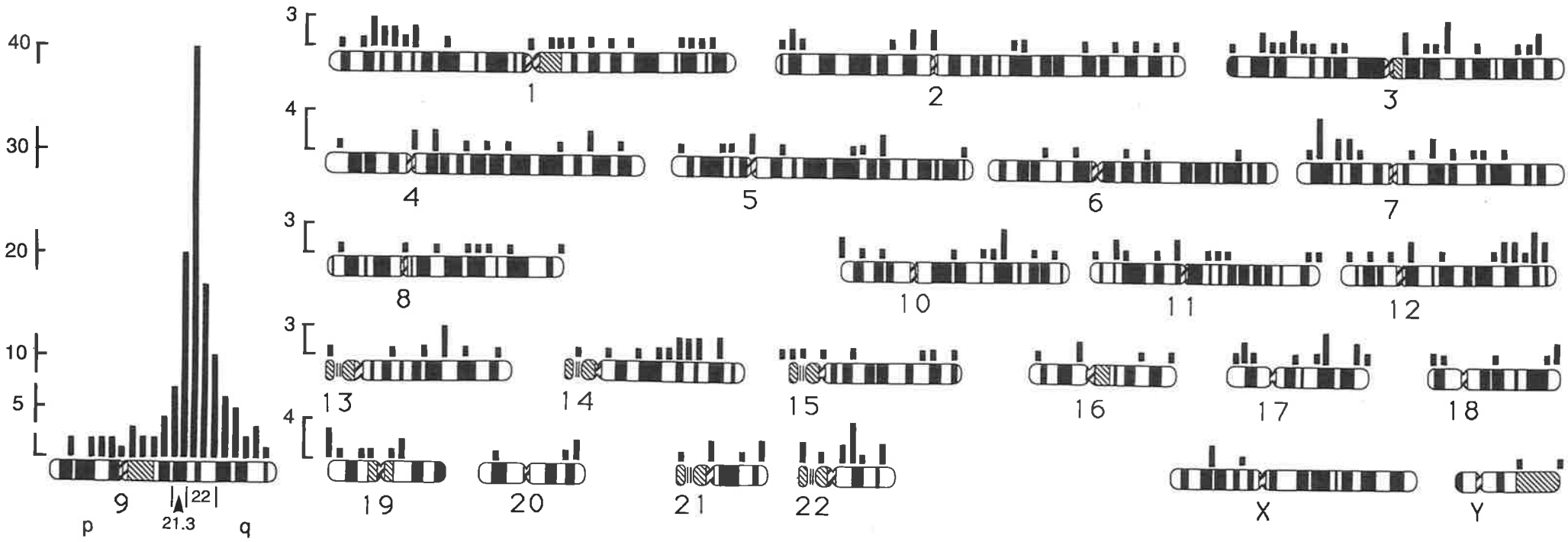


Figure 3.5 Distribution of grains scored over all human chromosomes, probed with the 800 bp cDNA fragment of GAS1. Two normal subjects: male and female, were used. The three tallest peaks are over the bands 9q21.3-q22; the probable location of *GAS1*. The very tall peak of 40 grains is located over sub-band 9q22.1 making it the most likely point location of *GAS1* (from Evdokiou *et al.*, 1993).

Figure 3.6 Distribution of grains scored over all human chromosomes, probed with the 400 bp cDNA, showing 16% of grains over 9q21.3-q22 out of a total of 441 grains

located over 9q22.1, makes this sub-band the most likely point location of *GAS1*. The 400bp fragment was a weak probe, producing only 16% of grains over 9q out of a total of 441 grains scored to all chromosomes. However this fragment was also localised to 9q22.1-q23, confirming the previous result (Fig 3.6). Background grains were not notably clustered although there was a small peak over chromosome 22, which was more prominent with the 400bp probe (Fig 3.6). However, there was no peak sufficiently substantial to indicate the presence of repeated or related genes or pseudogenes (Webb *et al.,* 1990a; Board *et al.,* 1989; Webb *et al.,* 1990b).

3.3.4 Confirmation of the location of *GAS1* to human chromosome 9

The assignment of *GAS1* to human chromosome 9 by *in situ* hybridisation was confirmed by the results of the PCR analysis (Fig. 3.7). The specificity of the PCR conditions was initially tested on genomic DNA from human cells and from the mouse and hamster fusion partners used to form the hybrid panel. The conditions were optimised so that only the human GAS1 gene is amplified (Fig. 3.7, lanes marked Human, Mouse, Hamster). Using these conditions, the oligomers specific for human *GAS1* were then utilised in PCR reactions on the complete DNA panel from the human/rodent somatic cell hybrids.

The 138 bp human GAS1 sequence was amplified in the hybrid containing a single human chromosome 9 and not in hybrids containing other human chromosomes. (Fig. 3.7, lane 9) The presence of chromosome 9 in the positive hybrid was confirmed by PCR using oligomers specific for the ABO blood group gene which produces the expected 181bp PCR product (Fig. 3.7, lane 9A). The ABO blood group gene has previously been mapped to chromosome 9 (Yamamoto *et al.,* 1990) and thus acts as a

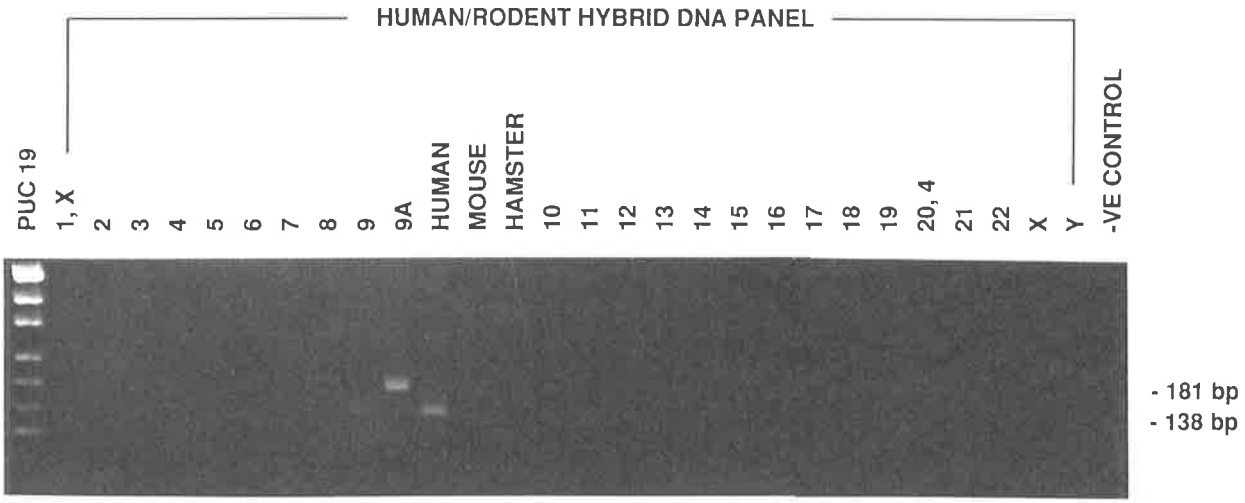


Figure 3.7 Products of PCR reactions on DNA from somatic cell hybrids. The agarose gels were stained with ethidium bromide to visualise the PCR products primed by oligonucleotides specific for human *GAS1* and *ABO* genes. The substrates for the PCR reaction were genomic DNAs from: Human lymphocytes; Mouse 3T6 fibroblast cells: Hamster RJK88 (lanes marked) and rodent/human hybrid cells (numbers marking the lanes correspond to the human chromosomes present). All hybrid DNAs were primed with the GAS1 oligomers except for the DNA in lane 9A, which was primed with oligomers specific to the *ABO* gene. The molecular weight standard (first lane from left) is PUC19 DNA digested with *Hpa*II. The negative control has no DNA present.

control for the presence of this chromosome in this hybrid. The only other lane which shows amplification of the *GAS1* specific PCR product, is the positive control which contains the entire human genome (Fig. 3.7, lane marked Human).

3.3.5 *GAS1* maps to a region frequently deleted in myeloid malignancies.

A total of 106 cases of myeloid malignancy with deletions of chromosome 9 have been reviewed (Mitelman, 1991). Of these, 89 were acute non-lymphocytic leukemia's (ANLL), 8 refractory anaemias, 2 chronic myeloid leukemias, 2 chronic myeloproliferative disorders, 3 myelodysplastic syndromes and 2 paroxysmal nocturnal haemoglobinurias. The 106 cases comprise 1.4% of the total published cases of myeloid malignancy, where some cytogenetic abnormality was detected. Deletions of 9q11-9q32 are recognised as recurrent abnormalities within ANLL (Trent *et al.,* 1989). Here, the data from these deletions is presented in detail (Fig 3.8), in an attempt to define the most commonly deleted chromosome band, and hence the region critical to leukemogenesis.

In Fig. 3.8, the extent of each deletion is indicated by assignment of the proximal and distal breakpoints to specific chromosome bands (Mitelman, 1991). Part or all of the chromosome band 9q22 is recorded as deleted in 82 of the 96 q-arm deletions. Given the limits to resolution in most chromosomal preparations used for oncological cytogenetics, a minimal common deletion is within band 9q22 (Fig. 3.8). This finding matches well with the point localisation of *GAS1* to 9q22.1.

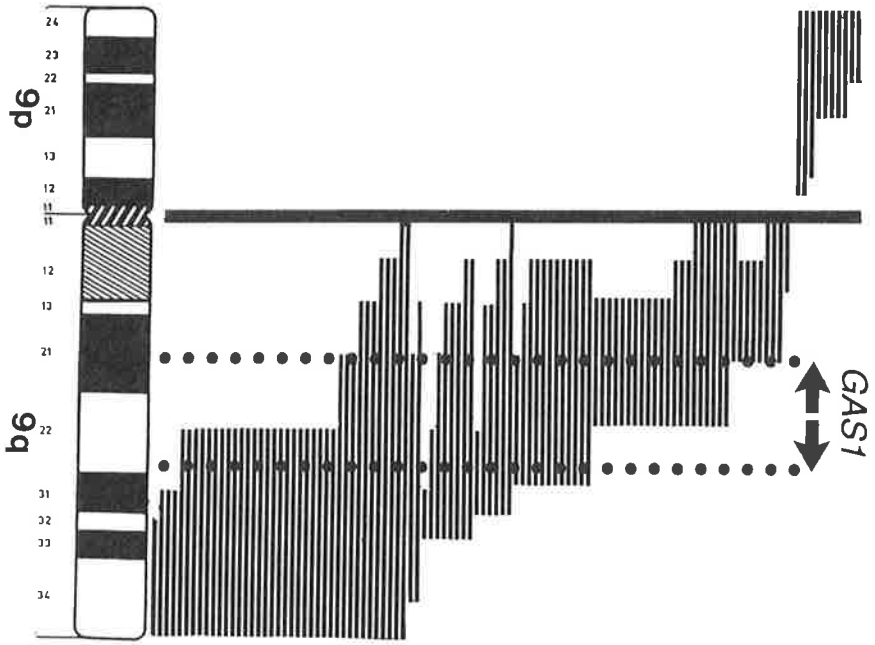


Figure 3.8 Cytogenetical deletions of chromosome 9 in 106 cases of myeloid malignancy (listed in text). Deletions in 96 cases are in the q arm (9q) Each deletion is here recorded as a horizontal line showing the extent of the deleted segment. The chromosome 9 ideogram, at the side of the figure, is from the 400 G-Band karyotype (ISCN, 1985). For each deletion, the points of chromosome breakage have been placed at the midpoints of the whole chromosome band cited: thus "del(9)(q22qter)" extends proximally as far as the centre of 9q22. All breakpoints are mapped in this fashion, which necessarily ignores sub-bands. The latter are not often resolvable in neoplastic karyotypes. Of the 96 q-arm deletions, 82 show at least partial deletion of 9q22. No other band is so frequently implicated, suggesting that a common deleted segment, and thus a locus critical to myeloid malignancy, is present within 9q22. This localisation lies within the region 9q21.3-q22, to which *GAS1* is assigned by *in situ* mapping (as indicated by the dotted lines) (from Evdokiou *et al.,* 1993).

3.5 DISCUSSION

GAS1 was the first gene to be mapped to both human chromosome 9 and mouse Chromosome 13. At the time of this localisation, no other genes were reported to be common to both murine chromosome 13 and human chromosome 9 and on this basis GAS1 appeared to be an "orphan" gene. "Orphan" genes are more normally located at the telomeres of chromosomes and not so far proximal as is the case for GAS1 on both human and murine Chromosomes (Bernard *et al.,* 1996). Although the finding of *Gas-1* on murine Chromosome 13 was unexpected at the time (Webb *et al.,* 1992), a recent map of mouse Chromosome 13 has now identified two other genes common to both mouse Chromosome 13 and human chromosome 9 (Justice and Stephenson, 1996). The first such gene is the Fanconi's anaemia group C (FACC) gene which has been mapped by linkage analysis to chromosome 9q and murine Chromosome 13 (Wevric *et al.,* 1993) and the second is the spleen tyrosine kinase (SYK) gene which by *in situ* hybridisation was localised to 9q22 in human and chromosome 13, bands B-C2 in the mouse. (Ku *et al.,* 1994).

The localisation of human *GAS1* to bands 9q21.3-q22 by *in-situ* hybridisation places the gene in a position which has been predicted to be of importance in leukemic initiation or progression (Sreekantaiah *et al.,* 1989); a claim based on the finding that this region is recurrently deleted, or otherwise rearranged in certain myeloid malignancies. Furthermore, the pattern of deletion (Fig 3.8) is typical of that observed at loci of known tumour suppressor genes. Such a pattern is characterised by diverse but overlapping deletions, with a broad range of breakpoints spread over a particular chromosome arm. Despite this spread, a zone of minimal common deletion is typically evident. For example, deletion of the retinoblasoma *(RB1)* gene, in myeloid malignancies, is

commonly of two types: del(13)(q12q14); or del(13)(q14q32) (Johnson *et al.,* 1985; Fitchett *et al.,* 1987). Here, the minimal common deletion lies within a zone of overlap in 13q14, which coincides with the point location of RB1, at 13q14.1 (Trent *et al.,* 1989). By a similar argument, two of the most common deletions illustrated for 9q (Fig 3.8) are del(9)(q13q22) and del(9)(q22qter), indicating a minimal common deletion within 9q22.

The localisation of the growth arrest-specific gene, *GAS1*, to the region 9q21.3-q22, and its ability to inhibit DNA synthesis, suggest GAS1 should be regarded as a putative tumour suppressor gene. Loss of this gene either by mutation or deletion may be important in the development of certain malignancies, including some of the myeloid leukemias.

CHAPTER 4

ANALYSIS OF GAS-1 IN NORMAL AND TRANSFORMED MOUSE CELLS

4.1 INTRODUCTION

Control of cellular proliferation involves the co-ordinate activities of a large number of genes that act in both a positive and negative manner. In cancer cells, the control of proliferation is frequently defective and many abnormalities in the genes that regulate the G1 to S transition have been described in tumours. The cell cycle-specific expression of Gas-1 and its preferential expression in quiescent cells could reflect requirements to initiate or sustain quiescence by blocking the pathway to cellular proliferation. Abnormalities that may lead to functional inactivation of the Gas-1 gene, such as deletions, translocations, and possible alterations in DNA methylation would allow a cell to escape from growth control restraints and possibly give rise to uncontrolled cellular proliferation and thus tumour formation.

Since Gas-1 could be a target for genetic alterations that might result in cancer, the mouse Gas-1 cDNA probe was used to investigate a range of mouse-derived tumour cell lines for possible mutations and/or alterations in the expression of Gas-1 mRNA. The presence of an *Ava*I restriction fragment length polymorphism (RFLP) in the Gas-1 gene reported by Colombo et al. (1989) was used to assess a variety of mouse tumour cell lines for possible loss of heterozygosity and rearrangements of the Gas-1 gene. Initial investigations suggested the presence of complex banding patterns in AvaI digested DNA of tumour cell lines when compared to normal tissues. However, during the course of these investigations, it became apparent that the different banding patterns seen in the *Ava*I-digested DNA samples were a result of differentially methylated Gas-1 alleles (*Ava*I is a methylation sensitive restriction endonuclease) and not an RFLP as previously reported. This finding lead to a broader investigation of the the possible role

of DNA methylation and in particular hypermethylation as a possible mechanism for inactivation of the Gas-1 gene in mouse tumour cell lines.

DNA methylation has been implicated as playing a role in the control mechanisms that govern chromatin structure and gene expression in mammals (Razin and Cedar, 1984) There are several lines of evidence that has led to this conclussion. (i) Tissue specific genes are transcriptionally silenced when they are methylated at critical sites in their promoters. Demethylation during development and in specific cell types is correlated with their treansition into a transcriptionally active state (Razin and Riggs, 1980; Razin and Cedar, 1991). Housekeeping genes however, are completely unmethylated in the promoter regions of all cell types (Bird *et al.,* 1985). (ii) Genes that are inactive in *vivo* can be reactivated with the potent demethylating agent, 5-azacytodine (Jones, 1985). (iii) Methylated genes introduced into fibroblasts in culture remain methylated and suppressed (Yisraeli *et al.,* 1988).

Based on the initial observations that the mouse Gas-1 gene is differentially methylated in normal and malignant cells, this study attempts to establish the possible role of DNA methylation as a possible mechanism in the control of Gas-1 expression.

4.2 MATERIALS AND METHODS

4.2.1 Demethylation of Gas-1 by treatment with azacytidine

NIH3T3 or Lewis lung carcinoma cells at 70% confluency were treated with 5-azacytidine (Sigma Co) at a final concentration of 3 μg/ml in DMEM culture medium for 24 hr. The azacytidine-treated cells were washed with phosphate buffered-saline and

then were allowed to recover in fresh medium with 10% fetal calf serum for a further 24 hr without 5-azacytidine. Cells were treated with a further dose of azacytidine (3 μg/ml) again for 24 hr. To achieve demethylation of the Gas-1 gene, this cycle of Aza-C treatment, followed by 24 hr recovery in the absence of the drug, was repeated four times. DNA and RNA were isolated from the treated cells and analysed by Southern blotting for alterations in the Gas-1 gene and by Northern blotting for changes in the expression of Gas-1 (sections 2.2.8 and 2.2.13 respectively)

4.3 RESULTS

4.3.1 Induction of Gas-1 mRNA in growth arrested cells

Using the mouse Gas-1 cDNA probe (PA-1), (2.1.10), the initial aim of these experiments was to confirm the observations that quiescent, serum-starved NIH3T3 cells express high levels of Gas-1 mRNA which is downregulated with in a few hours of activating the cells with 10% serum (Schneider *et al.*, 1988).

NIH3T3 cells were initially plated in 10% fetal calf serum and, when they were semiconfluent, the FCS concentration was reduced to 0.5%. Total RNA was extracted 24 hr later (2.2 12). Cultures were then returned to 10% FCS for various times, RNA extracted and analysed on Northern blots for expression of Gas-1 mRNA (2.2.13). Figure 4.1 shows an increase of the 3.0 kb Gas-1 mRNA during serum-starvation in medium with 0.5% FCS which is rapidly decreased to basal levels after the addition of 10% FCS. To study the accumulation of Gas-1 mRNA during density-dependent inhibition, NIH3T3 cells were plated in 10% FCS and maintained in the same medium

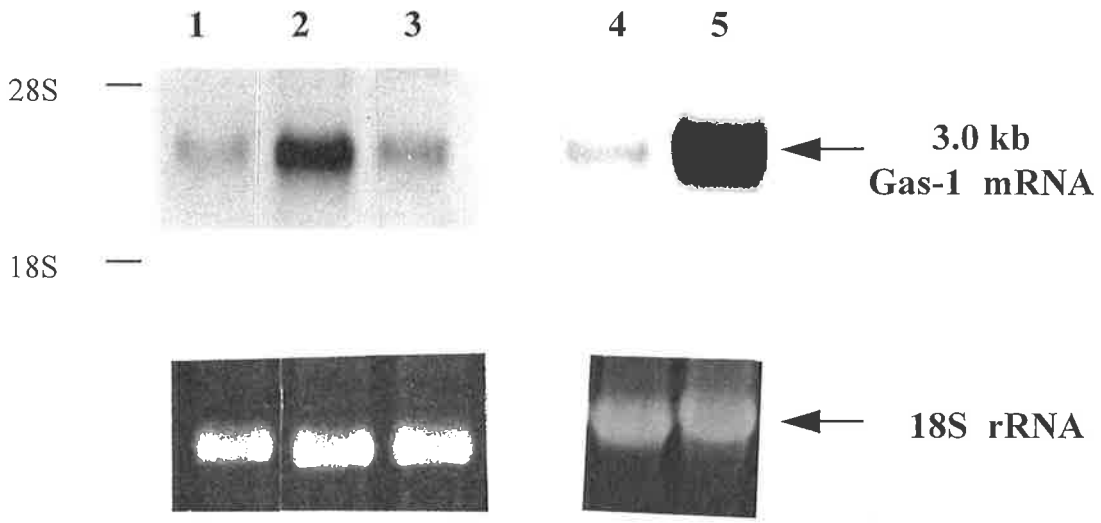


Figure 4.1 Analysis of Gas-1 mRNA expression in NIH3T3 mouse fibroblasts. RNA was extracted from subconfluent NIH3T3 cells growing asynchronously in the presence of 10% FCS (lanes 1 and 4), serum starved for 48 hrs in 0.5% FCS (lane 2), reactivated by the addition of 10% FCS for 6 hr (lane 3), or growth arrested by density dependent inhibition for 6 days in the presence of 10% FCS, (lane 5). Northern blotting using a mouse Gas-1 cDNA probe (PA-1) is shown in the upper panel and a parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel.

for 6 days, at which point cultures were confluent. An increase of Gas-1 mRNA was clearly evident in cells grown to confuency when compared to subconfluent cultures (Fig 4.1).

The preferential expression of Gas-1 mRNA in growth-arrested cells demonstrated by these experiments confirms the initial observations by Schneider *et al.* (1988) and further validated the authenticity of the Gas-1 probe.

4.3.2 *Gas-1* is abnormally methylated in mouse tumour cell lines.

To assess whether Gas-1 is a target for genetic alterations in cancer, several mouse tumour cell lines which originated from C57BL and Balb/c strains of mice were investigated by Southern analysis for rearrangements of the Gas-1 gene. DNA from the tumour cell lines and from normal mouse lung and spleen tissues was isolated using standard techniques (2.2.12), digested with the *Ava*I restriction endonuclease (2.2.3) and the resulting Southern blots hybridised with the Gas-1 cDNA probe (PA-1) (2.2.10).

Digestion of DNA with the *Ava*I restriction endonuclease (previously reported to detect an RFLP at the Gas-1 locus (Colombo *et al.,* 1989)) showed altered patterns of hybridisation in the mouse tumour cell lines when compared to the normal lung (Fig 4.2). The restriction activity of the *Ava*I enzyme is sensitive to the methylation status of the target DNA. *Ava*I recognises the sequence CCCGGG, however when the second C in the recognition sequence is methylated *Ava*I does not cut the DNA. The altered patterns observed may therefore result from modifications in the degree of DNA methylation of the Gas-1 gene in tumours. To assess this possibility, DNA was digested with *Hpa*II (a methylation-sensitive enzyme) or *Msp*I (which recognises the same

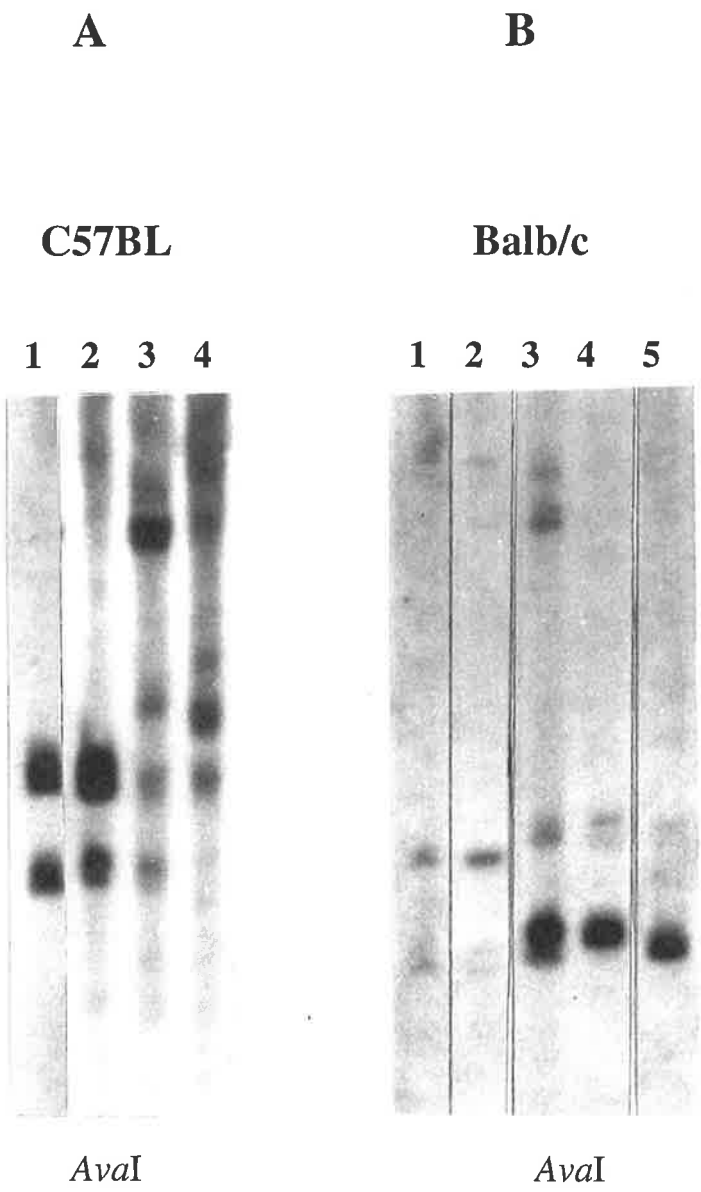


Figure 4.2 Southern analysis of Gas-1 in C57BL (A) and Balb/c (B) mouse tumour cell lines. DNA was digested with the *Ava*I restriction endonuclease and the southern blots hybridised with the mouse Gas-1 cDNA probe (PA-1). (A) DNA was isolated from normal tissues of lung (lanes 1) and spleen (lanes 2) and from mouse tumour cell lines of Lewis lung carcinoma (lane 3) and B16 melanoma (lane 4). (B) DNA was isolated from normal tissues of lung (lanes 1) and spleen (lanes 2) and from mouse tumour cell lines of X63 myeloma (lane 3), P3653 myeloma (lane 4) and OKM1 hybrydoma (lane 5). The variations in banding patterns observed in the different tissues are due to alterations of DNA methylation in the Gas-1 gene

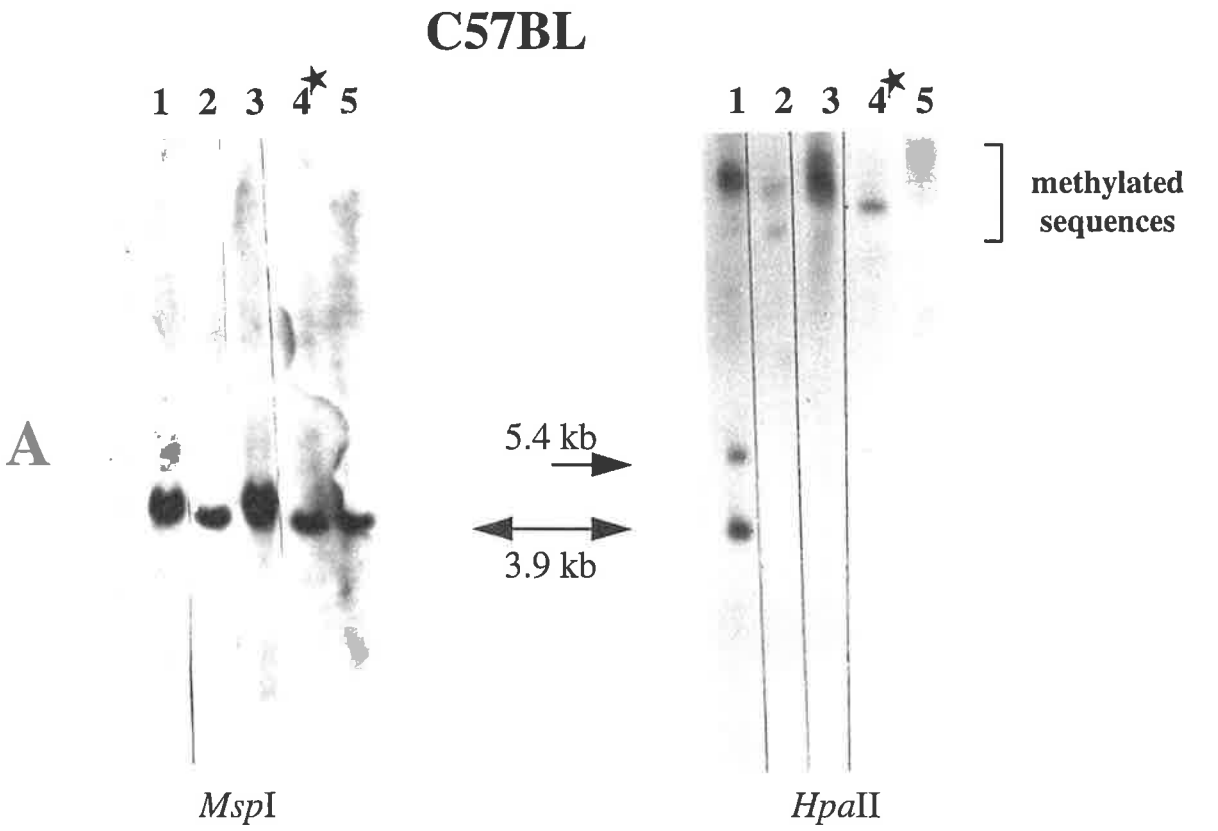
CCGG sequence as *Hpa*II but is not sensitive to methylation of the central cytosine) and analysed by Southern blots for the degree of DNA methylation in the Gas-1 gene. When compared to DNA from normal mouse lung, there was considerable hypermethylation of the Gas-1 gene in all the tumorigenic cell lines from both strains of mice, as recognised by the large uncut bands (Fig 4.3). The altered patterns seen in the *Ava*I-digested DNA could not therefore be attributed to rearrangements in the Gas-1 gene in tumours but rather to differences in the degree of Gas-1 DNA methylation. Incomplete digestion of the DNA was ruled out since the banding patterns observed were consistently present in independent experiments.

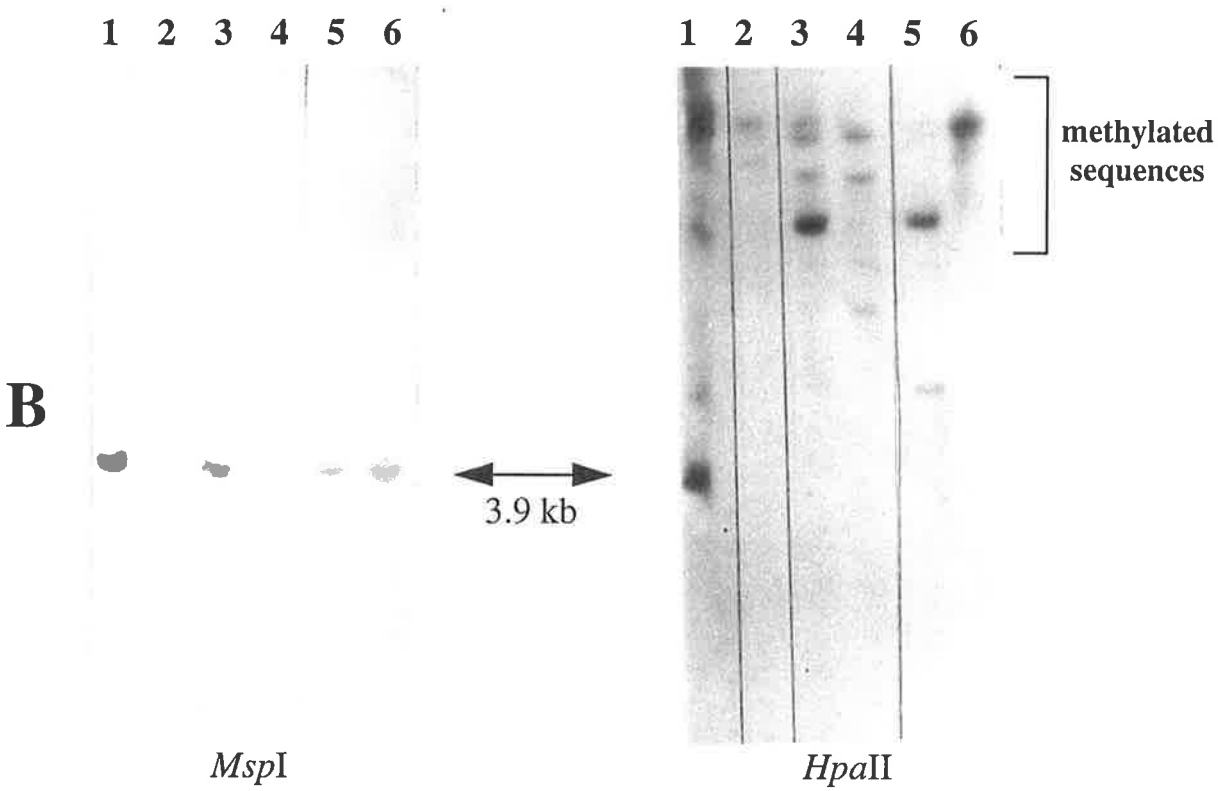
Digestion of DNA with the non-methylation sensitive enzyme *Eco*RI resulted in a single band of 2.7 kb on the autoradiograph and showed no obvious differences in the banding patterns from any of the tumour cell lines when compared to normal tissue (Fig 4.4). These results suggested the absence of any gross rearrangements of the Gas-1 gene in mouse tumours but highlighted differences in the degree of DNA methylation of the Gas-1 gene in normal and tumour DNA.

4.3.3 The methylation state of the Gas-1 gene does not change in serum stimulated cells.

There are numerous HpaII sites in the mouse Gas-1 gene and most are clustered at the promoter region (Fig 4.5A). The results of the altered patterns of DNA methylation seen above suggested a possible role for DNA methylation in the control of GAS1 expression. It was therefore, of interest to know, if the methylation state of Gas-1 changed when quiescent cultured cells are serum stimulated. NIH3T3 cells were initially plated at low density (5×10^5 cells/flask) in 10% FCS and cultured for 24 hr. The FCS concentration was reduced to 0.5% and total RNA and DNA was extracted 24 hr later

Figure 4.3 Southern analysis showing the methylation status of the Gas-1 gene in normal mouse tissues and tumour cell lines from C57BL (A) and Balb/c (B) strains of mice. DNA was digested with the *Msp*I (left panels) and *Hpa*II (right panels) restriction endonuclease and the southern blots hybridised with the mouse Gas-1 cDNA probe (PA-1). (A) DNA was isolated from normal tissues of lung (lanes 1) and spleen (lanes 2) and from mouse tumour cell lines of Lewis lung carcinoma (lanes 3 and 4) and B16 melanoma (lane 5). (B) DNA was isolated from normal tissues of lung (lanes 1) and spleen (lanes 2) and from mouse tumour cell lines of X63 myeloma (lane 3), P3653 myeloma (lane 4), OKM1 hybrodoma (lane 5) and NIH3T3 fibroblasts (lane 6) Hypermethylated sequences in the mouse tumour cell lines are recognised by the large uncut bands when compared to the pattern seen in the lung DNA. * DNA was isolated directly from Lewis lung carcinoma cells grown subcutaneously in C57BL mouse. The other tumour cell lines were grown in culture prior to DNA isolation.


C57BL
1 2 3 4 5
1 2 3 4 5
methylated sequences
A
5.4 kb
3.9 kb
MspI
HpaII


Balb/c
1 2 3 4 5 6
1 2 3 4 5 6
methylated sequences
B
3.9 kb
MspI
HpaII

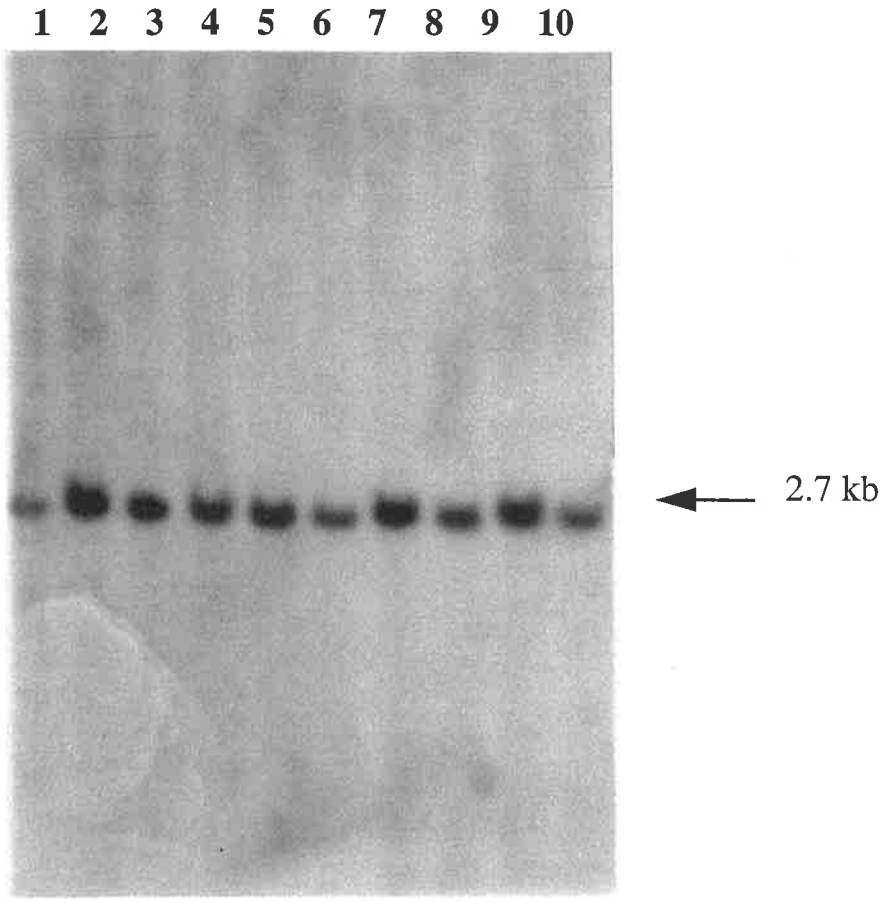
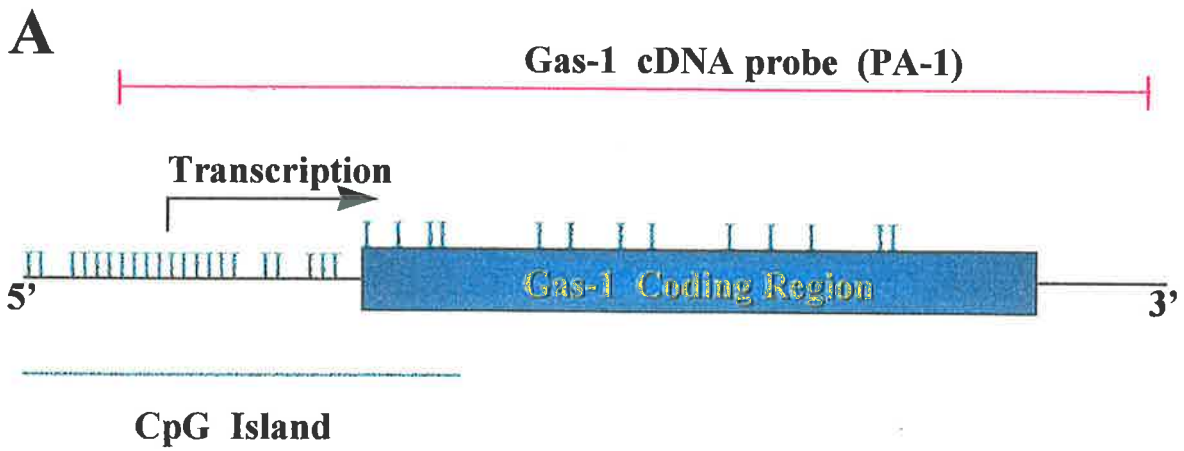
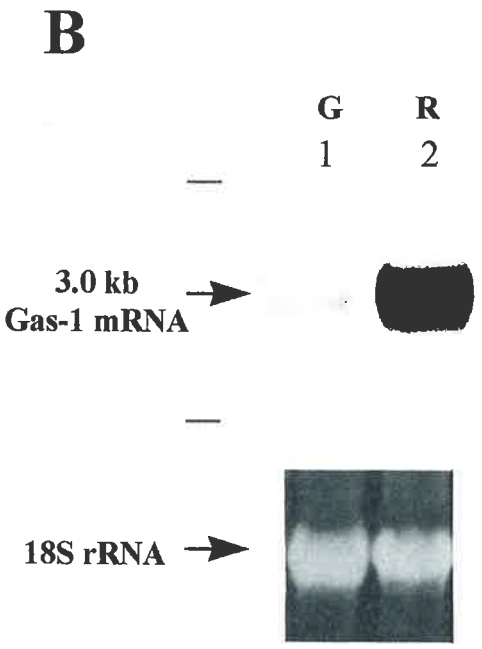
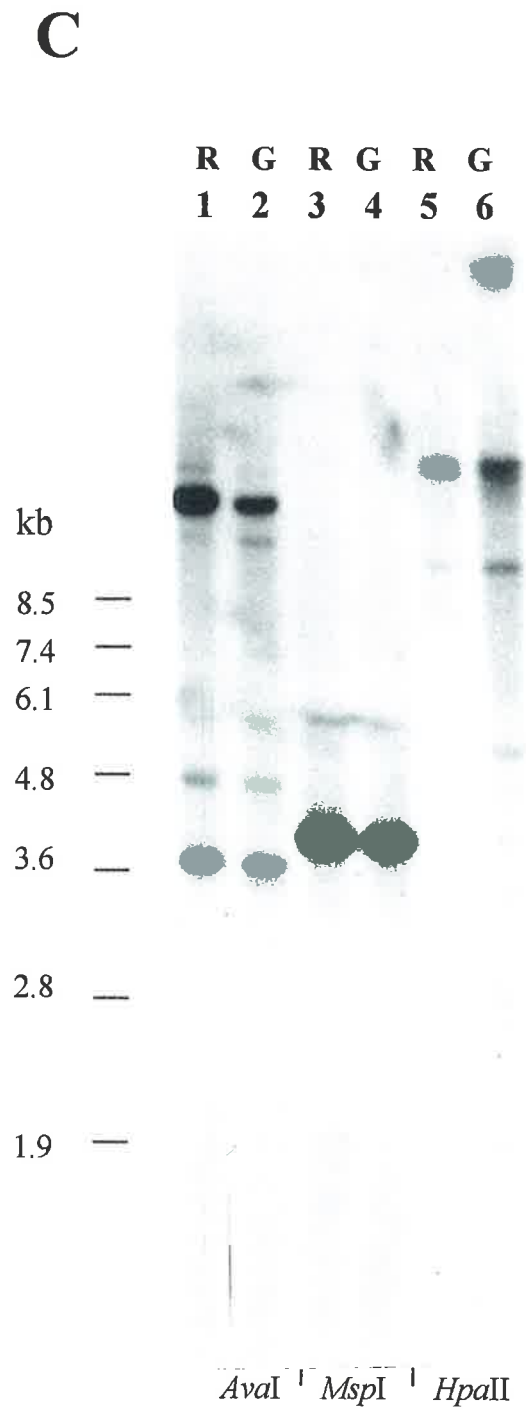


Figure 4.4 Southern blot analysis of Gas-1 in mouse tumour cell lines. DNA was digested with the methylation-insensitive restriction endonuclease *Eco*RI and the southern blot hybridised with the mouse Gas-1 cDNA probe (PA-1). DNA was isolated from NIH3T3 fibroblasts (lane 1), normal lung of C57BL (lane 2), Lewis lung carcinoma (lane 3), B16 melanoma (lane 4), normal lung of Balb/c (lane5), p815 hybridoma (lane 6), X63 myeloma (lane 7), p3653 hybridoma (lane 8), OKM3T3 (lane 9) and OKM1 (lane 10).

