Mutation Is Required To Activate the p53 Gene for Cooperation with the *ras* Oncogene and Transformation

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Previous experiments have brought into question which amino acid sequence of the p53 oncogene product should be considered wild type and whether the normal protein is capable of cooperating with the *ras* oncogene to transform cells in culture. To address these questions, a series of p53 cDNA-genomic hybrid clones have been compared for the ability to cooperate with the *ras* oncogene in transformation assays. From these experiments, it has become clear that the amino acid alanine at position 135, in either the genomic clone or the cDNA clone, failed to produce a p53 protein that cooperated with the *ras* oncogene and transformed cells. Replacing alanine with valine at this position in either the genomic or the cDNA clone activated for transformation in this assay. Using restriction enzyme polymorphisms in the p53 gene, it was shown that normal mouse DNA encodes alanine at position 135 in the p53 protein. Thus, mutation is required to activate the p53 protein for cooperation with the *ras* oncogene. After cotransfection with the activated *ras* gene, the genomic p53 DNA clone always produced more transformed cell foci (1.7-fold) than similar cDNA clones and these foci were more readily cloned (3.6-fold) into permanent cell lines. A series of deletion mutants of the genomic p53 clone were employed to show that the presence of intron 4 in the p53 gene was sufficient to provide much enhanced clonability of transformed foci from culture dishes. The presence of introns in the p53 gene constructions also resulted in elevated levels of p53 protein in the p53-plus-*ras*-transformed cell lines. Thus, qualitative changes in the p53 protein are required to activate p53 for transformation with the oncogene *ras*. Quantitative improvements of transformation frequencies are associated with the higher expression levels of altered p53 protein that are provided by having one of the p53 introns in the transforming plasmid.

p53 is a cellular protein that forms a stable complex with two different oncogene products produced by DNA tumor viruses, simian virus 40 (SV40) large T antigen and adenovirus type 5 E1B 55-kilodalton protein (14, 15, 26). The p53 in transformed cells containing these complexes is metabolically stable, displaying a half-life of many hours (19, 22). By contrast, in nontransformed cells, the half-life of p53 is 6 to 30 min (19, 22, 23), suggesting that stabilization of p53 correlates with transformation and complex formation with these viral proteins. Elevated levels of stable p53 are also found in many chemically transformed and tumor-derived cell lines (3, 4, 15). It has been proposed that elevation of p53 levels is partially responsible for the altered growth characteristics of these cell lines. Transfection of p53 cDNA clones into primary rodent cells results in immortalization of these cells (13, 25), and p53 can cooperate with the activated *ras* oncogene to transform primary rat embryo fibroblasts (REFs) in culture (6, 8, 20, 25).

Previous reports have clearly demonstrated that a genomic clone of p53 derived from a BALB/c mouse library (contained in LTRcG-val in this report) (6, 8) and several p53 cDNA clones (6, 8) were capable of cooperating with the *ras* oncogene to transform REFs. A p53 cDNA clone (11-4 or MSVcL-ala) isolated from embryonal carcinoma cells, however, (21) failed to cooperate with the *ras* oncogene in this assay (8, 12). Two differences exist between the 11-4 cDNA clone that failed to transform and the LTRcG-val genomic clone that cooperated with the *ras* oncogene. (i) The genomic clone contains introns allowing the possibility that alternate splicing patterns or enhanced expression of mRNAs plays a role in transformation; and (ii) at amino acid position 135, the genomic clone contains a valine codon,

whereas the 11-4 cDNA clone encodes alanine at that position. In addition, every murine and human cDNA of p53 that has been isolated to date, including several from the BALB/c genetic background (1, 13, 30), encodes alanine at amino acid position 135. This raised the possibility that the genomic clone LTRcG-val is a mutant and called into question what nucleotide sequence should be termed the wild-type gene.

To address these questions, several new p53 constructions have been produced. A genomic-cDNA hybrid clone with alanine at position 135 (LTRcG-ala) has been made, which differs from LTRcG-val by only one nucleotide. Second, a cDNA clone with valine at residue 135 (MSVcL-val) has been constructed and compared with MSVcL-ala, which encodes alanine at 135. A cDNA clone (MSV-AB) with just one intron (intron 4) has also been constructed. These DNA clones were transfected with the *ras* oncogene to test their transforming properties. The results clearly show that valine encoded at position 135 in the p53 gene, either in the clone with introns or in the cDNA clone, is required for p53 to cooperate with *ras* to produce transformation in REFs. The presence of introns in these constructions, even one intron, enhanced the frequency of transformation or focus production modestly (1.7-fold) but increased the ability to clone these foci into transformed cell lines by 3.6-fold. The cloned cell lines containing transfected p53 DNA molecules with introns produced higher average steady-state levels of p53 protein than p53-plus-*ras*-transformed cell lines containing p53 cDNAs. Thus, p53 introns, particularly intron 4, provide a quantitative advantage in producing clonable transformed cell foci, and valine is required at amino acid position 135 for transformation with these DNA clones. Alanine at that position, even with introns present, will not transform cells with *ras*.

