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Time-Dependent Maturation of the Simian Virus 40 Large T Antigen-p53 Complex Studied by Using Monoclonal Antibodies

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Newly synthesized simian virus 40 large tumor antigen (T Ag) slowly forms a stable complex with the host tumor antigen, "p53." By the use of immunological and temporal separations and inhibition of aggregation and processing by *A* locus mutation, we have distinguished specific steps in the reaction sequence leading to formation of the rapidly sedimenting oligomeric complex. The monoclonal antibody PAb101 bound only a fraction of the total soluble pulse-labeled T Ag bound by antitumor serum. After a chase, all T Ag had matured to the form recognized by PAb101. All p53 in the mouse line SVA31E7 was precipitated by the T Ag-specific monoclonal antibody PAb101, even after a short pulse, and is therefore entirely bound to mature T Ag. The p53-specific monoclonal antibody PAb122 precipitates nearly all of the mature T Ag recognized by PAb101, except *A* locus mutant T Ag, synthesized at the nonpermissive temperature. *A* locus mutation inhibited entry of newly synthesized T Ag into the oligomeric >28S complex of T Ag and p53.

The large tumor antigen (T Ag) of the DNA tumor virus simian virus 40 (SV40) immunoprecipitates with host proteins of the molecular weight range 48,000 to 55,000, known collectively as p53 (4, 18, 20, 21, 24). The coprecipitation of the viral and host proteins has been shown to be due to the presence of stable complexes (12, 13, 20, 21, 23). The host p53s are found at significantly elevated levels in cells transformed by RNA and DNA tumor viruses, chemical carcinogens, ionizing radiation, and "spontaneous events" (as reviewed in reference 17). The interaction of the viral transforming protein (reviewed in reference 33) T Ag with the host protein p53 may be a primary event in oncogenic transformation by the virus. Soon after infection of nonpermissive cells by SV40, p53 and T Ag accumulate coordinately (3, 22). The accumulation of p53 in nonpermissive cells is dependent on the function of the viral *A* locus (3, 22), which encodes T Ag, as is the formation of the complex between p53 and T Ag in SV40-transformed cells (12). Although p53 can be detected in SV40-infected permissive cells (9, 13, 24), T Ag appears to be less strongly and completely complexed to p53 in these cells (8, 23, 30). This may mean that the p53-containing complex plays a more significant role in the transforming infection than in the productive infection.

T Ag occurs in large, heterogeneous aggregates detectable by sucrose gradient centrifugation (2, 19, 28, 29) or by gel exclusion chromatography (14). These high-molecular-weight aggregates of T Ag have been shown to be due to both self-association of T Ag (1, 26) and binding of p53 to T Ag (9, 12, 20, 21, 23). T Ag is phosphorylated at multiple sites (11, 16, 34–36), and the more phosphorylated form(s) of T Ag is the form found bound to p53 in high-molecular-weight complexes (9, 12, 23). Maturation of T Ag to the highly phosphorylated, p53-bound, rapidly sedimenting form is a slow reaction (9, 12), allowing the separation of the various radiolabeled forms of T Ag as a function of time after synthesis. Monospecific (23, 25) and monoclonal antibodies (7, 13) have been used to localize p53 to the extranucleolar nucleoplasm of SV40-transformed cells, giving immunofluorescence indistinguishable from that characteristic of T Ag. This is consistent with the existence of the complex in the nucleus in vivo.

Further characterization and subfractionation of the various forms of T Ag and p53 should be greatly facilitated by the use of monoclonal antibodies specific to subfractions of T Ag. PAb122 reacts with a determinant of both native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-purified p53 common to the mouse, monkey, and human proteins (13) and consequently precipitates p53-bound T Ag. PAb101 (formerly called clone 412) recognizes a determinant of large T Ag present on

only part of the total T Ag in an extract of SV40-transformed cells (13). The PAb101-specific antigenic determinant has been further localized, by the use of fragments of T Ag encoded by adenovirus-SV40 hybrids, to the carboxy terminus of large T Ag (6). The possibility that a determinant on p53 might make a contribution to the PAb101-specific determinant was eliminated by PAb101 reactivity with T Ag fragments in adenovirus-SV40 hybrid virus-infected HeLa cells which do not have any antigen which reacts with PAb122 (6).

We set out to determine how subfractions of T Ag, immunologically defined by precipitation by T Ag-specific monoclonal PAb101, p53-specific monoclonal PAb122, or hamster antitumor serum, correlate with the biologically definable and biochemically and temporally separable subfractions of T Ag. We have found that newly synthesized T Ag recognized by antitumor serum undergoes a sequence of changes whereby it becomes reactive with the T Ag-specific monoclonal PAb101 and then, through binding with p53, becomes immunoprecipitable by the p53-specific monoclonal PAb122.

