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p53-DEPENDENT APOPTOSIS MODULATES THE CELLULAR RESPONSE TO ONCOGENES AND THE CYTOTOXICITY OF ANTICANCER AGENTS

By Scott William Lowe

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ABSTRACT

The p53 tumor suppressor gene is considered the most frequently mutated gene in human cancer, and p53 mutations have been associated with aggressive cancers, metastasis, and with poor prognosis. However, the molecular basis for the association between p53 mutation, advanced tumor stage, and poor prognosis remains unknown. In order to investigate the mechanisms whereby p53 suppresses neoplastic growth, transforming interactions between endogenous p53 and both viral and cellular oncogenes were analyzed in primary and established fibroblasts. In particular, p53-deficient mouse embryonic fibroblasts were used to systematically examine the role of p53 in regulating cellular responses to transfected oncogenes.

These studies establish a direct mechanism of tumor suppression by p53 involving the selective destruction of oncogene-expressing cells by apoptosis. Absence of p53 resulted in failure to execute the apoptotic program and allowed transformation of primary cells by a single oncogene. These studies demonstrate that (i) cellular p53 levels and stability increased upon introduction of the adenovirus early region 1A (E1A) oncogene into fibroblasts, (ii) p53 was required for E1A-associated apoptosis, and (iii) p53 suppressed transformation by E1A. While tumorigenicity was significantly enhanced by genetic changes that promoted cell survival, cells co-expressing E1A and *ras* oncogenes were highly tumorigenic but remained sensitive to apoptosis. Therefore, tumorigenic phenotypes apparently arise by various routes which alter the balance of growth, differentiation, and survival in different ways.

The involvement of p53 in eliminating oncogene-expressing cells accounts for the observation that p53 loss typically occurs late in tumor progression, and may be a factor in limiting metastatic spread or influencing the outcome of cancer therapy. In this regard, oncogene-associated apoptosis was triggered by treatment with ionizing radiation and several chemotherapeutic drugs, and p53 was required for efficient execution of the death program. Moreover, tumors derived from cells expressing endogenous

p53 typically regressed following treatment of animals with ionizing radiation, whereas tumors derived from p53-deficient cells were resistant and continued to grow. These data provide a molecular explanation for the association between p53 mutation and poor prognosis, and suggest a novel mechanism whereby tumor cells can acquire cross-resistance to anticancer agents.

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DEDICATION

To James L. Lowe, my father and best friend.

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CHAPTER 1

Introduction and Overview

Since the identification of p53 in 1979, a considerable amount of effort has been devoted to understanding its activity in both normal and neoplastic cells. p53 is unique among molecules involved in carcinogenesis, since both activating and inactivating mutations appear to promote oncogenic transformation. Thus, for many years, p53 was thought to be a dominant-acting oncogene. Only after nearly a decade of investigation did evidence emerge indicating that p53 was in fact a tumor suppressor gene. Presently, p53 is considered to be the most frequently mutated gene in human cancer, and intensified efforts have lead to a dramatic increase in our understanding of its function. Nevertheless, many questions remain. What biological processes are affected by p53 that limit neoplastic growth? How are these processes affected by p53 mutation during tumor progression?

Sections I-III of this discussion summarize what is presently known about p53, with particular emphasis on properties relevant to its activity as a tumor suppressor. Since data presented in subsequent chapters implies that p53 participates in the process of apoptosis, Section IV discusses features of apoptosis relevant to carcinogenesis. Finally, Section V discusses the experimental rationale and preliminary data that preceded the experiments presented in Chapters 2-5 and Appendix 1.

SECTION I: Historical perspective of p53: oncogene vs. tumor suppressor gene

Discovery of p53

p53 was first identified as a cellular protein bound to simian virus 40 (SV40) large T antigen and later, to an adenovirus early region 1B (E1B)-encoded protein (Lane and Crawford, 1979; Linzer and Levine, 1979). A related protein was also detected in SV40-free embryonic carcinoma cell lines

using antisera directed against SV40-infected cells (Linzer and Levine, 1979). Antisera to Balb/c Meth A cells (a chemically transformed mouse line) detected an antigen of 53 kD in several chemically and spontaneously transformed mouse lines with no known viral etiology (De Leo et al., 1979). The fact that viral oncogenes targeted a cellular protein whose expression was altered in other transformed cells suggested that p53 influenced oncogenic transformation. Increased p53 expression was observed in several human tumor lines, raising the possibility that p53 contributed to human cancer (Crawford et al., 1981).

Several observations associated p53 with positive growth control, implying that p53 accumulation could promote oncogenic transformation. First, p53 was expressed at relatively low levels in untransformed cell lines (e.g. 3T3; (Linzer and Levine, 1979; Oren et al., 1981)). Second, p53 expression in nontransformed cells appeared to be under cell cycle control (Milner and Milner, 1981; Reich and Levine, 1984). Thus, proliferating cells expressed higher p53 levels than quiescent cells (Milner and Milner, 1981), and mitogenic stimulation of quiescent lymphocytes or 3T3 cells enhanced p53 transcription prior to S phase entry (Milner and Milner, 1981; Reich and Levine, 1984). By contrast, the much higher levels of p53 observed in both SV40 and spontaneously transformed cells resulted from increased p53 protein stability (Oren et al., 1981; Reich et al., 1983; Gronostajski et al., 1984). Third, microinjection of p53-specific antibodies into quiescent 3T3 cells blocked cell cycle progression following serum stimulation. This observation implied that p53 activity was required for the transition from the resting to proliferating state ((Mercer et al., 1982; Mercer et al., 1984), reviewed in (Oren, 1985)).

p53 functions as a typical nuclear oncogene

Several similarities were noted between p53 and the c-*myc* oncogene. For example, both c-*myc* and p53 mRNA levels increased following mitogen stimulation of quiescent cells (Reich and Levine, 1984), and both were DNA-binding proteins (Luka et al., 1980; Lane and Gannon, 1983). Thus, p53 was characterized as a nuclear oncogene. The identification and cloning of several p53 cDNAs and genomic clones allowed researchers to directly test this hypothesis (reviewed in (Oren, 1985)). These studies demonstrated that p53, like *myc*, cooperated with activated *ras* oncogenes to transform primary rat embryonic fibroblasts (Eliyahu et al., 1984; Parada et al., 1984) and promoted the establishment of primary cells into immortalized lines (Jenkins et al., 1984). Furthermore, overexpression of p53 in the established RAT1 line made these cells highly tumorigenic, suggesting that the transforming activity of p53 was distinct from its immortalizing activity (Eliyahu et al., 1985).

The p53 gene is rearranged in several tumor lines

Since p53 accumulation was thought to promote oncogenic transformation, it was somewhat surprising that several tumor lines lacked p53 expression altogether. For instance, HeLa cells expressed undetectable levels of p53 protein, although the transcript was expressed at normal levels (Crawford et al., 1981; Matlashewski et al., 1986). In HL-60 cells, a human promyelocytic leukemia line, the p53 gene contained large deletions such that neither normal nor aberrant forms of p53 were expressed. However, HL-60 cells overexpressed c-*myc*, so it was assumed that the deficiency in p53 function was overcome by an alternative, related pathway (Wolf and Rotter, 1985).

In cells transformed either by Abelson virus or by Friend leukemia

virus, the p53 gene was frequently inactivated by virus insertion (Wolf et al., 1984a; Mowat et al., 1985; Oren, 1985). It was surprising that such a high frequency of independent tumors would contain an inactivated oncogene. Nevertheless, the tumorigenicity of p53-deficient lines was considerably lower than lines expressing p53 (Wolf et al., 1984a; Mowat et al., 1985), and re-introduction of p53 into a weakly tumorigenic Abelson virus-transformed line dramatically increased its ability to form lethal tumors (Wolf et al., 1984b). Thus, while it appeared that p53 inactivation conferred a selective advantage during neoplastic progression of these leukemias, the enhanced tumorigenicity of p53-expressing clones was consistent with the proposed action of p53 as a nuclear oncogene.

p53 genes are oncogenically activated by mutation

The gene transfer studies described above indicated that p53 overexpression was sufficient for cellular immortalization and *ras* co-transformation. Direct evidence supporting this view emerged from a study demonstrating that the immortalizing activity of p53 was conditional on the promotor/enhancer construct used to express the cDNA, such that weaker promoters were ineffective (Jenkins et al., 1985). However, certain mutations within the p53 coding sequence enhanced the immortalizing activity of the previously defective constructs. Each of these mutants encoded more stable proteins, implying that p53 accumulation and immortalization could occur by activating mutations within the p53 gene.

Not all p53 clones had transforming and immortalizing activities. For example, a p53 cDNA cloned from F9 cells (Pennica et al., 1984) failed to transform primary REFs in cooperation with *ras* oncogenes (Hinds et al., 1987; Finlay et al., 1988). Although this clone may have been a mutant, subsequent mutagenesis produced clones that cooperated with *ras* in transformation

assays (Finlay et al., 1988). The p53 proteins encoded by these novel mutants had extended half-lives and physically associated with hsc70, both characteristics of p53s encoded by other cDNAs. Since experimentally-induced mutations in the F9 clone were oncogenic, it was suggested that the F9 clone was actually "wild-type" p53. Presumably, the other clones had sustained activating mutations.

Studies comparing the F9 clone to an oncogenic clone derived from a genomic library provided definitive evidence that transforming clones were mutants (Hinds et al., 1989). The different biological activities of these clones were linked to a single nucleotide change at position 135; the F9 clone encoded an alanine while the genomic clone specified a valine. All other sequenced p53 genes encoded alanine at this position, and a restriction fragment length polymorphism indicated that the valine codon did not exist in mouse genomic DNA. This implied that wild-type p53 contained alanine at position 135 and was incapable of cooperating with *ras* in transformation. The fact that p53 clones containing such a diverse spectrum of point-mutations were active in transformation assays raised the possibility that these mutations abrogated normal p53 function (Eliyahu et al., 1988; Hinds et al., 1989).

Wild-type p53 suppresses oncogenic transformation

Nearly a decade after its discovery, the involvement of p53 in oncogenic transformation was re-evaluated. Unlike *myc* and other nuclear oncogenes, overexpression of the proto-oncogene was not sufficient for p53's transforming activity. Rather, mutation within the coding sequence was required for both p53 accumulation and oncogenic transformation. Furthermore, it was suggested that p53 was not an oncogene at all, but rather a tumor suppressor gene (Ben David et al., 1988; Hinds et al., 1989).

This suggestion was confirmed by experiments demonstrating that wild-type p53 suppressed oncogene-mediated focus formation in primary embryonic fibroblasts, including foci induced by *ras* and mutant p53, *ras* and *myc*, *ras* and E1A, and E1A and E1B (Finlay et al., 1989; Eliyahu et al., 1989). Of the foci obtained from transfections including wild-type p53, most did not express detectable levels of exogenous p53, and those that did had sustained mutations within the p53 gene (Finlay et al., 1989). The fact that overexpression of wild-type p53 inhibited focus formation by *ras* and mutant p53 alleles suggested that these transforming mutants inhibited wild-type p53 in a dominant-negative manner (Finlay et al., 1989; Eliyahu et al., 1989).

p53 suppresses neoplastic growth *in vivo*

The pattern of p53 mutation in human cancer was consistent with the proposed tumor suppressor activity of p53. Alterations in the p53 gene occurred frequently in colon carcinoma (Baker et al., 1989). The pattern of p53 mutation in these tumors was characteristic of a tumor suppressor gene: most tumors had acquired point mutations in one p53 allele in conjunction with loss of the other allele (Fearon and Vogelstein, 1990; Vogelstein, 1990). Indeed, re-introduction of wild-type p53 into several colon carcinoma lines dramatically reduced their growth potential, and no lines expressing wild-type p53 were obtained ((Baker et al., 1990). Moreover, mutant p53 alleles derived from these tumors were oncogenic in *ras* co-transformation assays, suggesting that these missense mutations conferred a selective advantage during tumor progression (Hinds et al., 1990). p53 appeared to suppress neoplastic growth in many tissues, since similar patterns of p53 mutation and allelic loss were subsequently observed in many other tumor types (Nigro et al., 1989; Takahashi et al., 1989).

Germ line mutations in p53 were shown to contribute to Li-Fraumeni syndrome, a familial cancer syndrome. While many familial cancer syndromes effect a specific tissue, Li-Fraumeni patients develop diverse mesenchymal and epithelial tumors. Thus, p53 appears to affect processes common to many cell types. In all families analyzed, tumor-susceptibility co-segregated with the inheritance of a mutant p53 allele (Malkin et al., 1990; Srivastava et al., 1990; Vogelstein, 1990), and tumors from these patients displayed loss of the remaining wild-type allele (Malkin et al., 1990). These observations strongly suggested that p53 functioned as a tumor suppressor in human cancer.

Gene disruption studies in mice confirmed that p53 was indeed a tumor suppressor gene (Donehower et al., 1992). Mice homozygous for the disrupted p53 allele developed normally, but were predisposed to lethal tumors. Therefore, in the context of the whole animal, the primary function of p53 was to suppress neoplastic growth.

Conclusions

Present evidence strongly supports the view that p53 functions as a tumor suppressor gene, and many of the original contradictions regarding p53 function are now readily explained. For example, the fact that the original p53 clones harbored mutations accounts for the observation that overexpression of these genes is oncogenic. These mutant p53 alleles--at least when sufficiently overexpressed--apparently inhibit wild-type p53 function. Accordingly, the observation that these alleles encode more stable proteins might simply reflect selection for forms that accumulate sufficiently to interfere with wild-type p53. However, the inhibitory effects of p53 mutants are probably incomplete, since many tumors also lose the remaining allele.

Nevertheless, several observations suggest that p53 is an atypical tumor suppressor gene. For example, re-introduction of mutant alleles into p53-deficient erythroleukemias enhances tumorigenicity (Wolf et al., 1985; Shaulsky et al., 1990). A similar result is obtained using p53-deficient fibroblast lines, implying that some p53 activities result from gain of function mutations (Dittmer et al., 1993). Moreover, the involvement of p53 in suppressing transformation does not readily explain why microinjection of p53-specific antibodies (Mercer et al., 1982; Mercer et al., 1984) or p53 antisense RNA (Shaulsky et al., 1990) inhibits proliferation. It should be noted, however, that p53-deficient primary mouse fibroblasts grow readily in culture (Donehower et al., 1992; Livingstone et al., 1992). Therefore, p53 function is not required for normal cell cycle progression.

SECTION II: p53 genes and proteins

Following the characterization of p53 as a tumor suppressor gene, an overwhelming volume of information has emerged concerning the role of p53 in normal and transformed cells. These efforts have increased our understanding of p53 function and have provided insight into both cancer etiology and tumor progression (see, for example (Hsu et al., 1991; Sidransky et al., 1992)). Areas of current investigation include: characterization of the spectrum and frequency of p53 mutations in human cancer (reviewed in (Harris and Hollstein, 1993), identification of p53 targets and regulators (Vogelstein and Kinzler, 1992; Pietenpol and Vogelstein, 1993), exploration of the mechanisms whereby viral oncogenes promote oncogenic transformation (reviewed in (Levine, 1990; Levine et al., 1991)), and understanding of the selective pressure for p53 mutation during tumor progression (Lane, 1992; Oren, 1992). The following section contains an overview of this information,

with particular reference to issues addressed in this thesis.

p53 and human cancer

p53 mutations contribute to neoplasia in many tissues. p53 mutations have been observed in most tumor types examined, including malignant melanoma (Stretch et al., 1991), soft tissue sarcomas (Toguchida et al., 1992b; Toguchida et al., 1992a), and cancers of the lung (Takahashi et al., 1989; Chiba et al., 1990; D'Amico et al., 1992; Takahashi et al., 1991), colon (Baker et al., 1989; Nigro et al., 1989; Shaw et al., 1991), bladder (Sidransky et al., 1991), prostate (Isaacs et al., 1991), breast (Nigro et al., 1989; Bartek et al., 1990; Varley et al., 1991), cervix (Crook and Vousden, 1992; Crook et al., 1992). liver (Hsu et al., 1991; Bressac et al., 1991), skin (Brash, et al., 1991), and certain lymphomas and leukemias (Gaidano et al., 1991). Taken together, these studies support the view that p53 is the most frequently mutated gene in human cancer. Although the spectrum and frequency of p53 mutations occurring in human malignancy implies that p53 affects processes common to many cell types, p53 is not required for normal growth and development (Donehower et al., 1992).

p53 structure and hot spots for mutation. The cloning and characterization of p53 genes from a variety of species (including human, monkey, mouse, rat, frog, and fish) has identified several conserved regions within p53 genes and proteins. Computer-assisted analysis of p53 amino acid sequences has not identified any conserved functional motifs that would shed light on its function (i.e. kinase domains, helix-loop-helix, etc.). Five homologous regions exist in all p53 sequences (Soussi et al., 1990). In human p53 (total of 393 amino acids), these domains correspond to amino acids 13-19 (I), 117-142 (II), 171-181 (III), 234-258 (IV), and 270-286 (V). Domains II-V are hydrophobic and are thought to form the core of the protein. In addition, all p53 genes encode molecules with an acidic amino terminus and a basic

carboxy-terminus (Oren, 1985). These regions may have functional significance with regard to the transcriptional activities of p53.

One striking feature of p53 in carcinogenesis is the diversity of mutations observed in tumors. While activating mutations in oncogenes (e.g. *ras*) generally occur at only a few positions throughout the molecule, p53 mutations have been observed in over 40 codons (Levine et al., 1991). This observation is consistent with the view that p53 mutations produce defective (rather than activated) proteins, but remains surprising given the apparent requirement for these mutants to suppress wild-type p53 function. Nevertheless, p53 mutations in human tumors almost uniformly occur within conserved regions II to V (hydrophobic core), consistent with the surmised importance of conserved domains for protein function ((Nigro et al., 1989), reviewed in (Levine et al., 1991)). Moreover, many mutant alleles encode p53s with characteristic antigenic properties, indicating that these proteins have similar conformational alterations (Finlay et al., 1988; Gannon et al., 1990). However, mutant p53 alleles can have distinct biological and biochemical properties (Hinds et al., 1990; Halvey et al., 1990; Hicks et al., 1991), reviewed in (Levine et al., 1991)).

While p53 mutations can occur at many places throughout the molecule, different cancers often display preferential patterns of mutation, including specific codons and/or nucleotide substitutions (reviewed in (Harris and Hollstein, 1993)). These mutational "hotspots" have implicated specific carcinogens in the etiology of a certain cancers. For example, p53 mutations in skin cancer show marked preference for codons containing pyrimidine dimers, consistent with mutagenic role of ultraviolet light (Brash, et al., 1991). Alternatively, p53 mutations in Li-Fraumeni syndrome occur

between codons 245-258, although the biological implications of this pattern are unknown (Vogelstein, 1990).

p53 mutations enhance tumor progression. Of particular interest is the involvement of p53 in colorectal cancer. The availability of tissue from all stages of this cancer (benign to malignant) has facilitated the identification of the genetic events that contribute to both the initiation and progression of this tumor (Fearon and Vogelstein, 1990). Malignant carcinomas frequently (>75%) show allelic loss on chromosome 17p, encompassing the p53 locus (Baker et al., 1989; Nigro et al., 1989; Shaw et al., 1991). Allelic loss often occurs in conjunction with point-mutations in the remaining p53 allele. By contrast, benign adenomas rarely show p53 loss or mutation (Vogelstein et al., 1988; Shaw et al., 1991). Since malignant carcinomas apparently arise from benign adenomas, p53 mutation is a late event in colon carcinogenesis that occurs during progression from benign to malignant stages (Fearon and Vogelstein, 1990). Point-mutations in p53 may provide a selective advantage during tumor progression, allowing an expanded population of cells and increasing the probability of subsequent allelic loss.

p53 mutations occur late in the progression of other tumor types, including melanoma (Stretch et al., 1991), brain cancer (Sidransky et al., 1992), gastric cancer (Yamada et al., 1991), prostate cancer (Bookstein et al., 1993), ovarian cancer (Mazars et al., 1991), multiple myeloma (Neri et al., 1993), and acute lymphoblastic leukemia (Yeargin et al., 1993). In some instances, p53 mutation has been observed in a metastasis specimens but not the primary tumor (Yamada et al., 1991). The specificity of p53 mutations for late stage tumors indicates that p53 loss is required for tumor progression rather than initiation. Consistent with this hypothesis, studies using p53-deficient mice indicate that endogenous p53 does not reduce the incidence of chemically-

induced skin polyps, but limits their progression to more malignant forms. Thus, polyps in p53-deficient mice were 10 times more likely to progress to malignant carcinomas (Kemp et al., 1993). p53 mutations are apparently poor tumor initiators in human cancer, since Li-Fraumeni patients develop surprisingly few tumors (averaging <2) with a typical onset of over thirty years (Vogelstein, 1990). This is in contrast to other familial cancer syndromes, in which patients generally develop many more tumors at a much younger age.

What could be the selective pressure for p53 mutation late in tumor progression? One possibility suggests that p53 becomes rate-limiting for tumor progression by changes that occur earlier in oncogenesis. For colon cancer, it has been suggested that:

> "Genetic alterations that occur during the progression of colorectal tumors may increase the sensitivity of cells to p53 inhibition, making wild-type p53 expression a key, rate-limiting factor for further growth and expansion. At this point, and not before, mutations in the p53 gene would confer a selective growth advantage to cells *in vivo*, which would explain the frequent occurrence of p53 mutations and allelic loss only in the more advanced stages of colorectal tumorigenesis." (Baker et al., 1990).

Alternative routes to p53 inactivation. Factors that inhibit p53 activity could mimic p53 mutation, and several contribute to human cancer. A remarkable illustration of alternative routes to p53 inactivation occurs in cervical carcinoma. Approximately 70% of these cancers contain sequences from human papilloma virus types 16 and 18 (Howley, 1991). The E6 protein encoded by these viruses promotes the degradation of p53 by a ubiquitin-dependent pathway (Werness et al., 1990; Scheffner et al., 1990). HPV-positive cells therefore express extremely low levels of p53 protein (e.g. HeLa cells; see above). p53 genes are uniformly wild-type in these cancers, apparently

because there is no selective pressure for p53 mutation. By contrast, all HPV-negative cancers harbor p53 mutations (Crook et al., 1991; Crook et al., 1992), implying loss of p53 function is essential for cervical carcinoma progression.

The cellular oncogene *mdm*-2 may also contribute to carcinogenesis by inhibiting p53 functions. *mdm*-2 is amplified in approximately one third of human sarcomas (Oliner et al., 1992). p53 mutation and *mdm*-2 amplification appear functionally redundant, since p53 mutations are not observed in sarcomas containing amplified *mdm*-2. Like HPV E6, MDM-2 physically associates with p53 (Momand et al., 1992). Although MDM-2 does not induce p53 degradation, it blocks several activities normally attributed to wild-type p53 (Momand et al., 1992; Finlay, 1993).

It seems probable that other trans-acting mechanisms will be identified that inhibit p53 activity. For example, several observations presented in this thesis suggest that, under appropriate circumstances, activation of the *bcl*-2 oncogene could mimic p53 mutation (see Chapters 2 and 3). Thus, while p53 mutation contributes to many forms of cancer, the incidence at which p53 function is lost may be significantly higher.

p53 interactions with viral oncoproteins

DNA tumor viruses and carcinogenesis. Much of what is known about p53 has emerged from studies of the small DNA tumor viruses. Several of these viruses can initiate tumors in animals, including certain adenoviruses, papilloma viruses, and SV40. In order to divert cellular enzymes for their own replication, viruses must foster a cellular environment permissive for DNA synthesis. Viral oncoproteins achieve this by mimicking signals that promote cell growth, or by inactivating factors that either limit proliferation or contribute to the differentiated state. Viral DNA occasionally integrates into host genome. If expressed in the absence of productive infection (i.e.

generation of more virions and cell lysis), viral oncoproteins can promote transformation. Since cellular activities altered during viral transformation may also contribute to spontaneous tumorigenesis, viral oncoproteins can uncover relevant processes in tumor initiation and progression.

Transforming interactions between viral oncogenes also provide models of multistep carcinogenesis. For example, adenovirus transformation requires the early region 1A (E1A) and 1B (E1B) genes (reviewed in(Flint, 1984; Branton et al., 1985)). The effects of these proteins on cell growth and transformation are not additive, but rather synergistic. Adenovirus E1A stimulates cell growth, but sustained proliferation requires co-expression of the E1B-encoded proteins (Rao et al., 1992). The E1B proteins do not increase the efficiency with which E1A initiates proliferation. Furthermore, in the absence of E1A, E1B has no detectable effect on cell growth (van den Elsen et al., 1983).

The transforming activities of several viral oncoproteins require physical interactions with cellular factors normally involved in growth control. The fact that unrelated viruses target the same cellular factors suggest that these molecules control rate-limiting processes in oncogenic transformation. The significance of these interactions is illustrated by the physical association between several viral oncoproteins (E1A, large T antigen, and HPV E7) and the product of the retinoblastoma tumor suppressor gene (Rb) (Whyte et al., 1988; DeCaprio et al., 1988; Dyson et al., 1989). In normal cells, Rb appears to negatively regulate cell cycle progression. By binding to Rb, viral oncogenes release the cell from negative growth controls and facilitate transformation. Consistent with this model, the Rb binding-domain is required for the transforming activity of E1A (reviewed in (Ruley, 1990)).

Viral oncoproteins target p53. Interactions between viral early region proteins and p53 also contribute to oncogenic transformation by human adenoviruses, SV40 and HPV (reviewed in (Levine, 1990; Levine et al., 1991)). Given the role of p53 as a tumor suppressor, the viral tumor antigens are thought to interfere with p53 functions that preclude transformation. The papilloma virus types 16 and 18 E6 protein forms a physical complex with p53 and promotes its proteolytic degradation by a ubiquitin-dependent pathway (Werness et al., 1990; Scheffner et al., 1990; Scheffner et al., 1993). Consequently, the interaction between p53 and the viral oncoprotein inactivates p53 function. This appears necessary for viral transformation, since non-oncogenic papilloma viruses (e.g. HPV-11) encode E6 proteins incapable of associating with p53 (Werness et al., 1990) or promoting p53 degradation (Scheffner et al., 1990). The evidence that large T antigen and $p55^{E1B}$ inactivate p53 is less convincing, however, because p53 levels and protein stability are increased in SV40 and adenovirus-transformed cells. Nevertheless, both large T antigen and $p55^{E1B}$ inhibit p53-mediated transcriptional activation (Farmer et al., 1992; Yew and Berk, 1992; Segawa et al., 1993), a property normally associated with wild-type p53 (see below).

Since oncogenic transformation frequently involves the loss of p53 function, it is surprising that p53 levels and stability are greatly increased in adenovirus and SV40 transformed cells. The stabilization of p53 associated with viral tumor antigens may simply be coincidental--perhaps p53 becomes inaccessible to the proteolytic enzymes. This view is supported by the observation that Ad-5 E1B sequesters p53 in a dense perinuclear structure (Zantema et al., 1985). However, the stability of nuclear p53 also increases in adenovirus type 12-transformed cells in the absence of stable interactions with Ad12 $p55^{E1B}$ (Zantema et al., 1985). Moreover, free and T antigen-associated

p53 have similar half-lives in SV40-transformed cells, implying that sequestration by large T antigen does not stabilize p53 (Deppert and Haug, 1986; Deppert and Steinmayer, 1989).

Although the significance of p53 stabilization has been obscure, the phenomenon may have implications with regard to p53 function or regulation. For example, since p53 may have both positive (as well as negative) effects on cell growth (Mercer et al., 1982; Shohat et al., 1987), tumor antigens could promote cell transformation by increasing p53 levels (van den Heuvel et al., 1990). Alternatively, p53 stabilization may reflect a cellular process that resists viral infection and/or transformation. Binding of the viral oncoprotein could allow transformation by countering the effects of increased p53 expression. Studies presented in Chapters 2 and 3 of this thesis support the latter model.

Biochemical properties of p53

Although the biochemical properties of p53 have been studied in considerable detail, it has been difficult to determine which activities are pertinent to tumor suppression. Since both mutant p53 and viral oncoproteins abrogate the ability of wild-type p53 to suppress neoplastic growth, these molecules are expected to disrupt or alter relevant p53 properties.

Immunochemistry. The availability of many p53-specific immunochemical reagents has facilitated the analysis of p53 cell biology and biochemistry. These antibodies display species specificity (e.g. PAb248; mouse), conformational-specificity (e.g. PAb246), or species-independent reactivity (e.g. PAb421) (Harlow et al., 1981; Yewdell et al., 1986). Monoclonal antibodies that bind to specific epitopes spanning murine p53 have been particularly useful, as have the conformational-specific antibodies PAb246

and PAb240 (Yewdell et al., 1986; Gannon et al., 1990). PAb246 recognizes an epitope that is specific to wild-type p53, such that mutant p53 proteins do not react (Finlay et al., 1988). Alternatively, PAb240 is specific for many mutant p53s, and is incapable of binding wild-type p53 (Gannon et al., 1990). In addition to providing valuable diagnostic reagents, the interaction between p53 and conformation-specific antibodies illustrates an important point; diverse p53 mutants encode proteins with similar conformational alterations.

Post-translational modifications. Post-translational modifications such as phosphorylation provide insight into potential mechanisms regulating p53 function. Although 53 is phosphorylated at several residues, few studies have correlated changes in p53 activity with site-specific phosphorylation. However, phosphorylation at both serine-315 and serine-389 may have relevance to p53 regulation in normal and transformed cells.

p53 can associate with $p34^{cdc2}$ *in vivo* (Sturzbecher et al., 1990; Milner et al., 1990), and is phosphorylated *in vitro* by $p34^{cdc2}$/cyclin A and B complexes at serine-315 (Bischoff et al., 1990; Sturzbecher et al., 1990). While these results provide a physical link between p53 and cell cycle control, p53 proteins containing alanine substitutions at codon 315 retain several properties normally associated with wild-type p53, including their ability to bind large T antigen and inhibit SV40 replication (Meek and Eckhart, 1990; Sturzbecher et al., 1990).

p53 is phosphorylated at serine-389 (Meek et al., 1990), a highly conserved residue (Soussi et al., 1990). Serine-389 is phosphorylated by casein kinase-II *in vitro*, and a related activity exists *in vivo* (Meek et al., 1990). Moreover, casein kinase II associates with p53 in insect cells (Herrmann et al., 1991), suggesting that phosphorylation by this enzyme occurs physiologically. p53 is also phosphorylated at this position by a DNA-dependent protein

kinase (Lees-Miller et al., 1990; Hupp et al., 1992). Phosphorylation of unmodified p53 by casein kinase II or DNA-dependent protein kinase dramatically enhances its affinity for p53-specific DNA sequences (Hupp et al., 1992). Furthermore, although overexpression of wild-type p53 can inhibit cell growth, p53 proteins containing alanine substitutions at codon 389 have no effect (Milne et al., 1992). Therefore, phosphorylation at this site may regulate p53 tumor suppressor activities. It should be noted, however, that a small polyribonucleotide can be covalently attached to serine-389 (Samad and Carroll, 1991), and it remains possible that the phenotype of the alanine-389 mutant reflects the absence of this RNA-linkage.

Oligomerization. p53 forms homo-oligomers both *in vitro* and *in vivo* (Kraiss et al., 1988; Eliyahu et al., 1988; Finlay et al., 1989; Milner et al., 1991). Moreover, wild-type and mutant p53 associate in the same complexes, providing a potential biochemical explanation for the dominant-negative effects of mutant p53 proteins (Eliyahu et al., 1988; Finlay et al., 1989; Friedman et al., 1990; Milner et al., 1991). Although p53 can form mono-, di- and tetra-oligomers, it appears as though the tetramers predominate *in vivo* (Friedman et al., 1993). p53 oligomerization requires a C-terminal domain that encompasses a predicted alpha-helical region and an adjacent basic region (Sturzbecher et al., 1992).

The normal function of p53 oligomerization remains obscure, since oligomerization mutants--at least when sufficiently overexpressed--retain several activities associated with wild-type p53. These include inhibition of SV40 replication, suppression of oncogene-induced focus formation, and the ability to function in transcriptional transactivation (Shaulian, et al., 1993; Sturzbecher, et al., 1992). By contrast, it appears that oligomerization is both necessary and sufficient for the trans-dominant action of mutant p53s. Thus,

the transforming activity of several mutants co-segregates with the oligomerization domain, and synthetic peptides encompassing this region inhibit wild-type p53 transactivation (Shaulian et al., 1992; Unger et al., 1993).

Subcellular localization. While p53 has been observed in both the cytoplasm and nucleus, it appears that nuclear localization is essential for transformation suppression (Shaulsky et al., 1990; Shaulsky et al., 1991). Several observations suggest that altered p53 subcellular localization contributes to oncogenic transformation. First, the adenovirus-5 $p55^{E1B}$ oncoprotein sequesters p53 outside the nucleus (Zantema et al., 1985). Second, p53 accumulates in the cytoplasm of certain breast cancers that harbor only wild-type p53 genes (Moll et al., 1992). Finally, many mutant p53 proteins are cytoplasmic (Martinez et al., 1991; Gannon and Lane, 1991; Zerrahn et al., 1992). However, exclusion from the nucleus is not the only mechanism whereby p53 mutation promotes transformation, since many mutant alleles encode nuclear proteins (Vogelstein and Kinzler, 1992).

DNA binding activity. Early studies demonstrated that p53 had an affinity for double-stranded DNA (Luka et al., 1980; Lane and Gannon, 1983; Steinmeyer and Deppert, 1988). High-affinity DNA binding requires the C-terminal basic region (Foord et al., 1991). Proteins encoded by mutant p53 genes display a much weaker affinity for DNA then does wild-type p53, suggesting that this property is relevant for p53 tumor suppression (Foord et al., 1991; Kern et al., 1991a). Interestingly, most activating mutations in p53 occur in the internal conserved regions, implying that mutations outside the DNA-binding domain can affect the affinity of p53 for DNA.

More recently, p53 has been shown to display high affinity for specific DNA sequences. The initial DNase-footprinting studies identified p53-specific sequences adjacent to the SV40 origin of replication (Bargonetti et al.,

1991), within the human ribosomal gene cluster (Kern et al., 1991b), and in the murine muscle creatine kinase gene enhancer (Zambetti et al., 1992). A consensus recognition site has been defined by comparison of sequences from a large number (18) of clones containing p53 binding sites (El-Deiry et al., 1992). Sequence-specific binding is probably required for tumor suppression, since mutant p53 proteins are incapable of these interactions. Furthermore, both mutant p53s and SV40 large T antigen prevent site-specific binding of wild-type p53 (Bargonetti et al., 1991; Kern et al., 1991b; Zambetti et al., 1992; Bargonetti et al., 1992).

DNA replication. The interactions between p53 and large T antigen provide insight into the relationship between p53 and DNA synthesis. Large T antigen regulates initiation and propagation of SV40 DNA replication both *in vivo* and *in vitro*. First, large T antigen binds the SV40 origin of replication and acts as a helicase. Second, T antigen interacts with DNA polymerase α to promote synthesis of SV40 DNA (Gannon and Lane, 1990).

Because SV40 replication is well-characterized, the effects of p53 on large T antigen functions have been analyzed in detail. Both human and murine p53 prevent T antigen-dependent replication *in vivo* and *in vitro* (Braithwaite et al., 1987; Sturzbecher et al., 1988; Wang et al., 1989; Friedman et al., 1990), apparently by inhibiting the initiation functions of large T antigen (Sturzbecher et al., 1988; Wang et al., 1989). Tumor-derived mutant p53s encode proteins that fail to suppress SV40 replication or bind T antigen. Moreover, mutant proteins inhibit suppression of SV40 replication by wild-type p53, probably by physically interacting with wild-type p53 (Friedman et al., 1990). It remains possible that p53 blocks DNA synthesis directly, by competing with DNA polymerase α for binding to large T antigen (Gannon and Lane, 1987). However, these observations are difficult to reconcile with

the fact that human cells expressing endogenous p53 can replicate SV40 DNA. Perhaps p53 levels under physiological circumstances are not sufficient to inhibit SV40 replication.

These observations suggest a model for p53 activity in normal cells (reviewed in (Levine et al., 1991)). Since p53 inhibits SV40 replication, it may perform similar functions in the regulation of cellular DNA synthesis in uninfected cells. This view predicts that p53 binds a T antigen homologue and suppresses its initiator activities. Thus, p53 mutants that fail to associate with this molecule might release the cell from negative growth controls and facilitate tumor progression. The existence of a cellular "T antigen" could explain why all mutant proteins fail to bind their viral counterpart.

Several lines of evidence suggest that p53 may regulate DNA replication in mammalian cells. First, overexpression of wild-type p53 has been shown block S phase entry (see below). Second, the original sequences demonstrated to specifically associate with p53 were adjacent to a viral (Bargonetti et al., 1991) and a putative cellular (Kern et al., 1991b) replication origin. Finally, the N-terminal region of p53 interacts with replication protein A (RPA). RPA binds DNA and promotes unwinding at replication origins. GAL4-p53 N-terminal fusion proteins activate RPA-dependent replication in vectors containing GAL4 recognition sites adjacent to the replication origin. In addition, full length p53 interacts with RPA and blocks DNA-binding (Dutta et al., 1993). While the *in vivo* significance of these observations has not been established, p53 interactions with RPA provide a provocative system for further investigation.

Transcriptional transactivation. Sequence-specific DNA binding by p53 may also regulate transcription. Prior to the identification of specific p53 binding sites in DNA, several studies suggested that p53 could function as a

transcription factor. For example, wild-type p53 fused to the GAL4 DNA binding domain was able to transactivate transcription from reporter constructs containing GAL4 binding sequences in both yeast and mammalian cells (Fields and Jang, 1990; Raycroft et al., 1990). Efficient transactivation required the N-terminal acidic domain of p53 (Fields and Jang, 1990). Moreover, fusion proteins derived from mutant p53 alleles were incapable of transcriptional transactivation (Raycroft et al., 1990; Raycroft et al., 1991).

p53 activates the transcription of several viral and cellular genes, apparently by binding specific DNA sequences. Transcription of the muscle creatine kinase gene is positively regulated by wild-type p53 due to the presence of a p53-response element in the muscle creatine kinase enhancer (Weintraub et al., 1991). DNase-footprinting studies identified a 50 base pair element within this region that confers p53-dependent transactivation upon a heterologous promoter in cultured cells (Zambetti et al., 1992). Mutant p53 proteins are unable to activate transcription in this assay. In another series of experiments, a DNA sequence capable of binding p53 *in vitro* was placed near a minimal promoter in a reporter gene construct (Kern et al., 1992). Transfection of wild-type p53 into cultured cells activates transcription of the reporter gene, and the reporter activity correlates with the number of p53 binding sites. Mutant proteins reduce p53-dependent transactivation in a dose-dependent manner, apparently by preventing wild-type p53 from binding to its response element. Wild-type p53 (but not mutant) activates transcription from similar constructs *in vitro*, indicating that transactivation is a direct effect (Farmer et al., 1992).

p53 may positively regulate the transcription of several other genes. Overexpression of wild-type p53 activates its own transcription, although this may be indirect (Deffie et al., 1993). *mdm*-2 expression is induced upon p53

overexpression, possibly as a negative feedback control of p53 activity (Wu et al., 1993; Barak et al., 1993). Efficient induction of transcription requires a p53-response element in the first intron of the *mdm*-2 gene (Wu et al., 1993; Barak et al., 1993), p53 activates transcription of *GADD45* through a p53-response element located in the third intron of the *GADD45* gene. Although its function is unknown, *GADD45* is preferentially expressed in growth-arrested cells following DNA damage (Papathanasiou et al., 1991). Finally, several approaches have identified a gene, *CIP1/WAF1* (Harper et al., 1993; El-Deiry et al., 1993), whose transcription is strongly induced by p53 (El-Deiry et al., 1993). It is not known whether this activation is direct.

Consistent with the putative role of p53 in negative growth control, p53 suppresses transcription of several genes that promote proliferation. These include proliferating cell nuclear antigen (PCNA) (Mercer et al., 1991), c-*fos* (Ginsberg et al., 1991), c-*jun* (Ginsberg et al., 1991), and interleukin-6 (Santhanam et al., 1991). p53 also has been shown to repress transcription of the *mdr*-1 (Chin et al., 1992) and *hsp*-70 genes (Agoff et al., 1993). Transcriptional repression apparently involves interactions between p53 and the basal transcription machinery or other transcription factors. For example, wild-type (but not mutant) p53 physically associates with TATA-binding protein *in vitro* (Seto et al., 1992), and inhibits transcriptional initiation from TATA-dependent promoters both *in vitro* and *in vivo* (Seto et al., 1992; Mack et al., 1993). In addition, physical interactions between p53 and the CAAT binding protein are required for p53-dependent repression of the *hsp*-70 promoter (Agoff et al., 1993).

All mutant p53 proteins are defective in transcription assays, implying that transcriptional control is important for p53 suppressor activities (reviewed in (Vogelstein and Kinzler, 1992)). Several additional observations

support this view. First, SV40 large T antigen, adenovirus E1B, and HPV E6 inhibit p53-mediated transactivation (Farmer et al., 1992; Yew and Berk, 1992; Lechner et al., 1992; Segawa et al., 1993). Moreover, the oncogenicity of $p55^{E1B}$ co-segregates with its ability to block p53-mediated transactivation (Yew and Berk, 1992). Second, the cellular oncoprotein MDM2 blocks p53-mediated transactivation (Momand et al., 1992), apparently by concealing its transactivation domain (Oliner et al., 1993). This interaction may inhibit p53 function *in vivo*, since *mdm*-2 amplification frequently occurs in tumors expressing wild-type p53 (Oliner et al., 1992).

It has been difficult to demonstrate which p53-regulated genes are relevant to p53 activities as a tumor suppressor. For example, p53 overexpression may induce indirect changes in gene expression that reflect differences in the growth state of the cell. The product of the *CIP1/WAF1* gene is presently the most likely molecule to mediate tumor suppression by p53. p53 activates *CIP1/WAF1* transcription, and the overexpression of *CIP1/WAF1* induces growth arrest and suppress transformation (El-Deiry et al., 1993). Moreover, the p21 protein product of *CIP1/WAF1* physically associates with cyclin-dependent kinase (cdk) complexes *in vivo* (Xiong et al., 1993; Harper et al., 1993) and inhibits cdk activity *in vitro* (Harper et al., 1993). Therefore, *CIP1/WAF1* provides a direct link between p53 transcriptional transactivation and an anti-proliferative response.

Transcription factor verses replication factor. A considerable amount of evidence implicates p53 in transcriptional control, but other studies suggest that p53 directly inhibits DNA replication. Nevertheless, the bias of evidence supporting a role for p53 as in transcriptional control may simply reflect current technological limitations in studying DNA replication (Oren, 1992). Perhaps p53 is involved in both processes, since both transcription and DNA

replication require an open DNA complex. This view is supported by recent studies demonstrating that transcriptional activation domains, including the N-terminal domain of p53, can also influence DNA replication by interacting with replication protein A (He et al., 1993; Li and Botchan, 1993; Dutta et al., 1993).

SECTION III: Mechanisms of tumor suppression by p53

Although p53 may inhibit DNA synthesis or participate in transcriptional regulation, less is known about how these processes prevent the initiation or progression of malignant tumors. On theoretical grounds, tumor suppressor genes may participate in negative growth control, cell survival, senescence, or differentiation. Remarkably, p53 has been implicated in each of these processes. Given this complex biology, it is essential to understand the physiological activities of p53 that create the selective pressure for p53 loss during tumor progression.

Many studies have addressed this issue by overexpressing wild-type or mutant p53 proteins to enhance or interfere with p53 functions, respectively. While such studies can reveal the possible consequences of activating or inactivating p53, the relevance of this approach to identifying circumstances in which endogenous p53 acts as a tumor suppressor is unknown. Furthermore, studies that overexpress mutant p53 proteins to inhibit wild-type p53 functions are inconclusive, because mutant p53 alleles are not simply dominant-negative inhibitors of wild-type p53 (Dittmer et al., 1993).

p53 and differentiation

Tumor cells typically appear less differentiated than the tissue from which they are derived, implying that the differentiated state limits neoplastic growth. Increases in p53 expression have been associated with differentiation,

and it is conceivable that p53 mutation could prevent or reverse the differentiation process. In normal lymphoid cells, endogenous p53 levels increase upon terminal differentiation (Kastan et al., 1991b). Wild-type p53 induces the expression of several B-cell specific markers upon introduction into a p53-deficient Abelson virus-transformed pre-B-cell line (Shaulsky et al., 1991). These more differentiated derivatives grow readily but are less tumorigenic than the p53-deficient line. In kidney cells, p53 can associate with the WT1 tumor suppressor, a transcription factor thought to function in kidney development (Maheswaran et al., 1993). Co-expression of p53 with WT1 alters transactivation from both p53-dependent and WT1-dependent test genes, raising the possibility that p53 modulates the differentiation program. However, p53 is not required for B cell or kidney differentiation, since p53-deficient mice display no obvious developmental defects (Donehower et al., 1992).

p53 and senescence

Cultured primary cells inevitably cease proliferation, apparently because they become nonresponsive to mitogens. This property, senescence, is intrinsic to primary cells in culture and may prevent aberrant growth. Cultured cells occasionally acquire mutations that allow them to overcome senescence and produce established lines capable of growing indefinitely (i.e. immortal). A number of viral and cellular oncogenes also facilitate the immortalization process (reviewed in (Ruley, 1990)). Cells derived from spontaneous tumors are almost always immortal, implying escape from senescence is essential for oncogenesis.

Several lines of evidence suggest that wild-type p53 participates in senescence of cultured cells. First, overexpression of "dominant-negative" p53 alleles can immortalize primary cells (Jenkins et al., 1984; Jenkins et al.,

1985). Second, the regions of SV40 large T antigen required to immortalize primary cells co-segregate with its ability to bind p53 (Peden et al., 1989; Zhu et al., 1991). Third, p53 mutations typically occur during establishment of embryonic fibroblasts into permanent lines (Harvey and Levine, 1991), and primary fibroblasts from mice containing disrupted p53 genes appear immortalized (Chapter 3 and (Harvey et al., 1993)). Finally, epidermal fibroblasts from Li-Fraumeni patients (which have endogenous p53 mutations) are readily established into permanent cell lines (Bishoff et al., 1990). While these studies strongly suggest that p53 is required for senescence of cultured cells, it remains to be determined how this phenomenon relates to tumor progression and/or p53 loss *in vivo*.

Suppression of cell growth by p53

Ectopic expression of wild-type p53 can inhibit proliferation, suggesting that p53 normally functions in negative growth control. Re-introduction of p53 into p53-deficient colon carcinoma and osteosarcoma lines inhibits clonal outgrowth, apparently by blocking S phase entry (Baker et al., 1990; Diller et al., 1990). These conclusions were based on the observation that most transfected cells contained G1 DNA content, with relatively few cells in S phase or G2/M. However, cell cycle analysis involved analyzing a small percentage of transiently transfected cells within a large population by flow cytometry, and purified cells were not placed back in culture. Thus, it remains possible that cell cycle progression was unaffected, and that high p53 levels were toxic to cells in S phase or G2/M.

Using inducible p53 expression vectors or temperature-sensitive p53 alleles, it has been conclusively demonstrated that p53 overexpression can inhibit proliferation (Mercer et al., 1991; Michalovitz et al., 1990; Martinez et al., 1991). Down-regulation of p53 activity allows proliferation to resume,

indicating that high p53 levels are not overtly toxic. Most studies suggest that p53 blocks S phase entry (Mercer et al., 1991; Martinez et al., 1991), but p53 may also inhibit proliferation in other cell cycle phases (Michalovitz et al., 1990). Although p53 homologues have not been identified in *S. cerevisiae*, p53 inhibits yeast cell cycle progression (Nigro et al., 1992). Thus, it appears that the cellular machinery involved in p53-mediated cell cycle arrest is highly conserved.

Induction of apoptosis by p53

p53 overexpression can induce cell death by an active process known as apoptosis, or programmed cell death. This activity was first demonstrated using a myeloid leukemia cell line expressing a only a temperature-sensitive p53 allele (Yonish-Rouach et al., 1991). Cell viability was unaffected at the restrictive temperature for wild-type p53 expression, but cells rapidly initiated apoptosis at the permissive temperature. Interleukin-6 suppressed apoptosis without affecting p53 levels. p53 overexpression induces apoptosis in other cells, including p53-deficient lines derived from colon carcinoma (Shaw et al., 1992), a Burkitt's lymphoma (Ramqvist et al., 1993), murine erythroleukemias (Ryan et al., 1993), a v-*myc* induced T cell lymphoma (Wang et al., 1993), and certain oncogenically-transformed primary rodent cells containing endogenous p53 (Debbas and White, 1993).

