Mutant p53 DNA Clones from Human Colon Carcinomas Cooperate with *ras* in Transforming Primary Rat Cells: A Comparison of the "Hot Spot" Mutant Phenotypes[1]

Philip W. Hinds,[2] Cathy A. Finlay, Robin S. Quartin, Suzanne J. Baker, Eric R. Fearon, Bert Vogelstein, and Arnold J. Levine[3]

Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, New Jersey 08544-1014 [P. W. H., C. A. F., R. S. Q., A. J. L.], and Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231 [S. J. B., E. R. F., B. V.]

Abstract

The majority of the p53 genes derived from human colorectal carcinomas contain point mutations. A significant number of these mutations occur in or around amino acids 143, 175, 273, or 281. Experiments presented here demonstrate for the first time that p53 DNA clones containing any one of these mutations cooperate with the activated *ras* oncogene to transform primary rat embryo cells in culture. These transformed cells produce elevated levels of the human p53 protein, which has extended half-lives (1.5–7 h), as compared to the wild-type human p53 protein (20–30 min). The p53 mutant with an alteration at residue 175 (p53-175H) binds tightly to the cellular heat shock protein, hsc70. In contrast, the p53 mutants possessing mutations at either residue 273 or 281 (p53-273H/281G) do not bind detectably to this heat shock protein and generally are less efficient at forming transformed foci in culture. The transformed cell lines are tumorigenic in nude mice. Thus, two classes of p53 mutant proteins can be distinguished: p53-175H, which cooperates with *ras* efficiently and binds to hsc70, and p53-273H/281G, which has a reduced efficiency of transformed foci formation and does not bind hsc70. This demonstrates that complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation, but rather it facilitates this function, perhaps by ensuring sequestration of the endogenous wild-type p53 protein. The positive effect on cell proliferation by these mutant p53 proteins is consistent with a role for activated p53 mutants in the genesis of colorectal carcinomas.

Introduction

The cellular protein p53 is often found at increased levels in transformed cells due to an increase in its metabolic stability (1, 2). This stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins (3–14), suggesting that alterations in p53 function may be involved in the process of cellular transformation. The participation of murine p53 in the transformation of cells in culture has been demonstrated by the ability of mutant, but not wild-type, p53 genes to cooperate with activated Ha-*ras* to transform primary rat embryo cells (8, 15–20). Recently, the wild-type murine p53 protein has been demonstrated to display properties of a suppressor of transformation (21, 22). Three distinct observations are consistent with this type of function for the wild-type p53 gene and gene product. First, the introduction of wild-type murine p53 into primary rodent cells along with two cooperating transforming genes (e.g., *ras* and E1A) results in a dramatic decrease in the number of transformed foci observed (21, 22). The remaining transformed cell lines were found to contain the murine p53 gene but either failed to express it (77% of the time) or produced high levels of an altered murine product (21). Thus, overexpression of the wild-type murine p53 protein appears to be detrimental to the process of transformation of cultured rat cells by oncogenes. Similarly, introduction of the wild-type human p53 gene into human tumor cells drastically inhibits their growth (23–25). Second, inactivation of the p53 gene has been associated with the development of Friend virus-induced erythroleukemia in mice (26). Many tumor cells derived from the spleens of mice infected with the Friend virus complex contain rearrangements or other mutations at the p53 gene locus, resulting in either loss of p53 synthesis or the production of altered proteins (27–30).

The third line of evidence consistent with the possibility that the wild-type p53 protein is a member of a group of proteins involved in suppression of transformation is the ability of p53 to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-*M*, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product (3, 6, 9, 31). Such a complex has been observed between $p105^{RB}$, the product of the retinoblastoma susceptibility gene, and T antigen (32), the adenovirus E1A protein (33), and the E7 protein of human papilloma virus 16 or 18 (34). These interactions between these viral proteins and $p105^{RB}$ are thought to inactivate a growth-suppressive function of $p105^{RB}$, thus mimicking deletions and mutations commonly found in the *RB* gene in tumor cells. In a similar fashion, oligomeric protein complex formation between these viral proteins and p53 may eliminate or alter the function of p53 (21).

Recently, the inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (35), including human colorectal carcinoma (36), human lung cancer (37, 38), chronic myelocytic leukemia (39), chronic myelogenous leukemia (40), and osteogenic sarcomas (41). More than 75% of colorectal tumors were found to contain allelic deletions in chromosome 17p, and the region common to all deletions contained the p53 gene (36). Most interestingly,

Received 8/2/90.
[1] This work was supported by Grant P01-CA41086-04 from the National Cancer Institute.
[2] Present address: Whitehead Institute, Nine Cambridge Center, Cambridge, MA 02142.
[3] To whom requests for reprints should be addressed.

when the remaining p53 genes from such tumors were sequenced, they were found to contain single nucleotide changes resulting in missense mutations (35, 36). The mutations clustered in four "hot spots" corresponding to the four most highly conserved portions of the gene. The clonal nature of these mutations is consistent with a tumor progression model in which (nonneoplastic) cells bearing a mutated p53 gene have a distinct proliferative advantage over normal cells due to interference with wild-type p53 function mediated by mutant p53 proteins (21, 36). The increased proliferative capacity of these cells would then increase the probability of a mutant cell suffering a second, inactivating mutation (gene conversion, mitotic recombination, or deletion) at the p53 locus. Such a cell, containing both mutations in the p53 locus, would then be able to express the fully neoplastic phenotype (21, 36). This model of tumor progression leads to the prediction that mutant human p53 genes containing the missense mutations derived from colorectal carcinomas should confer a selective advantage upon recipient cells. Furthermore, when overexpressed, these p53 genes may cooperate with activated *ras* to transform primary rodent cells in culture (*i.e.*, act like a dominant oncogene in this assay).

In this report, experiments are presented which demonstrate for the first time that different human p53 clones possessing mutations detected in colon carcinomas (*i.e.*, mutations at amino acid residues 143, 175, 273, or 281 out of 393 residues) cooperate with the activated *ras* oncogene to transform rat embryo fibroblasts in culture. All of the transformed cell lines derived from these assays produced the human p53 protein in elevated levels. The mutant p53 proteins had extended half-lives, from 1.5 to 7 h. The p53 mutant at residue 175 (p53-175H) produced more transformed cell foci (2.5–6-fold) than the p53-273H/281G mutants, and the p53-143A/175H proteins formed an oligomeric protein complex with the cellular heat shock protein hsc70. The p53-273H/281G mutant proteins did not detectably form complexes with hsc70. All transformed cell lines tested efficiently produced tumors in nude mice. Consistent with a previous report (42), the wild-type human p53 gene did not possess transforming activity in cooperation with *ras*. Thus, hsc70 binding is not strictly required for p53 activation and can only be used to predict a subset of p53 activating mutations. Despite this discordance between activation and hsc70 binding, a second site mutation, placing a frameshift into the p53-175H activated DNA clone at amino acid 328, produced a stable p53 protein fragment (p53-175Hfs) that no longer transformed rat cells with the *ras* oncogene and failed to bind or interact with the heat shock protein in these cells. Thus, an intact p53 carboxy-terminus appears to be required for transformation and for hsc70 binding to the p53-175Hfs missense mutant. The production of an activated p53 therefore appears to require the retention of certain p53 functions while inactivating a growth-suppressive function. hsc70 may serve to stabilize nonproductive complexes between mutant and wild-type p53 or between mutant p53 and a distinct mediator molecule. One such protein which may be sequestered by these complexes is a novel M_r 90,000 protein (p90), identified in these studies, which associates with all human p53 proteins produced in transformed cells except p53-175Hfs. Thus, p90 may represent a mediator of p53 function in cell transformation.