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The question of whether the alanine or valine codon is present in BALB/c mouse DNA normally (wild type) was addressed by assaying for the existence of a restriction site polymorphism created by the valine or alanine codons. Normal BALB/c (or other murine inbred strains) DNA encodes alanine at position 135 in the p53 protein, demonstrating that the failure to transform with the *ras* oncogene is a property of the wild-type allele. Surprisingly, the original LTRcG genomic clone of p53 (2) is a mutant clone activated for transformation.

These studies permit an assignment of several phenotypes to wild-type p53 and to a variety of mutations that activate p53 proteins for transformation with the *ras* oncogene. Wild-type p53 efficiently associates with the SV40 large T antigen, expresses the PAb246 epitope, and fails to interact strongly with the heat shock protein, hsc70. Several independently derived mutants of p53, which map between amino acids 120 and 230, (i) activate p53 to transform rat embryo cells with the *ras* oncogene, (ii) bind poorly, if at all, with the SV40 large T antigen, (iii) form complexes efficiently with hsc70, and (iv) no longer express the conformation-dependent epitope PAb246. That several diverse mutations across a large region of the p53 gene product all are able to activate the protein for transformation in cooperation with *ras* suggests the distinct possibility that these mutations are inactivating a wild-type p53 function, which then leads to transformation. Such a phenotype would be consistent with the idea that p53 might be a recessive oncogene or anti-oncogene.

MATERIALS AND METHODS

Construction of p53 cDNA-genomic hybrid clones. Plasmids LTRp53cG (here referred to as LTRcG-val), MSVcL (MSVcL-ala), and LTRcL (LTRcL-ala) were used as parent constructs (5, 8). LTRcG-val contains cDNA sequences that encode amino acid residues 1 to 13 and 330 to 390 and 88 base pairs (bp) of p53 3'-noncoding sequence. The remainder of the p53 sequences are of genomic origin. Enhancer and promoter activities are provided by the Harvey murine sarcoma virus (MSV) long terminal repeat (LTR), and a polyadenylation signal is provided by a 260-bp *BclI-BamHI* fragment of SV40. LTRcL was constructed by replacing the genomic *XhoI-SacII*-coding fragment of LTRcG-val with cDNA sequences from an F9 cDNA (21). The enhancer, promoter, and 3' sequences are thus identical to those of LTRcG-val, but LTRcL encodes alanine at residue 135, whereas the genomic sequence encodes valine at this position. MSVcL also contains F9 cDNA sequences but has an additional 199 bp of p53 3'-noncoding sequence and a 610-bp *MboI* fragment containing the SV40 small t splice site before the poly(A) site.

All plasmids were constructed by standard methods (16), using restriction enzyme conditions supplied by the manufacturer. Plasmid LTR-XK was made by replacing the *KpnI-SacII* fragment of the genomic LTRcG-val clone with the corresponding F9 cDNA sequence. Similarly, LTR-KS was made by replacing the genomic *XhoI-KpnI* fragment with the cDNA sequence. These plasmids were used to demonstrate that the transforming activity of the p53 clone was confined to the 5' *XhoI-KpnI* fragment of the BALB/c clone LTRcG. Thus, in this nomenclature, the last two letters of the construct name designate by restriction enzyme site the 5' and 3' boundaries of genomic DNA in the construct (T.-H. Tan, Ph.D. thesis, Princeton University, Princeton, N.J., 1987). The remaining plasmids (LTR-XA, MSV-AK, MSV-

AB, LTR-AP, MSVcL-val, and LTRcG-ala) were constructed by using a similar strategy, as is diagrammed in Fig. 1. Plasmids designated LTR contain 88 bp of p53 3'-noncoding sequence and 260 bp of SV40 sequence; those designated MSV contain 287 bp of p53 3' sequence and 850 bp of SV40 sequence. The AB, AP, MSVcL-val, and LTRcG-ala constructs, as well as MSVcL and LTRcG-val, were analyzed by the chemical sequencing method of Maxam and Gilbert (17) to confirm the presence of the correct codon 135 and to check the integrity of the novel junctions.

To produce murine p53 in COS cells, the plasmids MSVcG-ala/neo and MSVcG-val/neo were constructed. MSVcL-ala was digested with *EagI* and the resultant 5' overhang was filled in by using the Klenow fragment of DNA polymerase. The vector was then digested to completion with *SaII*. This results in the deletion of a 288-bp pBR322 fragment 3' to the SV40 polyadenylation site. An *XhoI-SnaBI* fragment of *dI52-91/G418R* (11) containing the G418R gene driven by the SV40 enhancer-promoter (and origin of replication) was ligated to the linearized p53 vector. The p53 coding sequences were then removed with *XhoI* and *StuI* and replaced with the corresponding genomic sequences from LTRcG-ala or LTRcG-val. The resultant 12.5-kilobase plasmids encode p53 and G418 resistance and contain an SV40 origin of replication.

