Cell, Vol. 57, 1083–1093, June 30, 1989, Copyright © 1989 by Cell Press

The p53 Proto-Oncogene Can Act as a Suppressor of Transformation

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Summary

DNA clones of the wild-type p53 proto-oncogene inhibit the ability of E1A plus *ras* or mutant p53 plus *ras*-activated oncogenes to transform primary rat embryo fibroblasts. The rare clones of transformed foci that result from E1A plus *ras* plus wild-type p53 triple transfections all contain the p53 DNA in their genome, but the great majority fail to express the p53 protein. The three cell lines derived from such foci that express p53 all produce mutant p53 proteins with properties similar or identical to transformation-activated p53 proteins. The p53 mutants selected in this fashion (transformation in vitro) resemble the p53 mutants selected in tumors (in vivo). These results suggest that the p53 proto-oncogene can act negatively to block transformation.

Introduction

The proto-oncogene product, p53, is a cellular protein expressed at low levels in nontransformed cells (Dippold et al., 1981; Benchimol et al., 1982; Thomas et al., 1983; Rogel et al., 1985). In contrast, in tumor-derived and transformed cell lines, the levels of p53 are often elevated (Crawford et al., 1981; Dippold et al., 1981; Benchimol et al., 1982; Rotter, 1983; Thomas et al., 1983; Koeffler et al., 1986). In particular, in cells transformed by SV40 or adenovirus type 5, p53 is found in stable complexes with the transforming proteins from these viruses, the SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979), and the E1B-55kd protein, respectively (Sarnow et al., 1982). The levels of p53 in cells containing these complexes are approximately 100-fold higher than in the nontransformed cell, and the half-life of p53 is correspondingly extended (from 20 min to 24 hr) (Oren et al., 1981; Reich et al., 1983). Therefore, the elevation of p53 levels has often been correlated with cellular transformation. The results of several studies have supported the hypothesis that increased levels of p53 effect changes in the phenotype of the normal cell; overexpression of p53 resulted in the immortalization of rodent cells (Jenkins et al., 1984; Rovinski and Benchimol, 1988) and, when assayed in conjunction with an activated *ras* gene, in the full transformation of primary rodent cells to a tumorigenic phenotype (Eliyahu et al., 1984; Parada et al., 1984). Recent studies, however, have demonstrated that the wild-type sequence of the proto-oncogene p53 (p53-wt) does not encode a transforming protein; i.e., no transformants are observed when primary rat embryo fibroblasts are cotransfected with a p53-wt plus an activated *ras* gene (Finlay et al., 1988; Eliyahu et al., 1988; Hinds et al., 1989). The confusion surrounding the transforming activities of p53 resulted from the existence of several independently derived p53 cDNAs and of at least one p53 genomic clone, all of which possess mutations that activate the p53 protein for cooperation with *ras* (Finlay et al., 1988; Eliyahu et al., 1988; Hinds et al., 1989). The mutations (the result of one or two single nucleotide changes) are localized between amino acids 130 and 234 (out of a total of 390 amino acids). In addition, linker insertion mutations in this same region also activate the p53 protein for transformation with the *ras* oncogene (Finlay et al., 1988). In general, the activated p53 proteins have several properties in common: they bind poorly or not at all to the SV40 large T antigen; they form complexes with the constitutively expressed member of the 70 kd heat shock family, hsc70; and they do not express an epitope localized between amino acid residues 88 and 109 that is recognized by the murine-specific, conformation-dependent, monoclonal antibody PAb246. These transformation-activating mutations span a region comprising approximately 25% of the p53 protein. These observations suggest that the transformation-activating mutations could result from a loss-of-function mutation of the p53 protein. These mutants, when overexpressed, could then act in a *trans*-dominant fashion to inactivate the endogenous wild-type p53, perhaps by the formation of nonfunctional multimeric complexes of proteins (Herskowitz, 1987). It has previously been clearly demonstrated that high levels of mutant p53 proteins are required for the efficient cloning and establishment of p53 plus *ras*-transformed cell lines (Hinds et al., 1989). The association between mutant p53 proteins and the endogenous wild-type p53 protein in a complex has also been previously observed (Rovinski and Benchimol, 1988; Eliyahu et al., 1988). Thus, inactivation of the p53-wt protein by an inactive mutant p53 protein in the same complex may actually be involved in the process of p53-mediated transformation.

The inactivation of p53 appears to play an important role in the development of Friend virus-induced erythroleukemias in vivo. Rearrangement of the murine p53 gene occurs in a high proportion of malignant cell lines derived from the spleens of Friend virus-infected mice (Mowat et al., 1985; Chow et al., 1987), resulting in the inactivation of p53 (Ben-David et al., 1988) or the synthesis of elevated levels of antigenically related truncated polypeptides (Rovinski et al., 1987; Munroe et al., 1988). These investigators have suggested that the inactivation of the p53 gene confers a selective advantage for the development of the tumorigenic phenotype. The rearrangement of the p53 gene has also been observed in 3/6 human osteogenic sarcomas (Masuda et al., 1987).

The inactivation of p53 functions could also be involved in the process of viral transformation. Recent studies have demonstrated that both the SV40 large T antigen and the E1A products from Ad5 form specific complexes with the

Table 1. Effect of Murine p53-wt on *ras* Plus p53-Activated Transformation of Rat Embryo Fibroblasts

	Number of Foci							
	Experiment Number							
Transforming Genes	1	2	3	4	5	6	7	Average
(A)								
ras + p53-wt	1	0	0	0	0	0	0	0.14
ras + p53-val_{135}	35	11	6	3	18	14	4	13.0
ras + p53-KH215	ND	20	13	13	27	32	11	19.3
ras + p53-wt + p53-val_{135}	1	0	0	0	0	1	0	0.28
ras + p53-wt + p53-KH215	ND	ND	1	3	1	0	0	1.0
ras + p53-FS + p53-val_{135}	ND	4	12	2	ND	ND	ND	6.0
ras + p53-FS + p53-KH215	ND	16	9	12	ND	ND	ND	12.3
(B)								
riboras + p53-wt	0	0	0					0
riboras + p53-val_{135}	23	12	25					20
riboras + p53-wt + p53-val_{135}	0	0	0					0

ND: not determined.

product of the cellular retinoblastoma (Rb) gene (DeCaprio et al., 1988; Whyte et al., 1988). Deletion or inactivation of the Rb gene predisposes individuals to the development of human malignant retinoblastoma (Friend et al., 1986; Lee et al., 1987), and abnormalities in the Rb locus have recently been detected in osteosarcomas (Friend et al., 1986), small cell lung cancers (Harbour et al., 1988), and in breast cancer cell lines (Lee et al., 1988). Thus, there is a precedent for viral transforming proteins binding to the product of a recessive oncogene, and it is possible that these complexes neutralize or alter the growth-regulating properties of the Rb gene product. The complexes that p53 forms with the SV40 large T antigen or the E1B-55kd protein, respectively, could be analogous to the complexes observed between the Rb gene product and T antigen or E1A. In fact, in cells expressing the Ad5-E1B-55kd protein, the p53-E1B complex is found localized to a filamentous body in the cytoplasm (Zantema et al., 1985), supporting the hypothesis that p53, normally found in the nucleus, is sequestered from other cellular proteins in this complex.

