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Tumor Suppressor p53: Analysis of Wild-Type and Mutant p53 Complexes

JO MILNER,* ELIZABETH A. MEDCALF, AND ALISTAIR C. COOK

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom

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It has been suggested that the dominant effect of mutant p53 on tumor progression may reflect the mutant protein binding to wild-type p53, with inactivation of suppressor function. To date, evidence for wild-type/mutant p53 complexes involves p53 from different species. To investigate wild-type/mutant p53 complexes in relation to natural tumor progression, we sought to identify intraspecific complexes, using murine p53. The mutant phenotype p53-246^0 was used because this phenotype is immunologically distinct from wild-type p53-246$^+$ and thus permits immunological analysis for wild-type/mutant p53 complexes. The p53 proteins were derived from genetically defined p53 cDNAs expressed in vitro and also from phenotypic variants of p53 expressed in vivo. We found that the mutant p53 phenotype was able to form a complex with the wild type when the two p53 variants were cotranslated. When mixed in their native states (after translation), the wild-type and mutant p53 proteins did not exhibit any binding affinity for each other in vitro. Under identical conditions, complexes of wild-type human and murine p53 proteins were formed. For murine p53, both the wild-type and mutant p53 proteins formed high-molecular-weight complexes when translated in vitro. This oligomerization appeared to involve the carboxyl terminus, since truncated p53 (amino acids 1 to 343) did not form complexes. We suggest that the ability of the mutant p53 phenotype to complex with wild type during cotranslation may contribute to the transforming function of activated mutants of p53 in vivo.

The tumor suppressor function of wild-type p53 is lost following activating mutation of the p53 gene. Interestingly, the mutated gene product appears to play an active role in tumor progression via a dominant negative effect (1, 6, 12, 22, 27). It has been proposed that the dominant effect of p53 mutants may reflect interaction of the mutant protein with wild-type p53, thereby inactivating the suppressor function of the latter. This suggestion gains credence from reports of mutant p53 binding to wild-type p53 in cells containing high-expression vectors for the mutant protein (5, 6, 24). However, in each case this evidence is based on murine/rat p53 protein complexes. We have now investigated the binding of mutant p53 with wild-type p53 within the same species, murine p53. In these studies, we have exploited the fact that activated mutants of murine p53 appear to be phenotypically distinct from the wild type; in particular, they frequently lack the epitope recognized by the monoclonal antibody PAb246 (12, 17, 26, 29). The mutant phenotype of p53 is further characterized by its reactivity with the monoclonal antibody PAb240 and its failure to react with PAb1620. Wild-type p53, on the other hand, is reactive with PAb246 and with PAb1620 but nonreactive with PAb240 (3, 9). Using these three antibodies to discriminate between wild-type and mutant forms of murine p53, we probed for mutant p53 bound to and coimmunoprecipitating with the wild type.

MATERIALS AND METHODS

Plasmids. The following plasmids were used: pSP65m53 (13), MSVcL-ala, and MSVcL-val (7). MSVcL-ala contains wild-type murine p53 cDNA with alanine at position 135 of the p53 polypeptide; MSVcL-val contains an activated mutant of murine p53 cDNA with valine replacing alanine at position 135 (12). For human p53, plasmid pSP65p53H8 was used; H8 is equivalent to the H1 form of human p53 (10). To obtain expression under the SP6 promoter, the cDNAs for p53$^{Ala\text{-}135}$ and p53$^{Val\text{-}135}$ were reconstructed by using pSP65m53. Briefly, the 3.15-kb *Xba*I-to-*Xho*I fragment from pSP65m53 was ligated with the 2.3-kb *Bam*HI-to-*Xho*I fragment of either MSVcL-ala or MSVcL-val. Following ligation, the products were used to transform *Escherichia coli* TG1, and colonies resistant to ampicillin were selected for analysis by size and restriction digests. The predicted pSP6p53-ala and pSP6p53-val constructs were characterized by loss of the *Xba*I site from the original plasmid pSP65m53, by the presence of the *Bam*HI site from the 2.3-kb fragment of plasmid MSVcL, and by the generation of 3.15- and 2.3-kb fragments after digestion with *Bam*HI and *Xho*I (Fig. 1).

Transcription and translation. The plasmids were amplified in *E. coli* TG1, purified, and used for transcription and translation of the p53 protein as detailed by Gamble and Milner (8). Truncated p53 was obtained by linearization with *Stu*I to cleave at nucleotides 1020 to 1036 at the carboxy terminus of the coding sequence for p53$^{Ala\text{-}135}$. Rabbit reticulocyte lysate was used for the translation step, and the immunology of the p53 protein was routinely tested by using the following panel of anti-p53 monoclonal antibodies: PAb421 (11), PAb242, PAb246, and PAb248 (29), RA3.2C2 (23), PAb200.47 (4), PAb1620 (cloned by Ball et al. [2]; characterized by Milner et al. [18]), and PAb240 (9). PAb1620 reacts with mouse and human p53 and is specific for the wild-type phenotype (3, 18). PAb240 is also reactive with mouse and human p53 but is specific for the mutant phenotype (3, 9). A single batch of rabbit reticulocyte lysate was used for the translations, since we have discovered that different batches of rabbit reticulocyte lysate can affect the immunological reactivity of the translated p53 polypeptide (3). The translated p53 was kept on ice (unless otherwise stated) and was used immediately in each experiment.

* Corresponding author.

