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Regulation of the Cellular p53 Tumor Antigen in Teratocarcinoma Cells and Their Differentiated Progeny

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F9 embryonal carcinoma cells express high levels of a 53,000-molecular-weight cellular tumor antigen called p53. When F9 cell cultures are treated with retinoic acid and dibutyryl adenosine 3',5'-phosphate, they differentiate, predominantly into endoderm-like cells. This differentiation is accompanied by a marked decrease in the levels of p53. The mechanism(s) responsible for this decline in the level of p53 in differentiated cells was investigated. The results demonstrate that the high levels of p53 in F9 cells relative to their differentiated progeny were not due to alterations in the stability or turnover of this protein. Rather, the regulation during differentiation involved a marked decrease in the amount of in vitro translatable p53 mRNA detected in the differentiated cell cultures. This mechanism is unlike the one operating during the simian virus 40 infection or transformation, where the increased levels of p53 are largely due to the increased stability of the p53 protein (Oren et al., Mol. Cell. Biol. **1**:101–110, 1981).

Many types of transformed cells express high levels of a cellular phosphoprotein termed p53 (2, 3, 6, 7, 16). This protein is also present in nontransformed cells, but at a much reduced level (0.1 to 0.01) (2, 8). Elevated levels of p53 have been observed in cells transformed by a large variety of agents, such as chemical carcinogens (3, 10), DNA tumor viruses (2, 6, 7, 9, 10), RNA tumor viruses (3, 16), and irradiation (3), as well as in spontaneous transformants (3, 10). Since the levels of p53 appear to be closely correlated with transformed growth features, it was of interest to understand the mechanisms leading to its accumulation in transformed cells. In a previous study in this series (14), the high levels of p53 detected in simian virus 40 (SV40)-infected or -transformed mouse cells were shown to be largely due to a prominent increase in the stability of p53 protein. Nontransformed cells and SV40-transformed cells both contained similar amounts of translatable p53 mRNAs. Because the SV40 large tumor antigen (T antigen) both regulates the level of p53 in transformed cells (8) and is physically complexed with p53 (5–7, 11, 12), the hypothesis was proposed that the SV40 T antigen-p53 complex results in greater stability of p53 and its accumulation (14).

The question of how p53 was regulated in cells not transformed by SV40, with no T antigen present, remained to be answered. For that reason the present study analyzed the regulation of p53 in F9 embryonal carcinoma cells. These cells, which are highly tumorigenic, contain high levels of p53 when compared with normal cells (7). Treatment of F9 cells with retinoic acid and dibutyryl adenosine 3',5'-phosphate (cAMP) causes them to differentiate, predominantly into endoderm-like cells (20, 22). Furthermore, retinoic acid treatment of mice challenged with embryonal carcinoma cells lengthens the survival time of the animals and increases differentiation in the tumor (21). The differentiation process is accompanied by a decline in the levels of p53. Infection of these differentiated cells with SV40 results in SV40 T antigen synthesis and an increase in the amount of p53 in infected cells. The results of experiments presented in this communication demonstrate that the stability of the p53 protein in both F9 cells and their differentiated progeny cells was about the same. However, the levels of translatable p53 mRNA were shown to decline after differentiation of F9 cells. This could result from either a decrease in the rate of transcription of the p53 gene or a post-transcriptional event. In either case, the levels of p53 in nontransformed and transformed cells appear to be regulated by different mechanisms during differentiation of F9 cells and in SV40-infected or -transformed cells.

MATERIALS AND METHODS

F9 embryonal carcinoma cells (1) were provided by S. Strickland. SV40 strain 776 was obtained from G. Khoury. Cell culture and virus infection were as described before (8). BALB/c 3T3 cells were provided by G. Todaro.

The F9 cell differentiation protocol was slightly

modified from a previous report (22). Cells were plated (3×10^5 per 100-mm plate) and immediately treated with 10^{-7} M retinoic acid and 10^{-3} M dibutyryl cAMP. After 3 days of treatment the cells were split 1:2 in the same medium, and they were used on the 4th day of differentiation.

