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# Two Distinct Mechanisms Regulate the Levels of a Cellular Tumor Antigen, p53

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The steady-state levels of p53 protein and p53 mRNA in transformed and nontransformed cells were examined to elucidate the mechanisms controlling expression of p53. mRNA levels were determined by Northern blot hybridization analysis, employing a p53-specific cDNA clone (M. Oren and A. J. Levine, Proc. Natl. Acad. Sci. U.S.A. **80**:56–59, 1983), and protein levels were determined by the Western blotting technique. Analysis of p53 mRNA revealed a single polyadenylated mRNA species migrating at ca. 18S. Levels of p53 mRNA in simian virus 40-transformed cell line (SVT2) and in an homologous nontransformed cell line (3T3) were equivalent, although the steady-state levels of p53 protein were 25- to 100-fold higher in the SVT2 cells than in the 3T3 cells. A study with a non-virus-transformed cell system revealed a different result. Embryonal carcinoma cells (F9) were found to have nearly 20-fold higher levels of p53 mRNA in comparison with differentiated benign progeny cells. In this system the difference in p53 mRNA levels corresponded to the difference in p53 protein levels. Pulse-chase experiments were performed to study the half-life of p53 protein in these four types of cells. The turnover of p53 protein occurred with biphasic kinetics. In addition, it was found that protein synthesis inhibitors placed in the medium during the chase period prevented the turnover of p53 protein in transformed cells, but not in nontransformed (3T3) cells. These results provide evidence that the regulation of p53 expression in cells can occur at the level of p53 mRNA abundancy or p53 protein stability depending upon the experimental system under study, and that a regulated degradation process controls the turnover of p53 protein.

Cells transformed by a wide variety of agents have been found to express elevated levels of a cellular protein termed p53 (4, 5, 10, 12, 18). This protein has also been detected in some nontransformed cell lines, such as BALB/c 3T3, or in primary fibroblasts, but at a level of 1/10 to 1/100 that found in homologous transformed cells (5, 7, 13). p53 has been termed a cellular tumor antigen because animals which bear certain tumors or which have been immunized with tumorigenic cells produce anti-p53 antibodies (5, 7, 8, 12).

Previous studies have indicated that the increased expression of p53 in transformed cells may be achieved by more than a single mechanism (13, 16, 17). However, limitations of the techniques employed placed certain reservations upon the conclusions which could be formulated. The procedures that were employed to estimate the levels of p53 protein involved metabolic labeling of p53 with radioactive amino acids and quantitating the immunoprecipitable p53 protein observed in sodium dodecyl sulfate (SDS)-polyacrylamide gel autoradiograms. This standard pulse-labeling protocol introduces inaccuracies in the measurement of relative protein levels (2). It does not, for example, account for differences in cellular amino acid pools or differences in protein half-lives, which in the case of p53 can be as short as 30 min (3T3 cells) or longer than 22 h (SVT2 cells) (16). To overcome these problems a Western blotting technique has been employed to estimate relative levels of p53 protein. In addition, the technique which was used to measure p53 mRNA levels in the past (16, 17) depended upon quantitating p53 mRNA with an in vitro rabbit reticulocyte translation system. This approach may not have reflected an accurate measure of p53 mRNA because translation controls operative in vivo may not be operative in vitro. Molecular cloning of a cDNA specific for murine p53 mRNA (15)

now permits a precise analysis of the steady-state levels of p53 mRNA, using the technique of Northern blot hybridization.

The results presented in this paper support previous studies (16, 17) and demonstrate that an alteration in either p53 protein stability or mRNA abundancy can lead to increased expression of p53 in transformed cells, depending upon the experimental system under study. In simian virus 40 (SV40)-transformed cells, the high levels of p53 are largely due to an increase in the stability of the protein. It has been hypothesized that the greater stability of p53 is due to its physical association with SV40 large tumor (T) antigen (13, 16). In contrast, the elevated levels of p53 found in the F9 embryonal carcinoma cell line appear to be primarily due to increased amounts of p53 mRNA, although the half-life of p53 protein was also found to be greater than that in 3T3 cells. In analyzing the half-life of p53 in F9 cells, it was found that protein synthesis was required for the degradation of the p53 protein. These data indicate that a regulated process is responsible for controlling p53 protein turnover.

