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Evidence for Free and Metabolically Stable p53 Protein in Nuclear Subfractions of Simian Virus 40-Transformed Cells

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To determine functional subcellular loci of p53, a cellular protein associated with cellular transformation, we analyzed the nucleoplasmic, chromatin, and nuclear matrix fractions from normal mouse 3T3 cells, from methylcholanthren-transformed mouse (MethA) cells, and from various simian virus 40 (SV40)-transformed cells for the presence of p53. In 3T3 and MethA cells, p53 was present in all nuclear subfractions, suggesting an association of p53 with different structural components of the nucleus. In 3T3 cells, p53 was rapidly turned over, whereas in MethA cells, p53 was metabolically stable. In SV40-transformed cells, p53 complexed to large tumor antigen (large T) was found in the nucleoplasmic and nuclear matrix fractions, as described previously (M. Staufenbiel and W. Deppert, Cell 33:173–181, 1983). In addition, however, metabolically stable p53 not complexed to large T (free p53) was also found in the chromatin and nuclear matrix fractions of these cells. This free p53 did not arise by dissocation of large T-p53 complexes, suggesting that stabilization of p53 in SV40-transformed cells can also occur by means other than formation of a complex with large T.

p53 is a cellular protein which is associated with cellular transformation in a variety of systems (for reviews, see references 3, 18, and 26). In normal cells, the closely restricted expression of p53 during cell proliferation strongly suggests an important role for p53 in the regulation of this process, especially in controlling initiation of cellular DNA synthesis (6, 14–17, 24). In these cells, p53 exhibits a very short half-life (5 to 20 min, depending on the cell system analyzed) and is present in very low quantities (for reviews, see references 3, 18, and 26). Many transformed cells, in contrast, express a p53 with a strongly increased half-life which is present in grossly elevated levels (for reviews, see references 3, 18, and 26). Thus, it seems likely that expression of elevated levels of p53 or metabolic stabilization of this protein or both are causally related to the process of transformation (for reviews, see references 3, 18, and 26).

So far, no distinct biochemical activity has been attributed to p53. Thus, a likely mode of p53 action in both normal and transformed cells is a regulatory interaction with cellular processes leading to cell proliferation. Because these complex processes require the participation of various structural systems of the cell, it may be assumed that p53 associates, at least temporarily, with various structural systems of the cell involved in cell proliferation. We have previously described a method which allows the analysis of various nuclear subfractions for the presence of regulatory proteins (30). Application of this method to determine the subnuclear distribution of the transforming protein of simian virus 40 (SV40), the SV40 large tumor antigen (large T), led to the identification and characterization of three distinct nuclear subclasses of large T: large T in the nucleoplasm, large T at the cellular chromatin, and large T associated with the nuclear matrix (29). In the present study, we similarly analyzed the subnuclear distribution and metabolic stability of p53 in normal BALB/c mouse 3T3 cells, in methylcholanthren-transformed BALB/c mouse (MethA) cells, and in the SV40-transformed BALB/c mouse tumor line mKSA, as well as in various other SV40-transformed cell lines. In addition to p53 complexed to large T, nuclear subfractions prepared from SV40-transformed cells were also analyzed for the presence of p53 not complexed to large T (free p53), since such p53 might have been missed in previous analyses (5, 11, 13). In view of the finding that in most transformed cells exhibiting raised levels of p53 this molecule is not stabilized by formation of complexes with any known tumor antigen (for a review, see reference 26), the demonstration of free p53 in SV40-transformed cells would be of considerable importance, since it would address the question of a possible mechanism(s) of p53 stabilization common to all transformed cells.

We demonstrate that, in all cells analyzed, p53 was found in various nuclear subfractions, suggesting a functional interaction of this molecule with different structural systems of the nucleus. p53 was rapidly turned over in normal 3T3 cells, but it was metabolically stable in transformed cells. SV40-transformed cells, in addition to p53 in complex with large T, also harbored p53 which was not complexed to large T (free p53). This free p53, like p53 in MethA cells, was metabolically stable and did not arise by dissociation of large T-p53 complexes. This raises the interesting possibility that p53 stabilization in SV40-transformed cells is not exclusively mediated by formation of complexes with large T, but rather that p53 can be metabolically stabilized in these cells by an alternative means similar to p53 stabilization in other, non-SV40-transformed cells.

MATERIALS AND METHODS

Cells. BALB/c mouse 3T3 cells, MethA cells (4), SV40-transformed BALB/c mouse tumor cell line mKSA (9), SV40-transformed rat cell line SVRE9 (23), and the SV40-*A*-gene-mutant-transformed rat cell lines FR(*tsA*58)A and FR(*tsA*58)R35 (22) were grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum.