Vector control plasmids were constructed by digesting constructs containing genomic p53 sequences with *XhoI*. The 5' overhang was filled in with Klenow enzyme and ligated to a 12-bp *EcoRI* linker. The substitution of an *EcoRI* site for the *XhoI* site was diagnostic of the desired frameshift mutation. These constructs shift out of frame at codon 13 and have a series of termination codons 110 bp downstream.

Transfection and establishment of transformed cell lines. Primary REFs were prepared and transfected with activated Ha-*ras* (9) and a p53 plasmid by the calcium phosphate procedure as previously described (8). At 12 to 14 days after transfection, transformed foci were scored and trypsinized in an attempt to establish permanent cell lines. Clonability is defined as the number of morphologically altered foci capable of expansion to permanent cell lines divided by the total number of foci initially cloned. For analysis of transient expression of p53 in REFs, transfection was carried out in an identical manner except that 15-cm-diameter dishes containing 2 to 3 million cells were transfected and harvested 48 h after transfection. Transfection of p53-neo constructs into COS cells was as previously described (27).

Analysis of protein in transformed cell lines. Metabolic labeling with ³⁵S and preparation of cell extracts from transformed lines or from cells transiently expressing p53 was performed as previously described (8). Proteins were immunoprecipitated, separated on 7.5% polyacrylamide, and subjected to fluorography or immunoblot analysis as previously described (8). Relative protein levels in immunoblot analysis were determined by densitometry of appropriately exposed autoradiograms.

Preparation of genomic DNAs and Southern blotting. Genomic DNA was extracted from tissue culture cells by lysis in situ, using 5 ml of lysis buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 5 mM NaCl, 0.5% sodium dodecyl sulfate [SDS], and 0.1 mg of proteinase K per ml) per confluent 10-cm-diameter dish. The lysate was incubated at 37°C for 3 to 18 h. After phenol extraction, the lysate was adjusted to 2 M ammonium acetate, and the nucleic acids were precipitated with 1 volume of cold ethanol. DNA from mouse tails was prepared by the SDS-proteinase K procedure as previously described (18). Restriction enzyme digests were al-

lowed to occur overnight, using conditions recommended by the manufacturer. After agarose gel electrophoresis, DNA was transferred to Biotrans nylon membrane by electroblotting at 30 V for 3 h in TAE (40 mM Tris acetate, 2 mM EDTA) with a Hoefer electroblotter. Membranes were baked under vacuum for 1 h, and the DNA was then cross-linked to the membrane by exposure to UV light for 90 s. Filters were prehybridized for 15 min in Church buffer (7% SDS, 1% bovine serum albumin, 1 mM EDTA, 0.25 M Na_2HPO_4 [pH 7.2]) followed by overnight incubation with 3 million cpm of ^{32}P -labeled probe per ml of Church buffer. After hybridization, filters were washed twice for 30 min each with $2\times$ SSC ($1\times$ is 150 mM NaCl, 15 mM sodium citrate)-0.1% SDS followed by one or two 30-min washes in $0.2\times$ SSC-0.1% SDS. Filters were dried briefly and exposed to Kodak XAR5 at -80°C , with one Kodak intensifying screen.

Preparation of probes. A 205-bp *Mbo*II fragment spanning codons 140 to 208 was prepared by digestion of F9 p53 cDNA with *Apa*LI and *Kpn*II, followed by digestion of the appropriate purified fragment with *Mbo*II. The *Mbo*II fragment was collected by using NA45 paper (Schleicher & Schuell) under conditions supplied by the manufacturer. The purified fragment was labeled with [^{32}P]dCTP ($>3,000$ Ci/mmol; ICN) by using the random hexamer priming method (7) to a specific activity of 1×10^9 to 5×10^9 cpm/ μg .

RESULTS

Activating mutations for transformation with p53. Previous experiments (6, 8, 20, 25) have identified several cDNA and genomic clones of p53 that cooperate with the oncogene *ras* and transform cells and some cDNA clones that fail to cooperate with *ras* and that do not transform REFs. Typically, these plasmid constructions were derived from several different laboratories (1, 2, 13, 21, 25, 30) and differed in their enhancer-promoter usage, the presence or absence of introns, and even amino acid sequence. It was not possible to distinguish among these variables or to determine their relative contributions to qualitative and quantitative effects upon transformation. The differences between these transforming and nontransforming p53 clones led to several questions. What is the wild-type sequence of the p53 gene and product? Does the wild-type product transform cells and cooperate with *ras*? Would overexpression of the wild-type protein result in cellular transformation? To address these questions, a series of DNA clones were made to permit direct comparisons of wild-type or mutant p53 genes or cDNAs in identical constructions. In these experiments, two variables were separated and compared for their contributions to the ability of p53 to cooperate with *ras* and transform cells in culture. (i) The presence of valine or alanine at amino acid position 135 of the p53 protein was examined in comparable cDNA clones or cDNA-genomic hybrid clones containing the p53 gene. (ii) The role of p53 introns in contributing to the quantitative level of p53 protein produced was examined and correlated with the efficiency with which p53-plus-*ras*-transformed cell clones were grown into permanent cell lines.