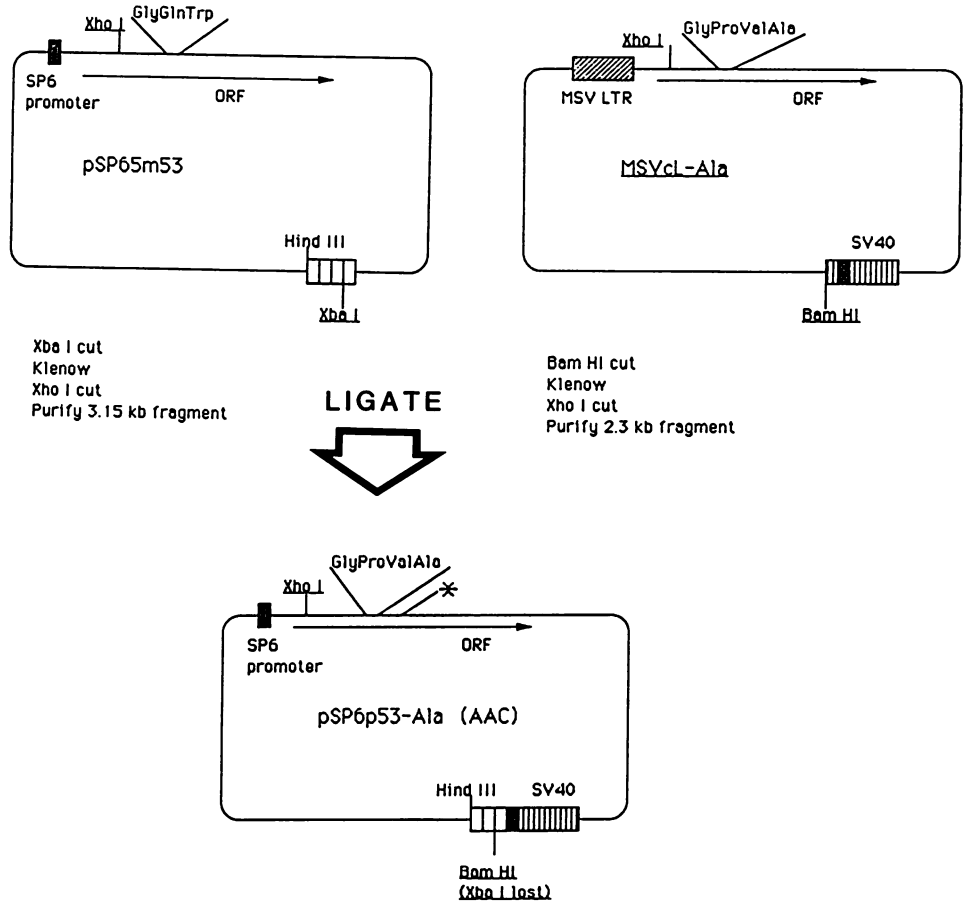

FIG. 1. Strategy used to obtain $p53^{Ala-135}$ and $p53^{Val-135}$ under expression controlled by the SP6 promoter. Plasmid pSP65m53 was cut with *Xba*I and *Xho*I to yield a 3.15-kb fragment containing the initial sequence of the p53 open reading frame (ORF) under control of the SP6 promoter. Plasmid MSVcL-ala was cut with *Bam*HI and *Xho*I to yield a 2.3-kb fragment containing the remaining p53 open reading frame contiguous with the initial p53 sequence in the 3.15-kb fragment generated from plasmid pSP65m53 (see above). These two fragments were ligated as indicated, with retention of the *Xho*I site from the original plasmids and of *Bam*HI from MSVcL-ala. The relative position of alanine 135 is indicated by an asterisk. The same strategy was used to construct pSP6p53-val.

Cell lines. The following murine cell lines were used: T3T3, a line of spontaneously transformed 3T3 cells kindly donated by David Lane; and 3T3tx, a line of 3T3 cells that underwent spontaneous transformation in our laboratory. These cell lines were chosen because they express different immunological forms of the p53 protein: T3T3 cells express $p53\text{-}246^{+}$, which is immunologically identical to the wild-type phenotype of murine p53 (see Fig. 4A); and 3T3tx cells express the mutant phenotype of p53, that is, $p53\text{-}246^{0}$ (see Fig. 4A). All lines were cultured in RPMI medium supplemented with newborn calf serum in an atmosphere of 10% carbon dioxide in air at 37°C. In general, the cells were not allowed to grow to more than 80% confluency and were subcultured at 1:10 for maintenance or at 1:3 for experimental analysis 24 h later.

Metabolic labeling. For the labeling of p53 translated in vitro L-[^{35}S]methionine (40.5 TBq/mmol; 555 MBq/ml) was added to the reticulocyte lysate to give a concentration of 5% (vol/vol). To this was added 1/10 volume of p53 mRNA. Translations were carried out for 1 h at 37°C and stopped by chilling on ice. For metabolic labeling, the cells were grown in 75-cm flasks and were labeled for 2 h at 37°C with 200 μCi of L-[^{35}S]methionine in 2 ml of methionine-free modified Eagle medium (Glasgow modification). Extracts were prepared by rinsing the monolayer with phosphate-buffered saline (PBS) and lysing the cells in situ in 2 ml of ice-cold lysis buffer, pH 8.0 (10 mM Tris hydrochloride, 0.14 M NaCl, 0.5% Nonidet P-40) for 30 min on ice. The lysate was then microfuged for 5 min at 4°C, and the supernatant was preadsorbed with *Staphylococcus aureus* (1/5 vol of a 10% suspension). After preadsorption for 10 min on ice, the *S. aureus* was pelleted, and the supernatant was used immediately for immunoprecipitation and immunoblotting.