Labeling of cells and in situ cell fractionation. A detailed description of the cell fractionation procedure, as well as a characterization of extracts and structures, has been given elsewhere (29, 30). Briefly, cells grown to confluency on plates were washed with KM buffer (10 mM morpho-

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linepropanesulfonic acid [MOPS], pH 6.8; 10 mM NaCl; 1.5 mM $MgCl_2$; 1 mM ethylene glycol-bis)β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid [EGTA]; 5 mM dithiothreitol; 10% glycerol) and lysed in KM buffer containing 1% Nonidet P-40 (NP-40) (nucleoplasmic extract). Nuclear structures still attached to the substratum (NP-40 nuclei) were extracted with KM buffer without dithiothreitol and EDTA, containing 100 μg of DNase I per ml (number D-5010, Sigma Chemical Co.) for 15 min at 37°C. An equal volume of 4 M NaCl was then added, and the incubation was continued for 30 min more at 4°C (chromatin extract). Nuclear matrices were solubilized in TK buffer (40 mM Tris hydrochloride, pH 9.0; 25 mM KCl; 5 mM dithiothreitol; 10% glycerol) containing 1% Empigen BB (Albright and Wilson, Ltd.) for 60 min at 4°C. All buffers contained 30 μg of aprotinin (200 KIU; Trasylol, Bayer) per ml, and, immediately after fractionation, phenylmethylsulfonyl fluoride was added to yield a concentration of 1 mM. The nucleoplasmic and nuclear matrix extracts were made 150 mM in NaCl and adjusted to pH 8.0 by the addition of 100 mM Tris hydrochloride, pH 8.0. To the Empigen BB extract (nuclear matrix extract), NP-40 was added to yield a final concentration of 2%. The chromatin extract was desalted on a small Sephadex G-25 column. These treatments helped to prevent degradation and denaturation of large T in the various extracts and allowed quantitative recovery of large T and p53 from these extracts by immunoprecipitation. Before fractionation, cells were labeled with [^{35}S]methionine (100 μCi per plate per ml) as described previously (29) and in the text.

Antibodies, immunoprecipitation, and SDS-PAGE. SV40 large T and large T-p53 complexes were immunoprecipitated with monoclonal antibody PAb108, which recognizes a non-denaturation-sensitive determinant at the very amino-terminal end of large T (8). p53 was immunoprecipitated with either monoclonal antibody PAb122 (7) or an antiserum directed against gel-purified, sodium dodecyl sulfate (SDS)-denatured p53 prepared in a fashion similar to that described by McCormick and Harlow (13). This antiserum had a titer on SV40-transformed cells of about 1:250 as analyzed by immunofluorescence microscopy, reacted specifically with p53 as analyzed by Western blotting (2), and quantitatively immunoprecipitated p53 (unpublished data).

All extracts were cleared by centrifugation at 130,000 × *g* for 30 min at 4°C. Extracts from SV40-transformed cells were then sequentially precipitated, first with 10 μl of purified PAb108 and then with 200 μl of settled protein A-Sepharose as described previously (10, 29). After removal of immune complexes bound to protein A-Sepharose by centrifugation at 2,000 × *g*, immunoprecipitations with PAb108 were repeated. Cleared extracts were then reprecipitated with either 10 μl of PAb122 or 10 μl of anti-SDS-denatured-p53 serum followed by 200 μl of settled protein A-Sepharose. Extracts from 3T3 and MethA cells were directly precipitated with PAb122. Samples were processed for SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed on SDS-polyacrylamide gels as described previously (10, 29).

RESULTS

Distribution and metabolic stability of p53 in nuclear subfractions of 3T3 and MethA cells. As determined by immunofluorescence microscopy, the main subcellular location of p53 in both 3T3 and MethA cells is the cell nucleus (for a review, see reference 3; also, unpublished data). To identify possible structural targets of p53 in these cells, we investigated its subnuclear distribution by using a previously described and characterized cell fractionation procedure (29, 30). Fractionation of cells in situ (29, 30) yields three extracts which, with regard to the distribution of nuclear p53, represent the following three different structural systems of the nucleus: (i) the nucleoplasm in the NP-40 extract, (ii) the solubilized chromatin in the DNase–high-salt extract, and (iii) the solubilized nuclear matrix proteins in the Empigen BB extract (29, 30).

Parallel cultures of 3T3 and MethA cells were labeled with [^{35}S]methionine for 1 h and either fractionated directly (pulse-labeled) or kept in normal growth medium for 4 h after the labeling period (pulse-chase-labeled) before fractionation. Nuclear fractions were immunoprecipitated with the p53-specific monoclonal antibody PAb122 (7), and immunoprecipitates were analyzed by SDS-PAGE as described in Materials and Methods. The subnuclear distribution and metabolic stability of p53 in 3T3 cells is shown in Fig. 1A; that for MethA cells is shown in Fig. 1B. p53 was present in all nuclear subfractions of both cell lines. However, although p53 in 3T3 cells was rapidly turned over and was no longer detectable after a 4-h-chase period (Fig. 1A), p53 in MethA cells was metabolically stable (Fig. 1B). In addition, no shift in subnuclear distribution of p53 was detectable in these cells during the chase period, indicating a relatively stable association of p53 with the various nuclear substructures.

