Excess Wild-Type p53 Blocks Initiation and Maintenance of Simian Virus 40 Transformation

KENJI FUKASAWA, GEORGE SAKOULAS, ROBERT E. POLLACK,* AND SUZIE CHEN

Department of Biological Sciences, Columbia University, New York, New York 10027

Wild-type (wt) murine p53 has been tested for its ability to block and reverse the transforming effects of simian virus 40 (SV40) large T antigen. Established and precrisis mouse cells overexpressing exogenously introduced wt p53 became resistant to SV40 transformation. The introduction of excess wt p53 into SV40-transformed precrisis cells reverted their transformed phenotype. However, the phenotype of SV40-transformed established cells was not reverted by excess wt p53. We conclude that an antioncogenic action of wt p53 is exerted during SV40 transformation and that in precrisis cells, the antitransforming action of wt p53 can be exerted both at initiation and during the maintenance of transformation.

The cellular phosphoprotein p53 was initially identified by its association with large T antigen in simian virus 40 (SV40)-transformed cells (18, 20). Subsequently, the transforming proteins of other DNA tumor viruses, such as the E1b 55-kDa protein of adenovirus (31) and the E6 protein of papillomavirus (38), were shown to form complexes with p53 as well. Early studies classified a genomic clone of p53 derived from normal mouse liver as a cellular oncogene because of the ability of this clone to cooperate with activated Ha-*ras* in inducing transformation of early-passage rodent cells (10, 16, 26), to enhance SV40 transformation efficiency (24), and to immortalize primary cells (15, 30). However, comparison with other clones of the murine p53 gene isolated from other normal cells showed that the oncogenic clone was a mutant with a base substitution that resulted in valine instead of alanine at amino acid position 135 (12). These comparisons also showed that the oncogenic potential of the mutated form was not shared by wild-type (wt) p53. Rather, exogenous wt p53 was found to have antitransforming activity, inhibiting transformation when cotransfected with *ras* plus E1A (12), *ras* plus *myc*, or E1A plus E1B (8). Consistent with its antitransforming role in transfection studies, wt p53 was found to be reduced or absent in a wide variety of naturally occurring human tumors, usually replaced by one or another mutant (mt) p53 allele. In particular, the gene for human p53 is located on the short arm of chromosome 17, a frequent site of allele loss in common cancers. Rearrangements and deletions in the p53 gene were found to occur frequently in human osteogenic sarcomas (21), in colon cancer (1, 25), in lung cancer (34), and in breast cancer (2).

We are interested in understanding how SV40 initiates and maintains the transformed phenotype. Results from studies on wt p53 transfection and on mutations of p53 genes commonly found in human tumors have converged to provide a testable hypothesis for the role of the p53-SV40-T antigen complex in the induction and maintenance of the transformed phenotype: that is, the complex inactivates wt p53 and thereby permits the transformed phenotype to be expressed. This hypothesis is supported by a report showing that certain mutant large T antigens, which are unable to complex with p53, have much lower transforming effi-

ciencies in rodent cells than in wild-type cells (27). We have tested those mutants for their transformation efficiency in Swiss 3T3 and C57L precrisis cells by low-serum screening. We observed that the transforming efficiencies of those mutants were more than 100-fold lower than those of the wild-type large T antigen in Swiss 3T3 cells and that they were unable to transform precrisis cells (12a). Taken together, these results suggest that the complex formation between large T antigen and p53 enhances the transforming efficiency of large T antigen. This hypothesis makes predictions that can be tested in cells carrying an excess amount of wt p53. With regard to initiation, it predicts that incoming SV40 T antigen during infection will not be able to titrate all of the excess wt p53 proteins produced by both endogenous and mostly exogenously added wt p53 genes, which should result in a reduced frequency of transformation by SV40. With regard to maintenance, introducing an exogenous wt p53 gene into an SV40-transformed cell should revert its descendants to a more normal phenotype in some or all of the parameters of transformation. We have tested these predictions. We find that mouse cell lines overexpressing wt p53 are significantly more resistant to SV40 transformation than are control Swiss 3T3 and C57L precrisis cells. Furthermore, we find that SV40 T antigen-transformed cell lines derived from C57L precrisis cells are considerably reverted by the expression of added wt p53 sequences. Reversion by excess wt p53 was limited to precrisis cells; the SV40 T antigen-transformed cell line derived from Swiss 3T3 cells failed to revert upon the introduction of wt p53. From these results, we confirm that an antitransforming function of wt p53 reported previously for other oncogenes also acts to interfere with SV40 large T antigen. Reversion can be exerted during either initiation or maintenance for precrisis cells but only during initiation for Swiss 3T3 cells.

MATERIALS AND METHODS

Cells. To isolate precrisis mouse fibroblast cells, an 8-week-old male C57L mouse (Jackson Laboratory, Bar Harbor, Maine) was sacrificed and a piece of dermis was removed from the abdomen. Tissue was washed in Dulbecco's modified Eagle medium (DMEM) and minced with 0.2 ml of trypsin in a 60-mm dish. A thin glass plate was placed over the tissues to enhance their attachment to the dish, and DMEM supplemented with 10% fetal calf serum (FCS;

---

* Corresponding author.

GIBCO) was overlaid. About 4 days later, when cells of fibroblastic morphology started to appear from the tissue, the glass plate and the tissues were removed. All of the adherent cells were cultured thereafter in DMEM–10% FCS supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). This medium is referred to as complete medium. Cultures were passaged according to the 3T3 protocol (35). Cultures were trypsinized and replated at 7.5 × 10⁵/100-mm dish every 3 days. This maintains cultures at a subconfluent density at all times. Precrisis cells used in this study were either passage 3 or passage 4 cells. All cells were grown in complete medium in an atmosphere containing 10% CO_2.

Plasmids. LTRp53cG (here referred to as pLTRcG-val) is a murine p53 cDNA genomic hybrid clone (9) that contains cDNA sequences encoding amino acid residues 1 to 13 and 330 to 390 and 88 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 long terminal repeat, and a polyadenylation signal is provided by a 260-bp *BclI-BamHI* fragment of SV40. LTRcG-ala, which encodes murine wt p53, is identical to pLTRcG-val except that there is an alanine instead of a valine at amino acid 135 (14). Plasmid pSVRI used for the transformation assay contains an entire wild-type SV40 DNA at the *EcoRI* site of pBR322 (6). Plasmid pSVlacO, used to generate the transformants to be tested for reversion, contains only an intact SV40 early region (4).

DNA transfection and selection. The calcium phosphate transfection procedure described previously (13) was used. For isolation of clones overexpressing either wt p53 or mt p53, Swiss 3T3 and C57L precrisis (passage 3) cells were plated 24 h before transfection at 8 × 10⁵ cells per 100-mm dish. Fresh medium was given 4 h before the addition of DNA. Cells were transfected with 20 µg of pLTRcG-ala (wt p53) or pLTRcG-val (mt p53) and 2 µg of a plasmid containing the neomycin resistance gene (pKoNeo) as the selectable marker (33). After 16 h, cells were washed with fresh DMEM and incubated in fresh complete medium. Forty-eight hours after transfection, cells were washed, trypsinized, split in a 1:3 ratio, and plated into selection medium consisting of complete medium supplemented with G418 (GIBCO) at 400 µg/ml for Swiss 3T3 cells or 300 µg/ml for C57L precrisis cells. Cells were maintained in selective medium, with changes of medium twice weekly.

For isolation of SV40-transformed C35SVB and 3T3SVB lines, Swiss 3T3 and C57L precrisis (passage 3) cells were transfected with 20 µg of pSVlacO. After 16 h, cells were washed with fresh DMEM and incubated in complete medium. Forty-eight hours after transfection, cells were washed, trypsinized, split in a 1:3 ratio, and plated into DMEM supplemented with 1% FCS. Four weeks later, dense foci were cloned. Transfection of pLTRcG-ala into C35SVB and 3T3SVB lines was carried out by the protocol used to isolate clones overexpressing wt p53. The selection medium used contained 400 µg of G418 per ml for both C35SVB and 3T3SVB lines. Three weeks after transfection, neomycin-resistant colonies were cloned.

Focus formation assay. Cells were plated at a density of 8 × 10⁵ cells per 100-mm dish 24 h prior to transfection. The cells were fed with fresh complete medium 4 h before transfection. The cells were transfected with 20 µg of pSVRI. After incubation for 12 h at 37°C, the cells were washed twice with medium and fed with fresh complete medium. The next day, the cells were trypsinized and replated at a density of 10⁵ cells per 60-mm dish. Medium

was changed to DMEM with 1% FCS 24 h later. The cells were fed twice a week and examined for the appearance of transformed foci 4 weeks later.