Immunoprecipitation and immunoblotting. Aliquots of in vitro translation products were diluted 1:100 with ice-cold lysis buffer and immunoprecipitated with anti-p53 monoclonal antibodies for 1 h. Rabbit anti-mouse immunoglobulin was used as second antibody to ensure quantitative immunoprecipitation. Immune complexes were collected on *S. aureus* and analyzed as detailed previously (8). Cell lysates were not diluted but otherwise were immunoprecipitated in an identical manner. The following monoclonal antibodies were used routinely: PAb421, PAb248, PAb246, PAb1620, and PAb240. The monoclonal antibody PAb419, directed against the large T antigen of simian virus 40 (11), was included as a negative control. Immunoprecipitated proteins

TABLE 1. Immunoreactivity of p53 translated in vitro at 37°C, using a single batch of rabbit reticulocyte lysate, and of p53 from T3T3 and 3T3tx cells

Plasmid or cell line	Immunoreactivity with given monoclonal antibody								T-antigen binding[a]
	PAb421	RA3.2C2	PAb242	PAb246	PAb248	PAb200.47	PAb1620	PAb240	
Plasmids									
pSP6p53-ala	+	+	+	+	+	+	+	0	+
pSP6p53-val	+	+	+	0	+	+	0	+	0
Cell lines									
T3T3	+	+	+	+	+	ND[b]	+	0	+
3T3tx	+	+	+	0	+	ND	0	+	0

[a] Binding of p53 to simian virus 40 large T antigen after mixing in vitro for 10 min at 20°C (20).
[b] ND, Not determined.

were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 15% minigels with a 5% stacking gel, and run at 100 V for approximately 1 h. For immunoblotting, the unfixed gels were assembled immediately (8). Otherwise, the gels were fixed for 30 min (50% methanol, 7% acetic acid), fluorographed for 15 min (Amplify; Amersham, U.K.), dried, and exposed to Kodak X-Omat S X-ray film at −70°C. Procedures for sequential immunoprecipitations have been described previously (17, 19). For immunoblotting, the nitrocellulose blots were blocked with 1% bovine serum albumin in PBS-azide at 37°C for 1 h. The blots were rinsed in PBS-azide and probed with PAb421 plus RA3.2C2; these monoclonal antibodies are directed against discrete epitopes on the p53 polypeptide (28). The blots were rinsed, and bound antibody was detected by using ^{125}I-protein A (diluted 1:500 in 0.1% ovalbumin in PBS-azide). The blots were rinsed and dried (8) and then exposed to X-ray film with an intensifying screen as described above.

Size fractionation of p53 proteins translated in vitro. The ability of p53 proteins to form complexes when translated in vitro was investigated by size fractionation of the ^{35}S-labeled p53 proteins. Proteins were fractionated by gel filtration on a Superose 6 column (30 by 2 cm), using the Pharmacia FPLC (fast protein liquid chromatography) system. The column was run at room temperature with a flow rate of 0.5 ml/min. The eluant buffer was the same as that used for the preparation of cell and reticulocyte lysate samples for immunoprecipitation (0.4 M NaCl, 20 mM Tris base, 0.5% Nonidet P-40). The column was prewashed with 50 ml of eluant buffer before loading of the sample of reticulocyte lysate, diluted in eluant buffer if necessary. Twenty 1.0-ml samples were collected, and 100-μl were aliquots withdrawn to determine the distribution of trichloroacetic acid (TCA)-precipitable [^{35}S]methionine. The distribution of ^{35}S-labeled p53 was determined by dividing the residual volume of the fractions into three 300-μl aliquots and immunoprecipitating with PAb421, PAb1620, and PAb240. Total TCA-precipitable counts in the original reticulocyte lysate were determined by adding 1 μl of the undiluted lysate to 1 ml of aqueous solution containing 5.25% hydrogen peroxide, 50 mM NaOH, and 0.34 M methionine. The mixture was incubated at 37°C for 15 min and subsequently precipitated with 1 ml of 25% TCA. It should be noted that storage of reticulocyte lysate samples at −70°C resulted in the generation of high-molecular-weight complexes containing p53 (see Results). Therefore, all size fractionation studies were carried out with fresh samples of reticulocyte lysate analyzed within 2 h of translation.

RESULTS

Characterization of the immunological reactivity of the p53 proteins. The immunoreactivity of native p53 proteins was assessed by immunoprecipitation against a panel of anti-p53 monoclonal antibodies (Table 1). The binding of p53 to the large T antigen of simian virus 40 has also been assessed (20; unpublished observations), and these results are included in Table 1.

When translated in vitro, wild-type p53$^{Ala-135}$ adopted the wild-type phenotype, reactive with PAb246 and PAb1620 but nonreactive with PAb240. The activated mutant p53$^{Val-135}$ allele is temperature sensitive, with promoter and suppressor effects on cell growth at 37.5 and 32.5°C, respectively (16). The conformation of the p53$^{Val-135}$ polypeptide is also temperature sensitive: at 37°C, p53$^{Val-135}$ adopts the mutant p53 phenotype, whereas at 30°C it is indistinguishable from wild-type p53 (20). For the purposes of this study, it was therefore important to ascertain that p53$^{Val-135}$ was expressed as the mutant phenotypic form before embarking on wild-type/mutant p53 binding studies. Accordingly, all translations in vitro were performed at 37°C, and the immunoreactivities of p53$^{Ala-135}$ and p53$^{Val-135}$ were checked in every experiment. In addition, a common stock of rabbit reticulocyte lysate was used to avoid any of the batch-to-batch variation that can affect p53 protein conformation (3). At 37°C, p53$^{Ala-135}$ and p53$^{Val-135}$ were respectively translated into the wild-type and mutant phenotypes of p53.

T3T3 and 3T3tx cells express equivalent levels of p53 protein. Although the p53 genotypes for these cell lines are not yet known, the two cell lines are a useful source of high levels of phenotypically wild-type and mutant p53 proteins (Table 1).