Activation of p53 for tumor suppression

Although forced overexpression of wild-type p53 can inhibit proliferation or kill cells by apoptosis, low levels of p53 are constitutively expressed in all cells without interfering with normal growth or survival. Thus, p53 expression or activity must increase under circumstances in which the protein functions as a tumor suppressor. This consideration raises

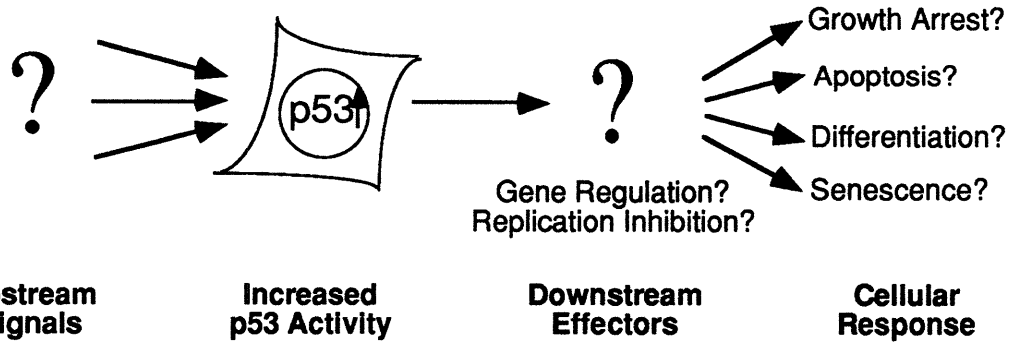


Figure 1. Model for p53 activity in tumor suppression.
p53 is activated by upstream signals that increase p53 levels or activity, leading to direct inhibition of DNA synthesis or altered gene transcription of growth-related genes. Downstream effectors may elicit the biological response. Depending on the circumstances (e.g. cell type, cell environment), p53 activation leads to growth arrest, senescence (i.e. irreversible growth arrest), or apoptosis. p53 mutation prevents either detection of upstream signals or activation of downstream effectors. In either case, the appropriate biological response is not activated.

several fundamental questions (see Figure 1). How is p53 *activated* and under what circumstances? What *signals* or *properties of tumor cells* (or potential tumor cells) are responsible for activation? What is the *fate* of cells in which p53 has been activated?

Since the biological consequences of p53 overexpression may not be physiological, it has been difficult to design experiments that adequately assess endogenous p53 activities. Null mutants are useful in this regard, since they allow direct analysis of gene function in a physiological context. Although many tumor cells have lost endogenous p53 expression, functional studies using these lines are limited by the lack of appropriate controls. Recently, the development of mouse strains carrying disrupted p53 alleles has permitted the isolation of cells--differing only in the status of the p53 gene--in which p53 function can be systematically analyzed. Using this approach, a model for p53 function in tumor suppression has rapidly emerged.

Genomic instability and gene amplification

Aneuploidy and genomic instability are common features of tumor cells. DNA amplification can be used as a measure of genomic instability; unstable cells are highly susceptible to DNA amplification while normal cells are not (Tlsty, 1990). p53 normally maintains genomic stability, since primary mouse embryonic fibroblasts lacking endogenous p53 rapidly become aneuploid upon continuous passaging in culture (Livingstone et al., 1992). CAD (trifunctional enzyme carbamoyl-P synthetase, aspartate transcarbamylase, dihydroorotase) gene amplification is required for cellular resistance to N-(phosphonacetyl)-L-aspartate (PALA), a uridine biosynthesis inhibitor. p53-deficient cells (including mouse embryonic fibroblasts derived from "knock-out" mice, immortalized fibroblasts derived from Li-Fraumeni patients, and human tumor lines) display an approximately 10^2 to 10^3-fold increase in PALA-resistant cells than do their wild-type p53 expressing counterparts (Livingstone et al., 1992; Yin et al., 1992). Furthermore, DNA amplification (and PALA resistance) is reduced to undetectable levels upon re-introduction of wild-type p53 (Yin et al., 1992).

PALA typically induces cell cycle arrest in G1, probably by activating a cell cycle checkpoint mechanism that limits proliferation in the absence of sufficient quantities of macromolecular precursors. p53-deficient cells continue to proliferate following PALA treatment, suggesting that p53 normally functions this G1 checkpoint (Livingstone et al., 1992; Yin et al., 1992). These studies suggest that p53 loss increases genomic instability by eliminating a G1 checkpoint and permitting inappropriate S phase entry. However, other mechanisms contribute to genomic instability, since tumor cells containing wild-type p53 genes are also unstable (Livingstone et al., 1992).

Cellular response to DNA damage

All cells possess repair mechanisms that reduce the frequency with which DNA damage produces mutation, and defects in these processes predispose individuals to cancer (reviewed in (Hartwell, 1992)). Cell cycle progression into S phase or mitosis prior to repair of genetic damage can fix mutations. Therefore, part of the repair process involves cell cycle checkpoints that arrest cells in either G1 or G2 following detection of DNA damage.

Increases in endogenous p53 levels and stability precede cell cycle arrest following DNA damage induced by ionizing radiation and other genotoxic agents (Maltzman and Czyzyk, 1984; Kastan et al., 1991a; Fritsche et al., 1993). Furthermore, p53-deficient human tumor lines fail to arrest in G1 following γ-irradiation, and re-introduction of wild-type p53 restores the checkpoint (Kastan et al., 1991a; Kuerbitz et al., 1992). Unlike their normal counterparts, embryonic fibroblasts derived from p53-deficient mice do not arrest in G1 following γ-irradiation (Kastan et al., 1992). The latter study demonstrates that p53 is required for this G1 checkpoint.

Ionizing radiation and other DNA damaging agents--many used in cancer chemotherapy--induce p53 accumulation by enhancing p53 stability (Maltzman and Czyzyk, 1984; Kastan et al., 1991a). Cells derived from cancer-prone individuals with ataxia-telangiectasia (AT) also fail to arrest following γ-irradiation, apparently because the products of the AT genes are required for p53 stabilization (Kastan et al., 1992). p53 accumulation activates *GADD45* transcription (Kastan et al., 1992), but it is unclear whether gene regulation by p53 actively contributes to G1 arrest. At present, the most likely candidate for a p53 effector is *CIP1/WAF1*, since $p21^{CIP1/WAF1}$ directly interacts with the cell cycle machinery (Xiong et al., 1993; Harper et al., 1993; El-Deiry et al., 1993).

This view predicts that transcription of *CIP1/WAF1* will not be induced following γ-irradiation of AT fibroblasts.

Guardian of the Genome model

The involvement of p53 in a cell cycle checkpoint has suggested a model to account for p53 action in suppressing oncogenic transformation (Lane, 1992). In this view, p53 is an essential component of a G1 checkpoint mechanism that limits cell cycle progression following DNA damage, presumably to allow sufficient time for DNA repair. p53 levels and stability increase upon detection of DNA damage leading to growth arrest, either directly or by transcriptional activation of target genes that block proliferation. In the absence of p53 function, as would occur upon p53 mutation or by p53 binding to cellular or viral oncogenes, p53 is unable to transactivate target genes or induce growth arrest. Thus, cells continue to proliferate following DNA damage, increasing the probability of mutation, some of which are oncogenic. Similarly, cells with defects in the G1 checkpoint are more likely to become aneuploid or amplify DNA (reviewed in (Hartwell, 1992)).

The "guardian of the genome" model provides explanations for the hypothetical questions posed above (Figure 2). p53 is *activated* for tumor suppression by changes in p53 protein stability leading to p53 accumulation. The *signal* responsible for p53 activation is DNA damage. Other molecules, which may include the products of the ataxia-telangiectasia genes, may be involved in detection of DNA damage or the stabilization of p53. The observation that caffeine blocks both growth arrest and p53 accumulation following γ-irradiation suggests that p53 stabilization is required for activation (Kastan et al., 1991a). No particular *properties of potential tumor cells* are required for p53 activation; indeed, the ability to increase p53 levels following DNA damage is intrinsic to all normal cells. Finally, the *fate* of cells that

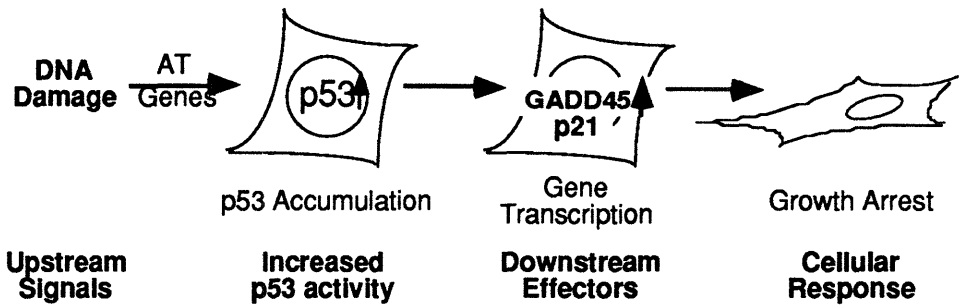


Figure 2. p53 and the cellular response to DNA damage.
DNA damage increases p53 protein stability owing to the activities of the ataxia-telangiectasia gene products. The resulting increase in p53 levels either directly or indirectly activates a G1 checkpoint and induces growth arrest, possibly through increased transcription of genes such as *GADD45* or *CIP1/WAF1* (p21). Growth inhibition allows DNA damage to be repaired and decreases the mutation frequency. Cells lacking p53 are unable to activate the G1 checkpoint following DNA damage; thus, cells continue to progress into S phase without sufficient time to repair damaged DNA. This increases the likelihood that cells will sustain oncogenic mutations, leading to tumor progression.

activate p53 is transient growth arrest, which is sustained until the damaged DNA is repaired.

At least several mutations are required for the malignant phenotype (Fearon and Vogelstein, 1990). Since p53 loss increases the rate at which cells acquire oncogenic mutations, the "guardian of the genome" model predicts that p53 mutation should be a potent initiator of tumorigenesis. Indeed, humans and mice carrying germline mutations in the p53 gene are predisposed to spontaneous tumors (Vogelstein, 1990; Donehower et al., 1992). This view also implies that p53 activity *indirectly* suppresses transformation. Thus, cells lacking p53 functions acquire additional mutations, and it is these mutations that directly influence the neoplastic phenotype.

Nevertheless, there are reasons to doubt that this indirect mechanism is the principle means by which p53 mutation contributes to carcinogenesis. First, p53 mutation is not an initiating event in most cancers, but rather appears to promote tumor progression. In colon cancer, p53 mutations are rarely observed in benign adenomas, but are associated with advanced stages of malignancy (see discussion in Section II). On average, tumor cells have sustained 2-3 other oncogenic changes prior to p53 mutation (Fearon and Vogelstein, 1990). In addition, p53-deficiency does not enhance either the initiation or promotion of carcinogen-induced benign skin polyps in mice, but dramatically increases their progression to malignant carcinomas (Kemp et al., 1993). Second, p53 may *directly* influence both the initiation and maintenance of transformed phenotypes. Thus, dominant-negative p53 alleles enable *ras* oncogenes to transform both primary and established cells (Hinds et al., 1989; Hicks et al., 1991). Transformed foci appear within days after gene transfer, and stable transformation requires continuous expression of mutant p53 (Zambetti et al., 1992). This requirement would be unnecessary if p53 loss had only increased the occurrence of other transforming mutations.

SECTION IV: Apoptosis as a potential mechanism of tumor suppression

Tumor suppressors have been viewed largely as molecules concerned with negative growth control. However, recent advances in the understanding of apoptosis, or programmed cell death, have suggested that regulation of cell survival may also influence tumorigenesis. Since mutations that repress the apoptotic program could facilitate tumor progression, it seems likely that several tumor suppressor genes will participate in apoptosis.

Characteristics of apoptosis

A formal description of apoptosis was forwarded in 1972 (Kerr et al., 1972). Apoptosis is now considered an essential process in normal development and tissue homeostasis, and defects in apoptotic programs contribute to several human diseases (reviewed in (Raff, 1992)). Apoptosis was initially defined by morphological criteria; cells undergoing apoptosis display cell shrinkage, loss of cell-cell contacts, membrane blebbing, chromatin condensation, and nuclear fragmentation (Kerr et al., 1972). Prior to loss of membrane integrity, dying cells are engulfed by macrophages or other surrounding cells. This process is distinct from necrosis, where cells burst and release their cytoplasmic contents into the surrounding environment. This difference may explain the physiological importance of apoptosis: apoptosis provides a mechanism to eliminate unwanted cells without initiating an inflammatory response. Thus, apoptosis has been sometimes referred to as "physiological" cell death, whereas necrosis has been described as "accidental" cell death (Raff, 1992; Vaux, 1993).

Cells undergoing apoptosis frequently activate an endonuclease that cleaves between nucleosomes, leading to eventual degradation of DNA to oligomers that are multiples of approximately 180-200 nucleotides (Wyllie, 1980; Wyllie et al., 1984). Although extensive DNA degradation would certainly lead to loss of viability, it may not be necessary for apoptosis, since some forms of apoptosis occur either prior to or without detectable DNA degradation (reviewed in (Fesus, 1993)).

Apoptosis and programmed cell death: requirements for gene function

Apoptosis is often equated with "programmed cell death", because it occurs by a gene-directed process that requires the active participation of the cell. Both RNA and protein synthesis inhibitors block apoptosis in cultured

thymocytes, suggesting that apoptosis is a cellular response to physiological stimuli that requires new gene expression (Wyllie et al., 1984; Sellins and Cohen, 1987). This simple interpretation is complicated by the observation that RNA and protein synthesis inhibitors have variable effects depending on the cell type and apoptotic stimuli (reviewed in (Fesus, 1993; Vaux, 1993)). For example, many cells become susceptible to tumor necrosis factor α-induced apoptosis upon treatment with cycloheximide (Laster et al., 1988). Although this may reflect the presence of a short-lived molecule that suppresses apoptosis, none has been identified.

Genetic studies in *C. elegans* provide direct evidence that apoptosis is a gene-directed process, as well as insight into the genetic mechanisms regulating apoptosis. The *ced*-3 and *ced*-4 genes are required for execution of the apoptotic program (Ellis and Horvitz, 1986). Consequently, animals harboring loss of function mutations in *ced*-3 or *ced*-4 possess extra cells. These additional cells remain viable and frequently differentiate. *ced*-4 encodes a protein that may bind calcium (Yuan and Horvitz, 1992), and *ced*-3 has structural similarities to the mammalian interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993). ICE may function similarly in mammalian cells, since overexpression can induce apoptosis (Miura et al., 1993). The *ced*-9 gene either directly or indirectly antagonizes the activities of *ced*-3 and *ced*-4 and has functional similarities to the mammalian *bcl*-2 oncogene (Hengartner et al., 1992). Thus, activating mutations in *ced*-9 prevent normal cell deaths from occurring. Loss of function mutations in *ced*-9 are associated with ectopic cell deaths in *C. elegans*, implying that apoptosis may occur by "default" in this organism.

Apoptosis is a common feature of malignant tumors.

In order to maintain tissue homeostasis, cell proliferation must equal

cell loss. Neoplastic growth occurs when cell proliferation exceeds cell loss. By comparing potential doubling times of tumors (estimated by mitotic index or thymidine incorporation) to observed growth rates it has been demonstrated that cell loss in tumors is often extremely large: in many tumors, the actual rate of tumor growth is less than 5% of the potential growth rate (based on the percentage of proliferating cells) (Wyllie, 1985). Consequently, factors that decrease the "cell loss factor" can dramatically enhance tumor progression.

Cell loss in tumors occurs predominantly from cell death by either necrosis or apoptosis. Tumor necrosis results primarily from hypoxia and nutrient deprivation, so necrotic cells typically are observed in zones at a distance from blood vessels (discussed in (Wyllie, 1985)). Although necrosis contributes significantly to tumor cell loss, it cannot explain the magnitude of cell death observed in many tumors (Kerr, et al., 1972). The role of apoptosis in determining net tumor growth has been largely overlooked, perhaps because cell apoptotic deaths are not localized. Dying cells shrink and are rapidly phagocytosed by macrophages or neighboring cells. However, apoptosis accounts for a large proportion of cell loss in tumors, particularly during periods of tumor regression (Searle, et al., 1975).

The susceptibility of normal and neoplastic cells to apoptosis has several implications with regard to cancer (Kerr, et al., 1972; Wyllie, 1985; Dive and Hickman, 1991; Wyllie, 1993). First, suppression of apoptosis in normal cells could cause hyperplasia, creating an expanded cell population from which cells acquiring oncogenic mutations could arise. Second, since apoptosis contributes significantly to the cell death in tumors, mutations that suppress apoptosis could promote tumor progression. Finally, the cytotoxicity of many anticancer agents may result from their ability to activate apoptosis.

Loss of apoptotic pathways could therefore reduce the effectiveness of cancer therapy.

Oncogenes can modulate apoptosis

***bcl*-2.** The molecular biology of apoptosis in human cancer was largely ignored until the *bcl*-2 oncogene was shown to modulate apoptosis. *bcl*-2 was first identified by its proximity to a common translocation breakpoint involved in many human lymphoid malignancies (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1985). These translocations deregulate *bcl*-2 expression by placing the oncogene under the transcriptional control of the immunoglobulin heavy chain promoter. Unlike most other oncogenes, *bcl*-2 has no obvious effect on proliferation (Vaux et al., 1988; Nunez et al., 1990) but instead enhances survival by inhibiting apoptosis (Vaux et al., 1988; Nunez et al., 1990; Hockenbery et al., 1990). Thus, deregulated expression of *bcl*-2 promotes neoplastic growth by preventing normal cell deletion.

Overexpression of *bcl*-2 inhibits most forms of apoptosis (Korsmeyer, 1992). Gene disruption studies indicate that *bcl*-2 is normally required for maintenance of lymphoid organs (Veis et al., 1993) and may function in an anti-oxidant pathway (Hockenbery et al., 1993). Given the numerous settings in which *bcl*-2 over-expression blocks cell death, it is surprising that *bcl*-2-deficient mice complete embryonic development (Veis et al., 1993). Perhaps this reflects functional redundancy, since *bcl*-2 is a member of a larger gene family (Maheswaran et al., 1993; Oltvai et al., 1993).

Adenovirus E1A and E1B. Interactions between the adenovirus E1A and E1B genes provide insight into the regulation of apoptosis in oncogenic transformation. The E1A oncogene promotes proliferation and S phase entry, probably by associating with cellular proteins involved in negative growth control (Whyte et al., 1988; Whyte et al., 1989). In addition to complementing

E1B activities in adenovirus transformation, E1A collaborates with activated *ras* oncogenes to transform primary cells (Ruley, 1983). The E1B-encoded proteins, $p19^{E1B}$ and $p55^{E1B}$, have no obvious effect on cell proliferation in the absence of E1A (van den Elsen et al., 1983), and do not substitute for *ras* in co-transformation with other oncogenes (e.g. *myc*) (Ruley, 1990).

The role of E1B in adenovirus transformation has been clarified by the observation that it, like *bcl*-2, inhibits apoptosis. This conclusion stemmed from the characterization of several adenovirus mutants (*cyt* and *deg*), which cause cytopathic effects and degradation of viral and chromosomal DNA (Takemori et al., 1968; D'Halluin et al., 1979). All *cyt* and *deg* mutants contain mutations within the viral E1B gene, including several that abolish $p19^{E1B}$ expression (White et al., 1984). Moreover, E1A expression is required for the *cyt* and *deg* phenotypes (White and Stillman, 1987), which resemble apoptosis (White et al., 1991). Thus, E1A induces apoptosis, which is countered by an E1B-encoded protein (Rao et al., 1992).

Regulation of apoptosis may explain the collaborative interactions between E1A and E1B in adenovirus transformation. E1A initiates cellular proliferation, but at the same time increases susceptibility to apoptosis (Rao et al., 1992). Consequently, proliferation cannot be sustained. E1A sequences required for apoptosis coincide with those required for induction of DNA synthesis (White et al., 1991), implying that these processes are linked. E1B, by countering apoptosis, allows sustained proliferation without directly influencing cell growth (Rao et al., 1992). While these studies do not rule out the possibility that E1B has additional activities, they suggest that inhibition of apoptosis enhances oncogenic transformation.

c-*myc*. Like E1A, the c-*myc* oncogene can promote both proliferation and apoptosis (Evan et al., 1992). Similarly, the regions of *myc* required for

apoptosis and proliferation are identical. Apoptosis associated with c-*myc* is particularly pronounced when cells are cultured in low serum concentrations or at high cell density. It has been suggested that *myc* "primes" cells for apoptosis (Wyllie, 1993). Thus, *myc*-overexpressing cells express the cellular machinery required for immediate execution of the death program, but "trigger" apoptosis only in response to growth-limiting conditions.

The fact that a cellular oncogene induces both proliferation and apoptosis suggests that activation and escape from apoptosis may be of general importance in tumor progression. Consistent with this hypothesis, *bcl*-2 blocks *myc*-induced apoptosis (Fanidi et al., 1992; Bissonnette et al., 1992) and collaborates with *myc* in oncogenic transformation of cultured cells (Vaux et al., 1988) and in transgenic animals (Strasser et al., 1990). Similar mechanisms may contribute to human cancer, since lymphoid cancers containing both deregulated *myc* and *bcl*-2 have been identified (Gauwerky, et al., 1989).

Proliferation verses cell death. The studies described above imply that cellular susceptibility to apoptosis modulates the transformed phenotype. At least two models can account for the association between proliferation and apoptosis. First, oncogenes such as *myc* and E1A may directly regulate both proliferation and apoptosis (see, for example, (Shi et al., 1992)). Since proliferation and apoptosis are incompatible, the activation of either process must involve synergism between oncogenes and other factors, perhaps the cellular environment. Negative regulators of apoptosis specifically interfere with the oncogene's apoptotic activity without affecting proliferation. Alternatively, apoptosis may reflect a cellular response to forced proliferation (see Chapters 2, 3, and (Rao et al., 1992)). In this view, the activity of the oncogene is solely mitogenic, and apoptosis ensues under circumstances where the proliferation is perceived as aberrant. Negative regulators of

apoptosis block activation (or implementation) of the cellular response. In either case, inactivation of apoptosis results in sustained proliferation and transformation.

Tumor suppressor genes and apoptosis

Neoplastic growth involves both activation of oncogenes and inactivation of tumor suppressor genes. As described above, oncogenes can enhance tumor progression either by promoting proliferation or suppressing apoptosis. Certain oncogenes that induce proliferation also promote apoptosis, suggesting that oncogenic changes that occur during tumor progression may increase cellular susceptibility to apoptosis. Since apoptosis is a common feature of malignant tumors, a significant growth advantage is conferred upon a cell that is able to circumvent the death program.

Oncogenes may promote apoptosis as part of their normal spectrum of activities or as a cellular response to forced proliferation. In the latter view, molecules involved in the cellular response are candidate tumor suppressor genes. In the presence of gene function, apoptosis occurs in response to aberrant proliferation--a veritable tumor suppressor activity. Upon inactivation of gene function, cells are unable to initiate apoptosis in response to oncogenic stimuli, leading to enhanced tumor growth and progression. Loss of function mutations occurring in such a tumor suppressor gene would not initiate tumorigenesis, but rather promote progression of tumors to more malignant states. Certainly, the p53 tumor suppressor gene has characteristics of such a molecule.

SECTION V: Thesis Overview

Oncogene collaborations and multistep carcinogenesis

Transformation of cultured cells to a tumorigenic state typically requires two or more genes acting in concert. For example, activated *ras* oncogenes transform primary cells (i.e. tissue explants that have not been passaged in culture) poorly, if at all, while genes such as *myc*, adenovirus early region 1A (E1A), simian-virus 40 (SV40) large T antigen, and certain mutant p53 alleles enable *ras* to transform primary cells to a tumorigenic state (reviewed in (Ruley, 1990)). Similarly, c-*myc*, E1A, large T antigen and mutant p53 alleles do not transform but facilitate the establishment of cells into lines capable of growing indefinitely in culture. While these results suggest that *in vitro* establishment is a necessary precondition for oncogenic transformation, *ras* is unable to transform the established REF52 cell line, demonstrating that *in vitro* establishment (i.e. immortalization) alone does not render cells susceptible to transformation by *ras* (Franza et al., 1986).

We have investigated transforming interactions between *ras* and other oncogenes in order to identify relevant biochemical interactions involved in normal growth control and transformation. REF52 cells are particularly useful in this regard, since they provide one of the tightest biological systems in which transformation by *ras* strictly requires a second, collaborating oncogene. Previous studies have suggested that *ras* fails to transform REF52 cells because continuous expression of even modest levels of oncogenic $p21^{H\text{-}ras}$ inhibits proliferation (Franza et al., 1986). Thus, E1A, SV40 large T antigen, and mutant p53 oncogenes collaborate with *ras* by circumventing cellular controls that cause *ras* to inhibit cell growth (Franza, et al., 1986; Hirakawa, et al., 1988; Hicks, et al., 1991). This hypothesis is supported by the observation

that REF52 cells transformed by *ras* and a temperature-sensitive SV40 large T antigen (*tsA58*) undergo growth arrest at the restrictive temperature for T antigen expression (Hirakawa, et al., 1988). By contrast, oncogenic transformation by "immortalizing" oncogenes such as E1A and c-*myc* may be limited by mechanisms that increase cellular susceptibility to apoptosis (see discussion in Section IV).

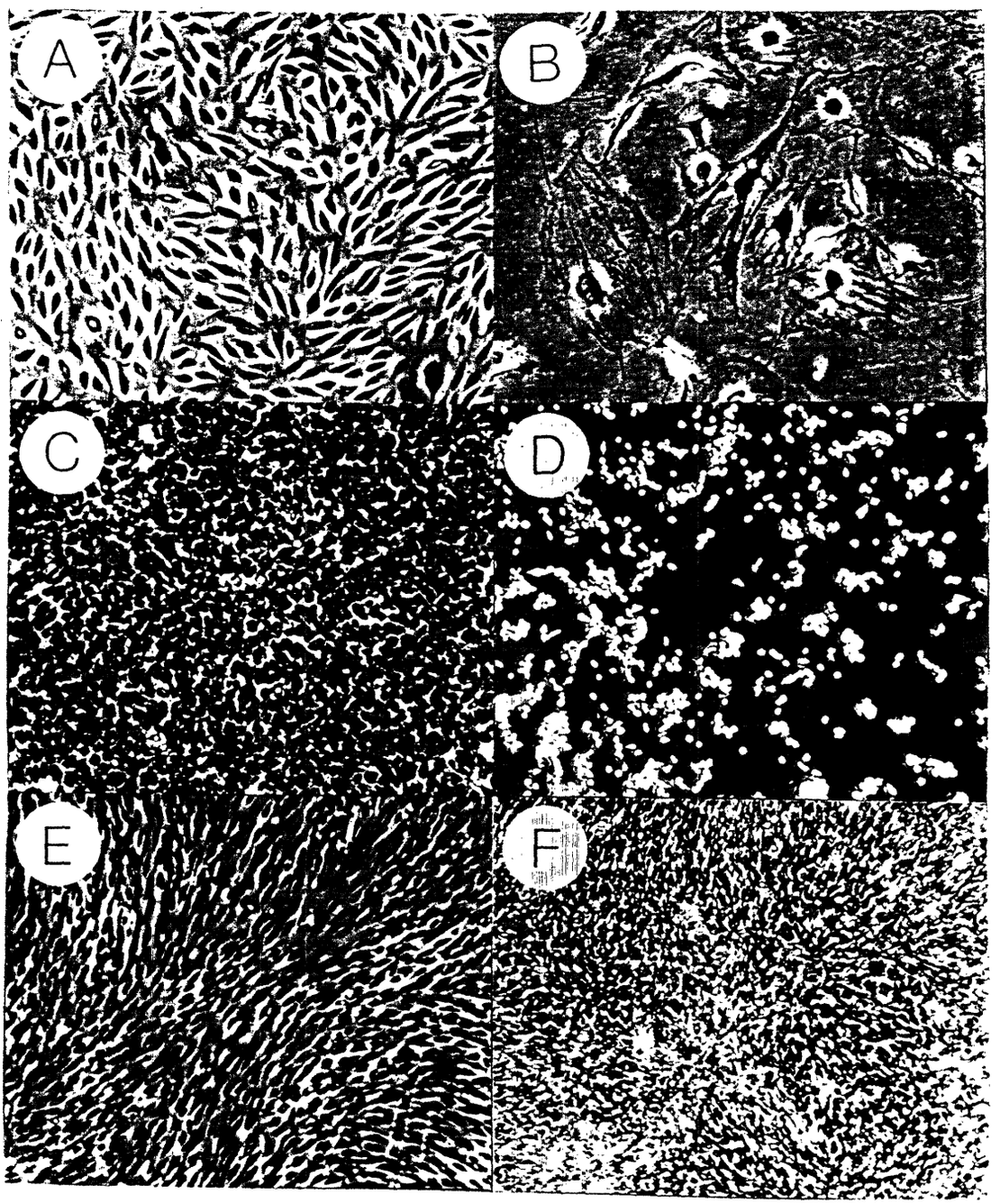
Preliminary observations

The research presented in this thesis originated from the studies investigating the cellular mechanisms that limit transformation by *ras* oncogenes. During the course of these studies, several observations suggested a series of experiments that would clarify the role of p53, E1A, and E1B in adenovirus transformation. Therefore, we began to focus on the cellular responses to E1A and its transforming interactions with other oncogenes. Nevertheless, these studies continued to investigate processes in which normal cells resist transformation by single oncogenes. The following is a brief description of the observations that provided the conceptual framework for this thesis.

***ras*-induces growth arrest of REF52 cells.** While conditional transformation of REF52 cells by *tsA58* provided a convenient means to regulate cellular responses to *ras* (Hirakawa, et al., 1988), the approach was limited since the cells were initially transformed. To conclusively demonstrate that growth arrest resulted from *ras* expression (rather than removal of large T antigen) it was necessary to develop a direct method to express oncogenic *ras* in normal REF52 cells. Retrovirus-mediated gene transfer has proven useful in this regard, since genes can be synchronously introduced into a relatively large number of cells and are expressed within hours of gene transfer.

Figure 3. Effects of T24 H-*ras* expression following retroviral-mediated gene transfer.

Cells were plated at a density of 2 x 10^5/150 mm dish and infected with either a *neo*-expressing (A, C, E) or *ras*-expressing (B, D, F) retrovirus at a high multiplicity of infection (5-10 cfu/cell). Photomicrographs were taken at 5 days post-infection. A and B, REF52 cells; C and D, 1A1 cells (REF52 clone expressing E1A); E and F, RAT2 cells.


A
B
C
D
E
F

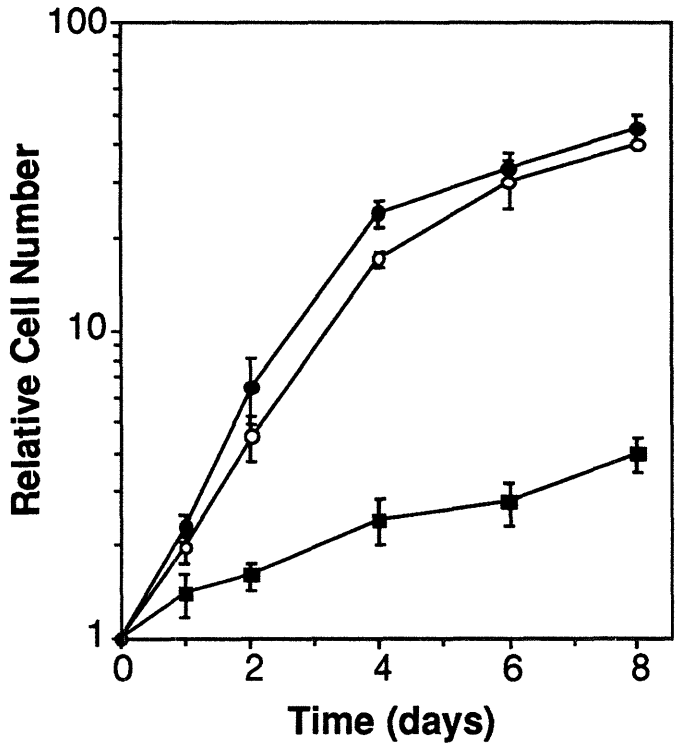


Figure 4. Growth of REF52 cells expressing T24 H-*ras* following retroviral gene transfer.

REF52 cells were plated at a density of 2 x 10^5/150 mm dish and infected with the *neo*-expressing (o) or *ras*-expressing retrovirus (■), or left uninfected (●). Cell numbers were determined at various times post-infection and normalized to the value on day 0. Each data point was obtained from at least 4 independent infections.

REF52 cells infected with a *ras*-expressing retrovirus (U3Histkras; containing the T24 H-*ras* allele) acquired a large, flat morphology, whereas infection of REF52 cells with a control retrovirus (U3Histkneo; (von Melchner and Ruley, 1989)) had no effect. Moreover, infection of E1A-expressing REF52 cells or RAT2 cells with the *ras* retrovirus rapidly induced morphological transformation (Figure 3). REF52 cells infected with control retroviruses continued to proliferate at a rate similar to uninfected controls; however, infection with the *ras* retrovirus inhibited cell growth (Figure 4), predominantly in the G1 phase of the cell cycle (Figure 5). Furthermore, *ras*-expressing cells were unable to enter S phase following serum depletion and

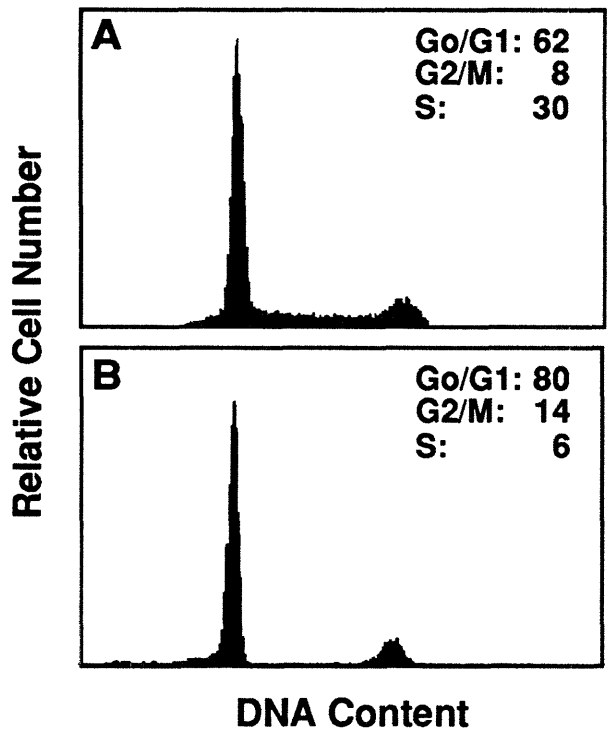


Figure 5. Cell cycle profile of *ras*-arrested REF52 cells.
REF52 cells were infected with the either *neo*-expressing or *ras*-expressing retrovirus. On the second day after infection, cells were trypsinized, stained with propidium iodide, and analyzed for DNA content by flow cytometry.

re-stimulation (Figure 6). These results indicated that *ras*-overexpression was indeed capable of arresting REF52 cell proliferation.

Is p53 involved in *ras*-induced arrest? Several observations suggested that p53 might participate in *ras*-induced growth arrest. First, mutant p53 alleles encoding dominant-negative proteins enabled *ras* to transform REF52 cells, thereby circumventing *ras*-induced arrest (Hicks et al., 1991). Second, overexpression of wild-type p53, like *ras*, can inhibit proliferation in G1 (Michalovitz et al., 1990; Mercer et al., 1990; Diller et al., 1990). If so, it seemed plausible that increases in endogenous p53 expression might accompany *ras*-induced growth arrest. Since E1A prevented *ras*-arrest, we hypothesized that E1A might block p53 accumulation.

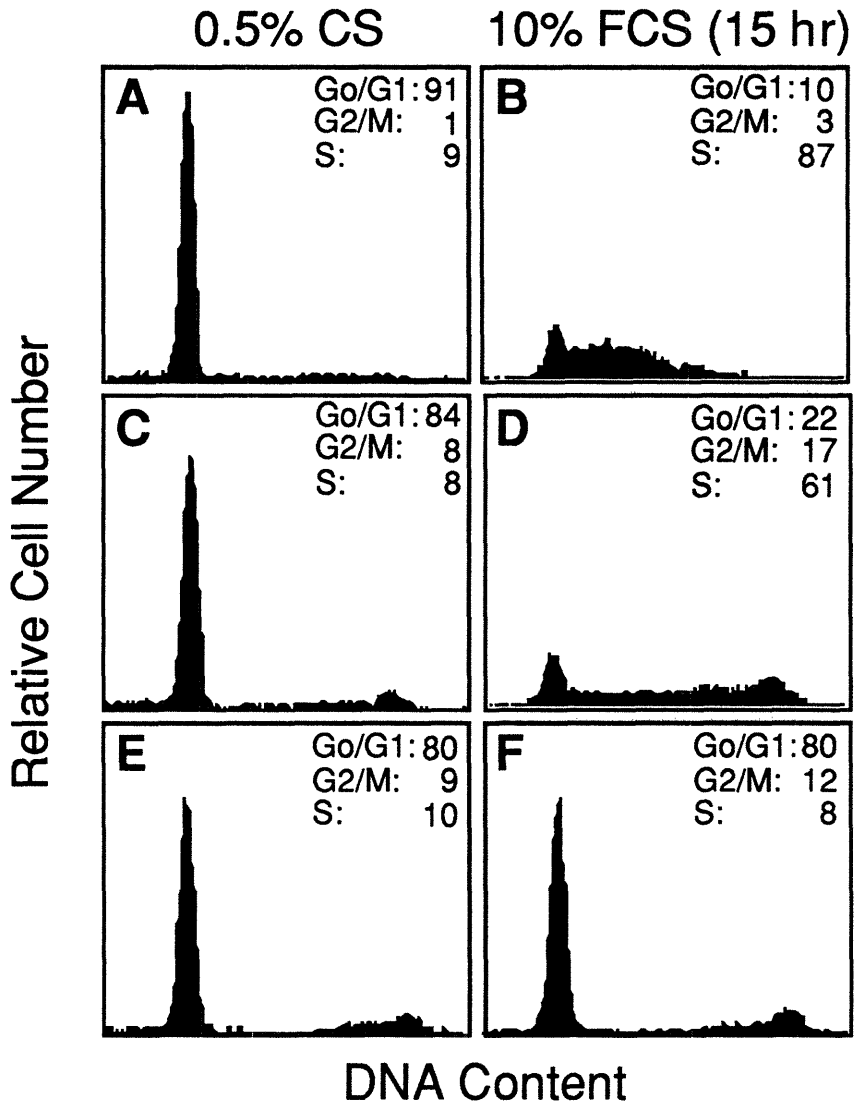
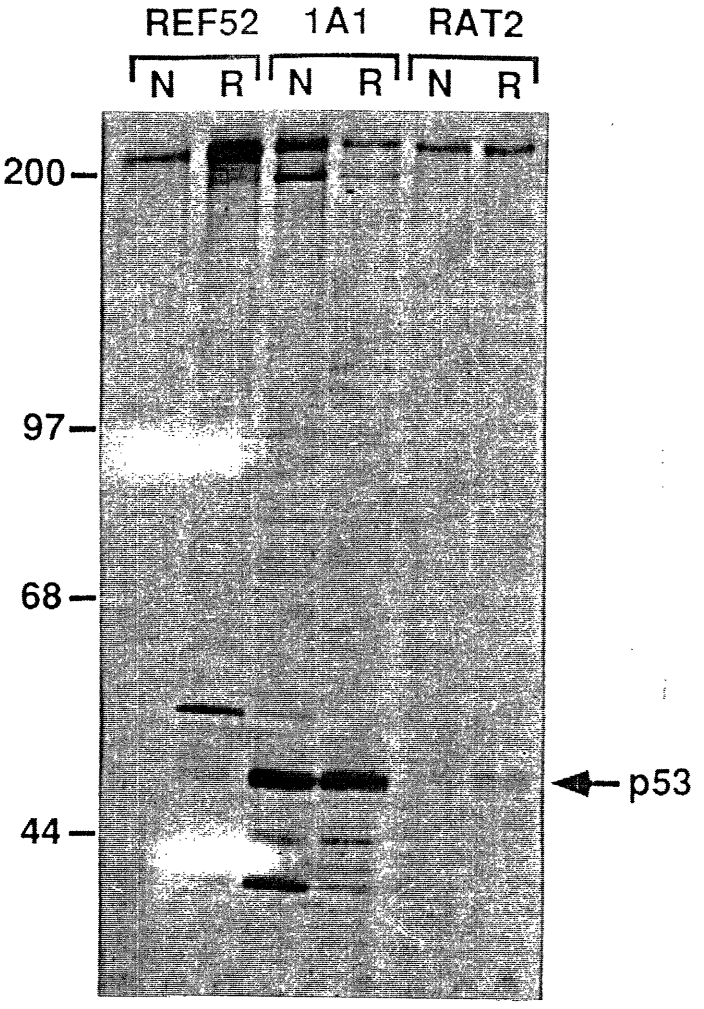


Figure 6. Serum stimulation of quiescent and *ras*-arrested REF52 cells.

REF52 cells were either left untreated (A, B), or infected with the *neo*-expressing (C, D) or *ras*-expressing (E, F) retroviruses. After 24 hours after infection, cultures were placed in medium containing 0.5% calf serum. After 48 hours of serum deprivation, cultures were re-stimulated with medium containing 10% FBS. Cells were analyzed for DNA content either prior to (A, C, E), or 15 hours after serum addition (B, D, F).

Figure 7. p53 levels in retrovirus-infected cells (following page).

REF52 cells, REF52 cells expressing E1A (1A1) and RAT2 cells were infected with viruses expressing either *neo* (N) or *ras* (R). p53 levels were estimated by immunoprecipitation of ^{35}S-lysates (see Chapter 2) 10 days after infection. The mobility of p53 is marked with an arrow at the right of the gel.


REF52
1A1
RAT2
N
R
N
R
N
R
200
97
68
44
p53

The results of this experiment were quite unexpected. *ras* had no obvious effect on p53 expression prior to or during cell cycle arrest. By contrast, E1A-expressing cells contained dramatically elevated p53 levels (Figure 7). Furthermore, p53 levels remained high in E1A-expressing cells, despite oncogenic transformation by *ras*. These results indicate that p53 accumulation was not required for *ras*-induced growth arrest. Rather, p53 expression was induced in response to E1A.

Paradox of E1A-induced p53 levels. Since oncogenic transformation frequently involves the loss of p53 function, it was paradoxical that p53 levels were increased in cells expressing E1A--an oncogene that promotes proliferation. Although p53 levels are elevated in adenovirus-transformed cells, the increase was thought to result from either the physical association between p53 and the adenovirus E1B protein or a secondary consequence of oncogenic transformation (Zantema et al., 1985; Jochemsen et al., 1987; Mak et al., 1988; van den Heuvel et al., 1990). However, our studies suggested that the increase might result from activities associated with E1A.

Why would increases in p53--the product of a tumor suppressor gene--occur in cells expressing E1A? A simple explanation for this phenomenon is that p53 accumulation reflects mutations within the p53 gene, which often encode more stable proteins (Finlay et al., 1988). However, this was apparently not the case (see Chapter 2). Alternatively, viral proteins may alter p53 function, such that elevated p53 levels promote oncogenic transformation (van den Heuvel et al., 1990). Finally, p53 accumulation may reflect a mechanism whereby cells attempt to resist viral transformation. Consistent with this possibility, REF52 cells tolerated E1A expression poorly, and frequently lost E1A altogether (see Chapter 2).

The latter hypothesis was supported by studies demonstrating that E1A can induce apoptosis (see Section IV). Since E1A increased p53 levels, it was possible that p53 accumulation might induce apoptosis. Moreover, the observation that E1B blocks apoptosis and collaborates with E1A in adenovirus transformation suggested that E1B might circumvent the effects of elevated p53. While p53 physically associates with $p55^{E1B}$, $p19^{E1B}$ (which does not interact with p53) was more efficient at protecting against E1A-induced apoptosis (Rao et al., 1992).

Nevertheless, the notion that apoptosis might involve p53 accumulation was reinforced by the demonstration that forced overexpression of p53 could induce apoptosis (Yonish-Rouach et al., 1991; Shaw et al., 1992). Although physiological situations in which p53 functioned in apoptosis remained undefined, the fact that E1A increased endogenous p53 levels suggested that E1A-associated apoptosis might represent the first such setting. Moreover, since p53 expression increased in response to an oncogene that was unable to transform alone, it seemed plausible that p53 accumulation was part of a cellular mechanism that resists oncogenic transformation. Therefore, we decided to investigate the role of p53 in E1A-induced apoptosis.

p53 accumulation and apoptosis

The observation that p53 levels increase in response to E1A provides the cornerstone for the studies presented here. Much of the experimentation was aimed at identifying both the causes and consequences of this phenomenon. Given our interest in defining mechanisms in which oncogenes interact in transformation, we were intrigued by the possibility that E1A-induced p53 expression might reveal fundamental clues as to how p53 functions as a tumor suppressor gene. From an intellectual standpoint,

we wished to investigate the following: Do increases in p53 levels represent a cellular response to aberrant proliferation (i.e. oncogenes) that enable cells to resist oncogenic transformation? Is p53 involved in apoptosis? Does apoptosis suppress transformation?

Chapter 2 describes the biochemical characterization of p53 accumulation in cells expressing E1A, with specific comparisons to p53 expression in cells co-expressing E1A and E1B. These studies demonstrated that p53 was stabilized in cells expressing E1A alone, and that the effects of E1A could account for the p53 stabilization observed in adenovirus-transformed cells. Moreover, p53 stabilization was associated with apoptosis, suggesting a physiological setting in which p53 was suppressing oncogenic transformation. Chapter 3 describes experiments designed to directly test this hypothesis. p53-deficient mouse embryonic fibroblasts were used to determine whether p53 was required for E1A-induced apoptosis. Like wild-type cells co-expressing E1A and E1B, p53-deficient cells expressing only E1A were resistant to apoptosis. p53-dependent apoptosis appeared to suppress transformation by E1A, since p53-deficient cells expressing E1A were tumorigenic. In contrast to E1B, activated *ras* oncogenes did not block E1A-associated apoptosis, but collaborated with E1A to transform cells to a highly tumorigenic state. Thus, transformation by E1A can occur by at least two distinct mechanisms, one involving escape from apoptosis (E1B) and another that compensates for apoptosis (*ras*).

The involvement of p53 in apoptosis has several implications for human cancer, particularly with regard to cellular responses to anticancer agents. Chapter 4 demonstrates that several anticancer agents trigger p53-dependent apoptosis in E1A-expressing cells. p53 was required for induction of apoptosis by γ-irradiation and several chemotherapeutic compounds,

implying that loss of p53 during tumor progression could produce cross-resistant tumors. Chapter 5 provides direct data implicating p53 in tumor response to γ-irradiation. Finally, Appendix 1 investigates whether p53 is required for apoptosis in other settings, specifically in the mouse thymus. Although several stimuli trigger apoptosis in primary thymocytes, p53-dependent apoptosis was activated only in response to γ-irradiation. Therefore, apoptosis can occur by p53-dependent and independent pathways.

In conclusion, although initially interested in the mechanisms that prevented *ras* from transforming cultured cells, we identified a mechanism that precludes transformation by E1A. Thus, p53 can suppress oncogenic transformation by modulating apoptosis. These studies provide a rationale for the observation that p53 loss occurs late in tumor progression, after other oncogenic mutations have occurred. Moreover, they suggest that the ability of tumor cells to trigger p53-dependent apoptosis may influence the effectiveness of cancer therapy, and provide a biological explanation for the prognostic significance of p53 mutation.

CHAPTER 2

Stabilization of the p53 tumor suppressor is induced by adenovirus E1A and accompanies apoptosis

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INTRODUCTION

p53 was identified as a cellular protein associated with simian virus 40 (SV40) large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979), and later, with the adenovirus-5 E1B ($p55^{E1B}$) and human papilloma virus types 16 and 18 E6 proteins (Sarnow et al., 1982; Werness et al., 1990). Although the gene was originally classified as a dominant-acting oncogene, present evidence indicates that p53 functions primarily as a tumor suppressor (Levine et al., 1991; Malkin et al., 1990; Srivastava et al., 1990; Donehower et al., 1992).

Mechanisms whereby p53 protects against neoplastic growth are unknown. Forced overexpression of wild-type p53 can suppress cell growth (Finlay et al., 1989; Eliyahu et al., 1989) and promote cell death by apoptosis (Yonish-Rouach et al., 1991; Shaw et al., 1992) However, the relevance of these experiments to the natural circumstances in which p53 participates in tumor suppression and/or apoptosis is unknown, since physiological changes in p53 levels or activity that might mediate cellular resistance to oncogenic transformation have not been identified.

Interactions between viral early region proteins and p53 also contribute to oncogenic transformation by human adenoviruses, SV40 and HPV (Levine, 1990). Given the role of p53 as a tumor suppressor, the viral tumor antigens are thought to interfere with p53 functions which preclude transformation. This is illustrated by the human papilloma virus E6 protein which promotes the proteolytic degradation of p53 (Scheffner et al., 1990). Similarly, stable complexes between p53 and $p55^{E1B}$ may inactivate p53 function by sequestering the protein outside the nucleus (Zantema et al., 1985) or by blocking interactions between p53 and other cellular targets (Yew and Berk, 1992).

Since oncogenic transformation frequently involves the loss of p53 function, it seems paradoxical that the stability and levels of p53 are greatly increased in adenovirus and SV40 transformed cells. Although the stabilization of p53 associated with viral tumor antigens may simply be coincidental, the phenomenon may have significance regarding p53 function or regulation. For example, p53 may have positive as well as negative effects on cell growth (Mercer et al., 1984; Shohat et al., 1987; Shaulsky et al., 1990; van den Heuvel et al., 1990) Thus, the tumor antigens could promote cell transformation, in part, by increasing the levels of p53. Alternatively, the stabilization of p53 may result from factors other than tumor antigen binding, possibly as part of a mechanism whereby cells attempt to resist viral transformation. Binding of the tumor antigen could allow transformation by countering the effects of increased p53 expression.

While evidence supporting this last model is limited, several studies suggest tumor antigen binding may not stabilize p53. First, p53 turnover decreases in adenovirus type 12-transformed cells, even though the protein does not form a stable, immunoprecipitable complex with Ad12 $p55^{E1B}$ (Zantema et al., 1985). Second, the half-lives of free and T antigen-associated p53 are similar in SV40-transformed cells (Deppert and Haug, 1986). In both cases, the effect of the tumor antigen on p53 turnover was attributed to metabolic changes associated with cell transformation, although neither study excluded the possibility of physical interactions between the tumor antigen and p53.