Immunoblotting. Subconfluent cell cultures were washed twice in phosphate-buffered saline (PBS) and then lysed in lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1 mM EDTA, 1% Nonidet p-40 [NP-40], 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The cell extract was cleared by centrifugation for 30 min at 100,000 × g at 4°C. The lysate containing ≈700 µg of total protein was denatured at 95°C for 5 min in sample buffer (2% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 60 mM Tris [pH 6.8], 0.01% bromophenol blue) and subjected to SDS-polyacrylamide gel electrophoresis. Fractionated proteins were then transferred to nitrocellulose sheets (Schleicher & Schuell). After transfer, blots were stained with 0.2% Ponceau S (Sigma) to visualize total protein transferred to the sheets. Blots were incubated in blocking buffer (5% [wt/vol] nonfat dry milk–0.1% Tween 20 in Tris-buffered saline [TBS]) for 2 h at room temperature and then with anti-p53 monoclonal antibody PAb421 for 2 h at room temperature. The blots were then rinsed in TBS and incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; Boehringer Mannheim) for 1 h at room temperature. The blots were then rinsed in TBS, and the antibody-antigen complex was visualized with bromochioroindolyl phosphate and nitroblue tetrazolium as substrates in 100 mM NaCl–5 mM MgCl₂–100 mM Tris (pH 9.5).

Immunoprecipitation. (i) Immunoprecipitation of metabolically labeled p53. Subconfluent monolayers of cells in 60-mm dishes (≈8 × 10⁵ cells) were washed three times with methionine-free DMEM and then labeled for 4 h in methionine-free DMEM containing 100 µCi of [³⁵S]methionine-cysteine (Tran³⁵S-label; ICN Biomedical) and 5% dialyzed FCS. Cells were washed three times with cold PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The cell extract was cleared by centrifugation for 30 min at 100,000 × g at 4°C. The pellet was discarded. The lysate was then precleared by incubation with normal rabbit serum, and nonspecific immune complexes were immunoprecipitated with *Staphylococcus aureus*. After preclearing, the lysates containing equal amounts of trichloroacetic acid-insoluble radioactivity were subjected to immunoprecipitation. After the addition of secondary antibodies (rabbit anti-mouse IgG [Sigma]), the immune complex was collected by incubation with *S. aureus*. The precipitates were washed three times in wash buffer (0.5 M NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% NP-40, 0.25% gelatin). The bound proteins were eluted by boiling for 5 min in 50 µl of sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The gels were treated with En³Hance (NEN, Dupont) and autoradiographed at −70°C on Kodak XAR-5 film.

(ii) Immunoprecipitation of wt p53 and immunoblotting for detection. Subconfluent monolayers of cells in 100-mm dishes were washed twice in cold PBS and then lysed in lysis buffer for 30 min on ice. The lysates were cleared by centrifugation for 30 min at 100,000 × g at 4°C. The lysates were then precleared as described above. The lysates containing 1.5 mg of total proteins were incubated with anti-p53 monoclonal antibody PAb246 overnight on rotating wheel at 4°C. The immune complex was collected as described above and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose sheets and immunoblotted with PAb421 as described above.

PCR amplification of p53 cDNA and sequencing. Total cellular RNA was extracted in the presence of guanidine isothiocyanate (7). Poly(A)$^+$ RNA was isolated from total RNA by messenger affinity paper (Hybond-mAP; Amersham) by the method suggested by the suppliers. Two micrograms of poly(A)$^+$ RNA was diluted into the final volume of 25 μl with the cDNA reaction mixture (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM MgCl$_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 4 mM sodium pyrophosphate, 1 mM each deoxynucleoside triphosphate, 25 U of RNase inhibitor, 1 μg of oligo(dT) primer [Promega], 30 U of avian myeloblastosis virus reverse transcriptase [Promega]). The reaction mixture was incubated at 42°C for 1 h. Ten microliters of the cDNA reaction mixture was amplified by the polymerase chain reaction (PCR), using a pair of primers. Pair A consisted of 5'-CAGGGTGTCACGCTTCTC CGAA-3' (bases −28 to −6 of mouse p53 cDNA) and 5'-TCAGCCCTGAAGTCATAAG-3' (complementary to the coding strand from +1223 to +1242). This primer pair covers an entire coding region of mouse p53 cDNA, including some 5' and 3' noncoding regions. Pair B consisted of 5'-ACT TACCAGGGCAACTATGGC-3' (+295 to +316) and 5'-AAAGCTGTCCCGTCCCAGAAG-3' (complementary to the coding strand from +781 to +802). The final volume for PCR was expanded to 100 μl with the PCR reaction mixture (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% gelatin, 100 pmol of each primer, 200 μM each deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase [Perkin Elmer Cetus]). After addition of mineral oil, the cDNA preparations underwent 30 PCR cycles (94°C, 1 min; 55°C, 2 min; 72°C, 3 min) linked to a final 7-min incubation at 72°C. The PCR products were run on a 1.0% agarose gel, and the corresponding bands were electroeluted. Depending on the recovery of amplified p53 cDNA, the electroeluted DNA was either directly sequenced or subjected to a further amplification for 30 cycles with a single primer (asymmetric PCR) to generate a single-stranded DNA sequencing template. In the latter case, reamplified cDNA was purified by an anion-exchange column (PCR purification kit; Qiagen) to remove all of the unincorporated deoxynucleoside triphosphates and primers. Sequencing was performed by the dideoxy method, using ^{32}P-end-labeled primers (primer pair B) and the TaqTrack sequencing system (Promega). Sequencing products were analyzed by autoradiography of denaturing 6% polyacrylamide gels.

Southern blot analysis. High-molecular-weight DNA was prepared from individual clones as described previously (3). DNA samples (25 μg) were digested to completion with the appropriate restriction enzymes (New England BioLabs) under conditions recommended by the supplier and fractionated on a 0.8% agarose gel in Tris-acetate-EDTA buffer. Gels were soaked at room temperature in 0.25 N HCl for 15 min, in 0.4 N NaOH–0.6 M NaCl for 30 min, and in 1.5 M NaCl–0.5 M Tris-HCl (pH 7.5) for 30 min. DNA was then transferred to GeneScreen Plus membranes (NEN, Dupont) according to the method suggested by the supplier. After transfer, the membranes were immersed in 0.4 N NaOH, rinsed in 0.2 M Tris-HCl (pH 7.5)–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and air dried. The membranes were then prehybridized in hybridization solution (50% formamide, 1% SDS, 10% dextran sulfate, 1 M NaCl) for 20 min at 42°C and hybridized for 16 h at 42°C in hybridization solution containing ≈10^6 cpm of random-priming heat-denatured probe and salmon sperm DNA (100 μg/ml). Hybridized blots were washed twice for 5 min at room temperature in 2× SSC, twice in 2× SSC–1% SDS for

30 min at 65°C, and twice in 0.1× SSC for 30 min at room temperature and then autoradiographed at −70°C on Kodak XAR-5 film in the presence of an intensifying screen.

Indirect immunofluorescence. Cells grown on coverslips were washed twice with PBS and fixed with 3.7% formaldehyde for 20 min at room temperature. After fixation, the cells were washed three times with PBS and permeabilized with 1% NP-40 in PBS for 5 min at room temperature. For T staining, coverslips were incubated with anti-T monoclonal antibody PAb416 and then with fluorescein-conjugated goat anti-mouse antibody (diluted 1:100). Coverslips were embedded on aqueous nonfluorescing mounting medium (Shandon). For actin staining, coverslips were incubated with rabbit anti-actin serum (diluted 1:40) and then with fluorescein-conjugated goat anti-rabbit IgG (diluted 1:50) and embedded on mounting medium. For both T staining and actin staining, antibody incubations were performed for 30 min at 37°C. Coverslips were washed three times with PBS after each incubation.

Northern (RNA) blot analysis. Total cellular RNA was extracted in the presence of guanidine isothiocyanate (7). For each sample, 20 μg of total RNA was heated for 5 min at 60°C in 50% (vol/vol) formamide, 6% (vol/vol) formaldehyde, and running buffer (20 mM 4-morpholinepropanylsulfonic acid [pH 7.5], 5 mM sodium acetate, 1 mM EDTA). The samples were then electrophoresed on an 0.8% agarose gel containing 6% (vol/vol) formaldehyde. The RNA was transferred onto a GeneScreen Plus membrane by the method suggested by the supplier. The membrane was then hybridized for 16 h at 42°C in hybridization buffer (50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate) containing ≈10^6 cpm of heat-denatured Swiss 3T3 mouse entire p53 cDNA (prepared by PCR amplification; see above) and 100 μg of denatured salmon sperm DNA per ml. Hybridized membranes were washed twice in 2× SSC for 5 min at room temperature, twice in 2× SSC–1% SDS for 30 min at 65°C, and twice in 0.1× SSC for 30 min at room temperature. Autoradiography was then performed at −70°C on Kodak XAR-5 film in the presence of an intensifying screen.

Colony formation in soft agarose. Analysis of revertant lines for anchorage-independent growth was performed in 60-mm dishes by overlaying 10^5 cells in DMEM containing 10% FCS and 0.3% (wt/vol) agarose (FMC BioProducts) onto a base of 0.5% (wt/vol) agarose prepared in the same medium. Colonies were allowed to form at 37°C for 14 to 21 days.

Growth curves. Cells were plated 5 × 10^4/35-mm dish in growth medium containing either 10 or 1% FCS. Every other day over a period of 12 days, the cells were harvested and resuspended in 0.04% trypan blue (GIBCO), and the number of live cells per dish was measured by hemacytometer counting. The number of cells per dish at each time point was determined by the average of duplicated dishes. Saturation density was measured for at least 3 days at the end of the growth experiment.

