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Stabilization of the p53 Transformation-Related Protein in Mouse Fibrosarcoma Cell Lines: Effects of Protein Sequence and Intracellular Environment

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The transformation-related protein p53 is normally very labile. The stability of p53 is significantly increased in a number of fibrosarcoma cell lines derived from mouse tumors induced by treatment with physical or chemical agents. In many instances, p53 stabilization is correlated with the ability to form a stable complex with the heat shock protein cognate hsc70. We describe a line in which p53 is very stable yet has no detectable interaction with hsc70. The inability to form such a complex probably resides in the primary structure of the endogenous p53, since introduction of other p53 variants into those cells resulted in the appearance of a p53-hsc70 complex. The factors affecting p53 stability were investigated by stable transfection experiments. The results indicated that the primary structure of the p53 protein is a major determinant of its turnover rate; different p53 variants were degraded at distinct and characteristic rates in a number of transformed cell types. However, at least one p53 variant was degraded differently in nontransformed BALB/c-3T3 than in transformed fibrosarcoma cells, demonstrating that the specific cellular environment can also affect the stability of p53.

p53 is a cellular phosphoprotein present in low amounts in proliferating nontransformed cells and in elevated levels in a variety of transformed cell types (reviewed in references 6, 21, 27, and 36). The aberrant overexpression of p53 was shown to affect cellular growth properties in a number of experimental systems. Thus, p53-overproducing plasmids can immortalize primary cells (19), transform primary cells in collaboration with Ha-*ras* (14, 30), induce replicative competence in growth-arrested fibroblasts (20), and increase the tumorigenicity of established cell lines (13, 41). These activities have led to the classification of p53 as an oncoprotein of the nuclear-acting group. Recently, however, it was shown that at least some of these activities are easily demonstrable only when mutant p53 is used, whereas wild-type p53 is much less effective, if at all (12, 15, 18). In fact, there is a growing body of evidence suggesting that wild-type p53 may actually interfere with neoplastic transformation and tumor induction (2, 15a).

In nontransformed cells, p53 has an extremely short half-life of 5 to 40 min (25, 28, 34, 35). The stability of p53 is markedly increased in various types of cells transformed by DNA tumor viruses (25, 28, 38, 44), RNA tumor viruses (3), and chemical carcinogens (16, 34) as well as in embryonal carcinoma cells (29, 34). In a number of cases, this stabilization is correlated with the formation of a complex between p53 and other proteins. Thus, in simian virus 40-transformed cells, p53 is bound to the viral large T antigen and is dramatically stabilized (25, 28). Similarly, p53 in complex with the adenovirus 58-kilodalton (kDa) E1b protein is also very stable (38, 44). Nevertheless, there is growing evidence that at least part of this stabilization may be due to indirect effects of the viral transformation rather than simply to the tight association between the proteins (10, 11, 44).

Some chemically transformed cells may present a similar example. In the Meth A cell line, derived from a methylcholanthrene-induced mouse fibrosarcoma tumor, p53 is also significantly more stable than in nontransformed fibroblasts (16, 34). In these cells, p53 is found in complex with another cellular protein of approximately 70 kDa (16, 31), which has been identified as the 70-kDa heat shock protein (hsp70) cognate (17, 32, 39), or hsc70. Analysis of cell lines derived by transfection with a mutant p53 gene has strongly suggested a direct correlation between the binding of p53 to hsc70 and the stabilization of the former (15). In fact, Meth A cells also express at least two distinct mutant forms of p53 (1, 5, 12). It is therefore conceivable that these mutations result in a tight binding of p53 to hsc70 and consequently lead to pronounced stabilization of the protein.

In this study, we addressed this issue by investigating the turnover of p53 in several mouse fibrosarcoma cell lines. Our results indicate that p53 stabilization is common in such tumor-derived lines. However, although there was often a good correlation between increased stability and binding to hsc70, there was at least one case in which these properties could be clearly uncoupled. Furthermore, we demonstrate that different variants of p53 are degraded at different, distinctive rates within the same cell type, whereas a single p53 variant exhibits greatly altered stabilities in different cell types. Hence, the stability of p53 is determined both by its primary structure and by cell-type-specific factors, which may be dependent on the transformation state of the cell.

MATERIALS AND METHODS

Plasmids. The p53 expression plasmid pLTRp53cG9 contains a chimera of p53 cDNA and genomic p53 DNA sequences under the control of the Harvey murine sarcoma virus long terminal repeat (13, 20). pLTRp53c5 was constructed by excising the *Xho*I-*Sac*II fragment from pLTRp53cG9 and replacing it by the Meth A-derived p53 cDNA clone pp53-176 (12, 43). pp53N-10 is a partial mouse p53 cDNA clone, originally cloned in λgt10 and subsequently subcloned into the *Eco*RI site of pBR322 (O. Eizen-

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berg and M. Oren, unpublished data). It contains a 1-kilobase cDNA insert, extending approximately from nucleotides 350 to 1350 of the p53 sequence (numbering as in reference 43). ptkgpt contains the *gpt* gene (26) linked to the herpesvirus thymidine kinase promoter (13).