Figure 4.5 Comparison of the level of expression of Gas-1 mRNA in NIH3T3 cells with changes in the methylation state of the Gas-1 gene during the transition from resting (R) to a growing state (G). (A) Schematic representation of the mouse Gas-1 gene and 5' promoter sequences. The Gas-1 cDNA probe (PA-1) used for Southern analysis is shown in the top. A dense clustering of CpG dinucleotides typical of a CpG island is shown as vertical lines. Since Gas-1 is an intronless gene, this diagram also represents the genomic structure of Gas-1. (B) Northern blot analysis showing expression of Gas-1 mRNA in (R) resting (lane 1, 0.5% FCS 48 hr) and (G) actively-growing (lane 2, 10% FCS) NIH3T3 cells. (C) Southern blot analysis of DNA isolated from the same cultures and digested with either *Ava*I (lanes 1 and 2), *Msp*I (lanes 3 and 4) or *Hpa*II (lanes 5 and 6) restriction endonucleases.


A
Gas-1 cDNA probe (PA-1)
Transcription
5'
Gas-1 Coding Region
3'
CpG Island


B
G
R
1
2
3.0 kb
Gas-1 mRNA
18S rRNA


C
R G R G R G
1 2 3 4 5 6
kb
8.5
7.4
6.1
4.8
3.6
2.8
1.9
AvaI
MspI
HpaII

(2.2 12). Cultures were then returned to 10% FCS and allowed to recover overnight at which point RNA and DNA was extracted. RNA from both populations of cells was analysed on Northern blots for expression of Gas-1 mRNA (Fig 4.5B), whereas DNA was digested with *Msp*I, *Hpa*II or *Ava*I restriction enzymes and analysed on Southern blots to assess the degree of DNA methylation of the Gas-1 gene, using the Gas-1 cDNA clone (PA-1) as the probe (Fig 4.5C). The level of expression of Gas-1 mRNA was elavated in serum starved cells whereas Gas-1 mRNA decreased significantly after serum stimulation. (Fig 4.5B). Southern blot analysis with the methylation-sensitive *Ava*I or *Hpa*II and the methylation-insensitive *Msp*I restriction enzymes (Fig 4.5C) has shown that, the restriction patterns of *Msp*I were different to those of *Ava*I and *Hpa*II, suggesting that the Gas-1 gene in NIH3T3 cells was methylated. However, the *Ava*I and *Hpa*II banding patterns of the resting (R) or actively growing (G) cells remained the same. These results suggested that the methylation state of the Gas-1 gene did not change in the transition from a quiescent to a stimulated state eventhough the transcriptional activity of Gas-1 was altered. These results suggested that DNA methylation does not appear to play a role in the regulation of Gas-1 expression in the transition from Go to S phase in the cell cycle.

4.3.4 Mouse tumour cell lines do not express Gas-1 mRNA

Aberrations in the expression of Gas-1 in transformed cells are well recognised. NIH3T3 cells transformed with v-ras, v-src, v-fos, or v-myc and rendered quiescent by confluence or serum starvation, all express extremely low levels of Gas-1 mRNA. Moreover, expression is constitutive and is not down-regulated with serum, implying that control of Gas-1 expression is abnormal in malignant cells (Ciccarelli *et al.,* 1990;

Del Sal *et al.,* 1992). To study the effects of malignancy on the expression of the Gas-1 gene, three mouse tumour cell lines, Lewis lung carcinoma, B16 melanoma, and X63 myeloma were analysed by Northern blotting for the presence of Gas-1 mRNA. The Northern blot (Fig 4.6) shows, in the first two lanes, the typical changes of Gas-1 mRNA expression during the cell cycle in NIH3T3 cells. Gas-1 mRNA accumulated in serum starved cells and was downregulated significantly after serum stimulation. However, expression of Gas-1 was absent in all three tumour cell lines analysed, even under conditions of growth arrest (0.5% FCS for 48 hrs). The lack of expression of Gas-1 in all three tumour cell lines correlated with the degree of DNA hypermethylation of the Gas-1 gene detected previously (Fig 4.3). When compared to the hypomethylated state of the Gas-1 gene in normal mouse lung tissue, which was previously described as the tissue with the highest expression of this gene, the hypermethylated state of Gas-1 in the tumour cell lines could be a contributing factor for the loss of Gas-1 expression in these malignant cells.

4.3.5 Demethylation by 5-azacytidine does not effect the expression of Gas-1 in Lewis lung carcinoma cells

Evidence indicating that DNA methylation inhibits gene expression has been obtained with the drug 5-azacytidine (5-azaC), a potent demethylating agent (Jones, 1985). AzaC is a cytosine analog and during DNA replication it incorporates into DNA and prevents the maintenance of the proper methylation state by inhibiting the activity of the maintenance methylase enzyme. When incorporated into DNA, it cannot be methylated and causes almost complete demethylation of genomic DNA (Jones and Taylor 1980, 1981; Creusot *et al.,* 1982). Treatment of a variety of different cell types with 5-azaC, results in selective activation of previously silent genes and cause changes

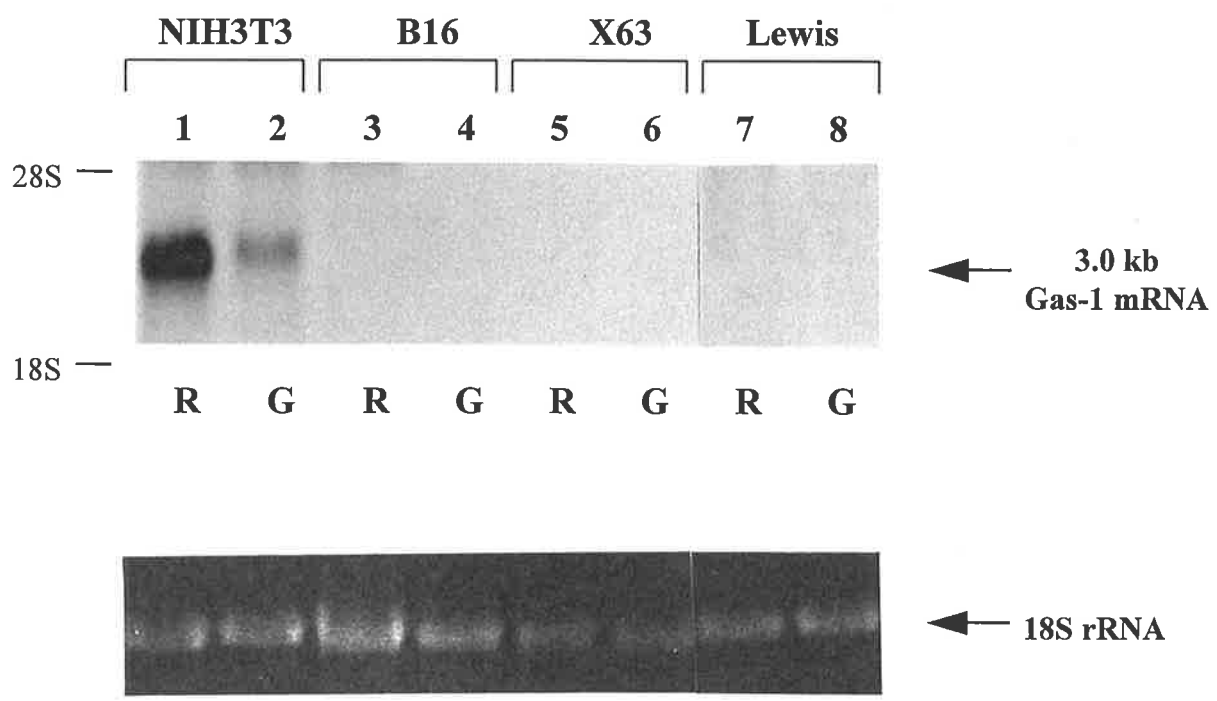
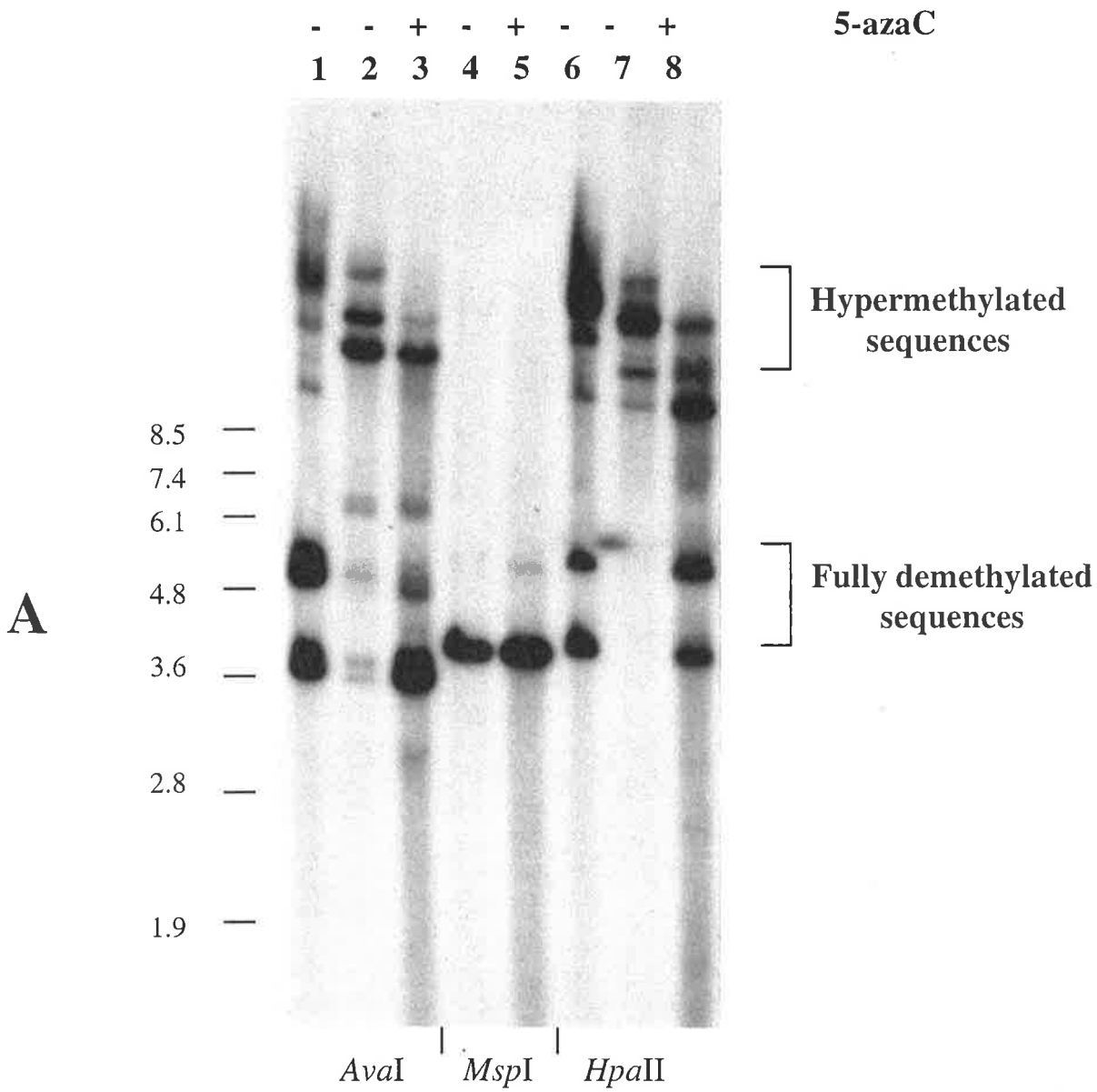


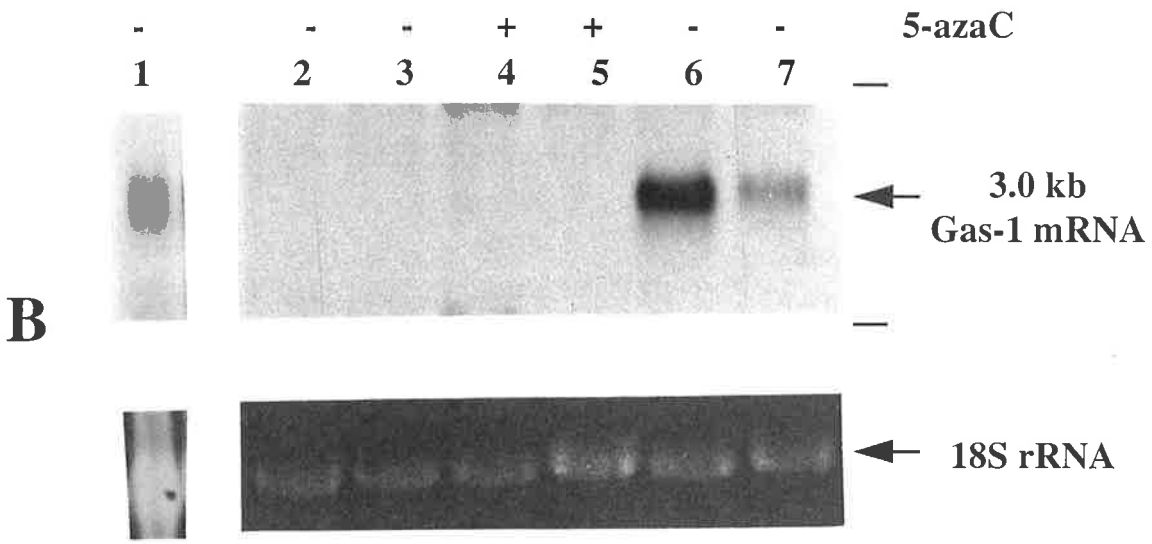
Figure 4.6 Absence of Gas-1 mRNA in mouse tumour cells. Northern blot analysis showing expression of Gas-1 mRNA in resting (R), cells (0.5% FCS 48 hr) and actively growing (G) cells (10% FCS). NIH3T3 cells (lanes 1 and 2), B16 melanoma (lanes 3 and 4), X63 myeloma (lanes 5 and 6) and Lewis lung carcinoma (lanes 7 and 8). Northern blotting using a Gas-1 cDNA probe (PA-1) is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel.

in phenotypic expression (Groudine *et al.,* 1981; Harri, 1982; Konieczny *et al.,* 1984). The lack of expression of Gas-1 mRNA observed in the mouse tumour cell lines analysed above, prompted an investigation for a possible role of DNA methylation as a mechanism for the loss of expression of Gas-1 in malignant cells. The mouse tumorigenic cell line, Lewis lung carcinoma fails to express Gas-1 mRNA, even when grown under conditions of growth arrest (Fig 4.6). The Gas-1 gene is also hypermethylated in Lewis lung carcinoma cells when compared to normal lung (Fig 4.3 and 4.7A). To investigate whether loss of expression of Gas-1 is a result of hypermethylation of the Gas-1 gene, the drug 5-azaC was used (4.2.1) in an attempt to demethylate Gas-1 and to assess the ability of the drug to reactivate expression of Gas-1 mRNA in these cells.

Following four rounds of treatment of Lewis lung carcinoma cells with the drug 5-azaC, the Gas-1 gene was extensively demethylated. The restriction patterns of both *Ava*I and *Hpa*II digested DNA from Lewis lung carcinoma cells became very similar to that of normal lung (Fig 4.7A). Normal lung expresses high levels of Gas-1 mRNA (Fig 4.7B, lane 1) and the demethylated pattern observed must therefore represent a transcriptionally active chromatin structure. If methylation were regulating the expression of Gas-1, demethylation of the Gas-1 gene in Lewis lung carcinoma cells should induce the expression of Gas-1 mRNA. However, Gas-1 mRNA could not be induced in the 5-azaC-treated cells (Fig 4.7B) despite extensive demethylation of the Gas-1 gene (Fig 4.7A). Taken together these results suggest that the lack of expression of Gas-1 in mouse tumours is not due to methylation of the Gas-1 gene.

Figure 4.7 Demethylation of the Gas-1 gene with 5-azaC fails to induce Gas-1 mRNA expression in the Lewis lung carcinoma cell line. (A) Southern blot analysis showing the methylation state of Gas-1 in normal lung (lanes 1 and 6), untreated Lewis lung carcinoma cells (lanes 2, 4, and 7) and 5-azaC-treated cells (lanes 3, 5, and 8). DNA samples were digested with either *Ava*I, *Msp*I or *Hpa*II restriction endonucleases and hybridised with a Gas-1 cDNA probe (PA-1). Hypermethylated and demethylated sequences are indicated. (B) Northern blot analysis examining the expression of Gas-1 mRNA in normal lung tissue (lane 1), and untreated Lewis lung carcinoma cells (lanes 2 and 3 in duplicate) and 5-azaC-treated cells (lanes 4 and 5 in duplicate). Lanes 6 and 7 contain RNA isolated from resting and actively-growing NIH3T3 cells respectively and were used as positive controls for the level of Gas-1 mRNA expression.


- - + - + - - +
5-azaC
1 2 3 4 5 6 7 8
8.5
7.4
6.1
4.8
3.6
2.8
1.9
Hypermethylated sequences
Fully demethylated sequences
A
AvaI
MspI
HpaII


- - - + + - -
5-azaC
1 2 3 4 5 6 7
3.0 kb Gas-1 mRNA
18S rRNA
B

4.4 DISCUSSION

The finding that the methylation state of the Gas-1 gene does not change when quiescent cultured cells are serum stimulated, is in accordance with the idea that the methylation state of DNA can only be changed during or after DNA replication. If the expression of a gene is to be regulated by modulation of DNA methylation, this kind of regulation can be exerted only after the gene is replicated in S phase. However, expression of Gas-1 is regulated before S phase (Schneider *et al.,* 1988) as it is the case for c-fos, c-jun, c-myc and other such genes which are regulated in the very early stages following serum stimulation. On this basis DNA methylation would not be expected, to play a role in the expression of Gas-1 during the cell cycle. Changes in DNA methylation that may lead to functional inactivation and loss of Gas-1 expression may however be more important in tumour progression.

The results presented here indicate that the Gas-1 gene is differentially methylated in a tissue-specific manner. DNA isolated from normal lung had a preference for the demethylated state of Gas1, as evident by the presence of the lower bands in the Southern blots (Fig 4.3). Gas-1 is not expressed at equal levels in all tissues of the mouse and expression is more abundant in lung tissue (Schneider *et al.,* 1988). The demethylated pattern of the Gas-1 gene observed in lung tissue would therefore represent a transcriptionally active state. None of the three tumour cell lines analysed, expressed any detectable levels of Gas-1 mRNA even under conditions that normally induce growth arrest. The lack of Gas-1 expression correlated with hypermethylation of Gas-1 in all three tumour cell lines when compared to the methylation state of Gas1 in normal mouse lung. These observations suggest that hypermethylation of Gas-1 could result in the loss of Gas-1 expression in tumours

The involvement of DNA methylation in the regulation of gene expression is well documented (reviewed in Doerfler 1983; Jones, 1990). Evidence that DNA methylation regulates the expression of specific genes was first derived from gene transfer experiments. Unmethylated exogenous genes stably transfected into cultured cells were consistently transcribed at a basal level. When DNA was methylated in vitro and then introduced into fibroblasts, this modification was maintained from generation to generation and the presence of these methyl groups on the transfected gene sequences was sufficient to inhibit their expression (Busslinger *et al.,* 1983). DNA methylation probably inhibits gene expression by affecting the protein-DNA interactions required for transcription. When DNA was introduced into cells by DNA-mediated gene transfer, unmethylated DNA adopted a *DNase*I-sensitive structure typical of other transcriptionaly active genes. However, fully-methylated sequences were placed in a *DNase*I-insensitive structure and were structurally similar to the inactive genes of the cell (Keshet *et al.,* 1986). These studies suggested that the presence of the methyl moieties are capable of altering the conformation of the DNA, thus affecting the accessibility of factors required for transcription.

Two models have been proposed that implicate a role for DNA methylation in the development of cancer. Firstly, changes in DNA methylation contribute to carcinogenesis by affecting the expression of proto-oncogenes and tumour suppressor genes. Global DNA hypomethylation in tumours is of selective advantage to the tumour cell through the increased expression of oncogenes (Feinberg and Vogelstein 1983; Vorce and Goodman 1991). Alternatively, high levels of expression of methyltransferase commonly seen in tumour cells (Kautiainen and Jones 1986) induces hypermethylation of specific loci and leads to silencing of tumour suppressor

genes.(Baylin *et al* 1991; El-Deiry et al 1991; Issa et al 1993; Ohtani-Fujita et al 1993). In contrast to the gene expression-mediated model, the mutation-mediated model proposes that DNA methylation could contribute to genetic instability through increases in point mutations. Deamination of methylated cytosine generates a thymine residue. The occurrence of C to T transition mutations is higher for methylcytosine than for unmethylated cytosine residues. Such a C to T transition accounts for a high percentage of the DNA polymorphisms found among normal individuals and by genetic mutations found in genetic disorders. Mutations of this nature are of significance in colon cancer, in which 47% of the characterised point mutations in the p53 tumour suppressor gene involve a C to T transition (Rideout *et al.,* 1990).

The nucleotide analog 5-azaC, is a strong inhibitor of DNA methylation and has been widely used to activate suppressed gene expression (Jones, 1985). In this study, Lewis lung carcinoma cells were treated with 5-azaC in an attempt to restore the expression of Gas-1. Although substantial demethylation of the Gas-1 gene occurred after treatment with 5-azaC, the expression of Gas-1 mRNA was not induced. The purpose of methylation of the Gas-1 gene remains unclear, as it does not appear to directly regulate gene expression. The results obtained here however, may be consistent with the idea that changes in DNA methylation may result in a transcriptionally active conformation for the Gas-1 gene, but for transcription to occur, appropriate *trans*-acting factors are also required which may not be present in all cell types and may be absent in the tumour cells (Keshet *et al.,* 1986; Lesley *et al.,* 1989). Such a mechanism is supported by gel retardation data which suggested that the absence of transcription factor(s) or the binding of these to specific Gas-1 promoter sequences is abnormal in transformed cells (Cowled, personal communication). These results also extend those of

Lesley and Jones (1989) who showed that extensive hypomethylation can induce a gene such as globin to take up a potentially active configuration but not necessarily to be transcribed. Finally it is possible that hypermethylation of Gas-1 is a consequence of malignancy and may bear no functional significance for the loss of Gas-1 expression.

CHAPTER 5

EXPRESSION OF GAS1 IN HUMAN CELLS

AND

GROWTH-SUPPRESSIVE ACTIVITY OF GAS1 IN NIH3T3 FIBROBLASTS

5.1 INTRODUCTION

At this stage of the study, a partial human GAS1 clone [181/HLEE] became available and thus the initial aim of these experiments was to analyse the pattern of expression of the human GAS1 gene in human fibroblasts and assess if this pattern of expression is similar to that previously seen with the mouse homologue.

During the course of these investigations, the full length cDNA of the human homologue to the mouse Gas-1 gene had been isolated (Del Sal *et al.,* 1994). Alignment of the putative human and mouse Gas-1 amino acid sequences revealed that the two proteins bear 82% amino acid identity whereas the 5' promoter regions share an 85% homology (de Martin *et al.,* 1993). Since cell cycle-control elements are commonly conserved between species, it is likely that the human and mouse Gas-1 genes have the same function. One approach to support the concept that GAS1 is directly involved in maintaining the quiescent state is to overexpress GAS1 ectopically by gene transfer techniques and assess its ability to induce growth arrest in a population of cells which are actively growing. Previous investigations of the growth suppressing effects of Gas-1 were based on the transient expression of the mouse Gas-1 gene in NIH3T3 fibroblasts by microinjection of a Gas-1 expressing plasmid and analysis of its *in vitro* growth properties (Dal Sal *et al.,* 1992). Here those studies are extended further by using the human GAS1 cDNA to generate stable GAS1-expressing cell lines. Unlike the previous transient expression experiments reported by Del Sal *et al.,* (1992), the current study allows a direct assessment of growth parameters after long-term overexpression of GAS1, including observation of morphological changes; a parameter not previously assessed.

In this study, the human GAS1 cDNA coding for the complete GAS1 protein was cloned in both sense and antisense orientations into constitutive and inducible mammalian expression vectors and transfected into NIH3T3 mouse fibroblasts. Stable transfectants capable of expressing high levels of exogenous GAS1 were propagated and characterised further for their ability to suppress growth in vitro, at a time when the endogenous levels of Gas-1 were low.

The activity of a particular gene may be effectively inhibited by creating antisense molecules that bind specifically to the mRNA coding for the target gene product, thereby interrupting the translation of that gene (Izant and Weintraub, 1984; Weintraub, 1990; Moffat, 1991). In this study, transfectants containing the human GAS1 cDNA, cloned in the antisense orientation, were assessed for their ability to exhibit a transformed phenotype when the endogenous levels of Gas-1 were downregulated at a time when the cell normally requires Gas-1 for growth arrest.

Since the expression of Gas-1 is linked to the cell cycle, it is possibly related to the control of the cycle. The gene product of GAS1 could be required to initiate or sustain growth arrest or be related to the control of differentiation. Determining the biological function of GAS1 may provide important insights into the control of cell proliferation.

5.2 MATERIALS AND METHODS

5.2.1 Plasmids and Clones:

A 1.8kb *Bgl*II-*Eco*RI fragment corresponding to nucleotides 188-2019 of the GAS1 cDNA sequence was excised from the 6.5 kb human GAS1 genomic clone (2.1.10) and ligated (2.2.5) into the *Bam*HI/*Eco*RI sites of Bluescript KS+ (Stratagene).

For inducible expression, a shorter 1.55 kb *Xba*I-*Nhe*I fragment (nucleotides 188-1724), containing the full open reading frame coding for the GAS1 protein (nucleotides 411-1444), was subcloned into the *Xba*I site of the dexamethasone-inducible expression vector pMAMneo (Clontech Inc). For constitutive expression of high levels of GAS1 mRNA, the same 1.55 kb fragment was cloned directly into the *Nhe*I site of the pRcCMV expression vector (Invitrogen Inc). For the generation of antisense GAS1 mRNA, the same fragment was cloned into the *Nhe*I site of the pRcCMV vector but in the reverse orientation. Figure 5.1 shows diagrammatically the strategy used for the generation of the recombinant vectors. Both expression plasmids contain the neomycin-resistance gene which allows positive selection of transfectant clones using the antibiotic, geneticin (G418: Gibco BRL). The vector/insert ligation product was transformed into competent *E coli* bacteria (2.2.6, 2.2.7) and plasmid DNA isolated by miniplasmid preparation (2.2.1). The identity and orientation of the recombinant vectors were confirmed by restriction endonuclease digestion (2.2.2) and PCR analysis (2.2.15) using each of the 5' vector-specific primers and a 3'downstream GAS1 primer (Table 5.1). Both vectors were isolated and purified in preparation for transfection using the Qiagen midi plasmid kit, according to the manufacturers instructions (2.2.1).

Table 5.1 Primers used to assess the presence of the GAS1 expression constructs in the transfected cell lines. The expected size of the PCR product for each primer set is also shown.

Primer	Sequence	Expected size (bp)
pMAMneo GAS1 (HP7)	5'-GGCTATCATCACAAGAGCGGAAC-3' 5'-GCAGCAGCGCCATCAGGCACAG-3'	367
5'CMV GAS1 (HP7)	5'-GAACCCACTGCTTAACTGGCTTAT-3' 5'-GCAGCAGCGCCATCAGGCACAG-3'	472

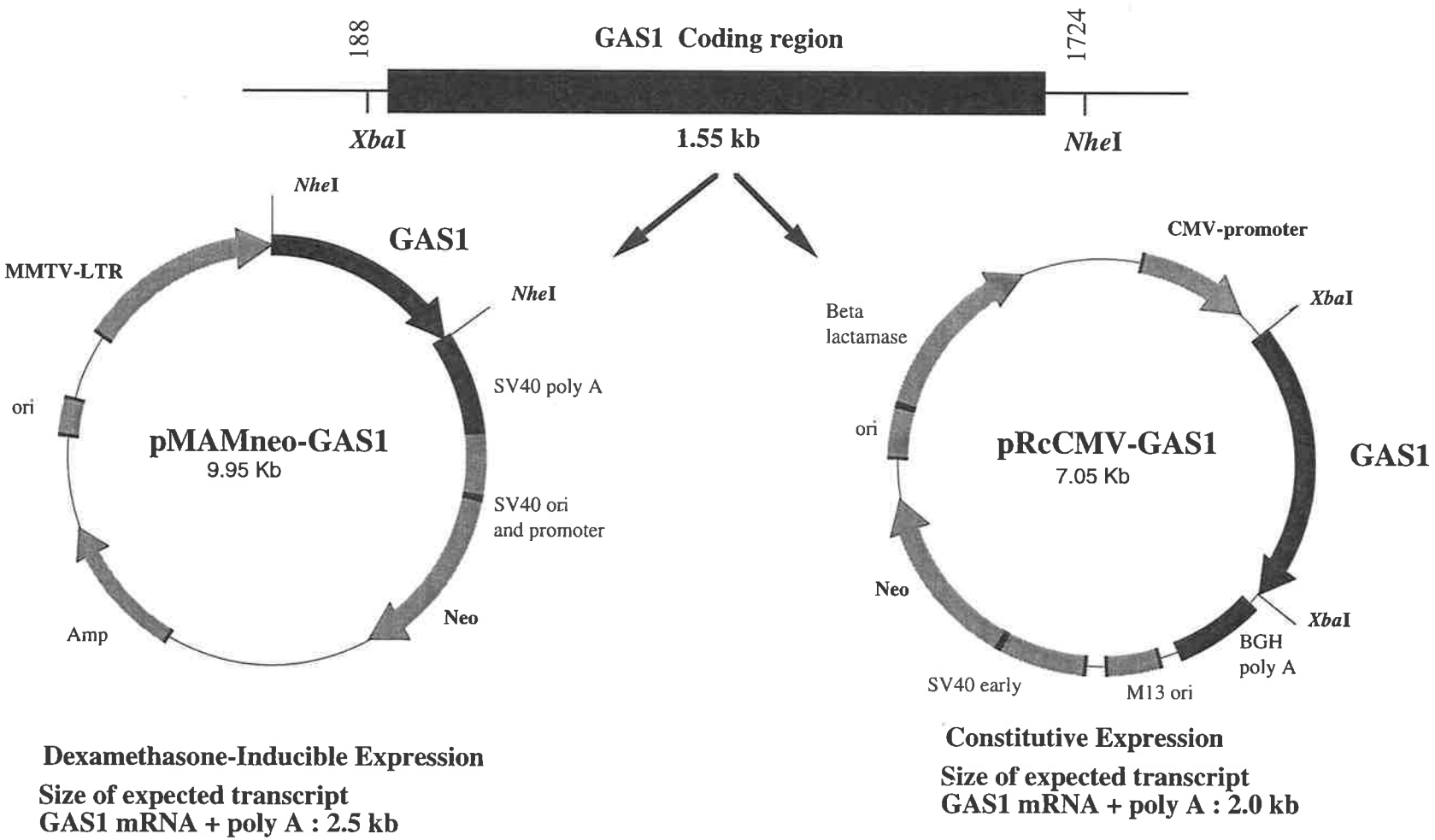


Figure 5.1 Cloning of the open reading frame coding for the GAS1 protein, into expression vectors. A 1536 bp fragment of the GAS1 cDNA was ligated into the NheI site of the pMAMneo plasmid vector to allow dexamethasone-inducible expression of GAS1 mRNA. For constitutive expression the same GAS1 cDNA fragment was ligated into the XbaI site of the pRcCMV plasmid vector. For antisense GAS1 mRNA expression the same fragment was ligated into the pRcCMV plasmid but in the antisense orientation.

5.2.1 Detection of GAS1 mRNA in IMR90 cells by DNase treatment and RT-PCR

RT-PCR was performed essentially as previously described (2.2.16) and modified to facilitate treatment of samples with DNase I. The DNase treatment was carried out in the same reaction mix as the reverse transcriptase step, prior to the addition of the reverse transcriptase. One microgram of RNA was isolated from growth arrested IMR90 fibroblasts (IMR90 cells were grown in 0.5% FCS and total RNA isolated 48 hr later), mixed with reverse transcriptase master mix, excluding both reverse transcriptase and a down-stream GAS1 specific primer (HP2), in a total volume of 40 µl. The master mix contained 5 mM $MgCl_2$ 10 mM Tris-Cl pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1 mm each dNTP, and 0.5 units of rRNAsin ribonuclease inhibitor. Contaminating DNA was removed from the RNA by adding 20 units of RNAase-free DNase I (Boehringer Mannheim) and incubating at 37°C for 30 min. The DNase reaction was terminated by heating to 95°C for 5 min and then cooling to room temperature for 5 min. Two microliters, or 50 units of AMV reverse transcriptase and 250 ng of the GAS1 downstream primer (HP2), were added to the mix and the reaction incubated for 30 min at 42°C. For amplification of GAS1, 5 µl of cDNA was subsequently used in PCR analysis using the following GAS1 specific primers, which produced a 128-bp PCR product.

GAS1 HP1	5'-CGGGACCAGATCTCGACAGCTGTT-3'
GAS1 HP2	5'-TCCTGCCCACTTGCATGAGTG-3'

To test the effectiveness of the DNase treatment, parallel RNA samples were spiked with 500 ng of genomic DNA isolated from IMR90 cells. PCR analysis was performed on samples before and after DNase treatment.

5.2.3 Transfections

NIH3T3 cells were seeded at 5 x 10^5 per 25 cm^2 flask and grown overnight. Cells were then transfected (2.2.21) with either the dexamethasone inducible GAS1-expressing plasmid, pMAMneo-GAS1 or the constitutive GAS1-expressing plasmid pRcCMV-GAS1. For control transfections, the same cells were transfected with the parental vectors. Transfectant populations were then selected by culturing cells in the presence of 400 μg/ml G418. After selection for 2 weeks, the number of G418-resistant colonies was counted and randomly-selected colonies were manually picked and propagated in the continual presence of 400 μg/ml G418 for subsequent analysis of growth parameters.

Genomic DNA from selected clones was isolated using standard techniques (Sambrook *et al.,* 1989) and the presence of the transfected GAS1 gene was confirmed by PCR analysis using the primers shown (Table 5.1). By using a 5' primer specific for plasmid sequences, amplification from the endogenous genomic Gas-1 gene was avoided and only the fragment from the transfected plasmid was amplified.

Total RNA was isolated from transfected cultures (2.2.12) and expression of the transfected GAS1 gene was assessed by Northern blotting (2.2.13). To demonstrate dexamethasone-inducible expression of GAS1, cells were incubated in DMEM culture medium in the presence of 1 x 10^{-6} M dexamethasone for 48 hr before isolation of RNA.

5.2.4 Analysis of growth rate

Transfectant clones were plated in 25 cm^2 culture flasks at 2 x 10^4 cells per flask in DMEM culture medium plus 400 μg/ml G418 in the presence or absence of 1 x 10^{-6} M dexamethasone. Cells were harvested by trypsinisation at the indicated times and counted in a haemocytometer. Results from each time point were from either triplicate or duplicate flasks.

5.2.5 Soft agar cloning assay

Antisense GAS1 (pRcCMV GAS1-antisense) transfectant cells (2 x 10^4 / well, 9.6 cm^2, culture plates) were mixed with 2 ml of 0.33% plating agar in DMEM and overlaid on 2 ml of base containing 0.5% agar in DMEM. Plates were incubated for 14 days at 37°C in 5% CO_2 and 100% humidity. The number of colonies per plate with a diameter of greater than 0.1 mm was counted using an inverted microscope.

5.3 RESULTS

5.3.1 Expression of GAS1 mRNA in human fibroblasts

In the previous chapter, it was shown that in NIH3T3 cells, expression of the mouse Gas-1 gene was induced under conditions of growth arrest. As an initial attempt to characterise the pattern of expression of the human homologue to the mouse Gas-1 gene, a partial human GAS1 cDNA clone GAS1[HLEE], was used as a probe in Northern blot analysis to monitor expression of GAS1 mRNA in human fibroblasts, cultured under different conditions. RNA was isolated from a number of human fibroblast cell lines including IMR90, SF1972 and MRC-5. Figure 5.2 shows that the

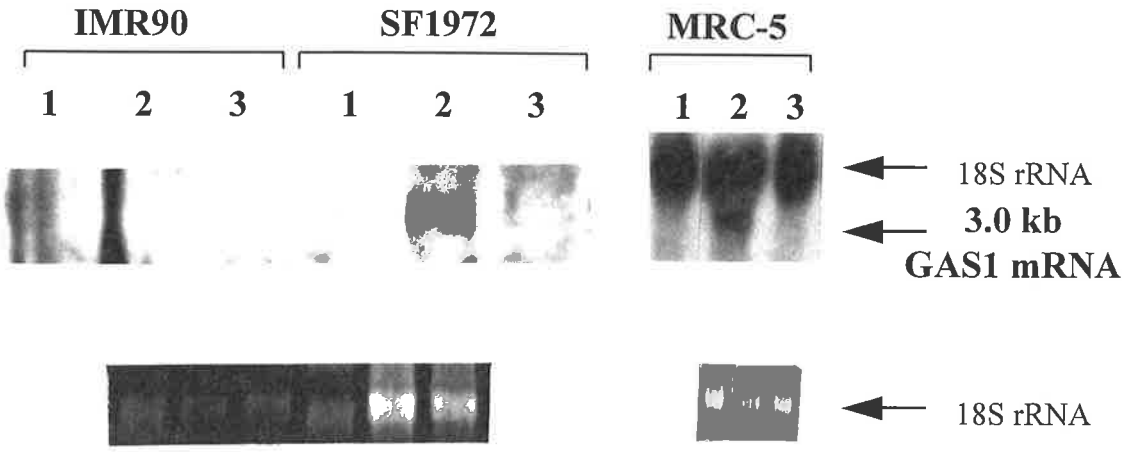


Figure 5.2 Analysis of GAS1 mRNA expression in a variety of human fibroblast cell lines. Northern blot analysis using a human GAS1 cDNA probe [HLEE] is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. RNA was extracted from cells growing asynchronously in the presence of 10% FCS (lanes 1), serum starved for 48 hr in 0.5% FCS (lanes 2), and reactivated to enter the cell cycle by the addition of 10% FCS for 6 hrs (lanes 3).

expression of human GAS1 is very low or undetectable in actively growing cells (10% FCS) of all cell lines examined. Expression of GAS1 mRNA is significantly increased in SF1972 and MRC-5 fibroblasts when arrested by serum deprivation (0.5% FCS for 48 hr). The level of GAS1 mRNA returned to basal levels and was undetectable within 6 hr after re-activating the cells with 10% FCS (Fig 5.2). In comparison to the level of expression of mouse Gas-1 mRNA detected in growth-arrested mouse fibroblasts (Fig 4.1), the level of expression of GAS1 mRNA detected here in human cells is low or absent. Expression of GAS1 was not detected in IMR90 cells even under conditions of growth arrest, suggesting that GAS1 mRNA was not expressed or was below the threshold level to be detected by Northern analysis. On this basis, it was of interest to know if GAS1 mRNA could be detected by RT-PCR.

5.3.2 Detection of GAS1 mRNA in IMR90 cells by RT-PCR

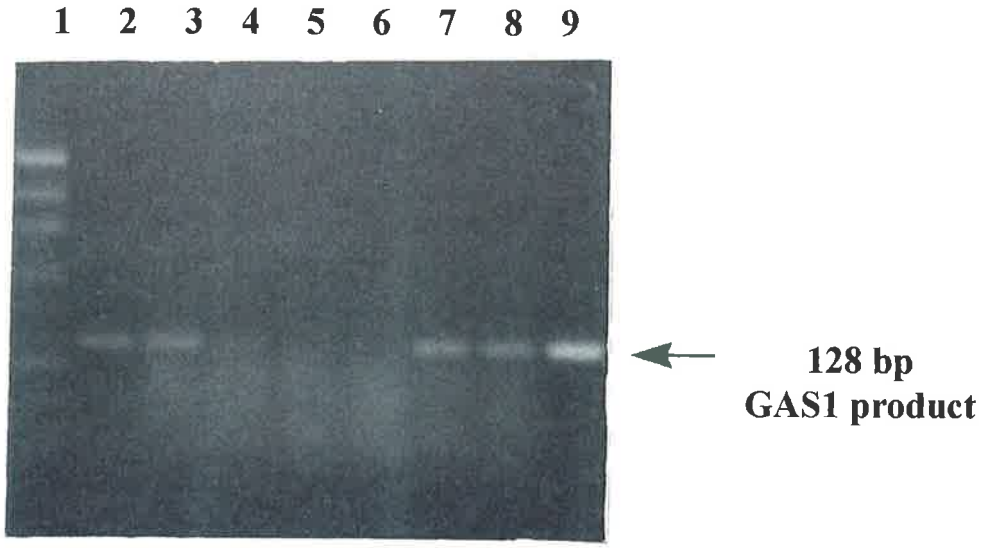
GAS1 is an intronless gene and this meant that RT-PCR could not be performed directly from the isolated RNA, since any contaminating DNA in the RNA preparation will also be amplified. Contaminating DNA was eliminated from total RNA isolated from growth arrested IMR90 cells by treatment with RNase-free DNase I (5.2.1) and as described in Dilworth and McCarrie (1992). Figure 5.3 shows the presence of contaminating DNA in the sample of total RNA that was not treated with DNaseI, as indicated by the amplification of the expected 128 bp product in the RNA-only lane (lane 4). DNaseI treatment of the RNA sample prior to reverse transcription effectively eliminated the contaminating DNA signal (no band in the DNase-treated RNA-only sample, lane 6), but leaves the cDNA signal intact (band in the DNase treated RNA sample after reverse transcription, lane 8). DNase treatment of a duplicate sample of

Figure 5.3 Detection of GAS1 mRNA by RT-PCR in growth arrested IMR90 cells and effects of pretreatment with DNaseI on the production of amplification products. One microgram of total RNA isolated from growth arrested IMR90 cells (0.5% FCS for 48 hr) with or without 500 ng of spiked genomic DNA was subjected to RT-PCR using GAS1 specific primers HP1 and HP2. HP2 was used as the downstream primer for the initiation of first strand cDNA synthesis.

1. *Hpa*II-digested pUC19 marker
2. DNA from peripheral blood, positive control
3. RNA spiked with 500 ng of DNA
4. RNA only — - RT
5. RNA spiked with 500 ng of DNA + DNaseI treatment
6. RNA only + DNaseI treatment
7. cDNA from RNA spiked with 500 ng of DNA — +RT
8. cDNA from RNA only
9. GAS1 [HLEE] plasmid positive control

Note amplification signal in both spiked and non-spiked sample as a result of contaminating DNA (lanes 3 and 4). The faint signal in the RNA-only sample (lane 4) indicates endogenous DNA contamination in the RNA preparation. PCR following treatment with DNaseI effectively eliminates the signal from contaminating DNA in both spiked and non-spiked samples (lanes 5 and 6). RT-PCR of DNaseI-treated samples detects the 128 bp specific GAS1 signal (lanes 7 and 8).