Presence of free (uncomplexed) and metabolically stable p53 in nuclear subfractions of mKSA cells. Parallel cultures of mKSA cells were pulse-labeled (1 h) and pulse-chase-labeled (1 and 4 h, respectively) with [^{35}S]methionine and subfractioned as described above. Nuclear extracts were first cleared from large T and large T-p53 complexes by immunoprecipitation with the large T-specific monoclonal antibody PAb108 (8). Analysis of these immunoprecipitates by SDS-PAGE (Fig. 2A) showed the nuclear subclasses of large T and large T-p53 complexes in pulse-labeled mKSA cells as described previously (29). Cleared nuclear extracts of pulse- and pulse-chase-labeled cells were then analyzed for the presence of p53 not complexed to large T by use of monoclonal antibody PAb122 directed against p53. The nucleoplasmic extract did not contain free p53, as shown in Fig. 2B, lanes marked N. However, a 53,000-molecular-weight protein (53K protein) was precipitated from the chromatin fraction (Fig. 2B, lanes G). Analysis of the nuclear matrix fraction (Fig. 2B, lanes NM) revealed a considerable amount of radiolabeled 53K protein, approximately equal to the amount of radiolabeled large T in this fraction (Fig. 2A, lane NM). To verify that the 53K protein precipitated from these extracts by monoclonal antibody PAb122 was authentic p53, these extracts were also precipitated with a monospecific, but polyclonal, antiserum prepared against gel-purified SDS-denatured p53 from mKSA cells (see Materials and Methods). This antiserum precipitated the same 53K protein from these extracts as did monoclonal antibody PAb122 (data not shown). Thus, we conclude that the 53K protein precipitated by both antibodies represents authentic p53.

The presence of free p53 in nuclear extracts was not restricted to mKSA cells. We have analyzed several other SV40-transformed mouse and rat cells, including VLM (32), C98 (28), and SVRE9 (23) (see below), for free p53. All SV40-transformed cell lines analyzed so far contained free p53, although there were some differences with regard to amount and subcellular distribution (data not shown).

Free p53 in mKSA cells was metabolically stable. No difference in either subnuclear location or amount of free p53

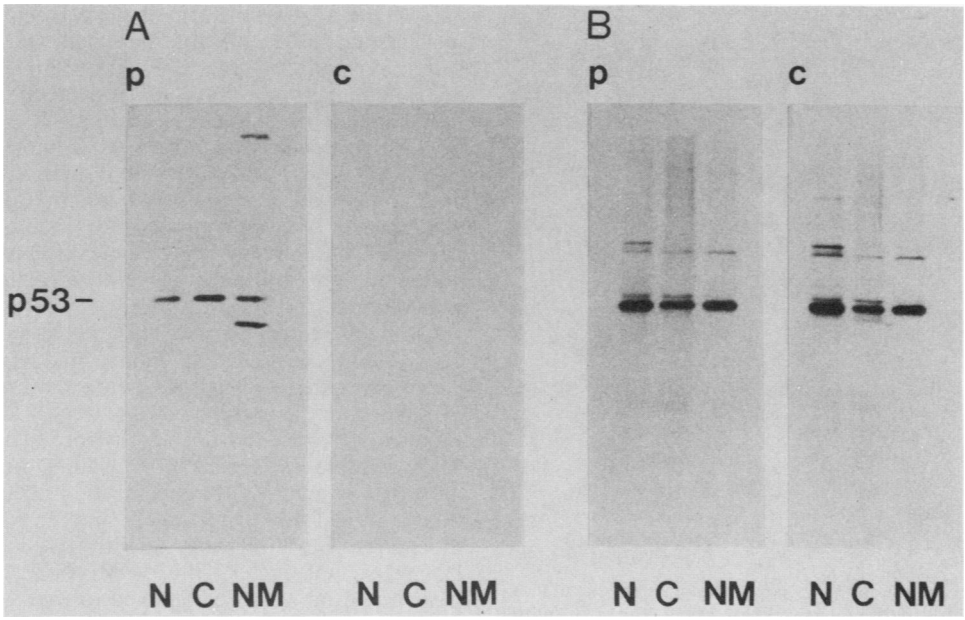

FIG. 1. Subcellular distribution and metabolic stability of p53 in 3T3 and MethA cells. Parallel cultures of 3T3 (A) and MethA (B) cells were labeled with [^{35}S]methionine for 1 h and subfractionated (pulse-labeled; gels p) or kept in normal growth medium for 4 h more (chased; gels c) before fractionation. Cell fractionation was carried out as described in Materials and Methods. Nuclear extracts were immunoprecipitated with monoclonal antibody PAb122. Immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography. Nuclear extracts were the nucleoplasmic extract (lanes marked N), the chromatin extract (lanes marked C), and the nuclear matrix extract (lanes marked NM).