If, as the above evidence suggests, inactivation of p53 renders the normal cell predisposed to becoming transformed, then conversely, overexpression of the p53-wt protein in a cell might prevent the process of transformation. To test this hypothesis, rat embryo fibroblasts were transfected with DNA clones expressing either *ras* plus p53-mutant ± p53-wt or *ras* plus E1A ± p53-wt, and the number of transformed foci was assayed. The results demonstrate that the p53-wt dramatically reduced the number of foci observed in these assays when compared with the number of transformants observed in transfection with either *ras* plus p53-mutant or *ras* plus E1A alone. In cultures transfected with *ras* plus p53-mutant plus p53-wt, the appearance of transformed foci was nearly completely eliminated, and if cultures were transfected with *ras* plus E1A plus p53-wt, the number of transformed foci was reduced, on average, 67%–80%. Thirteen foci transformed by *ras* plus E1A plus p53-wt were cloned, and the levels of murine p53 expressed were determined. In the great majority of these cell lines (10/13), the murine p53 gene was present but failed to be expressed, in 2/13 cell lines, elevated levels of p53 antigenically related polypeptides of altered size (deletion and fusion proteins) were detected, and in 1/13 cell lines, elevated levels of altered murine p53 protein were found. In each instance where murine p53 was expressed (3/3), the protein was found associated with hsc70 and, in two cases, the murine p53 that was made was not recognized by PAb246. Therefore, in cells cotransfected with activated oncogenes and wild-type p53, the wild-type p53 either fails to be expressed or mutant, activated forms of the murine p53 are selected for in such clones. Interestingly, the type of p53 mutants generated by these selections in vitro are similar to those observed in tumors in vivo (Mowat et al., 1985; Eliyahu et al., 1988). In contrast, if primary rat cells were transfected with *ras* plus E1A plus p53-mutant (val_{135} or KH215), an increased number of foci were generated and elevated levels of heat shock–associated mutant p53 were found in 15/15 cell lines examined. These results suggest that expression of activated mutant forms of p53 enhances the transformation process with other oncogenes.

The results of the above studies suggest that the proto-oncogene p53 should more appropriately be classified as a recessive oncogene. Overexpression of p53 can act to suppress transformation, and inactivation of this gene renders the cell susceptible to uncontrolled growth.

Results

Transfection with p53-wt Suppresses Transformation of Rat Embryo Fibroblasts by *ras* Plus p53-Mutant or by *ras* Plus E1A

Previous studies (Hinds et al., 1989) have suggested that at least one mechanism involved in *ras* plus p53-mutant transformation event could be the inactivation of the p53-wt function by the formation of oligomeric protein com-

Table 2. Effect of Murine p53-wt on *Ras* Plus E1A Transformation of Rat Embryo Fibroblasts

Transforming Genes	Number of Foci: Experiment Number 1	2	3	4	Average	Ratio to Number of *ras* + E1A Foci
ras + p53-wt	0	0	0	1	0	NA
ras + p53-val_{135}	49	6	15	35	26	NA
ras + E1A	72	34	76	94	69	1.0
ras + E1A + p53-wt	51	14	13	14	23	0.33
ras + E1A + p53-val_{135}	230	136	158	136	165	2.4
						Ratio to Number of Foci w/*ras* + SVE1A
ras + p53-wt	0	1	0	0	0	NA
ras + p53-val_{135}	15	35	18	14	20	NA
ras + SVE1A	84	92	102	107	96	1.0
ras + SVE1A + p53-wt	17	31	8	19	19	0.20
ras + SVE1A + p53-val_{135}	215	124	124	143	151	1.60

NA: not applicable.

plexes with hsc70 protein. The effects of increased p53-wt levels on the transformation of primary cells by *ras* plus p53-mutant were therefore investigated. In these assays, rat embryo fibroblasts were transfected with three genes, two genes previously shown to cooperate (*ras* plus p53-mutant) and the p53-wt gene, and the number of transformed foci was determined 12–14 days later. The results of these studies are presented in Table 1 and show that the addition of p53-wt reproducibly inhibits the formation of transformed foci in these assays. On average, 13–20 transformed foci are observed per experiment when cells are transfected with *ras* plus p53-val_{135} or with *ras* plus p53-KH215, two different activating p53 mutations, and the presence of additional p53-wt results in a decrease in these foci to 0–1 focus/assay. To confirm that the product of the p53-wt gene is required for this effect and that the decrease in transformants does not result from a reduction in effective p53-mutant concentrations as a consequence of promoter competition for transcription factors, these studies were repeated using the identical construct possessing a frameshift mutation at amino acid 13 of the p53 protein (p53-FS; Table 1). This frameshift eliminates the production of detectable p53 protein (Hinds et al., 1989), and the mutant frameshift construct has no effect on the transformation of REF by *ras* plus p53-mutant (Table 1). Doubling the concentration of p53-mutant also has no effect on these assays (data not shown).