HP1
Gas-1 Coding Region
5'
3'
HP2
128 bp


1 2 3 4 5 6 7 8 9
128 bp
GAS1 product

RNA that was spiked with 500 ng of genomic DNA eliminated 100% of the contaminating DNA and no amplification product was detected prior to reverse transcription (RNA samples spiked with DNA before and after DNase treatment; compare lanes 3 and 5). Following reverse transcription of the DNase treated sample, the expected 128 bp product is amplified from the synthesised cDNA (lane 7). The amount of DNA (500 ng) spiked represents a much higher level of contamination than that normally observed in RNA samples and the complete elimination of the spiked DNA suggested an efficient DNase treatment. On this basis, the signals observed after the RT-PCR of DNase treated RNA in both non-spiked and spiked RNA samples must have originated from the synthesised cDNA (lanes 7 and 8 respectively). These results demonstrate the presence of GAS1 mRNA in growth arrested IMR90 cells, which is detected by RT-PCR of DNase-treated RNA but is undetectable by Northern analysis.

5.3.3 Constitutive expression of GAS1 mRNA in NIH3T3 cells is antiproliferative

To investigate the effects of over-expression of GAS1 on cell growth, NIH3T3 mouse fibroblasts were transfected (2.2.21) with the constitutive pRcCMV-GAS1 expression plasmid or the control vector pRcCMV. Following selection for two weeks in the presence of the antibiotic G418, there was a nine fold decrease in the number of colonies that arose after transfection with the pRcCMV-GAS1 expression plasmid compared to the vector alone (Table 5.2).

Table 5.2: *Growth of NIH3T3-resistant colonies two weeks after transfection with plasmid constructs.*

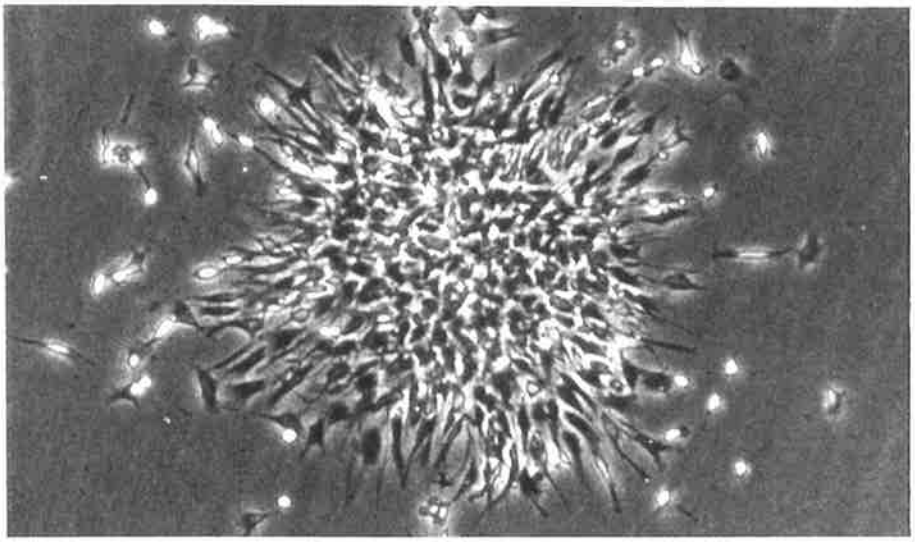
	Number of G418-resistant colonies
pRcCMV-vector	85
pRcCMV-GAS1-sense	9
pRcCMV-GAS1-antisense	100
pMAMneo-vector	45
pMAMneo-GAS1	43

Each result represents the average of the number of colonies in duplicate flasks.

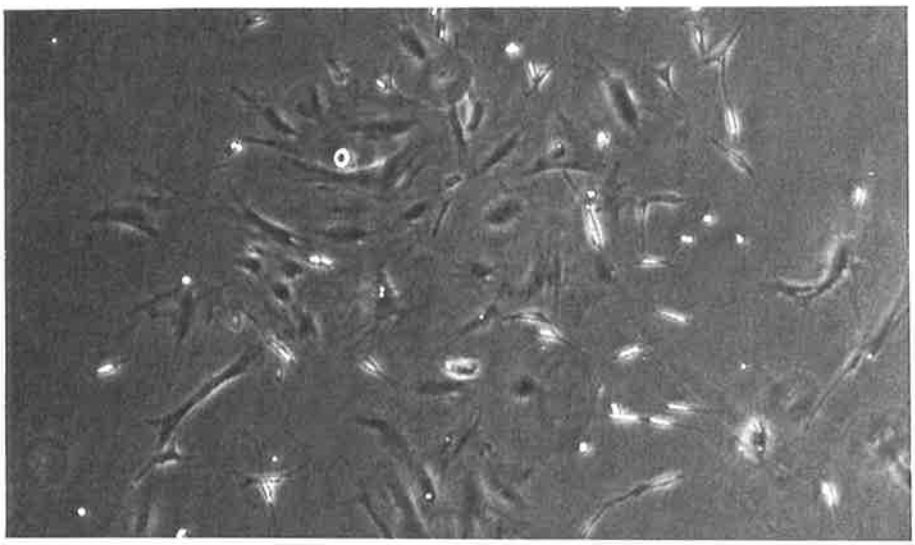
The G418-resistant colonies that arose following transfection with the pRcCMV-GAS1 plasmid were extremely small and the cells within these colonies were morphologically different when compared to those of the vector transfectants (Fig 5.4). The GAS1 transfected cells displayed a flat morphology with a large cytoplasm and resembled growth arrested cells or cells in crisis (Fig 5.4C). RNA could not be isolated from such a small number of cells, thus the expression of exogenous GAS1 was not assessed. This characteristic morphology was only present at the end of the two week selection period and disappeared thereafter when the cells were trypsinised and propagated as a mass culture for subsequent analysis. Northern analysis of RNA isolated from the mass culture, which was expanded and propagated after G418 selection, demonstrated that these cells did not express exogenous GAS1 mRNA. The reduced number of colonies that arose in the pRcCMV-GAS1 transfectants suggested that expression of GAS1 mRNA was antiproliferative and cells overexpressing GAS1 during the initial selection process were counterselected.

Figure 5.4 Photomicrograph of proliferating colonies arising after transfection of NIH3T3 cells and selection for two weeks in the presence of the antibiotic, G418. Colonies were observed and photographed through an inverted microscope. (A) NIH3T3 cells transfected with the pRcCMV-vector-only control (50x). (B) NIH3T3 cells transfected with the pRcCMV-GAS1-expressing plasmid (50x). (C) Magnification (100x) of (B) to show characteristic morphology of growth arrested cells in the same colony.

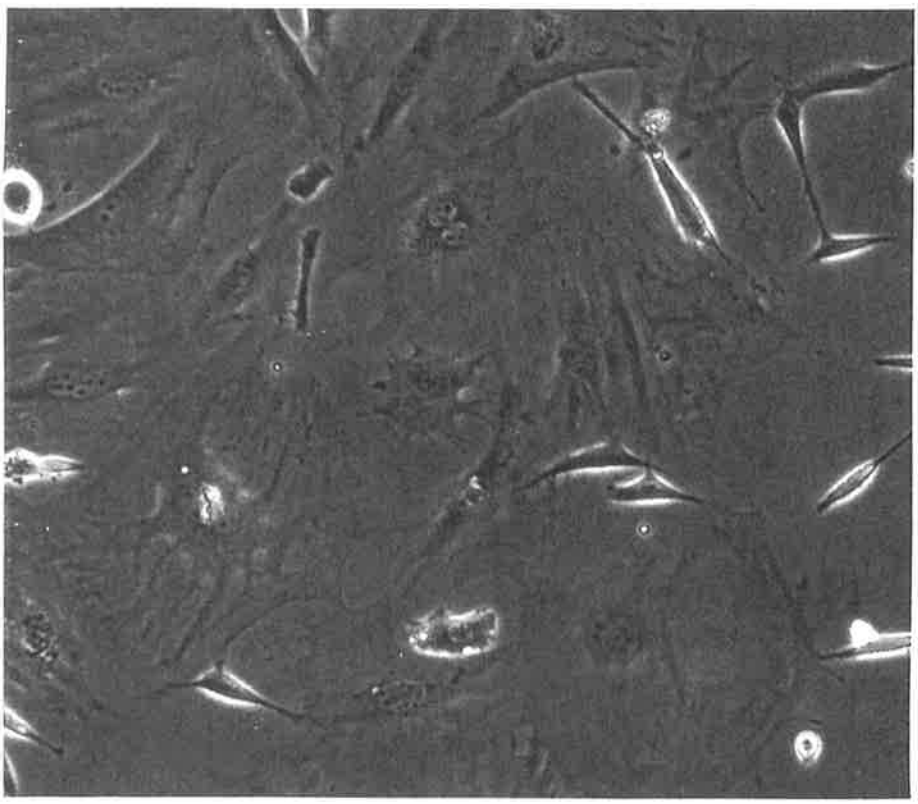
A
pRcCMV-vector



B
pRcCMV-GAS1



C
pRcCMV-GAS1



The transfectant cells which presumably expressed high levels of GAS1 during the initial period of selection were inhibited in their growth and did not propagate. Cells expressing a lower but tolerable level of GAS1 proliferated until GAS1 reached a threshold level able to induce growth arrest. This is depicted by the reduced size of the colonies and altered morphology of the cells. The loss of this morphology after trypsinisation would suggest that these cells were too fragile to survive the trypsinisation step and only those cells that lost GAS1 expression or contained only the neomycin resistance gene were able to propagate in the subsequent passages.

5.3.4 Dexamethasone-inducible expression of GAS1 in NIH3T3 fibroblasts leads to growth arrest.

The inability to isolate and propagate any stable clones that constitutively expressed high levels of exogenous GAS1 mRNA prevented a direct examination of the effects of overexpression of GAS1 in NIH3T3 cells. It was therefore necessary to use an inducible expression system to control the onset of GAS1 expression more precisely. NIH3T3 cells were transfected with an expression vector in which the dexamethasone-inducible mouse mammary tumour virus (MMTV)-derived promoter controls the expression of GAS1 and the SV40 early promoter constitutively expresses a cis-linked neomycin resistance gene (Fig 5.1). Following transfection with the pMAMneo-GAS1 construct, the cells were grown for two weeks in the presence of G418 to select for cells containing the plasmid and the number of colonies counted. From two independent transfection experiments, cells transfected with the GAS1 construct formed the same number of G418-resistant colonies as cells transfected with the vector-only construct (Table 5.2). Expression of the transfected GAS1 mRNA was induced in randomly-selected G418-resistant clones and in the mass culture by incubating growing cells for

48 hr in 5 x 10^{-8} M dexamethasone. Northern analysis detected variable levels of the 2.5 kb exogenous GAS1 transcript in the mass culture and in two individual clones (clones 3 and 6) (Fig 5.5).

Clone 3 which expressed the highest level of dexamethasone-inducible GAS1 mRNA, was analysed further for its ability to undergo growth arrest after the addition of dexamethasone to asynchronously-growing cells. Cells from clone 3 were plated overnight at 5 x 10^4 per flask in 10% serum and then were exposed to dexamethasone. Cells were trypsinised and counted at the indicated times (Fig 5.6). Within 6 days, clone 3 cells underwent growth arrest at densities that were more than 60% less than the corresponding uninduced cells (Fig 5.6B). The vector-transfected NIH3T3 cells showed no change in growth rate and therefore served as a control to demonstrate that this effect was due to the induction of GAS1 and not to the effects of dexamethasone (Fig 5.6A). In addition to a marked decrease in growth rate, induction of expression of GAS1 by dexamethasone also caused a marked change in morphology when compared to control cells. These cells displayed morphological characteristics resembling growth-arrested cells, (Fig 5.7B and C) and were similar in morphology to cells transfected with the constitutively-expressing GAS1 plasmid (compare morphology in Fig 5.4C and 5.7C). The characteristic changes in morphology were restricted to the GAS1-induced cells and were absent from both the uninduced and from control cells treated with dexamethasone (Fig 7A), suggesting that these effects were due to GAS1 and not due to the effects of dexamethasone.

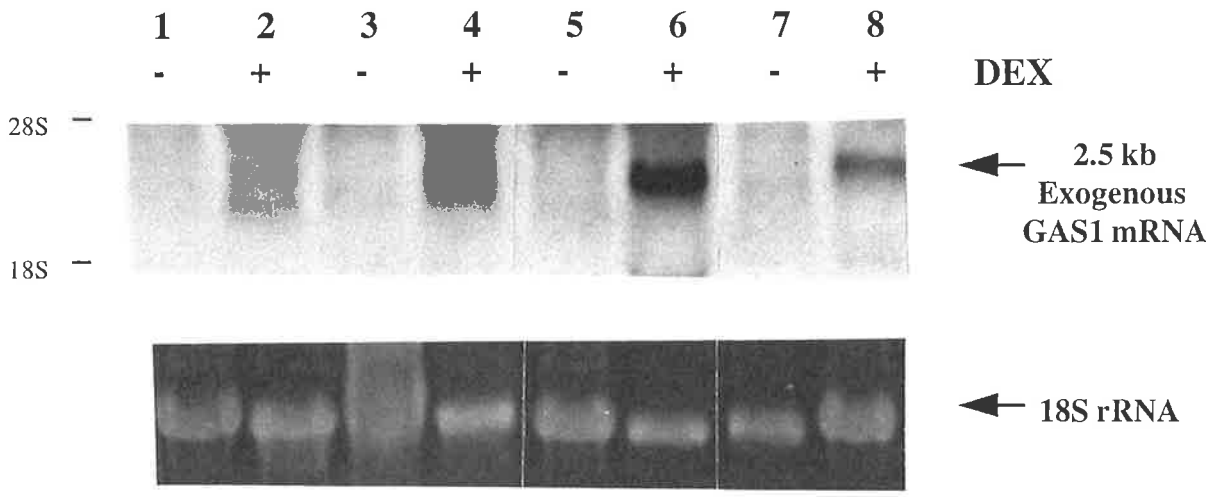
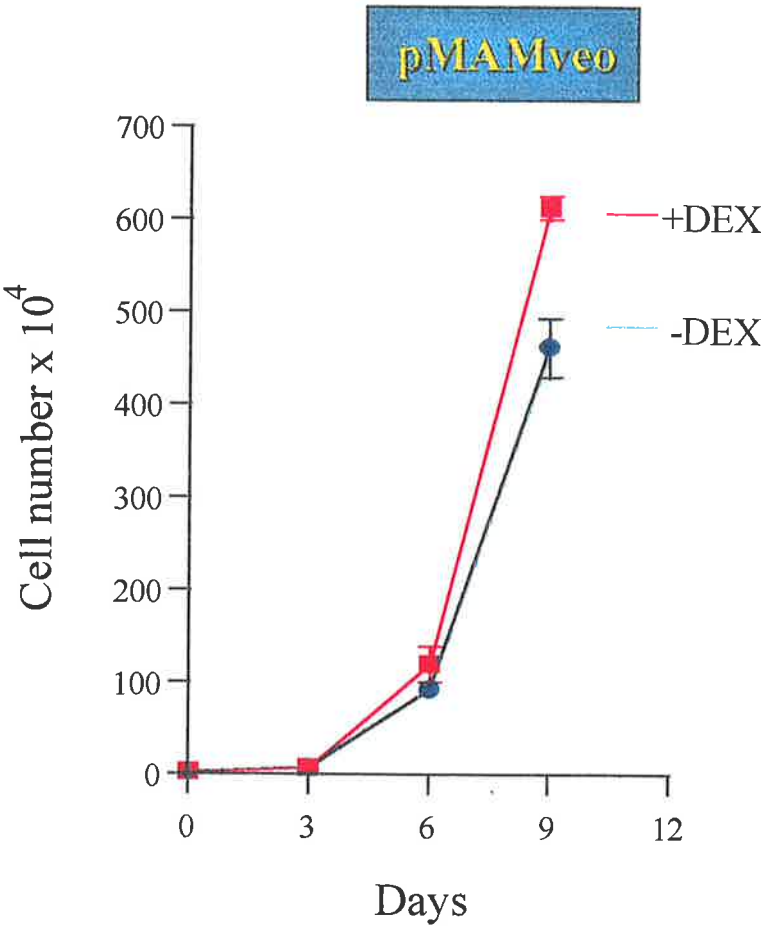


Figure 5.5 Induction of expression of GAS1 mRNA by dexamethasone in NIH3T3 cells transfected with the pMAMneo-GAS1 plasmid. Cells were incubated for 48 hrs in the absence (-DEX) or in the presence (+DEX) of 5 x 10^{-8} M dexamethasone. Northern blot analysis using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. pMAMneo vector only (lanes 1 and 2), pMAMneo-GAS1 mass culture (lanes 3 and 4), pMAMneo-GAS1 clone 3 (lanes 5 and 6) and pMAMneo-GAS1 clone 6 (lanes 7 and 8).

Figure 5.6 Inhibition of growth of NIH3T3 cells transfected with pMAMneo-GAS1. Cells were plated at 5 x 10^4 cells per 25 cm^2 flask in DMEM with 10% FCS in the absence (-DEX, blue line) or presence (+DEX, red line) of 1 x 10^{-6} M dexamethasone to induce expression of GAS1 mRNA. Cells were removed by trypsinization, collected and counted at the indicated times. (A) NIH3T3 cells transfected with pMAMneo-vector only. (B) NIH3T3-pMAMneo-GAS1 clone 3 cells. Each value is the mean ± standard error from duplicate flasks.

A


pMAMveo
700
600
500
400
300
200
100
0
Cell number x 10^4
+DEX
-DEX
0
3
6
9
12
Days

B

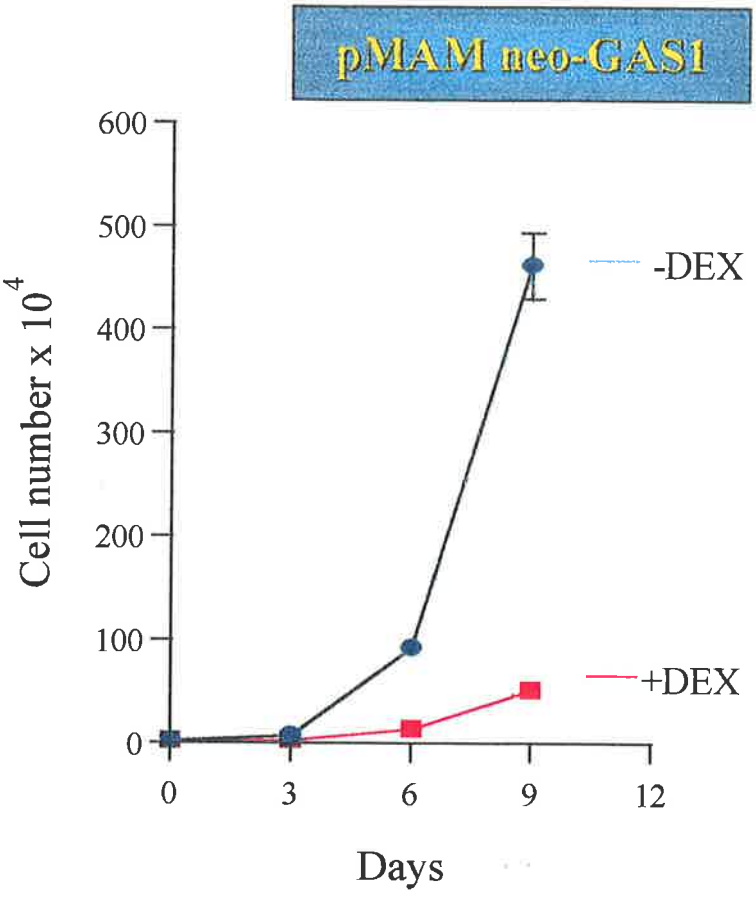
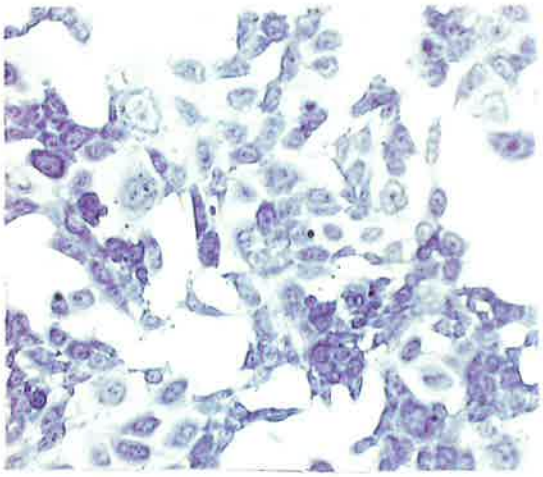

pMAM neo-GAS1
600
500
400
300
200
100
0
Cell number x 10^4
-DEX
+DEX
0
3
6
9
12
Days

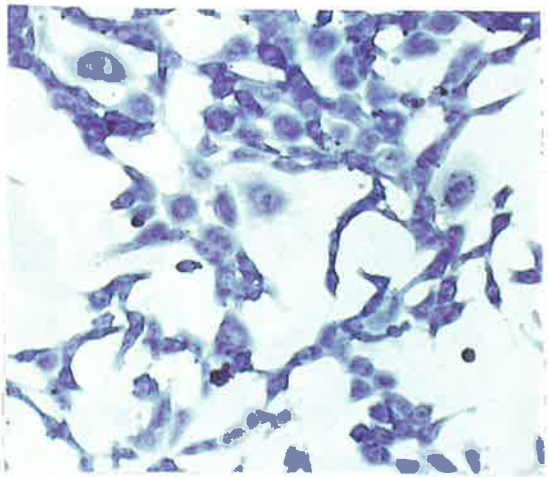
Figure 5.7 Characteristic morphology of NIH3T3-pMAMneo-GAS1 tranfected cells expressing GAS1 mRNA after induction with dexamethasone. Cells were plated at 5 x 10^4 cells per flask and grown for 72 hr in the absence (-DEX) or presence (+DEX) of 1 x 10^{-6} M dexamethasone. Cells were stained with Giemsa and photographed through an inverted microscope. (A) pMAMneo-vector transfectants. (B) pMAMneo-GAS1 clone 3 transfectants. (C) The same cells as in B (+DEX) but at 100x magnification.

A

pMAMneo-vector



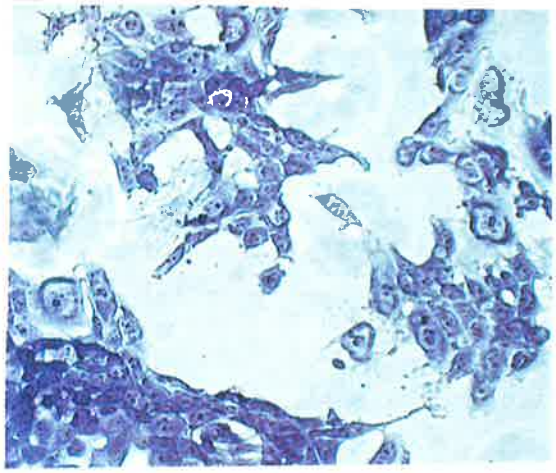
-DEX



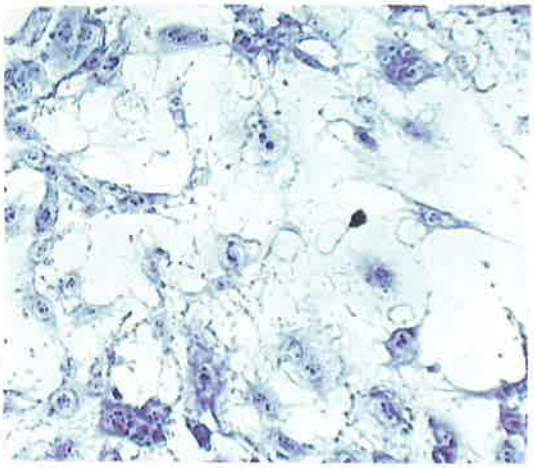
+DEX

B

pMAMneo-GAS1

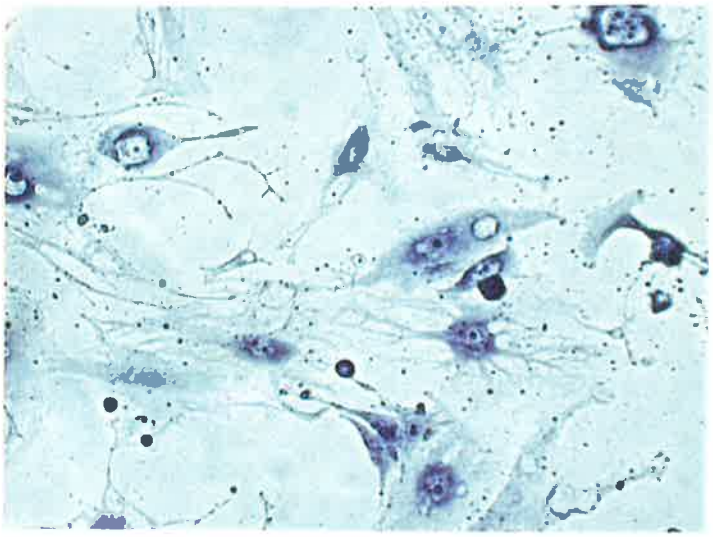


-DEX



+DEX

C

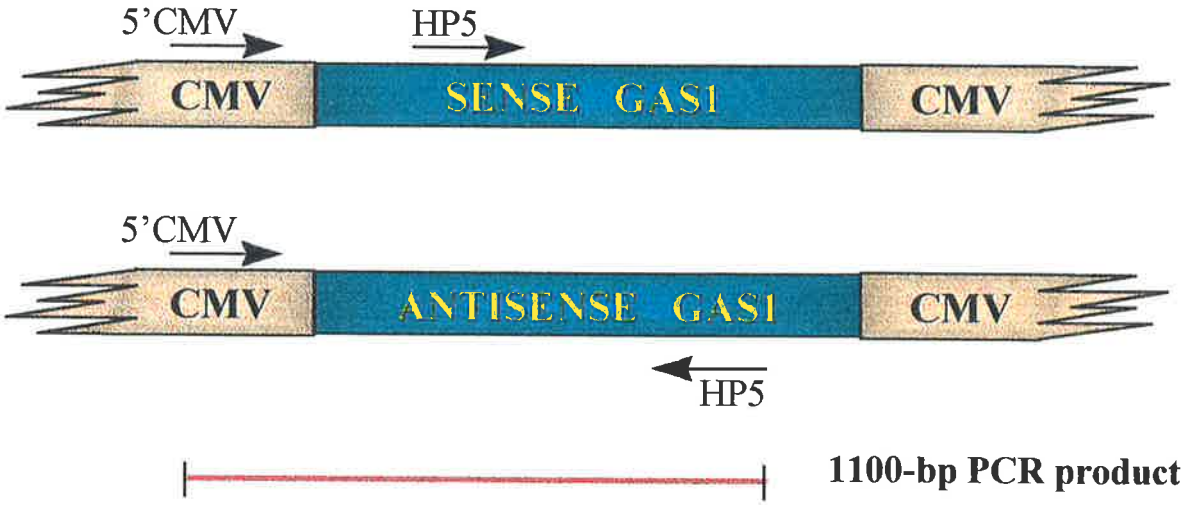


+DEX

5.3.5 Expression of antisense GAS1 mRNA in NIH3T3 fibroblasts.

To determine if antisense inhibition of expression of Gas-1 transforms normal fibroblasts, GAS1 cDNA was cloned into the constitutive pRcCMV plasmid vector in the antisense orientation (Fig 5.1) and transfected into NIH3T3 cells (5.2.2 and 2.2.21). Following selection for two weeks in the presence of G418, there was no significant difference in the number of colonies arising after transfection with the pRcCMV-GAS1 antisense expression plasmid when compared to the vector-only plasmid (Table 5.2). PCR analysis of DNA isolated from these cells confirmed the presence of the transfected antisense GAS1 plasmid (Fig 5.8). However, expression of antisense GAS1 mRNA could not be detected (Fig 5.9). Northern analysis of RNA isolated from the mass culture representing all clones containing the antisense GAS1 or the vector-only plasmids has shown that expression of the endogenous Gas-1 mRNA was inhibited. The Northern blot (Fig 5.9) shows, in the first three lanes, the typical changes of the endogenous expression of Gas-1 mRNA in vector-only transfected NIH3T3 cells grown under different conditions: Gas-1 mRNA was low in actively-growing cells (10% FCS) but was upregulated when cells were serum starved (0.5% FCS for 48 hr) or contact inhibited (grown to confluency in 10% FCS). However, in the antisense GAS1 transfectants, expression of the endogenous Gas-1 mRNA was not upregulated to the same level as in the control cells, suggesting that expression of antisense GAS1 mRNA effectively inhibited expression of endogenous Gas-1.

Transfection of NIH3T3 cells with the antisense GAS1 plasmid construct induced changes in morphology (Fig 5.10). Unlike the vector-transfected cells which displayed a normal morphology and were contact-inhibited when grown to confluency, the antisense GAS1 expresing cells grew in a criss cross fashion (Fig 5.10A) and failed



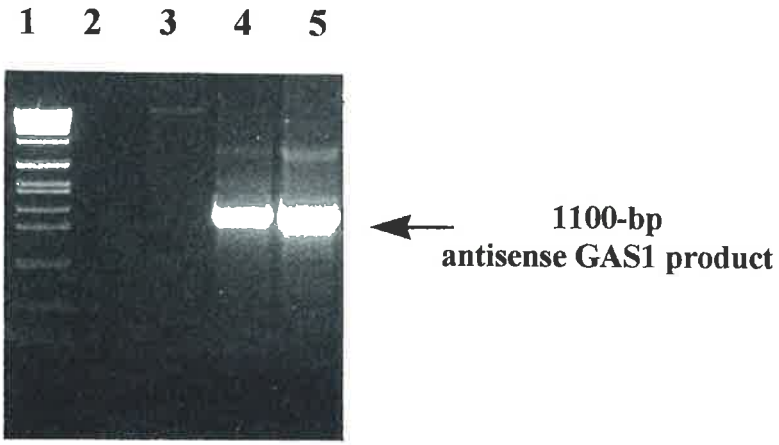


Figure 5.8 PCR analysis of DNA isolated from NIH3T3 cells transfected with the pRcCMV-vector-only or the pRcCMV-GAS1-antisense expression plasmids. The primers used were 5'CMV (upstream) and HP5 (downstream of the antisense GAS1 fragment). SPP-1 phage DNA digested with *Eco*RI (lane 1), negative control, no DNA (lane 2), pRcCMV-vector-only transfectants (lane 3), pRcCMV-GAS1-antisense transfectants (lane 4) and pRcCMV-GAS1-antisense plasmid was used as the positive control (lane 5). The expected 1100 bp amplification product is detected in the antisense GAS1 transfectants and not in the vector-only transfectants.

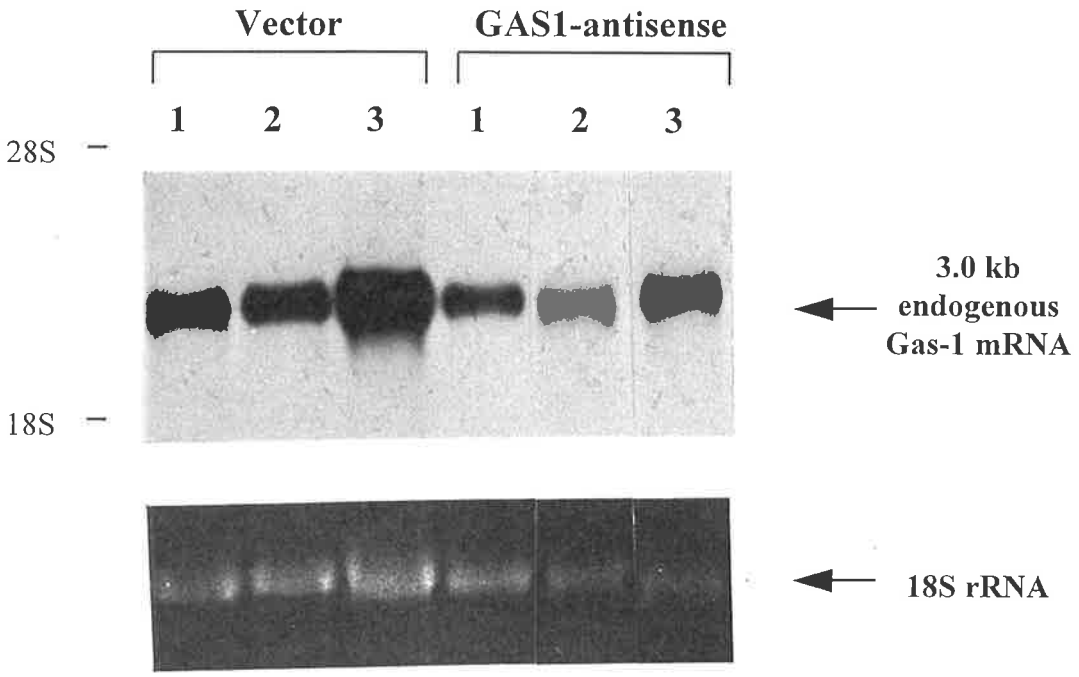
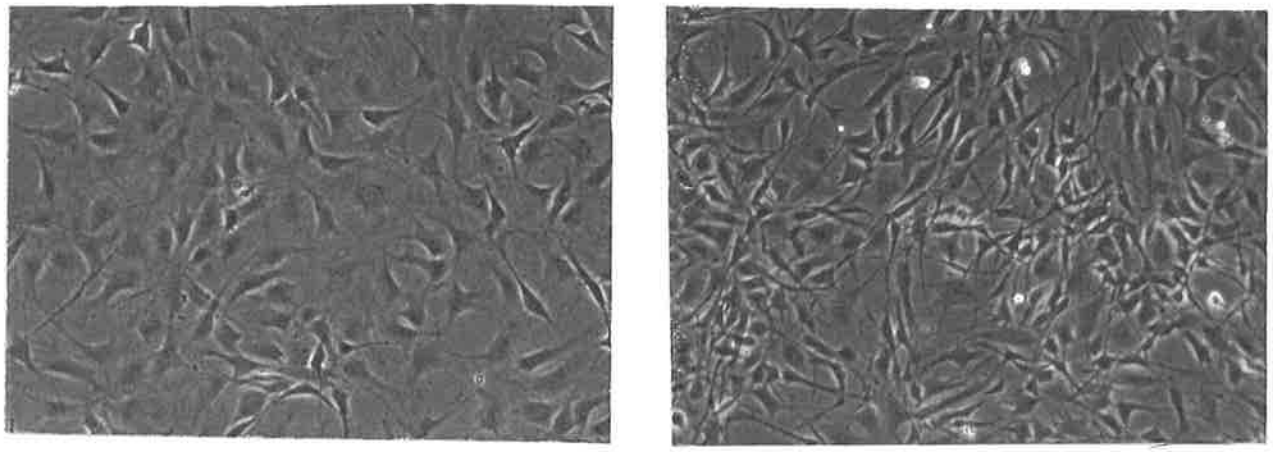


Figure 5.9 Expression of endogenous Gas-1 mRNA in NIH3T3 cells transfected with pRcCMV-GAS1-antisense or pRcCMV-vector-only plasmids. Cells were grown in the presence of 10% FCS (lanes 1), serum-starved for 48 hr in 0.5% FCS (lanes 2) or grown to confluency in 10% FCS (lanes 3). Northern blot analysis using a mouse Gas-1 cDNA probe (PA-1) is shown in the upper panel and a parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. Note that expression of the exogenous 2.0 kb antisense GAS1 mRNA could not be detected.

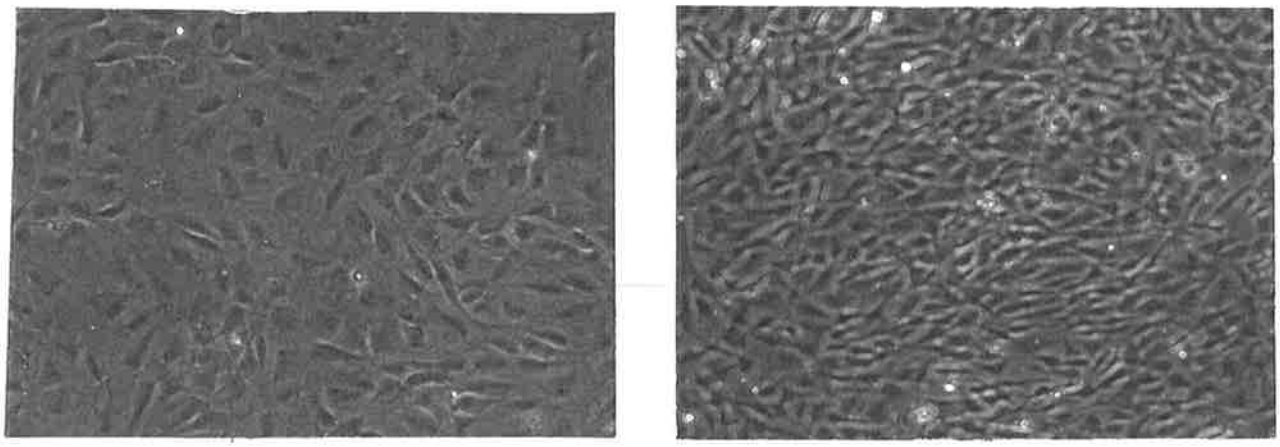
Figure 5.10 Morphological transformation of NIH3T3 cells transfected with the pRcCMV-antisense GAS1 plasmid. Cells were plated at 5 x 10^5 cells per 25 cm^2 flask in DMEM with 10% FCS. On days 3, 6 and 15, the cells were photographed through an inverted microscope (50x). In each case, the morphology of the cells was compared with the vector-only transfectants grown under the same conditions. (A) Morphology at day 3 of growth when cells are at subconfluent levels. (B) Morphology at day 6 of growth when cells approach confluency and vector-only transfectants are contact inhibited. (C) Morphology at day 15 of growth when cells have reached saturation densities. At this point there is a 2 fold increase in the number of antisense transfectant cells when compared to the vector only transfectants.

Vector GAS1-antisense

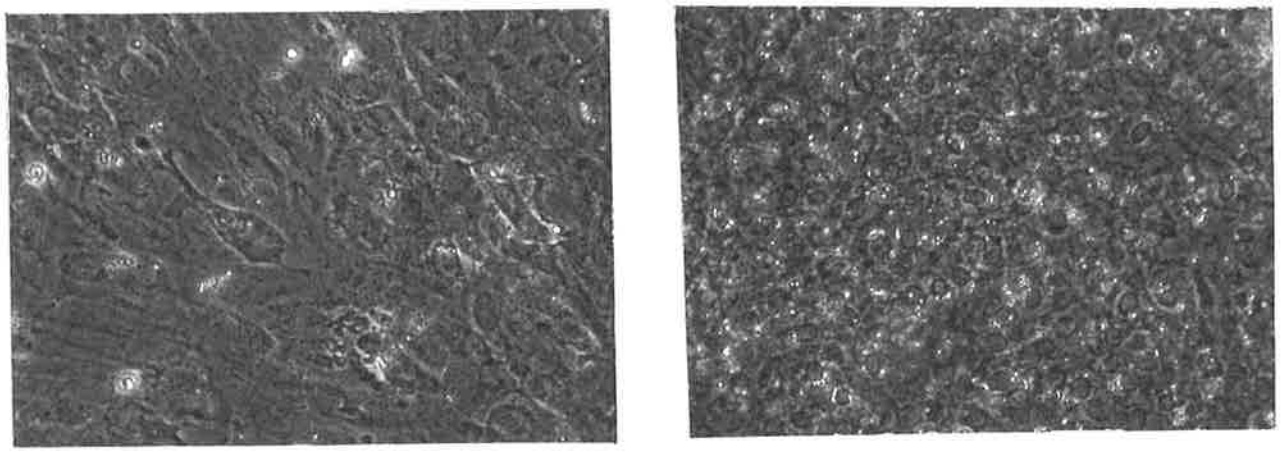
A 3 days : subconfluent



B 6 days : confluent



C 15 days : saturation

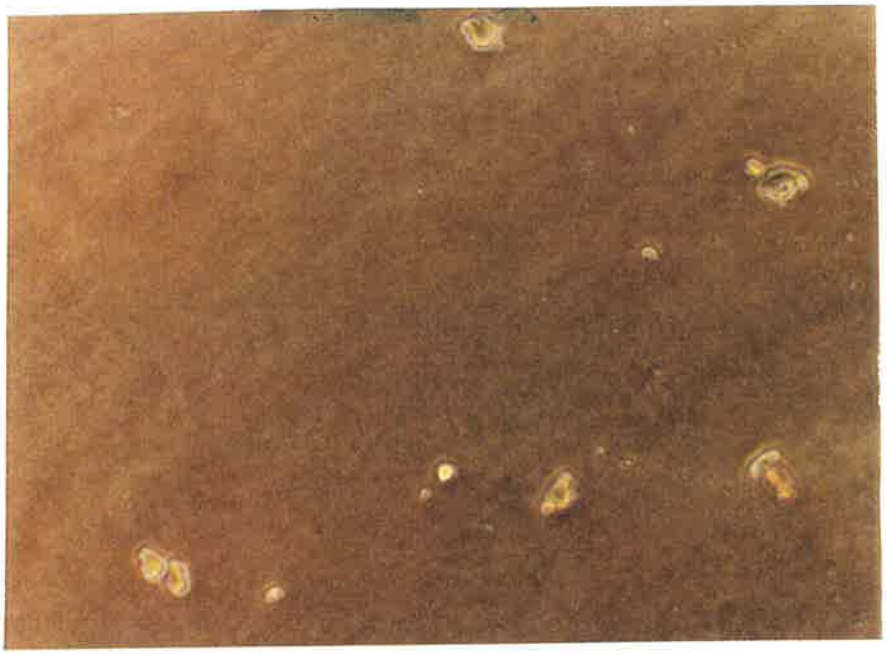


to undergo contact inhibition when grown for the same time (Fig 5.10B). At saturation densities, the GAS1- antisense transfectants grew as a multiple layer whereas the vector-only transfectants grew as a monolayer (Fig 5.10C). The mass culture was propagated further and the cells examined for development of transformed behaviour. When assessed for anchorage-independent growth, the antisense GAS1-expressing cells, failed to give rise to colonies in semisolid agar and remained as single cells like the vector control cells (Fig 5.11). Similarly when assessed for growth factor-independence by growing the cells in reduced serum (0.5% FCS), both antisense and vector control cells grew poorly under these conditions and could not be counted. When assessed for changes in growth rate in the presence of 10% FCS, the antisense GAS1 cells displayed a marked increase (2 fold) in the saturating cell densities at about day 15 of the growth curve but the rate of growth was unaltered during the exponential stage up to day 6 (Fig 5.12).

