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# Complex Formation of Simian Virus 40 Large T Antigen with Cellular Protein p53

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**We investigated the formation of native complexes between simian virus 40 large T antigen and the cellular protein p53 (T-p53) by using simian virus 40 *tsA*58-transformed mouse fibroblasts (*tsA*58 F2b). We observed that newly synthesized p53 bound to all structural subclasses of large T antigen detectable on sucrose density gradients. This led to various intermediates of T-p53 complexes which converted within 2 h into typical mature aggregates. The final levels of stable T-p53 complexes seemed to be determined by p53 rather than by large T antigen.**

The idea that the cellular p53 protein may be involved in the process of oncogenesis arose by virtue of its stable association with two different viral oncogenic proteins, simian virus 40 (SV40) large T antigen (14, 15, 17, 20) and the adenovirus 5 E1b 58-kilodalton protein (24). Recent reports demonstrated a direct role for p53 in cell transformation in that it was able to immortalize primary rodent cells and to transform these cells by complementing activated *ras* genes (5, 12, 23). Simian virus 40 large T antigen is a multifunctional regulatory protein essential for virus growth and cell transformation (for a review, see reference 29). This phosphoprotein occurs in various structural forms such as monomers and different homologous oligomers (2, 7, 9, 16) which arise by noncovalent self-assembly after posttranslational modulation of monomers (18, 19). In addition, large T antigen forms complexes with p53 which aggregate into extremely stable heterologous oligomers (2, 6, 16). Studies with temperature-sensitive simian virus 40 *A* gene mutants (*tsA*) showed that, in *tsA*-infected permissive cells at the nonpermissive temperature, large T antigen failed to convert into oligomers (7, 13, 21, 22) and to form complexes with p53 (7, 21) simultaneously. However, large T antigen from certain *tsA*58 mouse transformants is apparently only partially defective for these structural features (2, 9). In these studies it was postulated that large T antigen-p53 (T-p53) complexes may assemble after appropriate modification of large T antigen, whereas very recent data suggested that large T antigen binds very rapidly, probably without processing, to p53 (27).

In the present study we dealt with the questions (i) whether only a distinct structural subclass of large T antigen is able to bind p53 and (ii) whether intermediates of T-p53 complexes are detectable.

As already shown for large T antigen from *tsA*58-infected monkey cells (7, 13, 21, 22), large T antigen from *tsA*58-transformed BALB/c 3T3 mouse cells, the *tsA*58 F2b (3) used in the present study, was also heat sensitive for oligomerization and complexing with p53. Furthermore, we could demonstrate that oligomeric forms of large T antigen in *tsA*58 F2b cells formed at the permissive temperature remained stable after shift up to the nonpermissive temperature as already observed in *tsA*58-infected TC-7 cells (21).

These typical heat-sensitive properties of large T antigen in *tsA*58 F2b cells enabled us to study T-p53 complex formation under extreme conditions, i.e., binding of newly synthesized p53 at 39°C to old large T antigen preformed at 32°C. *tsA*58 F2b cells were cultured at 32°C, shifted up to 39°C for 2 h, and then radiolabeled with 40 μCi of [$^{35}$S]methionine for 2 h (Fig. 1). Cells were lysed at pH 9 with 0.5% Nonidet P-40 as described previously (21). Cell extracts were analyzed for T-p53 complexes and for free large T antigen by sequential immunoprecipitation with non-cross-reacting monoclonal antibodies specific for either p53 (PAb 122 [10]) or large T antigen (PAb 430 [11]). Immunoprecipitation of the labeled extract with monoclonal p53 antibody PAb 122 showed only a p53 band (Fig. 1A, a, lane 122). Newly synthesized large T antigen, but no p53 protein, was detected by subsequent immunoprecipitation with PAb 430 from the supernatant (Fig. 1A, a, lane 430). Since large T antigen synthesized and modified at 32°C remains stable at 39°C and if newly synthesized p53 binds to old large T antigen processed at 32°C, the immunoprecipitates shown in Fig. 1A, a, lanes 122 and 430 should also contain unlabeled large T antigen. Furthermore, in this case one would expect T-p53 complexes containing labeled p53 when PAb 430 was used first. Anti-large T antibody PAb 430 immunoprecipitated large T antigen and p53 (Fig. 1A, b, lane 430). PAb 122 detected no more labeled p53 in the supernatant (Fig. 1A, b, lane 122), suggesting that p53 newly synthesized at 39°C in fact binds completely to old, i.e., unlabeled, but not newly synthesized large T antigen.

