JOURNAL OF VIROLOGY, Nov. 1986, p. 817–821
0022-538X/86/110817-05$02.00/0

Vol. 60, No. 2

Fluctuation of Simian Virus 40 (SV40) Super T-Antigen Expression in Tumors Induced by SV40-Transformed Mouse Mammary Epithelial Cells

JANET S. BUTEL,[1]* CONNIE WONG,[1] AND BRADLEY K. EVANS[2]†

Departments of Virology and Epidemiology[1] and Neurology,[2] Baylor College of Medicine, Houston, Texas 77030

Received 28 April 1986/Accepted 16 July 1986

Higher-molecular-weight forms of the simian virus 40 (SV40) large tumor antigen (T-Ag), designated super T-Ag, are commonly found in SV40-transformed rodent cells. We examined the potential role of super T-Ag in neoplastic progression by using a series of clonal SV40-transformed mouse mammary epithelial cell lines. We confirmed an association between the presence of super T-Ag and cellular anchorage-independent growth in methylcellulose. However, tumorigenicity in nude mice did not correlate with the expression of super T-Ag. In the tumors that developed in nude mice, super T-Ag expression fluctuated almost randomly. Cell surface iodination showed that super T-Ag molecules were transported to the epithelial cell surface. The biological functions of super T-Ag remain obscure, but it is clear that it is not important for tumorigenicity by SV40-transformed mouse mammary epithelial cells. Super T-Ag may be most important as a marker of genomic rearrangements by the resident viral genes in transformed cells.

Aberrantly sized forms of the simian virus 40 (SV40) transforming protein, large tumor antigen (T-Ag), are commonly observed in SV40-transformed rodent cells. Higher-molecular-weight forms, designated collectively as super T-Ag, were first observed in transformed rat and mouse cell lines in 1979 (6, 16, 31, 32). An intriguing correlation was made between the presence of super T-Ag and the ability of the cells to exhibit anchorage-independent growth (2, 10), but the important question remains whether super T-Ag is central to the acquisition of a tumorigenic phenotype by SV40-transformed cells. One plausible hypothesis is that super T-Ag might confer a selective growth advantage on a transformed cell, thereby prompting the evolution of a malignant population. We recently derived a series of SV40-transformed mouse mammary epithelial cells (4) that provided an ideal set of closely matched, yet independent, cell lines with which to examine a potential relationship between super T-Ag and tumorigenic potential. The results of the present study provide convincing evidence that super T-Ag is not related to tumorigenicity in this system.

Properties of clonal transformed cell lines. It was important that clonal derivatives be used in this study to preclude the possibility of cell selection from a heterogeneous parental population during transplantation in vivo. Five clonal lines derived from the parental SV40-transformed mammary epithelial cells (4) by selection in methylcellulose were selected for analysis (Fig. 1). Three lines synthesized approximately equal amounts of wild-type (WT) T-Ag of 91,000 molecular weight and a super T-Ag that migrated with an apparent molecular weight of about 97,000 (lanes B, C, and F). One clonal line expressed only WT T-Ag (lane D), and another synthesized only a super T-Ag (lane E). Although the parental lines resulted from independent transforming events, the super T-Ag expressed by the clonal derivatives were of the same approximate size.

The growth characteristics of the clonal lines in vitro were determined. All grew to approximately equivalent saturation densities in the presence of either 1 or 10% fetal bovine serum in the media (data not shown), just as the parental lines had done (4). Four of the five clonal lines formed colonies when tested for anchorage-independent growth in methylcellulose (4) in the presence of 10% fetal bovine serum (Table 1). The line that expressed only WT-size T-Ag