One possible explanation for these results is that overexpression of p53-wt suppresses expression of the activated *ras* gene from its promoter. Identical results were obtained, however, if this experiment was repeated utilizing the activated *ras* gene under the control of the ribosomal L32 3A promoter (Table 1B). Thus, the nature of the promoter regulating the *ras* oncogene in these experiments does not alter the ability of wild-type p53 to inhibit p53 mutant plus *ras*-mediated transformation. In addition to this experiment, the levels of *ras* synthesized in REF cells transfected with *ras* plus E1A plus p53-mutant, p53 wild-type, or p53-FS DNA clones were measured. Cells transfected with either of these three combinations of *ras* plus p53 DNA clones were labeled with [^{35}S]methionine for 1–2 hr at 48 hr after these transfections. The *ras* protein produced was immunoprecipitated from soluble cell extracts, and the level of labeled *ras* protein was measured in an autoradiogram of the SDS–polyacrylamide gel of the immunoprecipitate. The p53 wild-type, mutant, and frameshift DNA clones had little or no effect upon the levels of *ras* protein produced by the cotransfected *ras* clone. When similar experiments were performed to determine if the wild-type p53 protein would inhibit the levels of p53-mutant protein produced by cotransfection of p53-wt and p53-mutant clones, the wild-type protein did not inhibit the level of the mutant protein produced in such cells (data not shown).

To determine if the inhibitory effects of p53-wt could be observed in transformation assays that did not directly involve p53-mutant proteins, REF were transfected with *ras* plus E1A ± p53-wt, and the number of transformants was assayed 2 weeks later. The results of these experiments are presented in Table 2 and demonstrate that expression of p53-wt also reduces the number of *ras* plus E1A-transformed foci observed in these assays by 30%–85%, depending on the experiment. Similar results were also observed utilizing E1A either under the control of the SV40 enhancer region (Table 2) or under the control of the regulatory region for the murine albumin gene (data not shown), suggesting that the results are independent of the enhancer–promoter regulating E1A gene expression.

Analysis of Murine p53 Expression in Cells Transformed by *ras* Plus SVE1A Plus p53-wt

Of particular interest was whether the *ras* plus SVE1A plus p53-wt transformants expressed the wild-type murine p53 protein. Transformed foci from two independent experiments of this type were cloned and expanded into transformed cell lines. These cell lines were initially screened

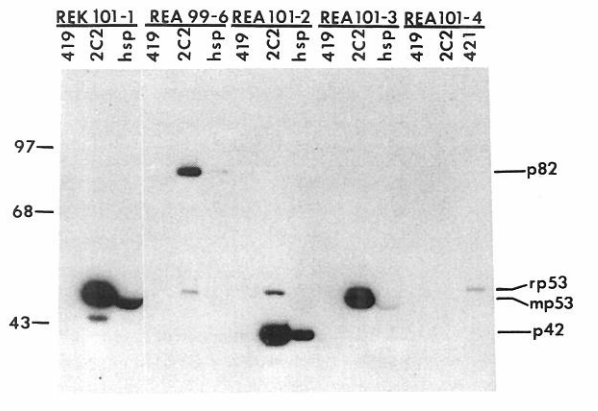

Figure 1. Immunoblot Analysis of p53 Proteins in Transformed Cell Lines

Equal aliquots of lysates of cells transformed by activated *ras* and SVE1A and p53 KH215 (REK 101–1) or by *ras* and SVE1A and p53-wt (REA 99–6, REA 101–2, REA 101–3, and REA 101–4) were immunoprecipitated with the anti-SV40 large T antigen monoclonal antibody PAb419 (419; a negative control for p53), anti-murine p53 monoclonal antibody RA3-2C2 (2C2), or with anti-hsp70 peptide antiserum (hsp). Immunoprecipitated proteins were separated on a 7.5% polyacrylamide gel and subjected to immunoblot analysis with the anti-p53 monoclonal antibody PAb421. REK 101–1 was run on the same gel, but not in an adjacent lane. Total cellular protein (in milligrams) in each aliquot was as follows: REK 101–1, 1.35; REA 99–6, 0.45; REA 101–2, 1.15; REA 101–3, 1.65; REA 101–4, 1.25. rp53 and mp53 indicate migration of rat and murine p53, respectively. p42 and p82 indicate migration of murine-p53-related proteins of 42 kd and 82 kd, respectively. Migration of molecular mass markers is indicated in kilodaltons.

by immunoprecipitation of ^{35}S-labeled cell lysates for the presence of E1A and murine p53. While the majority (11/13) of cell lines expressed readily detectable levels of E1A polypeptides (data not shown), 10/13 cell lines expressed no detectable murine p53, 2/13 cell lines expressed elevated levels of antigenically related p53 proteins of an altered size (42 kd and 82 kd), and 1/13 cell lines expressed elevated levels of murine p53. When the murine p53-related proteins from these three cell lines were immunoprecipitated with several different monoclonal antibodies, they all coimmunoprecipitated the hsc70. To confirm that these proteins were indeed altered murine p53 polypeptides and that the proteins were associated with hsc70, Western blot analysis was performed. Four cell lines, one that failed to express p53 (REA 101–4), one expressing the 42 kd protein (REA 101–2), one synthesizing the 82 kd protein (REA 99–6), and one expressing the 53 kd protein (REA 101–3), were incubated with several antibodies: PAb419 (a control monoclonal antibody that recognizes the SV40 T antigen [Harlow et al., 1981]); RA3-2C2 (a murine p53-specific monoclonal antibody [Rotter et al., 1980]); or anti-hsc70 serum (Hinds et al., 1987). The immunoprecipitated proteins were isolated and fractionated on SDS–polyacrylamide gels, transferred to Immobilon paper, and probed with PAb421 (a conformation-independent monoclonal antibody specific for p53 [Harlow et al., 1981]), followed by incubation with ^{125}I–protein A. The resultant autoradiogram of this blot is presented in Figure 1. All three polypeptides (p82, p42, and p53) were immunoprecipitated by RA3-2C2 and recognized by PAb421, which detect two distinct epitopes on the p53 protein, confirming that these are not unrelated proteins coimmunoprecipitated in the initial screen of these cell lines. Furthermore, each mutant form of p53 is associated with hsc70, as evidenced by the coimmunoprecipitation of murine p53-related proteins by anti-hsc70 serum. As expected, no murine p53 was observed in the cell line that failed to synthesize this protein. The endogenous rat p53 protein was also immunoprecipitated from this cell line with PAb421, a monoclonal antibody known to react with p53 from various species. Of interest is that in the cell lines expressing the murine mutant p53 proteins p42 and p82, a band that comigrates with rat p53 was coimmunoprecipitated by the RA3-2C2 monoclonal antibody, which is murine p53-specific. Such an association of mutant murine p53 proteins with the endogenous wild-type rat p53 has also been observed in other experiments (Eliyahu et al., 1988; Rovinski and Benchimol, 1988). To confirm that the coimmunoprecipitating band is indeed rat p53, unlabeled cell lysates were boiled prior to immunoprecipitation to separate any noncovalently associated proteins. The samples were then immunoprecipitated with RA3-2C2 or PAb421 and subjected to Western analysis as described above. Under these conditions, the rat p53 was no longer found associated with the murine p53-mutant protein (data not shown).