To find out whether complexing of newly produced p53 has to undergo time-dependent processing, we applied the same protocol as described for Fig. 1A but pulse-labeled cells for only 10 min after shift up to 39°C (Fig. 1B). This experiment indicated that p53 needs either no or at least very rapid processing for binding to large T antigen. Even under pulse-labeling conditions, no uncomplexed p53 protein was detectable (Fig. 1B, b, lane 122).

To prove directly that all p53 newly synthesized at 39°C in fact binds to old but not to labeled large T antigen, we repeated the experiment (shown in Fig. 1A and B), but instead of radiolabeling proteins we stained the gel with Coomassie blue. As expected, PAb 122 coprecipitated a reasonable amount of large T antigen (Fig. 1C, a, lane 122), and in the supernatant PAb 430 recognized almost equal amounts of supposedly free large T antigen (Fig. 1C, a, lane 430). Conversely, using PAb 430 first precipitated significantly more large T antigen, including both free and, most likely, p53-bound large T antigen (Fig. 1C, b, lane 430). PAb

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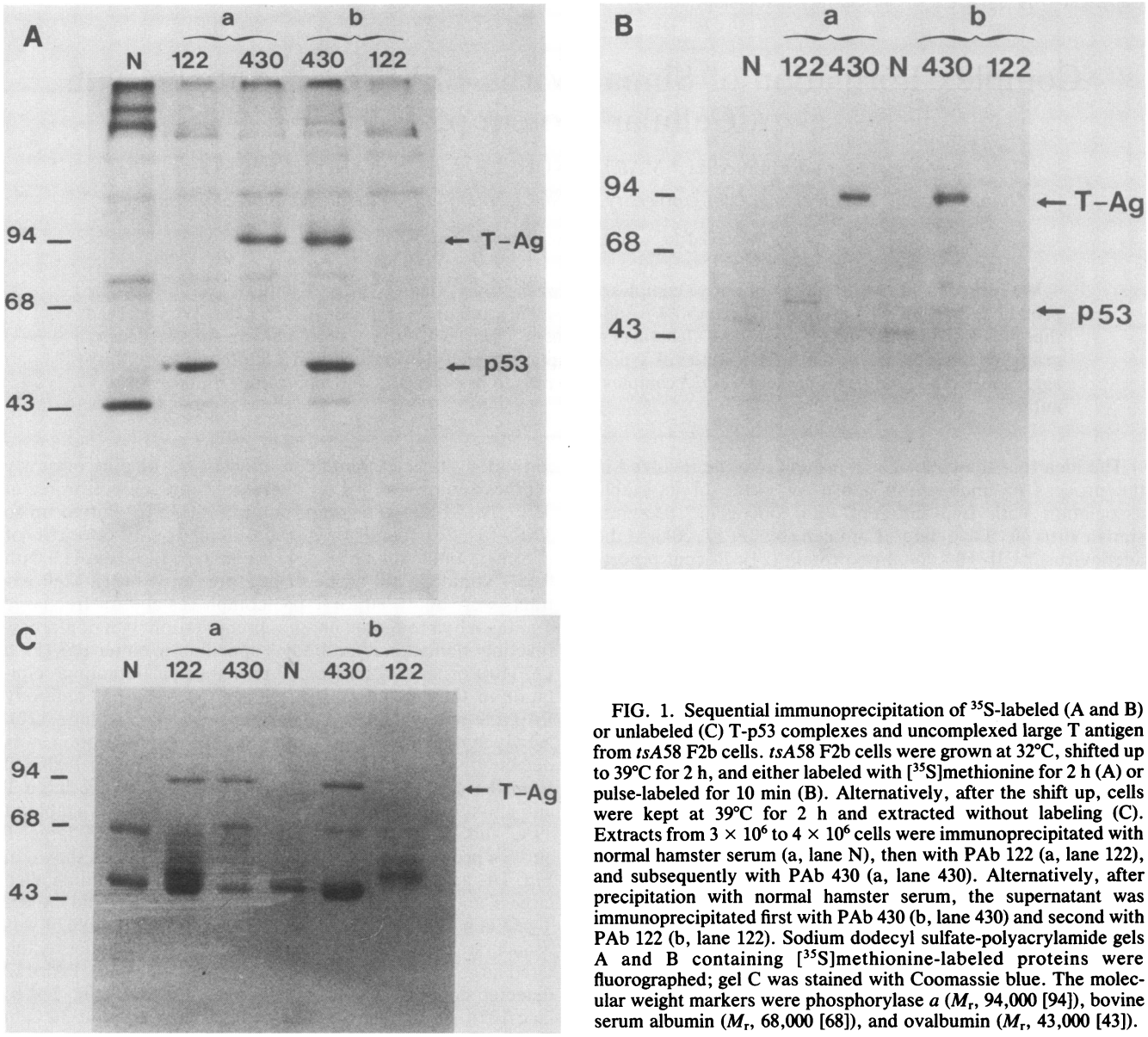


