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Accumulation of p53 in a Mutant Cell Line Defective in the Ubiquitin Pathway

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The wild-type p53 gene product plays an important role in the control of cell proliferation, differentiation, and survival. Altered function is frequently associated with changes in p53 stability. We have studied the role of the ubiquitination pathway in the degradation of p53, utilizing a temperature-sensitive mutant, *ts20*, derived from the mouse cell line BALB/c 3T3. We found that wild-type p53 accumulates markedly because of decreased breakdown when cells are shifted to the restrictive temperature. Introduction of sequences encoding the human ubiquitin-activating enzyme E1 corrects the temperature sensitivity defect in *ts20* and prevents accumulation of p53. The data therefore strongly indicate that wild-type p53 is degraded intracellularly by the ubiquitin-mediated proteolytic pathway.

The nuclear phosphoprotein p53 is a negative regulator of cell proliferation and transformation (2, 11, 14). In many established cell lines and human cancers, the gene is mutated. Such mutations commonly result in a p53 which has increased stability and markedly altered function and have been the subject of several studies (30, 31, 38, 57). Genetic instability indicated by gene amplification and impaired control of the G_1-S transition in the cell cycle are exhibited by cells homozygous for p53 mutations (reviewed in reference 23). A role for p53 in transcriptional control is shown by sequence-specific DNA binding (4, 15, 26, 28). Other studies have shown that wild-type p53 can affect expression of several genes and that specific binding of p53 to transcription factors, TATA-binding protein, and CCAAT-binding factor results in the altered transcription from the test promoters (1, 51). An association between mdm2 protein and p53 has been established, and the *mdm2* gene is known to undergo amplification in murine transformed cells and many human sarcomas (13, 40, 42). Transgenic mice with a complete loss of p53 protein develop normally but have a high frequency of tumors postnatally (10). Humans heterozygous for germ line p53 mutations have a high susceptibility to carcinogenesis (34).

The mechanism by which p53 is regulated intracellularly is not completely understood, although it is widely accepted that turnover of the protein is an important aspect. Normally, p53 is a short-lived protein with a half-life of about 30 min (16, 43, 46). DNA tumor viruses express gene products which bind the normal p53 and alter its properties. In the case of human papillomavirus type 16, for example, the half-life of p53 is decreased in complexes with E6 and p53 (50), whereas complexes with simian virus 40 (SV40) large T protein and adenovirus E1B p55 result in an increased half-life of p53 (46, 49). Mutations in SV40 large T protein which affect binding of p53 decrease the efficiency of SV40-mediated transformation of susceptible cells (32, 35, 56, 63).

Polyubiquitination of proteins has been shown to be involved in nonlysosomal proteolysis (as recently reviewed in reference 24). Ubiquitin (Ub), a small basic protein of 76 amino acids found in all eukaryotic cells, can be covalently linked to proteins. Proteins that are polyubiquitinated at a single site become targets for degradation. Ubiquitination is a multistep process. Ub-activating enzyme (E1) catalyzes the initial ATP-dependent step, resulting in the formation of an E1-bound ubiquitinyl adenylate. The activated Ub is then transferred to one of the several Ub-conjugating enzymes (E2) and subsequently to the targeted specific substrate proteins. Certain E2 enzymes require an additional enzyme, the Ub-protein ligase (E3), for the recognition of certain substrates and transfer of Ub (24). Two lines of indirect evidence suggest that the ubiquitination pathway may be involved in p53 turnover intracellularly. First, Scheffner et al. (50) have shown that E6-p53 complexes generated in vitro are more rapidly degraded in reticulocyte extracts and that ubiquitinated intermediates can be detected. Under their experimental conditions, however, free p53 was stable for more than 3 h. Second, Ceichanover et al. (6) have used rabbit reticulocytes to synthesize p53, N-Myc, c-Myc, and c-Fos proteins in vitro and examined their subsequent turnover. The degradation of all these proteins was ATP dependent and involved E1. Antibodies against E1 were inhibitory in a dose-dependent manner, and this effect was reversed by the addition of purified E1.