Ability of human and murine p53 proteins to oligomerize when mixed in vitro. The ability of native p53 proteins to form complexes when mixed in vitro was determined by mixing radiolabeled human p53 with unlabeled p53$^{Ala-135}$. Both proteins were expressed in vitro (see Materials and Methods); after translation, equal aliquots of each were mixed and incubated at 20°C for 15 min. The translation mix was subsequently diluted, and equivalent aliquots were immunoprecipitated with PAb246, with PAb1620, and with PAb240. Radiolabeled human p53 was clearly detectable in the immunoprecipitations with PAb246 (Fig. 2, lane 2). Since PAb246 is specific for murine p53 and does not react with human p53 (see, for example, Fig. 2, lane 1), this result demonstrates coimmunoprecipitation of human p53 in complex with wild-type murine p53. The conformation of the human p53 polypeptide was also wild type, shown by its lack

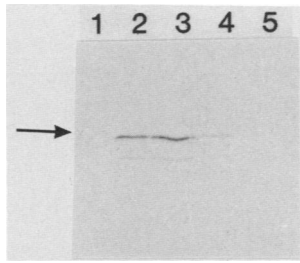

FIG. 2. Complex formation between human and murine p53 upon mixing in vitro. Equal aliquots of reticulocyte lysate, one containing ^{35}S-labeled human p53 and the second containing unlabeled murine p53$^{Ala-135}$, were mixed and incubated for 15 min at 20°C. After incubation, the mixed lysate was diluted and immunoprecipitated (see Materials and Methods). Lanes: 1, unmixed ^{35}S-labeled human p53 immunoprecipitated with PAb246; 2 to 5, mixed lysates with PAb246 (lane 2), PAb1620 (lane 3), PAb240 (lane 4), and PAb419 (lane 5). Proteins were resolved by 15% SDS-PAGE. ^{35}S-labeled p53 (arrow) was visualized by autoradiography; exposure time was 16 h at −70°C.

of reactivity with PAb240 (Fig. 2, lane 4). Thus, wild-type p53 proteins can form human/murine p53 complexes on mixing in vitro.

Wild-type p53$^{Ala-135}$ and mutant p53$^{Val-135}$ do not complex with each other when mixed posttranslationally. To test whether wild-type and mutant p53 phenotypes would also complex with each other when mixed in vitro, we looked for radiolabeled p53 coimmunoprecipitating with unlabeled p53 of the opposite phenotype. The conditions were identical to those described above for the formation of human/murine p53 complexes. However, no radiolabeled mutant p53 was coimmunoprecipitated with wild-type p53 when the wild-type-specific monoclonal antibody PAb246 was used for immunoprecipitation (Fig. 3A, lane 2), nor was radiolabeled wild-type p53 coimmunoprecipitated with mutant p53 when the monoclonal antibody PAb240 was used (Fig. 3B, lane 3). These results demonstrate that p53$^{Ala-135}$ and p53$^{Val-135}$ do not form wild-type/mutant complexes of p53 when mixed posttranslationally in vitro.

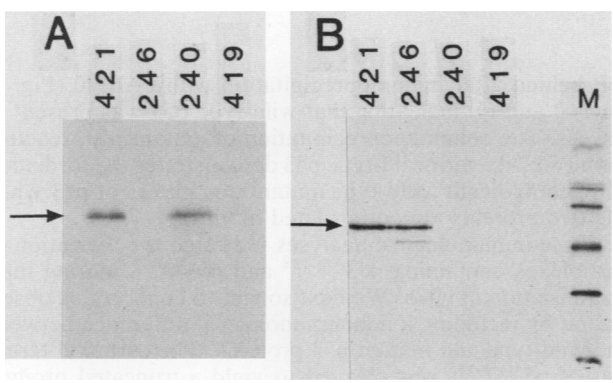

FIG. 3. Complex formation between different variants of murine p53 mixed in vitro. Equal aliquots of reticulocyte lysate containing the two p53 variants were mixed and incubated for 15 min at 20°C. After incubation, the mixed lysates were diluted and immunoprecipitated (see Materials and Methods). (A) ^{35}S-labeled mutant p53$^{Val-135}$ mixed with unlabeled wild-type p53$^{Ala-135}$; (B) ^{35}S-labeled wild-type p53$^{Ala-135}$ mixed with unlabeled p53$^{Val-135}$. Immunoprecipitating antibodies (PAb-) are as indicated. Proteins were resolved by 15% SDS-PAGE. ^{35}S-labeled p53 marker and ^{14}C-labeled molecular weight markers were run for each gel. ^{35}S-labeled p53 (arrow) was visualized by autoradiography; exposure time was 24 h for both panels.

Wild-type and mutant phenotypes of p53 from T3T3 and 3T3tx cells do not complex when mixed in vitro. We next examined the ability of p53 variants expressed in vivo to complex with each other when mixed in vitro. For this purpose, the T3T3 and 3T3tx cell lines were used as sources of phenotypic wild-type and mutant p53, respectively (Fig. 4A). The 3T3tx cells were metabolically labeled with [^{35}S]methionine before lysis. The T3T3 cells were not labeled. Equivalent aliquots of cell lysates were mixed at 20°C. After mixing for various times, there was no evidence of radiolabeled p53-246^0 coimmunoprecipitating with p53-246$^+$ (immunoprecipitations with PAb246 or with PAb1620; Fig. 4B, lanes 3 and 4).

Both 3T3tx and T3T3 cells contained high levels of p53, and the p53-246^0 from 3T3tx cells had a slightly slower electrophoretic mobility than did p53-246$^+$ from the T3T3 cells (Fig. 4C). These different mobilities allowed us to probe for complexes of the two p53 forms by a second approach, immunoblotting following immunoprecipitation of p53 from the mixed-cell lysates. Immunoblots of p53 immunoprecipitated with PAb421 or with PAb248 showed a doublet for p53 (Fig. 4C, lanes 1 and 2), representing the slower and faster mobilities of the two p53 variants (the monoclonal antibodies PAb421 and PAb248 detect both forms of p53; Table 1). However, only the faster-migrating form, p53-246$^+$, was detectable following immunoprecipitation with PAb246 or with PAb1620 (Fig. 4C, lanes 3 and 4), and only the slower-migrating form, p53-246^0, was detectable following immunoprecipitation with PAb240 (Fig. 4C, lane 5). In Fig. 4C, lane 4, the faint band comigrating with p53-246^0 was also detectable in the negative control (not shown) and probably represents a background band of ^{35}S-labeled protein.