The mutant p53 proteins that arose from transformation of *ras* plus SVE1A plus p53-wt were further analyzed by immunoprecipitation of [^{35}S]methionine-labeled cell lysates using four different anti-p53 monoclonal antibodies that recognize four distinctly different epitopes across the p53 molecule. PAb242 (Yewdell et al., 1986) recognizes an epitope spanning amino acids 9–25 (Wade-Evans and Jenkins, 1985), PAb246, a conformation-dependent monoclonal antibody (Yewdell et al., 1986), recognizes an epitope spanning amino acids 88–109 (Wade-Evans and Jenkins, 1985), RA3-2C2 recognizes amino acids 157–192 (Wade-Evans and Jenkins, 1985), and PAb421 recognizes amino acids 370–378 (Wade-Evans and Jenkins, 1985) out of a total of 390 amino acids. Both the 42 kd and the 53 kd proteins are recognized by 242, 2C2, and 421 (Figure 2). These proteins are not recognized by PAb246, which is characteristic of mutant transformation-activated p53 proteins (Finlay et al., 1988). P82 is recognized by all four monoclonal antibodies (Figure 2). The nature of the alterations in the p53 gene that gave rise to these proteins is currently under investigation. Southern blot analysis of the DNA in these cell lines demonstrated that all 13 cloned cell lines contained a murine p53 gene or genes. In the case of the cell lines containing p42 and p82, no obvious structural alterations in the transfected gene were observed. These cell lines typically possess multiple copies of the transfected genes and changes that may have occurred in a single copy, for example, would likely have gone undetected.

Analysis of Murine p53 Expression in Cells Transformed by *ras* Plus SVE1A Plus p53-Mutant

p53 proteins that are activated for transformation with *ras* do not suppress the transformation of REF by *ras* plus

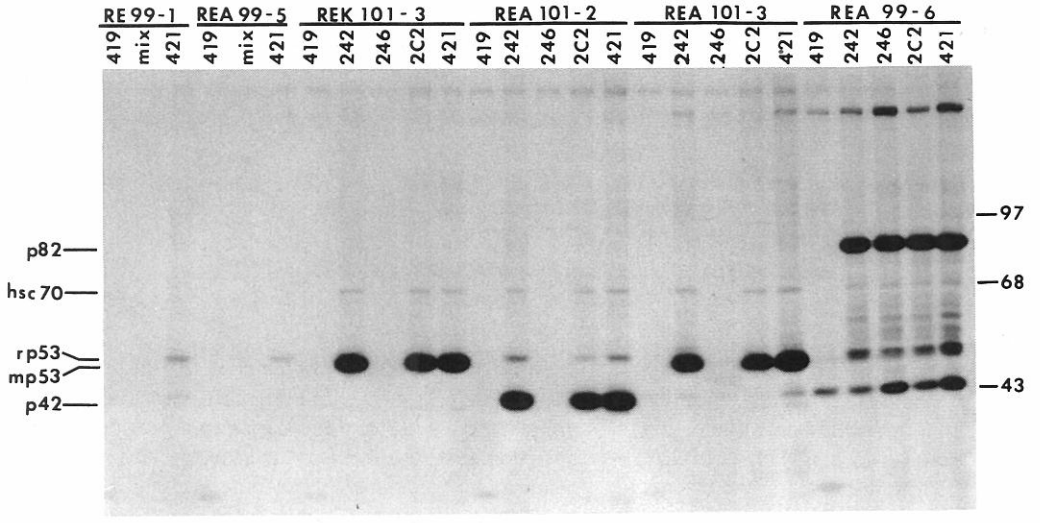

Figure 2. Epitope Analysis of Mutant p53 Proteins

Cells transformed by activated *ras* and SVE1A (RE 99–1), *ras* and SVE1A and KH215 (REK 101–3), or by *ras* and SVE1A and p53-wt (REA 99–5, REA 101–2, REA 101–3, and REA 99–6) were metabolically labeled with [^{35}S]methionine. Equal aliquots representing 9 × 10^6 TCA-precipitable counts for REA 99–6 or 3 × 10^6 counts for the remaining cell lines were subjected to immunoprecipitation with the anti-SV40 large T antigen monoclonal antibody PAb419 (419; a negative control antibody); anti-murine p53 monoclonal antibodies PAb242 (242), PAb246 (246), or RA3–2C2 (2C2); anti-p53 monoclonal antibody PAb421 (421) or a mixture of 242, 246, and 2C2 (mix). Migration of rat p53 (rp53) and murine p53 (mp53) is indicated. p42 and p82 indicate migration of murine-p53-related proteins. hsc70 indicates the constitutively produced member of the 70 kd family of heat shock proteins. Migration of molecular mass markers is indicated in kilodaltons.

E1A. Instead, on average, there was a reproducible increase of 60%–140% in the number of transformed foci when cells were transfected with *ras* plus E1A plus p53-val_{135} (when compared with the number observed with *ras* plus E1A alone) (Table 2). Similar increases were noted if cells were transfected with *ras* plus E1A plus p53-KH215 (data not shown). These increases could, on average, be explained if there were two populations of cells in the culture, one population which was transformed by *ras* plus p53-mutant and one population which was transformed by *ras* plus E1A. To examine this question and to provide a control for the previous experiments, 15 independent cell lines transformed by *ras* plus SVE1A plus p53-mutant (val_{135} or KH215) were examined for the expression of E1A and murine p53. The cell lines were metabolically labeled with [^{35}S]methionine for 1 hr or 2 hr, then the soluble protein extracts prepared from these cells were immunoprecipitated with PAb419, RA3-2C2, or M73 (an E1A-specific monoclonal antibody [Harlow et al., 1985]). The results of this analysis from representative cell lines are presented in Figure 3. All four cell lines expressed E1A and elevated levels of murine p53, comparable to those