In order to determine whether p53 is indeed degraded by the Ub pathway in intact cells, we have exploited one of several temperature-sensitive (ts) mutants of BALB/c 3T3, *ts20*, which is shown in the present study to have a defect in that pathway. We demonstrate that the mutant accumulates p53 at the restrictive temperature but not at the permissive temperature. Furthermore, correction of the ts defect by introduction of the human gene for E1 into *ts20* blocks the accumulation of p53. The data presented here strongly support the idea that the Ub pathway regulates the turnover of wild-type p53 intracellularly, as in cell-free systems.

MATERIALS AND METHODS

Cell lines. Isolation and characterization of the ts phenotype of mutants *ts2* and *ts20* from wild-type BALB/c 3T3 clone *A31* have been previously described (27, 52, 62). Mutant *ts22* included in the present study was isolated previously in experiments which also yielded *ts20*. A 6-thioguanine (TG)-resistant clone deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase (*hprt*$^-$) was isolated from *ts20* in 10^{-5} M TG and designated *ts20TG*^{R}. This cell line does not grow in HAT medium (DF-10 medium supplemented with hypoxanthine

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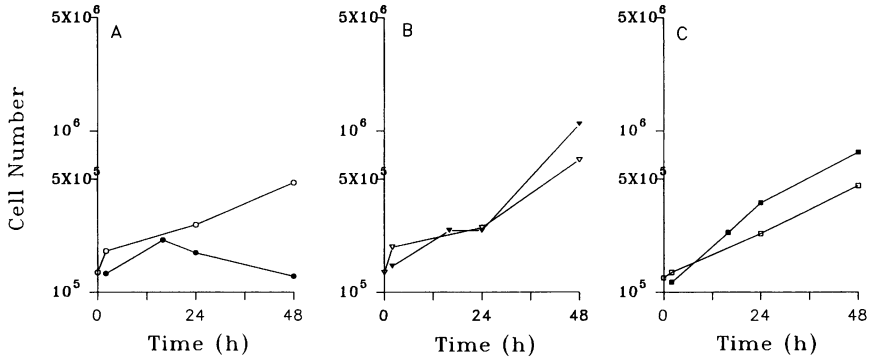

FIG. 1. Temperature-dependent growth of cells. Cells were seeded at 1×10^5 to 2×10^5 cells per 60-mm-diameter dish at 35°C for 16 h (0 h in the figure) before being shifted to 39°C (●, ▼, ■) or reincubated at 35°C (○, ▽, □). At 4, 16, 24, and 48 h thereafter, cells were trypsinized and the number of cells was determined with a Royco cell counter. The cell lines used were *ts20TG*^{R} (○, ●) (A), *H38-5* (▽, ▼) (B), and wild-type *A31* (□, ■) (C).

[5×10^{-5} M]–aminopterin [5×10^{-7} M]–thymidine [1×10^{-5} M]). Cells were maintained at 35°C in a humidified incubator with 7.5% CO_2 in DF-10 medium (Dulbecco-Vogt medium and Ham's F-10 medium [50:50, vol/vol]) supplemented with 10% newborn calf serum. The permissive and nonpermissive temperatures for mutant cell lines were 35 and 39°C, respectively.

Plasmids. p53A6.6 containing the whole polyomavirus genome cloned at the *Bam*HI site in pAT153 was propagated and harvested from DH-1 (*dam*$^+$) bacteria as previously described (9, 29).

Construction of pUCHG-M1. A plasmid with two dominant selectable markers for mammalian cells (*hph*, encoding the enzyme hygromycin B phosphotransferase, confers resistance to the cytotoxic drug hygromycin B [5], and *gpt*, encoding xanthine-guanine phosphoribosyltransferase, permits cellular proliferation in the presence of mycophenolic acid if the medium is supplemented with xanthine [41]) was constructed by introducing the *Bam*HI fragment containing *gpt* from pSV2*neo*SV*gpt* (53) into pUC18/HG generated by E. Liu (formerly at the University of California, San Francisco) and provided by M. B. Small of our department. The resultant plasmid, pUCHG-M1 (7.4 kb), expresses *gpt* with transcriptional signals derived from SV40 and *hph* from the herpes simplex virus thymidine kinase promoter.