Overall, these results demonstrate that the wild-type and mutant p53 phenotypes, expressed in vivo or in vitro, do not coimmunoprecipitate following mixing in vitro. Thus, in their native states, these immunologically variant forms of p53 do not appear to have any binding affinity for each other in vitro.

Cotranslational formation of wild-type/mutant p53 complexes. It is possible that the generation of wild-type/mutant p53 complexes requires cotranslational interaction between the nascent polypeptides. Such interaction would be feasible in cells expressing one wild-type allele and one mutant allele for p53.

To investigate this possibility, equivalent amounts of p53 mRNAs were cotranslated in vitro. Translations were at 37°C, and the ability of p53 to form complexes under these conditions was examined by size fractionation using FPLC gel filtration (see Materials and Methods). Freshly translated p53 resolved into four size fractions (Fig. 5C). Peak 4 eluted in fraction 14 and is presumed to contain monomeric p53, since p53 proteins prepared as for SDS-PAGE also eluted in fraction 14 (not shown). Bovine serum albumin (molecular mass, 68 kDa) coeluted with p53; thus, the apparent molecular weight of monomeric p53 on gel filtration is higher than that observed on SDS-PAGE. The highest-molecular-weight form of p53 peaked in fraction 5 (peak 1), with intermediate forms in fractions 7/8 (peak 2) and fractions 10/11 (peak 3). An additional peak of TCA-precipitable counts was observed at fraction 18. This peak did not contain immunoprecipitable p53 and may represent complexes containing [^{35}S]methionyl-tRNA.

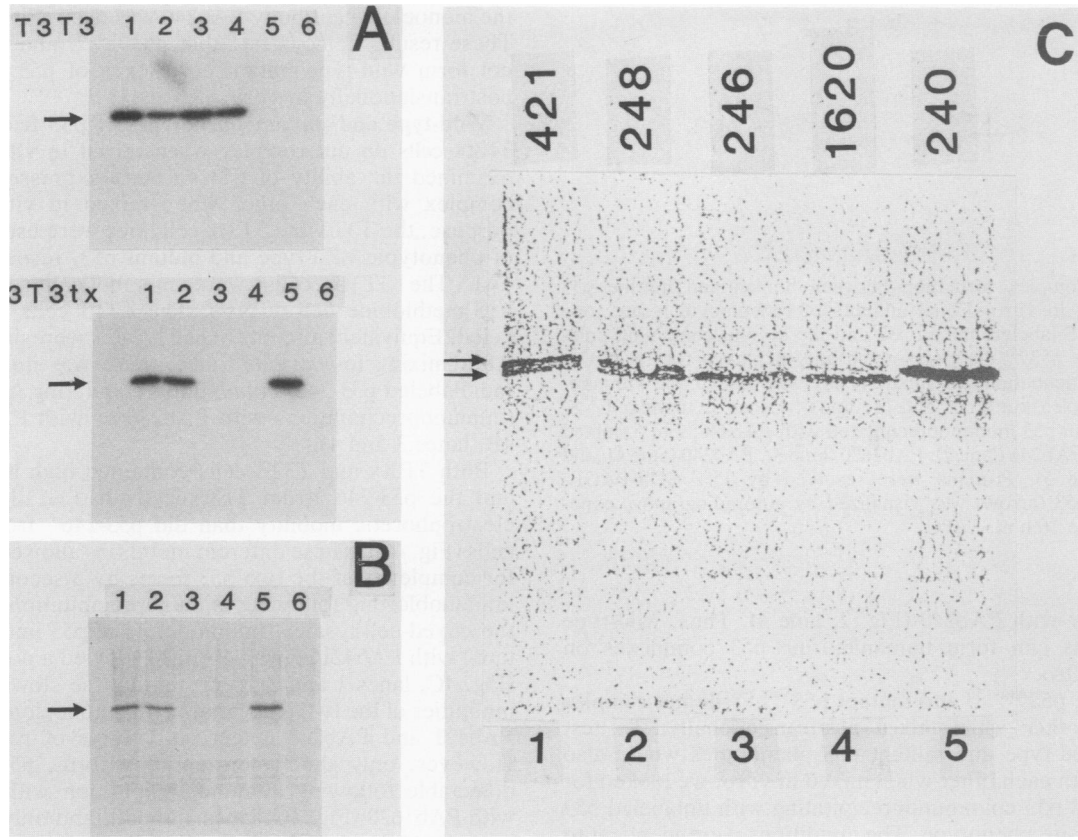

FIG. 4. Characterization of p53-246^{+} and p53-246^{0} from T3T3 and 3T3tx cell lines and probing for complexes after mixing in vitro. Immunoreactivity of p53 was determined by immunoprecipitation of p53 with specific monoclonal antibodies and visualization by immunoblotting (A). Binding of p53-246^{+} with p53-246^{0} was investigated by mixing of ^{35}S-labeled 3T3tx lysate (p53-246^{0}) with nonlabeled T3T3 lysate (p53-246^{+}) for 15 min at 20°C (B). The immunoprecipitated proteins from the lysate mix were also examined by immunoblotting. The p53-246^{+} variant from T3T3 cells has a slightly faster electrophoretic mobility than does p53-246^{0} from the 3T3tx cells, and this was apparent when the immunoblots were exposed to X-ray film with tin foil instead of an intensifying screen (C); the p53 doublet is indicated by a double arrow. Bands of ^{35}S-labeled background proteins are also visible. Lanes: 1, PAb421; 2, PAb248; 3, PAb246; 4, PAb1620; 5, PAb240; 6, PA6419. All samples were resolved by using minigels; the autoradiograph in panel C has been enlarged approximately twofold to show the p53 doublet.