RESULTS

Isolation of cell lines overexpressing wt p53. To test the antitransforming function of wt p53, we first isolated Swiss 3T3 and C57L precrisis cells overexpressing exogenous wt p53 by cotransfecting pLTRcG-ala and a plasmid containing the neomycin resistance gene (pKoNeo) as a selectable marker at a 10:1 ratio. Four weeks after transfection, neomycin-resistant colonies were cloned and analyzed for stable
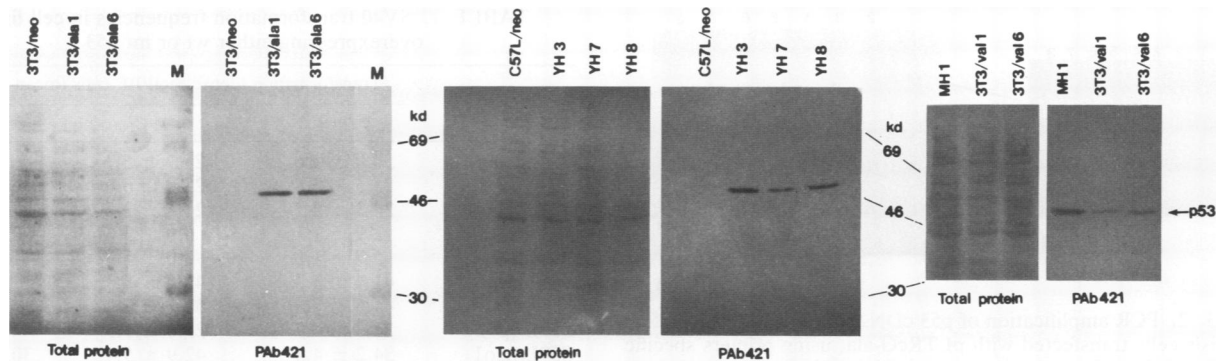
FIG. 1. Expression of p53 in Swiss 3T3 and C57L precrisis cells transfected with a wt p53 plasmid (pLTRcG-ala) or an mt p53 plasmid (pLTRcG-val). Cell extracts containing ≈700 μg of proteins were run on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose sheets. The blots were first stained with Ponceau S to visualize total protein transferred (left of each set). The same blots were then probed with anti-p53 monoclonal antibody PAb421 (right). The low level of p53 expressed in control normal cells (3T3/neo and C57L/neo) was not detectable in the immunoblot procedure used. Both types of cells transfected with either the wt or mt p53 plasmid overexpress p53.

integration of an exogenous p53 gene(s) by Southern blot and for p53 expression by immunoblot. Five wt p53 overexpressers (three clones derived from C57L precrisis cells [YH lines] and two derived from Swiss 3T3 cells [3T3/ala lines]) were maintained in culture for further studies. The immunoblot of those lines obtained by using anti-p53 monoclonal antibody PAb421 is shown in Fig. 1. The endogenous p53 expressed in 3T3/neo and C57L/neo control lines was undetectable under the stringency of the immunoblot used, while all of the wt p53-transfected lines overexpressed p53.

To test whether overexpressed p53 proteins came from an exogenously transfected plasmid, we PCR amplified cDNA prepared from those lines, using primers specific for the p53 cDNA transcribed from transfected plasmids. One primer is the sequence located inside the p53 coding region (+295 to +316 of p53 cDNA), and the other is the sequence near the BclI site of a BclI-BamHI fragment of the SV40 genome (bases 2742 to 2761 in the SV40 genome), which is used in plasmid LTRcG-ala for a polyadenylation signal. If those cell lines express the transfected p53 genes, their p53 cDNAs should be amplified by these primers. The intactness of cDNA prepared was confirmed by PCR amplification of p53 cDNA with primers +295 to +316 and +718 to +802 of murine p53 cDNA. These primers are not specific; they amplify p53 cDNA of both endogenous and exogenous origins. The PCR amplified an intact 0.5-kb p53 cDNA in all cDNA preparations, confirming their intactness, which were later used for sequencing (see below). The products of PCR amplification using primers specific to the p53 cDNA of exogenous origin were first analyzed by ethidium bromide staining (Fig. 2a). The positive control, in which the plasmid used in transfection (pLTRcG-ala) was used for a template for PCR amplification, shows amplified DNA with the predicted size of 3.6 kb. No cDNA from C57L/neo or 3T3/neo was amplified with these primers (lanes 2 and 6), as expected. However, cDNA from all wt p53 overexpressers was successfully amplified and gave the predicted 1.1-kb cDNA (lanes 3, 4, 5, 7, and 8). To confirm that amplified cDNAs (or DNA for the positive control) were from p53, they were probed with an entire Swiss 3T3 p53 cDNA (Fig. 2b). These amplified species were indeed from p53, and we concluded that cell lines transfected with pLTRcG-ala express stably integrated plasmid DNA.

We isolated two additional sets of cell lines: lines overexpressing mt p53 encoding valine instead of alanine at amino

acid position 135 (3T3/val lines derived from Swiss 3T3 cells and MH lines from C57L precrisis cells) and lines derived from neomycin-resistant colonies isolated after cotransfection with pKoNeo and pBR322 (3T3/neo and C57L/neo lines). Some of 3T3/val lines spontaneously became transformed during cell culture, probably due to excess amounts of mt p53, as observed by others (9, 17). To be able to test the response to SV40 of cells producing an excess of mt p53, we subcloned neomycin-resistant lines (3T3/val1 and 3T3/val6) that maintained a flat, untransformed morphology during passaging. Although PCR sequencing confirmed that these 3T3/val lines express transfected mt p53, the level of p53 expression was lower than in MH lines derived from precrisis cells (Fig. 1).

We used rapid PCR sequencing (5) to exclude the possibility that a transfected or endogenous wt p53 gene might have undergone a mutation(s) resulting in the oncogenic activation. cDNA was prepared from poly(A)+ RNA of 3T3/ala, YH, and Swiss 3T3 cells. Parental C57L precrisis cells were not examined, since early-passage precrisis cells are unlikely to have undergone mutational activation of p53. Amplified p53 cDNAs were then electrophoresed, electroeluted, and sequenced by using primers of p53 cDNA sequences +295 to +316 and +783 to +803, which encompass the known mutational activation region, amino acid residues 130 to 234 (11). All wt p53-transfected clones expressed only wild-type sequences in the mutational activation region of p53, confirming that the endogenous and exogenous p53 genes did not undergo any activational mutations through the isolation process, although mutation outside of this region cannot be ruled out. In particular, the p53 cDNA sequence of Swiss 3T3 cells from bp +316 to +783 was the same as the previously reported murine wt p53 cDNA sequence (28). This result indicates that immortalization of Swiss 3T3 cells cannot have been due to mutational activation within this region of a precrisis cell p53 gene. We also tested the turnover of p53 in Swiss 3T3 cells, since it has been reported that wt p53 has a short half-life of 10 to 30 min, while all of the mt p53 species that are activated for transformation have half-lives of 2 to 12 h (for a review, see reference 19). We found that the half-life of p53 in Swiss 3T3 cells was 20 to 30 min, characteristic of wt p53 (data not shown), further supporting the conclusion that p53 present in Swiss 3T3 cells is wild type.

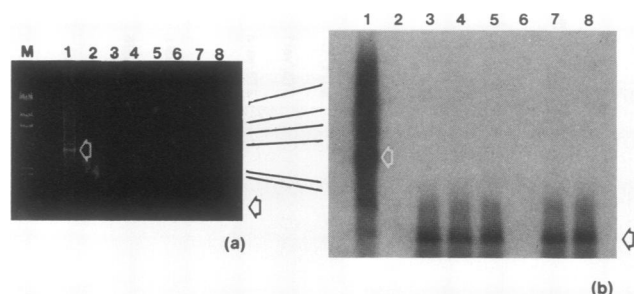**Overexpression of wt p53 lowers SV40 transformation effi-**

FIG. 2. PCR amplification of p53 cDNA of Swiss 3T3 and C57L precrisis cells transfected with pLTRcG-ala, using primers specific to p53 cDNA of exogenous origin. The cDNA, prepared from poly(A)$^+$ RNA of control normal cells (3T3/neo and C57L/neo) and pLTRcG-ala-transfected cell lines, was PCR amplified by using primers 5'-ACTTACCAGGGCAACTATGGC-3' (the sequence +295 to +316 of murine p53 cDNA) and 5'-ACCTCTACAAT GTGGTATG-3' (the sequence 2742 to 2761 of the SV40 genome). The latter primer sequence appears only in cDNA of exogenous origin (pLTRcG-ala); p53 cDNA of exogenous but not endogenous origin should be amplified specifically with these primers. For details, see Materials and Methods. (a) Ethidium bromide staining of the amplified cDNA. After 30 cycles of PCR amplification, a small aliquot of the PCR reaction mixture was directly analyzed in an 0.8% agarose gel stained with ethidium bromide. As the positive control, 1 ng of the plasmid used for the transfection (pLTRcG-ala) was used as the PCR template (lane 1). PCR-amplified DNA with a predicted size of 3.6 kb is indicated by the white arrow. The cDNA from control normal cells (C57L/neo [lane 2] and 3T3/neo [lane 6]) was not amplified by the primers used. The predicted 1.1-kb cDNA (black arrow) was amplified in all of the wt p53 overexpressers: YH3 (lane 3), YH7 (lane 4), YH8 (lane 5), KH1 (lane 7), and KH7 (lane 8). HindIII-digested fragments of lambda DNA were used as molecular weight markers. The size of fragments are (from the top) 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb. (b) Southern blot analysis of PCR-amplified cDNA. The amplified cDNA (or DNA for pLTRcG-ala) analyzed in panel a was blotted onto GeneScreen Plus membranes and probed with an entire Swiss 3T3 p53 cDNA. The amplified 3.6-kb DNA hybridized with the probe is indicated by the white arrow, and the 1.1-kb cDNA is indicated by the black arrow. The order of lanes and samples is same as in panel a.

ciency. To test the ability of wt p53 to interfere with the transforming function of SV40, we transfected pSVRI, encoding an entire wild-type SV40 genome, into the wt p53 overexpressers YH and 3T3/ala. As controls, the same plasmid was transfected into parental 3T3 Swiss and C57L cells and into 3T3/neo and C57L/neo lines. The same transfection was also done to the mt p53-overexpressing 3T3/val and MH lines. The transfected cells were cultured in medium containing 1% FCS. Four weeks after transfection, the number of dense foci was scored (Table 1). Comparison between original parental cells (Swiss 3T3 and C57L precrisis cells) and clones cotransfected with the neomycin resistance gene and pBR322 (3T3/neo and C57L/neo) shows that the neomycin selection process had a small enhancing effect on subsequent SV40 transformation (Table 1).