The present study analyzed p53 expression in normal and transformed REF52 cells. Although established as a permanent line, REF52 cells resemble primary cells in that transformation can require two or more oncogenes acting in concert (Ruley, 1990). For example, REF52 cells are not transformed

by either *ras* or adenovirus E1A individually, but are oncogenically transformed by combinations of the two oncogenes (Franza et al., 1986). SV40 large T antigen and dominant transforming forms of p53 also transform in collaboration with *ras* (Hirakawa and Ruley, 1988; Hicks et al., 1991). Since p53 is the only known cellular gene product with this activity, we analyzed the effects of various oncogenes on cellular p53 expression. Quite unexpectedly, the half-life of endogenous p53 was extended in all cells expressing adenovirus-5 E1A. Moreover, the stabilization of p53 was associated with the selective loss of E1A-expressing cells by a process resembling apoptosis. While having no additional effect on p53 turnover, E1B protected cells against the toxic effects of E1A.

METHODOLOGY

Cell culture

REF52 cells were maintained in Dulbecco's Modified Eagle's medium (DME) supplemented with 5% fetal bovine serum (FBS), 5% calf serum (CS), penicillin (50 units/ml), and streptomycin (50 μg/ml). REF52 cells expressing Adenovirus Type 5 (Ad5) E1A (e.g. clone 1A1) or transformed by T24 H-*ras* and E1A (e.g. clone RNA7) have been described (Franza et al., 1986). Clones R53/4 and 52LTR/6 expressed mutant p53 genes ($p53^{pro193}$ and $p53^{val135}$, respectively (Hicks, et al., 1991)) and were obtained from Dr. Michael Mowat (Manitoba Institute of Cell Biology). Ψ2 cells producing the tsA58-3 and LJ-12S retroviruses (Jat and Sharp, 1989) were maintained on DME containing 10% CS.

Gene transfer

Retroviruses expressing the Ad5 E1A 12S cDNA were isolated following transfection of pLJ-12S into Ψ2 cells. LJ-12S expresses a 12S E1A

cDNA and a *neo* gene from the viral long terminal repeat (LTR) and an SV40 early promoter, respectively (Dr. Mark Timmers, unpublished). Cells expressing LJ-12S were isolated in media supplemented with 0.4 mg/ml G418.

The *tsA58*-3 retrovirus expresses a temperature sensitive large T antigen (*tsA58*) and *neo* (Jat and Sharp, 1989). To introduce SV40 large T antigen into clones already expressing *neo*, 5×10^6 *tsA58*-3 producer cells were g-irradiated for 27 minutes (3000 rads) and co-cultivated with 2×10^3 target cells for 3 days at 37°C in the presence of 2 μg/ml polybrene. Colonies of infected target cells arose by 10 days at 37°C, while the irradiated producer cells detached from the plate. Colonies were pooled and transferred to the permissive temperature for large T expression (33°C) for at least 3 days prior to analysis. No cells remained in parallel cultures containing only irradiated producer cells.

Ad5 E1A and E1B genes were introduced into cells by calcium phosphate co-precipitation. p1AHygro (Ad5 E1A linked to a hygromycin B resistance gene) was co-transfected with p5XX (Ad5 XbaI-XhoI genomic E1B fragment) and stable transfectants were isolated in medium containing 100 μg/ml hygromycin B. Alternatively, p5XX was introduced into 1A1 cells by co-transfecting pY3 (Blochinger and Diggelmann, 1984) and selecting for hygromycin B resistance. Stable lines were isolated and expanded in 25 μg/ml hygromycin B. For transient expression studies cells were transfected with pCH110 and either p1Aneo (Franza et al., 1986) or pBluescript (Stratagene) plasmid DNAs (15 and 5 μg/ml, respectively).

Immunoprecipitation

Monoclonal antibodies PAb419, PAb421, and M73 react with SV40 large T antigen, p53, and Adenovirus E1A, respectively (Harlow et al., 1981; Harlow et al., 1985). PAb240 reacts with most dominant-transforming forms of p53 but

does not recognize wild-type p53 (Gannon et al., 1990). 13D2 reacts with the $p55^{E1B}$ product and was obtained from Dr. Eileen White (Rutgers University). Cellular proteins were labeled with 100 μCi/ml ^{35}S-Express protein labeling mix (New England Nuclear) for either 4 or 18 hours in methionine-free medium (Flow Labs) containing 5% dialyzed FBS. For 18 hour labelings, 5% normal DME was included. Cell lysates were prepared (Hinds et al., 1987), normalized to equivalent trichloroacetic acid precipitable counts (usually 2 x 10^7 cpm), and cleared of non-specific IgG binding proteins (Harlow et al., 1986). p53, large T antigen, and E1B [Hinds, 1987 #58] and E1A proteins (Harlow et al., 1986) were precipitated, fractionated on 10% SDS-polyacrylamide gels and visualized by fluorography. Signal intensities were quantified using a Molecular Dynamics PhosphorImager and ImageQuant software.

For pulse-chase experiments, 5 x 10^5 cells were seeded into 100 mm dishes and allowed to adhere overnight. Dishes were washed twice with phosphate-buffered saline (PBS) and incubated in the presence of methionine-free medium for 2 hours. Cultures were pulse-labeled for 1 hr with 200 μCi ^{35}S-labeled amino acids, washed twice with PBS, and chased for various times with normal growth medium supplemented with 10 mM unlabeled methionine. At various intervals, cells were lysed and the amount of labeled p53 was determined by immunoprecipitation as described above.

Immunofluorescence

For immunofluorescence experiments, cells were seeded on glass cover slips at sub-confluent densities and allowed to adhere overnight. The cells were washed twice with PBS and fixed in freshly prepared formaldehyde solution (4% w/v paraformaldehyde and 0.4% v/v picric acid in PBS). After 15 minutes at room temperature, the fixed cells were washed in PBS, permeablized in methanol-acetone (1:1) for 2 minutes, and washed again in

PBS. To minimize non-specific staining, the cover slips were pre-incubated in PBS containing 5% goat serum for 1 hour at room temperature. The primary antibody (PAb421 hybridoma tissue culture supernatant diluted 1:10 in PBS-5% goat serum), was applied to the cover slips and the samples were incubated for 2 hours at 37°C. Subsequently, the cover slips were washed in PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (1:200 dilution in PBS-5% goat serum, Calbiochem) for 20 minutes at 37°C. Finally, the samples were washed extensively in PBS-0.2% Tween 20 and mounted in PBS-glycerol (1:3) containing 0.1% (w/v) p-phenlyenediamine (Sigma). Control cultures were treated identically, except the primary antibody was omitted. Transiently transfected cells were analyzed in a similar manner except anti-β-galactosidase (Promega) and mixtures of primary (PAb421, PAb248 and PAb246 for p53 or M58 and M73 for E1A) and secondary (rhodamine-anti-rabbit IgG and fluoroscein-anti-mouse IgG) antibodies were used.

Cell Viability

Cells were seeded at 3 x 10^4 cells/60 mm dish in 10% FCS or 0.5% CS. At various times thereafter, floating and adherent cells were pooled and viability was assessed by trypan blue exclusion. Fresh media was added 3.5 days after seeding. At least 200 cells were counted for each determination. Degradation of genomic DNA was taken as evidence of cell death by apoptosis. Cells were seeded at 2 x 10^5 cells/150 mm dish in 10% FCS or 0.5% CS. After 3 days, genomic DNA was isolated from pools of floating and adherent cells and analyzed by agarose gel (1%) electrophoresis.

RESULTS

p53 levels are elevated in cell lines expressing adenovirus E1A

p53 expression was analyzed in REF52 clones following immunoprecipitation using a broadly-reactive anti-p53 antibody (PAb421). Cells expressing adenovirus E1A (1A1) and cells transformed by T24 H-*ras* and E1A (e.g. RNA7) expressed significantly more p53 than the parental cell line (Figure 1A). On average, the p53 levels in E1A-expressing clones were 10-fold higher than in REF52 cells, and were similar to the levels expressed in 293 cells, a human cell line expressing adenovirus E1A and E1B. In 293 cells, a protein of approximately 55 kD co-immunoprecipitated with p53, indicating that p53 was complexed to Ad5 $p55^{E1B}$ (Sarnow et al., 1982).

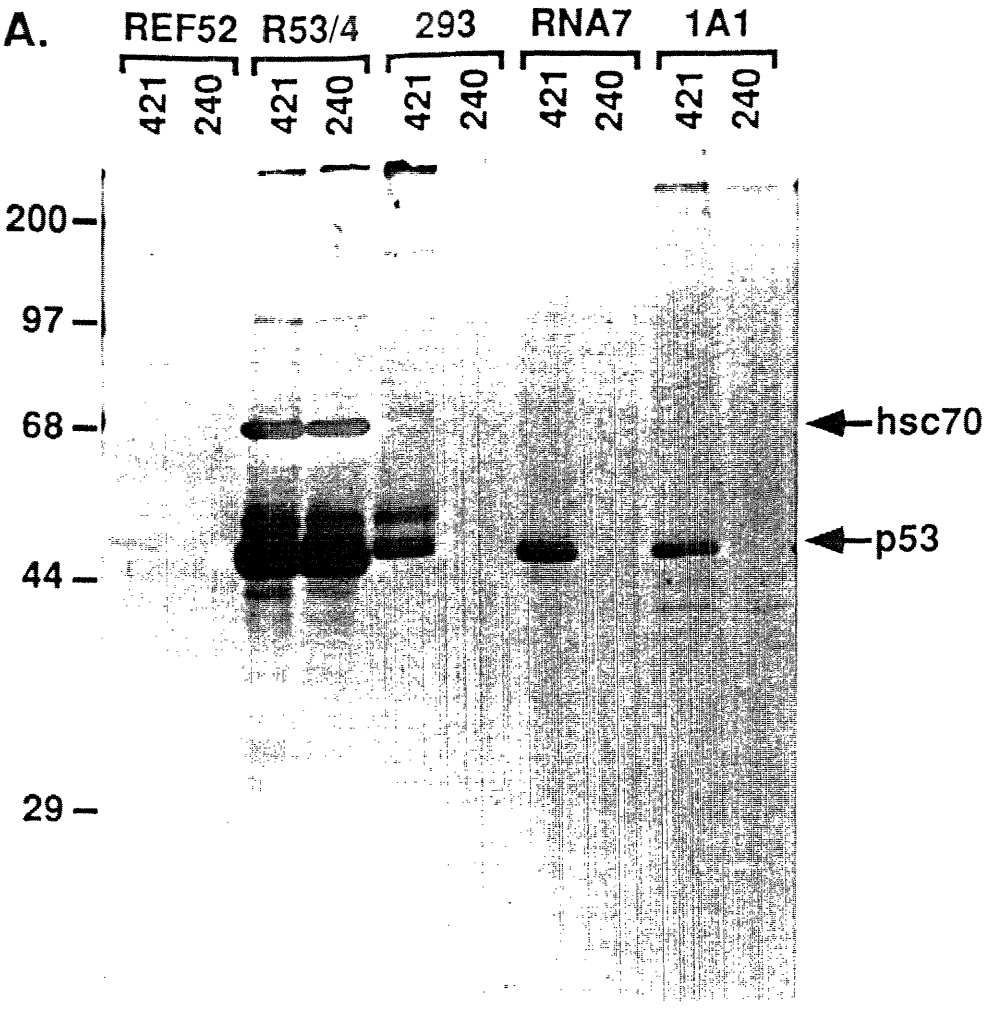
Steady-state levels of p53 are frequently elevated in naturally occurring tumor cells and in oncogenically-transformed cell lines. In most cases, the increase results from point mutations that stabilize the protein (Finlay et al., 1988; Eliyahu et al., 1988; Hinds et al., 1989). Many, but not all, of the mutant proteins share similar conformational alterations: they express an epitope recognized by PAb240 (Gannon et al., 1990) and bind hsc70, a constitutively expressed member of the heat shock family (Pinhasi-Kimhi et al., 1986). For example, p53 from R53/4 cells (which express T24 H-*ras* and a mutant p53 gene, $p53^{pro193}$) complexed to a 70kD protein and was immunoprecipitated with PAb240 (Figure 1). In contrast, the p53 expressed in REF52, RNA7, and 1A1 cells neither associated with hsc70 nor reacted with PAb240 (Figure 1A) suggesting that the p53 was wild-type in conformation.

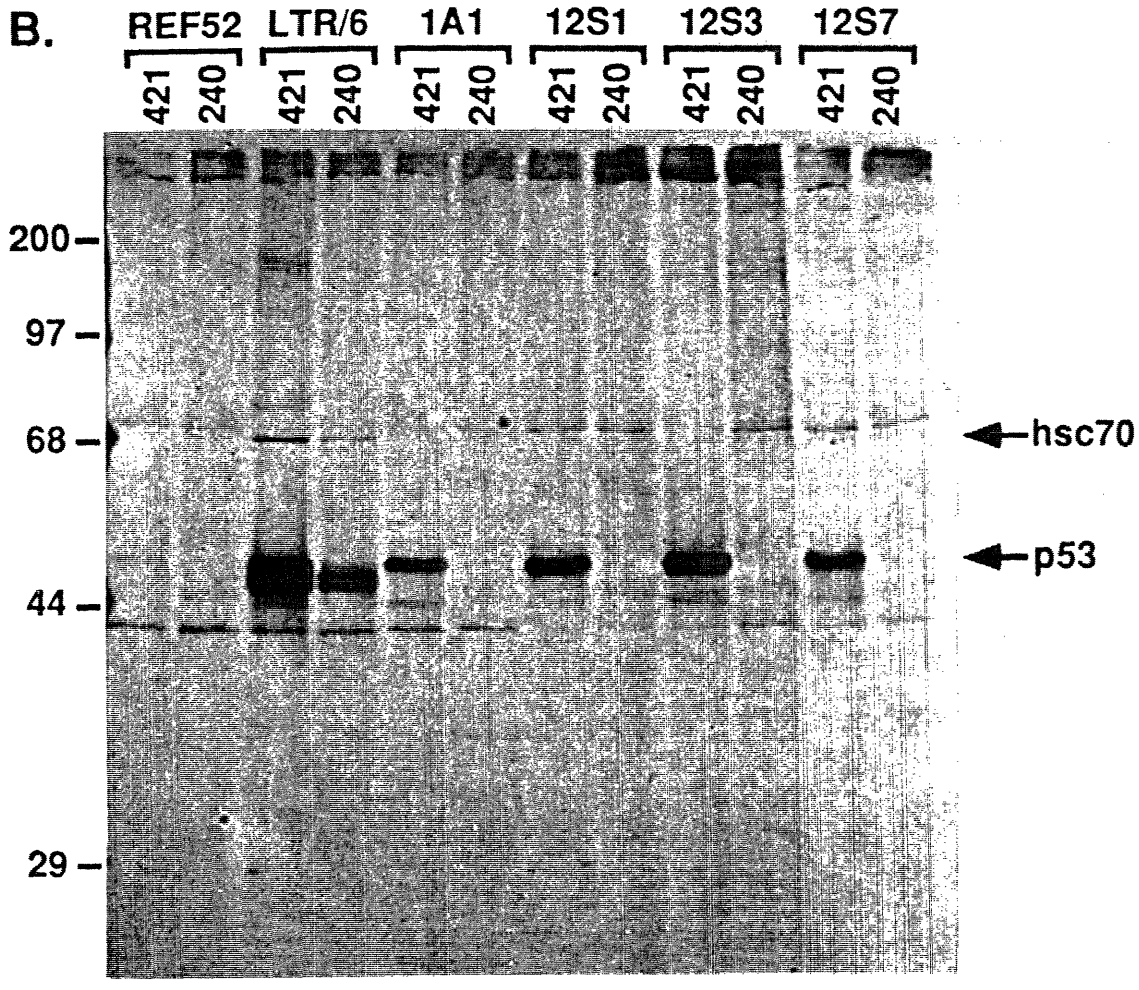
Increased p53 expression requires only the 12S E1A product

Differential splicing generates two E1A transcripts, designated 12S and 13S, which encode proteins of 243 and 289 amino acids, respectively. Sequences required for oncogenic transformation in collaboration with

Figure 1. p53 levels in REF52 cells expressing adenovirus E1A.

Cells were labeled for 4 hours. with ^{35}S-labeled amino acids and p53 was immunoprecipitated using either PAb421 (421) or PAb240 (240). The immunoprecipitated proteins were separated on 10% SDS polyacrylamide gels and visualized by fluorography. (A) p53 levels in cell lines expressing the entire Ad5 E1A gene (next page). (B) p53 levels in cell lines infected with a retrovirus expressing the 243R E1A protein, analyzed after minimal selection in culture (less than 20 population doublings) (second page). REF52, a rat embryo fibroblast line; R53/4, a REF52 clone transformed with T24 H-*ras* and p53^{pro193}; 293, a human adenovirus-transformed cell line; RNA7, REF52 cells transformed by T24 H-*ras* and E1A; 1A1, a REF52 clone expressing Ad5 E1A; LTR/6, REF52 clone expressing p53^{val135}; 12S1, 12S3, and 12S7, REF52 clones expressing the 243R E1A protein. p53 and hsc70 are marked with arrows. The mobility of molecular weight standards (in kilodaltons) are shown at the left of each gel.


A.
REF52
R53/4
293
RNA7
1A1
421
240
200
97
68
44
29
hsc70
p53


B.
REF52
LTR/6
1A1
12S1
12S3
12S7
421
240
200
97
68
44
29
hsc70
p53

activated *ras* oncogenes are contained within the amino-terminal domain shared by the 289R and 243R proteins (Ruley, 1990). Clones 1A1 and RNA7 express both the 243R and 289R proteins (Franza et al., 1986). Therefore, to determine whether the 243R protein was sufficient to increase p53 levels, a 12S cDNA was introduced into REF52 cells by retrovirus-mediated gene transfer, and p53 expression was assessed immediately after a sufficient number of cells were obtained for metabolic labeling (1×10^6 cells, obtained after 20 population doublings). p53 levels in the 12S E1A-expressing cells (clones 12S1, 12S3, and 12S7) were 10 times higher than the parental cell line (Figure 1B), indicating that the 243R protein was sufficient for p53 induction. Unlike $p53^{val135}$, none of the cellular p53s formed complexes with hsc70 or reacted with PAb240.

Nuclear localization of p53 in E1A-expressing cells

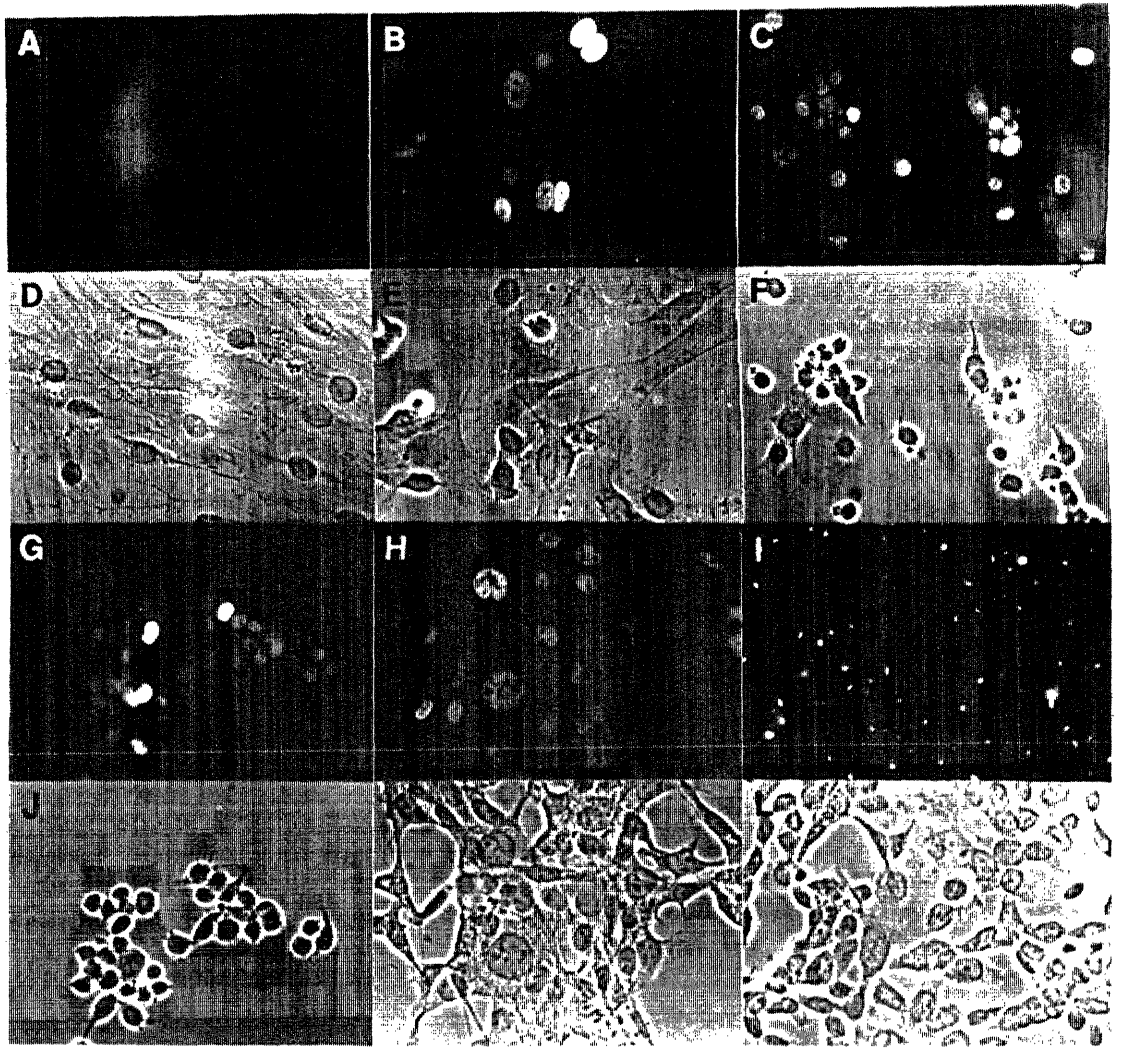
The intracellular localization of p53 was examined by immunofluorescent staining (Figure 2). The staining pattern in REF52 cells was relatively weak, and predominantly nuclear. Moreover, most cells in the population expressed similar levels of p53. Likewise, the p53 in cells expressing the entire E1A gene, only 12S E1A, T24 H-*ras* and E1A, T24 H-*ras* and 12S, E1A was predominantly nuclear. However, anti-p53 fluorescence was considerably more intense and heterogeneous than in normal REF52 cells, suggesting that individual cells expressed variable levels of p53. Finally, cells transformed by E1A and E1B also expressed high levels of p53, but the protein localized within distinct perinuclear regions (Zantema et al., 1985).

Elevated p53 levels in cells transiently expressing E1A

p53 expression was monitored in primary mouse embryo fibroblasts by immunofluorescent staining 36-40 hours after transferring E1A genes by DNA-mediated gene transfer (Figure 3). A β-galactosidase gene (pCH110) was

Figure 2. Localization of p53 in E1A-expressing cells.

Cells were stained with PAb421 and FITC-labeled goat anti-mouse IgG. (A, D) REF52 cells; (B, E) 1A1, clone expressing Ad5 E1A; (C, F) 12S1, clone expressing a 243R E1A cDNA; (G, J) RNA9, clone transformed by E1A and T24 H-*ras;* (H, K) r12S-2, clone transformed by 12S E1A and T24 H-*ras;* (I, L) 1A1XX8, cell line derived following transfer of Ad5 E1B into 1A1 cells. Fluorescent images are shown above phase-contrast photomicrographs of the same field.


A
B
C
D
E
F
G
H
I
J

introduced together with either E1A or control plasmids, to identify cells that had acquired exogenous DNA. The cells were stained with rabbit anti-β-galactosidase antibody and with murine antibodies specific for either E1A or p53, together with a mixture of anti-rabbit IgG (rhodamine) and anti-mouse IgG (fluoroscein) secondary antibodies. Approximately 10% of the transfected cells expressed both E1A and β-galactosidase (Figure 3A & B), and of these, 25-50% expressed discernibly higher levels of p53 (Figure 3C & D). In contrast, higher levels of p53 were not induced in cells transfected with control plasmids (e.g.. Bluescript) and pCH110 (Figure 3E & F). Occasional cells which displayed anti-p53 fluorescence were present in both transfected and untransfected cultures and did not stain with anti-β-galactosidase. Similar results were also obtained following the transfer of E1A genes into REF52 and NIH 3T3 cells (Figure 4), and high p53 levels were induced in HeLa cells infected with adenovirus-5 mutants lacking early region 1B, as judged by western blot analysis (Dr. Eileen White, personal communication). Thus, the induction of p53 by E1A is rapid and occurs in a variety of cell types.

Binding of SV40 large T antigen to p53 in cells expressing E1A

Oncogenic p53 variants lose the ability to bind SV40 large T antigen (Braithwaite et al., 1987; Wang et al., 1989). Various clones were infected by retroviruses expressing SV40 large T antigen, and then analyzed for complexes containing both p53 and large T antigen (Figure 5). As expected, p53 levels in REF52 cells were elevated following the transfer of large T antigen, and p53 was quantitatively precipitated by using antibodies against large T antigen (PAb419). In contrast, introduction of SV40 large T antigen into E1A-expressing cells (e.g.. clone 1A1) had little additional effect on p53 levels, even though all of the p53 coprecipitated with large T antigen. Thus, while SV40 large T antigen and E1A independently increase p53 levels,

Figure 3. Induction of p53 following transient expression of E1A.

Primary mouse embryo fibroblasts were co-transfected with pCH110 and either p1Aneo (A-D) or pBluescript (E, F). After 36-40 hours the cells were incubated with a rabbit anti-β-galactosidase antibody and with murine antibodies specific for either E1A or p53 and stained with a mixture of anti-rabbit IgG (rhodamine) and anti-mouse IgG (fluoroscein) secondary antibodies. Cells in the same field were photographed to show anti-β-galactosidase fluorescence (A, C and E) and either anti-E1A (B) or anti-p53 fluorescence (D, F).

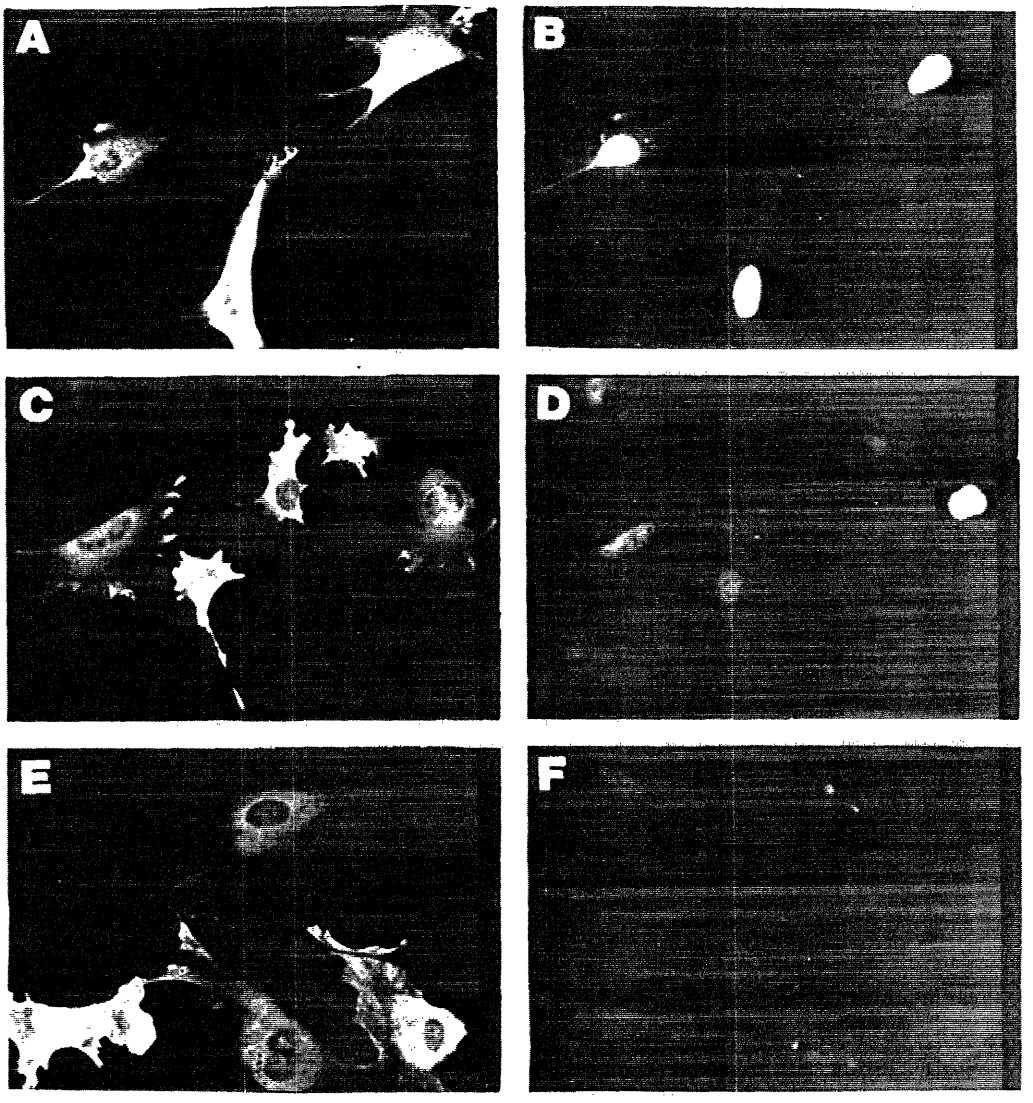

A
B
C
D
E
F

Figure 4. Induction of p53 in 3T3 cells following introduction of E1A.

NIH 3T3 cells were transfected with either p1Aneo (A, C) or pBluescript (B, D). Forty hours after transfection, cells were incubated with murine monoclonal antibodies specific for either E1A (A, B) or p53 (C, D) followed by FITC-conjugated anti-mouse IgG. Photomicrographs are from representative fields.

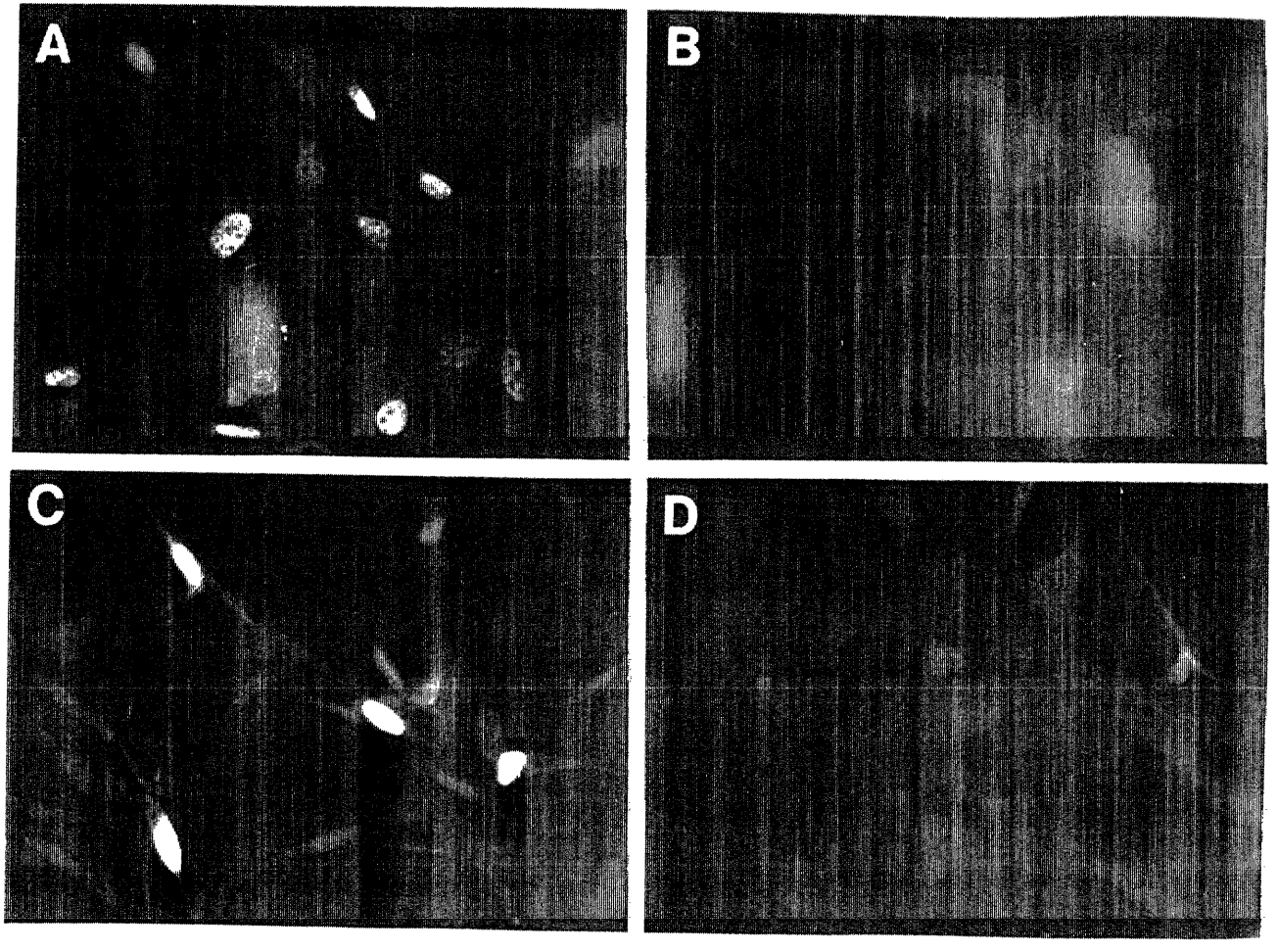
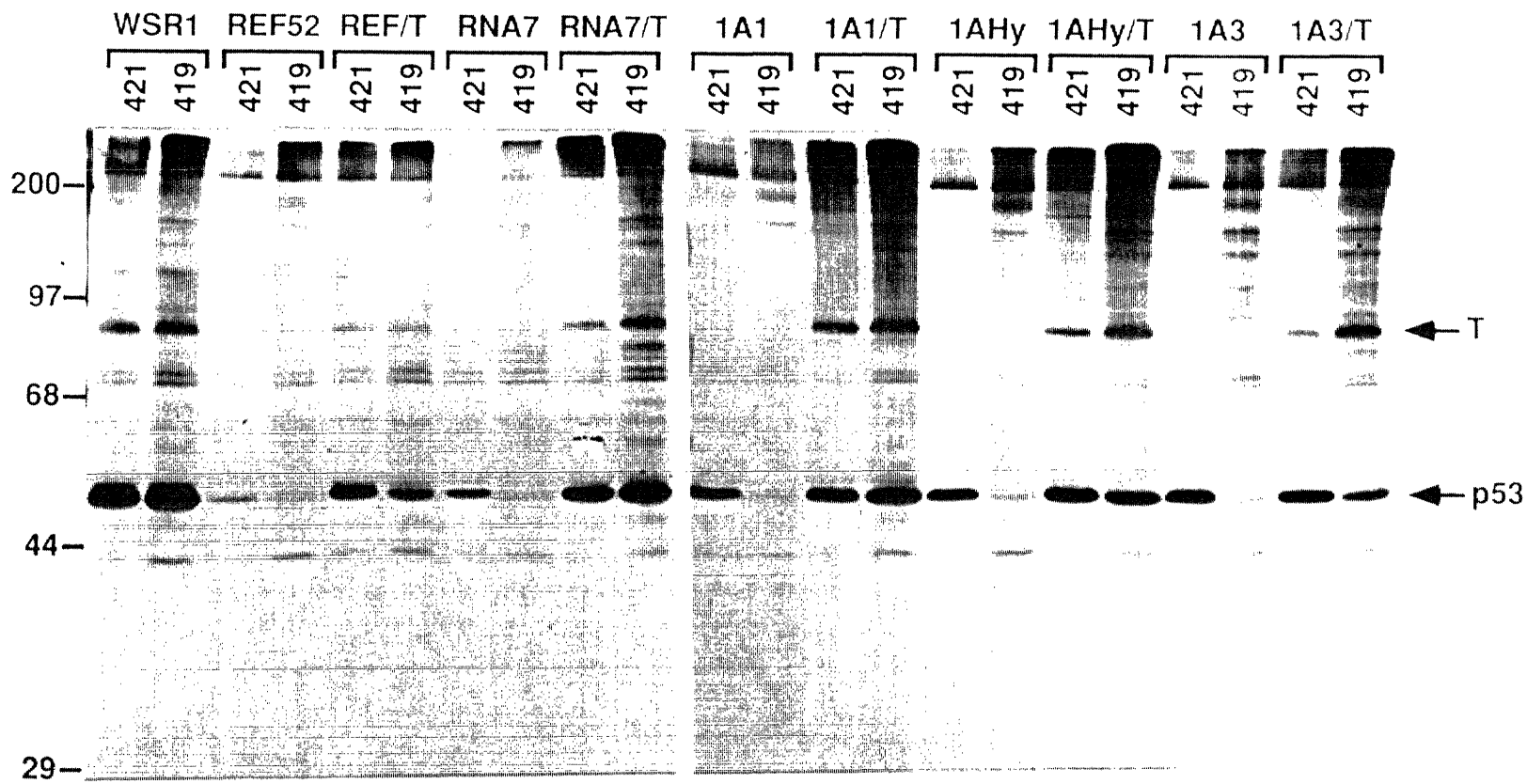

A
B
C
D

Figure 5. Interactions between p53 and SV40 large T antigen in cells expressing E1A .

A temperature-conditional SV40 large T antigen (*tsA*58) was introduced into REF52 cells and clones expressing E1A. Cells were labeled for 4 hours with 100 μCi/ml ^{35}S-labeled amino acids at 33°C, and p53/T antigen complexes were precipitated using monoclonal antibodies against p53 (PAb421) and large T antigen (PAb419). WSR1, REF52 clone transformed by wild-type large T antigen and T24 H-*ras*; RNA7, clone transformed by T24 H-*ras* and E1A; 1A1, 1AHy, and 1A3, clones expressing Ad5 E1A. REF/T, RNA7/T, 1A1/T, 1AHy/T, and 1A3/T are cell populations derived from the same clones following infection with a retrovirus vector expressing *tsA*58. Bands corresponding to SV40 large T antigen (T) and p53 are indicated with arrows. The mobility of molecular size markers are shown on the left.


WSR1
REF52
REF/T
RNA7
RNA7/T
1A1
1A1/T
1AHy
1AHy/T
1A3
1A3/T
421
419
200
97
68
44
29
T
p53

the effects are not additive. Moreover, the p53 stabilized by E1A appears to maintain a wild-type conformation, capable of binding T antigen.

Binding of $p55^{E1B}$ to p53 in cells expressing E1A

These observations suggest that the elevated levels of p53 in adenovirus-transformed cells might result largely, if not entirely, from E1A expression. To examine whether E1B had any additional effect on p53 levels or protein stability, cell lines expressing both E1A and E1B were constructed. Plasmids encoding Ad5 E1A and Ad5 E1B were simultaneously introduced into REF52 cells by co-transfection. Alternatively, E1B was introduced into cells already expressing E1A (clone 1A1). Clones were isolated in hygromycin, expanded, and analyzed after a limited number of passages.

p53 and $p55^{E1B}$ were independently immunoprecipitated from ^{35}S-methionine-labeled cell lysates. All clones expressing E1A and E1B contained $p53/p55^{E1B}$ complexes, yet p53 levels were no higher than in cells expressing E1A alone (Figure 6). A protein of approximately 155kD associated with the $p53\text{-}p55^{E1B}$ complex in cells expressing both E1A and E1B. This protein co-immunoprecipitated with antibodies to either p53 or $p55^{E1B}$; therefore, recovery of the 155 kD protein did not result from antibody crossreaction. The 155 kD protein was not associated with p53 in REF52 cells or in cells expressing E1A or T24 H-*ras* and E1A, suggesting that the protein bound specifically to $p53/p55^{E1B}$ complexes.

Stabilization of p53 in cells expressing E1A

Levels of p53 gene transcripts and rates of protein turnover were measured to determine the mechanism whereby p53 levels were increased. As judged by northern blot analysis, E1A had no obvious effect on p53 gene transcription. The levels of p53 transcripts in cells transformed with T24 H-*ras* and E1A and cells expressing E1A or 12S E1A where comparable to those

Figure 6. Interactions between p53 and $p55^{E1B}$ in cells expressing E1A.

Cells were labeled for 18 hours with 100 μCi/ml ^{35}S-labeled amino acids, and p53/ $p55^{E1B}$ complexes were precipitated using PAb421 (421) and 13D2, an antibody to $p55^{E1B}$. 1AHy, clone expressing Ad5 E1A; 293, human cells expressing Ad5 E1A and E1B; 1AXX3, 1AXX6, 1AXX7, clones derived following co-transfection of E1A and E1B into REF52 cells; 1A1XX6, 1A1XX8; cell lines derived from clone 1A1 following introduction of E1B. The mobilities of p53, $p55^{E1B}$, and a co-precipitating 155kD protein are marked with arrows, and the mobilities of molecular size markers are indicated at the left.

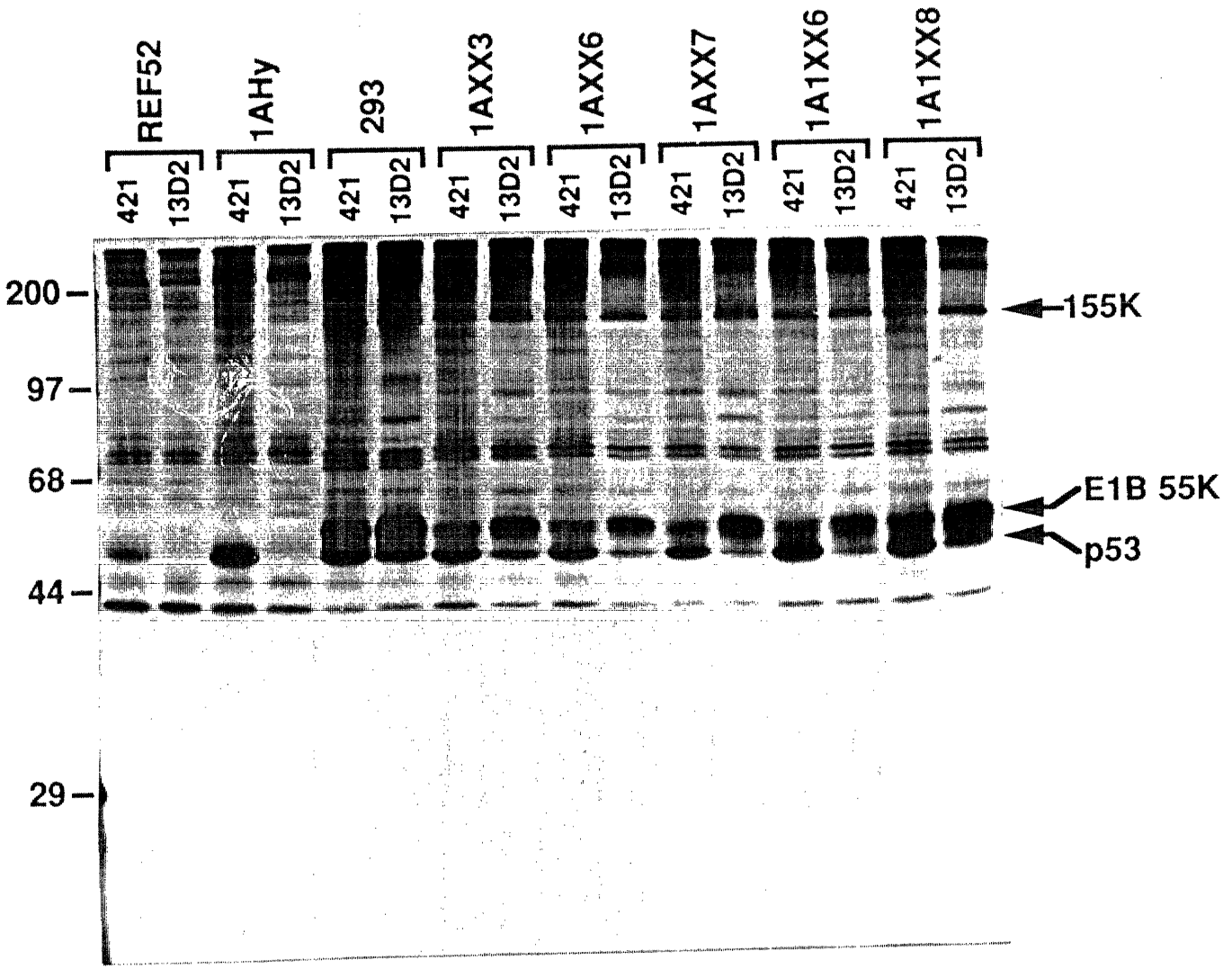
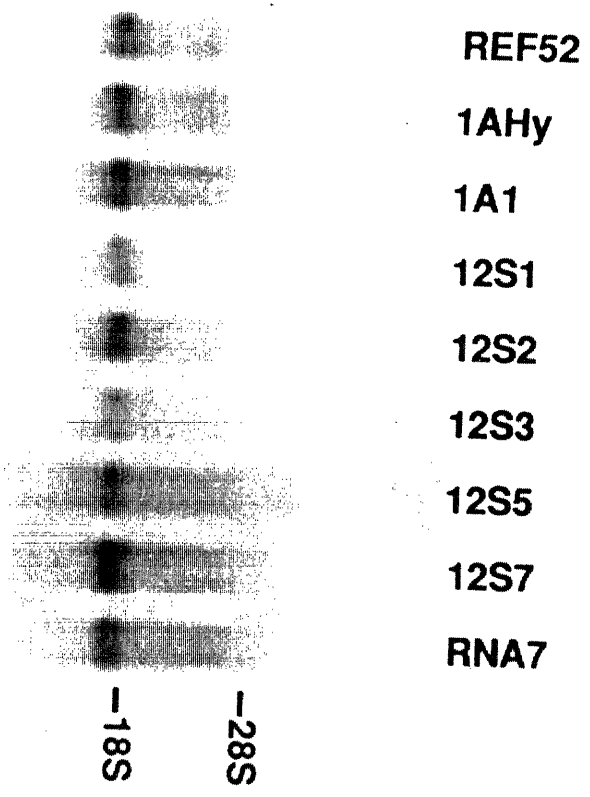

REF52
1AHy
293
1AXX3
1AXX6
1AXX7
1A1XX6
1A1XX8
421
13D2
200
97
68
44
29
155K
E1B 55K
p53

Figure 7. p53 gene expression.

p53 transcripts were detected by northern blot analysis of total RNA following separation on a 1% agarose gel. A ^{32}P-labeled rat p53 cDNA fragment was used as a probe. 1AHy and 1A1, REF52 cells containing Ad5 E1A; 12S1, 2, 3, 5, and 7, clones expressing a 12S E1A cDNA; RNA7, cells transformed by T24 H-*ras* and Ad5 E1A. The locations of the 28S and 18S ribosomal RNAs are shown at the right.


REF52
1AHy
1A1
12S1
12S2
12S3
12S5
12S7
RNA7
18S
28S

present in normal REF52 cells (Figure 7). Therefore, E1A did not appear to effect p53 transcription.

To examine p53 turnover, cells were labeled with ^{35}S-methionine for 1 hour and chased with excess unlabeled methionine for various times and levels of labeled p53 were monitored by immunoprecipitation. Autoradiograms illustrating p53 turnover in representative clones are shown in Figure 8. The half-life of p53 in the parental line was 20-30 minutes, consistent with previously reported values for wild-type protein (Gronostajski et al., 1984; Reich and Levine, 1984). In contrast, p53 in clones expressing E1A were 5-10 times more stable, with half-lives exceeding 2 hours. Moreover, p53 half-lives in cells expressing E1A and those expressing both E1A and E1B were not significantly different. This indicates that E1A is responsible for the increased stability of p53 in at least some cells transformed by human adenoviruses. However, we cannot exclude the possibility that E1B has an independent (but non-additive) effect on p53 levels.

Elevated p53 levels require continuous E1A expression

E1A is selected against, such that clones transfected with E1A expression plasmids frequently lose the gene during serial passaging unless selection is maintained for a linked drug resistance gene or unless the cells are transformed by a collaborating oncogene (e.g. *ras* or E1B). In the present study, cells were allowed to lose E1A in order to study the association between E1A expression and high p53 levels. Clone 1A1, derived from a *neo* resistant colony following transfer of p1Aneo (Franza et al., 1986), was passaged twice weekly in the absence of G418. The cells quickly (3 weeks, approximately 20 population doublings) lost the epithelial morphology characteristically associated with E1A, and became indistinguishable from the parental cells (Figure 9). Loss of E1A expression in the resulting cell population (designated

Figure 8. Stabilization of p53 in cells expressing E1A.

Cells were incubated for 2 hours with media lacking methionine, labeled for 1 hr with 100 μCi/ml ^{35}S-labeled amino acids, washed twice with PBS and chased with excess unlabeled methionine. Cells were lysed at various times, and p53 was immunoprecipitated using PAb421. 1A1 and 1AHy, clones expressing Ad5 E1A; 1AXX3, REF52 clones derived by co-transfecting E1A and E1B; 1A1XX8, cell line derived from 1A1 following transfer of E1B.