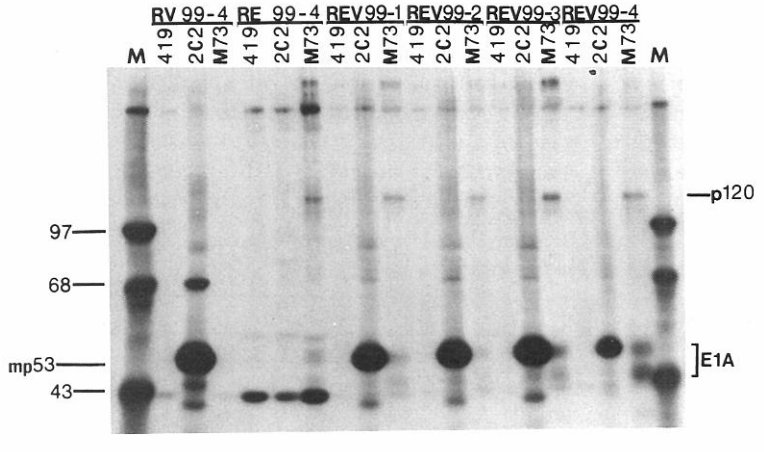

Figure 3. Analysis of E1A and Murine p53 Expression in Transformed Rat Cell Lines

Four cell lines transformed by activated *ras* plus SVE1A plus p53-val_{135}, REV99–1, REV99–2, REV99–3, and REV99–4 were metabolically labeled with [^{35}S]methionine and aliquots of cell lysates representing 1/3 of each plate were immunoprecipitated with PAb419 (419), RA3-2C2, an anti-murine-p53 monoclonal antibody (2C2), or M73, an anti-E1A monoclonal antibody. Shown for comparison are the levels of p53-val_{135} synthesized in a cell line transformed by *ras* plus val_{135} (RV 99–4) and the levels of E1A synthesized in a cell line transformed by *ras* plus SVE1A (RE 99–4). TCA precipitable material in each aliquot ranged from 0.6 × 10^7 cpm for REV 99–2 to 2.3 × 10^7 cpm for RE 99–4. Migration of murine p53 (mp53), the E1A polypeptides (E1A) and a 120 kd protein coimmunoprecipitating with E1A (p120) are indicated: M = molecular mass markers and is indicated in kilodaltons.

Table 3. Analysis of Rat Cells Transformed with *Ras* Plus E1A and Mutant or Wild-Type Murine p53 Genomic Clones

I. *ras* + SVE1A + p53-mutant (val_{135} or KH215)
   A. 15/15 lines express high levels of murine p53 in complex with hsc70.
   B. 13/15 lines express easily detectable E1A.

II. *ras* + SVE1A + p53-wt
   A. 10/13 lines express no detectable murine p53.
   B. 2/13 lines express high levels of murine p53 of altered mobility (p42 + p82).
   C. 1/13 lines expresses high levels of murine p53, which is bound to hsc70.
   D. 11/13 lines express easily detectable E1A.

observed in cells transformed by *ras* plus p53-val_{135} alone (see control lanes). In total, 15/15 cell lines expressed high levels of the murine p53-mutant protein, and 13/15 cell lines expressed readily detectable levels of E1A. Therefore, it does not appear that there are two distinct populations of E1A plus *ras* and p53 plus *ras* in these REF cultures. These triple transfections of REF cells result in a modest increase in transformed foci that contain and express all three oncogenes. The mutant p53 clone is selected for and the wild-type p53 clone is selected against in all of these experiments. The results of the above analyses on cells transformed by *ras* plus SVE1A plus p53-wt and by *ras* plus SVE1A plus p53-mutant are summarized in Table 3.

The Effects of p53-wt Expression in an Immortalized Rodent Cell Line, Rat-1

In contrast to primary cells, Rat-1 cells are transformed by transfection with an activated *ras* gene alone (Land et al., 1983). To determine the effects of p53-wt on *ras* transformation of Rat-1 cells, Rat-1 cells were transfected with *ras* plus p53-wt or with *ras* plus p53-val_{135}, and the number of foci were counted 2 weeks later. Although less dramatic, the results (see Table 4) parallel those in primary cells. Cotransfection of *ras* plus p53-wt reduced the number of foci approximately 25% (when compared with *ras* alone) and cotransfection of *ras* plus p53-val_{135} resulted in a 65% increase in the number of transformants (when compared with *ras* alone). The minimal effect of p53-wt on Rat-1 transformation may result from an increased ability of this cell line to eliminate genes detrimental to its cell growth. Following cotransfection of Rat-1 cells with SVneo plus p53-wt, ten G418-resistant colonies were expanded into cell lines and analyzed for murine p53 expression. No cell lines expressed murine p53 protein, and Southern analysis showed 8/10 cell lines did not carry the gene and 2/10 cell lines had less than one copy per cell (data not shown). In contrast, cotransfection of Rat-1 cells with SVneo plus p53-val_{135} resulted in 6/10 cell lines expressing elevated levels of murine p53, and previous studies have demonstrated that Rat-1 cells expressing high levels of this mutant protein are more tumorigenic (Eliyahu et al., 1985) than the parent Rat-1 cell line.

The Effects of p53-wt and Mutant Gene Expression upon the Plating Efficiency of Primary Rat Embryo Fibroblasts

The results presented in the previous sections clearly show that the wild-type p53 gene product is incompatible with the growth of E1A plus *ras* transformed cells or even the nontransformed immortalized rodent cell line, Rat-1. One hundred percent of these cell lines failed to express or retain the p53 gene or gene product. To examine the effect of the wild-type p53 gene product upon primary cells in culture, the plating efficiencies of these cells, cotransfected with p53 cDNAs or genomic clones and a neomycin resistance gene (neo), were measured. The wild-type p53 gene does not immortalize such cells in culture. Thus, the relative plating efficiencies of these cells are a sensitive measure for comparing the effects of the wild-type and mutant p53 gene products in primary cells in culture. Primary rat embryo fibroblasts transfected with PBR322 plus neo (10:1 ratio) and p53-wt cDNA ± neo (10:1 ratio) showed similar plating efficiencies, with the p53-wt cDNA providing a slight stimulation in the number of colonies produced (Table 5). The mutant cDNA clone or mutant genomic clone of p53 produced a significant (3- to 7-fold) stimulation in plating efficiencies of these cells (Table 5) and many of these colonies (30%–50%) were able to readily form permanent cell lines in culture. This same p53-wt cDNA clone cotransfected with the E1A plus *ras* clones (in a 1:1:1 ratio) was able to reduce the number of transformed foci from 80%–87% in experiments that confirmed the results in Tables 2 and 3. Thus, the p53 cDNA