## MATERIALS AND METHODS

**Cell cultures.** BALB/c murine cells 3T3 and 3T12 and the SV40-transformed 3T3 derivative SVT2 were provided by G. Todaro (1). The methylcholanthrene-transformed cell line (Meth A) and the embryonal carcinoma cell lines (F9, MH) were provided by F. Ruddle and M. Sherman, respectively. Cells were maintained in Dulbecco modified Eagle medium containing 10% calf serum or 10% fetal bovine serum as described previously (16, 17). F9 cell cultures were differentiated in vitro by treatment with $10^{-7}$ M retinoic acid and $10^{-3}$ M dibutyryl cyclic AMP (17, 20).

**Isolation of RNA.** Cells were washed in phosphate-buffered saline and lysed at 0°C in lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.5], 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5% Nonidet P-40). The extract was centrifuged at 4°C for 10 min at 10,000 rpm (Beckman J2-21, Rotor JA-20). SDS and EDTA were added to the supernatant fraction to achieve final concentrations of 0.5% and 5 mM, respectively. The extract was treated with phenol-chloroform, and the RNA was precipitated in ethanol.

Polyadenylic acid-containing [poly(A)$^+$] RNA was selected by chromatography over oligodeoxythymidylic acid-cellulose (Sigma Chemical Co.).

**Northern blot hybridization.** RNA samples were denatured in 2.2 M formaldehyde–50% formamide–0.2 M morpholinepropanesulfonic acid–50 mM sodium acetate–1 mM EDTA (pH 7.0) at 70°C for 5 min (6, 11). Samples were electrophoresed in a 1% agarose–formaldehyde–morpholinepropanesulfonic acid–buffered gel. RNA species were transferred to nitrocellulose and hybridized with nick-translated plasmid DNA pp53-208 (15) essentially by the method of Thomas (21) with the omission of dextran sulfate.

**Protein analysis.** Procedures for the preparation of [$^{35}$S]methionine-labeled cell extracts, immunoprecipitation, and polyacrylamide gel electrophoresis were as described previously (13). The specific anti-p53 monoclonal antibodies used were from hybridoma PAb421 (8). Either preimmune murine sera or antibodies from an unrelated hybridoma cell line were used as normal antibody controls. The translation inhibitors used in the pulse-chase analyses were either cycloheximide at 50 μg/ml or anisomycin at 100 μM (Sigma).

Radioimmunoelectrophoresis (Western blot hybridization) was performed essentially as described by Burnette (3) with the exception that immunoprecipitates, instead of crude extracts, were applied to the gels. Radioiodinated *Staphylococcus aureus* protein A was purchased from New England Nuclear Corp.

## RESULTS

**Steady-state levels of p53 mRNA.** The Northern blot hybridization technique was used to examine relative levels of p53 mRNA in transformed and nontransformed cells (6, 11). Equal amounts (4 μg) of poly(A)$^+$ mRNA were isolated from growing cell cultures, electrophoresed through formaldehyde-denaturing agarose gels, and then blotted onto nitrocellulose. RNA species immobilized on the nitrocellulose support were hybridized with a radioactive p53-specific cDNA clone, pp53-208 (15). In all murine cell lines examined, a single poly(A)$^+$ mRNA species hybridized with pp53-208, migrating just ahead of an 18S rRNA marker (Fig. 1 and 2). The level of p53 mRNA from 3T3 cells was approximately equivalent to the level found in SV40-transformed 3T3 cells (SVT2) (Fig. 1). Densitometer tracing of the autoradiogram gave a quantitative confirmation of the results (Table 1). The increased level of p53 protein (25- to 100-fold) found in SVT2 cells compared with that found in 3T3 cells (12) cannot be due to different levels of p53 mRNA expression. 3T12 and Meth A cell lines which are transformed and tumorigenic, but do not contain SV40 DNA sequences, possess about twofold more p53 mRNA than do 3T3 or SVT2 cells (Fig. 1, Table 1). These data suggest that a different mechanism may operate to enhance p53 levels in these cells. We have used another nonviral transformation system, namely, F9 embryonal carcinoma cells, to examine this idea.