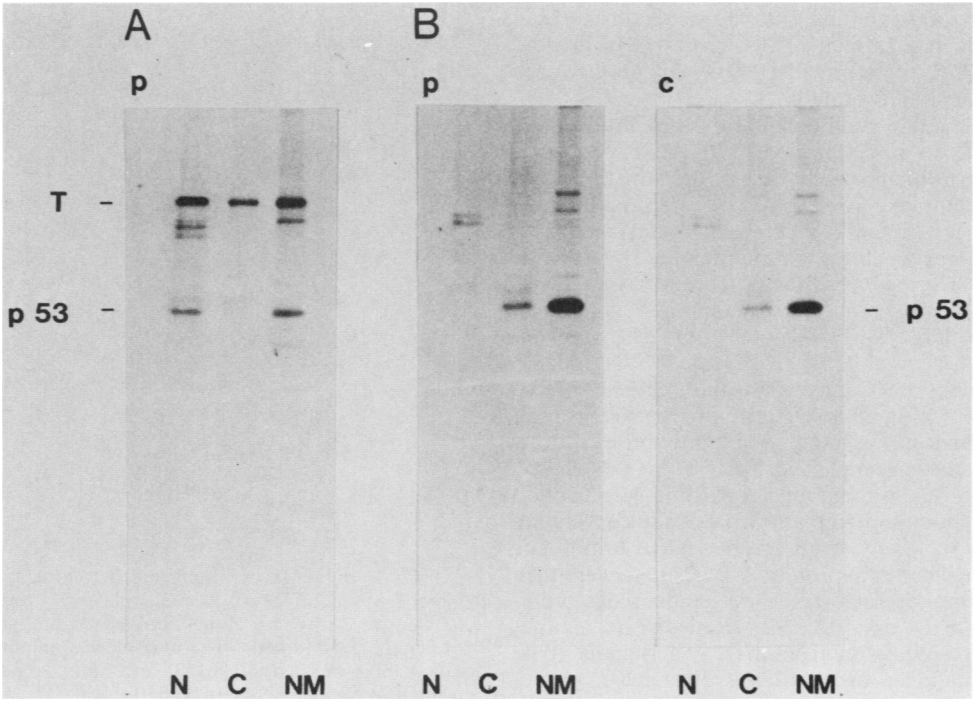

FIG. 2. Distribution of large T subclasses and of free p53 in nuclear extracts of mKSA cells. Parallel cultures of mKSA cells were pulse-labeled (gels marked p) and pulse-chase-labeled (gels c) labeled with [^{35}S]methionine and subfractionated as described in the legend to Fig. 1 and in Materials and Methods. Nuclear extracts were first analyzed for large T and large T-p53 complexes by quantitative immunoprecipitation with monoclonal antibody PAb108 (A). Extracts cleared from large T and large T-p53 complexes were then analyzed for free p53 with monoclonal antibody PAb122 (B). Immunoprecipitates were analyzed as described in the legend to Fig. 1. Designation of nuclear extracts is as described in the legend to Fig. 1.

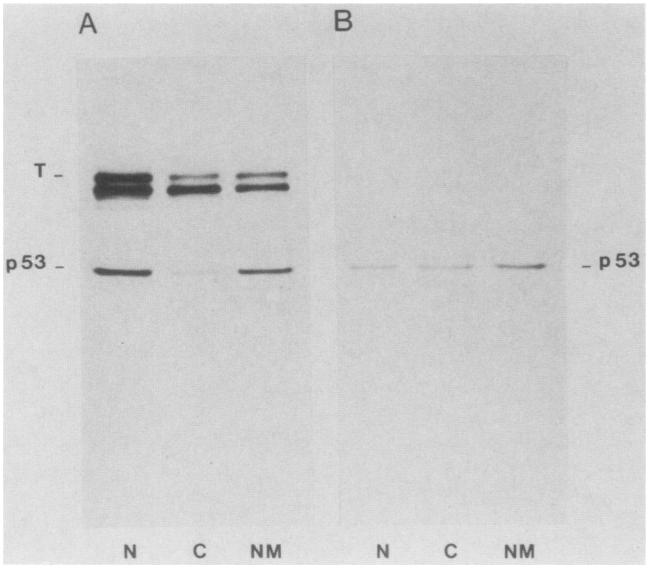

FIG. 3. Presence of free p53 in the nucleoplasmic extract of SVRE9 cells. SVRE9 cells were pulse-labeled for 1 h with [^{35}S]methionine and then subfractionated. Nuclear extracts were first cleared from large T and large T-p53 complexes by immunoprecipitation with PAb108 (A) and then analyzed for free p53 with PAb122 (B). Immunoprecipitates were analyzed as described in the legend to Fig. 1. Designation of nuclear extracts is as described in the legend to Fig. 1.

could be detected between pulse-labeled (Fig. 2B, gel p), or pulse-chase-labeled (Fig. 2B, gel c) cells. We have also analyzed the metabolic stability of free p53 in other SV40-transformed cells and have found it to be stable, as is p53 in mKSA cells (data not shown). Thus, free p53 in nuclear subfractions of SV40-transformed cells and of MethA cells exhibits similar metabolic stability.

Effects of fractionation conditions on the stability of large T-p53 complexes. An obvious caveat to the experiments described above was the possibility that within the living cell all p53 might be complexed to large T, i.e., that the free p53 observed in our experiments might have dissociated from large T-p53 complexes during fractionation. To test this possibility, we performed a series of control experiments and analyzed the effects of our fractionation conditions on the stability of large T-p53 complexes, as described below.

Dilution. It might be argued that the relatively large dilution of large T-p53 complexes during preparation of the nuclear extracts and during immunoprecipitation might dissociate p53 from these complexes. To test this possibility, we incubated immunopurified nucleoplasmic large T-p53 complexes in the immunoprecipitate with various extraction buffers, added in relatively large volumes (4 to 6 ml). The immune complexes bound to protein A-Sepharose were then recovered, and the supernatants were sequentially reprecipitated with PAb108 and PAb122, respectively. Neither large T nor p53 was recovered from these supernatants (data not shown), indicating a high stability of at least nucleoplasmic large T-p53 complexes when diluted.

Nucleoplasmic free p53. Although mKSA cells and a variety of other SV40-transformed cell lines did not contain free p53 in the nucleoplasmic fraction, several SV40-transformed rat cell lines exhibited free and metabolically stable p53 in the nucleoplasmic extract. This is documented by the analysis of the SV40-transformed rat cell line SVRE9 (23) for nuclear subclasses of large T and large T-p53 complexes (Fig. 3A) and for free p53 (Fig. 3B). In these cells, a considerable portion of p53 in the nucleoplasmic extract was found not to be complexed to large T (Fig. 3B, lane N). Because nucleoplasmic large T-p53 complexes in these cells (Fig. 3A, lane N) were resistant to dilution (see above; also, data not shown), it may be assumed that the free p53 in the nucleoplasm of these cells did not arise by dissociation of nucleoplasmic large T-p53 complexes, given the relatively mild conditions for nucleoplasmic extraction (see Materials and Methods).