FIG. 1. Sequential immunoprecipitation of $^{35}$S-labeled (A and B) or unlabeled (C) T-p53 complexes and uncomplexed large T antigen from *tsA*58 F2b cells. *tsA*58 F2b cells were grown at 32°C, shifted up to 39°C for 2 h, and either labeled with [$^{35}$S]methionine for 2 h (A) or pulse-labeled for 10 min (B). Alternatively, after the shift up, cells were kept at 39°C for 2 h and extracted without labeling (C). Extracts from $3 \times 10^6$ to $4 \times 10^6$ cells were immunoprecipitated with normal hamster serum (a, lane N), then with PAb 122 (a, lane 122), and subsequently with PAb 430 (a, lane 430). Alternatively, after precipitation with normal hamster serum, the supernatant was immunoprecipitated first with PAb 430 (b, lane 430) and second with PAb 122 (b, lane 122). Sodium dodecyl sulfate-polyacrylamide gels A and B containing [$^{35}$S]methionine-labeled proteins were fluorographed; gel C was stained with Coomassie blue. The molecular weight markers were phosphorylase *a* ($M_r$, 94,000 [94]), bovine serum albumin ($M_r$, 68,000 [68]), and ovalbumin ($M_r$, 43,000 [43]).

122 precipitated no complexed large T antigen from the supernatant (Fig. 1C, b, lane 122). Unlabeled p53 was not detectable on these gels mainly because of the presence of unlabeled heavy chains of immunoglobulins running, like mouse p53 protein, close to an apparent molecular weight of about 50,000. Taken together, these data confirmed the hypothesis that newly made p53 binds at 39°C to old large T antigen processed at 32°C. Hence, large T antigen seems to be temperature sensitive for appropriate modification but not for the mechanism of binding p53.

Next we analyzed the rapid binding of p53 to mature large T antigen in more detail under temperature shift up or shift down conditions by sucrose density gradient centrifugation. *tsA*58 F2b cells cultured at 32°C were first shifted up to 39°C for 2 h and then either pulse-labeled with 100 μCi of [$^{35}$S]methionine for 10 min or, additionally, chased for 2 h with medium containing unlabeled methionine and then subjected to density gradient centrifugation as previously described (21). The gradient fractions were analyzed for p53 by using PAb 122. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels which were fluorographed as described by Bonner and Laskey (1). Distinct amounts of pulse-labeled p53 sedimented around 14S, and slightly more was found at the bottom of the gradient (Fig. 2A). Disappearance of the 14S forms during a 2-h chase suggested that these complexes are intermediate forms which convert slowly into mature forms (23S and beyond) (Fig. 2B). Expectedly, no labeled large T antigen comigrated with the intermediate forms around 14S or with the mature forms sedimenting at 23S and beyond. From the data presented in Fig. 1A, B, and C, one would expect that these forms consist of labeled p53 and unlabeled large T antigen synthesized at 32°C. Indeed, Coomassie blue staining of the gel clearly demonstrated large T antigen and p53 in complexes (data not shown). In another control experiment, gradient fractions were immunoprecipitated with PAb 430, which produced the same sedimentation profile for p53 as that obtained with PAb 122 (data not shown).