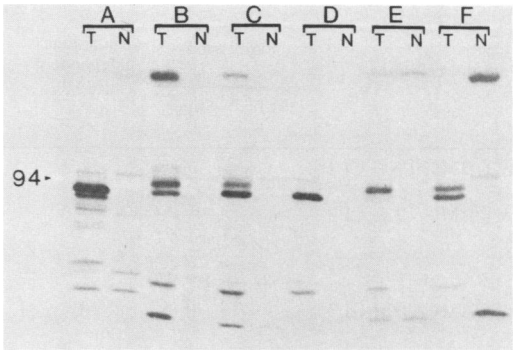

FIG. 1. Expression of SV40 large T-Ag in clonal lines of SV40-transformed mouse mammary epithelial cells. About 10^7 cells of each line were labeled with 100 μCi of [^{35}S]methionine for 3 h and disrupted with a Nonidet P-40 solution, and the clarified lysate was incubated with anti-T-Ag ascites fluid from SV40 tumor-bearing hamsters (T) or with normal hamster serum (N). Immune complexes were adsorbed with *S. aureus* Cowan 1, and the antigens were eluted and analyzed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Lanes: A, SV40-infected TC-7 cells; B, SV40 WT/BMG/19-C Cl 1 cells; C, SV40 WT/BMG/19-C Cl 3 cells; D, SV40 WT/BMG/20-B Cl 3 cells; E, SV40 WT/BMG/21-8 Cl 4 cells; F, SV40 WT/BMG/22-3 Cl 5 cells. The position of the 94,000-molecular-weight phosphorylase *a* marker is indicated on the left. Note the presence of super T-Ag in lanes B, C, E, and F.

* Corresponding author.
† Present address: The Rockefeller University, New York, NY 10021.

TABLE 1. Anchorage-independent growth in vitro and tumorigenicity in nude mice of SV40-transformed mammary epithelial clonal cell lines

Cell line	Presence of super T-Ag	Anchorage independence[a]		Tumorigenicity[b]			
		Passage tested	Colony formation in methylcellulose (%)	Passage tested	No. of tumors/no. of animals inoculated (%)	Latent period (wk)	
						Avg	Range
SV40 WT/BMG/19-C Cl 1	+	15	3.6	10	0/5 (0)		
SV40 WT/BMG/19-C Cl 3	+	17	9.5	8	2/5 (40)	22.5	21–24
SV40 WT/BMG/20-B Cl 3	0	17	<0.001	13	4/5 (80)	10.0	6–14
SV40 WT/BMG/21-8 Cl 4	+	16	18.0	11	5/5 (100)	7.4	6–10
SV40 WT/BMG/22-3 Cl 5	+	17	5.1	15	1/5 (20)	16.0	

[a] Results obtained when 10% fetal bovine serum was added. None of the cell lines formed colonies when 1% fetal bovine serum was added.
[b] Groups of five nude mice were injected subcutaneously with 2×10^6 cells (per animal) of each cell line. The mice were observed twice weekly for tumor development for 5 months.

(20-B Cl 3) had lost the capacity for anchorage-independent growth, confirming previous reports of an association between the presence of super T-Ag and anchorage independence (2, 10).

Tumorigenicity of SV40-transformed mammary epithelial cells in nude mice. Athymic nude mice (HSD:athymic mice-*nu* BR; Harlan Sprague Dawley, Inc., Madison, Wis.) were used to test the tumorigenic potential of the clonal cell lines. Groups of five female mice (5 to 6 weeks old) were inoculated subcutaneously with 2×10^6 cells per animal for each cell line and were observed for 5 months (Table 1). The frequency of tumor induction varied from 0 to 100%. The length of the latent periods before tumor appearance ranged from an average of 7.4 weeks after inoculation to 22.5 weeks. Tumorigenicity did not correlate with the expression of super T-Ag by the inoculated cells. One of the most tumorigenic lines, 20-B Cl 3, did not express super T-Ag, whereas the nontransplantable line, 19-C Cl 1, did synthesize super T-Ag.

