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Activating Mutations for Transformation by p53 Produce a Gene Product That Forms an hsc70-p53 Complex with an Altered Half-Life

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The 11-4 p53 cDNA clone failed to transform primary rat fibroblasts when cotransfected with the *ras* oncogene. Two linker insertion mutations at amino acid 158 or 215 (of 390 amino acids) activated this p53 cDNA for transformation with *ras*. These mutant cDNAs produced a p53 protein that lacked an epitope, recognized by monoclonal antibody PAb246 (localized at amino acids 88 to 110 in the protein) and preferentially bound to a heat shock protein, hsc70. In rat cells transformed by a genomic p53 clone plus *ras*, two populations of p53 proteins were detected, PAb246$^+$ and PAb246$^-$, which did or did not bind to this monoclonal antibody, respectively. The PAb246$^-$ p53 preferentially associated with hsc70, and this protein had a half-life 4- to 20-fold longer than free p53 (PAb246$^+$). These data suggest a possible functional role for hsc70 in the transformation process. cDNAs for p53 derived from methylcholanthrene-transformed cells transform rat cells in cooperation with the *ras* oncogene and produce a protein that bound with the heat shock proteins. Recombinant clones produced between a Meth A cDNA and 11-4 were tested for the ability to transform rat cells. A single amino acid substitution at residue 132 was sufficient to activate the 11-4 p53 cDNA for transformation. These studies have identified a region between amino acids 132 and 215 in the p53 protein which, when mutated, can activate the p53 cDNA. These results also call into question what the correct p53 wild-type sequence is and whether a wild-type p53 gene can transform cells in culture.

The nuclear oncogene p53 is often expressed at elevated levels in tumor-derived as well as virally and chemically transformed cell lines (4, 6, 17). The levels of p53 in nontransformed cells are quite low, and the half-life of the protein is short (6 to 30 min) (19, 25, 26). In simian virus 40 (SV40)- and adenovirus-transformed cells, p53 is found in an oligomeric protein complex with the SV40 large T antigen (16, 17) or the adenovirus E1b 55,000-M_r (55K) protein, respectively (29). In these virus-transformed cell lines, the p53 levels are as much as 100-fold higher than in nontransformed counterparts, and the half-life of the protein is correspondingly extended (19, 25). It has been suggested that the elevation of p53 levels is involved in the process of viral transformation and that the increased amounts result from the stabilization of p53 in these oligomeric protein complexes. Increased levels of p53 have also been implicated in alterations in the growth control of primary rodent cells. Elevated levels of p53 resulted in the immortalization of primary cells (14) or, when assayed in conjunction with an activated *ras* gene, in the full transformation of rat embryo fibroblasts (7, 21). Pinhasi-Kimhi et al. (24) and Hinds et al. (13) recently demonstrated that in p53-plus-*ras*-transformed cells, p53 is found in oligomeric protein complexes with a member(s) of the mammalian 70K heat shock protein (hsp70) family. Similar p53-p70 complexes have been observed in several transformed cell lines expressing elevated levels of p53 (10, 23, 28, 31). Furthermore, a p53 mutant protein which preferentially binds to the constitutively expressed member of the hsp70 family, hsc70 (32), possesses enhanced transforming activity compared with the parent protein (13). These observations suggested the possibility that hsc70 could bind to p53, extend its half-life, and increase the levels of this protein in a cell. This might then contribute to transformation of these cells.

The experiments presented in this communication test this hypothesis, and the results suggest that the p53-hsc70 complex may well play a role in the regulation of p53 levels in transformed cell lines. The relative transforming activity of a p53 cDNA derived from F9 embryonal carcinoma cells (11-4) and five linker insertion mutants made from the parental cDNA was assayed. Two mutants which preferentially bound hsc70 and not PAb246 possessed transforming activity. Pulse-chase experiments demonstrated that the mutant proteins possessed an extended half-life in the transformed cell lines. Similar results were obtained in cell lines transformed by a p53 cDNA genomic hybrid clone, LTRp53cG (8), plus *ras*. Comparative analysis of 11-4 and a p53 cDNA clone derived from Meth A cells (M-8) (1) demonstrated that a single nucleotide change could activate 11-4 for transformation. In all instances, the activating qualitative changes were in the N-terminal 215 amino acids of p53, and in every cell line examined, p53 was bound to a 70K protein in the transformed cells. These results suggest that qualitative changes in p53 contribute to quantitative increases in p53 levels and that the p53-hsc70 complex may play a role in these quantitative changes by extending the half-life of the mutant p53 protein.

MATERIALS AND METHODS

Plasmids. LTRp53cG contains genomic p53 sequences under the direction of the Harvey murine sarcoma virus (HaMSV) long terminal repeat (LTR) (8). p11-4 contains a murine p53 cDNA under the direction of the SV40 promoter/enhancer (32). MSVcL contains the murine p53 cDNA from p11-4 under the HaMSV promoter and was constructed by ligating the *Xho*I-*Bam*HI cDNA fragment of p11-4 with