As Table 1 shows, we observed an enhancement of SV40 transformation efficiency in mt p53-expressing cells (3T3/val and MH), confirming results of earlier studies (9, 10, 24, 26). In distinction, lines overexpressing wt p53 (3T3/ala and YH) were all significantly resistant to SV40 transformation (Table 1). In Swiss 3T3 cells overexpressing wt p53, transformed foci were reduced in frequency by sevenfold on average compared with 3T3/neo controls. The transformed foci in C57L precrisis cells overexpressing wt p53 were reduced in

TABLE 1. SV40 transformation frequencies in cell lines overexpressing either wt or mt p53

Cell line	Transformation (dense foci)/10^6 cells (mean ± SE)		
	Expt 1	Expt 2	Expt 3
Swiss 3T3	30.0 ± 5.6	25.7 ± 5.3	ND^a
3T3/neo1	25.6 ± 3.8	25.0 ± 6.6	26.0 ± 3.9
3T3/neo2	24.3 ± 2.2	21.4 ± 2.7	22.9 ± 3.3
3T3/ala1	2.9 ± 1.1	5.6 ± 3.6	3.6 ± 1.4
3T3/ala6	5.7 ± 1.8	3.0 ± 1.3	1.7 ± 1.3
3T3/val1	41.4 ± 3.3	48.5 ± 5.3	ND
3T3/val6	44.5 ± 7.3	52.7 ± 7.2	ND
C57L (p4)	21.3 ± 4.7	25.0 ± 7.3	ND
C57L/neo1	34.2 ± 4.4	42.9 ± 5.4	30.9 ± 3.5
C57L/neo2	40.0 ± 4.4	41.7 ± 6.0	ND
YH3	5.0 ± 2.0	5.5 ± 2.0	3.3 ± 1.4
YH7	1.0 ± 0.9	1.4 ± 1.8	1.4 ± 1.3
YH8	0.7 ± 0.7	0	0.9 ± 0.3
MH1	152.0 ± 13.1	179.0 ± 14.3	ND

^{a} ND, not done.

frequency by between 10- and 100-fold compared with C57L/neo lines. Several additional lines of Swiss 3T3 and C57L precrisis cells overexpressing wt p53 yielded similar results. The average reduction in transformation frequency of Swiss 3T3 cells was 10-fold, while that of C57L precrisis cells was more than 30-fold. Thus, resistance appears to be more complete in precrisis cells than in established cells. This difference has been reported for other oncogenes, in which the antitransforming action of wt p53 is more dramatic in precrisis cells than in established cells (8, 11).

To obtain further information about this difference in wt p53's activity in precrisis and established cells, we cloned rare dense foci which arose from YH7 and 3T3/ala6 lines transfected with SV40 plasmid pSVRI. All dense clones that we isolated expressed large T antigens, as confirmed by both T staining and immunoblot (data not shown). Surprisingly, pSVRI-transfected YH7 (YH7A-H) clones derived from dense foci became quite flat after a few passages in culture, losing their initial, transformed phenotype. On the other hand, the transformed phenotype of all clones derived from pSVRI-transfected 3T3/ala6 (3T3/ala6A to E) was stable to subcloning. The observed distinction in stability of the transformed phenotype was supported by measurements of the saturation density of these subclones (Table 2). YH7-derived clones, although showing a saturation density higher than that of the control (C57L/neo), had a low saturation density comparable with that of the control established 3T3/neo cells.

Isolation of SV40-transformed lines for studies of reversion by introduction of exogenous wt p53. Although the results described above indicate that in precrisis cells both initiation and maintenance of transformation are blocked by overexpression of wt p53 and that in established cells only initiation is blocked, studies of transformation frequency cannot strictly distinguish between an effect on initiation and one on maintenance. To make this distinction, we tested wt p53's ability to revert SV40-transformed cells to a more normal phenotype. Only if the action of wt p53 operates on the maintenance of transformation will reversion be detected.

To increase the sensitivity of a SV40-transformed cell to any reverting effects of wt p53, we isolated transformants with low-number copies of large T antigen sequences. Both Swiss 3T3 and C57L precrisis cells were transfected with a plasmid encoding the early region of the wild-type SV40

TABLE 2. Saturation densities of clones derived from dense foci in pSVRI-transfected YH7 and 3T3/ala6 lines

Cell line and clone	Saturation density[a] in 10% FCS (10^4 cells/cm²)
C57L/neo1 (control)	3.8
YH7 (parental)	3.4
YH7A	12.6
YH7B	10.2
YH7C	13.8
YH7D	10.1
YH7E	12.5
YH7F	13.6
YH7G	11.9
YH7H	8.2
3T3/neo1 (control)	8.4
3T3/ala6 (parental)	9.3
3T3/ala6A	>40.0[b]
3T3/ala6B	>40.0[b]
3T3/ala6C	>40.0[b]
3T3/ala6D	>40.0[b]

[a] Measured as described in Materials and Methods.
[b] After cell density reaches ≈4×10^5 cells per cm², cells peel off plates.

genome. Transfected cells were maintained in medium containing 1% FCS. Four weeks after transfection, several dense foci from each cell line were cloned and analyzed for stable integration and copy number of large T antigen sequences by Southern blot (Fig. 3a) and for the expression of large T antigens by T staining (Fig. 3b) and immunoblot (Fig. 3c and d). Clones 3T3SVB, derived from Swiss 3T3 cells, and C35SVB, derived from C57L precrisis cells, have a low number of functional copies of large T antigen sequence. Those lines express similar amounts of large T antigens (Fig. 3c and d). We examined the endogenous p53 gene products in these two clones by sequential immunoprecipitation using monoclonal antibodies PAb246 and PAb421. PAb246 distinguishes between wt p53 (PAb246⁺ PAb421⁺) and mt p53 (PAb246⁻ PAb421⁺). Cell extracts were immunoprecipitated with PAb246, and then supernatants were immunoprecipitated with PAb421, which recognizes both wt and mt p53 (Fig. 4). Since all of the p53 expressed in the two cell lines was immunoprecipitated with PAb246, it is unlikely that the wt p53 of these cells was mutated during transformation.

Reversion of transformed precrisis cell line by wt p53. To test the ability of wt p53 to revert the transformed cells to a more normal phenotype, C35SVB, derived from C57L pre-
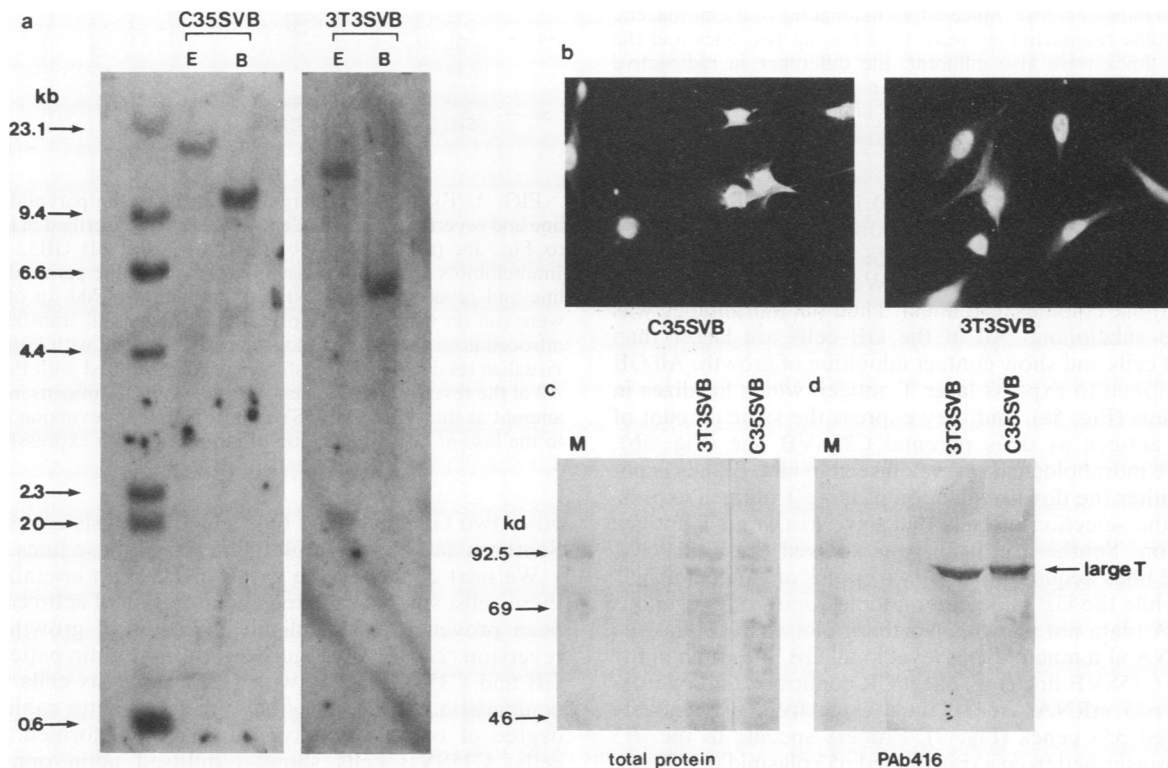