All of the size fractionation studies were carried out with freshly translated p53, since we observed that storage at −70°C resulted in the generation of high-molecular-weight complexes of p53 with loss of monomeric p53 (Fig. 5A). Please note that sample collections shown in Fig. 5A were started later than for those shown in Fig. 5B to F (sample 1 in Fig. 5A is equivalent to sample 5 in the other panels).

p53$^{Ala-135}$ and p53$^{Val-135}$ gave similar profiles on gel filtration (Fig. 5B), and when the two mRNAs were cotranslated, the size fractionation profile was essentially unaltered (Fig. 5C). Clearly, the cotranslated proteins were able to form higher-order complexes, as indicated by peaks 1 to 3. We next asked whether complexes containing wild-type and mutant p53 phenotypes were formed when p53$^{Ala-135}$ and p53$^{Val-135}$ were cotranslated. Following cotranslation, p53 species reactive with both PAb246 and PAb240 were clearly evident (Fig. 6, lanes 2 and 3). Sequential immunoprecipitations showed that PAb246 removed most of the cotranslated p53 proteins and that subsequent immunoprecipitation with PAb240 detected little residual mutant p53 (Fig. 6, lanes 2 and 5). This finding indicates that mutant p53 reactive with PAb240 was largely coimmunoprecipitated with the wild type. In the reciprocal sequence, residual wild-type p53 was left behind after immunoprecipitation with PAb240 (Fig. 6, lanes 3 and 6), indicating that wild-type p53 was present in excess. The coimmunoprecipitation of mutant p53, reactive with PAb240, with wild-type p53 demonstrates the formation of phenotypically wild-type/mutant complexes of p53 when the two proteins are cotranslated in vitro.

These immunological analyses indicated the formation of complexes containing p53$^{Ala-135}$ and p53$^{Val-135}$ during their cotranslation in vitro. We next sought to confirm this observation by including a nonimmunological difference between the wild-type and mutant p53 proteins. The carboxyl terminus of p53$^{Ala-135}$ was cleaved to yield a truncated product with a predicted length of 343 amino acids (see Materials and Methods). Given its lower molecular weight, the truncated p53 was clearly distinguishable from full-length p53 following gel electrophoresis and, as expected, lacked the epitope for PAb421, which maps to amino acids 370 to 378 (Fig. 7A, lane 1). Truncated p53$^{Ala-135}$ still adopted the wild-type phenotype when translated in vitro and was reactive with PAb246 but not with PAb240 (Fig. 7A, lanes 3 and 4). It was also reactive with PAb1620. When this truncated form of wild-type p53 was cotranslated with mutant p53$^{Val-135}$, no complex formation between the two proteins was observed