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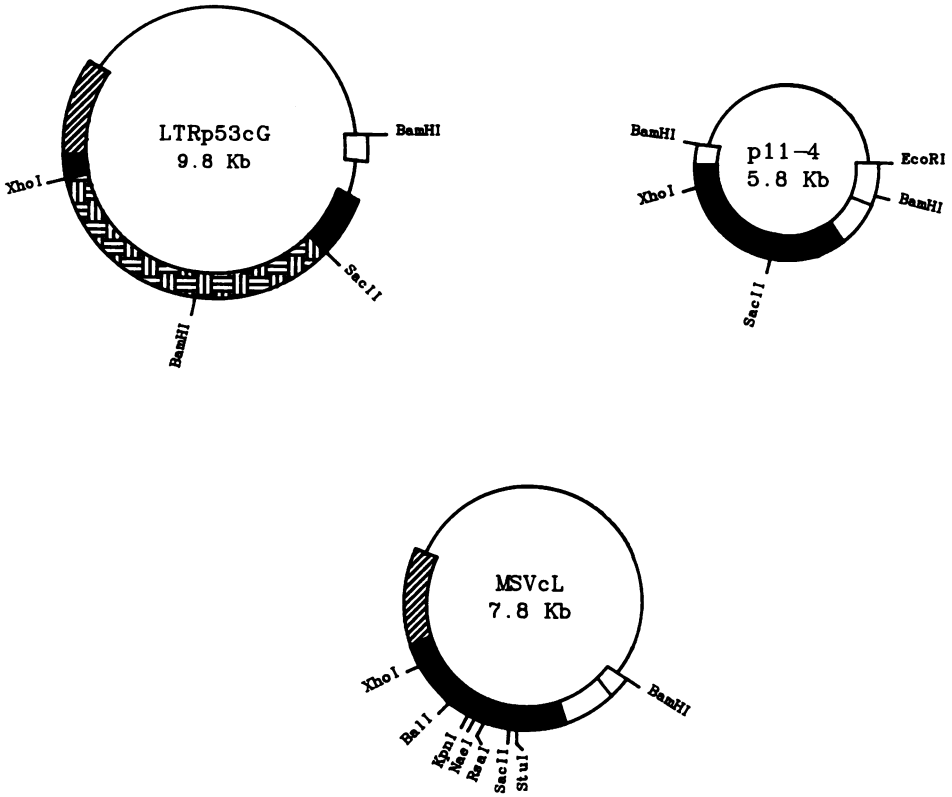

FIG. 1. Plasmids used in p53 transformation. LTRp53cG (8) contains a genomic-cDNA hybrid p53 sequence under the direction of the HaMSV LTR, and p11-4 (32) contains cDNA sequences encoding p53 under the direction of the SV40 promoter-enhancer. Thin line, Vector (pBR322) sequences; open bar, SV40 sequences; dashed bar, HaMSV LTR; solid bar, cDNA sequences; cross-hatched bar, genomic sequences. MSVcL contains the 11-4 cDNA under direction of the HaMSV LTR from LTRp53cG and was constructed as described in Materials and Methods.

the *Xho*I-*Bam*HI vector fragment of LTRp53cG (Fig. 1). Five mutant cDNAs (BH158, KH215, NH222, RH233, and SH344) originally constructed in p11-4 (32) were also placed under the control of the HaMSV promoter as described above.

LTRM-8 and LTR11-4 were constructed by replacing the *Xho*I-*Sac*II p53 fragment of LTRp53cG with the *Xho*I-*Sac*II cDNA fragment of either p53-M-8 (1) or p11-4, respectively. LTR11-4 recombinants (PD3, PD5, and PD7) were constructed by replacing the *Xho*I-*Apa*LI, *Apa*LI-*Kpn*I, or *Kpn*I-*Stu*I cDNA fragment of LTR11-4 with the corresponding cDNA fragment of LTRM-8.

Transfections. The results presented in Table 1 were obtained by transfecting 3×10^5 secondary or tertiary rat embryo fibroblasts with 1.25 μg of an activated *ras* gene (T24) (9) plus 1.25 μg of the p53 test plasmid with 10 μg of salmon sperm DNA as carrier as described previously (13). The results presented in Figure 5 were obtained by transfecting 5×10^5 cells with 5 μg of an activated *ras* gene (EJ6.6) (20) plus 5 μg of the test plasmid with 2.5 μg of BALB/c mouse liver DNA as carrier, as described previously (7).

Tumorigenicity. Fisher 344 rats (5 days old) were injected subcutaneously with 10^6 cells transformed by either LTRp 53cG plus *ras* or MSVKH215 plus *ras*. Four LTRp53cG-plus-*ras*- and three MSVKH215-plus-*ras*-transformed cell lines were tested, and in all instances, tumors were detectable within 5 to 8 days (unpublished observations). Each cell line was assayed in two rats. Tumors were never observed (up to 2 months) following injection of secondary rat embryo fibroblasts.

Cell lines, labeling, and immunoprecipitation. All cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were labeled with 100 μCi of [^{35}S]methionine (ICN) in 2 ml of methionine-free DMEM plus 2% dialyzed FBS for 2 h at 37°C. Following labeling, cells were washed with ice-cold phosphate-buffered saline and scraped from the plate. Cell pellets were stored at −80°C.

Immunoprecipitations were performed as described previously (13) except that 50% protein A–Sepharose (Sigma) was used in place of fixed staphylococcal protein A. Incubation of protein A-Sepharose and antigen-antibody complex was continued for at least 2 h at 4°C with constant agitation. Following immunoprecipitation, immunoprecipitates were separated on 7.5% polyacrylamide gels. Gels were prepared for fluorography by sequential immersion in 30% methanol–10% acetic acid (30 min), H_2O (three times for 10 min each), and 1 M sodium salicylate–5% glycerol (20 min). Dried gels were exposed to Kodak XAR-5 film at −80°C. Immunoblotting was performed as described (13).

Protein half-life determination. Plates (10 cm) containing equivalent numbers of cells from the cell line under investigation were labeled with [^{35}S]methionine for 1 h. Following labeling, the radioactive medium was removed, and the cells were washed twice with DMEM plus 10% FBS (chase medium). Plates were then refed with 10 ml of DMEM plus

10% FBS and incubated for specific periods of time. At the end of the specified chase period, cells were scraped from the plate, pelleted at 4°C, and stored at −80°C. Equivalent amounts of trichloroacetic acid (TCA)-precipitable counts were immunoprecipitated and subjected to electrophoresis as described above. Following exposure of the fluorographed gels to film, gel slices corresponding to p53 bands were excised, placed in 20-ml scintillation vials, and rehydrated by exposure to 0.25 ml of H_2O for at least 0.5 h at 37°C. When the gel slice was fully swollen, 1.5 ml of Protosol (New England Nuclear) was added to each vial, and incubation at 37°C was continued for 2 h. Finally, 10 ml of Biofluor scintillation fluid was added, and the vials were placed at room temperature for 1 to 2 days. The amount of ^{35}S-labeled protein released from the gel slice was determined by scintillation counting for 10 min. Counts per minute in the p53 band immunoprecipitated from a nonchased sample were taken as 100%, and one half-life was determined as the time point at which 50% of the zero-time counts remained.