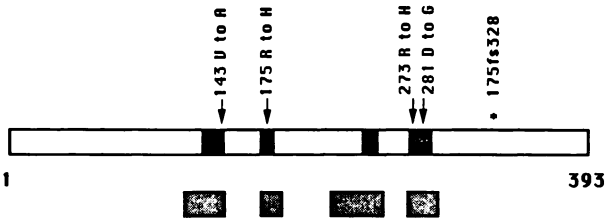

Fig. 1. Schematic diagram of the human p53 protein. *Filled boxes* within the *open bar*, regions frequently mutated in human tumors (see text). *Shaded boxes below bar*, domains of high cross-species identity (59). *Arrows*, missense mutations in each of the DNA clones used in the study. *Asterisk*, the point mutation resulting in a shift of reading frame.

Results

Mutant Human p53 DNA Clones Cooperate with Activated *ras* to Transform Primary Rat Embryo Fibroblasts. To test the hypothesis that mutant human p53 genes derived from colorectal carcinomas are activated for transformation with *ras*, several mutant human cDNA[4] clones derived from this cancer tissue (35, 36) were cloned into an expression vector containing the cytomegalovirus promoter-enhancer region. Because previous experiments had demonstrated that the efficiency of focus formation and cell line establishment was higher in murine p53 constructs containing introns than in similar constructs without them (19, 20), these constructs were designed to also contain human p53 introns 2–4. The "hot spot" p53 gene mutations derived from colon carcinomas used in this study were in clones p53-175H (an arginine to histidine mutation at residue 175), clone p53-273H (an arginine to histidine alteration at residue 273), and p53-281G (an aspartic acid to glycine mutation at residue 281) (see Fig. 1). Clone p53-WT contained a wild-type sequence of human p53 in the same vector. An activated murine mutant p53 cDNA clone, KH215 (8, 43), served as a positive control.

These partial cDNA-genomic clones of p53 plus the activated *ras* oncogene were cotransfected into primary rat embryo fibroblasts. In each set of transfections, transformed foci were scored 2–3 weeks later in duplicate cell cultures. The results of five representative experiments are presented in Table 1. In the absence of a p53 insert (bluescript, Table 1), only a minimal level of foci induced by an activated *ras* gene was observed (0.5 foci/experiment). The wild-type p53 DNA clone did not produce foci above this background level. No transformed cell lines could be cloned from the only four *ras* plus p53-WT foci which were observed in these assays, thus confirming that the wild-type human p53 protein [as shown previously for the wild-type murine p53 (8, 20)] is not capable of transformation in this assay (Table 1). The p53-175H clone produced an average of 11 foci/experiment (23-fold higher than background), and these foci were efficiently cloned into permanent cell lines which, in the p53 plus *ras* assay, is a critical test for the biological activity of p53 mutant clones (8, 19, 21). The

[4] The abbreviations used are: cDNA, complementary DNA; RGF, rat embryo fibroblast(s); bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium.

Table 1 Transformed foci and cloning efficiency of human mutant p53 plus *ras*-transformed rat embryo fibroblasts

Clones	No. of foci/ experiment	Average no. of foci	Cloning efficiency (%)
Bluescript alone	0, 0, 0, 0, 0	0	
Bluescript + *ras*	1, 1, 0, 0.5, 0	0.5	
p53-WT + *ras*	2, 0, 0, 0, 0	0.4	0/4 (0)
p53-175H + *ras*	14, 16, 7.5, 15, 5	11.5	7/12 (58)
p53-273H + *ras*	13, 6, 2.5, 2, 0	4.7	3/10 (30)
p53-281G + *ras*	2.5, 5, 1.5, 0.5, 0	1.9	2/10 (20)
p53-175Hfs + *ras*	1.5, 1, 1, 0, 0	0.7	0/2 (0)
CMV-KH215 + *ras*	16, 30, 17.5, 20, 9	18.5	(21)[a]

[a] The cloning efficiency of foci transformed by *ras* + KH215 (under the control of the Harvey murine sarcoma virus long terminal repeat) was previously determined (8).

other two clones with p53 "hot spot" mutations observed in colon carcinoma, p53-281G and p53-273H, showed a reduced activity in these assays, with 2.4–6-fold less foci (when compared to p53-175H) produced by these p53 mutant clones. These results were reproducible from assay to assay and appear to represent a significant decrease in transforming activity, especially with the p53-281G clone. Even though the number of foci was reduced with p53-273H or p53-281G plus *ras*, some of the clones derived from these foci were fully transformed and were successfully grown into cell lines (Table 1). The mutant murine p53 cDNA clone, CMV-KH215 (Table 1), produced p53 plus *ras*-transformed foci more efficiently than any of the human p53 clones, indicating that the activated human p53 genes may be less transforming than the activated murine p53 genes in a rat cell.

A human p53 cDNA clone, p53-c143A, containing a missense mutation at residue 143 (valine to alanine) (36), was also tested for the ability to cooperate with *ras* in the transformation of REF cells. The wild-type human p53 cDNA clone, p53-cWT, served as a control. The average number of transformed foci per experiment when p53-c143A plus *ras* were transfected into REF cells was 4.0, and this was only 2-fold above the background of foci in these experiments. The low number of transformed foci in these experiments most likely resulted from the lack of introns in the p53 gene. Increased numbers of foci were observed (30–400% increase) in assays involving p53-273H plus introns when compared to assays with p53-273H minus introns (data not shown). Similarly, the efficiency of cloning transformed foci from p53-c143A plus *ras* transfections (6 of 27 foci produced permanent cell lines, 22%) was lower than similar p53 mutant expression vectors with introns (19) but was significantly above the p53-cWT (without introns) plus *ras* results (a background number of foci with 1 of 17 foci producing a permanent cell line or 5% cloning efficiency).

Expression of Human p53 Protein in Transformed Rat Embryo Cells. To investigate the involvement of human p53 proteins in the formation of transformed cell lines, the expression and properties of the p53 proteins produced in these transformed cell lines were examined. The parental REF cells, the one established cell line derived from a p53-cWT transfection, three *ras* plus p53-c143A-derived cell lines, and two *ras* plus p53-175H-derived cell lines were labeled with [^{35}S]methionine, and the soluble protein extracts were incubated with antibodies specific for the human p53 protein (Ab2), total human plus rat p53 proteins (PAb421) (44), or the hsp70 proteins (7). The immunoprecipitates were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Parental REF cells, in the absence of exogenous p53 clones, produced very low levels of rat p53 protein that was only reactive with PAb421 and not Ab2, reflecting the specificity of Ab2 for human p53 protein (Fig. 2*A*, *Lanes 2* and *3*). In contrast, the three cell lines transformed by *ras* and p53-c143A expressed high levels of a slower migrating form of p53 which reacted both with Ab2 and PAb421 (Fig. 2*A*, *Lanes 8, 9, 11, 12, 14, 15*). A similar species of p53 was produced in cell lines transformed by *ras* plus p53-175H (Fig. 2*B*). The slower electrophoretic migration of these proteins and the binding of these proteins to human p53-specific antibodies identified these proteins as human p53. Immunoprecipitation with these anti-p53 monoclonal antibodies also resulted in the coimmunoprecipitation of an M_r 70,000 protein which, in previous studies with the murine p53 protein, was identified as the hsc70 heat shock protein (8). Immunoprecipitation of the same extracts with anti-hsp70 peptide antisera resulted in the precipitation of hsc70 and the coimmunoprecipitation of an M_r 53,000 protein (Fig. 2*B* for *ras* plus p53-175H cells, and Fig. 4*C* for *ras* plus p53-c143A cells), demonstrating that both activated p53-c143A and p53-175H mutant human p53 proteins form a complex with hsc70, as is the case for numerous activated mutant murine p53 proteins (7, 11, 14). Immunoblot analysis confirmed that anti-hsp70 antibodies coimmunoprecipitated human p53 from these *ras* plus mutant p53-transformed cell lines (data not shown).