Table 4. Effect of p53-wt on *ras* Transformation of Rat-1 Cells

	Number of Foci				
	Experiment Number				
Transforming Genes	1	2	3	Average	Ratio to *ras* + PBR322
ras + PBR322	283	205	116	201	1.0
ras + p53-wt	206	148	93	149	0.75
ras + p53-val_{135}	394	385	205	328	1.63

Table 5. The Effect of p53-wt on the Plating Efficiency of Primary Rat Embryo Fibroblasts

Clones (10:1 Ratio)	Colonies/10^6 Cells[a]	Ratio
(A)		
PBR322 + neo	7.2	1.0
p53-wt cDNA + neo	10.6	1.47
KH215 cDNA + neo	24.5	3.40
p53 genomic-val + neo	48.9	6.79
(B)		
p53 genomic FS + neo	14.7	1.0
p53 genomic wt + neo	10.5	0.71
p53 genomic-val + neo	>90	>6.12

[a] These results are the average of two experiments where the ratio of p53 cDNA or genomic clones to the neo-clone was 10:1 on a weight basis.

encoding the wild-type gene product differentially effects the plating efficiency of primary cells and transformed cells in culture. The p53 genomic clone, which expresses higher levels of the p53 gene product when compared with the cDNA clones (Hinds et al., 1989), was cotransfected with a neo gene clone (at a 10:1 ratio) into primary rat embryo fibroblasts. This clone decreased the plating efficiency of such cells by 29% when compared with the cotransfection of a p53 frameshift mutant (p53 genomic FS) clone with neo (Table 5). Under these same conditions, the p53-wt genomic clone plus neo (10:1 ratio) decreased the plating efficiency of KH215 plus *ras*-transformed cells by 93% (P. Hinds, unpublished data). This is a minimum of a 3-fold differential between primary cells and transformed cells because the residual transformed clones from such experiments (Tables 2, 3, and 4) were shown in every case not to express the p53-wt products, lose the gene for p53-wt, or harbor a mutant p53 gene.

These experiments demonstrate two important points: First, the p53-wt cDNA (moderate expression levels) and genomic clones (high expression levels) have modest effects (30%–47%) upon primary rat embryo fibroblasts tested for plating efficiencies. These same clones significantly reduce the transformation of REF and the plating efficiency of KH215 plus *ras*-transformed cells (80%–93%). No such cell lines (100%) contain and express the wild-type p53 gene or gene product. Second, the mutant p53 cDNA and genomic clones increase the plating efficiencies (3-fold for cDNA and 7-fold for the genomic) of primary rat embryo fibroblasts (Table 5). Many of these colonies (30%–50%) can be cloned to produce permanent rat cell lines expressing these mutant p53 gene products (C. Finlay, unpublished data).

Discussion

Previous studies (Hinds et al., 1987; Finlay et al., 1988; Hinds et al., 1989) have led to the conclusion that a wide variety of mutations spanning a large region (25% of the coding sequences) of the p53 gene all activate this gene for transformation with the *ras* oncogene. This has led to the hypothesis that mutations that activate for transformation may be mutations that result in a loss of function of the p53 proto-oncogene (Hinds et al., 1989). The great majority of such activating mutations have several properties in common: the p53 protein usually binds poorly to the SV40 large T antigen; it binds very well to the hsc70 cellular protein; and the p53 protein has undergone a conformational change as detected by the loss of an epitope (at amino acids 88–109) seen by the monoclonal antibody PAb246 (Finlay et al., 1988). These observations have led to the hypothesis that inactive, mutant p53 proteins cooperate with *ras* in transformation events by acting in a *trans*-dominant fashion to inactivate the function of normal, wild-type p53 (of rat origin) in a REF cell. The mutant murine p53 protein may do this by entering into a complex with hsc70 and wild-type rat p53, and thereby inactivating a function of the wild-type p53 protein. Such complexes

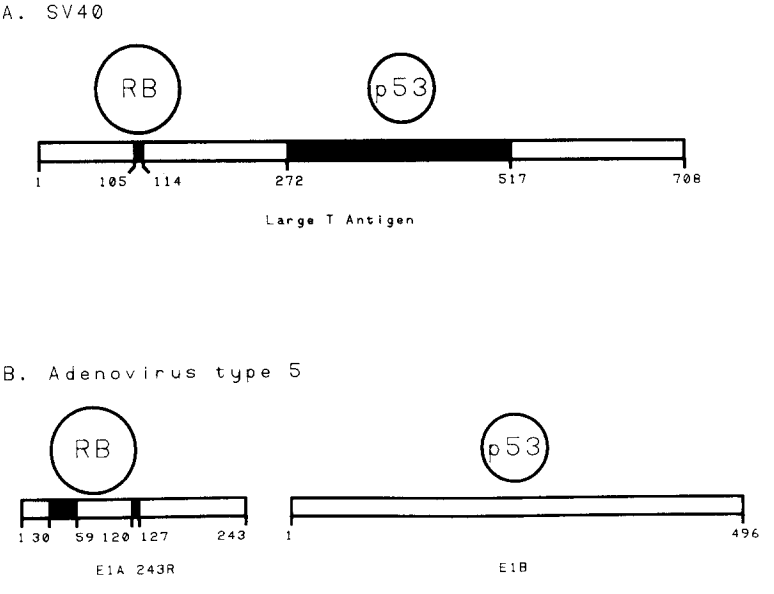

Figure 4. Schematic Representation of the Interaction between the Retinoblastoma Protein, p53, and Viral Antigens

Black boxes represent proposed sites of interaction between the SV40 large T antigen or adenovirus E1A 243R protein and the cellular proteins retinoblastoma (Rb) and p53 (DeCaprio et al., 1988; Schmeig and Simmons, 1988; Moran, 1988; Whyte et al., 1989). These regions and E1B are required for full transformation of primary cells by these viruses, demonstrating the correlation between Rb and p53 binding and cellular transformation despite the separation of function(s) in the adenovirus E1 region as compared with the SV40 large T antigen.

have been demonstrated in this study and others (Eliyahu et al., 1988; Rovinski and Benchimol, 1988). Previous studies (Hinds et al., 1989) showed that high levels of mutant p53 protein are required to efficiently cooperate with the *ras* oncogene and produce transformed cells. This is expected for the *trans*-dominant inactivation of wild-type function in an oligomeric protein complex (Herskowitz, 1987). Based upon this hypothesis, wild-type p53 would act as a suppressor of transformation in the p53-mutant plus *ras* assay. The work presented here shows that that is indeed the case. The wild-type p53 clone almost completely eliminates the ability of p53-mutant clones plus *ras* to transform REF cells.

