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Expression of Wild-Type p53 Is Not Compatible with Continued Growth of p53-Negative Tumor Cells

PENNY JOHNSON,[1] DAWN GRAY,[1] MICHAEL MOWAT,[2] AND SAMUEL BENCHIMOL[1]*

Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario M4X 1K9,[1] and Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba R3E 0V9,[2] Canada

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Inactivation of the cellular p53 gene is a common feature of Friend virus-induced murine erythroleukemia cell lines and may represent a necessary step in the progression of this disease. As well, frequent loss or mutation of p53 alleles in diverse human tumors is consistent with the view of p53 as a tumor suppressor gene. To examine the significance of p53 gene inactivation in tumorigenesis, we have attempted to express transfected wild-type p53 in three p53-negative tumor cell lines: murine DP16-1 Friend erythroleukemia cells, human K562 cells, and SKOV-3 cells. We found that aberrant p53 proteins, which differ from wild-type p53 by a single amino acid substitution, were expressed stably in these cells, whereas wild-type p53 expression was not tolerated. The inability of p53-negative tumor cell lines to support long-term expression of wild-type p53 protein is consistent with the view that p53 is a tumor suppressor gene.

Friend virus-induced murine erythroleukemia provides a model of multistage carcinogenesis characterized by an early stage during which there is a polyclonal expansion of infected but nonmalignant erythroid progenitor cells and a later stage in which there is clonal expansion of malignant cells (9, 55). At least three genetic changes are associated with Friend virus-induced leukemogenesis. First, gp55, the membrane glycoprotein encoded by the *env* gene of Friend spleen focus-forming virus, can bind to the erythropoietin receptor and may mimic the action of erythropoietin, causing prolonged proliferation of infected cells in the absence of the growth factor (33). Second, the cellular *Spi-1* gene is frequently activated as the result of spleen focus-forming provirus integration adjacent to this locus (39). In erythroleukemias induced solely by Friend murine leukemia virus, a second, distinct locus, *Fli-1*, is a frequent integration site for the virus (7). Finally, inactivation of the cellular p53 gene is a common feature of erythroleukemias induced either by the Friend virus complex or by Friend murine leukemia virus alone (6, 40).

Tumorigenic Friend cell lines (FCLs) can be generated from malignant spleen cells in the late stage of the disease. Independent FCLs exhibit a variety of mutations in the p53 gene, including deletions (8, 14, 42, 50), proviral insertions (8, 27), and point mutations (41), which result in complete extinction of p53 expression, expression of truncated p53 polypeptides, or expression of abnormal p53 protein. In all FCLs that have been examined, mutation of one p53 allele is accompanied either by mutation of the homologous allele (27) or, more frequently, by complete absence of the homologous allele. The consequences of these diverse mutations in the p53 gene may be equivalent, resulting in functional inactivation of p53 (5, 41). These mutations, which occurred in vivo during the progression of Friend disease (14, 40), appear to confer a selective growth advantage to virus-infected cells and may therefore constitute a necessary step for development of malignant Friend disease.

Selection against wild-type p53 expression is not restricted to murine erythroleukemia. Deletion of one p53 allele together with missense mutation of the remaining allele is a common feature of human lung (43, 54), colon (3, 43), and breast (43) carcinomas. Rearrangements of the p53 gene have also been detected in osteogenic sarcomas (37), in the blast crisis stage of chronic myelocytic leukemia (CML) (1, 31, 36), and in hepatocellular carcinoma cells (12). Thus, the p53 gene appears to be a target for mutation in a wide range of malignancies.

p53 alleles carrying missense mutations, particularly those isolated from various tumor cell lines, including FCLs, can cooperate with an activated *ras* gene to transform early-passage rat embryo fibroblasts (REFs) (17, 19, 28, 45, 49) or adult rat WAXI cells (30), possibly through a dominant negative mechanism (26). Wild-type p53 not only lacks this transforming activity (17, 22, 28) but also appears to suppress transformation by other oncogenes in gene transfer experiments (18, 21). Overall, these data suggest that p53 protein may be a negative regulator of cell growth that is inactivated in the course of certain malignancies and which should therefore be classified as the product of a tumor suppressor gene.

Gene replacement provides another approach to demonstrate tumor suppression function. This has been demonstrated for the wild-type *Rb* gene (11, 29) and for chromosome 11 markers associated with Wilms tumor (52, 57). Introduction of the *Rb* gene into *Rb*-negative tumor cells resulted in suppression of the tumorigenic phenotype (11, 29). In this study we wished to examine the effects of introducing a wild-type murine p53 gene into a p53-negative FCL and a wild-type human p53 gene into two human tumor cell lines that do not express endogenous p53 protein or RNA. We demonstrate that wild-type p53 expression is incompatible with continued growth of transformed cells in tissue culture.

MATERIALS AND METHODS

Cell lines. Three p53-negative transformed cell lines were used as recipients in gene transfer experiments: FCL DP16-1 (40), CML cell line K562 (2), and human ovarian adenocarcinoma cell line SKOV-3 (13). Cell lines were grown in

* Corresponding author.

minimal essential medium (MEM) supplemented with antibiotics and 10% fetal calf serum (FCS) (Bockneck, Inc.).

Recombinant plasmids. pMR53 contains a mutant murine p53 gene (Arg-193 to Pro) in the pUC18 cloning vector; p11-4 (kindly provided by A. Levine, Princeton University) encodes a wild-type murine p53 cDNA under the transcriptional control of the simian virus 40 (SV40) early-gene promoter-enhancer; pECM53 contains a wild-type murine p53 cDNA in the SV40-derived expression vector pECE (20); pβw contains a wild-type murine cDNA cloned into the expression vector pHβAPr-1-neo (24); and pEW53-6 contains a wild-type murine p53 gene, isolated from normal BALB/c mouse liver DNA as a single 16-kb *Eco*RI fragment, cloned into pECE (27a). pECH53 contains a wild-type human p53 cDNA (38) in pECE; pED-1 differs from pECH53 in having a point mutation in the human p53 cDNA that changes Cys-135 to serine; pβHwt contains human wild-type p53 cDNA cloned into the pHβAPr-1-neo expression vector. pSV_2neo and pHMR272 were used as selectable markers.