In contrast to these results, the p53 mutant cell lines derived from p53-273H plus *ras* transfections produced p53 proteins that were expressed at high levels in these transformed cell lines but showed no detectable interactions with the hsc70 heat shock proteins (Fig. 3). Similar results were obtained with p53-281G plus *ras*-transformed cell lines (data not shown). Immunoprecipitation with either p53-specific monoclonal antibodies or hsp70-specific antisera failed to detect a p53-hsc70 complex in these cells.

These data demonstrate that high levels of mutant human p53 are detected in p53 plus *ras*-transformed cell lines, but interaction with the rat hsc70 is dependent upon the type of mutation. p53 proteins with mutations at residues 143 or 175 interact with hsc70, whereas residue 273 or 281 mutants do not bind detectably to hsc70.

Interestingly, the one cell line established from a *ras* plus wild-type human p53 transfection was also found to express human p53, as demonstrated by the ability of this protein to react with Ab2 (Fig. 2*A*, *Lane 5*). In contrast to mutant human p53 expressed in transformed cells, the wild-type human p53 protein was found at much lower levels (equal to that produced by the endogenous rat p53 gene) and did not bind detectably to hsc70 (Fig. 2*A*, *Lanes 5* and 6). Despite these differences between wild-type and mutant p53 proteins in transformed rat cells, the expression of these proteins did result in one common observation. In all cases, when human p53 was immunoprecipitated with PAb421 or Ab2, an M_r 90,000

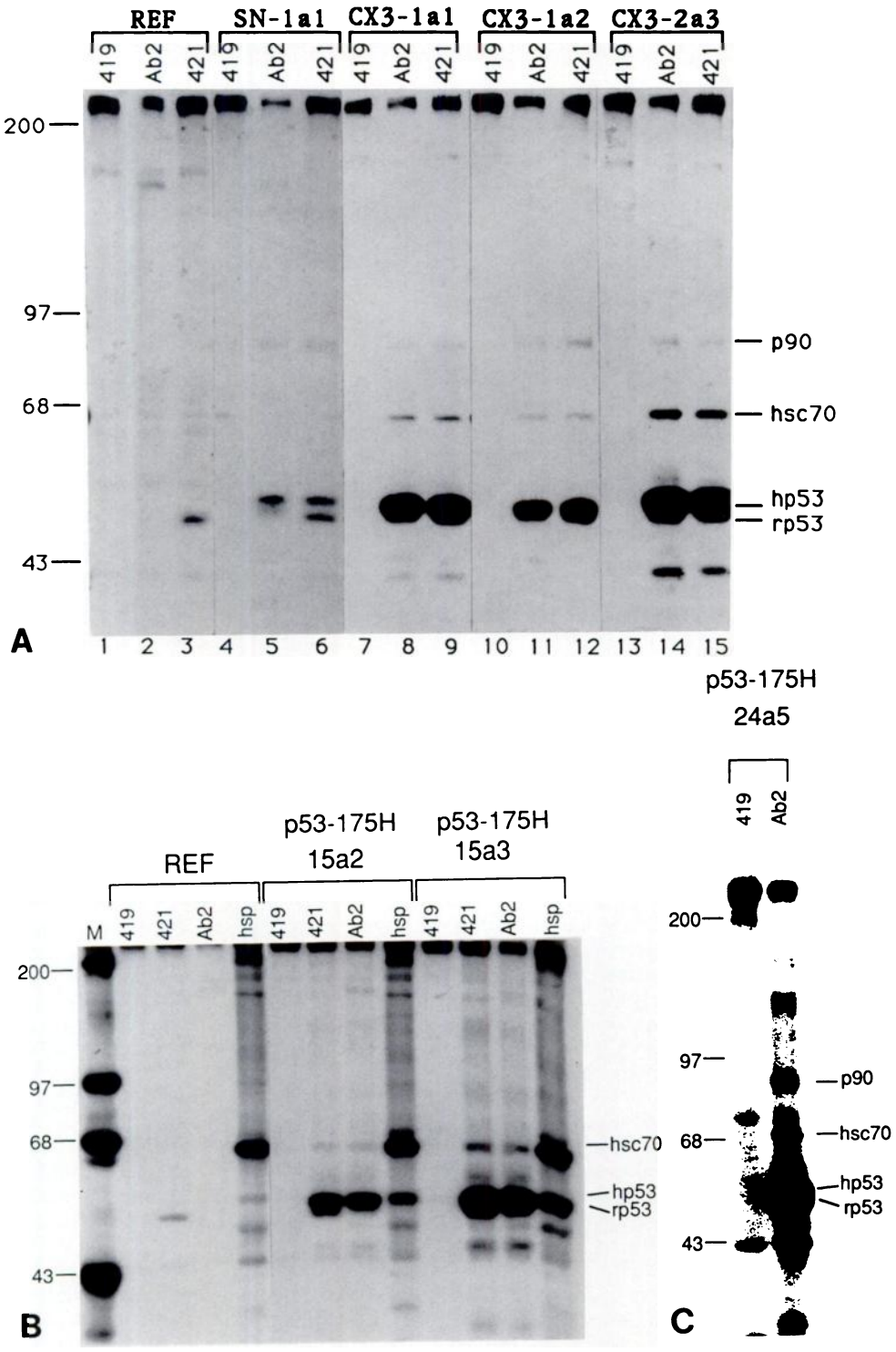

Fig. 2. Expression of p53-cWT, p53-c143A, and p53-175H in transformed rat embryo fibroblasts. *A*, primary rat embryo fibroblasts (*REF*) or the transformed cell lines SN-1a1 (p53-cWT-1a1) (transfected with wild-type human p53 and *ras*), Cx3-1a1 (p53-c143A-1a1), Cx3-1a2 (p53-c143A-1a2), or Cx3-2a3 (p53-c143A-2a3) (transfected with the mutant p53-c143A and *ras*) were metabolically labeled with [^{35}S]methionine, and equal amounts of trichloroacetic acid-insoluble protein (1.5×10^6 cpm) were subjected to immunoprecipitation and gel electrophoresis as described in "Materials and Methods." Antibodies used were PAb419 (*419*), a negative control specific for SV40 large T antigen (not present in these cells); PAb421 (*421*), which recognizes both primate and rodent p53; and Ab2, which is human p53 specific. The migrations of rat p53 (*rp53*), human p53 (*hp53*), *hsc70*, and a novel coimmunoprecipitating *M*, 90,000 protein (*p90*) are shown. Molecular mass markers are indicated at *left* in kilodaltons. The autoradiogram is a 7-day exposure. *B*, proteins were immunoprecipitated from 2×10^6 cpm lysates of metabolically labeled REF and the transformed cell lines p53-175H-15a2 and p53-175H-15a3 (transfected with the mutant p53-175H and *ras*). Antibodies were as described in *A*, but an immunoprecipitation with anti-hsp70 (*hsp*) was also included. The autoradiogram is a 4-day exposure. *C*, proteins were immunoprecipitated from a lysate of cell line p53-175H-24a5 and immunoprecipitated with PAb419 or Ab2. The autoradiogram is a 9-day exposure.