Cells and transfections. Meth A fibrosarcoma cells (9) were a gift of V. Rotter. UV2237-IP3 cells (33) were a gift of A. Raz. IB9 cells (7) were a gift of L. Eisenbach. Clones 6 and 26 are derived from foci induced by transfection of primary rat embryo cells with a p53 overexpression plasmid plus activated Ha-*ras* (14, 32). Clone IIB4 is derived from Rat-1 cells transfected with pLTRp53cG9 (13). All lines were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. Transfection of cells was by the calcium phosphate coprecipitation method as described previously (14). The DNA coprecipitate was left on the cells for 4 to 6 h, followed by a glycerol shock (10% glycerol in Dulbecco modified Eagle medium plus 10% fetal calf serum) for 2 min. Cells were cotransfected with 10 μg of each pertinent plasmid and 1 μg of ptkgpt, and colonies appearing in the presence of mycophenolic acid (20 μg/ml) were expanded into cell lines and maintained in the presence of the drug.

Immunoprecipitation and protein analysis. Cells were labeled with [^{35}S]methionine for 30 min and analyzed immediately (pulse) or incubated for additional time periods with nonradioactive medium (chase). In other cases, cells were continuously labeled with [^{35}S]methionine for 4 h. Cell extracts were prepared as described previously (23). Equal amounts of trichloroacetic acid-insoluble radioactivity were subjected to immunoprecipitation, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12.5% gel (23). Gels were fluorographed with 2,5-diphenyloxazole–dimethyl sulfoxide, dried, and exposed to Agfa Curix X-ray film. To quantitate the results, autoradiograms were scanned with the aid of a densitometer (model 620; Bio-Rad Laboratories).

S1 nuclease analysis. S1 nuclease analysis was performed as described by Berk and Sharp (4). The probe was prepared by labeling the 3′ end of the ~1,300-nucleotide *Pvu*I-*Ban*I fragment of plasmid pp53N-10 DNA, using the Klenow fragment of *Escherichia coli* DNA polymerase I. Cytoplasmic RNA was prepared from the cell lines as previously described (28) and hybridized to the labeled fragment. The hybridization mixture (20 μl) contained 100,000 cpm of radioactive probe, 20 μg of RNA, 80% (vol/vol) formamide, 400 mM NaCl, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 6.0), and 1 mM EDTA. Hybridization was carried out for 20 h at 52°C and was terminated by rapidly diluting the mixture to 200 μl with S1 nuclease reaction buffer (250 mM NaCl, 30 mM sodium acetate [pH 4.6], 1 mM zinc sulfate, 5% glycerol, 5 μg of heat-denatured salmon sperm DNA). Then 200 U of S1 nuclease (Boehringer Mannheim Biochemicals) was added, and the solution was incubated for 30 min at 37°C. After extraction with phenol and ethanol precipitation, the samples were subjected to electrophoresis on a 5% polyacrylamide gel containing 8 M urea, followed by autoradiography.

RESULTS

Stabilization of p53 in several fibrosarcoma lines. One of the cell lines in which p53 has been studied in most detail is the Meth A fibrosarcoma line (8). In these cells, p53 has a greatly elevated half-life (16, 34) and is found in complex with hsc70 (17, 32, 39). To determine whether these features of p53 represent a more general phenomenon, we investigated the levels and stabilities of p53 in several mouse fibrosarcoma cell lines. In addition to Meth A, the analysis included IB9, also derived from a methylcholanthrene-induced fibrosarcoma (7), and UV2237-IP3 (referred to here as IP3), which originates from a murine fibrosarcoma line induced by UV irradiation (33). Cells of each line, as well as nontransformed BALB/c 3T3 cells, were either labeled for 30 min and analyzed directly (Fig. 1A) or labeled for 30 min and then chased in the presence of nonradioactive methionine for 5 h (Fig. 1B). In all fibrosarcoma lines, p53 turned over at a much slower rate than in the nontransformed cells. In agreement with previous reports (16, 34), Meth A p53 was very stable and coprecipitated with the hsp70 cognate, hsc70 (the increased intensity of the hsc70 band after the chase probably reflects the relatively slow entry of newly synthesized hsc70 into complex with p53). IB9 cells exhibited an intermediate rate of p53 turnover, as observed before (35), and a reduced, though still detectable, amount of coprecipitating hsc70. These data supported a causal relationship between the complex with hsc70 and the apparent stabilization of p53 (15). However, this correlation did not hold true for IP3 cells; although p53 was apparently very stable in these cells, there was no evidence for any coprecipitation with hsc70.

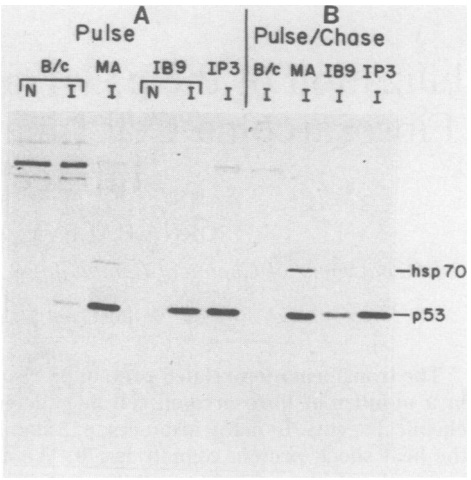

FIG. 1. Pulse-chase analysis of p53 in various transformed lines. Nontransformed BALB/c 3T3 (B/c) and the tumor-derived mouse fibrosarcoma lines Meth A (MA), IB9, and IP3 were labeled for 30 min with [^{35}S]methionine (40 μCi/60-mm-diameter dish) and then either analyzed immediately (A) or subjected to a 5-h chase (B). Equal amounts of trichloroacetic acid-insoluble radioactivity were reacted with either control hybridoma culture medium (N) or anti-p53 monoclonal antibody PAb421 (I). Precipitated polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. On the right are indicated the positions of p53 and the hsp70 cognate protein (referred to as hsc70 in the text).