As a first step, genomic-cDNA hybrid clones that encode either alanine at amino acid 135 (LTRcG-ala) or valine at that position (LTRcG-val) were each transfected into REFs in culture along with the *ras* oncogene clone. Both the number of foci that appeared and the efficiency of cloning these foci are presented in Fig. 1. Clearly, the presence of a valine at position 135 in the protein activates for transformation and

alanine at that position in the same expression vector fails to produce foci with the *ras* oncogene. Similarly (Fig. 1), the p53 cDNA with valine at position 135 (MSVcL-val), but not the same cDNA with alanine (MSVcL-ala), transforms REFs in conjunction with *ras*, producing clonable foci. Thus, valine in position 135 of the p53 protein is sufficient to activate a cDNA. This result, for the first time, eliminates the possibility (8) that the LTRcG-val genomic clone transformed cells with *ras* by using alternative splicing. In addition, it had been noted previously (8) that the genomic clone LTRcG-val cooperated with the *ras* oncogene to produce transformed foci more efficiently. Both the number of foci (1.7-fold) and the efficiency of cloning these foci to produce a permanent cell line (3.6-fold) were higher when introns were present in the p53 clones (Fig. 1). A series of recombinant DNA plasmids composed of a portion of the genomic clone LTRcG-val and the nontransforming cDNA MSVcL-ala (Fig. 1) demonstrated that the enhanced efficiency of cloning transformed foci into permanent cell lines can be mapped to the presence of a single intron (Fig. 1, clone AB), the fourth intron of the p53 gene.

Enhanced efficiency of cloned foci produced by p53 intron-containing plasmids is due to elevated levels of p53 protein. To investigate the mechanisms by which the presence of a p53 intron can increase the efficiency of producing cloned foci (Fig. 1), the possibility that such clones produce higher levels of p53 protein was investigated. The steady-state level of p53 expressed in cloned cell lines derived from foci was determined by Western immunoblot analysis. The murine-specific monoclonal antibody 2C2 (24) was used to immunoprecipitate only the mouse p53 from these transformed rat cells. In addition, PAb246 (29) was used because it has been shown previously that two conformational forms of p53 are present in these cells, PAb246⁺ and PAb246⁻, and that the PAb246⁻ form is closely correlated with transforming activity (8). After immunoprecipitation of p53 with these two antibodies, the proteins were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose paper, and probed with PAb421, a distinct, conformation-independent monoclonal antibody (10). Figure 2 presents the Western blot analysis for four cell lines: cG T33B4, which was made with LTRcG-val (eight introns present) plus *ras*; AB T60-4, which was transformed with clone AB (only intron 4, see Fig. 1) plus *ras*; cLv T647B and cLv T648B, which were transformed by MSVcL-val (no introns) plus *ras*. The same amounts of protein in crude extracts of p53 were used for these immunoprecipitation experiments, and previous experiments (22) have demonstrated that this Western blot procedure is a quantitative assay for the steady-state levels of p53. Clones transformed with LTRcG-val produce large levels of murine p53 (Fig. 2, lane 2), some of which (80%; Fig. 2, lane 3) (8) is PAb246⁺. In contrast, cell lines transformed by the cDNA clone MSVcL-val and *ras* produce much lower steady-state levels of p53 (ranging from 2- to >10 -fold less; representative lines are shown in Fig. 2, lanes 7 to 12). Figure 2 also demonstrates that cell lines derived from a p53 clone containing only intron 4 (AB T60-4; lanes 4 to 6) have the same steady-state levels of p53 as lines transformed by LTRcG-val (with eight introns and *ras*). The cell line shown (AB T60-4) is typical of four of five lines examined; the fifth line contains about 50% of the p53 found on the average in lines transformed by LTRcG-val (all eight introns) plus *ras*. The presence of a p53 intron in the p53 DNA plasmid results in p53-plus-*ras*-derived foci that are cloned into permanent cell lines with an efficiency 360 to 460% greater than that of cells transformed by p53 cDNA