RESULTS

Relative transforming activity of p53 mutants in cooperation with an activated *ras* gene. The p53 cDNA clone 11-4 (22), derived from F9 embryonal carcinoma cells, is unusual among p53 cDNA clones in that it fails to transform primary rat cells when cotransfected with the *ras* oncogene (13). With identical vectors and enhancer-promoter constructions, the 11-4 cDNA failed to cooperate with *ras* and form foci under conditions in which other p53 cDNAs or a genomic clone of p53 succeeded in transforming these cells. The goal of this first set of experiments was to determine whether the p53 11-4 cDNA could be activated for transformation with *ras* by creating mutational changes in the cDNA. Linker insertion mutations were constructed in the parental cDNA by the insertion of a *Hin*dIII linker into a convenient restriction site and are named, for example, KH215 when a *Hin*dIII linker has been inserted into the *Kpn*I site at amino acid 215 (of a total of 390 amino acids) (32). The relative transforming activity of the parental cDNA (11-4) and the mutants was assayed by cloning the parental cDNA and the mutants into expression vectors containing either the SV40 promoter/enhancer or the HaMSV LTR (Fig. 1) and cotransfecting the cDNAs with an activated *ras* gene into secondary rat embryo fibroblasts. The number of transformed foci was determined 2 weeks later (Table 1). Cotransfection of *ras* with LTRp53cG, a cDNA genomic hybrid murine clone (8) (Fig. 1), resulted in an average of 37 foci per experiment, and the morphologically transformed foci were readily clonable into transformed cell lines which are tumorigenic in young syngeneic animals. These results, consistent with previous reports of p53 genomic clones, showed transforming activity in cooperation with a *ras* oncogene (7, 21). In contrast, the parental 11-4 cDNA, whether under the control of the SV40 enhancer (11-4) or the HaMSV LTR (MSVcL), yielded few foci, none of which were clonable. These results indicate that the 11-4 parental cDNA was incapable of producing stable transformants in this assay. Two mutants derived from 11-4, those with linker insertions at 158 and 215, under the control of the SV40 early promoter, also showed minimal activity. A focus of cells transformed by SVKH215 plus *ras* was cloned, however, into a transformed cell line which expressed murine p53. This indicated that KH215 possessed activity in the assay. This was confirmed when these mutants were assayed under the control of the HaMSV LTR. Following cotransfection with either MSVKH215 plus *ras* or MSVBH158 plus *ras*, there was a six- to ninefold increase in the number of foci, and most important, these transformed foci were able to be cloned and produce cell lines with a reasonable frequency (10 to 20%). These results are consistent with the previously observed enhanced transcriptional strengths of the Moloney murine sarcoma virus LTR (15) and the Moloney murine leukemia virus LTR (5) relative to the SV40 early promoter in NIH 3T3 cells. Three MSVKH215-plus-*ras*-transformed cell lines were tumorigenic in young syngeneic rats with a latency period of 5 to 7 days, a time frame equivalent to that observed in young rats injected with cell lines transformed by the p53 genomic LTRp53cG plus *ras* (unpublished observations). Stable transformants were not derived from cotransfections with several additional mutants (those with linker insertions at amino acid 222, 233, or 344). These results demonstrate that, when the mutant cDNAs are expressed under the control of a strong promoter, qualitative changes in the parental 11-4 cDNA at amino acids 158 and 215 reproducibly yield p53 proteins with enhanced transforming activity in these assays.

Immunological evidence for the association of p53 protein with hsc70. The parental protein and these mutants were initially characterized by expressing the 11-4 parental cDNA and the mutant cDNAs under the control of the SV40 promoter in COS monkey cells (32). Under these conditions, the parental protein and several of the mutants (those with linker insertions at amino acids 222, 233, and 344) were found in a complex with the SV40 large T antigen, and in addition, the murine proteins were recognized by a variety of monoclonal antibodies specific for p53. Two mutants, those with linker insertions at amino acids 158 and 215, failed to bind to the SV40 large T antigen; instead, the mutant proteins were found preferentially bound to a 68K to 70K

TABLE 1. Transformation

Transforming sequences	No. of foci/ experiment (mean)	No. clonable/ no. tested (%)	No. tumorigenic/ no. tested
ras + LTRp53cG	52, 13, 34, 13, 23, 23 (37)	12/17 (70)	8/8
ras + p11-4	0, 0, 2, 2, 0, 0 (<1)	0/1 (0)	ND[a]
ras + SVBH158	ND, 0, 0, 0, ND, ND (0)	ND	ND
ras + SVKH215	3, 0, 3, 1, 0, 2 (1.5)	1/3 (33)	ND
ras + MSVcL	8, 0, 2, 0, 0, 2 (2)	0/11 (0)	ND
ras + MSVBH158	17, 6, 8, 7, 4, 10 (9)	4/34 (12)	ND
ras + MSVKH215	20, 5, 12, 6, 4, 6 (9)	6/29 (21)	6/6
ras + MSVNH222	12, 3, 6, 5, 2, 4 (5)	0/14 (0)	ND
ras + MSVRH233	7, 1, 4, 3, 0, 1 (3)	0/9 (0)	ND
ras + MSVSH344	3, 6, 7, 1, 0, 3 (3)	0/11 (0)	ND

[a] ND, Not determined.