To determine more precisely the turnover rate of p53 in IP3 cells, a detailed pulse-chase experiment was performed. The half-life of the protein in these cells was well above 8 h (Fig. 2), which is significantly longer than the 4 to 6 h observed in Meth A cells (16, 34; unpublished results).

Expression by IP3 cells of hsc70 that can bind p53. One trivial explanation for the absence of a detectable p53-hsc70 complex in IP3 cells is that the latter do not express this heat shock protein cognate. In a preliminary attempt to address this issue, the total protein profile of IP3 cells was investigated by sodium dodecyl sulfate-polyacrylamide gel electro-

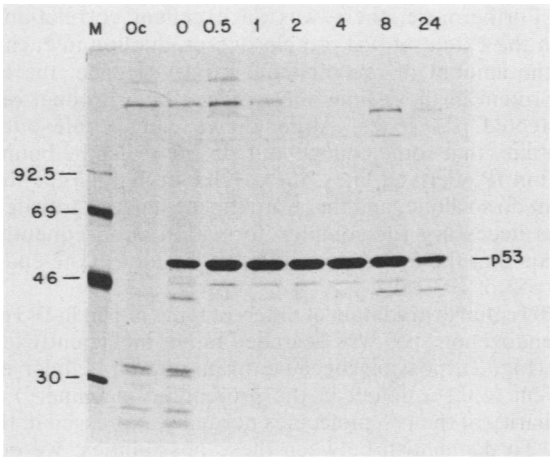

FIG. 2. Pulse-chase analysis of p53 in IP3 cells. IP3 mouse fibrosarcoma cells were labeled for 30 min with [^{35}S]methionine (40 μCi/60-mm-diameter dish) and then chased for the periods (in hours) indicated at the top. Proteins were analyzed as described in the legend to Fig. 1. M, Molecular weight markers; Oc, control hybridoma culture medium. Numbers on the left indicate the molecular sizes (in kilodaltons) of the marker polypeptides.

phoresis. A polypeptide exactly comigrating with hsc70 was clearly visible in IP3 cells, in quantities comparable to those found in Meth A cells (data not shown).

Further evidence for the presence of hsc70 in IP3 cells and for the absence of a complex between this protein and p53 was obtained from the experiment shown in Fig. 3. Extracts from Meth A and IP3 cells were immunoprecipitated with two anti-p53 monoclonal antibodies: PAb421, which recognizes both free and complexed p53, and PAb246 (42), which usually recognizes only p53 that is not bound to hsc70 (39, 40). As reported previously (24), very little p53 could be precipitated by PAb246 from Meth A extracts. On the other hand, both antibodies precipitated p53 from IP3 extracts. Figure 3 also depicts the proteins precipitated from the two

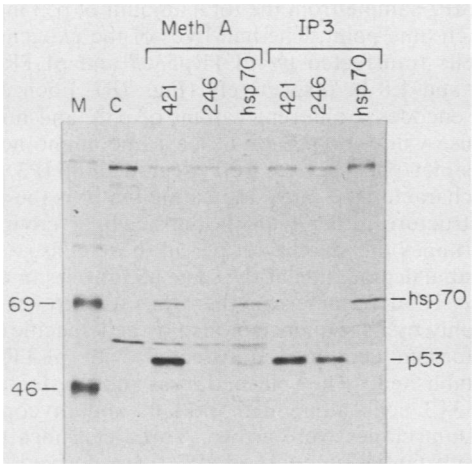

FIG. 3. Analysis of p53 and hsc70 in fibrosarcoma lines. Meth A and IP3 cells were labeled for 4 h with [^{35}S]methionine. Equal amounts of acid-insoluble radioactivity were reacted with the following antibodies: anti-p53 monoclonal antibody PAb421 (421), anti-p53 monoclonal antibody PAb246 (246), and anti-hsp70 polyclonal antibody (hsp70). C, Meth A extract immunoprecipitated with control hybridoma culture medium; M, molecular size markers (indicated in kilodaltons on the left).

cell lines by polyclonal anti-hsp70 antiserum (17). It should be noted that this antiserum is not specific for hsc70 (17) and may therefore potentially detect any member of the 70-kDa heat shock protein family that is constitutively expressed in the cells. The term hsc70 is therefore used below in a generic sense rather than to refer to the product of one particular gene. In this experiment, the antibody was used in limiting quantities, and comparison with the total amount of hsc70 in the extract indicated that it precipitated only a relatively small fraction of this protein (data not shown). Nevertheless, it was evident that IP3 cells did express an apparently normal-size form of this protein. Furthermore, the anti-hsp70 antibody could coprecipitate p53 from Meth A but (within the level of detection) not from IP3 (Fig. 3).