Labeled cell extracts and cytoplasmic RNA were prepared as previously described (14).

In vitro protein synthesis followed published procedures (14). In some instances, the reticulocyte lysate was only mildly treated with micrococcal nuclease. In our hands, this resulted in a more efficient translation of the p53 mRNA.

Immunoprecipitation procedures were as described previously (8). Polyacrylamide gel electrophoresis and autoradiography were described by Oren et al. (14).

RESULTS

Levels of p53 in F9 cells and their differentiated progeny. Murine embryonal carcinoma cells, such as the F9 cell line, are transformed and highly tumorigenic. Under normal tissue culture conditions F9 cells maintain a characteristic non-differentiated morphology and grow in typical multilayer clumps (Fig. 1A). Treatment of these cells with retinoic acid and dibutyryl cAMP results in dramatic morphological and biochemical changes (Fig. 1B) reflecting the differentiation of these cells into endoderm-like cells (22).

F9 cells contain high levels of the p53 cellular tumor antigen when compared with nontransformed cells such as BALB 3T3 (7). A good correlation has been established between elevated levels of p53 and the transformed phenotype (2–4, 6, 7, 9, 10, 16). For this reason experiments were designed to determine the levels of p53 expressed in F9 cells and their differentiated progeny. F9 cell cultures and similar cultures treated with retinoic acid and dibutyryl cAMP for 4 days (as in Fig. 1) were each split into two groups. One group of F9 cells and differentiated cells was infected with SV40, and the second set was mock infected. After 20 h all of the cell cultures were labeled with [^{35}S]methionine for 4 h. Soluble protein extracts were prepared from these, and equal amounts of radioactive protein from each culture were incubated with a monoclonal antibody directed against p53. The immunoprecipitates were collected, washed, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (8). The autoradiogram from this preparation is presented in Fig. 2. In each case, normal serum (N in Fig. 2) did not detect any specific proteins. The mock-infected F9 cells contained five- to sixfold-higher levels of p53 than their differentiated counterparts. SV40 infection of the differentiated cells, but not of the F9 cells, resulted in the synthesis of SV40 T antigen and an increase in the level of p53 compared with the mock-infected differentiated F9 cell progeny. Since a p53 monoclonal antibody was used in this experiment, the SV40 T antigen (in F9 Diff, SV40, of Fig. 2) was detected by virtue of its physical interaction or complexing with p53 (5–7, 11, 12). In addition, the synthesis and detection of SV40 T antigen in the differentiated cells was yet another measure of the differentiated state of the retinoic acid-treated cell culture because SV40 early gene products are not expressed in F9 cells (17, 18).

Densitometer tracings of the autoradiogram in Fig. 2 demonstrated that the p53 levels detected in F9 differentiated cell cultures declined 5.7-fold when compared with the untreated F9 cell cultures. This should be considered a minimum estimate because the differentiated F9 cell cultures still contain residual undifferentiated F9 cells. Based upon the ability of F9 cells, but not the differentiated cells, to produce colonies in agar, it is estimated that about 5% of the cells in the differentiated culture were undifferentiated F9 cells.

Regulation of p53 in differentiated progeny cells. The mechanisms involved in regulating the levels of p53 in F9 and differentiated cells were investigated. In SV40-infected or -transformed cells, the half-life of detectable p53 protein is much longer than in normal cells (14). To determine whether altered protein stability played a role in the F9 differentiated cell system, a pulse-chase experiment was performed. F9 cell cultures and F9 cell cultures treated with retinoic acid and dibutyryl cAMP were labeled with [^{35}S]methionine for 1 h. At the end of this time, the cell cultures were washed and refed with medium containing unlabeled methionine. The cells were incubated at 37°C for 0 h (end of the pulse period) and for 0.75, 1.5, 2.5, 3.5, 6 and 10 h (as the chase period). At each of these times, a culture was harvested, soluble protein was extracted, and p53 levels were analyzed by immunoprecipitation and gel electrophoresis as described previously. Figure 3 presents the autoradiogram of this pulse-chase experiment. Although the differentiated cells contain lower levels of p53 when compared with the F9 cells (in all samples at all times), the half-life of p53, calculated from the densitometer tracings, was about the same in F9 cells and the differentiated cells (approximately 3.5 h in both cases). This is clearly different from the situation found in normal 3T3 cells where the half-life of p53 was 20 to 30 min and in SV40-transformed cells where the p53 half-life was greater than 22 h (15).