monkey protein (32). Additionally, these two mutants did not bind to PAb246, a murine-specific, conformation-dependent monoclonal antibody (34) which recognizes an epitope spanning amino acids 88 to 109 (33). This epitope is located more than 50 amino acids away from the insertion mutations, indicating that the mutant proteins has altered conformations. Prior experiments have demonstrated that in LTRp53cG-plus-*ras*-transformed cell lines, p53 is found complexed to hsc70 (13, 24). To determine whether the KH215 and BH158 mutant proteins bind to hsc70 in the transformed cell lines, two of these lines, one transformed by BH158 plus *ras* and the other with KH215 plus *ras*, were labeled with [^{35}S]methionine and the cell lysates were immunoprecipitated with the murine-specific antibodies RA3-2C2 (27) or PAb246 or with a rabbit antiserum specific for members of the mammalian hsp70 family (13). The results demonstrate that both cell lines were expressing elevated levels of murine p53 (Fig. 2). Immunoprecipitation with RA3-2C2 yielded a coimmunoprecipitating protein of 74K corresponding to hsc70, and conversely, immunoprecipitation with anti-hsp70 serum coimmunoprecipitated a 53K protein which comigrated with authentic p53. As previously observed in COS monkey cells, neither mutant protein bound to PAb246. Western blot analysis confirmed that these two proteins were p53 and hsc70 (unpublished results).

These results indicated that the presence of the p53-hsc70 complex is associated with the inability of p53 to bind to

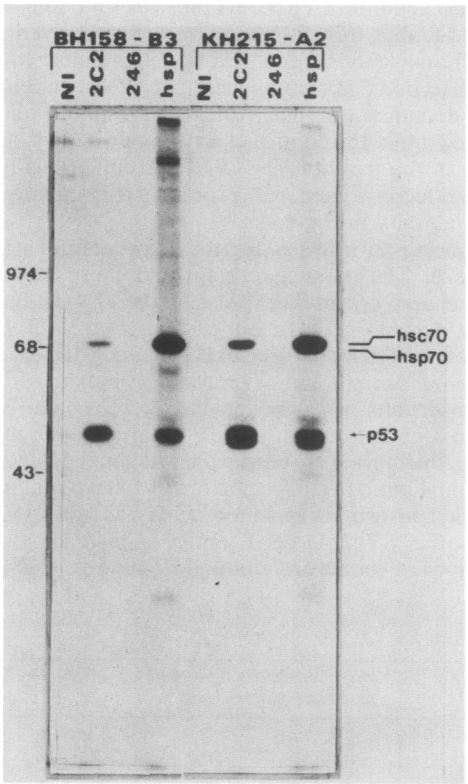

FIG. 2. Immunoprecipitation of p53 and hsp70 proteins from p53-plus-*ras*-transformed cell lines. Equivalent amounts of TCA-insoluble radioactivity (2×10^6 cpm) from [^{35}S]methionine-labeled extracts of the BH158-plus-*ras*-transformed cell line BH158-B3 or the KH215-plus-*ras*-transformed cell line KH215-A2 were subjected to immunoprecipitation with control nonimmune rabbit serum (NI), murine p53-specific monoclonal antibodies RA32C2 (2C2) and PAb246 (246), and anti-hsp70 antiserum (hsp). Molecular size markers are indicated (in kilodaltons).

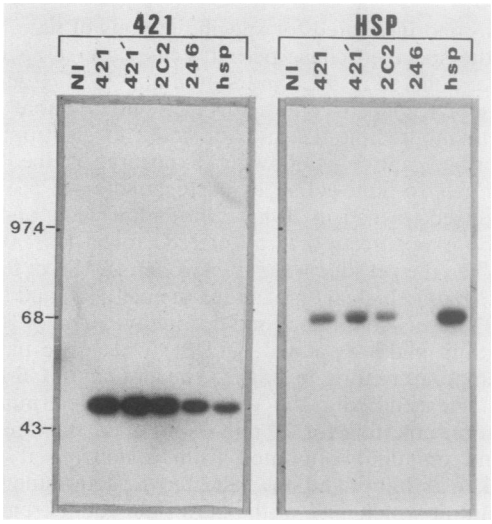

FIG. 3. Immunoblot of p53 and hsp70 proteins immunoprecipitated by anti-p53 and anti-hsp70 antibodies. Cell lysates of LTRp53cG-plus-*ras*-transformed cell line A1 containing 500 μg of soluble protein were immunoprecipitated with control nonimmune rabbit serum (NI), anti-p53 monoclonal antibody PAb421 (421), anti-murine p53 monoclonal antibodies RA32C2 (2C2) and PAb246 (246), and anti-hsp70 antiserum (hsp). Lanes marked 421′ were washed with water instead of RIPA buffer in the final step of immunoprecipitation. Immunoprecipitates were loaded on two sets of wells on a single 7.5% polyacrylamide gel. After electrophoresis, the separated proteins were transferred to nitrocellulose. One filter, representing one set of lanes, was incubated with PAb421 (421) and the other was incubated with anti-hsp70 antiserum (HSP). Blotted filters were then incubated with ^{125}I-protein A. Molecular size markers are indicated (in kilodaltons).

PAb246. We therefore examined whether p53 present in an LTRp53cG (genomic clone)-plus-*ras*-transformed cell line bound to PAb246. Unlabeled cell lysates were immunoprecipitated with PAb421 (12), RA3-2C2, PAb246, or anti-hsp70 serum, and the pellets were analyzed on SDS-polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose and then blotted with either PAb421 or anti-hsp70 serum. The relative levels of p53 and hsc70 immunoprecipitated by these antibodies are shown in Fig. 3. As previously observed, hsc70 was coimmunoprecipitated by PAb421 and RA3-2C2 (13). Only a portion (30 to 40%) of murine p53, however, was immunoprecipitated by PAb246. Furthermore, very low or nondetectable levels of hsc70 were coimmunoprecipitated by PAb246. These results demonstrate that there are two populations of p53 in an LTRp53cG-plus-*ras*-transformed cell line, one which can bind PAb246 (PAb246$^+$) and the rest which cannot bind PAb246 (PAb246$^-$).