5.4 DISCUSSION

Since the mouse and human GAS1 genes share strong homologies in the nucleotide and amino acid sequences (Del Sal *et al.,* 1994), it was predicted that they would have the same function. The results presented here were the first to demonstrate that the human GAS1 gene is preferentialy expressed in growth arrested human fibroblasts and its pattern of expression is similar to the pattern of expression previously seen with the mouse homologue (Chapter 4). Human GAS1 mRNA was induced in serum starved fibroblast cells and was rapidly down-regulated when the same cells were activated to proliferate by the addition of serum. An antibody against GAS1 protein was

A **Vector**



B **GAS1-antisense**

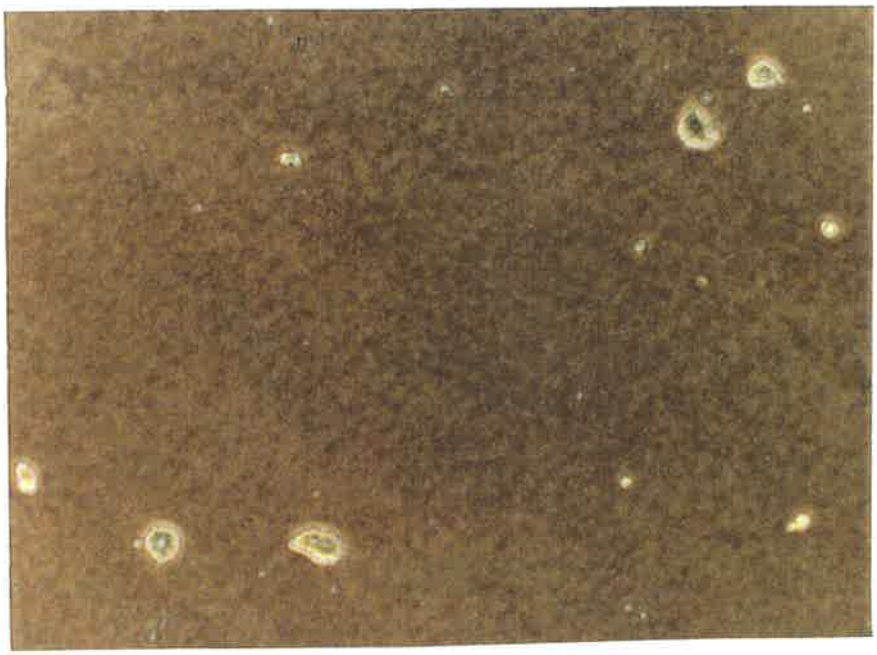


Figure 5.11 Failure of antisense GAS1-transfected cells to form colonies in semisolid agar. (A) NIH3T3 cells transfected with the pRcCMV-vector only. (B) NIH3T3 cells transfected with pRcCMV-GAS1-antisense.

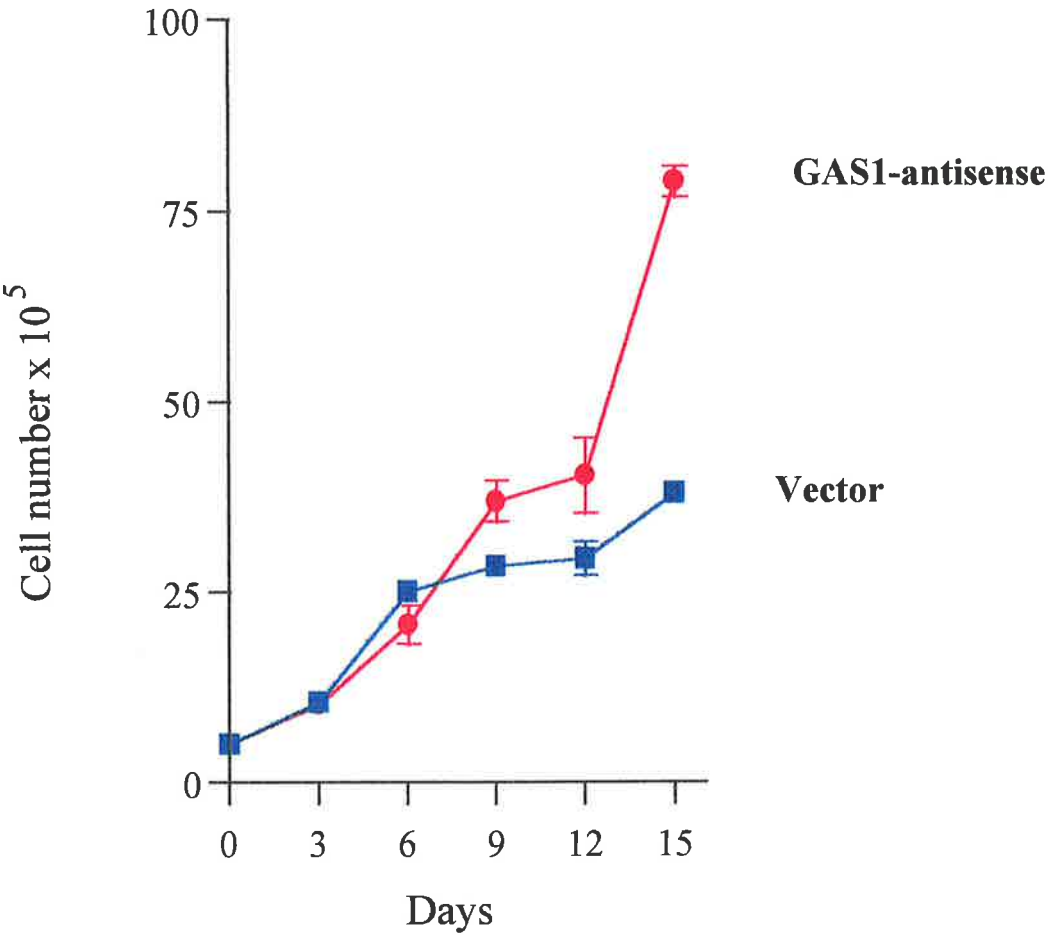


Figure 5 12 *In vitro* growth rate analysis of NIH3T3 cells transfected with pRcCMV-GAS1 antisense. Cells were plated at 5 x 10^5 cells per 25 cm^2 flask in DMEM with 10% FCS and propagated at 37 ^{0}C. On days 3, 6, 9, 12, and 15 cells were removed by trypsinisation and counted. Points; mean of duplicate flasks; *bars;*SE.

not available, so we were unable to determine if a similar pattern of expression holds true for the GAS1 protein. When compared to the high level of expression of mouse Gas-1 mRNA that is normally seen in growth-arrested mouse NIH3T3 cells (Chapter 4), the level of expression of GAS1 detected here in human cells does not appear to be up-regulated to the same extend. This would suggest that only small increases in the expression of GAS1 in human cells are required for biological activity resulting to growth arrest.

The expression of GAS1 mRNA could not be detected in growth-arrested IMR90 cells using conventional Northern analysis. Therefore, an RT-PCR based method was developed to detect expression of GAS1 in these cells. RT-PCR provides an effective method for detecting very small amounts of a specific mRNA sequence in a sample of total RNA. However the presence of contaminating DNA originating from the total RNA isolation method poses a problem, in that it is an equally effective template for amplification of the desired sequence as the resulting cDNA. One method to distinguish PCR products amplified from contaminating genomic DNA versus mRNA derived cDNA is to use primers that span an exon-exon junction in the DNA. This way, the presence of an intron in the genomic DNA template will yield a larger amplification product than that arising from the intronless cDNA (Mivechi and Rossi, 1990). However, GAS1, is an intronless gene, making this approach inapplicable. It was therefore necessary to eliminate contaminating genomic DNA directly from samples of RNA prior to RT-PCR using DNase treatment. The results of the RT-PCR demonstrated the presence of GAS1 mRNA in growth arrested IMR90 cells, whereas it could not be detected by conventional Northern analysis. The PCR-based method developed in this study provides an efficient way in which, low levels of expression of GAS1 could be detected. The growth arrest-specific pattern of expression of human GAS1 described in

this study was also confirmed by Del Sal *et al.*(1994). It was shown that the expression of human GAS1 was low in gowing human embryonic fibroblasts (HEF), while it was significantly increased when cells were arrested either by serum deprivation or by density dependent inhibition. A similar pattern of expression was also obtained in Western analysis using an anti-Gas-1 antibody (Del Sal *et al.,* 1994).

One phenotype of a gene that inhibits growth is the inability of cells expressing high levels of the gene product in transfection experiments to propagate. Expression of growth-inhibitory genes would be counterselected in this assay, whereas genes that have either no affect or a growth-stimulatory effect would be expressed at high levels in the propagating cells. To test this possibility, NIH3T3 fibroblasts were transfected with a plasmid construct that constitutively expressed high levels of GAS1 mRNA. The number of G418-resistant colonies that were recovered after transfection with the GAS1 plasmid was almost 9-fold lower than following transfection with the vector-only or with the antisense GAS1 constructs. The low number of GAS1 transfectants meant that RNA could not be isolated from these clones and thus we were unable to assess if the exogenous GAS1 transcript was expressed. However, the small size of the G418-resistant colonies and, more importantly, the pronounced morphological changes displayed by the GAS1-transfected cells suggested that the exogenous GAS1 gene was expressed. When the GAS1-transfected cells were propagated further as a mass culture, the morphology of these cells reverted to a normal phenotype and was similar to that of the vector-only control cells. The "giant" cells present in the GAS1 transfected colonies were no longer visible in the subsequent passage, probably because they were too fragile to survive the trypsinisation process. In addition, Northern analysis failed to detect the exogenous GAS1 transcript in the subsequent passage of the mass culture. These results suggested that high levels of expression of GAS1 were antiproliferative and these cells

must have either slowed growth, had growth-arrested or undergone cell death. Initially, the cells which presumably expressed high levels of GAS1 mRNA were unable to tolerate such levels and therefore resulted in cell death during the early stages of the selection period. The lower number of colonies that arose after complete selection supported this assumption and suggested that the GAS1-expressing cells were counterselected. However, the cells that did propagate following trypsinisation have most likely originated from cells that selectively lost the GAS1 gene while retaining the neomycin resistance gene, or expressed levels of GAS1 below a threshold detectable by Northern analysis and below a threshold critical for altered biological activity. The results presented here are consistent with selection pressure against the continued over-expression of exogenous GAS1 in NIH3T3 cells.

The growth-suppressive activity of GAS1 was confirmed using an inducible expression plasmid. In contrast to the constitutive transfection experiments, transfection with the dexamethasone-inducible GAS1 construct avoided the selection pressure against cells expressing GAS1. As expected, there was no significant difference in the size or number of G418-resistant colonies between GAS1 or vector-only transfectants since the inducer was absent during G418 selection. Stable GAS1 transfectant cell lines were therefore propagated. Expression of GAS1 was induced with dexamethasone and clones were analysed for growth parameters. Expression of the transfected GAS1 mRNA was highly inducible and variable levels of GAS1 mRNA were detected in a number of clones after the addition of dexamethasone. Exogenous GAS1 mRNA was not detected in the absence of dexamethasone, suggesting that the MMTV promoter was not leaky in the absence of the inducer. One clone that expressed the highest level of dexamethasone-inducible GAS1 mRNA was analysed further. This clone displayed a significant reduction in growth rate when compared to the vector control cells. In

addition, there were also pronounced morphological changes in cells induced to express GAS1 after the addition of dexamethasone. GAS1-expressing cells were flat with a very large cytoplasm and were similar in appearance to the "giant" cells previously seen in the cells transfected with the constitutive GAS1-expressing plasmid. Dexamethasone treatment of the vector-only control cells had no effect on either the growth rate or morphology. This result, together with the similarities in morphology of the cells expressing constitutive- and dexamethasone-inducible-GAS1 in the two independent transfection experiments suggested, that the altered morphology and the suppression of growth observed in the GAS1-expressing cells were due to the growth inhibitory effects of GAS1 and not due to the effects of dexamethasone.

The findings reported here establish that the human GAS1 gene, when overexpressed in NIH3T3 cells, is able to block proliferation in an asynchronously growing population of cells. These results were independently confirmed by the subsequent findings reported by Del Sal *et al.* (1992) who showed that transient overexpression of the mouse Gas-1 gene blocks cell proliferation and does not allow the Go-S-phase transition in serum stimulated quiescent fibroblasts. The study presented here, has also shown that cells overexpressing exogenous GAS1 display remarkable morphological changes characteristic of growth-arrested cells or cells in crisis. This result was not possible in the microinjection experiments of Dal Sal *et al.* (1992). The results presented here of the growth suppressive effects of the human GAS1 gene clearly demonstrate that the human GAS1 gene product is actively involved in mediating growth suppression in a similar manner to the mouse homologue.

This study has also demonstrated, that inhibition of expression of endogenous Gas-1 by the antisense GAS1 plasmid induces a change in phenotype characteristic of transformed behaviour. Antisense GAS1 transfected cells grew in a criss cross manner

and failed to undergo contact inhibition when grown to cnfluency. The antisense GAS1 cells grew to a higher saturation density but their growth rate was unaltered during the exponential growth phase. This is consistent with the idea that antisense GAS1 would not be expected to have an effect in inhibiting Gas-1 function during the exponential phase, since expression of the endogenous Gas-1 mRNA is low and at basal levels at this stage. However, inhibition of endogenous Gas-1 would be expected to have an effect at a time when the endogenous levels of Gas-1 mRNA are upregulated by confluence during the later stages of the growth curve. Expression of antisense GAS1 at this particular stage inhibited the up-regulation of endogenous Gas-1 and prevented cells from contact inhibition, hence the observed increase in saturation density. However, when assessed for limited growth factor requirements, the antisense GAS1 transfected cells showed no difference in their ability to grow in low serum when compared to vector-only transfectants. In fact, both populations of cells grew poorly under these conditions. Similarly, the antisense GAS1 transfected cells failed to produce colonies in semisolid agar, as did the vector control cells, suggesting they had not developed anchorage-independence, another measure of transformed behaviour. These results demonstrated that antisense inhibition of expression of Gas-1 was insufficient to fully transform NIH3T3 cells. However this may be related to the efficiency of antisense inhibition, since only a moderate reduction of the endogenous Gas1 mRNA was observed in these experiments. Because of the low efficiency by which the antisense GAS1 plasmid reduced the endogenous levels of Gas-1, this reduction was not considered sufficient to carry out studies in nude mice.

One of the problems that has often been associated with antisense experiments is the inability to document antisense RNA transcripts in the transfected cells even though a pronounced biological effect is often observed (Kassid *et al.,* 1989; Nishikura *et al.,*

1987). This case was no exception since at no time we were able to demonstrated that the antisense GAS1 mRNA was expressed despite the presence of the antisense GAS1 plasmid. Nevertheless, these first efforts demonstrate that antisense GAS1 can partially, though not completely, lead to cellular transformation at least in NIH3T3 cells and that additional genetic events may be required for the cells to develop a fully-transformed phenotype.

CHAPTER 6

TUMOUR-SUPPRESSIVE ACTIVITY OF GAS1 IN HUMAN CELL LINES

6.1 INTRODUCTION

The findings reported in the previous chapter established that the human GAS1 gene has growth inhibitory activity and when ectopically over-expressed is able to induce growth arrest in NIH3T3 mouse fibroblasts. Gene products with growth-inhibitory properties may frequently act as tumour suppressors. The retinoblastoma (RB) and p53 genes are prime examples of genes which inhibit cellular proliferation and are frequently mutated or deleted in a wide range of human cancers (reviewed in: Velculescu *et al.,* 1996 and Cordon-Cardo *et al.,* 1995). Over-expression of wild-type (wt) p53 inhibits the growth of both normal and transformed cells *in vitro* (Merlo *et al.,* 1994) and inhibits tumorigenicity in a nude mouse assay, of a range of human tumour cell lines (Baker *et al.,* 1990; Mercer *et al.,* 1990; Takahashi *et al.,* 1992). Similarly, over-expression of wild-type RB in tumour cells, which contain mutant or deleted RB, suppressess proliferation and tumorigenicity (Huang *et al.,* 1988; Takahashi *et al.,* 1991). Following transfection of RB- or p53-expressing gene constructs, proliferation *in vitro* of transformed cells is blocked just prior to or at the restriction point in late G1, before the G1-S transition (Lin *et al.,* 1992; Livingston *et al.,* 1993), emphasising the central importance of the activity of growth-controlling genes in maintaining a normal phenotype. In addition, protein products of transforming DNA tumour viruses like the SV40 large T antigen (Jiang *et al.,* 1993), adenovirus E1B (Yew *et al.,* 1992) and papilloma virus type 16 E6 (Mietz *et al.,* 1992) transforming proteins bind and inactivate RB or p53 (reviewed in: Vousden *et al.,* 1995). Similarly the MDM2 proto-oncogene product interacts physically and functionally with both RB and p53 gene products and can inhibit their growth-regulatory capacity (Oliner *et al.,* 1992). The

inactivation of growth-inhibitory genes, as may be the case for *GAS1* by a range of mechanisms, may contribute to the multi-step development of the malignant phenotype.

Overexpression of the mouse Gas-1 gene by microinjection of a Gas-1-expressing plasmid into normal and oncogene-transformed NIH3T3 cells (with the exception of SV40-transformed NIH3T3 cells), blocks proliferation of actively-growing cells and inhibits entry by quiescent cells into S phase when activated by serum (Del Sal *et al.,* 1992). Recently it was shown by Del Sal *et al.* (1994) that over-expression of human GAS1 suppresses proliferation in the T24 bladder carcinoma and in the A549 lung adenocarcinoma cell lines, but not in the osteosarcoma-derived cell line, SAOS-2, nor in the adenovirus-type-5-transformed cell line, 293. The GAS1-resistant cell line, SAOS-2, does not contain any functional RB or p53 gene products, suggesting that the activity of these genes may have a role in mediating the growth-inhibitory activity of GAS1. Similarly, transformation by adenovirus type 5 may inactivate RB and p53 in the 293 cell line, resulting in resistance to GAS1. Overexpression of Gas-1 in NIH3T3 fibroblasts blocks proliferation in a p53-dependent manner but the transcriptional activation function of p53 is not required for Gas-1-induced growth arrest. However, the presence of a functional RB protein did not seem to be required for responsiveness to Gas-1 (Del Sal *et al.,*1995).

In this study, stable GAS1-expressing cell lines were generated to investigate further the growth-suppressive activity of GAS1 *in vitro* and to test the ability of GAS1 to suppress the growth of human tumours in nude mice. The A549 lung adenocarcinoma and the HTD114 cell lines were selected because they failed to express endogenous GAS1 mRNA, even when propagated under conditions leading to growth arrest. The use of the A549 lung adenocarcinoma cell line, in these investigations and those of Del Sal *et al.* (1994) was coincidental. A549 lung adenocarcinoma cells express wild type

p53 (Lehman *et al.,* 1991). This study has shown that overexpression of GAS1 inhibited the proliferation of A549 cells *in vitro* and suppressed tumorigenicity in nude mice. In contrast, the HTD114 cells were refractory to the effects of overexpression of GAS1 despite the presence of wild type p53 in these cells. The possible mechanisms for the failure of HTD114 cells to respond to overexpression of GAS1 are investigated.

6.2 MATERIALS AND METHODS

6.2.1 Cell lines used for transfections.

The cell lines used in this study were: (a) A549 lung adenocarcinoma cell line, which contains wild-type p53 (Lehman *et al.,* 1991) and was obtained from the American Type Culture Collection (ATCC); (b) HTD114 (an APRT-negative sub-line of the HT1080 fibrosarcoma cell line), previously described by Stambrook *et al.* (1983); (c) HT1080wtp53 was provided by Dr R Reddel and contains wild type p53 (Sharma *et al.,* 1993) (d) HT1080 6TGc5 was provided by Dr E. Stanbridge, contains mutant p53 and was described by Benedict *et al.* (1984). All four cell lines were maintained in a Dulbecco's Modified Eagles medium (DMEM), pH7.4, supplemented with 10% fetal calf serum. Cells were grown at 37^{o}C in a water saturated 5% CO_2 atmosphere and were subcultured 3 times per week after trypsinisation with 0.05% trypsin/0.53 mM EDTA.

6.2.3 Transfections.

Transfections were performed with 10 μg of either vector DNA or GAS1 plasmid constructs (5.2.2) and 20 μg of inert herring sperm DNA (Sigma) as the carrier, using a standard calcium phosphate precipitation method (2.2.21). Twenty four hours after transfection, the DNA precipitate was removed by washing the monolayers with fresh culture medium and the cells were allowed to recover in standard culture medium for a further 24 hr. Transfectant populations were then selected by culturing cells for 2 weeks in the presence of 400-800 μg/ml G418. The number of G418-resistant colonies was counted and individual colonies were manually picked and propagated in the continued presence of G418 for subsequent analysis. Genomic DNA was isolated from the G418-resistant clones using standard techniques and the presence of the transfected GAS1 gene confirmed by PCR analysis using the primers and strategy previously outlined (2.2.22).

6.2.4 Tumorigenicity in nude mice.

Cell lines (10^6 cells in 0.1ml) were injected subcutaneously into the dorsal surface of 6-8-week old male Balb/c athymic nude mice. A549 cells containing the inducible human GAS1 construct were maintained in 1 x 10^{-6} M dexamethasone for 48 hr prior to implantation to induce expression of GAS1 mRNA. The mice were given 0.28 mg/L dexamethasone in palatable drinking water (Narayanan *et al.*, 1992) and the amount of water consumed was monitored periodically. Mice injected with HT1080 and HTD114 transfectant clones were not given any dexamethasone. The volume of each tumour was measured weekly using callipers and animals were sacrificed when tumours reached an approximate size equivalent to 1000 mm^3. Tumour volumes were calculated

using, as an approximation, the formula for volume of a hemi-ellipsoid: $\pi(\text{Width}^2 \text{ X Length})/6$.

6.2.5 Analysis of tumours.

Mice were sacrificed by cervical dislocation, tumours were dissected out under sterile conditions and chopped finely into standard culture medium. Suspensions of tumour cells were transferred into 25 cm^2 flasks containing standard culture medium supplemented with 400 μg/ml G418 to select against contaminating normal mouse cells. Cells were expanded and analyzed for the presence and expression of the transfected GAS1 gene as described (5.2.2).

6.3 RESULTS

6.3.1 Constitutive expression of GAS1 in A549 cells.

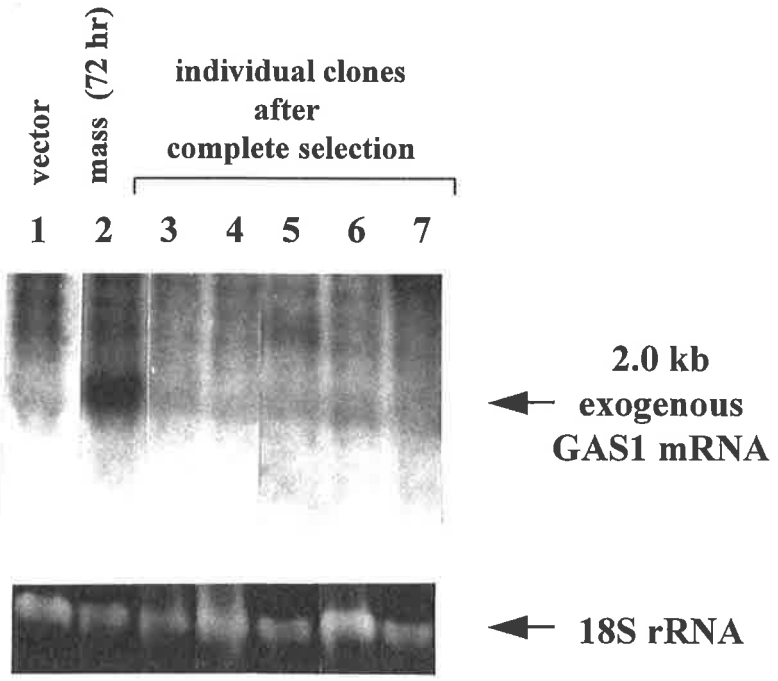
A549 lung adenocarcinoma cells were transfected with the constitutive pRcCMV-GAS1 expression plasmid or the control vector pRcCMV. Seventy two hours after transfection, total RNA was isolated and assayed by Northern blotting for expression of GAS1 mRNA. The 2.0 kb exogenous transcript was detected in cells transfected with the GAS1 plasmid but was absent in cells transfected with the control vector (Fig 6.1A). No morphological differences were detectable between the GAS1-expressing cells and control cells. Following selection for two weeks in G418, 3-5 fold fewer colonies arose in two independent experiments after transfection with pRcCMV-

Figure 6.1 Constitutive expression of exogenous GAS1 mRNA and detection of the exogenous plasmid in A549 cells transfected with pRcCMV-GAS1.

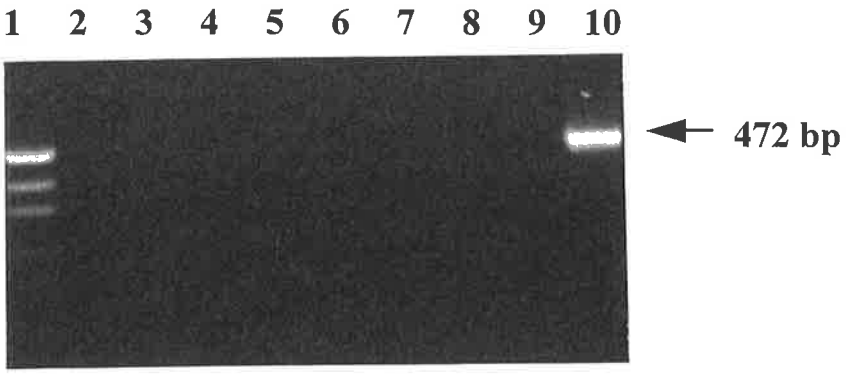
(A) Northern blot analysis using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. A549 cells were transfected with pRcCMV control vector-only (lane 1) or pRcCMV-GAS1 (lane 2) and RNA from the mass cultures was harvested 72 hr post transfection. Lanes 3-7 contain mRNA isolated from five randomly-selected pRcCMV-GAS1 clones after complete selection for three weeks in G418.

(B) PCR amplification of a 472 bp fragment from the transfected GAS1 plasmid in the same five clones and a mass culture after complete selection in G418. *Hpa*II-digested pUC19 marker (lane 1), pRcCMV-GAS1 clones (lanes 2-6), mass culture of pRcCMV-GAS1 cells after complete selection (lane 7), vector-only transfectant mass culture (lane 8), negative control with no DNA (lane 9), and positive control containing the pRcCMV-GAS1 plasmid used for the transfections (lane 10)

A


vector
mass (72 hr)
individual clones after complete selection
1 2 3 4 5 6 7
2.0 kb exogenous GAS1 mRNA
18S rRNA

B


1 2 3 4 5 6 7 8 9 10
472 bp

GAS1 compared to vector alone (Table 6.1). As an additional control, the same GAS1 fragment, cloned into pRcCMV in an antisense orientation, produced approximately the same number of colonies as the vector (Table 6.1). Although some G418-resistant colonies arose following transfection with pRcCMV-GAS1, the reduced number suggested that expression of GAS1 mRNA inhibited the proliferation of A549 cells.

Table 6.1 Growth of G418-resistant colonies two weeks after transfection with GAS1 plasmid constructs

CONSTRUCT	A549 (wtp53)		HTD114 (wtp53)		HT1080 (wtp53)		HT1080 6TGc5 (mutp53)	
	1	2	1	2	1	2	1	2
pRcCMV	98	110	120	161	207	223	305	
pRcCMV-GAS1	26	22	115	148	54	92	288	
pRcCMV-GAS1 (antisense)	88							
pMAMneo	21	45	55		78		148	
pMAMneo-GAS1	22	43	45		84		198	

Cells were transfected with plasmid vectors as shown and selection in G418 allowed to proceed for 2 weeks. The number of colonies in each flask was counted under an inverted microscope. In some cases the experiments were independently duplicated

Any colonies that arose after transfection of GAS1 may have selectively lost expression of the transfected GAS1 gene, while retaining a functional neomycin resistance gene. To test this prediction, five randomly-selected clones were examined for the presence and expression of the transfected GAS1 gene. Northern analysis

demonstrated that these clones did not express exogenous GAS1 mRNA (Fig. 6.1A), although transient high levels of expression of GAS1 had been detected in the mass culture 72 hr after transfection. PCR analysis of genomic DNA from the same G418-resistant clones showed that the transfected pRcCMV-GAS1 plasmid was present 72 hours after transfection but was absent in the completely selected G418-resistant individual clones (Fig. 6.1B). Taken together, these results strongly suggest that the continued expression of GAS1 mRNA is incompatible with proliferation of A549 cells.

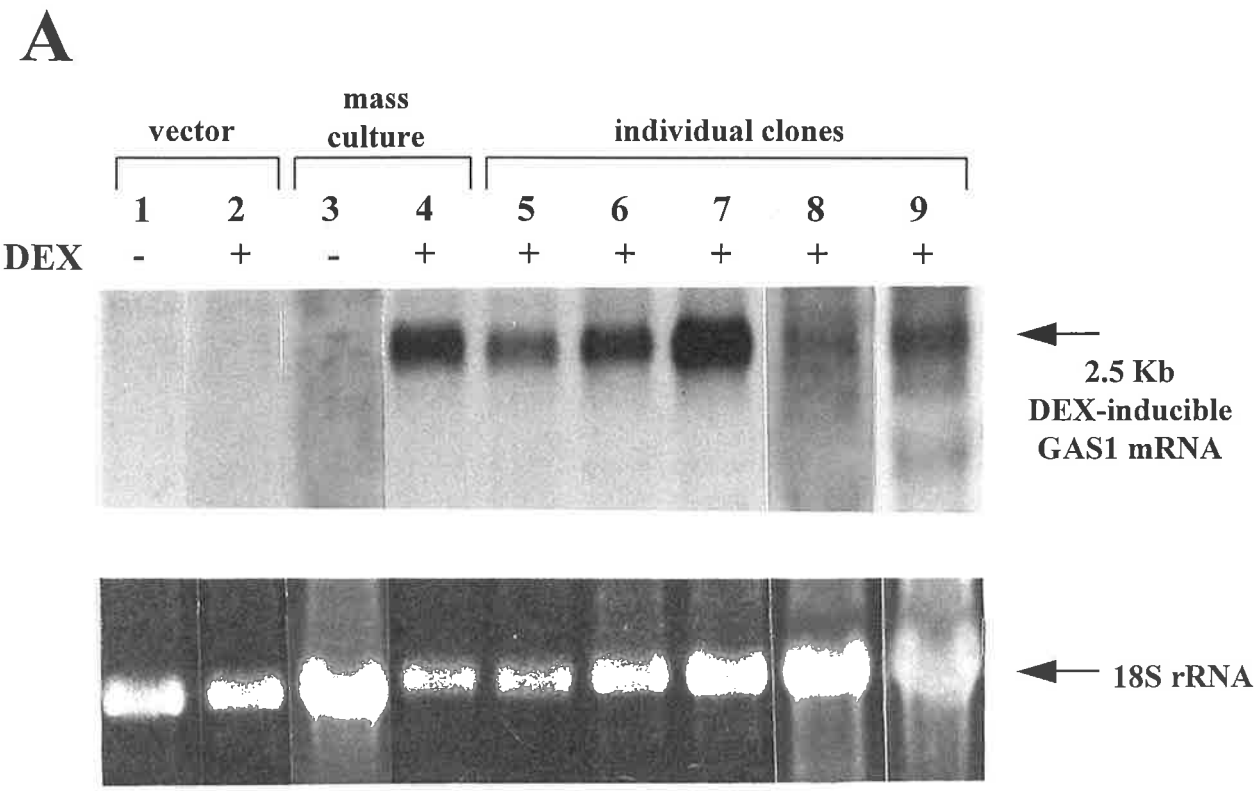
6.3.2 Dexamethasone-inducible expression of GAS1 mRNA.

Since we were not able to isolate and propagate any clones that constitutively expressed high levels of GAS1 mRNA, the response of A549 cells to over-expression of GAS1 could not be examined. It was therefore necessary to use an inducible vector system to limit expression of GAS1 to defined times. The pMAMneo vector was chosen, in which a promoter derived from the dexamethasone-inducible mouse mammary tumour virus (MMTV), controls the expression of GAS1 mRNA. A549 cells were transfected with the pMAMneo-GAS1 construct, propagated for 2 weeks in the presence of 400 μg/ml G418 and the number of colonies counted. Cells transfected with the GAS1 construct formed the same number of G418-resistant colonies as cells transfected with the vector only (Table 6.1). Five randomly selected clones and a mass culture of G418-resistant clones were examined for the presence and expression of the exogenous GAS1 gene. Expression of the transfected GAS1 mRNA was induced by incubating the clones for 48 hr in 5 x 10^{-8} M dexamethasone. Northern analysis detected variable levels of the 2.5 kb exogenous GAS1 transcript in the mass culture and in all five clones analysed (Fig 6.2A). PCR analysis of genomic DNA demonstrated the presence of the pMAMneo-GAS1 plasmid construct in all five clones and the mass

Figure 6.2 Induction of expression of GAS1 mRNA by dexamethasone in A549 cells transfected with the pMAMneo-GAS1 plasmid.

(A) RNA was isolated from G418-resistant clones transfected with either the pMAMneo or pMAMneo-GAS1 vectors and incubated for 48 hr in the absence or in the presence of 5×10^{-8} M dexamethasone. Northern blot analysis using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. Vector-only control transfectants (lanes 1 and 2), RNA isolated from the mass culture transfected with pMAMneo-GAS1 (lanes 3 and 4) and pMAMneo-GAS1 clones A1-A5 (lanes 5-9).

(B) PCR amplification of a 367 bp DNA fragment from the pMAMneo-GAS1 plasmid. *Hpa*II-digested pUC19 marker (lane 1), pMAMneo-GAS1 clones A1-A5 (lanes 2-6), mass culture transfected with pMAMneo-GAS1 (lane 7), negative control with no DNA (lane 8) positive control containing the pMAMneo-GAS1 plasmid used for the transfections (lane 9).


A
vector
mass culture
individual clones
1 2 3 4 5 6 7 8 9
DEX - + - + + + + + +
2.5 Kb DEX-inducible GAS1 mRNA
18S rRNA
B
1 2 3 4 5 6 7 8 9
367 bp

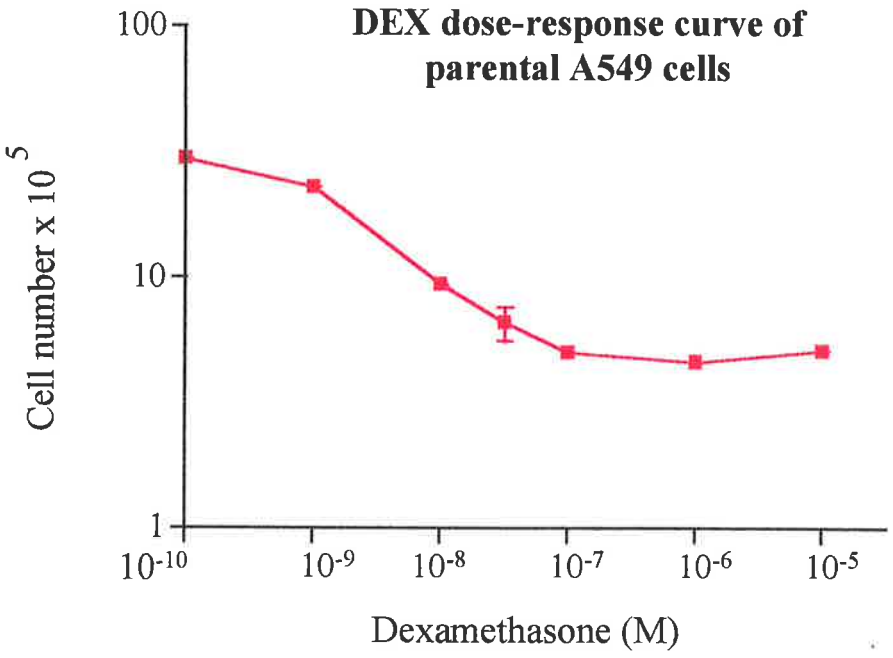
culture (Fig. 6.2B). There was no detectable expression of GAS1 in the mass culture in the absence of dexamethasone (Fig 6.2A, lane 3), indicating that, the MMTV promoter was not leaky and there was no transcription from the GAS1 cDNA in the absence of dexamethasone. GAS1-transfectant cell lines could therefore be propagated and expression of GAS1 induced for further analysis.

Treatment with dexamethasone alone inhibited the proliferation of parental A549 cells over a range from 1 x 10^{-9} to 5 x 10^{-8} M with little additional inhibition at higher concentrations to 1 x 10^{-6} M (Fig 6.3A). To determine the lowest concentration of dexamethasone capable of inducing expression of GAS1 without affecting growth of parental cells, a dexamethasone dose-response analysis was performed. Cells were seeded in duplicate 25cm^2 flasks at a concentration of 5 x 10^5 cells /flask in the presence of increasing concentrations of dexamethasone. RNA was isolated 48 hr later and analysed for expression of the exogenous GAS1 gene by Northern blotting. Expression of the exogenous GAS1 gene was detected following treatment with a dexamethasone concentration as low as 1 x 10^{-8} M and was concentration-dependent, with the highest levels of expression seen with 1 x 10^{-6} M dexamethasone (Fig. 6.3B). However, incubation of the GAS1-transfectant cells with 1 x 10^{-9} M dexamethasone did not induce any detectable expression of GAS1 mRNA although it did partially inhibit proliferation of parental A549 cells. Therefore, in subsequent experiments, a concentration of dexamethasone was used (1 x 10^{-6} M) in which no further growth suppression of parental cells occurred and in which maximal expression of exogenous GAS1 was detected. This would allow differentiation between growth suppression induced by GAS1 in addition to the baseline suppression induced by dexamethasone alone.

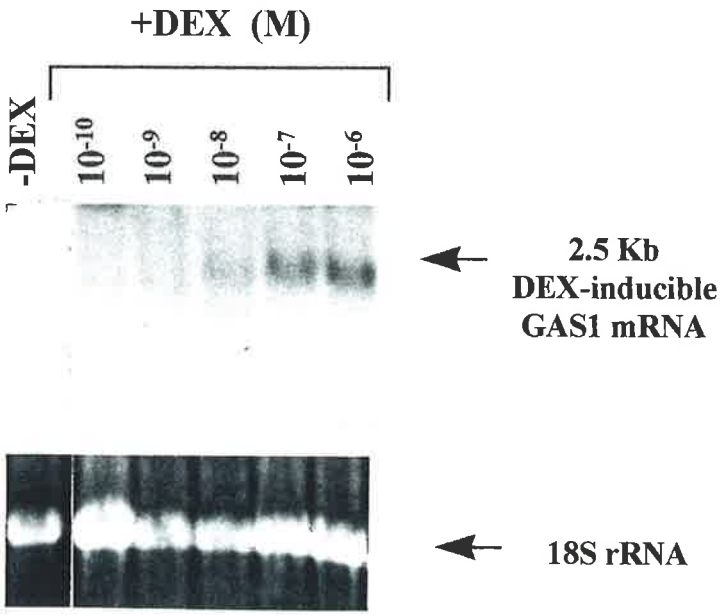
Figure 6.3 (A) Inhibition of growth of parental A549 cells by dexamethasone. Cells were plated at 2 x 10^4 cells per 25cm^2 flask in DMEM with 10% FCS. On the second day, dexamethasone was added at the indicated concentrations. On day 7, cells were removed by trypsinization, collected and counted. *Points;* mean of duplicate flasks; *bars;* S.E.

(B) Effect of concentration of dexamethasone on the expression of GAS-1 mRNA. A G418-resistant clone (A5) of A549 cells transfected with pMAMneo-GAS1, was incubated with a range of dexamethasone concentrations (1 x 10^{-10} M - 1 x 10^{-6} M) Twenty-four hours later, RNA was harvested and expression of GAS1 mRNA analysed by Northern blotting (upper panel). Parallel ethidium bromide staining pattern of control 18S rRNA is shown in the lower panel.

A


DEX dose-response curve of parental A549 cells
Cell number x 10^5
100
10
1
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
Dexamethasone (M)

B


+DEX (M)
-DEX
10^-10
10^-9
10^-8
10^-7
10^-6
2.5 Kb
DEX-inducible
GAS1 mRNA
18S rRNA

Cells were seeded at a concentration of 5 x 10^4 cells per $25cm^2$ flask in the presence or absence of 1 x 10^{-6} M dexamethasone and grown for 7 days. The cells were then harvested by trypsinization and counted in a hemocytometer. Induction of expression of GAS1 by dexamethasone resulted in a significant reduction in cell number (40%-50%) in the three GAS1-transfectant clones compared to the controls (Fig. 6.4A). No differences in morphology were evident between the GAS1-expressing and control cells. Thus, although a marked inhibition of proliferation of A549 cells occured in the presence of dexamethasone alone, by using appropriate vector-only controls, we were able to detect additional suppression of growth in the GAS1-expressing transfectant cells when compared to those cells transfected with the vector alone.

Expression of exogenous GAS1 by A549 clones A3 and A5 was monitored following culture in the continued presence of 1 x 10^{-6} M dexamethasone from 24 hr to 15 days. The exogenous GAS1 transcript could easily be detected 24 hours after induction of expression with dexamethasone, but decreased, until it was no longer detectable after 15 days of continuous induction (Fig. 6.4B). Presumably, due to the antiproliferative activity of GAS1, a negative selection pressure existed against cells which continued to express GAS1 and sub-lines of cells arose that no longer expressed GAS1.

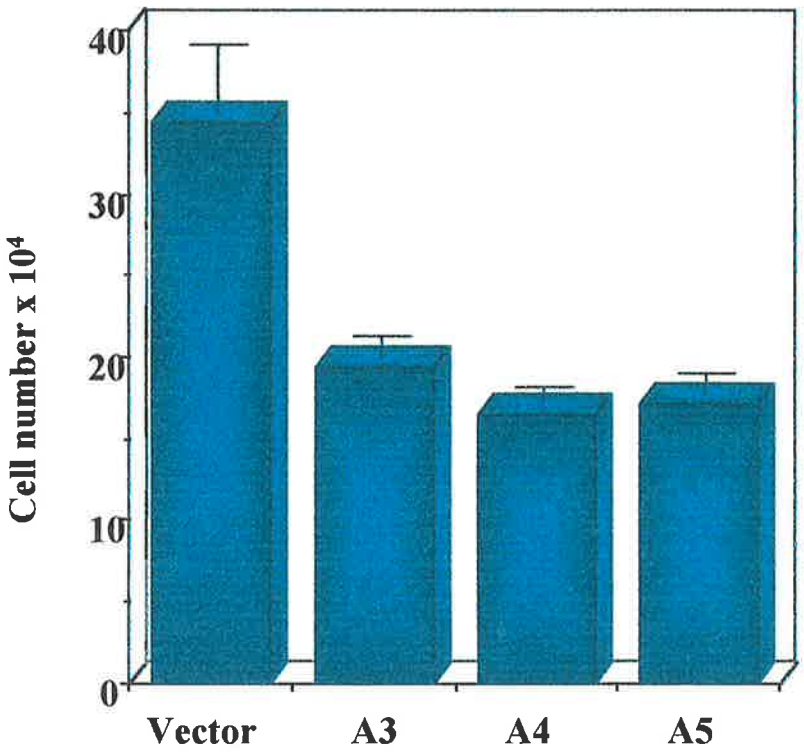
6.3.3 Growth of A549-GAS1 transfectant colonies in semi-solid agar

To assess any alterations in transformed behaviour, the ability of the pMAMneo-GAS1-transfected A549 clone A3 to grow colonies in semi-solid agar was analysed (5.2.5). Cells were plated out in 0.33% agar and cultured for two weeks in the presence of 5 x 10^{-8} M dexamethasone. The number of colonies greater than 0.1mm was then

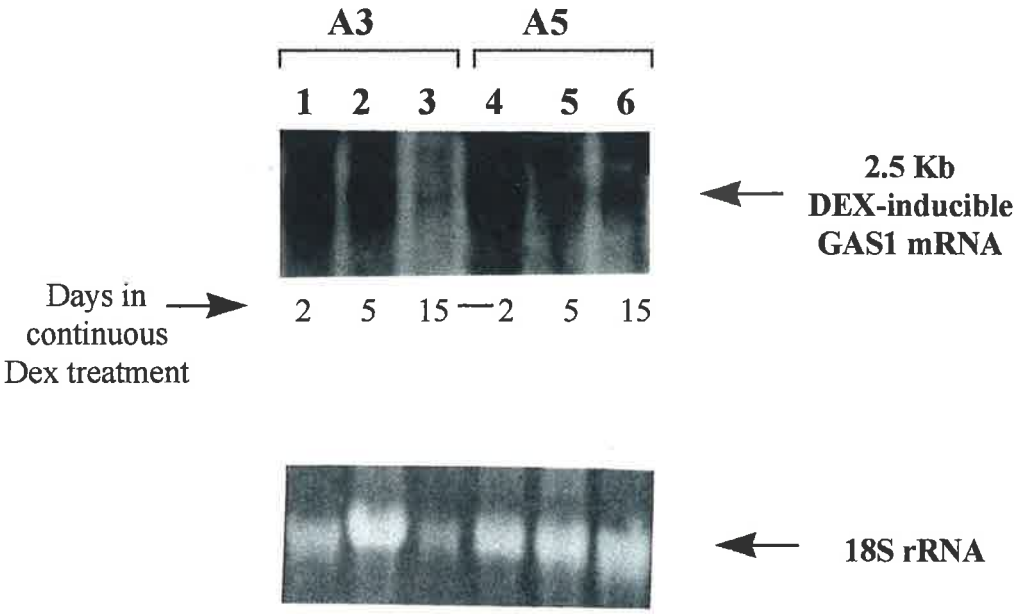
Figure 6.4 (A) GAS1-induced inhibition of growth of A549-pMAMneo-GAS1 transfectant clones A3, A4 and A5. Cells were plated at 5 $x10^4$ cells per $25cm^2$ flask in DMEM with 10% FCS in the presence of 1 x 10^{-6} M dexamethasone to induce expression of GAS1 mRNA. On day 7, cells were removed by trypsinization, collected and counted. *Columns:* mean of duplicate flasks, *Bars*, S.E.