Although the findings presented above demonstrated the presence of substantial amounts of hsc70 in IP3 cells, they left open the possibility that this hsc70 was structurally altered and consequently incapable of stably binding p53. Alternatively, it could be argued the IP3 cells constitute a cellular environment that is incompatible with the formation of a p53-hsc70 complex. To address these issues, IP3 cells were stably transfected with plasmids encoding different types of mouse p53. The plasmids used were pLTRp53cG9, which contains a chimera between p53 cDNA and genomic DNA (13, 20), and pLTRp53c5, which carries p53 cDNA derived from Meth A cells (12). The p53 encoded by pLTRp53cG9 carries a single amino acid substitution (alanine to valine at position 135), whereas that encoded by pLTRp53c5 carries two substitutions: glutamic acid to glycine at position 168, and methionine to isoleucine at position 234 (1, 5, 12). Rat fibroblasts transfected with either of these plasmids exhibit a complex between the exogenous p53 and hsc70 (15, 17, 32; D. Michalovitz and M. Oren, unpublished observations). After cotransfection of IP3 with ptkgpt (see Materials and Methods) and each of the two plasmids, mycophenolic acid-resistant colonies were expanded into cell lines and analyzed by immunoprecipitation with the anti-p53 monoclonal antibody PAb421. Of the lines shown in Fig. 4A, two derived from transfection with pLTRp53cG9 expressed high amounts of the transfected p53 (lanes 1 and 2) and at least one expressed rather low levels of this protein (lane 3); one of the lines derived with pLTRp53c5 made detectable amounts of the exogenous p53 (lane 8). In all cases, synthesis of the transfected p53 was accompanied by the formation of a complex with hsc70, and the amount of hsc70 in complex was in good correlation with the abundance of the transfected protein.

One interpretation could be that the apparent binding defect in IP3 cells resulted from a lowered binding affinity of hsc70 for p53 rather than an absolute lack of binding. This interpretation, however, predicts that the amounts of hsc70 coprecipitated by anti-p53 antibodies in IP3-derived lines will still be substantially lower than in other cell types expressing comparable amounts of p53. This was clearly not the case (Fig. 4B), as is particularly obvious from comparison of the IP3-derived lines shown in lanes 2 and 3 and lines clone 6 (lane 1) and IIB4 (lane 4), all of which had been transfected with the same p53 expression plasmid, pLTRp53cG9. Thus, the hsc70 of IP3 cells is clearly capable of binding tightly to p53, and the cellular environment in this line does not seem to restrict the formation of such a complex.

One inherent assumption of these studies is that the increased levels of p53 in the transfected lines indeed represent the expression of the transfected plasmids rather than a massive induction of endogenous p53. Unfortunately, there

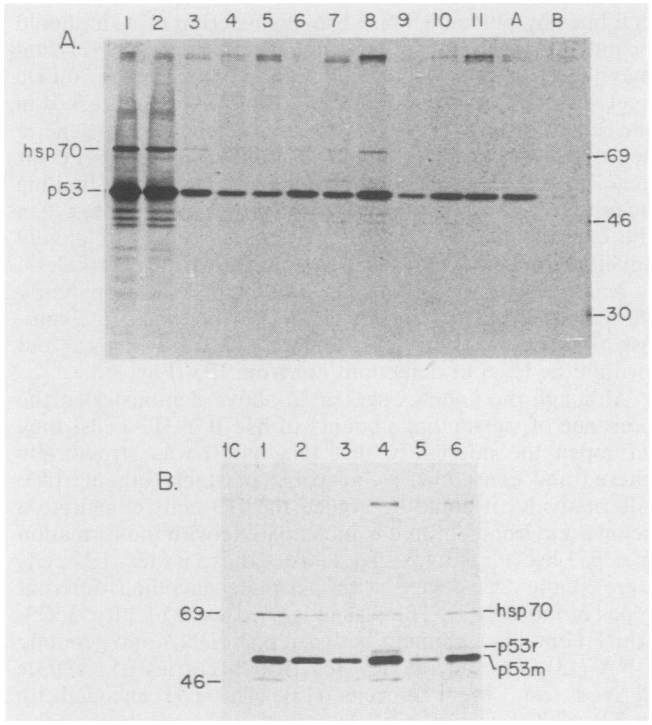

FIG. 4. Expression of p53 in IP3-derived cell lines. IP3 cells were stably cotransfected with either pLTRp53cG9 or pLTRp53c5 and the dominant selectable marker plasmid ptkgpt. Mycophenolic acid-resistant clones were isolated and expanded into lines. After labeling for 4 h with [^{35}S]methionine, samples containing equal amounts of acid-insoluble radioactivity were immunoprecipitated as described in the legend to Fig. 1. (A) Analysis of a representative group of transfected lines, indicated by numbers at the top. Lanes A and B depict extracts of a line established from IP3 with ptkgpt alone, reacted with PAb421 and control hybridoma culture medium, respectively. Lanes: 1 to 7, lines established from a transfection with pLTRp53cG9; 8 to 11, lines established from a transfection with pLTRp53c5. (B) Comparison between IP3-derived lines and other, previously described p53-overproducing cell lines. Lanes: 1C and 1, clone 6 cells (32); 2 and 3, IP3-derived lines, corresponding to lanes 1 and 2, respectively, of panel A; 4, IIB4 (13); 5, Meth A; 6, clone 26 (14). p53m and p53r correspond to mouse and rat p53, respectively. The sample in lane 1C was reacted with control hybridoma culture medium rather than with PAb421.