To examine the possibility that the levels of p53 mRNA change during differentiation of F9 cells, total cytoplasmic RNA was extracted from untreated and retinoic acid- and dibutyryl

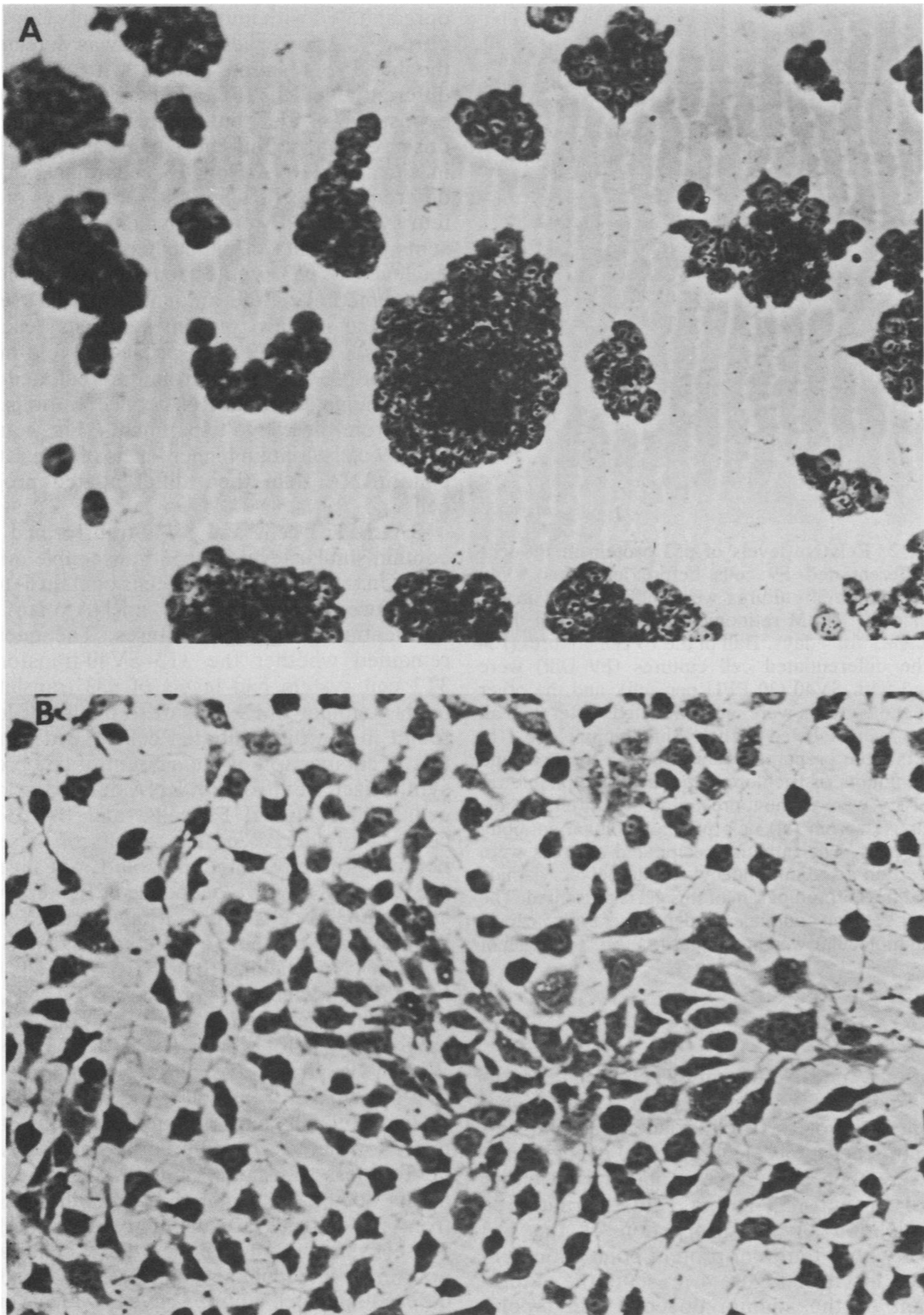

FIG. 1. Morphology of F9 cells and their differentiated progeny. (A) F9 cells grown as described in the text and (B) F9 cells after 4 days of growth in 10^{-7} M retinoic acid and 10^{-3} M dibutyryl cAMP. Cells were fixed in methanol and stained with Giemsa.

cAMP-treated F9 cell cultures. These RNA preparations were added to a reticulocyte in vitro protein translation system (15) to prime protein synthesis. First, conditions were established so that [^{35}S]methionine incorporation into the total proteins synthesized in vitro with each RNA preparation was proportional to the amount of RNA priming the reaction. With these

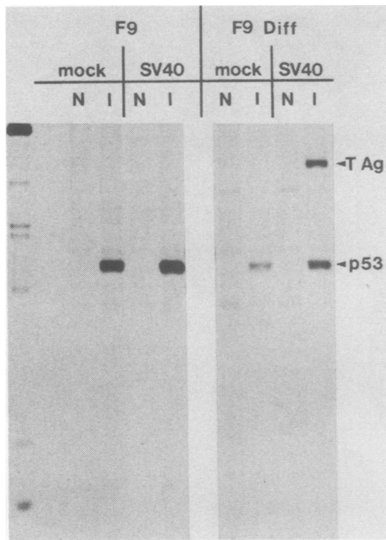

FIG. 2. Relative levels of p53 protein in F9 cells and differentiated F9 cells before and after SV40 infection. F9 cell cultures were differentiated in the presence of 10^{-7} M retinonic acid and 10^{-3} M dibutyryl cAMP for 4 days. Half of the F9 cell cultures (F9) and the differentiated cell cultures (F9 Diff) were infected with SV40 (30 PFU per cell), and the other half of the cultures were mock infected. After 20 h all cultures were labeled with [^{35}S]methionine for 4 h. Extracts were prepared as described in the text, and equal amounts of trichloroacetic acid-precipitable radioactivity were immunoprecipitated with either normal mouse serum (N) or a p53 monoclonal antibody RA3-2C2 (I). The immunoprecipitated samples were analyzed on a sodium dodecyl sulfate-polyacrylamide gel, and the autoradiogram of this gel is presented. The leftmost gel lane contains an adenovirus type 5 capsid protein molecular weight standard. T Ag, T antigen of SV40.