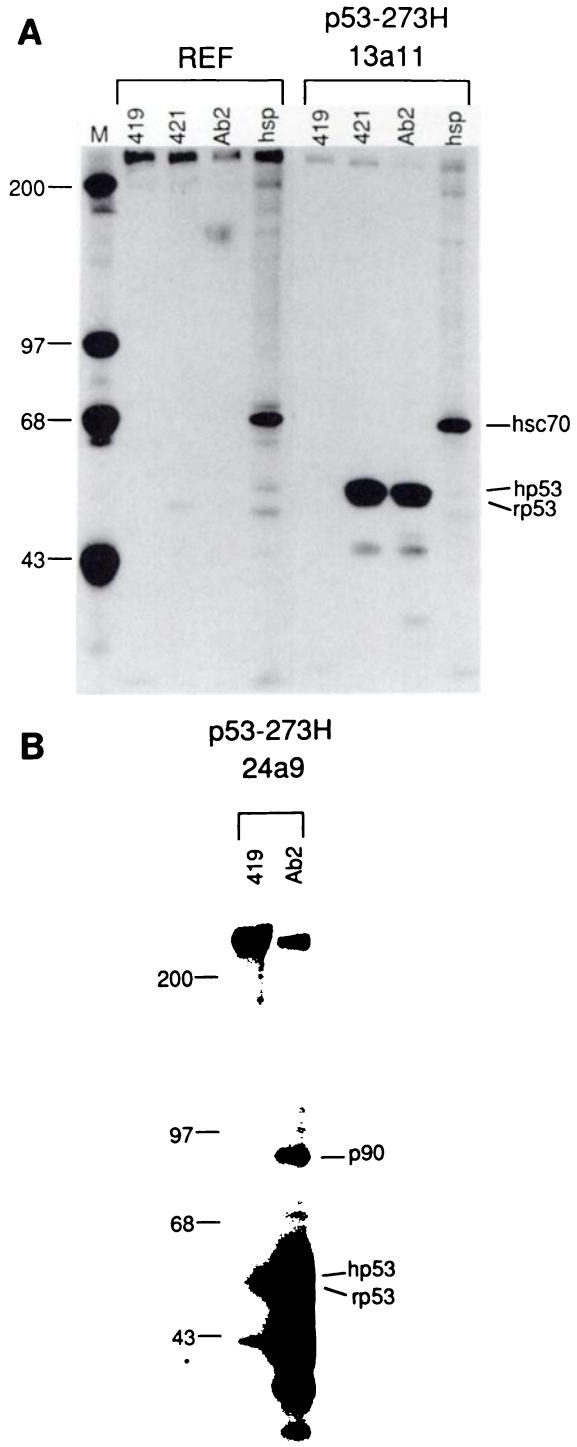

Fig. 3. Expression of p53-273H in transformed rat embryo fibroblasts. *A*, primary rat embryo fibroblasts (*REF*) or the transformed cell line p53-273H-13a11 (transfected with mutant p53-273H and *ras*) were metabolically labeled with [^{35}S]methionine, and equal amounts of trichloroacetic acid-insoluble protein (1×10^6 cpm) were immunoprecipitated with PAb419 (*419*), PAb421 (*421*), Ab2, or anti-hsp70 antisera (*hsp*) (see legend to Fig. 2) and separated by electrophoresis. The migrations of molecular mass markers hsc70, human p53 (*hp53*), and rat p53 (*rp53*) are indicated. The autoradiogram is a 3.5-day exposure. *B*, a lysate of cell line p53-273H-24a9 was immunoprecipitated with PAb419 or Ab2. p90, human p53, and rat p53 are indicated. The proteins migrating at *M*, 40,000–45,000 are probably breakdown products of p53 which have been shown to react with PAb421 in immunoblots (8, 19, 43).

protein was coimmunoprecipitated (Figs. 2*A*, 2*C*, and 3*B*, p90; also see Fig. 4*C*). The fact that two p53-specific antibodies (Ab2, PAb421) directed against distinct epitopes both coimmunoprecipitated this *M*, 90,000 protein and failed to do so in primary REF cells suggests that the *M*, 90,000 protein is in a complex with the human p53 protein in the transformed rat cell lines. An *M*, 85,000–90,000 protein with identical properties, which coimmunoprecipitates with murine p53, has been observed in many different transformed REF or Rat-1 cells overexpressing mutant murine p53.[5] The identity of this *M*, 90,000 protein is presently under investigation.

Mutant Human p53 Has an Extended Half-Life in Transformed Rat Embryo Cells. To further characterize the human p53 proteins produced in transformed REF cells, the half-lives of the mutant or wild-type p53 proteins in these cells were determined. This was carried out by using a pulse-chase protocol as described previously (8). The results of the experiments using the p53-cWT or p53-c143A cDNA clones in transformed cells are presented in Fig. 4. The p53 wild-type human protein had a short half-life of about 20 min in these cells, whereas the p53-c143A mutant protein had an extended half-life of about 90 min (Fig. 4*B*). A longer exposure of Fig. 4*B* demonstrates the associated stable hsc70 protein and the labile associated p90 protein which either turns over rapidly or dissociates from this protein complex with time (Fig. 4*C*).

Table 2 reviews the properties of the human wild-type or mutant p53 proteins produced by the cDNA or cDNA-genomic clones of p53 in the transformed REF cells. It is clear from these studies that each of the human mutant p53 proteins differed from the wild-type p53 in ability to cooperate with *ras* and promote transformation and that wild-type p53 protein is kept at low levels in part by its short half-life. All four human p53 mutants were stable and expressed at higher levels in these cells. The p53-175H and p53-c143A mutants bind to the cellular hsc70 protein, whereas the p53-273H and p53-281G mutants do not bind to hsc70. These latter p53 mutants are in general poorer (2.5–6-fold) at producing transformed foci (Table 2) than the p53-175H mutant.

Second Site Mutations in the Transforming p53 Protein Can Eliminate the Transforming Ability of the Protein. Activating mutations in the p53 gene, produced either *in vitro* or *in vivo*, cluster between amino acid residues 118 and 307 (8, 35). It has been suggested that the mutant p53 protein binds to the wild-type p53 protein in a transformed cell, and that this complex inactivates the wild-type function of p53 (21, 36). This suggests further that a fragment of an activated p53 protein might act in a similar *trans*-dominant fashion to confer a loss of function upon wild-type p53 protein. During the cDNA cloning and polymerase chain reaction amplification of the human p53 mutants from colon carcinomas, a second site mutation was artifactually created in one of the p53-175H clones, creating a double mutant called p53-175Hfs. This mutant has a frameshift mutation at amino acid 328. This produces a protein with 15 missense amino acids (not in p53) for a total of 342 residues. Thus, this protein has a deletion of 65 amino acids from the car-

[5] P. Hinds, unpublished data.