is presently no tool to distinguish directly between the endogenous and transfected mouse p53 proteins in these lines. We therefore took advantage of the structural differences between the p53 mRNA molecules generated by the resident gene and the transfected recombinant p53 gene. Most notably, the mRNA encoded by both pLTRp53cG9 and pLTRp53c5 misses almost the entire 3′ untranslated region of p53 mRNA. Cytoplasmic RNA was isolated from several p53-overproducing transfected lines and subjected to S1 nuclease analysis (Fig. 5). The radiolabeled probe used for these experiments was derived from a partial p53 cDNA clone, pp53N-10. The middle region of that probe corresponds to sequences that were present only in full-length endogenous mRNA, not in the mRNA transcribed off the transfecting plasmids (Fig. 5C). Thus, hybridization with transfection-derived mRNA should yield a protected fragment of only 546 nucleotides, as compared with the ~700-nucleotide fragment protected by the endogenous transcripts. All three IP3-derived overexpressors indeed synthesized the expected transfection-derived mRNA (Fig. 5A). Furthermore, there was an excellent correlation between the extent of p53 protein overproduction in each line and the amount of recombinant mRNA. Hence, the extra p53 protein in those lines must indeed be a product of the transfected p53 genes. Although we cannot rule out the possibility that some endogenous p53 may also be bound to hsc70 in IP3-derived lines, the results strongly argue that it cannot do so alone, and therefore the presence of transfected p53 is necessary for complex formation. Consequently, it appears that the failure to bind hsc70 resides in the endogenous p53 of IP3 cells.

Differential degradation of different types of p53 in IP3 cells. The endogenous p53 was degraded rather inefficiently in IP3 cells (Fig. 2), possibly because of an altered cellular environment (e.g., a defect in the proteolytic machinery) or a peculiarity of the p53 molecules normally expressed in these cells. To distinguish between these possibilities, we determined the turnover rate of p53 in the transfection-derived lines described in Fig. 4A. We performed a series of pulse-chase experiments with untransfected IP3 cells, IP3 cells transfected only with ptkgpt, and IP3 cells successfully transfected with either pLTRp53cG9 or pLTRp53c5 (Fig. 6). Whereas almost no p53 was degraded in 8 h in IP3 cells, either untransfected or transfected with ptkgpt alone, there was a substantial decrease in the amount of the protein even after 4 h in the pLTRp53cG9 transfectant; results for the pLTRp53c5 line were intermediate.

To obtain a more quantitative assessment of these results, the autoradiograms were densitometrically scanned and the decay curves were plotted (Fig. 7). We observed in some experiments an increase in labeled p53 during the first hour of chase, which probably reflects a relatively slow rate of replacement of the radioactive methionine by the unlabeled amino acid in the free and tRNA bound intracellular pools. To make the calculations more precise, the 1-h chase point was taken as the starting value for each of the plots. Whereas in IP3 cells the half-life of p53 (endogenous plus exogenous) was very long, it was about 7.5 h in the pLTRp53c5 line and only about 2.2 h in the pLTRp53cG9 line (Fig. 7A). In Fig. 7B, the putative rate of decay of the exogenous p53 only was calculated by subtracting the amount of p53 in the corresponding IP3 sample from the total amount of p53 in a given line at each time point. The half-lives of the exogenous p53 in IP3 cells transfected by pLTRp53c5 and pLTRp53cG9 were 4.2 and 1.8 h, respectively (Fig. 7B). Each of these plasmids encodes a different variant of p53, and both vary from mouse wild-type p53 by at least one amino acid (15). Thus, distinct p53 species were degraded in IP3 cells at distinct, characteristic rates, indicating that it is the primary protein structure rather than the intracellular environment that determines the stability of p53 in these cells.

Differential degradation of the same p53 species in different cell lines. To determine whether the stability of p53 is dictated only by its sequence or also by cell-specific factors, nontransformed cell lines transfected with pLTRp53cG9 were established. The plasmid was cotransfected into BALB/c 3T3 cells along with ptkgpt, and mycophenolic acid-resistant clones were grown. Three cell lines that expressed substantial amounts of p53 were derived. On the basis of the mRNA analysis (Fig. 5B), it is concluded that these high amounts of p53 truly represent transcription from the transfected plasmids. A stability analysis of p53 in one such line is presented in Fig. 8; the two other lines displayed essentially identical patterns (data not shown). It is clear that in these nontransformed cells, the exogenous p53 was turning over very rapidly. A more detailed kinetic study revealed