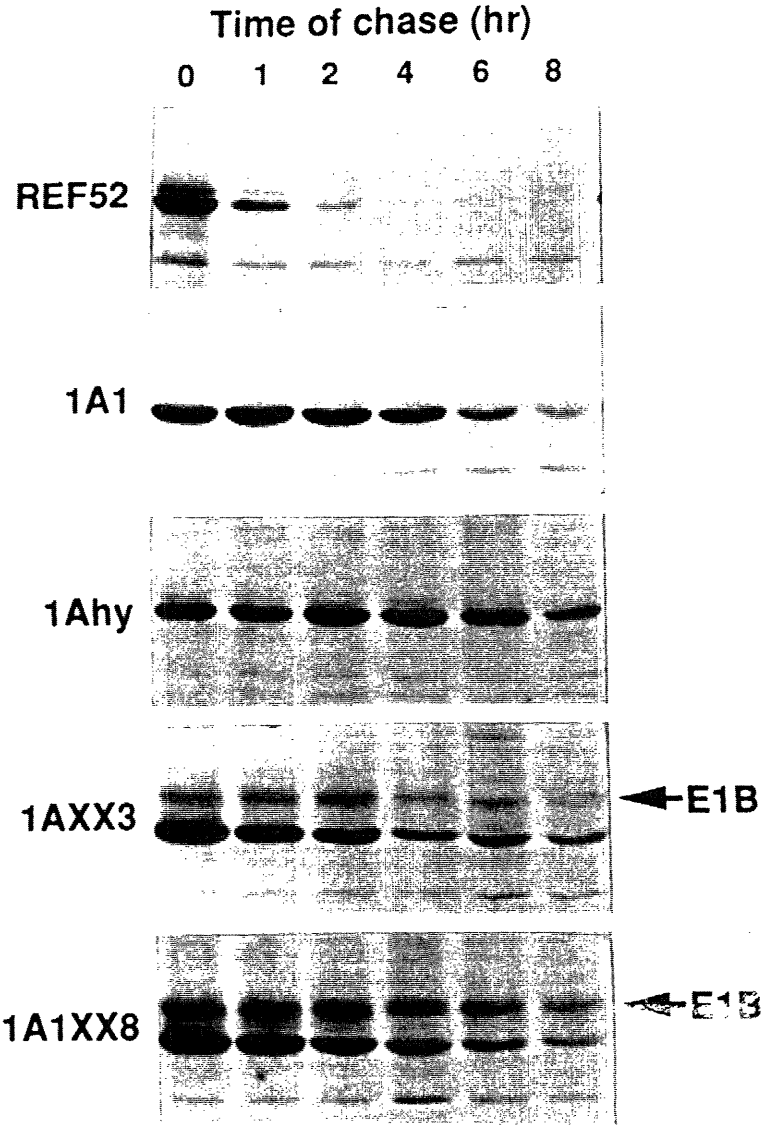
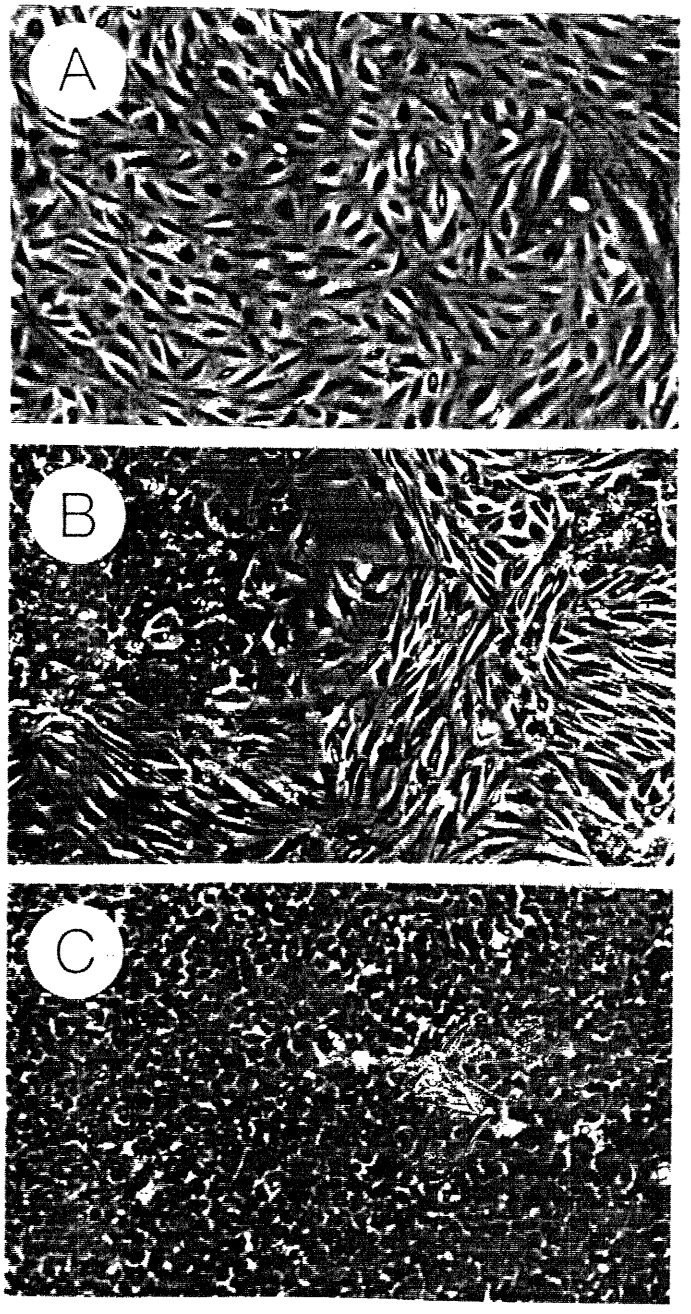

Time of chase (hr)
0
1
2
4
6
8
REF52
1A1
1Ahy
1AXX3
E1B
1A1XX8
E1B

Figure 9. Morphology of revertant 1A1 cells.

Clone 1A1 was sub-cultured (1:8) every 3 days in the presence or absence of G418. (A) normal REF52 cells, (B) 1A1 cells after approximately 12 doublings in the absence of G418 (the field illustrates a mixed population of REF52-like and 1A1-like cells), (C) 1A1 cells maintained in G418.


A
B
C

$1A1_{rev}$) was demonstrated by immunoprecipitation using an E1A-specific monoclonal antibody (Figure 10, right panel). Similarly, p53 levels in $1A1_{rev}$ cells were 10-fold lower than the original 1A1 clone, declining to the levels observed in normal REF52 cells (Figure 10, left panel). These results indicate that the stabilization of p53 requires continuous E1A expression.

Cells expressing E1A undergo apoptosis.

Recently, both E1A and p53 have been shown to induce apoptosis (Yonish-Rouach et al., 1991; Rao et al., 1992; White et al., 1992; Shaw et al., 1992). We therefore examined E1A-expressing REF52 cells for signs of apoptosis; namely, reduced viability of cells grown at high densities or in media containing low serum and degradation of genomic DNA. Cultures of E1A expressing cells contained significant numbers of dead cells when maintained in 10% FBS, as judged by trypan blue dye exclusion (Figure 11A). In contrast, the viability of either REF52 cells or cells transformed by E1A and E1B was nearly 100%. The viability of cells transformed by T24 H-*ras* and E1A was higher than cells expressing E1A alone, except that cell viability frequently declined at high cell densities (not shown). Death of E1A-expressing cells was particularly pronounced in media containing 0.5% calf serum. Although E1B protected against the loss of cell viability associated with E1A, T24 H-*ras* did not.

Genomic DNA was isolated from various clones 3 days after seeding in media containing 10% FBS or 0.5% CS, and analyzed by agarose gel electrophoresis. DNA from E1A expressing clones generated a "ladder" characteristic of internucleosomal cleavage Figure 11B). DNA degradation was particularly pronounced when cells were maintained in low serum. Cells transformed T24 H-*ras* and E1A also contained degraded DNA when

Figure 10. Elevated p53 levels requires continuous E1A expression.

Cells were labeled for 18 hours with ^{35}S-labeled amino acids. p53 was precipitated using either PAb421 (421) or PAb240 (240) and E1A polypeptides were precipitated using a monoclonal antibody, M73. REF52, the parental rat embryo fibroblast line; 1A1, REF52 cells expressing E1A and $1A1_{rev}$, derived by passaging 1A1 cells for approximately 20 population doublings (6 passages) in the absence of G418 selection. Autoradiograms illustrating levels of p53 (left) and E1A (right) are shown. The mobilities of p53, E1A and molecular size markers are indicated.

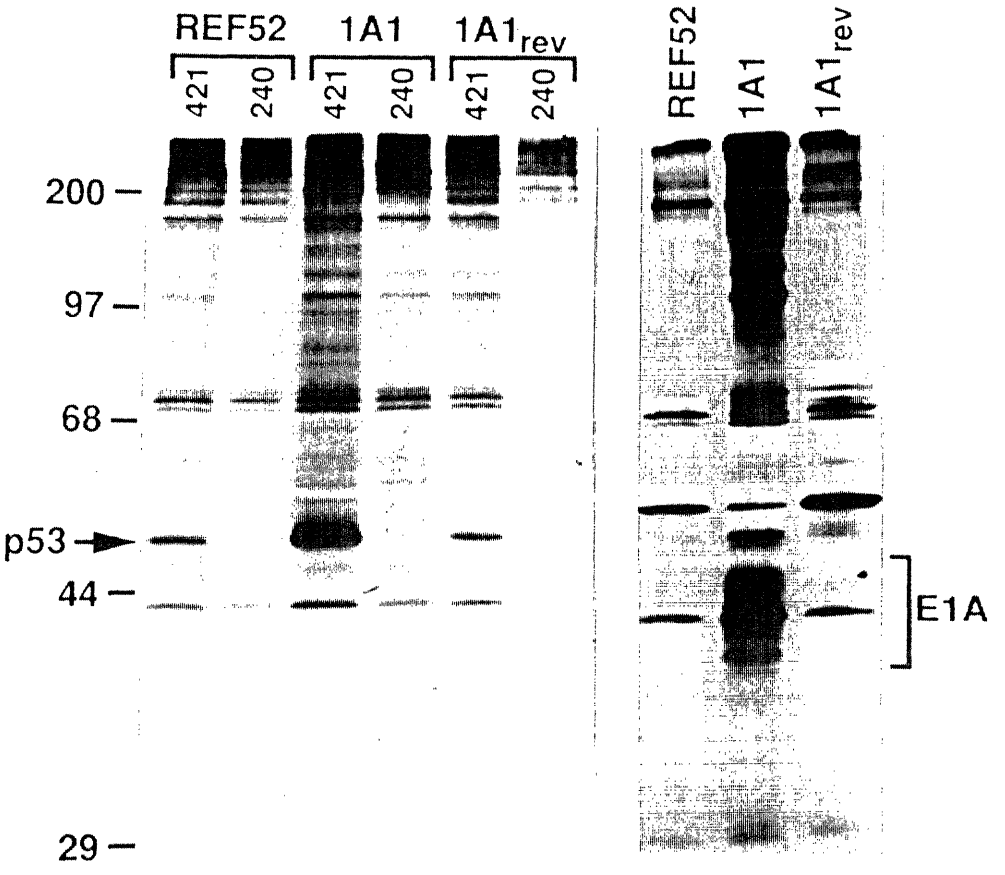
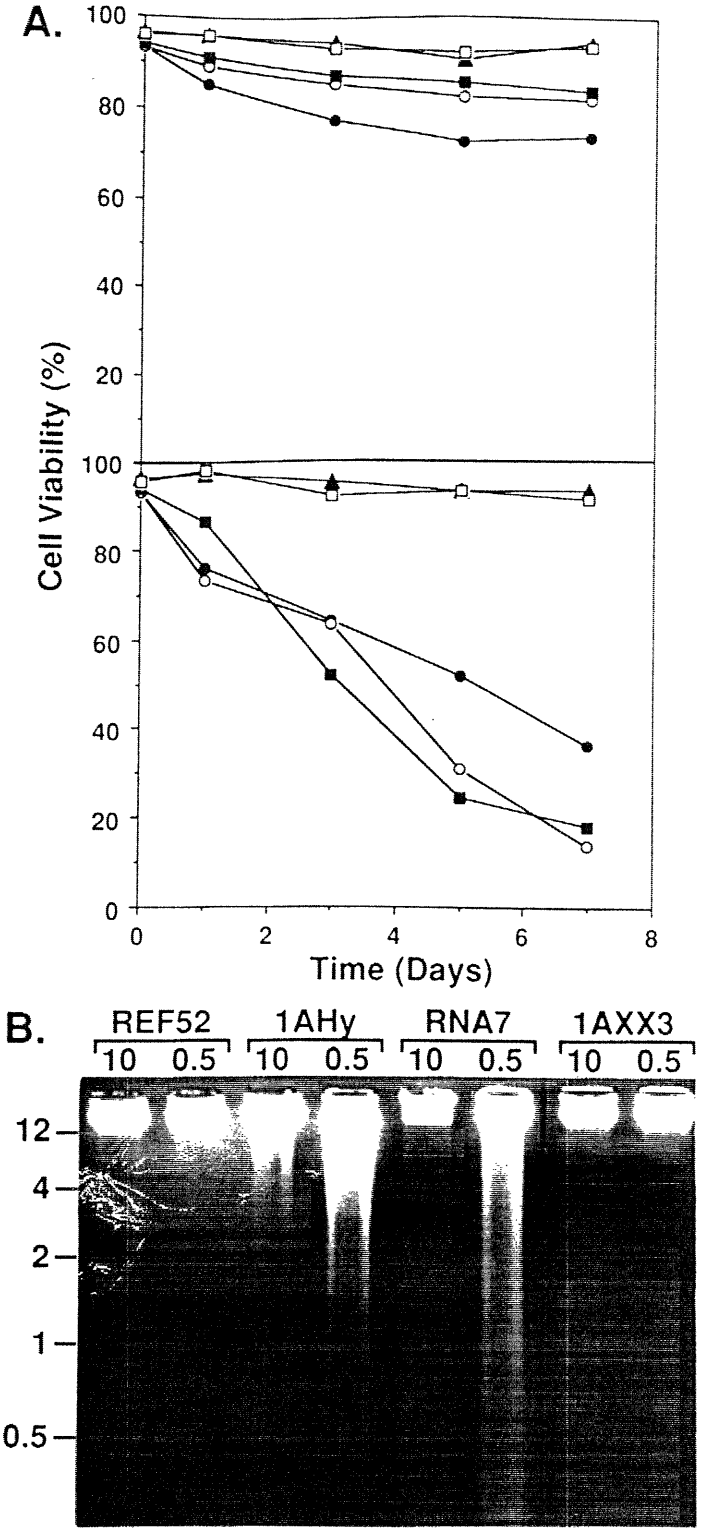

REF52
1A1
1A1rev
421
240
421
240
421
240
200
97
68
p53
44
29
REF52
1A1
1A1rev
E1A

Figure 11. E1A-induced apoptosis.

A. Cells (3×10^4 cells/60 mm dish) were seeded in 10% FCS (top) or 0.5% CS (bottom). At various times, the viability of floating and adherent cells was assessed by trypan blue exclusion. Fresh media was added 3.5 days after seeding. REF52 (triangle); Ad5 E1A (closed circle); 12S E1A (open circle); T24 H-*ras* + E1A (closed circle); Ad5 E1A + Ad5 E1B (open square). Each point represents the average from three independent cell clones. At least 200 cells were counted for each determination.

B. Cells (2×10^5 cells/150 mm dish) were seeded in 10% FCS or 0.5% CS. After 3 days, genomic DNA was isolated from both floating and adherent cells, fractionated by agarose gel electrophoresis and stained with ethidium bromide. REF52, parental line; 1AHy, clone expressing Ad5 E1A; RNA7, clone transformed by T24 H-*ras* and E1A; 1AXX3, clone transformed by E1A and E1B.


A.
Cell Viability (%)
100
80
60
40
20
0
0
2
4
6
8
Time (Days)
B.
REF52
1AHy
RNA7
1AXX3
10
0.5
12
4
2
1
0.5

cultured in low serum; whereas, no DNA degradation was observed in normal REF52 cells or cells transformed by E1A and E1B.

DISCUSSION

E1A stabilizes p53 protein

The stabilization of p53 in cells transformed by adenovirus type 5 has been thought to result from physical interactions with the p55 E1B tumor antigen or from changes associated with oncogenic transformation. However, the present study demonstrates that the metabolic stabilization of p53 can occur in untransformed cells expressing E1A alone. Indeed, neither transformation by *ras* nor binding of $p55^{E1B}$ or SV40 large T antigen extended the half life of p53 above that observed in cells expressing E1A alone.

The mechanism whereby E1A increases the half-life of p53 is unknown. The effect is presumably indirect since the two proteins are not known to interact physically (Egan et al., 1988; Whyte et al., 1989). Moreover, the effects of SV40 large T antigen and E1A on p53 turnover were not additive. While the stabilized p53 was unaltered with regard to electrophoretic mobility, subcellular localization or interactions with monoclonal antibodies and viral tumor antigens, we cannot exclude the possibility that p53 is modified in a manner that makes the protein less susceptible to proteolytic degradation. Alternatively, E1A may affect pathways involved in p53 turnover.

The levels of 53 have been measured in cells expressing different segments of adenovirus early region 1, and have been correlated with the levels of E1B (Zantema et al., 1985; Jochemsen et al., 1987; Mak et al., 1988; van den Heuvel et al., 1990). Consequently, stabilization of p53 has been attributed to interactions with $p55^{E1B}$ and or changes associated with

transformation. However the correlation between p53 and E1B levels is probably indirect, since E1A expression is typically much higher in cells cotransfected with E1A and E1B than in cells transfected with E1A alone (van den Elsen et al., 1983; Senear and Lewis, 1986; Jochemsen et al., 1987; Yoshida et al., 1987). Both cis- and trans-acting effects of E1B on E1A transcription have been reported (Natarajan, 1986; Herrman et al., 1987; Jochemsen et al., 1987; Yoshida et al., 1987). The phenomenon may also reflect selection against cells expressing higher levels of E1A in the absence of E1B (Figure 8).

Stabilized p53 is indistinguishable from wild-type p53

Although conformational changes may stabilize p53, several observations indicate that the p53 induced by E1A is structurally wild-type and therefore is biochemically competent to function as a tumor suppressor. First, p53 levels were elevated in all clones expressing E1A, including those analyzed soon after transfer of E1A. These included cells (primary mouse embryo fibroblasts, mouse 3T3 cells and HeLa cells) known to contain only wild type p53 genes. Second, the p53 was localized to the nucleus and did not associate with hsc70 or react with PAb240. Third, all of the p53 was capable of binding SV40 large T antigen. Fourth, p53 levels reverted to normal as cells lost E1A, whereas, it would be highly unlikely that the cells could concomitantly lose mutant p53 genes. Finally, mutant p53 genes enable *ras* to transform REF52 cells (Hicks et al., 1991), an unlikely interaction if the cells already contain mutant p53.

Is p53 stabilization involved in E1A-associated apoptosis?

Recently, E1A has been found to induce a cytotoxic phenotype resembling apoptosis (White et al., 1991; White et al., 1992; Rao et al., 1992). The phenomenon accounts for the DNA degradation (*deg*) and cytocidial (*cyt*) phenotypes associated with adenovirus strains containing mutations in the

19kD E1B protein and probably contributes to the enhanced sensitivity of E1A-expressing cells to killing by tumor necrosis factor-α (TNF-α). Thus, the 19kD E1B and Bcl-2 proteins each protect cells from E1A-induced apoptosis, and $p19^{E1B}$ protects against killing by TNF-α. Similarly, a significant proportion of REF52 cells expressing E1A are nonviable, such that the E1A gene is quickly lost during serial passaging. Cell death is particularly pronounced in low serum and is accompanied by DNA degradation, as is characteristic of cells undergoing apoptosis.

Several observations suggest that p53 stabilization is involved in E1A-induced apoptosis. First, both E1A and wild-type p53 induce apoptosis (White et al., 1991; White et al., 1992; Rao et al., 1992; Yonish-Rouach et al., 1991; Shaw et al., 1992). Second, p53 stabilized by E1A appears structurally wild-type. Third, p53 levels revert to normal as cells lose E1A. Fourth, E1B physically associates with p53 and allows cells to tolerate E1A. This also suggests that the stabilization of p53 is not simply a consequence of DNA degradation (Maltzman and Czyzyk, 1984; Kastan et al., 1991) or apoptosis. Finally, in an accompanying report (Debbas and White, 1993), $p19^{E1B}$ was found to protect cells from p53-induced apoptosis while a dominant-negative form of p53 ($p53^{val135}$) allowed E1A to transform in the absence of E1B.

Myc, like E1A and dominant transforming forms of p53, can promote the establishment of primary cells into permanent lines and transform primary cells in collaboration with *ras* oncogenes (Ruley, 1990). While Myc can also induce apoptosis (Evan et al., 1992), E1A activities involved in both transformation and apoptosis in REF52 cells appear to be independent of c-*myc*,. In particular, c-*myc* transcription is unaffected by E1A and (Kohl and Ruley, 1987), and the levels of c-*myc* transcripts decline when cells expressing E1A are exposed to conditions (low serum) that promote apoptosis (M.

Ragozzino and H.E.R. unpublished). It will be important to test whether the half-life of p53 changes during Myc-induced apoptosis.

ras oncogenes do not abolish E1A-associated apoptosis

It is perhaps significant that T24 H-_ras_ did not completely protect against E1A-induced apoptosis, even though E1A is not selectively lost from cells co-transformed with T24 H-_ras_. Presumably, a selective advantage provided by _ras_ and/or transformation compensates for cell losses due to E1A. Since cells transformed by _ras_ and E1A are highly tumorigenic, escape from apoptosis appears to be neither a prerequisite for, nor a consequence of, oncogenic transformation in vitro.

Role of E1B in adenovirus transformation

Two region E1B proteins, $p55^{E1B}$ and $p19^{E1B}$, separately collaborate with E1A to transform cultured cells and contribute to the oncogenicity of human adenoviruses (Bernards et al., 1986; Barker and Berk, 1987; White and Cipriani, 1990). Both E1B proteins protect against the consequences of p53 stabilization. Binding of $p55^{E1B}$ blocks p53 functions (Yew and Berk, 1992) and sequesters the protein from the nucleus (Zantema et al., 1985). $p19^{E1B}$, and to a lesser extent $p55^{E1B}$, protect against E1A-induced apoptosis (White et al., 1992; Rao et al., 1992) and $p19^{E1B}$ protects against p53-induced apoptosis (Debbas and White, 1993). Thus, an important, if not the primary, oncogenic role of both E1B proteins is to counter cellular responses to E1A (i.e. stabilization of p53 and associated apoptosis) that preclude transformation by E1A alone. This would also explain why no transforming activities have been attributed to E1B in the absence of E1A (van den Elsen et al., 1983).

p53 stabilization may suppress transformation

In summary, p53 turnover can be regulated in response E1A. This represents the first example of a physiological setting in which high levels of

endogenous p53 are induced in response to an oncogenic challenge, with the apparent consequence of suppressing transformation. Similarly, the stabilization of native p53 and associated apoptosis may provide a defense against the progression naturally occurring tumors *in vivo*.

CHAPTER 3

Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells

This work is in press: Lowe, S. W., Jacks, T., Housman, D. E. & Ruley, H. E. (1994) Proc Natl Acad Sci USA.

INTRODUCTION

The p53 tumor suppressor is the most frequently mutated gene in human tumors (Levine et al., 1991). Presently, the only model to account for the action of p53 as a tumor suppressor views p53 as a "guardian of the genome" (Kastan et al., 1992; Lane, 1992). According to this model, p53 is an essential component of a DNA damage control system which, when operating normally, reduces the likelihood that cells will sustain oncogenic mutations. This view stems from the observations that p53 expression and stability are induced in cells exposed to DNA-damaging agents (Kastan et al., 1991), leading to either cell cycle arrest (which may facilitate DNA repair (Kastan et al., 1992)) or cell death by apoptosis (Lowe et al., 1993b; Clarke et al., 1993). Failure to activate p53 expression following DNA damage may account for the high cancer incidence in individuals with ataxia-telangiectasia (Kastan et al., 1991), and in mice lacking p53 (Lowe et al., 1993b).

Nevertheless, there are reasons to doubt that this indirect mechanism is the only means by which p53 mutation contributes to cancer. First, loss of p53 typically occurs late in tumor progression, after oncogenic mutations have already occurred (Fearon and Vogelstein, 1990; Stretch et al., 1991; Mazars et al., 1991; Yamada et al., 1991; Sidransky et al., 1992). Second, mutant p53 alleles, which can inhibit normal p53 function (Vogelstein and Kinzler, 1992), enable *ras* oncogenes to transform both primary and established cells (Hinds et al., 1989; Hicks et al., 1991). Transformed foci appear within days, and stable transformation requires continuous expression of mutant p53 (Zambetti et al., 1992). Thus, p53 may directly influence both the initiation and maintenance of transformed phenotypes. This view is supported by studies concerning the transforming interactions between the adenovirus early region 1A (E1A) gene and other oncogenes. Primary cells are not

transformed by E1A alone, but are transformed to a tumorigenic state with combinations of E1A and either adenovirus E1B or activated *ras* oncogenes (Ruley, 1990). We recently demonstrated that p53 levels and stability increase in response to E1A, and suggested that stabilized p53 suppressed transformation by enhancing apoptosis (Lowe and Ruley, 1993). Consistent with this hypothesis, proteins that either block p53 transactivation (adenovirus $p55^{E1B}$ and mutant p53) or protect against E1A-associated apoptosis (adenovirus $p19^{E1B}$ and mutant p53) can collaborate with E1A in oncogenic transformation (Yew and Berk, 1992; Vogelstein and Kinzler, 1992; Rao et al., 1992; Debbas and White, 1993).

Although suggestive, the evidence that p53 directly suppresses oncogenic transformation is circumstantial, since it has not been possible to assess the physiological activities of endogenous p53. While forced overexpression of wild-type or mutant p53 can reveal the possible consequences of activating or inactivating p53 function, the relevance of this approach to circumstances in which endogenous p53 acts as a tumor suppressor is unknown. Certainly, proteins not normally involved in cell proliferation might suppress cell growth or viability when sufficiently overexpressed. Furthermore, mutant p53 alleles can transform p53-deficient cells, indicating they are not simply dominant-negative suppressors of wild-type p53 (Dittmer et al., 1993).

In this study, transforming interactions between endogenous p53 and transfected oncogenes were analyzed using embryonic fibroblasts derived from mice carrying disrupted p53 genes. Since the recipient cells differed only in their p53 status, differences between cellular responses to exogenous genes could be unambiguously attributed to p53 function. Embryonic fibroblasts also provide a well-characterized model of multistep carcinogenesis in which

oncogenic transformation typically requires two or more oncogenes acting in concert (Ruley, 1990). These studies indicate that p53 can directly suppress oncogenic transformation by its involvement in apoptosis. Consequently, p53 loss allows transformation of primary cells by a single oncogene.

METHODOLOGY

Cells, plasmids, and gene transfer

$p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were obtained from 12.5 day embryos derived from crosses between mice with a disrupted p53 allele (T.J. and R. Weinberg, unpublished). Cells were cultured in DME containing 10% fetal bovine serum (FBS), and used between passages 3 and 5. p1AHygro contained the adenovirus-5 E1A gene (nucleotides 1-1834) inserted into pY3 (expressing hygromycin phosphotransferase (Blochinger and Diggelmann, 1984)). pT24neo encodes a *ras* oncogene (T24 H-*ras*) (Franza et al., 1986) and p5XX encodes the entire E1B gene. pLTRKH215 and pLTRcGala expressed a dominant-transforming p53 allele ($p53^{KH215}$) and mouse wild-type p53, respectively (Hinds et al., 1989). Stable lines expressing E1A were generated by calcium phosphate precipitation (Franza et al., 1986) using 1 μg p1AHygro and a 10-fold molar excess of pT24neo, p5XX, pLTRKH215, or pLTRcGala. Alternatively, pY3 was used at an equivalent molar amount to 1 μg p1AHygro. For each precipitation, the total mass of DNA was adjusted to 20 μg using pBluescript. After transfection, cultures were sub-cultured into medium containing either 100 μg/ml ($p53^{-/-}$ MEFs) or 15 μg/ml ($p53^{+/+}$ and $p53^{+/-}$ MEFs) Hygromycin B (Sigma), concentrations which were determined empirically. After 2-3 weeks, isolated clones from each transfection were expanded and colony numbers were estimated after crystal violet staining.

Viability assays

E1A-expressing colonies are morphologically distinct from normal MEFs, and display E1A-specific immunofluorescence staining (see below). Three weeks after transfection, colonies were marked and transferred to medium containing 0.1% FBS. Colonies were photographed at various times and scored for regression after 72 hours. Alternatively, cell lines were seeded at 1-2 x 10^6 cells/100mm plate, and subsequently transferred to medium containing 0.1% FBS. At various times, adherent and non-adherent cells were pooled for viability measurements by uptake of fluorescein isothiocyanate (FITC) and flow cytometry (Shi, et al., 1990). Cells were incubated in medium containing 40 mg/ml FITC for 15 minutes at 37°C. Subsequently, cells were washed in PBS, and fixed in 5% formalin (v/v), and analyzed by flow cytometry. At least 10^5 cells were analyzed for each determination.

For analyzing DNA fragmentation, low molecular weight DNA was purified from pools of adherent and non-adherent cells 24 hours after transfer to 0.1% FBS. Cells were washed in phosphate buffered saline (PBS), and resuspended in ice cold buffer containing 0.15M NaCl, 10mM Tris (pH 7.4), 2 mM $MgCl_2$, and 1mM DTT. Nonidet-P40 was added to a final concentration of 0.5% (v/v) and the samples were incubated on ice for 30 minutes. Nuclei were isolated by centrifugation, resuspended in buffer containing 0.35M NaCl, 10mM Tris (pH 7.4), 2mM $MgCl_2$, and 1mM DTT, and incubated on ice for 15-30 minutes. The nuclei were then removed by centrifugation, and the supernatant was extracted with phenol and chloroform. The low molecular weight DNA was recovered by ethanol precipitation. Samples were resuspended in 20 µl Tris-EDTA and treated with RNase A for 30 minutes prior to electrophoresis on 1% agarose gels.

p53 expression and cell cycle analysis

E1A, p53, and β-galactosidase-specific immunofluorescent staining was performed described in Chapter 2. p53 half-life was estimated by p53 immunoprecipitation of ^{35}S-labeled cell lysates as described in Chapter 2. Western blot analysis was performed using lysates derived from 10^6 cells of each sample. For each sample, cells were washed in PBS and lysed in Laemmli buffer (Harlow and Lane, 1988). The proteins were separated on 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked and probed with a pool of p53-specific monoclonal antibodies (PAb421, PAb240, and PAb248) (Yewdell et al., 1986; Gannon et al., 1990). p53 was detected using an alkaline phosphatase-conjugated secondary antibody and a chemiluminescent substrate ((Haldi and Guarente, 1989; Isaacs et al., 1991); Lumi-Phos 530, Boehringer-Mannheim)).

Cell cycle analysis

For cell cycle analysis, cells were plated at 1-2 x 10^6 cells/100mm dish and were incubated with 5-bromo-2'-deoxyuridine (BrdU) for 4 hours, beginning 14 hours after transfer to 0.5% FBS. Subsequently, cultures were washed to remove dead cells, and the adherent cells were collected after treatment with trypsin and prepared for flow cytometry (van Erp et al., 1988). The cells were washed in PBS and fixed in 70% ethanol for 30 minutes at -20°C. Subsequently, the cells were centrifuged, resuspended in 1 ml of 0.2 mg/ml pepsin (Sigma) in 2N HCl, and incubated for 30 minutes at room temperature. After neutralizing the samples with 3 ml 0.1M $Na_2B_40_7$, the cells were washed with PBS followed by PBS supplemented with 0.5% Tween20 and 2% FBS (PTS). The cells were resuspended in 100 µl of PTS containing 5 µl of an FITC-conjugated anti-BrdU antibody (Boehringer Mannheim) and incubated for 30 minutes at room temperature. The cells were then washed

with PTS, resuspended in 0.5 ml PTS containing 0.5 mg/ml RNase A and 50 μg/ml propidium iodide (Calbiochem), and incubated for at least 30 minutes at room temperature. All samples were passed though 70 μm mesh prior to FACS analysis. FACS analysis was performed on a FACStar Plus (Becton Dickinson) flow cytometer. The percentage of cells in each phase of the cell cycle (at the end of the BrdU labeling period) was estimated using the MODFIT program (Verity Software House). The co-efficient of variance for each fit was usually less than 5%. The percentage of cells incorporating BrdU during the 4 hour pulse was estimated from the log red fluorescence and log green fluorescence plot using the disp2D program (Becton Dickinson).

Tumorigenicity experiments

Tumorigenic potential was measured by the ability of cells to form progressive tumors in athymic nude mice. To avoid selection for a transformed phenotype, hygromycin-resistant clones were expanded minimally prior to injection. 4-6 week male nude mice (Swiss nu/nu, Taconic) were injected with 2 x 10^6 cells and monitored for tumors at the injection sites for approximately 9 months. Tumors were scored positive when they became clearly visible (~2mm diameter).

RESULTS

Introduction of E1A into $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ mouse embryo fibroblasts

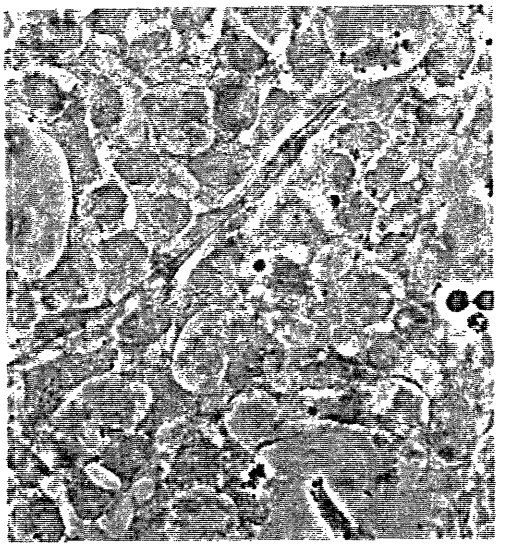
Both E1A and mutant p53 alleles facilitate immortalization of primary cells in culture (Houweling et al., 1980; Jenkins et al., 1984). As an initial step in analyzing interactions between E1A and endogenous p53, we compared the ability of E1A (alone, or with other oncogenes) to promote clonal outgrowth. E1A was introduced into $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs using p1AHygro, a plasmid that co-expresses adenovirus-5 E1A and hygromycin

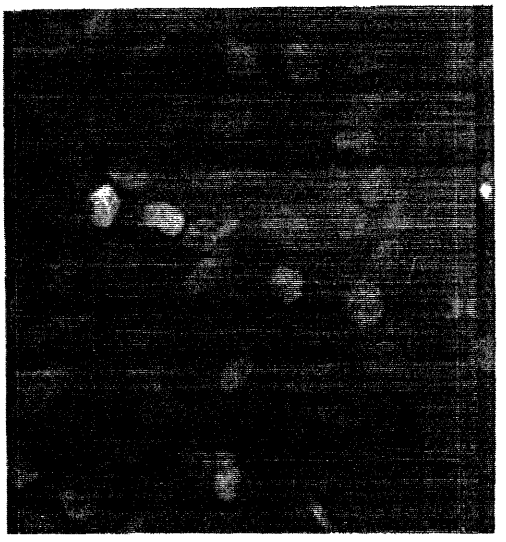
phosphotransferase. Thus, colonies arising in hygromycin B had a high probability of expressing E1A. E1A-specific immunofluorescence of a representative hygromycin B-resistant colony is shown in Figure 1. A plasmid expressing only hygromycin phosphotransferase (pY3) was used to assess the effects of endogenous p53 genes on clonal outgrowth. While $p53^{+/+}$ and $p53^{+/-}$ MEFs transfected with pY3 produced very few colonies (3 and 22/10^6 cells, respectively), $p53^{-/-}$ MEFs generated many colonies (466/10^6 cells) (Figure 2). Thus, the absence of endogenous p53 resulted in efficient clonal outgrowth.

The ability of E1A to produce colonies correlated with p53 dosage. E1A was inefficient at promoting clonal outgrowth in $p53^{+/+}$ MEFs; transfection of p1AHygro averaged only 8 colonies/10^6 cells (Figure 2A). A 5-10 fold increase in colonies was obtained when p1AHygro was co-transfected with plasmids expressing either adenovirus-5 E1B, T24 H-*ras*, or a mutant p53 allele. $p53^{+/-}$ MEFs express less p53 than wild-type MEFs (Livingstone et al., 1992), and transfection of p1AHygro into $p53^{+/-}$ cells produced many more colonies than in $p53^{+/+}$ MEFs (254/10^6 cells). Still, colony numbers increased approximately two-fold when E1A was co-introduced with either E1B, T24 H-*ras*, or mutant p53 (Figure 1B). Transfection of p1AHygro into $p53^{-/-}$ MEFs generated as many colonies as any oncogene combination (526/10^6 cells). Wild-type p53 significantly reduced colony numbers in $p53^{-/-}$ MEFs (Figure 2C), and of those that did emerge and were analyzed, none (0/3) expressed detectable p53 immunofluorescence (not shown). Since endogenous p53 levels are increased in response to E1A (Lowe and Ruley, 1993) and p53 overexpression causes either growth arrest or apoptosis (Levine et al., 1991; Yonish-Rouach et al., 1991), the increase in p53 levels that accompanies E1A expression may suppress clonal outgrowth.

Figure 1. E1A-specific immunofluorescence of cells transfected with p1AHygro following selection in Hygromycin B.

p1AHygro was introduced into $p53^{+/+}$ MEFs and E1A-expressing colonies were selected in hygromycin B. Approximately 3 weeks later, colonies were analyzed for E1A-expression by immunofluorescent staining. Top, phase contrast photomicrograph of a representative colony; bottom, E1A-specific immunofluorescence of the same field.





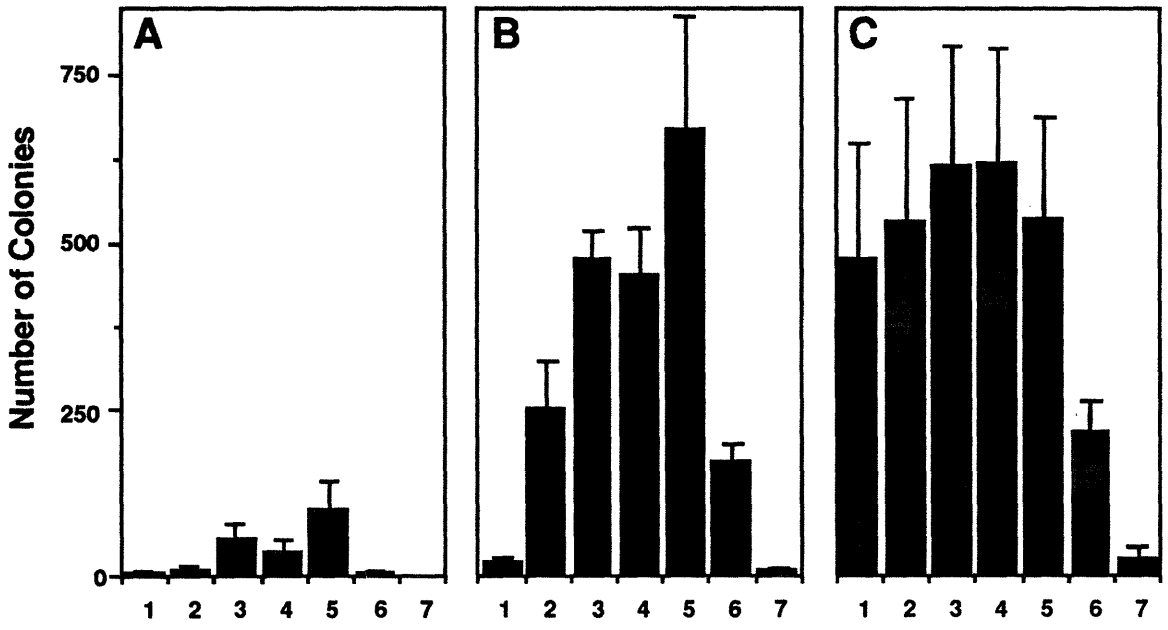


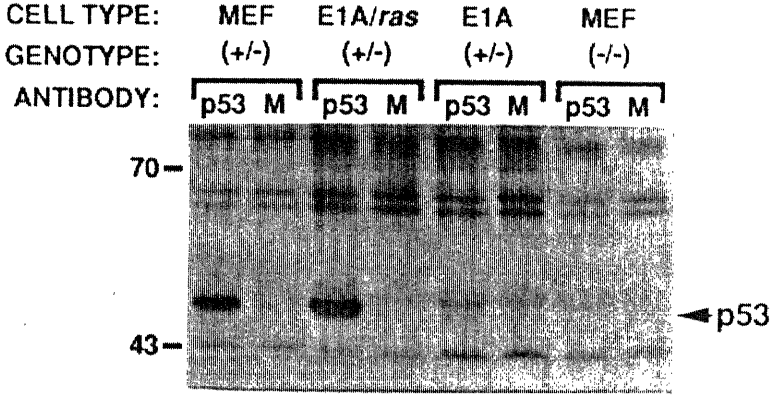
Figure 2. Introduction of exogenous genes into MEFs.

Transfection of various plasmids into (A) $p53^{+/+}$, (B) $p53^{+/-}$, and (C) $p53^{-/-}$ embryonic fibroblasts (10^6 cells/transfection). The transfected plasmid(s) expressed the following: column 1, hygromycin phosphotransferase; 2, E1A; 3, E1A and E1B; 4, E1A and T24 H-*ras*; 5, E1A and a mutant p53; 6, E1A and wild-type p53; and 7, no DNA. The E1A expression vector (p1AHygro) co-expressed the adenovirus-5 E1A gene and hygromycin phosphotransferase, allowing isolation of E1A-expressing colonies in medium containing hygromycin B. Colony numbers were estimated approximately 2 weeks after transfection. The values represent the average and standard deviation determined from at least 3 transfections.

Attempts to expand E1A-expressing colonies into stable cell lines revealed striking differences among fibroblasts of the three genotypes. Most $p53^{-/-}$ colonies expressing E1A (12 of 15) or hygromycin phosphotransferase (3 of 4) were established into permanent lines. By contrast, E1A-expressing colonies derived from $p53^{+/+}$ MEFs rarely reached a size suitable for transfer (500-1000 cells), and none (0/3) could be established. $p53^{+/+}$ colonies were established when E1A was co-expressed with either E1B (5/6), T24 H-*ras* (6/10), or mutant p53 (9/16). Although many colonies of E1A-expressing $p53^{+/-}$ MEFs were obtained (see Figure 1B), only one clone (1/10) produced a

Figure 3. p53-immunoprecipitation of clone 1A.B1.

Clone 1A.B1 was incubated with 100 μCi/ml ^{35}S-labeled methionine for 2 hours. Immunoprecipitations were performed on cell lysates using a p53-specific (p53; PAb421) and control (M; PAb240) antibody as described in Chapter 2. Immunoprecipitations from untransfected $p53^{+/-}$ and $p53^{-/-}$ MEFs, and a $p53^{+/-}$ clone co-expressing E1A and T24 H-*ras* (which was readily established into a permanent line) are shown for comparison.


CELL TYPE:
MEF
E1A/ras
E1A
MEF
GENOTYPE:
(+/-)
(+/-)
(+/-)
(-/-)
ANTIBODY:
p53 M
p53 M
p53 M
p53 M
70
43
p53

permanent line. Immunoprecipitation analysis indicated this clone did not express p53 (Figure 3). Therefore, in the absence of other oncogenes, the combination of E1A and endogenous p53 was incompatible with long term growth.

p53-dependent death of cells expressing E1A

E1A can induce apoptosis, particularly following serum depletion, and E1B inhibits apoptosis (Rao et al., 1992; Lowe and Ruley, 1993). Since E1A increases p53 levels (Lowe and Ruley, 1993) and p53 is necessary for some forms of apoptosis (Lowe et al., 1993b; Clarke et al., 1993), we tested whether p53 was required for E1A-associated cell death. Although stable lines co-expressing E1A and endogenous p53 were not established, the availability of unexpanded colonies provided a means to analyze the effects of E1A on cell viability. Therefore, E1A-expressing colonies (with or without E1B) were marked, transferred to medium containing 0.1% fetal bovine serum (FBS), and inspected for growth or regression. Although the untransfected fibroblasts remained viable in 0.1% FBS (Figure 4), the majority of $p53^{+/+}$ and $p53^{+/-}$ colonies expressing E1A completely regressed by 72 hours after serum withdrawal (8/8 and 23/25 colonies regressed, respectively; illustrated in Figure 5). In contrast, $p53^{-/-}$ colonies remained viable in 0.1% FBS (2/25 colonies regressed) and many continued to grow. Although E1B enhanced the viability of $p53^{+/+}$ colonies expressing E1A in 0.1% FBS, the increase was less than the effect of p53 absence (6/12 colonies regressed). Therefore, p53 deficiency substituted for E1B in suppressing E1A-associated cell death. Because multiple clones were analyzed prior to significant growth in culture, resistance to death was not due to genetic alterations occurring upon clonal expansion.

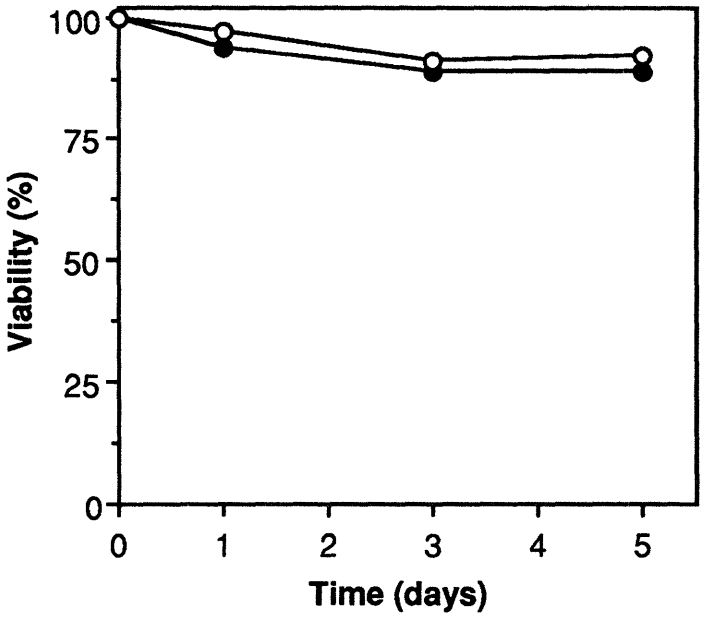


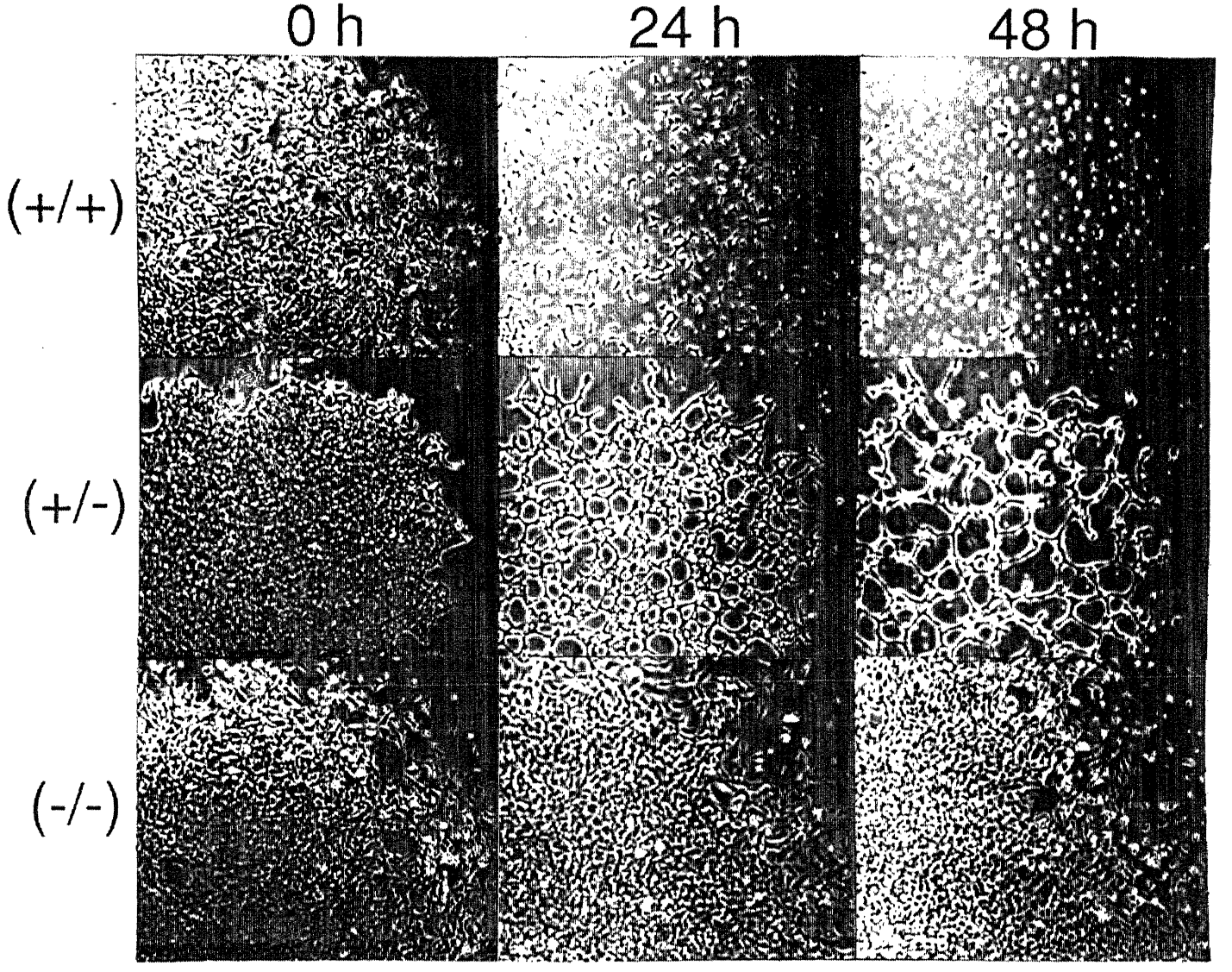
Figure 4. Viability of untransfected MEFs in 0.1% FBS.
$p53^{+/+}$ (closed circles) and $p53^{-/-}$ (open circles) MEFs were transferred to medium containing 0.1% FBS. At the indicated times, cell viability was determined from pools of adherent and non-adherent cells by the trypan blue exclusion method. At least 200 cells were counted for each determination.

ras oncogenes do not inhibit p53-dependent apoptosis

Like E1B, T24 H-*ras* allowed establishment of E1A-expressing colonies containing endogenous p53. To test whether T24 H-*ras* also prevented p53-dependent cell death, cell lines expressing E1A were transferred to medium containing 0.1% fetal bovine serum (FBS) and cell viability was measured by flow cytometry (see representative data in Figure 6) at various times thereafter (Figure 7). While all cells remained viable in 10% FBS, $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras* died rapidly in 0.1% FBS. $p53^{+/-}$ cells co-expressing E1A and T24 H-*ras* also died in 0.1% FBS, but less rapidly than wild-type cells. In contrast, all $p53^{-/-}$ lines remained viable in low serum, as did $p53^{+/+}$ cells co-expressing E1A and E1B. Cells undergoing apoptosis typically activate an endonuclease that cleaves between nucleosomes, resulting in degradation of genomic DNA (Wyllie, 1980). $p53^{+/+}$ cells co-

Figure 5. Regression of E1A-expressing colonies upon serum depletion.

p1AHygro was introduced into $p53^{+/+}$ (+/+), $p53^{+/-}$ (+/-), and $p53^{-/-}$ (-/-) MEFs and colonies were isolated by selection in hygromycin B. After 3 weeks, E1A-expressing colonies were marked and photographed at the indicated times following transfer to medium containing 0.1% FBS (without hygromycin B).