FIG. 3. Evidence that transfected cell lines have acquired a low number of copies of SV40 DNA and that these integrated sequences express SV40 T antigen. (a) Southern blot analysis of stably integrated large T antigen sequences in SV40 large T-transformed Swiss 3T3 and C57L precrisis cells (3T3SVB and C35SVB lines, respectively). High-molecular-weight DNA from those cell lines was digested with EcoRI (E) or BamHI (B). Samples (25 µg) were electrophoresed on 0.8% agarose gels, blotted onto GeneScreen Plus membranes, and probed with an entire SV40 genome. HindIII-digested fragments of lambda DNA were used as molecular weight markers. (b) 3T3SVB and C35SVB lines stained for SV40 large T antigens. The cells were fixed with 3.7% formaldehyde. SV40 large T antigens were detected with anti-T monoclonal antibody PAb416 and stained with fluorescein-conjugated goat anti-mouse antibody. Magnification is ×500. (c and d) Immunoblot of large T antigen expressed in 3T3SVB and C35SVB lines. Cell extracts containing ≈700 µg of proteins were run on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose sheets. The blots were first stained with Ponceau S to visualize the total proteins transferred (c). The same blots were then probed with anti-large T monoclonal antibody PAb416 (d). Both cell lines have acquired a low number of copies of SV40 DNA and express similar amounts of SV40 large T antigens.
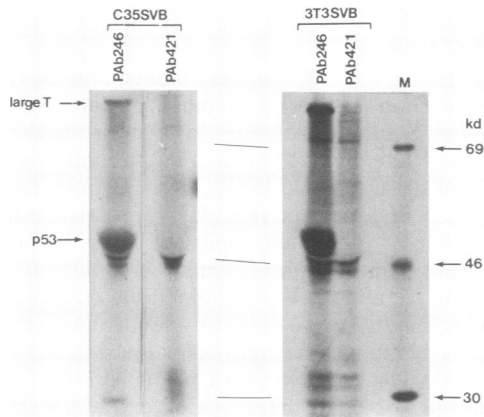
FIG. 4. Sequential immunoprecipitation of p53 from SV40-transformed cell lines, 3T3SVB and C35SVB, derived from Swiss 3T3 and C57L precrisis cells. Cells were labeled with [^{35}S]methionine-cysteine. Cell extracts were immunoprecipitated sequentially first with the wt p53-specific monoclonal antibody PAb246. After PAb246-p53 immune complexes were collected, the supernatant was incubated with monoclonal antibody PAb421 against both wt and mt p53. Immunoprecipitates were analyzed on 7.5% SDS-polyacrylamide gels. After immunoprecipitation with PAb246, no detectable p53 remained in lysates from both transformants. These results demonstrate the presence of PAb246$^+$ wt p53 and the absence of PAb246$^-$ mt p53. Immunoprecipitations on these two lines were performed independently. Since different amounts of trichloroacetic acid-insoluble radioactivities were used for the two lines and the exposure times were also different, the difference in radioactive densities of immunoprecipitates between two lines does not indicate the relative amounts of p53 and large T antigen expressed by them.
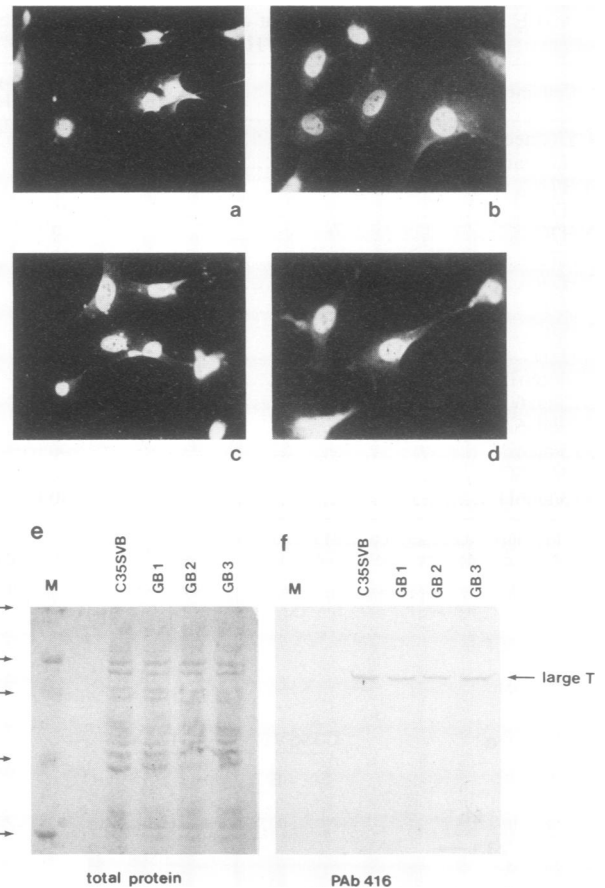

FIG. 5. Expression of large T antigens in the parental C35SVB line and revertants. (a to d) Cells T stained as described in the legend to Fig. 3b. (a) C35SVB; (b) GB1; (c) GB2; (d) GB3. (e and f) Immunoblots of large T antigens expressed in the parental C35SVB line and revertants. Cell extracts containing ≈700 μg of proteins were run on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose sheets. The blots were first stained with Ponceau S to visualize total proteins transferred (e) then probed with PAb416 (f). All of the revertants continue to express large T antigens in the same amount as the parental C35SVB line. Thus, the reversion is not due to the loss or down-regulation of large T antigen expression.

crisis cells, was cotransfected at 20:1 with pLTRcG-ala and pKoNeo. Neomycin-resistant colonies appeared about 3 weeks after transfection. About 60% of transfected colonies showed a flattened morphology. We cloned and amplified three of those colonies (GB lines). Their flat morphology was stable to subcloning. All of the GB cells are larger than C35SVB cells and show contact inhibition of growth. All GB lines continue to express large T antigen which localizes in the nucleus (Fig. 5a), and they express the same amount of large T antigen as does parental C35SVB line (Fig. 5b). Thus, the morphological reversion seen in all GB lines is not due to either the down-regulation of large T antigen expression or the selection of cells that have lost large T antigen expression. Southern blot analysis showed that both GB1 and GB2 lines acquired one to two copies of exogenous p53 DNA, while the GB3 line had multiple copies of exogenous p53 DNA (data not shown). Northern blot analysis showed p53 mRNA at a much higher level in all GB lines than in the parental C35SVB line (Fig. 6a). PCR confirmed that overexpressed p53 mRNAs in GB lines came from exogenously introduced p53 genes (Fig. 7). Primers specific to the p53 cDNA transcribed from a transfected p53 plasmid (pLTRcG-ala) failed to amplify p53 cDNA of the C35SVB line (Fig. 7a, lane 2), while they amplified p53 cDNA of all GB lines, yielding the amplified cDNA with the predicted size of 1.1 kb (lanes 3 to 5). Those PCR-amplified sequences were shown to be p53 by Southern blot using Swiss 3T3 p53 cDNA as a probe (Fig. 7b). Assay of wt p53 protein in the three GB lines by immunoprecipitation using wt p53-specific monoclonal antibody PAb246 showed that all GB lines overexpress wt p53 proteins compared with the C35SVB line and that the GB3 line expresses the protein at a higher level than do the

other two GB lines (Fig. 6b), which is consistent with both Southern and Northern blot analysis of these lines.