DNA transfer. DP16-1 and K562 were electroporated with p53-bearing plasmid DNA and a plasmid encoding a drug resistance marker gene, using a Bio-Rad electroporator and capacitance extender at a setting of 200 to 300 V, 500 or 960 μF, for one pulse. A total of 3×10^6 cells were washed and resuspended in HBS buffer (15). DNA (total of 20 to 40 μg) was linearized with an appropriate restriction enzyme (Boehringer-Mannheim Corp.), extracted with phenol-chloroform, precipitated in ethanol, and resuspended in an appropriate volume of HBS. DNA in HBS was added to the cells and incubated at room temperature for 10 min prior to electroporation. After electroporation, the cells were allowed to recover for 10 min and then slowly reintroduced into MEM plus 10% FCS. At 24 h after electroporation, cells were seeded into 1.3% methylcellulose at a density 0.5×10^5 to 1×10^5 cells per ml in MEM supplemented with 20% FCS and Geneticin (Sigma Corp.) at 0.4 mg/ml or hygromycin (Sigma) at 0.2 mg/ml for K562 cells or 0.6 mg/ml for DP16-1 cells.

Samples (10 μg total) of p53-bearing DNA, pSV_2neo, and NIH 3T3 carrier DNA were introduced into 5×10^5 SKOV-3 cells by the method of calcium phosphate coprecipitation (23, 58). At 24 h posttransfection, cells were trypsinized and split into six tissue culture dishes (60 mm; Nunc Inc.) in MEM–10% FCS–Geneticin (2 mg/ml). After 2 weeks of growth in selective media, colonies were scored, picked, and expanded for protein, DNA, and RNA analysis. Similarly, early-passage REFs were cotransfected with p53-bearing plasmid DNA and pSV_2neo and selected in Geneticin (0.4 mg/ml). Individual colonies were analyzed by polymerase chain reaction (PCR) amplification for the presence of transfected p53 sequences.

p53 protein labeling and immunoprecipitation. Cells were labeled for 1 h at 37°C with 0.2 mCi of [^{35}S]methionine in 1 ml of MEM lacking methionine or 1.0 mCi of $^{32}P_i$ in 1 ml of MEM lacking phosphate. Cells were lysed for 30 min on ice in 0.3 to 0.6 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris [pH 8.0], 0.5 mM phenylmethylsulfonyl fluoride). The cell debris was removed by centrifugation, and the remaining supernatant was precleared by incubation overnight with 5 μg of nonspecific immunoglobulin G2a murine monoclonal antibody, followed by addition of 0.5 ml of a 10% suspension of Formalin-treated *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring), which were then removed by centrifugation. The supernatant was immunoprecipitated with appropriate antibodies: PAb419, a control monoclonal antibody against SV40 large T antigen (25); PAb421 (25), a pan-specific monoclonal antibody against p53; PAb246 (59), a murine p53-specific monoclonal antibody that recognizes an epitope present on wild-type p53 but not generally on mutant forms of p53; RA3 2C2 (16, 48), a murine p53-specific monoclonal antibody; and PAb1801 (4), a monoclonal antibody specific to human p53. Immune complexes were collected with 50 μl of *S. aureus* cells, washed twice in NET-gel buffer (150 mM NaCl, 5 mM EDTA [pH 8.0], 50 mM Tris [pH 7.4], 0.05% Nonidet P-40, 0.02% sodium azide, 0.25% gelatin), and then eluted into 30 μl of sample buffer (2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol, 25 mM Tris [pH 6.8]) plus 0.1 M dithiothreitol by heating at 70°C for 15 min. Samples were loaded onto a 12.5% polyacrylamide gel in the presence of SDS. Electrophoresis was at 35 mA. Dried gels were exposed to Eastman Kodak XAR-5 film.

Isolation of DNA and RNA. Subconfluent cells were resuspended in 4 M guanidinium isothiocyanate (35), which was layered onto a 5.7 M CsCl cushion and centrifuged 16 h at 36,000 rpm. DNA was harvested from the middle third of the gradient, and RNA was harvested from the pellet at the bottom of the centrifuge tube. DNA was dialyzed against 10 mM Tris (pH 8.0)–1 mM EDTA (pH 8.0), extracted twice with phenol and twice with phenol-chloroform, precipitated with ethanol, and analyzed by restriction enzyme digestion and Southern blot analysis. RNA was extracted with phenol-chloroform and chloroform, precipitated with ethanol, and analyzed by Northern (RNA) blot analysis.

Amplification of transfected DNA by PCR. The presence of transfected pED-1 DNA in K562 clones was determined using PCR (51). Briefly, 2×10^4 cells were resuspended in 30 μl of distilled H_2O and boiled for 10 min. Then 1 μl of proteinase K (Sigma Corp.) at 10 mg/ml was added to the cell lysates, which were incubated for 2 h at 55°C. The proteinase K was inactivated by boiling the cell lysates again for 10 min. DNA in the lysates was then amplified in a reaction mixture containing 67 mM Tris hydrochloride (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 6.8 μM EDTA, 10 mM 2-mercaptoethanol, bovine serum albumin (170 mg/ml), and 1.5 mM each deoxynucleoside triphosphate. Thirty cycles of denaturation (94°C for 1 min), annealing (65°C for 1 min), and elongation (72°C for 2 min) were carried out in the presence of 2.5 U of *Taq* DNA polymerase (Perkin Elmer Cetus Corp.), using specific primers JS-3 (antisense, human exon 11, GAGGGAGAGATGGGGGTGGGAGGCTGTC) and JS-6 (sense, human exon 5, CCCATCCTCACCATCATCA CACTGGAAG). This amplification was expected to produce a 420-bp fragment from pED-1 but not from endogenous genomic p53 sequences. The products of amplification were electrophoresed on a 1% agarose gel, and the DNA was visualized by ethidium bromide staining.

Similarly, the presence of pEW53-6 was analyzed in clones arising from coelectroporation into FCL DP15-1 by PCR amplification using the pEW53-6 plasmid-specific primers 53-7 (antisense, mouse exon 1, AAGCATTCAGG) and PECEP-1 (sense, polylinker, CTAAGCTTGTCGACGG TACCC). Individual REF colonies were analyzed for the presence of plasmid-borne p53 sequences by using specific primers: pECH53 was detected by using primers JS-3 and JS-6, and pEW53-6 was detected by using 53-7 and PE-CEP-1.

RESULTS

Transient wild-type p53 expression assay. We used recombinant plasmids capable of expressing either mouse or hu-

man p53 to investigate the effects of wild-type p53 expression in transformed cells that do not express endogenous p53 protein or RNA. pEW53-6 contains the entire p53 gene cloned from BALB/c mouse liver DNA into the SV40-derived expression vector pECE. pβw contains a murine wild-type p53 cDNA cloned downstream of the human β-actin promoter in the vector pHβAPr-1-neo. pECH53 and pβHwt contain the wild-type human p53 cDNA in the expression vectors pECE and pHβAPr-1-neo, respectively.