F9 cells are transformed and highly tumorigenic, but after treatment with retinoic acid and dibutyryl cyclic AMP they differentiate into benign endoderm-like cells (17, 20). The level of p53 protein in F9 cells has been found to be very high in comparison with that in their differentiated progeny cells as assayed by [$^{35}$S]methionine incorporation (17). Steady-state levels of p53 mRNA in these cells were compared by a Northern blot hybridization analysis (Fig. 2). F9 cells contained ca. 20-fold higher levels of p53 mRNA than did differentiated cells (Table 1). In contrast with the previous results, this difference in p53

mRNA abundancy can account for the differences observed in p53 protein levels (17). The mechanisms regulating p53 expression in this system are therefore probably quite unlike those operating in the SV40-transformed cell-3T3 cell system.

**Steady-state levels of p53 protein.** We have employed the Western blotting technique (3) to estimate steady-state levels of p53 protein in various cell lines. Cell extracts were prepared and immunoprecipitated with either unrelated monoclonal antibodies (n) or anti-p53 monoclonal antibodies ($\alpha$) (8). The immunoprecipitates were electrophoresed on SDS-polyacrylamide gels and then electrophoretically transferred to a sheet of nitrocellulose paper. The immobilized antigens on the nitrocellulose were visualized by reaction with anti-p53 antibodies and the subsequent binding of $^{125}$I-radioiodinated *S. aureus* protein A to the immune complexes.

Since some cells express low levels of p53, it was advantageous to immunoprecipitate p53 before samples were applied to SDS gels. By this procedure, greater amounts of total cellular protein from cell lines containing low levels of p53 could be analyzed without overloading a gel lane with excess total protein. Less total protein was

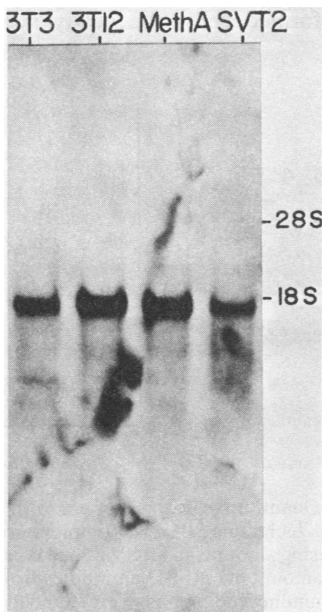


FIG. 1. Northern blot hybridization of BALB/c murine p53 mRNA with pp53-208 cDNA clone. Equal amounts (4 μg) of poly(A)$^+$ mRNA from each cell line were applied to a formaldehyde-denaturing 1% agarose gel. RNA species were immobilized on nitrocellulose and hybridized with a nick-translated radioactive probe, pp53-208 (15). The migration of rRNA molecular weight size markers is indicated.

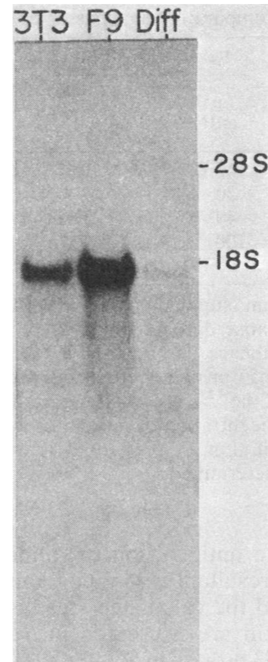


FIG. 2. Northern blot hybridization analysis of p53 mRNA from several murine cell types. Equal amounts (4 μg) of poly(A)$^+$ mRNA from each cell type were electrophoresed and analyzed as described for Fig. 1. F9 cultures differentiated in the presence of $10^{-7}$ M retinoic acid and $10^{-3}$ M dibutyryl cyclic AMP for 4 days are represented as Diff.

used from cells containing high levels of p53 protein. In this way both types of samples could be analyzed on the same blot. To arrive at a relative level of p53 for each cell line, the intensity of the $^{125}$I-protein A bound to p53 antibody was determined by densitometer tracing or by scintillation counting. This level was then corrected for the different amounts of cellular protein extract used in the initial immunoprecipitate (see Table 1, footnote *b*). Consequently, the intensity of the p53 band seen in the autoradiogram was not necessarily proportional to the calculated relative level of p53 protein (see Fig. 4; Table 1). That this procedure is able to detect and quantify p53 levels was shown in a reconstructed dose-response experiment. p53 was immunoprecipitated from increasing amounts of a cellular extract and analyzed by the Western blotting technique. The resultant p53 signal on the autoradiogram was proportional to the amount of p53 immunoprecipitated (Fig. 3).