FIG. 1. Diagram of p53 cDNA-genomic hybrid clones. Clones were constructed from LTRcG-val (5), which contains BALB/c genomic p53 sequences, and MSVcL-ala (8), which contains a p53 cDNA derived from F9 cells (21). The methods used to construct the hybrids are described in Materials and Methods. Briefly, the clones are named for the restriction sites bordering the sequences derived from genomic DNA. Thus, clone AK contains genomic sequences from the *Apa*LI site at codon 120 to the *Kpn*I site at codon 215. For clarity, only the boundary restriction sites are shown in XA, AK, AB, AP, and MSVcL-val. val or ala indicate the amino acid encoded by codon 135. The number of foci derived by cotransfection of these constructs with activated *ras* (with the number of experiments in parentheses) and the clonability of those foci (as defined in Materials and Methods) are listed. ND, Not determined; --, not applicable. A, *Apa*LI; B, *Bal*I; K, *Kpn*I; P, *Pvu*II; S, *Sac*II; X, *Xho*I.

constructs (no introns) and *ras* (Fig. 1). The cell lines containing transfected p53 genes with introns express as much as 10-fold-higher levels of p53 (Fig. 2) as their counterparts transformed by p53 cDNAs. The cell lines used for the experiments shown in Fig. 2 all have approximately the same copy number of murine p53 plasmids integrated into the rat cell DNA (10 to 20 copies per cell); therefore, the increased levels of p53 protein in these cell lines are due to the influence of introns upon production, stability, or transport of the p53 mRNA. It is difficult to see how an intron would affect the stability of a protein directly without affecting its primary sequence.

As observed previously with the transformed cell lines containing p53 plasmids with introns (8), cells transformed by p53 cDNA clones (lines cLv T647B and cLv T648B) each produce two forms of p53, PAb246⁺ and PAb246⁻ (Fig. 2). It is now clear that these alternative forms of p53 do not arise from alternative splicing or alterations in primary sequence. Furthermore, the percentage of each of these two distinct p53 configurations in a cell line can vary widely (Fig. 2) (8)

FIG. 2. Immunoblot analysis of p53 proteins in p53-plus-*ras*-transformed cell lines. Lysates of cell lines transformed by activated *ras* and LTRcG-val (cG T33B4), MSV-AB (AB T60-4), or MSVcL-val (cLv T647B and cLv T648B) were subjected to immunoprecipitation with nonimmune tissue culture supernatant (NI) or monoclonal antibodies 2C2 or 246. Precipitated proteins were subjected to immunoblot analysis with PAb421 as described in Materials and Methods. For lines cG T33B4 and AB T60-4, 1 mg of total protein derived from 4 to 5 million cells was subjected to immunoprecipitation. p53 proteins were immunoprecipitated from 2 mg of total protein derived from 7 to 10 million cells for lines cLv T647B and cLv T648B. Murine p53 is indicated (arrow).

FIG. 3. Southern blot of BALB/c genomic DNA. High-molecular-weight DNA from the LTRcG-val-plus-ras-transformed cell line cG T33B4 or from four individual BALB/c mice (B/C1, B/C2, B/C3, B/C4) was digested to completion with *HphI*. A partial *HphI* restriction map of the p53 gene is shown at the top. Distances between *HphI* cut sites (H) are indicated. Hv represents an *HphI* site present in p53 clones encoding valine at residue 135 but not in clones encoding alanine. An *MboII* cDNA fragment used as a probe (▨) will hybridize to a 250-bp fragment common to genes encoding either alanine or valine and to a 142-bp fragment in valine-encoding genes. In the absence of a valine codon at residue 135, the probe will hybridize to a 642-bp fragment. The results of a Southern blot using the *MboII* probe are shown at the bottom; fragment sizes are indicated in base pairs. The 450-bp band is derived from the p53 pseudogene (30).

and must be an inherent characteristic of the cell line and the cellular environment.

Finally, the demonstration that introns in a p53 plasmid produced a cell line derived from p53-plus-ras cooperation that contains higher levels of p53 than similar cell lines expressing p53 from genes without introns has been observed in a large number (>20) of such cell lines analyzed over the past 2 years. The examples shown in Fig. 2 are representative of these results.

Val-135 is not encoded in the BALB/c mouse genome. The amino acid substitution which results in the different transforming abilities of the BALB/c genomic clone (LTRcG-val) and the F9 cDNA clone (MSVcL-ala) may be due to a polymorphism in the genomic BALB/c DNA used to derive these clones. Alternatively, this difference may be due to a mutation in one of these two clones. To determine if the valine codon is present in BALB/c genomic DNA from a normal mouse, we took advantage of the fact that the presence of the valine codon results in a recognition site for the restriction enzyme *HphI*. This site is missing in genes which encode alanine at residue 135. Genomic DNA from a cell line transformed by LTRcG-val plus *ras* (cG T33B4) or from four individual BALB/c mice was digested with *HphI* and transferred to nylon membrane. As diagrammed in Fig. 3, a cDNA probe homologous to sequences 1 to 205 bp 3' to the *HphI* site in question would hybridize to fragments of 640 and 250 bp if the *HphI* site was not present. In the presence of Val-135, the 640-bp fragment is cleaved to a 500- and a 140-bp fragment, only the latter of which will hybridize to the probe. Thus, the presence of a 140-bp *HphI* fragment and the absence of the 640-bp fragment is diagnostic of Val-135. Figure 3 shows that although the 140-bp fragment is present