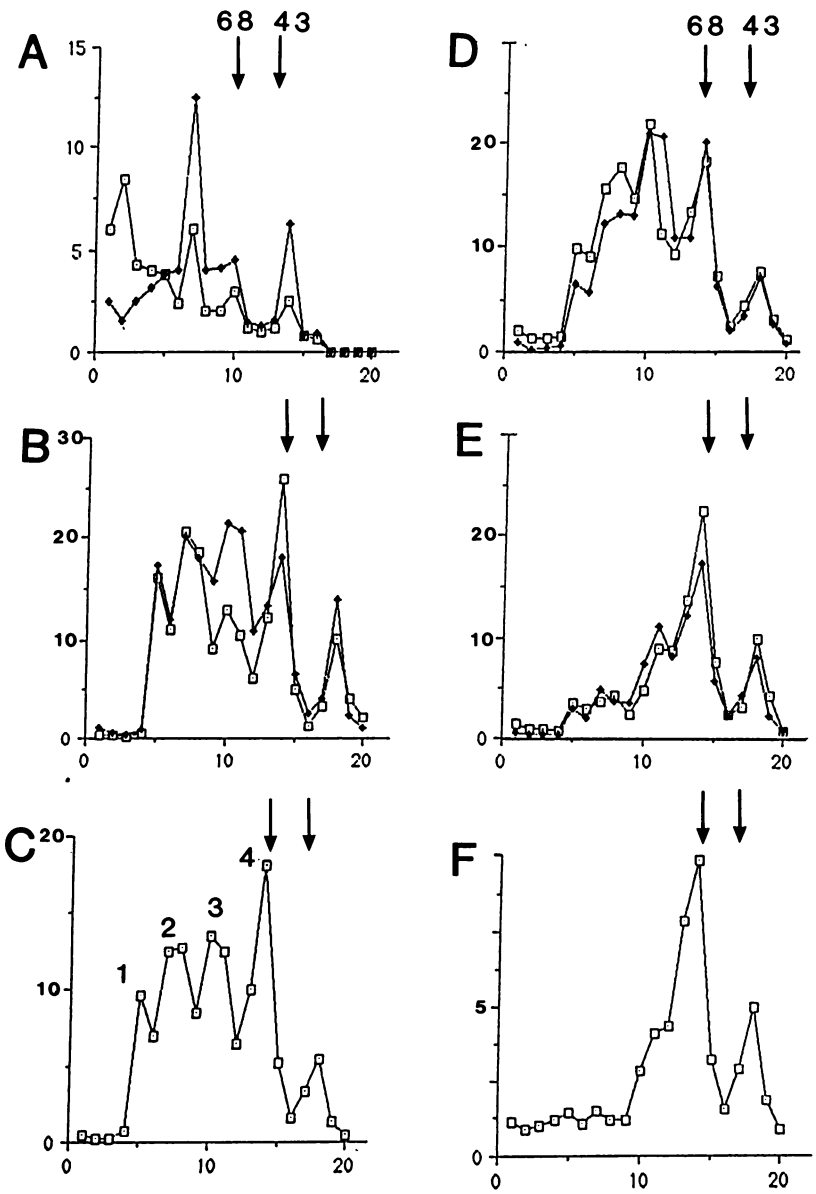

FIG. 5. Size fractionation of ^{35}S-labeled p53 proteins translated in vitro, using rabbit reticulocyte lysate (see Materials and Methods for details). The TCA-precipitable counts associated with each fraction are presented, 10^4 counts per minute on the x axis and fraction number on the y axis. (A) $p53^{Ala-135}$ (⊡) and $p53^{Val-135}$ (■) fractionated separately following storage at −70°C; (B) freshly translated $p53^{Ala-135}$ (⊡) and $p53^{Val-135}$ (■) fractionated separately; (C) $p53^{Ala-135}$ and $p53^{Val-135}$ cotranslated; (D) $p53^{Ala-135}$ cotranslated with human p53 (⊡) and $p53^{Val-135}$ cotranslated with human p53 (■); (E) $p53^{Ala-135}$ cotranslated with truncated $p53^{Ala-135}$ (⊡) and $p53^{Val-135}$ cotranslated with truncated $p53^{Ala-135}$ (■); (F) truncated $p53^{Ala-135}$ translated alone. With the exception of those shown in panel A, all samples were fractionated within 2 h of translation, without freeze-thawing. The paired data sets in panels B, D, and E were obtained from parallel translations, and a common stock of rabbit reticulocyte lysate was used for all translations. Aliquots from the same translations were used for panel C and Fig. 6, and for panel E and Fig. 7. The elution points of 68- and 43-kDa size markers are indicated; the 200-kDa marker eluted in fractions 10/11. The four peaks from which p53 was immunoprecipitable are indicated in panel C; peak 4 appeared to represent monomeric p53 (see text).

(Fig. 7B, lane 2). Similarly, cotranslation of full-length $p53^{Ala-135}$ with truncated $p53^{Ala-135}$ also failed to show any evidence of complex formation between the two defined proteins (Fig. 7C, lane 2). Size fractionation of the truncated $p53^{Ala-135}$ revealed that most of the protein was present in the monomeric form (Fig. 5F), suggesting that amino acids 343 to 390 of p53 contain a domain(s) involved in the generation of p53 complexes. Interestingly, when the truncated $p53^{Ala-135}$ was cotranslated with either $p53^{Ala-135}$ or $p53^{Val-135}$, the ability of the full-length proteins to form complexes was also reduced (Fig. 5E).

DISCUSSION

The p53 gene is regulatory for normal cell proliferation, and recent genetic studies indicate a tumor suppressor function for p53 (1, 6, 12, 22, 27; for a review, see reference 15). This observation suggests that p53 may also function as

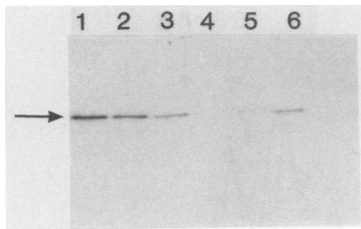

FIG. 6. Analysis of $p53^{Ala-135}$ and $p53^{Val-135}$ following their cotranslation in vitro. Equal aliquots of the p53 mRNAs were cotranslated in the presence of [^{35}S]methionine. The translated products were divided into aliquots and immunoprecipitated with PAb421 (lane 1), PAb246 (lane 2), PAb240 (lane 3), and PAb419 (lane 4). The presence of residual p53-246^0, uncomplexed with p53-246$^+$, was examined by reimmunoprecipitating the supernatant from the PAb246 immunoprecipitation with PAb240 (lane 5). In the reciprocal analysis, the supernatant from the PAb240 immunoprecipitation was reimmunoprecipitated with PAb246 to probe for residual p53-246$^+$ (lane 6). The primary immunoprecipitations were quantitative for each monoclonal antibody. Proteins were resolved on a 15% minigel; the autoradiograph was exposed for 16 h. p53 is indicated by an arrow.

a suppressor for normal cell growth, although this remains to be established. The tumor suppressor function of p53 can be lost following mutation, and target sites for activating mutations of human p53 tend to be clustered within highly conserved domains of the p53 gene (22). The mutant p53 allele contributes to the process of cell transformation. An understanding of the dominant effect of mutant p53 on tumor progression will be important to our understanding of cell growth control in general and of p53 function in particular.

In this study, we have examined the binding affinity of mutant p53 for the wild-type protein. Several groups have noted that endogenous p53 is coimmunoprecipitated with murine p53 in rat cells transfected with cDNA for mutant murine p53 (5, 12, 24). This finding has led to the hypothesis

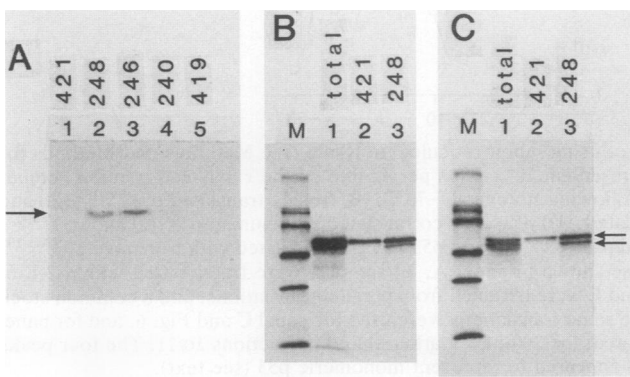

FIG. 7. Analysis of $p53^{Ala-135}$ and $p53^{Val-135}$ following cotranslation with truncated $p53^{Ala-135}$ in vitro. (A) Immunoreactivity of the truncated $p53^{Ala-135}$ when translated alone. Lanes: 1, PAb421; 2, PAb248; 3, PAb246; 4, PAb240; 5, PAb419. The cotranslations of $p53^{Val-135}$ with truncated $p53^{Ala-135}$ (B) and of $p53^{Ala-135}$ with truncated $p53^{Ala-135}$ (C) were immunoprecipitated with PAb421 (lanes 2) and with PAb248 (lanes 3). Lanes M, ^{14}C-labeled high-molecular-mass markers (200, 92, 69, 43, 30, and 14 kDa); lanes 1, aliquots of total p53. All lanes contained equivalent loadings. The lower p53 bands in panels B and C represent the truncated $p53^{Ala-135}$; the upper bands represent full-length p53 (arrows). The sharpness of the bands was improved by using aluminum foil backing to the X-ray film during exposure. Exposure times were 16 h for panel A and 22 h for panels B and C.

