THE p53 TUMOR SUPPRESSOR GENE: FUNCTION OF WILD-TYPE INACTIVATION IN TUMOR PROGRESSION

BY

GEOFFREY G. HICKS

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Physiology
University of Manitoba
Winnipeg, Manitoba

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for my wife, Cindy,

and our parents,
Madeleine and Harold Hicks,
Donna and Ed Anhalt,

for everything.

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I find myself transformed from a high-spirited graduate with only good intentions to offer, to being a National Cancer Institute of Canada Post-Doctoral Fellow studying at MIT. I wish to extend my deepest thanks to all of those who have helped me to realize the potential and opportunities I find myself now enjoying.

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faith

ABSTRACT

p53 is a cellular encoded nuclear phosphoprotein whose function was originally believed to be oncogenic in nature. We now realize that this gene encodes a tumor suppressor or antioncogene. In the course of this thesis work, we have experimentally investigated mechanisms of wild-type p53 inactivation and how this event is involved in the transformation process. A series of three projects characterize the loss of p53 tumor suppressor activity by two major mechanisms: complete loss of the p53 gene by genomic rearrangement, and functional loss of wild-type p53 by a dominant negative mutation. In the first project we molecularly cloned both p53 alleles of a Friend erythroleukemic cell line. These p53 genes were found to have been grossly rearranged during the progression of the disease. We determined that both p53 alleles were inactivated by the viral insertion of the Friend Murine leukemia virus. This strongly supported the hypothesis that p53 inactivation was an important step in the progression of the disease. We next examined the hypothesis that point mutations in the p53 gene could functionally inactivate endogenous wild-type p53 in a dominant negative manner. Using REF52 fibroblast cells, we demonstrated that cotransfection of a mutant p53 gene with the T24/EJ *ras* gene was able to rescue these cells from a *ras*-induced growth arrest and lead to full trans-formation. Cotransfection of a full length genomic wild-type p53 gene totally lacked this activity. Further, we showed that expression of a temperature-sensitive p53 mutant, p53val^{135}, actively maintains the loss of negative growth regulation in these cells when it is in the mutant form. A switch to the wild-type p53 conformation

results in growth arrest at G_1/S and G_2/M. Inactivation of p53 by point mutations and allelic loss or rearrangements is now found in every type of human cancer studied.

Review of the Literature

INTRODUCTION

The number of changes required to transform a normal cell to full malignancy is unknown. It is generally agreed that such events would include both the acquisition of activation events in cellular genes which would promote the transformed phenotype, and the loss of other cellular genes which would normally control this growth. Perhaps the most satisfying model for human cancer is Vogelstein's model of colorectal carcinoma where it is not so much the sequence of events *per se*, as it is the accumulation of oncogene activations and tumor suppressor gene inactivations specific to the progression of colorectal carcinoma (Figure 1; Fearon and Vogelstein, 1990). The mechanisms inactivating the p53 tumor suppressor gene in particular, and the function these events have in tumor progression, is the subject of this thesis work.

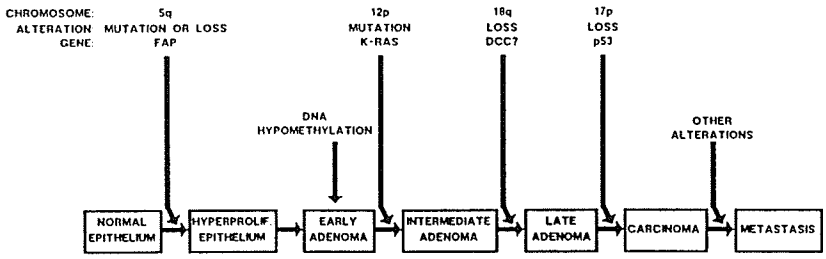

Figure 1. A genetic model for colorectal tumorigenesis. Tumorigenesis proceeds through a number of genetic alterations beginning with a genetic predisposition (loss of 5q in familial adenomatous polyposis), epigenetic changes (hypomethylation patterns and ensuing aneuploidy), activation of oncogenes (K-*ras*), and loss of antioncogenes (*RB* and p53). The order of these changes is not invariant and it is the accumulation of events which appears critical. (Taken from Fearon and Vogelstein, 1990).

HISTORICAL PERSPECTIVE

The entire field of oncogenes and antioncogenes (tumor suppressor genes) spans a very short time frame of 15 years. Although there had been for years research investigating the transforming potential of DNA tumor viruses like the simian virus 40 (SV40) and RNA retroviruses like the Rous sarcoma virus, it wasn't until 1976 that the modern age of oncogenes began. It started when the avian sarcoma virus transforming gene (v-*src*) was identified as being derived from the sequences of a cellular gene (c-*src*)(Stehelin *et al.*, 1976). Soon after, differences between the viral and cellular sequences were integrally linked to the oncogenic potential of the viral protein over the cellular counterpart from which it was derived (Brugge *et al.*, 1977). The study of retroviruses lead not only to discovery of new oncogenes, but quite often identified the mechanism of their activation (mutation, deletion, overexpression, etc). This advanced our understanding to a level that might not otherwise have existed. In fact, it is only recently that oncogenes were identified by other methods. The cloning of the first human oncogene, T24/EJ *ras*, stunned both the scientific and popular presses as it was reported that a single point mutation at codon 12 was entirely responsible for its oncogenic activation (Tabin *et al.*, 1982; Reddy *et al.*, 1982). This reshaped our thinking about the potential for a very small number of oncogenes to control the entire process of cellular transformation. Finally, at a time when scientists were being overwhelmed with oncogenes and acronyms, Weinberg and Ruley proposed their model of transformation requiring that at least two types of oncogenes cooperatively transform primary cells in culture:

one from a nuclear and immortalizing family; and the other from a cytoplasmic and transforming family (Land *et al.*, 1983; Ruley, 1983). This model not only directed the field of oncogenes for years, but helped an enormous number of people to enter and understand it.

At the same time, however, this dominant oncogene model of transformation, and the zealous appeal of its biology, overshadowed increasing evidence of chromosomal abnormalities and losses correlated with human cancers. These observations had in fact been around long before the era of oncogenes began. The theory that loss of a gene function was an important step in oncogenesis was first proposed by Harris in 1969. They showed that the hybridization of a malignant cell with a normal cell resulted in the loss of the malignant phenotype (Harris *et al.*, 1969). These now famous somatic cell hybridization experiments showed that genes from a normal cell could replace those lost in a malignant one, returning the hybrid to a state responsive to normal growth regulation. Hence mutations in malignant cells might be considered recessive, not dominant. Knudson soon followed with his classical "two-hit" theory in which all types of retinoblastoma involved two seperate mutations, one to each allele (Kudson, 1971). In this theory he argued that sporadic retinoblastoma arose by both mutations occuring somatically in the same retinal precursor cell, while in heritable retinoblastoma, one of the mutations was already germinal. Retinoblastoma occurs following the recessive loss of a single gene product, resulting in malignancy. Knudson, himself, coined the phrase "antioncogene" to emphasize that these kinds of genes were the antithesis of the dominantly-acting oncogenes

(Knudson, 1983). "Antioncogene" is now used interchangeably with "tumor suppressor" gene, which more aptly describes its function.

The identification of most human tumor suppressor genes was the result of a combination of cytogenic studies showing a consistent chromosomal loss with a particular neoplastic disorder, and molecular biology showing homozygosity of restriction fragment length polymorphisms in a gene correlated with the potential to clinically present with a certain cancer. The cloning of tumor suppressor genes was done by linkage studies, chromosome walking, and a labor intensive *tour de force* (for a review, see Stanbridge, 1990).

The first recognized antioncogene to be cloned was the retinoblastoma susceptibility gene (*RB*) found on chromosome 13q14 (Friend *et al.*, 1986). *RB* appears to be a negative growth regulator whose function is regulated post-translationally by phosphorylation, in a cell cycle dependent manner (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989). Deletion or mutatation of *RB* is found in many other cancers such as lung, breast, prostate, colorectal, and bladder (Lee *et al.*, 1988; Vogelstein *et al.*, 1988; Yokota *et al.*, 1988; Varley *et al.*, 1989; Weston *et al.*, 1989; Bookstein *et al.*, 1990). The presumed inactivation of *RB* by several DNA tumor virus proteins has also supported its role as a negative growth regulator (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989). Direct evidence of *RB* as a growth regulator and tumor suppressor came with the reintroduction of the *RB* gene into *RB* negative cell lines, where the transformed phenotype was lost (Huang *et al.*, 1988; Bookstein *et al.*, 1990). The strong similarities between *RB* and p53 will become apparent in the following sections.

Other cloned tumor suppressor genes, Wilms' and DDC, differ from *RB* and p53 in one important manner; they are expressed in a tissue specific manner and therefore are involved with the progression of specific cancer types. The Wilms' tumor suppressor gene, cloned from chromosome 11p13 encodes a zinc-finger protein and is likey a kidney specific transcription factor (Call *et al.*, 1990; Gessler *et al.*, 1990; Harber *et al.*, 1990). The DCC gene (Deleted/Detected in Colorectal Carcinoma) encodes a gene which is related to the integrin family and is believed to be involved in cell-cell interactions (Fearon *et al.*, 1990).

In the introduction to the thesis, the evolution of p53s biological identity first as a tumor antigen, then an oncogene, and finally a tumor suppressor gene, will be chronicled. This will then be followed by a review of the p53 gene and protein, and finish with a characterization of p53 in human cancers.

GENESIS OF A SUPPRESSOR GENE

p53: A tumor antigen.

p53 was first reported over ten years ago as a protein precipitating with antisera directed against tumors induced by the simian virus 40 (SV40) (Linzer and Levine, 1979; Lane and Crawford, 1979). It was found tightly oligomerized with the SV40 derived large T antigen protein in cells that were infected or transformed by this virus (Linzer and Levine, 1979; Lane and Crawford, 1979). Elevated levels of the p53 protein were then also

observed in cells transformed chemically or spontaneously (Deleo *et al.*, 1979; Linzer and Levine, 1979; McCormick and Harlow, 1980). It became clear that p53 was a cellularly encoded gene product, and it was in its own right to be considered a transformation associated antigen.

As a tumor antigen, p53 was characterized in a number of ways. Foremost, by its elevated expression found in a variety of tumor cells which had been induced or derived in very different manners (Deleo *et al.*, 1979; Kress *et al.*, 1979; Simmons *et al.*, 1980; Rotter *et al.*, 1980 and 1981; Dippold *et al.*, 1981; Crawford *et al.*, 1982). Expression of p53 in normal cells was barely detectable except when cells were undergoing rapid proliferation, such as those in the spleen or developing embryo (Mora *et al.*, 1980 and 1982; Jay *et al.*, 1980; Rotter *et al.*, 1980; Chandrasekaran *et al.*, 1981; Benchimol *et al.*, 1982). The marked drop in detectable p53 following such normally regulated proliferation was observed in the induced differentiation of F-9 embryonal carcinoma (Oren *et al.*, 1982) and Friend erythroleukemia cells (Shen *et al.*, 1983; Ben-Dori *et al.*, 1983). This suggested that a normal role for p53 may lie in the regulation of proliferation, and therefore overexpression of p53 could maintain a dedifferentiated state characteristic of the transformed cell. This interpretation was consistent with the very short half-life of the p53 protein mediated by rapid degradation (Oren *et al.*, 1981), cell cycle regulated expression and function in G_1 (Milner and Milner, 1981), and a role in initiating DNA synthesis (Mercer *et al.*, 1982; Campisi *et al.*. 1982).

Secondly, as a tumor antigen aberrant overexpression of p53 by tumor cells can be immunogenic, resulting in antisera to p53 isolated from tumor

bearing animals (Linzer and Levine, 1979; DeLeo *et al.*, 1979; Rotter *et al.*, 1980). This has also been reported in the serum of human cancer patients (Crawford *et al.*, 1982; Caron de Fromentel *et al.*, 1987). This point was raised in support of a selective advantage for transformed cell lines found totally lacking p53 expression. Indeed, we have evidence in our lab that tumor cells over-expressing mutant forms of p53 are more susceptible to natural antibody mediated cytolysis than are tumor cells not expressing p53 (Chow and Mowat, unpublished results). However, p53 positive cell lines are much more tumorigenic (Wolf *et al.*, 1984a and 1984c; Mowat *et al.*, 1985). What role the immunogenic properties of aberrant p53 expression may play *in vivo* selection or tumorigenicity remains unclear. It was hoped that detection of p53 overexpression may be of a clinical advantage in the diagnosis of progressive cancers, however, strong correlative ties between the detection of mutant p53 and prognosis remains weak (Ostrowski *et al.*, 1991).

p53: An oncogene.

With the cloning of the first p53 cDNA (Oren and Levine, 1983; Czosnek *et al.*, 1984) it quickly became evident that p53 could act dominantly in gene transfer experiments to complement *ras* in the full transformation of rodent fibroblasts (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). Further, p53 demonstrated the ability to immortalize primary rodent fibroblasts (Jenkins *et al.*, 1984; Rovinski and Benchimol, 1988) and enhance the tumorigenic phenotype of cell lines (Wolf *et al.*, 1984c; Eliyahu *et al.*, 1985; Kelekar and Cole, 1986; Tuck and Crawford, 1989a). It was on the basis of these *in vitro* studies that p53 was grouped with

the tumor virus antigens, SV40 large T antigen and adenovirus 5 E1A, and the cellular oncogenes, *myc*, *myb*, and *fos*, as an immortalizing nuclear oncogene.

Characterization of these p53 transformed lines confirmed there was great increases in the expression of p53 protein compared to the levels observed in untransformed controls. This was not too surprising as the expression vectors were designed to do just that, but it was important to show that the oncogenic conversion of a dominant oncogene lie in the aberrant expression of the gene product, usually by overexpression. This was shown experimentally when an increase in the transformation frequency was observed by use of a RSV-LTR instead of the SV40 early enhancer to increase p53 expression in a promoter dependent manner (Jenkins *et al.*, 1985). Jenkins went further to show that an "activating" mutation in the p53 gene itself, would also increase the transformation frequency of the SV40 early enhancer construct (Jenkins *et al.*, 1985). In addition, there was a series of papers from Wolf and Rotter which described an Abelson virus-transformed line that did not express p53. This cell line was much less tumorigenic than its sister cell lines which did express p53, forming only small tumors which eventually regressed (Wolf *et al.*, 1984a and 1984b). Reintroduction of p53 by transfection, resulted in a fully transformed cell line (Wolf *et al.*, 1984c). The tumorigenic enhancement of p53 was shown in other systems (Eliyahu *et al.*, 1985) and to extend all the way to the metastatic phenotype (Pohl *et al.*, 1988). Finally, overexpression of p53 was found to reduce the requirement for serum or PDGF for proliferation, another hallmark of transformation (Kaczmarek *et al.*, 1986; Gai *et al.*, 1988).

These experiments where overexpression or activation of p53 resulted in cells which acquire the ability to immortalize, transform cooperatively with an activated *ras* gene, enhance tumorigenicity, and grow in reduced serum conditions, appeared to strongly support the nuclear oncogene model for p53.

p53: A target for mutation.

Results not consistent with the oncogene model were demonstrated using cell lines of Friend virus (*FV*)-induced erythroleukemia (Mowat *et al.*, 1985). It was reported that 50% of the tumor lines studied had large increases in the expression of p53, and another 20% appeared to overexpress truncated forms of the protein. Further, this alteration in the expression of the p53 oncogene appeared to be selected for during the progression of the disease. However, in the remaining 30% of the cell lines isolated there was no detectable p53 expression, and this appeared to be the result of gross genomic rearrangements of the gene (Mowat *et al.*, 1985). Some of these rearrangements were later shown to be the direct result of integration of the Friend virus into the coding sequences of the p53 gene (Hicks and Mowat, 1988; Ben-David *et al.*, 1988). In order support a dominant oncogene role for p53, it was argued to be a situation similar to Wolf *et al.*, where the p53 overexpressing cell lines were more tumorigenic. Of course, we now recognize this to be the first evidence of p53 as an antioncogene, where the loss of p53 activity was selected for during the progression of the disease. This inactivation of p53 by allele loss during progression is now supported in several human tumor systems including breast cancer (Mackay *et al.*, 1988), colon cancer (Baker *et al.*,

1989), astrocytomas (James *et al.*, 1989), lung cancer (Yokota *et al.*, 1987; Takahashi *et al.*, 1990), osteosarcomas (Masuda *et al.*, 1987), and chronic myelogenous leukemia in blast crisis (Borgstrom *et al.*, 1982; Ahuja *et al.*, 1989). Hence, selection for the loss of p53 does appear to be an advantage in tumor progression.

The discrepancy between *in vitro* oncogenic activity and *in vivo* deletions of p53 has since been resolved by the realization that the p53 alleles used in the aforementioned immortalizing and transforming assays and those reported in *FV*-induced erythroleukemia p53-positive lines all contain point mutations. Although these mutations do not map to any frequent or common residue, they do fall within highly conserved regions of the protein (Soussi *et al.*, 1987; Eliyahu *et al.*, 1988; Jenkins and Sturzbecher, 1988a; Nigro *et al.*, 1989; Munroe *et al.*, 1990). The p53 gene appears to be a target for mutation, and unlike its antioncogene counterpart, *RB*, these are usually missense mutations.

Despite the apparent randomness of p53 missense mutations, they generally result in a common conformational change in the protein identified by the loss of an epitope for monoclonal antibody PAb246 (Yewdell *et al.*, 1986; Milner and Cook, 1986; Sturzbecher *et al.*, 1987; Clarke *et al.*, 1988; Finlay *et al.*, 1989). Such mutations lead to overexpression of a stable mutant p53 protein with the ability to oligomerize with both itself and wild-type p53 (wtp53) proteins (Eliyahu *et al.*, 1988; Kraiss *et al.*, 1988; Rovinski and Benchimol, 1988; Gannon *et al.*, 1990). These data support the model that the oncogenic activity of mutant p53 proteins may be to effectively eliminate any wild-type p53 activity in the cell by a dominant negative mechanism (Herskovitz, 1987).

Hence, wtp53 activity is lost by an increased expression of a more stable but nonfunctional mutant p53. In addition, any existing wtp53 is sequestered into nonfunctional oligomers with the mutant proteins.

p53: A target for tumor virus proteins.

It is now well established that tumor virus encoded proteins from SV40, adenoviruses, and human papilloma viruses type 16 or 18, tightly bind the antioncogene products of both p53 and *RB* (Figure 2; Linzer and Levine, 1979; Lane and Crawford, 1979; Sarnow *et al.*, 1982; Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989; Werness *et al.*, 1990). This event is thought to functionally parallel the loss of wtp53 seen in many human tumors by mutation or deletion. Direct evidence that these interactions are inactivating for p53 came recently where it was reported that E6 binding to p53 promotes an ubiquitin-directed proteolytic destruction of the p53 protein (Scheffner *et al.*, 1990). As the common target cell of all these viral infections are somatic cells of the adult, it is believed by binding of these two tumor suppressor gene products, the host cell will be relieved of its antiproliferative state and stimulate cell growth and division (reviewed in Green, 1989; Levine, 1990; Lane and Benchimol, 1990).

The functional consequences of these interactions to the tumor virus protein has also greatly contributed to our understanding of how the tumor suppressive functions of both p53 and *RB* may operate normally in the cell. Using SV40 large T antigen as the best studied example, the binding domains for p53 and *RB* have been shown to overlap with sequences known to be essential for transformation by the large T antigen (Srinivasan *et*

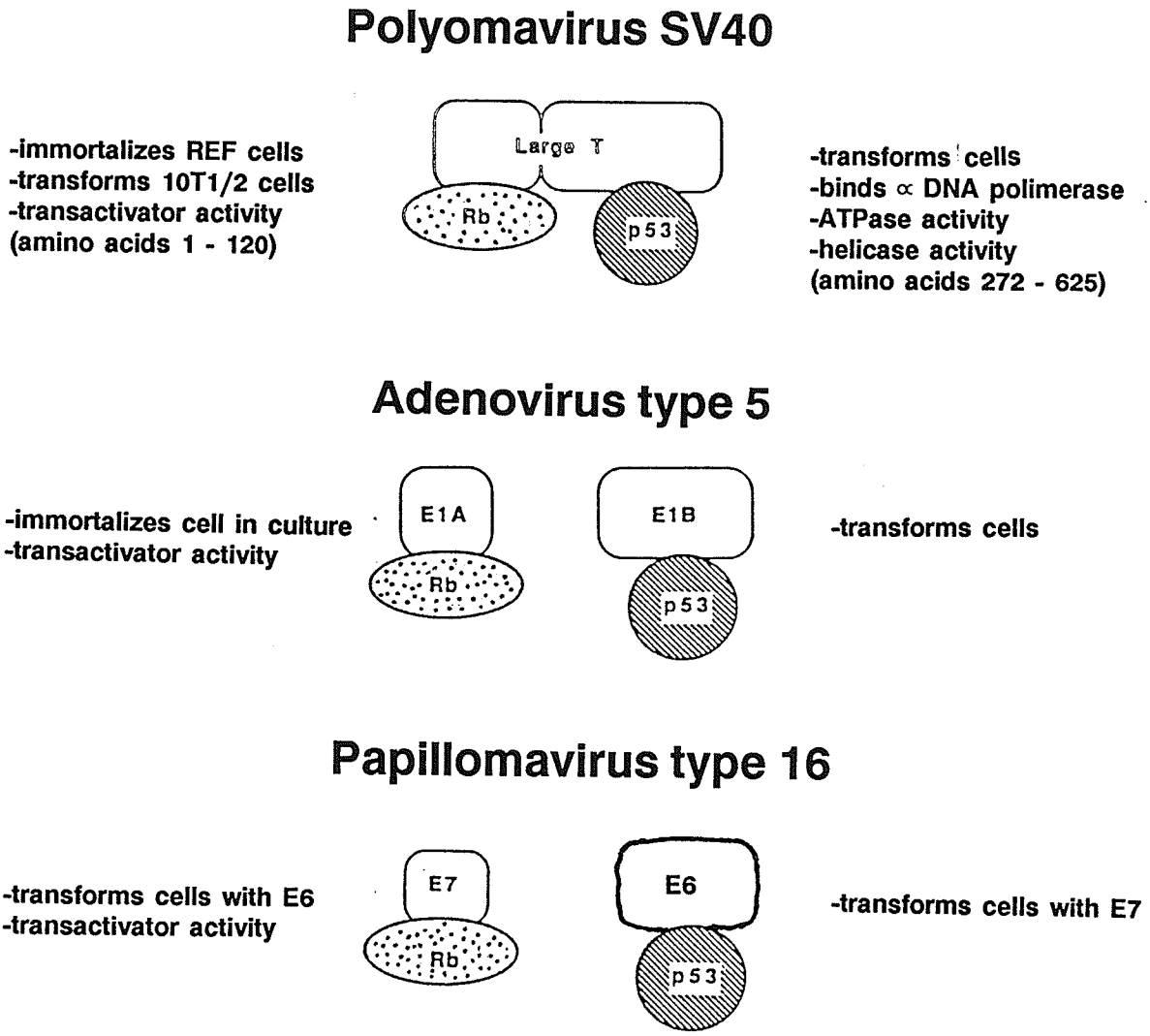

Figure 2. Tumor virus protein targeting of antioncogenes. Complexes between the oncogenes of DNA tumor viruses and cellular antioncogenes are shown. Transforming activities attributed to the tumor virus protein sequences of the anti-oncogene binding domains are outlined to the side. (Taken from Lane and Benchimol, 1990, and from Levine and Momand, 1990).

al., 1989). Further, SV40 large T antigens which fail to bind p53, also fail to transform REF52 cells and primary mouse embryo fibroblasts or show a marked reduction in this activity in C3H-10T½ cells (Peden *et al.*, 1989).

The mechanisms for these biological consequences may be enlightened by other studies which show that murine p53 binding to the SV40 large T antigen prevents the binding of α DNA polymerase to the viral protein (Gannon and Lane, 1987) as well as the blocking of DNA replication (Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988; and Wang *et al.*, 1989). These experiments suggest a possible role of p53 as a tumor suppressor with the initiation of DNA synthesis.

A second major feature of the DNA tumor virus interactions is that they appear to neutralize both p53 and *RB*. The parallels between these two tumor suppressor genes are striking. An important question to be addressed is whether the tumor suppressive function of these two anti-oncogenes is redundant or cooperative.

p53: A tumor suppressor.

With a bonefide wild-type p53 sequence determined, the wtp53 gene could now be tested directly in the same activated *ras* transformation cooperativity assay as its mutated p53 counterparts had five years earlier. Three closely published reports revealed that wtp53 expressing plasmids could suppress the transforming potential and inhibit focus formation of either viral E1A-, cellular *myc*- or mutant p53-plus-*ras* in triple-transfections of primary rodent fibroblasts (Hinds *et al.*, 1989; Finlay *et al.*, 1989; Eliyahu *et al.*, 1989). Further, in the rare

transformations that were isolated it was demonstrated that the introduced wtp53 allele was either not expressing, or had become mutated itself! Tumor suppression was also clearly observed by the introduction of wtp53 into transformed cells (Baker *et al.*, 1990; Mercer *et al.*, 1990a; Johnson *et al.*, 1991). These experiments not only clearly demonstrate a wtp53 tumor suppressor activity but indicate that the wtp53 antiproliferative activity is very powerful and strongly selected against in transformed cells.

Overexpression of wtp53 in fibroblasts results in a reversible growth arrest primarily in late G_1, although growth arrest in other parts of the cell cycle is also observed (Michalovitz *et al.*, 1990). This pattern of growth arrest in response to wtp53 overexpression is also demonstrated using inducible p53 constructs (Mercer *et al.*, 1990b). Further studies have shown that negative regulation of the cell cycle by p53 is dependent on wild-type conformation and this is integrally associated with cellular localization to the nucleus during G_1 (Ginsberg *et al.*, 1990; Shaulsky *et al.*, 1990a; Gannon & Lane, 1991, Martinez *et al.*, 1991). These studies suggest that p53 conformation and cellular localization are important for its action in the control of the cell cycle (Cook and Milner, 1990; Milner and Watson, 1990; Shaulsky *et al.*, 1990a).

The mechanism of p53 tumor suppressing activity is likely to be directly related to its activity as a negative growth regulator and its implication in cell cycle regulation. Expression of p53 is itself under cell cycle control (Milner and Milner, 1981; Reich *et al.*, 1984) and it has been found to interact with the cell cycle regulator *cdc2* kinase (Addison *et al.*, 1990; Bischoff *et al.*, 1990; Sturzbecher *et al.*, 1990).

Finally, recent evidence has begun to identify transactivation by p53 (Fields and Jang, 1990; Raycroft *et al.*, 1990) and p53 responsive elements (Weintraub *et al.*, 1991) which suggests that the ultimate regulation by p53 may be at the gene transcription level.