in the cell line cG T33B4, containing LTRcG-val as expected (lane 1), there is no evidence of this fragment in BALB/c genomic DNA. Further, although the 640-bp fragment (no *HphI* site) is clearly present in BALB/c DNA, this fragment is absent from the control digest containing LTRcG-val-transformed cell DNA (lane 1). Additional experiments have also failed to detect the Val-135 codon in the genomic DNAs of two additional BALB/c mice, two DBA2/J $\times$ C57BL/6/J mice, or in F9 cells which come from 129J mice. Finally, the genomic DNA used to make the library from which LTRcG-val was cloned has been analyzed by *HphI* digestion and also shows no evidence of the presence of the Val-135 codon (M. Oren, personal communication). Therefore, the sequence which encodes Val-135 must have arisen as a mutation, either in individual cells used to make the library from which LTRcG-val was cloned or as an artifact of cloning.

The absence of the codon encoding Val-135 in genomic DNAs from several mice suggests that Ala-135 represents the wild-type codon. This finding is consistent with previous empirical arguments used to support the contention that the F9 cDNA best represents wild-type p53 (8). We thus conclude that wild-type p53 is a protein of 390 amino acids with alanine at position 135. Phenotypically, wild-type p53 therefore binds to all murine-specific monoclonal antibodies, including PAb246, binds to SV40 large T antigen, does not bind to hsc70, and fails to transform primary rat cells in cooperation with activated *ras* (8, 27).

Wild-type and mutant p53s produce different steady-state levels of protein in transiently transfected REFs. In addition to a requirement for mutation to activate p53 for transformation, high levels of protein are required for efficient cloning and establishment of p53-plus-ras-transformed cell lines (see Fig. 2). The high levels of p53 protein in transformed cells are due, in part, to increased metabolic stability of p53 protein itself in transformed lines (8). Therefore, a mutation like Ala to Val at position 135 in the protein may be required to stabilize p53 sufficiently to allow an increase in the steady-state level of a functionally wild-type protein. Alternatively, transformation may result from mutations which alter the biological activity of p53. In this case, high steady-state levels may be due to secondary causes such as juxtaposition to a strong enhancer-promoter (MSV LTR) which may, in turn, override the cellular machinery responsible for the short half-life of p53. To assess the role of the Ala-to-Val mutation in altering the properties of transforming p53 protein, the LTRcG-val and LTRcG-ala DNA clones were transfected into primary REF cultures by using the calcium phosphate precipitation technique. The steady-state levels of murine-specific p53 protein were determined at 48 h after transfection by Western blot analysis after immunoprecipitation of p53 by the 2C2 murine-specific antibody. Figure 4 presents the results of this experiment. The LTRcG-val DNA clone produces readily detectable levels of p53 (Fig. 4, lane 4), but no detectable p53 is produced from the LTRcG-ala clone (lane 2). The Ala-to-Val mutation, therefore, appears to be important in elevating the level of p53 in transfected cells.

The absence of a detectable p53 protein in the wild-type LTRcG-ala-transfected cell (Fig. 4) requires that alternate methodology be used to prove that this clone can synthesize a normal p53 protein. To demonstrate this, an SV40 origin of replication was inserted into MSVcG-ala, MSVcG-val, and into two additional clones, MSVcG-fs-ala and MSVcG-fs-val, which are frameshift mutations that destroy the production of these p53 proteins. These four constructions were then used to transfect COS 7 cells which express the

FIG. 4. Immunoblot analysis of p53 protein produced in transient transfection of REFs. Two to three million REFs were transfected with the genomic p53 constructs LTRcG-ala (cG-ala), LTRcG-val (cG-val), LTRfscG-ala (fs-ala), or LTRfscG-val (fs-val) as described in Materials and Methods. The latter constructs introduced a frameshift at amino acid 13 and terminated shortly thereafter. At 48 h after transfection, cells were harvested and lysed. Half of each extract was subjected to immunoprecipitation with nonimmune monoclonal supernatant (NI); the other half was immunoprecipitated with anti-murine p53 RA32C2 (2C2). The immunoprecipitated proteins were prepared for immunoblotting with PAb421 as described in Materials and Methods.