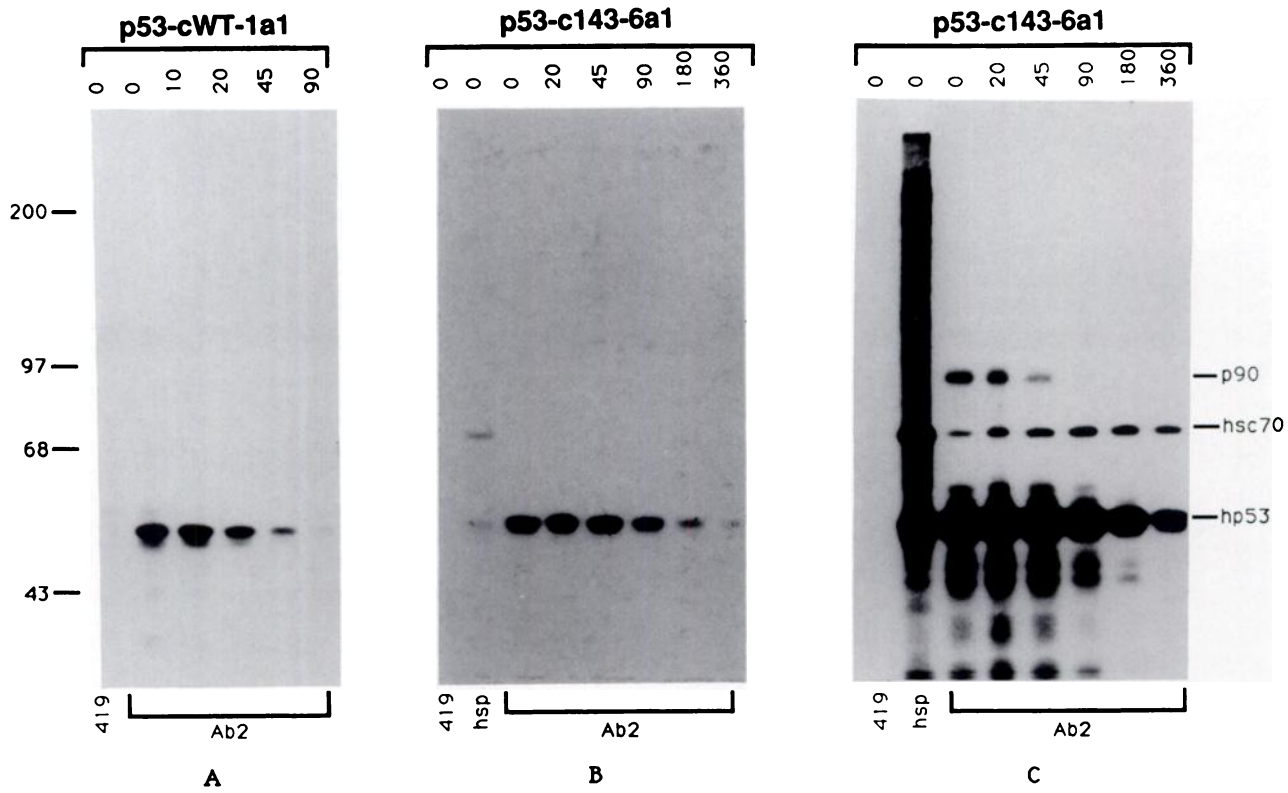

Fig. 4. Determination of p53 protein half-life in p53 plus *ras*-transformed REF. Transformed cell lines p53-cWT-1a1 and p53-c143-6a1 were labeled with [^{35}S]methionine for 1 h and chased in unlabeled media for 0, 10, 20, 45, 90, 180, or 360 min prior to immunoprecipitation with PAb419 (*419*), anti-hsp70 antisera (*hsp*), or Ab2 (see legend to Fig. 2). Equal amounts of acid-precipitable counts were immunoprecipitated, separated on the same polyacrylamide gel, and exposed to film for (*A*) 12 days, (*B*) 16 h, or (*C*) 4 days. Molecular mass markers are indicated in kilodaltons (*A*), and the migrations of human p53 (*hp53*), hsc70, and p90 are shown (*C*).

boxy-terminal end. Because this mutant also carries the residue 175 mutation, it was tested for the ability to transform REF cells with *ras*. When these experiments were carried out, the p53-175Hfs clone failed to cooperate with *ras* and transform REF cells (Table 2). The possibility remained that the p53-175Hfs protein was very unstable and therefore incapable of cooperating in the transformation assay. To examine the properties of the p53-175Hfs protein, a triple transfection of REF cells was undertaken with adenovirus E1A plus *ras* plus p53-175Hfs clones. Transformed foci were readily cloned into cell lines which were tested for expression of the p53-175Hfs protein. The cells were labeled with [^{35}S]methionine, and the p53-175Hfs protein was immunoprecipitated using Ab2, the human specific antibody. This protein was detected (Fig. 5) in these cells and, as expected, migrated more rapidly than the endogenous rat p53. In addition, PAb421 (which recognizes an epitope at amino acids 370–378) failed to detect this protein (Fig. 5). The half-life of the p53-175Hfs protein, when measured in these cells, was approximately 1 h (Table 2), 3 times longer than wild-type but less than the parent protein p53-175H. Interestingly, p53-175Hfs lacks the p53-175H characteristic of binding to the hsc70 protein. In addition,

Table 2 Properties of human p53 mutant proteins

Clone	Mutation (residue)	Relative transformation frequency	$t_{½}$ protein	hsc70 bound	p90 bound	Tumors in nude mice
p53-cWT	Wild-type	0	20 min	–	+	+
p53-c143A	143	1.6	1.5–2 h (3 lines)	+	+	+
p53-WT	Wild-type	0	ND[a]	ND	ND	ND
p53-175H	175	11.5	3.6–6.4 h (2 lines)	+	+	+
p53-273H	273	4.7	7 h (1 line)	–	+	+
p53-281G	281	1.9	1.4 h (1 line)	–	+	ND
p53-175fs	175, fs328	0	1 h[b] (1 line)	–	–	ND

[a] ND, not determined.
[b] Half-life estimated in cell lines expressing *ras* + E1A + mutant p53.