THE p53 GENE AND PROTEIN

p53: gene structure.

p53 is a single copy gene found only in the vertebrate genome. It is located on chromosome 17p13 in humans (Benchimol *et al.*, 1985; Isobe *et al.*, 1986; Mcbride *et al.*, 1986; Miller and Cook, 1986) and on chromosome 11 in mice (Czosnek *et al.*, 1984; Rotter *et al.*, 1984). The mouse genome also contains an inactive, processed pseudogene found on murine chromosome 14 (Zakut-Houri *et al.*, 1983; Czosnek *et al.*, 1984). The p53 gene is contained in 11 exons with a striking degree of conservation in the coding sequence of five domains (Fig. 3; Soussi *et al.*, 1990). Although these domains will be discussed in greater detail elsewhere, there are other important salient features of the gene itself. First, Exon 1 is entirely noncoding. It has been suggested that the dyad symmetry found in this 5' untranslated region forms a putative stem and loop structure which may be involved in regulation of two major start sites for transcription (Oren *et al.*, 1983; Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985; Tuck and Crawford, 1989b). Second, there is a very large first intron. Although the significance of this long intron remains little understood, it has been found rearranged in some human tumors (Masuda *et al.*, 1989) leading to speculation of changes in mRNA transcription or stability, and the

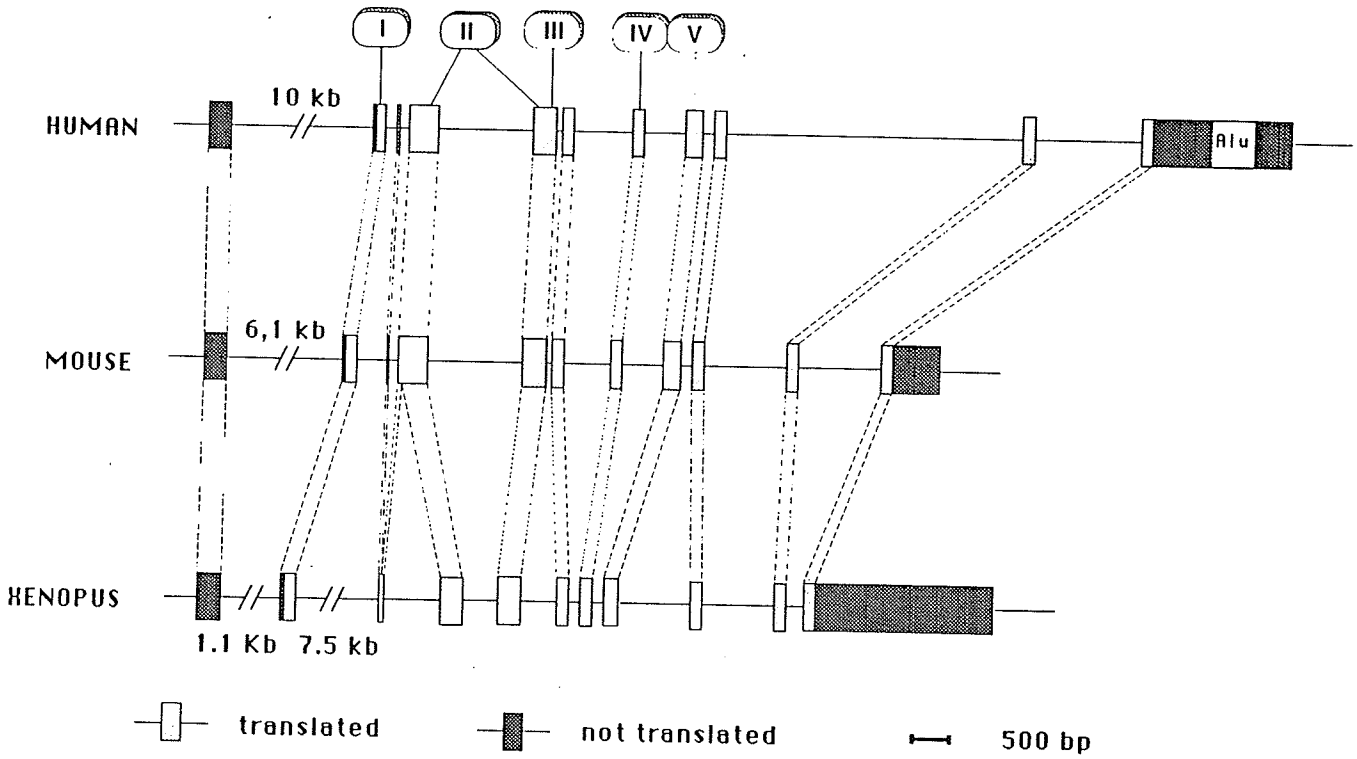

Figure 3. Genomic organization of p53 genes. The genomic organization of p53 genes for three species is shown. Exons are depicted by boxes and untranslated sequences are shown in a darker shade. Roman numerals I through V show the conserved protein domains. (Taken from Soussi *et al.*, 1990).

identification of a second possible promoter (Reissman *et al.*, 1988 and 1989). Thirdly, the p53 promoter is a very unusual RNA polymerase II promoter in that it does not have a TATA box, G/C rich sequences, or a CAAT box (Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985; Lamb and Crawford, 1986). A downstream helix-loop-helix motif has been described which suggests that the expression of p53 may be regulated by the *myc/myoD* family of transcriptional activators (Ronen *et al.*, 1991). Finally, expression of the gene is enhanced by intron 4 sequences. It has been known that p53 cDNA constructs which included intron sequences showed dramatic increases in p53 expression and *ras*-cotransformation (Hinds *et al.*, 1989). Sequences found in intron 4 have been shown to enhance the expression of p53 in a tissue specific manner and to enhance the expression of other genes in *cis*-constructs (Brinster *et al.*, 1988; Lozano and Levine, 1991). A similar mechanism has also been reported in the cell cycle specific regulation of the proliferating nuclear antigen mRNA (Ottavio *et al.*, 1990).

p53: the protein.

The identification of five highly conserved domains (six including the C-terminus) was possible with the cloning and sequencing of the frog p53 gene (Soussi *et al.*, 1987), and supported further with rat (Soussi *et al.*, 1988b), chicken (Soussi *et al.*, 1988a), rainbow trout (Soussi *et al.*, 1990) and monkey p53 sequences (Rigaudy and Eckhardt, 1989). These domains are outlined in Figures 3, 4, and 5.

Analysis of the p53 protein in terms of its charge distribution, hydropathic profile, and predicted secondary structure reveals a high

degree of conservation throughout the entire protein. This can be generally divided into three regions: 1) acidic helical N-terminus (amino acid residues 1 - 100) containing a high number of acidic residues, very few basic amino acids, and an unusually high proline content believed to be responsible for its molecular weight of 53 kD on SDS-PAGE gels instead of the predicted 44 kD. 2) hydrophobic region (amino acid residues 100 - 300) containing few charged residues and three hydrophobic regions which interestingly corresponding to conserved domains II, IV, and V. 3) basic helix-coil-helix C-terminus (amino acids 300 - 393) containing a high charge density and a very hydrophilic profile (Figure 4)(Karlin and Brendel, 1990). This results in a predicted tertiary structure with the amino and carboxy termini located on the protein surface and the hydrophobic region, containing the conserved domains II through V, in the interior of the molecule.

These conserved domains, of course, are the target areas for mutations which result in a protein with a common conformational change (discussed in both the preceding and the following sections). The consequences of this conformational change at the protein level are many and will be briefly outlined here and in Figure 4. First, the conserved domains III, IV, and V of p53 are important for the association of murine p53 with the SV40 large T antigen, specifically binding with the wild-type p53 conformation (Tan *et al.*, 1986; Jenkins *et al.*, 1988a). While the binding domains for adenovirus E1B and papillomavirus E6 proteins have not yet been characterized, there is some evidence that the binding ability of E1B is also p53 dependent (Braithwaite and Jenkins, 1989). Second, the region is also important for the single- and double-stranded DNA binding

activity of wild-type p53 (Lane and Gannon, 1983; Steinmeyer and Deppert, 1988; and Kern *et al.*, 1991); mutant p53 proteins will still bind, but abnormally (Kern *et al.*, 1991). A binding potential resticted to the mutant confomation is with the cellular heat shock proteins (Pinhasi-Kimhi *et al.*, 1986; Hinds *et al.*, 1987; Sturzbecher *et al.*, 1987 and 1988; Ehrhart *et al.*, 1988; Finlay *et al.*, 1988; Romano *et al.*, 1989; Soussi *et al.*, 1989; Halevey *et al.*, 1989). While it has been suggested that this interaction may serve an oncogenic potential in terms of increasing the stabilty of p53 or cellular localization, it more likely reflects the normal function of heat shock proteins to marshal aberrent expression of hydophobic protein domains exposed in the mutant proteins (Pelham, 1986 and 1988). Another p53 function restricted to conformation is the potential of p53 as a transcriptional activating factor. Although as few as the first 73 (Fields *et al.*, 1990) or 160 (O'Rourke *et al.*, 1990) amino acids are sufficient to transactivate as *GAL4* fusion products, Lozano and coworkers have shown that the wild-type conformation is necessary for this activity when all the conserved domains are included in the constructs (Raycroft *et al.*, 1990). Finally, p53 and SV40 large T antigen have recently been shown to be associated with microtubule associated proteins in transformed cells; an activity also attributed to *RB* and $pp39^{mos}$ (Maxwell *et al.*, 1991; Szekely *et al.*, 1991; Zhou *et al.*, 1991).

Post-translational modification of p53 is exclusively by a high level of phosporylation on serine and threonine residues (Samad *et al.*, 1986; Meek and Eckhart, 1988). While there is evidence that enhanced phosphorylation of p53 is related to transformation (Sheidtmann and Haber, 1990) and that increased phophatase activity to specific residues is

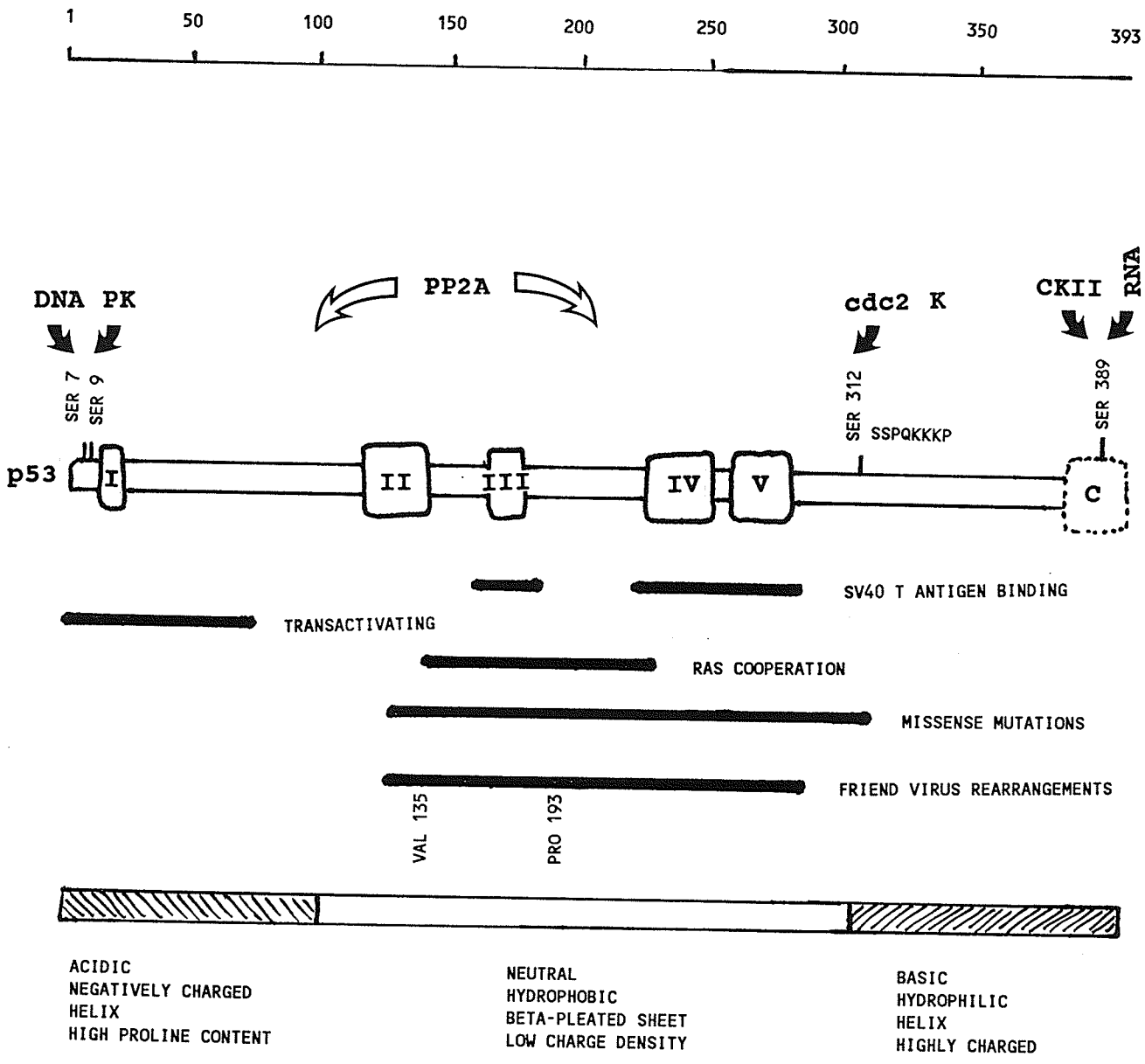

Figure 4. The p53 protein and functional features. The p53 protein is cartooned in the center of the diagram with the conserved domains indicated by Roman numerals. Position of amino acid residues and analysis of the primary amino acid sequence is given at the top and bottom of the figure, respectively. Regions of the p53 protein responsible for a number of biological functions are identified by horizontal bars below the p53 protein. Above it, serine residues which are substrates of known protein kinases are identified: DNA PK, DNA activated protein kinase; cdc2 K, *cdc2* kinase; CK II, casein kinase II. PP2A, protein phosphatase 2A, is shown with open arrows to indicate its specific phospho-substrates are not yet known. The reported attachment site of a RNA molecule is also shown. (Please refer to the text for references).

inhibitory (Sheidtmann *et al.*, 1991), there is also evidence that phosphorylation patterns are not different in primary versus transformed cells (Patschinsky and Deppert, 1990). With the recent identification of specific serine residues and the kinases involved, the importance of p53 phosphorylation will soon be much better understood. This group includes the *cdc2*-kinase phosphorylation of serine 312 (Addison *et al.*, 1990; Meek and Eckhart, 1990; Miller *et al.*, 1990; Sturzbecher *et al.*, 1990) and its potential regulation of the adjacent nuclear localization signal (Dang and Lee, 1989; Hunt, 1989; Addison *et al.*, 1990; Shaulsky *et al.*, 1990b; Silver, 1991); casein kinase II phosphorylation of serine 389 (Meek *et al.*, 1990; Herrmann *et al.*, 1991); and a DNA-activated protein kinase phosphorylation of serines 7 and 9 (Lees-Miller *et al.*, 1990).

Although phosphorylation is basically the major post-translational modification of p53, two others have been reported. One is a small RNA molecule found covalently linked to serine 386 of a murine p53 (equivalent to serine 389 in human)(Samad *et al.*, 1986). The other is a conserved sequence for N-glycosylation at Asn residue 239. Glycosylation of p53 has never been reported, but it is interesting that this residue is highly conserved and is a site for frequent mutation (Takahashi *et al.*, 1989; Hollstein *et al.*, 1991).

p53 AND HUMAN CANCER

Mutation of the p53 gene is the single most common genetic alteration in human cancer. The normal p53 allele is found on chromosome 17p13. Although genomic rearrangements, deletions, or total loss of the p53 allele have been reported in human cancers (Table 1), by far the most

common lesion is a missense point mutation found within a 600 bp region coding for codons 110 to 307 (Figure 5; Hollstein *et al.*, 1991). The significance of missense mutations in the contribution of mutant p53 to cancer is fortified by the observation that aneuploidy is usually the most common mechanism in neoplastic related genetic aberrations (Dellarco *et al.*, 1985). In addition, point mutations which lead to the loss of the retinoblastoma tumor suppressor gene are very different in nature, almost always being nonsense mutations which result in a total loss of gene product (Levine and Momand, 1990). Careful examination of the hundreds of already described point mutations in the p53 gene should provide important information into the etiology of p53 and various human cancers, the mechanisms of mutation, and the possible identification of critical amino acid residues. The types of human cancers where p53 mutations have been described are summarized Table 2.

One can overview these allelic mutations to the p53 gene in human cancers and draw generalitites specific to cancer types and mutagenic mechanisms. For example, allelic loss leading to homozygosity is usually characterized by the remaining p53 allele coding for a mutant p53. As an event, this appears to randomize in the types of cancers it is found in, and is more likely to be the product of increased aneuploidy than involvement in a specific cancer progression. This is in contrast to genomic rearrangements and allelic losses leading to a null phenotype. These events do appear to have a more common cancer type, leukemias and osteosarcomas. Interstingly, these are cell types which normally have a high level of proliferation in the adult; the selection for complete loss

Table 1. Summary of p53 Rearrangements and Cancer Type.

Cancer Type	Allele Lesion	Reference Source
Bladder	loss to homo.	Oka *et al.*, 1991.
Bone	rearrangement	Masuda *et al.*, 1987; Miller *et al.*, 1990.
Breast	loss to homo.	Varley *et al.*, 1991.
Esophagus	loss to homo.	Meltzer *et al.*, 1991.
Kidney	loss to homo.	Oka *et al.*, 1991.
Leukemia	rearrangement	Longo *et al.*,1990; Marshal *et al.*,1990; Ahuja *et al.*, 1989;
	loss to null	Wolf *et al.*, 1984b;
Lung	loss to homo.	Takahashi *et al.*, 1989.
Neuronal	deletion	Azoui *et al.*, 1989; Menon *et al.*, 1990.

Table 2. Summary of p53 Mutations and Cancer Type.

Cancer Type	Reference Source
Brain	Nigro *et al.*, 1989; Mashiyama *et al.*, 1991.
Bladder	Oka *et al.*, 1991.
Breast	Nigro *et al.*, 1989; Prosser *et al.*, 1990; Bartek *et al.*,1990; Varley *et al.*, 1991. Davidoff *et al.*, 1991.
Cervical	Crook *et al.*, 1991.
Colon	Nigro *et al.*, 1989; Baker *et al.*, 1989 & 1990; Rodrigues *et al.*, 1990.
Esophagus	Nigro *et al.*, 1989; Baker *et al.*, 1989 & 1990; Hollstein *et al.*, 1990; Rodrigues *et al.*, 1990; Meltzer *et al.*, 1991.
Kidney	Oka *et al.*, 1991.
Liver	Bressac *et al.*, 1990 & 1991; Hsu *et al.*, 1991; Hosono *et al.*, 1991.
Lymphoma/ Leukemia	Cheng *et al.*, 1990; Gaidano *et al.*, 1991;
Lung	Takahashi *et al.*, 1989, Nigro *et al.*, 1989. Chiba *et al.*, 1990.
Sarcoma	Nigro *et al.*, 1989; Mulligan *et al.*, 1990; Menon *et al.*, 1990; Romano *et al.*, 1989.

of p53 may be quite different from the selection for missense mutations described in other cancer types.

A critical evaluation of all 280 of the known point mutations found in the p53 gene of various cancer cells was recently published and is presented in Figure 5. 98% of these are somatically derived mutations occuring with a 90% frequency in codons which have been conserved over all 5 species described. Several general patterns are begining to emmerge: frequent non-clustered G:C to T:A substitutions (resulting in G to T transversion) are found in the tobacco related lung and esophageal cancers; mutation of codon 249 is common in hepatocellular carcinoma, but differences in the mechanisms by which it is acheived can be linked to individuals from distinct high incedence regions; and transitions in CpG dinucleotide (Rideout *et al.*, 1990) contributes as the highest specific mutation found in all cancer types.

Finally, a very small percentage of mutations identifies an incredibly exciting new discovery of germ line p53 mutations. This contributes to a familial predisposition to many cancer types, known as Li-Fraumeni syndrome (Li and Fraumeni, 1969; Chang *et al.*, 1987; Malkin *et al.*, 1990; Srivasta *et al.*, 1990).

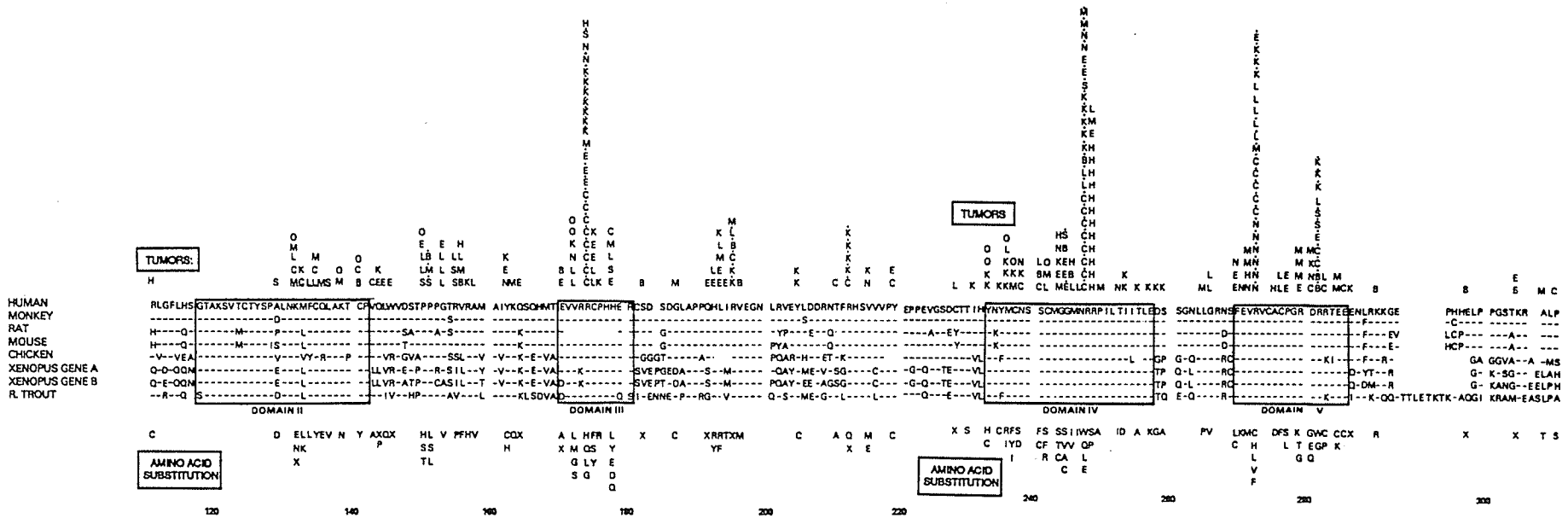

Figure 5. Localization of p53 base substitutions in human cancer. Symbols at the top refer to cancer types: B, bladder; C, colon; E, esophagus; H, liver; K, leukemias and lymphomas; L, lung; M, breast; N, brain; O, ovary; and S, sarcoma. A period directly above the tumor symbol indicates that the mutation is a transition at a CpG dinucleotide. Single-letter amino acid abbreviations below p53 sequences designate substituted residues in the tumors, X refers mutations which resulted in a stop codons. (Taken from Hollstein *et al.*, 1991).

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Loss of Wild-Type p53 Activity by Gene Inactivation is Virally Mediated

FOREWORD

The following chapter is the full-paper format of a manuscript published in the *Journal of Virology*: Geoffrey G. Hicks, and Michael Mowat. 1988. Integration of Friend murine leukemia virus into both alleles of the p53 oncogene in an erythroleukemic cell line. J. Virol. **62**:4752-4755.

Dr. Mowat has previously found that during the *in vivo* progression of Friend Virus (*Fv*) induced erythroleukmia there were several changes in the expression of the p53 oncogene. In 50% of the erythroleukemic cell lines derived there was tremendous increases in the expression of p53 compared to normal spleen cells. This was consistent with a role for p53 as a dominant acting nuclear oncogene. About a third of the erythroleukemic cells lines however, showed a loss of p53 expression (Mowat *et al.*, 1985). We were convinced that the complete loss of p53 found in these cell lines was as important to the development of *Fv* erythroleukemia as the aberrant overexpression of p53 seen in the other lines. We then chose to molecularly clone the rearranged p53 genes to understand the mechanism for the loss of p53 expression and its relative importance in the progression of *Fv* erythroleukemia.

This paper provided the first evidence that the gross rearrangements seen in the p53 gene was due to the integration of the helper virus, Friend murine leukemia virus. Targeting of the p53 gene and subsequent inactivation by the transforming viral agent itself, lent strong support to the importance of loss of p53 in the *in vivo* progression of the erythroleukemia. Indeed, such a model has been further fortified by the demonstration that spleen focus-forming virus, a second component of the

Friend virus complex, also inactivates the p53 by integration during the progression of the disease *in vivo* (Ben-David *et al.*, 1988 and 1990b).

An important interpretation of these data discussed in our paper was to address the point of how inactivation of p53 could be considered oncogenic since p53 was overexpressed in the other 50% of more tumorigenic Friend erythroleukemic cell lines. In review of the current literature at the time, we noted that demonstrated oncogenic p53 genes were in fact point mutated, and that:

> "mutant p53 protein may act to inactivate the wild-type p53 protein or gene function. The dominant-acting p53 oncogene in these situations may therefore be similar to a loss of p53 activity by retrovirus insertion."

This has since been proven true and the mechanisms of mutated p53 oncogenicity will be discussed in subsequent chapters. However it is important to this discussion to note that p53 genes from the other Friend virus-induced p53 expressing lines have been shown to possess dominant acting immortalizing and transforming potential (Rovinski *et al.*, 1987; Rovinski and Benchimol, 1988; Munroe *et al.*, 1990); and that specific mutations in the p53 gene from these cell lines have been identified (Munroe *et al.*, 1990). Further, transgenic mice expressing a mutant p53 allele are predisposed to the development of lung carcinomas, bone and soft tissue sarcomas, lymphoid malignancies and an increased susceptibility to Friend virus-induced erythroleukemia (Lavigueur *et al.*, 1989 and 1991).

The loss of wild-type p53 expression in Friend virus-induced erythroleukemia has been shown to be one of a small number of events which lead to the tumorigenic conversion of spleen cells. The initial stage of the disease, which is marked by a massive nontumorigenic proliferation, is

a result of the spleen focus-forming virus *env* gene product, gp55, binding to and stimulating the erythropoietin receptor (Hoatlin *et al.*, 1990; Li *et al.*, 1990). This results in growth factor independent proliferation of the infected cells. Transformation, however, occurs with Friend virus-mediated inactivation of p53, and integration into adjacent loci of the *ets* related cellular *Spi-1* genes (Moreau-Gachelin *et al.*, 1988 and 1989) or *Fli-1* (Ben-David *et al.*, 1990a). These latter integrations occur with a high frequency and thus it appears that it is an accumulation of all three events which is important.