SV40 large T antigen. The SV40 large T antigen replicates these p53 DNA clones to high copy number in COS 7 cells, and when p53 protein is produced it will bind to the SV40 large T antigen. This complex formation is thought to stabilize p53 (15, 19). These four plasmids were transfected into COS 7 cells, and the cells were labeled for 2 h with [35 S]methionine at 48 h after transfection. The soluble cell protein extracts were incubated with a series of p53 monoclonal antibodies (PAb421, PAb242 [29], PAb246, PAb248 [29]) which recognize p53 epitopes at four distinct locations across the protein (28). In addition, PAb419, an SV40 large T antigen-specific monoclonal antibody, was used to examine the association of SV40 T antigen with p53. The autoradiograms that demonstrate the expression of p53 protein in COS 7 cells transfected with MSVcG-val or MSVcG-ala DNA clones are presented in Fig. 5. PAb242, PAb246, and PAb248 recognize only the murine p53 protein made in these monkey cells and detect both the Val and Ala forms of this protein. The wild-type p53 protein (Ala) and, to a lesser extent, the mutant p53 protein (Val) bind to the SV40 large T antigen as evidenced by its coimmunoprecipitation (Fig. 5). PAb241 recognized both the monkey (upper band) and mouse p53 protein from mutant and wild-type p53 and coimmunoprecipitated the SV40 large T antigen. PAb419 immunoprecipitated the SV40 large T antigen and monkey and mouse p53

FIG. 5. Immunoprecipitation of murine p53 from transiently transfected COS 7 cells. Two to three million COS 7 cells in a 15-cm-diameter dish were transfected with MSVcG-ala/neo, MSVcG-val/neo, MSVfscG-ala/neo or MSVfscG-val/neo. These constructs contain genomic p53 genes and frameshifts as described in the legend to Fig. 4. At 48 h after transfection, the cells were labeled with [35 S]methionine and lysed. Aliquots containing 10^7 trichloroacetic acid-precipitable counts were subjected to immunoprecipitation with control nonimmune hybridoma supernatant (NI), anti-SV40 large T antigen monoclonal antibody PAb419 (419), anti-p53 monoclonal antibody PAb421 (421), or anti-murine specific monoclonal antibodies PAb242 (242), PAb246 (246), and PAb248 (248). The immunoprecipitated proteins were separated on 7.5% polyacrylamide and fluorographed with 1 M sodium salicylate-5% glycerol. The positions of SV40 large T antigen (T Ag), monkey p53 (p53 mk), and murine p53 (p53 ms) are indicated. M, Molecular mass markers (in kilodaltons).

proteins. The frameshift mutations placed in the valine (MSVfscG-val)- or alanine (MSVfscG-ala)-containing DNA clones failed to produce detectable p53 proteins in this assay. Thus, the association of p53 protein with the SV40 large T antigen in monkey cells abrogates the instability of the wild-type MSVcG-ala-produced protein seen in REFs. These data suggest that the Ala-to-Val mutation at position 135 in the protein is directly responsible for the stabilization of p53 seen in these transformed rat cell lines. This result and conclusion does not eliminate the possibility that the Ala-to-Val mutation also alters the biological activity of the p53 protein, as opposed to solely stabilizing a functional entity.

DISCUSSION

In a series of experiments carried out in several laboratories over the past 4 years (6, 8, 13, 20, 24), a p53 genomic clone from a BALB/c liver DNA library and many different independently derived p53 cDNAs were all shown to cooperate with the *ras* oncogene and to transform rat cells in culture. One p53 cDNA clone (11-4 from embryonal carcinoma cells of 129/J mice) (21) failed to cooperate with *ras* and transform REFs in culture (8, 12). Because of the use of different enhancer-promoters, constructions of cDNAs, the presence or absence of introns in these constructions, and even amino acid sequence differences between clones, the interpretation of these diverse results remained speculative. To eliminate these variables, two distinct differences between the genomic clone LTRcG-val that transformed rat cells and a cDNA clone (MSVcL-ala) that failed to cooperate with *ras* to transform rat cells were examined in identical constructions. First, cDNA clones with a single amino acid difference, valine or alanine in position 135 of the protein, were compared. The DNA clone encoding valine at that position transformed primary REFs with *ras*, the alanine containing DNA clone did not. Second, the role of the presence of introns in these clones was examined. It was not necessary to have introns in these cDNA clones to transform cells in culture, but the presence of introns in a clone containing valine at position 135 enhanced the number of foci modestly (1.7-fold) and dramatically increased the efficiency of cloning foci to produce permanent cell lines (3.7-fold). Clearly then, a mutation at amino acid 135 in the protein has either activated or deactivated this clone for transformation. The presence of an intron, and intron 4 of the p53 gene in particular, increased the efficiency of transformation. That intron 4 from the p53 gene is somehow special in this process can be seen by comparing the use of the SV40 small t antigen splice in construction MSVcL-val versus the addition of intron 4 in clone MSV-AB (Fig. 1). Transformed rat cell lines produced with MSV-AB-plus-*ras* are cloned 3.7-fold more efficiently than are MSVcL-val-plus-*ras* cells. Furthermore, transformed rat cell lines containing p53 clones with introns (or only intron 4) express up to 10-fold more p53 protein than do rat lines transformed with p53 cDNA clones without introns (Fig. 2). Clearly, intron 4 of the p53 gene can have a dramatic effect on the level of expression of p53 protein, and one might expect that this would be due to either an enhanced rate of transcription or an increased efficiency of transport or stability of the p53 mRNA in these cells. Correlated with this is the biological property of efficient cloning of a focus to produce a permanent cell line.