A comparative analysis of p53 levels was performed with as little as 0.1 mg of protein extract from SVT2 cells and as much as 4 mg of protein extract from 3T3 cells. In each case an excess amount of p53-specific antibodies, with

TABLE 1. Comparative analysis of p53 expression

| Cell type | Relative level (%)[a] of: p53 mRNA | p53 protein[b] | Half-life (h) of p53 protein |
|---|---|---|---|
| SVT2 | 15 (22) | 100 | >22 |
| 3T3 | 20 | 4 (1) | 0.4 |
| Meth A | 40 | ND[c] | 5 |
| F9 | 100 | 20 | 3 |
| F9 (Diff) | 5 (9) | 1 | 3 |

[a] Values from supplementary experiments which deviate from those derived from Fig. 1, 2, and 4 are given in parentheses.

[b] Levels of p53 protein were calculated by dividing the intensity of the $^{125}$I-protein A signal of the Western blot by the concentration of protein used in the initial immunoprecipitation.

[c] ND, Not determined.

regard to the antigen concentration, was employed. The resultant autoradiograms are shown in Fig. 4, and the calculated steady-state levels of p53 protein are presented in Table 1. The amount of p53 protein in SVT2 cells is expressed as 100%, and relative levels in other cell lines have been calculated from that standard. The nontransformed 3T3 cell line possessed about 4% of the amount of p53 detected in the SV40-transformed cell line, although as seen in the previous section the relative levels of p53 mRNA were about the same. F9 embryonal carcinoma cells contained a relative level of p53 protein equal to 20% of that observed in SVT2 cells, whereas after differentiation of F9 cells into benign progeny endoderm the level of p53 protein fell to about 1% of that observed in SVT2 cells. The difference in p53 protein levels between F9 and differentiated cells is largely accounted for by the difference seen in p53 mRNA levels (about 20-fold). These two experimental systems (SVT2-3T3; F9-differentiated) employ two different mechanisms for controlling the expression of p53 protein (Table 1). Another cell line included in this study was the embryonal carcinoma line MH. It is rare among the murine cell lines in that although it is tumorigenic, it possesses very low levels of both p53 mRNA and p53 protein.

**Intracellular p53 protein turnover.** SV40 infection or transformation of murine cells has previously been shown to increase the half-life of p53 protein from about 30 min to greater than 22 h (16). This resistance of p53 to the normal degradative processes could be due to its physical association with SV40 T antigen (10, 12, 13). In non-virus transformed cells such as F9 embryonal carcinoma cells or Meth A cells (5) the half-life of p53 is also increased, relative to 3T3 cells, to about 3 and 5 h, respectively (17). As yet, no one has identified any cellular protein like SV40 T-antigen associated with p53 in a protein-protein oligomeric complex that could afford p53 protection from degradative enzymes. It is possible that the normal mechanisms of p53 protein degradation or posttranslational modification are altered in these transformed cells, thereby increasing the half-life of p53 protein. To investigate this idea, the turnover of p53 in nontransformed versus transformed cells was examined in more detail by pulse-chase experiments. Tissue culture cells were pulse-labeled with [$^{35}$S]methionine for 1 h and then incubated in nonradioactive (chase) medium for various time periods. Cell extracts were prepared, and p53 protein was immunoprecipitated from the same amount of trichloroacetic acid-insoluble radioactive counts for each time point. Immunoprecipitates were then examined after SDS-gel electrophoresis. The resultant autoradiograms are shown in Fig. 5, and a graphic analysis of these data from densitometer tracings is given in Fig. 6.

The results show that the half-life of p53 in the transformed cells is much longer than it is in 3T3 cells. In addition, the decay of p53 does not appear to be simply exponential with respect to time. This is reflected in the bimodal nature of the decay curves. In both the nontransformed and transformed cells, the newly synthesized