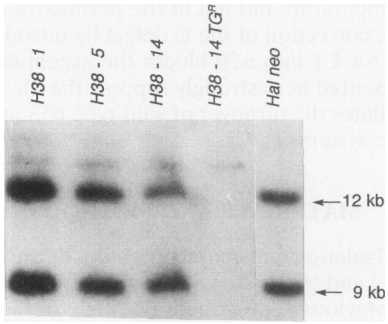

FIG. 2. Southern blot analysis of primary transfectants *H38-1*, *H38-5*, and *H38-14*, back-selected primary transfectant *H38-14TG*^{R}, and the human cell line Hal*neo*. HMW DNA (10 μg) from each cell line was digested with *Bam*HI, electrophoresed on a 0.8% agarose gel, transferred to a Nytran membrane and probed with ^{32}P-labelled 3.5-kb human cDNA for E1. The sizes of the bands are indicated.

Construction of pSG-E1. For the expression of cDNA for the human Ub-activating enzyme E1 in mammalian cells, DNA encoding E1 was released from the vector pGEM7zf(+) (21) (a gift from A. L. Schwartz and P. Handley of the Washington University School of Medicine, St. Louis, Mo.) by *Bam*HI and *Hin*dIII and directionally cloned into the mammalian expression vector pSG5 (Stratagene) cut with the same restriction enzymes.

Transfections with human genomic DNA and pSG-E1. Stable correction of the ts defect in mutant cells was obtained by the calcium phosphate-mediated DNA transfer technique (19, 59) with cotransfection of pUCHG-M1 with human genomic DNA or pSG-E1. High-molecular-weight (HMW) DNA was prepared from Hal*neo*, an SV40-transformed human fibroblast line developed in our laboratory (47), as described previously (59) except that the solution was passed through a 25-gauge needle four times before coprecipitation. Linearized pUCHG-M1 DNA (2.5 μg, cut with *Nar*I) and 22.5 μg of genomic DNA were coprecipitated and added to *ts20TG*^{R} cells for 4 h. Dishes were refed with DF-10 medium and allowed to grow at 35°C for 3 days. Cells from a set of dishes (12 to 20 dishes per set) were distributed in twice the number of dishes for selection of the Hyg^R phenotype (hygromycin B [Boehringer Mannheim Biochemicals], 175 μg/ml). Cultures were shifted to 39°C after 8 days, and incubation was continued until colonies (>4 mm diameter) were isolated. Fourteen temperature-resistant (ts$^+$) colonies were isolated from a total of 1.4×10^8 transfected cells (see Table 1); all but one were *gpt*$^+$ as well. The individual clones were propagated in DF-10 medium

TABLE 1. Correction of ts growth defect in various mutants

Recipient cell line	Donor DNA[a]	Total cells	No. of colonies	
			Hyg^R[b]	ts$^+$[c]
ts20TG^R	HMW[d]	1.4×10^8	4,360	14
ts20TG^R	pSG-E1[e]	2.0×10^6	45	19
ts20B	pSG-E1	1.0×10^6	10	4
ts22A	pSG-E1	1.5×10^6	23	8
tsE2	pSG-E1	1.5×10^6	19	9

[a] Cells were transfected by pUCHG-M1 and donor DNA as described in Materials and Methods.
[b] Colonies in hygromycin B at 35°C.
[c] Colonies in hygromycin B at 39°C.
[d] HMW genomic DNA from Hal*neo*.
[e] E1 cDNA in pSG5.