We have previously argued that the valine in position 135 of the LTRcG-val genomic clone is caused by a mutation because every murine or human cDNA clone isolated to date

has alanine at that position (8). The experiments presented here prove that assertion by demonstrating that the codon for valine is not present in DNA from BALB/c mice, DBA mice, or 129 mice. Indeed, analysis of the DNA in the library from which the original p53 genomic clone was obtained also suggests that the valine codon in position 135 is the product of a mutation and does not exist in the mouse genome (M. Oren, personal communication). Thus, it is now clear that the wild-type sequence has alanine at position 135 in the p53 protein. All p53 cDNAs that cooperate with *ras* and transform REFs in culture have mutations that cluster between amino acids 120 and 230 (out of 390 amino acids) in this protein (6, 8). The cDNA that fails to transform rat cells with the *ras* oncogene, 11-4 or MSVcL-ala, is identical to the BALB/c genomic clone in every amino acid except the valine-to-alanine change discussed here. The sequence of amino acids encoded by the 11-4 cDNA is the only primary sequence for murine p53 that has been isolated twice from two different sources, i.e., F9 embryonal carcinoma cells of 129 mice (21) and normal T cells of BALB/c mice (1). These two cDNA clones fail to cooperate with *ras* to transform cells in culture (8, 12; M. Oren, personal communication). All other cDNAs isolated to date have an altered protein sequence and transform cells in cooperation with *ras* (6, 8, 13). Finally, the p53 protein produced by cDNA clone 11-4 behaves most like the murine wild-type p53 protein from mouse cells. The 11-4 p53 binds tightly and efficiently with the SV40 large T antigen and does not bind detectably with the hsc70 protein. This 11-4 p53 is efficiently recognized by the PAb246 monoclonal antibody (PAb246⁺ conformation) (27). Mutants of 11-4 that cooperate with *ras* (8) or other transforming cDNAs isolated independently in several laboratories (1, 6) produce proteins that bind to the SV40 large T antigen poorly or not at all, prefer to bind to hsc70 (8, 12, 27), and express exclusively or at a significant level the PAb246⁻ conformation (fail to bind PAb246) (8, 12, 27). On the basis of the work presented in this report, it is clear that the wild-type p53 protein cannot cooperate with *ras* to transform REFs even when the wild-type p53 is expressed with the same enhancer-promoter in a construction with p53 introns present. This optimal construction for p53 mRNA still produces a wild-type protein in REFs that is at a very low, indeed undetectable, level. The p53 wild-type protein made from the same DNA clone binds to SV40 T antigen, presumably resulting in metabolic stability and easy detection of the transfected protein in COS 7 cells. The alanine-to-valine mutation at position 135 thus appears to stabilize the p53 protein in rat cells.

Although higher levels of mutant p53 are achieved by the Ala-to-Val-activating mutation, this finding does not mean that the biological function of p53-valine mutants is not also altered. Indeed, a surprising number of different mutational events in the p53 protein, between amino acids 120 and 230 in the protein, activate p53 for cooperation with *ras* (6, 8). While it is not clear whether each of these mutations stabilizes the protein, this observation suggests the possibility that the mutant p53 protein needs to be inactivated for some function and that a number of different mutations can do this. The inactivated p53 protein could then act in a *trans*-dominant fashion in these rat cells to alter the function of wild-type rat p53. One of the predictions of this hypothesis is that the mutant mouse p53 would act in a complex containing the rat p53 protein. This, in fact, has been observed (25; M. Oren, personal communication). This model also predicts that the loss of wild-type p53 in some transformed cells would favor a transformation event. That

is precisely the interpretation given to recent experiments studying erythroleukemias in mice (25). If this is the case, the wild-type p53 protein expressed in some cell lines (permanent or transformed cell lines) would be antithetical to the growth of cells in culture. Cells transfected with wild-type p53 would fail to grow or would die. Indeed, experiments designed to test this idea have shown that the wild-type murine p53, in permanent rat cell lines, but not the mutant p53 cDNA-genomic hybrid clones are selected against in their expression of p53 or the retention of this gene in growing cell lines. If this idea proves to be correct, p53 would more properly join the ranks of the recessive oncogenes. The SV40 large T antigen would then bind to p53 to eliminate its functioning in SV40-transformed cells and not to increase its levels and enhance its function. Clearly, only additional experimentation can sort out these concepts and indicate the correct answer.

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