MATERIALS AND METHODS

Cells. SVA31E7 mouse cells transformed by wild-type SV40 were derived from BALB/c 3T3 cells by Y. Ito (National Cancer Institute, Bethesda, Md.). Mouse cells transformed by a *tsA* virus (A21) were isolated by J. Hiscott and V. Defendi (15) after transformation of BALB/c mouse embryo fibroblasts with the *tsA58* virus of P. Tegtmeyer et al. (32). The methylcholanthrene-derived BALB/c mouse tumor cells line (Meth A) was obtained from A. DeLeo (5). Cells were grown in Dulbecco modified Eagle antibiotic-antimycotic (ABAM; medium with 10% calf serum and 1% GIBCO).

Antisera and monoclonal antibodies. Hamster antitumor serum was prepared by injection of SV40-transformed hamster tumor cells (H6590B; obtained from V. Defendi, New York University, New York City) into 3- to 4-week-old hamsters. This serum was tested with extracts of Meth A cells and shown not to bind p53 in the absence of T Ag. The mouse hybridoma lines PAb122 (originally clone 122) and PAb101 (originally clone 412) were derived by E. Gurney et al. (13). The T Ag-specific monoclonal antibody PAb101 was used as cell culture medium. The p53-specific monoclonal antibody PAb122 was used as cell culture medium or as ascites fluid from pristane-primed mice injected with 3×10^6 cells per mouse.

Sucrose gradient centrifugation and immunoprecipitation of cell extracts. Cell extracts for sucrose gradient sedimentation were prepared by freeze-thawing whole cells in 0.01 M Tris-hydrochloride (pH 9.0)–1.0 mM dithiothreitol–0.14 M NaCl buffer at a cell/buffer ratio of 1:2.5 ml. Cell extracts were clarified by centrifugation for 20 min at 16,000 × *g* in a Sorvall centrifuge at 4°C. Clarified extracts (0.35 ml) were applied to a 5-ml linear 5 to 20% sucrose gradient (with 0.2 ml of 60% sucrose pads). The sucrose gradient was made up in a buffer: 10 mM Tris-hydrochloride (pH 7.4)–1 mM dithiothreitol–0.14 M NaCl. Centrifugation was for 12.5 h at 23,000 rpm in a Beckman SW50.1 rotor at 4°C. Fractions (ca. 0.27 ml) were collected from the bottoms of the gradients. External rRNA S value markers were centrifuged on parallel gradients.

Gradient fractions were subjected to clearing precipitation with 5 μl of either normal hamster or normal mouse serum and 20 μl of a 10% suspension of *Staphylococcus aureus* and then centrifuged for 2 min in an Eppendorf centrifuge. The supernatants were then immunoprecipitated: the even-numbered fractions with either 5 μl of hamster antitumor serum, 5 μl of PAb122 ascites, or 100 μl of PAb101 culture medium, and the odd-numbered fractions with the corresponding normal serum, followed by 20 μl of *S. aureus*. The incubation times and washing were done as previously described (24).

SDS-PAGE and fluorography. SDS-PAGE and fluorography of sucrose gradient fractions were carried out as previously described (12), except that 7.5 to 10% (20 by 20 cm) acrylamide gradient gels were used.

Sequential immunoprecipitation. Sequential immunoprecipitation of cell extracts was executed as described previously (13). Samples were analyzed on 7.5 to 15% gradient polyacrylamide gels, with autoradiography as described previously (13). The relative amounts of T Ag and p53 in different samples were calculated from the exposure times necessary to give equivalent bands of moderate density on Kodak XAR X-ray film.

RESULTS

Maturation of the rapidly sedimenting T Ag-p53 complex. The abilities of the p53-specific monoclonal antibody PAb122 and the T Ag-specific PAb101 to precipitate the sedimenting forms of T Ag and p53 were compared with that of hamster antitumor serum. SVA31E7 cells were pulse labeled for 30 min before analysis by sedimentation, immunoprecipitation, SDS-PAGE, and fluorography (Fig. 1). After a short pulse label, antitumor serum detected T Ag sedimenting in a broad distribution between the top and bottom of the gradient, but the T Ag distribution was skewed toward the top of the gradient (Fig. 1A). The newly synthesized host protein p53 cosedimented with very little newly synthesized T Ag (as opposed to previously synthesized T Ag) after a pulse label, but sedimented toward the bottom of the gradient. Immunoprecipitation with the p53-specific monoclonal antibody PAb122 detected p53 in the same fractions of the gradient in which p53 was detected by antitumor serum (Fig. 1B). However, the p53-specific monoclonal PAb122 precipitated very little radiolabeled T Ag bound to p53 after a pulse label. Both (i) the difference in sedimentation rates of p53 and the majority of T Ag and (ii) the failure of the anti-p53 monoclonal antibody to precipitate much T Ag indicate that most newly synthesized T Ag and p53 are not complexed to each other. The T Ag-specific monoclonal antibody PAb101 immunoprecipitat-