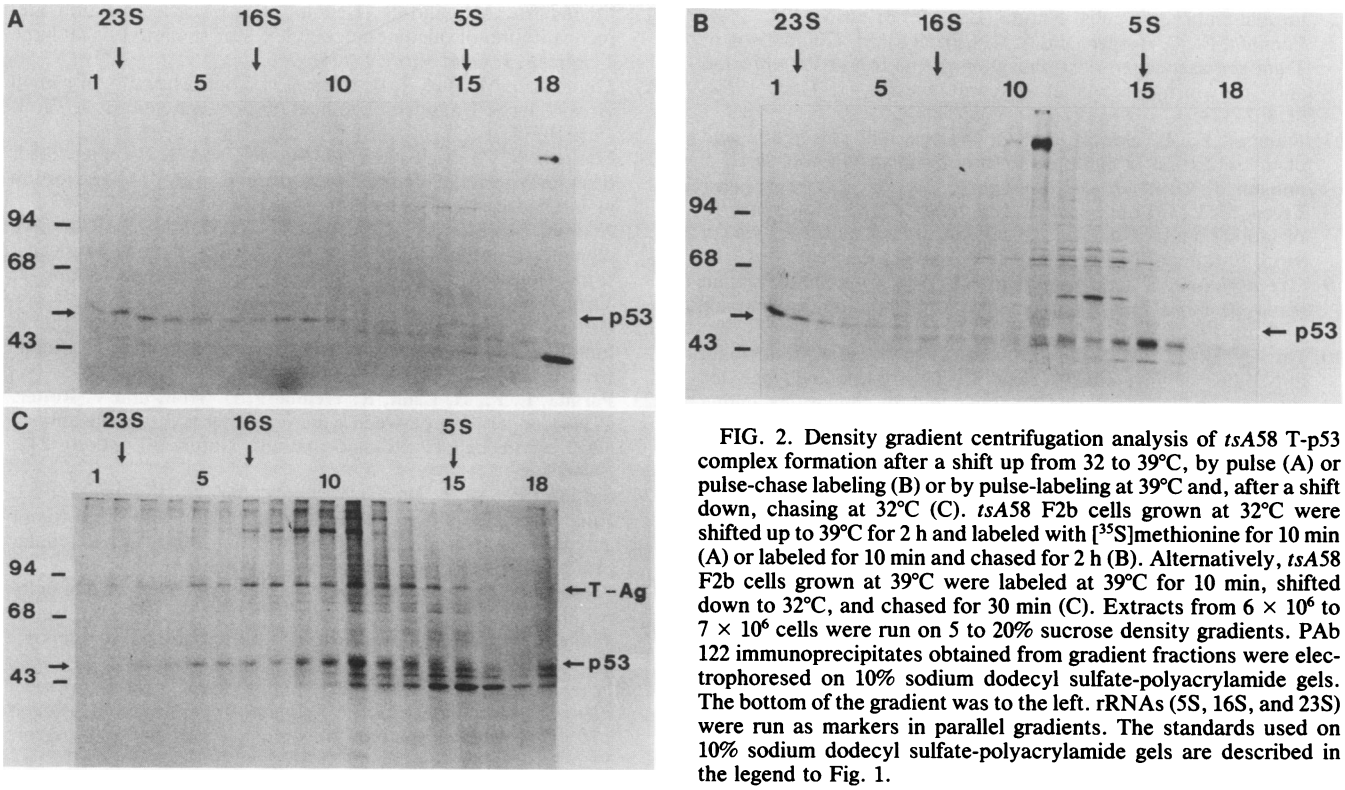


FIG. 2. Density gradient centrifugation analysis of *tsA*58 T-p53 complex formation after a shift up from 32 to 39°C, by pulse (A) or pulse-chase labeling (B) or by pulse-labeling at 39°C and, after a shift down, chasing at 32°C (C). *tsA*58 F2b cells grown at 32°C were shifted up to 39°C for 2 h and labeled with [$^{35}$S]methionine for 10 min (A) or labeled for 10 min and chased for 2 h (B). Alternatively, *tsA*58 F2b cells grown at 39°C were labeled at 39°C for 10 min, shifted down to 32°C, and chased for 30 min (C). Extracts from $6 \times 10^6$ to $7 \times 10^6$ cells were run on 5 to 20% sucrose density gradients. PAb 122 immunoprecipitates obtained from gradient fractions were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels. The bottom of the gradient was to the left. rRNAs (5S, 16S, and 23S) were run as markers in parallel gradients. The standards used on 10% sodium dodecyl sulfate-polyacrylamide gels are described in the legend to Fig. 1.