To ensure that these vectors were capable of expressing the predicted p53 protein, the recombinant plasmids were transfected into recipient cells, and protein expression was monitored 48 or 72 h later by metabolic labeling and immunoprecipitation. Plasmids encoding wild-type murine p53 were transfected into SV40-transformed monkey COS-1 cells. Monoclonal antibodies specific for mouse p53 (RA3 2C2 and PAb246) detected expression of wild-type p53 protein from the transfected p53 gene or cDNA in these cells (Fig. 1). PAb246 recognizes a conformation-dependent epitope on normal murine p53 protein that is commonly missing on mutant forms of the protein. These antibodies were unable to recognize the endogenous monkey p53 protein in cell extracts prepared from mock-transfected COS-1 cultures (Fig. 1A, mock). Coprecipitation of SV40-large T antigen and monkey p53 was observed with RA3 2C2 and PAb246 only in cells transfected with murine wild-type p53 DNA, indicating that one or both of these polypeptides was bound to mouse p53 protein in the transfected cells. The presence of protein complexes was also demonstrated by coimmunoprecipitation of mouse p53, monkey p53, and large T antigen with PAb419 directed against large T antigen and PAb421 directed at both primate and rodent p53.

pECH53 and pβHwt, which encode wild-type human p53, were transfected into the p53-negative ovarian adenocarcinoma cell line SKOV-3, and transient expression was monitored as described above. p53 synthesis was detected by immunoprecipitation with PAb421 and by PAb1801, a monoclonal antibody specific for primate p53 (Fig. 1B). Synthesis of p53 protein continued for up to 120 h after transfection (Fig. 1B, lanes 9 and 10).

Electroporation of p53 DNA into p53-negative FCL DP16-1. Plasmid pMR53 carries a mutant p53 gene isolated from FCL CB7 (40) and has been described previously (41, 49). A single G-to-C transversion in the p53 coding sequence predicts a change in the amino acid residue at position 193, from arginine to proline (41). pMR53 and pEW53-6, a plasmid encoding wild-type murine p53, were separately electroporated into DP16-1 cells together with either pSV_2neo (conferring resistance to G418) or pHMR272 (conferring resistance to hygromycin) to facilitate isolation of clones that had incorporated foreign DNA. Both p53 alleles of the recipient cell line, DP16-1, are rearranged; consequently, no endogenous p53 protein is expressed in these cells (40). DP16-1 cells electroporated with plasmids encoding mutant or wild-type p53 were plated into methylcellulose supplemented with G418 or hygromycin. Colonies of drug-resistant cells were isolated, expanded into cell lines, and assessed for (i) protein and RNA expression of the plasmid-borne p53 gene and (ii) presence of the transfected DNA (Table 1). All 18 drug-resistant colonies examined from three independent electroporations of pMR53 into DP16-1 expressed the mutant p53 protein abundantly (Fig. 2, lanes 1 to 4). pMR53-generated p53 protein was recognized by the monoclonal antibody PAb421 but not by PAb246, as expected of a mutant p53 polypeptide.

The efficiency of introducing pEW53-6 into DP16-1 cells was markedly lower than the efficiency of introducing pMR53. Although a similar number of G418-resistant colonies was produced following coelectroporation of pSV_2neo with pMR53 or pEW53-6, Southern blot analysis of DNA from several clones revealed that only 3 of 15 clones had incorporated pEW53-6 DNA (Fig. 3A), compared with 18 of 18 electroporated with pMR53. None of the three transfected clones expressed p53 protein. One of the clones expressed a truncated p53-related transcript, whereas another clone expressed no detectable p53 transcript (Fig. 3B). The third clone was not examined for RNA. Structural alterations in the transfected DNA were not detected by Southern blot analysis.

Coelectroporation of DP16-1 cells with pSV_2neo and p11-4 or pECM53, two cDNA expression vectors encoding wild-type mouse p53, failed to generate G418-resistant colonies containing wild-type p53 cDNA sequences. None of nine clones examined resulting from electroporation with pECM53 and none of five clones examined from electroporation with p11-4 carried the transfected p53 cDNA, as determined by Southern blot analysis. Together, these data suggest that the DP16-1 cells support the long-term expression of mutant p53 polypeptides but not wild-type p53 protein and that these cells may eliminate genes detrimental to their growth.

The transforming activity of certain mutant p53 alleles may be the result of dominant negative mutations (3, 5, 21, 32, 53). Mutant p53 polypeptides may act in *trans* to render the endogenous wild-type p53 protein functionally inactive. One example of this proposed interaction is provided by the mutant p53 allele present in pMR53, which is able to immortalize and, in cooperation with *ras*, transform primary rat embryo cells in the presence of endogenous wild-type rat p53 (49). We reasoned that if the aberrant p53 protein encoded by pMR53 could also act in *trans* to neutralize wild-type mouse p53 protein, its synthesis in DP16-1 cells might confer resistance to expression of a transfected wild-type p53 gene. Accordingly, pMR53 was electroporated along with pHMR272 into DP16-1 cells. Eight hygromycin-resistant clones were picked at random; all eight expressed high levels of aberrant p53 protein ($PAb421^+$ $PAb246^-$) encoded by pMR53. One of these clones was selected as a recipient cell for further study and designated DP16-1 1.7. To increase the likelihood of maintaining a transfected wild-type p53 sequence in DP16-1 1.7 cells, an expression vector, pβw, that encodes both murine wild-type p53 and the *neo* gene was used. In six G418-resistant clones examined, there was no detectable expression of wild-type ($PAb246^+$) p53 protein, whereas the mutant p53 protein continued to be expressed abundantly. Four of these clones were examined for the presence of DNA by Southern analysis. Although all four clones contained the *neo* gene, only two clones contained murine wild-type p53 cDNA sequences apparently intact; the wild-type p53 cDNA appeared to be rearranged in a third clone and missing in the fourth clone (data not shown). To confirm the apparent inability of mutant murine p53 protein to act in *trans* to neutralize wild-type murine p53 protein, we turned to another FCL, DP15-1, which synthesizes high levels of endogenous, aberrant ($PAb246^-$) p53 protein. DP15-1 cells were coelectroporated with pEW53-6 and pSV_2neo. Eleven G418-resistant clones from this electroporation were analyzed by PCR amplification (as described in Materials and Methods) for the presence of plasmid-borne p53 sequences. Four of the eleven carried the exogenous wild-type p53 sequences. However, none of the four expressed wild-type ($PAb246^+$) p53 protein (data not shown).