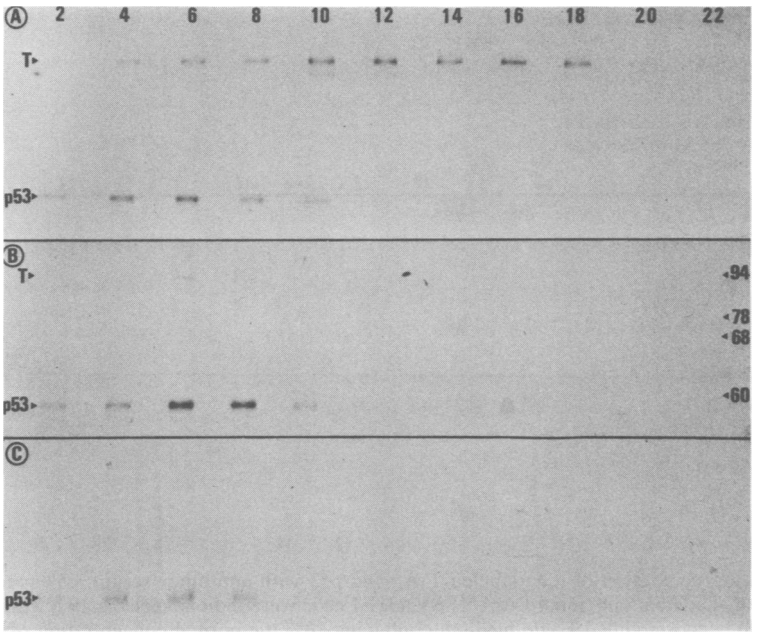

FIG. 1. Immunoprecipitation of pulse-labeled T Ag and p53 with antitumor serum, p53-specific monoclonal PAb122, and T Ag-specific monoclonal PAb101. SVA31E7 cells were starved for 1 h in methionine-free medium and then pulse labeled for 30 min with [^{35}S]methionine (68 μCi/ml). Clarified cellular extracts were centrifuged through parallel 5 to 20% sucrose gradients. Fractions were collected from the bottoms of the tubes. Even fractions were immunoprecipitated with immune serum or monoclonal antibody, and odd fractions were immunoprecipitated with the corresponding normal control. Immunoprecipitation was done with hamster antitumor serum (A), PAb122 ascites fluid (B), or PAb101 medium (C). Bottoms of the gradients are to the left.

ed a similar peak of p53 toward the bottom of the gradient, with very faint bands of cosedimenting T Ag (Fig. 1C; see Fig. 3A). Because the PAb101 is specific for T Ag, this result suggests that the pulse-labeled p53 precipitated because it was complexed to T Ag synthesized before the pulse. This result also shows that the T Ag-specific monoclonal PAb101 did not recognize that fraction of T Ag sedimenting toward the top of the gradient and not complexed with p53. This pulse-labeled, slowly sedimenting species has previously been shown to be precipitable by monospecific antiserum against T Ag purified by immunoprecipitation and SDS-PAGE (12). This form is also recognized by the T Ag-specific monoclonal antibody PAb100, formerly called clone 7 (data not shown).

This complex pattern of sedimentation and immunoreactivity was greatly simplified when the SVA31E7 cells were radiolabeled for 19 h with [^{35}S]methionine and then chased for 3 h in the absence of radiolabel. After this long label and chase, T Ag and p53 cosedimented, and the distributions and intensities of the precipitated bands after immunoprecipitation by antitumor serum, p53-specific monoclonal PAb122, or T Ag-specific monoclonal PAb101 were indistinguishable (Fig. 2). This result shows that (i) the T Ag and p53 were in complexes having the same sedimentation rates and that (ii) monoclonal antibody against either polypeptide precipitated the other from the same fractions, indicating they were complexed.

It is then evident that the T Ag-specific monoclonal PAb101 recognizes T Ag radiolabeled for long periods and sedimenting in high-molecular-weight complexes with p53. It does not, however, recognize T Ag radiolabeled for short periods and sedimenting as a monomer or small T Ag oligomer. As it has previously been shown that pulse-labeled T Ag increases in sedimentation coefficient with a chase (9, 10, 12), we postulated that the T Ag-specific monoclonal PAb101 should precipitate progressively more T Ag with a chase. Figure 3 illustrates an experiment in which SVA31E7 cells were radiolabeled with [^{35}S]methionine for 30 min and then chased for 0, 1, 2.5, or 7.5 h, the extracts were sedimented on parallel sucrose gradients, and the fractions were immunoprecipitated with the T Ag-specific monoclonal PAb101. The position of the peak of p53 changed very little throughout the chase period. However, the intensity of the bands of T Ag underwent a pronounced increase. T Ag was barely detectable after the 30-min pulse (Fig. 3A), but increased in intensity with time, until