conditions, the RNAs from F9 cells and differentiated cells were translated in vitro. Equal amounts of protein (trichloroacetic acid-insoluble counts per minute) synthesized in each reaction were then immunoprecipitated with either normal or anti-p53 serum. This permitted a determination of the proportion of the p53 protein within the total protein population synthesized in vitro. Figure 4 presents the autoradiogram of the sodium dodecyl sulfate-polyacrylamide gel used to analyze these immunoprecipitates. As a control, the relative levels of p53 synthesized in vivo by F9 cell cultures and differentiated cell cultures are also presented (Fig. 4, in vivo, F and R). In this experiment, about a fourfold difference was detected in p53 levels in vivo between F9 and the differentiated cells. p53 protein was synthesized in vitro with RNA from F9 cells and was immunoprecipitated with immune serum only (Fig. 4, in vitro, F). A lower level of p53 was detected in the in vitro reaction primed with RNA from differentiated cells (Fig. 4, in vitro, R). In longer exposures of this autoradiogram it could be observed that sixfold-more p53 was synthesized in vitro with RNA from F9 cells than from the differentiated cells when the total levels of protein synthesized were equivalent. Figure 5 presents the results of similar experiments where the levels of p53 synthesized in vitro are plotted as a function of the amount of RNA used to prime the in vitro protein synthesis reaction. The RNA from F9 cells primed 10-fold-higher levels of p53 than RNA from differentiated cells over a range of primer RNA concentrations. It is clear from these two experiments (Fig. 4 and 5) that F9 cells contain higher levels of translatable p53 mRNA than their differentiated progeny cells.