(B) Continual induction of expression of Gas1 mRNA by dexamethasone. A549-pMAMneo-GAS1 transfectant clones A3 (lanes 1-3) and A5 (lanes 4-6) were grown in the presence of 1 x 10^{-6} M dexamethasone and RNA isolated after 2 days (lanes 1 and 4), 5 days (lanes 2 and 5) and 15 days (lanes 3 and 6). Northern blot analysis using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel.

A


Cell number x 10⁴
40
30
20
10
0
Vector
A3
A4
A5

B


A3
A5
1 2 3 4 5 6
2.5 Kb
DEX-inducible
GAS1 mRNA
Days in
continuous
Dex treatment
2 5 15 2 5 15
18S rRNA

counted under an inverted microscope. No difference was observed in the number of colonies formed by the GAS1 clone A3 when compared to the vector-transfected controls

Table 6.2 Soft agar assay of A549 cells transfected with pMAMneo-GAS1

Cell line	No. of colonies expt 1	expt2
A549 pMAMneo-vector only	600	700
A549 pMAMneo-GAS1 clone A3	556	605

The number of colonies in each dish was counted under an inverted microscope. The values represent the total number of colonies from two experiments.

6.3.4 Suppression of Tumourigenicity of A549 GAS1-transfectant cells *in vivo*

To assess the effect of induced over-expression of GAS1 on the growth of tumours *in vivo*, A549 pMAMneo-GAS1 transfectant clones A3 and A5 were injected subcutaneously into the backs of male athymic nude mice (6.2.4). To induce expression of GAS1 mRNA, cells were cultured *in vitro* for 48 hr in the presence of 1 x 10^{-6} M dexamethasone before injection into the mice. Expression of exogenous GAS1 in the transplanted A549 cells was induced by the addition of 0.28 mg/L dexamethasone to the drinking water (Narayanan *et al.,* 1992). The mice drank approximately 3ml water/day, resulting in a dose of dexamethasone of approximately 30 ng/g body weight per day. The water was palatable to the mice. Both GAS1-transfectant cell lines, A3 and A5 showed profound suppression of tumour formation and reduction in growth rate over a

period of 13 weeks when compared to the vector-only controls (Fig. 6.5A). The number of tumours arising from the GAS1-expressing clones A3 and A5 was noticeably lower (60% and 50% respectively) than the control group (90%) (Table 6.3). Tumours that grew from either GAS1-transfectant clones developed 2-4 weeks later than tumours arising from the vector-only control cells.

Table 6.3 Tumorigenicity of A549 GAS1-expressing clones in nude mice

A549 clones	Number of tumours
pMAMneo-vector only	9/10
pMAMneo-GAS1 clone A3	8/13
pMAMneo-GAS1 clone A5	5/10

Nude mice were injected subcutaneously with 10^6 cells and examined for the presence of palpable tumours. The mice were given dexamethasone (0.28 mg/L) in the drinking water (Narayanan et al., 1992) for continual induction of GAS1 in the transfectant cells and were followed for 3 months.

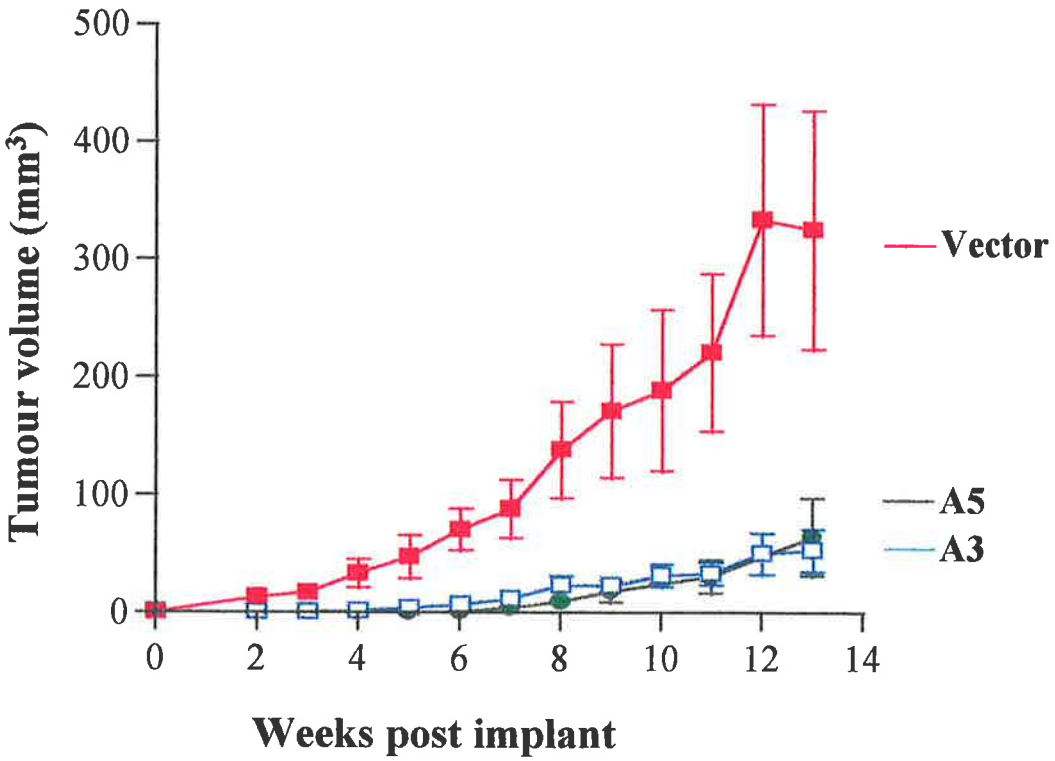
The reduced number of tumours, the extended latency period and the decrease in growth rate of tumours arising from the GAS1 transfectants all suggest that expression of GAS1 inhibits the tumorigenicity of A549 lung carcinoma cells. The tumours that eventually arose from the GAS1-transfectant lines could be derived from variant cells in which expression of the transfected GAS1 mRNA had ceased. To test this possibility, DNA and RNA were isolated directly from tumours arising from clones A3 or A5 and examined for the presence of the transfected GAS1 cDNA by PCR analysis and for expression of GAS1 mRNA by Northern analysis. GAS1 mRNA was undetectable in a tumour recovered from clone A5 (Fig. 6.5B). However, insufficient RNA was recovered

Figure 6.5 (A) Tumour growth measured weekly *in vivo* of A549 cells transfected with pMAMneo-GAS1. Two GAS1-expressing clones A3 and A5 were injected into 13 and 12 mice respectively and 10 mice were injected with the pMAMneo control vector transfectant cells. Mice were given 0.28 mg/L dexamethasone in the drinking water for the duration of the experiment. *Points:* mean tumor volume, *bars*, S.E. □ pMAMneo-GAS1-A3, ● pMAMneo-GAS1-A5. ■ pMAMneo.

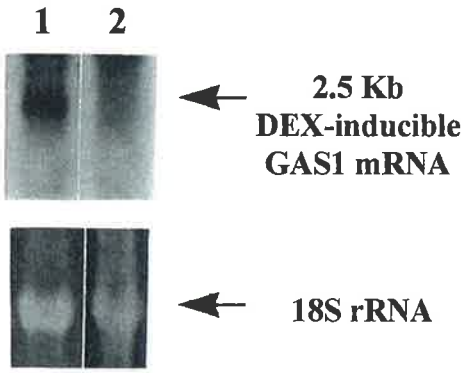
(B) Northern blot analysis of RNA isolated from one of the resulting tumors at week 13, arising from the transfectant clone pMAMneo-GAS1-A5 (lane 2). Lane 1, RNA isolated from the same clone of cells incubated in 1 x 10^{-6} M dexamethasone for 48hr, just prior to injection into the mice. Northern blot analysis using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel.

(C) Detection of the transfected GAS1 plasmid by PCR. DNA was isolated from transfectant clones before injection and from the resulting tumours in nude mice at week 13. *Hpa*II-digested pUC19 marker (lane 1), negative control with no DNA (lane 2), pMAMneo-GAS1 clones A3, A4, A5 prior to injection (lanes 3, 4, and 5) respectively, tumours 1 and 2 from clone A5 (lanes 6 and 7) and tumours 1 and 2 from clone A3 (lanes 8 and 9).

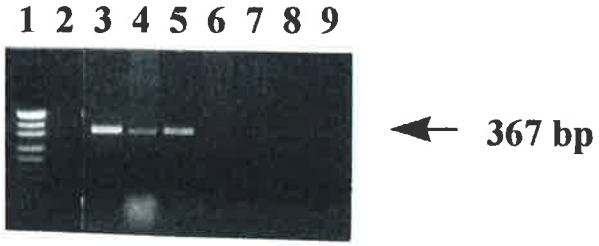
A


500
400
300
200
100
0
Tumour volume (mm³)
0
2
4
6
8
10
12
14
Weeks post implant
Vector
A5
A3

B


1
2
2.5 Kb
DEX-inducible
GAS1 mRNA
18S rRNA

C


1 2 3 4 5 6 7 8 9
367 bp

from the other tumours to allow analysis of expression of GAS1 mRNA. PCR analysis showed that the tumours arising from clone A5 no longer contained the pMAMneo-GAS1 plasmid sequences, while those that arose from clone A3 were only very weakly positive for the transfected GAS1 gene, presumably due to few remaining transfected cells (Fig 6.5C). These results suggest that suppression of tumorigenicity is dependent on the continued expression of the GAS1 gene and tumours only arise in nude mice when expression of GAS1 ceases in the injected cells.

6.3.5 Transfection of GAS1 into HTD114 fibrosarcoma cells

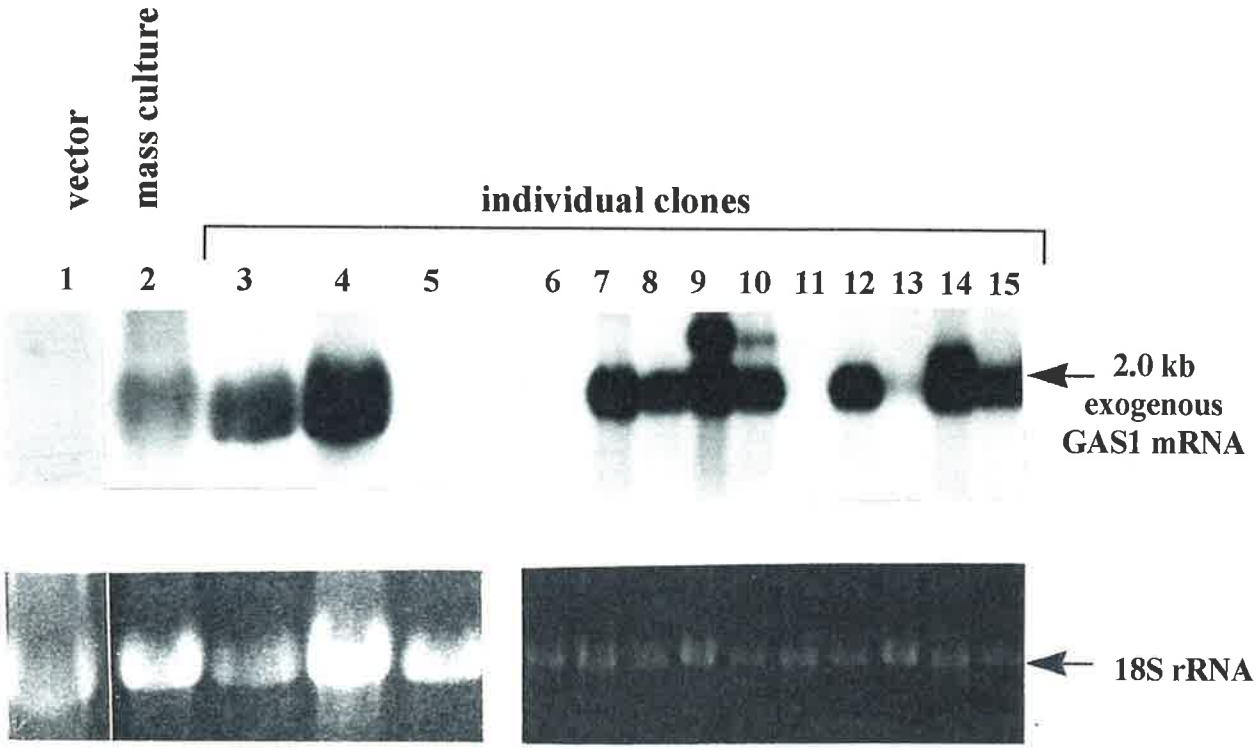
In contrast to the growth-suppressive effect of GAS1 upon A549 cells, over-expression of GAS1 in HTD114 fibrosarcoma cells transfected with the constitutively-expressing pRcCMV-GAS1 plasmid had no effect on either the *in vitro* or *in vivo* growth properties of these cells. Transfection of the pRcCMV-GAS1 plasmid construct into HTD114 cells resulted in the same number of G418-resistant colonies as cells transfected with the control vector alone (Table 6.1). Northern analysis of thirteen randomly-selected clones, as well as the mass culture, showed high levels of expression of exogenous GAS1 mRNA in all except two clones (Fig. 6.6A). Long-term culture of representative clones indicated that this high level of expression of GAS1 mRNA was stably maintained for more than 12 months. The GAS1-expressing cells were morphologically indistinguishable from control cells and showed no significant difference in growth rates *in vitro* (Fig. 6.6B), despite continued high levels of expression of exogenous GAS1. There was also no change in the ability of the GAS1-transfectant cells to form colonies in semi-solid agar (Table 6.4)

Figure 6.6 HTD114 cells transfected with pRcCMV-GAS1.

(A) Northern blot analysis of RNA isolated from 13 randomly selected G418-resistant clones (lanes 3-15) and from the mass culture (lane 2) of HTD114 cells transfected with pRcCMV-GAS1. Lane 3 and lane 4 represent clones 2 and 15 respectively which were subsequently used for *in vitro* growth analysis and assay of tumorigenicity in nude mice. Lane 1 represents RNA isolated from the mass culture transfected with the vector only. Northern blotting using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel.

(B) *In vitro* growth rate analysis of HTD114 cells transfected with pRcCMV-GAS1. Cells were plated at 2 $x10^4$ cells per $25cm^2$ flask in DMEM with 10% FCS and propagated at 37°C. On days 3, 6 and 9, cells were removed by trypsinization and counted. *Points;* mean of duplicate flasks; *bars;* S.E. ■ vector-only transfected cells, ● pRcCMV-GAS1-transfected clone HTD2, □ clone HTD15

A


vector
mass culture
individual clones
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
2.0 kb
exogenous
GAS1 mRNA
18S rRNA

B

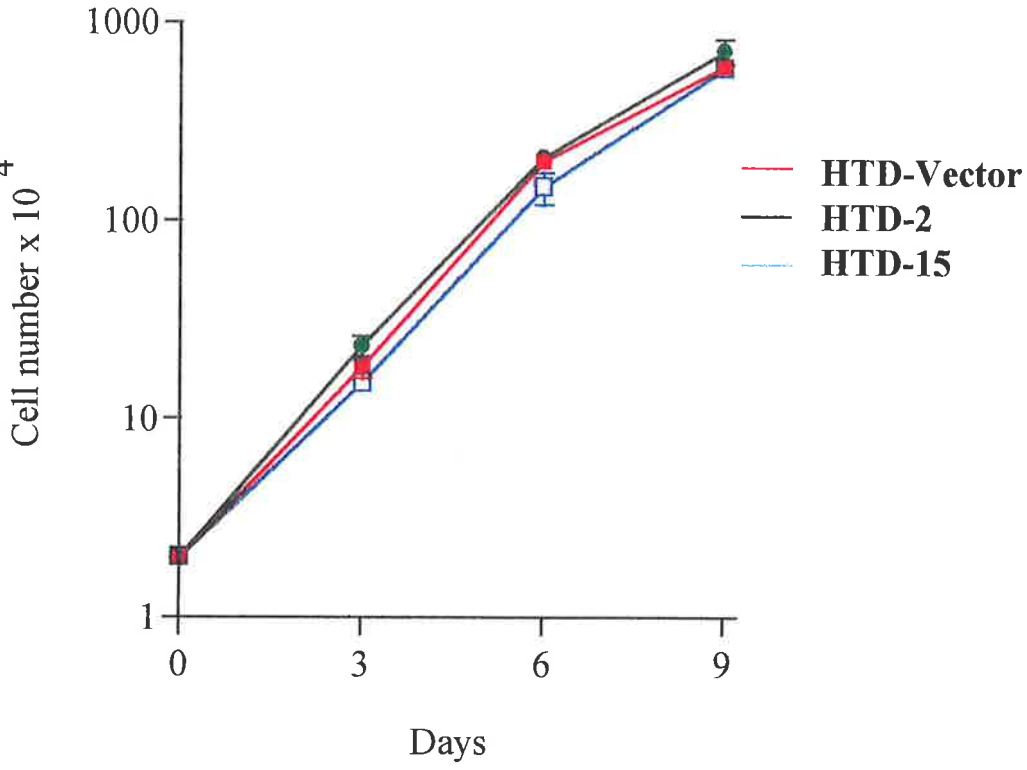

1000
100
10
1
Cell number x 10 4
0
3
6
9
Days
HTD-Vector
HTD-2
HTD-15

Table 6.4 Soft agar assay of HTD114 cells transfected with pRcCMV-GAS1

Cell line	Number of colonies	
pRcCMVneo-vector only	223	262
pRcCMV-GAS1 clone HTD2	200	232
pRcCMV-GAS1 clone HTD15	189	240

The number of colonies in each dish was counted under an inverted microscope. The values represent the total number of colonies from two experiments.

When two pRcCMV-GAS1-transfectant HTD114 clones, HTD2 and HTD15, and control vector-only cells were injected subcutaneously into 6-8 week old male Balb/c athymic nude mice, tumours were produced in 100% of animals with a latency of one week (Table 6.5) and grew similarly (Fig. 6.7A). When the tumours arising from the GAS1-transfectant clones were re-established in culture in the presence of G418, the exogenous GAS1 transcript was detected at approximately the same level as that of the original cell line used for the implants (Fig. 6.7B). Taken together, these results clearly show that, unlike the A549 cells, HTD114 cells are resistant to the growth- and tumour-suppressive effects of overexpression of GAS1 and there is no selection pressure against the propagation of the GAS1-transfectant HTD114 cells.

Table 6.3 Tumorigenicity of HTD114 GAS1-expressing clones in nude mice

HTD114 clones	Number of tumours
pRcCMVneo-vector only	5/5
pRcCMV-GAS1 clone HTD2	5/5
pRcCMV-GAS1 clone HTD15	5/5

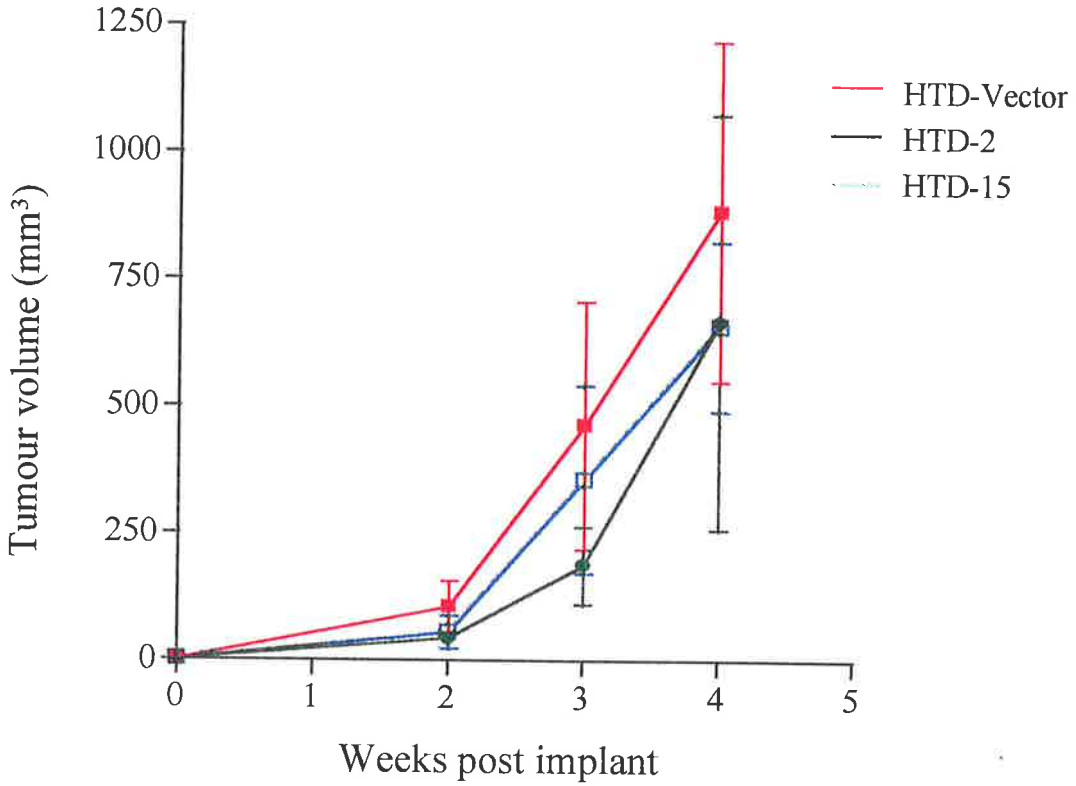
All mice injected with HTD114 cells had developed tumours within 7 days

Figure 6.7 Tumorigenicity of HTD114 GAS1 transfectants *in vivo*

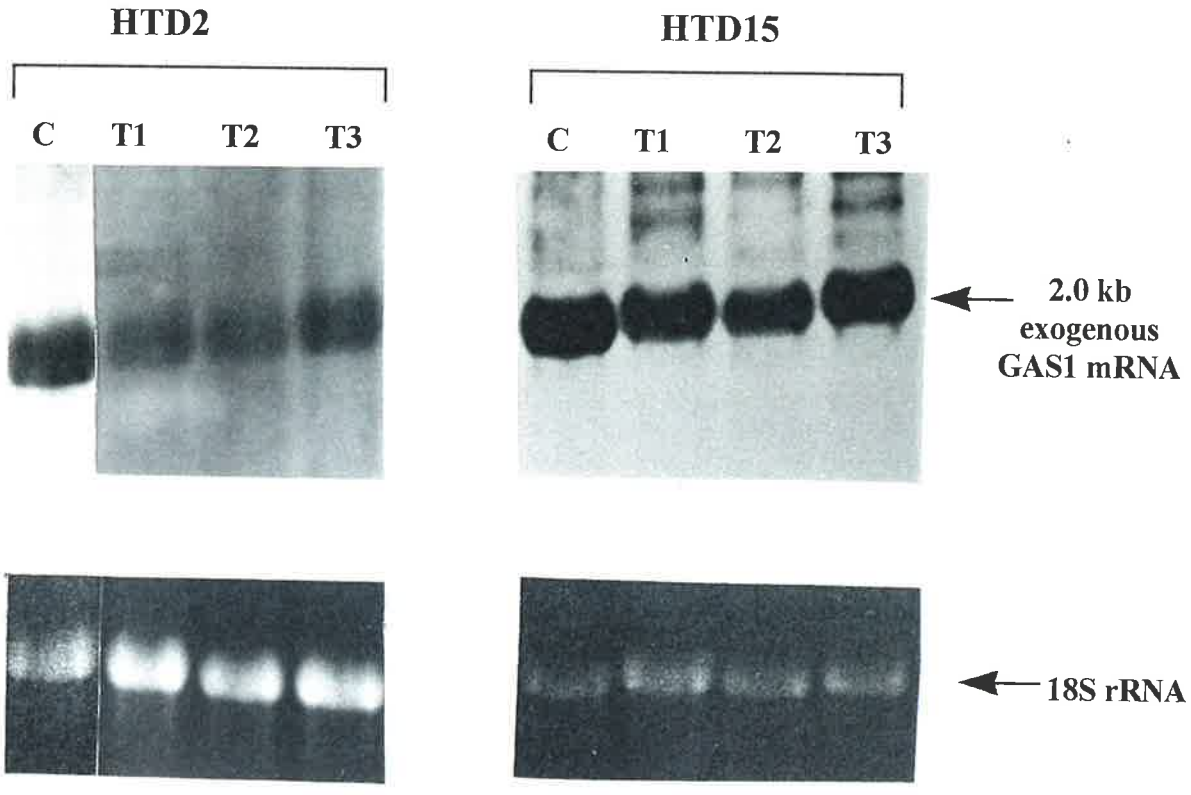
(A) *In vivo* tumour growth of HTD114 cells transfected with pRcCMVneo-GAS1. Cells (1×10^6 in 0.1ml) were injected subcutaneously into the back of Balb/c nude mice. The tumour volume in individual animals was measured at weekly intervals for 4 weeks. Five animals per group were used. *Points;* mean volumes of tumours, *bars;* S.E. ■ vector-only transfectants, ●pRcCMV-GAS1-transfected clone HTD2, □ clone HTD15.

(B) Expression of GAS1 mRNA in tumours arising from GAS1-transfectant HTD114 cells. Three tumours T1, T2, and T3 arising from clones HTD2 and HTD15 were excised at the end of week 4, manually disrupted and briefly cultured in the presence of 400 μg/ml G418. RNA was isolated and assessed by Northern blotting for the presence of the exogenous GAS1 transcript. Lanes marked C, RNA isolated from both clones prior to implantation whereas, T1, T2, and T3 is RNA from tumours arising in three mice implanted with clone HTD2 and tumours from clone HTD15. Northern blotting using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel

A


1250
1000
750
500
250
0
Tumour volume (mm³)
0
1
2
3
4
5
Weeks post implant
HTD-Vector
HTD-2
HTD-15

B


HTD2
HTD15
C
T1
T2
T3
C
T1
T2
T3
2.0 kb
exogenous
GAS1 mRNA
18S rRNA

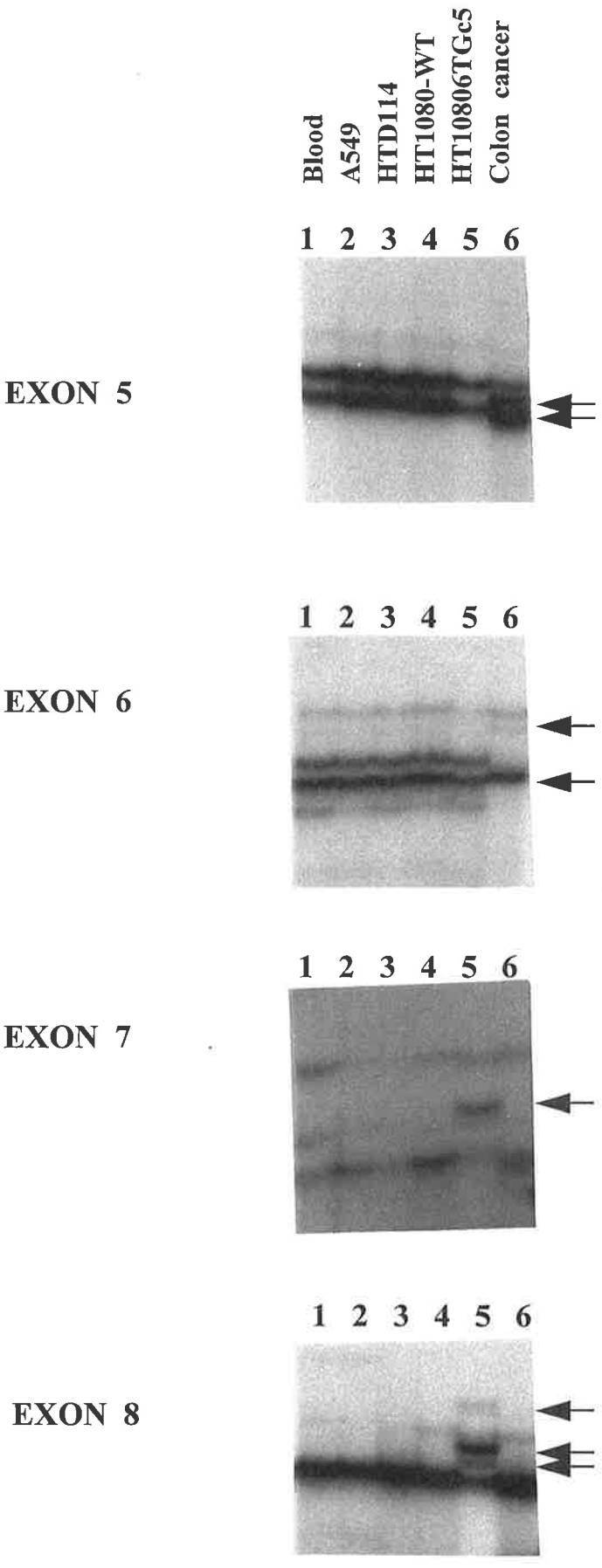
6.3.6 p53 status of HTD114 cells

Previous studies (Del Sal *et al.,* 1995) suggested that, in mouse fibroblasts, Gas-1 requires functional p53 for its growth-suppressive activity and that cell lines harbouring mutant p53, or those that are null for the p53 gene, are refractory to overexpression of Gas-1. The GAS1-resistant phenotype of HTD114 fibrosarcoma cells suggested that these cells may contain mutations in the p53 gene. However, single strand confirmation polymorphism analysis (2.2.23) of exons 5, 6, 7 and 8 of the p53 gene, carried out on DNA isolated from various cells showed no mobility shifts in any of the p53 exons from HTD114 cells (Fig 6.8) or from A549 cells, which are known to express wild-type p53 (Lehman *et al.,* 1991). Conversely, mobility shifts were detected in positive control samples of DNA isolated from human colon carcinomas which were known to harbour p53 mutations in each of the exons tested (Fig 6.8). To confirm the results of the SSCP analysis, the same PCR fragments generated from HTD114 cells were directly sequenced (Appendix I). No mutations were detected in any of the exons analysed.

6.3.7 Expression of MDM2 in HTD114 cells

Although the entire p53 gene was not screened for mutations, it seemed unlikely that mutations in the p53 gene were causing the lack of response of this cell line to the growth-suppressive activity of GAS1. Alternatively, other proteins, such as MDM2, which interact with p53, might functionally inactivate p53 in HTD114 cells, leading to a lack of response to over-expression of GAS1. The MDM2 oncogene product is a functional antagonist of wt p53 (reviewed in Picksley and Lane, 1993). Over-expression of MDM2 protein inactivates both the growth-inhibitory and apoptotic functions of wt p53 by directly binding to p53 and inhibiting its transcriptional-activating or repressing

Figure 6.8 PCR-SSCP analysis of exons 5, 6, 7 and 8 of the p53 gene. Lanes 1, DNA isolated from peripheral blood of a normal subject, parental A549 (lanes 2), parental HTD114 (lanes 3), parental HT1080 wt p53 (lanes 4), HT10806TGc5 (lanes 5), and positive control DNA isolated from colon cancer patients known to harbour p53 mutations in the corresponding exons (lanes 6). Shifted bands are indicated by arrows. Note that the HT10806TGc5 cell line (lanes 5) contains mutations in exons 7 and 8 of the p53 gene as previously reported in Anderson *et al.* (1994). No mutations are detected in the other cell lines except the positive control.


Blood
A549
HTD114
HT1080-WT
HT10806TGc5
Colon cancer
1 2 3 4 5 6
EXON 5
1 2 3 4 5 6
EXON 6
1 2 3 4 5 6
EXON 7
1 2 3 4 5 6
EXON 8

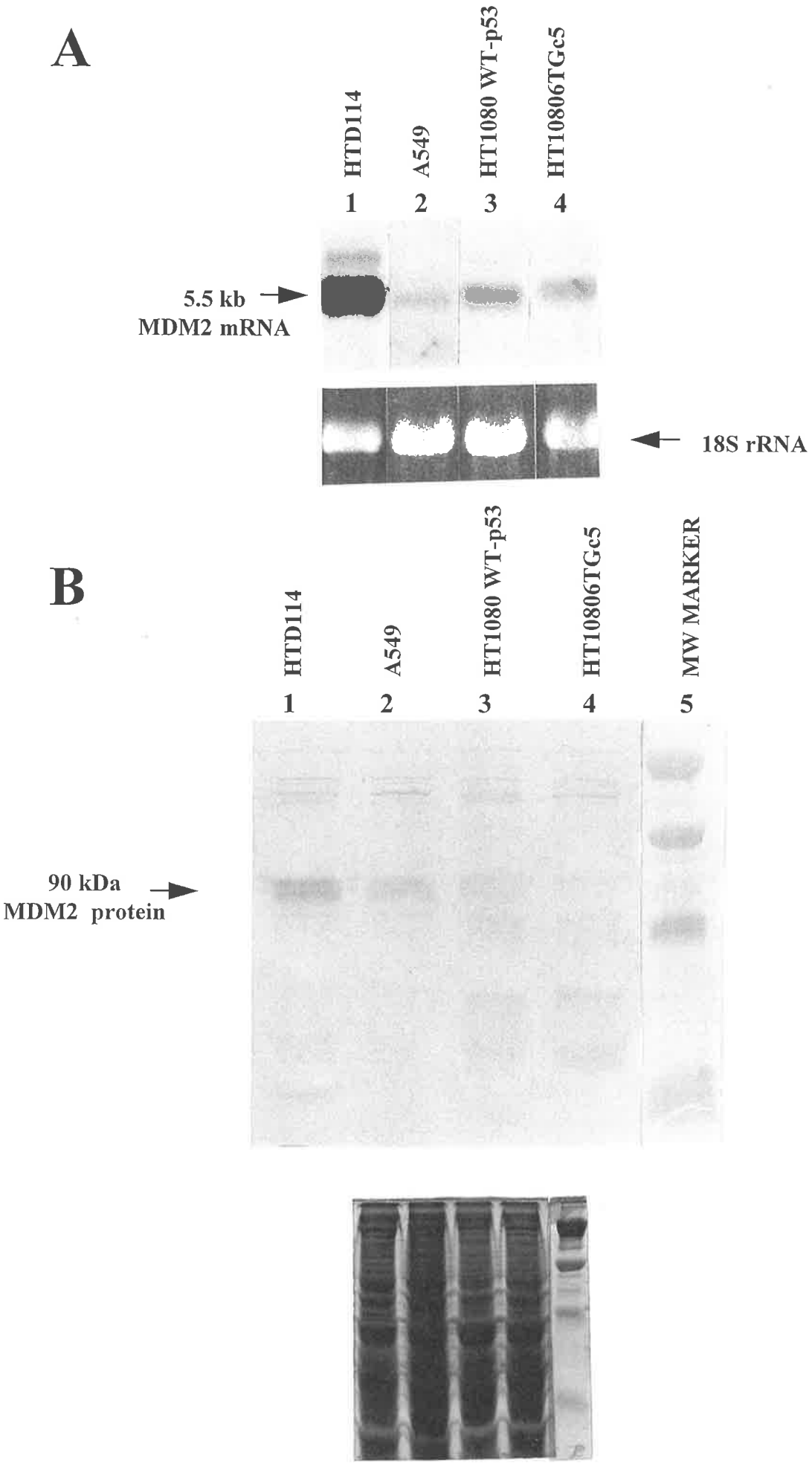
functions (Finlay *et al.,* 1993; Knippschild *et al.,* 1995; Martin *et al.,* 1995; Otto and Deppert, 1993; Chen *et al.,* 1995).

Since the HTD114 cell line was derived from a human fibrosarcoma, and MDM2 is frequently amplified and/or overexpressed in a large proportion of sarcomas (Oliner *et al.,* 1992; Leach *et al.,* 1993), it was possible that the GAS1-resistant phenotype of HTD114 cells could be due to inactivation of p53 mediated by over-expression of the MDM2 protein. To test this, the levels of MDM2 mRNA in both HTD114 and A549 cells were assessed by Northern analysis of RNA isolated from both cell lines. MDM2 mRNA was barely detectable in A549 cells, whereas HTD114 cells contained approximately 20 fold higher amounts of MDM2 mRNA than A549 cells, as assessed by densitometric analysis (Fig. 6.9A). Similarly, Western analysis demonstrated that the level of MDM2 protein was significantly elevated in HTD114 cells when compared to A549 cells (Fig. 6.9B). However, Southern analysis failed to show any evidence of MDM2 gene amplification in HTD114 cells (Fig. 6.10), suggesting that the increased levels of MDM2 mRNA and protein are not a direct result of gene amplification, but rather due to altered transcriptional regulation or possibly increased stability of MDM2 mRNA and protein. The inability of HTD114 cells to respond to overexpression of GAS1 therefore may be related to the elevated levels of MDM2, which can functionally inactivate p53. These cells would now behave as if they are mutant for p53.

Figure 6.9 Levels of MDM2 mRNA and protein.

(A) Northern blot analysis of expression of MDM2 mRNA. Blots were probed with an MDM2 cDNA probe, (upper panel) and parallel ethidium bromide staining pattern of control 18S rRNA is shown in the lower panel. HTD114 cells (lane 1), A549 cells (lane 2), HT1080-wtp53 (lane 3) and HT10806TGc5 (lane 4).

(B) Expression of MDM2 protein. Equal amounts of cell lysate protein (lower panel) were analysed by Western blotting with an antibody directed against human MDM2 (top panel). HTD114 cells (lane 1), A549 cells (lane 2), HT1080 wtp53 (lane 3), HT10806TGc5 (lane 4) and protein molecular weight markers (lane 5).


A
HTD114
A549
HT1080 WT-p53
HT10806TGc5
1
2
3
4
5.5 kb
MDM2 mRNA
18S rRNA
B
HTD114
A549
HT1080 WT-p53
HT10806TGc5
MW MARKER
1
2
3
4
5
90 kDa
MDM2 protein

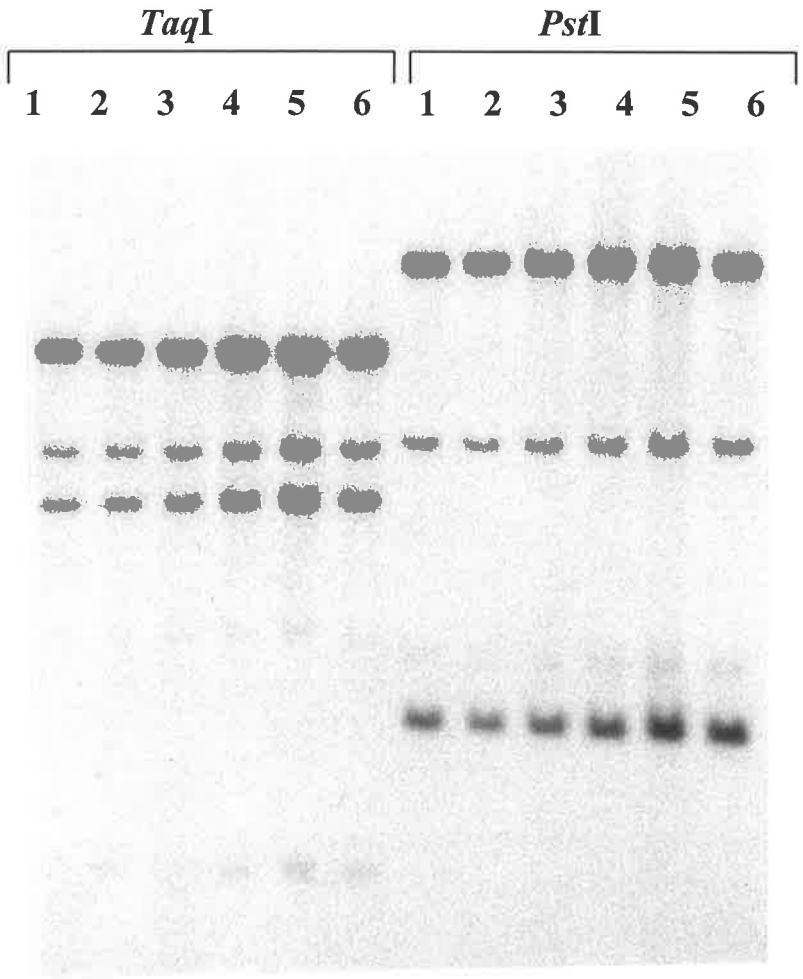


Figure 6.10 Southern blot analysis of the MDM2 gene. DNA was digested with *Taq*1 or *Pst*1 restriction enzymes and the Southern blot probed with MDM2 cDNA. Lanes 1 and 2, DNA isolated from peripheral blood of two normal individuals, HTD114 cells (lanes 3), A549 cells (lanes 4), HT1080 wtp53 cells (lanes 5), and HT10806TGc5 cells (lanes 6).

6.3.8 Transfection of GAS1 in HT1080 cells with wild-type or mutant p53 and low levels of MDM2

To define further the role of MDM2 and p53 in mediating the lack of response of HTD114 cells to over-expression of GAS1, two other HT1080 sub-lines were also examined for their sensitivity to GAS1-induced growth suppression. HT1080 6TGc5 contains point mutations in exons 7 and 8 in both copies of the p53 gene. The p53 protein synthesised by this cell line has an extended half-life, which is often associated with mutated forms of p53 (Anderson *et al.,* 1994). A second subline of HT1080 was also obtained in which p53 immunoprecipitation analysis with conformation-specific antibodies has shown the presence of wild type p53 (Whitaker *et al.,* 1995) and SSCP analysis demonstrated that exons 5-9 of the p53 gene were wild-type (Sharma *et al.,* 1993).

SSCP analysis of exons 5, 6, 7 and 8 of the p53 gene in the p53-wt subline of HT1080 failed to detect any mutations (Fig 6.8 lanes 4), suggesting that the p53 gene in these cells was probably wild-type. However, unlike the HTD114 cells, Northern analysis demonstrated that levels of MDM2 mRNA were relatively low and comparable to those detected in A549 cells (Fig. 6.9A). Similarly, levels of MDM2 protein were not elevated in HT1080wt p53 cells (Fig. 6.9B). On this basis, if elevated levels of MDM2 in HTD114 cells were responsible for the resistance to the growth-suppressive activity of GAS1, the HT1080 subline with normal levels of MDM2 and wild-type p53 should be growth-arrested following overexpression of GAS1.