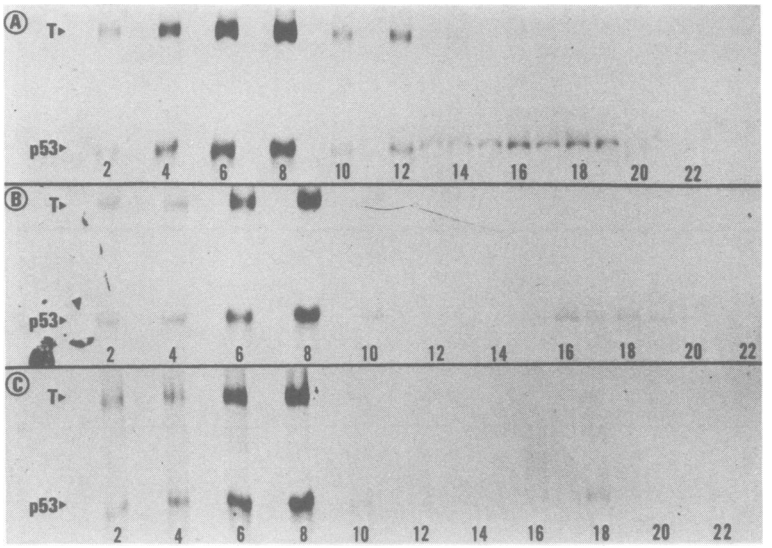

FIG. 2. Immunoprecipitation of long-labeled T Ag and p53 with antitumor serum, p53-specific monoclonal PAb122, and T Ag-specific monoclonal PAb101. SVA31E7 cells were radiolabeled for 19 h with [^{35}S]methionine (20 μCi/ml) in Dulbecco modified Eagle medium containing 10% the normal concentration of methionine, the medium was removed, and the cells were incubated for another 3 h in complete Dulbecco modified Eagle medium. Clarified cellular extracts were centrifuged through parallel 5 to 20% sucrose gradients. Fractions were collected from the bottom of the tubes. Even fractions were immunoprecipitated with immune serum or monoclonal antibody, and odd fractions were immunoprecipitated with the corresponding normal serum. Immunoprecipitation was done with hamster antitumor serum (A), PAb122 ascites fluid (B), or PAb101 medium (C).

the bands of T Ag were of greater intensity than the p53 bands (Fig. 3D). This indicates that T Ag was converted from a form which was not bound by the T Ag-specific monoclonal PAb101 to one which was specifically bound, because the pulse-labeled cells were chased in unlabeled medium. This conclusion is substantiated by experiments involving the different methodology of sequential immunoprecipitation (discussed below).

Sequential immunoprecipitation of free T Ag and p53-complexed T Ag. Free and complexed p53 and T Ag have been studied by a second method, involving serial immunoprecipitation with one monoclonal antibody until no more antigen is precipitable by that antibody, and then immunoprecipitation of the supernatant with either antitumor serum or another monoclonal antibody. When SVA31E7 cells were radiolabeled with [^{35}S]methionine for 15 min, all of the detectable p53 precipitated with the p53-specific monoclonal PAb122, whereas none of the T Ag was precipitated by PAb122 after the 15-min pulse (Fig. 4, lane a). Very little of the T Ag precipitated from the supernatant with antitumor serum (Fig. 4, lane d) was precipitated with the T Ag-specific monoclonal PAb101 (Fig. 4, lane e). In contrast, when used first, PAb101 precipitated all of the p53 (Fig. 4, lane f), indicating that it was all bound to T Ag, and none was subsequently precipitated from the supernatants with the p53-specific monoclonal PAb122 (Fig. 4, lane j). With the 15-min pulse radiolabel, a substantial portion of the T Ag was not precipitated by the T Ag-specific monoclonal PAb101, but was precipitated by antitumor serum (Fig. 4, lane i), in contrast to the almost complete precipitation by PAb101 after a 2-h label and complete precipitation after a 20-h label and chase (data not shown). These results, like those obtained by using sucrose gradient centrifugation (Fig. 1 and 2), indicate that most newly synthesized T Ag is not bound to p53 and does not precipitate with the p53-specific monoclonal PAb122, but does precipitate with the p53-specific monoclonal after a long label. In marked contrast to newly synthesized T Ag, these results indicate that all p53, even after a short radiolabel, is bound to T Ag.