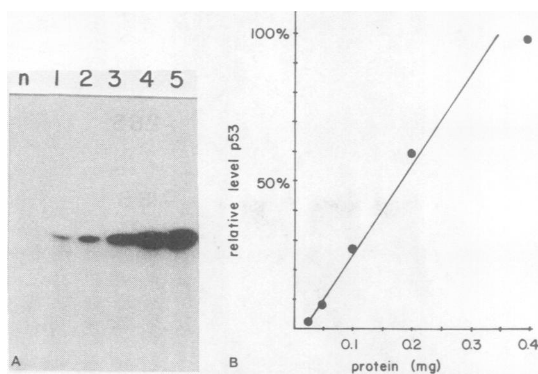


FIG. 3. Quantitative analysis of p53 with the Western blotting technique. P53 was immunoprecipitated from increasing amounts of an SVT2 cell extract, using a standard amount of anti-p53 antibodies from PAb421 (8). The immunoprecipitates were applied to SDS-polyacrylamide gels and then transferred to nitrocellulose. The nitrocellulose was reacted with anti-p53 antibodies and $^{125}$I-conjugated protein A. (A) Autoradiogram of the blot; (B) graphic representation of the p53 signal as determined by densitometer tracing. Lane n, Control immunoprecipitation with 100 $\mu$g of protein; lanes 1 through 5, immunoprecipitation of p53 with 25, 50, 100, 200, and 400 $\mu$g of protein, respectively.

p53 appears to be refractory to degradation shortly after its synthesis (labeling). The refractory period also appears to be proportional to the half-life of the p53 protein in each of these cell lines.

To eliminate the possibility of continued incorporation of [$^{35}$S]methionine from labeled cellular pools during the early phases of the chase period, protein synthesis inhibitors were included in the nonradioactive chase medium. This protocol produced a surprising result. Inclusion of translation elongation inhibitors, cycloheximide (50 μg/ml) or anisomycin (100 μM), resulted in the stabilization of p53 in the F9 and Meth A cells, but not in the nontransformed 3T3 cells. The exemption of 3T3 cells from this stabilizing effect was not due to an insufficient time for the drugs to affect protein translation. Within 5 min after cycloheximide treatment, [$^{35}$S]methionine incorporation into total protein was reduced to at least 70% as judged from total protein trichloroacetic acid-precipitable radioactivity (data not shown). Thus there is a differential effect of translational inhibitors upon the regulation of p53 degradation or turnover in 3T3 cells as contrasted with F9 or Meth A cells (Fig. 5 and 6).

## DISCUSSION

The steady-state level of any cellular protein is determined by both its rate of synthesis and its rate of degradation. As shown in this study, the amount of p53 protein expressed by a cell can be

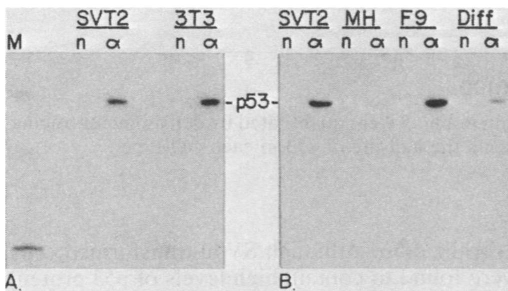


FIG. 4. Western blot analysis of p53 protein from murine cells. A defined amount of protein from different cellular extracts was used in an immunoprecipitation analysis with either normal sera (n) or anti-p53 monoclonal antibodies (α) from hybridoma PAb421 (8). Immunoprecipitates were run on SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose. The immobilized antigens were then reacted with anti-p53 antibodies (PAb421) and $^{125}$I-conjugated protein A. (A) Immunoprecipitation from SVT2 (0.1 mg of protein) and 3T3 (4 mg of protein) cells. M is a molecular weight size marker protein lane. (B) Immunoprecipitation of proteins from SVT2 (0.1 mg), MH (1 mg), F9 (1 mg), and differentiated (Diff; 2.5 mg) cells.