Transfection of the constitutively-expressing pRcCMV-GAS1 plasmid into the HT1080wt p53 sub-line resulted in a 3-4 fold reduction in the number of G418-resistant colonies 2 weeks after selection when compared to the vector-only control cells (Table 6.1). The reduced number of transfectant clones obtained from HT1080wtp53 cells

suggests that overexpression of GAS1 inhibits the proliferation of these cells in a similar manner to that observed in GAS1-sensitive A549 cells. Northern analysis of RNA isolated from the mass culture of GAS1-transfected HT1080wtp53 cells showed that the exogenous GAS1 transcript was initially expressed after selection for two weeks in G418 but expression was unstable and was lost when cells were subcultured (Fig 6.11). The tumorigenic potential of these cells could not be assessed in nude mice since the untransfected HT1080wtp53 cell line did not form any tumours in nude mice even after 3 months.

In the HT1080 sub-line, HT1080 6TGc5, which contains point mutations in exons 7 and 8 of the p53 gene (Anderson *et al.,* 1994), SSCP analysis confirmed the presence of these mutations (Fig 6.8 lanes 5). Northern and Western blotting demonstrated that, unlike HTD114, HT1080 6TGc5 did not over-express MDM2 mRNA (Fig. 6.9A) or protein (Fig. 6.9B). When the HT1080 6TGc5 cell line was transfected with the constitutively-expressing pRcCMV-GAS1 plasmid, there was no reduction in the number of G418-resistant colonies (Table 6.1), as with the HTD114 cell line. Northern analysis demonstrated that the G418-resistant HT10806TGc5 cells expressed high levels of exogenous GAS1 mRNA that was stable for at least 4 passages (Fig 6.11). Taken together, these results show that the HT1080 6TGc5 cell line, containing mutant p53 and normal levels of MDM2, is resistant to the growth-inhibitory activity of GAS1.

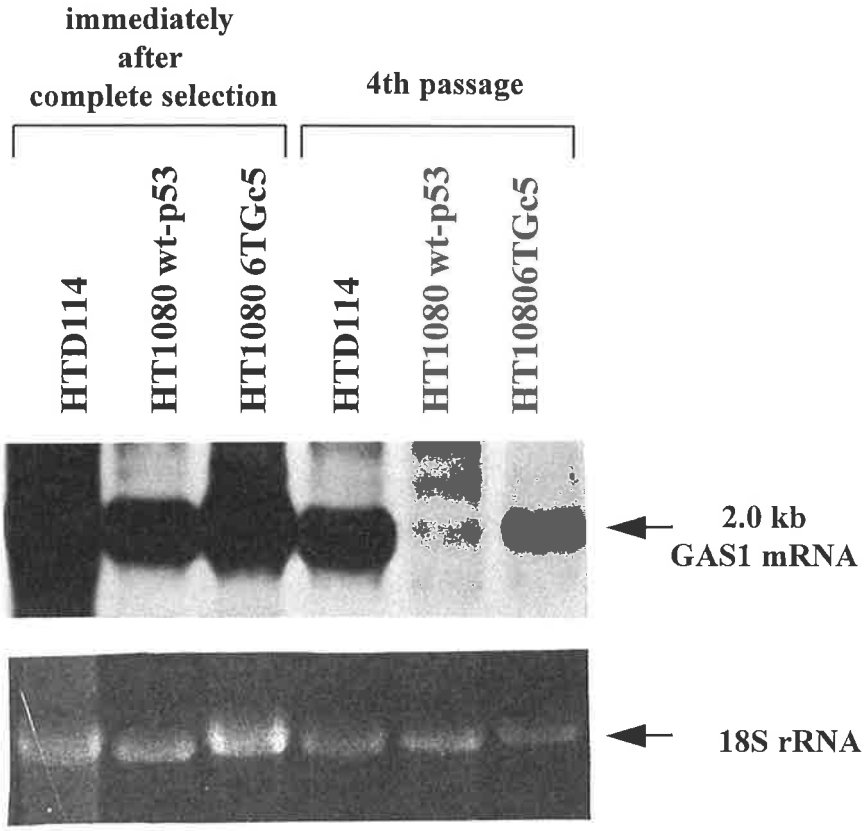


Figure 6.11 Northern analysis of RNA isolated from mass cultures of pRcCMV-GAS1-transfected cell lines immediately after 2 weeks of complete selection in G418 and after the fourth passage. Northern blotting using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. At the fourth passage the expression of GAS1 mRNA in the HT1080 wt-p53 cell line is greatly reduced whereas expression of GAS1 is maintained in the other cell lines.

6.4 DISCUSSION

Using transient microinjection techniques, overexpression of the growth arrest-specific gene product, GAS1, inhibits the entry of cells into S phase and inhibit DNA synthesis (Del Sal *et al.,* 1992; Del Sal *et al.,* 1994). In this study, we have extended these previous investigations by using both constitutive and inducible expression systems to generate stable GAS1-transfectant human cell lines. The cell lines used in this study were selected because they failed to express endogenous GAS1 mRNA, even when propagated under conditions leading to growth arrest. These lines were therefore suitable to investigate further the growth-suppressing activity of GAS1 *in vitro* and to test the ability of GAS1 to suppress the growth of human tumours in nude mice.

Stable transfectant A549 cell lines could not be generated that expressed GAS1 mRNA under the control of the strong constitutive CMV promoter, impliying that expression of GAS1 mRNA was antiproliferative in A549 cells. Some GAS1 mRNA was detected 72h after transfection, showing that the pRcCMV-GAS1 plasmid was capable of expressing GAS1 mRNA but, due to strong negative selection pressure, any colonies that arose were derived from non-GAS1-expressing cells. These results are reminiscent of the previous findings (Chapter 5) in which transfection of the constitutive GAS1 expressing plasmid in NIH3T3 cells failed to give rise to G418 resistant colonies that expressed exogenous GAS1 mRNA. To generate stable cell lines capable of expressing GAS1, the dexamethasone-inducible pMAMneo plasmid was therefore selected. The promoter of this plasmid was not active in the absence of dexamethasone thereby avoiding problems associated with leakage of transcription in the absence of the inducer. The same number of G418-resistant colonies arose in the

GAS1 transfectants as in the vector controls and no GAS1 mRNA was detected in the GAS1-transfectants in the absence of dexamethasone. When dexamethasone was added to the GAS1-transfectant cell lines, GAS1 mRNA was expressed at high levels and there was a marked reduction in growth rate of the cells above that observed due to dexamethasone alone. These results are consistent with the suppression of proliferation observed in the transient GAS1 expression experiments of Dal Sal *et al.,* 1992, 1994). When assessed for anchorage indepenent growth, the GAS1-expressing cells showed no change in their ability to grow colonies in semi-solid agar. Since there is a strong selection pressure against the continued propagation of GAS1-expressing A549 cells in long-term liquid culture medium, it is unlikely the colonies that grew in semi-solid agar after two weeks were still synthesising any GAS1 mRNA. Therefore it was not possible, using this protocol, to determine if over-expression of GAS1 will alter the ability of tumour cells to form colonies in semi-solid agar. Although an antibody against the GAS1 protein did exist, it was however not available for our use in this study. Attempts to generate a polyclonal GAS1 antibody by Chiron Mimotopes Peptide Systems were unsuccesfull and the resulting antibody was non-specific. We were therefore unable to determine if the transfectant cells expressed GAS1 protein and this appeared to be a major limitation throughout the present study. However, when the cells expressed GAS1 mRNA, a marked inhibition in growth rate was observed, so it can be deduced that the plasmid constructs are capable of coding for a functional GAS1 protein.

This study has also demonstrated that the tumorigenicity of GAS1-A549 transfectant cells was partially suppressed when compared to control vector-transfected cells. Fewer mice developed tumours, there was a marked reduction in tumour volume and also an increase in the tumour latency in the GAS1-expressing clones analysed. The small tumours that eventually arose, did not express exogenous GAS1 mRNA. PCR

analysis showed that the GAS1 plasmid construct had been lost by the cells or, alternatively, that the tumours had arisen from a small sub-set of the cells which did not contain the transfected GAS1 gene. Although mice were injected with cell lines arising from a single GAS1-expressing colony, it is possible that, during propagation, a small sub-set of cells arose that did not contain the GAS1 gene but presumably still contained the G418-resistance gene. Suppression of the tumorigenic phenotype may therefore rely on the continued expression of GAS1 and tumours grow only when GAS1 expression is lost, strongly suggesting that the GAS1 gene product has tumour-suppressive activity.

In contrast to A549 cells, the HTD114 subline was completely resistant to the growth- and tumour-suppressive activities of GAS1. Del Sal *et al.* (1995) showed that overexpression of Gas-1 in NIH3T3 fibroblasts, induces growth arrest via a wt p53-mediated pathway and lack of wt p53 blocks the growth-inhibitory effects of Gas-1. SSCP analysis and direct DNA sequencing of exons 5, 6, 7 and 8 of the p53 gene strongly suggested that HTD114 cells contain wt p53. The presence of mutations in other regions of the p53 gene, apart from exons 5-8, were not excluded in this study. However, these are expected to be rare. Since HTD114 cells over-express MDM2 it is unlikely to also contain mutations in the p53 gene. In a series of sarcomas, both p53 mutation and over-expression of MDM2 were observed but not in the same tumour (Leach *et al.*, 1993). Similarly, direct sequencing of p53 cDNA from a series of glioblastomas failed to detect any mutations in those tumours that showed amplification and over expression of MDM2. However, Cordon-Cardo *et al.* (1994) reported that a small sub-set of sarcomas overexpressed both MDM2 and mutant p53 but it is likely that mutation of p53 and overexpression of MDM2 in the same cell is a rare event.

HT1080 cells with wt p53 were growth-arrested following over-expression of GAS1. However, if the p53 was mutant, proliferation of the cells was unaffected by

over-expression of GAS1 as observed in HT1080 6TGc5 cells. The presence of a wt p53 gene product could also be negated by over-expression of MDM2, as observed in HTD114 cells. MDM2 physically interacts with wt p53 protein to functionally inactivate it (reviewed in Picksley and Lane, 1993). Overexpression of MDM2 inhibits both the G1 arrest function of p53 and also the ability of wt p53 to induce apoptosis (Finlay, 1993; Chen *et al.,* 1996). For these effects to occur, it is critical that MDM2 binds to p53 and inhibits the transcriptional activation and repression functions of p53 (Chen *et al.,* 1995; Momand *et al.,* 1992). It is not yet clear whether complex formation between MDM2 and p53 is necessary to inhibit GAS1-mediated growth suppression in HTD114 cells. Since the growth-arresting activity of GAS1 requires wt p53 but does not involve the transactivation functions of p53 (Del Sal *et al.,* 1995), it is possible that MDM2 is inducing resistance to GAS1 by either inhibition of the transrepression functions of p53 or some other unknown mechanism. MDM2 also physically sequesters p53 in the cytoplasm of cells to prevent any p53-mediated events in the nucleus (Knippschild *et al.,* 1996). Alternatively, other p53-independent activities of MDM2, including its intrinsic transformation activity (Dubs-Poterszman *et al.,* 1995) and direct activation of the S-phase-inducing transcription factors, E2F1/DP1 (Otto and Deppert, 1993) may be involved in stimulating growth of HTD114 cells and preventing the growth- and tumour-suppressive activity of GAS1.

This study has confirmed the growth-suppressive activity of GAS1 and provided the first direct biological evidence that GAS1 can inhibit the growth of tumours *in vivo*, thereby acting as a tumour suppressor gene. It has also demonstrated that the GAS1-mediated growth-suppressing signalling pathway may be inhibited by inactivation of wild-type p53 by the MDM2 oncogene product suggesting that the balance in favour of free and functional p53 is important in mediating GAS1 growth suppression. One way

to varify this, would be to downregulate MDM2 levels in the HTD114 cells using antisense techniques and then test the ability of GAS1 to suppress growth (Chapter 7).

CHAPTER 7

IDENTIFICATION AND CHARACTERISATION OF AN ALTERNATIVELY-SPLICED VARIANT OF THE MDM2 ONCOGENE PRODUCT

7.1 INTRODUCTION

Overexpression of the mouse Gas-1 gene in NIH3T3 fibroblasts blocks proliferation in a p53-dependent manner. Cells containing mutant p53 or which are null for the p53 gene, fail to enter growth arrest after ectopic expression of Gas-1 both in asynchronously growing cultures and during synchronous Go-to-S phase transition (Del Sal *et al.*, 1995). These findings were supported further by investigations on human cell lines described in the previous chapter.

Mechanisms by which wild type p53 is able to regulate cell cycle progression is thought to be via transcriptional activation of target genes involved in the cascade of events that lead to growth arrest (Crook *et al.*, 1994; Pientenpol *et al.*, 1994). At least one of the p53-induced genes, *waf1*, is required for G1 arrest (Dulic *et al.*, 1994; El-Deiry *et al.*, 1993). However, Del Sal *et al.* (1995) has shown that the transcriptional activation function of p53 is not required for *Gas-1* mediated growth arrest and that *waf1* is not one of the genes upregulated by overexpression of *Gas-1*. It was therefore proposed that overexpression of *Gas-1* specifically modulates the transcriptional repression functions of p53, leading to growth suppression or alternatively, the *Gas-1* overexpression-dependent signalling pathway could modulate the interactions of wild type p53 with other proteins, thus resulting in growth suppression (Del Sal *et al.*, 1995).

The p53 protein interacts directly with the MDM2 oncoprotein (Momand *et al.*, 1992). The binding of MDM2 to the amino terminus of p53, inactivates p53 and removes growth regulation by this protein in a manner similar to that seen in virally-induced cancers in which the viral proteins bind to and inhibit p53 function. (Oliner *et al.*, 1992). In addition, MDM2 interacts with another tumour suppressor gene product, the Rb protein (Xiao *et al* 1995). These observations have suggested that overexpression

of MDM2 is another mechanism by which p53 growth suppressive function can be abrogated.

The findings reported in the previous chapter suggested that the failure of HTD114 cells to undergo growth arrest after overexpression of GAS1 could be related to the abnormally-elevated levels of MDM2 in these cells. To define further the role of overexpression of MDM2 in the abrogation of GAS1 -mediated growth-suppression in HTD114 cells the following was considered. If overexpression of MDM2 in HTD114 cells inhibits GAS1-mediated growth suppression, then downregulation of the endogenous levels of MDM2 using, an antisense strategy should increase the sensitivity of these cells to exogenous GAS1.

7.2 MATERIALS AND METHODS

7.2.1 Cloning of the antisense MDM2 fragment into the pRcCMVneo constitutive expression vector

For the generation of antisense MDM2 mRNA, a 949 bp *Not*I fragment of the MDM2 c14-2 clone, corresponding to nucleotides 1-949 of the human MDM2 cDNA, was cloned into the *Not*I site of the pRcCMVneo constitutive expression vector in the reverse orientation (Fig. 7.1). The vector/insert ligation product was transformed into *E coli* bacteria (2.2.6, 2.2.7) and plasmid DNA isolated by miniplasmid preparation (2.2.1). The identity and orientation of the recombinant plasmid was confirmed by restriction endonuclease digestion (2.2.2). The recombinant plasmid DNA was isolated and purified in preparation for transfection using the Qiagen midi plasmid kit (2.2.1). The GAS1-expression vector pRcCMV-GAS1 used for the co-transfection in this study has been described previously (5.2.2).

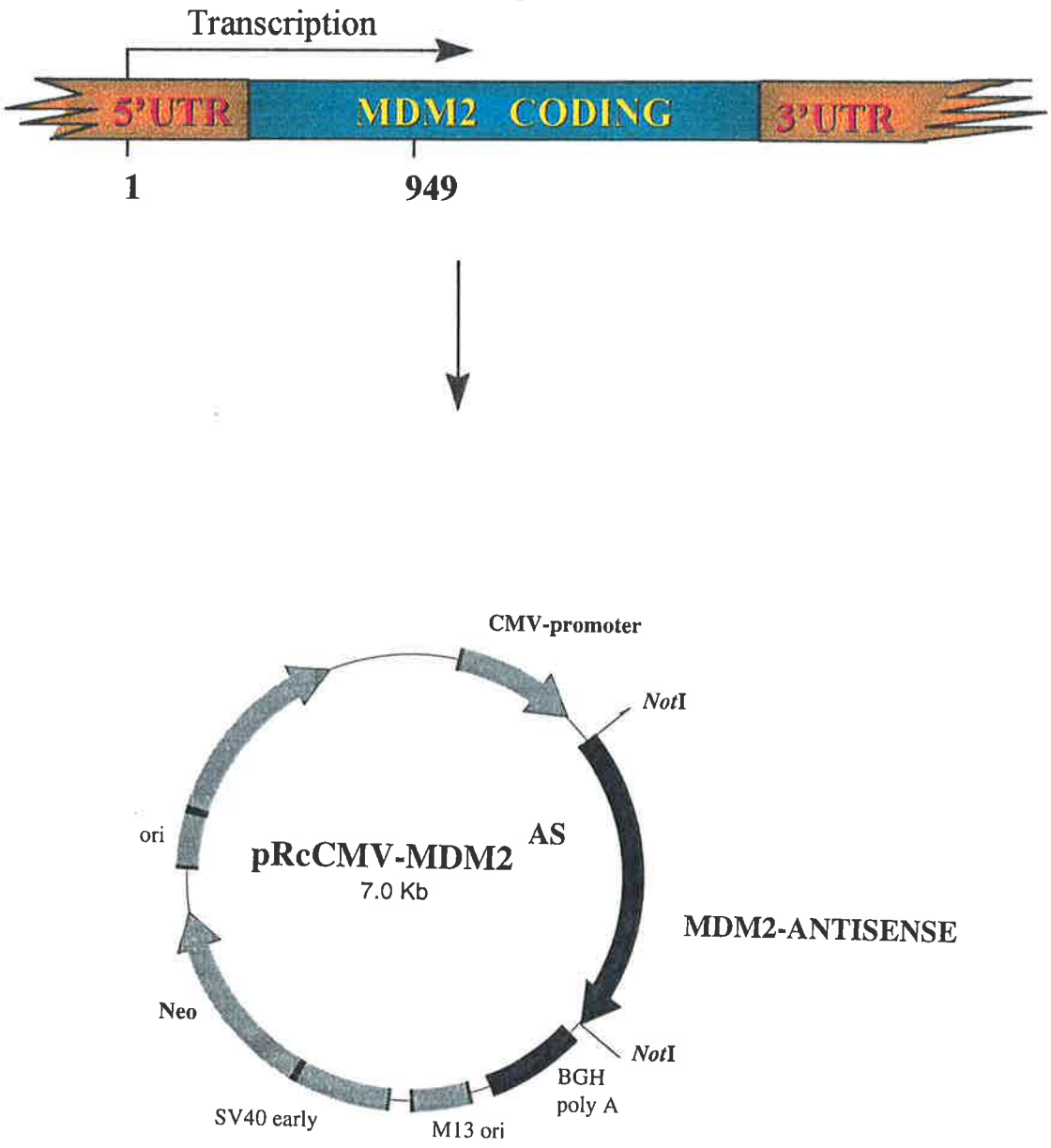


Figure 7.1 Cloning of the antisense MDM2 cDNA fragment into the plasmid vector, pRcCMVneo. A 949 bp *Not*I fragment from clone C14-2 (Oliner *et al.*, 1992) corresponding to nucleotides 1-949 of the human MDM2 cDNA, was ligated into the *Not*I site of the pRcCMVneo expression vector in the reverse orientation.

7.2.2 Transfection of HTD114 cells

HTD114 cells were seeded at 5 x 10^5 cells per 25 cm^2 flask and grown overnight. Cells were then transfected (2.2.21) with either:

(a). pRcCMV-vector only

(b) pRcCMV-GAS1

(c) pRcCMV-MDM2-antisense

(d) pRcCMV-GAS1 + pRcCMV-MDM2-antisense

Transfectant populations were selected by culturing cells in the presence of 400 μg/ml of G418. Two weeks after transfection, the number of G418-resistant colonies was counted and ten randomly-selected colonies from each transfection were manually picked and propagated in the continual presence of G418 for subsequent analysis. Total RNA was isolated from each of the mass cultures representing all selected clones and also from each of the individual clones. RNA isolated from the mass culture of the vector-only transfectants served as the control. Expression of the transfected GAS1 gene was assessed by Northern blotting (2.2.13) using the GAS1 cDNA fragment used to generate the recombinant expression vector as the probe. Similarly, the degree of inhibition of the endogenous levels of MDM2 mRNA by the antisense MDM2-expressing plasmid was assessed using the 949 bp *Not*I MDM2 cDNA fragment as the probe.

7.2.3 Nested RT-PCR for the detection of the MDM2 alternatively-spliced variant

Total RNA was extracted and used as a template for first-strand cDNA synthesis by a random primer extension method as previously described (2.2.16) and modified to

facilitate the use of random hexamers in place of oligo-dT or a specific downstream primer. Two micrograms of total RNA was reverse-transcribed into single-stranded cDNA at 42°C for 1 hr in a 20 µl reaction containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100, $MgCl_2$, 1 mM each dNTP, 500 ng of random hexamers (Bresatec), 10 units RNAsin and 30 units of AMV reverse transcriptase. A 1515-bp MDM2 fragment was amplified using the following nested PCR primers (Sigalas *et al.*, 1996) to ensure specificity (Fig. 7.2).

EXTERNAL

MDM2-EXT1 5'-CTGGGGAGTCTTGAGGGACC-3'

MDM2-EXT2 5'-CAGGTTGTCTAAATTCCTAG-3'

INTERNAL

MDM2-INT1 5'-CGCAAAACCCCGGGCAGGCAAATGTGCA-3'

MDM2-INT2 5'-CTCTTATAGACAGGTCAACTAG-3'

The PCR reaction was carried out for 35 cycles of: 92°C for 1 min, 55°C for 1 min and 72°C for 2 min. Total reaction volume was 50 µl and comprised of 5µl of the cDNA, 100 ng of each of the oligonucleotide primers, 0.8 mM dNTP's, 2.5 mM $MgCl_2$ in the PCR buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C) and 1% Triton X-100. Each PCR reaction contained 1 unit of Taq DNA polymerase (Promega). Amplification was followed by an extension at 72°C for 5 min. The nested PCR reactions were carried out in two stages, with 2 µl of the first-round of PCR reaction

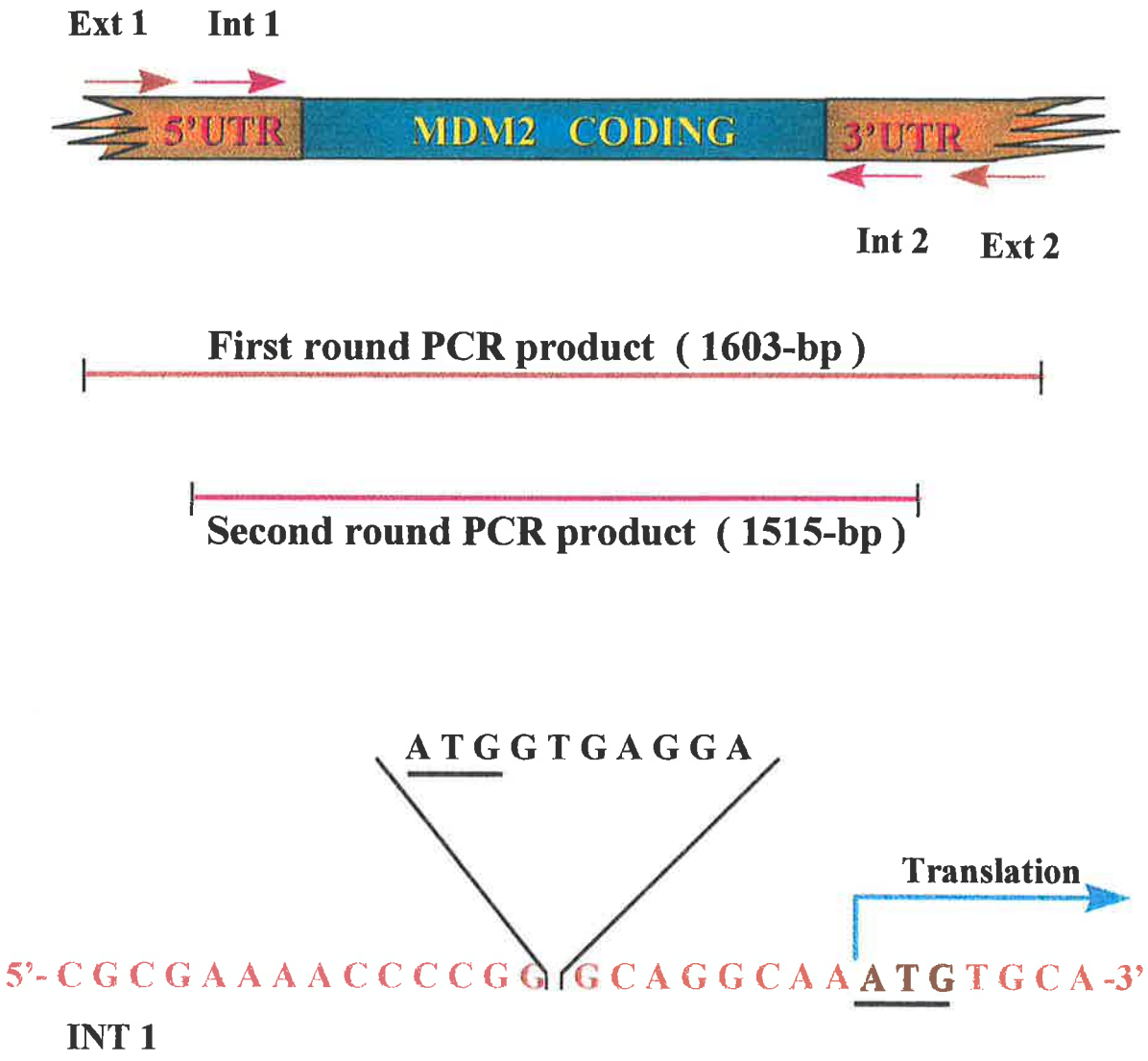


Figure 7.2 Position of the nested PCR primers in relation to the coding region of the MDM2 gene. Both external (EXT) and internal (INT) primers cover the entire coding region of the MDM2 gene. To ensure specificity, the products generated using the external primers were seeded into the second round PCR reaction with the internal primers. For a full length transcript, a 1603 bp fragment is amplified with the external primers and 1515 bp fragment is amplified using the internal primers. The 5' INT primer is a long primer and was synthesised to specifically remove 10 bp of the MDM2 published sequence. This results in the deletion of the first ATG which could give rise to false translational products and only the correct ATG codon is utilised.

with the external primers being seeded into the second-round reaction with the internal primers. The products of the second-round of PCR reactions with the internal primers were visualised on 1.5% agarose gels. The alternatively-spliced variant of MDM2 generated after the second round reactions with the internal primers, was separated by electrophoresis on low melting temperature agarose, the appropriate band cut out from the gel and the PCR product purified using the Wizard PCR preparation DNA purification kit (Promega).

7.2.4 Sequencing of the MDM2 alternatively-spliced variant.

The purified 269 bp alternatively-spliced variant of MDM2 was cloned directly into the pGEM-T vector (2.2.5) and the sense and antisense universal primers of pGEM (2.1.11) were used to sequence the cloned fragment in both directions using the Stratagene Cyclist Exo$^-$*pfu* DNA sequencing kit with α–^{32}P-dATP (2.2.21). The products of the sequencing reactions were separated by electrophoresis on 6% polyacrylamide sequencing gels (2.2.22). The sequence obtained from the cloned PCR product was aligned with the published MDM2 cDNA sequence (Oliner *et al.,* 1992), using the Genejockey DNA analysis software package.

7.3 RESULTS

7.3.1 Transfection with pRcCMV-GAS1: Colony numbers and expression of GAS1 mRNA

HTD114 cells transfected with the GAS-1-expressing plasmid pRcCMV-GAS1 formed the same number of G418-resistant colonies as cells transfected with the control vector pRcCMV (Table 7.1). The mass culture representing all of the clones, demonstrated the presence of the 2.0 kb exogenous GAS1 mRNA (Fig. 7.3), whereas 8 of the 10 individual clones expressed variable levels of GAS1 mRNA. In addition to the 2.0 kb GAS1 transcript, some of the clones also expressed transcripts of different sizes, suggesting the presence of multiple constructs in the same clone which may have become rearranged during integration into the host genome (Fig. 7.3 lanes 6 and 7). The differing size of transcripts of GAS1 in some of the clones would suggest that these are distinct clones.

Table 6.3 Number of HTD114 G418-resistant colonies two weeks after transfection with plasmid constructs

	Number of G418-resistant colonies	
	expt 1	expt 2
pRcCMVneo-vector only	68	93
pRcCMV-GAS1	80	68
pRcCMV-MDM2-antisense	25	33
pRcCMV-GAS1 + pRcCMV-MDM2-antisense	72	95

HTD114 cells were transfected with the plasmid constructs as shown and selection in G418 allowed to proceed for 2 weeks. Each figure represents the total number of colonies from duplicate flasks from 2 independent transfection experiments

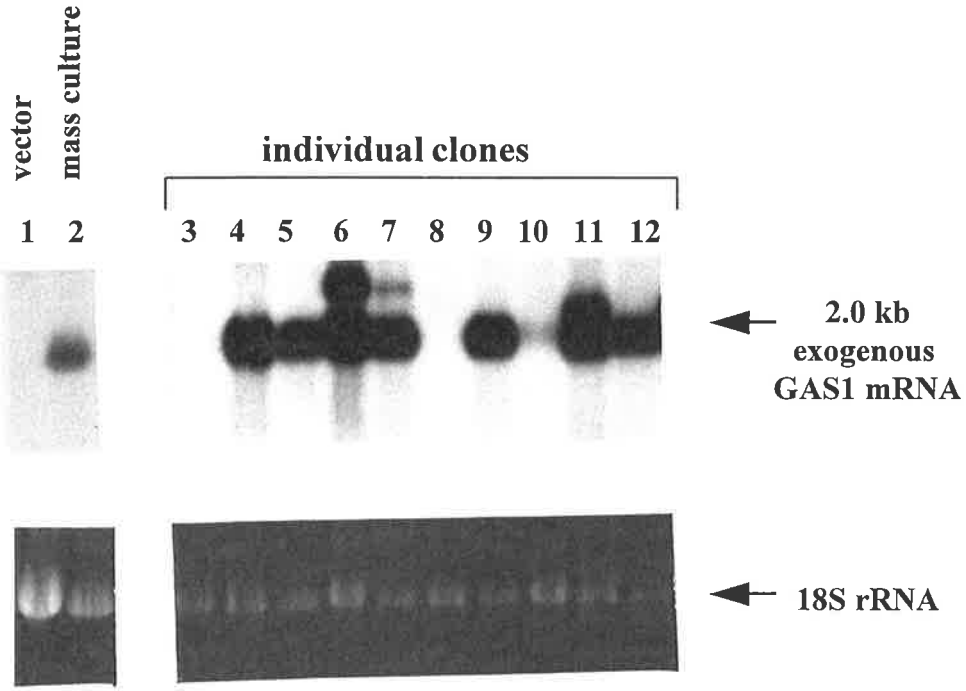


Figure 7.3 Expression of exogenous GAS1 mRNA in HTD114 cells transfected with the pRcCMV-GAS1 expression vector. Northern blot analysis using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. HTD114 cells were transfected with pRcCMV control vector-only (lane 1) or pRcCMV-GAS1, mass culture, (lane 2). Lanes 3-12 contain mRNA isolated from ten randomly-selected pRcCMV-GAS1 clones.

7.3.2 Transfection with pRcCMV-MDM2-antisense: Colony numbers and inhibition of expression of the endogenous MDM2 mRNA

HTD114 cells transfected with the antisense MDM2-expressing plasmid pRcCMV-MDM2 antisense resulted in more than 2.5 fold fewer G418-resistant colonies than cells transfected with the control vector pRcCMV (Table 7.1). The significance of the lower number of colonies with the antisense plasmid is not yet clear, but may be related to inhibition of the oncogenic activity of MDM2. Antisense MDM2 downregulates the endogenous levels of MDM2 protein and this should lead to elevated levels of unbound p53 which is growth-suppressive, hence the lower transfection efficiency. Using the 949 bp MDM2 cDNA fragment as a probe, Northern analysis of RNA isolated from the mass culture transfected with pRcCMV-MDM2-antisense, detected a 5.5 kb transcript which is characteristic of the normal endogenous full length MDM2 mRNA (Fig. 7.4) (Oliner *et al.*, 1992). Similarly, Northern analysis of ten individual clones displayed variable levels of the endogenous MDM2 mRNA which, in some clones was significantly reduced (Fig. 7.4 lane 4) or absent (Fig 7.4 lane 7), suggesting, the antisense MDM2-mediated inhibition was effective in some, but not all clones. At no time during these experiments, was the presence of the expected 1.3 kb antisense MDM2 mRNA demonstrated. However, the significantly reduced levels of the endogenous MDM2 mRNA observed in some of the clones suggested that the antisense construct was expressed. The failure to detect antisense mRNA is one of the problems often associated with antisense experiments. This finding is reminiscent of the results previously discussed (Chapter 5, section 5.3.5) in which expression of antisense GAS1 had a pronounced biological effect in NIH3T3 cells without being able to detect the antisense mRNA.

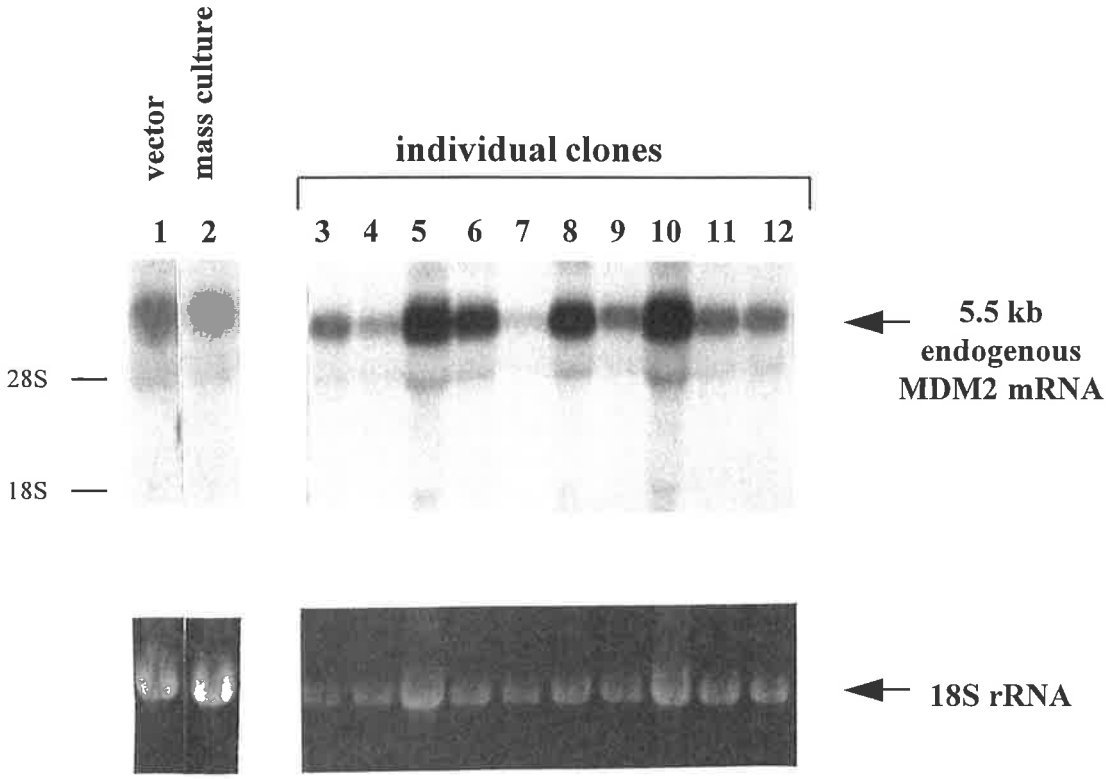
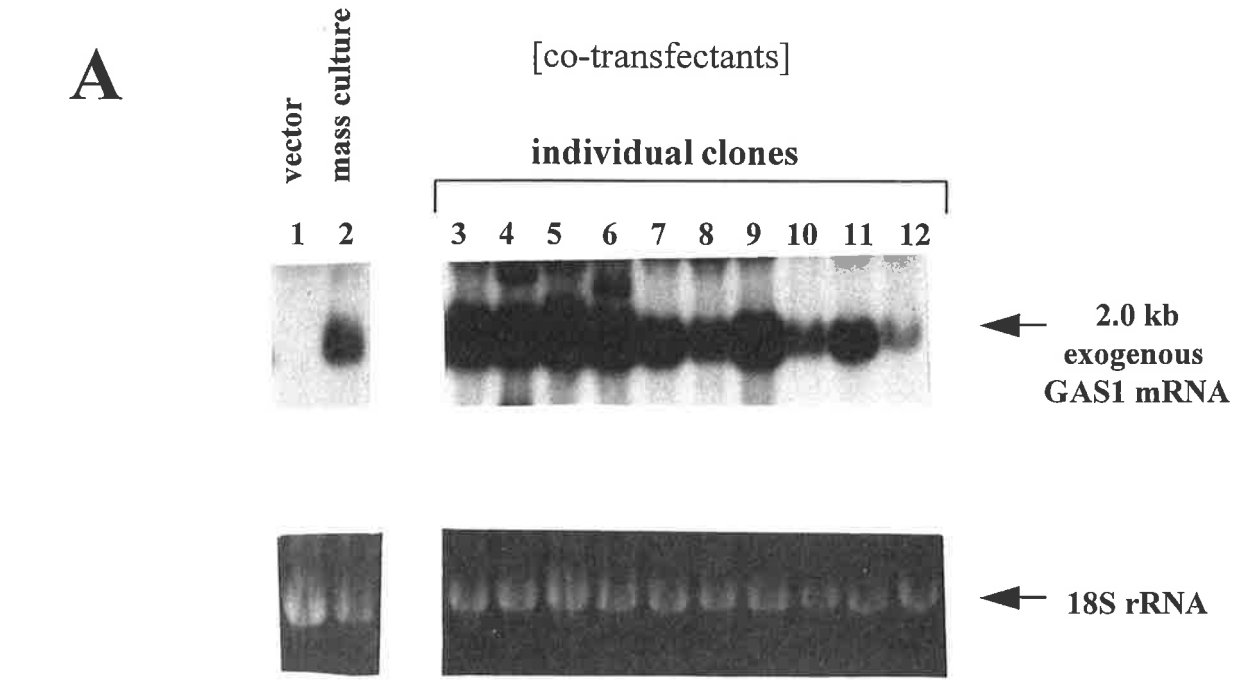


Figure 7.4 Inhibition of expression of the endogenous levels of MDM2 mRNA in HTD114 cells transfected with the pRcCMV-MDM2 antisense expression vector. Northern blot analysis using an MDM2 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. RNA was isolated from HTD114 cells transfected with pRcCMV control vector-only (lane 1) or pRcCMV-MDM2 antisense, mass culture, (lane 2) Lanes 3-12 contain mRNA isolated from ten pRcCMV-MDM2 antisense clones. The MDM2-antisense mRNA could not be detected in any of the transfectants.

7.3.3 Co-transfection of HTD114 cells with pRcCMV-GAS1 and pRcCMV-MDM2-antisense induces the expression of a variant MDM2 transcript

Following selection for two weeks in the presence of G418, there was no reduction in the number of G418-resistant colonies in HTD114 cells co-transfected with pRcCMV-MDM2-antisense and pRcCMV-GAS1 plasmid constructs when compared to the vector-alone transfectants (Table 7.1). Northern analysis, using a GAS1 cDNA fragment as the probe showed high levels of expression of exogenous GAS1 mRNA in all ten clones including the mass culture (Fig. 7.5A). To assess if the endogenous levels of MDM2 were adequately inhibited by the antisense MDM2-expressing plasmid, the same Northern blot was stripped free of the first probe (2.2.11) and re-hybridised using the 949 bp MDM2 cDNA fragment as a probe. The endogenous levels of the 5.5 kb MDM2 mRNA were significantly lower in some of the clones when compared to the vector-only transfectants (Fig. 7.5B) suggesting that the antisense MDM2 construct was effective in inhibiting the endogenous levels of MDM2 in a similar manner to that observed in the MDM2-antisense only transfectants (Fig. 7.4). Although there was a reduction in the amount of endogenous MDM2 mRNA, these cells remained refractory to overexpression of GAS1 as assessed by the failure of GAS1 to cause a reduction in colony number. This result was contrary to our expectations and suggested that there was no selection pressure against the growth of HTD114 cells overexpressing GAS1 and antisense MDM2 despite the reduced level of MDM2 mRNA. However, three of the clones, including the mass culture, now expressed a variant MDM2 transcript that was detected as a 2.2 kb band on the Northern blot (Fig. 7.5B). Densitometric analysis demonstrated that the level of expression of the 2.2 kb variant MDM2 transcript in the three individual clones was more than twenty fold higher when compared to the full

Figure 7.5 (A) Expression of GAS1 in HTD114 cells co-transfected with the pRcCMV-GAS1 and pRcCMV-MDM2 antisense expression vectors. Northern blot analysis using a GAS1 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. HTD114 cells were transfected with pRcCMV control vector-only (lane 1) or pRcCMV-GAS1 + pRcCMV-MDM2 antisense, mass culture, (lane 2). Lanes 3-12 contain mRNA isolated from ten randomly-selected pRcCMV-GAS1+ pRcCMV-MDM2 antisense co-transfected clones. (B) Inhibition of expression of the endogenous levels of MDM2 in HTD114 cells transfected with the pRcCMV-GAS1 + pRcCMV-MDM2 antisense expression vectors and expression of the 2.2 kb variant MDM2 transcript. The same Northern blot as in (A) was rehybridised using an MDM2 cDNA probe which is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. The order of the tracks is the same as in (A). The strong hybridisation signal in the mass culture and in the three clones corresponds to the 2.2 kb MDM2 variant transcript. Bands in lane 6 and 9 are due to the previous 2.0 kb GAS1 mRNA signal which was not efficiently washed off the blot prior to hybridisation with the MDM2 probe. Note these bands are positioned slightly lower than the 2.2 kb MDM2 variant mRNA signal.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mass culture
[co-transfectants]
individual clones
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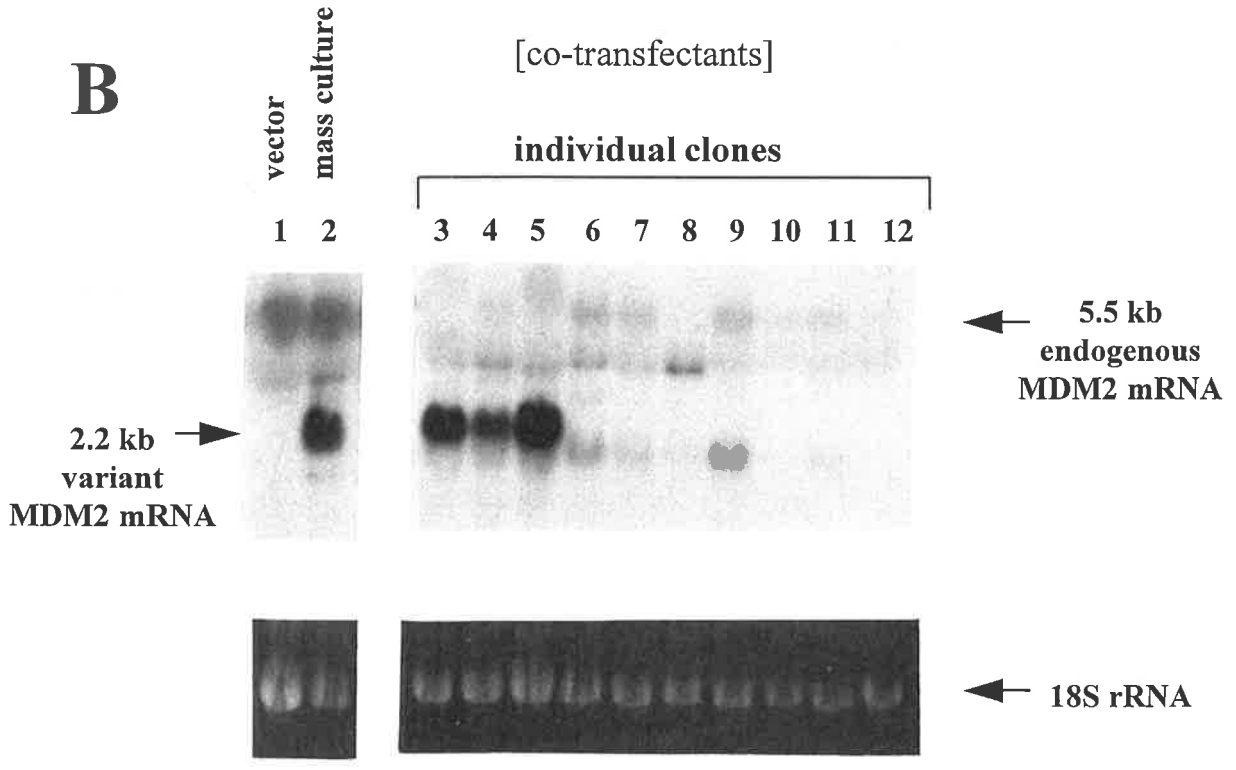
2.0 kb
exogenous
GAS1 mRNA
18S rRNA


B
vector
mass culture
[co-transfectants]
individual clones
1 2 3 4 5 6 7 8 9 10 11 12
2.2 kb
variant
MDM2 mRNA
5.5 kb
endogenous
MDM2 mRNA
18S rRNA

length transcript. Although not evident in the mass culture (Fig. 7.5B lane 2), the three individual clones that expressed the variant transcript had virtually undetectable levels of the full length transcript (Fig. 7.5B lanes 3, 4 and 5). The variant transcript was expressed only in the co-transfectants and its expression could not be detected in either the vector-only, GAS1-only or the MDM2-antisense-only transfectants. Figure 7.6 shows that the 5.5 kb full length MDM2 transcript is present in the mass cultures of all transfected populations. However, the 2.2 kb MDM2 variant transcript was present only in the co-transfectants. These results suggested that expression of the variant is perhaps a result of the synergistic effects of both antisense MDM2 and overexpression of GAS1 in some but not all clones.