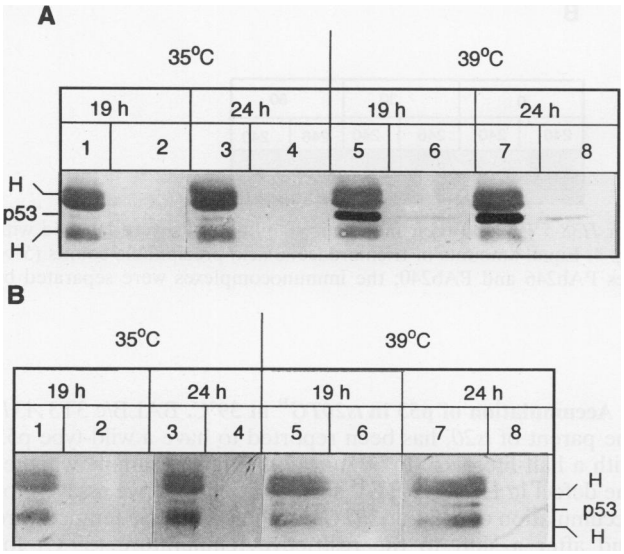

FIG. 3. Accumulation of p53 in *ts20TG*^{R} at 39°C. Extracts containing equal amounts of proteins from *ts20TG*^{R} (A) or *H38-5* (B) were prepared in immunoprecipitation buffer as described in Materials and Methods and separated by SDS-PAGE with (lanes 1, 3, 5, and 7) or without (lanes 2, 4, 6, and 8) prior precipitation using monoclonal antibody PAb421, transferred to nitrocellulose, and probed with PAb421. Samples (550 μg) were analyzed by immunoprecipitation, and 30-μg samples were analyzed directly. The blot was probed with goat antibody to mouse immunoglobulin G conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals), and proteins were visualized as described previously (47). H corresponds to the heavy chains of immunoglobulins.

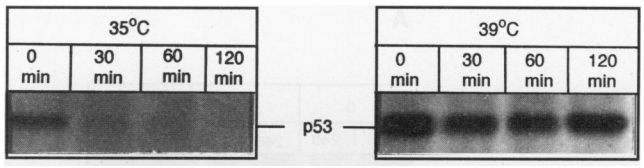

FIG. 4. Metabolic stability of p53 in *ts20TG*^{R}. Cells were labelled with [^{35}S]methionine for 60 min (pulse) as described in Materials and Methods, and lysates were prepared and processed as described in the legend to Fig. 3. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. A total of 3.5 × 10^6 trichloroacetic acid-precipitable counts per lane were used.

at 35°C prior to back-selection in TG to verify linkage of pUCHG-M1 and the ts$^+$ phenotypes. Seven were identified, and one (*H38-5*) was chosen for detailed study. In the pUCHG-M1/pSG-E1 cotransfection, *Nar*I-cut linear pUCHG-M1 (1.8 μg) mixed with pSG-E1 (18.75 μg; supercoiled) was used. The selection procedure was followed as described above.

Transient transfection. Cells were seeded at 5 × 10^5 cells per 100-mm-diameter dish, and 16 h later, 1 μg of p53A6.6 DNA and 9 μg of carrier DNA (salmon sperm DNA) were transfected by the calcium phosphate coprecipitation technique as described above. After 16 h at 35°C (time zero), the cells were either shifted to 39°C for 24 h or reincubated at 35°C. The viral DNA was isolated by Hirt extraction, digested with *Sal*I and *Dpn*I, and analyzed by Southern blots as described previously (9).

SDS-PAGE, Western blots (immunoblots), and immunoprecipitation. Exponentially growing cells were seeded at 5 × 10^5 cells per 100-mm dish at 35°C. Sixteen hours later, the cells were either shifted to 39°C or reincubated at 35°C. At various times thereafter, the cells were washed twice with cold phosphate-buffered saline and lysed with 1 ml of immunoprecipitation buffer (25 mM Tris [pH 7.5], 100 mM NaCl, 2% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, aprotinin [50 μg/ml], 50 μM leupeptin) as described previously (47). Extracts containing equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with or without prior precipitation by using monoclonal antibody PAb421 (a gift of C. Prives of Columbia University, New York, N.Y.). To generate radiolabelled proteins, cells were prepared as described above except that the cells were washed with methionine-free medium, incubated for 60 min in methionine-free medium, and then labelled with [^{35}S]methionine (58 μCi/ml) for 60 min (pulse). Cultures were harvested (0 min) or reincubated in complete medium for various times (30, 60, and 120 min) with a 50-fold excess of cold methionine (chase). Lysates were prepared and processed as described above. Immunoprecipitates from extracts were collected by using monoclonal antibodies PAb240 and PAb246 (Oncogene Science Inc., Uniondale, N.Y.) and PAb421 against p53 and analyzed by SDS-PAGE and fluorography as previously described (45). The quantitation of p53 was determined by densitometric scanning (Molecular Dynamics model 300B computing densitometer).