Friend virus-induced erythroleukemia has proved to be an invaluable model system for our current understanding of how the loss of wild-type p53 through both gene inactivation and mutation, functions as one of a series of oncogenic events.

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Integration of Friend Murine Leukemia Virus into Both Alleles of the p53 Oncogene in an Erythroleukemic Cell Line

Geoffrey G. Hicks and Michael Mowat*

The Manitoba Institute of Cell Biology and
the Department of Physiology, University of Manitoba,
Winnipeg, Manitoba, Canada, R3E 0V9
phone (204) 787-4139

Running Title: p53 oncogene rearrangements

*corresponding author

ABSTRACT

The Friend virus-transformed erythroleukemic cell line DP16-9B4 has undergone a complex rearrangement of the p53 oncogene and lacks any detectable expression of the p53 protein. We report here characterization of both p53 alleles in this cell line and identify independent integrations of Friend murine leukemia virus sequences into the coding region of both alleles.

INTRODUCTION

There is increasing evidence that the cellular encoded protein p53 plays a role in the transformation process (reviewed in reference 4; 36). Gene transfer of the murine p53 gene can immortalize early-passage rodent cells in culture (15) and the p53 gene can complement the *ras* oncogene in an immortalization-transformation assay using primary rodent cells (7,15,37). Also, elevated levels of p53 has been found to increase tumorigenicity of certain cell lines (6,18,31,53). Therefore the gene encoding p53 can be considered an oncogene.

We are interested in understanding the role p53 plays in the multi-stage erythroleukemia induced by Friend leukemia virus. The early stage of Friend leukemia is characterized by an increase in the number of erythroid cells in the spleen and bone marrow (48,49). These cells can terminally differentiate and have a limited life span (48). The second stage of the disease is characterized by the appearance of cells capable of forming tumors and growing as permanent cell lines (9,52). These cells can be detected and isolated by their ability to form macroscopic colonies in semi-solid culture medium or spleen colonies in irradiated *Sl/Sl*^{d} mice (23,24). Elevated levels of p53 protein can only be detected in the cell lines isolated from the late stage of the disease and not in the enlarged spleens (31,43).

Friend virus complex consists of two viruses, the replication defective spleen focus forming virus (SFFV) and a replication competent virus Friend murine leukemia virus (F-MuLV) (49). Several studies have shown that the SFFV is responsible for the induction of spleen foci, erythroid

proliferation and splenomegaly found during the early stages of Friend leukemia (for a review, see references 45,48,49). The helper virus F-MuLV induces splenomegaly, severe anemia and erythroleukemia only when injected into new born Balb/c or Swiss mice (22,50). Recently it has been shown that the SFFV is found integrated into a common region in 95% of erythroleukemic cell lines (29). Previous experiments using helper free stocks of SFFV, were able to show that SFFV could induce the early stages of Friend disease but not the late stage (2). These results suggest a role for the helper virus F-MuLV in the emergence of tumor forming cells that appear in the late stages of Friend leukemia (2). What is not clear from these results is whether the helper virus just functions to allow SFFV spread or plays a direct role in tumor progression.

Several leukemia and tumor cell lines with rearrangement of the p53 gene have been described (3,26,31,53,54,55). These rearrangements result in loss of p53 protein or production of truncated forms of the p53 protein. We have described several Friend induced erythroleukemic cell lines that have undergone rearrangement of the p53 oncogene (3,31,42). One cell line, which did not make p53 protein, showed a complex rearrangement of both alleles of the p53 oncogene (31). To understand the mechanism for these rearrangements we have molecularly cloned both p53 alleles from this cell line. We show in this study that the helper virus F-MuLV has integrated independently into both alleles of the p53 gene.

MATERIALS AND METHODS

Cells.

Cells lines DP16-9B3 and DP16-9B4 were established from independent methylcellulose colonies derived from the spleen of a Friend virus infected DBA/2 mouse as described (31). Cells were maintained in alpha minimal essential medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.).

DNA isolation and molecular hybridization.

High molecular weight DNA was isolated as previously described (11,30). DNA samples were digested to completion with the appropriate restriction endonucleases (Boehringer Mannheim, Dorval, Que. or Pharmacia, Dorval, Que.), fractionated on 0.8% agarose gels and transferred to nylon filters (Biorad, Mississauga, Ont.) with 0.4 M NaOH as described (39). Filters were hybridized to a [^{32}P] labelled nick-translated probe (40)(2×10^8 cpm/μg) for 16 h in 50% formamide, 20% poly-ethylene glycol, at 42°C (39). After hybridization, filters were washed in 2 × SSPE [1 × SSPE is 0.15 M NaCl plus 0.01 M $Na_2H_2PO_4 \cdot H_2O$ (pH 7.4) plus 0.5 mM EDTA (pH 8.0)] plus 0.1% sodium dodecyl sulfate (SDS) (twice for 15 min at room temperature) followed by 0.1 × SSPE plus 0.1% SDS (twice for 30 min at 65°C). Autoradiography was performed at -70°C with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) and Cronex Lightning Plus intensifying screens (Dupont, Lachine, Que.).

Molecular Cloning.

Genomic DNA from DP16-9B4 was digested to completion with *Eco*RI. DNA greater than 23 kilo-base-pair (kb) was enriched for by preparative gel electrophoresis and p53 sequence containing fragments determined by Southern blot analysis (47) of a portion of the gel hybridized with the *Bgl*II-*Pst*I fragment of the mouse p53 cDNA clone p27.1a (16). This region was excised and electroluted in 0.5 × TBE (1 × TBE is 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). Bacteriophage λDNA was isolated as outlined by Maniatis *et al.* (25). Size fractionated DNA was ligated to the gel electrophoresis purified arms of λL47.1 DNA (21) previously digested with *Eco*RI (17,25). The resulting recombinants were packaged *in vitro* (Promega BioTech, Mississauga, Ont.) (14) and plated on *Escherichia coli* WA803 (56). λL47.1 was grown in NZCM media (NZCM media is per liter: 10 g casein hydrolysate, 5 g NaCl, 1 g casamino acids, 2 g $MgSO_4 \cdot 7H_2O$, pH 7.5), which yielded small recombinant viral plaques. We found that the use of yeast extract in the media reduced the plaque size considerably. The lambda library was screened with the p27.1a probe as previously described (25).

Probes.

The p53 exon 1 probe is a 725 base-pair (bp) *Eco*RI-*Hind*III fragment from the 16 kb *Eco*R1 DNA fragment containing the intact p53 gene from a Friend virus-transformed erythroleukemia cell line, CB7 (42). The p53 intron 1 probes 5, 7, and 6 are the *Hind*III-*Xho*I, *Xho*I-*Hind*III, and *Hind*III-*Xho*I fragments, respectively, from the same p53 gene (provided by Alan

Bernstein). The p53 cDNA probe is a 900 bp *Bgl*II–*Pst*I fragment from the mouse p53 cDNA clone 27.1a (16).

The F-MuLV full length probe is an 8.5 kb fragment of plasmid p57–F-MuLV (35) representing the F-MuLV clone 57 (from A. Oliff). The F-MuLV envelope probe is an 830 bp *Bam*HI fragment derived from plasmid pHC6 (a gift from A. Oliff; 34). The Friend SFFV full length probe is pSFFV, derived from a molecularly cloned Friend SFFV (57). The SFFV-*env* probe is from plasmid pYY320 containing an 810 bp *Pvu*II fragment from the molecularly cloned SFFV (41). The intracisternal-A-particle long terminal repeat probe is plasmid p322IAP as previously described (provided by N. Hozumi; 12).

RESULTS

DP16–9B4 is an erythroleukemic cell line previously described as not expressing p53 protein and being less tumorigenic when injected into syngeneic mice, compared with a normal p53-expressing erythroleukemic cell line DP16–9B3 isolated from the same mouse (31). Southern blots of DP16–9B4 genomic DNA digests, using a p53 cDNA as a probe (16), revealed a loss of the normal 17.4 kb *Eco*RI fragment and the appearance of a novel *Eco*RI fragment greater than 23 kb (Fig. 1). Digestion with *Bgl*II revealed four bands of 16.6 kb, 14.3 kb, 8.4 kb, and 6.1 kb, compared with 18kb for the normal p53. The 3.1 kb *Eco*RI and the 2.3 kb *Bgl*II fragments represent a processed pseudogene which is inactive (58).

To understand in more detail the molecular mechanisms which led to the rearrangement of the p53 gene in DP16–9B4, we chose to clone both re-

arranged alleles. 400 p53 positive clones were identified from a recombinant λL47.1 library of 8×10^5 phage. In the preliminary restriction analysis of 14 clones, two common banding patterns emerged indicating that both rearranged alleles were cloned. A pair of recombinants from each group were randomly chosen. Lines D and E of Fig. 2 show the restriction maps of clones 713/533 and 711/523, respectively. The fragments generated by various endonucleases were compared with those of a cloned p53 gene (CB7) from an erythroleukemic cell line (42), and of the published data of Beinz *et. al.* (1) and Wolf and Rotter (55). Southern blots were also used to assist in identification of fragments by hybridization to a panel of p53 probes as indicated (line A of Fig. 2). The exploded view reveals the new cleavage sites introduced by the foreign DNA sequences. The last known restriction sites or p53-hybridizing sequences of a normal p53 gene are indicated above. Both rearrangements appear to be the result of an integration of foreign sequences: in clones 713/533 an insert of ≈3.5 kb with an apparent loss of a region containing exon 10, and in clones 711/523, an insert of ≈4.5 kb with an apparent loss of a region containing exons 5 to 7. Although there is hybridization of the p53 cDNA probe to at least exon 9 of clones 711/523, it is not clear whether a portion or all of exon 8 remains intact.

To test whether these rearrangements may be virally mediated, we subjected a duplicate set of Southern blots to a panel of endogenous and exogenous viral probes. These were comprised of the intracisternal A particle long terminal repeat (12), and SFFV (57), SFFV-*env* (57), F-MuLV (35), F-MuLV-*env* (34) probes respectively. p57-FMuLV contains the entire viral genome of the helper virus clone 57 (35). It was the only member of

this panel which specifically hybridized to the recombinant clones. Southern blots of *Kpn*I digests of the clones are shown in Fig. 3. The blot shown in the left panel of Fig. 3 was probed with p27.1a, which contains the cDNA sequences of exon 4 through exon 11 (16). In the right panel of Fig. 3, a duplicate Southern blot was probed with p57-FMuLV (34). The 1.2 kb hybridizing fragment of clones 713/533 and the 5.0 kb fragment of clones 711/523 indicate the inclusion of F-MuLV sequences into the respective *Kpn*I fragments. A restriction map of the F-MuLV is shown in line F of Fig. 2 for comparison.

The 713/533 clones have an inserted sequence which hybridizes to the entire helper virus probe but not specifically to the envelope region. From the restriction map of the helper virus and that of the insert, it seems likely that these sequences represent the first 2.0 kb of the 5′ end of F-MuLV and loss of the envelope and 3′ long terminal repeat region. The rearrangement of the second allele, represented by clones 711/523, is considerably more complex. The first 600 bp of the insert contain a new *Kpn*I site and it also hybridizes to the helper virus probe. From this hybridization and the restriction map of the helper virus, these sequences are likely to represent a portion of either the 5′ or 3′ long terminal repeat, both of which contain the only *Kpn*I sites. The remaining 2.8 kb of the insert 3′ to this does not hybridize to either the helper virus or p53 cDNA probes; it may therefore represent 3′ p53 intron sequences.

The rearrangements of both of the p53 alleles in the cell line DP16-9B4 appear to be complex and the result of as many as four independent events. It seems clear however, that the primary event was the integration of the helper virus, F-MuLV, and that the inactivation of

both functional alleles was a result of this. Secondary events resulted in deletion of most of the viral sequences as well as p53 coding exons.

DISCUSSION

Previous work using helper virus (F-MuLV) free stocks of Friend spleen focus forming virus ($SFFV_P$) showed that SFFV was sufficient to induce the early stages of Friend disease characterized by erythroid cell proliferation, but failed to result in the latter stage of the disease, characterized by the appearance of transplantable immortal erythroleukemia cells (2). These results implicate a direct role for F-MuLV in the appearance of the truly malignant cells that appear late in the disease. The replication competent retroviruses which do not have an oncogene as part of their genomes cause tumors by an insertional mutagenesis mechanism (for review, see references 33 and 51). This usually results in promoter or enhancer insertion resulting in the constitutive expression of the proto-oncogene or a truncated form of the gene product (33,51). It is generally believed that the presence of proviruses in the same region in several independent tumors indicates that these particular sequences play a role in tumor progression. The first example of a gene to be activated by provirus insertion was the c-*myc* proto-oncogene in avian leukosis virus-induced lymphomas (13). Viral integrations that inactivate a gene may also play a role in tumor progression, if there is a selective advantage for loss of this gene (10,27).

In the present study, we have found that F-MuLV had integrated into the coding sequence of both alleles of the p53 gene in the Friend virus-

induced erythroleukemic cell line, DP16-9B4. It is clear that these rearrangements have occurred *in vivo* during the progression of Friend virus-induced leukemia (31) and are not artifacts of molecular cloning. First, the p53 rearrangements in DP16-9B4 have also been found in other cell lines independently isolated from the same spleen (31). Second, Southern blot analysis of DNA from DP16-9B4 using an F-MuLV-*env*-specific probe revealed only a single *env*-hybridizing fragment, which did not comigrate with the rearranged alleles (3). This indicates that internal viral deletions existed in the p53 gene of the original DNA and that there are at least three F-MuLV integration sites in the DP16-9B4 genome. Finally, the phage library was not amplified, and several clones with the same restriction pattern were isolated. Consistent with our finding is the previously published example of Moloney leukemia virus integrating into the p53 gene of an Abelson virus-induced lymphoma cell line. The integrated provirus also contained a 3.0 kb insertion of foreign sequences into the Moloney murine Leukemia virus *pol* and *env* regions (54). The significance of the insertions or deletions within viral sequences remains to be understood.

There have been other examples of inactivation of the p53 gene by rearrangements. The human myeloid leukemia cell line HL60 had undergone major deletions in both alleles of the p53 gene (55). One acute promyelocytic leukemia patient showed rearrangement of one p53 allele and loss of the other (38). Recently, rearrangement of the p53 gene has been reported in three of six osteogenic sarcoma tumors and three of five osteogenic sarcoma cell lines, as well as one Hodgkin's lymphoma cell line (26).

At the moment, it is not known what the selective advantage inactivation of the p53 gene confers on cells. A possible advantage the p53-negative cells may have over cells expressing p53 is the ability to escape the host immune response. The p53 protein was originally identified as a tumor antigen (5) and is found in the plasma membrane (28) and on the cell surface (44). Lung and breast carcinoma patients (4) and tumor-bearing animals have been shown to make antibodies against p53 protein (5,46). It is possible that cells expressing large amounts of an altered p53 protein on their cell surface could be subject to an immune response. Hence, cells that do not express the altered p53 protein would then have a selective advantage.

Alternatively, if p53 were acting as a "tumor suppressor" then inactivation of the gene may play a role in transformation. This argument is met with opposition from gene transfer experiments, in which the p53 gene can act a dominant oncogene (6,7,15,18,37,42), and experiments in which cell lines not making p53 protein are less tumorigenic that p53-positive cells when injected subcutaneously (31,53). However, it has recently been shown that p53 genes capable of immortalizing primary cells have undergone activating mutations (8). This results in p53 proteins which then bind heat-shock protein hsp70 and lose an epitope to the monoclonal antibody PAb246 (8). Further, Munroe and Benchimol have suggested that these mutations may allow the mutant p53 gene to act in a dominant negative manner. The mutant p53 protein may act to inactivate the wild-type p53 protein or gene function (32). The dominant-acting p53 oncogene in these situations may therefore be similar to a loss of p53

activity by retrovirus insertion. Experiments are now underway to test these hypotheses.

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FIG. 1.

Southern blot analysis of the p53 gene in Friend cell lines from mouse DP16. Genomic DNA from cell lines DP16-9B3 and DP16-9B4 was digested with *Eco*RI or *Bgl*II, fractionated by electrophoresis in 0.8% agarose gels, transferred to nylon membranes, and probed with nick-translated murine p53 cDNA 27.1a (16).

FIG. 1.

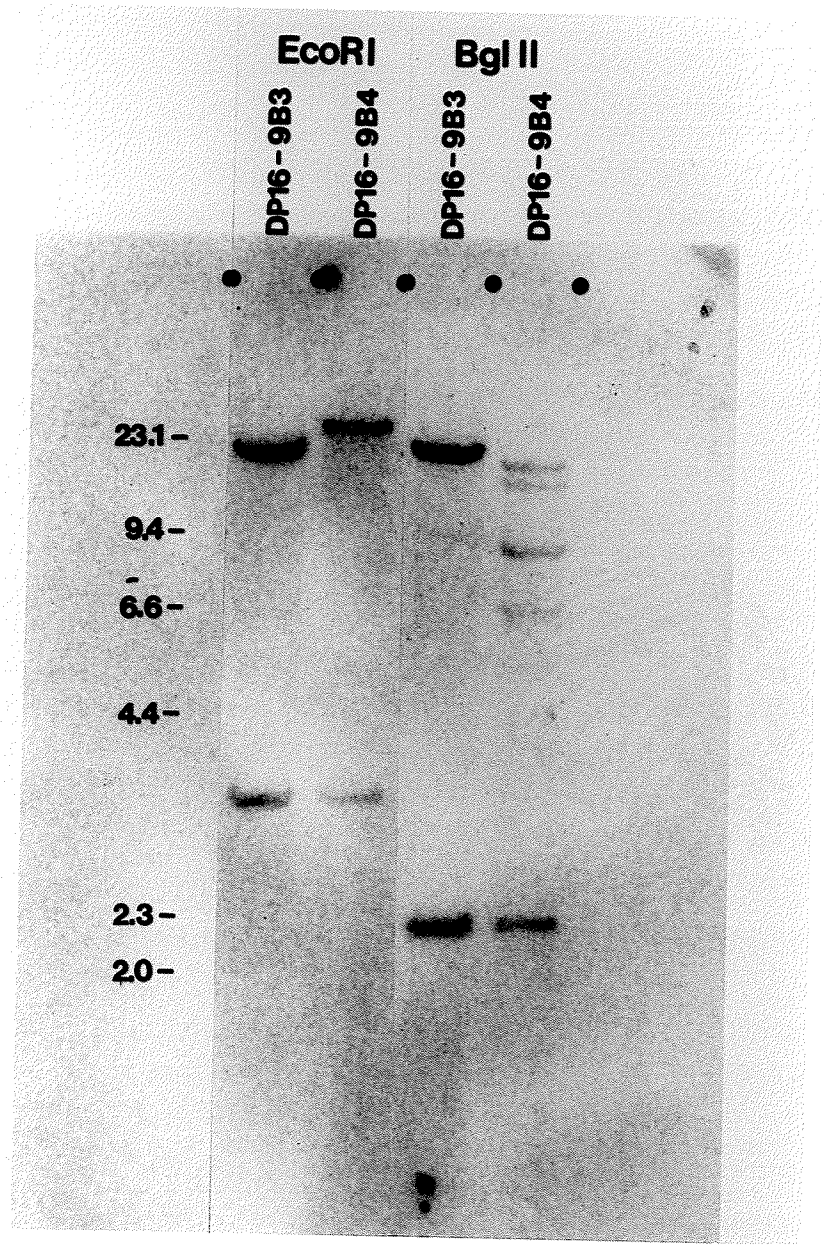

EcoRI
Bgl II
DP16-9B3
DP16-9B4
DP16-9B3
DP16-9B4
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-

FIG. 2.

Restriction enzyme maps of normal and rearranged p53 genes. (A) Regions of the p53 probes used. (B and C) Restriction map of the mouse p53 gene, taken from published data (1,36,55), exons as determined by Bienz *et al.* (1) are depicted as solid boxes. (C) Cleavage sites for enzymes *Eco*RI (R), *Bam*HI (B), *Bgl*II (G), *Kpn*I (K), and *Pvu*II (V) for the genomic clone of the murine erythroleukemia cell line CB7 p53 gene (43). (D and E) The genomic clones 713 and 533 have an insert of ≈3.5 kb (D), while the genomic clones 711 and 523 have an insert of ≈4.8 kb (E). The approximate sites of integration are indicated by the last known restriction sites or p53-hybridizing sequences. The exploded view indicates the size and restriction pattern of the inserted foreign sequences. (F) Physical map of the F-MuLV genome, derived from the published data of A. Oliff, W. Koch, and R. Friendrick (19,20,34).

FIG. 2.

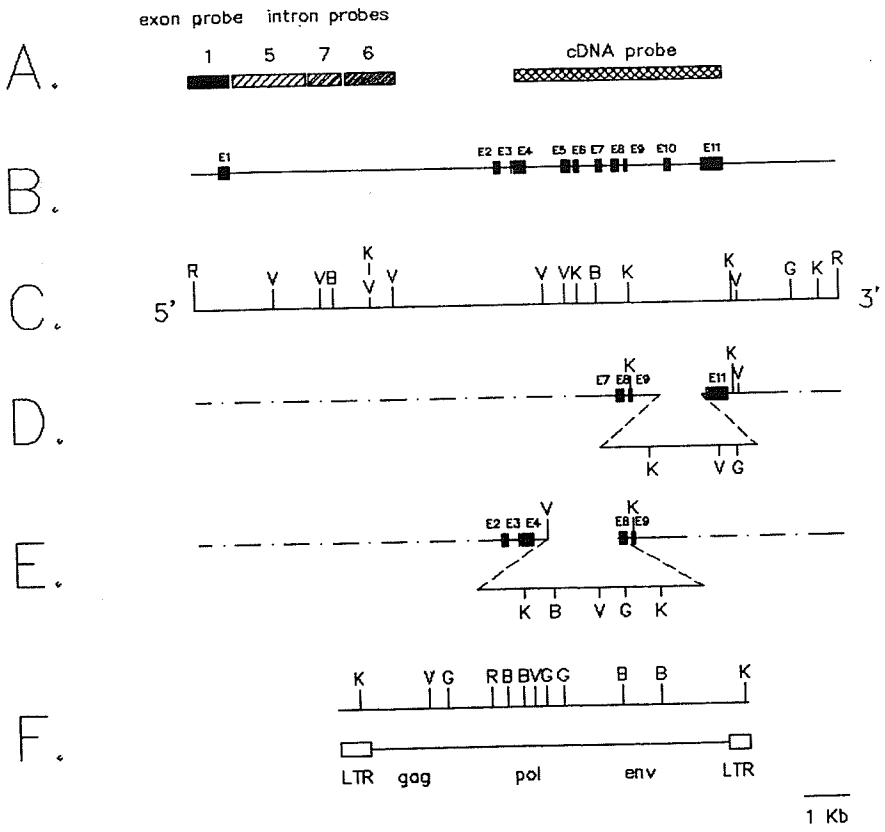
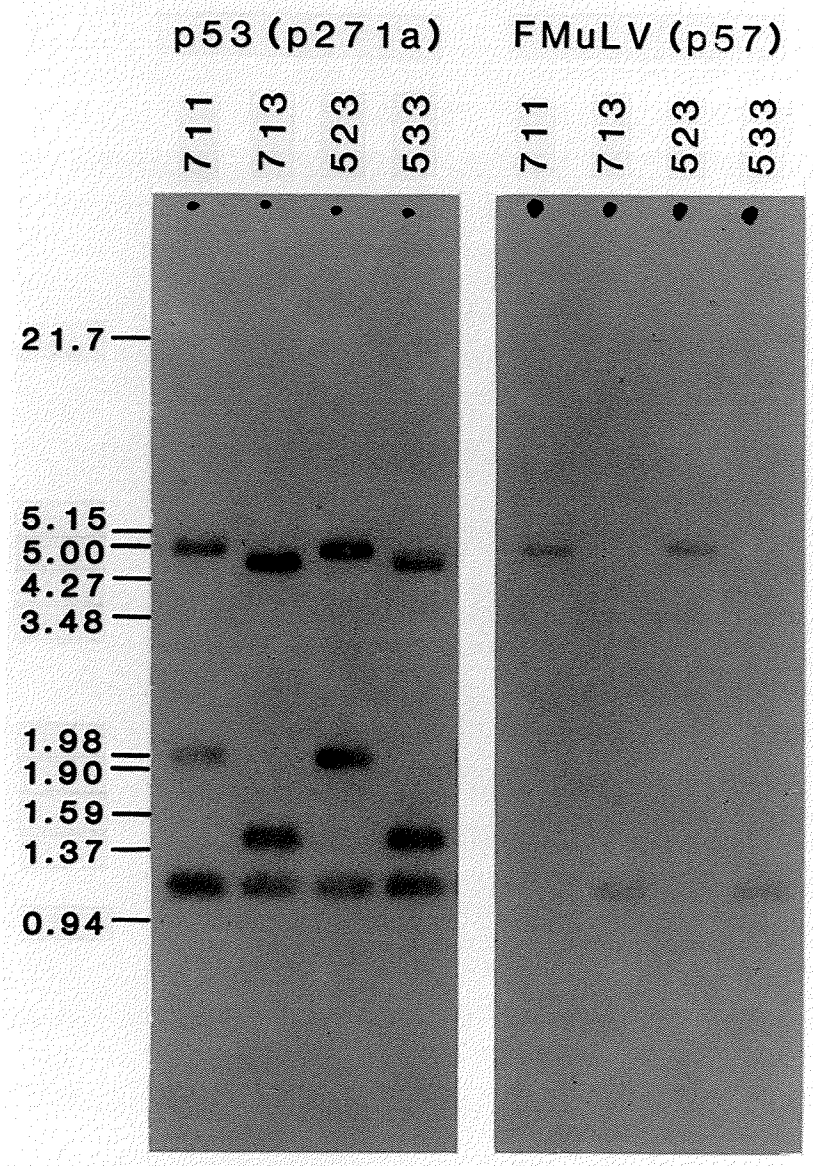

exon probe
intron probes
1
5
7
6
cDNA probe
A.
B.
C.
D.
E.
F.
E1
E2 E3 E4
E5 E6 E7 E8 E9
E10
E11
5'
3'
R
V
VB
K
V
V
V
VK
B
K
K
V
G
K
R
E7 E8 E9
E11
K
V G
E2 E3 E4
V
E8 E9
K B V G K
K V G R B B V G G B B K
LTR
gag
pol
env
LTR
1 Kb

FIG.3.

Southern blot analysis of the λL47.1 recombinant clones 711, 713, 523, and 533. The recombinant inserts were isolated from λL47.1 vector arms by *Eco*RI digestion followed by two rounds of preparative gel electrophoresis. The purified insert DNA digested with *Kpn*I, fractionated on duplicate 1.0% agarose gels, transferred to nylon membranes, and probed with either nick-translated murine p53 cDNA 27.1a (16) or F-MuLV viral DNA derived from p57-F-MuLV (34), as indicated.