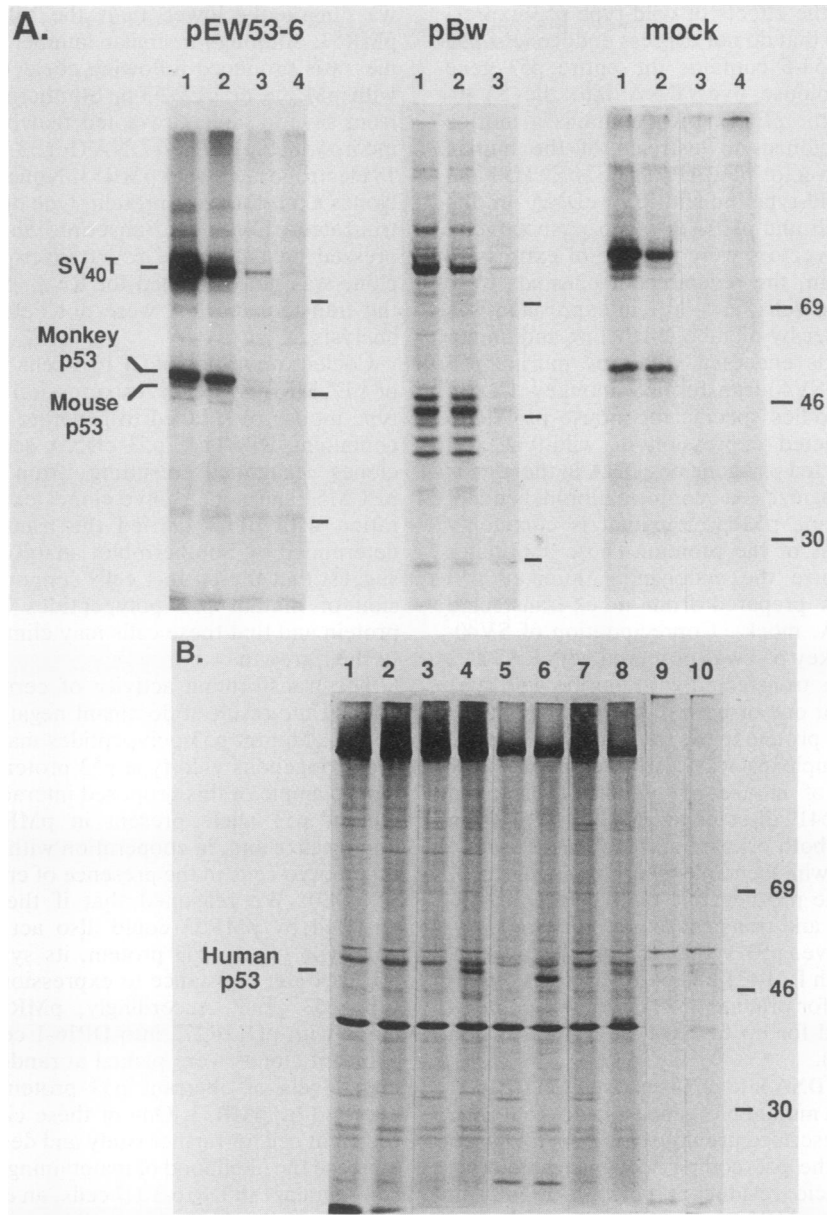

FIG. 1. Transient expression of wild-type p53 in COS-1 and SKOV-3 cells. (A) COS-1 cells were transfected with pEW53-6 or pβw encoding wild type murine p53 or were mock transfected; proteins were labeled 72 h later with $^{32}P_i$ and immunoprecipitated with the following monoclonal antibodies as described in Materials and Methods: PAb419 (lanes 1), a monoclonal antibody specific for SV40 large T antigen; PAb421 (lanes 2), a monoclonal antibody recognizing murine and primate p53; and PAb246 (lanes 3) and RA3 2C2 (lanes 4), monoclonal antibodies specific for murine p53. (B) SKOV-3 cells were transfected with pECH53 (lanes 3 and 4) or pβHwt (lanes 7 to 10), both encoding human wild-type p53, or were mock transfected (lanes 1 and 2); proteins were labeled with [^{35}S]methionine at 72 and 120 h (lanes 9 and 10) posttransfection and immunoprecipitated with PAb419 (lanes 1, 3, 5, 7, and 9) and PAb421 (lanes 2, 4, 6, 8, and 10). The acute myelogenous leukemia cell line OCIM2 (44), which expresses human p53, was labeled as a positive control (lanes 5 and 6).

Expression of p53 in K562 and SKOV-3 cells. In the next series of experiments, we wished to determine whether other transformed cells were similar to DP16-1 and DP15-1 in their inability to support long-term expression of wild-type p53. The human CML-derived cell line K562 and the human ovarian adenocarcinoma cell line SKOV-3 were used as recipients in gene transfer experiments. Neither of these cell lines expresses endogenous p53 protein or p53 transcripts (34, 47; our unpublished observations). pECH53 (wild-type human p53 cDNA), pED-1, a derivative plasmid carrying a single point mutation that converts the cysteine residue at position 135 to serine, and pMR53 were individually electroporated into K562 cells together with pHMR272 as a selectable marker.

Two of five hygromycin-resistant colonies resulting from electroporation of pMR53 into K562 cells expressed high levels of the mutant murine protein (Fig. 2, lanes 5 to 10). The three remaining clones did not carry the transfected pMR53 DNA by Southern analysis. In addition, pED-1, encoding an aberrant human p53 protein, was expressed in 1

TABLE 1. Expression of p53 in p53-negative cell lines

Recipient cell line	Plasmid	No. of clones expressing p53 protein/ no. of clones examined[a]
DP16-1 (FCL)	pEW53-6 (murine wild-type gene)	0/3
	pMR53 (Arg-193 to Pro)	18/18
K562	pECH53 (human wild-type cDNA)	0/17
	pED-1 (Cys-135 to Ser)	1/1
	pMR53	2/2

[a] Only clones that had incorporated transfected p53 sequences as assessed by Southern blot or PCR analysis are included. Southern blot analysis was not performed on the 18 DP16-1-derived clones resulting from electroporation with pMR53, since all expressed the predicted foreign p53 protein.

of 11 clones examined (Fig. 2, lanes 11 and 12). Only the expressing clone contained the transfected cDNA by PCR analysis (data not shown). The efficiency of stably incorporating two separate plasmids into K562 cells by electroporation was much lower than for DP16-1. The efficiency of transformation using electroporation is known to vary among different cell types and species (46); in particular, the efficiency of introducing two unlinked genes can differ widely (10, 56).