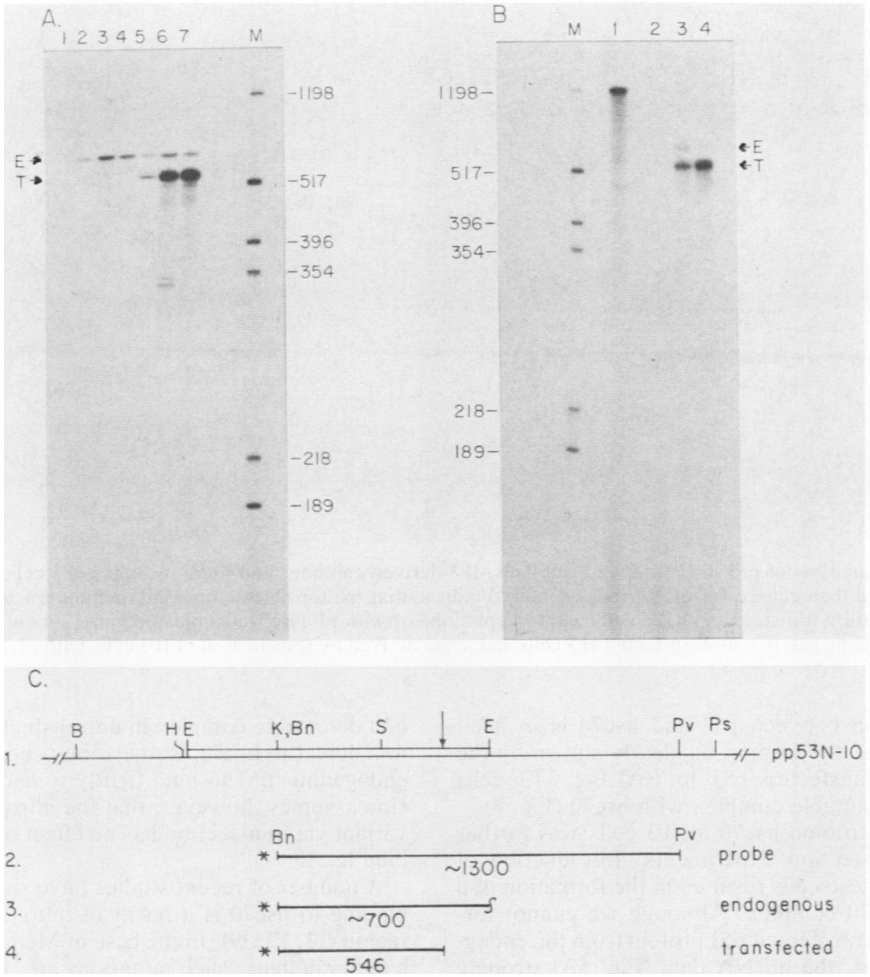

FIG. 5. S1 nuclease analysis of p53-specific mRNA from transfected lines derived from IP3 (A) or BALB/c 3T3 (B). The end-labeled ~1,300-nucleotide *Pvu*I-*Ban*I fragment was hybridized with cytoplasmic RNA from various lines and subjected to S1 nuclease analysis as described in Materials and Methods. The products were separated on a 5% polyacrylamide gel containing 8 M urea. (A) Lanes: 1, 20 μg of yeast tRNA; 2, untransfected IP3 cells; 3, IP3 cells transfected with pLTRp53cG but with no detectable expression of p53 (Fig. 4, lane 4); 4, IP3 cells transfected with ptkgpt alone (Fig. 4, lane A); 5, IP3 cells transfected with pLTRp53c5 (Fig. 4, lane 8); 6 and 7, IP3 transfected with pLTRp53cG9 (Fig. 4, lanes 2 and 1, respectively). (B) Lanes: 1, 20 μg of yeast tRNA; 2, untransfected BALB/c 3T3 cells; 3, IP3 cells transfected with pLTRp53cG9; 4, BALB/c cells transfected with pLTRp53cG9; M, DNA size markers (end-labeled *Hin*fI-digested pSP65 DNA). Numbers in the margins indicate the lengths (in nucleotides) of the labeled restriction fragments. Arrows mark the positions of the DNA fragments derived from protection of the probe by the endogenous (E) and transfection-derived (T) p53 mRNAs. (C) Structures of the probe and of the expected protected DNA fragment. (1) Structure of plasmid pp53N-10, used for preparation of the probe (see Materials and Methods). Symbols: ▭, p53 cDNA; ——, pBR322 DNA. The position indicated by the arrow corresponds to the 3′ end of p53-specific DNA in plasmids pLTRp53cG9 and pLTRp53c5 as well as in the mRNA encoded by them in transfected cells. Abbreviations for restriction sites: B, *Bam*HI; Bn, *Ban*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*I; S, *Sac*II. (2) Structure of the probe used for S1 nuclease analysis. *, Labeled end of the molecule. (3) Fragment corresponding to protection of the probe by the endogenous mouse p53 mRNA. (4) Fragment corresponding to protection of the probe by the transfection-derived mRNA, encoded by either pLTRp53cG9 or pLTRp53c5.

a half-life of less than 30 min (not shown). This value is very similar to that reported for the endogenous p53 in BALB/c 3T3 cells (25, 28, 34). Hence, the same species of p53 can be degraded at remarkably different rates in different cell types, indicating a role for cell-specific factors in determining the stability of this protein.

DISCUSSION

In this work, we investigated the turnover of p53 in three mouse fibrosarcoma lines. In all three cases, p53 was found in much higher levels than in nontransformed fibroblasts, in large part because of the stabilization of p53.