Expression of SV40 T-Ag in tumors from nude mice. Tumors that developed in the nude mice were excised aseptically when they attained a diameter of 10 to 30 mm. A portion of each tumor was trypsinized with 0.1% Enzar T-trypsin (ICN Pharmaceuticals, Inc., Cleveland, Ohio) to establish cells in culture. Dissociated cells were collected from a series of trypsinizations, pelleted at low speed, suspended, seeded into tissue culture flasks, and cultured as previously described (4). The remainder of each tumor was homogenized at moderate speed for 2 min in an Omni-mixer (Ivan Sorvall, Inc., Norwalk, Conn.) and extracted. A detergent solution (50 mM Tris hydrochloride [pH 9.0]–100 mM NaCl–1% Nonidet P-40–1% Trasylol) was added to each tumor pellet to make a final 25% suspension and allowed to extract for 2 h at 4°C; the supernatant fluid was collected

TABLE 2. Fluctuation of SV40 super T-Ag expression in SV40-transformed mouse mammary epithelial clonal cell lines and derivative tumors in nude mice

Parental cell line	T-Ag expression		Anchorage-independent growth	Tumorigenicity (%)	Nude mouse tumor	Detection of SV40 super T-Ag by indicated method in[a]:			
						Cell line			Tumor extract
	WT	Super				Metabolic labeling with [^{35}S]Met	Immunoblot	Surface labeling with ^{125}I	Immunoblot
SV40 WT/BMG/19-C Cl 1	+	+	+	0					
SV40 WT/BMG/19-C Cl 3	+	+	+	40		+	+	+	
					1	ND	+	ND	ND
					2	+	+	+	+
SV40 WT/BMG/20-B Cl 3	+	0	0	80		0	0	0	
					1	0	0	0	0
					2	+	+	+	±
					3	+	+	0	ND
					4	ND	ND	ND	+
SV40 WT/BMG/21-8 Cl 4	0	+	+	100		+	+	+	
					1	0	0	0	0
					2	0	0	0	0
					3	+	+	+	0
					4	ND	0	ND	ND
					5	ND	±	ND	ND
SV40 WT/BMG/22-3 Cl 5	+	+	+	20		+	+	+	
					1	+	+	+	+

[a] Tumors were excised aseptically and minced. A portion was extracted, and the remainder was established as cell cultures. All the tumors expressed WT T-Ag. ND, Not determined; ±, small amount of super T-Ag.

after clarification for use in immunoprecipitation experiments. None of the serum samples from tumor-bearing nude mice, taken at the time of sacrifice, contained T antibody able to react with nuclear T-Ag in SV40-infected monkey kidney cells, as determined by immunofluorescence (4).

Tumor cell lines established in culture were analyzed for the expression of SV40 T-Ag. Cells were labeled for 3 h with 100 to 200 μCi of L-[^{35}S]methionine (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per culture and disrupted with the detergent solution indicated above; the extracts were immunoprecipitated with T antibodies from tumor-bearing hamsters (18) or with normal hamster serum, the immune complexes were adsorbed with Formalin-fixed *Staphylococcus aureus* Cowan 1, and the precipitated proteins were analyzed by 8 to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18, 32). Unexpectedly, the tumor cell lines were found to vary from the parental cells with respect to the expression of T-Ag species; the presence of super T-Ag fluctuated almost randomly. For example, although the parental 21-8 Cl 4 cell line expressed only super T-Ag, all the derivative tumor cell lines expressed WT-size T-Ag and several of the tumors (3 of 5) did not contain any super T-Ag (Fig. 2A and Table 2). The converse phenomenon also occurred. Whereas the parental 20-B Cl 3 cell line did not express a super T-Ag, most of the derivative tumors (3 of 4) contained super T-Ag (Fig. 2B and Table 2). The coprecipitation of cellular protein p53 was apparent for all the parental and derivative tumor cell lines, but since all the tumor cell lines were found to contain WT T-Ag, it is not possible to conclude whether p53 was complexed with the super T-Ag molecules.