0 h
24 h
48 h
(+/+)
(+/-)
(-/-)

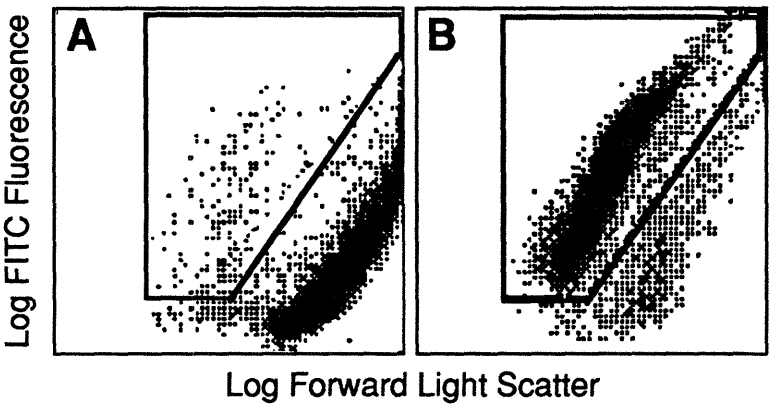


Figure 6. Analysis of cell viability by uptake of FITC and flow cytometry. Adherent and non-adherent cells were pooled, incubated with FITC, and analyzed by flow cytometry. Quantitation was performed using Coulter software. (A) untreated MEFs co-expressing E1A and T24 H-*ras* (5% non-viable); (B) MEFs co-expressing E1A and T24 H-*ras* 72 hours after transfer to 0.1% FBS (89% non-viable).

expressing E1A and T24 H-*ras* contained large amounts of degraded DNA after transfer to 0.1% FBS, whereas cells lacking p53 or expressing E1B did not (Figure 8). The degraded DNA was present in multiples of approximately 180-200 base pairs, consistent with internucleosomal DNA cleavage and cell death by apoptosis.

p53 expression and cell proliferation during apoptosis

Wild-type cells co-expressing E1A and T24 H-*ras* contained elevated p53 levels, resulting from a 5-10 fold increase in protein stability (Figure 9A). The increase was probably due to E1A effects, since transient introduction of E1A (but not T24 H-*ras*) increased p53 levels (Figure 10). Interestingly, p53 levels in cells co-expressing E1A and T24 H-*ras* were higher than MEFs treated with 5 Gy ionizing radiation (a treatment that also stabilizes p53 (Kastan et al., 1991)) (Figure 9B). However, p53 levels did not increase further upon transfer to 0.1% FBS, indicating that high p53 levels were not sufficient for apoptosis.

Since E1A sequences required for apoptosis are identical to those required for induction of DNA synthesis (White et al., 1991), we tested

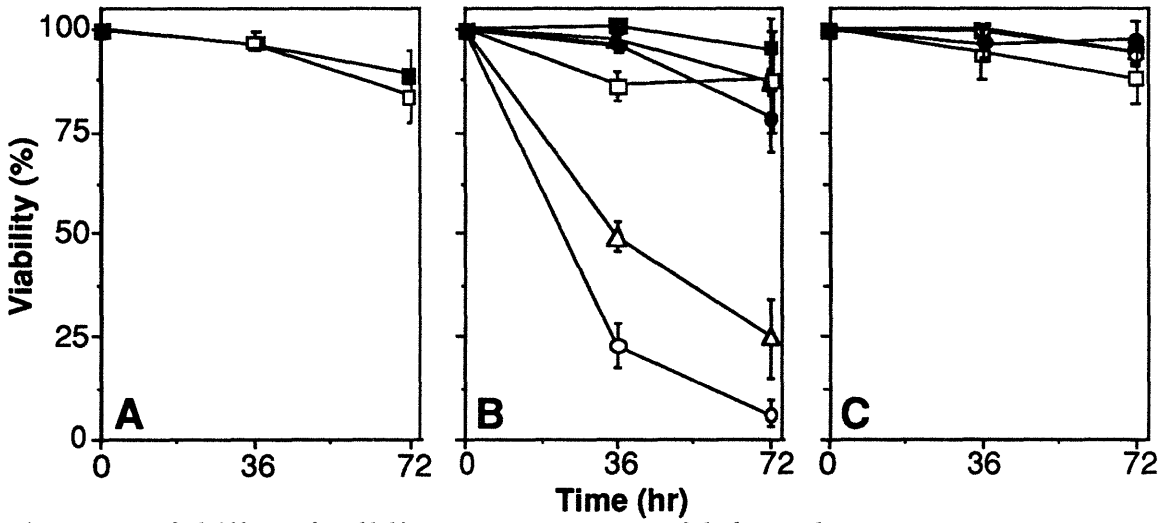


Figure 7. Viability of cell lines upon serum withdrawal.

Viability of cells expressing E1A (A), E1A and T24 H-*ras* (B), and E1A and E1B (C) in media containing either 10% FBS (closed symbols) or 0.1% FBS (open symbols) was measured by uptake of FITC and flow cytometry. Cell lines were derived from $p53^{-/-}$ (squares), $p53^{+/-}$ (triangles), and $p53^{+/+}$ (circles) MEFs. Each values represents the average and standard deviation obtained from at least 3 independent clones, and were normalized to the percentage of viable cells in each population at the start of the experiment (generally >90%).

whether p53-dependent apoptosis might be triggered by unscheduled proliferation. $p53^{+/+}$ and $p53^{-/-}$ MEFs and their E1A-expressing derivatives were incubated with 5-bromo-2'-deoxyuridine (BrdU) for 4 hours beginning 14 hours after transfer to medium containing 0.5% FBS. Cells were analyzed for DNA content (by propidium iodide staining) and DNA synthesis (by BrdU incorporation) using multiparameter flow cytometry. These data are illustrated in Figure 11 and summarized in Table 1. $p53^{+/+}$ MEFs arrested rapidly upon serum withdrawal (Figure 11A, B), although $p53^{-/-}$ fibroblasts exited the cell cycle more slowly (Figure 11 C, D). In 0.1% FBS, cells expressing E1A continued to proliferate with no reduction in BrdU-positive cells, even though many $p53^{+/+}$ cells had initiated apoptosis (compare Figure 11B and 11F). Although these results do not distinguish between the effects of E1A or T24 H-*ras*, cells that tolerate E1A expression alone also proliferate after serum

Figure 8. Analysis of low molecular weight DNA from cells incubated in 0.1% FBS.

Low molecular weight DNA was isolated from 2 x 10^6 cells 24 hours after transfer to 0.1% FBS. DNA was resolved on 1% agarose gels and visualized by ethidium bromide staining. Since samples were normalized by cell number, viable cells contained almost no low molecular weight DNA.

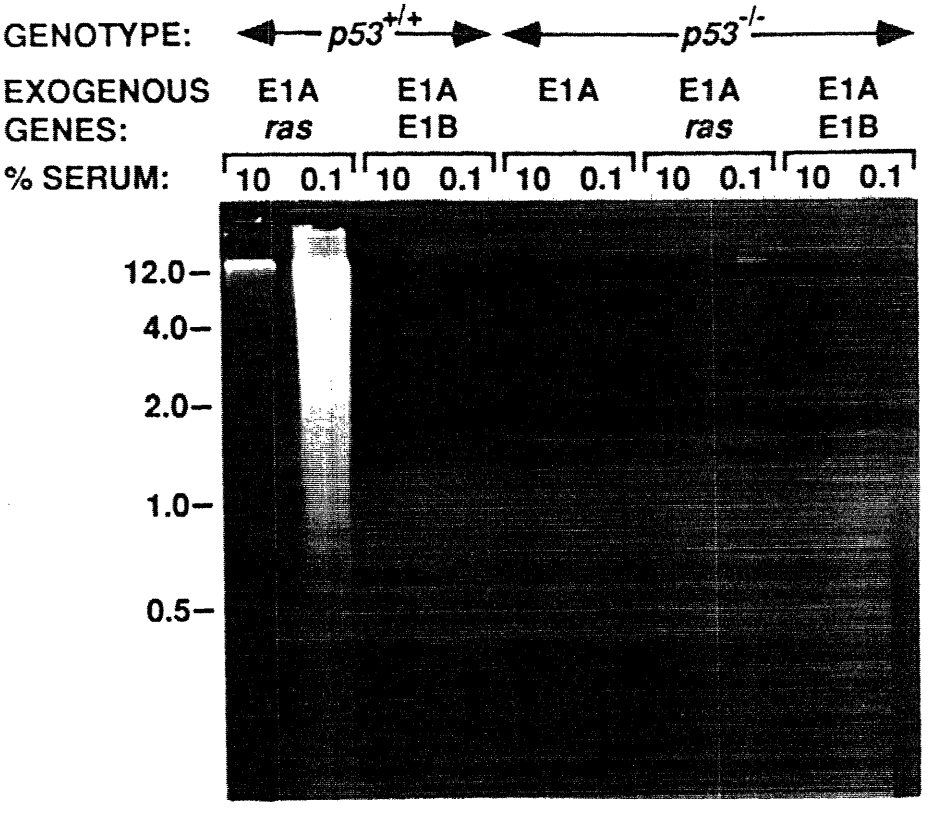

GENOTYPE:
p53+/+
p53-/-
EXOGENOUS GENES:
E1A ras
E1A E1B
E1A
E1A ras
E1A E1B
% SERUM:
10 0.1 10 0.1 10 0.1 10 0.1 10 0.1
12.0
4.0
2.0
1.0
0.5

Figure 9. p53 expression in cells co-expressing E1A and T24 H-*ras* during apoptosis.

A) Cells were incubated for 2 hours with methionine-deficient medium, labeled for 1 hr with 100 μCi/ml ^{35}S-labeled amino acids, and chased with excess unlabeled methionine. At various times, cells were lysed and p53 was immunoprecipitated using PAb421. p53 half-life was estimated following quantitation of the p53 signal by phosphorimager. The p53 half-life was approximately 0.5 hours in untransfected MEFs and 4-5 hours in cells co-expressing E1A and T24 H-*ras*.

B) p53 levels were estimated by western blot using cell lysates derived from 10^6 cells. Lane 1, $p53^{-/-}$ MEFs; lane 2, $p53^{+/+}$ MEFs; lane 3, $p53^{+/+}$ MEFs 8 hours following exposure to 5 grays ionizing radiation; lanes 4 and 5, untreated $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras*; lanes 6-8, $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras* at 1, 4, and 8 hours (respectively) after transfer to 0.1% FBS.

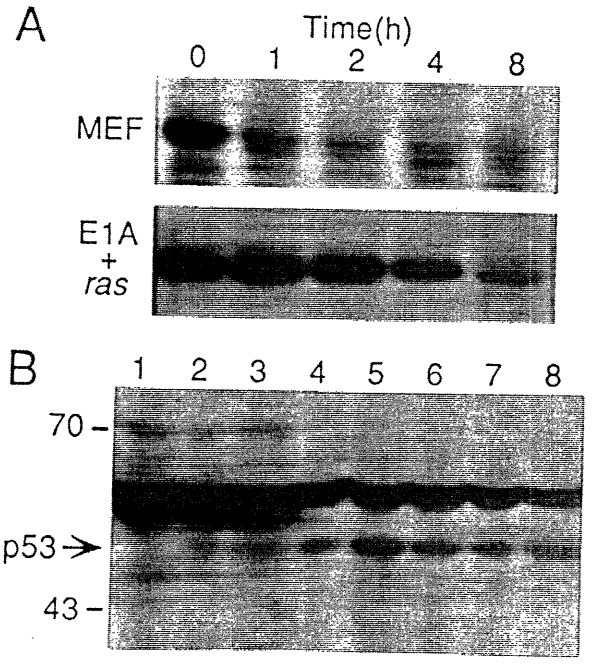
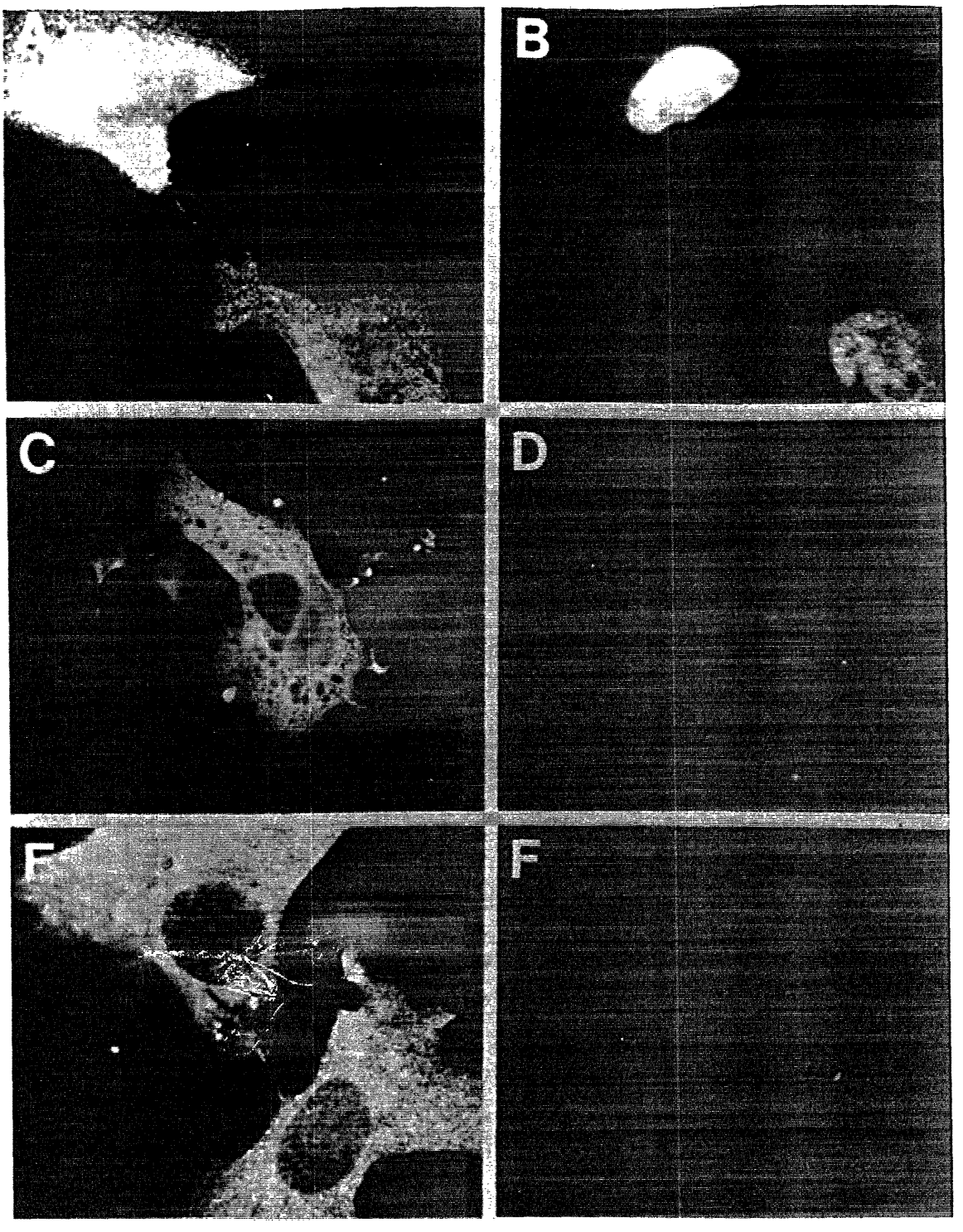

A
Time(h)
0
1
2
4
8
MEF
E1A
+
ras
B
1
2
3
4
5
6
7
8
70 –
p53
43 –

Figure 10. p53 expression following transient transfection of E1A, T24 H-ras, and hygromycin phosphotransferase.

Primary mouse embryo fibroblasts were co-transfected with pCH110 and either plasmids expressing E1A (A, B), T24 H-*ras* (C, D) or hygromycin phosphotransferase (E, F). After 36-40 hours the cells were incubated with a rabbit anti-β-galactosidase antibody and with murine antibodies specific for either E1A (A, C, E) or p53 (B, D, F). Coverslips were subsequently stained with a mixture of anti-rabbit IgG (rhodamine) and anti-mouse IgG (fluoroscein) secondary antibodies. Anti-β-galactosidase fluorescence and anti-p53 fluorescence in are from the same field (compare A and B, C and D, E and F, respectively).


B
C
D
F
F

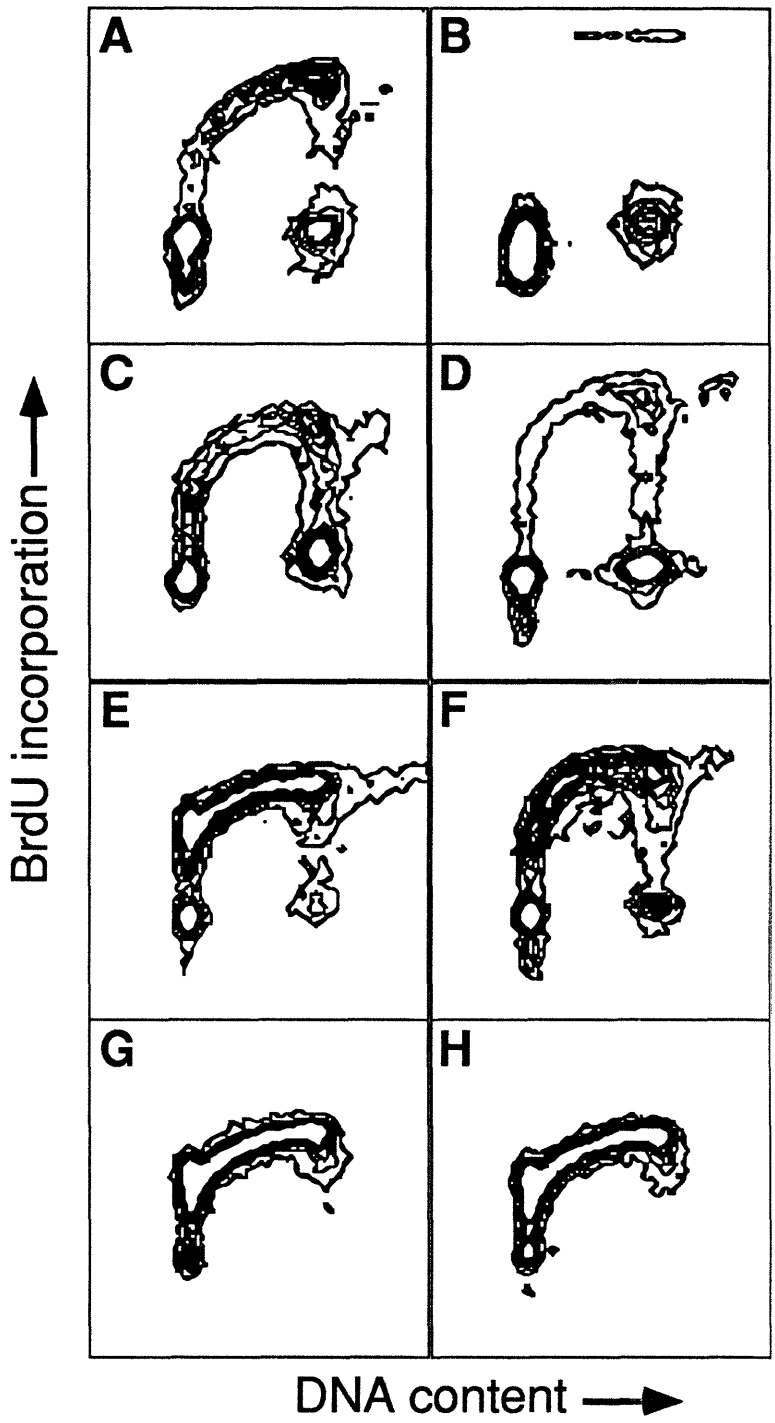


Figure 11. Cell cycle analysis by flow cytometry.

$p53^{+/+}$ (A, B) and $p53^{-/-}$ (C, D) MEFs, or $p53^{+/+}$ (E, F) and $p53^{-/-}$ (G, H) cell lines co-expressing E1A and T24 H-*ras* were incubated in either 10% FBS (A, C, E, and G) or 0.5% FBS (B, D, F, and H). 14 hours later, cells were incubated in BrdU for 4 hours, and cell proliferation was assessed by DNA content (propidium iodide staining) and incorporation of BrdU (using a FITC-anti-BrdU antibody) by flow cytometry.

Table 1. Cell cycle progression following serum depletion.

	Cell Type		Percent of Total			
Treatment	Genes	p53	G0/G1	S	G2/M	% BrdU
none	none	+/+	61+/-3	17+/-4	22+/-5	28+/-4
	none	-/-	40+/-5	21+/-9	39+/-4	46+/-13
	E1A+*ras*	+/+	34+/-3	47+/-3	19+/-1	74+/-1
	E1A	-/-	27+/-1	52+/-2	21+/-2	79+/-1
	E1A + *ras*	-/-	23+/-2	62+/-2	15+/-0	89+/-2
0.5% FBS	none	+/+	77+/-4	3+/-1	20+/-4	5+/-3
	none	-/-	52+/-4	9+/-1	40+/-6	19+/-1
	E1A+*ras*	+/+	34+/-0	40+/-2	26+/-1	69+/-2
	E1A	-/-	30+/-3	52+/-3	18+/-2	74+/-2
	E1A + *ras*	-/-	27+/-4	57+/-4	15+/-1	86+/-3

Untransfected MEFs and various E1A-expressing clones were transferred to medium containing 0.5% FBS and incubated with BrdU as described in Methodology. Cellular proliferation was assessed by DNA content (propidium iodide staining) and incorporation of BrdU (using a FITC-anti-BrdU antibody) by multiparameter flow cytometry. The percentage of cells in each phase of the cell cycle was estimated by computer analysis of the propidium iodide fluorescence. The percentage of cells synthesizing DNA during the 4 hour pulse was estimated from the amount of BrdU incorporation. The data represent the average and standard deviation from 3 independent experiments.

withdrawal, suggesting that E1A is sufficient to circumvent growth arrest (M. Raggozino and H.E.R., unpublished observations). Therefore, p53-dependent apoptosis coincided with continued cell cycle progression under conditions that would normally suppress cell growth.

p53-deficient cells expressing E1A are tumorigenic

Since apoptosis provides a mechanism whereby p53 can act as a tumor suppressor (Yonish-Rouach et al., 1991; Lowe et al., 1993b; Clarke et al., 1993), we investigated whether reduced susceptibility to apoptosis would increase tumorigenic potential. Nude mice were injected with $p53^{+/+}$ and $p53^{-/-}$ cells and monitored for tumors at the sites of injection (Table 2). $p53^{-/-}$ cells expressing E1A were tumorigenic, with latency periods similar to $p53^{+/+}$ cells

Table 2. Tumorigenicity of E1A-expressing cells

	p53 genotype					
	(-/-)			(+/+)		
Genes[a]	clone	frequency[b]	latency[c]	clone	frequency[b]	latency[c]
none	MEF	0/4	NA	MEF	0/4	NA
Hygro	3	0/4	NA			
	6	0/4	NA			
E1A	1	2/4	194			
	3	2/4	136			
	4	1/4	133			
	6	3/4	144			
E1A/E1B	2	3/4	117	4	0/4	NA
	4	2/4	117	5	2/4	125
	6	3/4	140	6	0/2	NA
E1A/*ras*	6	4/4	8	1	4/4	14
	8	4/4	6	2	3/4	16
	9	3/4	8	3	4/6	21

[a]exogenous genes introduced into MEFs. [b]number of tumors/number of sites injected. [c]number of days from injection of cells to tumor detection. Clones derived from $p53^{+/+}$ (+/+) or $p53^{-/-}$ (-/-) MEFs or untransfected cells were introduced into athymic nude mice (2×10^6 cells) and monitored for tumors at the sites of injection. Tumors were scored positive when they became clearly visible (~2 mm diameter).

co-expressing E1A and E1B. Untransfected MEFs and $p53^{-/-}$ MEFs expressing only hygromycin phosphotransferase were not tumorigenic, indicating that tumorigenicity required E1A. Because all clones were derived from drug-resistant colonies, no prior selection for a transformed phenotype was imposed. Therefore, abrogation of p53-dependent apoptosis permits transformation of MEFs by E1A alone, and the absence of p53 substituted for E1B in transformation.

The latency of tumors derived from $p53^{-/-}$ cells co-expressing E1A and T24 H-*ras* was significantly less than tumors derived from wild-type cells (Table 2). Nevertheless, endogenous p53 did not abolish the tumorigenicity of cells co-expressing E1A and T24 H-*ras*; tumors appeared after approximately

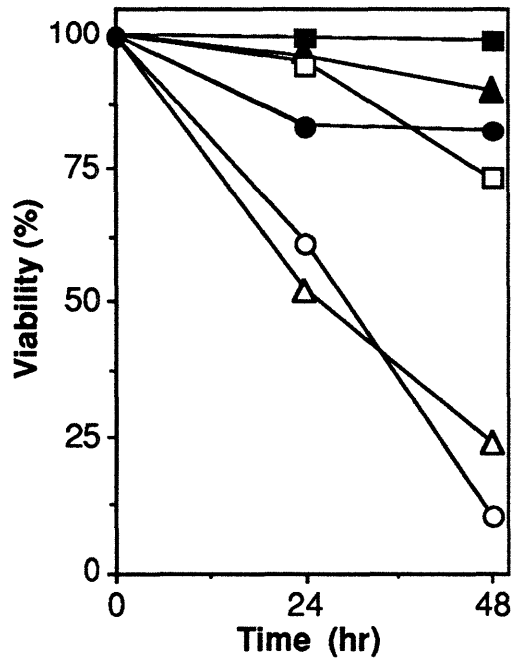


Figure 12. Viability of tumor cells following serum withdrawal.

Cells derived from a $p53^{+/+}$ MEFs tumor co-expressing E1A and T24 H-*ras* (clone 1AR.C3) were dispersed with trypsin and re-established in culture. Subsequently, cells were either maintained in 10% FBS (closed circles) or transferred to medium containing 0.1% FBS (open circles). Viability was measured by trypan blue exclusion at various times thereafter. Circle, clone 1AR.C3; triangles, tumor cells derived from clone 1AR.C3; squares, clone 1AR.A8 ($p53^{-/-}$ line co-expressing E1A and T24 H-*ras*).

2 weeks. Since wild-type cells transformed by E1A and T24 H-*ras* were sensitive to apoptosis *in vitro* yet highly tumorigenic, it was possible that tumors arose from resistant variants. To test this possibility, tumor cells derived from a $p53^{+/+}$ clone co-expressing E1A and T24 H-*ras* were re-established in culture, and transferred to medium containing either 10% or 0.1% FBS. The tumor-derived cells lost viability as rapidly as the original clone, indicating that tumorigenicity did not result from mutations that suppressed apoptosis (Figure 12). These observations indicate that escape from apoptosis is not a prerequisite for either transformation or tumor growth.

DISCUSSION

Direct suppression of transformation by p53

The present study establishes a direct mechanism of tumor suppression in which p53 participates in the destruction of aberrantly growing cells by apoptosis. We show that p53 levels and stability increase in response to E1A, p53 is required for E1A-associated apoptosis, and p53 suppresses oncogenic transformation by E1A. This mechanism of tumor suppression suggests that p53 mutations, which typically occur late in tumor progression (Fearon and Vogelstein, 1990; Stretch et al., 1991; Mazars et al., 1991; Yamada et al., 1991; Sidransky et al., 1992), could enhance the survival of cells expressing oncogenes activated early in tumor progression. p53 loss may also increase the likelihood that cells acquire oncogenic mutations by allowing inappropriate cell proliferation following DNA damage (Kastan et al., 1992). In either case, p53 action is required to protect the organism from the deleterious consequences of genetic damage.

The effects of p53-deficiency on the cell growth and survival were surprisingly dose-dependent. Thus, $p53^{+/-}$ cells transfected with either E1A or E1A and T24 H-*ras* formed almost as many colonies as the $p53^{-/-}$ cells, and $p53^{+/-}$ cells transformed by *ras* and E1A displayed an intermediate level of resistance to apoptosis. These observations imply that mutations leading to partial loss of p53 functions could allow the growth of expanded cell populations from which p53-deficient variants might arise.

Immortalization by p53 loss or E1A

Adenovirus E1A and mutant p53 alleles facilitate the immortalization of primary cells (Houweling et al., 1980; Jenkins et al., 1984; Harvey and Levine, 1991). In this study, p53-deficiency had a greater effect on the growth potential of embryonic fibroblasts than any of the oncogenes tested, suggesting

that p53 loss may be sufficient for immortalization. By contrast, while E1A promotes colony outgrowth, the establishment of permanent cell lines appears to require additional genetic changes (Zerler et al., 1986). Our results indicate that p53 loss and escape from E1A-associated apoptosis contributes to immortalization by E1A.

Role of E1B in adenovirus transformation

Two region E1B products, $p55^{E1B}$ and $p19^{E1B}$, separately collaborate with E1A to transform cultured cells (Bernards et al., 1986). We show in this study that p53 loss and E1B have equivalent effects on cell growth, survival, and transformation. Thus, the primary role of E1B is to bypass E1A-induced p53 stabilization, which precludes transformation by E1A alone. Two E1B-encoded proteins are involved, since $p55^{E1B}$ binds p53 and $p19^{E1B}$ prevents apoptosis upon p53 overexpression (Rao et al., 1992; Debbas and White, 1993).

p53 stabilization may "prime" cells for apoptosis

Although E1A-associated apoptosis occurs during normal propagation of cells, cell death is greatly enhanced upon removal of growth factors. Similarly, *myc*-expressing fibroblasts lose viability when exposed to environmental conditions that normally limit proliferation (Evan et al., 1992). It has been suggested that *myc* "primes" cells for apoptosis; thus, *myc*-expressing cells are able to immediately execute the apoptotic program while normal cells are not (Wyllie, 1993). However, the ultimate fate of the cell--proliferation or apoptosis--is determined by environmental signals. We suggest that p53 stabilization is part of one mechanism whereby oncogenes prime cells for apoptosis.

Elevated p53 levels are probably necessary for suppressor activity, since p53 is normally expressed at low levels without adversely affecting cell growth or survival (Levine, et al., 1991). However, p53 stabilization is not

sufficient for apoptosis, since exposure of normal cells to ionizing radiation also stabilizes p53 but induces growth arrest without apoptosis (Kastan et al., 1992). E1A promotes proliferation despite high p53 levels, suggesting that E1A prevents p53-dependent growth arrest. Similarly, failure of E1A-expressing cells to undergo p53-dependent growth arrest following γ-irradiation accompanies apoptosis (Lowe et al., 1993a). Thus, stimuli that normally limit proliferation instead induce apoptosis in cells unable to respond appropriately due to the expression of an oncogene. In this manner, p53 could function as part of a general mechanism to selectively destroy aberrantly growing cells.

Apoptosis as a mechanism of tumor suppression

The present study demonstrates that tumorigenicity can be significantly enhanced by genetic changes that promote cell survival. A similar mechanism accounts for co-transformation by *myc* and *bcl*-2 (Bissonnette et al., 1992; Fanidi et al., 1992). While the involvement of p53 in *myc*-associated apoptosis has not been examined, both activation and escape from apoptosis appear to be of fundamental importance to multistep carcinogenesis and tumor progression. In contrast, cells transformed by E1A and T24 H-*ras* are highly tumorigenic yet remain sensitive to apoptosis, even when passaged as tumors and placed back in culture. Therefore, escape from apoptosis is neither a prerequisite for, nor a consequence of, oncogenic transformation. This is perhaps not surprising since apoptosis is a common feature of malignant tumors (Wyllie, 1985).

We suggest that cells can acquire tumorigenic phenotypes by various routes which alter the balance of growth, differentiation and survival in different ways. Oncogenes such as $p19^{E1B}$ and *bcl*-2 block apoptosis directly, whereas the enhanced growth rate of *ras*-transformed cells may simply

compensate for cell losses due to apoptosis. *ras* co-transformation may also protect cells from environmental conditions that trigger apoptosis, for example, through the production of autocrine growth factors. In either case, tumor growth can occur while the cells remain genotypically susceptible to apoptosis. This may be a factor in limiting tumor progression and metastatic spread and, as described elsewhere, appears to modulate the cytotoxicity of anticancer agents (Lowe et al., 1993a).

ACKNOWLEDGMENTS

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CHAPTER 4

p53-dependent apoptosis modulates the cytotoxicity of anticancer agents

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INTRODUCTION

Both radiation and chemotherapy have had a significant impact on the treatment of cancer. A major impediment to successful therapy, however, is the failure of some tumor types to respond to either form of treatment and the appearance of resistant cell populations upon relapse of an originally responsive malignancy. Consequently, the underlying basis of cellular resistance to anticancer agents has been the focus of much experimental study. In general, these investigations have examined how chemotherapeutic agents reach their intracellular targets or the molecular nature of the drug-target interaction (Chin et al., 1993; Chabner and Myers, 1989). For example, high levels of expression of the *mdr*-1 gene have been shown to limit the intracellular concentration of various chemotherapeutic compounds and may contribute to multidrug resistance (Chin et al., 1993).

A more complete understanding of cellular resistance to cancer therapy may require the elucidation of the mechanisms by which anticancer agents cause cell death. Since ionizing radiation and many chemotherapeutic agents induce DNA damage or cause disruptions in DNA metabolism, the tumor-specific cytotoxicity of these agents has been attributed to their genotoxic effect on actively proliferating cells. However, in many cases, the cellular damage caused by active doses of these agents is not sufficient to explain the observed toxicity (Dive and Hickman, 1991; Chabner and Myers, 1989).

In recent years, the exploration and understanding of the process of programmed cell death, or apoptosis, has forced a reconsideration of the mechanism whereby tumor cells can acquire or lose sensitivity to cytotoxic treatments. Apoptosis has been described as "physiological" cell death, since it is a genetically determined cellular program essential for normal development and maintenance of tissue homeostasis (Raff, 1992). Cells

undergoing apoptosis display shrinkage, loss of cell-cell contact, chromatin condensation, and internucleosomal degradation of DNA (Wyllie, 1980). Many toxic stimuli have been shown to induce apoptosis, even at doses or concentrations insufficient to cause general metabolic dysfunction (Lennon et al., 1991; Dive and Hickman, 1991). These results suggest that divergent types of cellular damage may lead to the generation of a common signal(s) that initiates the cell death program. Accordingly, the ability of tumor cells to detect cellular damage and activate the apoptotic response may determine the ultimate success of cancer therapy.

There is mounting evidence that the expression of oncogenes can sensitize cells to apoptosis. Both the adenovirus early region 1A (E1A) gene and c-*myc* can increase cellular susceptibility to programmed cell death, particularly under conditions of low serum concentration or high cell density (Rao et al., 1992; Evan et al., 1992). Overexpression of these genes can also confer susceptibility to apoptosis induced by several anticancer agents, including tumor necrosis factor-α (Chen et al., 1987), etoposide (Fanidi et al., 1992), and other compounds used in chemotherapy (Lotem and Sachs, 1993). Thus, the sensitivity of tumor cells to chemotherapeutic regimens may be accentuated by their inappropriate expression of oncogenes. In the present study, we examine the response of primary mouse embryo fibroblasts and their E1A-expressing derivatives to radiation and several chemotherapeutic compounds. Although the parental fibroblasts were largely resistant to these agents, E1A-expressing cells rapidly underwent apoptosis following treatment with relatively low doses of ionizing radiation, 5-fluorouracil, etoposide, or adriamycin.

Recently, we and others have demonstrated a crucial role for the p53 tumor suppressor gene in the execution of some forms of apoptosis (Lowe et

al., 1993b; Clarke et al., 1993). The production of animals with homozygous inactivation of the p53 gene (T.J. and R.A. Weinberg, unpublished) has permitted us to develop cell populations in which the p53 function can be examined in a systematic manner. Utilizing this strategy, we have explored the effects of p53 expression on normal and oncogene-expressing cells following irradiation and treatment with various chemotherapeutic agents. The results of these studies are clear: p53 is required for the efficient activation of apoptosis following irradiation or treatment with chemotherapeutic compounds. Thus, the absence of p53 expression leads to a dramatic increase in cellular resistance to these agents, implying that tumor cells can acquire drug and radiation resistance through mutations that interfere with apoptosis. In particular, the status of the p53 gene, which is mutated in a high percentage of human cancers (Hollstein et al., 1991; Levine et al., 1991), may be an important determinant of the efficacy of many treatment protocols.

METHODOLOGY

Cells and cell culture

$p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ mouse embryonic fibroblasts used in this study have been previously described (Livingstone et al., 1992; Kastan et al., 1992). Clones expressing E1A and various other oncogenes were generated by calcium phosphate co-precipitation (Chapter 3). All cells were maintained in DME containing 10% fetal bovine serum (FBS) supplemented with penicillin and streptomycin (growth media). In general, MEF cultures were used between passages 3 and 5.

Colony regression assays

p1AHygro, which encodes the adenovirus-5 E1A gene and hygromycin

phosphotransferase, was transfected into $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs by calcium phosphate co-precipitation (Chapter 3). Thus, colonies arising in hygromycin B had a high probability of co-expressing E1A. E1A-expressing colonies have a distinct morphology that distinguishes them from normal or hygromycin-resistant MEFs, and have been shown to express E1A by immunofluorescent staining. Approximately 3 weeks after transfection, cultures were transferred to normal growth medium (without Hygromycin B), and E1A-expressing colonies were marked and treated with 5 Gy ionizing radiation using a Gammacell 40 irradiator equipped with a ^{137}Cs source. Alternatively, colonies were incubated in the presence of 1 μM 5-fluorouracil (Sigma), 0.2 μM etoposide (Sigma), or 0.2 μg/ml adriamycin (provided by F. Haluska). Selected colonies were photographed at various times thereafter. All colonies were scored for significant regression 72 hours after treatment, except for colonies incubated in adriamycin, which were scored for regression after 24 hours.

Dose-response assays

For irradiation experiments, exponentially growing cells were detached from plates and adjusted to 10^6 cells/ml. Samples were irradiated for different times, and 1 ml of each cell suspension was added to 100 mm dishes containing normal growth medium. Cell viability was assessed 36 hours after irradiation by pooling adherent and non-adherent cells and measuring uptake of fluorescein isothiocyanate (FITC) by FACS analysis (Shi et al., 1990; Chapter 3). At least 10^5 cells were measured for each determination. Cells used in chemical cytotoxicity experiments were plated at 1-2 x 10^6 cells/100mm dish, allowed to adhere, and incubated with various concentrations of 5-fluorouracil, etoposide, adriamycin, or sodium azide (Sigma). Cell viability was determined 24 hours following treatment.

Apoptosis assays

Chromatin structure was visualized by staining fixed cells with 2,4-diamidino-2-phenylindole (DAPI) 8-10 hours after treatment with anticancer agents. Fragmentation of cellular DNA was measured 24 hours after treatment as described (White et al., 1984; Chapter 3). Adherent and non-adherent cells were pooled, washed in phosphate buffered saline (PBS), and resuspended in ice cold buffer containing 0.15M NaCl, 10mM Tris (pH 7.4), 2 mM $MgCl_2$, and 1mM DTT. Nonidet-P40 was added to a final concentration of 0.5% (v/v) and the samples were incubated on ice for 30 minutes. Nuclei were isolated by centrifugation, resuspended in buffer containing 0.35M NaCl, 10mM Tris (pH 7.4), 2mM $MgCl_2$, and 1mM DTT, and incubated on ice for 15-30 minutes. The nuclei were then removed by centrifugation, and the supernatant was extracted with phenol and chloroform. The low molecular weight DNA was recovered by ethanol precipitation. Samples were resuspended in 20 µl Tris-EDTA and treated with RNase A for 30 minutes prior to electrophoresis on 1% agarose gels.

Cell cycle analysis

Cellular proliferation was assessed by DNA content and incorporation of 5-bromo-2'-deoxyuridine (BrdU) by multiparameter flow cytometry (van Erp et al., 1988). Cells were plated in growth medium at 1-2 x 10^6 cells/100mm dish, allowed to adhere, and exposed to 5 Gy ionizing radiation. 14 hours after treatment, BrdU (Amersham) was added and the cultures were incubated at 37°C for an additional 4 hours. Cultures were washed twice with PBS to remove dead cells, and the adherent cells were collected after treatment with trypsin. The cells were washed in PBS and fixed in 70% ethanol for 30 minutes at -20°C. Subsequently, the cells were centrifuged, resuspended in 1 ml of 0.2 mg/ml pepsin (Sigma) in 2N HCl, and incubated

for 30 minutes at room temperature. After neutralizing the samples with 3 ml 0.1M $Na_2B_4O_7$, the cells were washed with PBS followed by PBS supplemented with 0.5% Tween20 and 2% FBS (PTS). The cells were resuspended in 100 µl of PTS containing 5 µl of an FITC-conjugated anti-BrdU antibody (Boehringer Mannheim) and incubated for 30 minutes at room temperature. The cells were then washed with PTS, resuspended in 0.5 ml PTS containing 0.5 mg/ml RNase A and 50 µg/ml propidium iodide (Calbiochem), and incubated for at least 30 minutes at room temperature. All samples were passed though 70 µm mesh prior to FACS analysis. FACS analysis was performed on a FACStar Plus (Becton Dickinson) flow cytometer. The percentage of cells in each phase of the cell cycle (at the end of the BrdU labeling period) was estimated using the MODFIT program (Verity Software House). The co-efficient of variance for each fit was usually less than 5%. The percentage of cells incorporating BrdU during the 4 hour pulse was estimated from the log red fluorescence and log green fluorescence plot using the disp2D program (Becton Dickinson).

RESULTS

Previous studies have shown that functional p53 is required for the G_1 cell cycle arrest in fibroblasts exposed to ionizing radiation (Kastan et al., 1992) and for the initiation of apoptosis in irradiated mouse thymocytes (Lowe et al., 1993b; Clarke et al., 1993). In a separate study, we used wild-type and p53-deficient mouse embryonic fibroblasts to demonstrate that apoptosis associated with expression of the adenovirus E1A oncogene also is dependent on p53 (Chapter 3). E1A-associated apoptosis is observed during normal propagation of cells, but the level of cell death dramatically increases when cells were maintained in low concentrations of serum. Since conditions of

growth limitation appear to trigger cell death in E1A-expressing cells, we sought to determine whether other treatments known to inhibit proliferation might also lead to p53-dependent apoptosis.

E1A-expressing cells rapidly undergo p53-dependent cell death following exposure to ionizing radiation.

Due to the difficulty in establishing cell lines that continuously express E1A and endogenous p53 (Chapter 3), we analyzed cell viability using unexpanded colonies derived following introduction of E1A into early passage mouse embryo fibroblasts (MEFs). Specifically, $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs were transfected with p1AHygro, a plasmid co-expressing both the adenovirus-5 E1A oncogene and hygromycin phosphotransferase, and E1A-expressing colonies were selected in hygromycin B. In a separate series of experiments, a plasmid encoding the adenovirus E1B gene (which counteracts the effects of p53 (Debbas and White, 1993; Lowe and Ruley, 1993)) was co-introduced with p1AHygro. After approximately 3 weeks in hygromycin-containing medium, E1A-expressing colonies were marked and inspected for regression after exposure to 5 grays (Gy) ionizing radiation.

The viability of colonies exposed to ionizing radiation is illustrated in Figure 1 and summarized in Table 1. While irradiation of untransfected fibroblasts had no effect on cellular viability (see below), virtually all $p53^{+/+}$ and $p53^{+/-}$ colonies expressing E1A rapidly degenerated. By contrast, $p53^{-/-}$ colonies expressing E1A were resistant to irradiation, indicating that cell death required p53 function. Although not as effective as the absence of p53, co-expression of E1B protected E1A-expressing $p53^{+/+}$ cells from death following irradiation.

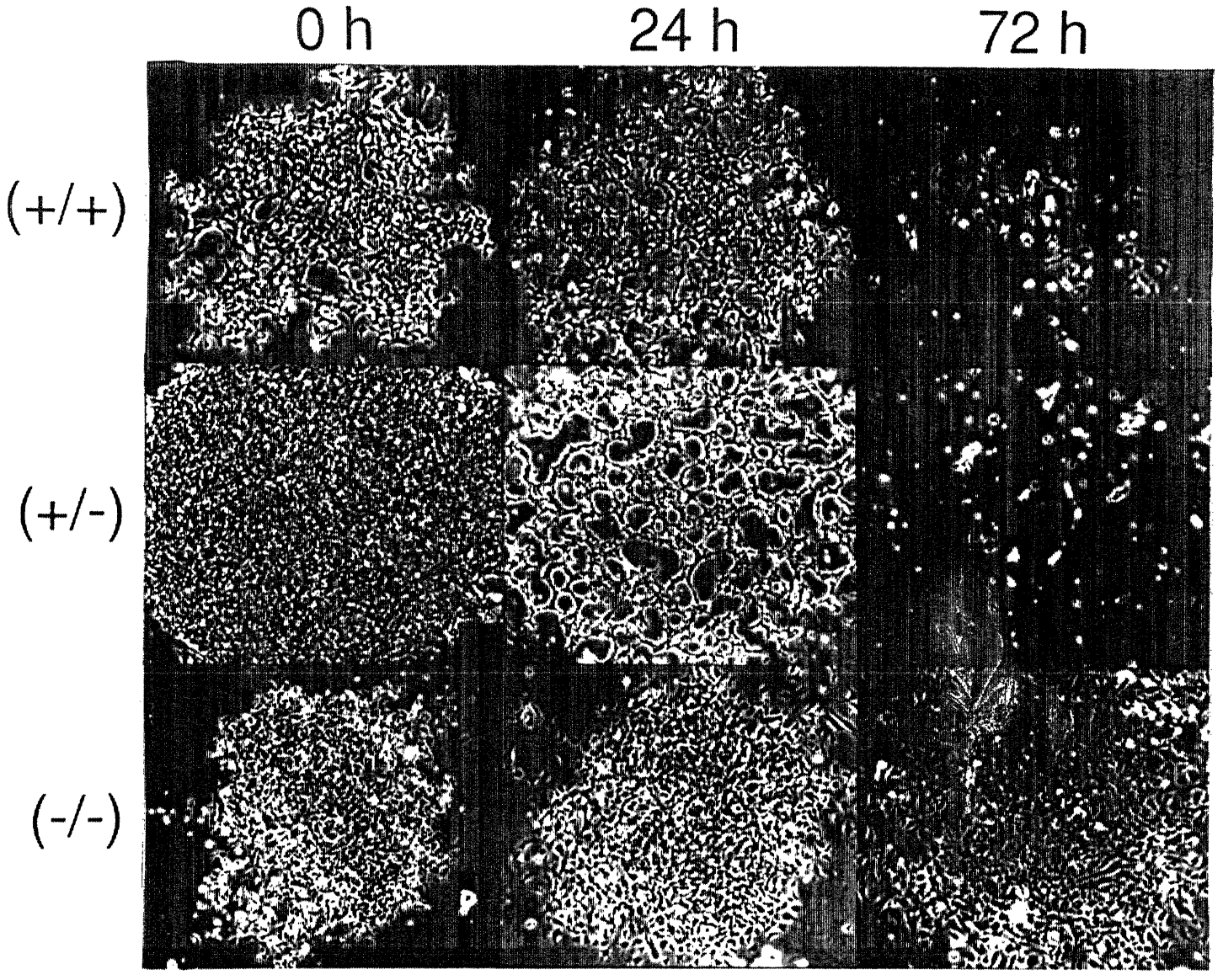

0 h
24 h
72 h
(+/+)
(+/-)
(-/-)

Figure 2. Colony regression after treatment with chemotherapeutic agents.

p1AHygro was introduced into $p53^{+/+}$ (+/+), $p53^{+/-}$ (+/-), and $p53^{-/-}$ (-/-) MEFs and colonies were selected in hygromycin B. E1A-expressing colonies were marked and photographed 72 hours after treatment with 1 μM 5-fluorouracil or 0.2 μM etoposide. Alternatively, colonies were treated with 0.2 μg/ml adriamycin and photographed 24 hours after treatment. All experiments were performed in the absence of Hygromycin B.