FIG. 3.


p53 (p271a)
FMuLV (p57)
711
713
523
533
711
713
523
533
21.7
5.15
5.00
4.27
3.48
1.98
1.90
1.59
1.37
0.94

Loss of Wild-type p53 Activity by a Dominant Negative Mutation Allows Activated *ras* Overexpression and Full Transformation

FOREWORD

The following manuscript was published in *Molecular and Cellular Biology*: Geoffrey G. Hicks, Sean E. Egan, Arnold H. Greenberg, and Michael Mowat. 1991. Mutant p53 tumor suppressor alleles release *ras*-induced cell cycle growth arrest. Mol. Cell. Biol. 11:1344-1352.

Although the data of the previous chapter argues strongly that complete loss of endogenous p53 expression appears to be a selected for oncogenic event, it did not explain the overwhelming evidence available at the time showing that overexpression of p53 protein was dominantly transforming. This discrepancy has since been resolved by the realization that the p53 alleles used in the aforementioned immortalizing/transforming assays and those reported in Friend virus-induced erythroleukemic p53 positive lines, all contain point mutations (Eliyahu *et al.*, 1988; Munroe *et al.*, 1990; Jenkins and Sturzbecher, 1988). Although these mutations do not map to a common residue, they do fall within highly conserved regions of the protein and generally result in a common conformational change in the protein (Yewdell *et al.*, 1986; Milner and Cook, 1986; Soussi *et al.*, 1987; Sturzbecher *et al.*, 1987; Finlay *et al.*, 1988). Such mutations lead to the overexpression of a stable mutant p53 protein with the ability to oligomerize with both itself and wild-type p53 (wtp53) proteins (Eliyahu *et al.*, 1988; Kraiss *et al.*, 1988, Rovinski and Benchimol, 1988). These data support the model that the oncogenic activity of mutant p53 proteins may be to effectively eliminate any wild-type p53 activity in the cell through a dominant negative mechanism (Hershowitz, 1987; Rovinski and Benchimol, 1988). Hence, wtp53 activity is lost by an increased

expression of a more stable, but non-functional, mutant p53 which may still compete for natural p53 target interactions. In addition, any wtp53 protein available is sequestered into non-functional oligomers with the mutant proteins. Further, it is now well established that many tumor virus proteins tightly bind the anti-oncogene product of p53 (Linzer and Levine, 1979; Lane and Crawford, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). This event is thought to functionally parallel the loss of wtp53 seen in many human tumors by mutation or deletion.

At the time we undertook the following experiments, it was not clear whether any of the available p53 cDNA or genomic clones coded for a wtp53 protein. We decided to clone our own full length genomic wild-type p53 gene from the spleen of a BALB/c *nu*/+ mouse. To assay for transforming or suppressing activity, we chose the rat embryo fibroblast line REF52 model system. Unlike most established fibroblast systems, REF52 cells are refractory to transformation by the *ras* oncogene alone because even very small increases in the expression of an activated *ras* gene induces growth arrest (Franza *et al.*, 1986). Using a *ras* cotransfection transformation assay would not only assay for a true collaborating activity, but reduce the background transformation to near zero. Although several viral transforming genes, including SV40 large T antigen, had been shown able to rescue REF52 cells from *ras*-induced lethality (Franza *et al.*, 1986; Hirakawa and Ruley, 1987), no corresponding cellular gene or activity had been identified. Our original hypothesis would argue that the SV40 large T antigen's affinity for binding wtp53 served to effectively eliminate any endogenous wtp53, and that this loss of wtp53 is the cooperating oncogenic event. The hypothesis would then predict that introduction of exogenous

mutant p53 genes would also rescue *ras*-induced growth arrest in a similar dominant negative manner. Further, introduction of a wild-type p53 gene would not complement or rescue this *ras*-induced growth arrest.

In this manuscript, we present evidence that the wild-type activity of the tumor suppressor gene p53 is involved in the negative growth regulation of this model system. Moreover, it clearly demonstrates how the introduction of a dominant negative mutant p53 protein can release cells from a normal tumor suppressive response induced by the aberrant expression of an oncogenic proliferative inducing signal. Finally, high levels of *ras* overexpression coincident with this mechanism of p53 inactivation, are related to the tumorigenicity of these cells.

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Mutant p53 Tumor Suppressor Alleles Release ras Induced Cell Cycle Growth Arrest

Geoffrey G. Hicks, Sean E. Egan[1], Arnold H. Greenberg, and Michael Mowat*

Manitoba Institute of Cell Biology and the University of Manitoba
100 Olivia St., Winnipeg, MB, Canada, R3E 0V9
phone (204) 787-4139
fax (204) 783-6875

Running Title: p53 and negative growth regulation

*corresponding author.
[1]Present address: Whitehead Institute, Cambridge, MA 02142.

ABSTRACT

Overexpression of an activated *ras* gene in the rat embryo fibroblast line REF52 results in growth arrest at either the G_1/S or G_2/M boundary of the cell cycle. Both the DNA tumor virus proteins simian virus 40 large T antigen and adenovirus 5 E1a are able to rescue *ras* induced lethality and cooperate with *ras* to fully transform REF52 cells. In this report, we present evidence that the wild-type activity of the tumor suppressor gene p53 is involved in the negative growth regulation of this model system. p53 genes encoding either a p53val^{135} or p53pro^{193} mutation express a highly stable p53 protein with a conformation dependent loss of wild-type activity and the ability to eliminate any endogenous wild-type p53 activity in a dominant negative manner. In cotransfection assays, these mutant p53 genes are able to rescue REF52 cells from *ras*-induced growth arrest, resulting in established cell lines which express elevated levels of the *ras* oncoprotein and show morphological transformation. Full transformation, as assayed by tumor formation in nude mice, is found only in the p53pro^{193}-plus-*ras* transfectants. These cells express higher levels of the *ras* protein than do the p53val^{135}-plus-*ras* transfected cells. Transfection of REF52 cells with *ras* alone or a full-length genomic wild-type p53 plus *ras* results in growth arrest and lethality. Therefore, the selective event for p53 inactivation or loss during tumor progression may be to overcome a cell cycle restriction induced by oncogene overexpression (*ras*). These results suggest that a normal function of p53 may be to mediate negative growth regulation in response to *ras* or other proliferative inducing signals.

INTRODUCTION

p53 is now considered to be a tumor suppressor gene (reviewed in reference 35). Originally, p53 appeared to be a dominant acting nuclear oncogene by virtue of its ability to immortalize rodent fibroblasts (28,52), complement *ras* in transformation of primary rat embryo cells (15,28,45), and enhance the tumorigenic phenotype of cell lines (14,31,60,65). It was on the basis of these *in vitro* studies that p53 was grouped with simian virus 40 (SV40) large T antigen, adenovirus 5 (Ad5) E1A, and *myc* as an immortalizing oncogene. On the other hand, results from our laboratory and others indicated that p53 is inactivated in several tumor systems (6,25,37,42,51,63,64). Selection for the loss of p53 also appeared to be an advantage in tumor progression. These results were in conflict with the dominant nuclear oncogene model.

The discrepancy between *in vitro* and *in vivo* systems has since been resolved by the realization that the p53 alleles used in the aforementioned immortalizing and transforming assays and those reported in Friend Virus (FV)-induced erythroleukemia p53-positive lines all contain point mutations. Although these mutations do not map to a hot spot, they do fall within highly conserved regions of the protein (for a review, see reference 29; 3,43,44) and generally result in a common conformational change in the protein identified by the loss of an epitope for monoclonal antibody PAb246 (10,19,40,56,66). Such mutations lead to overexpression of a stable mutant p53 protein with the ability to oligomerize with both itself and wild-type p53 (wtp53) proteins (12,21,33,51). These data support the model that the oncogenic activity of mutant p53 proteins may

be to effectively eliminate any wild-type p53 activity in the cell through a dominant negative mechanism (24). Hence, wtp53 activity is lost by an increased expression of a more stable but nonfunctional mutant p53 which may still compete for natural p53 target interactions. In addition, wtp53 is sequestered into nonfunctional oligomers with the mutant proteins. Further, it is now well established that many tumor virus proteins tightly bind the antioncogene product of p53 (reviewed in reference 36). This event is thought to functionally parallel the loss of wtp53 seen in many human tumors by mutation or deletion (3,58).

Consistent with these results, the wild-type p53 gene has recently been shown to possess tumor-suppressing activity (13,18,26). The mechanism of p53 tumor-suppressing activity is likely to be directly related to its activity as a negative growth regulator. p53 has been implicated in cell cycle regulation. It's expression is under cell cycle control (41,48) and recently it has been found to interact with the cell cycle regulator *cdc2* kinase (1,7,57). Recent evidence has also demonstrated that wtp53 has antiproliferative activity (4,30,38,39).

Negative growth regulation in the rat embryo fibroblast line REF52 can be induced by the T24/EJ *ras* oncogene (20). Expression of this transfected gene to levels higher than 10 to 30% of the endogenous rat p21 level results in growth arrest and subsequent lethality of the cell (20). REF52 cells can be "rescued" from *ras*-mediated cell cycle arrest by cotransfection of SV40 large T antigen, Ad5 E1a, and to limited extent transcriptionally activated c-*myc* (20,27,32). While these nuclear oncogenes are nontransforming in REF52 cells by themselves, in cotransfections with *ras* the tumor virus genes allow for a 10- to 100-fold

increase in oncogenic *ras* expression and full transformation. The ability of REF52 cells to tolerate high levels of *ras* is dependent on the stable expression of the cooperating viral nuclear oncogene. In temperature-sensitive large T antigen-plus-*ras* transfectants, switch to the nonpermissive temperature results in cell cycle growth arrest at either the G_2/M or G_1 point in the cell cycle (27). Ridley et al. have shown similar results in Schwann cells; these cells also growth arrest at G_1 and G_2/M in response to elevated levels of T24/EJ *ras* and can be rescued by the transfected activity of large T antigen (49).

At the moment, it is not clear whether the ability of these tumor virus nuclear oncogenes to induce tolerance to high levels of *ras* expression in REF52 cells is due to a block in the negative growth control pathway or to an enhancement of downstream positive signals. An example of the latter complementing activity is the ability of c-*myc* to cooperate with *ras* in REF52 cell transformation (32). This cooperation, however, was dependent on vectors that coexpress a transcriptionally enhanced c-*myc* linked to the *ras* gene and occurs with a frequency 20 to 30 times less than for E1a-plus-*ras* transfections. Therefore, it is likely not working through the same mechanisms as the tumor virus proteins. We hypothesized that the viral protein cooperation was more likely through the loss of a cellular tumor suppression mechanism. Hence, we were interested in determining whether REF52 cells could be rescued from *ras*-induced growth arrest by a dominant transforming mutant p53 allele that would eliminate the tumor-suppressing and antiproliferative activities of the endogenous wtp53 through a similar mechanism. In this report, we clearly demonstrate that the expression of a mutant p53 allele can efficiently circumvent the

negative growth regulation induced by high levels of T24/EJ *ras* expression and allow transformation of REF52 cells. Further, the ability of REF52 cells to tolerate high levels of *ras* expression cannot be facilitated by the expression of the endogenous p53 or by an introduced wtp53 allele.

MATERIALS AND METHODS

Cells and transfection.

Low-passage REF52 cells (20) were maintained in Dulbecco's alpha minimum essential media supplemented with 10% fetal bovine serum. REF52 cells were seeded at a density of 10^6 cells per 100-mm dish 24 h prior to transfection. Plasmids were cotransfected by the calcium phosphate method (Cellfect; Pharmacia); precipitates where removed 8 to 10 h post-transfection and then glycerol shocked for 2 min. At 24 h post-transfection, cells were transferred from the original 100-mm plate to eight 150-mm plates and drug selected in either hygromycin B (100μl/ml; Sigma) or G418 (400μg/ml; Geneticin; GIBCO) 24 h later. Colony formation was determined at 14 to 21 days posttransfection and cell lines were derived from well isolated colonies and subcloned.

Genomic cloning and plasmids.

pEW53.3 contains the genomic 16-kb p53 gene isolated from the spleen of a normal BALB/c mouse. Molecular cloning of this allele was done as detailed previously (25). Restriction enzyme analysis of several isolated *Eco*RI DNA cloned inserts revealed banding patterns identical to each other and the genomic murine p53 clone CB7 (52). The *Eco*RI insert from one such

clone, λHX5-2, was subcloned into the expression vector pECE (16) in both the forward orientation, pEW53.6, and reverse orientation, pEW53.3, with respect to the SV40 early promoter.

Plasmid pLTRp53cG9 encodes a hybrid mouse genomic p53-cDNA construct (14) which expresses a mutant p53val^{135} protein from a Harvey murine sarcoma virus long terminal repeat promoter and was a gift from M. Oren. Plasmid pEC53 encodes a full-length genomic murine p53 gene isolated from the FV-transformed erythroleukemic cell line CB7 (52). This plasmid encodes a mutant p53pro^{193} protein (43). The activated *ras* expression plasmid, pEJ, encodes the genomic T24/EJ *ras* allele from its own promoter (54). pΔ2005 encodes the SV40 large T tumor antigen and the neomycin resistance gene and was a gift from M. Bastin (2). Finally, either plasmid pY3 for hygromycin resistance (8) or pSV$_2$neo for geneticin resistance (55) was used as a drug-selectable marker.

Immunoprecipitation and Western Immunoblotting.

p53 immunoprecipitations were performed as previously described (52) with the following variations. [^{35}S]methionine labelled cells (3 h) were lysed for 30 min in 1.0 ml lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 50μg aprotinin [Sigma]) on ice with intermittent shaking. Cellular debris was removed by centrifugation, and the supernatant was precleared by a 40-min incubation in a resuspended pellet of a 10% suspension of crude *Staphylococcus aureus* (250μl per sample, washed; Sigma). Following recentrifugation, 10^7 trichloroacetic acid precipitable counts of lysate supernatant was immunoprecipitated for 20 min with the appropriate monoclonal antibody: PAb122, a pan-specific

monoclonal antibody against p53 (22); PAb421, a pan-specific monoclonal antibody against p53 (23); PAb246, a murine p53-specific monoclonal antibody sensitive to a conformational epitope on wtp53 protein but generally not reactive with mutant p53 protein species (66); RA3.2C2, a murine p53-specific monoclonal antibody (50); and either PAb419, a monoclonal antibody against SV40 Large T antigen (23) or 2μg of immunoglobulin G2a murine polyclonal antibody (Sigma) as a control. Immune complexes were collected by a subsequent 40-min incubation with 100μl of a 3% suspension of protein A-Sepharose beads (Pharmacia). Samples were loaded onto a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate and electrophoresed at 35mA. Fixed gels were treated with Enhance (Dupont), dried, and exposed to Eastman Kodak XAR-5 film with Lightening-Plus screens at -70°C.

ras Western immunoblots were performed as outlined previously (59). Blots were then incubated at 4°C overnight with PAb[ras]10 (Dupont), a *ras* pan-specific monoclonal antibody, and visualized by alkaline phosphatase conjugation (Vectastain; Vector).

RESULTS

Mutant p53 rescues from *ras*-induced growth arrest.

If the endogenous rat wild-type p53 is involved in the mechanism of oncogenic *ras*-induced cell cycle arrest, one would predict that loss of wtp53 activity through the introduction of a p53 mutant should relieve the cell of this negative regulation. A second prediction of this model would

be that introduction of an exogenous wtp53 allele would not rescue REF52 cells; rather, it would induce a similar growth arrest.

To this end we have molecularly cloned a full-length genomic murine wtp53 gene from the spleen of a BALB/c *nu*/+ mouse. In Cos cell transient transfection assays, both plasmids express a p53 protein precipitable by the p53-specific monoclonal antibodies pAb122, pAb421, pAbRA3.2C2, and pAb246 (unpublished results). We then tested these alleles in the classic immortalization and *ras* cotransfection assays of primary rat embryo fibroblasts. While both the p53val^{135}- and p53pro^{193}- encoding plasmids exhibited these activities, pEW53.3 and pEW53.6 lacked any immortalizing or *ras* cooperating properties (data not shown). These results are consistent with recently published data for pEW53 or other wtp53-expressing plasmids in negative growth regulation or tumor suppression experiments (13,18,26,43). Considering these results together with the biological activities described in this report, we believe that these plasmids encode a true murine wtp53 under the control of its own promoter (pEW53.3) and subject to normal processing. To control for differences in the wtp53 plasmid constructs, we used the plasmid pEC53 (p<u>E</u>CE FV-transformed cell line <u>C</u>B7 genomic p<u>53</u> gene), which encodes a p53pro^{193} conformational mutant that has been extensively characterized (42,43,51).

Table 1 shows the results of three independent experiments in which several genes were cotransfected with *ras* into REF52 cells and subsequently assayed for their ability to rescue these cells from *ras*-induced growth arrest. Transfection of SV40 large T antigen plus *ras* results in a colony formation almost equal to the transfection efficiency (as determined by the frequency of drug-selectable colonies in a drug

selectable marker alone transfection). These colonies have a highly transformed phenotype and cloned cell lines are easily attained. This is in contrast to transfection of the T24/EJ *ras*-encoding plasmid (pEJ) alone into REF52 cells, which consistently results in very low colony formation (<0.5% of the transfection frequency). These colonies rarely go beyond the 20-cell stage and are never able to form stable cloned cell lines. This data is consistent with previously published results showing SV40 large T antigen is able to rescue REF52 cells normally growth arrested by expression of oncogenic *ras* (20,27). Transfection of the p53val^{135}-encoding plasmid pLTRp53cG9 alone does not appear to alter the transfection frequency or the phenotype. This too is consistent with previously published observations for the transfection of other immortalizing nuclear oncogenes alone into the already established REF52 cell line (20,27,32).

Both p53 mutant genes were able to rescue REF52 cells from *ras*-induced lethality. The p53val^{135}-encoding plasmid, pLTRp53cG9, rescued REF52 cells with an efficiency slightly lower than that of the large T antigen. Transfection of pEC53 in the p53pro^{193}-plus-*ras* assay, however, yielded roughly 15% of the number of colonies of its p53val^{135}-plus-*ras* counterpart. As transfection conditions were identical (including molar equivalents of each plasmid), this finding likely reflects a difference in stable integration of the much larger genomic allele in the case of pEC53.

The colony forming ability of wtp53 plus *ras* was no different than the *ras* alone frequency, indicating that wtp53 is not able to rescue REF52 cells from *ras*-induced growth arrest. The same results were found with the transfection of the wtp53 alone, which reduced the number of drug-

selectable colonies to nearly zero. Only 3 cell lines were isolated from which nonrefractile cell lines were established. These lines were similar to the parental line in morphology and subsequently shown by Southern analysis not to have incorporated the p53 or *ras* plasmids.

Finally, there are distinct morphological phenotypes of these various cell lines. Figure 1 shows phase-contrast photomicrographs of typical transfectant morphologies. The parental line REF52 is flat, retaining the characteristic fibroblastic shape and very large cytoplasmic content (Fig. 1A). The p53val^{135}-plus-*ras* transfectants possess a more distinct nucleus and show a dramatic drop in cytoplasmic content (Fig. 1B). Although they retain the star/diamond shape of a fibroblast, they are much smaller and refractile. REF52 cells transfected with the p53pro^{193} plus *ras* are for the most part similar to the latter cell type, but have an interesting subpopulation of highly amorphous cells exhibiting high levels of cell ruffling, scattering, and pseudopodal extensions (Fig. 1C). Under conditions of extensive passaging or variable plating densities, most subclones of these lines retained the expression of this amorphous sub-population (data not shown). The SV40 large T antigen-plus-*ras* cells were the most refractile with morphology characteristics of the transformed phenotype (Fig. 1D).

p53 versus *ras* expression.

We next determined whether the ability to rescue *ras*-induced growth arrest in REF52 cells is dependent upon the level of expression of the cotransfected genes. Immunoprecipitations of extracts from four large T-plus-*ras* [^{35}S]methionine labelled cell lines are shown in figure 2B. Both

SV40 large T antigen and p53 coprecipitate in almost equimolar amounts with the p53 specific monoclonal antibody pAb421 (lanes B). Similarly, coprecipitation of both proteins is observed with use of a large T antigen-specific monoclonal antibody, pAb419 (lanes C). The increased stability of p53 in these cells can be explained by their bound state to the SV40 large T antigen. The protein band seen at the apparent molecular weight of p35 kd is an unrelated immunological cross-reactive protein previously characterized (23).

Expression of p53 in the mutant p53 cotransfections was next examined. All p53val^{135}-plus-*ras* cell lines derived from individual colonies showed elevated expression of the introduced p53 gene. While there is some variation between cell lines in terms of the relative increases in exogenous p53 expression, all clones show great increases in absolute levels of p53 over the normal low expression of endogenous rat p53 (Fig. 2A, lanes B). Mutant p53 proteins can complex with the cellular encoded heat shock proteins. This is the presumed identity of the coprecipitating protein seen at apparent molecular sizes of 68 to 72 kDa, as previously described (47). All p53pro^{193}-plus-*ras* transfected cell lines showed significantly enhanced p53 protein levels (Fig. 2C). In addition the p53pro^{193} proteins appear to coprecipitate several other cellular proteins with the apparent molecular weights of p70, p60, and p34.

To determine that these observed protein levels were reflective of the cell lines' steady-state p53 expression, we metabolically labelled the cells for a period of 18 h (greater than 50 times the normal endogenous p53 half-life). In cell lines transfected with either p53 mutant,

virtually all the p53 expressing the pan-reactive PAb421 epitope also expressed the murine specific RA3.2C2 epitope. Further, the lysates showed little reactivity with the murine wild-type antibody PAb246 (Fig. 3). These results suggest that the majority of the p53 expressed in the p53val^{135} or p53pro^{193} transfectants is the exogenous mutant p53. We then took the supernatant of the RA3.2C2 immunoprecipitation and subjected it to a second precipitation with RA3.2C2 followed by PAb421 (Fig. 3). The final pan-reactive PAb421 signal was stronger than the preceding murine-specific one, presumably indicating an increased steady-state expression of the endogenous rat p53. This result is consistent with the dominant negative model where endogenous p53 protein levels are increased by their oligomerization with the mutant p53.

We next examined the expression of the *ras* protein. Large increases in the expression of activated *ras* protein in the large T-plus-*ras* transfectant lines Δ2005/1 and Δ2005/3 were detected (Fig. 4). Increased expression of T24/EJ *ras* in REF52 cells is typically accompanied by a reduction or complete loss of the endogenous *ras*. This is visualized by the loss of the normal doublet (processed and unprocessed forms) seen in the parental REF52 cell line and the increased expression of the EJ *ras* running as a band between them.

With the exception of the cell line, 52LTR/6, all p53val^{135}-plus-*ras* lines showed increased levels of the EJ *ras* protein. Despite the variation in the levels of p53val^{135} seen in the transfectants (Fig. 2A), all transfectants express the same level of EJ *ras*. 52LTR/6 was the only cell line to show just endogenous *ras* expression. This line was subsequently determined not to have stably integrated any copies of the EJ

ras gene. It is interesting to note that this line retained the same morphology and characteristics of the parental line.

The p53pro^{193}–plus–*ras* transfectants (R53/1,–4,–9, and –11), however, show increases in the expression of EJ *ras* equal to or greater than the levels seen in the large T–plus–*ras* lines (Fig. 4). It is important to note that these significant increases in *ras* protein are coincident with the significant increases of p53pro^{193} seen over that of the p53val^{135} lines.

p53-plus-*ras* complementation to full transformation is allele specific. We next determined whether tumorigenicity was affected by the levels of *ras* expression between the p53val^{135}–, p53pro^{193}–, and large T antigen–plus–*ras* transformant lines. Table 2 shows the tumor frequency and latency period of the various transfectants. Only one out of nine p53val^{135}–plus–*ras* transformed lines had any capacity for tumor formation. 52LTR/11 was very sporadic in its ability to form tumors; the tumors were relatively small and had a very lengthy latency. The p53pro^{193}–plus–*ras* transfectants, on the other hand, readily formed tumors when injected subcutaneously into nude mice. The tumorigenicty of these cell lines, as measured by the latency period of tumor formation, was slightly slower than that seen with the T antigen–plus–*ras* transfectants.

DISCUSSION

In this study, we provide experimental evidence that the p53 tumor suppressor gene participates in the *ras*-induced negative growth regulation in REF52 cells. Consistent with previous reports, we have shown here that large T-plus-*ras* transformation is very effective in rescuing REF52 cells from *ras*-induced lethality. Immunoprecipitation of either large T or p53 in these transfectants coprecipitates nearly equal amounts of the other. Furthermore, binding of wtp53 protein by large T antigen is coincident with an increased tolerance to the expression of a transfected *ras* gene and a high degree of tumorigenicity.

If the SV40 large T antigen is binding wtp53 to remove its activity as a negative growth regulator, we predicted that introduction of mutant p53 genes in a p53-plus-*ras* transfection of REF52 cells would replace the transforming activity of large T. We find that introduction of either the p53val^{135} or p53pro^{193} allele plus *ras* into REF52 cells is able to rescue these cells from *ras*-introduced lethality. These transfectants are further characterized by increased expression of the introduced mutant p53 and oncogenic *ras* genes and by a morphological transformation. Quite unexpectedly, we discovered only the p53pro^{193} allele is able to complement *ras* to full transformation of the REF52 cells, as measured by tumorigenicity in nude mice.

Although the p53val^{135}-plus-*ras* transfectants show varying degrees of increased expression in mutant p53 and an extremely limited capacity to form tumors when injected subcutaneously in nude mice, all show a similar modest increase in the expression of the T24/EJ protein. As we were

unable to isolate any cell lines from *ras*-alone transfections, our results further confirm the extreme sensitivity of REF52 cells to even very low levels of oncogenic *ras*. Hence, p53val^{135} does permit a significant increase in the expression of T24/EJ *ras*. Further, this rescue occurs at a very high percentage of the transfection frequency. Expression of mutant p53 in the p53pro^{193}-plus-*ras* transfectants is greatly increased over that seen with the p53val^{135} mutant. These increases in the expression of p53pro^{193} is associated with several p53-coprecipitating proteins and large increases in *ras* expression. It is very likely that the tumorigenicity of p53pro^{193}-plus-*ras* transfectants is due to the difference in *ras* expression over the p53val^{135}-plus-*ras* transfectants.

Transfection of wtp53-plus-*ras* results in a similar growth arrest seen by *ras* alone. It is important to emphasize that the same observations were made in the wtp53-alone transfections. The aberrant expression of wtp53 in REF52 cells is strongly selected against. This is consistent with a wtp53 antiproliferative effect seen in other model systems using either the same wtp53 construct (30) or others (4,38,39) and suggests a major role for p53 in negative growth regulation.

Several questions arise from these data. First, are the differences seen between the two mutant p53s a result of increased expression of one over the other, or is it more integrally linked to the difference in the specific mutations themselves? The most direct explanation would be that the p53pro^{193} gene or plasmid construct simply expresses higher levels of p53. The increased activity contributed by a highly expressed mutant p53 allows a greater *ras* tolerance in REF52 cells. This may be through a more

efficient sequestering of endogenous wild-type p53 or through an increased competition for wtp53 targets, or both.