Of 40 hygromycin-resistant clones examined following coelectroporation of K562 with pECH53 and pHMR272, 17 contained the transfected wild-type p53 cDNA sequences. This was demonstrated by Southern blot analysis of *Sma*I-digested DNA, which revealed a p53 cDNA-specific fragment of 1.4 kb (Fig. 4A and B). However, further examination of these 17 clones by Southern blot analysis of *Pvu*II-digested DNA, which should yield a 970-bp hybridizing fragment, revealed that sequences 5′ to the cDNA encompassing the SV40 promoter-enhancer region of the expres-

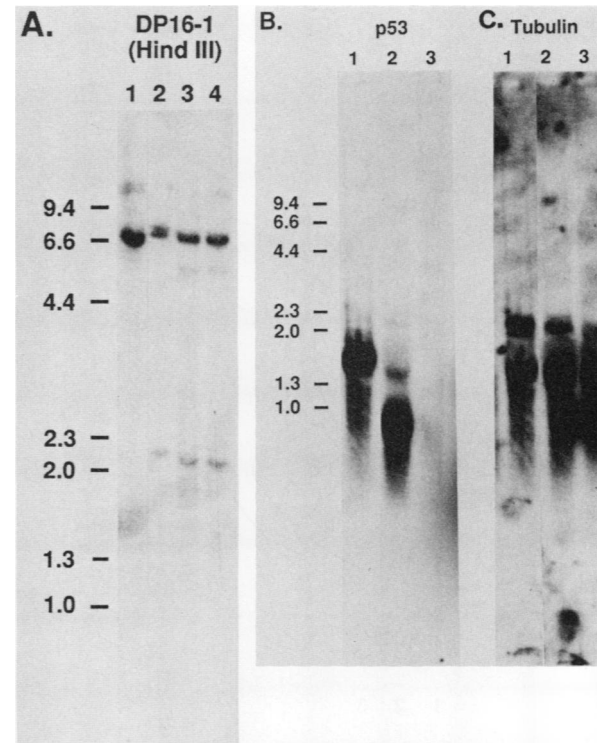

FIG. 3. DNA and RNA analysis of independent clones of DP16-1 cells electroporated with pEW53-6. (A) Southern blot analysis of three DP16-1 clones containing the wild-type p53 gene of pEW53-6. DNA from the three clones (lanes 2 to 4) and from parental DP16-1 cells (lane 1) was digested with *Hin*dIII. A p53 cDNA fragment used as probe will hybridize to a 2-kb fragment derived from the pEW53-6 plasmid that is not present on the rearranged endogenous p53 alleles of DP16-1 cells. (B) RNA from two of the three clones shown in panel A was hybridized to a p53 cDNA probe (lanes 2 and 3). RNA from FCL DP15-1, known to express p53 protein, was included as a control (lane 1). The same filter was reprobed with a tubulin-specific probe (C).

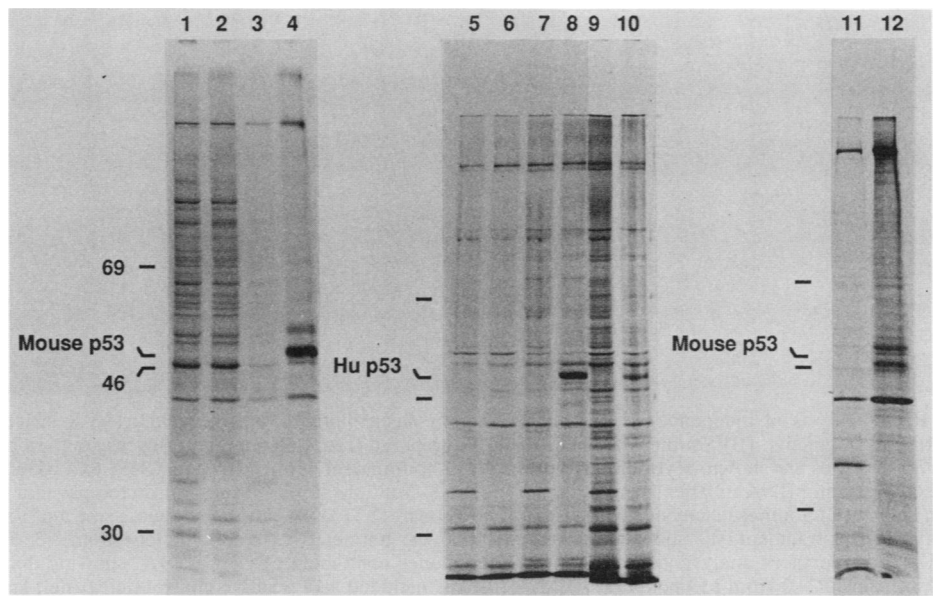

FIG. 2. Stable expression of mutant p53 in DP16-1 and K562 cells. Cells were coelectroporated with plasmids encoding mutant p53 protein and a selectable marker. Colonies were expanded, and the proteins of independent clones were labeled with [^{35}S]methionine and immunoprecipitated with PAb419 (lanes 1, 3, 5, 7, 9, and 11) or PAb421 (lanes 2, 4, 6, 8, 10, and 12). Lanes: 1 and 2, DP16-1 cells; 3 and 4, DP16-1 cells electroporated with pMR53; 5 and 6, K562 cells; 7 and 8, OCIM2 cells (positive control); 9 and 10, K562 cells electroporated with pED-1; 11 and 12, K562 cells electroporated with pMR53.