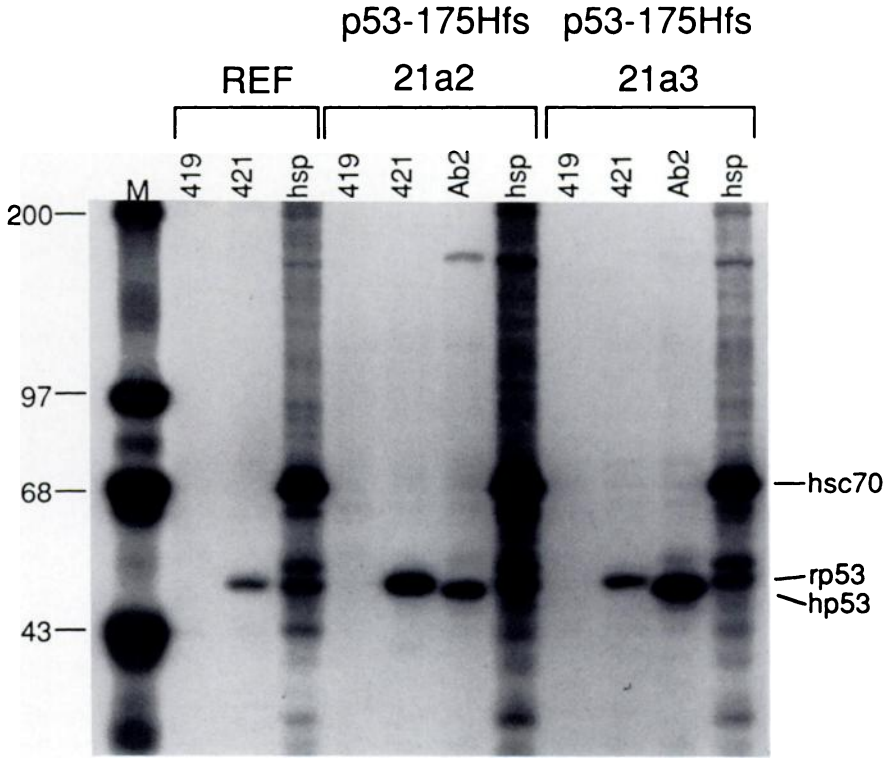

Fig. 5. Expression of p53-175Hfs in transformed REF. Primary rat embryo fibroblasts (*REF*) or the transformed cell lines p53-175Hfs-21a2 or p53-175Hfs-21a3 (transfected with p53-175Hfs plus E1A plus *ras*) were metabolically labeled with [^{35}S]methionine, and equal amounts of labeled protein (2.7×10^6 cpm) were immunoprecipitated with PAb419 (*419*), PAb421 (*421*), Ab2, or anti-hsp70 antisera (*hsp*) (see legend to Fig. 2) and electrophoretically separated. The migrations of molecular mass markers, hsc70, rat p53 (*rp53*), and human p53 (*hp53*) are indicated. The autoradiogram is a 7-day exposure.

no evidence of complex formation was seen between p53-175Hfs and rat p53 or p90. These data suggest that the p53 carboxy-terminus is helpful or even required for stabilizing hsc70 interactions and that this interaction may contribute to the ability of the p53-175H mutant to transform cells in culture.

Discussion

The results presented here demonstrate for the first time that mutant human p53 cDNAs or cDNA-genomic hybrid clones derived from colon carcinomas can behave as dominant oncogenes and cooperate with the *ras* oncogene in transforming rat embryo fibroblasts. Four different missense mutations at amino acid residues 143, 175, 273, and 281 each contributed to the transformed phenotype. In all of these cases, the human p53 mutant protein was produced in high levels in the transformed cell at least in part due to the extended half-lives of these mutant proteins. Seventy-five percent of human colon carcinomas have mutations at the p53 locus, and over 50% of the missense mutations have been localized to the three "hot spots" at or surrounding the amino acid residues represented by the mutants tested in this study (35, 36).[6] These three mutant p53 DNA clones have characteristic and reproducible, but different, transformation frequencies, indicating that these mutations are not equivalent in their phenotypes. Also striking is the fact that p53-175H and p53-c143A mutant proteins bind to hsc70 in the transformed cell, whereas the p53-273H and p53-281G mutant proteins do not detectably interact with or bind to hsc70. Previous experiments have shown that some mutant murine p53 proteins have an altered conformation (7, 8), and it has been suggested that such altered p53 molecules bind to hsc70 and sequester wild-type p53 in a complex that blocks proper folding, assembly, or localization of p53 (45). It is thought that the heat shock 70 family of proteins is involved in protein folding and assembly of protein complexes (quaternary structures) (46). hsc70 has recently been shown to transiently associate with many newly synthesized polypeptides during translation, dissociating upon the proper folding of the proteins. Treatments that result in proteins which cannot be properly folded greatly extend the association of newly synthesized proteins with hsc70 (47). Thus, p53-c143A and p53-175H may represent mutant proteins which never fold correctly and thus retain their affinity for hsc70. If wild-type p53 is recruited into this complex, the p53-mutant-hsc70-p53-wild-type complex would poison the function of wild-type p53. Interestingly, this cannot be the case for the p53-273H and p53-281G mutants. The p53-273H human mutant protein can associate with the rat p53 protein and form an oligomeric complex as demonstrated by coimmunoprecipitation with the human specific Ab2 antibody (Fig. 3).

The p53-175Hfs mutant protein fails to transform and fails to bind to the hsc70 protein even though it is fairly stable (1 h half-life) and produced at elevated levels in *ras* plus E1A plus p53-175Hfs-transformed REF cells. This observation is consistent with a role for the hsc70 protein in transformation by p53-c143A or p53-175H missense mutant proteins; however, the p53-175Hfs mutant protein also does not appear to interact with either the wild-type rat p53 protein or the p90 protein. It is improbable that the domain deleted in p53-175Hfs is directly re-

[6] B. Vogelstein, unpublished observations.

sponsible for interaction with these three proteins, but rather, it is likely that one of the interactions facilitates the others. Experiments designed to elucidate the interdependence of these associations are in progress.

In contrast to transfections with mutant human p53, transfections with activated *ras* plus wild-type human p53 resulted in a very low frequency of focus formation, and only one focus could be cloned into an established cell line. Furthermore, this sole transformed REF cell line expressed a protein with characteristics of endogenous wild-type p53 in that the protein is found at low levels, possesses a short half-life, and does not associate with hsc70. This cell line may be derived from a rare *ras*-transformed cell that fortuitously expresses wild-type human p53. Such *ras*-only transformed cells have been found to arise at a low frequency (48). Thus, it appears likely that mutation of human p53 activates a dominant transforming function that is not detectable in wild-type p53.

In summary, these results clearly demonstrate that all of the human p53 mutant proteins differ from the wild-type human p53 protein by having extended half-lives, by being expressed at higher levels in these cells, and by possessing the ability to transform cells in culture. These data support the suggestion that mutation of p53 on one allele could have a growth-promoting phenotype *in vivo*, which expands the number of cells with such mutations and favors the selection of a second mutational event (deletion or gene conversion) in the cancer cells. The observation that many tumor cells retain only the mutant p53 allele suggests that these mutant proteins are not fully dominant over the wild-type allele, or that an additional proliferative advantage is conferred on cells in the total absence of wild-type p53 (49). Such a positive effect of mutant p53 on cell proliferation in the absence of wild-type p53 could be an intrinsic function of the molecule or could be mediated by titration of cellular proteins other than endogenous p53, for example, via the protein p90.

Materials and Methods

Plasmids and Antibodies. The human p53 DNA sequences were expressed under the control of the human cytomegalovirus promoter-enhancer in a vector derived from plasmid BCMGNeo-mIL2 (50) by excising the human β-globin sequences and bovine papilloma virus sequences with *Bam*HI and *Not*I. In addition, the *Xho*I site was changed to a *Bam*HI site by linker addition. An 1800-bp *Xba*I fragment of wild-type human p53 cDNA (51) was cloned into the *Bam*HI site to create plasmid p53-cWT. Wild-type is defined as the sequence reported by three independent studies and includes a proline/arginine polymorphism at amino acid 72 (51–53). Plasmid p53-cWT codes for proline[72]. Mutant construct p53-c143A was created by insertion of an 1800-bp *Xba*I cDNA fragment, containing the valine[143] to alanine change, derived from a nude mouse xenograft (Cx3) of a primary colorectal carcinoma (36). In addition, the construct codes for arginine at amino acid 72. Intron-containing constructs (p53-WT, p53-175H, p53-175Hfs, p53-273H, and p53-281G) were created by exchanging a 1500-bp *Nco*I genomic fragment, spanning codons 1–160, coding for proline at residue 72, and containing introns 2–4 (42), for the corresponding 475-bp *Nco*I cDNA fragment. The 1300-bp mutant cDNA fragments for p53-175H and p53-281G were derived from xenografts of colon tumors Cx22A, and Cx7A, respectively (35). p53-175H codes for the arginine[175] to histidine mutation. p53-281G codes for the aspartic acid[281] to glycine mutation. p53-175Hfs has an *in vitro* derived deletion at codon 328 (TTC to TC) in addition to the arginine[175] to histidine mutation, and codes for a 342-residue protein, including 15 non-sense carboxy-terminal amino acids. The 1700-bp cDNA fragment for p53-273H, coding for the arginine[273] to histidine mutation, was derived from an epidermoid carcinoma cell line (54).