RESULTS AND DISCUSSION

Correction of *ts20TG*^{R} by DNA sequences encoding the Ub-activating enzyme E1. We have isolated a series of ts mutants of BALB/c 3T3 (*ts2*, *ts20*, and *ts22*) which are defective in cell and polyomavirus DNA syntheses (27, 52, 62). HMW human DNA was used to correct the ts growth defect in a TG-resistant subline of *ts20* (*ts20TG*^{R}) by a coselection strategy with a plasmid, pUCHG-M1, which we constructed for this purpose. Transfectants were enriched at 35°C in hygromycin B, and ts$^+$ clones were isolated upon the shift to 39°C. Subsequent passage of these ts$^+$ clones at 35°C followed by back-selection in TG permitted us to distinguish those clones which were corrected by the HMW human DNA from those with reversions at the ts locus. Figure 1 shows the growth properties of the cell lines. *ts20TG*^{R} shows an increase for 16 h followed by a decline in the number of cells (Fig. 1A) and thymidine incorporation (data not shown), as previously reported for *ts20* (62); however, *H38-5* grows equally well at both temperatures (Fig. 1B), as does the wild-type parental cell line *A31* (Fig. 1C).

We previously have shown that cell hybrids derived from fusion of BALB/c 3T3-derived *ts2*, *ts20*, and *ts22* and mouse L cell-derived *tsA1S9* did not complement growth at the nonpermissive temperature (27). Furthermore, a human X chromosome-linked gene corrected the ts defect in *ts2* (27), *ts20* (62), and *tsA1S9* (reference 61 and references therein). McGrath and coworkers (36) noted a marked similarity between the sequences encoding the yeast UBA1 protein and the human sequence which corrected *tsA1S9*, suggesting that a defect in the Ub-activating enzyme E1 was responsible for the ts defect in *tsA1S9*. We therefore determined whether our primary transfectants carried the human E1 sequences. Using oligonucleotide primers derived from the sequence of the human A1S9 cDNA clone, we found amplification of a 1.2-kb fragment in all the temperature-resistant clones tested in PCR experiments. The back-selected ts clones did not amplify the fragment, confirming the loss of the E1 sequences (data not shown). Use of the same primers with the cDNA yielded a

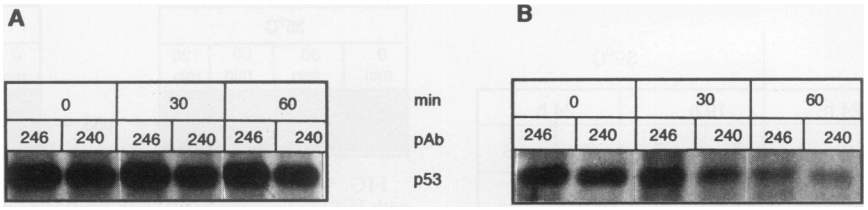

FIG. 5. Immunoprecipitation of different forms of p53 in *ts20TG*^{R} (A) or *H38-5* (B) and their metabolic stability. Cells were labelled with [^{35}S]methionine for 60 min and chased for 30 or 60 min as described for Fig. 4. Equal amounts of trichloroacetic acid-precipitable counts (5×10^6 cpm) were sequentially immunoprecipitated with monoclonal antibodies PAb246 and PAb240; the immunocomplexes were separated by SDS-PAGE and visualized by fluorography as described for Fig. 4.

fragment of 360 bp, suggesting the presence of an intron in the genomic fragment. To further document this result, genomic DNA from the primary transfectants *H38-1*, *H38-5*, and *H38-14* and the human cell line Hal*neo* was digested with *Bam*HI and the Southern blot was probed with the 3.5-kb human cDNA for E1. It hybridized with two fragments of 12 and 9 kb in all three primary transfectants (Fig. 2). Moreover, a ts clone isolated after back-selection in TG (*H38-14TG*^{R}) did not show any hybridization, indicating the loss of the corresponding human sequences.

To verify that sequences encoding E1 rather than some other (unknown) linked sequences were in fact responsible, we cotransfected the full-length cDNA encoding human E1 (pSG-E1) with pUCHG-M1 into each of the BALB/c 3T3 ts mutants. As shown in Table 1, all three mutants were effectively corrected for the ts defect. Although the number of ts$^+$ colonies from different cell lines varied, the ratios of ts$^+$ to Hyg^R transfectants were very similar. The fraction of temperature-resistant colonies in the Hyg^R clones overall is 130 times higher with the cDNA vector (0.42) than the fraction with human genomic DNA (0.0032). We also found that correction of the ts defect in *ts20TG*^{R} restored the ability to support polyomavirus DNA synthesis at the nonpermissive temperature (data not shown).