To detect higher amounts of intermediates in low-molecular-weight forms, we set up another type of experiment. To avoid the presence of old, high-molecular-weight forms of large T antigen, we pulse-labeled cells grown at 39°C for 10 min with [$^{35}$S]methionine and then shifted them down to a low temperature (32°C) and chased them for 30 min. The p53 running with large T antigen sedimented in a wide profile ranging from 7S up to ≥23S. One can assume that all detectable forms of large T antigen, including monomers, are able to complex with p53. Furthermore, we learned that large T antigen synthesized at 39°C was rapidly, i.e., within 30 min at 32°C processed to regain the ability to form complexes with p53.

Recovery of T-p53 complex formation after a shift down to the permissive temperature excludes the possibility that large T antigen synthesized at the nonpermissive temperature is rapidly degraded and therefore inactive in binding to p53. Large T antigen, which was coprecipitated with p53 under these conditions, sedimenting around 7S, may represent the smallest T-p53 complex with a 1:1 stoichiometry (8).

By using the specific monoclonal antibody PAb 101, which recognizes only mature large T antigen, it was shown by Carroll and Gurney (2) that p53 binds to mature large T antigen. According to Schmieg and Simmons (27), this specific maturation occurs within 5 to 15 min, whereas the oligomerization of large T antigen in various permissive and nonpermissive cell lines proceeds more slowly within 1 and 2 h (7, 19). According to our present results, pulse-labeled p53 binds preferentially to high-molecular-weight forms of large T antigen. However, under appropriate experimental conditions p53 binds to nearly all structural forms of large T antigen detectable on sucrose density gradients. Nearly all intermediates finally converted into high-molecular-weight forms, sedimenting at 23S and beyond (Fig. 2).

Both large T antigen and p53 are modified by phosphorylation at multiple positions (4, 26). Analysis of the phosphorylation of large T antigen in a complex with p53 in our laboratory has revealed, in contrast to previous reports (6, 9, 16), that the p53-complexed subset of large T antigen is considerably less phosphorylated than unbound large T antigen (28). These observations are in agreement with results reported for the E1b 58-kilodalton protein from adenovirus 5-transformed cells, which is also less phosphorylated in the p53-complexed than the free form (25). However, it has yet to be determined whether the low phosphorylation of large T antigen is a specific prerequisite for complex formation with p53 or whether complexed large T antigen is either protected against further phosphorylation or even more susceptible to dephosphorylation.

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## LITERATURE CITED

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
2. **Carroll, R. B., and E. G. Gurney.** 1982. Time-dependent maturation of the simian virus 40 large T antigen-p53 complex studied by using monoclonal antibodies. J. Virol. **44**:565–573.
3. **Christensen, J. B., and W. W. Brockman.** 1982. Effects of large and small T antigens on DNA synthesis and cell division in simian virus 40-transformed BALB/c 3T3 cells. J. Virol. **44**:574–585.
4. **Crawford, L. V.** 1983. The 53,000-dalton cellular protein and its role in transformation. Int. Rev. Exp. Pathol. **25**:1–49.
5. **Eliyahu, D., A. Raz, P. Gruss, D. Givol, and M. Oren.** 1984. Participation of p53 cellular tumour antigen in transformation of