BALB 3T3 cells and SV40-transformed cells contain similar levels of p53 translatable mRNA (14). On the other hand, F9 cells contain 6- to 10-fold-more p53 translatable mRNA than cells differentiated from F9 cultures. The question remained whether the 3T3–SV40-transformed 3T3 cell system had levels of p53 translatable mRNA similar to F9 cells or to the lower levels found in the differentiated cells. To determine this, a comparison of the amount of p53 protein synthesized in vitro with RNA derived from F9 cells, differentiated F9 cells, and BALB 3T3 cells was undertaken. The results of these experiments are presented in Table 1. The levels of p53 detected by in vivo labeling of F9, differentiated, and 3T3 cells with [^{35}S]methionine for a 4-h period are presented for comparison. Based upon in vivo labeling, the BALB 3T3 cells have 6- to 7-fold-lower levels of p53 than F9 cells, and the cells differentiated from F9 cell cultures have 2.5- to 7-fold-lower levels of p53 protein. With in vitro translation of RNA from these cells as a measure of translatable p53 mRNA, BALB 3T3 and F9 cells have about equal levels of this mRNA, whereas the differentiated F9 cells had 6.5-fold-less translatable mRNA. These experiments demonstrate that the cells differentiated from F9 cultures are distinctly different from 3T3 cells, even though both of these cells have a nontransformed phenotype. Both 3T3 and the differentiated cell cultures have low levels of p53 but for different reasons, i.e., protein turnover in 3T3 and lower levels of translatable p53 mRNA in F9 differentiated cells.

DISCUSSION

p53 is a cellular protein (6, 7, 19) whose high levels have been correlated with transformation by a variety of agents (2, 3, 6, 7, 9, 10, 16). The regulation of the levels of p53 in nontransformed