Accumulation of p53 in *ts20TG*^{R} at 39°C. BALB/c 3T3 *A31*, the parent of *ts20*, has been reported to have a wild-type p53 with a half-life of 20 to 30 min (20, 40). To examine whether the defect in E1 in *ts20TG*^{R} affected p53 levels, we assayed for accumulation of p53 in *ts20TG*^{R} at the permissive temperature and after a shift to the restrictive temperature (39°C) for various times as shown in Fig. 3A. p53 was assayed by immunoblot after immunoprecipitation of cell extracts with PAb421 (22). Low levels are detected in *ts20TG*^{R} at 35°C, as expected; however, there is a marked increase in the band at 19 and 24 h after the shift to 39°C. We also analyzed extracts without prior concentration by immunoprecipitation. As expected, p53 is undetectable at 35°C, whereas it is evident at 39°C. The level of the unconcentrated extract (Fig. 3A, lane 8) is comparable to that of the concentrated sample at 35°C (lane 1 or 3), indicating at least an 18-fold accumulation. Further experiments have shown that increases in p53 can be detected by 4 to 6 h after the shift to 39°C, and it continues to accumulate progressively up to 24 h at 39°C (data not shown). *H38-5*, corrected by the human gene encoding E1, does not show accumulation at either temperature (compare lanes 1 and 3 with 5 and 7 in Fig. 3B).

Accumulation of p53 is due to decreased turnover. We then sought to determine whether this effect was due to decreased

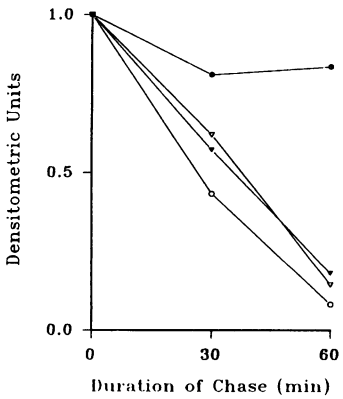

FIG. 6. Degradation of total p53 from *ts20TG*^{R} (○, ●) or *H38-5* (▽, ▼) at 35°C (○, ▽) or 39°C (●, ▼). Labelling of cells and immunoprecipitation of extracts were done as described for Fig. 4 except that the p53 in each extract was precipitated by using a combination of PAb240, PAb246, and PAb421. The immunocomplexes were separated by SDS-PAGE, visualized by fluorography, and quantitated by densitometric scanning. The values at different times are presented relative to the values at time zero (end of pulse) for each cell line.

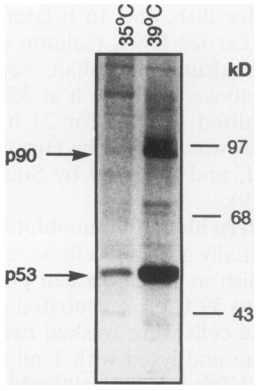

FIG. 7. Association of p53 with cellular protein p90 in *ts20TG*^{R}. The cells were labelled with [^{35}S]methionine for 3 h as described for Fig. 4. Cells were lysed in 1 ml of buffer composed of 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin (50 μg/ml), and 50 μM leupeptin. Immunoprecipitates were washed five times with 5% sucrose–1% Nonidet P-40–0.5 M NaCl–50 mM Tris (pH 7.5)–5 mM EDTA as described by Momand et al. (40). Lysates (5×10^6 cpm) were immunoprecipitated with PAb421, separated by SDS-PAGE, and detected by fluorography as described for Fig. 4.

turnover, as expected for a defect in Ub-mediated degradation. At 35°C and after the shift to 39°C for 6 h, cells were pulse labelled for 60 min with [^{35}S]methionine and chased for up to 2 h. Radiolabelled p53 was determined by immunoprecipitation with monoclonal antibody PAb421 followed by separation by SDS-PAGE and fluorography. The data in Fig. 4 show that p53 is stable in *ts20TG*^{R} at 39°C for at least 2 h but is rapidly degraded at 35°C as expected.