The results of several control experiments confirmed that the two populations of p53 observed in LTRp53cG-plus-*ras*-transformed cells (PAb246$^+$ and PAb246$^-$) did not result from an inefficient association of PAb246 antibody with p53. First, the addition of more PAb246 antibody to an extract previously incubated with PAb246 did not immunoprecipitate additional p53 (less than 1 to 2% of the total p53 level was observed). When PAb421 was added to the same extract, the remainder of the p53 in the extract was immunoprecipitated (60 to 70% of the total p53 in the cell in this example). Second, when more PAb246 antibody was added to an extract previously incubated with this antibody and the additional PAb246 was incubated at 4°C overnight (in-

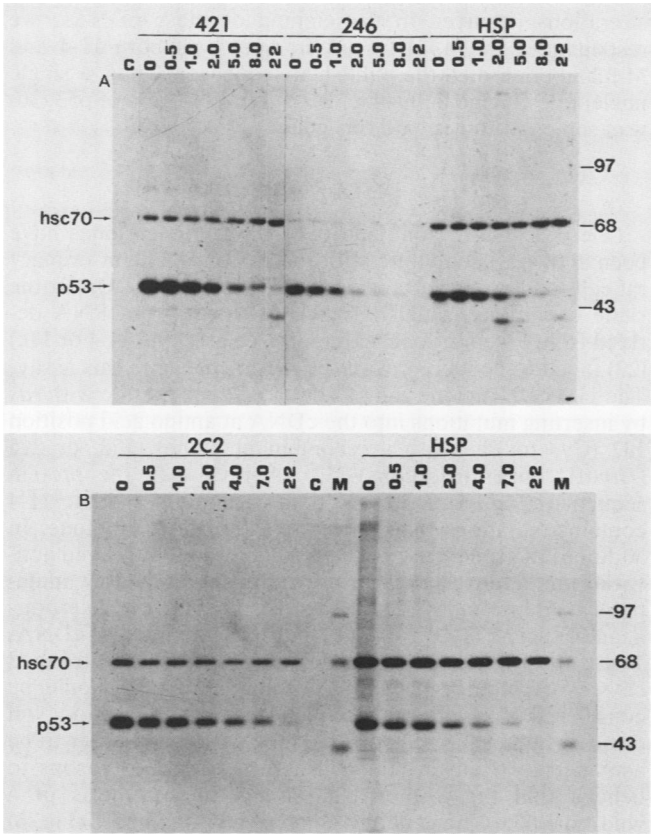

FIG. 4. Determination of p53 half-life in p53-plus-*ras*-transforme-d cell lines. (A) LTRp53cG-plus-*ras*-transformed cell line A1 was pulse-labeled and chased as described in Materials and Methods. Chase times were for 0, 0.5, 1.0, 2.0, 5.0, 8.0, and 22 h. Equivalent amounts of TCA-insoluble radioactivity (10^6 cpm) from each time point were immunoprecipitated with PAb421 (421), PAb246 (246), or anti-hsp70 antiserum (HSP). TCA-insoluble counts (10^6 cpm) from the unchased (0) time point were also immunoprecipitated with control nonimmune rabbit serum (C). Molecular size markers are indicated (in kilodaltons). (B) MSVKH215-plus-*ras*-transformed cell line A2 was pulse-labeled and chased for 0, 0.5, 1.0, 2.0, 4.0, 7.0, and 22 h. Immunoprecipitations from 5×10^6 cpm were performed with RA32C2 (2C2), anti-hsp70 antiserum (HSP), and control nonimmune rabbit serum (C; zero time only). Lanes M, Molecular size markers (in kilodaltons).

creasing the time to reach antigen-antibody equilibrium), no additional p53 bound to PAb246. Therefore, neither increased concentrations nor time revealed additional PAb246$^+$ p53 molecules. Furthermore, the PAb246$^+$ p53 and PAb246$^-$ p53 were different with regard to their association with hsc70, and in the next section it will become clear that these two forms of p53 had very different half-lives in the same cell (were physiologically distinct forms).

Turnover of murine p53 in p53-plus-*ras*-transformed cell lines. Previous reports have suggested that the elevated levels of p53 in SV40-transformed cell lines can result from the stabilization of p53 in the p53-large T antigen protein complexes (19, 25). To investigate whether the p53 present in the p53-hsc70 complex possessed an increased half-life, the turnover of p53 in LTRp53cG-plus-*ras*- and KH215-plus-*ras*-transformed cell lines was examined by pulse-chase experiments. Cells were pulsed with [^{35}S]methionine for 1 h and then chased for various periods of time in the presence of excess unlabeled methionine. The p53 or hsc70 was then immunoprecipitated from equivalent amounts of TCA-precipitable counts for each time point. Representative autoradiographs are presented in Fig. 4A and B. The results were quantitated for these and several additional cell lines (Table 2) by solubilizing the gel slices containing p53 and counting them in a scintillation counter. In three independently derived cell lines produced by cotransfection of the LTRp53cG genomic clone plus *ras* (Table 2, lines A1, B3, and B4), between 40 and 80% of the p53 protein produced reacted with PAb246 (was PAb246$^+$). The half-life of PAb246$^+$ p53 was between 15 and 45 min (Table 2). This is comparable to the half-life of rat p53 in secondary rat embryo fibroblasts (data not presented). The remainder of the p53 in these cells, PAb246$^-$ or hsc70-associated p53, had a half-life that was 4- to 20-fold longer than the p53 that was not associated with hsc70 (Table 2). The half-life of p53 associated with hsc70 was determined in two ways: by coimmunoprecipitation with anti-hsp70 sera and by determining total labeled p53 in the cells with PAb421 and subtracting counts due to p53 free of hsc70 (PAb246$^+$). These two estimates agreed quite well. This analysis demonstrates that the half-life of the total p53 protein in these cells (PAb421) was derived from at least two components or species of p53; PAb246$^+$ with a short half-life and PAb246$^-$ with a longer half-life. The PAb246$^-$ p53 was bound to hsc70. Because the percentage of p53 bound to hsc70 (PAb246$^-$) can vary between cell lines (40 to 80%), the relative contribution to the total p53 half-life can vary. It is interesting that in B4 cells, in which only 20% of the p53 was