Sequential immunoprecipitations were also carried out with extracts of SVA31E7 cells radiolabeled for 30 min and then chased for 0 or 1.5 h (Fig. 5). The p53-specific monoclonal PAb122 precipitated about one-third of the T Ag labeled during the pulse (no chase) and about 75% of the labeled T Ag after the chase, indicating that newly synthesized T Ag gradually formed complexes with p53. Even after a 4.5-h

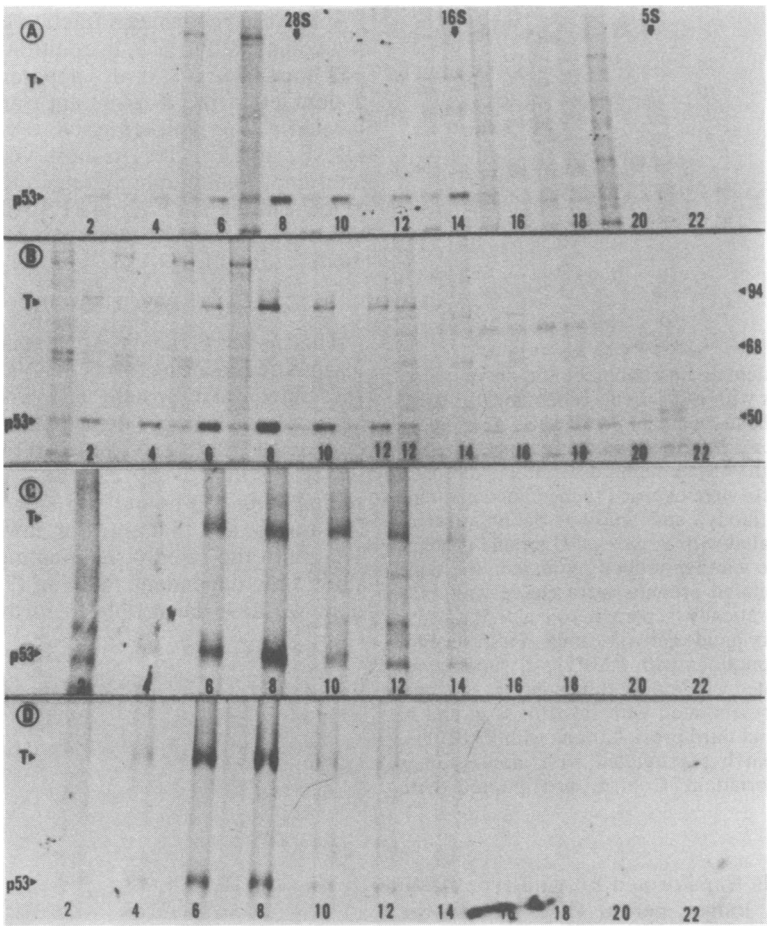

FIG. 3. Detection of pulse-labeled T Ag by T Ag-specific monoclonal PAb101 as a function of the duration of chase. SVA31E7 cells were starved for 1 h in methionine-free medium and then radiolabeled with [^{35}S]methionine (104 μmCi/ml) in methionine-free medium for 30 min, followed by chases for the indicated periods in complete Dulbecco modified Eagle medium. Clarified extracts were centrifuged through parallel 5 to 20% sucrose gradients. Fractions were collected from the bottoms of the tubes and immunoprecipitated with either PAb101 (even numbers) or control (odd numbers). Fraction 12 was duplicated in gradient B. The chase periods were: no chase (A), 1 h (B), 2.5 h (C), or 7.5 h (D).

chase (data not shown), about 5% of the labeled T Ag was not in complexes with p53. The T Ag-specific monoclonal PAb101, in contrast, precipitated about 75% of the T Ag labeled during the pulse (no chase) and all of the labeled T Ag after the 1.5-h chase, indicating more rapid formation of the T Ag determinant recognized by PAb101.

Precipitation of *tsA* mutant T Ag by PAb101. The time course experiments illustrated in Fig. 1, 2, and 3 showed that the rapidly sedimenting p53-complexed T Ag immunoprecipitated by the p53-specific monoclonal antibody PAb122 also comprises part of that fraction of T Ag recognized by the T Ag-specific monoclonal PAb101. The pulse experiments showed that there is a transient form of T Ag not recognized by PAb101, a form which ultimately chases into T Ag recognized by PAb101. The serial titration experiments further suggested that there is a third form of T Ag recognized by PAb101 but not bound to p53 and therefore not precipitated by the p53-specific monoclonal PAb122 (13; Fig. 4, lane e, and Fig. 5, lane e). The presence of this third form of T Ag precipitable by PAb101 but not PAb122 is substantiated by experiments with the A21 cell line (15), a BALB/c line transformed by the *tsA58* mutant of SV40 (32). These cells were radiolabeled at either the permissive (33°C) or nonpermissive temperature (39.5°C). It is evident that the proteins labeled for 6 h at the permissive temperature of 33°C showed a pattern of immunoprecipitation with antitumor serum, PAb122, and PAb101 monoclonal antibodies (Fig. 6A, B, and C) very similar to that