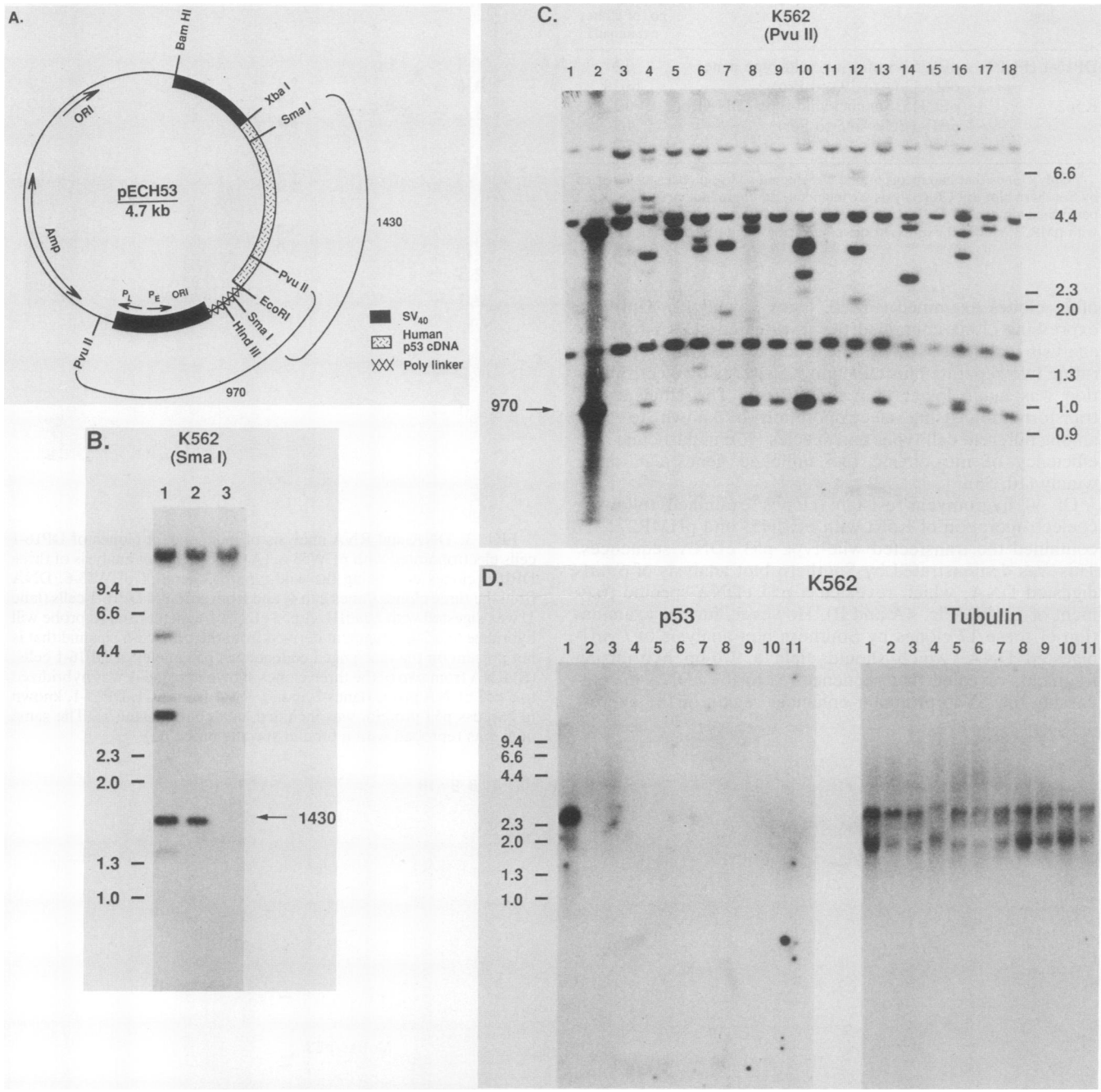

FIG. 4. DNA and RNA analysis of independent clones of K562 cells electroporated with pECH53. (A) Restriction map of pECH53 showing relevant restriction fragments. (B) Southern analysis of DNA prepared from independent drug-resistant clones and digested with *Sma*I. Three representative clones are shown. Hybridization of the 1,430-bp fragment derived from pECH53 to a p53 cDNA probe indicates the presence of transfected plasmid DNA in lanes 1 and 2 but not in lane 3. Multiple copies of the transfected plasmid DNA may be present in the sample shown in lane 1. (C) Southern analysis of *Pvu*II-digested DNA from 17 DNA-positive clones, using a p53-specific cDNA probe. The expected 970-bp hybridizing fragment (see above and text for details) was not detected in certain clones (lanes 3, 5, 7, 12, and 14). Lane 1 is parental K562 DNA. (D) Northern analysis of independent K562 clones containing pECH53 DNA, showing that none expressed the expected human p53 transcript. RNA from p53-positive OCIM2 cells was included as a positive control (left panel, lane 1). The same filter was reprobed with a tubulin-specific probe (right panel).

TABLE 2. Effect of wild-type p53 on plating efficiency of SKOV-3 cells

Plasmid	No. of G418-resistant colonies/ 5×10^5 cells transfected		
	Expt 1	Expt 2	Expt 3
pHβAPr-1-neo (control)	144	28	
pβHwt (human wild-type cDNA)	46	0	
pECE + pSV_2neo (control)	78	53	12
pECH53 + pSV_2neo (human wild-type cDNA)	0	0	3
pED-1 + pSV_2neo (human mutant cDNA)			62

sion vector were missing in 5 of 17 clones (Fig. 4C). Most important, K562 clones, which appeared to contain a completely intact wild-type p53 cDNA as well as vector-derived transcriptional control sequences, did not express p53 protein or p53 transcripts (Fig. 4D). The ability of the pECE-derived expression vector pED-1 to support long-term expression of mutant p53 polypeptide in K562 cells demonstrated that SV40 transcriptional control sequences are active in K562 cells and that a single amino acid change in the wild-type p53 protein is sufficient to permit long-term expression in K562 cells.

SKOV-3 is a human adenocarcinoma cell line that does not express endogenous p53 protein (Fig. 1B) or mRNA (data not shown). SKOV-3 cells grow as monolayers and can be transfected with DNA by calcium phosphate coprecipitation. We found that human wild-type p53 protein was expressed transiently in SKOV-3 cells after transfection of pECH53 or pβHwt (Fig. 1B). We next wanted to determine whether wild-type p53 could be expressed stably in these cells. pECH53 plus pSV_2neo or pβHwt alone was transfected into SKOV-3 cells, and stable clones were selected by growth in G418-supplemented medium. pβHwt carries both the human wild-type p53 cDNA, under the transcriptional control of the human β-actin promoter, and the gene conferring resistance to G418. Thus, G418 resistance would be dependent on stable incorporation of a plasmid capable of expressing wild-type p53 protein.