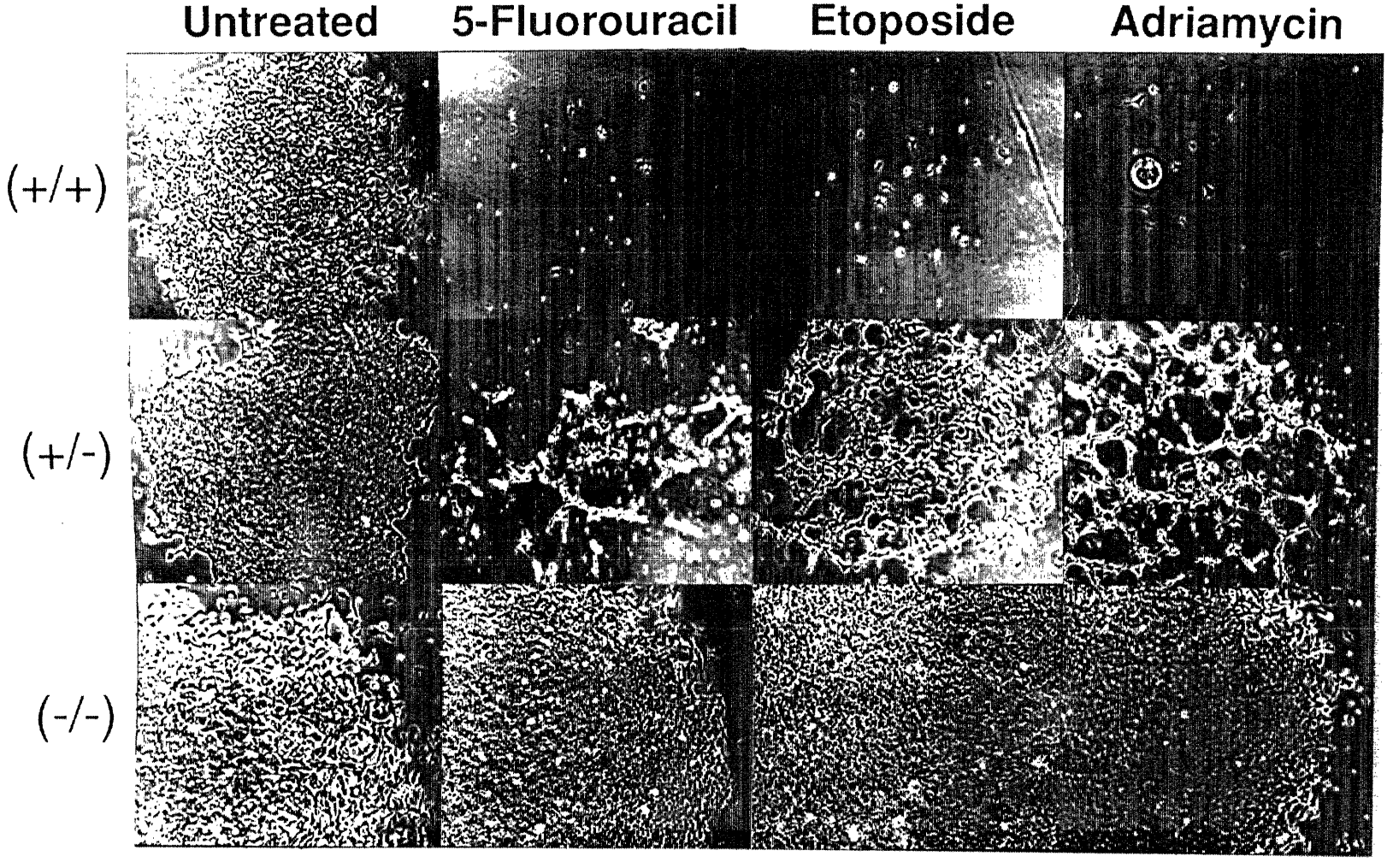

Untreated
5-Fluorouracil
Etoposide
Adriamycin
(+/+)
(+/-)
(-/-)

Table 1. Viability of E1A-expressing colonies after γ-irradiation or treatment with chemotherapeutic agents.

			Colony Viability (72 h)[c]		
Treatment	Genes[a]	p53[b]	Regressing	Resistant	% Resistant
Radiation	E1A	(+/+)	5	1	**17**
(5 Gy)	E1A	(+/-)	25	0	**0**
	E1A	(-/-)	1	24	**96**
	E1A + E1B	(+/+)	4	9	**69**
	E1A + E1B	(+/-)	9	16	**64**
	E1A + E1B	(-/-)	ND	ND	ND
5fluorouracil	E1A	(+/+)	5	0	**0**
(1μM)	E1A	(+/-)	23	2	**8**
	E1A	(-/-)	1	26	**96**
Etoposide	E1A	(+/+)	5	1	**17**
(0.2 μM)	E1A	(+/-)	20	5	**20**
	E1A	(-/-)	0	25	**100**
Adriamycin	E1A	(+/+)	3	0	**0**
(0.2 μg/ml)	E1A	(+/-)	22	3	**12**
	E1A	(-/-)	0	25	**100**

p1AHygro (with or without an E1B expression vector) was transfected into $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs and colonies were selected in hygromycin B. Approximately 3 weeks after transfection, the colonies were marked and exposed to 5 Gy ionizing radiation, 1μM 5-fluorouracil, 0.2 μM etoposide, or 0.2 μg/ml adriamycin in the absence of Hygromycin B. Colonies were inspected by microscopy for significant regression and cell death 72 hours after initiating treatment (as shown in Figure 1 and 2), except for colonies incubated in adriamycin which were scored after 24 hours.

Genotoxic compounds used in cancer chemotherapy induce p53-dependent cell death in cells expressing E1A.

A variety of genotoxic compounds used as chemotherapeutic agents also increase p53 levels and can cause growth inhibition (Kastan et al., 1991; Fritsche et al., 1993). Several of these compounds were tested for their ability to induce p53-dependent cell death in E1A-expressing cells using the colony regression assay described above. 5-fluorouracil (anti-metabolite), etoposide

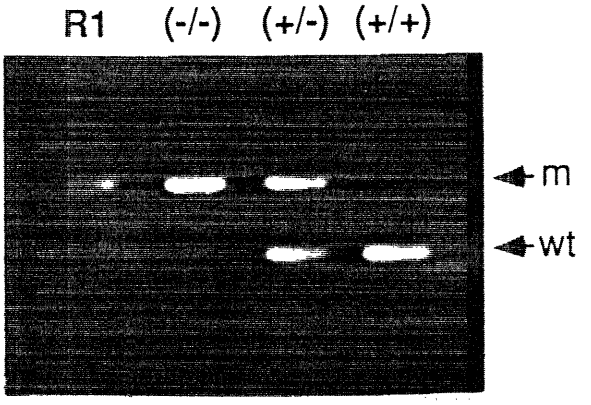
(topoisomerase II inhibitor) and adriamycin (intercalating agent) were chosen for these experiments because they have different intercellular targets (Chabner and Myers, 1989). As observed following irradiation, $p53^{+/+}$ and $p53^{+/-}$ colonies that expressed E1A displayed a remarkable sensitivity to each of these agents: the majority of colonies completely regressed within 72 hours of treatment (Figure 2 and Table 1). Again, cell death required p53 function, since $p53^{-/-}$ cells were resistant to all treatments. p53-deficient colonies did show some degree of regression after several days in adriamycin. The vast majority of E1A-expressing colonies derived from $p53^{+/-}$MEFs degenerated completely following treatment with various genotoxic compounds; however, a small number retained viability even after 6 days. By using a polymerase chain reaction assay that distinguishes between mutant and wild-type p53 alleles, three out of four resistant colonies were shown to have lost the wild-type allele, and therefore had become deficient for p53 (Figure 3). Taken together, these data indicate that E1A increased cellular sensitivity to several chemotherapeutic agents and that the ensuing cell death was dependent on a functional p53 gene.

Anticancer agents trigger p53-dependent apoptosis in cells transformed by E1A and T24 H-*ras*.

E1A is unable to oncogenically transform embryonic fibroblasts alone, but collaborates with either the adenovirus E1B gene or activated *ras* alleles to transform primary cells to a tumorigenic state (Ruley, 1990). While E1B inhibits p53-dependent apoptosis such that p53-deficient cells are transformed by E1A alone (Chapter 3), the basis for *ras* co-transformation with E1A is unknown. Thus, cells co-expressing T24 H-*ras* and E1A are highly tumorigenic but susceptible to apoptosis upon serum withdrawal (Chapter 3). Since oncogenically transformed fibroblasts provide an experimental

Figure 3. p53 status in viable E1A-expressing colonies derived from $p53^{+/-}$ MEFs following treatment with chemotherapeutic agents.

DNA was isolated from an E1A-expressing colony derived from $p53^{+/-}$ cells previously incubated in 1.0 μM 5-fluorouracil for 1 week (designated R1). p53 status was determined by a polymerase chain reaction assay that distinguishes between the wild-type and disrupted p53 alleles. As controls, the identical assay was performed on untransfected MEFs derived from $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs. Produces were separated on 1% agarose gels, the location of the null-specific (m) and wild-type-specific (wt) bands are designated to the right of the gel.


R1
(-/-)
(+/-)
(+/+)
m
wt

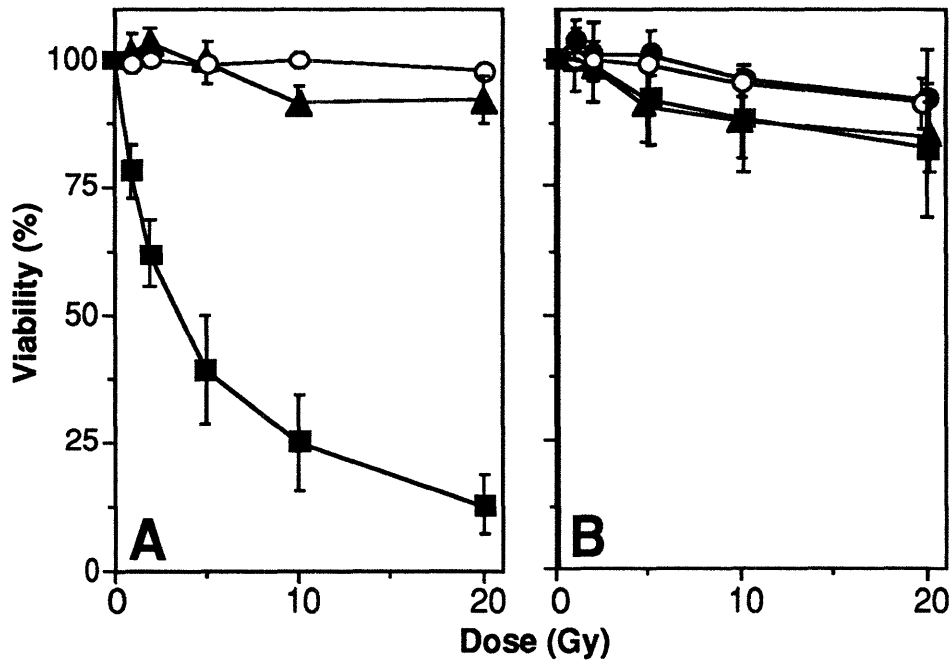


Figure 4. Viability wild-type and p53-deficient cells after irradiation.

The viability of cells expressing (A) or lacking (B) endogenous p53 was measured by FITC uptake and flow cytometry 36 hours after treatment with the indicated dose of ionizing radiation. Each point represents the average and standard deviation obtained from at least 3 independent clones. All values were normalized to the relative viability of the corresponding untreated controls from the same experiment (generally greater than 90%). Open circles, untransfected MEFs; closed circles, $p53^{-/-}$ cells expressing E1A; squares, cells co-expressing E1A and T24 H-*ras*; triangles, cells expressing E1A and E1B.

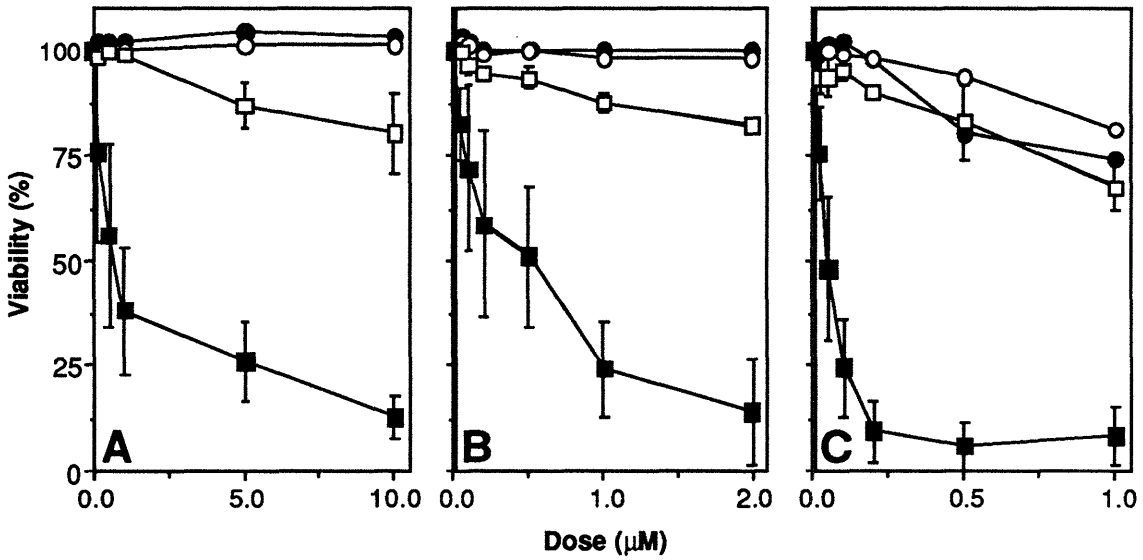
system analogous to naturally occurring tumors, we examined the effects of various anticancer agents on transformed lines expressing E1A.

Like cells expressing E1A alone, exposure of $p53^{+/+}$ cells transformed by E1A and T24 H-*ras* to ionizing radiation caused a dose-dependent decrease in viability, with significant death occurring at doses as low as 1 Gy. Radiation treatment had a minimal effect on all $p53^{-/-}$ lines, and most cells retained viability after treatment with 20 Gy (Figure 4). Although we have not extensively analyzed long-term viability following irradiation, $p53^{-/-}$ cells co-expressing E1A and T24 H-*ras* irradiated with 5 Gy displayed no significant

loss of growth or tumorigenic potential (see Chapter 5). $p53^{+/-}$ cells co-expressing E1A and T24 H-*ras* died following irradiation, but to a considerably lesser extent than wild-type cells (not shown). However, ionizing radiation had no effect on the viability of either $p53^{+/+}$ or $p53^{-/-}$ untransfected MEFs, or $p53^{+/+}$ lines that co-expressed E1B with E1A. It is noteworthy that doses of ionizing radiation sufficient to kill $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras* cause only transient growth arrest in untransfected fibroblasts ((Kastan et al., 1991; Kastan et al., 1992) and see below).

Similarly, $p53^{+/+}$ cells oncogenically transformed by E1A and T24 H-*ras* were extremely sensitive to low concentrations of 5-fluorouracil, etoposide, and adriamycin (Figure 5). Cell death required p53, since $p53^{-/-}$ cells co-expressing E1A and T24 H-*ras* were largely resistant to these treatments. The differences in concentrations required for half-maximal killing of $p53^{+/+}$ and $p53^{-/-}$ cells co-expressing E1A and T24 H-*ras* were greater than 20-fold in this assay. No decrease in viability was observed in the untransfected MEFs of either p53 genotype following exposure to 5-fluorouracil or etoposide, even at doses as high as 100 μM 5-fluorouracil. However, the viability of $p53^{-/-}$ cells co-expressing E1A and T24 H-*ras* began to decline at higher concentrations of these two drugs, and the viability of all cells declined with increasing concentrations of adriamycin. Thus, at sufficiently high concentrations, these agents can cause cell death in a p53-independent manner.

During apoptosis, loss of membrane integrity is typically preceded by chromatin condensation and internucleosomal cleavage of genomic DNA (Wyllie, 1980). As visualized by staining with 2,4-diamidino-2-phenylindole (DAPI), significant numbers of $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras* contained condensed chromatin and fragmented nuclei within 8 hours following treatment with the various anticancer agents (Figure 6). In



F

igure 5. Viability of wild-type and p53-deficient cells after treatment with chemotherapeutic agents.

Viability of the untransfected MEFs and $p53^{+/+}$ and $p53^{-/-}$ clones co-expressing E1A and T24 H-*ras* was estimated by FITC uptake and FACS analysis 24 hours after treatment with the indicated concentrations of 5-fluorouracil (A), etoposide (B), and adriamycin (C). Each point represents the average and standard deviation obtained from at least 3 independent clones. All values were normalized to the relative viability of the corresponding untreated controls from the same experiment (generally greater than 90%). Closed circles, untransfected $p53^{+/+}$ MEFs; open circles, untransfected $p53^{-/-}$ MEFs; closed squares, $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras*; open squares, $p53^{-/-}$ cells co-expressing E1A and T24 H-*ras*.

contrast, $p53^{-/-}$ populations rarely contained cells with altered chromatin structure. After irradiation, $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras* contained large amounts of low molecular weight DNA, which produced a characteristic "ladder" on agarose gels (Figure 7A). The degraded DNA was present in oligomers that were multiples of approximately 180-200 base pairs, suggesting internucleosomal cleavage. Cells lacking p53 or expressing E1B did not contain degraded DNA after exposure to ionizing radiation. Treatment with low doses of 5-fluorouracil, etoposide, and adriamycin also induced DNA fragmentation in $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras*, but not in p53-deficient cells (Figure 7B). These data suggest that cell death in E1A-

Figure 6. Analysis of chromatin structure following treatment with anticancer agents.

$p53^{-/-}$ (A, C, E) and $p53^{+/+}$ (B, D, F) cells transformed by E1A and T24 H-*ras* were treated with anticancer agents and chromatin structure was visualized by staining with DAPI 8 hours later. Cells and genotypes were treated as follows: (A) untreated, $p53^{-/-}$; (B) 1 μM 5-fluorouracil, $p53^{+/+}$; (C) 5 Gy ionizing radiation, $p53^{-/-}$; (D) 5 Gy ionizing radiation, $p53^{+/+}$; (E) 0.2 μg/ml adriamycin, $p53^{-/-}$; (F) 0.2 μg/ml adriamycin, $p53^{+/+}$.

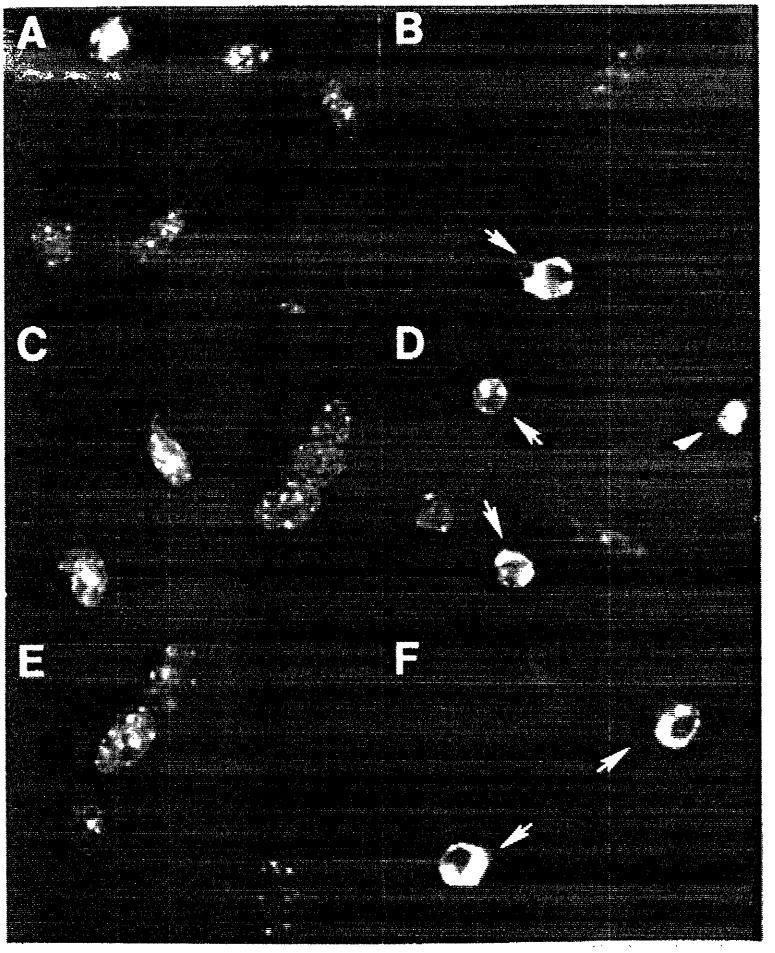
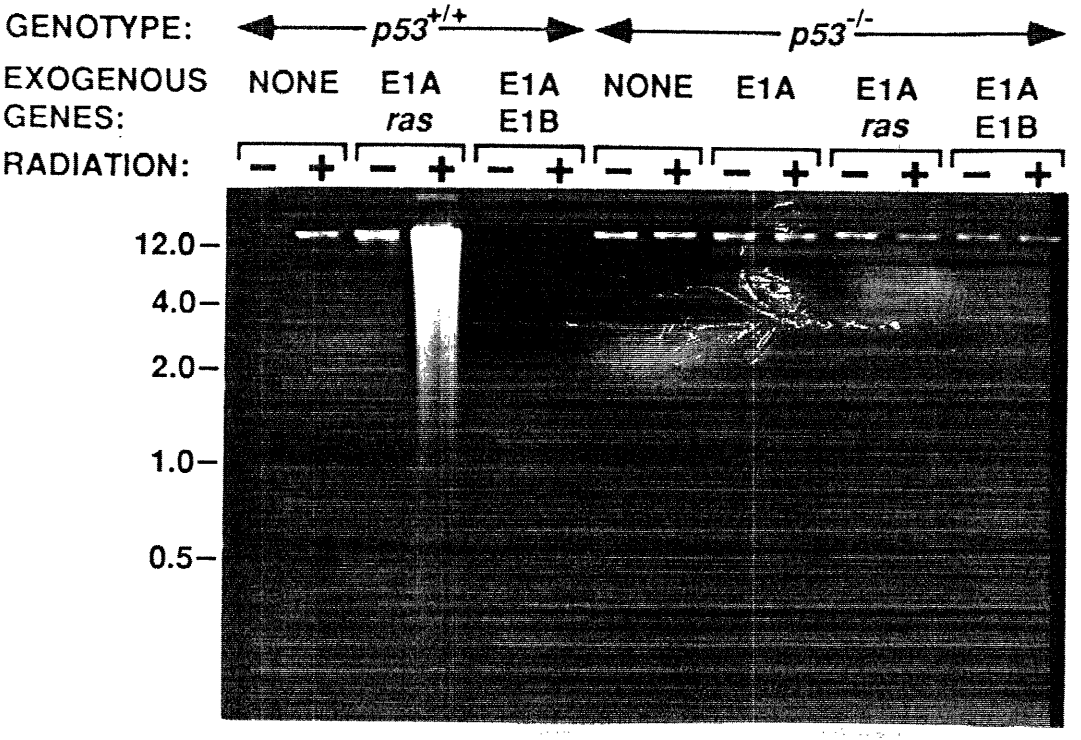
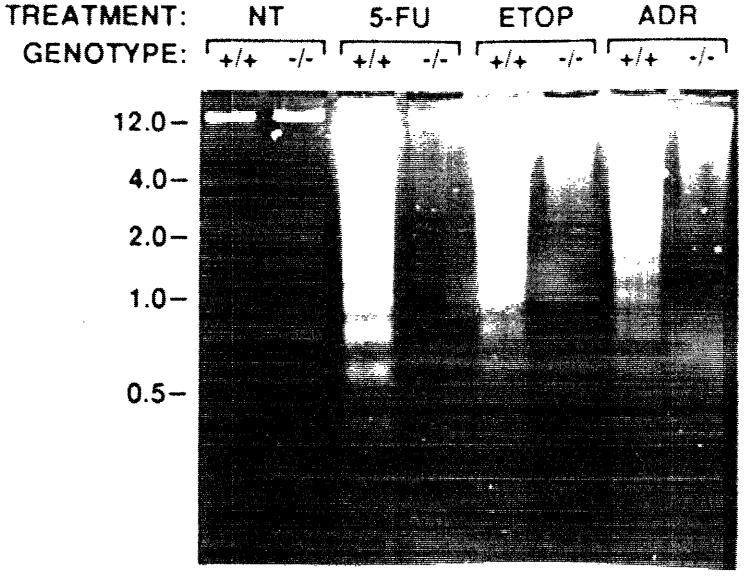

A
B
C
C
D
E
F

Figure 7. Analysis of low molecular weight DNA from cells exposed to ionizing radiation or treated with chemotherapeutic agents.

Low molecular weight DNA was isolated from 2 x 10^6 cells 24 hours after exposure to ionizing radiation (A) or treatment with chemotherapeutic agents (B). Cells were irradiated with 5 Gy (+) or incubated in media containing 1 μM 5-fluorouracil (5-FU), 0.2 μM etoposide (ETOP), or 0.1 μg/ml adriamycin (ADR). In B, only cells $p53^{+/+}$ (+/+) or $p53^{-/-}$ (-/-) cells co-expressing E1A and T24 H-*ras* were analyzed. DNA was resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. Note that viable cell populations do not contain significant quantities of low molecular weight DNA.


GENOTYPE:
p53+/+
p53-/-
EXOGENOUS GENES:
NONE
E1A ras
E1A E1B
NONE
E1A
E1A ras
E1A E1B
RADIATION:
– + – + – + – + – + – + – +
12.0
4.0
2.0
1.0
0.5


TREATMENT:
NT
5-FU
ETOP
ADR
GENOTYPE:
+/+ -/- +/+ -/- +/+ -/- +/+ -/-
12.0
4.0
2.0
1.0
0.5

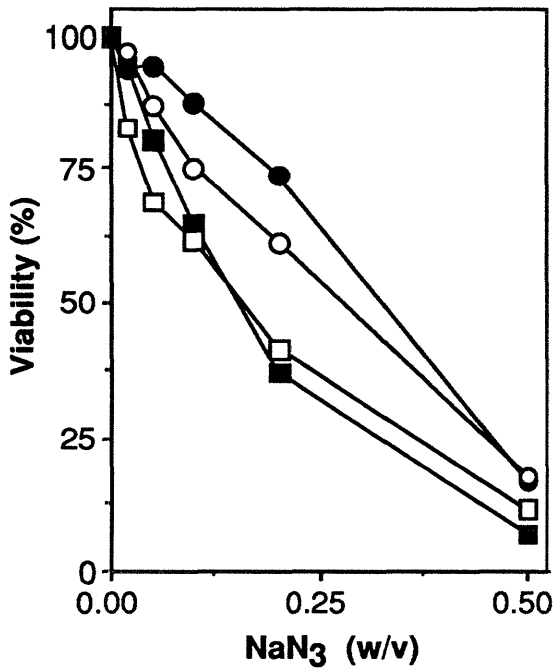


Figure 8. Viability of cells treated with sodium azide.
Cell lines co-expressing E1A and T24 H-*ras* (squares) and the untransfected MEFs (circles) were incubated in various concentrations of sodium azide ($p53^{+/+}$, closed symbols, $p53^{-/-}$, open symbols). Cell viability was estimated by uptake of FITC and FACS analysis 12 hours after initiating treatment. Each data point represents the average value derived from 2 independent clones.

expressing cells resulted from a common cellular response (apoptosis) to these treatments rather than from the genotoxic action of the agents themselves.

p53 is not required for cell death following treatment with sodium azide.

Since cells expressing E1A undergo p53-dependent apoptosis following serum depletion (Chapter 3), irradiation, and treatment with various chemotherapeutic compounds, it was of interest to determine whether co-expression of p53 with E1A made cells sensitive to any toxic treatment. Untransfected MEFs and various clones co-expressing E1A and T24 H-*ras* were treated with sodium azide, an electron transport poison. Sodium azide-treated cells displayed a similar dose-dependent decrease in viability whether

or not they expressed endogenous p53, although untransfected fibroblasts were less sensitive than cells co-expressing E1A and T24 H-*ras* (Figure 8). Thus, sodium azide-induced death did not require p53 function.

E1A expression allows cells to bypasses p53-dependent growth arrest following irradiation.

Treatment with DNA damaging agents causes growth inhibition in embryonic fibroblasts and other cell types (Kastan et al., 1991; Fritsche et al., 1993), and the G_1 cell cycle arrest that occurs following irradiation requires p53 function (Kastan et al., 1992). Thus, introduction of E1A into embryonic fibroblasts altered the cellular response to γ-irradiation from growth arrest to apoptosis. E1A sequences required for apoptosis are identical to those required for E1A-induced activation of DNA synthesis (White et al., 1991). Furthermore, wild-type cells expressing E1A continue to cycle in mitogen-deficient medium, even as they die by apoptosis (Chapter 3). These data suggest that p53-dependent apoptosis is triggered by unscheduled proliferation. Therefore, we investigated the effects of E1A and p53 expression on cell cycle progression following exposure to ionizing radiation.

Cell cycle progression was assessed in cells exposed to ionizing radiation or 5-fluorouracil by 5-bromo-2'-deoxyuridine (BrdU) incorporation and measurement of cellular DNA content. $p53^{+/+}$ and $p53^{-/-}$ MEFs and E1A-expressing derivatives were incubated with BrdU for 4 hours beginning 14 hours after treatment. This represents a period when the proliferation of irradiated fibroblasts is maximally inhibited (Kastan et al., 1991) and when $p53^{+/+}$ cells expressing E1A have initiated apoptosis. The relative amounts of cells in each phase of the cell cycle were estimated from the overall DNA content (as measured by propidium fluorescence) and the percentage of cells synthesizing DNA during the 4 hour BrdU pulse.

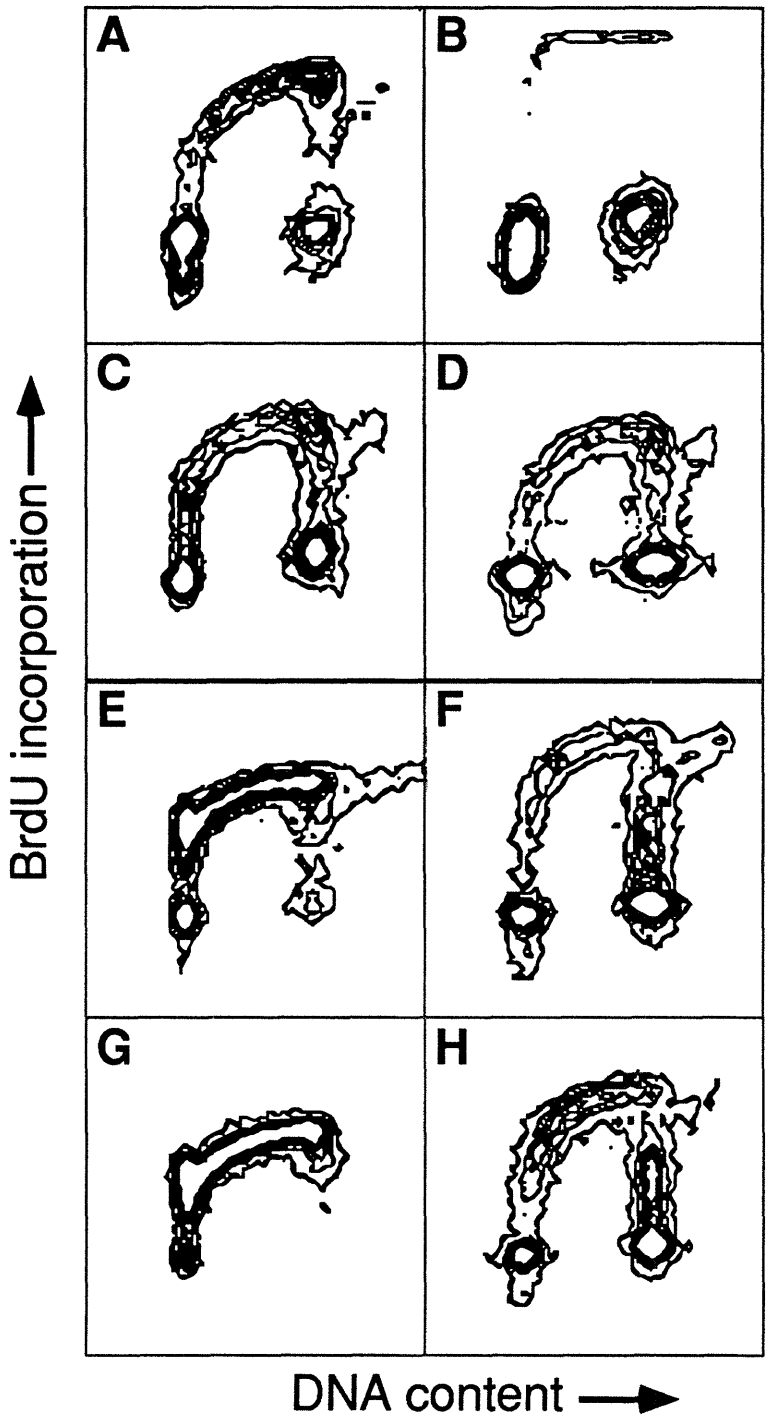


Figure 9. Cell cycle analysis of irradiated cells by flow cytometry.

$p53^{+/+}$ (A, B) and $p53^{-/-}$ (C, D) MEFs, or $p53^{+/+}$ (E, F) and $p53^{-/-}$ (G, H) cell lines co-expressing E1A and T24 H-*ras* were left untreated (A, C, E, and G) or irradiated with 5 Gy (B, D, F, and H). 14 hours after treatment, cells were incubated in 5-bromo-2'-deoxyuridine (BrdU) for 4 hours. Cell proliferation was assessed by DNA content (propidium iodide staining) and incorporation of BrdU (using a FITC-anti-BrdU antibody) by multiparameter flow cytometry.

Table 2. Cell cycle progression following exposure to ionizing radiation.

	Cell Type		Percent of Total			
Treatment	**Genes**	**p53**	**G0/G1**	**S**	**G2/M**	**% BrdU**
none	none	+/+	61+/-3	17+/-4	22+/-5	28+/-4
	E1A+*ras*	+/+	34+/-3	47+/-3	19+/-1	74+/-1
	none	-/-	40+/-5	21+/-9	39+/-4	46+/-13
	E1A	-/-	27+/-1	52+/-2	21+/-2	79+/-1
	E1A + *ras*	-/-	23+/-2	62+/-2	15+/-0	89+/-2
5 Gy	none	+/+	70+/-1	4+/-2	27+/-1	6+/-2
	E1A+*ras*	+/+	22+/-2	16+/-3	62+/-4	45+/-3
	none	-/-	35+/-3	18+/-5	47+/-2	43+/-13
	E1A	-/-	15+/-2	22+/-1	63+/-2	70+/-5
	E1A + *ras*	-/-	19+/-6	33+/-6	48+/-10	57+/-4

Untransfected MEFs and various clones were treated with 5 Gy ionizing radiation and incubated with BrdU as described in Methodology. Cell proliferation was assessed by DNA content (propidium iodide staining) and incorporation of BrdU (using a FITC-anti-BrdU antibody) by multiparameter flow cytometry. The percentage of cells in each phase of the cell cycle was estimated by computer analysis of the propidium iodide fluorescence. The number of cells synthesizing DNA during the 4 hour pulse was estimated from the amount of BrdU incorporation. The data represent the average and standard deviation from 3 independent experiments.

As shown in Figure 9 and summarized in Table 2, a 5-fold decrease in cells incorporating BrdU was observed in $p53^{+/+}$ MEF cultures treated with ionizing radiation, and cells arrested predominantly in the G_1. Irradiated $p53^{-/-}$ MEFs continued to synthesize DNA and accumulated in G_2/M, consistent with the involvement of p53 in radiation-induced G_1 (but not G_2) arrest (Kastan et al., 1992). Cells expressing E1A continued to synthesize DNA following treatment with ionizing radiation whether or not they expressed endogenous p53 (Table 2). Thus, E1A prevented $p53^{+/+}$ cells from arresting in G_1 following irradiation. However, neither E1A nor the combination of E1A and T24 H-*ras* prevented irradiated cells from accumulating in G_2/M (Table 2). Although these results do not rule out the possibility that T24 H-*ras* was responsible for circumventing cell cycle arrest, REF52 cells, which tolerate

E1A expression alone (Chapter 2), also continued to proliferate following irradiation (not shown). Therefore, as observed following serum depletion (Chapter 3), E1A bypasses p53-dependent growth arrest following exposure to ionizing radiation.

DISCUSSION

The cytotoxicity of anticancer agents may reflect their ability to induce programmed cell death

Although the primary cellular targets of many anticancer agents have been identified, less is known about the processes leading to the selective death of cancer cells (Eastman, 1990; Dive and Hickman, 1991). Because ionizing radiation and many chemotherapeutic compounds induce DNA damage or cause disruptions in DNA metabolism, cell death is frequently attributed to the genotoxicity of these agents in actively proliferating cells. However, treatment with radiation and most chemotherapeutic agents results in dramatic changes in cellular gene expression (Holbrook and Fornace, 1991; Fornace, 1992) and, in many cell types, induces apoptosis (Kaufmann, 1989; Barry et al., 1990; Lennon et al., 1991; Dive and Hickman, 1991; Sen and D'Incalci, 1992). These observations suggest that the cytotoxic action of many anticancer agents involves a genetically-determined mechanism that requires the active participation of the target cell. Unfortunately, a better understanding of this process has been hampered by the inability to obtain mutants in the cell death program (Eastman, 1990).

Oncogenes and p53 can modulate the cytotoxicity of anticancer agents

The present study demonstrates that ionizing radiation and several chemotherapeutic agents trigger apoptosis in cells expressing the E1A oncogene, and identifies a molecule, the p53 tumor suppressor, that is

required for efficient activation of the cell death program. Treatment of E1A-expressing fibroblasts with relatively low doses of either ionizing radiation or chemotherapeutic compounds rapidly induced apoptosis, while having little or no effect on the viability of untransfected fibroblasts or E1A-expressing cells lacking p53. The specificity of these agents for cells co-expressing E1A and endogenous p53 was not due to the their more active proliferation, since $p53^{-/-}$ cells expressing E1A grew more rapidly but were resistant. Overexpression of the c-*myc* oncogene also sensitizes cells to apoptosis (Evan et al., 1992). Like cells expressing E1A, *myc*-associated apoptosis is enhanced by several chemotherapeutic agents (Fanidi et al., 1992; Lotem and Sachs, 1993), although the involvement of p53 in this process has not been directly examined. Taken together, these data indicate that the cytotoxic action of many anticancer agents is largely determined by the genotype of the cell rather than the genotoxicity of the agent.

Since oncogenes can sensitize cells to apoptosis, alterations in cell cycle regulation may be necessary to activate the cell death program. It is noteworthy that both ionizing radiation and many chemotherapeutic agents induce apoptosis in a manner indistinguishable from that caused by depriving cells of growth factors. This observation is consistent with the model described elsewhere, which views oncogene-associated apoptosis as a cellular response to unscheduled or aberrant proliferation (Lowe et al., 1994). Our data demonstrate that E1A-expressing cells continue to proliferate following serum depletion (Lowe et al., 1994) and exposure to ionizing radiation. Thus, various stimuli that would normally cause growth arrest instead induce apoptosis in cells unable to respond appropriately due to the expression of an oncogene. Although the signal that triggers apoptosis is not known, the fact that different types of cellular damage induce similar changes

in p53 turnover and gene expression (Holbrook and Fornace, 1991; Fornace, 1992) suggests a common response to stress, not unlike the bacterial SOS response (Walker, 1987). Thus, p53 may normally act to suppress growth while the cell attempts repairs, and promote apoptosis in cells that continue to proliferate.

Increases in p53 levels are associated with p53-dependent apoptosis

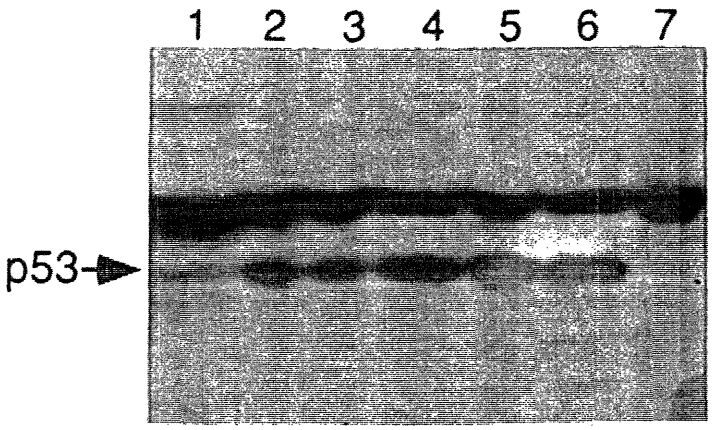
The stability of p53 protein is increased in cells expressing E1A (or E1A and T24 H-*ras*), resulting in elevated p53 levels (Lowe and Ruley, 1993; Lowe et al., 1994). Since normal cells express low levels of p53 without adversely effecting cell survival, p53 stabilization may be necessary for apoptosis. Nevertheless, p53 induction is not sufficient for cell death. For example, irradiation of normal cells also stabilizes p53, but causes cell cycle arrest without apoptosis (Kastan et al., 1992). Furthermore, cells transformed by E1A and T24 H-*ras* express stabilized p53 but are viable under normal culture conditions, and p53 levels do not increase further when apoptosis is triggered by serum depletion (Lowe et al., 1994) or γ-irradiation (Figure 10).

p53-independent mechanisms

Several studies have demonstrated that not all forms of apoptosis require p53 (Lowe et al., 1993b; Clarke et al., 1993). In this study, p53-independent cytotoxicity was more pronounced at relatively high doses of radiation or chemotherapeutic drugs. Similarly, established cell lines known to lack p53 expression undergo apoptosis upon treatment with chemotherapeutic agents, but the process requires higher concentrations than used here. (Kaufmann, 1989; Lennon et al., 1991; Sen and D'Incalci, 1992). Thus, cells that sustain sufficient damage may undergo apoptosis regardless of their p53 status. Since p53 can suppress immortalization (Harvey and Levine, 1991; Lowe et al., 1994), it would not be surprising if many established cells

Figure 10. p53 levels in cells co-expressing E1A and T24 H-ras following irradiation.

p53 levels were determined by Western blot using lysates derived from 10^6 cells. The blot was probed with a pool of p53-specific monoclonal antibodies (PAb421, PAb240, and PAb248), and p53 was visualized by chemiluminescence as described in Methodology. Cell lysates were derived from: untransfected MEFs (lane 1); untreated $p53^{+/+}$ cells co-expressing E1A and T24 H-*ras* (lane 2), or treated with 0.1% FBS (lanes 3, 4) or 5 Gy ionizing radiation (lanes 5, 6) (lanes 3 and 5 are from 4 hours after treatment; lanes 4 and 6 are from 10 hours after treatment); untreated $p53^{-/-}$ cells co-expressing E1A and T24 H-*ras* (lane 7).


1 2 3 4 5 6 7
p53

acquire defects in p53-dependent apoptosis. This could explain why higher concentrations of chemotherapeutic agents are required to induce apoptosis in most established lines (Sen and D'Incalci, 1992), and the inability to obtain cross-resistant mutants with defects in apoptotic pathways (Eastman, 1990). The present study utilized early passage mouse embryo fibroblasts which differed only in their p53 status. Since many experiments were performed on unexpanded colonies, resistance to cell death was not due to genetic alterations that might be selected for upon clonal expansion.

Apoptosis and tumor suppression by p53

Although this report has focused on the cellular response of oncogene-expessing cells to anticancer agents, these data reflect a more general mechanism by which p53 may function as a tumor suppressor. We have suggested that the involvement of p53 in oncogene-associated apoptosis represents a direct mechanism whereby p53 eliminates abnormally growing cells (Lowe et al., 1994). In the absence of p53, oncogene-expressing cells are unable to trigger the death program in response environmental signals. While these signals can arise from drugs that interfere with cellular homeostasis or cause DNA damage, they also may originate from mitogen-deprivation, high cell density, or changes in cellular micro-environment that might accompany metastasis. Furthermore, loss of p53 may increase the likelihood that cells will acquire further mutations (Kastan et al., 1992; Lane, 1992). Thus, for multiple reasons, cells undergoing p53 mutation or loss would have a selective advantage during tumor progression.

Cross-resistance to anticancer agents involving inhibition of p53-dependent apoptosis

Since it appears that the cytotoxicity of many anticancer agents involves a common genetic program, lesions in apoptotic pathways could

generate a cross-resistant phenotype. The present study demonstrates that loss of p53 function enhances cellular resistance to a variety of agents used in cancer therapy. Although cellular sensitivity and resistance were more thoroughly examined in the complete absence of p53 function, hemizygous cells displayed an intermediate level of resistance to anticancer agents. Point mutations leading to the expression of dominant-negative p53 alleles may have a similar effect, and their intrinsic ability to inhibit wild-type p53 function may contribute to variability in cellular response to anticancer agents. Factors which modulate p53 function could also influence cellular resistance to anticancer agents. These factors include the *mdm*-2 oncogene (Momand et al., 1992; Oliner et al., 1992), the human papilloma virus E6 protein (Scheffner et al., 1990; Crook et al., 1992), or as described here, the adenovirus E1B gene. Conversely, mutations that activate genes that normally suppress apoptosis might also contribute to drug resistance. Indeed, overexpression of the *bcl*-2 oncogene rescues *myc*-expressing cells from apoptosis occurring upon serum withdrawal and etoposide treatment (Fanidi et al., 1992).

Implications for human cancer

The data presented here provide a rationale for understanding the response to radiation and chemotherapy in human tumors. The ability to achieve a significant therapeutic index differentiating normal cells from tumor cells may be a consequence of genetic alterations that accompany malignant transformation, which lowers the threshold at which cell injury triggers apoptosis. Furthermore, the vulnerability of tumor cells to radiation or chemotherapy is greatly reduced by mutations that abolish p53-dependent apoptosis. Our data suggest that p53 status in tumor cells may be a strong

determinant of response to treatment with either chemotherapy or radiation. How well does this view correspond to clinical experience?

Three types of clinical patterns are notable. First, there are a number of tumor types in which a high percentage of primary tumors have acquired p53 mutations. These include malignant melanoma (Stretch et al., 1991), and cancers of the lung (Takahashi et al., 1989; Chiba et al., 1990; D'Amico et al., 1992; Takahashi et al., 1991), colon (Fearon and Vogelstein, 1990), bladder (Sidransky et al., 1991), prostate (Isaacs et al., 1991) and cervix (Crook and Vousden, 1992; Crook et al., 1992). In general, patients with these tumors respond poorly to treatment with either radiation or chemotherapy. Furthermore, in many of these tumor types, the presence of p53 mutation correlates with poor prognosis (Davidoff et al., 1991; Thorlacius et al., 1993; Sun et al., 1992; Horio et al., 1993; Visakorpi et al., 1992; Jaros et al., 1992). Second, there are tumors that rarely exhibit p53 mutations at presentation. Included in this group are testicular cancer (Heimdal et al., 1993), Wilm's tumor (J. Pelletier, personal communication) and childhood acute lymphoblastic leukemia (Gaidano et al., 1991; Jonveaux and Berger, 1991). In these forms of cancer, chemotherapeutic intervention is extremely effective. Third, upon relapse of acute lymphoblastic leukemia, failure of therapy correlates with the occurrence of mutations in the p53 gene (Felix et al., 1992; Yeargin et al., 1993) Furthermore, in several tumor types, p53 mutations have been identified in relapse specimens that were not present in the primary tumor (Hayashi et al., 1991; Sidransky et al., 1992; Felix et al., 1992; Neri et al., 1993). Indeed, these observations suggest that there may be a strong correlation between a tumor's p53 status and patient response to chemotherapy or radiation. While this view must represent only a part of the rationale for treatment response, it suggests that a more detailed examination

of the relationship between p53 status and response to radiation and chemotherapy may be important for developing more effective therapeutic intervention.

ACKNOWLEDGMENTS

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CHAPTER 5

The p53 tumor suppressor gene enhances tumor response to γ-irradiation in a transplanted tumor model

This work is ongoing.

INTRODUCTION

The use of radiation and chemotherapy in the treatment of human malignancy has had a significant impact on prolonging the disease free interval and, to a lesser extent, the overall survival rate (Pitot, 1987). However, anticancer agents are frequently ineffective; some tumor types fail to respond to either form of treatment or become nonresponsive upon tumor relapse. Since the identification of therapeutic agents and establishment of treatment regimens has been empirical, the mechanisms that determine their effectiveness are largely unknown. Thus, the development of better therapeutic agents may require: (i) the identification of factors that determine the tumor-specific action of anticancer agents, (ii) the elucidation of the biological and biochemical mechanisms responsible for their cytotoxic action, and (iii) understanding the molecular basis for cross-resistance. In the absence of a molecular understanding of these processes, one of the major objectives of clinical cancer research has been the identification of prognostic factors that could determine the type or aggressiveness of cancer therapy required (for one example, see (Harris et al., 1992). Since the failure of tumors to respond to treatment reflects the ultimate basis for "poor prognosis", it is possible that prognostic indicators may provide insight into biological mechanisms determining therapeutic effectiveness.