normal embryonic cells. Nature (London) **312**:646–649.
6. **Fanning, E., C. Burger, and E. G. Gurney.** 1981. Comparison of T antigen-associated host phosphoproteins from SV40-infected and -transformed cells of different species. J. Gen. Virol. **55**:367–378.
7. **Fanning, E., B. Nowak, and C. Burger.** 1981. Detection and characterization of multiple forms of simian virus 40 large T antigen. J. Virol. **37**:92–102.
8. **Freed, M. I., I. Lubin, and D. T. Simmons.** 1983. Stoichiometry of large T antigen and p53 in complexes isolated from simian virus 40-transformed rat cells. J. Virol. **46**:1061–1065.
9. **Greenspan, D. S., and R. B. Carroll.** 1981. Complex of simian virus 40 large tumor antigen and 48,000-dalton host tumor antigen. Proc. Natl. Acad. Sci. USA **78**:105–109.
10. **Gurney, E. G., R. O. Harrison, and J. Fenno.** 1980. Monoclonal antibodies against simian virus 40 T antigens: evidence for distinct subclasses of large T antigen and for similarities among nonviral T antigens. J. Virol. **34**:752–763.
11. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39**:861–869.
12. **Jenkins, J. R., K. Rudge, and G. A. Currie.** 1984. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature (London) **312**:651–654.
13. **Kuchino, T., and N. Yamaguchi.** 1975. Characterization of T antigen in cells infected with a temperature-sensitive mutant of simian virus 40. J. Virol. **15**:1302–1307.
14. **Lane, D. P., and L. V. Crawford.** 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature (London) **278**:261–263.
15. **Linzer, D. I. H., and A. J. Levine.** 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell **17**:43–52.
16. **McCormick, F., and E. Harlow.** 1980. Association of a murine 53,000-dalton phosphoprotein with simian virus 40 large-T antigen in transformed cells. J. Virol. **34**:213–224.
17. **Melero, J. A., D. T. Stitt, W. F. Mangel, and R. B. Carroll.** 1979. Identification of new polypeptide species (48–55 K) immunoprecipitable by antiserum to purified large T antigen and present in SV40-infected and -transformed cells. Virology **93**:466–480.
18. **Montenarh, M., and R. Henning.** 1983. Disaggregation and reconstitution of oligomeric complexes of simian virus 40 large T antigen. J. Gen. Virol. **64**:241–246.
19. **Montenarh, M., and R. Henning.** 1983. Self-assembly of simian virus 40 large T antigen oligomers by divalent cations. J. Virol. **45**:531–538.
20. **Montenarh, M., M. Kohler, G. Aggeler, and R. Henning.** 1985. Structural prerequisites of simian virus 40 large T antigen for the maintenance of cell transformation. EMBO J. **4**:2941–2947.
21. **Montenarh, M., M. Kohler, and R. Henning.** 1984. The oligomerization of simian virus 40 large T antigen is not necessarily suppressed by temperature-sensitive *A* gene lesions. J. Virol. **49**:658–664.
22. **Osborn, M., and K. Weber.** 1975. SV40: T antigen, the A function and transformation. Cold Spring Harbor Symp. Quant. Biol. **39**:267–276.
23. **Parada, L. F., H. Land, R. Weinberg, D. Wolf, and V. Rotter.** 1984. Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation. Nature (London) **312**: 649–651.
24. **Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine.** 1982. Adenovirus E1b-58 Kd tumor antigen and SV40 large tumor antigen are physically associated with the same 53 Kd cellular protein in transformed cells. Cell **28**:387–394.
25. **Sarnow, P., C. A. Sullivan, and A. J. Levine.** 1982. A monoclonal antibody detecting the adenovirus type 5 E1b-58 Kd tumor antigen: characterization of the E1b-58 Kd antigen in adenovirus-infected and -transformed cells. Virology **120**:510–517.
26. **Scheidtmann, K. H., M. Hardung, B. Echle, and G. Walter.** 1984. DNA-binding activity of simian virus 40 large T antigen correlates with a distinct phosphorylation state. J. Virol. **50**:1–12.
27. **Schmieg, F. I., and D. T. Simmons.** 1984. Intracellular location and kinetics of complex formation between simian virus 40 T antigen and cellular protein p53. J. Virol. **52**:350–355.
28. **Stürzbecher, H.-W., M. Mörike, M. Montenarh, and R. Henning.** 1985. Relationship of phosphorylation to the oligomerization of SV40 T antigen and its association with p53. FEBS Lett. **180**:285–290.
29. **Tooze, J. (ed.).** 1981. The molecular biology of tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.