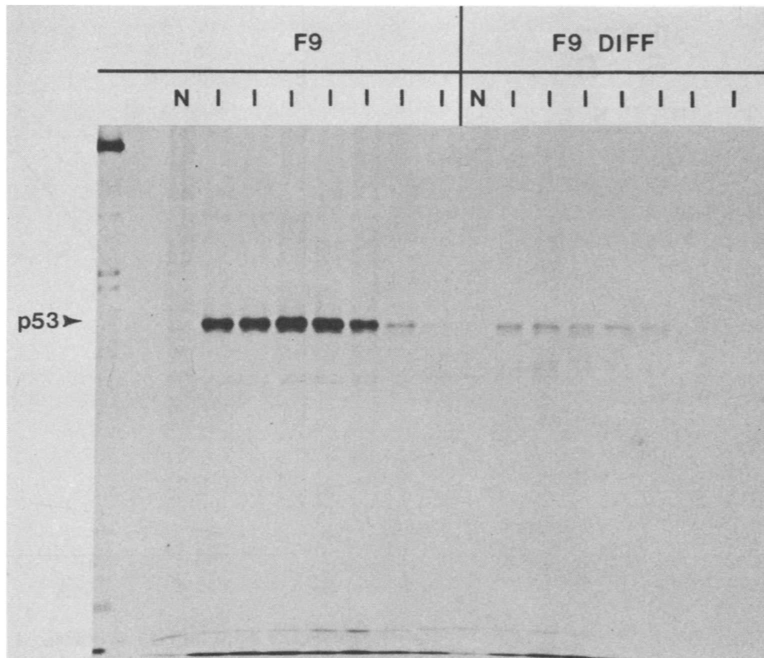

FIG. 3. Pulse-chase experiment of the p53 protein stability in F9 cells and F9 differentiated cells. F9 cell cultures (F9) and F9 differentiated cell cultures (F9 Diff) (see Fig. 1 and 2) were labeled with [^{35}S]methionine for 1 h. At the end of the labeling period, the cultures were washed with medium containing a fivefold excess of nonradioactive methionine (150 μg/ml). The cell cultures were then incubated at 37°C in this medium for chase periods of 0 h (at the end of the 1-h pulse-label) and 0.75, 1.5, 2.5, 3.5, 6, and 10 h. The soluble cell proteins were extracted and immunoprecipitated with either normal mouse serum (N) or a p53 monoclonal antibody RA3-2C2 (I). The left gel lane contains an adenovirus type 5 capsid protein molecular weight marker. The next lanes from left to right are normal serum (N) and immune serum (I) from the chase periods of 0, 0.75, 1.5, 2.5, 3.5, 6, and 10 h for the F9 and F9 Diff cultures.

and transformed cells has now been studied in two different systems: (i) SV40-infected or -transformed cells versus nontransformed 3T3 cells and (ii) F9 embryonal carcinoma cells before and after differentiation in culture. By labeling these cells in culture with [^{35}S]methionine, higher levels of p53 have been found in SV40-infected or -transformed cells and F9 cell cultures than were found in either 3T3 cells or cells differentiated from F9 cell cultures (14; this communication). Although higher levels of p53 are consistently observed in these transformed cells when compared with their nontransformed, non-tumorigenic counterparts, the mechanisms regulating the level of p53 differ in these two systems. The levels of p53 translatable mRNA extracted from 3T3 cells or SV40-transformed 3T3 cells were about equal. In the 3T3 cells, p53 was synthesized, but the protein had a short half-life of about 20 to 30 min. SV40-transformed 3T3 cells, on the other hand, produced p53 protein that was stable over a 22-h period. Because the SV40 large T antigen is required for regulating the high levels of p53 in transformed cells (8) and is physically associated with p53 in solution (5–7, 11, 12), it has been suggested that the SV40 T antigen-p53 complex stabilizes the turnover of p53 protein (14). This is in striking contrast to the mechanism regulating p53 in F9 cells and their progeny differentiated cells. In both F9 cells and their differentiated counterpart, the half-life of p53 was about the same (about 3.5 h). However, the level of p53 translatable mRNA was 6- to 10-fold lower in differentiated cells compared with the parent F9 cultures.

Some caution should be used in interpreting these data. In vitro translation assays with a reticulocyte system measure the level of translatable mRNA, but this may not reflect the absolute level of p53 RNA complementary in base sequence to that gene. There could be translational controls operative in vivo that are not reflected in vitro. Secondly, the pulse-chase experiments to measure the turnover or half-life of p53 rely on antibody to p53 to detect this protein. Failure to detect p53 might reflect loss of the protein during extraction procedures or an altered state of p53 no longer able to interact

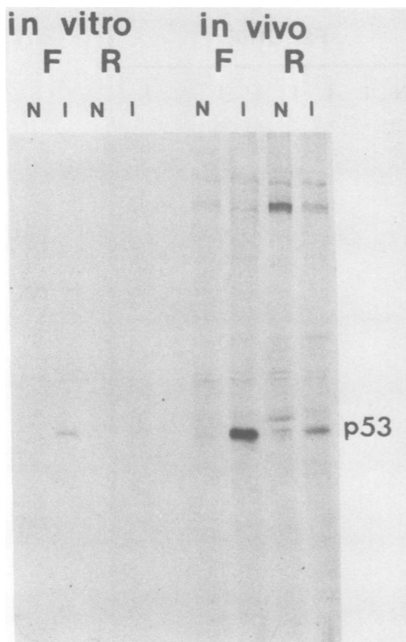

FIG. 4. Levels of p53 protein and p53 translatable mRNA in F9 and differentiated cell cultures. F9 cell cultures (F) and F9 differentiated cell cultures (R, since they are retinoic acid treated) were labeled with [^{35}S]methionine for 4 h. The soluble proteins were extracted and immunoprecipitated with either normal serum (N) or immune serum (I) to measure the levels of p53 in vivo, as described in Fig. 2. Duplicate F9 and differentiated cell cultures were used to extract cytoplasmic RNA which was used to prime an in vitro reticulocyte translation assay. Equal amounts of the [^{35}S]methionine-labeled proteins synthesized in vitro from F9 or differentiated F9 cell RNAs were then immunoprecipitated with normal or immune serum and analyzed in a similar fashion. In vitro refers to p53 synthesized by the reticulocyte translation system. In vivo refers to p53 synthesized in cell culture.