Alternatively, the differences in the p53 mutations could be responsible for the differences in *ras* expression. Recent data from Moshe Oren's lab have demonstrated that the p53val^{135} mutant encoded by pLTRp53cG9 is temperature sensitive in terms of the stability of its conformational change (39). It is possible there is a significant subpopulation of the p53val^{135} proteins which will retain wild-type antiproliferative activity or be stabilized in a wild-type conformation by appropriate substrates. Such a state of equilibrium between mutant p53val^{135} and wtp53val^{135} proteins would explain how the mutant p53val^{135} can rescue REF52 cells from *ras*-induced lethality but that a residual wild-type p53val^{135} activity can hold *ras* expression to levels which are below the threshold of full transformation. The delicate nature of this balance would be further supported by the limited transient capacity of the p53val^{135}-plus-*ras* cell line 52LTR/11 to form tumors.

Finally, one needs to address the point that the Ad5 E1a protein, which does not bind p53, is as effective as SV40 large T in the rescue and full transformation of REF52 cells by *ras* (20,53,61). One explanation is that both E1a and large T antigen bind the retinoblastoma susceptibility gene product RB, another well characterized antioncogene. It may be that both p53 and RB affect a similar negative growth regulation pathway or work in concert with one another. This would explain why p53pro^{193}-plus-*ras* transfectants which express the same levels of *ras* as the large T antigen-plus-*ras* transfectants, are slightly less tumorigenic. Alternatively, the E1a protein coprecipitates several other proteins in

the cells it transforms (62). It may be that one of the other E1a-coprecipitating proteins is involved in the same suppression effect as p53 but interacts with a common downstream target. We are currently investigating these possibilities.

Mutation in the *ras* oncogene is associated with many human neoplasia (For a review, see reference 5). The activation of *ras* genes has often been shown to be an early event in the multistep model of tumor progression, and its expression is often suppressed in these early stages (9,17,34,46). Increased levels of *ras* oncoprotein expression are strongly associated with increasing levels of tumorigenicity and ultimately, metastasis formation (11). It is likely that the subsequent event which allows high *ras* expression would be the loss of a tumor suppressor gene or its activity. The antiproliferative and tumor-suppressing activities of the normal wtp53 is now well founded in experimental model systems. Further, the inactivation of wtp53 by mutation or deletion has been reported in almost every human neoplasia examined and is often a late event in tumor progression (17). We have demonstrated here that the concomitant expression of a dominant-acting mutant p53 gene can override the normal negative growth regulation in REF52 cells induced by the aberrant expression of an activated *ras*. This rescue is characterized by a tolerance to very high levels of *ras* expression and full transformation. These data support the model that the normal tumor suppression mechanism of wtp53 is through negative growth regulation induced by aberrant oncogene expression.

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FIG. 1.

Phase-contrasted photomicrographs of parental REF52 cells (A), p53val^{135}-plus-*ras* cell line 52LTR/4 (B), p53pro^{193}-plus-*ras* cell line R53/4 (C), SV40 large T antigen-plus-*ras* cell line 52Δ2005/1 (D). All exposures and fields are the same except for panel A, which was exposed twice as long to visualize the cytoplasm.

FIG. 1

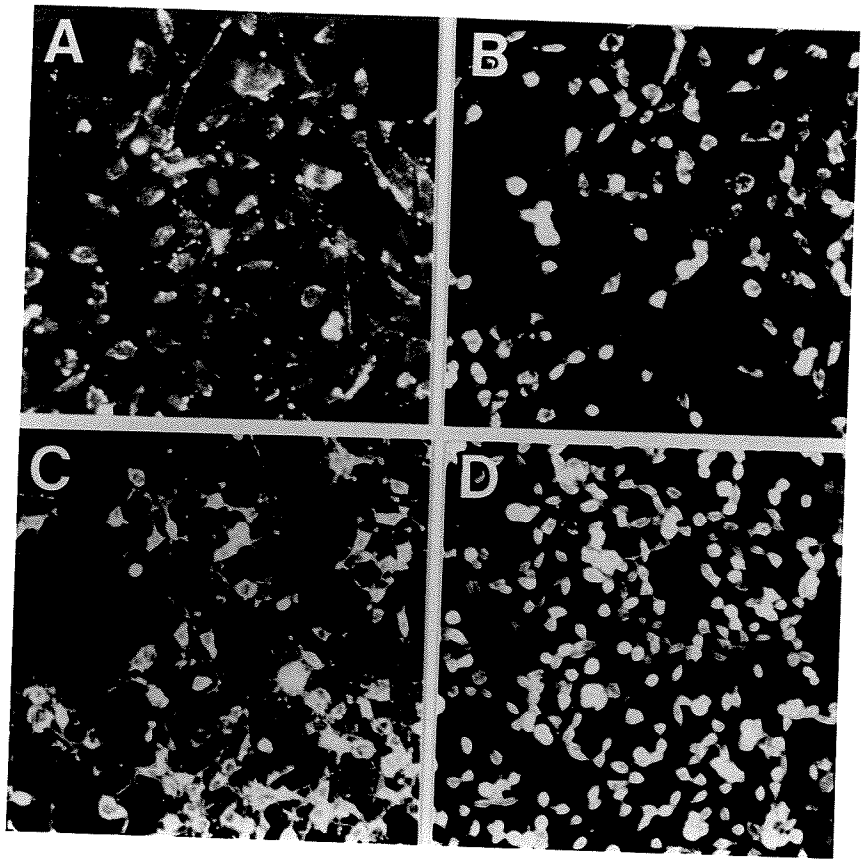

FIG. 2.

p53 expression in stably established transfectant cell lines derived from experiments 2 and 3 (shown in table one). Cells were metabolically labelled with [^{35}S]methionine for 3 h and cell lysates were prepared. In panel A, six p53val^{135}-plus-*ras* transfectant cell lines extracts were immunoprecipitated with either: IgG2a antibody (control) (lanes A), PAb122 (pan-specific p53 monoclonal antibody) (lanes B), or PAb246 (wild-type p53 conformation-dependent, murine-specific, p53 monoclonal antibody) (lanes C). In panel B, four SV40 large T antigen-plus-*ras* transfectant cell lines extracts were immunoprecipitated with either: IgG2a antibody (control) (lanes A), PAb421 (pan-specific p53 monoclonal antibody that recognizes the same epitope as PAb122) (lanes B), or PAb419 (SV40 large T antigen-specific monoclonal) (lanes C). Panel C shows both the normal very low expression of rat p53 in the parental line, REF52, as well as two cell lines from the p53pro^{193}-plus-*ras* transfections. Precipitating antibodies as in panel B.

FIG. 2A

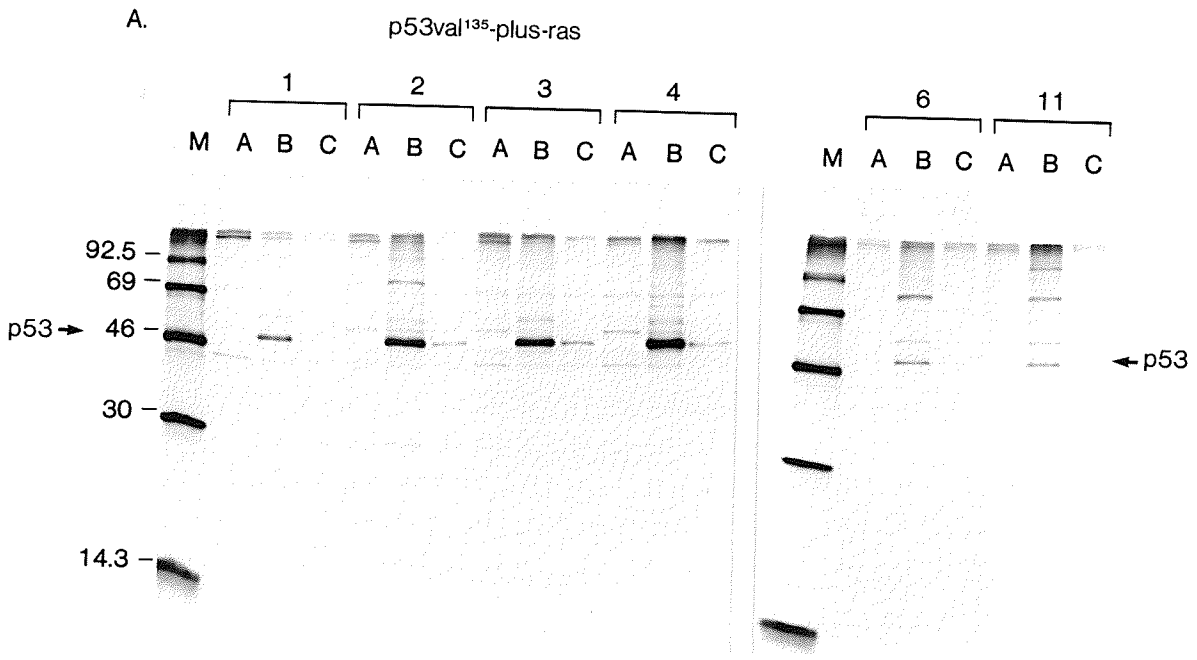
A.
p53val135-plus-ras
1
2
3
4
6
11
M A B C A B C A B C A B C M A B C A B C
92.5
69
p53
46
30
14.3
p53

FIG. 2B

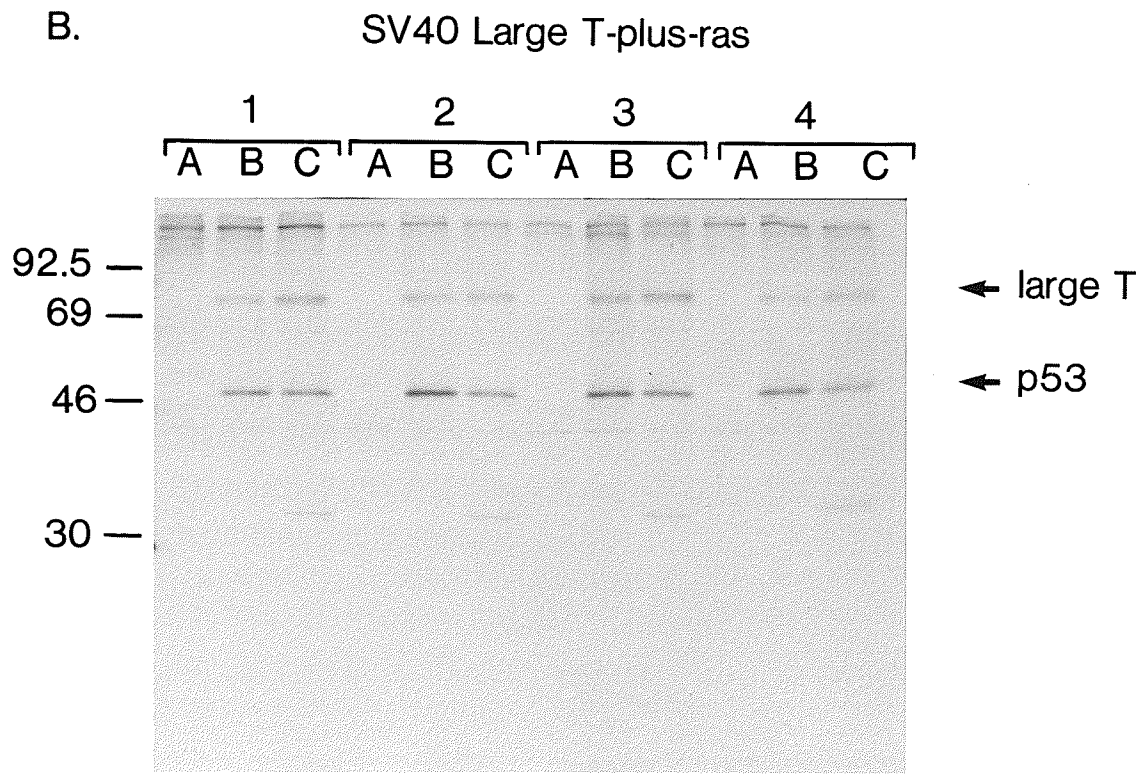
B.
SV40 Large T-plus-ras
1
2
3
4
A B C A B C A B C A B C
92.5
69
46
30
large T
p53

FIG. 2C

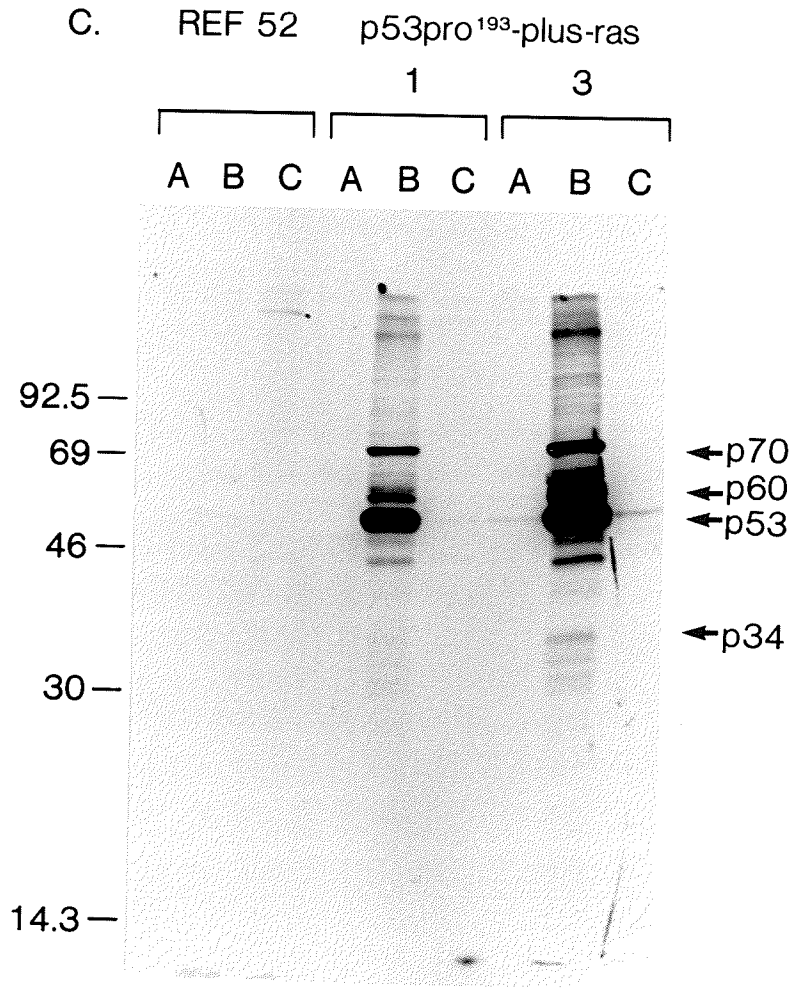
C.
REF 52
p53pro193-plus-ras
1
3
A B C A B C A B C
92.5
69
46
30
14.3
p70
p60
p53
p34

FIG. 3.

Steady-state characterization of p53 expression. REF52, p53val^{135}, p53val^{135}-plus-*ras*, and p53pro^{193}-plus-*ras* cell lines (REF52, 52LTR/6, 52LTR/4, and R53/4, respectively) were metabolically labelled with [^{35}S]-methionine for 18 h to approximate a steady-state labelling for p53. Prepared extracts were then immunoprecipitated with (left to right) control monoclonal antibody PAb419 (C), p53 pan-reactive monoclonal antibody PAb421 (421), p53 murine-specific monoclonal antibody RA3.2C2 (2C2), supernatant from the previous RA3.2C2 precipitation reprecipitated with monoclonal antibody RA3.2C2 (2C2), supernatant from second RA3.2C2 precipitation reprecipitated with monoclonal antibody PAb421 (421), and p53 murine wild-type conformation-specific monoclonal antibody PAb246 (246).

FIG. 3

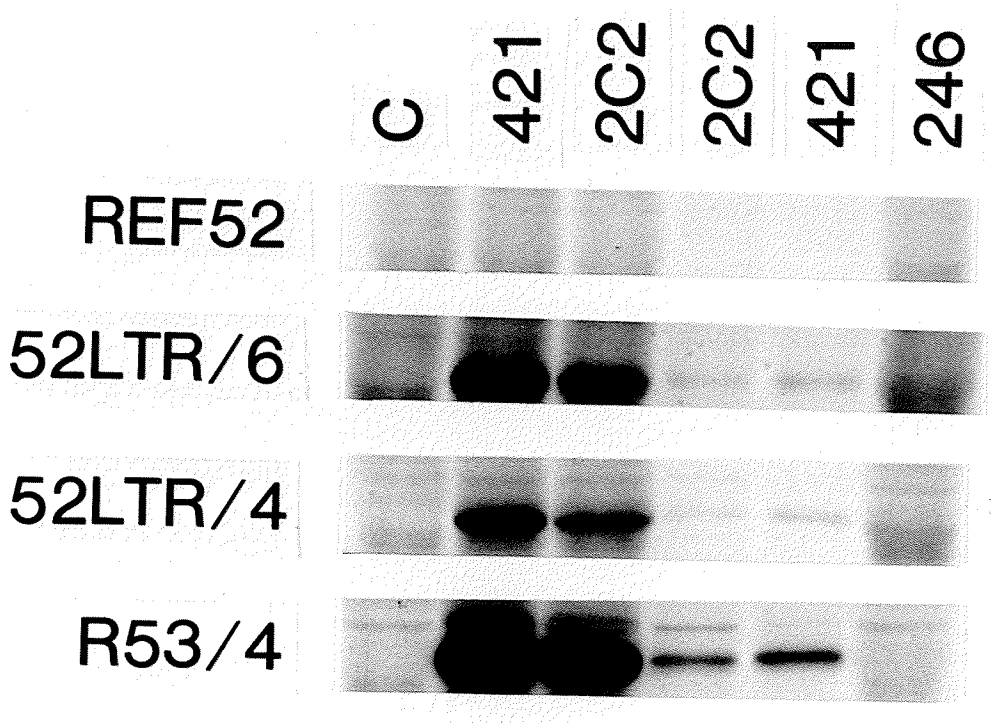

FIG. 4.

Western Blot analysis of *ras* expression. Cell lines were harvested and adjusted to a final concentration of 10^7 cells per ml. Cell lysates were prepared, and 50μl samples were subjected to polyacrylamide gel electrophoresis and Western blotting. Blots were incubated with *ras* antibody (pAb[*ras*]10; Dupont) and visualized using alkaline phosphatase conjugates. REF52 and 52PY3/1 lanes show normal endogenous *ras* expression of the parental line and transfection control, respectively. p53val^{135}-plus-*ras* cell lines (52LTR/4,-6, and -11; RLTR/1,-2, and -3) and p53pro^{193}-plus-*ras* cell lines (R53/1,-4,-9, and -11) are also shown. 52Δ2005/2 is an SV40 large T antigen-plus-*ras* cell line, and NGRG5 is a positive control line which expresses high levels of v-Ha-*ras*.

FIG. 4

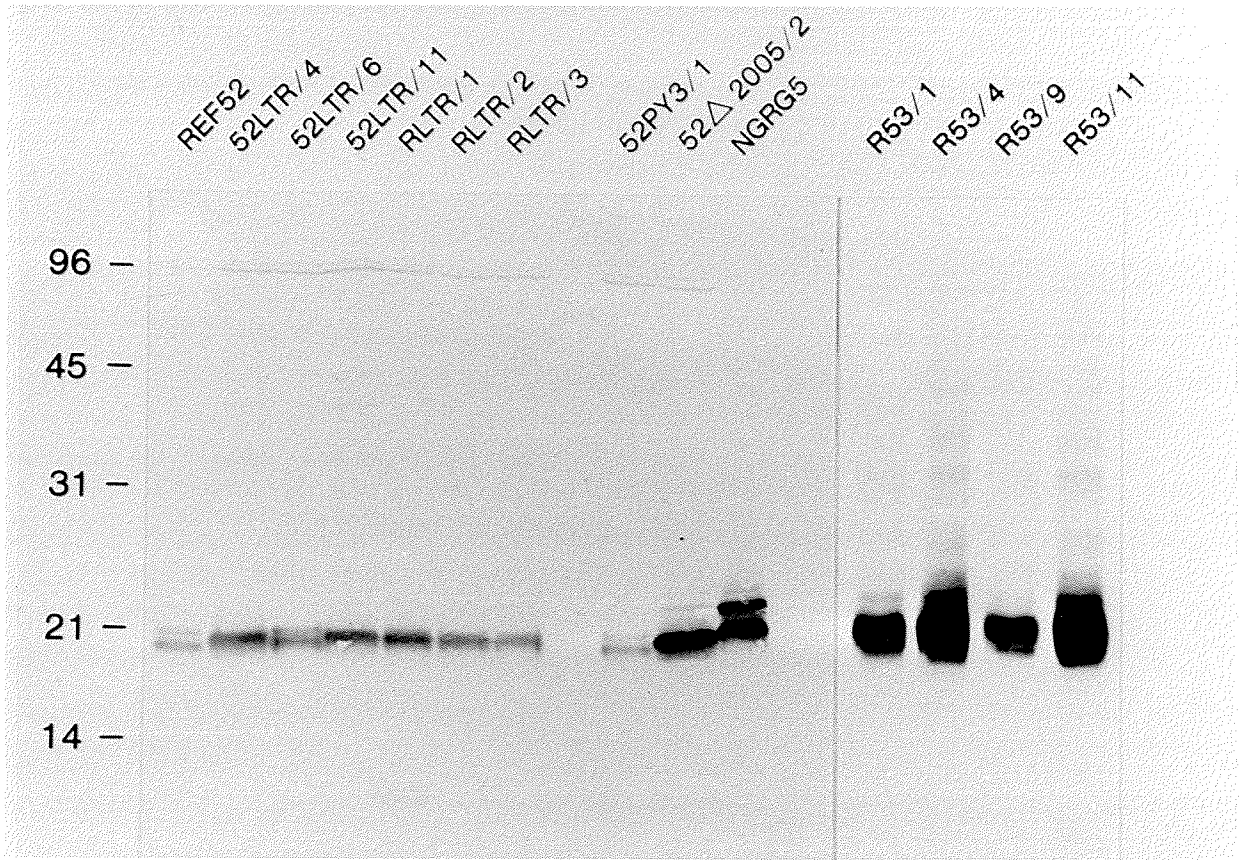
REF52
52LTR/4
52LTR/6
52LTR/11
RLTR/1
RLTR/2
RLTR/3
52PY3/1
52△2005/2
NGRG5
R53/1
R53/4
R53/9
R53/11
96
45
31
21
14

TABLE 1. p53 rescue from *ras*-induced growth arrest

Transfected Plasmids and Genes: pEJ, genomic T24/EJ ras allele; pΔ2005, SV40 large T antigen; pY3, hygromycin resistance gene; pSV2neo, neomycin resistance gene; pLTRp53cG9, $p53val^{135}$ (mutant murine p53); pEC53, $p53pro^{193}$ (mutant murine p53 full-length genomic clone); pEW53.3, wtp53 (murine p53 full-length genomic clone); pEW53.6, wtp53 (murine p53 full-length genomic clone). All transfections were coprecipitated with the indicated drug-selectable marker. All plasmids used in these transfections were standardized to equimolar equivalents of the p53 coding sequence of pLTRp53cG9 (5μg of DNA). **Colony Formation**: number of drug-selected colonies per transfection. **Stable Line**: ability to establish a stable cell line from a single colony. **nd**: not done.

TABLE 1. p53 rescue from ras-induced growth arrest

Experiment One: Transfected Plasmids	(Genes)	Colony Formation	Morphology	Stable Line
pEJ	(ras alone)	≤5	flat	nd
pY3	(drug resistance)	87	flat	nd
pLTRp53cG9	($p53val^{135}$ alone)	44	flat	nd
pLTRp53cG9 + pEJ	($p53val^{135}$ + ras)	21	refractile	nd

Experiment Two: Transfected Plasmids	(Genes)	Colony Formation	Morphology	Stable Line
pEJ	(ras alone)	≤5	flat	no
pδ2005 + pEJ	(large T + ras)	159	refractile	yes
pY3	(drug resistance)	186	flat	yes
pLTRp53cG9 + pEJ	($p53val^{135}$ + ras)	98	refractile	yes

Experiment Three: Transfected Plasmids	(Genes)	Colony Formation	Morphology	Stable Line
pEJ	(ras alone)	≤5	flat	no
pSV2neo	(drug resistance)	225	flat	yes
pLTRp53cG9 + pEJ	($p53val^{135}$ + ras)	200	refractile	yes
pEC53 + pEJ	($p53pro^{193}$ + ras)	35	refractile	yes
pEW53.3	(wtp53 alone)	≤5	flat	no
pEW53.3 + pEJ	(wtp53 + ras)	≤5	flat	no
pEW53.6 + pEJ	(wtp53 + ras)	≤5	flat	no

TABLE TWO. Allele specificity of tumorigenicity[a]

Cell Line	Tumor frequency (latency [wk])
pY3/SV_2neo	
pY3/1	0/8
ECW/1	0/6
ECW/2	0/6
ECW/3	0/6
T + *ras*	
52Δ2005/1	6/6 (1)
52Δ2005/2	6/6 (1)
$p53val^{135}$ + *ras*	
52LTR/1	0/8
52LTR/2	0/8
52LTR/3	0/8
52LTR/4	0/14
52LTR/6	0/14
52LTR/11	3/14 (10)
RLTR/1	0/6
RLTR/2	0/6
RLTR/3	0/6
$p53pro^{193}$ + *ras*	
R53/1	5/6 (3)
R53/4	5/6 (3)
R53/11	6/6 (3)

[a] Tumoriginicity was determined by injection of 5×10^5 cells subcutaneously into BALB/c nude mice. The genes transfected and the names of cloned cell lines expressing them are given. Tumor frequency is the number of tumors formed over the number of injections. All mice were sacrificed at the end of 13 weeks or when tumor growth was judged to be progressive. Latency is the average number of weeks from the original injection to the observation and palpatable confirmation of a subcutaneous tumor at the site of injection.

Loss of Wild-Type p53 Activity by a Dominant Negative Mutation is Temperature Dependent

FOREWORD

The following manuscript is currently being prepared for submission to *Oncogene*: Geoffrey G. Hicks, Nancy Stewart, Frixos Paraskevas, and Michael Mowat. 1991. Expression of mutant p53val^{135} actively blocks negative growth regulation in p53 rescued REF52 *ras* transfectants.

The discovery that p53val^{135} is temperature-sensitive for mutant or wild-type p53 conformation (Michalovitz *et al.*, 1990) enabled us to directly test the "reintroduction" of a wtp53 into the already well characterized p53val^{135}-plus-*ras* cell lines of the previous chapter. In the following manuscript, we will show that expression of the mutant p53val^{135} conformation is necessary to actively maintain the loss of *ras*-induced growth arrest in these REF52 cells. Further, we will characterize the nature of this growth arrest by cell cycle analysis.