7.3.4 The variant transcript is a novel alternatively-spliced variant of MDM2

To determine the identity of the 2.2 kb variant transcript, total RNA was isolated from the mass culture and from one of the individual clones which expressed the highest level of the variant 2.2 kb transcript (clone 1). Reverse transcription followed by PCR amplification using the external primers which span the complete coding region of MDM2, amplified the expected 1603 bp fragment which corresponds to the full-length MDM2 transcript in all samples including the vector only transfectants (Fig. 7.7). Additional PCR products were also amplified in all samples and were the same products present in the genomic DNA control (Fig. 7.7A lane 8) suggesting amplification from non-specific targets in the genome. To ensure specificity of amplification from the MDM2 cDNA, 2 µl of the products of the first round of PCR was added to a second round of PCR and amplified using the internal primers (7.2.3). In addition to the expected 1515 bp transcript, a shorter transcript of 269 bp was also amplified in clone 1

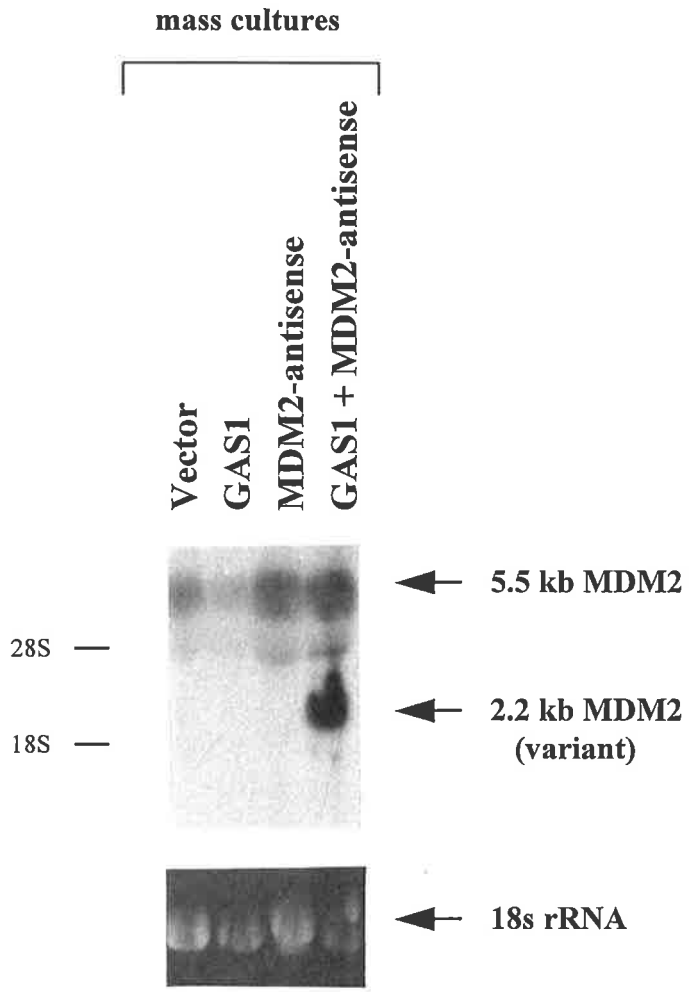


Figure 7.6 The 2.2 kb MDM2 variant transcript is expressed only in the co-transfectants. Northern blot analysis using an MDM2 cDNA probe is shown in the upper panel and parallel ethidium bromide staining pattern of control 18S rRNA in the lower panel. RNA was isolated from the mass cultures of HTD114 cells transfected with pRcCMV-vector, pRcCMV-GAS1, pRcCMV-MDM2 antisense and pRcCMV-GAS1 + pRcCMV-MDM2 antisense co-transfectants.

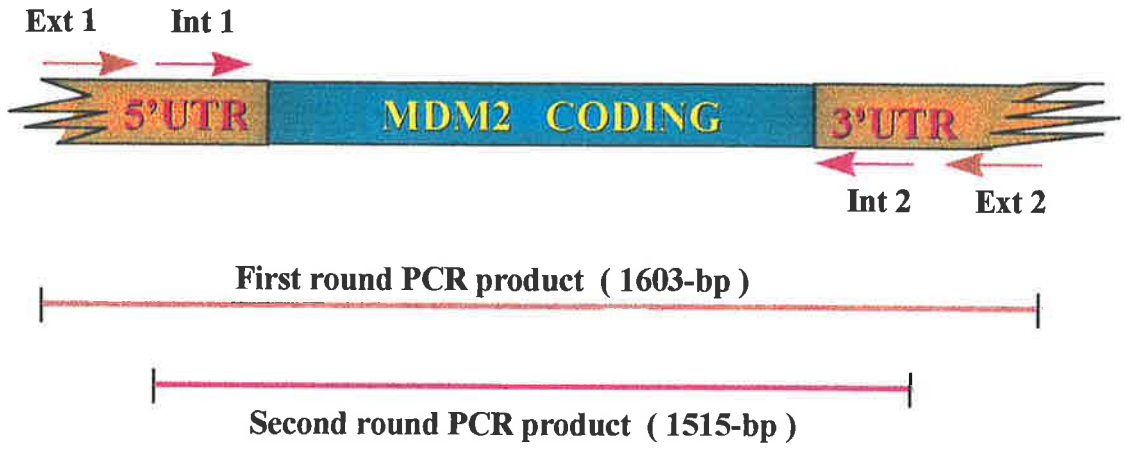
Figure 7.7 Agarose gel electrophoresis of RT-PCR products using nested PCR primers that span the complete coding region of MDM2 showing multiple size products in the HTD114 co-transfectants.

(A) Schematic representation of the position of the nested PCR primers in relation to the coding region of the MDM2 gene. See methods for details

(B) PCR products generated after the first round of PCR using the external primers showing amplification of the full length 1603 bp MDM2 transcript and the presence of non-specific amplification products. *Eco*RI-digested SPP-1 marker (lanes 1), pRcCMVneo vector mass culture (lanes 2), pRcCMV-GAS1 mass culture (lanes 3) pRcCMV-MDM2 antisense mass culture (lanes 4), pRcCMV-GAS1 + pRcCMV-MDM2 antisense mass culture (lanes 5), pRcCMV-GAS1 + pRcCMV-MDM2 antisense clone 1 (lanes 6), negative control with no DNA (lanes 7), and genomic DNA from HTD114 cells used in the first round of PCR amplification showing non-specific products arising from DNA (lane 8)

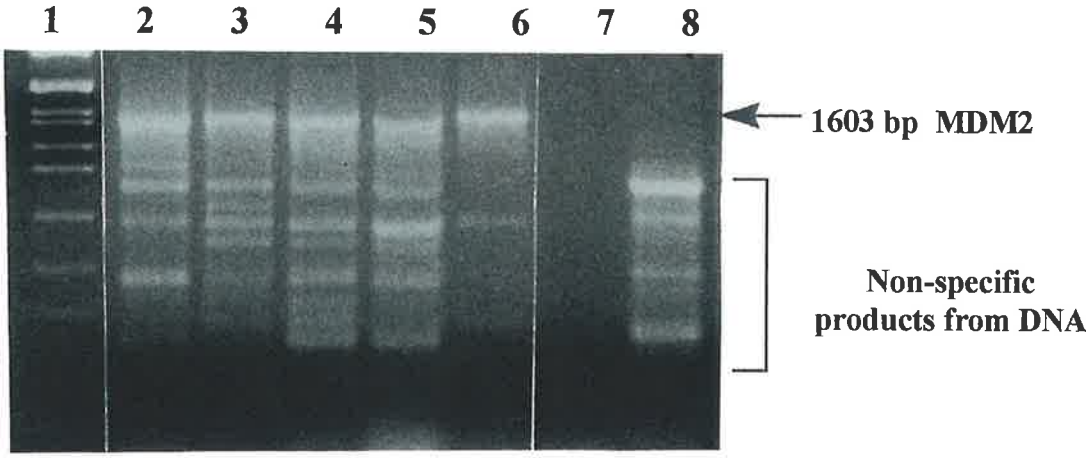
(C) In addition to the full length transcript (1515 bp), a 269 bp shorter alternatively-spliced variant of MDM2 was also amplified in clone 1 of the co-transfectants (lane 6). To ensure specificity, 2 µl of the first round of PCR were seeded into the second round and amplified using the internal primers. The order of the lanes is the same as in (B) without the genomic DNA control.

A



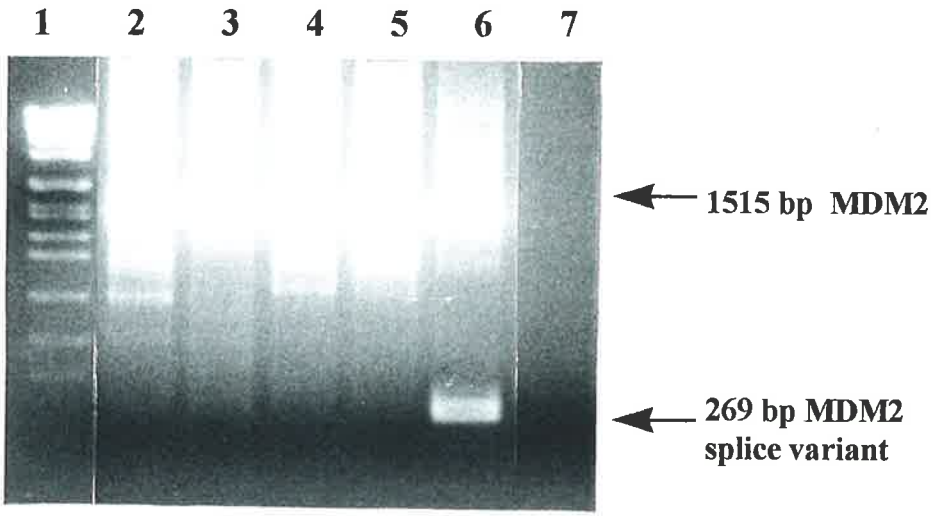
B

[First round PCR with external primers]



C

[Second round PCR with internal primers]



of the co-transfectants but was absent from the vector-only control cells, the GAS1-only or the MDM2 antisense-only transfectants (Fig. 7.7B). Although the 2.2 kb MDM2 variant transcript was detected on Northern blots from the mass culture of the co-transfectants (Fig. 7.5B, lane 2), RT-PCR failed to amplify the same 269 bp short variant as clone 1 and only the 1515 bp transcript was present. This may be related to the ratio of the variant transcript to that of the full-length transcript within the different populations of cells. The Northern blot (Fig. 7.5B) suggested that only 30% of the cells within the mass culture express the 2.2 kb variant transcript since only three of the ten clones were shown to express it. Clone 1 is a pure clone which was isolated from a single colony and therefore 100% of the cells should express the variant MDM2 mRNA. The ratio of the variant transcript to that of the full length MDM2 transcript is higher in clone 1 than in the mass culture (Fig 7.5B) and would therefore represent a more abundant target for PCR amplification of the variant transcript.

7.3.5 The MDM2 alternatively-spliced variant contains most of the p53 binding domain but is devoid of DNA binding motifs

To determine the identity of the variant MDM2 transcript, the RT-PCR product generated from clone 1 was cut from the gel, purified (2.2.4), cloned into pGEM-T vector (2.2.5) and sequenced (7.2.4) using the *Exo⁻pfu* DNA sequencing kit. This clone was also sequenced independently by The Flinders University sequencing facility and the data is shown in Appendix II. Alignment of this sequence with the published MDM2 cDNA sequence revealed a 269 bp alternatively-spliced variant of MDM2, [AEV] which contained a substantial part (nucleotides 1-171 and nucleotides 1422-1473) of the MDM2 cDNA, with a large section of the sequence between the two regions missing,

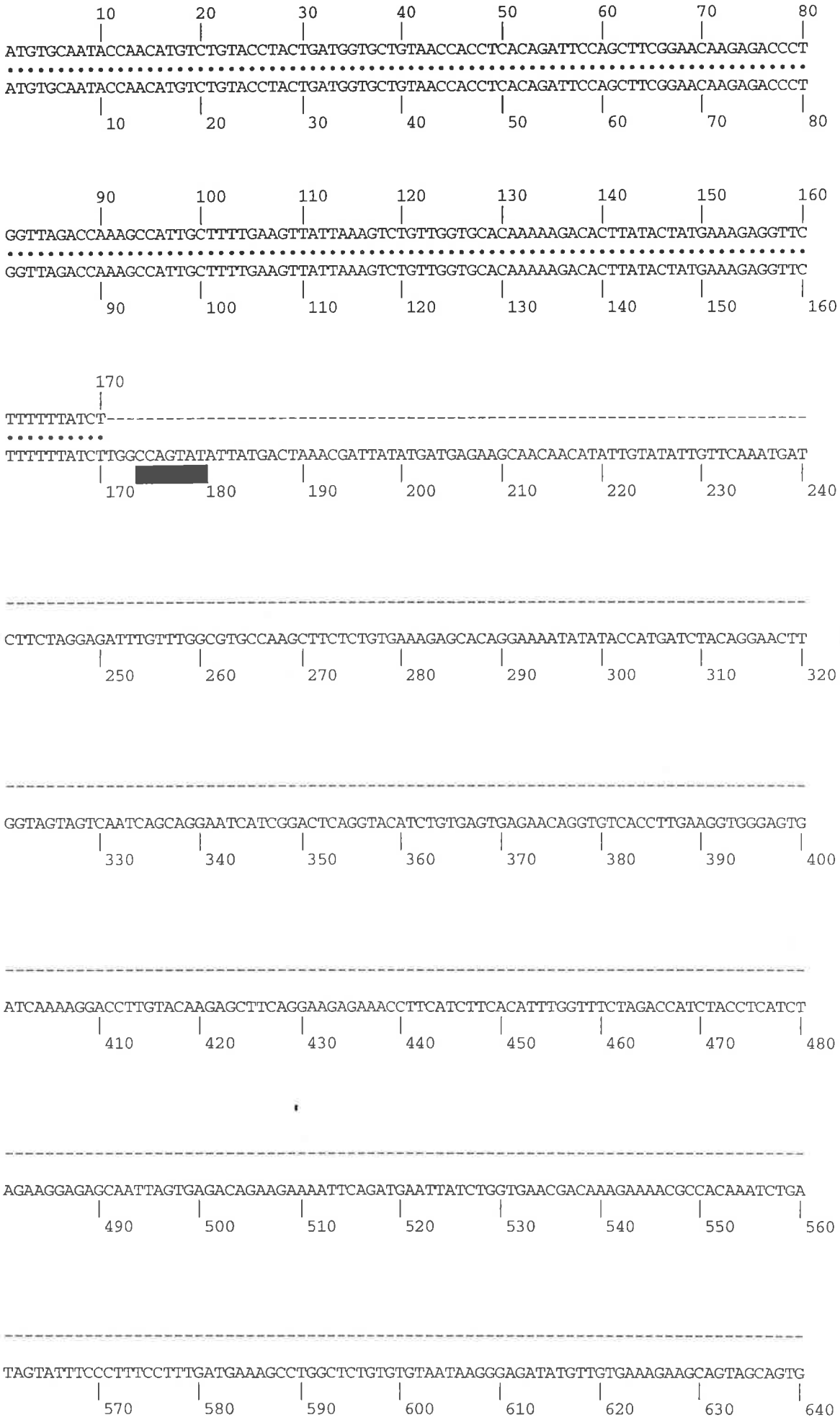
suggesting that the short RT-PCR product corresponded to an alternatively-spliced transcript of MDM2 (Fig 7.8). Analysis of the genomic structure of MDM2 suggested that the splicing occured within exon 5, joining into the nearly the end of exon 12 (Fig 7.9). An important observation is the presence of a seven bp sequence (CCAGTAT) at both, the donor and acceptor sites that make up the splicing junction (Fig. 7.8). The importance of this sequence is not yet clear but may indicate a recognition signal for splicing. The alternatively-spliced variant contains a large part of the p53 binding domain but does not contain the putative nuclear localisation signal (codon 181-185), an acidic domain (codons 223-274), a central zinc finger motif (305-322) or a carboxy-terminal "RING finger" zinc binding motif (codons 438-478) (Chen *et al.*, 1993; Boddy *et al.*, 1994; Sigalas *et al.*,1996). Comparison of this variant with the other five recently identified alternatively-spliced variants of MDM2 reported by Sigalas *et al* (1996) suggested that the variant reported here is a novel and a previously unrecognised spliced variant and is the smallest of the variants identified to date.(Fig 7.10).

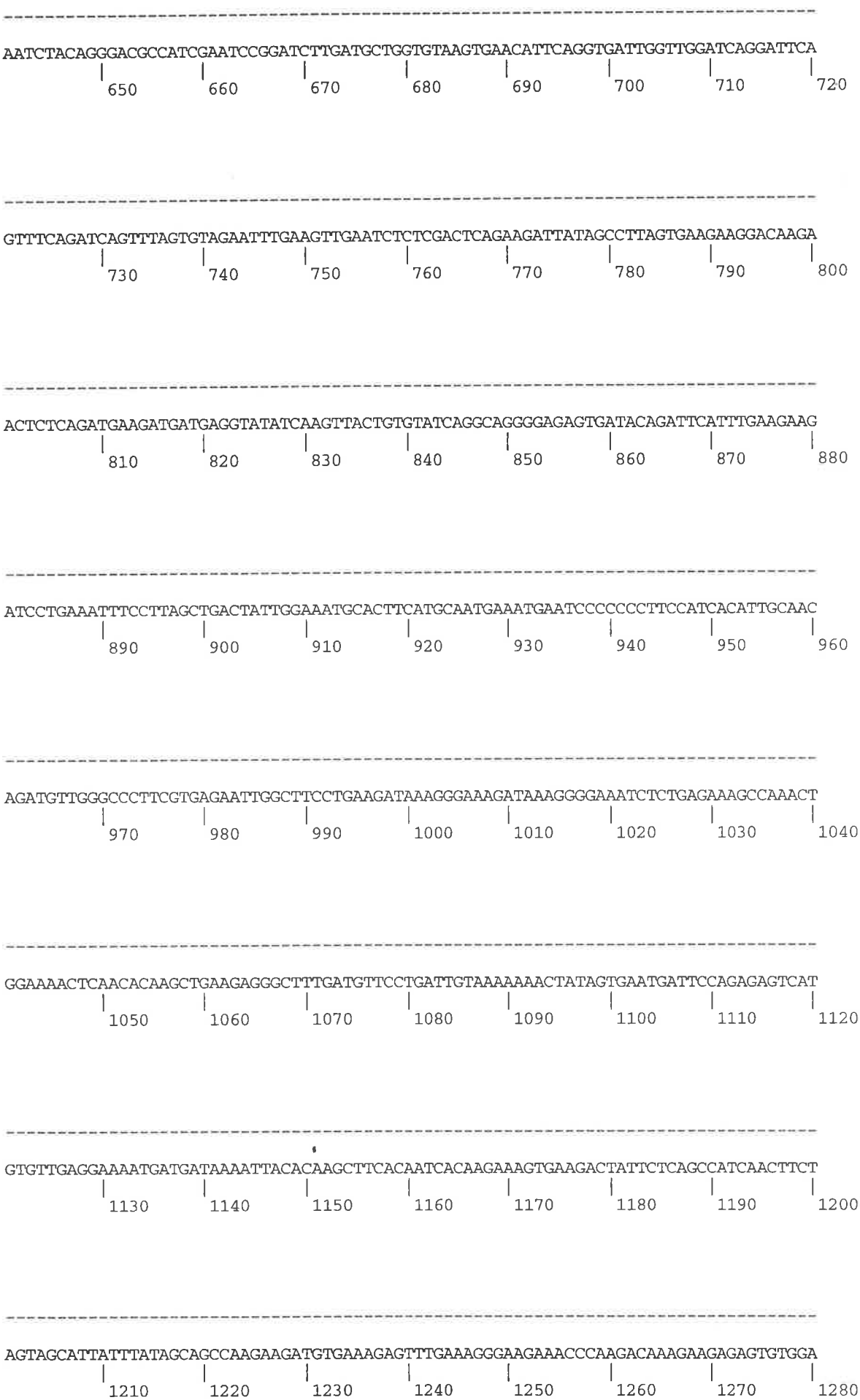
7.4 DISCUSSION

The studies reported in this chapter describe the characterisation of a previously unrecognised alternatively-spliced variant of the MDM2 oncogene product in HTD114 cells. Multiple sized MDM2 proteins have previously been identified in murine and human cells (Olson *et al.*, 1993; Barak *et al.*, 1994; Haines *et al.*, 1994; Cudas *et al.*, 1995) and it has also been reported that multiple-sized transcripts are detected by MDM2 probes in mouse and human cells (Oliner *et al.*, 1992; Barak *et al.*, 1994). Recently, five alternatively-spliced MDM2 gene transcripts were detected in a range of

Figure 7.8 Sequence alignment of the alternatively-spliced variant (top) with the published (Oliner *et al.*, 1992) full length (bottom) coding region of the MDM2 gene. The 7-bp-CCAGTAT- repeat units near the splicing sites are shown underlined.

MDM2 [AE-V] variant (top), MDM2 full length (bottom)

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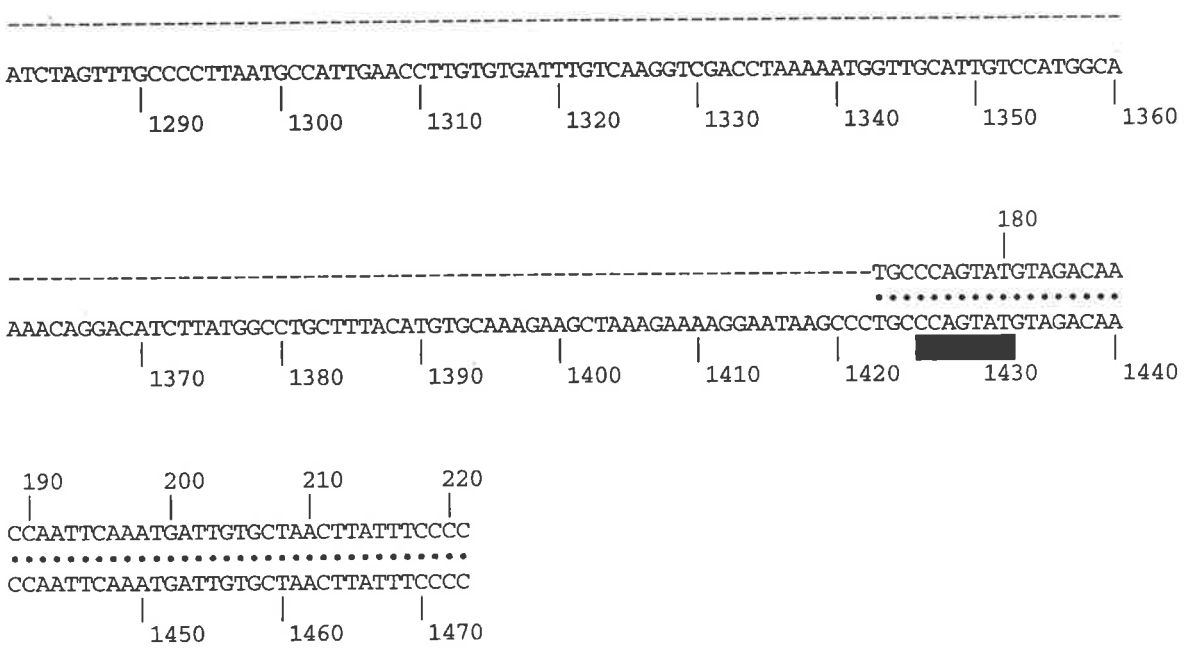
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1460
1470

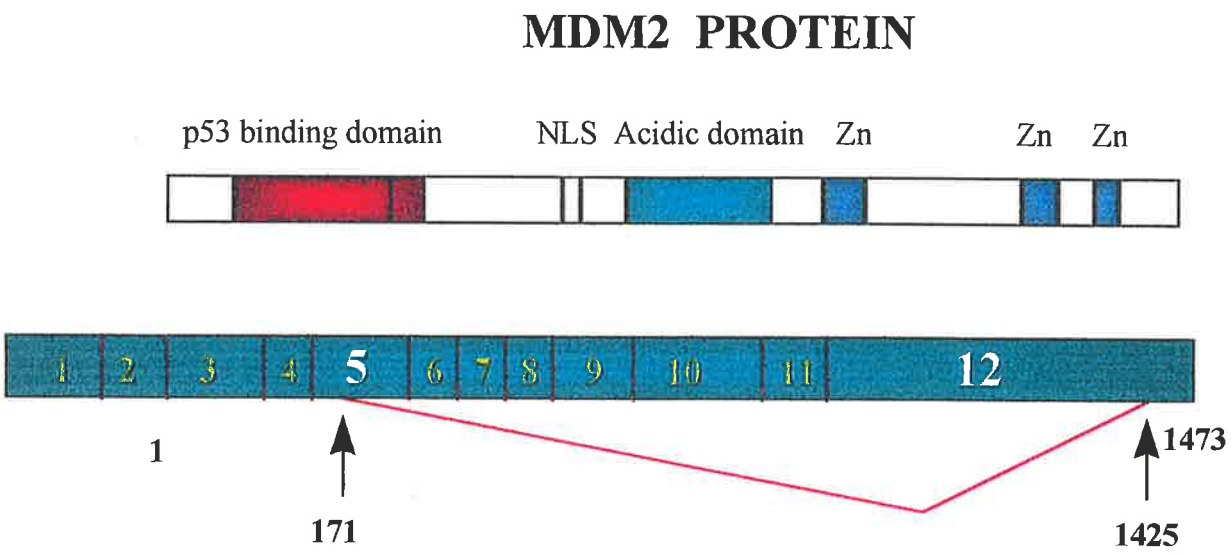

Figure 7.9 Genomic organisation of the MDM2 gene showing the location of exons and the position where splicing occurs in exons 5 and 12 to give rise to the variant MDM2 product. The exons are depicted as boxes and numbered 1-12 amid the intronic sequences (horizontal lines)

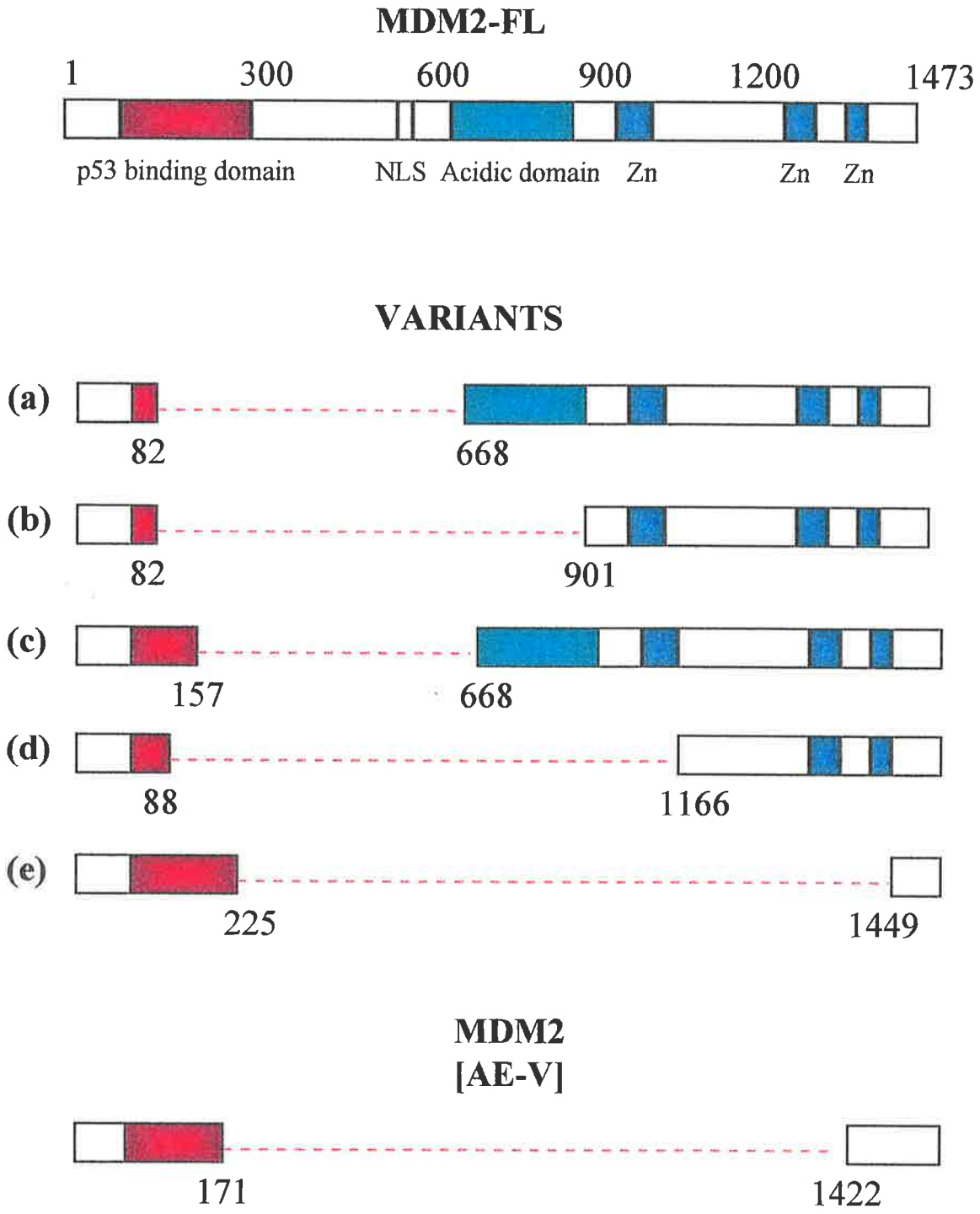

Figure 7.10 Summary of the sequencing data for the alternatively spliced transcript of MDM2, [AE-V] identified in this study, and comparison with the coding region of the full-length transcript and the previously identified spliced variants reported in Sigalas *et al.* (1996). The functional domains of the MDM2 protein are indicated: NSL, nuclear localisation signal; Zn, zinc finger.

human cancers but were absent in normal tissues (Sigalas *et al.*, 1996). The presence of these variants correlated with the late-stage and high-grade of ovarian and bladder carcinomas. Four out of the five alternatively spliced variants were lacking p53-binding domain sequences whereas the shortest variant contained most of the p53 binding domain but was devoid of any other putative DNA binding features of the MDM2 protein. The four variants which lacked regions of the N-terminal, which have previously been defined to be necessary for p53 binding (Chen *et al.*, 1993), were able to transform NIH3T3 cells, suggesting that their transforming ability was not mediated by functional inactivation of p53 protein but was related to the DNA binding ability of MDM2 to other as yet unknown targets. In the case of the shortest variant of MDM2 which contained most of the p53 binding domain and which was missing the DNA binding regions, transforming activity could be mediated by binding to p53 protein and inactivating the transcriptional activation and/or reppression functions of p53. These observations suggested the presence of both p53-dependent and p53-independent transforming functions of the MDM2 oncogene product (Sigalas *et al.*, 1996). The variant reported here is even smaller than the variant reported by Sigalas *et al* (1996) and represents a novel variant which contains most of the p53 binding domain but is missing all other DNA binding regions. If this variant has any transforming ability, it must be dependent on binding to the p53 protein. Whether the variant identified here binds to p53 or has any transforming ability has not yet been determined and would form the basis of future studies.

Although there was a reduction in the level of endogenous MDM2 mRNA in the co-transfectants, these cells remained refractory to overexpression of GAS1 as assessed by the failure to cause a reduction in the number of G418-resistant colonies after transfection. However these cells now expressed high levels of the alternatively-spliced

variant of MDM2. It is possible that the growth-suppressing signals of GAS1 are overridden by the efficient expression of the alternatively-spliced variant of MDM2. The function of the variant could be to inhibit the activity of any elevated levels of free p53 generated, due to the antisense-mediated down-regulation of the full length MDM2. Based on these observations, the following model is proposed: (i) GAS1 requires p53 to mediate its growth suppressing function. (ii) HTD114 cells overexpress MDM2, and most of the p53 protein within these cells is likely to exist as a complex with the MDM2 protein. (iii) The proportion of p53 bound to MDM2 determines if p53 is functionally inactivated by MDM2. (iv) The effects of antisense MDM2 is to release p53 from the p53-MDM2 complex thus changing the balance in favour of free and functional p53 protein within the cell. (v) The GAS1-suppressive signalling pathway now becomes operational. (vi) The cell overcomes the GAS1-mediated growth suppressive signals by efficiently expressing the alternatively-spliced variant of MDM2, which binds to and inactivates the free p53 protein. (vii) The cells thereby maintain their transform phenotype and remain refractory to overexpression of GAS1. (viii) Expression of the alternatively-spliced variant is dependent on the synergistic effects of antisense MDM2 and GAS1 overexpression.

This study has identified two downstream elements, MDM2 and the AE-V variant, which may be involved in modulating the cascade of events that lead to growth arrest mediated by GAS1.

CHAPTER 8

GENERAL DISCUSSION

8.1 DISCUSSION

Tumour suppressor genes have been identified on the basis of an association between neoplasia and the loss of function of both copies of the gene (reviewed in Stanbridge, 1990; Mitchell, 1991; Marshall, 1991). While the existence a large number tumour suppressor genes is predicted on the basis of such associations, only relatively few have been cloned to date including *RB1* (retinoblastoma: Friend *et al.,* 1986; Hamel *et al.,* 1992), p53, (Oren *et al.,* 1983) *WT1* (Wilm's tumour: Call *et al.,* 1990; Rose *et al.,* 1990), *DCC* ("deleted in colon cancer": Fearon *et al.,*1990), and *NF1* (Neurofibromatosis: Wallace *et al.,* 1990). A potential role for GAS1 as an S-phase inhibitor (Del Sal *et al.,* 1992), possibly analogous to the retinoblastoma protein, implies that aberrations of *GAS1* may be predicted in tumours. However, unlike the nuclear localisation of RB1, *GAS1* encodes a membrane protein, possibly a receptor for cell adhesion molecules that may be involved in contact inhibition or in anchorage of cells to the extracellular matrix. Given these properties, a more apt analogy for GAS1 may be the DCC tumour suppressor gene product. The base sequence of the DCC gene codes for a protein with sequence similarity to neural cell adhesion molecules and other related cell surface glycoproteins (Fearon *et. al.*, 1995). Additional evidence that genes for cell adhesion molecules might be tumour suppressors comes from the observation that defective differentiation of some colorectal carcinoma cell lines results from altered collagen receptors (Pignatelli and Bodmer, 1988).

The principal aim of this study was to determine if GAS1 is a tumour suppressor gene. The chromosomal localisation by *in situ* hybridisation of GAS1 to mouse Chromosome 13 and, more importantly, to human chromosome 9 at bands q21.3-q22, provide the first evidence that GAS may be a candidate tumour suppressor gene. The

human GAS1 gene maps in a region of the genome which is highly significant as a site of a number of potential tumour suppressor genes. Extensive search of the literature has shown that genetic alterations in this region of chromosome 9q have previously been implicated as early events in the progression of myeloid malignancy (Sreekantaiah *et al.,* 1989).

Typically, mutation of tumour suppressor genes leads to both sporadic and familial tumours which may occur in very different tissues. Loss of function of the RB1 gene for example, results in both familial and sporadic retinoblastoma and somatic loss of the wild type alleles is observed in sporadic myeloid malignancies (Johnson *et al.,* 1985; Fitchett *et al.,* 1987). In an analogous fashion, the existence of familial malignancies which map to 9q could be predicted. Such is indeed true of gene(s) which causes predisposition to the nevoid basal cell carcinoma syndrome (NBCCS). NBCCS is an autosomal dominant disorder which predisposes to basal cell carcinomas of the skin, medulloblastomas, ovarian fibromas and a variety of other tumours. The gene for NBCCS has been mapped by linkage analysis to 9q22.3-q31 (Gailani *et al.,* 1992; Farndon *et al.,* 1992; Chenevix-Trench *et al.,* 1993) and loss of heterozygosity of markers from this region in sporadic BCCs indicates that is most likely to be the site of a candidate tumour suppressor gene (Gailani *et al.,* 1992; Chenevix-Trench *et al.,* 1993). Due to its location and its putative action as a tumour suppressor gene, the GAS1 gene was analysed as a candidate for the NBCCS gene. Using two-colour fluorescence *in situ* hybridisation, the GAS1 gene was found to map outside the interval which, by genetic analysis, has been shown to contain the NBCCS gene. GAS1 was therefore excluded as the gene responsible for NBCCS. This work was carried out in collaboration with a group from the Queensland Institute of Medical Research, Brisbane, Australia and the results were published in Wicking *et al.* (1995). The candidate gene responsible for the

nevoid basal cell carcinoma syndrome has recently been identified as the human homologue of the Drosophila segment polarity gene named PTC. The PTC gene is mutated in NBCCS patients and in related tumours (Hahn *et al.,* 1996a). The human PTC gene was localised by *in situ* hybridisation to human chromosome 9q22.3 (Hahn *et al.,* 1996b).

In addition to being frequently deleted in myeloid malignancies, the region of chromosome 9 containing the GAS1 gene also contains several genes known to be mutated in cancer predisposition syndromes. These include the genes for multiple self-healing squamous epithelioma (Goudie *et al.,* 1993), Fanconi's anaemia group C (Kwiatowski *et al.,* 1993), Xeroderma pigmantosum group A (Henning *et al.,* 1992). A high incidence of rearrangements of the long arm of chromosome 9 was also noted in a series (8 out of 15) of colorectal carcinomas. The break points were localised between 9q13-9q34, with clustering at 9q22 in 5 cases (Konstantinova *et al.,* 1991) which is the point location of *GAS1* (Evdokiou *et al.,* 1993). Rearrangements of 9q in colon carcinoma were also frequently observed by Reichman *et al.* (1985). However, they did not define the location of the 9q breakpoints.

Loss of heterozygosity on chromosome 9 is the most common genetic alteration identified in bladder tumours and is present in all stages and grades. Several reports have presented evidence for at least two tumour suppressor loci on chromosome 9 (Ruppert *et al.,* 1993). One or more tumour suppressor genes are believed to be located on 9p with other genes located on 9q at a region where *GAS1* was localised. However, in a recent report, no mutations of *GAS1* were detected by SSCP screening of 10 bladder cancer cell lines and 14 primary bladder cancers, suggesting that *GAS1* may not be a target for mutation in bladder carcinoma (Simoneau *et al.,* 1996).

The mapping of GAS1 to human chromosome 9 at q21.3-q22, provides a useful tool for further analysis for the possible involvement of GAS1 in cytogenetically-defined clinical conditions which may relate to the function of the gene. This region of the human genome is considered to be a fragile site and one which is found to be frequently deleted in acute leukemia (Mitelman, 1991). Loss of this gene either by mutation or deletion may be important in the development of certain malignancies, including some of the myeloid leukemias.

The results presented in chapter four of this thesis indicate that the mouse Gas-1 gene is differentially methylated in a tissue specific manner. Expression of Gas-1 is abundant in lung tissue and the degree of hypomethylation of Gas-1 correlates well with a transcriptionally-active state. A number of mouse tumour cell lines were shown to lack expression of Gas-1 mRNA even when grown under conditions that normally induce growth arrest. The lack of expression of Gas-1 mRNA in the tumour cell lines could not be attributed to gross rearrangements of the Gas-1 gene but was well correlated with the degree of DNA methylation at the Gas-1 locus. When compared to normal lung, all tumour cell lines displayed extensive hypermethylation of the Gas-1 gene. These observations suggested that hypermethylation of *Gas-1* may be a contributing factor for the loss of expression seen in tumours. However, treatment of the Lewis lung carcinoma cell line with the nucleotide analog 5-azaC, failed to induce expression of Gas-1 despite extensive demethylation of the gene. Taken together, these results suggest that demethylation of the Gas-1 gene is not, by itself sufficient to activate transcription and that other trans-acting factors may be required for transcription of Gas-1 to occur. Presumably, such trans-acting factors are not present in all cell types and may be absent in tumour cells.

The results presented in chapter five provide strong evidence to support the hypothesis that the normal function of the human GAS1 gene is related to the control of growth. When overexpressed in NIH3T3 fibroblasts, GAS1 is able to suppress proliferation of these cells *in vitro* and morphological changes characteristic of growth arrest were also noted in cells overexpressing the exogenous GAS1 gene. These results confirm the previous findings reported by Del Sal *et al.* (1992) who showed that transient overexpression of the mouse Gas-1 gene in NIH3T3 fibroblasts blocks cell proliferation and does not allow the Go to S-phase transition. To determine if antisense inhibition of expression of Gas-1 transforms NIH3T3 fibroblasts, an antisense strategy was used to block Gas-1 expression at a time when the cells require Gas-1 for growth arrest. The results obtained demonstrated that antisense inhibition of expression of Gas-1 induces a change in phenotype where cells grow to a higher saturation density and fail to show contact inhibition. However, antisense inhibition of expression of Gas-1 is insufficient to transform the cells, suggesting that additional genetic events are required for the development of a fully-malignant phenotype.