Chromatin-associated free p53. It has been reported that large T-p53 complexes in mouse and rat cells withstand treatment with 2 M NaCl (13). However, to provide further evidence that the presence of free large T and free p53 in our chromatin extract did not result from dissociation of large T-p53 complexes caused by high ionic strength, we determined the minimal salt concentration necessary for extraction of large T and p53 from chromatin. We found that both molecules were already quantitatively extracted from DNase-treated nuclei with 0.4 M NaCl. Large T and p53 were found not to be complexed to each other even at this relatively low ionic strength (data not shown). Therefore, one can assume that they are not complexed to each other at this nuclear substructure.

Nuclear matrix-associated free p53. Since Empigen BB

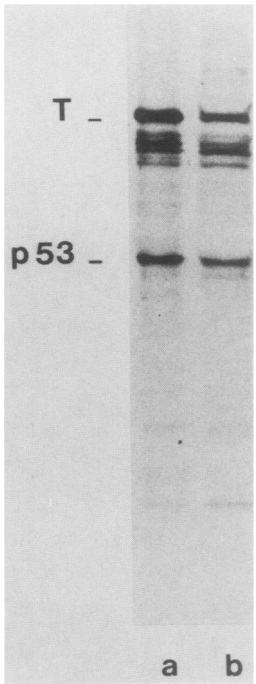

FIG. 4. Influence of Empigen BB treatment on the stability of large T-p53 complexes. A nucleoplasmic extract of mKSA cells labeled for 2 h with [^{35}S]methionine was prepared as described in Materials and Methods. The extract was immunoprecipitated with monoclonal antibody PAb108, and immune complexes bound to protein A-Sepharose were split in half. One half was analyzed directly by SDS-PAGE (lane a), and the other half (lane b) was reincubated in TK buffer containing 1% Empigen BB (see Materials and Methods). After 1 h of incubation at 4°C, immune complexes bound to protein A-Sepharose were processed for SDS-PAGE, and immune complexes were analyzed as described in the legend to Fig. 1.

effectively solubilizes cellular structural proteins, including intermediate filaments and the nuclear matrix (29, 30), it was important to demonstrate that Empigen BB treatment did not dissolve large T-p53 complexes. This was intrinsically difficult for nuclear matrix-associated large T-p53 complexes, since they were obtained after solubilization of this structure by Empigen BB. However, two arguments strongly favor our interpretation that free p53 at the nuclear matrix did not arise from dissociation of large T-p53 complexes. (i) Empigen BB treatment does not dissociate isolated large T-p53 complexes. Nucleoplasmic large T-p53 complexes were immunopurified by precipitation with monoclonal antibody PAb108 and protein A-Sepharose. The purified immune complexes were then treated with Empigen BB in the same buffer used to solubilize the nuclear matrix (see Materials and Methods). Untreated (Fig. 4, lane a) and Empigen BB-treated (Fig. 4, lane b) immune complexes were then analyzed by SDS-PAGE. Although the Empigen BB treatment dissociated some immune complexes from protein A-Sepharose, the ratio of large T to p53 in the Empigen BB-treated immunoprecipitate (Fig. 4, lane b) was similar to the ratio in the untreated one (Fig. 4, lane a), indicating that the large T-p53 complex is not sensitive to Empigen BB treatment. In addition, we were able to reprecipitate the large T-p53 complexes solubilized during Empigen BB treatment of immune complexes after addition of fresh antibody and protein A-Sepharose and found that large T and p53 were still complexed to each other (data not shown). We similarly treated large T-p53 complexes isolated from the nuclear matrix fraction. Again, Empigen BB treatment did not dissociate large T-p53 complexes (data not shown). (ii) Nuclear matrix-associated large T-p53 complexes and nuclear matrix-associated free p53 exhibit a different extraction behavior. The nuclear subfractions obtained by any fractionation procedure can be only operationally defined. Therefore, the presence of regulatory proteins like large T and p53 in a certain nuclear subfraction may depend on the exact fractionation conditions applied. In our fractionation protocol, cellular chromatin proteins are extracted with high salt concentrations (2 M NaCl) after the relaxing of cellular DNA loops by treatment of isolated nuclei with DNase I (29, 30) (see Materials and Methods). We found that this treatment maximally preserved the structure of the residual nuclear matrix (30; unpublished data). Alternatively, however, DNase I treatment of isolated nuclei is either omitted or applied after high-salt-concentration extraction in various published procedures for the isolation of nuclear matrices (for a review, see reference 1). We analyzed the effect of high-salt-concentration extraction of isolated NP-40-treated nuclei without prior DNase I treatment upon extraction of large T-p53 complexes and of free p53 from SV40-transformed cells. mKSA cells were pulse labeled with [^{35}S]methionine for 1 h, and the nucleoplasmic extract was prepared as described above. NP-40-treated nuclei were then directly extracted with 2 M NaCl in KM buffer, without prior DNase treatment. Residual nuclear matrices were dissolved with Empigen BB as described above. Nuclear extracts were then sequentially immunoprecipitated with PAb108 to isolate large T and large T-p53 complexes, followed by immunoprecipitation with PAb122 to recover free p53. The result is shown in Fig. 5. High-salt-concentration extraction of NP-40-treated nuclei without prior DNase I treatment in one step completely extracted the chromatin plus the nuclear matrix-associated subclasses of large T (Fig. 5A, lanes C and NM). This treatment, however, did not extract free p53 associated with