Cotransfection of pECH53 with pSV_2neo, in comparison with pSV_2neo alone, consistently reduced the number of G418-resistant colonies in three independent experiments. This inhibition of stable transformation was not seen with pED-1; in fact, expression of pED-1 appeared to enhance the survival of transfectants (Table 2). Thus, expression of wild-type p53 but not mutant p53 inhibits growth of SKOV-3 cells. Results of experiments comparing pβHwt with the parental vector pHβAPr-1-neo were consistent with these observations. pβHwt, a single plasmid that expresses both normal p53 protein and resistance to G418, generated fewer colonies than did the parental plasmid which lacks p53 coding sequences. Indeed, only one transfection with pβHwt resulted in growth of G418-resistant colonies. Five of these colonies were expanded and analyzed for DNA content. Southern blot analysis using a human p53-specific probe revealed that the p53 cDNA was present in only one of these clones (the expected 3.5-kb *Nco*I fragment in Fig. 5B, lane 6, and *Eco*RI junction fragment in Fig. 5C, lane 12); further analysis with a probe for the *neo* gene showed that it was present as the expected 830-bp *Nco*I fragment in all five clones (in multiple copies) (Fig. 5). Thus, selective loss of wild-type p53 sequences coupled with retention of *neo* sequences required for growth in drug-supplemented medium had occurred on the same plasmid.

Expression of wild-type p53 in REFs. To ensure that the failure to generate stable clones expressing wild-type p53 protein in transformed cells was not due to general cytotoxicity of overexpressed wild-type p53, early-passage REFs were cotransfected with plasmids encoding wild-type p53 and pSV_2neo at a molar ratio of 10:1. If high levels of wild-type p53 protein were mediating a nonspecific toxicity in cells, the number of G418-resistant colonies resulting from cotransfection of wild-type p53 with pSV_2neo should be lower than in controls transfected with the parental plasmid and pSV_2neo. The results of two independent transfections (Table 3) showed no significant difference in the number of G418-resistant colonies obtained. Although a 10-fold molar excess of p53-bearing plasmid was used in these cotransfection experiments, we wished to confirm the presence of exogenous wild-type p53 DNA in the G418-resistant colonies. Therefore, several colonies resulting from transfections with pEW53-6, pECH53, and pECE were analyzed for the presence of plasmid-borne p53 sequences by PCR amplification, using primers specific for the exogenous DNA. Four of four colonies transfected with pEW53-6 and two of two transfected with pECH53 yielded the expected PCR amplification products, whereas colonies that had been transfected with the parental vector lacking p53 sequences did not yield any amplification products, as expected.

DISCUSSION

The data presented here demonstrate that stable expression of wild-type p53 is incompatible with continued growth of transformed cells. While wild-type human and murine p53 could be expressed in transformed cells in short-term expression assays, attempts to detect long-term expression of wild-type p53 protein in any of three p53-negative transformed cell lines failed. In contrast, stable expression of mutant forms of murine and human p53 protein, differing from the corresponding wild-type sequence by a single amino acid residue, was readily detected in these same transformed cell lines.

Our data demonstrate that p53-negative tumor cells respond to introduction of plasmid-borne wild-type p53 in two ways. One involves the selective loss of clones expressing p53, while the other involves mechanisms that restrict expression of the introduced wild-type p53 sequence. Both types of response are evident in DP16-1 transfectants. Transfection with either pEW53-6, p11-4, or pECM53, all encoding wild-type p53, rarely led to stable integration of the exogenous wild-type p53 DNA. In the few cases in which integration occurred, the wild-type protein was not detected; one clone expressed a truncated transcript, while the other expressed no detectable p53 transcript. In contrast, when this cell line was transfected with the pMR53 plasmid encoding mutant p53 protein, stable expression was seen in all clones that we examined. Both pEW53-6 and pMR53 contain an intact p53 gene, including the native p53 gene promoter, transcription initiation site, introns, and untranslated regions. Surprisingly, no significant difference in the number of drug-resistant colonies was observed when the drug resistance plasmid was transfected alone or cotransfected with wild-type p53 or with mutant p53. It is possible that DP16-1 cells are able to eliminate genes detrimental to their growth, as suggested previously by Finlay et al. (21) for Rat-1 cells transfected with a wild-type p53 gene.

Previously we demonstrated that the aberrant murine p53

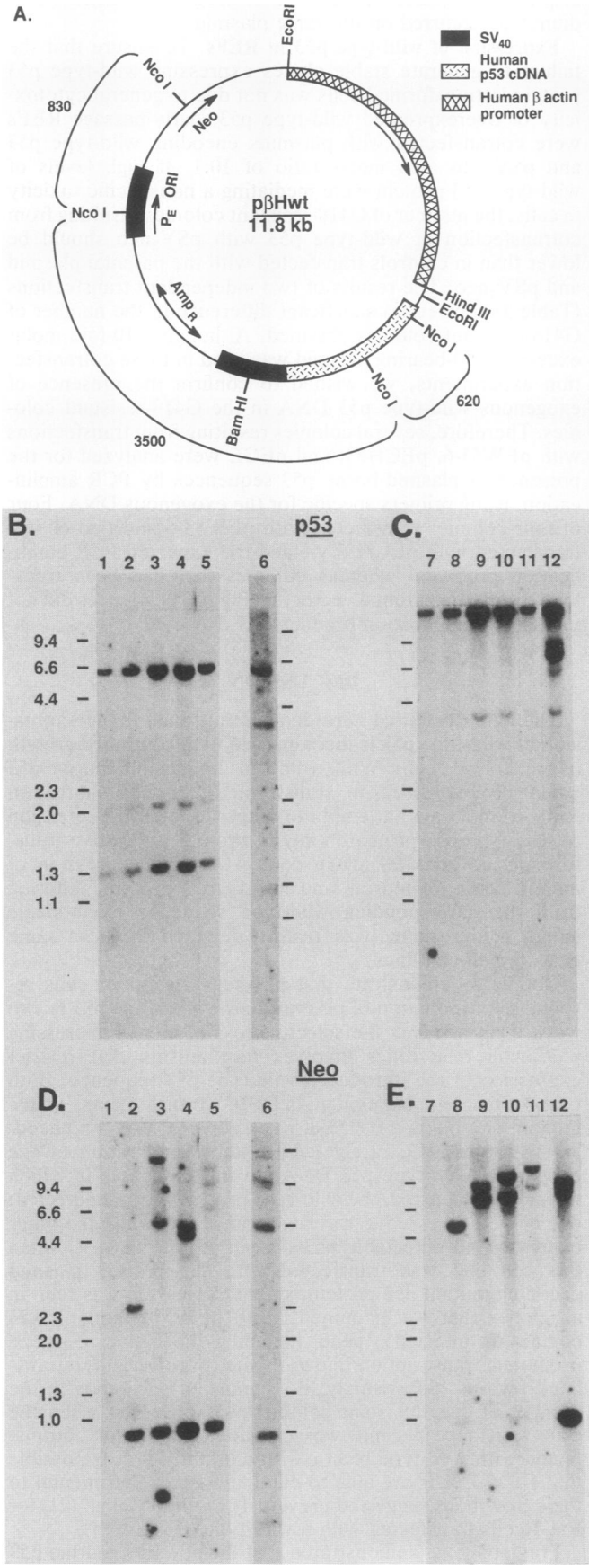

TABLE 3. Effect of wild-type p53 on plating efficiency of REFs

Plasmid	No. of G418-resistant colonies[a]	
	Expt 1	Expt 2
pEW53-6 + pSV$_2$neo (mouse wild-type gene)	62	
pECH53 + pSV$_2$neo (human wild-type cDNA)	43	89
pECE + pSV$_2$neo (control)	64	100
pβw (mouse wild-type cDNA)	64	
pHβApr-1-neo (control)	62	