The antiproliferative effects of GAS1 were not only restricted to NIH3T3 fibroblasts but were also observed in human tumour cell lines. Overexpression of GAS1 inhibited the proliferation of A549 lung adenocarcinoma cells *in vitro* and suppressed tumorigenicity of these cells in nude mice. GAS1 also inhibited proliferation of an HT1080 fibrosarcoma subline with wild type p53 and normal levels of MDM2. In contrast, over-expression of GAS1 in the HT1080 fibrosarcoma subline HTD114, had no effect on either the *in vitro* or *in vivo* growth properties of these cells. Further characterisation of the HTD114 cell line revealed the presence of a wild type p53 gene suggesting that the failure of these cells to respond to overexpression of GAS1 was not due to functional inactivation of p53 protein by mutation of the p53 gene. However,

HTD114 cells expressed elevated levels of the MDM2 oncogene product which is known to inhibit the growth suppressive function of p53. The HT1080 6TGc5 subline with mutant p53 and normal levels of MDM2 was also refractory to GAS1. Taken together, these findings suggest that GAS1 suppresses the growth and tumorigenicity of tumour cells in a p53 dependent manner and that overexpression of MDM2 may represent an alternative mechanism to p53 mutation, by which cells become refractory to the effects of GAS1. This study has therefore provided the first direct evidence that GAS1 can inhibit the growth of tumours *in vivo*, thereby acting as a tumour suppressor gene. In addition this study has identified another downstream element, MDM2, that is involved in the cascade of events that lead to growth arrest mediated by GAS1.

In an attempt to clarify the role of overexpression of MDM2 in HTD114 cells in the inhibition of GAS1-mediated growth suppression, this study has also identified a previously unrecognised and novel spliced variant of the MDM2 oncogene product. Whether this variant has any biological function waits to be determined. However, in the context of these investigations, it is speculated that expression of the variant may be a consequence of the synergistic effects of overexpression of GAS1 and antisense MDM2-induced inhibition of endogenous MDM2. Expression of this variant may be a defensive response by which cells overcome the growth-suppressing signals imposed by overexpression of GAS1. Induction of expression of this variant may therefore be a consequence of specific events occurring in cells in response to various signals

In summary the results presented in this thesis have established the growth-suppressive activity of the human GAS1 gene and provided the first direct evidence that GAS1 can inhibit the growth of tumours. In addition, this study demonstrated that the antiproliferative effects of GAS1 are mediated by a p53 dependent pathway and that

functional inactivation of p53 by either mutation and/or overexpression of the MDM2 oncogene product inhibits the GAS1 mediated growth-suppression.

8.2 FUTURE DIRECTIONS

The human GAS1 gene is located at a chromosomal site which is considered to be a fragile site and which is frequently deleted in myeloid malignancy. Whether the inactivation or the lack of expression of this gene, induced by the chromosomal deletion or translocation, has a role in determining malignant transformation in acute leukemia with del(9q) remains to be clarified. In a small preliminary study, we have not yet detected any gross deletions in the GAS1 gene in cells from patients with acute myeloid leukemia, The lack of clinical samples prevented a direct examination of the involvement of GAS1 in myeloid malignancy and this will need to be addressed in future studies. If *GAS1* is a tumour suppressor gene, both copies of the gene should be non-functional in malignant cells, as is generally the case for both RB1 and p53 Accordingly, the intention in future studies would be to analyse the sequence of the presumably single allele remaining in those cases of myeloid malignancy showing overt deletions of 9q21.3-22.

The functional role of the product of GAS1 as a potential cell surface receptor has not yet been established. Future studies should try to identify ligands for GAS1 or alternatively, investigate the association of the GAS1 protein with other interacting cell surface or cell matrix proteins. These investigations should provide insights as to the possible mechanisms of the signal transduction pathways that lead to GAS1-mediated growth suppression originating from the cell membrane.

Further characterisation of the previously unrecognised alternatively-spliced variant of MDM2 [AE-V] presents the challenge of defining its function and oncogenic potential and should form the basis of future studies.

A further area of investigation should include knockout experiments in which the GAS1 gene is homozygously inactivated in mice. These studies will determine the importance of GAS1 in embryonic growth and development. If GAS1 is essential for the control of cellular proliferation then embryonic lethality would be predicted. Alternatively, if viable progeny are obtained, it will be of interest to determine if these will predispose to tumour formation in a similar manner to that observed with *p53* and *RB1* knockouts.

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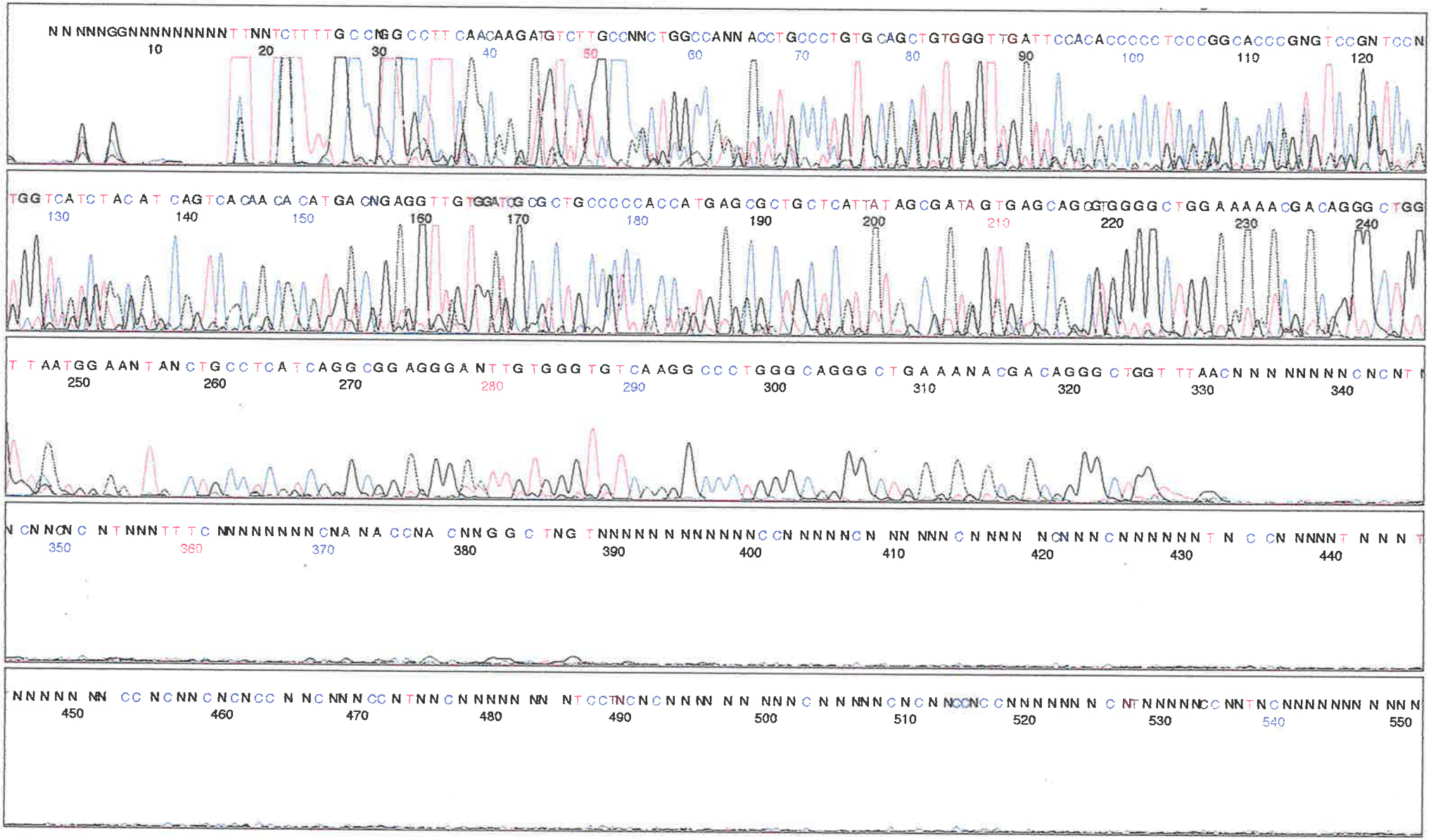
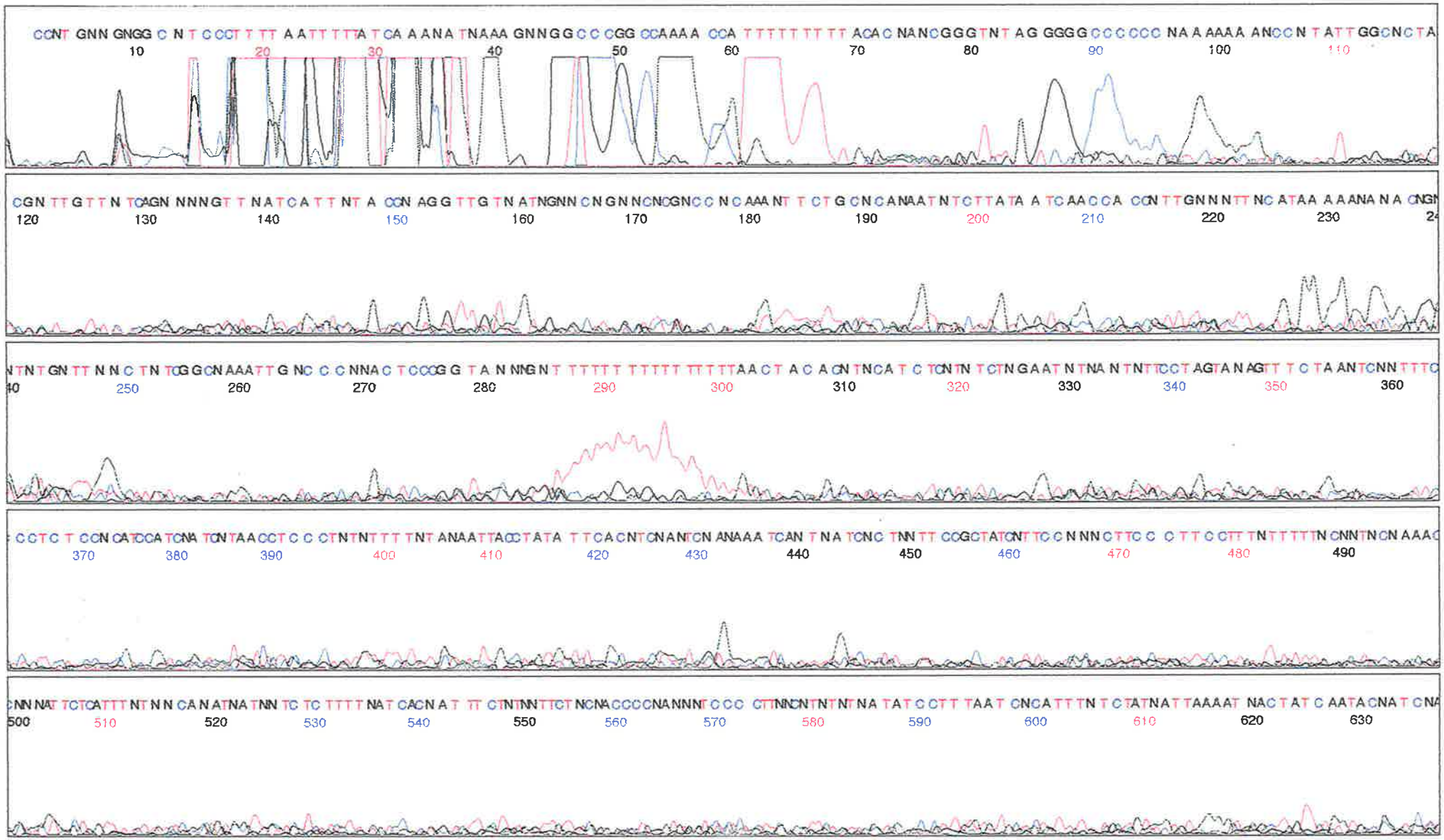
APPEDICES

APPENDIX I

Raw sequencing data of exons 5, 6, 7, and 8 of the p53 gene in HTD114 cells



Raw sequencing data (sense direction) of exon 5 of the p53 gene as sequenced using the p53 Exon 5 sense primer (2.1.11)



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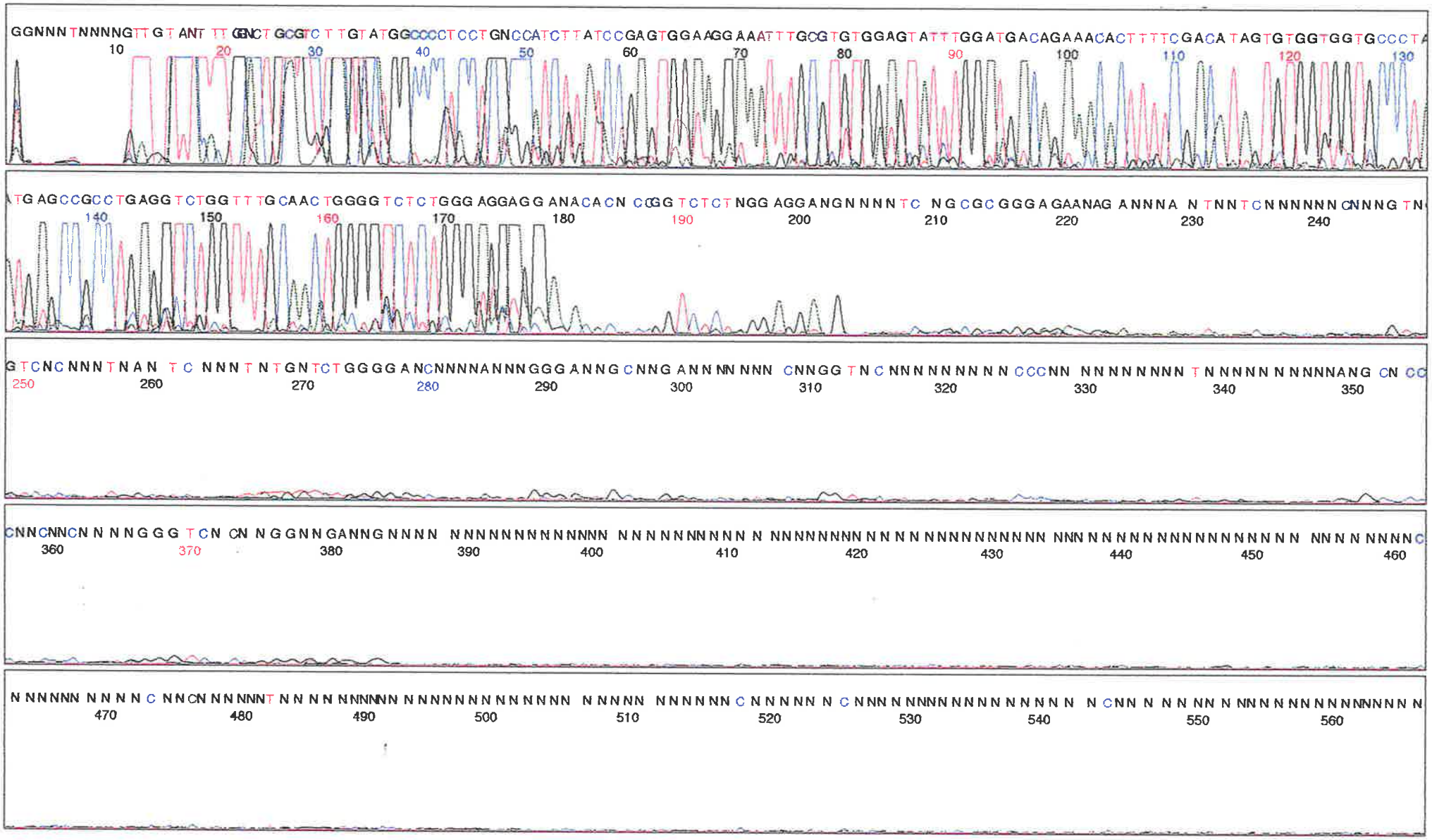
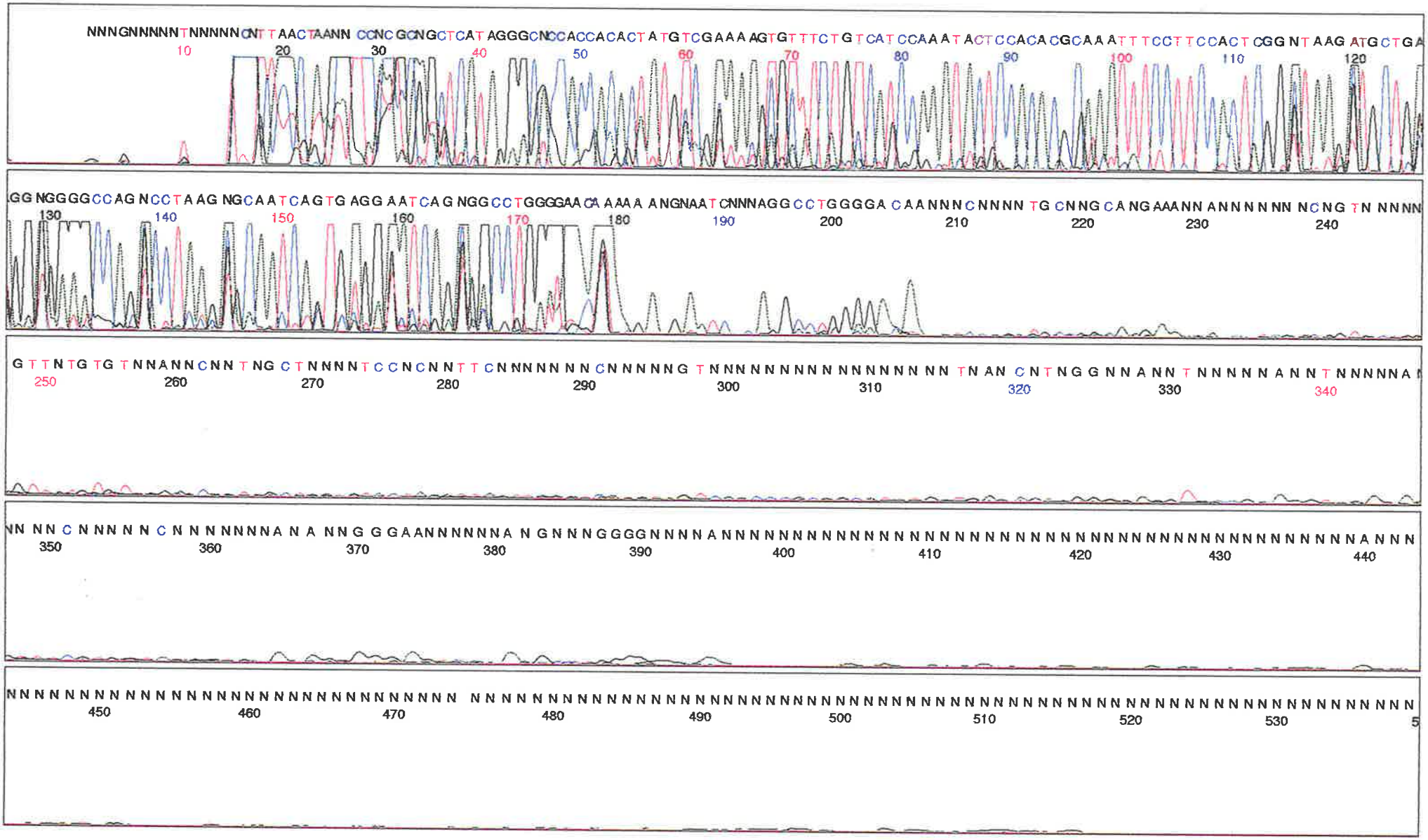


Raw sequencing data (sense direction) of exon 6 of the p53 gene as sequenced using the p53 Exon 6 sense primer (2.1.11)



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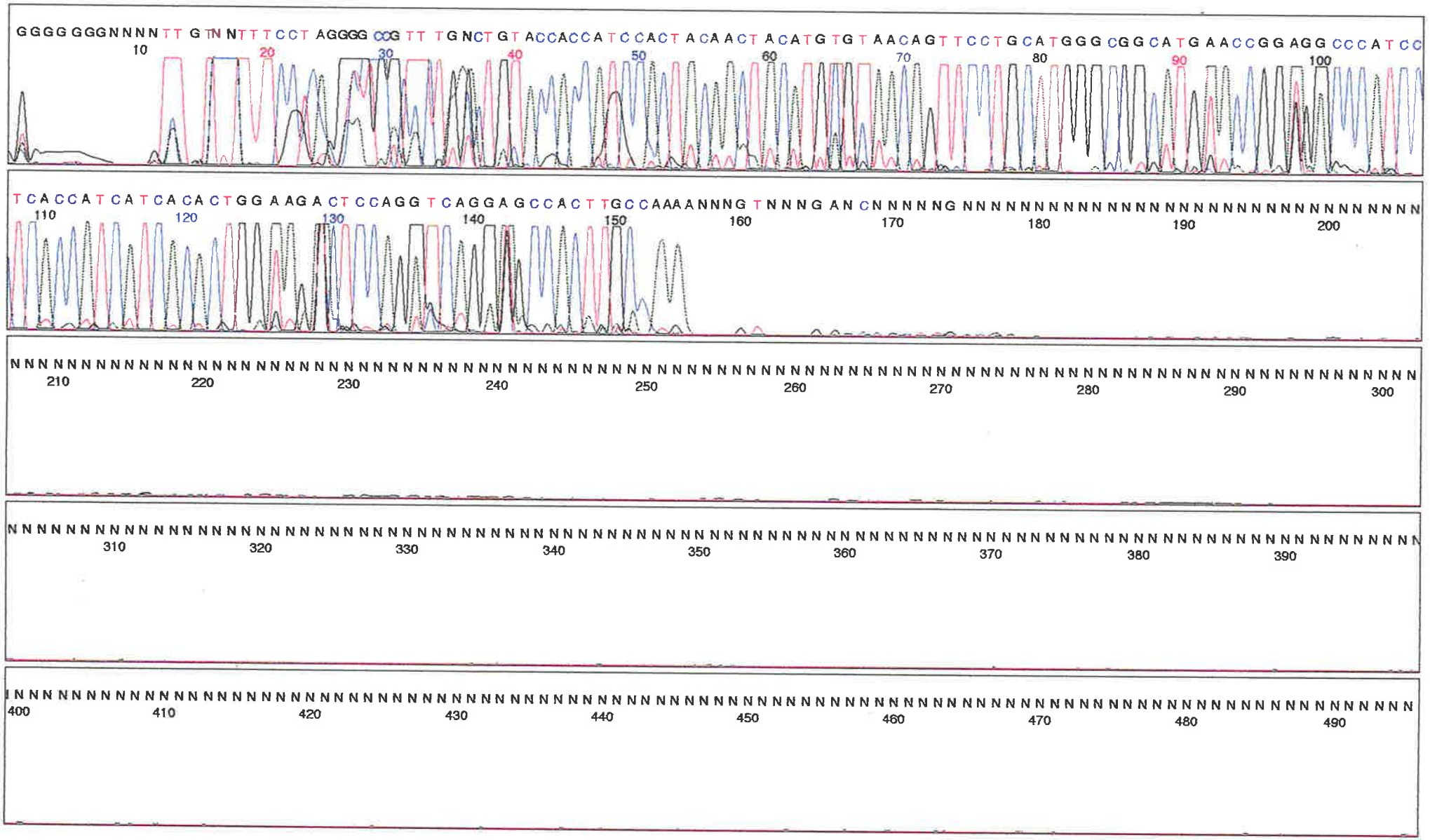
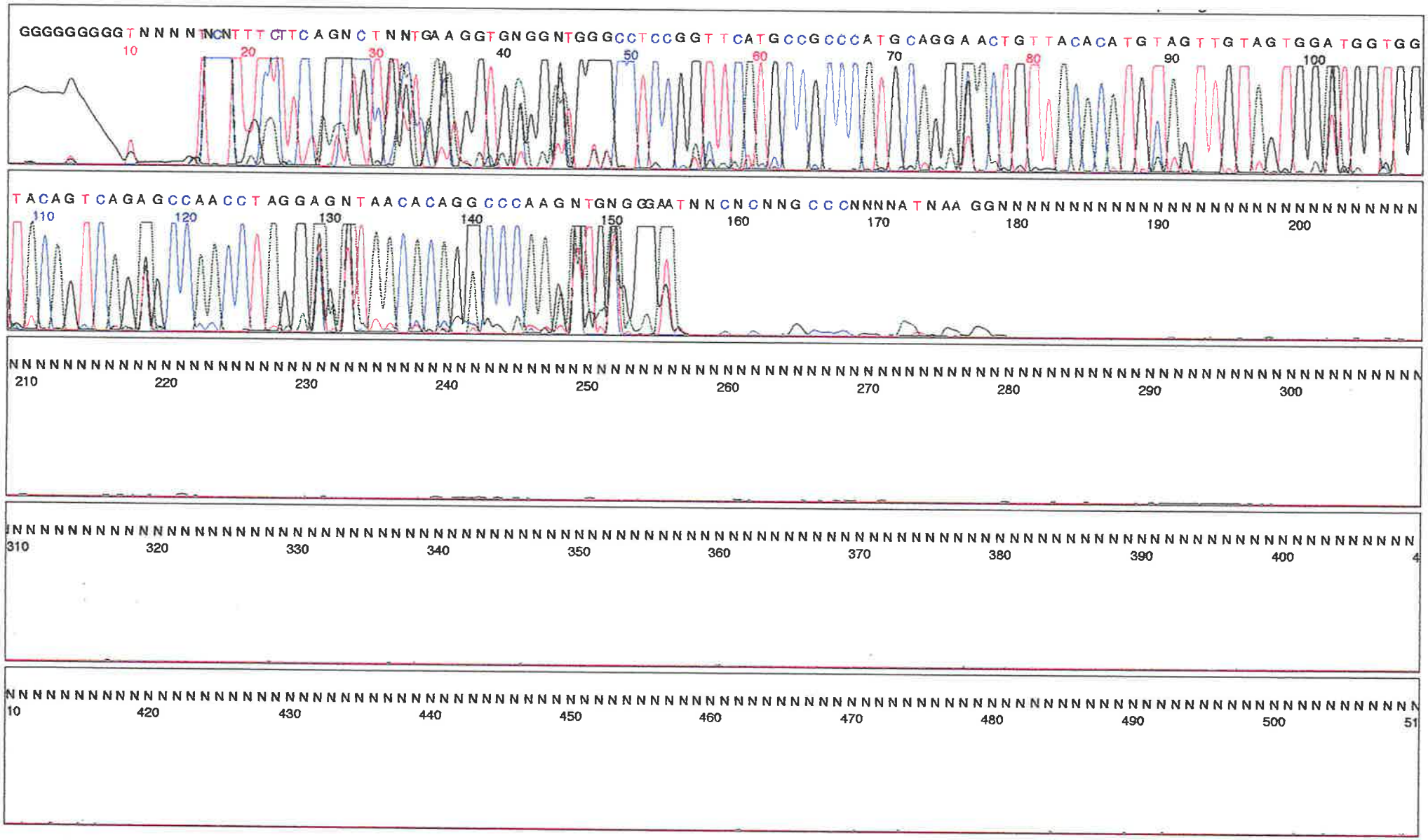


Raw sequencing data (sense direction) of exon 7 of the p53 gene as sequenced using the p53 Exon 7 sense primer (2.1.11)



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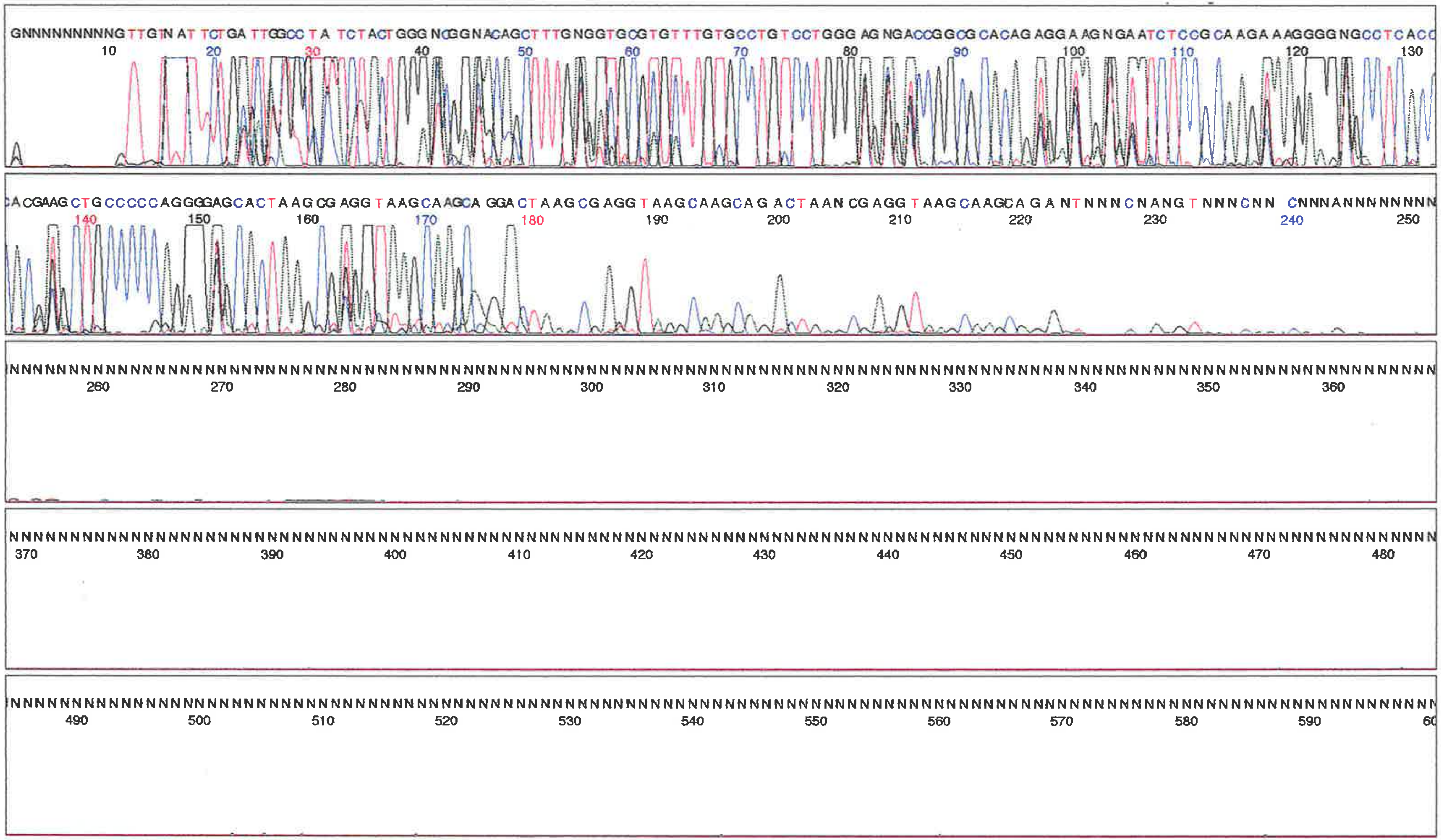
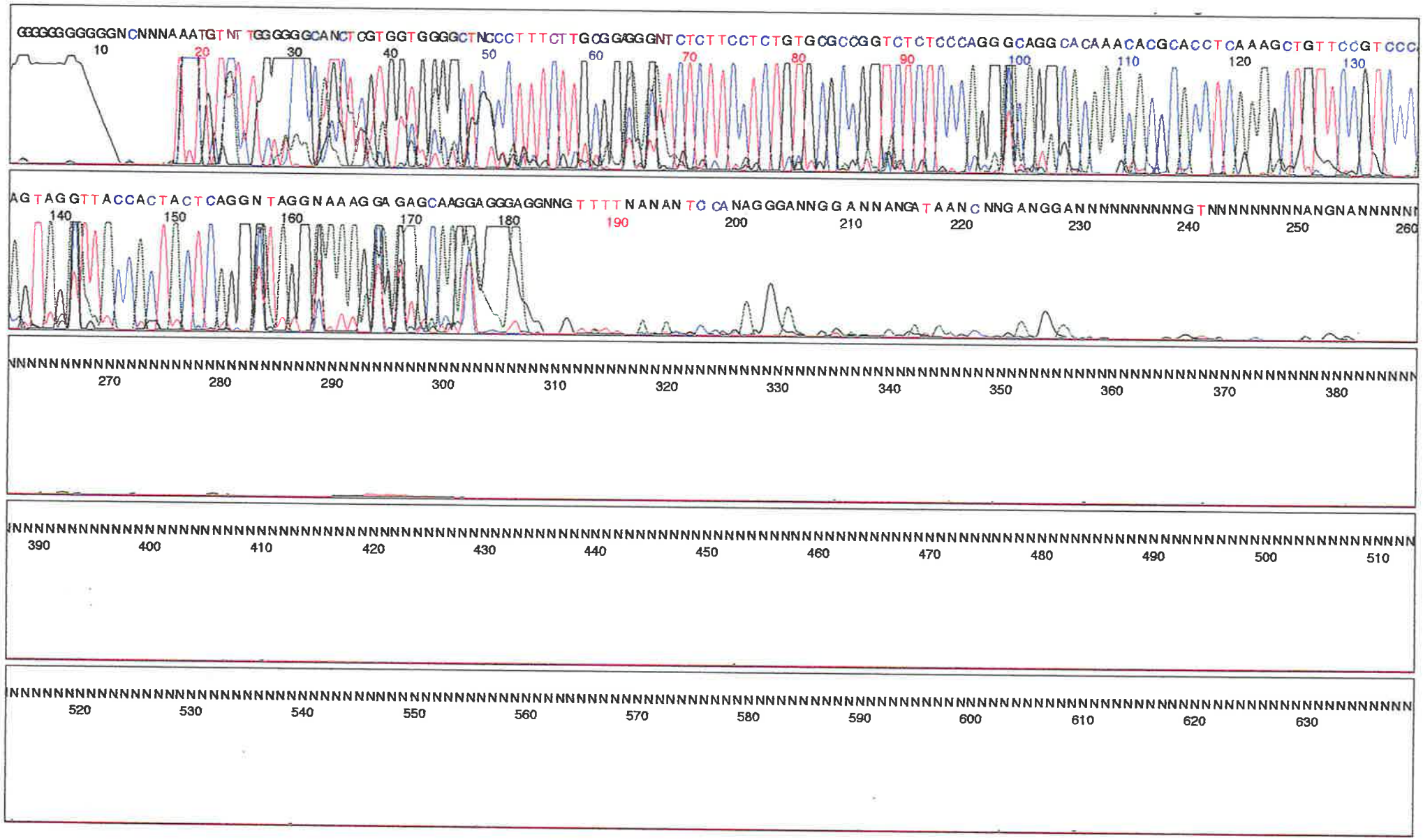


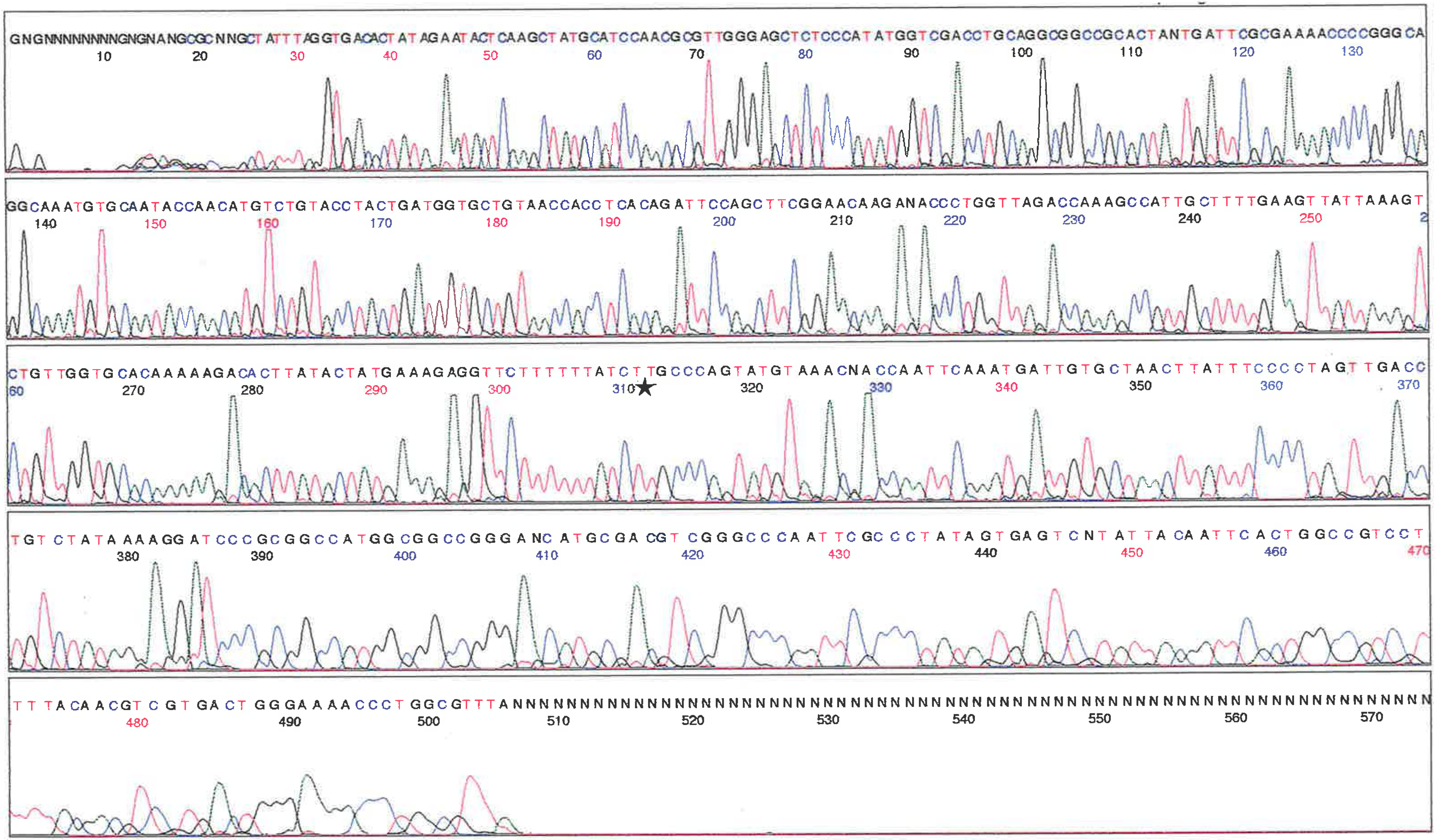
Raw sequencing data (sense direction) of exon 8 of the p53 gene as sequenced using the p53 Exon 8 sense primer (2.1.11)



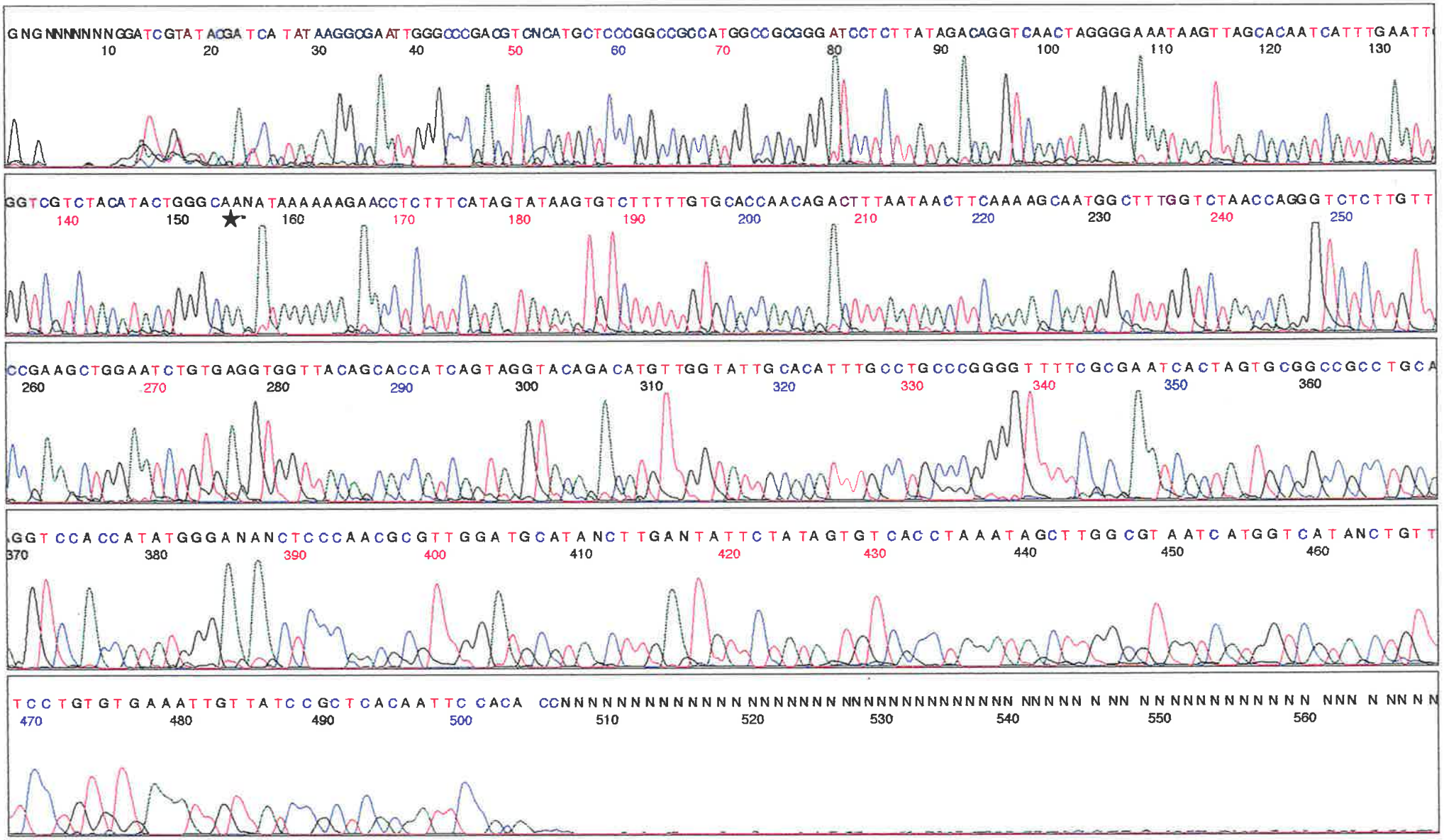
Raw sequencing data (anti-sense direction) of exon 8 of the p53 gene as sequenced using the p53 Exon 8 antisense primer (2.1.11)

APPENDIX II

Raw sequencing data of the MDM2 [AE-V] alternatively-spliced variant. The splicing junction is indicated with an asterisk *



Raw sequencing data (sense direction) of the MDM2 [AE-V] as sequenced using a pGEM universal primer. The splicing junction is inicated by an asterisk *



Raw sequencing data (anti-sense direction) of the MDM2 [AE-V] as sequenced using a pGEM universal primer. The splicing junction is inicated by an asterisk *