Michalovitz, D., O. Halevy, and M. Oren. 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell **62**:671-680.

Expression of Mutant p53val^{135} Actively Blocks Negative Growth Regulation in p53 rescued REF52 *ras* Transfectants

Geoffrey G. Hicks[1], Nancy Stewart, Frixos Paraskevas,
and Michael Mowat*

Manitoba Institute of Cell Biology and the Department of Physiology,
University of Manitoba, 100 Olivia St., Winnipeg, MB, Canada R3E 0V9

*corresponding author
[1] Present address: MIT Center for Cancer Research, Cambridge, MA 02139

ABSTRACT

Over expression of an activated *ras* gene in the rat embryo fibroblast line REF52 results in growth arrest at either the G_1/S or G_2/M boundaries of the cell cycle. We have previously reported that p53 genes encoding a p53val^{135} mutation are able to rescue REF52 cells from *ras*-induced growth arrest resulting in established cell lines which express elevated levels of the *ras* oncoprotein and show morphological transformation. Expression of the mutant p53val^{135} conformation has been shown to be temperature dependent. We report here that the ability to rescue REF52 cells from *ras*-induced growth arrest is temperature dependent. This arrest is coincident with a loss of the monoclonal antibody PAb240 mutant conformation sensitive epitope and an increased expression of the monoclonal antibody PAb246 wild-type conformation sensitive epitope, with little change in the total p53 or *ras* protein levels. While cells expressing mutant-p53val^{135} alone arrest primarily at G_1/S when shifted to the wild-type-p53val^{135} conformation, cells expressing mutant-p53val^{135} plus *ras* are also driven into early S phase, arresting before G_2. These results indicate that rescue from *ras*-induced growth arrest is actively maintained by the expression of mutant p53 in a dominant negative manner.

INTRODUCTION

It is now clear that wild-type p53 (wtp53) is a tumor suppressor gene characterized by its tumor-suppressing (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Hinds *et al.*, 1989), and antiproliferative activity (Baker *et al.*, 1989; Baker *et al.*, 1990; Mercer *et al.*, 1990a; Michalovitz *et al.*, 1990; Johnson *et al.*, 1991)(for a review, see: Lane and Benchimol, 1990; Levine *et al.*, 1991). Although the mechanisms of its action as a negative growth regulator remain unclear, it is likely to be directly related to its phosphorylation by the cell cycle regulator, *cdc2* kinase (Addison *et al.*, 1990; Bishoff *et al.*, 1990; Sturzbecher *et al.*, 1990), and its recent demonstration of transcriptional activation (Fields & Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990) and DNA specific binding (Steinmeyer & Deppert, 1988; Kern *et al.*, 1991).

Alterations of p53 is the single most common neoplastic lesion in human cancer (Vogelstein, 1990). Loss of wtp53 has been observed classically as a tumor suppressor gene by allelic inactivation through deletion or rearrangement (Wolf *et al.*, 1984a; Masuda *et al.*, 1987; Baker *et al.*, 1989; Takahashi *et al.*, 1990). More often, p53 alterations are missense mutations found in highly conserved coding regions of the protein (reviewed in Jenkins & Sturzbecher, 1988). This generally results in a common conformational change of the protein which is detectable by differences in immunoreactivity; the loss of an epitope for the monoclonal antibody PAb246 (Milner & Cook, 1986; Yewdell *et al.*, 1986; Finlay *et al.*, 1988; Sturzbecher *et al.*, 1987) and expression of a new epitope for the monoclonal antibody PAb240 (Gannon *et al.*, 1990). The conformational

change is associated with overexpression of a more stable mutant p53 protein, the ability to oligomerize with both itself and wtp53 proteins, and to alter the conformation of newly translated wtp53 protein (Eliyahu *et al.*, 1988; Kraiss *et al.*, 1988; Rovinski & Benchimol, 1988; Gannon *et al.*, 1990; Milner *et al.*, 1991; Milner & Medcalf, 1991). Such mutations are frequently oncogenic with immortalizing (Jenkins *et al.*, 1984; Rovinski *et al.*, 1988), transforming (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984), and tumor enhancing activity (Wolf *et al.*, 1984b; Eliyahu *et al.*, 1985; Kelekar & Cole, 1986; Tuck & Crawford, 1989).

The observation that a p53val^{135} mutant is temperature sensitive for the wild-type or mutant conformation (Michalovitz *et al.*, 1990) has permitted conformation-dependent activities of p53 to be tested directly. When the p53val^{135} protein is switched to the wild-type conformation, it is able to conditionally suppress transformation and inhibit proliferation of transfected rat embryo fibroblasts (Michalovitz *et al.*, 1990). Overexpression of wtp53 in these fibroblasts results in a reversible growth arrest primarily in late G_1, although growth arrest in other parts of the cell cycle were observed. This pattern of growth arrest in response to wtp53 overexpression was also demonstrated using inducible p53 constructs (Mercer *et al.*, 1990b). Further studies have shown that negative regulation of the cell cycle by p53 is wild-type conformation dependent and this is integrally associated with cellular localization to the nucleus during G_1 (Ginsberg *et al.*, 1990; Gannon & Lane, 1991; Martinez *et al.*, 1991). These reports support evidence which suggests that p53s conformation and cellular localization are the normal regulators of wtp53

during the cell cycle (Cook & Milner, 1990; Milner & Watson, 1990; Shaulsky *et al.*, 1990).

Negative growth regulation in the rat embryo fibroblast line REF52 can be induced by the T24/EJ *ras* oncogene (Franza *et al.*, 1986). Expression of this transfected gene to levels higher than 10 to 30% of the endogenous rat p21 level results in growth arrest and subsequent lethality of the cell (Franza *et al.*, 1986). This arrest can be rescued by cotransfection of either Adenovirus 5 E1A or simian virus 40 (SV40) large T antigen (Franza *et al.*, 1986; Hirakawa & Ruley, 1988). Hirakawa and Ruley demonstrated that a temperature-sensitive SV40 large T antigen could rescue REF52 cells from *ras*-induced lethality in a temperature dependent manner; at nonpermissive temperatures the cells arrested primarily in G_2/M and also in G_1/S. In a recent publication, we have demonstrated that p53, a cellular encoded gene, could also rescue REF52 cells from *ras*-induced growth arrest. Introduction of exogenous point mutated p53 alleles were able to abrogate the negative growth regulation induced by *ras*, while normal endogenous or introduced exogenous wtp53 alleles could not (Hicks *et al.*, 1991). We report here that loss of REF52 cells' sensitivity to negative growth regulation induced by T24/EJ *ras* is actively maintained by overexpression of mutant p53.

MATERIALS AND METHODS

Cells and Culture.

REF52 cells are an established rat embryo fibroblast line (Franza *et al.*, 1986) and were a gift from H. Earl Ruley. The following lines were derived in our laboratory and contain transfected genes which stably express the proteins indicated in parentheses as follows: LTR/1 ($p53val^{135}$ plus T24/EJ *ras*); LTR/4 ($p53val^{135}$ plus T24/EJ *ras*); LTR/6 ($p53val^{135}$); R53/4 ($p53pro^{193}$ plus T24/EJ *ras*); and Δ2005/2 (SV40 large T antigen plus T24/EJ *ras*) (Hicks *et al.*, 1991). Cell line TSR-5 is a REF52 transfectant that stably expresses temperature-sensitive SV40 large T antigen plus T24/EJ *ras* (Hirakawa and Ruley, 1988). The temperature-sensitive SV40 large T antigen is permissive at 32.5°C but not at 39°C; it is encoded by plasmid pZIPtsA58 (Jat *et al.*, 1986). $p53val^{135}$ is temperature-sensitive for either a wild-type p53 conformation at 32.5°C or a mutant p53 conformation at 39°C; it is encoded by plasmid pLTRp53cG6 and described in further detail elsewhere in this text.

Low passage REF52 cells and all cell lines were maintained in Dulbecco's alpha minimum essential media supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C, unless otherwise indicated.

Cell Cycle Analysis.

Cells were seeded and incubated as indicated. Monolayers of cells were washed twice with cold phosphate buffered saline (PBS), trypsinized, and washed twice more with cold TSE (TSE is 0.100 M Tris-HCl, 0.070 M NaCl,

0.005 M EDTA; adjusted to pH 7.5, and filter sterilized). Final cell pellets (1 to 2 $\times$ 10^6 cells) were resuspended into 750 μl cold TSE and fixed by drop-wise addition of 2.0 ml of 95% ethanol (-20°C) while gently vortexing, and stored overnight (4°C). Fixed cells were stained for DNA analysis by the propidium iodide method (Krishan *et al.*, 1975). Briefly, cells were washed twice with cold TSE and then resuspend by gentle vortexing into 1.0 ml of PI Solution (PI Solution is per 100 ml: 25 mg of propidium iodide (Sigma), 100 mg of sodium citrate, 10 mg of RNase A (Sigma), and 0.1% Triton X 100). Cells were incubated for at least 2 h in darkness prior to being passed through a 41 micron filter (Spectramesh; Spectrum) and analyses by flow cytometry on a Coulter EPICS -Profile II. 12.5 $\times$ 10^3 to 25 $\times$ 10^3 cells were sampled during each analysis. DNA content was determined by fluorescence and each analysis was normalized to the relative 2N DNA content of the G_1 peak. Distribution of the S-phase was determined by a broadened second degree polynomial (Dean, 1980) using Multicycle™ software (Phoenix Flow Systems). The scale of the cell number, ordinate axis, is determined by the relative distribution of fluorescent events, maximizing the cell cycle profile.

Immunoprecipitation and Densitometric Analysis.

p53 immunoprecipitations were performed as previously described (Hicks *et al.*, 1991). Briefly, [^{35}S]methionine labelled cells (1 h) were lysed for 30 min in 1.0 ml lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 50μg aprotinin [Sigma]) on ice with intermittent shaking. Cellular debris was removed by centrifugation and the supernatant was pre-cleared by a 40-min incubation in a resuspended pellet of a 10% suspension

of crude *Staphylococcus aureus* (250 μl per sample, washed; Sigma). Following recentrifugation, 10^7 trichloroacetic acid precipitable counts of lysate supernatant was immunoprecipitated for 20 min with the appropriate monoclonal antibody: PAb421, a pan-specific monoclonal antibody against p53 (Harlow *et al.*, 1981); PAb240, a mutant p53 conformation specific monoclonal antibody (Gannon *et al.*, 1990); PAb246, a murine p53-specific monoclonal antibody sensitive to a conformational epitope on wtp53 protein but generally not reactive with mutant p53 protein species (Yewdell *et al.*, 1986); and either PAb419, a monoclonal antibody against SV40 Large T antigen (Harlow *et al.*, 1981) or 2μg of immunoglobulin G2a murine polyclonal antibody (Sigma), as a control. Immune complexes were collected by a subsequent 40 min incubation with 100μl of a 3% suspension of protein A Sepharose beads (preswollen and washed; Pharmacia). Samples were loaded onto a 12.5% polyacrylamide gel in sample buffer and in the presence of sodium dodecyl sulfate, and electrophoresed at 35mA. Fixed gels were treated with Enhance (Dupont), dried, and exposed to Eastman Kodak XAR-5 film with Lightening-Plus screens at -70°C.

Densitometric scanning of autoradiographs were performed on BioRad model 620 Video Densitometer, and integrated using BioRad 1D Analyst™ software.

RESULTS

p53val^{135} rescue is temperature dependent.

If p53val^{135} expression is rescuing REF52 cells from growth arrest induced by the expression of T24/EJ *ras* in an active manner, and presumably through a dominant negative mechanism, we predicted that a temperature shift to the wild-type conformation of p53val^{135} should relieve this activity and induce growth arrest. All cell lines studied grew exponentially under normal incubation conditions (37°C), and with similar rates. When the temperature was shifted to 32.5°C, the parental REF52 cell line maintained an exponential rate of growth albeit slightly slowed (Fig. 1A). This pattern was also seen in REF52 cells which have been fully transformed by either SV40 large T antigen plus *ras* or p53pro^{193} plus *ras* (Fig. 1B). In the cell line LTR/4, where *ras*-induced growth arrest has been rescued by the temperature-sensitive p53val^{135}, there is a dramatic drop in the growth potential (Fig. 1C). Although the majority of the cells appear to have growth arrested, there remains a sub-population of cells which are able to continue cell division at a greatly reduced rate. This likely reflects a "leaky" nature of the temperature-sensitive wild-type-p53val^{135} conformation. Indeed, in one of the six LTR/4 experiments, the growth curve declined steadily from the original 4×10^4 cells. Finally, cell line LTR/6, which was transfected with only p53val^{135}, shows a growth arrest similar to the LTR/4 cells following temperature shift.

p53 epitope expression.

Changes in the expression of wtp53 conformation or mutant p53 conformation can generally be detected by the monoclonal antibody PAb246 or PAb240, respectively. We then wished to use the expression of these epitopes to both confirm a temperature-dependent conformational change in p53val^{135}, as well as to follow the dynamics of the epitope expression immediately following the temperature-shift, by densitometric analysis. The expression of each epitope, taken from parallel immunoprecipitations of the same metabolically labelled cell extract, is expressed as a percentage of the total p53 content (having been normalized to the PAb421 epitope, which is a p53 pan-specific monoclonal antibody and should therefore represent all the p53 in the cell). One should bear in mind that p53val^{135} transfected REF52 cells express ≈15 fold higher levels of p53 than does the normal parental line (Hicks *et al.*, 1991).

At 24 h post-temperature-shift, LTR/4 (p53val^{135} plus *ras*) showed a drop in the relative PAb240 expression from 75% to 53%. This is characterized by a steady decline observed over the 24 h period (Fig. 2A). At the same time the relative PAb246 epitope expression rose by a modest difference of 6% to 11%. LTR/6 (p53val^{135}) showed similar change, but with more dramatic differences. Relative PAb240 expression went from 70% to 32.6%, and relative PAb246 expression rose from 14% to 36%.

It is of interest to note that these changes were not continuously gradual. In both cell lines without *ras*, REF52 and LTR/6, the relative expression of PAb240 transiently rose at 6 h before declining to the lower 24 h level. This spiking is coincident with a dramatic drop in the

relative PAb246 expression in both LTR/4 and LTR/6 at 6 h before rising to levels above the initial ones.

DNA and cell cycle analysis of growth arrest.

We were interested to determine at what stage in the cell cycle these cell lines were arrested. Growth arrest was induced by incubation of the cells at the nonpermissive temperature for 4 days, followed by propidium iodide staining and DNA analysis on an EPICS-II fluorescence activated cell sorter (Coulter).

For the parental cell line we observed a normal cell cycle distribution at 39°C. At 32.5°C, the parental cell line maintained a normal distribution showing a slight increase in S and G_2 events indicative of a reduced temperature (Fig. 3A). This normal distribution was also observed for the p53val^{135}-plus-*ras* transfectant cell line LTR/4 at 39°C, where the conformation of the p53val^{135} is primarily mutant (Fig. 3B). Following temperature shift, these cells become growth arrested and we observe either one of two cell cycle arrest profiles. In the first situation, arbitrarily named LTR/4A, we see a primarily healthy population of cells which fix and stain easily. These cells appear to arrest primarily in G_1 (Fig. 2B). Cell cycle analysis indicates a small increase in the percentage of cells also arrested in G_2; noted in Table 1 by an increased percentage of cells with a G_2 DNA content as well as an increase in the distribution of these fluorescent events around G_2, represented statistically by an increased coefficient of variation (*CV*). Also, there is a significant drop in the number of cells in S phase, and most of these are distributed early after G_1/S (Fig. 3B). In the second situation,

LTR/4B, we observe almost an entire arrest at G_1/S (Fig. 3B). This pattern of arrest at G_1/S and collapse into early S phase is also seen in cell line LTR/1, another p53val^{135}-plus-*ras* transformed REF52 cell line (Fig. 3C). It should be noted that most cells of this B-type population appeared viable after 4 days of incubation, although there was an increased number of dead cells as well as a morphology consistent with difficulty in trypsinizing, fixing, and an increased stickiness of the cells.

To help elucidate whether the reasons for the differences seen in the A-type and B-type cell cycle profiles may be related to the expression of wild-type-p53val^{135}, T24/EJ *ras*, or both, we then examined the cell line LTR/6, which overexpresses p53val^{135} but expresses only the endogenous *ras* genes. This cell line consistently gave A-type cell cycle profiles, growth arresting primarily at G_1/S but also in G_2/M (Fig. 3D). Conversely, a cell line which was transfected with a nontemperature-sensitive p53 mutant and activated T24/EJ *ras* genes, did not growth arrest and maintained normal cell cycle profiles at both temperatures (Fig. 3E).

Finally, we examined the temperature-sensitive p53val^{135}-plus-*ras* induced growth arrest compare to the temperature-sensitive SV40 large T antigen-plus-*ras* induced growth arrest. We analysed such a cell line, TSR-5, a gift from H. Earl Ruley, and previously characterized (Hirakawa and Ruley, 1988). Again we observed a high percentage of cells which had growth arrested in G_1 and collapsed into early S phase (Fig. 3G). A major difference, however, was the much greater number of cells which were growth arrested in G_2, 36.4%, compared to any other temperature-sensitive p53val^{135} arrested cell lines (Table 1). This G_2/M arrest by the temperature-sensitive-large T-plus-*ras* transfectant cell line is

consistent with previously published results (Hirakawa and Ruley, 1988). To control for expression of a wild-type SV40 large T antigen, a SV40 large T-plus-*ras* transformed REF52 cell line transfected and isolated under the same conditions as all the p53val^{135} transfectants reported here, was observed to have a normal cell cycle profile at 32.5°C (Δ2005/2, Fig. 3F).

DISCUSSION

In this report we demonstrate that REF52 cells transfected with a temperature-sensitive (*ts*-) p53val^{135} plus T24/EJ *ras* are conditionally rescued from *ras*-induced growth arrest. Moreover, this release from negative growth regulation is actively maintained by overexpression of the mutant p53val^{135}. Overexpression of wild-type p53 conformation in these cell lines at the non-permissive temperature results in growth arrest. This cell cycle arrest appears to manifest itself into either of two patterns: arrest primarily at G_1/S but also at G_2/M, or arrest at G_1/S and early S phase with few cells at G_2/M. Both patterns are different from the growth arrest observed primarily at G_2/M in *ras* transformed REF52 cells conditionally rescued by a *ts*-SV40 large T antigen. These results show that expression of a mutant p53 protein can actively release the cell in a dominant negative manner from negative growth regulation induced by aberrant oncogene expression. Further, it suggests that the antiproliferative activity of p53 may be involved in regulation of the cell cycle at more points than simply transit through G_1/S and initiation

of DNA synthesis, as previous results have suggested (Mercer *et al.*, 1990b; Michalovitz *et al.*, 1990; Martinez *et al.*, 1991).

The impact of this data is strengthened by a full appreciation of the subtle differences of the REF52 cell line and the derivation of the cell lines examined here. Unlike most other established rodent embryo fibroblast systems, transfection of an activated *ras* oncogene alone will not transform REF52 cells (Franza *et al.* 1986). The expression of an activated *ras*, even in very small increases, results primarily in growth arrest at G_2/M but also at G_1/S and is followed by subsequent lethality (Franza *et al.*, 1896; Hirakwa *et al.*, 1987). Similar results induced by an activated *ras* have also been reported in Schwann cells (Ridley *et al.*, 1988). In humans, the activation of *ras* has often been shown to be an early event in the multistep model of tumor progression, and its expression is usually suppressed in these early stages (Paterson *et al.*, 1987; Bremner & Balmain, 1990; Fearon & Vogelstein, 1990; Kumar *et al.*, 1990). Hence, REF52 cells provide a good model system to study what events may be necessary to rescue normal cells from this *ras*-induced negative growth regulation permitting high *ras* expression and full transformation.

A second important difference is the way in which the p53val^{135}-plus-*ras* REF52 transformants were originally derived. Whereas most other mutant p53-plus-*ras* cell lines are isolated in a focus formation assay, and therefore under selective pressure for a full *ras*-transformed phenotype (Eliyahu *et al.*. 1989; Finlay *et al.*, 1989; Hinds *et al.*, 1989; Michalovitz *et al.*, 1991), our cell lines were derived in colony formation assays, where the selection pressure is simply for the ability of

transfectants to be able to express *ras* without *ras*-induced growth arrest (Hicks *et al.*, 1991). Under these conditions, we find that the p53val^{135} encoding gene is able to rescue REF52 cells from *ras*-induced growth arrest, and although the cell lines appear morphologically transformed, they are rarely tumorigenic in nude mice assays. It is not clear whether this may be due to the fact that they still express a significant percentage of p53 in the wild-type (PAb246^{+}) conformation; or it is intrinsic to a cell specific expressionof the p53val^{135} mutation.

A third component regards the proposed differences between dominant negative and dominant positive contributions of p53 mutations in transformation (reviewed in Michalovitz *et al.*, 1991). This hypothesis recognizes the difficulty in separating oncogenic contributions by p53 mutations which may either: 1) serve simply to inactivate wtp53 activity by the overexpression of a mutant p53 protein already deficient in wtp53 activity, and with the ability to oligomerize any existing wtp53 into nonfunctional oligomers resulting in the loss of p53 tumor suppressive functions – a dominant negative mutation; or 2) mutations may also serve to enhance oncogenic signalling through increased and deregulated interactions with normal p53 substrates – a dominant positive mutation.

With these three points in mind, combined with the data presented in this report demonstrating that the rescue from *ras*-induced growth arrest is conditional to the overexpression of the mutant p53val^{135}, we believe we have identified cells in which the dominant-negative contributions of a mutant p53 are clearly identified. Moreover, such a dominant negative mutation of p53 which actively releases the cell from the negative growth regulation induced by aberrant oncogene expression does not alone confer

full transformation, as determined by tumor growth in nude mouse assays. It is important to note that this is not simply a consequence of REF52 cells being refractory to transformation by p53, as p53pro^{193} plus *ras* transfections will fully transform these cells (Hicks *et al.*, 1991). Full transformation is correlative with much higher levels of T24/EJ *ras* expression, as well as several novel p53-coprecipitating proteins (Hicks *et al.*, 1991).

Characterization of the growth arrest in p53val^{135} transfected REF52 cells can be first examined in LTR/6 cells (no T24/EJ *ras*). Overexpression of *ts*-p53val^{135} at 32.5°C results in growth arrest primarily at G_1/S (Fig. 3D). This is consistent with previously published wtp53 antiproliferative activity, where overexpression of the same p53val^{135} or an inducible wtp53 construct results in a reversible growth arrest at G_1/S (Michalovitz *et al.*, 1990; Mercer *et al.*, 1990b). Like these authors, we find that although growth arrest induced by wtp53 is primarily at G_1/S, it can be seen at other parts of the cell cycle to a lesser degree, most notably at G_2/M in REF52 cells.

This type of growth arrest, characteristic of the antiproliferative activity of an overexpressed wtp53, was also observed in the p53val^{135}-plus-*ras* transfectant cell line LTR/4 (Fig. 3B). However, there was a second phenotype of LTR/4 growth arrested cells also observed. In this situation, the cells not only arrested at G_1/S, but a great many of them arrested in early S-phase (Fig. 3B). This could be interpreted as a failure to arrest at the G_1/S restriction point, or an inappropriate initiation of DNA synthesis (or both). This phenotype was also observed in another p53val^{135}-plus-*ras* transfected cell line, LTR/1 (Fig. 3C).

The reason for two phenotypes may in part be due to a "leakiness" in the expression or action of the wild-type p53 conformation resulting in a threshold effect between a strong wtp53 antiproliferative block at G_1/S or a weaker block which is overpowered by the oncogenic effects of an activated *ras*. There are clues that the expression of *ras* is involved in the latter phenotype. First, this phenotype was never observed in the growth arrested LTR/6 cells (p53val^{135} only), both in terms of the difference in cell cycle arrest profiles or the increased number of growth arrested cells which appeared to be a lethal event (as determined by eosin yellow exclusion and cellular morphology). Second, the significant increases seen in the broadening peaks of assigned G_1 and G_2 DNA content (coefficient of variance, Table 1) is indicative of increased aneuploidy (Alanen *et al.*, 1989; McFadden *et al.*, 1990), which is also a characteristic of activated *ras* expression (Hayag *et al.*, 1990). Third, this same pattern is observed in the G_1 and S-phase of the temperature-sensitive SV40 large T antigen-plus-*ras* transfectants.

It is not clear, however, what is directly responsible for the differences observed between *ts*-p53val^{135} and *ts*-SV40 large T antigen growth arrested cells. The simplest explanation is that it is due to relative overexpression of wtp53 in the p53val^{135}-plus-*ras* cells or the relative overexpression of T24/EJ *ras* in the SV40 large T antigen-plus-*ras* cells. While the overexpression of wtp53 has already been discussed, it is important to reemphasize that the cellular localization of p53 is also conformation dependent (Ginsberg *et al.*, 1990; Gannon and Lane, 1991; and Martinez *et al.*, 1991). If the normal function of wtp53 is involved in regulating the cell cycle at G_1/S, it is possible that the growth arrest

we observe in many other parts of the cell cycle is the result of inappropriate overexpression of a wild-type conformation p53val^{135}.

An argument for the overexpression of T24/EJ *ras* directing the differences in growth arrest patterns would be supported by previous reports of activated *ras* expression regulating the transit through the G_1/S and G_2/M restriction points of the cell cycle (Durkin & Whitfield, 1986 and 1987) and more specifically, *ras* regulating high levels of M-phase kinase (*cdc2*-kinase) activity (Daar *et al.*, 1991). This would explain a *ras*-induced growth arrest at G_2/M if normal growth regulation responses were still active. Because over expression of mutant p53val^{135} can release this negative growth regulation and permit elevated T24/EJ *ras* expression, it is likely that these control pathways have a common link. Given that p53 has also been implicated in *cdc2*-kinase interactions (Addison *et al.*, 1990; Bischoff *et al.*, 1990; Sturzbecher *et al.*, 1990), it is not unreasonable to speculate that the both p53val^{135} and T24/EJ *ras* may be regulating the control of the cell cycle through *cdc2*-kinase, downstream and upstream respectively. Further, it argues that p53 may have a regulatory role in the cell cycle outside of G_1/S transit and initiation of DNA synthesis, similar to that reported for the retinoblastoma susceptibility gene product *RB* (Buchovich *et al.*, 1989; Chen *et al.*, 1989; Cooper and Whyte, 1989). This will be the topic of future investigations.

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FIG. 1.

Temperature-dependent growth and survival of transfected REF52 cells. 4×10^4 log phase cells were seeded onto 60-mm dishes and incubated at either 37°C (•) or 32.5°C (○), as indicated. Growth potential was determined by trypsinization and counting of the number of viable cells in parallel cultures of each cell line at the times shown. Each cell line and the transfected genes are indicated. Standard error is the result of six independent experiments.