The molecular mechanisms leading to this stabilization are still unclear. In two cell lines, Meth A and IB9, there is a good correlation between the extended half-life of p53 and its association with the 70-kDa heat shock protein cognate hsc70. These facts support the notion that the tight binding of hsc70 may play a direct role in extending the half-life of p53, as suggested by the direct comparison of bound and unbound p53 in a transformed rat cell line (15). Nevertheless, there are clear exceptions to this rule, as exemplified by the IP3 line. In IP3 cells, p53 is very stable, yet there is no detectable binding to hsc70. Hence, the stabilization of p53 must be effected in these cells through a mechanism that does not depend on tight binding to hsc70. Furthermore, the

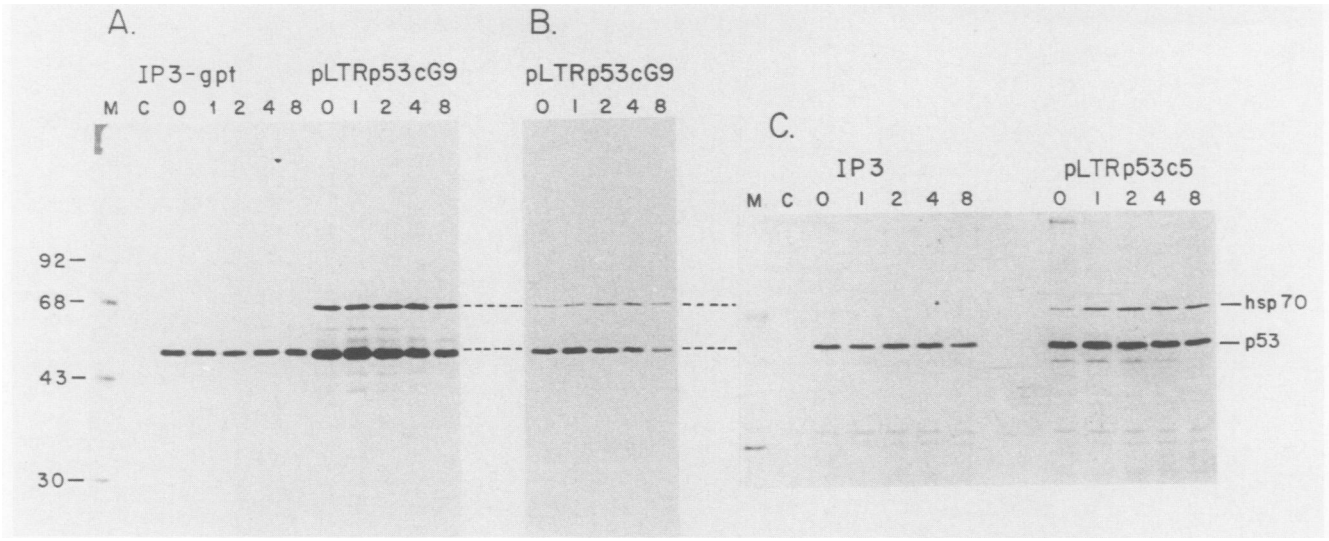

FIG. 6. Pulse-chase analysis of p53 in IP3-derived cell lines. IP3-derived cell lines and nontransfected IP3 cells were labeled for 30 min with [^{35}S]methionine and then chased for the periods (in hours) indicated at the top. Extraction and immunoprecipitation were done as for Fig. 1. (A) Lines derived by transfection of IP3 cells with ptkgpt alone or with pLTRp53cG9 plus ptkgpt (17-h autoradiographic exposure); (B) 3-h exposure of the same gel; (C) nontransfected IP3 cells and a line derived by transfection of IP3 cells with pLTRp53c5 (17-h exposure).

mere tight association between p53 and hsc70 is in itself insufficient to stabilize the former, as clearly shown by the rapid turnover of transfected p53 in BALB/c 3T3 cells despite the easily detectable complex with hsc70 (Fig. 8).

The failure of p53 to bind hsc70 in IP3 cells was further investigated by transfection experiments. Introduction of exogenous p53 into these cells resulted in the formation of a conspicuous p53-hsc70 complex. Although we cannot formally distinguish the transfected p53 protein from the endogenous IP3 counterpart, the mRNA data (Fig. 5A) strongly argue that the exogenous p53 is responsible for the formation of these complexes. These findings indicate that the absence of a detectable complex in untransfected IP3 cells is not due to a defect in hsc70. Rather, they suggest an inability of the endogenous p53 to bind tightly to hsc70. The latter conclusion assumes, however, that the introduction of a given p53 variant via transfection has no effect on its intrinsic ability to bind hsc70.

A number of recent studies have suggested that enhanced binding to hsc70 is a result of mutations in the p53-coding region (12, 17, 39). In the case of Meth A cells, which exhibit a tight complex, such mutations are, in fact, known to exist (1, 12). One would therefore speculate that IB9 cells also express a mutant p53 protein, although the mutation in this case has a milder effect on the interaction with hsc70. The

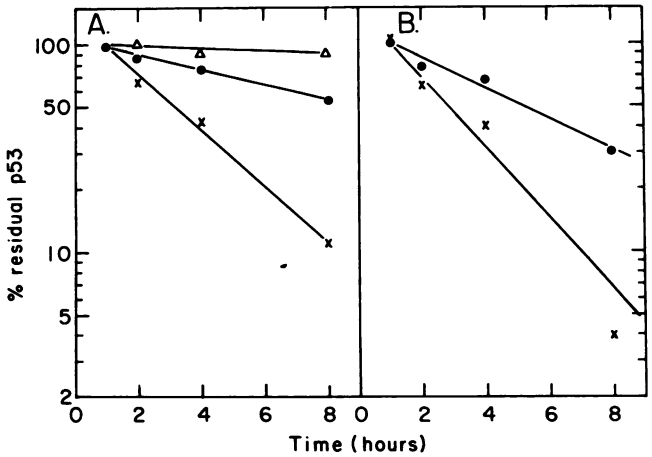

FIG. 7. Stability of p53 in IP3-derived lines. The p53 bands in the autoradiogram shown in Fig. 6 were scanned and quantitated (see Materials and Methods). (A) Relative intensity of each band (calculated as peak area), starting from the 1-h chase period; (B) residual exogenous p53, calculated by subtracting the amount of p53 in nontransfected IP3 cells at each time point from the total amount of p53 in each transfected line. Symbols: ● and ×, cells transfected with pLTRp53c5 and pLTRp53cG9, respectively; △, parental IP3 cells.