Plasmid CMVKH215 was constructed by replacing the 1800-bp human cDNA fragment of p53-cWT with a 2300-bp *Bam*HI fragment from plasmid MSVKH215 (8), containing a mutant murine p53 and SV40 polyadenylation site. Bluescript plasmid was purchased from Stratagene; plasmid pT24 expresses the activated Ha-ras protein (55), and plasmid p1A, a gift of T. Shenk (Princeton University), expresses the adenovirus type 5 E1A protein.

The monoclonal antibodies used were PAb419, specific for the large T antigen of SV40 virus (44); PAb421, specific for the carboxy-terminus of many species of p53 including human, mouse, rat, rabbit, and monkey (44); and Ab2, originally called PAb1801 (56), which is amino-terminal, human p53-specific (Oncogene Science, Inc.). The anti-heat shock protein (hsp)70 rabbit antiserum was raised against an 21-amino acid peptide corresponding to the carboxy-terminus of a human M_r 70,000 heat shock protein (7).

Cell Culture and Transfection. All cells were maintained in DMEM supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified, 5% CO_2 atmosphere. Primary rat embryo fibroblasts were prepared from 14–15-day-old Fischer 344 rat embryos. Primary or secondary REF were plated at 3×10^5 cells/10-cm dish. These cells were then transfected by the calcium phosphate procedures of either Graham and van der Eb (57) or Chen and Okayama (58), with comparable results, using DNA concentrations previously described (7). Transformed foci were scored by staining after 14–21 days in culture, and selected foci from parallel transfections were cloned by trypsinization.

Immunoprecipitation and Half-Life Analyses. REF and transformed cell lines were starved for methionine for 1 h and then metabolically labeled for 2 h with 100–200 μCi of [^{35}S]methionine Translabel (ICN) at 50 μCi/ml in methionine-free DMEM containing 2% dialyzed fetal bovine serum. Cells were collected by scraping, washed in phosphate-buffered saline, and stored at −80°C prior to lysis. Total protein cell lysates were prepared and 10^6–10^7 trichloroacetic acid-precipitable counts were subjected to immunoprecipitation as previously described (21). Immunoprecipitated proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels, which were treated with sodium salicylate and dried prior to fluorography, as has been described (8). Half-life analyses were carried out on protein lysates from cells labeled with [^{35}S]methionine for 1 h and chased in a time course to 6 h in DMEM containing excess (2.2 mM) cold methionine. Following immunoprecipitation and electrophoretic separation, labeled p53 protein was released from gel slices and counted as described (8). Half-life values were determined by logarithmic regression analyses.

Tumorigenicity. Established cell lines derived from transformed foci were assayed for tumorigenicity by s.c. injection of $3-5 \times 10^6$ cells/flank into athymic nude mice. Parental REF cells never formed tumors under these conditions, whereas the transformed cells produced palpable tumors within 2 weeks.

Acknowledgments

We thank R. Johnson and A. K. Teresky for technical assistance and K. James for help with the manuscript.

References

1. Oren, M., Maltzmann, W., and Levine, A. J. Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol. Cell. Biol., *1:* 101-110, 1981.

2. Reich, N. C., Oren, M., and Levine, A. J. Two distinct mechanisms regulate the levels of a cellular tumor antigen, p53. Mol. Cell. Biol., *3:* 2143-2150, 1983.

3. Linzer, D. I. H., and Levine, A. J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell, *17:* 43-52, 1979.

4. Crawford, L. V., Pim, D. C., Gurney, E. G., Goodfellow, P., and Taylor-Papadimitriou, J. Detection of a common feature in several human tumor cell lines—a 53,000-dalton protein. Proc. Natl. Acad. Sci. USA, *78:* 41-45, 1981.

5. Dippold, W. G., Jay, G., DeLeo, A. B., Khoury, G., and Old, L. J. p53 transformation-related protein: detection by monoclonal antibody in mouse and human cells. Proc. Natl. Acad. Sci. USA, *78:* 1695-1699, 1981.

6. Lane, D. P., and Crawford, L. V. T antigen is bound to a host protein in SV40-transformed cells. Nature (Lond.), *278:* 261-263, 1979.

7. Hinds, P. W., Finlay, C. A., Frey, A. B., and Levine, A. J. Immunological evidence for the association of p53 with a heat shock protein hsc70, in p53-plus-*ras*-transformed cell lines. Mol. Cell. Biol., *7:* 2863-2869, 1987.

8. Finlay, C. A., Hinds, P. W., Tan, T.-H., Eliyahu, D., Oren, M., and Levine, A. J. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol. Cell. Biol., *8:* 531-539, 1988.

9. Sarnow, P., Ho, Y. S., Williams, J., and Levine, A. J. Adenovirus E1B-58Kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54Kd cellular protein in transformed cells. Cell, *28:* 387-394, 1982.

10. Gronostajski, R. M., Goldberg, A. J., and Pardee, A. B. Energy requirement for degradation of tumor-associated protein p53. Mol. Cell. Biol., *4:* 442-448, 1984.

11. Pinhasi-Kimhi, O., Michalovitz, D., Ben-Zeev, A., and Oren, M. Specific interaction between the p53 cellular tumor antigen and major heat shock proteins. Nature (Lond.), *320:* 182-185, 1986.

12. Ruscetti, S. K., and Scolnick, E. M. Expression of a transformation related protein (p53) in the malignant stage of Friend virus-induced diseases. J. Virol., *46:* 1022-1026, 1983.

13. Pinhasi, O., and Oren, M. Expression of the mouse p53 cellular tumor antigen in monkey cells. Mol. Cell. Biol., *4:* 2180-2186, 1984.

14. Sturzbecher, H-W., Chumakov, P., Welch, W. J., and Jenkins, J. R. Mutant p53 proteins bind hsp72/73 cellular heat shock-related proteins in SV40-transformed monkey cells. Oncogene, *1:* 201-211, 1987.

15. Jenkins, J. R., Rudge, K., and Currie, G. A. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature (Lond.), *312:* 651-654, 1984.

16. Parada, L. F., Land, H., Weinberg, R. A., Wolf, D., and Rotter, V. Cooperation between gene encoding p53 tumor antigen and *ras* in cellular transformation. Nature (Lond.), *312:* 649-651, 1984.

17. Rovinski, B., and Benchimol, S. Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. Oncogene, *2:* 445-452, 1988.

18. Eliyahu, D., Raz, A., Gruss, P., Givol, D., and Oren, M. Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. Nature (Lond.), *312:* 646-649, 1984.

19. Hinds, P. W., Finlay, C. A., and Levine, A. J. Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. J. Virol., *63:* 739-746, 1989.