Figure 1A

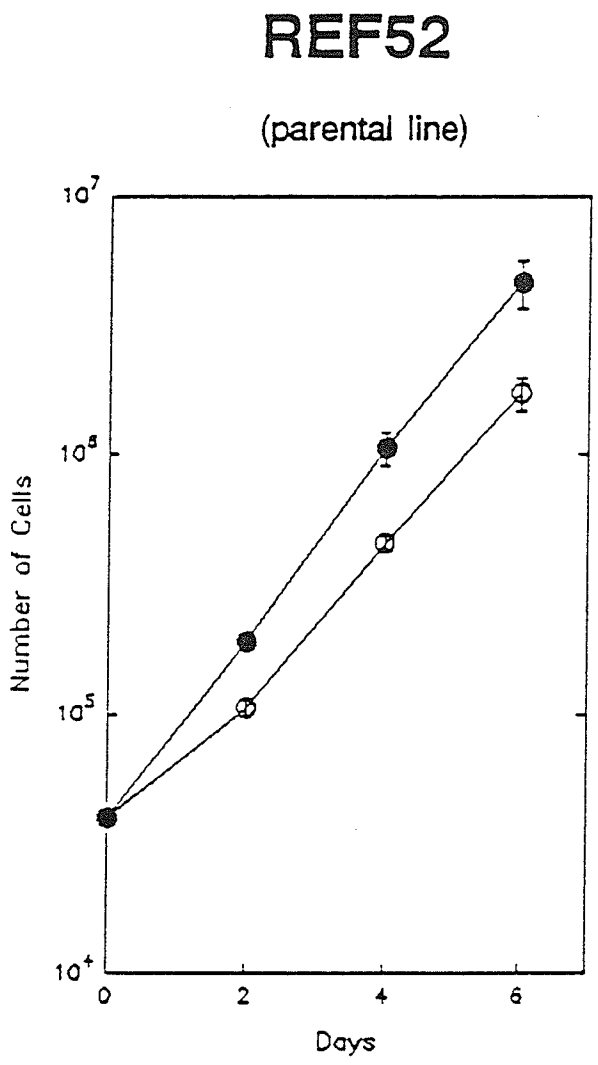

REF52
(parental line)
Number of Cells
10^7
10^6
10^5
10^4
0
2
4
6
Days

Figure 1B

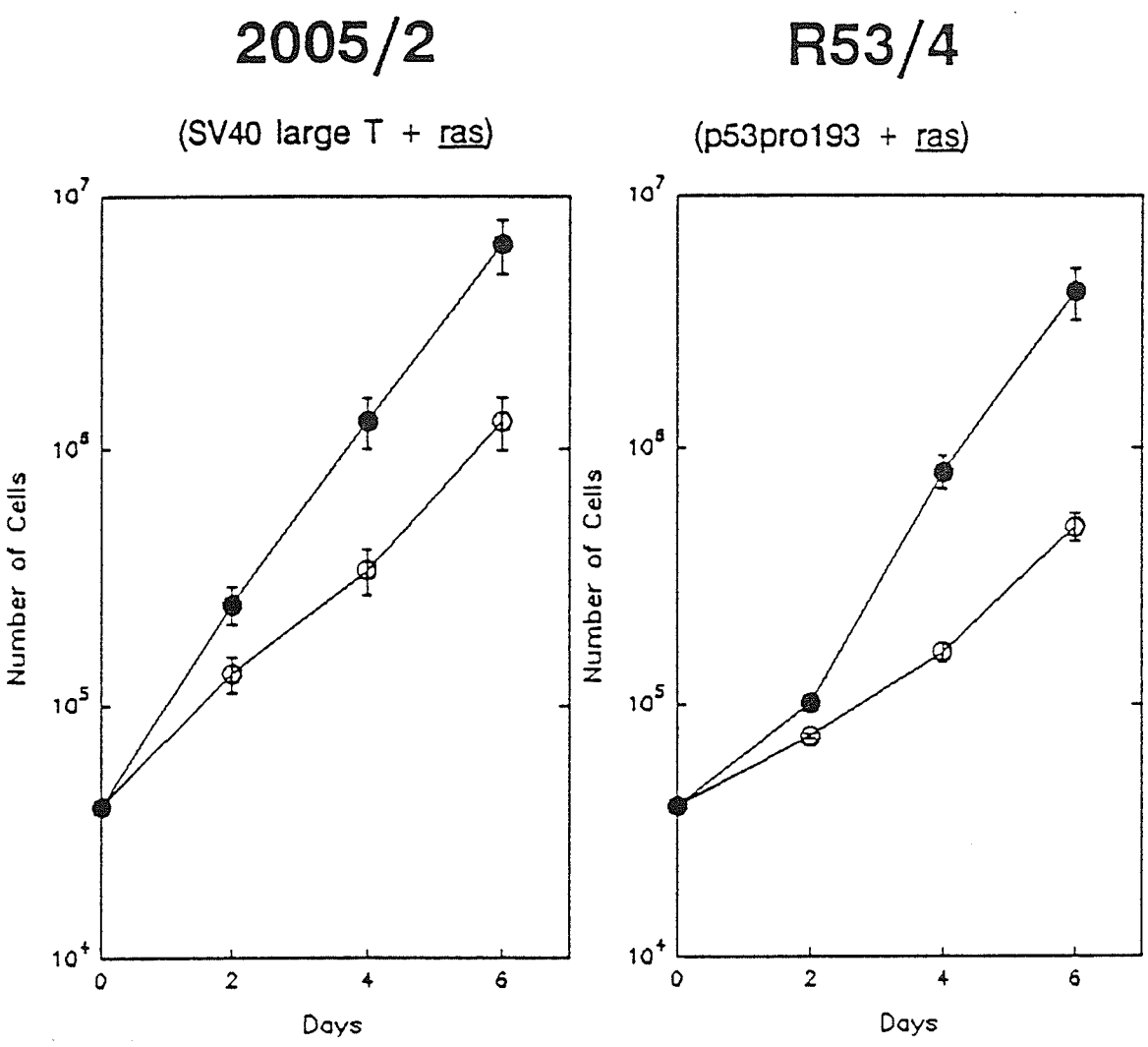

2005/2
(SV40 large T + ras)
R53/4
(p53pro193 + ras)
Number of Cells
Days
10^7
10^6
10^5
10^4
0
2
4
6

Figure 1C

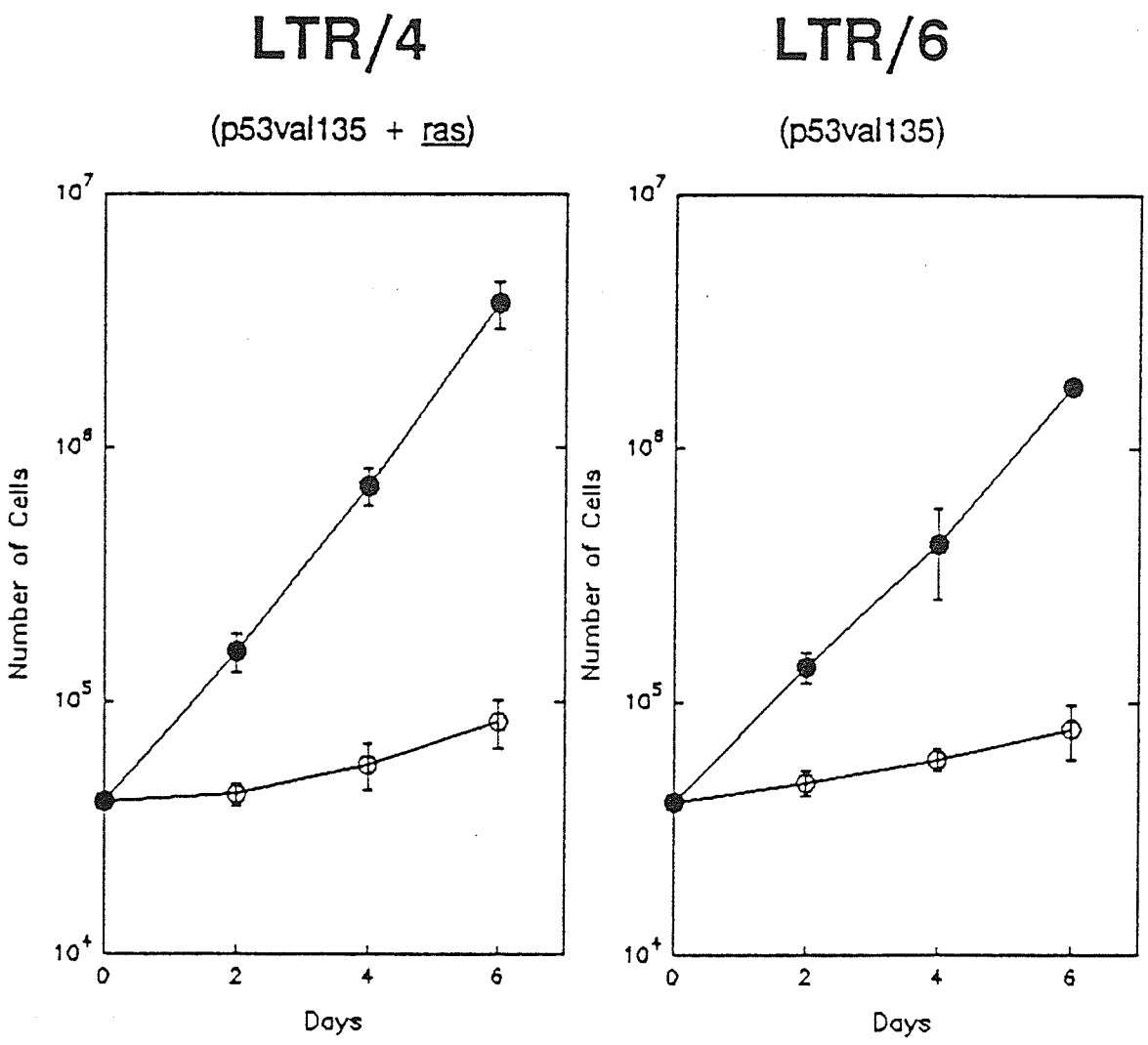

LTR/4
(p53val135 + ras)
LTR/6
(p53val135)
Number of Cells
10^7
10^6
10^5
10^4
0
2
4
6
Days

FIG. 2.

p53 epitope expression. 5×10^5 REF52 (▲), LTR/4 (■), or LTR/6 (●) cells were incubated for 24 h at 37°C prior to being temperature-shifted to 32.5°C; time 0 h. Metabolically labelled cell lysates were immunoprecipitated, run on SDS-PAGE, and the ensuing autoradiographs desitometrically analyzed. Shown here is the relative expression of PAb240 and PAb246 epitopes (panels A and B, respectively) which have been normalized to the PAb421 epitope expression at time points up to 24 h post-temperature-shift. Open symbols indicate the relative epitope expression of nonshifted cultures at 24 h.

Figure 2

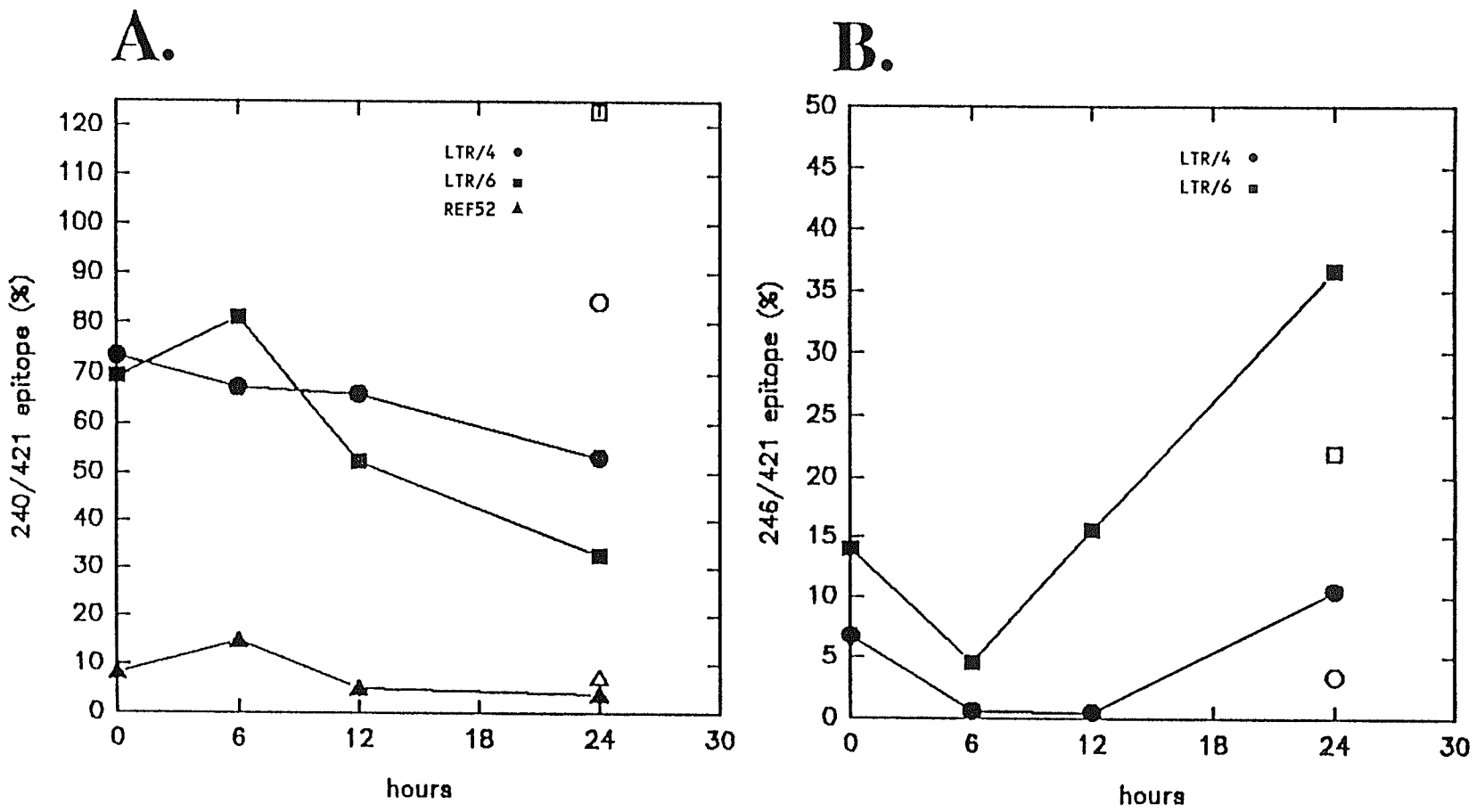
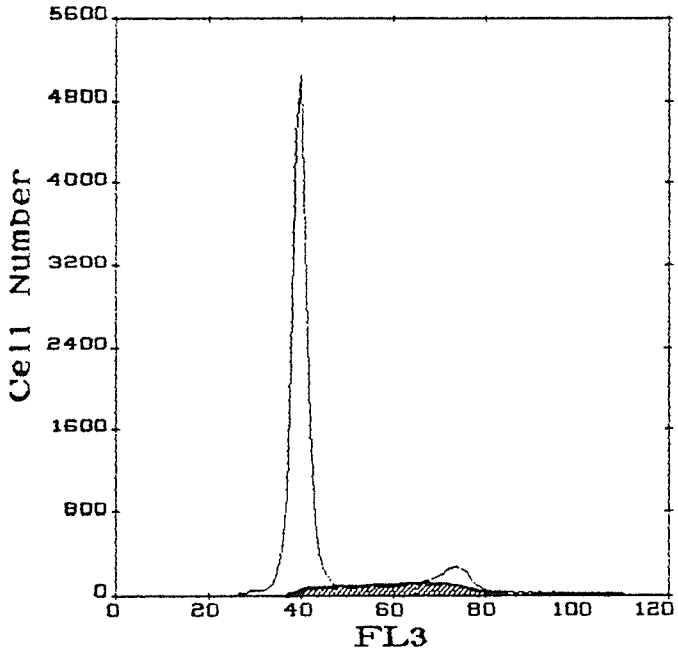

A.
B.
LTR/4
LTR/6
REF52
240/421 epitope (%)
246/421 epitope (%)
hours
hours

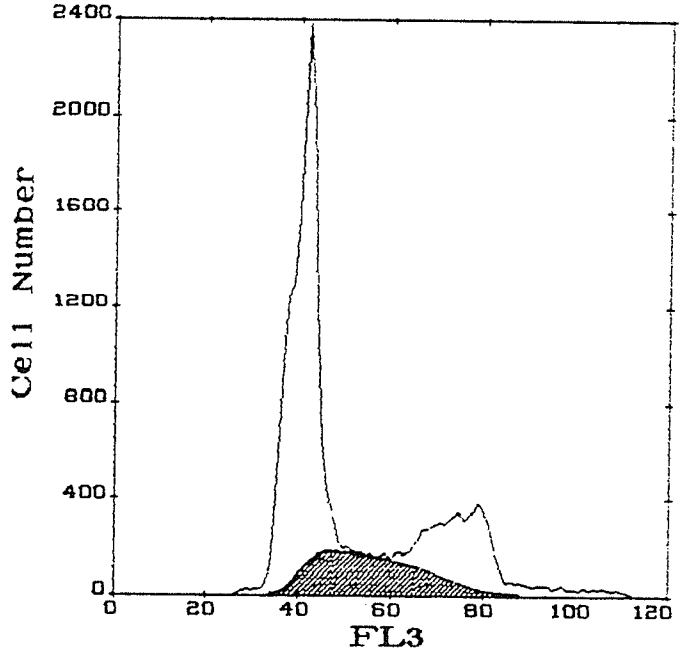
Figure 3.

DNA analysis. Cell lines were incubated at the indicated temperatures and times, fixed, stained with propidium iodide, and analyzed on a Coulter EPICS II for DNA content. DNA fluorescence data were cell cycle analyzed using Multicycle™ soft-ware (Phoenix Flow Systems). Distribution of S-phase is shown as a filled in curve and determined as described in materials and methods. Percentage distribution can be found in Table 1.