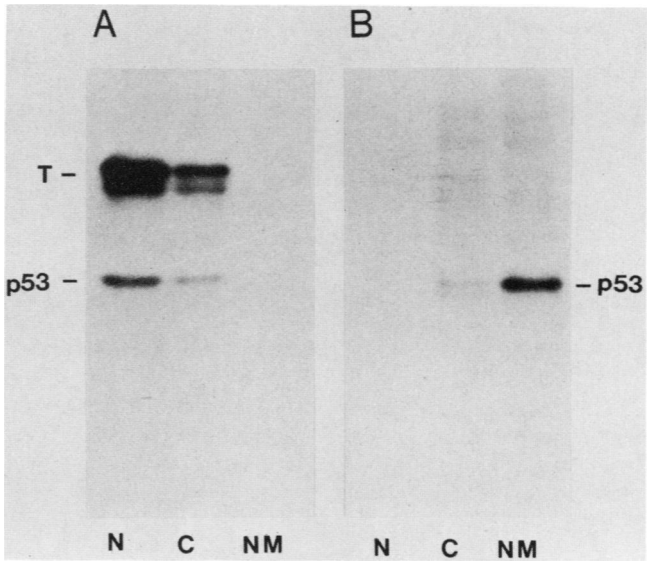

FIG. 5. Different extractability of nuclear matrix-associated large T and large T-p53 complexes and of nuclear matrix-associated free p53. mKSA cells were labeled with [^{35}S]methionine for 1 h, and a nucleoplasmic extract (lanes marked N) was prepared as described in Materials and Methods. NP-40-treated nuclei were then directly extracted with KM buffer containing 2 M NaCl without prior DNase I treatment (lanes marked C). Residual nuclear matrices were solubilized with TK buffer containing 1% Empigen BB (lanes marked NM). Nuclear extracts were first analyzed for large T and large T-p53 complexes by use of PAb108 (A) and then were analyzed for free p53 with PAb122 (B). Immune complexes were analyzed as described in the legend to Fig. 1.

the nuclear matrix (Fig. 5B, lanes C and NM). This p53 was released only from residual nuclear matrices after solubilization of these structures with Empigen BB (Fig. 5B, lanes C and NM). Thus, p53 at the nuclear matrix in complex with large T exhibits an extraction behavior different from that of free p53 associated with this structure, strongly indicating that complexed and free p53 at the nuclear matrix constitute different pools of this molecule.

Free p53 in rat cells transformed by an SV40 *A* gene mutant. Since it is conceptually difficult to rule out with certainty the dissociation of large T-p53 complexes by biochemical controls alone, we have analyzed the subnuclear distribution of free p53 in SV40-transformed cells not expressing the nuclear matrix-associated subclass of large T. We recently found that various rat cells transformed by SV40 *A* gene mutants did not express nuclear matrix-associated large T (W. Deppert, unpublished data). Therefore, we analyzed FR(*tsA*58)A cells (rat F111 cells transformed by *tsA*58, expressing a temperature-sensitive phenotype for transformation [22]) and FR(*tsA*58)R35 cells (rat F111 cells transformed by *tsA*58, expressing a non-temperature-sensitive phenotype for transformation [22]) for the presence of large T-p53 complexes and of free p53 in various nuclear subfractions after pulse-chase-labeling. Parallel cultures were kept at the permissive growth temperature (32°C) for at least 3 days, and pulse-labeled (1 h) or pulse-chase-labeled (4 h) with [^{35}S]methionine. The cultures were then subfractionated by our standard subfractionation protocol, and nuclear subfractions were analyzed by sequential immunoprecipitation with PAb108 for large T and large T-p53 complexes, followed by immunoprecipitation with PAb122 for free p53. The distribution of large T and large T-p53 complexes in