TABLE 2. Half-life of p53

Transforming sequences and cell line	Half-life (min)				PAb246$^+$[b] (% of total)
	Total p53[a]	PAb246$^+$[b]	PAb246$^-$[c]	hsc associated[d]	
LTRp53cG + *ras*					
A1	105	45	185	150	40
B3	50	20	120	—[e]	65
B4	160	15	>360	315	80
MSVKH215 + *ras*					
4	110	—	—	—	0
A2	80	—	—	100	0
C3	140	—	—	120	0

[a] Determined with PAb421 or RA32C2.
[b] Determined with PAb246.
[c] Result calculated by subtracting counts immunoprecipitated by PAb246 from counts immunoprecipitated by PAb421 (or RA32C2).
[d] Determined by counts coimmunoprecipitated by anti-hsp70 sera.
[e] —, Not determined.

PAb246$^-$ and bound to hsc70, the longer half-life of this p53 species (315 to 360 min) compensated for the low percentage. This contrasts with A1 cells, in which 60% of the p53 was PAb246$^-$ (bound to hsc70) but had a half-life of 150 to 185 min. Thus, the total p53 pool in these two cell lines had similar half-lives (105 to 160 min). The cell lines transformed by KH215 plus *ras* produced only PAb246$^-$ p53 molecules which bound to hsc70. The half-life of this p53 mutant protein was two- to eightfold greater than the PAb246$^+$ p53 from transformed cell lines (Table 2). KH215 p53, however, had a shorter half-life than genomic p53 that was PAb246$^-$ (LTRp53cG) (Table 2). The net result was that the half-life of the total p53 protein pool in LTRp53cG- or KH215-plus-*ras*-transformed cells was approximately the same and, in both instances, was greater than that of p53 in nontransformed primary cells (15 to 30 min).

Mapping of an activating mutation in a Meth A p53 cDNA. Cells derived from a Meth A fibrosarcoma possess elevated levels of p53, and the p53 in these cells has a half-life of approximately 4 h (25). Furthermore, Meth A p53 is found in a complex with a 68K murine protein in Meth A cells (10, 23), preferentially binds a 68K monkey protein in COS cells (23), and does not bind PAb246 (18). A cDNA derived from Meth A cells has also been shown to transform Chinese hamster embryo fibroblasts in cooperation with an activated *ras* gene (7). To confirm that a Meth A cDNA is activated relative to 11-4, the transforming activity of a cDNA (M-8) (1) under the control of the HaMSV LTR was assayed on early passage rat embryo fibroblasts with an activated *ras* gene (Fig. 5). M-8 possessed activity in this assay. To map the location of the sequences responsible for the transforming activity of LTR M-8, cDNA-cDNA hybrid clones were constructed between 11-4 and M-8 (Fig. 5), and the relative transforming activities of the hybrid clones were assayed. The only fragment found to confer transforming activity to 11-4 was the *Apa*LI (amino acid 120)-*Kpn*I (amino acid 215) fragment of M-8 (Fig. 5). A comparative examination of the sequences of 11-4 (p53-17c) and M-8 (1) (Fig. 6) revealed the presence of a single amino acid change (Cys to Phe) at position 132. To eliminate the possibility that additional alterations occurred in the cloning of these cDNAs, we resequenced the *Apa*LI-*Kpn*I fragments of both 11-4 and M-8 and confirmed the published sequences. Thus, a single nucleotide change in this highly conserved region of p53 (30) was sufficient to activate this gene.

DISCUSSION

One p53 cDNA clone and two p53 genomic clones have been shown to cooperate with the *ras* oncogene in primary rat cells and produce transformed cell foci (7, 21). Under the same conditions, a cDNA clone produced from RNA derived from F9 embryonal carcinoma cells (clone 11-4 or 17c) (22) failed to cooperate with the *ras* oncogene in this assay. The 11-4 cDNA clone can be activated to cooperate with *ras* by inserting mutations into the cDNA at amino acid position 132 (Cys to Phe with a recombinant clone), 158, or 215 (*Hin*dIII linker insertions) (32). A review of the protein sequence (Fig. 6) indicates that it is unlikely that 11-4 contains mutations that inactivate a transforming clone. In addition, the specificity of these transformation-related activating mutations derives from the observation that mutations at amino acids 132, 158, and 215 activate but insertions at amino acid 222, 232, or 344 fail to activate the 11-4 cDNA. These observations bring up the question of which published cDNA or genomic nucleotide sequence of p53 should be considered the wild-type sequence. This conclusion will decide whether the p53 gene must be mutated in order to be activated for transformation. There are several reasons to believe that the 11-4 cDNA directs the synthesis of a wild-type p53. The genomic clone p53cG or Ch53-7 (Fig. 6) was cloned from normal BALB/c mouse liver DNA (3) and should be a strong candidate for the wild-type nucleotide sequence. The genomic clone Ch53-7 has a single amino acid difference (Ala to Val at amino acid 135) from the p53-17c (11-4 cDNA) that fails to transform rat cells with *ras* (Fig. 6). This alteration most likely represents a mutation in the Ch53-7 (p53cG) genomic clone, since none of the six addi-