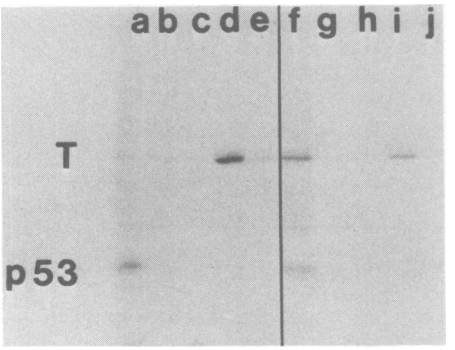

FIG. 4. Sequential immunoprecipitation of pulse-labeled extracts with p53-specific monoclonal PAb122 and T Ag-specific monoclonal PAb101. SVA31E7 cells were starved for 1 h in methionine-free medium, and then labeled with [^{35}S]methionine at 500 μCi/ml for 15 min. Cell extracts were exposed to medium containing monoclonal antibody, and antibody-bound proteins were reprecipitated with *S. aureus*. Unbound proteins were exposed to another antibody sample in the same manner. Precipitated proteins were eluted with SDS and electrophoretically separated on a 7.5 to 15% gradient polyacrylamide gel. a, b, and c, First, second, and third precipitations with PAb122; d, supernatant from c precipitated with mouse tumor serum; e, supernatant from c precipitated with PAb101. f, g, and h, First, second, and third precipitations with PAb101; i, supernatant from h precipitated with mouse tumor serum; j, supernatant from h precipitated with PAb122.

shown by cells transformed by wild-type SV40 labeled for a longer period (Fig. 2). At the nonpermissive temperature, in contrast, the sedimentation properties of T Ag were changed markedly. As described previously (12), the mutant T Ag sedimented as a broad distribution between 5 and 28S, a distribution of sedimenting forms not corresponding to that of the >28S peak of p53 (Fig. 6D). This distribution of the two proteins after labeling for 5 h at 39.5°C is nearly indistinguishable from that of 30-min pulse-labeled wild-type T Ag and p53 (Fig. 1A). The p53-specific monoclonal PAb122 imunoprecipitated p53 as a form sedimenting as the usual >28S, but precipitated very little radiolabeled T Ag (Fig. 6E), again a pattern very similar to that given by cells transformed by wild-type SV40 and pulse labeled (Fig. 1B). However, when T Ag-specific PAb101 was used to immunoprecipitate a parallel gradient, a strikingly different pattern was obtained. PAb101 precipitated T Ag in a broad distribution across the gradient (Fig. 6F), a pattern similar to that given by antitumor serum with mutant T Ag labeled at 39.5°C (Fig. 6D) and not at all like the patterns obtained with the T Ag-specific monoclonal PAb101 precipitation of pulse-labeled protein (Fig. 1C and 3A). This indicates that the T Ag-specific monoclonal PAb101 can recognize a fraction of mutant T Ag not complexed to p53, in addition to recognizing p53-bound T Ag. It is also apparent that p53 was a doublet in the *tsA* mutant-transformed cells labeled at the nonpermissive temperature (Fig. 6D, E, and F). This is best visualized in the antitumor immunoprecipitate, although it is also evident in the PAb122 and PAb101 immunoprecipitates. This may represent a posttranslational modification of p53.

DISCUSSION

This paper presents the first analysis of the various sedimenting forms of SV40 T Ag and the associated host protein p53 with monoclonal antibodies. These results substantiate our previous results (12) and those of others (9, 23), indicating that T Ag can be found in rapidly sedimenting p53-bound and slowly sedimenting free forms, and that entry of newly synthesized T Ag into the rapidly sedimenting complex is a slow, time-dependent reaction (9, 12). In addition, we have been able to further dissect the