Mutant forms of p53 resulting from missense mutations which change a conformational epitope that can be differentiated by monoclonal antibodies have been reported. Thus, p53 molecules expressed from the wild-type gene are selectively recognized by PAb246, whereas many mutant forms are recognized by PAb240 (16, 60). In order to determine whether there is a preferential accumulation of one or the other form of p53, cells were pulse labelled with [^{35}S]methionine and chased as in the previous experiment, and the cell extracts were subjected to sequential immunoprecipitation by PAb246 followed by PAb240. The results in Fig. 5 show the presence of both immunologically different forms of p53 in *ts20TG*^{R} (Fig. 5A) and *H38-5* (Fig. 5B) at 39°C. Both forms of p53 persisted in *ts20TG*^{R} at 39°C but not at 35°C or in *H38-5* at either temperature (data not shown). Pooled data for all the immunological forms are shown in Fig. 6, demonstrating a half-life of 30 min for all unstable p53s. Thus, p53 is degraded by the Ub pathway regardless of the epitope conformation. The higher initial levels of both wild-type and putative mutant forms in *ts20TG*^{R} at 39°C need not be due to increased rates of synthesis, since a relatively long labelling period was used for the pulse (1 h) to ensure adequate incorporation into the other samples.

We considered and rejected other explanations for the accumulation of p53 in *ts20TG*^{R} at 39°C. First, a ts murine p53 which has a PAb240-reactive epitope at ≥37.5°C and a PAb246-reactive epitope at 32.5°C has been reported (37, 39). If there were such a temperature-dependent form of p53 in *ts20*, it should also have been present in *H38-5*, since both of these cell lines would be expected to contain the same alleles for p53. However, the data show that *H38-5* does not have a stable p53 at 39°C. Second, the PAb240-reactive form of p53 is often found associated with cellular heat shock protein hsp70, possibly resulting in its stabilization (25, 39, 44, 54, 55). We have looked for the presence of such a complex of p53 and hsp70 by using immunoprecipitation with anti-hsp70 antibody. Although there was hsp70 in the immunoprecipitate, there was almost no p53 (data not shown). Therefore, the accumulation of p53 in *ts20TG*^{R} at 39°C is not due to its association with hsp70. On the other hand, immunoprecipitation of p53 from radiolabelled cells at 39°C does efficiently coprecipitate the cellular protein p90, encoded by *mdm2* (3, 40), as shown in Fig. 7 and confirmed by immunoprecipitation with a monoclonal antibody to mdm2 (a gift of A. Levine, Princeton University) (data not shown).

Our data demonstrate that wild-type p53 accumulates in *ts20TG*^{R} at the restrictive temperature. Although BALB/c 3T3 *A31* is reported to have a wild-type p53, our cell line has been passaged for many generations and multiply recloned in the course of selection of the ts and TG-resistant phenotypes. Nonetheless, p53 is unstable at 35°C in *ts20* as expected for a wild-type allele. Furthermore, the p53 which accumulates at the restrictive temperature is reactive with antibody PAb246. The data therefore strongly support the idea that wild-type p53 accumulates because of the inhibition of the ubiquitination pathway. Mutant p53 may similarly be affected in *ts20TG*^{R}, although this conclusion is based solely on the immunological criteria used in this study. The putative mutant form is not more stable than the wild type in *ts20TG*^{R} at 35°C and *H38-5* at 35 and 39°C. Induction of a configuration reactive with PAb240 has been reported to occur in vitro (39); however, we consider this an unlikely explanation in itself. Although the extracts used for the immunoprecipitation experiments (Fig. 5) were frozen prior to analysis, other experiments using fresh extracts gave similar results. The findings described in this paper are consistent with prior in vitro studies and an earlier report that p53 is degraded in BALB/c 3T3 by an ATP-dependent system (20). Although the data presented here demonstrate that p53 is accumulated in cells that are defective in Ub-dependent proteolytic systems, the specific steps remain to be determined. Target sequences for ubiquitination have been described elsewhere (24), and one such example, the PEST sequences, has been identified in p53 (48). In view of the high level at which p53 accumulates in *ts20TG*^{R} at 39°C, it should be a useful cell line in assessing the role of such sequences and defining intermediates in p53 degradation. We would also predict that *ts20* will be a useful test cell system for assessing whether other growth-regulatory proteins are degraded by the ubiquitination pathway. Ubiquitination has been shown to be involved in chromatin structure (histones H2A and H2B [7]), cell cycle regulation (17, 18), DNA repair (12), and peroxisomes (58), as well as other pathways (24). Hence, many pathways are expected to be affected by temperature shift, consistent with the pleiotropic phenotype observed in *ts20* and other ts BALB/c 3T3 cell lines mutant in E1 (8, 27, 33, 52, 62).

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