Figure 3A

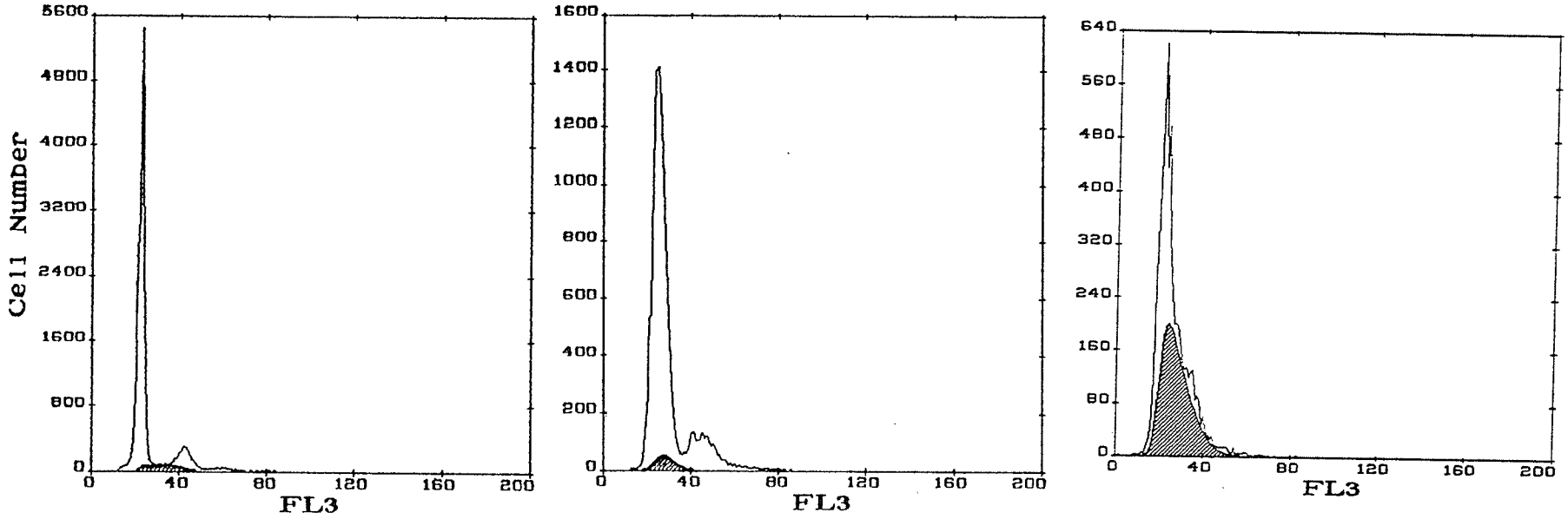


REF52 - 39'C



REF52 - 32.5'C

Figure 3B

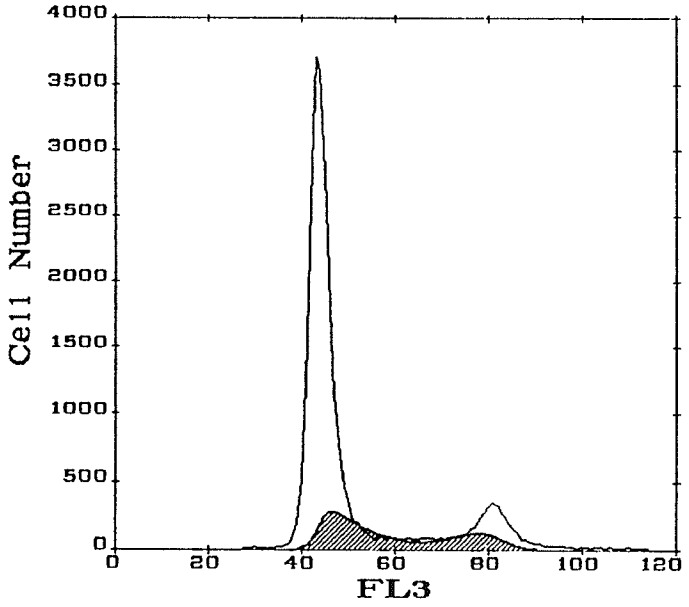


LTR/4 - 39'C

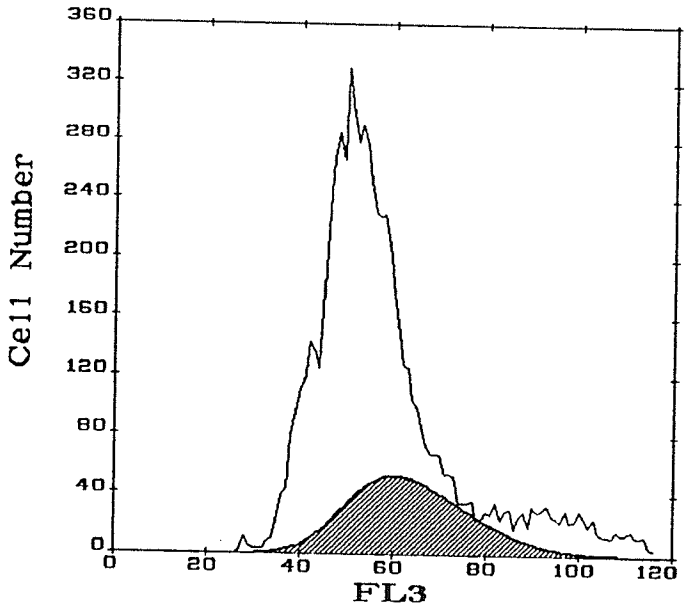
LTR/4A - 32.5'C

LTR/4B - 32.5'C

Figure 3C

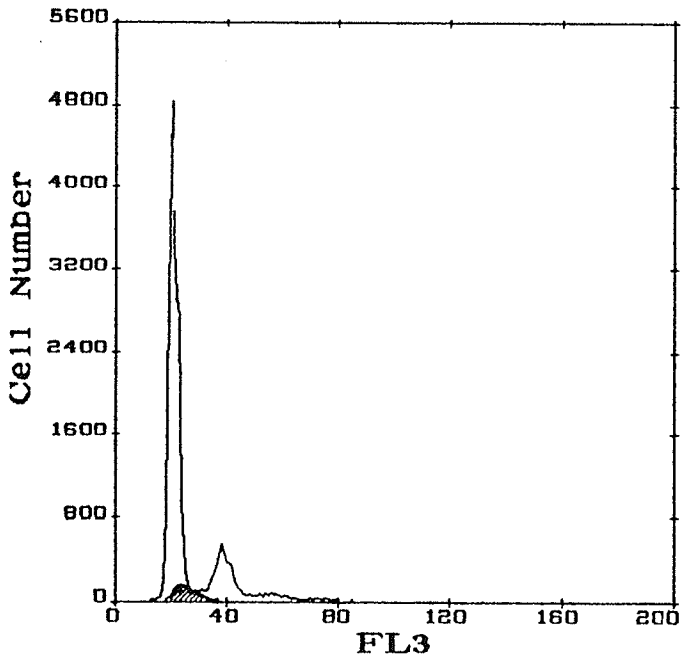


LTR/1 - 39'C

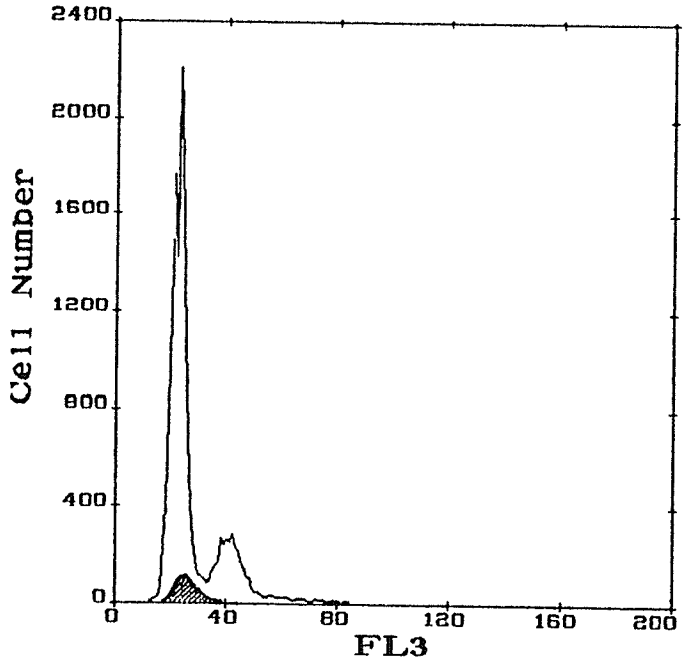


LTR/1B - 32.5'C

Figure 3D

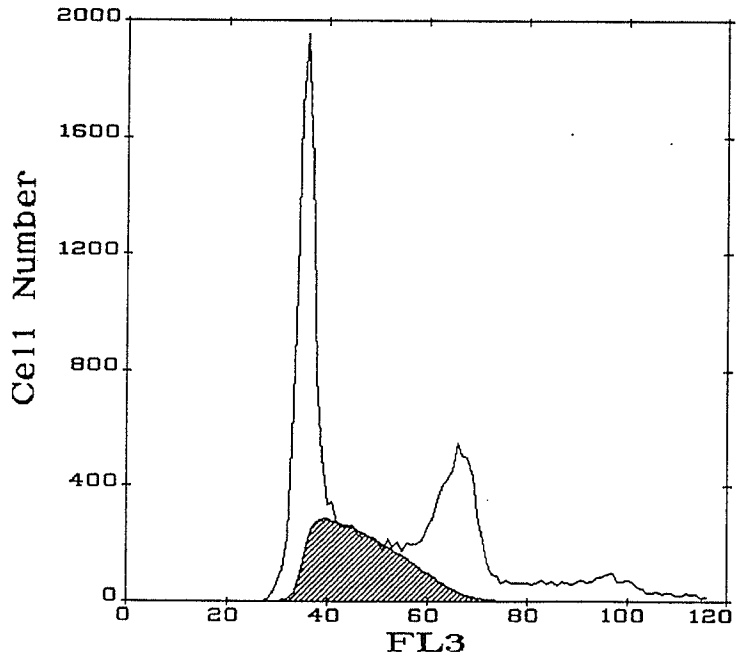


LTR/6 - 39'C

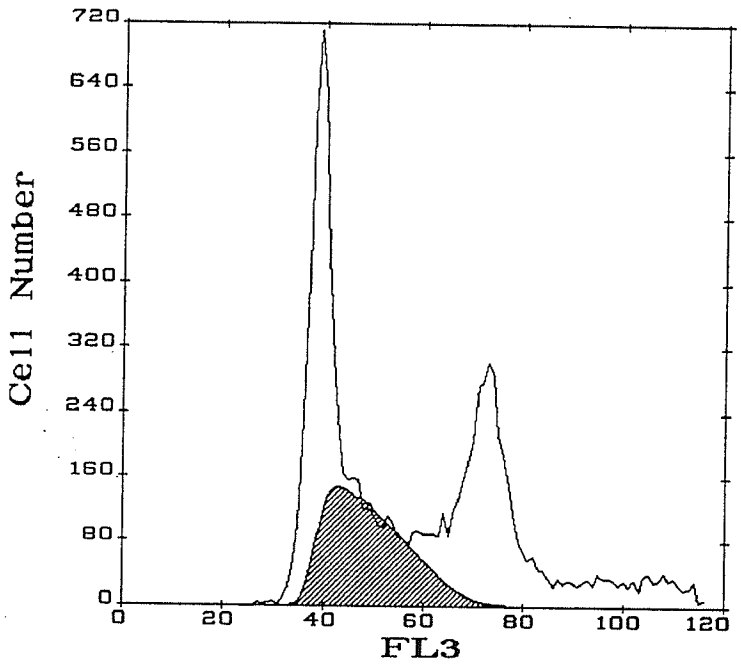


LTR/6 - 32.5'C

Figure 3E

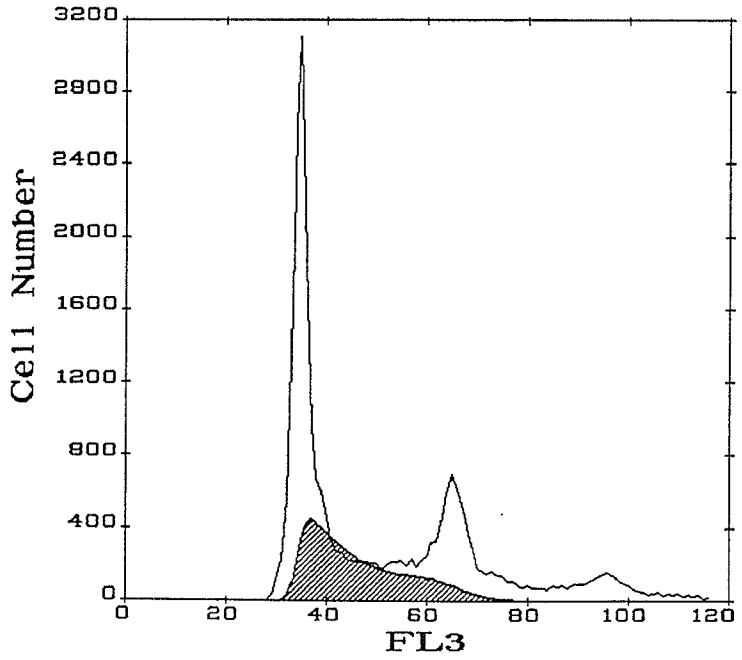


R53/4 - 39'C

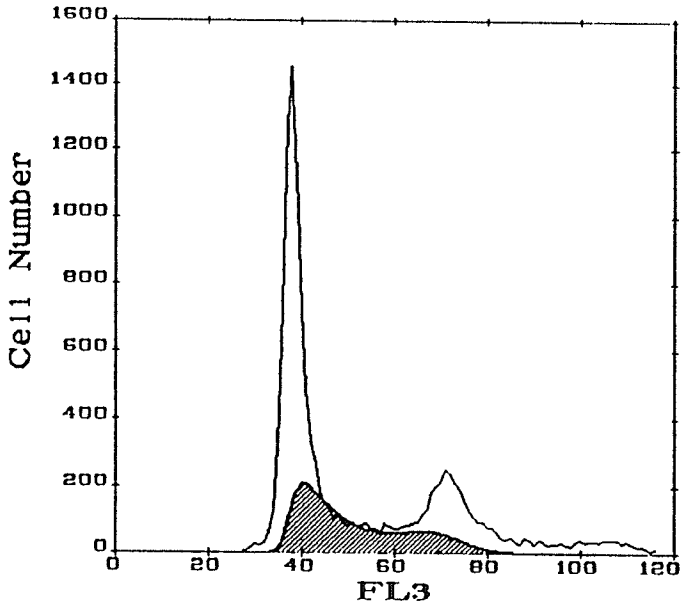


R53/4 - 32.5'C

Figure 3F

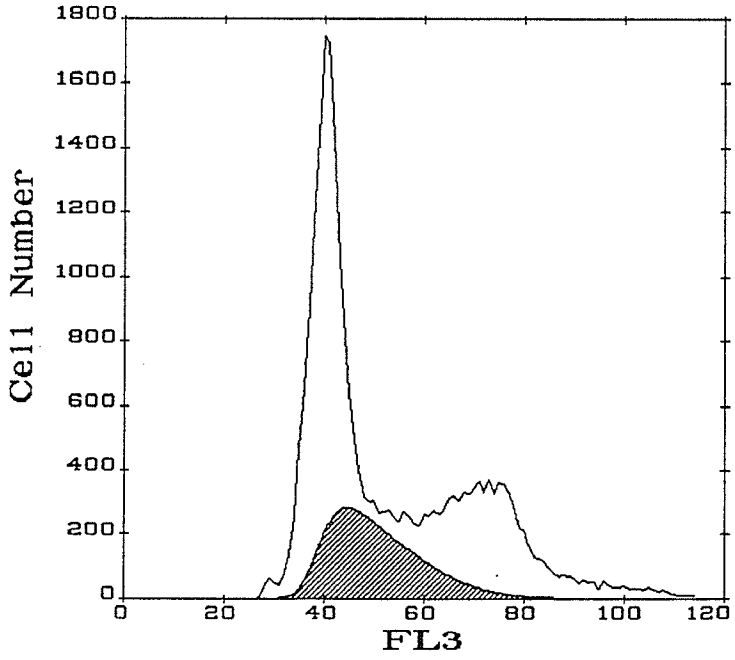


2005/2 - 39'C

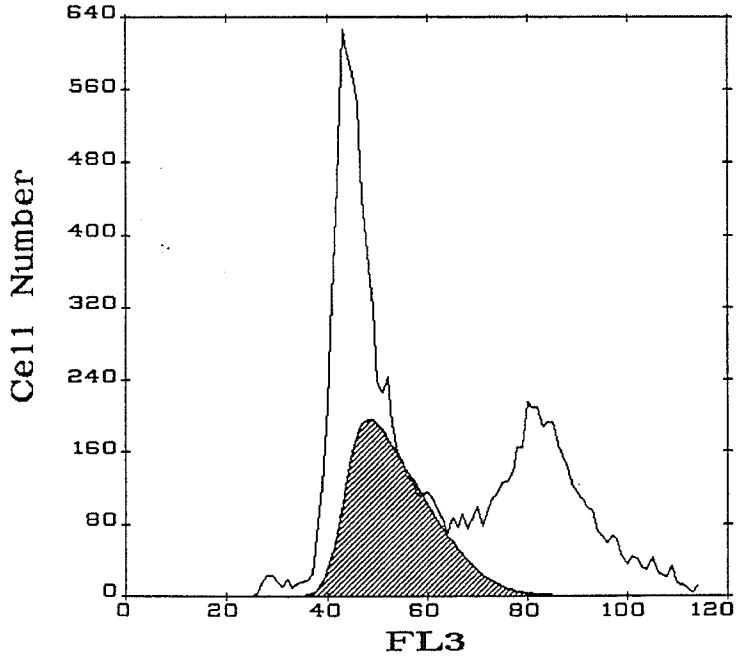


2005/2 - 32.5'C

Figure 3G



TSR-5 - 32.5'C



TSR-5 - 39'C

TABLE 1. Cell cycle analysis[a]

Cell Line	transfected genes	°C[b]	growth arrest[c]	G_1 (*CV*)[d]	S	G_2 (*CV*)[d]
REF52	parental line	39.0	no	81.4% (4.1)	17%	1.3% (4.7)
REF52	parental line	32.5	no	57.6% (7.2)	22.1%	20.3% (8.7)
LTR/4	$p53val^{135}$ + *ras*	39.0	no	62.4% (6.6)	7.7%	10.6% (7.9)
LTR/4A[e]	$p53val^{135}$ + *ras*	32.5	yes	78.7% (11.8)	4.4%	16.9% (16.3)
LTR/4B	$p53val^{135}$ + *ras*	32.5	yes	41.0% (10.9)	54.0%	4.3% (19.7)
LTR/1	$p53val^{135}$ + *ras*	39.0	no	69.6% (4.5)	22.9%	7.5% (4.7)
LTR/1B	$p53val^{135}$ + *ras*	32.5	yes	66.1% (14.8)	22.7%	11.2% (12.9)
LTR/6	$p53val^{135}$	39.0	no	76.6% (8.1)	6.4%	17.0% (9.0)
LTR/6	$p53val^{135}$	32.5	yes	72.3% (11.4)	7.0%	20.7% (10.1)
R53/4	$p53pro^{193}$ + *ras*	39.0	no	40.2% (4.8)	32.4%	27.4% (6.9)
R53/4	$p53pro^{193}$ + *ras*	32.5	no	32.8% (5.3)	31.5%	35.9% (7.8)
Δ2005/2	large T + *ras*	39.0	no	43.1% (4.2)	35.0%	21.9% (8.0)
Δ2005/2	large T + *ras*	32.5	no	47.9% (4.7)	33.6%	18.5% (7.5)
TSR-5	*ts*-large T + *ras*	32.5	no	45.1% (7.9)	24.3%	30.6% (13.9)
TSR-5	*ts*-large T + *ras*	39.0	yes	31.2% (6.6)	32.4%	36.4% (10.5)

[a] EPICS-II™ (Coulter) DNA fluorescence data was analyzed for cell cycle distribution (Multicycle™; Phoenix Flow Systems, La Jolla, CA).
[b] Temperature at which cells were incubated (4 d) prior to DNA analysis.
[c] General indication of growth potential of the cell line at the time of DNA analysis, as determined by growth curves (see text).
[d] *CV* is the relative distribution of fluorescent events around the 2N DNA (G_1) and 4N DNA (G_2) means (coefficient of variation).
[e] Cell line designations, A and B, reflect two varying patterns of DNA analysis consistently observed for the same growth arrested $p53val^{135}$-plus-*ras* transfected REF52 cell lines in different experimental trials.

Summary and Future Directions

SUMMARY AND FUTURE DIRECTIONS

In the course of this thesis work, I have experimentally investigated mechanisms of wild-type p53 inactivation and how this event is involved in the transformation process. A series of three projects characterize the loss of p53 tumor suppressor activity by two major mechanisms: 1) complete loss of the p53 gene by genomic rearrangement (Hicks and Mowat, 1988); and 2) functional loss of wtp53 by a dominant negative mutation (Hicks *et al.*, 1991a & 1991b). Inactivation of p53 by point mutations and allelic loss or rearrangements is now found in every human cancer studied (Hollstein, 1991; Levine *et al.*, 1991). In this summary, I will discuss the contributions of this thesis to our understanding of p53's role in transformation, and future directions of study.

In the first chapter, I have investigated the apparent paradox of significant changes in the p53 expression of Friend virus (*Fv*)-induced erythroleukemic cell lines. 30% of the cell lines isolated did not express any detectable p53 due to gross rearrangements of the p53 gene (Mowat *et al.*. 1985). This was in contrast to 50% of isolated cell lines which greatly overexpressed p53 protein, an activity consistent with a dominant oncogene model of p53. We initially hypothesized that the loss of p53 expression was under an equal selective pressure and contributed just as significantly to the progression of *Fv*-induced erythroleukemia as did overexpression of p53 in other cell lines did. To this end, I molecularly cloned both rearranged alleles of a p53 negative cell line, DP16-9B4, in the hope that an understanding of the mechanism of the gene rearrangement would provide a better understanding to the importance of

this event. We discovered that both p53 alleles of this cell line were rearranged by the integration of the *Fv* helper virus, Friend Murine Leukemia Virus (FMuLV)(Hicks *et al.*, 1988). This was strong evidence linking the inactivation of p53 as an event contributing to the progression of the disease. It was supported by two reports from our Toronto collaborators showing that the *Fv* replication defective virus, Spleen Focus-Forming Virus (SFFV), had integrated into the p53 gene of several other p53 negative cell lines (Ben-David *et al.*, 1988); and secondly, p53 negative lines were not clonally related to the p53 overexpressing lines isolated from the same mouse (Chow *et al.*. 1987). Together, these reports identified the p53 gene as a common target for insertional inactivation during the *in vivo* progression of *Fv*-induced erythroleukemia. By definition, acting as an antioncogene.

At this time it was becoming apparent that the p53 alleles used in the *in vitro* immortalizing, transforming, and tumor enhancing experiments, all had point mutations (Eliyahu *et al.*, 1988; Jenkins and Sturzbecher, 1988; Munroe *et al.*, 1989). Apart from a common oncogenic-like activity, these mutations generally resulted in a common conformational change associated with an increased half-life and the ability to oligomerize with other p53 molecules (Eliyahu *et al.*, 1988; Kraiss *et al.*, 1988; Rovinski and Benchimol, 1988). We then tested a second hypothesis that point mutated p53 genes were acting in a dominant negative fashion to inactivate any existing wtp53 activity. This would be functionally equivalent to a total loss of p53 seen in the genomic rearrangements of *Fv* cell lines. In the second paper presented in this thesis, we assayed three p53 genes for the ability to cotransform REF52 cells with an activated *ras* gene;

- two we had cloned ourselves, a full length genomic wild-type gene and a full length genomic p53pro^{193} gene isolated from a *Fv* cell line, and the third, the classic p53val^{135} cDNA hybrid gene used in all the original cotransformation experiments. REF52 cells provide a unique fibroblast system for study because overexpression of the *ras* gene alone results in growth arrest and lethality in these cells (Franza *et al.*, 1986). Rescue of these cells from *ras*-induced lethality and full transformation could be achieved with the cotransfer of SV40 large T antigen or adenovirus E1A genes (Franza *et al.*, 1986; Hirakawa and Ruley, 1987), less effectively with c-*myc*, the traditional *ras*-cooperating nuclear oncogene (Kohl and Ruley, 1987). In essence, we were not only assaying for the ability of a mutant p53 over a wild-type p53 to cotransform rat embryo fibroblasts with an activated *ras* oncogene, but assaying for such an activity that would be achieved by the release of the cell from its normal negative growth regulation.

Indeed, this is what we observed. The mutant p53 alleles were able to rescue REF52 cells from *ras*-induced growth arrest while the wild-type allele, alone and with *ras*, lacked this activity, invoking a similar growth arrest (Hicks *et al.*, 1991a). This was one of the first experiments which directly linked the overexpression of a mutant p53 with the loss of an assayable tumor suppressor-like activity, namely the negative growth regulation and arrest induced by *ras*. What we didn't expect to find was that although both mutant p53 alleles rescued the REF52 cells, only p53pro^{193} was able to fully transform REF52 cells. The p53pro^{193}-plus-*ras* transformed cells were shown to have much higher levels of T24/EJ *ras* expression than the p53val^{135}-plus-*ras* cell lines, and

monoclonal antibodies specific to p53 detected several p53-associated proteins in p53pro^{193}-plus-*ras* cells which do not precipitate with p53val^{135}-plus-*ras* cell extracts.

The discovery that p53val^{135} is temperature-dependent for its mutant or wild-type conformation (Michalovitz *et al.*, 1990) permitted us to "reintroduce" wtp53 into the p53val^{135}-plus-*ras* cell lines simply by shifting the temperature. In the experiments outlined in the third chapter, we show that the rescue of REF52 cells is temperature-dependent for the expression of mutant p53. A shift to the wild-type conformation temperature results in the growth arrest of these cells primarily at G_1/S, consistent with other reports of the antiproliferative activity of wtp53 (Michalovitz *et al.*, 1990; Mercer *et al.*, 1990a). However, this block isn't as exclusive as other groups may suggest. We can observe cells that have arrested at all other points of the cell cycle to a lesser degree, most notably in early S-phase and at G_2/M (Hicks *et al.*, 1991b). This arrest is different than the arrest of REF52 cells observed primarily at G_2/M when temperature-sensitive (*ts*) SV40 large T antigen-plus-*ras* cells are at the nonpermissive temperature (Hirakawa and Ruley, 1987). One should bear in mind that the *ts*-p53val^{135}-plus-*ras* cells are arrested in a situation of abnormally high wtp53 overexpression, while the *ts*-SV40 large T-plus-*ras* cells are arrested in the presence of much higher levels of an activated *ras*. Nevertheless, the data presented here clearly shows that the release of REF52 cells from *ras*-induced growth arrest is conditionally dependent on the overexpression of a mutant p53. Although it remains unclear how direct the relationship of these two mechanisms of negative growth regulation might be, it is likely that they are in some

way linked by a common regulation of the cell cycle. A model involving the cell cycle regulator, *cdc2* kinase, will be discussed in more detail shortly.

There is an important question raised by these experiments and those reported recently in the literature. Is the loss of wtp53 by a dominant negative mutation truly functionally equivalent to the null phenotype? Evidence supporting the normal wild-type function of p53 as a tumor suppressor gene or inhibitor of transformation has been shown quite convincingly in rodent embryo fibroblast systems (Finlay *et al.*, 1989; Eliyahu *et al.*, 1989; Hinds *et al.*, 1989; Michalovitz, 1990) as well as in tumor cells (Baker *et al.*, 1990; Mercer *et al.*, 1990a; Johnson *et al.*, 1991). This is consistent with a complete loss of p53 expression in the Friend model system and in many human tumor derived cells and cell lines (reviewed in INTRODUCTION and in Hollstein *et al.*, 1991). With the exception of many leukemia and bone neoplasia, however, the majority of human cancer cells are found with a single missense mutation in the p53 gene. In many cases this is accompanied by a loss of heterozygosity or reduction to homozygosity of the mutant encoding allele, again satisfying Knudsons' two-hit hypothesis for antioncogene inactivation (Knudson, 1971). In a number of cases though, the normal p53 allele remains and still expresses a normal wtp53 (Nigro *et al.*, 1989; Davidoff *et al.*, 1991; Hollstein, 1991; Levine *et al.*, 1991). It has been proposed that a single "hit" missense mutation in p53 may be enough to knock-out the remaining allele wtp53 by a dominant negative mechanism (Rovinski and Benchimol, 1988). We have shown, as part of this thesis work, that introduction a single missense mutated p53 allele is sufficient to abrogate the wild-type

activity of both normal endogenous alleles in a dominant negative manner, thereby permitting the aberrant expression of an activated *ras* oncogene (Hicks *et al.*. 1991a, Hicks *et al.*, 1991b). Further, that loss of negative growth regulation in these cells is actively maintained by the expression of the mutant p53, and that negative growth regulation and growth arrest can be initiated in these cells by the expression of exogenous wild-type p53 alleles (Hicks *et al.*, 1991a; Hicks *et al.*, 1991b).

Hence, a strong argument can be made to support p53 inactivation by point mutation as being functionally equivalent to the complete loss of p53. This certainly is true in the *in vitro* fibroblast model systems we and others have studied, but one should keep in mind that most *in vivo* derived tumor cells with a mutant p53 gene expressed are found reduced to homozygosity (Hollstein *et al.*, 1991; Levine *et al.*, 1991). Further, in some cell lines there still remains a strong selective pressure to lose the wtp53 allele, despite the concomitant overexpression of a mutant p53 protein (Finlay *et al.*, 1989; Johnson *et al.*, 1990). The extension of the dominant negative model of p53 inactivation as a "one hit" mechanism in the *in vivo* transformation process should still be carefully considered.

This doesn't rule out the possibility that overexpression of mutant p53 protein may have an intrinsic oncogenic potential of their own apart from their ability to inactivate wtp53 function. Such mutations would function in a dominant positive manner, or be said to have a gain in function (Halevy *et al.*, 1990; Michalovitz *et al.*, 1991). One might consider evidence for this activity the ability of a mutant p53 to enhance the tumorigenicity of a transformed cell which has already lost both p53

alleles or is already free of tumor suppression (Wolf *et al.*, 1984; Eliyahu *et al.*, 1985). Further, not all p53 mutations derived from tumor cells act with the same transforming and wtp53 binding properties ascribed to p53val^{135}. For example p53cys^{270} can enhance oncogene-mediated transformation of fibroblasts, but binds wtp53 weakly, while a p53phe^{132} binds wtp53 quite effectively, but is weakly transforming (Halevy *et al.*, 1990). Indeed, we have observed that p53val^{135} can bind wtp53 and release REF52 cells from negative growth regulation, but is not transforming. Finally, some p53 negative cell lines appear to be refractory to the reintroduction of mutant p53 alleles (Benchimol, personal communication), which is again inconsistent with a function of mutant p53 to simply inactivate wtp53. Although a more direct testing of this hypothesis will undoubtedly be done soon, we should be careful to consider which p53 mutations are exhibiting what activities in the systems we are studying now. This will likely be a crucial point in understanding the differences between cancers which tend to eliminate p53 altogether and those that tend to select for expression of mutated p53, and secondly, the difference between germ line p53 mutations that predispose Li-Fraumeni syndrome patients to many cancers as an initial event (Malkin *et al.*, 1990; Srivastava *et al.*, 1990), and the somatic cell p53 mutations which tend to be a much later, and post transformation event in tumor progression.

Taking this into account, experiments are now underway in our lab which are taking a few new directions. First, we are investigating the hypothesis that p53pro^{193} in its full transformation of REF52 cells may indeed be acting in a dominant positive manner. We suspect that if this were true, it is likely involved with one or more of the p53-

transformation associated proteins, p60 and p34, that we find coprecipitating with p53-specific monoclonal antibodies (Hicks *et al.*, 1991a). We have already ruled out the possibility that p60 may simply another heat shock protein, and are focussing our efforts to determine whether or not these proteins are *cdc2* kinase (p60 cyclin and $p34^{cdc2}$). Our suspicions are not based solely on the apparent molecular weights, but are supported by previous reports of p53's interaction with *cdc2* kinase *in vitro* (Addison *et al.*, 1990; Bishoff *et al.*, 1990; Sturzbecher *et al.*, 1990) and that the antiproliferative effects of wtp53 overexpression (Michalovitz, 1990; Mercer *et al.*, 1990b) strongly suggest that p53 may normally be involved in the cell cycle progression through G_1/S, which is controlled by *cdc2* kinase. Further, our own results suggest that wild-type p53 conformation $p53val^{135}$-plus-*ras* cells appear to arrest in other parts of the cell cycle, most notably at G_2/M (Hicks *et al.*, 1991b). Finally, oncogenic *ras* has been shown to regulate *cdc2* kinase activity in a positive control of the cell cycle (Durkin and Whitfield, 1986 and 1987; Daar *et al.*, 1991). It is possible that a common mechanism which may directly link the induced negative growth regulation of p53 and *ras* is through *cdc2* kinase. Oncogenic forms of *ras* may interfere with the normal upstream regulation of *cdc2* kinase activation, while mutated p53 proteins inappropriately effect the normal downstream signaling by an active *cdc2* kinase.

We have also initiated another project which will more directly test the biological consequences of p53 and *cdc2* kinase interactions using full length genomic p53 genes with and without mutations at the putative *cdc2* kinase serine phosphorylation site. These mutants will be assayed in the

REF52 cell cotransformation assay for their ability to rescue from *ras*-induced growth arrest and transformation. Ensuing cell lines will be characterized for phosphorylation patterns and cellular localization of p53. We hope that these experiments will help elucidate the normal mechanisms of p53's negative growth regulation and identify how loss of these functions or specific interactions with other cellular proteins can contribute to the transformation process.

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Curriculum Vitae

GEOFFREY GORDON HICKS 16/9/91

BORN: August 21, 1961
Saskatoon, Saskatchewan

PRESENT ADDRESS: MIT Center for Cancer Research
Building E17 - 517
Cambridge, MA 02139
(617) 253-0264

REASEARCH AND RELATED EXPERIENCE:

May, 1982 - August, 1982, Canadian Heart Foundation - University of Manitoba, Department of Biochemistry, Summer Research Student.

September, 1982 - April, 1983, University of Manitoba, Department of Microbiology - Research Project Student.

September to April, 1983 - 1991, University of Manitoba, Department of Chemistry - Lab Demonstrator - Intermediate Biochemistry 2.235.

September, 1985 - December, 1988, University of Manitoba, Department of Physiology/ Manitoba Institute of Cell Biology - Masters Student

September to April, 1988 - 1991, University of Manitoba, Department of Chemistry, - Grader/Marker - Intermediate Biochemistry 2.235.

January, 1989 - September, 1991, University of Manitoba, Department of Physiology/ Manitoba Institute of Cell Biology - PhD Student

AWARDS AND SCHOLARSHIPS:

1991 - 1993 National Cancer Institute of Canada Fellowship.

1991 Faculty of Medicine ABI Major Award for Research in Molecular Biology.

1990 - 1991 University of Manitoba Graduate Fellowship

1989 Department of Physiology, University of Manitoba, 1st Place Research Poster Competition.

1987 - 1988 Manitoba Cancer Treatment and Research Foundation Scholarship

1986 - 1987 Manitoba Cancer Treatment and Research Foundation Scholarship

1982 Canadian Heart Foundation Scholarship

PUBLICATIONS:

PAPERS:

G.G. Hicks, and M.R. Mowat. Integration of Friend Murine Leukemia Virus into both Alleles of the p53 Oncogene in an Erythroleukemic Cell Line. J. Virol. 62:4752-4755, 1988.

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