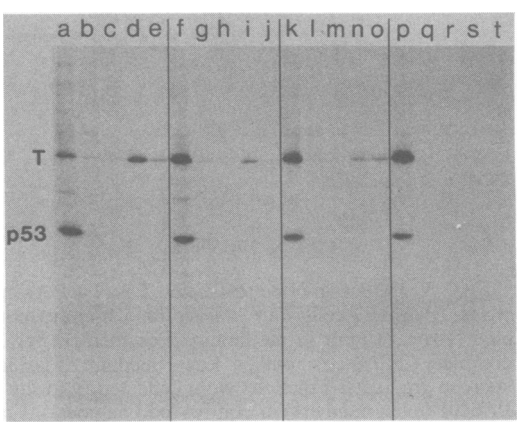

FIG. 5. Sequential immunoprecipitation of ^{35}S-labeled extracts with p53-specific monoclonal PAb122 and T Ag-specific monoclonal PAb101: pulse label ± chase. SVA31E7 cells were starved for 1 h in methionine-free medium and then pulse labeled for 30 min with [^{35}S]methionine (550 μCi/ml). Extracts were made immediately (a to j) or after 90 min of chase in complete medium (k to t). Cell extracts were exposed to medium containing monoclonal antibody, and antibody-bound proteins were precipitated with *S. aureus*. Unbound proteins were exposed to another antibody sample in the same manner. Precipitated proteins were eluted with SDS and electrophoretically separated on a 7.5 to 15% gradient polyacrylamide gel. a, b, and c, and k, l, and m, First, second, and third precipitations with PAb122; d, and n, supernatants from c and m precipitated with mouse tumor serum; e and o, supernatants from c and m precipitated with PAb101. f, g, and h and p, q, and r, First, second, and third precipitations with PAb101; i and s, supernatants from h and r precipitated with mouse tumor serum; j and t, supernatants from h and r precipitated with PAb122.

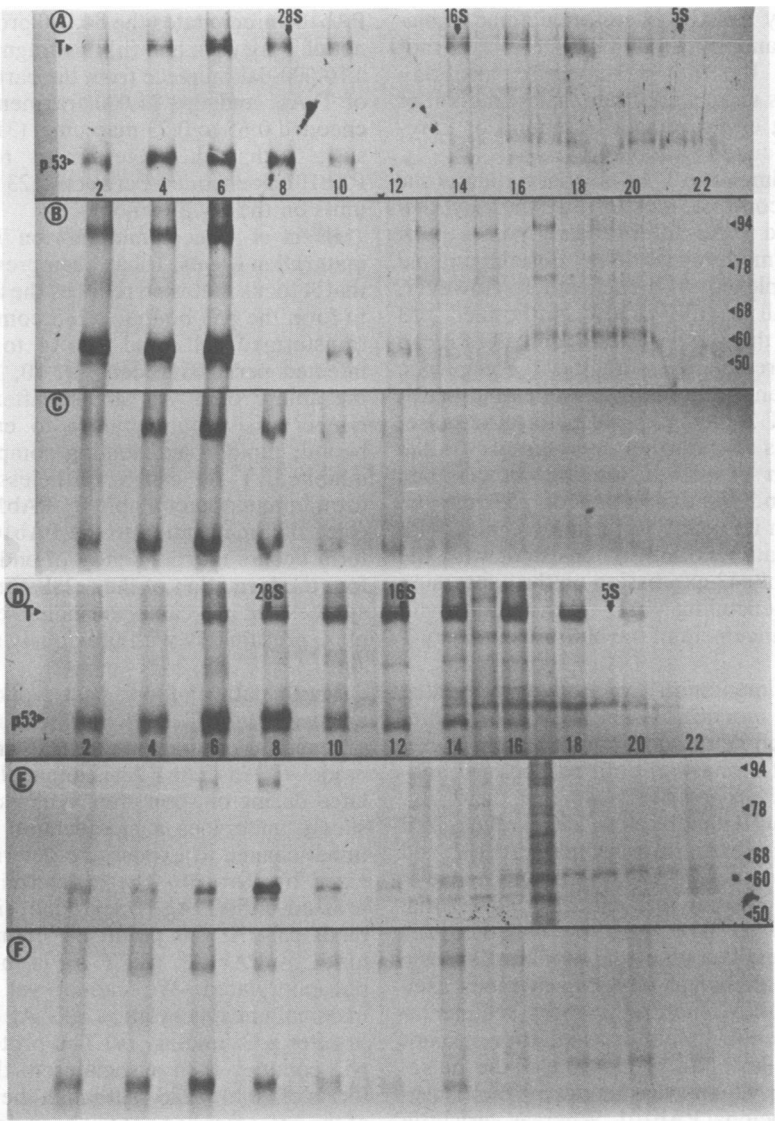

FIG. 6. Effect of *tsA* mutation on detection of T Ag by the T Ag-specific monoclonal antibody PAb101 and the p53-specific monoclonal antibody PAb122. SV40 *tsA58*-transformed mouse embryo fibroblasts (i) maintained at 33°C or (ii) shifted to 39.5°C for 14 h were radiolabeled with [^{35}S]methionine (60 μCi/ml) for 6 h at 33°C or for 5 h at 39.5°C. After centrifugation of extracts through parallel 5 to 20% sucrose gradients, even fractions were precipitated with immune sera and odd fractions with control serum. Radiolabeled at 33°C: immunoprecipitation with antitumor serum (A), precipitation with PAb122 (B), and precipitation with PAb101 (C). Radiolabeled at 39.5°C: precipitation with antitumor serum (D), precipitation with PAb122 (E), and precipitation with PAb101 (F).

maturation process through our ability to separate the different forms of T Ag and p53 both physically and in time of appearance. We have been aided in this delineation of the steps in complex maturation by the T Ag-specific monoclonal antibody PAb101, which binds only a subset of T Ag (13), a subset defined here as "mature" T Ag.