Similarly, the wild-type p53 clone reduces the ability of E1A plus *ras* oncogenes to transform REF cells when the three gene constructs are cotransfected. The foci that are produced in this experiment express the E1A proteins and all fail to express the wild-type murine p53 proteins even though all the clones contain the murine p53 DNA clone in their genomes (by Southern blot analysis). In fact, 10/13 lines analyzed fail to express the wild-type murine p53 protein at all. The remaining three cell lines each express a mutant form of the murine p53 protein that binds to hsc70, sequesters the wild-type rat p53 protein in such a complex, and, in two cases, fails to express the PAb246 epitope. The lack of this epitope is characteristic of the transformation-activated p53 mutants (Finlay et al., 1988). These data strongly support the hypothesis that the wild-type p53 protein acts to suppress or inhibit transformation by p53 mutants or E1A and *ras*.

As a control for these experiments, p53 mutant clones were cotransfected with SVE1A plus *ras* into REF cells. The presence of the activated mutant p53 clones enhanced (60%–140%) the number of foci produced. Virtually all of these cloned cell lines derived from such foci contained E1A (13/15 at a minimum) and p53 (15/15) expressed at high levels. Clearly, mutant p53 clones are selected for in E1A plus *ras* transformation assays with REF cells while wild-type p53 expression is selected against in the same assays.

The mechanism by which the wild-type p53 protein acts to suppress the p53-mutant or E1A plus *ras* transformation events is not clear. Changing the promoters and enhancers that are regulating E1A or *ras* did not affect the results of the p53 wild-type suppression of these oncogene activities. This may indicate that the wild-type p53 protein is not negatively regulating these oncogenes at the level of the promoter–enhancer elements. The p53 wild-type DNA clone and the corresponding protein made by it does not reduce the levels of p53-mutant protein or activated *ras* protein made by DNA clones cotransfected with the wild-type p53 DNA. Similarly, the wild-type p53 protein has little or no effect upon the level of mutant p53 directed by transfection of a genomic mutant p53 clone. Thus, it does not appear that the wild-type p53 protein acts to negatively regulate the transient or immediate levels of the activating oncogene products produced during the transformation event. More information will be needed to determine the mechanism by which p53-wt proteins inhibit these transformation events.

Expression of high levels (p53 genomic clone) or moderate levels (p53 cDNA clone) of the wild-type p53 gene products (Hinds et al., 1989) in E1A plus *ras*-transformed cell lines, or even nontransformed, permanent cell lines like Rat-1, is not compatible with growth of these cells. The wild-type cDNA or genomic clones reduce (80%–93%) the transforming efficiencies of such cells, and cell lines that are derived from the remaining colonies fail to contain or express the wild-type p53 gene product (100% negative). The expression of high levels of wild-type p53 in primary rat embryo fibroblasts (genomic clone) reduces the plating efficiency of such cells by about 30% while more moderate p53 levels (cDNA clones) had little or no effect (a stimulation of 47%) upon the plating efficiencies of primary rat cells. This level of variation may well be within experimental error of this system with only marginal effects of p53 in these cells. On the other hand, mutant p53 genomic clones and cDNA clones clearly stimulated the efficiency of plating of primary rat embryo fibroblasts in a clone-dependent fashion from 3- to 7-fold (300%–700%). These experiments (Table 5) clearly demonstrate the differential effects of wild-type p53 protein in primary rat embryo fibroblasts and some transformed rat embryo fibroblast cell lines. Expression of high levels of the wild-type p53 protein may thus have inhibitory effects upon a number of permanent cell lines in culture. Four out of five p53-cDNAs obtained from such cell lines contained activating mutations for transformation (Finlay et al., 1988). It is clear, however, that some cell types and cell lines (embryonal carcinoma cells, F9; T cells or thymocytes) can tolerate high levels of the wild-type p53 proteins with no adverse effects upon cell viability and growth (Linzer and Levine, 1979; Finlay et al., 1988). The differentiated state of a cell or other important variables may well control the cellular response to increased levels of the wild-type p53 protein.

Based upon these results, there is a clear need to reinterpret the meaning of the binding of p53 to the SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979) and the adenovirus E1B-55kd protein (Sarnow et al., 1982). These viral tumor antigens may bind to p53 so as to inactivate its functions in the cell and, in so doing, predispose the cell to a transformation event. In this context, the p53 and the retinoblastoma protein are very similar. The SV40 large T antigen encodes two domains detected by both structural and functional (genetic) studies that are involved in transformation of cells in culture (Srinivasan et al., 1989). One of these localized in the NH_2-terminal half of the proteins (out of 708 amino acids in this protein) can immortalize rat cells in culture (Colby and Shenk, 1982; Sompayrac and Danna, 1985) and contains a domain (amino acids 105–114) required for binding to the retinoblastoma protein in cells (DeCaprio et al., 1988). The second domain required for transformation has been localized between amino acids 325 and 625 (Srinivasan et al., 1989) and significantly overlaps the region implicated in binding to the p53 protein (amino acids 272–517) (Schmeig and Simmons, 1988). Thus, the two domains of the SV40 large T antigen associated with p53 and the retinoblastoma proteins are involved in transformation of

cells in culture. Similarly, the adenovirus E1A protein, involved in immortalization of cells in culture (Houweling et al., 1980; Ruley, 1983), binds to the Rb protein (Whyte et al., 1988) while the adenovirus E1B-55kd protein, which is also required for transformation (Gallimore et al., 1974; Graham et al., 1974; Jones and Shenk, 1979), binds to the p53 protein. As schematically depicted in Figure 4, adenoviruses appear to have divided the interactions seen in two different domains of the SV40 large T antigen into two separate proteins.