We next examined the cytoskeletal actin organization of these cells, since the degree of restoration of actin cables has been proven to be a reliable indicator of growth control reversion (29, 36, 37). Figure 8 shows the actin pattern of the GB and C35SVB lines, with C57L precrisis cells used for comparison. All GB lines have restored actin cables to the degree of organization typical of untransformed precrisis cells. C35SVB cells show a diffused actin organization characteristic of transformed cells (29, 36, 37). Consistent with the actin pattern, untransformed precrisis and Swiss 3T3 cells do not grow in soft agar (Table 3). Where as the C35SVB line grows well in soft agar, the ability of the GB lines to grow without anchorage is almost completely lost. We determined the growth curves of the GB and C35SVB lines in 1 and 10% FCS. Doubling times and saturation densities derived from growth curves are summarized in Table 3. All GB lines grow more slowly and have lower saturation densities than does the parental C35SVB line.
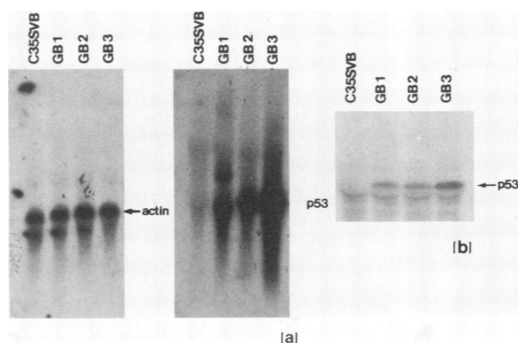
lal

(a)

(b)

FIG. 6. Expression of wt p53 in the C35SVB parental line and revertants. (a) Northern blot analysis of p53 mRNA in the parental C35SVB line and revertants. A 25-μg sample of total RNA was loaded on a 0.8% agarose gel containing 0.66 M formaldehyde. The two identical gels were run in parallel. The blots were probed with either beta-actin or an entire Swiss 3T3 p53 cDNA. GB lines all contain an excess of p53 mRNA. (b) Immunoblot of PAb246-immunoprecipitated wt p53 in the C35SVB line and revertants. Cell lysates containing ≈1.5 mg of proteins were immunoprecipitated with PAb246. Immunoprecipitates were run on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose sheet. The blot was then probed with PAb421, revealing an excess of wt p53 protein in revertant lines. Although the immunoblot here does not detect p53 expressed in the C35SVB line, the detection of p53 in that cell line depends on the stringency used for immunoblotting. The stringency is defined by the extent of dilution of antibodies in the blocking buffer and the composition of the blocking buffer. We used a very high stringency for the purpose of distinguishing exogenous from endogenous p53. At a lower stringency, p53 expressed in the C35SVB line can be clearly detected (see Fig. 9 and 10).
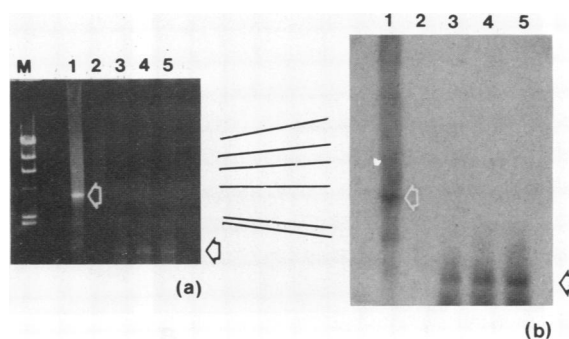
FIG. 7. PCR amplification of p53 cDNA prepared from the parental C35SVB line and revertants, using primers specific to the p53 cDNA of exogenous origin. The cDNA, prepared from poly(A)$^+$ RNA of the C35SVB line and all of the revertants, was PCR amplified as described in the legend to Fig. 2. (a) Ethidium bromide staining of amplified cDNA. The primers specific to p53 cDNA of exogenous origin amplified the positive control DNA (pLTRcG-ala; see legend to Fig. 2) and yielded the predicted 3.6-kb DNA (lane 1, white arrow). While these primers failed to amplify endogenous p53 cDNA of the parental C35SVB line (lane 2), they amplified transfected cDNA of p53 of all of the revertants and yielded an amplified cDNA of the predicted 1.1 kb in size (lane 3 to 5, black arrow). (b) Southern blot analysis of PCR-amplified cDNA. The amplified cDNA (or DNA for the positive control) analyzed in panel a was blotted onto GeneScreen Plus membranes and probed with an entire Swiss 3T3 p53 cDNA. The amplified 3.6-kb DNA of the positive control is indicated by the white arrow, and the 1.1-kb cDNA of the revertants is indicated by the black arrow. The order of lanes and samples is same as in panel a.

Reversion to low saturation density is most marked in GB3, which overexpresses exogenous wt p53 to the greatest extent. Reversion with respect to growth rate and saturation density of the GB lines makes them similar to Swiss 3T3 cells; they have not reverted in these assays to the phenotype of the parental C57L precrisis cells. Taken together, these data show that reversion of the transformed phenotype by introduction of wt p53, although not complete in regard to all parameters of transformation, is stable and considerable in precrisis cells.

Revertants contain wt p53 not bound to large T antigen. We next examined whether the overexpression of wt p53 in GB lines resulted in availability of p53 species unbound to large T antigen. According to the hypothesis that large T-p53 complex formation inactivates wt p53's antitransforming function, those p53 proteins free of large T would be able to exert their antitransforming function and contribute to the reversion. Thus, the revertants should contain large T-free p53 proteins. Cell lysates were prepared from the C35SVB parental line and the revertants. Portions of the lysates containing equivalent amounts of total proteins were analyzed with an immunoblot doubly probed with anti-large T monoclonal antibody PAb416 and anti-p53 monoclonal antibody PAb421 (Fig. 9a). Portions of the same lysates used in the immunoblot were subjected to immunoprecipitation with anti-large T monoclonal antibody to remove large T antigens and any proteins interacting with large T antigens from the lysates. After collection of the immune complexes, supernatants were analyzed in immunoblots doubly probed with PAb416 and PAb421 (Fig. 9b). After removal of large T and large T-binding proteins, no p53 in the lysate of the C35SVB line was detected, showing that all p53 expressed in this line

was bound to large T antigen. However, a large amount of p53 in all revertants was still detectable, showing the availability of large T-free p53 proteins.

Nonreversion of SV40-transformed Swiss 3T3 cells after introduction of wt p53. Once transformed by SV40, established Swiss 3T3 cells are not reverted by the introduction of exogenous wt p53. For example, the 3T3SVB line cotransfected with wt p53 and pKoNeo yielded no neomycin-resistant colonies with a flattened morphology. We extended our attempts to revert Swiss 3T3 transformants by introducing wt p53 into three other SV40-transformed Swiss 3T3 cell lines. All failed to give colonies with a flattened morphology. The failure of wt p53 plasmid to revert 3T3-derived transformants raised the question of whether the neomycin-resistant transfected cells were in fact expressing transfected wt p53. Seven randomly chosen neomycin-resistant 3T3SVB colonies were immunoblotted with PAb421. Six of those clones overexpress p53 compared with the parental 3T3SVB line (data not shown). To examine whether overexpressed p53 was wild type, four p53-overexpressing clones (C2, C4, C5, and C7) were subjected to PAb246 (anti-wt p53)-PAb421 (anti-wt plus mt p53) sequential immunoprecipitation. We found that all detectable p53 expressed in the four wt p53-overexpressing clones appeared to be PAb246$^+$, as no PAb246$^-$ PAb421$^+$ mt p53 could be detected after two rounds of PAb246 immunoprecipitation (data not shown). Therefore, we concluded that the four clones overexpressed wt p53, even though none showed any sign of reversion. We then examined the availability of large T-free p53 in the four wt p53-overexpressing clones. Portions of cell lysates prepared from the 3T3SVB parental line and those four clones were immunoblotted with both PAb416 (anti-T) and PAb421 (anti-p53) (Fig. 10a). Large T antigens and all proteins
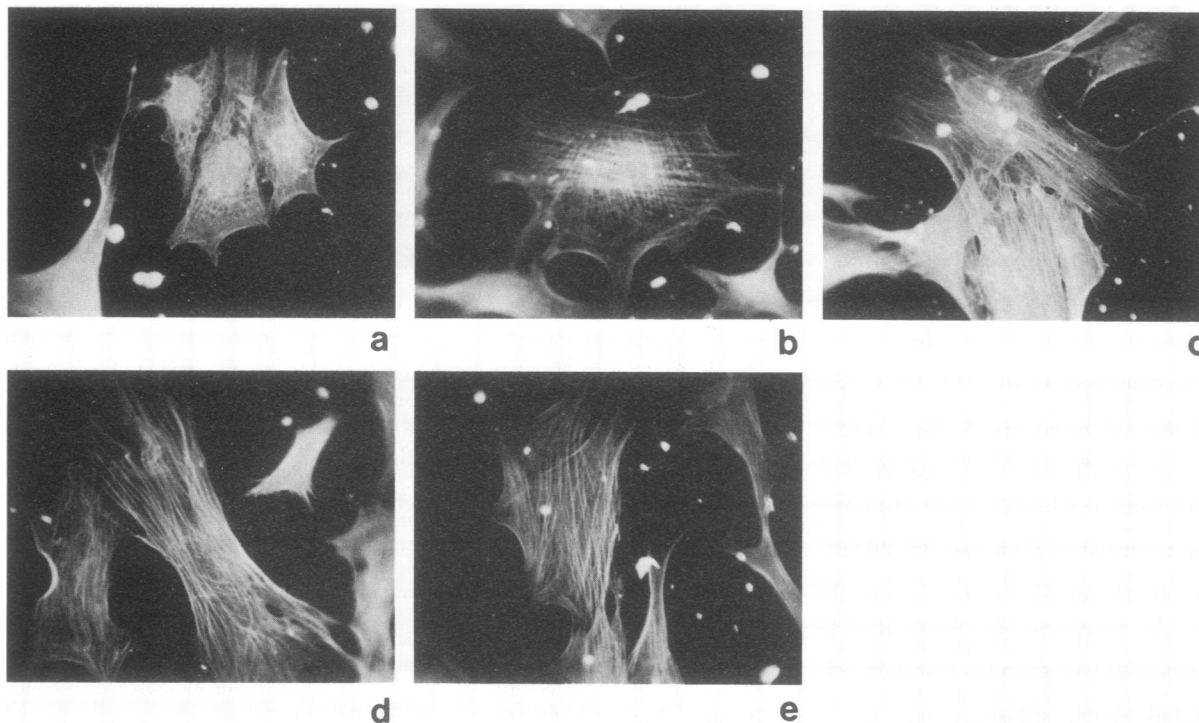
FIG. 8. Actin patterns of the C35SVB line and revertants. Cells were fixed with 3.7% formaldehyde, detected with rabbit antiactin serum, and stained with fluorescein-conjugated goat anti-rabbit IgG. Magnification is ×500. (a) C35SVB line; (b) C57L precrisis cell; (c) GB1; (d) GB2; (e) GB3. All revertant lines regained organized actin cables.