Mutations in the p53 tumor suppressor gene are associated with aggressive cancers (Baker et al., 1989; Ichikawa et al., 1992; Mazars et al., 1992; Tsuda and Hirohashi, 1992; Bookstein et al., 1993; Donghi et al., 1993; Fagin et al., 1993; Neri et al., 1993), metastasis (Yamada et al., 1991; Crook and Vousden, 1992; Marchetti et al., 1993), and with poor prognosis (Crook and Vousden, 1992; Thompson et al., 1992; Thor et al., 1992; Horio et al., 1993; Riou et al., 1993). Because p53 is considered the most frequently mutated gene

in human cancer (Hollstein et al., 1991; Levine et al., 1991), considerable effort has been invested into understanding its function. However, the molecular basis for the association between p53 mutation, advanced tumor stage, and poor prognosis remains unknown. We have recently demonstrated a role for p53 in modulating apoptosis--or programmed cell death--in response to oncogenes (Lowe and Ruley, 1993; Lowe, et al., 1994) and anticancer agents (Lowe, et al., 1993a; Lowe, et al., 1994b). These studies suggest that certain oncogenes activated during tumor progression might sensitize cells to p53-dependent apoptosis. Thus, p53 mutations could increase the threshold with which tumor cells activate the death program in response to both physiological stimuli and anticancer agents, suggesting a biological basis for the prognostic significance of p53 mutation.

While *in vitro* cell culture models provide simple systems for studying anticancer agent cytotoxicity and apoptosis, their relevance to tumor response *in vivo* remains unknown. Complicating factors *in vivo* can influence the cellular response to anticancer agents (Hellman, et al., 1988). For example, oxygen in the tumor environment is significantly reduced relative to its availability in cell culture. Since radiation-induced toxicity requires oxygen, hypoxia may reduce the cellular response to γ-irradiation *in vivo*. Thus, tumor vascularization can dramatically influence the response of tumors to ionizing radiation. For chemotherapeutic agents, it is often difficult to translate concentrations of a drug used in cell culture to *in vivo* doses, since the effective concentration a tumor receives is determined by a multitude pharmokinetic factors (Chabner and Myers, 1989). These factors make it difficult to demonstrate that mechanisms causing cell death *in vitro* are responsible for tumor regression *in vivo*.

In order to determine whether p53 status can influence tumor response to anticancer agents, we were interested in establishing a well-controlled model for tumor progression in which the role of p53, apoptosis, and tumor response could be studied in a systematic manner. The effects of p53 on tumor progression have been characterized following transplantation of p53-deficient fibroblasts containing tranfected oncogenes into athymic nude mice. Several considerations suggested that this system would be useful for assessing the effects of p53 on tumor response to anticancer agents. First, primary mouse embryo fibroblasts transformed by E1A and activated *ras* oncogenes provide a model of genetic changes that spontaneously occur during tumorigenesis (Ruley, 1983). Using embryonic fibroblasts derived from normal and p53-deficient mice, we have shown that cells transformed by E1A and *ras* are highly tumorigenic in athymic nude mice, but are susceptible to p53-dependent apoptosis *in vitro* (Lowe, et al., 1993a; Lowe, et al., 1994). Second, cells remain sensitive to apoptosis even after being passaged as tumors and placed back in culture, indicating that tumor growth does not require mutations that suppress apoptosis (Lowe, et al., 1994). Consistent with this observation, apoptosis is a common feature of malignant tumors (Wyllie, 1985). Finally, tumors are easily monitored for growth or regression following subcutaneous injection of cells into athymic nude mice.

In the present study, animals harboring tumors derived from $p53^{+/+}$ and $p53^{-/-}$ embryonic fibroblasts were treated with ionizing radiation, and tumors were monitored for growth or regression. Tumors derived from wild-type cells generally regressed in response to ionizing radiation--some disappearing completely--prior to eventual regrowth. By contrast, tumors derived from p53-deficient cells never regressed significantly. While some $p53^{-/-}$ tumors remained stationary for several days, others continued their

pre-treatment growth rate. These results indicate that p53 status and susceptibility to p53-dependent apoptosis can determine tumor responsiveness to treatment with anticancer agents.

METHODOLOGY

Cells and Injections

$p53^{+/+}$ and $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were obtained from 12.5 day embryos derived from crosses between mice with a disrupted p53 allele (T. Jacks and R. Weinberg, unpublished). Cells were cultured in DME containing 10% fetal bovine serum (FBS), and transfected between passages 3 and 5. The generation of lines co-expressing E1A and an activated *ras* oncogene (T24 H-*ras*) have been described previously (Chapter 2). Cells were passaged minimally in culture prior to injection in order to minimize selection for mutations that suppressed apoptosis.

For injection, cells were detached from tissue culture plates with trypsin, washed in phosphate buffered saline (PBS), and resuspended at a cell density of 8×10^6 cells/ml in PBS. Athymic nude mice (Taconic) were injected with 2×10^6 cells/site (0.25 ml) at two separate sites (each rear flank). Mice were used between ages 6 weeks and 6 months. In general, tumor latency was significantly reduced in younger mice; however, tumor response to γ-irradiation was similar.

Tumor size measurements

Upon the appearance of solid tumors, tumor volumes were estimated from caliper measurements of tumor length (L) and width (l) according to the standard formula adopted by the National Cancer Institute $((L \times l^2)/2)$. In general, tumors were allowed to grow until the volume of the tumor was 0.15-0.5 cm^3 prior to treatment. This represents a volume when tumor

growth is relatively constant; tumors are large enough to measure accurately, yet small enough to monitor the progression of non-responding tumors for at least two weeks. Since animals were injected at two sites, it was not uncommon that one tumor would arise significantly before the other. Therefore, in order to minimize the number of mice, tumors were occasionally treated outside this size range.

Irradiation protocols

When tumors reached an appropriate size, the mice were treated using several irradiation protocols. These included single fraction total body irradiation using 5 Gy and 7 Gy. These doses represent typical doses used in treating human cancer and were shown to induce p53-dependent apoptosis *in vitro* (Chapter 4). For mice, 7 Gy is the highest tolerated dose that can be used for total body irradiation, with a mortality rate of approximately 30% (Hall, 1988). 5 Gy is tolerated somewhat better by mice. In addition, a fractionation scheme was employed involving multiple treatments of low-dose irradiation over a period of several days. Empirical studies have indicated that 10 fractions of 2 Gy, over a period of approximately 2 weeks, induces the same biological toxicity as a single dose of 5 grays (Hall, 1988).

RESULTS

Mouse embryonic fibroblasts transformed by E1A and activated *ras* oncogenes provide an experimental system analogous to naturally occurring tumors. E1A and T24 H-*ras* (*ras*) have been introduced into embryonic fibroblasts derived from wild-type mice ($p53^{+/+}$) or mice homozygous for disrupted p53 genes ($p53^{-/-}$) (Chapter 2). $p53^{+/+}$ and $p53^{-/-}$ lines are virtually identical except for the status of p53; consequently, differences in tumor response to γ-irradiation can be directly attributed to p53 function. Moreover,

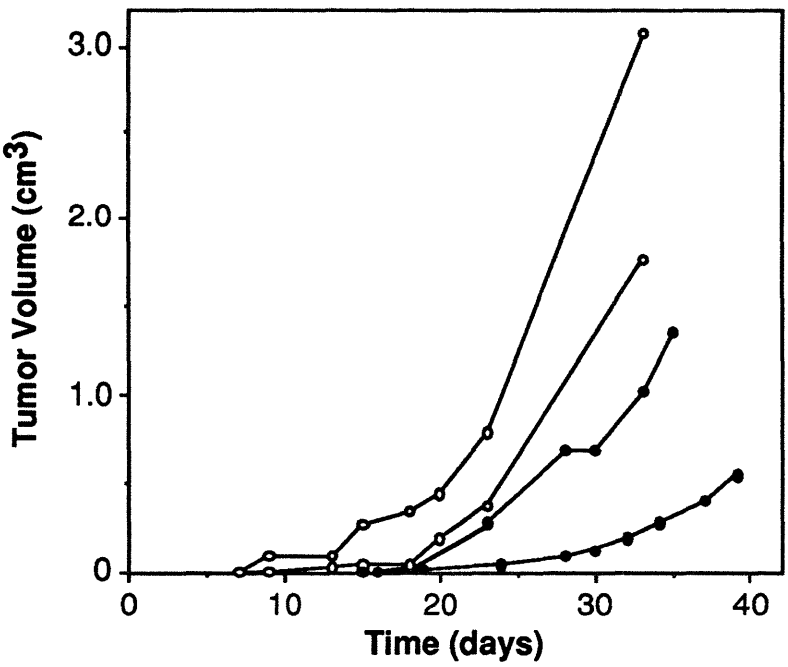

Figure 1. Tumor growth following subcutaneous injection of cells into athymic nude mice.

Embryonic fibroblasts transformed by E1A and T24 H-*ras* were injected into athymic nude mice (2 x 10^6 cells/site) and tumor volumes were estimated as described in Methodology. Open circles; tumors derived from $p53^{-/-}$ cells; closed circles; tumors derived from $p53^{+/+}$ cells.

because these cells are oncogenically transformed regardless of their p53 status, selective pressure for other mutations is minimized.

Baseline tumor growth

To establish a baseline for tumor growth in this system, athymic nude mice were injected with either $p53^{+/+}$ or $p53^{-/-}$ cells and tumor size was monitored. $p53^{-/-}$ tumors were detected earlier and grew faster than $p53^{+/+}$ tumors (Figure 1). Upon detection of a solid tumor, growth often proceeded slowly for a period of several days, but typically increased by the time tumors reached 0.15 cm^3. Tumors continued to expand at a relatively constant rate up to quite large volumes (>4 cm^3 for $p53^{-/-}$ tumors). This indicated that changes in tumor growth following γ-irradiation could be attributed to the effects of

treatment rather than complicating factors such as limiting nutrient supply. These observations suggested that treatment of tumors between 0.15-0.5 cm^3 would provide a good window for measuring both tumor growth and regression.

Tumor latency

In agreement with earlier studies (Chapter 3), $p53^{+/+}$ cells formed tumors less frequently and with a longer latency than $p53^{-/-}$ cells (Table 1). Mice injected with $p53^{-/-}$ cells developed tumors at all injected sites, with a latency averaging 9 days. The latency of tumors derived from $p53^{+/+}$ cells was three times longer that for $p53^{-/-}$ cells (27 days). However, both the frequency and latency of tumors derived from $p53^{+/+}$ cells was quite variable. Thus, one clone (1AR.C8) formed tumors with a relatively high frequency (9 of 13 sites injected), while the other (1AR.C10) was poor at forming tumors (2 of 9 sites injected).

Irradiation of animals harboring tumors

Animals harboring tumors were treated with various doses of γ-irradiation and tumors were monitored for growth or regression. Tumors derived from one $p53^{+/+}$ clone (1AR.C8) regressed upon irradiation following all treatment protocols (Table 1). Using single fraction schemes, tumor volumes generally decreased to less than 10% of the original volume within 1 week of treatment (Figure 2 and Table 1). In several instances, all visible evidence of the tumor disappeared. However, in all mice that survived radiation treatment, tumors eventually relapsed. In those mice that were re-treated, tumors again regressed, indicating that cells surviving the original treatment had not acquired mutations that conferred radiation resistance (Figure 3). In the only informative instance, the tumor response upon re-

Table 1. Effect of p53 on tumor latency and response to ionizing radiation.

			Volume[b]					
			7 Gy		5 Gy		Fractionation[c]	
Clone	Tumor	Latency[a]	7 d	14 d	7 d	14 d	7 d	14 d
C8	396	22	11	74				
(+/+)	397	21	10	51				
	384	16	30	90				
	380	30			0	84		
	378L	47			0	64		
	385L	24					0	6
	385R	28					0	0
	927L	19					0	0
	927R	16					18	18
C10	378R	30			100	152		
(+/+)	386	44					74	54
	AVE:	**27**	**17**	**72**	**33**	**100**	**18**	**16**
	S.D.:	**10**	**9**	**16**	**47**	**38**	**29**	**20**
A8	399L	10	428	764				
(-/-)	399R	16	92	194				
	376L	7	199	470				
	383L	4	243	374				
	383R	4	174	424				
	377L	7			386	439		
	377R	10			240	488		
	382L	7			263	730		
	388L	10					203	281
	388R	10					167	170
	387L	19					188	227
A9	400L	7	221	597				
(-/-)	400R	12	226	404				
	376R	4	375	1458				
	381R	7			213	339		
	381L	7			138	310		
	382R	7			420	1024		
	389L	10					100*	NA
	389R	10					120*	NA
	387R	19					133	133
A4	941L	4					168	224
(-/-)	941R	4					177	198
	AVE:	**9**	**245**	**586**	**277**	**555**	**173**	**206**
	SD:	**4**	**101**	**365**	**98**	**250**	**22**	**47**

NA, not applicable

Legend to Table 1

[a]days between injection of cells (see Methodology) and tumor detection (.002cm^3).

[b]Tumor volumes are presented as the percentage of the pre-treatment volume.

[c]animals were given 2 Gy /day for 12 days (no treatment was given on days 6 and 7).

*volumes from 5 days after treatment initiation were used, since mice died shortly thereafter.

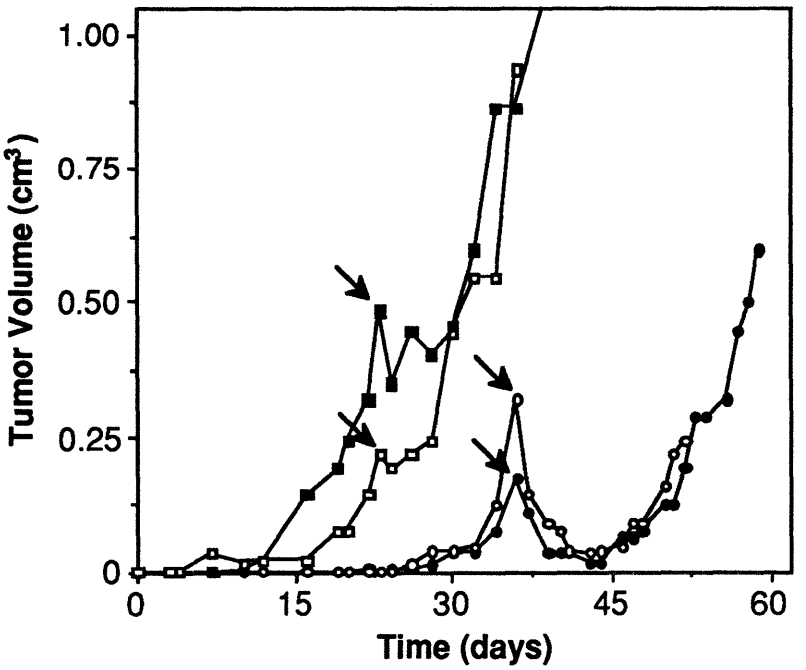

Figure 2. Single fraction irradiation of animals harboring tumors derived from $p53^{+/+}$ and $p53^{-/-}$ cells.

Embryonic fibroblasts transformed with E1A and T24 H-*ras* were injected subcutaneously into athymic nude mice and tumor volumes were monitored (circles, $p53^{+/+}$; squares, $p53^{-/-}$). When tumors had reached an appropriate volume (arrows), animals were treated with 7 Gy ionizing radiation. Tumor volumes were monitored for a period of more than 2 weeks after treatment. Open squares; tumor #400L; closed squares; tumor #399R; open circles, tumor #397; closed circles, tumor #396.

treatment was significantly reduced (Figure 3A). Thus, the response period was shorter and relapse occurred more rapidly. A second $p53^{+/+}$ clone (1AR.C10) produced very few tumors when injected into nude mice (2/9). The tumors that did arise grew slowly, and neither displayed rapid regression following treatment. However, the single dose protocol produced a significant lag in tumor expansion, and the multiple fraction protocol produced a 50% reduction in volume during the course of treatment (Table 1).

Remarkably, tumors derived from $p53^{-/-}$ animals displayed very little response to single fraction irradiation (Table 1 and Figure 2). Although some

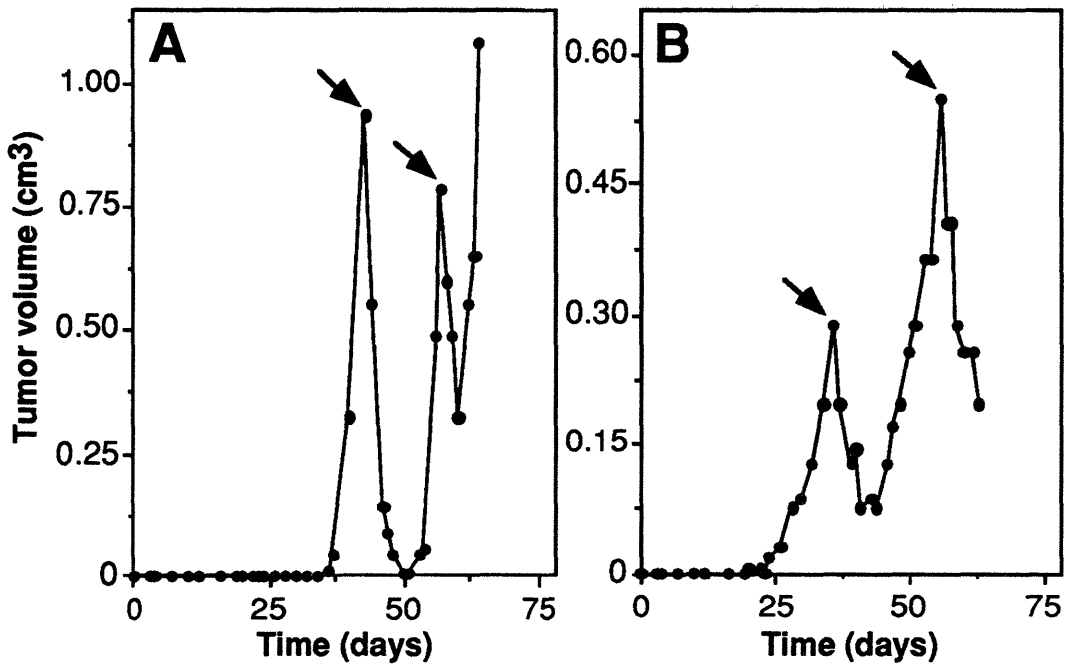

Figure 3. Retreatment of animals after tumor relapse.

Tumors derived from $p53^{+/+}$ cells transformed by E1A and T24 H-ras were treated with (A) 5 Gy ionizing radiation (tumor #380) or (B) 7 Gy ionizing radiation (tumor #384) and monitored for tumor regression. Upon tumor relapse, animals were retreated with the original dose. The arrows indicate the times of radiation treatment. In B, tumor volumes could not be monitored further because the animal developed acute radiation toxicity.

tumors displayed a transient shrinkage or slowed growth, in 13 of 14 instances tumor volumes had increased by 1 week following treatment. In several cases, tumors continued growing at their pre-treatment rate (not shown). Interestingly, fractionation schemes were superior in controlling the growth of $p53^{-/-}$ tumors, although tumors never regressed (Figure 4). It is worth noting that the absence of p53 has no influence on the transient G2 arrest that occurs following treatment of cultured cells with ionizing radiation (Kastan et al., 1992). Perhaps continuous low dose irradiation maintains cell cycle arrest in G2.

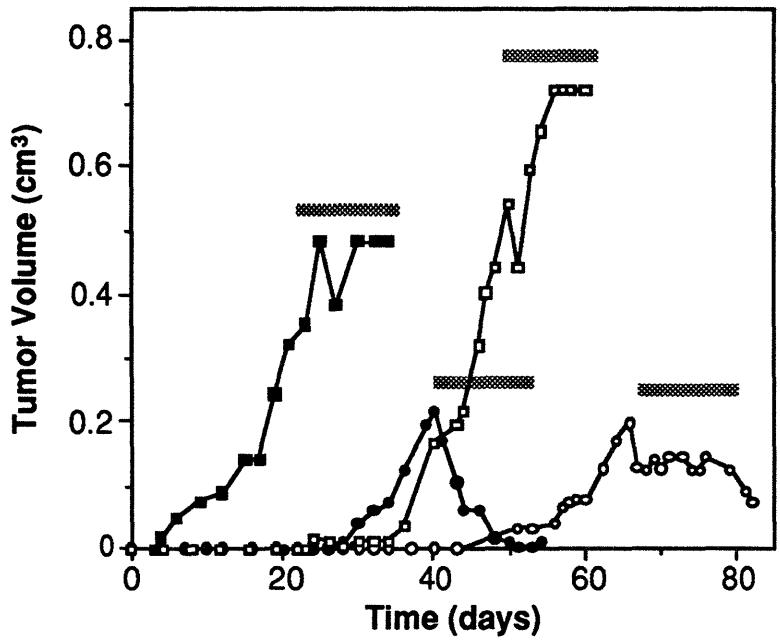

Figure 4. Multiple fraction irradiation of mice harboring tumors derived from $p53^{+/+}$ and $p53^{-/-}$ cells.

Embryonic fibroblasts transformed with E1A and T24 H-*ras* were injected subcutaneously into athymic nude mice and tumor volumes were monitored (circles, $p53^{+/+}$; squares, $p53^{-/-}$). When tumors had reached an appropriate volume, animals were treated with ten fractions of 2 Gy ionizing radiation (with days 6 and 7 left untreated). The shaded bars over each tumor indicate the treatment window. Where possible, tumor volume was monitored for a period of more than 2 weeks. In all cases, the mice suffered acute radiation toxicity and were sacrificed at two weeks. Open squares; tumor #941L; closed squares; tumor #387R; open circles, tumor #386 ; closed circles, tumor #385R.

DISCUSSION

p53 mutations in human cancer have been associated with poor prognosis. In the present study, we demonstrate that p53 status can influence tumor response to γ-irradiation. Thus, tumors derived from cells expressing p53 generally regressed upon γ-irradiation, whereas p53-deficient tumors continued to expand. *In vitro* studies indicate that the differential response of these tumors may reflect the inability of p53-deficient cells to trigger apoptosis (Chapter 3). However, these experiments are ongoing, and further

characterization of this system will be required to confirm this hypothesis. Nevertheless, these studies provide a potential molecular explanation for the association between p53 mutation and poor prognosis.

Although p53-deficient tumors were uniformly non-responsive to ionizing radiation, some $p53^{+/+}$ tumors also responded poorly following treatment. Both tumors that displayed a reduced response were derived from the same $p53^{+/+}$ clone. This clone was susceptible to apoptosis following treatment with ionizing radiation in cell culture. Perhaps complicating factors in the tumor environment modulate the ability of p53-expressing cells to activate apoptosis in response to γ-irradiation. Alternatively, since escape from apoptosis could promote tumor expansion as well as influence radiosensitivity (Chapter 3), cells may acquire mutations during the course of tumor progression (or, in this case, during expansion in cell culture) that lead to radioresistance. Indeed, resistant tumors may have acquired p53 mutations--either before or after introduction into animals--that reduced their susceptibility to apoptosis in response to ionizing radiation. Moreover, p53 mutations confer a selective advantage to tumor cells (Sidransky et al., 1992), indicating that cells acquiring p53 mutations could readily become the predominant cell type. It will be interesting to analyze the p53 genes derived from resistant tumors to determine whether reduced response to γ-irradiation correlates with p53 mutation.

In addition to γ-irradiation, several chemotherapeutic agents induce p53-independent apoptosis *in vitro*. In this study, we concentrated on irradiation protocols, primarily because γ-radiation is not complicated by pharmokinetic factors that influence drug delivery. These factors make it is difficult to extrapolate *in vitro* drug concentrations to effective doses *in vivo*. Nevertheless, will be important to determine whether p53 status also affects

tumor response to chemotherapeutic agents. In this regard, preliminary studies indicate that adriamycin causes tumor regression in a p53-dependent manner.

The experimental system used in this study allowed direct comparison of tumors derived from cells expressing or lacking p53. Although this study assessed response to radiation in *bona fide* tumors, extension of these results to spontaneous tumors is somewhat premature. It will be important to determine whether the response of spontaneous tumors can be influenced by p53 status, and whether tissues other than those of fibroblast origin behave similarly. However, similar experimental systems may be useful in developing more effective treatment protocols.

ACKNOWLEDGMENTS

These studies were performed in collaboration with Drs. Stephen Bodis and David Fischer from the Dana Farber Cancer Institute. M. McCurrach provided valuable technical assistance with the animals and tumor measurements.

CHAPTER 6

General Discussion

SECTION I: Apoptosis and tumor suppression by p53'

The studies presented in this thesis establish a direct mechanism of tumor suppression in which p53 participates in the destruction of aberrantly growing cells by an apoptotic process. First, p53 levels and stability increase in response to E1A. p53 accumulation contributes to E1A-associated cell death, since fibroblasts which lack p53 can express E1A without undergoing apoptosis. Finally, p53 suppresses transformation by E1A, as demonstrated by the fact that $p53^{-/-}$ cells are transformed to a tumorigenic state by E1A alone. This mechanism of tumor suppression suggests that p53 mutations, which typically occur late in tumor progression, could enhance the survival of cells expressing oncogenes activated early in tumor progression.

The fact that p53 is expressed in all normal cells without adversely affecting cell growth or survival implies that p53 activity must be regulated, particularly under circumstances that limit neoplastic growth. Given this consideration, several fundamental questions were posed concerning p53 and its activities as a tumor suppressor gene (see Chapter 1): How is p53 *activated* and under what circumstances? What *signals* or *properties of tumor cells* (or potential tumor cells) are responsible for p53 activation? What is the *fate* of cells in which p53 has been activated?

The involvement of p53 in the apoptotic response to oncogenes suggests a model for p53 activity during tumor progression (see Figure 1). In this view, the *signal* responsible for p53 activation is an oncogenic stimulus that causes forced or deregulated proliferation. In response, p53 is *activated* for tumor suppression by changes in p53 protein stability that allow p53 accumulation. The subsequent *fate* of cells is determined by environmental factors. Thus, cells expressing stabilized p53 continue to proliferate in a permissive growth environment, but

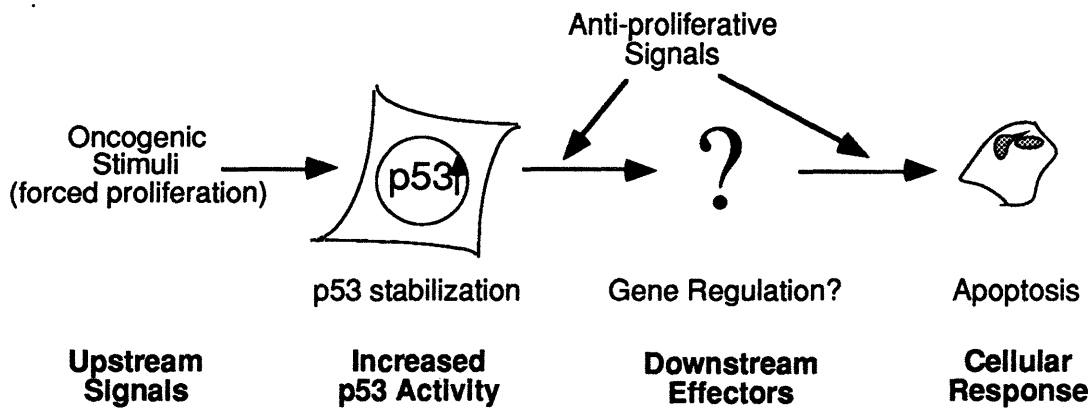

Figure 1. p53-dependent apoptosis in apoptotic response to oncogenes. Oncogenic stimuli can induce a cellular response leading to increases in p53 protein stability. p53 accumulation enhances susceptibility to apoptosis, such that cells are immediately able execute the apoptotic program in response to anti-proliferative signals (e.g. serum deprivation, DNA damage). Factors associated with the anti-proliferative environment either directly or indirectly synergize with stabilized p53 to activate downstream effectors and promote apoptosis. Loss of p53 function would enhance the survival of oncogene-expressing cells (even in an anti-proliferative environment) and allow tumor progression.

trigger apoptosis under conditions where further growth is perceived as aberrant. Since cells expressing "activated" p53 are highly susceptible to apoptosis, cells acquiring p53 mutations have a strong selective advantage. In the absence of the apoptotic pathway, neoplastic growth continues unchecked.

It also has been suggested that p53 loss increases the likelihood that cells acquire oncogenic mutations by allowing inappropriate proliferation following DNA damage (Kastan et al., 1992; Lane, 1992). In either case, p53 can be compared to a "molecular policeman" whose action is required to protect the organism against the deleterious consequences of genetic damage. However, the involvement of p53 in oncogene-associated apoptosis suggests a mechanism by which p53 functions *directly* in tumor suppression, and accounts for the observation that p53 mutations are typically observed late in tumor progression. By contrast, the involvement of p53 in the cellular response to DNA damage

indirectly suppresses transformation, by reducing the occurrence of oncogenic mutations. This may initiate neoplastic growth in patients with several heritable cancer syndromes (e.g. Li-Fraumeni syndrome), and accounts for the accelerated loss of genomic stability occurring late in tumor progression (Kastan et al., 1992; Livingstone et al., 1992; Yin et al., 1992).

The requirement for p53 in the cellular response to DNA damage accounts for the observation that p53-deficient mice develop normally, since p53 is not required for normal cell cycle progression but rather functions in a cell cycle checkpoint (Kastan et al., 1992). Similarly, p53 is not required for apoptosis during embryonic development. The involvement of p53 in apoptosis becomes apparent, however, upon expression of an oncogene. Apoptosis is particularly pronounced following serum depletion or treatment with genotoxic compounds. In mouse thymocytes, p53-dependent apoptosis is observed in response to DNA damage, but not physiological stimuli (Appendix I). Thus, p53-dependent growth arrest and apoptosis may be limited to pathological situations that are not prevalent during embryogenesis.

p53 stabilization

The importance of controlling tumor suppressor activities is analogous to that of proto-oncogenes, where tight regulation allows proto-oncogenes to be expressed in normal cells without causing cell transformation. Similarly, normal cells tolerate p53 expression because its activity is regulated. p53 levels increase both in response to oncogenes or following DNA damage, with the consequence of either directly or indirectly suppressing transformation. In both circumstances p53 expression is regulated at the level of protein turnover.

It remains possible that p53 stabilization is not a cause but a consequence of "activated" p53. For example, p53 could become stabilized by circumstances that increase its affinity for DNA, thereby sequestering it from proteases.

However, it seems unlikely that DNA-binding accounts for p53 stabilization, since adenovirus-5 $p55^{E1B}$ sequesters p53 outside the nucleus without affecting p53 accumulation in response to E1A (Chapter 2).

Ionizing radiation and E1A may stabilize p53 by similar mechanisms, since the effects of each treatment on p53 levels are not additive (Chapter 4). It is possible that E1A, like γ-irradiation, induces p53 stability by causing double-stranded breaks in DNA, perhaps as a consequence of premature S phase entry. Alternatively, p53 stabilization may occur by separate signal transduction pathways. The existence of distinct pathways for p53 stabilization has been demonstrated by the different genetic requirements for p53 accumulation in response to ultraviolet light and ionizing radiation (Khanna and Lavin, 1993). In this regard, E1A induces p53 accumulation in HeLa cells (E. White, personal communication) whereas γ-irradiation does not (Fritsche et al., 1993). The increase in p53 stability occurring upon γ-irradiation may require the ataxia-telangiectasia (AT) gene products ((Kastan et al., 1992); for contradictory data, see (Lu and Lane, 1993)) and p53 accumulation is blocked by caffeine (Kastan et al., 1991), It will be important to determine whether E1A can induce p53 in AT fibroblasts or in caffeine-treated cells.

Decreased p53 turnover could result from modifications in either p53 structure or the cellular environment. E1A could stabilize p53 by either directly or indirectly suppressing cellular proteolytic activities. Alternatively, E1A could either directly or indirectly alter p53 structure and reduce its susceptibility to proteolytic degradation. Ubiquitination appears to *destabilize* p53 in papilloma virus E6-expressing cells (Scheffner et al., 1990; Scheffner et al., 1993), but whether a similar mechanism regulates p53 stability in normal cells or in response to E1A is unknown. Interestingly, E1A induces p53 accumulation in E6-expressing HeLa cells (E. White, personal communication), implying that the

mechanism in which E1A stabilizes p53 is dominant to E6-facilitated destabilization (alternatively, E1A may simply suppress E6 expression). It is conceivable that other structural modifications affect p53 stability. p53 is both phosphorylated and linked to a short ribonucleotide, but the association between these modifications and p53 stability has not been tested. However, the stabilized p53 in E1A-expressing cells has no obvious conformational alterations, and is unaltered with regard to electrophoretic mobility, subcellular localization, or interactions with monoclonal antibodies and viral tumor antigens (Chapter 2).

Priming and triggering of apoptosis

Although E1A-associated apoptosis occurs during normal propagation of cells, cell death is significantly enhanced following treatment with anti-proliferative agents (Chapters 2-4). Moreover, p53 accumulates in proliferating cells, and p53 levels do not increase further under apoptotic conditions (Chapters 3 and 4). This suggests that p53 stabilization is not sufficient for apoptosis, rather, apoptosis appears to require both stabilized p53 and an anti-proliferative signal.

p53 stabilization may be part of the mechanism in which oncogenes "prime" cells for apoptosis (for a discussion of "priming" see (Wyllie, 1993)). Thus, E1A-expressing cells are able to immediately execute the apoptotic program whereas normal cells are not. Cells expressing E1A and stabilized p53 remain viable under conditions conducive to growth, but lose viability when exposed to anti-proliferative stimuli. Nevertheless, increased p53 stability is not sufficient to prime cells for apoptosis, since both p53 overexpression and γ-irradiation (which also stabilizes p53) frequently induce cell cycle arrest. E1A enables cells to proliferate despite the presence of high p53 levels, and presumably cells become primed for apoptosis by the combination of E1A and stabilized p53.

Several observations are consistent with the view that apoptosis requires elevated p53 levels, a growth-inhibitory environment, and forced proliferation. First, increases in p53 levels and stability are tightly associated with apoptosis in response to E1A and following γ-irradiation of mouse thymocytes (Chapters 2 and 3; Appendix I). Second, cell death is triggered by anti-proliferative stimuli, including serum depletion and genotoxic agents. In other experiments, p53-dependent apoptosis was triggered in cells expressing E1A by colchicine, a microtubule inhibitor that blocks mitosis (data not shown). Third, regions of E1A required for apoptosis correspond to those that stimulate cell proliferation (White et al., 1991). It will be important to determine whether these same regions are sufficient for p53 stabilization. Fourth, E1A circumvents cellular controls that limit proliferation. Thus, p53 levels in E1A/T24 H-*ras* expressing cells are as high as those that occur in growth-arrested fibroblasts following γ-irradiation (Chapter 3). Although irradiated fibroblasts expressing stabilized p53 undergo transient growth arrest, cells co-expressing E1A and T24 H-*ras* grow rapidly (Chapters 3 and 4). Moreover, cells expressing E1A continue to proliferate upon transfer to mitogen-poor medium or following γ-irradiation, even as they die by apoptosis (Chapters 3 and 4). Taken together, these observations suggest that p53 functions as part of a general mechanism to selectively destroy aberrantly growing cells.

Growth Arrest or Apoptosis?

Forced overexpression of p53 can induce either growth arrest or apoptosis (see, for example, (Michalovitz et al., 1990; Yonish-Rouach et al., 1991)). Similarly, physiological stimuli that stabilize p53 have been associated with both growth inhibition and apoptosis (Chapter 4; Appendix I; (Kastan et al., 1992)). In these studies, E1A altered the cellular response to stabilized p53 from growth arrest to apoptosis. For example, treatment of normal fibroblasts with ionizing

radiation stabilizes p53 and induces p53-dependent growth arrest (Chapter 4; Kastan et al., 1992). The E1A oncogene, and perhaps other oncogenes as well, bypasses p53-dependent G1 arrest following γ-irradiation, leading to aberrant proliferation and p53-dependent apoptosis. Thus, stimuli that normally limit proliferation instead induce apoptosis in cells unable to respond appropriately due to the expression of an oncogene.

The observation that E1A bypasses p53-dependent growth arrest suggests that p53 is primarily concerned with negative growth regulation, and is consistent with the notion that apoptosis is a consequence of abnormal growth. Thus, fibroblasts lacking p53 display no overt susceptibility to apoptosis, but have a greatly enhanced proliferative capacity as defined by the ability to grow at clonal densities (Chapter 3). p53-deficient cells cycle faster, require less time to pass through G1, and leave the cycling state more slowly than wild-type MEFs (Chapter 3). Thus, in fibroblasts, the involvement of p53 in apoptosis is only apparent in oncogene-expressing cells [However, note that primary thymocytes are susceptible to p53-dependent apoptosis in response to ionizing radiation (Appendix 1)].

How does stabilized p53 induce growth arrest? The recent cloning of *CIP1/WAF1* has provided a direct link between p53 accumulation and growth inhibition. In normal fibroblasts, increases in p53 levels activate *CIP1/WAF1* transcription (El-Deiry et al., 1993). Subsequently, the *CIP1/WAF1* gene product, p21, associates with the cylin dependent kinases (cdks) and inhibits their activity (Harper et al., 1993). Since active cdk4/cyclin D complexes phosphorylate the retinoblastoma gene product (Rb) and release the E2F transcription factors for initiation of S phase (Ewen et al., 1993), p53 could arrest cell growth, in part, by indirectly suppressing cdk activity and Rb phosphorylation. However, E1A sequesters Rb, and therefore cells contain free E2F even though G1-specific

cyclin-dependent kinases are inactive. This may explain why E1A-expressing cells bypass p53-dependent growth arrest following γ-irradiation.

How does stabilized p53 induce apoptosis? p53 could induce apoptosis directly, by regulating genes involved in the apoptotic process. In this view, the genes regulated by p53 in E1A-expressing cells must be distinct from those in normal cells (which lead to growth inhibition). Perhaps stabilized p53 expressed in growing cells synergizes with a factor normally not present in p53-arrested cells to regulate apoptotic genes. Free E2F is a candidate for such a factor, since E2F would normally be sequestered in p53-arrested cells.

Alternatively, stabilized p53 may regulate the same set of genes under circumstances that lead to both growth arrest and cell death, and apoptosis occurs as a result of distinct downstream factors. Consistent with this view, it has been suggested that p53-induced apoptosis results from an incompatible combination of growth-promoting and growth-inhibitory stimuli (Yonish-Rouach et al., 1993). A molecular basis for "conflicting signals" has been described above: cells expressing E1A (and stabilized p53) can induce *CIP1/WAF1* and suppress cdk activity, but since Rb is sequestered, this inhibition has no effect on the availability of free E2F. Thus, cells contain inactive cyclin-dependent kinases and "active" E2F, a situation that does not exist in normal cells. This may trigger apoptosis, perhaps by synergistic action of "inactive" cdk (or the absence of active cdk) and "active" (i.e. free) E2F. In cells lacking p53 or in adenovirus-transformed cells--which are resistant to apoptosis--p21$^{CIP1/WAF1}$ is not associated with cdk/cyclin complexes (Xiong et al., 1993). Therefore, these cells contain active cyclin-dependent kinases and free E2F, which is compatible with proliferation.

Does p53-dependent apoptosis occur only in response to E1A?

The above sections propose a model whereby p53 participates in a

mechanism to eliminate aberrantly growing cells. While this view is based solely on cellular responses to a viral oncogene, the principles identified here may have general relevance for oncogenic transformation and tumor progression. For example, cell proteins that interact with E1A regions that promote apoptosis may normally serve as distal targets of signal transduction pathways by which external stimuli regulate cell growth and survival (White et al., 1991; Ruley, 1990). By interfering with these activities, E1A mimics cellular activities involved in spontaneous transformation.

Like E1A, certain cellular oncogenes induce both proliferation and apoptosis, including c-*myc* and the *E2A-PBX1* encoded protein (Evan et al., 1992; Dedera et al., 1993). While the involvement of p53 in *myc* and *E2A-PBX1*-associated apoptosis has not been thoroughly examined, several observations suggest that *myc*-induced apoptosis may involve p53. First, dominant-negative p53 alleles synergize with *myc* in *ras*-co-transformation of primary cells (Eliyahu et al., 1989). Second, overexpression of these p53 mutants in certain myeloid leukemia lines reduces *myc*-induced sensitivity to genotoxic drugs (Lotem and Sachs, 1993). Third, activation of c-*myc* and p53 mutation commonly occur in the same tumors, particularly in Burkitt's lymphoma (Gaidano et al., 1991). This observation is reminiscent of the incompatibility between E1A and endogenous p53 expression (Chapters 2 and 3). Finally, preliminary studies indicate that p53-deficient mouse embryonic fibroblasts are resistant to *myc*-induced apoptosis (data not shown).

The fact that a viral oncogene induces p53-dependent apoptosis suggests that p53 stabilization and apoptosis may represent a natural defense against viral infection. Indeed, the human immunodeficiency virus induces apoptosis upon acute infection (Laurent-Crawford, et al., 1991), and both Epstein-Barr virus and Sindbis virus express viral proteins that modulate apoptosis (Gregory, et al.,

1991; Levine, et al., 1993). However, the involvement of p53 in these processes has not been examined. E1A sequences required for apoptosis map to conserved region 1, and related sequences exist in both SV40 large T antigen and HPV E7 (Ruley, 1990). Thus, it would not be surprising if E7 or certain T antigen mutants (encoding conserved region 1 but lacking the p53 binding domain) also induce apoptosis.

p53 is also required for radiation-induced apoptosis in primary mouse thymocytes (Appendix 1; Clarke et al., 1993). Again, p53 accumulation accompanies apoptosis (Appendix 1). p53-dependent apoptosis appears specific for agents that damage DNA, since other stimuli (such as glucocorticoids) induce apoptosis in a p53-independent manner. This may protect the organism from the potentially deleterious consequences of aberrant T cell receptor rearrangement, by eliminating cells containing broken DNA ends that might otherwise produce chromosomal rearrangements. Thymocytes are extremely susceptible to apoptosis, but unlike fibroblasts expressing E1A, immature thymocytes do not proliferate. Thus, p53-dependent apoptosis in thymocytes may represent a distinct mechanism from E1A-associated apoptosis in fibroblasts. However, it remains possible that proliferation-associated changes accompany radiation-induced apoptosis in thymocytes. Identification of additional activities involved in both processes will be required to clarify this issue.

Other direct mechanisms of tumor suppression by p53

The observation that E1A stabilizes p53 arose from studies investigating *ras*-induced cell cycle arrest (see Chapter 1). p53 may participate in this process, since both E1A (which prevents p53-dependent arrest following γ-irradiation, see Chapter 4) and dominant-negative p53 alleles circumvent *ras*-induced arrest. Consistent with this view, activated *ras* oncogenes are unable to promote clonal outgrowth in wild-type embryonic fibroblasts, but morphologically transform

p53-deficient cells (not shown). In should be noted that, unlike in E1A-expressing cells, increases in *CIP1/WAF1* activity in *ras*-expressing cells could inhibit Rb phosphorylation and prevent release of E2F. Indeed, this model accounts for the observation that E1A-induced apoptosis is dominant to *ras* arrest, since E1A blocks p53-dependent growth arrest but *ras* does not prevent apoptosis (Chapters 3 and 4).

While p53 accumulation was not observed following *ras*-induced arrest (Chapter 1), increases in p53 levels may not be required to maintain the arrested state. For example, UV irradiation of fibroblasts stabilizes p53 and induces growth arrest, but p53 levels decline long before cells resume proliferation (Lu and Lane, 1993). Alternatively, increases in p53 levels may not be necessary for *ras*-induced arrest. Nevertheless, p53-dependent arrest in response to oncogenes may represent another direct mechanism of tumor suppression by p53.

In addition, p53 could directly inhibit neoplastic growth by its involvement in cellular senescence. In these studies, p53 loss appeared sufficient for immortalization (Chapter 3). Therefore, p53 mutation may release the cell from normal growth controls that limit proliferative capacity, allowing unlimited population expansion. It is presently unclear what factors activate p53 for cellular senescence, or even whether senescence involves p53 stabilization.

SECTION II: Implications of p53 mutation and escape from apoptosis

p53 mutation and tumor progression

Since tumor expansion is determined by the balance between cell division and cell loss, factors that influence either proliferation or viability could have a dramatic effect on tumor progression. Therefore, increases in cellular susceptibility to apoptosis limit neoplastic growth, while mutations that suppress apoptosis promote tumor expansion (see discussion in Chapter 1). In these

studies, p53-dependent apoptosis has been established as a cellular mechanism that limits tumor expansion. Perhaps high cell density or limiting concentrations of growth factors, conditions that trigger apoptosis in cell culture, also are prevalent in the emerging tumor. Thus, p53 mutations prevent neoplastic cells from sensing growth-limiting factors and allow proliferation to continue unchecked. As discussed above, this accounts for the association between p53 mutation and malignant progression (see also, (Kemp et al., 1993)).

It is often assumed that p53 mutations promote neoplastic transformation by a dominant-negative mechanism. Indeed, forced overexpression of mutant p53s can inhibit wild-type function (Kern et al., 1992). It is unclear, however, whether all mutant p53s function as dominant-negative inhibitors under physiological circumstances. In these studies, the effects of p53-deficiency on the cell growth and survival were surprisingly dose-dependent (Chapters 3 and 4; Appendix 1), implying that point-mutations leading to partial loss of p53 functions may provide a selective advantage in the absence of a dominant-negative effect. This could allow the growth of an expanded cell population from which p53-deficient variants arise.

p53 mutations and metastasis

Tissue homeostasis and integrity may be determined, in part, by factors that inform cells of their environment. The location and concentrations of these molecules may influence net tissue expansion or confine cells to specific tissues. It has been suggested that apoptosis is activated when concentrations of these factors ("survival factors") decrease below a certain threshold, for example, when cells outgrow their nutrient supply or migrate to a foreign environment (Raff, 1992). However, during metastasis, tumor cells become able to tolerate foreign environments. Thus, the susceptibility of tumor cells to apoptosis may limit metatstatic spread.

Since p53 may participate in the process by which environmental factors influence apoptosis (Chapter 2 and 3), p53 mutation could increase the likelihood of metastasis. Indeed, p53 mutation has been associated with metastasis in several tumor types. This is particularly striking in gastric cancer, where p53 mutations were observed in the metastases, but not primary tumors, of several patients (Yamada et al., 1991).

p53 mutation and tumor response to cancer therapy

It is becoming more apparent that anticancer agents elicit their cytotoxic effects by triggering apoptosis. Similarly, mutations affecting cell death pathways may be responsible for non-responsive tumors or tumor relapse. These studies suggest that certain oncogenes may sensitize cells to apoptosis, which can be triggered by anticancer agents in a p53-dependent manner (Chapter 4). p53 mutations may influence tumor response to anticancer agents and ultimately patient prognosis by decreasing the efficiency with which these agents trigger apoptosis. Indeed, the absence of p53 eliminated the response of tumors to ionizing radiation using a transplanted tumor model (Chapter 5).

It is presently unknown whether p53-dependent apoptosis influences tumor response to anticancer agents in human cancer. Although discussed extensively in Chapters 4 and 5, it is worth re-stating that p53 mutations are: (i) more prevalent in tumor types that are notoriously refractile to treatment, (ii) are associated with poor prognosis, and (iii) have been correlated with tumor relapse. Although these studies are suggestive, they fall far short of proving that p53-dependent apoptosis influences tumor response to cancer therapy.

Nevertheless, the situation in acute lymphoblastic leukemia (ALL) is particularly compelling. The original studies examining the frequency p53 mutation in ALL were contradictory: several groups detected very low frequencies of p53 mutation in ALL (Gaidano et al., 1991; Jonveaux and Berger,

1991), while another study suggested p53 mutation was a common event (Yeargin et al., 1992). This discrepancy was clarified when it became apparent that the specimens containing p53 mutations were from relapse patients, whereas the specimens lacking p53 mutations were obtained at tumor presentation (prior to chemotherapy) (Yeargin et al., 1993). In the only published instance where presentation and relapse specimens were analyzed from the same patient, a p53 mutation was observed only in the relapse specimen (Felix et al., 1992). Moreover, it was noted that this patient did not respond further to therapy. In 6 of 8 similar comparisons, p53 mutations were associated with tumor relapse (M. Haas, personal communication). While these studies were not designed to associate p53 mutation with tumor response to anticancer agents, it is striking that p53 mutations would be specific for relapse specimens. Thus, the only cells that survived the initial therapy must have possessed or acquired a mutation within the p53 gene.

In order to develop better treatment and diagnosis of human cancer, it will be necessary to determine whether p53 and apoptosis contribute to tumor response to anticancer agents. Present studies related to this issue suffer from several limitations. First, no study has been designed to correlate tumor response with p53 status. Second, many studies do not adequately take into account tumor stage when determining the frequency of p53 mutations in a given tumor type. Since p53 mutations typically occur late in tumor progression, this information is essential. Third, p53 accumulation has been equated with p53 mutation. Thus, p53 immunochemical staining of paraffin sections has been used extensively to assess mutation frequencies. However, in situations this has been examined, considerable disagreement exists whether p53 accumulation reflects mutation (for two opposing views concerning p53 accumulation and mutation in breast cancer, see (Thompson et al., 1992; Harris et al., 1992)). Finally, the

limitations of human research have prevented comparison of p53 status from the same tumor at different stages (e.g. primary tumor vs. metastasis; presentation vs. relapse specimen) as recently described for ALL. Such comparisons are clearly the most informative, and further examination of p53 status in these types of specimens will be required to resolve this issue.

SECTION III: Mechanisms of transformation by E1A and cooperating oncogenes

In order to identify mechanisms by which p53 suppresses transformation, we examined the role of p53 in regulating the cellular response to transfected oncogenes. These studies addressed the following questions: Does the presence of endogenous p53 suppress transformation by individual oncogenes, specifically E1A? Do oncogenes that collaborate with E1A in transformation circumvent transformation suppression by endogenous p53? Finally, does apoptosis play a direct role in suppressing oncogenic transformation?