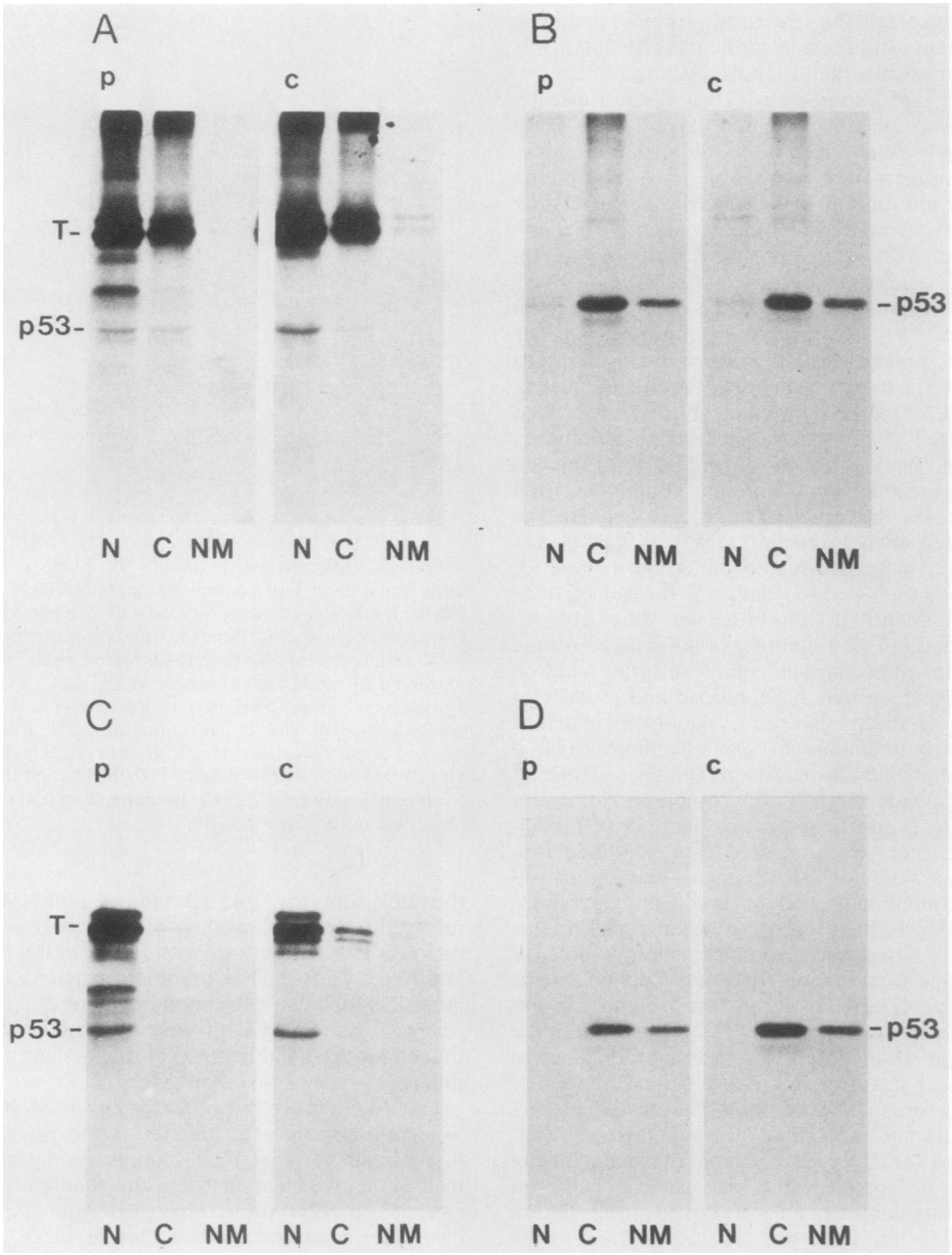

FIG. 6. Distribution of nuclear subclasses of large T and of free p53 in nuclear subfractions of the SV40-*tsA*-mutant-transformed cell lines FR(*tsA*58)R35 and FR(*tsA*58)A. Parallel cultures of FR(*tsA*58)R35 and of FR(*tsA*58)A cells were pulse-chase-labeled with [^{35}S]methionine as described in the text and in the legend to Fig. 1 and then subfractionated. Nuclear subfractions were first analyzed for large T and large T-p53 complexes by use of PAb108. Cleared nuclear extracts then were analyzed for free p53 by use of PAb122. Immune complexes were analyzed as described in the legend to Fig. 1. (A and B) Analysis of FR(*tsA*58)R35 cells. (A) Gels p and c were overexposed (twice) compared with the other panels to visualize the small amount of nuclear matrix-associated large T. (C and D) Analysis of FR(*tsA*58)A cells.

nuclear subfractions of pulse-labeled (gel p) and pulse-chase-labeled (gel c) FR(*tsA*58)R35 cells is shown in Fig. 6A. Only the nucleoplasmic and the chromatin-associated nuclear subclasses of large T were present in these cells in reasonable amounts. The extremely small amount of nuclear matrix-associated large T detectable in these cells (less than 0.1% of total large T) was seen only after prolonged exposure of the gel, as shown in Fig. 6A. Very little p53 was found to be complexed to large T in these cells, even in the nucleoplasmic fraction (Fig. 6A, lanes marked N). The nucleoplasmic fraction also did not contain free p53 (Fig. 6B, lanes marked N), but the majority of this molecule was present in a free and metabolically stable form in the chromatin and in the nuclear matrix fraction (Fig. 6B). Thus, free p53 in these cells exhibited a subnuclear distribution similar to that of p53 in SV40 wild-type-transformed cells (Fig. 2B), independent of the distribution of large T and large T-p53 complexes. Similarly, free p53 in FR(*tsA*58)A cells (Fig. 6D) was also found in a metabolically stable form in the chromatin and the nuclear matrix fraction, although the distribution of large T and large T-p53 complexes was quite different (Fig. 6C); after the pulse-labeling (Fig. 6C, gel p),

the vast majority of large T was present in the nucleoplasmic fraction (lanes marked N), very little (less than 1%, as determined by scanning the fluorogram) was associated with the cellular chromatin (lanes marked C), and only trace amounts were recovered from the nuclear matrix fraction (lanes marked NM). During the 4-h chase period, the amount of chromatin-associated large T increased to about 5% (Fig. 6C; gel c, lane C). However, large T still could not be detected at the nuclear matrix (Fig. 6C, gel c, lane NM). Thus, these experiments demonstrated that, in both cell lines, free p53 could be detected in nuclear subfractions containing only trace amounts of large T, arguing for a subnuclear distribution of free p53 independent of that of large T and large T-p53 complexes. In addition, it is also extremely unlikely that the minimal amounts of large T found in vivo, e.g., in the nuclear matrix fraction of these cells, could have complexed all of the p53 recovered as free p53 after fractionation. Therefore, these experiments provide additional and independent strong evidence for the presence of p53 in SV40-transformed cells which is metabolically stable but not complexed to large T.