associating with large T antigens were removed from the portions of the same lysates used in the immunoblot in Fig. 10a by immunoprecipitation with anti-T monoclonal antibodies. After collection of the immune complexes, supernatants were analyzed by an immunoblot doubly probed with PAb416 and PAb421 (Fig. 10b). No p53 in the 3T3SVB parental line was detected after removal of large T antigens and proteins associating with large T antigens, indicating that all p53 in 3T3SVB is bound to large T antigen. However, all four clones showed a significant amount of p53, indicating that a large amount of T-free p53 is present in those clones.

TABLE 3. Biological properties of C57L precrisis cells, Swiss 3T3 cells, the C35SVB line, and flat revertants

Cell line	Doubling time (h)[a] in:		Saturation density (10^4 cells/cm^2)[b] in:		% CFE[c]	Actin cables[d]
	10% FCS	1% FCS	10% FCS	1% FCS		
C57L (passage 4)	53.7	75.3	3.1	1.0	<0.1	+
Swiss 3T3	37.0	56.6	5.6	1.7	<0.1	+
C35SVB	20.7	24.2	—[e]	—	14.0	−
GB1	38.3	58.6	13.0	6.2	0.27	+
GB2	29.3	61.8	13.5	4.2	0.32	+
GB3	31.9	51.2	9.1	4.1	0.35	+

[a] Determined from growth slopes in the initial logarithmic phase.
[b] Measured for at least 3 days at the end of the growth experiment (see Materials and Methods).
[c] CFE, colony-forming efficiency (the ratio of soft agar colonies (>100 cells per colony) to viable cells seeded, expressed as a percentage).
[d] Estimated by indirect immunofluorescence (Fig. 8).
[e] —, saturation density not observed; cells peeled off plates.

Thus, the 3T3SVB line cannot be reverted despite the significant availability of large T-free p53 in the cell.

We next considered the possibility that the inability of wt p53 to revert the 3T3SVB line might be the consequence of activational mutation in the cell line's endogenous p53 gene that did not alter the PAb246 epitope of the protein. We sequenced p53 cDNA (+316 to +783) from the 3T3SVB line and found that the 3T3SVB line expressed only a wt p53 sequence, one identical to that of the parental Swiss 3T3 cells (data not shown). The p53 cDNA sequences of two other SV40-transformed cell lines derived from Swiss 3T3 cells also were wild type, confirming our sequential immunoprecipitation results. Both lines of work show that SV40 did not transform a preexisting minority of 3T3 cells which already had suffered activational mutations in their p53 genes, and while it remains possible that a very minor population of 3T3 cells in culture undergo activational mutations in their p53 genes, we have shown that SV40 does not selectively transform such a minority population.

DISCUSSION

In this study we examined whether an antitransforming function was exerted by wt p53 in the SV40 transformation process and, if so, whether initiation of transformation or maintenance of the transformed phenotype, or both, was affected. We examined both precrisis early-passage mouse cells derived from adult C57L mouse dermal tissue and the mouse established cell line Swiss 3T3. Since mutations found in the region between amino acids 130 and 234 of mouse p53 have been reported to induce immortalization of primary cells (15, 30), others have hypothesized that the establishment of Swiss 3T3 cells may require a mutation(s) in
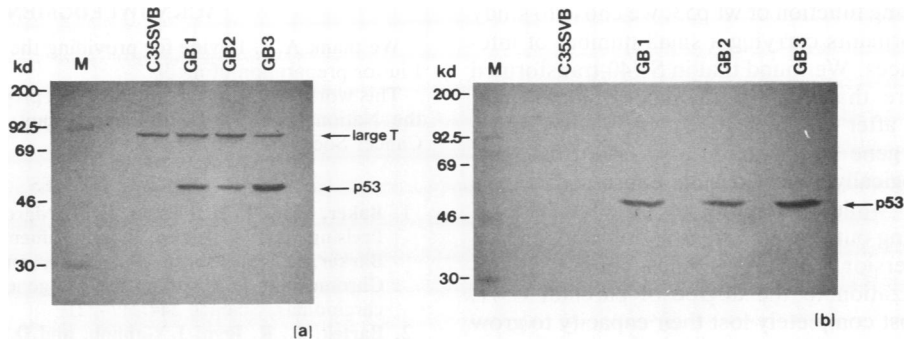
FIG. 9. State of p53 expressed in the parental large T-transformed C35SVB line and revertants. (a) Immunoblot of both large T antigen and p53 in the parental C35SVB line and revertants. Cell extracts containing ≈700 μg of proteins were run on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose sheets. The blots were probed with both PAb416 (anti-T) and PAb421 (anti-p53). (b) Detection of large T-free p53 in the C35SVB line and revertants. Cell extracts containing ≈1.2 mg of proteins were incubated with PAb108 and PAb416 (both are anti-large T monoclonal antibodies), and large T antigens and proteins associating with large T antigens were immunoprecipitated. The supernatants were then concentrated with a Centricon-10 (Amicon), run on a 7.5% SDS-polyacrylamide gel, and transferred onto nitrocellulose sheets. The blots were probed with both PAb416 and PAb421. Both immunoblots were performed with a lower stringency. After removal of large T antigens and proteins that complex with large T antigens, p53 in the parental C35SVB line is no longer detectable. However, p53 in all of the revertants is still detectable, showing the availability of large T-free p53 in the revertants.

the endogenous p53 gene(s). We found that the cDNA sequence from bp +316 to +783, which encompasses the region susceptible to the activational mutation, was wild type in Swiss 3T3 cells. Furthermore, p53 expressed in Swiss 3T3 cells was of a wild type that was shown to have a very short half-life of 20 to 30 min. From this finding we concluded that the establishment of Swiss 3T3 cells did not require mutation in the activation-susceptible region of p53 genes.

We isolated lines overexpressing wt p53 from precrisis cells and from Swiss 3T3 cells and tested their susceptibility to SV40 transformation. We found that both sets of lines overexpressing wt p53 were significantly more resistant to SV40 transformation than were controls and that the anti-transforming function of wt p53 seems more significant in precrisis cells than in established cells. This view is consistent with earlier reports showing the antitransforming function of wt p53 in the presence of the activated *ras* plus mt p53 to be more significant in precrisis cells than in established

cells (8, 11). Furthermore, the transformed phenotype of the clones isolated from dense foci that arose in SV40-transfected precrisis wt p53 overexpressers is unstable, while the phenotype in SV40-transfected established wt p53 overexpressers is stable to subcloning. Transformation by SV40 proceeds through a series of transient events termed initiation to a stable set of changes termed maintenance of the transformed phenotype. Our results suggest that the overexpression of wt p53 interferes with both initiation and maintenance of SV40-mediated transformation in precrisis cells but only with initiation in established cells. To further clarify whether the inhibition of transformation by wt p53 is exerted in only one of these phases or both, we examined whether wt p53 would revert any SV40-transformed cells to a more normal phenotype. We reasoned that reversion would be direct evidence that wt p53 could block maintenance of the transformed phenotype.