20. Eliyahu, D., Goldfinger, N., Pinhasi-Kimhi, O., Shaulsky, G., Skurnik, Y., Arai, N., Rotter, V., and Oren, M. Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene, *3:* 313-321, 1988.

21. Finlay, C. A., Hinds, P. W., and Levine, A. J. The p53 proto-oncogene can act as a suppressor of transformation. Cell, *57:* 1083-1093, 1989.

22. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. USA, *86:* 8763-8767, 1989.

23. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. U., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science (Wash. DC), *249:* 912-915, 1990.

24. Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., Vogelstein, B., and Friend, S. H. p53 suppresses the growth of osteosarcoma cells and blocks cell cycle progression. Mol. Cell. Biol., *10:* 5772-5781, 1990.

25. Mercer, W. F., Shields, M. T., Amin, M., Suave, G. J., Appella, E., Ullrich, S. J., and Romano, J. W. Antiproliferative effects of wild-type human p53. J. Cell. Biochem., *14C:* 285, 1990.

26. Mowat, M. A., Cheng, A., Kimura, N., Bernstein, A., and Benchimol, S. Rearrangements of the cellular p53 gene in erythroleukemia cells transformed by Friend virus. Nature (Lond.), *314:* 633-636, 1985.

27. Chow, V., Ben-David, Y., Bernstein, A., Benchimol, S., and Mowat, M. Multistage Friend erythroleukemia: independent origin of tumor cells with normal or rearranged p53 cellular oncogene. J. Virol., *61:* 2777-2781, 1987.

28. Rovinski, B., Munroe, D., Peacock, J., Mowat, M., Bernstein, A., and Benchimol, S. Deletion of 5'-coding sequences of the cellular p53 gene in mouse erythroleukemia: a novel mechanism of oncogene regulation. Mol. Cell. Biol., *7:* 847-853, 1987.

29. Munroe, D. G., Rovinski, B., Bernstein, A., and Benchimol, S. Loss of a highly conserved domain on p53 as a result of gene deletion during Friend virus-induced erythroleukemia. Oncogene, *2:* 621-624, 1988.

30. Ben-David, Y., Prideaux, V. R., Chow, V., Benchimol, S., and Bernstein, A. Inactivation of the p53 oncogene by internal deletions and/or retroviral integration in erythroleukemic cell lines induced by Friend leukemia virus. Oncogene, *3:* 179-185, 1988.

31. Werness, B. A., Levine, A. J., and Howley, P. M. The E6 proteins encoded by human papillomavirus types 16 and 18 can complex p53 *in vitro*. Science (Wash. DC), *248:* 76-79, 1990.

32. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J-Y., Huang, C-H., Lee, W-H., Marsilio, E., Paucha, E., and Livingston, D. M. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell, *54:* 275-283, 1988.

33. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins binds to the retinoblastoma gene product. Nature (Lond.), *334:* 124-129, 1988.

34. Münger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., and Howley, P. M. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J., *8:* 4099-4105, 1989.

35. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. Mutations in the p53 gene occur in diverse tumour types. Nature (Lond.), *342:* 705-708, 1989.

36. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., Bakamura, Y., White, R., and Vogelstein, B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science (Wash. DC), *244:* 217-221, 1989.

37. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. Science (Wash. DC), *246:* 491-494, 1989.

38. Iggo, R., Gatter, K., Barted, J., Lane, D., and Harris, A. L. Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet, *335:* 675-679, 1990.

39. Ahuja, H., Bar-Eli, M., Advani, S. H., Benchimol, S., and Cline, M. J. Alterations in the p53 gene and the clonal evolution of the blast crisis of chronic myelocytic leukemia. Proc. Natl. Acad. Sci. USA, *86:* 6783-6787, 1989.

40. Kelman, Z., Prokocimer, M., Peller, S., Kahn, Y., Rechavi, G., Manor, Y., Cohen, A., and Rotter, V. Rearrangements in the p53 gene in Philadelphia chromosome positive chronic myelogenous leukemia. Blood, *74:* 2318-2324, 1989.

41. Masuda, H., Miller, C., Koeffler, H. P., Battifora, H., and Cline, M. J. Rearrangement of the p53 gene in human osteogenic sarcomas. Proc. Natl. Acad. Sci. USA, *84:* 7716-7719, 1987.

42. Tuck, S. P., and Crawford, L. V. Overexpression of normal human p53 in established fibroblasts leads to their tumorigenic conversion. Oncogene Res., *4:* 81–96, 1989.

43. Tan, T-H., Wallis, J., and Levine, A. J. Identification of the p53 protein domain involved in formation of the simian virus 40 large T antigen-p53 protein complex. J. Virol., *59:* 574–583, 1986.

44. Harlow, E., Craford, L. V., Pim, D. C., and Williamson, N. M. Monoclonal antibodies specific for Simian Virus 40 tumor antigen. J. Virol., *39:* 861–869, 1981.

45. Levine, A. J. The p53 protein and its interactions with the oncogene products of the small DNA tumor viruses. Virology, in press, 1990.

46. Rothman, J. E. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell, *59:* 591–601, 1989.

47. Beckmann, R. P., Mizzen, L. A., and Welch, W. J. Interaction of hsp70 with newly synthesized proteins: implications for protein folding and assembly. Science (Wash. DC), *248:* 850–854, 1990.

48. Spandidos, D. A., and Anderson, M. L. M. A study of mechanisms of carcinogenesis by gene transfer of oncogenes into mammalian cells. Mutat. Res., *185:* 271–291, 1987.

49. Wolf, D., Harris, N., and Rotter, V. Reconstitution of p53 expression in a non-producer Ab-MuLV-transformed cell line by transfection of a functional p53 gene. Cell, *38:* 119–126, 1984.

50. Karasuyama, H., Tohyama, N., and Tada, T. Autocrine growth and tumorigenicity of interleukin 2-dependent helper T-cells transformed with IL-2 gene. J. Exp. Med., *169:* 13–25, 1989.

51. Zakut-Houri, R., Bienz-Tadmour, B., Givol, D., and Oren, M. Human p53 cellular tumor antigen: cDNA sequence and expression in COS cells. EMBO J., *4:* 1251–1255, 1985.

52. Matlashewski, G., Lamb, P., Pim, D., Peacock, J., Crawford, L., and Benchimol, S. Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. EMBO J., *3:* 3257–3262, 1984.

53. Lamb, P., and Crawford, L. Characterization of the human p53 gene. Mol. Cell. Biol., *5:* 1379–1385, 1986.

54. Harlow, E., Williamson, N. M., Ralston, R., Helfman, D. M., and Adams, T. E. Molecular cloning and *in vitro* expression of cDNA clone for human cellular tumor antigen p53. Mol. Cell. Biol., *5:* 1601–1610, 1985.

55. Goldfarb, M., Shimizu, K., Perucho, M., and Wigler, M. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature (Lond.), *296:* 404–409, 1982.

56. Banks, L., Matlashewski, G., and Crawford, L. Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. Eur. J. Biochem., *159:* 529–534, 1986.

57. Graham, F. C., and van der Eb, A. J. A new technique for the assay of infectivity of human adenovirus type 5 DNA. Virology, *52:* 456–467, 1973.

58. Chen, C., and Okayama, H. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol., *7:* 2745–2752, 1987.

59. Soussi, T., Fromental, C. D., Mechali, M., May, P., and Kress, M. Cloning and characterization of a cDNA from *Xenopus laevis* coding for a protein homologous to human and murine p53. Oncogene, *1:* 71–78, 1987.