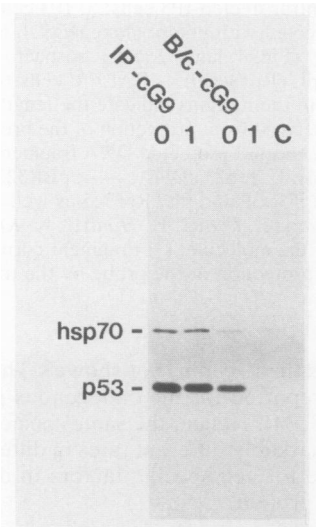

FIG. 8. Pulse-chase analysis of p53 in BALB/c 3T3 and IP3-derived cell lines stably cotransfected with pLTRp53cG9 and ptkgpt (B/c-cG9 and IP-cG9, respectively). Mycophenolic acid-resistant lines, overexpressing p53, were subjected to pulse-chase analysis as described in the legend to Fig. 1 except that the chase was only for 1 h. C, IP-cG9 (pulse) immunoprecipitated with control hybridoma culture medium.

situation in IP3 cells is less clear, since no complex with hsc70 is seen. In light of two observations, we nevertheless suggest that this line also carries a mutant p53 gene. First, the very high stability of endogenous p53 in IP3 cells is not due to a general inefficiency of the p53-degrading system, since other types of p53 are degraded faster when introduced into these cells. Furthermore, IP3 p53 is recognized quite efficiently by the monoclonal antibody PAb246. This antibody, although capable of binding wild-type p53, does so much less efficiently than PAb421 (24; O. Pinhasi-Kimhi, unpublished data). The fact that IP3 p53 appears to be recognized quite well by both antibodies also argues against its being of the wild-type sequence. Alterations in the half-life of p53 and probably in its primary structure therefore seem to be frequent features in mouse fibrosarcoma cell lines, suggesting that they may be involved in the induction or development of the corresponding tumors. Alternatively, these changes in p53 may be able to confer a growth advantage on the tumor-derived lines once they are placed in culture.

Finally, we attempted to assess in a more general way the factors controlling the rate of p53 turnover in different cell types. In agreement with previous reports (18, 37, 39), the primary structure of p53 itself plays a crucial role in determining its half-life. Two types of p53 were introduced into IP3 cells: Meth A-derived p53 encoded by pLTRp53c5, which carries two point mutations (1, 5), and the p53 encoded by plasmid pLTRp53cG9, carrying a single point mutation in the coding region (12, 15). Interestingly, the protein encoded by each of the two plasmids displayed in IP3 cells a turnover rate strikingly similar to that exhibited by the same p53 species in previously studied systems. The p53 encoded by pLTRp53c5 in IP3 cells has an estimated half-life of 4.2 h (Fig. 7), which is practically indistinguishable from the value obtained for the resident p53 in the original Meth A cells (16, 34; O. Halevy, unpublished data). Similarly, the p53 directed by pLTRp53cG9 in IP3 cells has a calculated half-life of 1.8 h, which is very similar to that measured in rat embryo fibroblasts transformed by a combination of this plasmid and Ha-*ras* (15; D. Eliyahu and D. Michalovitz, unpublished data). Both types of p53 were degraded in IP3 cells more rapidly than was the endogenous species, which may carry another, independent mutation (see above). Thus, different variants of p53 turn over at different, distinctive rates within the same cells.

The primary structure of p53 is not, however, the only determinant of its stability (Fig. 8). Although these BALB/c 3T3 cells express the same p53 variant as do the IP3 cells transfected with pLTRp53cG9, they degrade it much more rapidly. This is not because of the absence of a complex with hsc70, which is visible in these cells. Furthermore, the rate of synthesis of p53 in these cells is as high as in the IP3 transfectants, indicating that the mere overproduction of p53 does not result in stabilization of the protein. Rather, these results imply that the fate of any given species of p53 is also affected by the specific intracellular environment in which it is expressed. It is noteworthy that the p53 encoded by pLTRp53cG9 is very rapidly degraded only in the nontransformed BALB/c 3T3 cells, whereas it is markedly more stable in several types of transformed cells. It is therefore conceivable that the proteolytic machinery responsible for disposing of p53 is altered when cells become transformed; this notion is also supported by other studies, mostly involving simian virus 40-transformed cells (10, 11; E. Reihsaus, M. Kohler, S. Kraiss, M. Oren, and M. Montenarh, submitted for publication). In this respect, it is noteworthy that in transformed cells, p53 is found in high-molecular-weight oligomeric forms, a feature not detectable in nontransformed cells (22); this extensive oligomerization may play a role in the stabilization of p53. Clearly, the relationship between p53 stabilization and neoplastic transformation is an issue of great interest and one that may provide further insight into the molecular mode of action of this protein.

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