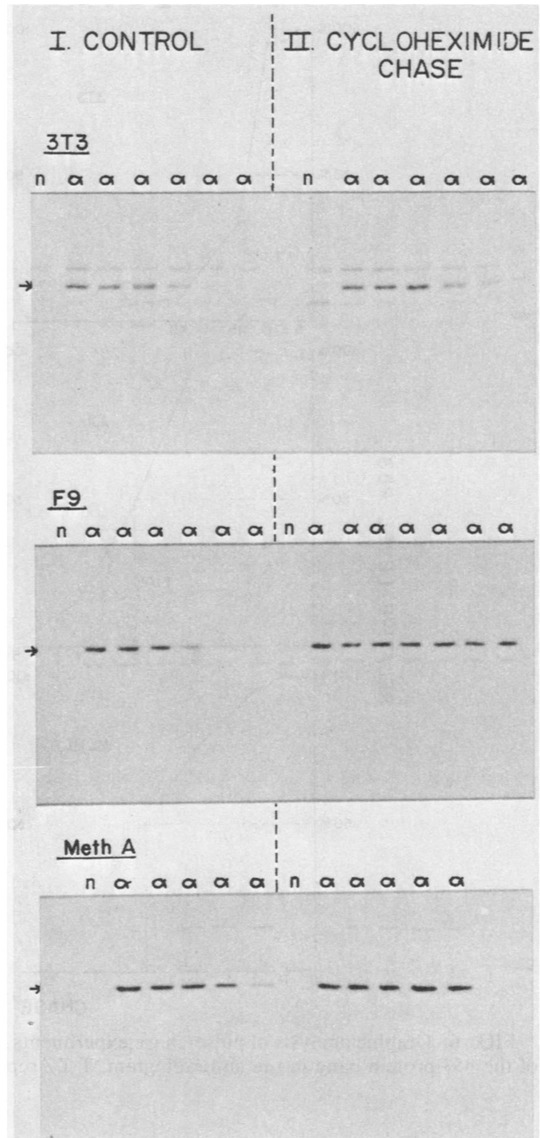


FIG. 5. Intracellular breakdown of p53 protein in 3T3, F9, and Meth A cells. n, Normal sera; α, anti-p53 monoclonal antibodies from PAb421. I. Control: Cells were pulse-labeled with [$^{35}$S]methionine for 1 h and chased in nonradioactive medium for various times. At each chase time point the same amount of radioactive material was immunoprecipitated with anti-p53 antibodies (8). Immunoprecipitates were analyzed for p53 levels on SDS-polyacrylamide gels. The chase periods for 3T3 were 5, 10, 15, 15, 45, and 90 min; those for F9 were 0, 1, 2, 3, 4, and 5 h; those for Meth A were 0, 1, 2, 4, and 9 h. II. Cycloheximide chase: A pulse-chase experiment was employed as described above except that cycloheximide (50 μg/ml) was included in the chase medium. This amount of cycloheximide was determined to inhibit >95% of radioactivity incorporated into proteins.

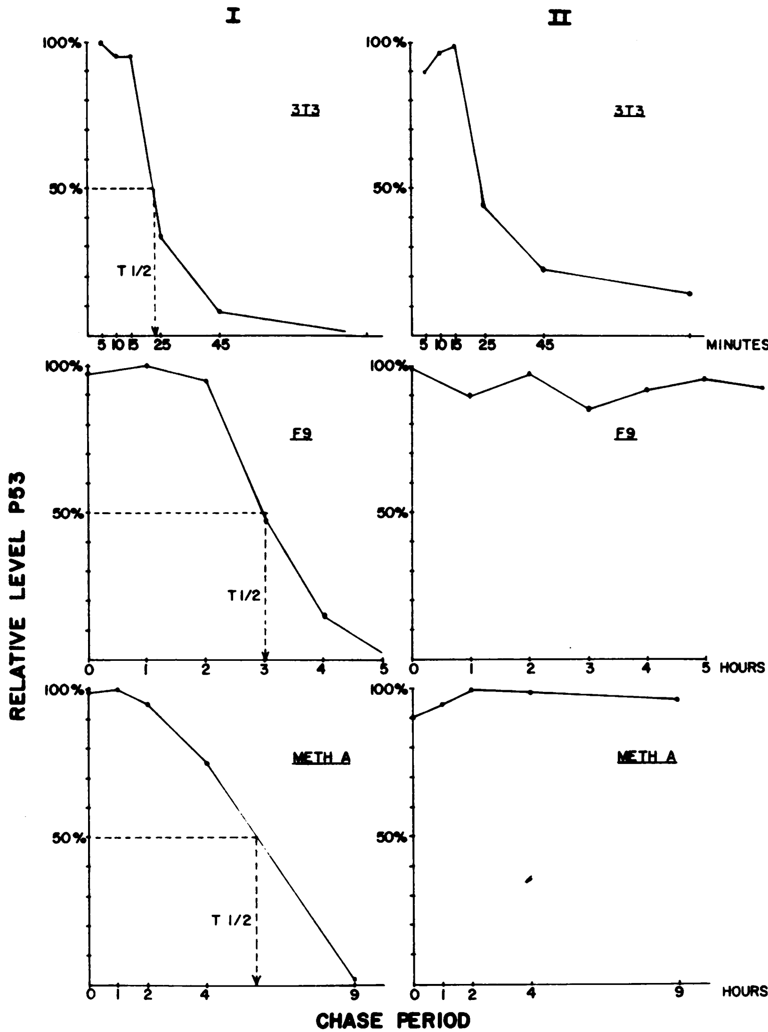


FIG. 6. Graphic analysis of pulse-chase experiments. Data in Fig. 5 were quantitated by densitometer tracing of the p53 protein band in the autoradiogram. T 1/2 represents the half-life of p53 in each cell type.