In addressing these questions, these studies provide insight into the biological and biochemical basis for transforming interactions between E1A and both viral and cellular oncogenes. Consider the following: (i) p53 was required for E1A induced apoptosis (Chapters 3 and 4), (ii) p53-dependent apoptosis was associated with increases in p53 levels and stability (Chapters 2 and 3), (iii) high p53 levels were not sufficient for apoptosis, rather, apoptosis was associated with aberrant cell cycle progression (Chapters 3 and 4), (iv) E1B had no additional effect on p53 stability (Chapter 2), but suppressed apoptosis (Chapters 2 and 3), (v) absence of p53 substituted for E1B in promoting growth, survival, and transformation with E1A (Chapters 3 and 4), and (vi) activated *ras* oncogenes do not block p53-dependent apoptosis (Chapters 2-5), but collaborate with E1A to transform cells to a highly tumorigenic state (Chapters 2 and 5).

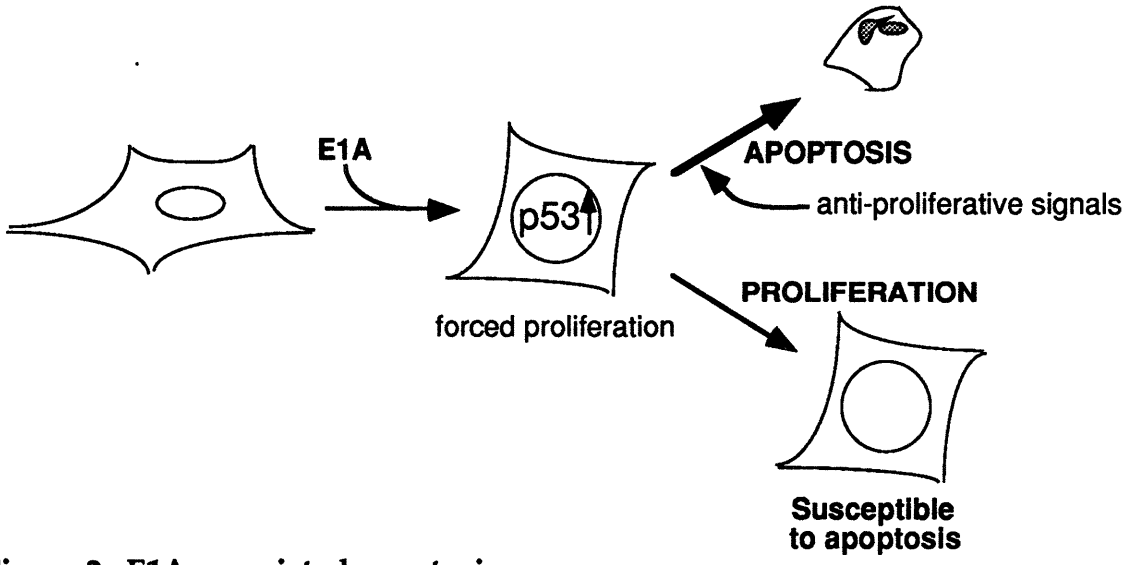

Figure 2. E1A-associated apoptosis.

E1A expression induces proliferation and, at the same time, increases p53 protein stability. Cells expressing stabilized p53 are "primed" for apoptosis; they can continue to proliferate in a permissive environment but trigger apoptosis in an anti-proliferative environment. Even under normal culture conditions, cells expressing E1A alone are highly prone to apoptosis, such that permanent cell lines cannot be established.

p53 and E1A-associated apoptosis

One view of p53 function in E1A-associated apoptosis is illustrated in Figure 2 (see discussion above). Cells expressing E1A contain stabilized p53 and are susceptible to p53-dependent apoptosis. Cells expressing stabilized p53 can either proliferate or initiate apoptosis, and apoptosis is enhanced by anti-proliferative stimuli such as serum deprivation or genotoxic agents. For cells expressing E1A alone, apoptosis occurs during the normal propagation of cells. Thus, co-expression of E1A and endogenous p53 is largely incompatible with long term growth. In REF52 cells expressing E1A, cells lose E1A expression (Chapter 2); in E1A-expressing MEFs, p53 expression is selected against (Chapter 3). Cellular susceptibility to p53-dependent apoptosis apparently suppresses transformation by E1A, since p53-deficient cells are transformed by E1A alone (Figure 3).

Although considered an "immortalizing" oncogene, E1A was inefficient at promoting colony outgrowth in normal embryonic fibroblasts (Chapter 3). Alternatively, stable cell lines expressing E1A were readily established from p53-deficient cells, while the only E1A-expressing line obtained cells containing endogenous p53 suffered p53 loss during clonal expansion. It has been suggested that immortalization by E1A requires additional genetic changes (Zerler et al., 1986). These results suggest that these changes involve p53 mutation and escape from E1A-associated apoptosis.

Transformation involving escape from p53-dependent apoptosis

The interactions between E1A and p53 have particular relevance to the role of E1B in adenovirus transformation (Figure 3). These studies suggest that the elevated p53 levels observed in adenovirus-transformed cells are due neither to E1B binding nor oncogenic transformation, but reflect a cellular response to E1A expression. While having no additive effect on p53 levels, E1B protects against p53-dependent apoptosis and enables E1A to transform. In this regard, the absence of p53 substituted for E1B activities, and E1B provided no significant advantage to p53-deficient cells. Thus, the primary function of the E1B proteins in adenovirus transformation--and perhaps adenovirus replication as well--is to counteract the effects of stabilized p53.

Several other genes that cooperate with E1A in oncogenic transformation of normal cells appear to block p53-dependent apoptosis. These include the *bcl*-2 oncogene (Rao et al., 1992) and mutant p53 alleles (Debbas and White, 1993). Taken together, these data suggest that inhibition of p53-dependent apoptosis occurs by at least two mechanisms. Cooperative interactions between E1A and either $p55^{E1B}$ or mutant p53 proteins probably involve physical interactions with p53 that inhibit transcriptional transactivation (Yew and Berk, 1992; Kern et al., 1992; Zambetti et al., 1992; Farmer et al., 1992). Alternatively, the $p19^{E1B}$ and *bcl*-2

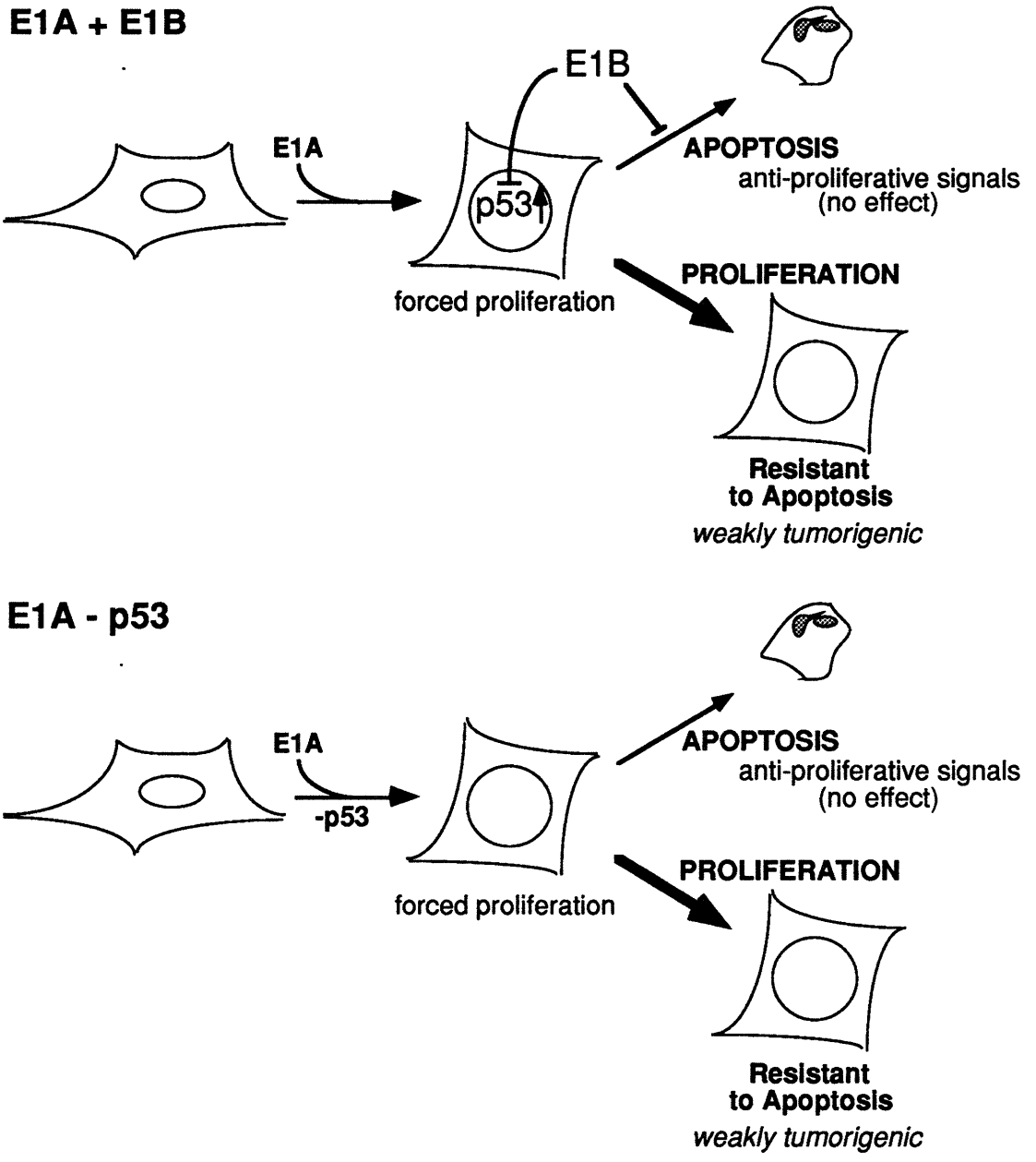

Figure 3. Oncogenic transformation involving escape from p53-dependent apoptosis.

E1B, while having no effect on E1A-induced p53 stabilization, inhibits p53-dependent apoptosis. E1B prevents apoptosis by two distinct mechanisms: by direct binding to p53 ($p55^{E1B}$) and by interfering with the apoptotic pathway ($p19^{E1B}$). Thus, cells proliferate rather than undergo apoptosis, are non-responsive to anti-proliferative signals, and are weakly tumorigenic. The primary function of both E1B proteins is to inhibit p53-dependent apoptosis, since p53 absence substitutes for E1B in promoting growth, survival, and transformation of E1A-expressing cells.

oncogenes act downstream of p53 by interfering with the apoptotic pathway (Rao et al., 1992). The latter mechanism is apparently more efficient, since $p19^{E1B}$ is better than $p55^{E1B}$ at blocking apoptosis (Rao et al., 1992).

Oncogenic transformation involving *ras* oncogenes

The transforming interactions between E1A and activated *ras* oncogenes are distinct from those involved in adenovirus transformation. While E1B suppresses apoptosis, cells transformed by E1A and T24 H-*ras* are highly tumorigenic yet remain sensitive to apoptosis. *ras* p21s normally are involved in signal transduction pathways that propagate external growth signals from the cell surface to the nucleus. It is possible that the potent proliferative activities of activated *ras* oncogenes compensate for cell loses due to E1A (Figure 4). Consistent with this, E1A-expressing cells transformed by *ras* grow faster than their E1B-expressing counterparts, both in cell culture and as tumors. Nevertheless, anti-proliferative stimuli are capable of inducing p53-dependent apoptosis in the presence of activated *ras* oncogenes, suggesting that cell death is not simply due to lack of growth or survival factors.

Alternate routes to transformation.

These studies demonstrate that oncogenic transformation can occur by distinct mechanisms, which alter the balance of growth, survival and transformation in different ways. This is perhaps not surprising, since neoplastic transformation can involve a plethora of activated oncogenes and inactivated tumor suppressor genes. One route to transformation involves forced proliferation and inactivation of the apoptotic response. However, escape from apoptosis is neither a prerequisite for, nor a consequence of, oncogenic transformation. Thus, cells can be highly tumorigenic but susceptible to apoptosis. The latter mechanism implies that tumor growth can occur while cells

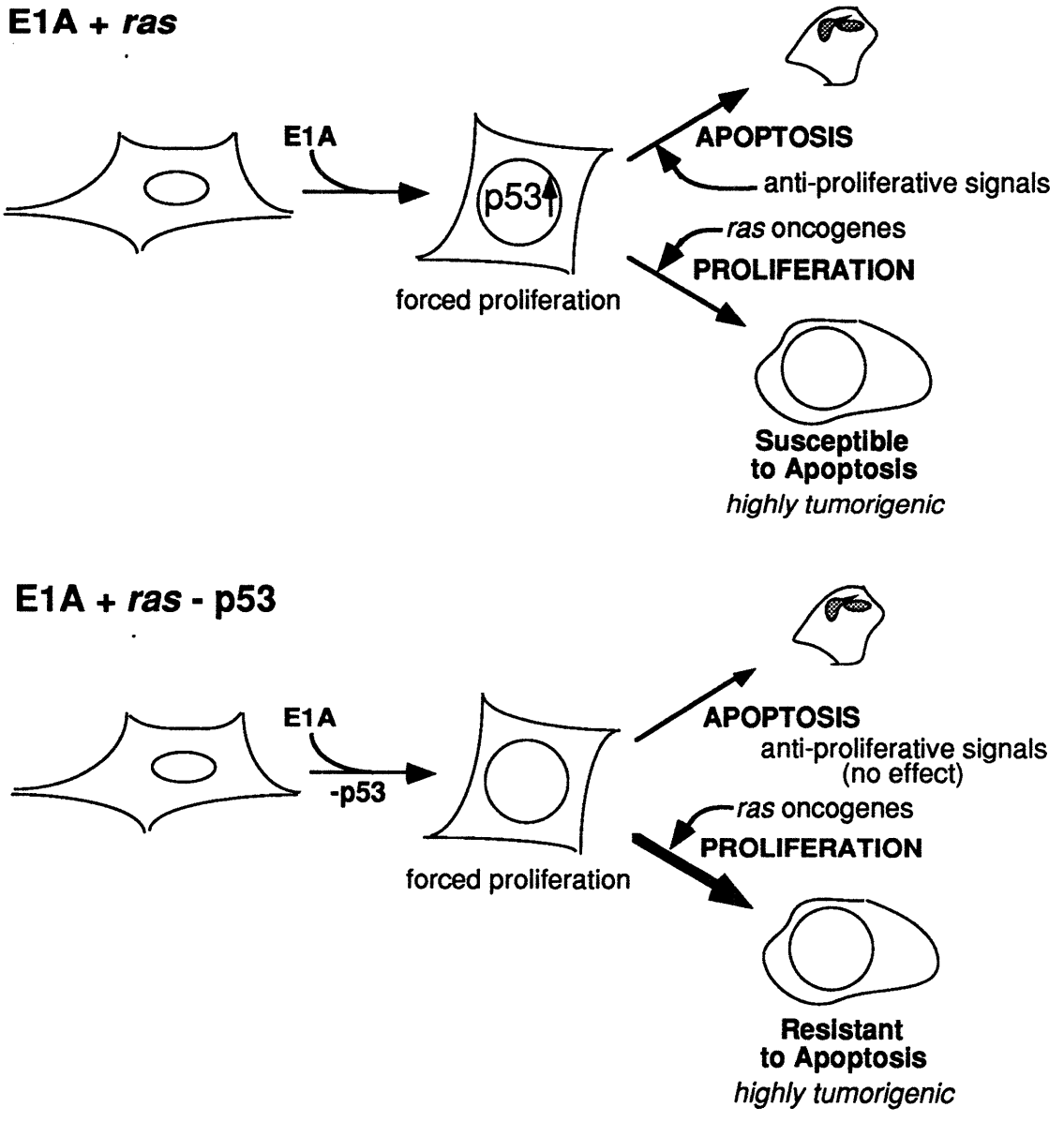

Figure 4. Transformation involving *ras* oncogenes.

ras oncogenes have no effect on E1A-induced p53 stabilization or the ability of anti-proliferative signals to trigger p53-dependent apoptosis in E1A-expressing cells. However, *ras* compensates for cell losses due to E1A, perhaps by providing a strong proliferative signal. Thus, cells co-expressing E1A and *ras* are highly tumorigenic yet remain sensitive to apoptosis. Since susceptibility to apoptosis requires p53 stabilization, apoptosis cannot be triggered in p53-deficient cells. Under these circumstances, rapid proliferation continues without apoptosis.

remain genotypically susceptible to apoptosis, and accounts for the observation that apoptosis is a common feature of malignant tumors.

SECTION IV: Experimental approach

The observations and conclusions of this thesis were strengthened by the use of an experimental approach that, until recently, has been impossible in mammalian systems. The production of animals with homozygous inactivation of the known genes has permitted the isolation of cell populations in which gene function can be examined in a systematic manner. Analysis of gene function in cells derived from "knock-out" mice combines the power of classical genetic approaches (i.e. null mutants) with the strengths of mammalian cell culture systems (e.g. gene transfer technology, cell biology).

In this study, embryonic fibroblasts derived from mice containing disrupted p53 genes were used to study the role of p53 in regulating cellular responses to transfected oncogenes. Because these cells were essentially identical except for the status of their p53 genes, differences in cellular responses could be directly attributed to p53 function. Similar analysis of embryonic fibroblasts derived from mice deficient for other genes should provide further insight into the mechanisms of normal and neoplastic growth.

SECTION V: Future directions

Much of the experimentation described here was directed at demonstrating that p53 is involved in apoptosis. Now that this is apparent, it will be important to develop a better understanding of other molecules involved in the apoptotic program. Presently, little is known about the molecular pathways leading to p53 stabilization or factors downstream of p53 that elicit the biological response. What is the cellular lesion(s) responsible for the decision to activate the death program? What detects cellular damage or stress? How does

aberrant growth and/or cellular damage increase p53 stability? Does p53 *mediate* apoptosis (i.e. directly regulate apoptotic genes) or is apoptosis indirect? What additional activities are involved in the apoptotic response? How does E1A alter the cellular response to stabilized p53 from growth arrest to apoptosis? This last question is of particular interest, since a molecular understanding of this process may provide insight into the regulation of cell cycle checkpoints. In addition, it will be important to determine what other oncogenes induce p53-dependent apoptosis (e.g. c-*myc*).

These studies may have relevance to human cancer, particularly with regard to mechanisms that determine the cytotoxicity of anticancer agents. p53-dependent apoptosis was required for effective killing by several anticancer agents in cell culture and p53 status influenced tumor response to γ-irradiation. A much more detailed analysis of p53 mutation during tumor progression and upon tumor relapse will be required to determine whether similar mechanisms influence tumor progression and response in human cancer. If so, systems analogous to those described in Chapters 4 and 5 may be useful in identifying novel anticancer drugs, including agents that restore p53 activities or activate apoptosis in a p53-independent manner.

APPENDIX 1

p53 is required for radiation-induced apoptosis in mouse thymocytes

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INTRODUCTION

The p53 tumor suppressor gene is the most widely mutated gene in human tumorigenesis (Hollstein et al., 1991; Levine et al., 1991). p53 encodes a transcriptional activator (Fields and Jang, 1990; Raycroft et al., 1990; Farmer et al., 1992; Kern et al., 1992; Zambetti et al., 1992) whose targets may include genes that regulate genomic stability (Livingstone et al., 1992; Yin et al., 1992), the cellular response to DNA damage (Kastan et al., 1991; Kastan et al., 1992), and cell cycle progression (Michalovitz et al., 1990; Martinez et al., 1991). Introduction of wild-type p53 into cell lines which have lost endogenous p53 function can cause growth arrest (Mercer et al., 1990; Diller et al., 1990; Baker et al., 1990) or induce a process of cell death known as apoptosis (Yonish-Rouach et al., 1991; Shaw et al., 1992).

During normal development, self-reactive thymocytes undergo negative selection by apoptosis (MacDonald and Lees, 1990), which can also be induced in immature thymocytes by other stimuli, including exposure to glucocorticoids (Wyllie, 1980) and ionizing radiation (Sellins and Cohen, 1987). Although normal negative selection involves signaling through the T cell receptor (MacDonald and Lees, 1990), the induction of apoptosis by other stimuli is poorly understood. We have investigated the requirement for p53 during apoptosis in mouse thymocytes. We report here that immature thymocytes lacking p53 die normally when exposed to compounds that may mimic T cell receptor engagement and to glucocorticoids but are resistant to the lethal effects of ionizing radiation. These results demonstrate that p53 is required for radiation-induced cell death in the thymus but is not necessary for all forms of apoptosis.

METHODOLOGY

p53-deficient animals

Mutant mice used in these experiments carried a germline disruption of the p53 gene (T.J. and R. Weinberg, unpublished) that was made by gene targeting in D3 embryonic stem cells (Gossler et al., 1986). The mutation consists of a replacement by the bacterial *neo* gene of p53 sequences between exons 2 and 6; immunoprecipitation analysis has confirmed that the mutation eliminates production of p53 protein (Livingstone et al., 1992). The mutation is carried on a hybrid (C57BL/6 x 129/sv) genetic background. Although genetic background is known to influence the sensitivity of thymocytes to treatments such as irradiation (Mori et al., 1992), we obtained consistent results from all animals within a given genotype.

Viability of isolated thymocytes

Thymocytes were prepared from animals between 5 to 8 weeks by removing the thymus and dispersing cells in PBS. In preliminary studies, >50% of untreated thymocytes spontaneously underwent apoptosis during the course of a typical experiment (24 hours), making it difficult to determine the effects of the experimental treatments. Therefore, culture conditions were optimized to maximize the viability of untreated cells. Since fluctuations in pH during thymocyte manipulation prior to treatment could diminish viability, medium containing 25mM HEPES (pH 7.2) was compared to standard bicarbonate buffered medium. Also, serum concentrations were varied in order to maximize thymocyte viability. At all serum concentrations, thymocyte viability was greater in HEPES-buffered medium (between 2% and 10% FBS), although viability declined in medium containing 20% FBS (Figure 1).

Cell density was varied to maximize viability during the 24 hour treatment period. Surprisingly, cell density had little effect on viability in 5%

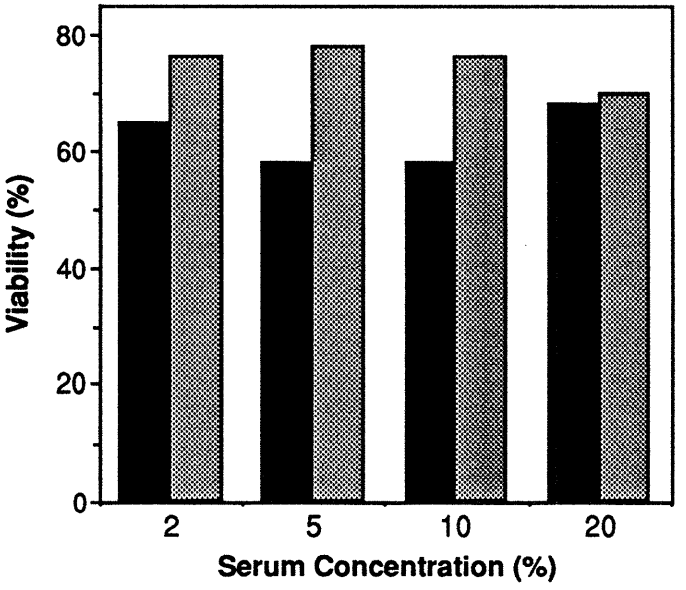


Figure 1. Effect of buffers and serum concentration on thymocyte viability.

Thymocytes were isolated from a wild-type animal (6 weeks old), adjusted to a density of 1 x 10^6/ml, and incubated in media containing either bicarbonate buffer (black) or HEPES buffer (shaded) and the indicated concentration of FBS. Cell viability was measured at 24 hours by FITC uptake and flow cytometry.

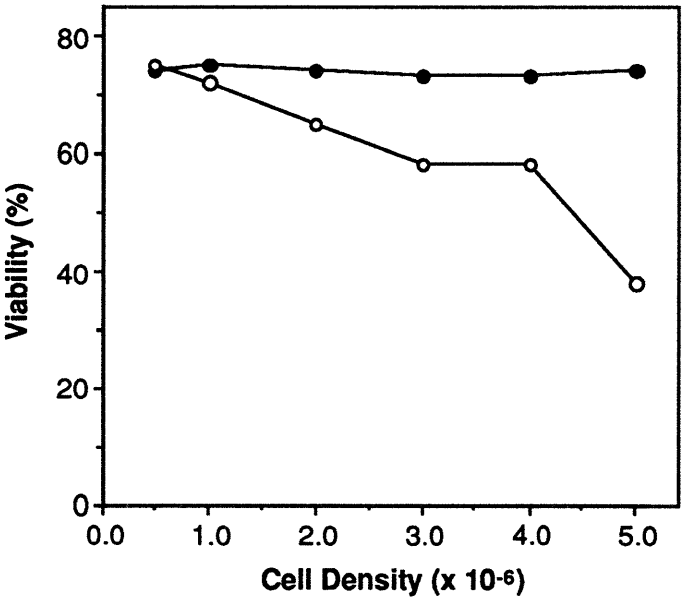


Figure 2. Effect of cell density on thymocyte viability.

Thymocytes were isolated from a wild-type mouse (6 weeks), adjusted to the indicated density, and incubated in HEPES-buffered DME supplemented with either 5% FBS (closed circles) or 10% FBS (open circles). After 24 hours at 37°C, cell viability was estimated by uptake of FITC and flow cytometry.

FBS, but viability declined significantly with increasing cell density in 10% FBS (Figure 2). Based on these results, all experiments used DME supplemented with 5% fetal bovine serum and 25 mM HEPES (pH 7.2) at 2 x 10^6 cells/ml. All thymocytes were isolated from mice age 4.5-7 weeks. At time zero, cultures were treated as described, transferred to 16mm wells, and incubated at 37°C. The relative amounts of nonviable cells were determined at various times by uptake of fluoroscein isothiocyanate (FITC) and flow cytometry (see Chapter 3 and (Shi et al., 1990)). A representative example of flow cytometry data from a viable and non-viable population is shown in Figure 3. Cell death by apoptosis was confirmed by analysis of genomic DNA (Wyllie, 1980). Irradiation was performed with a GammaCell 40 equipped with a ^{137}Cs source.

Thymocyte apoptosis *in vivo*

Thymocytes were recovered from mice 48 hours after treatment with 0.5 mg dexamethasone (administered by intraperitoneal injection in phosphate buffered saline, PBS), gamma irradiation (500 rad), or no treatment and stained with phycoerythrin-conjugated anti-CD4 and FITC-conjugated anti-CD8 antibodies (anti-L3T4 and anti-Lyt 2, Becton Dickinson). Multiparameter analysis of live cells was carried out on a FACStar Plus (Becton Dickinson). Dead cells were excluded by staining with propidium iodide and by gating of forward and side scatter of light during FACS analysis. The relative contributions of CD4 and CD8-expressing subpopulations were estimated using the Disp2D program (Becton Dickinson). Total genomic DNA was analyzed from 10^6 thymocytes isolated 10 hours after the treatments described above, according to the protocol of Barry and Eastman (Barry and Eastman, 1993).

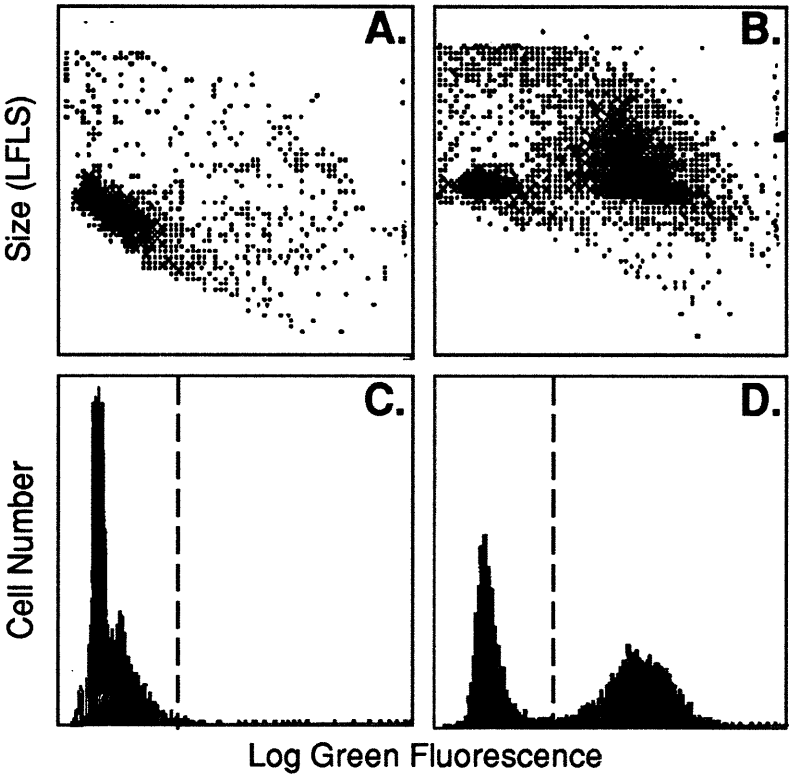


Figure 3. Flow cytometry of viable and non-viable thymocytes.

Thymocytes were isolated from a wild-type animal treated with 5 Gy ionizing radiation. Cell viability was determined by uptake of FITC and flow cytometry. Dead cells have increased green fluorescence. (A, B) dot plots illustrating cell size (LFLS, log forward light scatter) versus green fluorescence from representative samples. (A) untreated thymocytes at time 0; (B) 18 hours after treatment with 5 Gy ionizing radiation. Histograms of the same samples are illustrated directly below (C and D). The percentage of viable cells was determined from histograms using Coulter software. The dashed line delineates the cutoff between viable and non-viable cells.

Western blot analysis

Thymocytes were isolated as described above, except the cultures were incubated in 75 cm^2 tissue culture flasks (10 ml/flask). For each sample, 2×10^7 cells were treated, washed in PBS, and lysed in Laemmli buffer (Harlow and Lane, 1988). The proteins were separated on 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked and probed with a pool of p53-specific monoclonal antibodies (PAb421, PAb240, and PAb248) (Yewdell et al., 1986; Gannon et al., 1990). p53 was detected using an

alkaline phosphatase-conjugated secondary antibody and a chemiluminescent substrate ((Haldi and Guarente, 1989; Isaacs et al., 1991); Lumi-Phos 530, Boehringer-Mannheim).

RESULTS

p53-dependent and independent apoptosis *in vitro*

Because of the potential involvement of p53 in inducing apoptosis (Yonish-Rouach et al., 1991; Shaw et al., 1992; Kern et al., 1992; Lindquist, 1992), we studied cell death in thymocytes derived from mice carrying a germline disruption of the p53 gene (T. J. and R. Weinberg, unpublished). Thymocytes were isolated from p53 homozygous mutant, heterozygous, and wild-type animals and subjected *in vitro* to various treatments known to induce apoptosis. There were no significant differences between viability of untreated thymocytes over the time period of the experiments (Figure 4); on average, viability of untreated cells was approximately 70% at 24 hours. Although treatment with phorbol ester/calcium ionophore (which may mimic engagement of the T cell receptor (Crabtree, 1989)) and dexamethasone induced death with similar kinetics in thymocytes of all three genotypes (Figure 5A, B), p53-deficient cells displayed a dramatic resistance to the effects of ionizing radiation (Figure 5C). Moreover, the p53-deficient thymocytes remained viable following doses up to 2000 centiGray (cGy); wild-type cells were susceptible to treatment with as little as 100 cGy (Figure 6). In addition, at all doses and times examined, cells isolated from heterozygous animals displayed intermediate viability compared to wild-type and homozygous mutant animals (Figures 5 and 6).

Apoptosis in vivo

We further tested the effects of dexamethasone and γ-irradiation on thymocyte survival *in vivo*. Thymocytes isolated from treated animals were

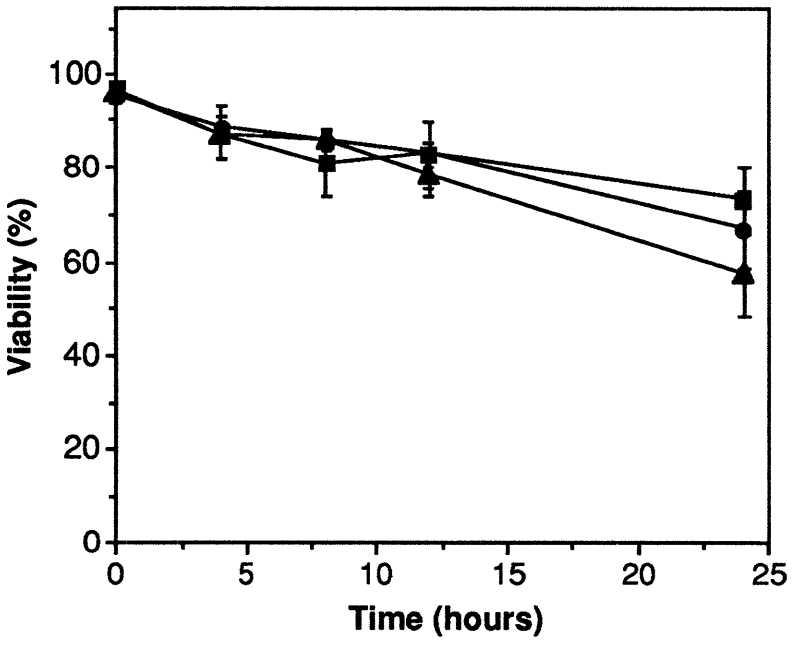


Figure 4. Viability of untreated thymocytes in vitro.

Thymocytes were isolated from $p53^{+/+}$ (circles), $p53^{+/-}$ (triangles), and $p53^{-/-}$ (squares) animals and incubated in normal growth medium (HEPES-buffered DME supplemented with 5% FBS). At various times, cell viability was determined by uptake of FITC and flow cytometry.

examined for the presence of the cell-surface markers CD4 and CD8 using two-color flow cytometry. Thymuses from untreated normal and mutant animals contained approximately 75-80 % immature, $CD4^+CD8^+$ cells, which are susceptible to apoptosis (Smith et al., 1989). Forty-eight hours following treatment with dexamethasone, all thymuses sustained a significant reduction in cell numbers which could be attributed to selective loss of $CD4^+CD8^+$ cells (Figure 7A, B). Similarly, thymuses from wild-type animals exposed to gamma radiation contained a low percentage of $CD4^+CD8^+$ cells (Figure 7A, B). In contrast, irradiation of p53 homozygous mutant animals caused only minor reductions in $CD4^+CD8^+$ cells (Figure 7A, B).

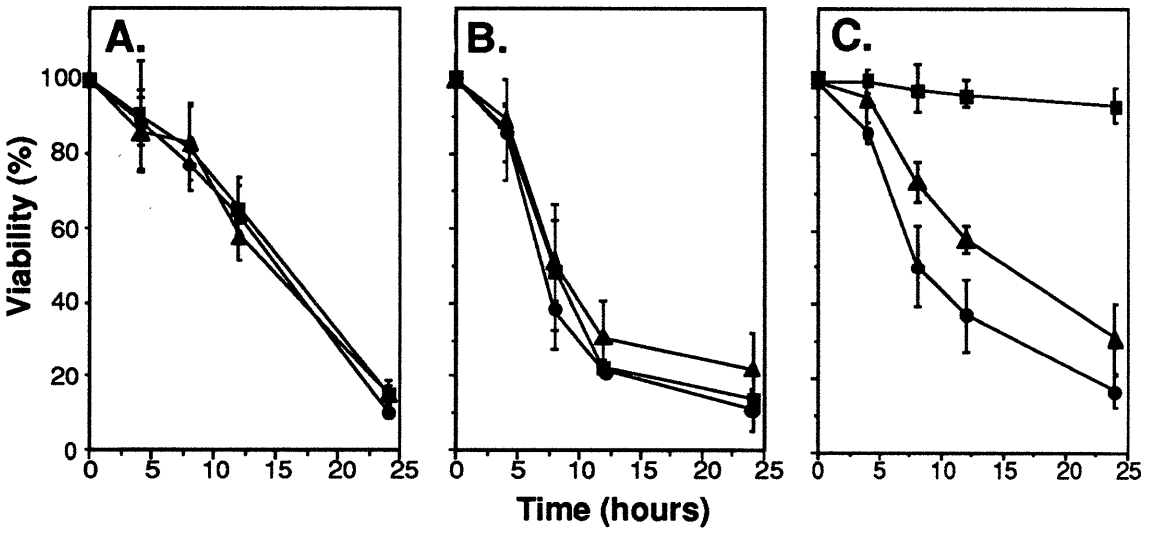


Figure 5. Induction of apoptosis in isolated thymocytes.

Thymocytes were treated with (A) 10 nM phorbol ester (phorbol 12-myristate 13-acetate, PMA) and 500 nM calcium ionophore (A23187), (B) 1 μM dexamethasone, or (C) 5.0 Gy ionizing radiation, and viability was assessed at various times thereafter. Thymocytes were isolated from $p53^{-/-}$ (squares), $p53^{+/-}$ (triangles), and $p53^{+/+}$ (circles) animals. Values represent the average viability from 4 independent experiments with standard deviations; each experiment compared cells derived from one mutant, one heterozygote, and one wild-type animal and were normalized to untreated samples from the same animal. Two experiments utilized littermates derived from F1 crosses.

Conditions that induced cell death produced the internucleosomal degradation of thymic DNA, which is indicative of apoptosis (Wyllie et al., 1984) (Figure 7C). This characteristic "DNA ladder" was not evident following irradiation of homozygous mutant animals (Fig. 3*c*). Consistent with the data from *in vitro* experiments, mice that were heterozygous for the p53 mutation were less susceptible than wild-type mice to the effects of ionizing radiation, both in survival of $CD4^+CD8^+$ cells and extent of DNA laddering (Figure 7 B,C).

p53 levels during apoptosis

Given the apparent requirement for p53 function in radiation-induced apoptosis of thymocytes, we examined steady-state level of p53 protein in wild-type cells following exposure to ionizing radiation. Consistent with findings in

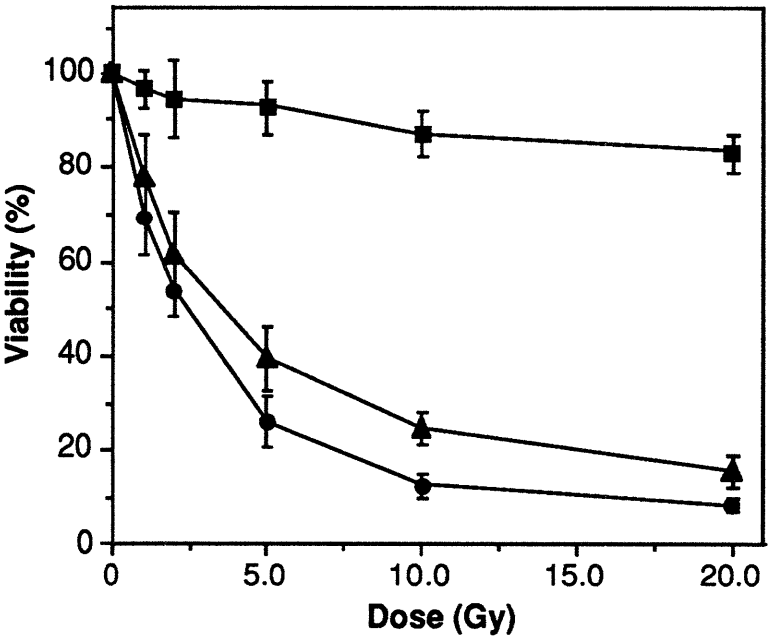
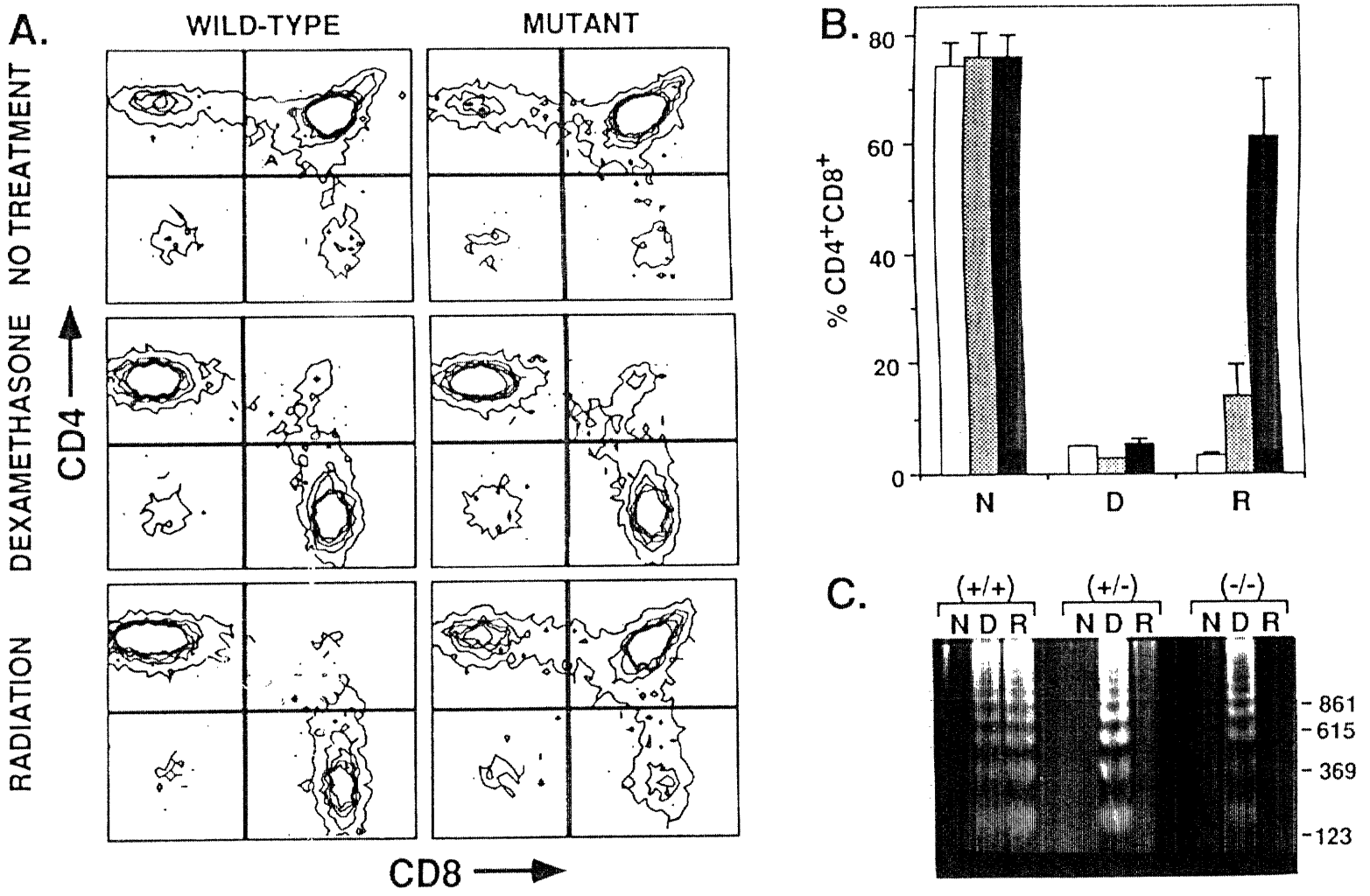


Figure 6. Viability of isolated thymocytes treated ionizing radiation.

Thymocytes were isolated from $p53^{-/-}$ (squares), $p53^{+/-}$ (triangles), and $p53^{+/+}$ (circles) animals and treated with the indicated doses of ionizing radiation. Viability was assessed after 20 hours by FITC uptake and flow cytometry. Values represent averages from 3 independent experiments and are normalized to the amount of viable cells remaining in untreated cultures derived from the same animal.

Figure 7. Thymocyte apoptosis *in vivo* (next page)

p53 homozygous mutant, heterozygous, and wild-type animals were treated with dexamethasone or ionizing radiation and isolated thymocytes examined for the cell surface expression of CD4 and CD8 (after 48 hours) and the condition of genomic DNA (after 10 hours). A, Two-color immunofluorescence contour plots from FACS analysis of CD4 and CD8 surface expression in wild-type and p53 homozygous mutant mice. B, Mean percentage of surviving $CD4^+CD8^+$ thymocytes from $p53^{+/+}$ (□), $p53^{+/-}$ (▩), and $p53^{-/-}$ (■) mice 48 hours following treatment with dexamethasone (D), gamma irradiation (R), or no treatment (N). C, agarose gel electrophoresis of total thymus DNA from wild-type (+/+), p53 heterozygous (+/-), and p53 homozygous mutant (-/-) mice 10 hours following treatment with dexamethasone (D), gamma irradiation (R), or no treatment (N). The position of molecular size standards (in nucleotides) is shown at right.


A.
WILD-TYPE
MUTANT
NO TREATMENT
DEXAMETHASONE
RADIATION
CD4
CD8
B.
% CD4+CD8+
80
60
40
20
0
N
D
R
C.
(+/+)
(+/-)
(-/-)
N D R
N D R
N D R
861
615
369
123

other cell types (Kastan et al., 1991; Kastan et al., 1992; Flint, 1984; Sarnow et al., 1982), irradiation of thymocytes caused a dramatic increase in p53 levels. The accumulation of p53 protein was apparent within 1 hour (Figures 8), before significant degradation of DNA (not shown; (Sellins and Cohen, 1987)). In contrast, treatment with phorbol ester/calcium ionophore and dexamethasone resulted in little or no increase in p53 levels.

DISCUSSION

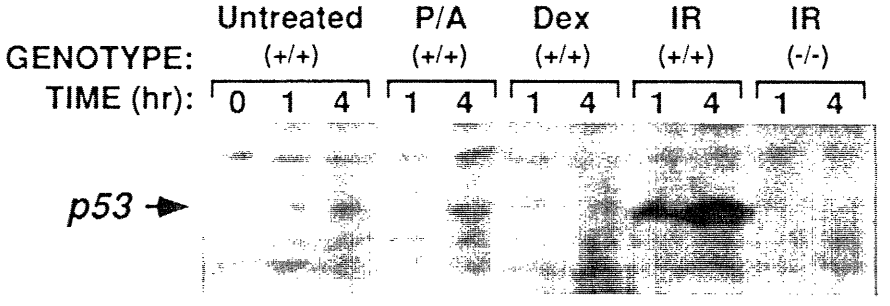
p53 dependent and independent apoptosis

These results establish the involvement of p53 in a cell death pathway, specifically radiation-induced apoptosis in the thymus. Equally important, these data demonstrate that apoptosis can also occur in the absence of p53 function. Thus, cell death in the thymus can be subdivided into at least two distinct pathways, one requiring p53 and one that is p53 independent. The existence of multiple apoptotic pathways in the thymus has been suggested from the analysis of *bcl-2* transgenic mice (Sentman et al., 1991; Strasser et al., 1991). Furthermore, the apparently normal development of mice homozygous for a p53 mutation (ref. (Donehower et al., 1992), and T.J. and R. Weinberg, unpublished) suggests that p53 is not required for cell death in many, perhaps most, instances.

p53 has been implicated in controlling a checkpoint during the G1 phase of the cell cycle that may monitor the state of the DNA before entry into S phase (Kastan et al., 1992; Lane, 1992). For example, p53-deficient fibroblasts fail to arrest transiently in G1 after gamma irradiation, although they still pause normally in G2 (Kastan et al., 1992). A rapid accumulation of p53 precedes G1 arrest in fibroblasts (Kastan et al., 1991; Kastan et al., 1992) and, as shown here, radiation-induced apoptosis in thymocytes. Thus, the different cellular responses (apoptosis versus G1 arrest) may result from the activation of distinct

Figure 8. p53 levels in isolated thymocytes undergoing apoptosis (next page).

Thymocytes were isolated from wild-type (+/+) and homozygous mutant (-/-) animals and treated with cytotoxic agents. At 1 and 4 hours following treatment, p53 levels were determined by Western blot. The treatment and genotype of the cells are indicated above the appropriate lanes: normal tissue culture media (untreated); PMA + A23187 (P/A); dexamethasone (dex); ionizing radiation (IR). The time (in hours) after treatment is indicated over each lane.


Untreated
P/A
Dex
IR
IR
GENOTYPE:
(+/+)
(+/+)
(+/+)
(+/+)
(-/-)
TIME (hr):
0 1 4
1 4
1 4
1 4
1 4
p53

target genes by p53. Alternatively, activation of the same target genes in the two cell types could have different consequences. The fact that elevated levels of p53 can lead to the initiation of apoptosis is consistent with earlier studies that demonstrated a link between p53 expression and cell death (Yonish-Rouach et al., 1991; Shaw et al., 1992), and it is possible that many conditions that lead to an accumulation of p53 could induce apoptosis. Those stimuli which cause apoptosis in thymocytes in the absence of p53 function may utilize other transcription factors to activate the same set of "cell death" genes.

Apoptosis and tumor suppression

The data presented here define another mechanism by which p53 can act as a tumor suppressor gene. It has been proposed that the mutational inactivation of p53 during tumorigenesis might allow the further accumulation of oncogenic mutations, due to the removal of an important G1 checkpoint (Kastan et al., 1992; Lane, 1992). In thymocytes, and perhaps in other cell types as well, the absence of p53 function can lead to inappropriate cell survival after γ-irradiation. The failure to eliminate cells that have incurred DNA damage could lead to the selection of cells that have undergone neoplastic transformation. Note that among the various tumor types that occur in p53 homozygous mutant mice, lymphoma is by far the most common ((Donehower et al., 1992) and T.J. and R. Weinberg, unpublished), and the four cases of this tumor that have been examined from our p53-deficient mice have consisted predominantly of $CD4^+CD8^+$ cells (T.J., unpublished results). Thus, like *bcl-2* activation (Tsujimoto, 1984; Sentman et al., 1991; Strasser et al., 1991), the inactivation of p53 may contribute to tumorigenesis through an inhibition of apoptosis.

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APPENDIX 2

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