Rapidly sedimenting complex. After a long label and chase, both p53 and T Ag cosediment as a >28S complex which is immunoprecipitable by antitumor serum, the T Ag-specific monoclonal PAb101, or the p53-specific monoclonal PAb122 indistinguishably. Serial titration experiments showed that all p53 labeled with [^{35}S]methionine is in the complex. Nearly all T Ag labeled in a pulse is in the complex after a long chase. However, a small fraction of T Ag

recognized by PAb101 (therefore defined as mature T Ag) and by antitumor serum was not precipitated by the p53-specific antibody PAb122. This suggests further that a fraction of mature T Ag either remains unbound or is reversibly associated with p53.

Slowly sedimenting T Ag and formation of the complex. In contrast, most of the newly synthesized p53 and T Ag, after labeling with a short pulse of [^{35}S]methionine, do not cosediment and are not complexed with each other. However, essentially all of the newly synthesized p53 sediments at the >28S position, and all of it can be immunoprecipitated with the T Ag-specific monoclonal antibody PAb101, indicating that it is bound to T Ag synthesized before the pulse. This suggests two further hypotheses: (i) the concentration of p53 is limiting on complex formation, and (ii) expression of p53 may be dependent on its ability to form a complex with T Ag. This latter notion is consistent with the dependence of p53 expression on the expression of the T Ag-encoding viral *A* gene (3, 22) and with the enhancement of the half-life of p53 by T Ag (27).

In the sedimentation experiments presented here, neither monoclonal antibody immunoprecipitated much of the newly synthesized T Ag. However, after chasing, both monoclonal antibodies bound progressively more of the T Ag, indicating that (i) it matures to a form recognized by the T Ag-specific monoclonal PAb101 and that (ii) it becomes bound to p53 and hence is precipitated by the p53-specific monoclonal PAb122. We do not yet know whether this maturation entails a chemical modification or a conformational change. The two methods used for these studies, sucrose gradient sedimentation and sequential immunoprecipitation, did not agree on the absolute proportion of the pulse-labeled T Ag which was bound by the T Ag-specific monoclonal PAb101. Whereas very little of the pulse-labeled T Ag from the gradients was bound by PAb101, about 75% of it was immunoprecipitated directly from extracts. This may reflect the 10-fold higher dilution of the antigens from the gradients. However, both methods agree in indicating that a large fraction of T Ag is not recognized after a pulse, but all of it is recognized after a subsequent chase. We then conclude that the determinants recognized by the T Ag-specific monoclonal PAb101 are maturation specific.

It is interesting to note that the determinant recognized by the T Ag-specific monoclonal PAb101 has been shown to occur in the carboxy-terminal fragment of T Ag (0.28 to 0.17 map units) encoded by the adenovirus-SV40 hybrid AD2ND1 (6). The determinant can be further localized by employing the observations that (i) PAb101 precipitates the 84,000 proteolytic product of T Ag (13); and (ii) this fragment is missing a 10,000-dalton piece from the carboxy terminus of T Ag, and the 84,000 fragment is therefore encoded 0.65 to 0.23 map units (31). This would suggest that the determinant recognized by PAb101 is encoded between 0.23 and 0.28 map units on the SV40 genome.

Effect of *A* locus mutation on T Ag and p53 maturation events. It has been previously shown that *A* locus mutation reduces the ability of T Ag to form the p53-bound, >28S complexes (12) in transformed cells and the 14 to 16S form in infected permissive cells (9, 10, 18). Although the ability of T Ag labeled after shift to the nonpermissive temperature to enter the p53-bound, rapidly sedimenting complex is largely inhibited, T Ag can nevertheless mature to a form immunoprecipitable by PAb101. This indicates that maturation to the PAb101-recognized form occurs independently of and probably before the formation of the >28S complex. Newly synthesized p53 can nevertheless enter the complex, presumably with previously synthesized T Ag.

These data and our past work (12, 13) are consistent with T Ag and p53 undergoing a series of reactions outlined in the following speculative model. (i) Nascent T Ag is minimally phosphorylated during or soon after synthesis. (ii) It then slowly undergoes a modification or conformational change to expose the determinant recognized by PAb101. These determinants are expressed until T Ag is degraded. (iii) T Ag then binds p53. At this point, the complex is recognized by PAb122. (iv) T Ag is then maximally phosphorylated. We cannot yet say whether maximal phosphorylation of T Ag occurs before or after p53 binding. (v) The protomeric T Ag-p53 complex then associates to the oligomeric forms of 20 to >28S. Although the stochiometry of the protomer has not yet been determined, the ratio of p53 to T Ag is uniform in the various sedimenting forms of the complex (12). We surmise that T Ag alone may oligomerize, either before or after binding to p53. Steps (iii) to (v) are inhibited by the *tsA* mutation.

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