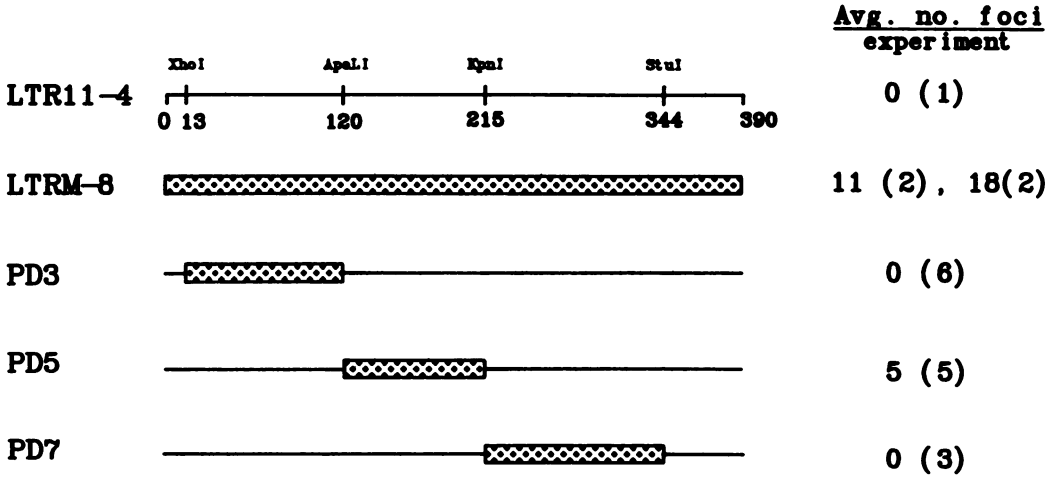

FIG. 5. Activation of p53 cDNA clone 11-4 by recombination with p53 cDNA clone M-8. LTR11-4 contains p53 cDNA sequences from the F9 embryonal carcinoma cell line (thin line) (22). LTRM-8 contains p53 cDNA sequences from cDNA clone M-8 (bar) derived from the methylcholanthrene-induced sarcoma cell line Meth A (1). Recombinants PD3, PD5, and PD7 were constructed and used in p53-plus-*ras* transformation assays as described in Materials and Methods. The restriction endonuclease recognition sites used to construct the recombinants are indicated for LTR11-4, as is the position in the amino acid sequence which is specified by the recognition site. The average number of foci occurring in individual experiments are indicated; the number in parentheses represents the number of transfections within a given experiment.

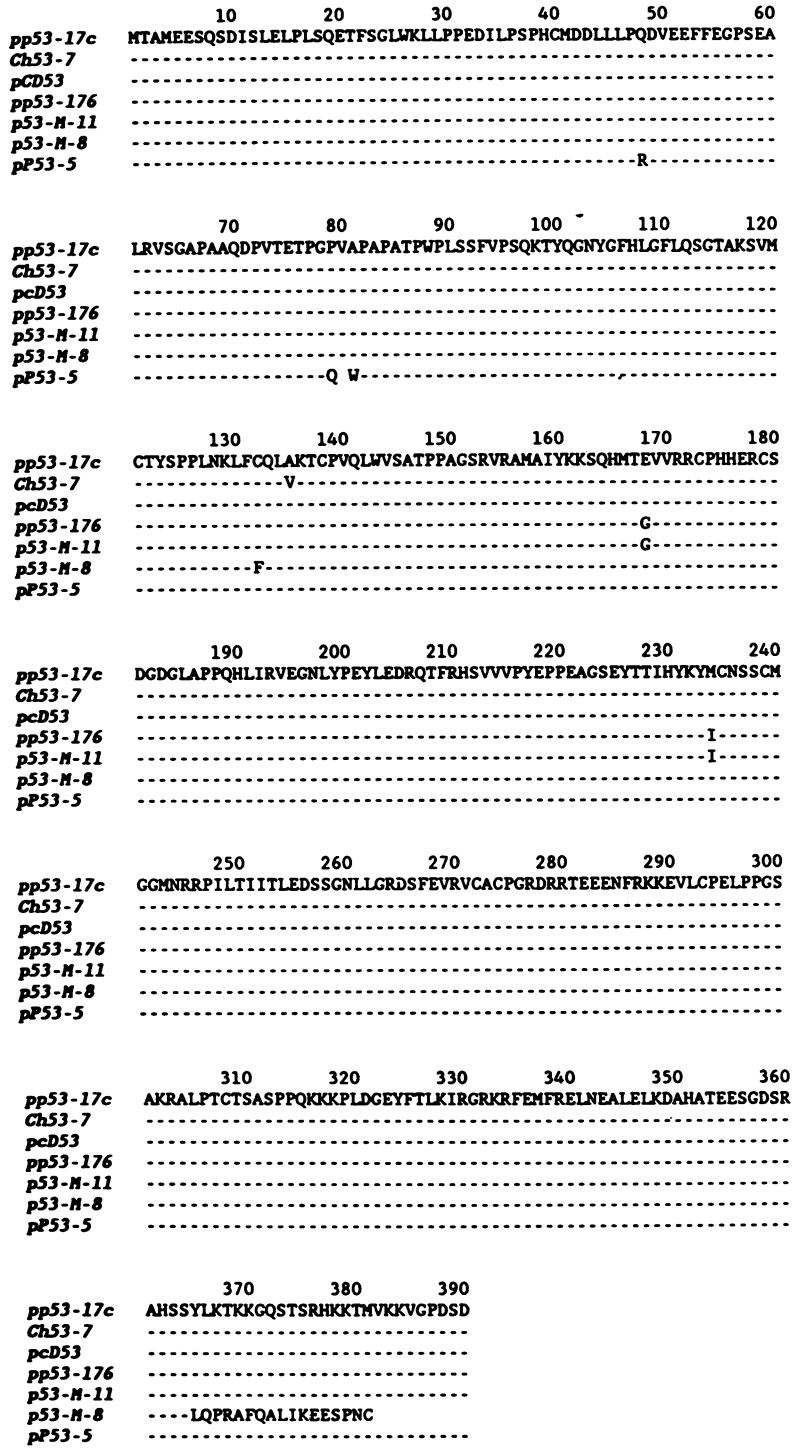

FIG. 6. Homology between published murine p53 sequences. Sequences are compared with the p53 protein encoded by the F9 embryonal carcinoma-derived p53 cDNA pp53-17c (22). Dashes indicate identity; differences between sequences are indicated as the substituted amino acid. Sequences used are as follows. Ch53-7, p53 genomic sequence from normal BALB/c mouse liver (3); pCD53, concanavalin A-stimulated mouse T cells (1); pp53-176, methylcholanthrene-induced tumor cell line Meth A (35); p53-M-11 and p53-M-8, Meth A (1); pP53-5, SV40-transformed mouse fibroblast cell line SVA31E7 (14).