This is not the first time that the hypothesis that the wild-type p53 proto-oncogene can act to suppress tumorigenesis has been suggested. Benchimol and his colleagues (Mowat et al., 1985; Chow et al., 1987; Rovinski et al., 1987; Ben-David et al., 1988; and Munroe et al., 1988) have documented rearrangements of murine p53 genes in tumors. In some of these tumors, there was a failure to express p53 and, in other tumors, altered p53 molecules were detected. Most interesting, however, was the appearance of truncated p53 proteins (44–46 kd range). Further, transfection of REFs with a clone encoding a full-length p53 derived from a murine erythroleukemic (Rovinski and Benchimol, 1988) cell line resulted in immortalized cell lines that expressed high levels of murine p53 bound to heat shock (70 kd) proteins. The experiments presented in this paper provide a way to recapitulate the events occurring in vivo and the generation of mutant p53 proteins similar to those observed in vivo (see Table 3).

While the experiments presented and discussed here are consistent with an activation of the p53 oncogene by a loss of function mutation, other experiments remain inconsistent with this simple form of this hypothesis. The clearest contradiction comes from an experiment where an activated, mutant p53 clone (Parada et al., 1984) was transfected into L12 cells, which are an Abelson murine leukemia virus transformed cell line in which no p53 protein is detectable. The clones of cells expressing this altered p53 protein consistently caused lethal tumors in mice, whereas the parental cell line with no p53 caused the development of tumors that later regressed (Wolf et al., 1984). A similar observation has been made by Mowat et al. (1985). In the absence of an endogenous wild-type p53 protein in L12 cells, this experiment suggests a positive or gain of function activity for the activating mutation. It remains a real possibility that p53 transformation-activating mutations, while acting in a *trans*-dominant manner to eliminate some wild-type p53 functions, also maintain a function that can act in yet another way to promote tumorigenesis, immortalize some cell lines, or enhance the plating efficiency of a cell type. The experiments presented in Table 5 in this paper clearly demonstrate some of these properties that are interpretable as a gain of function. Only additional studies will sort out these questions.

Experimental Procedures

Plasmids

LTRp53cG (here referred to as p53-val$_{135}$) is a murine p53 cDNA genomic hybrid clone (Eliyahu et al., 1985), which contains cDNA sequences encoding amino acid residues 1–13 and 330–390, and 88 bp of p53 3′ noncoding sequence. The remainder of the p53 sequences are of genomic origin. Enhancer and promoter activity is provided by the Harvey murine sarcoma virus long terminal repeat, and a polyadenylation signal is provided by a 260 bp BclI–BamHI fragment of SV40. p53 wild-type (wt) has been previously described and is identical to p53-val$_{135}$ except that there is an alanine at amino acid 135 (Hinds et al., 1989). p53 KH215 is identical to p53-wt except that an in-frame decameric HindIII linker has been inserted into the KpnI site at amino acid 215–216 of the murine p53 sequence. The KH215 mutation has been demonstrated to activate p53 for cooperation with an activated *ras* gene (Finlay et al., 1988). p53-FS contains an out-of-frame EcoRI linker at a XhoI site at amino acid 13 (Hinds et al., 1989). p1A was the generous gift of Dr. Thomas Shenk, Princeton University, and contains the Adenovirus type 5 E1A EcoRI–SacI fragment. SVE1A was constructed by deleting the E1A promoter region (nucleotides 193–548) and replacing the E1A promoter with the PvuII–HindIII fragment containing the enhancer–promoter region of an origin defective SV40. Most assays utilized an activated *ras* gene (T24) (Goldfarb et al., 1982). Riboras was the kind gift of Dr. Etta Eskridge and Dr. Thomas Shenk and contains the 2nd and 3rd exons of an activated *ras* gene (EJ6.6; Parada et al., 1982) under the control of the ribosomal L323A promoter (Dudov and Perry, 1984, 1986).

Cell Culture and DNA Transfections

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Primary rat embryo fibroblasts (REF) were prepared from 14- to 15-day-old Fisher 344 rat embryos, and 3×10^5 primary, secondary, or tertiary REF were transfected on day 1 by the calcium phosphate procedure (Graham and van der Eb, 1973) with 1.25 μg of each plasmid plus 10 μg of salmon sperm DNA as carrier as previously described (Hinds et al., 1987). Transformed foci were scored 12–14 days later. Rat-1 cells were seeded at 1.5×10^5 cells in a 60 mm dish and were transfected on day 1 as previously described (Hinds et al., 1987) with 1.5 μg of an activated *ras* gene (T24) plus 1.5 μg of the test gene plus 10 μg of salmon sperm DNA as carrier. Transformed foci were determined 12–14 days later.

For coselection experiments, secondary or tertiary rat embryo fibroblasts were seeded at a density of 1×10^6 cells/10 cm dish and were cotransfected on day 1 with 0.5 μg of pSV2neo (Southern and Berg, 1982) plus 5 μg of the test plasmid. Following transfection, the cells were trypsinized and $1–2 \times 10^6$ cells were seeded into medium containing 500–600 μg/ml of Geneticin G-418 (GIBCO). The cells were refed every 4–5 days. Ten to fourteen days later, the colony number was determined.

Immunoprecipitations

Transformed cell lines were metabolically labeled with 50 μCi/ml of [^{35}S]methionine translabel (ICN) in 2 ml of methionine-free DMEM plus 2% dialyzed FBS for 1–2 hr at 37°C. Following labeling, cells were rinsed with ice-cold phosphate-buffered saline and scraped from the plate. Cell pellets were stored at −80°C.

Immunoprecipitations were performed as previously described (Hinds et al., 1987), except that 50% protein A–Sepharose (Sigma) was used in place of fixed staphylococcal protein A. Incubation of protein A–Sepharose and antigen–antibody complex was continued for at least 2 hr at 4°C with constant agitation. Following immunoprecipitation, immunoprecipitates were separated on 7.5% polyacrylamide gels. Gels were prepared for fluorography by sequential immersion in 30% methanol–10% acetic acid (30 min), H_2O (three times for 10 min each), and 1 M sodium salicylate–50% glycerol (20 min). Dried gels were exposed to Kodak XAR-5 film at −80°C. Immunoblotting was performed as described (Hinds et al., 1987) except that Immobilon (Millipore) was used instead of nitrocellulose.

Acknowledgments

The authors thank A. K. Teresky for technical help and K. James for preparation of the manuscript. This research was supported by a grant from the National Cancer Institute, P01-CA41086-03.

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Received December 27, 1988; revised April 14, 1989.

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Note Added in Proof

Similar experiments and results described in this publication have been obtained by M. Oren and his colleagues.