On the basis of the hypothesis that the complex formation between SV40 large T antigen and p53 results in inactivation
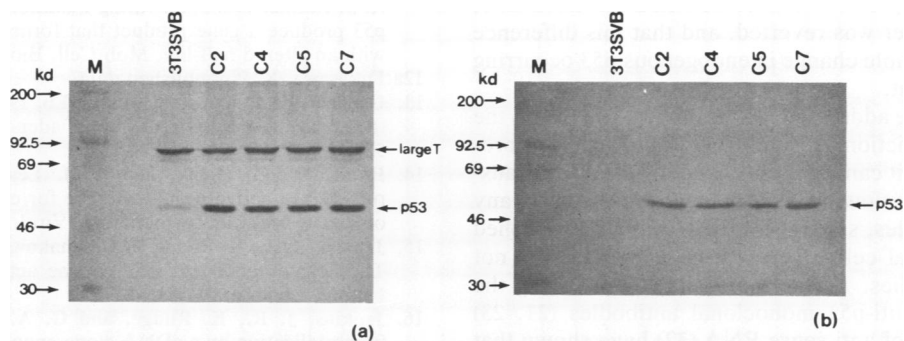

FIG. 10. State of p53 expressed in the parental large T-transformed 3T3SVB line and clones transfected with a wt p53 plasmid. (a) Immunoblot of both large T antigen and p53 in the parental 3T3SVB line and clones overexpressing wt p53. Cell extracts containing ≈700 μg of proteins were run on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose sheets. The blots were doubly probed with PAb416 (anti-T) and PAb421 (anti-p53). (b) Detection of large T-free p53 in the 3T3SVB line and wt p53-overexpressing clones. Cell extracts containing ≈1 mg of proteins were incubated with anti-large T monoclonal antibodies (PAb108 and PAb416), and large T antigens as well as proteins associating with large T antigens were immunoprecipitated. The supernatants were then analyzed as described in the legend to Fig. 9b. After removal of large T antigen and its associating proteins, p53 in the parental 3T3SVB line is no longer detectable. However, p53 in all the clones is still detectable, showing the availability of large T-free p53 in those clones.

of the antitransforming function of wt p53, we chose to study reversion in transformants carrying a small number of integrated SV40 sequences. We found that in SV40-transformed precrisis cells, more than half of the neomycin-resistant colonies recovered after cotransfection with wt p53 and a neomycin-resistant gene had acquired a revertant flat morphology. Morphologically reverted cells expressed a large amount of wt p53 while continuing to produce large T antigen as well, ruling out down-regulation of T antigen as a mechanism of reversion. Revertant lines completely restored actin organization to the degree of parental C57L precrisis cells, almost completely lost their capacity to grow in soft agar, and showed a significantly slower doubling time and a lower saturation density than did the parental SV40-transformed cells. The revertants were further analyzed for the state of p53 expressed. While all of the p53 species expressed in the parental large T antigen transformant are bound to large T antigens, a large amount of p53 in the revertants are large T-free, unbound species. This observation further supports the hypothesis as to the transforming function of the large T-p53 complex. The fact that reversion of SV40-transformed C57L precrisis cells by wt p53 was considerable but incomplete indirectly suggests that the inactivation of wt p53 by large T antigen, while necessary for SV40 to transform cells, may not be the sole mechanism employed by SV40 large T antigen.

As we expected, colonies derived from several different SV40-transformed Swiss 3T3 lines failed to revert upon wt p53 transfection. They are unable to revert morphologically even though a significant amount of large T-free wt p53 is available in the cells. In established cells, wt p53 apparently can exert an antitransforming function solely at the initiation stage, while in precrisis cells, wt p53 can exert an antitransforming function at either the initiation or maintenance stage. We conclude that even if SV40 successfully initiates the transformation in precrisis cells, wt p53 may block the maintenance of transformation, while in established cells, SV40 can complete any transformation it initiates despite the presence of excess wt p53. This difference may account for the previous observations that the antitransforming function of wt p53 is far more significant in precrisis cells than in established cells. It is worth noting that the wt p53 added to our SV40-transformed cells was able to detect a molecular difference between precrisis and established transformants, since only the former was reverted, and that this difference is not itself a detectable change in endogenous p53 occurring during establishment.

The question to be addressed next is how wt p53 exerts the antitransforming function. Assuming wt p53 to be an antiproliferative molecule, it cannot simply function as an antitransforming molecule by slowing or stopping the cell cycle of any cell in which it resides, since the growth of both established and precrisis normal cells overexpressing wt p53 was not blocked in our studies. Furthermore, studies involving the microinjection of anti-p53 monoclonal antibodies (21, 23) and the expression of anti-sense RNA (32) have shown that the presence of p53 is required for normal cells to proceed through the cell cycle. To clarify the missing link between the antitransforming function of wt p53 and the capacity of normal cells to progress through the cell cycle in the presence of excess wt p53, we have begun to study the responses of cells producing an excess of wt p53 to various growth-signaling stimuli.

REFERENCES

1. **Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. van Tuinen, D. H. Ledbetter, D. F. Barker, Y. Nakamura, R. White, and B. Vogelstein.** 1989. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science **244**:217–221.
2. **Bartek, J., R. Iggo, J. Gannon, and D. P. Lane.** 1990. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene **5**:893–899.
3. **Blanck, G., S. Chen, and R. E. Pollack.** 1983. Integration, loss and reacquisition of defective viral DNA in SV40-transformed mouse cell lines. Virology **126**:413–428.
4. **Brown, M., J. Figge, U. Hansen, C. Wright, K.-T. Jeang, G. Khoury, D. M. Livingston, and T. M. Roberts.** 1987. lac repressor can regulate expression from a hybrid SV40 early promoter containing a lac operator in animal cells. Cell **49**:603–612.
5. **Carothers, A. M., G. Urlaub, J. Mucha, D. Grunberger, and L. Chasin.** 1990. Point mutation analysis in a mammalian gene: rapid preparation of total RNA, PCR amplification of cDNA, and Taq sequencing by a novel method. Biotechniques **7**:494–499.
6. **Chen, S., D. S. Grass, G. Blanck, N. Hoganson, J. L. Manley, and R. E. Pollack.** 1983. A functional simian virus 40 origin of replication is required for the generation of a super T antigen with a molecular weight of 100,000 in transformed mouse cells. J. Virol. **48**:492–502.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
8. **Eliyahu, D., D. Michalovitz, S. Eliyahu, O. Pinhasi-Kimhi, and M. Oren.** 1989. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. USA **86**:8763–8767.
9. **Eliyahu, D., D. Michalovitz, and M. Oren.** 1985. Overproduction of p53 antigen makes established cells highly tumorigenic. Nature (London) **316**:158–160.
10. **Eliyahu, D., A. Raz, P. Gruss, D. Givol, and M. Oren.** 1984. Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. Nature (London) **312**:646–649.
11. **Finlay, C., P. W. Hinds, and A. J. Levine.** 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell **57**:1083–1093.
12. **Finlay, C., P. W. Hinds, Y.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine.** 1988. 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.
12a.**Fukasawa, K.** Unpublished data.
13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
14. **Hinds, P., C. Finlay, and A. J. Levine.** 1989. Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. J. Virol. **63**:739–746.
15. **Jenkins, J. R., K. Rudge, P. Chumakov, and G. A. Currie.** 1985. The cellular oncogene p53 can be activated by mutagenesis. Nature (London) **317**:816–817.
16. **Jenkins, J. R., K. Rudge, and G. A. Currie.** 1984. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature (London) **312**:651–654.
17. **Kelekar, A., and M. D. Cole.** 1986. Tumorigenicity of fibroblast lines expressing the adenovirus E1a, cellular p53, or normal c-*myc* genes. Mol. Cell. Biol. **6**:7–14.
18. **Lane, D. P., and L. V. Crawford.** 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature (London) **278**:261–263.
19. **Levine, A. J.** 1990. The p53 protein and its interactions with the

oncogene products of the small DNA tumor viruses. Virology 177:419–426.

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

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

22. Mercer, W. E., C. Avignolo, and R. Beserga. 1984. Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. Mol. Cell. Biol. 4:276–281.

23. Mercer, W. E., D. Nelson, A. DeLeo, D. Old, and R. Beserga. 1982. Microinjection of monoclonal antibody to protein p53 inhibits serum-induced DNA synthesis in 3T3 cells. Proc. Natl. Acad. Sci. USA 79:6309–6312.

24. Michalovitz, D., D. Eliyahu, and M. Oren. 1986. Overproduction of protein p53 contributes to simian virus 40-mediated transformation. Mol. Cell. Biol. 6:3531–3536.

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

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

27. Peden, K. W. C., A. Spinivasan, J. M. Faber, and J. M. Pipas. 1989. Mutants with changes within or near a hydrophobic region of simian virus 40 large tumor antigen are defective for binding cellular protein p53. Virology 168:13–21.

28. Pennica, D., D. V. Goeddel, J. S. Hayflick, N. C. Reich, C. W. Anderson, and A. J. Levine. 1984. The amino acid sequence of murine p53 determined from a cDNA clone. Virology 134:477–482.

29. Pollack, R., M. Osborn, and K. Weber. 1975. Patterns of organization of actin and myosin in normal and transformed cultured cells. Proc. Natl. Acad. Sci. USA 72:994–998.

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

31. Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine. 1982. Adenovirus E1b-58 kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 Kd cellular protein in transformed cells. Cell 28:387–394.

32. Shohat, O., M. Greenberg, D. Reisman, M. Oren, and V. Rotter. 1987. Inhibition of cell growth mediated by plasmids encoding p53 anti-sense. Oncogene 1:277–283.

33. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1:327–341.

34. Takahashi, T., M. M. Nan, I. Chiba, M. J. Birrer, R. X. Rosenberg, M. Vinocour, M. Levitt, H. Pass, A. F. Grazdar, and J. D. Minna. 1989. p53: a frequent target for genetic abnormalities in lung cancer. Science 246:491–494.

35. Todaro, G., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17:299–313.

36. Verderame, M., D. Alcorta, M. Egnor, K. Smith, and R. Pollack. 1980. Cytoskeletal F-actin patterns quantitated with fluorescein isothiocyanate-phalloidin in normal and transformed cells. Proc. Natl. Acad. Sci. USA 77:6624–6628.

37. Weber, K., E. Lazarides, R. Goldman, A. Vogel, and R. Pollack. 1974. Localization and distribution of actin fibers in normal, transformed and revertant cells. Cold Spring Harb. Symp. Quant. Biol. 39:363–369.

38. Werness, B. A., A. J. Levine, and P. M. Howley. 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248:76–79.