tional murine p53 cDNA sequences published to date (Fig. 6) have valine in position 135; all of the murine p53 sequences have alanine at that position. Additionally, all of the human cDNA clones have alanine at that position, and this is a very conserved region of the p53 protein (30). The genomic clone Ch53-7 was resequenced multiple times when isolated and truly has valine at position 135 (3). Interestingly, position 135 is in the region of activating mutations (amino acids 132, 158, and 215) for the p53 gene. The second reason to think that the 11-4 cDNA directs the synthesis of wild-type p53 is that

when transfected into COS monkey cells, a 53,000-dalton protein is produced which efficiently binds with the SV40 large T antigen and is recognized by five different murine p53 monoclonal antibodies (32). These five antibodies recognize distinctly different epitopes over the entire sequence of this protein (32, 34). When the genomic p53 clone was analyzed in this manner, the p53 protein produced bound to T antigen poorly and associated with the monkey 68K protein (hsp family) (23). Thus, the genomic clone produces p53 with a phenotype that is similar to the KH215 and BH158 insertion mutations of 11-4 cDNA. The third reason to believe that the 11-4 cDNA contains the wild-type sequence of p53 is that another cDNA clone, pcD53 (Fig. 6), derived from concanavalin A-stimulated T cells, has the identical sequence as 11-4 (1). The results presented here would predict that the pcD53 clone, unlike many other p53 cDNAs, will also fail to transform rat cells in cooperation with the *ras* oncogene. The second prediction derived from this reasoning is that the insertion of valine at amino acid 135 into the 11-4 cDNA clone should (like the genomic clone) activate it for transformation with *ras*. This is presently being tested. Finally, the implication of this line of reasoning is that several independent cDNA clones of p53 obtained from several sources contain activating mutations for transformation. Both p53-M-8 and p53-M-11 are derived from RNA taken from cell lines established from methylcholanthrene-induced tumors; in these instances, the activating mutations contributing to oncogenesis could have been selected for in the p53 gene.

Although qualitative changes in p53 are important in determining the transforming activity of this gene, the levels of p53 protein are also important variables in these transformation assays. The genomic p53 clone produces an 8 to 10-fold-higher level of p53 than that directed by the cDNA clones. The presence of introns in the genomic clone may increase the level of mRNA and be an important part of the LTRp53cG activity. This is currently under investigation.

The second major point made in this study is the possible role of the heat shock 70K proteins in p53-mediated transformation. All of the p53 cDNA activating mutations for transformation with *ras* produce a p53 protein that preferentially binds to or interacts with the hsc70 protein in transformed cells. The same p53 mutants (BH158 and KH215) also fail to express the PAb246 epitope which maps to amino acids 88 to 109. This observation suggests that these mutant proteins have a conformational change. Alternatively, the association of hsc70 with p53 may directly mask this epitope. The genomic p53 clone LTRp53cG in p53-plus-*ras*-transformed cell lines produces two categories of p53 proteins, $PAb246^+$ and $PAb246^-$. The $PAb246^-$ p53 proteins preferentially associate with hsc70 and have a longer half-life (4- to 20-fold greater) than the $PAb246^+$ p53 that is not complexed with hsc70. This p53-hsc70 complex was a consistent feature of all of the p53-plus-*ras*-transfected cell lines. Of 9 independent LTRp53cG-plus-*ras*-transformed rat cell lines, 11 LTRp53cG (transfected alone)-rat cell lines and 11 KH215-plus-*ras*-transformed rat cell lines analyzed, all had p53-hsc70 complexes. Consistent with this line of reasoning is the observation that the insertion into 11-4 cDNA of a 285-base-pair fragment encoding a single nucleotide change from a Meth A p53 cDNA clone (which produces a p53 protein that has transforming activity) was sufficient to activate the parental clone for transformation. Although transformed cell lines have not yet been derived from these foci and analyzed for the presence of the p53-hsc70 complex, the linker insertion mutations at amino acid 158 or 215 are clearly sufficient to alter both the transforming activity and binding properties of p53 to the hsp70 family.

The correlation between increased transforming activity and binding of p53 to hsc70 in these mutant p53 proteins does not prove that the hsc70 complex is involved in the transformation events. Even the fact that the hsc70-p53 complex contained a cellular species of p53 with a longer than average half-life does not prove a functional role for this class of p53 in the cell. These experiments do provide the first evidence that heat shock proteins, like hsc70, can affect the level or half-life of an oncogene product like p53. The analogy to the hsp70-related protein in *Escherichia coli*, DnaK, is striking. The induction of the *E. coli* heat shock response is mediated by σ^{32} levels (11). DnaK modulates the heat shock response by reducing σ^{32} levels via a posttranscriptional mechanism (11), perhaps at the level of protein stability (2). *dnaK* mutants are unable to achieve this downregulation of σ^{32} (and thus the heat shock response); instead, these mutants contain a more stable σ^{32} (11). Thus, DnaK may regulate transcriptional responses by causing a posttranscriptional decrease in σ^{32} levels. The reason this analogy is of interest is that DnaK, which has up to 50% amino acid homology with hsc70, also binds specifically to p53 when p53 protein is produced in *E. coli* (C. Clark and A. J. Levine, unpublished observations). Perhaps p53-hsc70 complexes will be shown to regulate growth responses in a similar fashion.

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