that the mutant protein may inactivate the suppressor function of wild-type p53, and this indeed might be the case in experimentally transfected cell lines. However, to assess this hypothesis in relation to natural tumor progression, it is necessary to demonstrate that mutant and wild-type p53 of the same species can also form complexes. Although it has been reported that p53 is able to oligomerize (14), this same report does not include evidence for the formation of p53-p53 oligomers.

Using gel filtration, we have now demonstrated that p53 also forms complexes when expressed in vitro. The actual concentration of most proteins translated in reticulocyte lysate is low, and therefore the ability of p53 to form complexes under such conditions suggests the involvement of relatively high binding affinities. This view is consistent with the report that aggregation of p53 synthesized in vivo is a very rapid process (14). The complexes formed in vitro may contain cellular components in addition to p53; indeed, this might explain the apparently high molecular mass of "monomeric" p53 (68 kDa; see Results).

The carboxyl terminus of p53 presumably contains sequences necessary for p53 complex formation, since truncated $p53^{Ala-135}$ (predicted amino acids 1 to 343) failed to form high-molecular-weight complexes (Fig. 5F). It was interesting that this truncated form of p53 also reduced the ability of cotranslated full-length p53 proteins to form complexes (Fig. 5E); this might be due to a disruptive effect of the truncated protein on cotranslated full-length p53. For example, in addition to the carboxy terminus, the oligomerization of p53 may involve amino acids contained within the truncated $p53^{Ala-135}$ polypeptide. If the carboxyl terminus of p53 is necessary for the stabilization of p53 oligomers, the truncated polypeptide may form unstable complexes with full-length p53. In this way, the presence of the truncated $p53^{Ala-135}$ might interfere with the normal formation of stable oligomers of the full-length p53 protein.

Human p53 formed wild-type/wild-type complexes with murine p53 when mixed in vitro. This formation of the interspecific human/mouse complexes of p53 is consistent with earlier reports of interspecific complexes of rat and murine p53 proteins in transfected cell lines (5, 6, 24). The human/murine p53 complexes elute in the high-molecular-weight peak 1 (unpublished observations), suggesting that these interspecific complexes of human and mouse p53 are formed by association of preexisting low-molecular-weight forms of p53.

The wild-type and mutant phenotypes of native murine p53 proteins showed no binding affinity for each other when mixed in vitro (Fig. 3 and 4). The lack of affinity between wild-type and mutant p53 conformations was observed for p53 proteins translated in vitro and also from cell lines. It is possible that the p53 proteins were already involved in high-affinity interactions at the time of mixing and that this precluded further complex formation. Alternatively, the full-length wild-type and mutant p53 phenotypes may simply have no affinity for each other. Indeed, it is clear that the two p53 phenotypes differ in their affinities for simian virus 40 large T antigen and for cellular proteins of the heat shock protein 70 (hsp70) family (7, 26). Moreover, the affinity between human and murine p53 appears to be restricted to proteins in the wild-type conformation (Fig. 2), since wild-type human p53 does not complex with the mutant phenotype of murine p53 upon mixing in vitro (unpublished observations).

Once synthesized, the wild-type and mutant phenotypes of p53 appear to have no affinity for each other when mixed in vitro. However, when mRNAs encoding wild-type and mutant p53 proteins were cotranslated in vitro, we obtained

evidence for wild-type/mutant p53 protein complexes. The complexes appeared to contain both wild-type and mutant phenotypic forms of p53, as judged by sequential immunoprecipitations. These assays showed that most of the p53 reactive with PAb240 (specific for the mutant form of p53) was coimmunoprecipitated with wild-type p53 (see Results). Size fractionation of the cotranslated proteins showed the same molecular profile as for the wild-type and mutant proteins translated separately (Fig. 5B and C). This result suggests that the formation of wild-type/mutant p53 proteins complexes is not due to aggregation into abnormally high molecular weight complexes. As yet we have no explanation for the cotranslational requirements in the binding of mutant with wild-type p53. Interestingly, a similar requirement has been indicated for the interaction of hsp70 with mutant p53 (25). The formation of wild-type/mutant p53 complexes may require one or both p53 species present as nascent polypeptide.

Wild-type p53 is a suppressor of abnormal cell proliferation. Activated mutants of p53 not only promote cell proliferation but in some way override the suppressor function of the wild-type allele. In the case of $p53^{Val-135}$, it is possible to discriminate between the suppressor and promoter effects of the p53 gene on cell proliferation by temperature, with promoter function at 37.5°C and suppressor function at 32°C (16). The conformation adopted by the $p53^{Val-135}$ polypeptide is also temperature sensitive and corresponds to the mutant phenotype at 37°C and the wild-type phenotype at 30°C (20). Thus, p53 appears to have two functional states which are determined by the conformation of the p53 polypeptide. The suppressor function of wild-type $p53^{Ala-135}$ may be compromised in cells containing a mutant allele of p53, since we have now demonstrated that cotranslation with activated $p53^{Val-135}$ allows the formation of phenotypically wild-type/mutant p53 complexes. The two p53 phenotypes differ in their binding affinities for other proteins (see above) and also in their localization within the cell (18a; 21). It follows that mutant p53 in complex with the wild type may interfere with the normal localization and targeting of wild-type p53. The result could be partial or complete loss of wild-type function.

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