DISCUSSION

In this study, we determined potential structural targets of p53 in the nuclei of normal 3T3 cells and of various transformed cells. In both normal 3T3 and MethA-transformed cells, p53 was found in the chromatin and in the nucleoplasmic and nuclear matrix fractions, as defined by our cell fractionation procedure (29, 30). However, because nuclear fractions obtained by cell fractionation can be only operationally defined, the precise interaction of p53 in vivo with the cellular chromatin and with the nuclear matrix is still not clear and will have to be further elucidated by functional analyses. Nevertheless, our results indicate that, at least by virtue of different extractability, p53 is subcompartmented in the nuclei of both normal and transformed cells, suggesting a functional interaction of this molecule with nuclear structures involved in cell proliferation. In this respect, it is interesting that p53 occupies the same nuclear sites as SV40 large T (29), which also is implicated in modulating the control of cellular proliferation (for a review, see reference 12). As reported previously, p53 in normal 3T3 cells was rapidly turned over, whereas p53 in the MethA cells was metabolically stable, leading to elevated levels of p53 in these cells (9, 19, 20, 24).

A more complex situation was observed in SV40-transformed cells. In these cells, a significant portion of p53 is complexed to large T (for a review, see references 3, 25, and 26). Large T-p53 complexes were found mainly in the nucleoplasmic and nuclear matrix fractions, although some cell lines (and also mKSA cells under certain growth conditions) can, in addition, exhibit large T-p53 complexes in the chromatin fraction (unpublished data). Up to now, it had been assumed that the formation of large T-p53 complexes was the prevailing mechanism operating in these cells for stabilizing p53 (5, 11, and 25) and it had been suggested that this process might play an important functional role in cell transformation (for a review, see references 3, 11, 25, and 26). The experiments presented in this study do not argue against the latter assumption. However, we provide evidence for the existence of an additional mechanism for stabilizing p53 in SV40-transformed cells leading to the generation of metabolically stable p53 not complexed to large T. Although it is unknown at present whether this form of p53 is complexed to a cellular protein, as suggested previously (6, 21), or exists in a free form, we have, for the sake of simplicity, designated this form free p53 to distinguish it from p53 in complex with large T. This free p53 was identified by sequential immunoprecipitations of nuclear subfractions of SV40-transformed cells with a monoclonal antibody directed against large T to remove large T and large T-p53 complexes, followed by immunoprecipitation with a monoclonal antibody directed against p53. Inherent in this technique, however, are several problems which potentially could produce artificial results due to fractionation conditions, dilution during immunoprecipitation, etc. Therefore, it was vital to provide further evidence that the free p53 detected in our analyses was not being generated by dissociation of large T-p53 complexes. Aside from biochemical controls, in which we showed that our fractionation and immunoprecipitation conditions had no detectable effect on the stability of large T-p53 complexes, three other independent lines of evidence strongly support our conclusion of the presence of free p53 in SV40-transformed cells. (i) Several SV40-transformed cell lines, including rat SVRE9 (Fig. 3), contained free and metabolically stable p53 in the nucleoplasmic fraction. It is extremely unlikely that the mild conditions used for preparing the nucleoplasmic extract (hypotonic buffer, pH 6.8, 1% NP-40; see Materials and Methods) should lead to dissociation of large T-p53 complexes. (ii) Large T-p53 complexes and free p53 at the (operationally defined) nuclear matrix exhibited a different extraction behavior. If NP-40-treated nuclei were extracted with 2 M NaCl without prior DNase I treatment, chromatin- and nuclear matrix-associated large T and large T-p53 complexes were extracted in one step, whereas free p53 at the nuclear matrix was not extracted under these conditions and was only solubilized by Empigen BB treatment of the residual nuclear matrices. (iii) Free and metabolically stable p53 was also found in nuclear subfractions of cells transformed by an SV40-*A*-gene mutant which contained only minute amounts of large T in these fractions. This renders it extremely unlikely that the free p53 detected in these fractions had been artificially generated by dissociation of large T-p53 complexes and argues for a large T-independent subnuclear distribution of p53. Thus, the evidence provided in this study strongly favors the interpretation that p53 stabilization in SV40-transformed cells can be achieved not only by formation of a complex with large T but also by stabilization of p53 in a free form, reminiscent of p53 stabilization in other, non-SV40-transformed cells, such as MethA cells. This raises the exciting possibility of a common cellular process for p53 stabilization in transformed cells independent of the formation of a complex with a viral tumor antigen like SV40 large T. This interpretation of our data is in line with the recently published observation that formation of a complex of p53 and the adenovirus E1b 58K tumor antigen (27) depends on the adenovirus serotype used for transformation (31). However, in adenovirus-transformed cells in which p53 is not complexed to the E1b 58K tumor antigen, p53 is also metabolically stable (31).

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