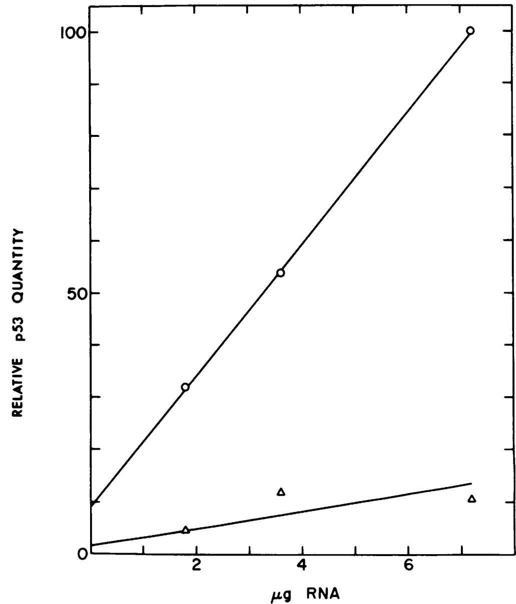

FIG. 5. Levels of p53 synthesized in an in vitro translation system by RNA from F9 and differentiated cell cultures. Cytoplasmic RNA from F9 cell cultures and differentiated cell cultures was extracted as in Fig. 4 and used to prime an in vitro reticulocyte translation system. [^{35}S]methionine-labeled p53 was isolated, and the amount synthesized by three RNA concentrations from F9 or differentiated cells was determined as in Fig. 2. Densitometer tracings of the autoradiograms (as in Fig. 2 to 4) provided the area under each peak. The level of p53 synthesized in vitro by RNA from F9 cells was normalized to 100%, and all other levels were calculated from that. (O) RNA from F9 cell cultures; (Δ) RNA from differentiated cell cultures.

with antibody. In spite of these reservations the dramatically different results with the 3T3–SV40-transformed 3T3 cell and F9 differentiated cell systems clearly demonstrate differences in the regulation of p53 in these two systems.

The F9 differentiated cell system utilizes the same cell cultures to demonstrate a decline in the levels of detectable p53 corresponding to a reversion of the transformed and tumorigenic phenotype. In an analogous set of experiments, p53 has been detected in mouse embryos and is lost, or the levels of this protein decline upon further differentiation (13). Higher levels of p53 can be restored in differentiated F9 cells infected with SV40. In these cells, SV40 T antigen is physically complexed with p53 (Fig. 2), and the levels of translatable p53 mRNA remain low (like the differentiated cells), whereas the half-life of p53 increases from about 3.5 h to greater than 24 h (M. Oren and N. Reich, unpublished results). The higher levels of p53 in SV40-infected F9 differentiated cells appear to be due to a

TABLE 1. Relative levels of the p53 protein in F9, differentiated F9, and 3T3 cells

Cells	Level of p53 labeled in vivo (%)	Level of translatable p53 mRNA in vitro (%)
F9	100[a]	100[a]
Differentiated F9	23, 44, 23, 13, 17[b]	14, 16[b]
BALB 3T3	15	92

[a] Normalized to 100% in each experiment.

[b] The numbers presented represent the results from five independent experiments labeled in vivo and two independent experiments labeled in vitro.

decrease in protein turnover and not to enhanced levels of p53 mRNA. Thus, the levels of p53 in a cell are regulated in at least two different ways, by p53 translatable mRNA levels and by protein turnover or alteration. Further studies will be required to choose between transcriptional regulation, RNA processing controls, or even nontranslatable p53 mRNAs as the regulator of the level of p53 in F9 and F9 differentiated cells.

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