[a] Average per 5 × 10^5 cells transfected.

protein encoded by pMR53 could immortalize normal REFs and complement an activated *ras* gene to transform these cells, possibly by a dominant negative mechanism. Mutant p53 protein may act in *trans* to render the endogenous rat p53 protein functionally inactive either through the formation of mutant/wild-type p53 heteroligomers or by competitive inhibition of the normal interactions between wild-type p53 protein and its targets in the cell. We reasoned, therefore, that the expression of mutant p53 protein encoded by pMR53 in DP16-1 cells might confer tolerance to wild-type p53 expression. This expectation, however, was not realized. In a related experiment, long-term expression of a wild-type p53 gene was not detected in DP15-1 Friend erythroleukemia cells, which overexpress endogenous aberrant p53 protein. The inability of DP16-1 and DP15-1 cells to express a wild-type p53 gene in the presence of mutant p53 protein may reflect the situation that arises during development of Friend disease in vivo. In all FCL that have sustained a mutation in the p53 coding sequence and consequently express an aberrant p53 polypeptide, there is loss of the corresponding normal allele. Our results demonstrate that expression of wild-type p53 in these cells is incompatible with cell growth even in the presence of elevated levels of mutant p53 protein.

The response of SKOV-3 cells to the introduction of wild-type p53 was easier to evaluate. Cotransfection experiments clearly demonstrated a reduction in the number of drug-resistant colonies as a result of introducing the wild-type p53 gene. Thus, expression of normal p53 in SKOV-3 cells resulted in inhibition of growth. In most cases, drug-resistant colonies that survived the introduction of wild-type p53 failed to express p53 as the result of specific deletion of the p53 cDNA sequences. In contrast, no such inhibitory effect on growth was seen with mutant p53. It is important to note that expression both of the mutant polypeptide encoded by pED-1 and of the normal p53 protein encoded by pECH53 was directed by the same (SV40) promoter-enhancer. Thus, the different effects on transformed cells observed with mutant and normal p53 protein cannot be ascribed to differences in the level of gene expression.

In the experiments described here, three different promot-

FIG. 5. Deletion of plasmid-derived p53-specific sequences in transfected SKOV-3 clones. (A) Restriction map of plasmid pβHwt. Relevant restriction sites are indicated. (B to E) Southern analysis. Five stable, drug-resistant clones of SKOV-3 cells transfected with pβHwt (lanes 2 to 6) and the parental SKOV-3 cells (lanes 1) were analyzed by Southern analysis of *Nco*I-digested (B and D) and *Eco*RI-digested (C and E) DNA, hybridized with a human p53-specific probe (B and C), and then rehybridized with a probe specific for the *neo* gene (D and E).

ers were used to direct p53 transcription: the human β-actin gene promoter, the SV40 early-gene promoter-enhancer, and the native murine p53 gene promoter. These promoters may be expected to have different efficiencies for directing transcription, yet we observed no differences in their abilities to support long-term p53 expression. The use of heterologous promoters to direct p53 expression raises an important question: Does overexpression of wild-type p53 result in general nonspecific cytotoxicity? To address this issue, we introduced wild-type p53 expression constructs into normal cells (REFs). The expression vectors used were the same as those used for transfer into the p53-negative tumor cells. The number of drug-resistant colonies obtained in a long-term assay after cotransfer of wild-type p53 DNA sequences and a drug resistance marker gene into normal, p53-positive cells was similar to the number of colonies obtained with the drug resistance plasmid alone. When a similar type of plating experiment was performed with the p53-negative tumor cell line SKOV-3, a different result was obtained, namely, suppression of colony growth by wild-type p53. This result argues that the inability of cells to sustain the continuous presence of p53 protein is a property of p53-negative tumor lines. A second experiment addresses the issue of nonspecific toxicity. In a transient expression assay, in which wild-type human p53 cDNA under the control of the strong human β-actin promoter was introduced into the p53-negative SKOV-3 cell line, we were able to measure p53 protein synthesis directed by the cDNA for up to 5 days posttransfection by metabolic labeling and immunoprecipitation. Thus, SKOV-3 cells expressing a foreign wild-type p53 cDNA remain viable and metabolically active for at least 5 days. This finding again argues against generalized toxicity. These cells have either limited or no growth potential, since they do not have the capacity for sufficient cell divisions to give rise to macroscopic colonies.

Two conclusions may be drawn from these observations. First, p53-negative tumor cells cannot sustain the continuous presence of wild-type p53 protein. Although the genetic basis for lack of p53 expression in DP16-1 cells as well as in numerous other FCLs is clearly established (reviewed in references 6, 40, and 41), the mechanism(s) that prevents expression of p53 protein and mRNA in K562 or SKOV-3 tumor cells is not known. Loss of p53 expression, however, appears to be significant in determining the neoplastic phenotype of these cells, since reinstatement of wild-type p53 expression is incompatible with their continued growth. Second, the mutation giving rise to the altered (Arg-193 to Pro) murine p53 protein encoded by pMR53 occurred in a p53 allele isolated from an FCL, and the mutation giving rise to the altered (Cys-135 to Ser) human p53 protein encoded by pED-1 was detected in fresh blast cells obtained from a patient with acute myelogenous leukemia (52a). These mutations in their natural host may therefore have played a significant role in the development of malignancy, since the aberrant proteins have an effect on p53-negative tumor cells markedly different from that of the wild-type protein. These observations indicate that an important functional property of wild-type p53 is altered by these two specific point mutations.

Overall, these data demonstrate the inability of p53-negative tumor cell lines to support long-term wild-type p53 expression and are consistent with the view of p53 as a tumor suppressor gene.

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