regulated by p53 mRNA levels or p53 protein stability or both.

Previous analyses have used the incorporation of radioactive amino acids to estimate levels of p53 protein (4, 13, 16, 17). However, there were serious limitations in interpreting these data based upon the experimental protocols employed (2). For this reason the Western blotting technique was used to measure relative steady-state levels of p53 protein in a variety of cell lines (Fig. 4). In most cases there was a good correlation between the level of p53 protein and the transformed state of the cell, although notable exceptions have been found (MH cells). A comparative analysis of the levels of p53 mRNA in various cells was performed by Northern blot hybridization. Although SV40-transformed cells were found to contain high levels of p53 protein in comparison with nontransformed 3T3 cells, this difference was not reflected in the level of mRNA (Table 1). Therefore, it would appear that the rate of synthesis of p53 protein is similar in both cell lines. As shown previously (16), however, the rate of p53 degradation is altered drastically. In contrast to this experimental system, the differentiation of F9 embryonal carcinoma cells into benign endoderm-like cells results in a decrease of p53 protein levels which appears to be accounted for by a decrease in the abundance of p53 mRNA (Fig 2). The control of p53 expression in differentiating F9 cells could be at the level of the rate of mRNA synthesis or the

stability of p53 mRNA. In either case, it is distinctly different from the SV40-transformed cell system (Table 1).

Experiments designed to follow the kinetics of p53 protein turnover detected a bimodal rate of p53 protein decay (Fig. 5 and 6). One interpretation of these data is that newly synthesized p53 protein is more refractory to degradation than is the p53 protein synthesized before it. This refractory time period could represent the time of a functional association of p53 with other proteins involved in regulating cellular functions.

The stabilizing effect that protein synthesis inhibitors have upon p53 turnover in F9 and Meth A cells suggests the existence of a selective or regulated process for p53 breakdown. In the case of F9 cells, the half-life of the putative degradation enzyme or cofactor must be less than 3 h to account for the effect upon p53 turnover (Fig. 6). Studies of the mechanisms of intracellular protein breakdown indicate that degradation of most short-lived proteins is probably nonlysosomal in nature (9). Their degradation, in contrast to the breakdown of long-lived proteins, does not appear to be influenced by inhibitors of protein synthesis (9). The degradation of the p53 protein therefore appears to be regulated differently in nontransformed (3T3) and transformed (F9, Meth A) cells. Further studies will be required to elucidate this aspect of the control of p53 expression.

Experiments presented in this study (Table 1) indicate that two mechanisms, mRNA abundancy and the half-life of p53 protein, independently or concomitantly result in enhanced levels of p53 protein in transformed cells. In the case of SV40-transformed cells compared with 3T3 cells, the level of p53 mRNA is the same in both cell types. Here, enhanced p53 protein levels result from a much greater half-life of p53 protein in the transformed cells (Table 1). This is probably due to the stabilizing effect of the physical association of p53 with the SV40 large tumor antigen (10, 12, 13). Similarly, adenovirus-transformed cells may have enhanced levels of p53 protein because of the complex found between the viral E1b-58Kd protein and p53 (19). However, F9 embryonal carcinoma cells, when compared with their differentiated benign cells, have 20-fold more p53 mRNA and p53 protein (Table 1). In this case, the steady-state levels of p53 appear to be regulated at the mRNA level (transcription or turnover of mRNA). Regulation of p53 at the level of transcription has previously been noted in lymphocytes by Milner and Milner (14).

F9 cells, as well as Meth A cells, possess both an enhanced level of p53 mRNA and a longer protein half-life in comparison with 3T3 cells. The combined effect of both p53 mRNA abundancy and protein stability may thereby result in higher steady-state levels of p53 protein in some transformed cells.

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