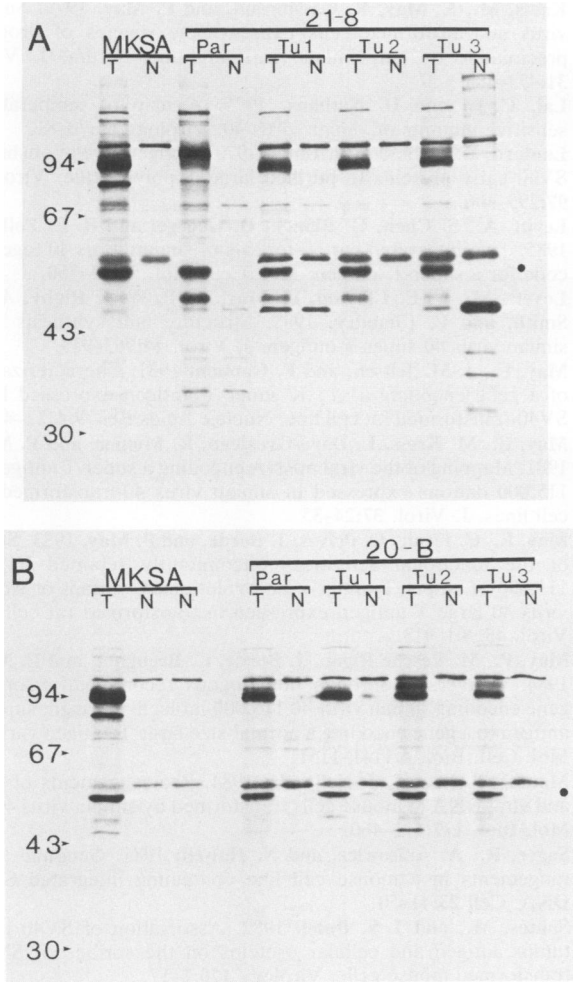

FIG. 2. Expression of SV40 large T-Ag in cell lines derived from tumors induced in nude mice by SV40-transformed mammary epithelial cell lines. Cultured cells were labeled with 200 μCi of [^{35}S]methionine and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described in the legend to Fig. 1. SV40-transformed BALB/c mouse kidney mKSA-Asc (MKSA) cells (15, 27–29) were included for control purposes. The positions of molecular weight markers (in thousands) are indicated on the left (94, phosphorylase *a*; 67, bovine serum albumin; 43, ovalbumin; 30, carbonic anhydrase). Symbol: ●, p53. (A) SV40 WT/BMG/21-8 Cl 4 cells. Note the absence of WT T-Ag in the parental cell line (Par) and its reappearance in all three derivative tumor cell lines (Tu 1, Tu 2, Tu 3). (B) SV40 WT/BMG/20-B Cl 3 cells. Note the absence of super T-Ag in the parental cell line (Par) and its reappearance in two of the derivative tumor cell lines (Tu 2 and Tu 3).

Because the conditions used might not have been optimal for labeling super T-Ag molecules, unlabeled extracts from each original tumor, as well as from the established cell lines, were analyzed by the immunoblot procedure (30). That technique confirmed the presence or absence of super T-Ag as determined in the metabolic labeling experiments (Table 2). The results with the original tumor extracts showed that cell selection during culture in vitro did not alter the T-Ag results.

SV40 T-Ag is known to be present on the plasma membrane of transformed cells (5, 13, 27, 32). Surface iodination experiments were performed on selected cell lines by using a lactoperoxidase-catalyzed reaction (27, 33) and revealed that it is possible to iodinate super T-Ag at the cell surface. The general pattern emerged that, if super T-Ag was synthesized by a cell line, some super T-Ag molecules were present at the cell surface (Table 2).

Functions of super T-Ag. The results (Table 2) do not support a functional role for super T-Ag in tumorigenicity by mammary epithelial cells. Rather, the expression of super T-Ag appears to fluctuate and may or may not be observed in tumors that develop. This rules out the possibility that an aberrant T-Ag has to be generated to foster those rare conversions of SV40-transformed mouse cells (34) to a malignant phenotype. Super T-Ag have been found to be transformation positive, able to bind cellular protein p53, and able to bind viral DNA (with the same alignment on SV40 origin DNA as that of WT T-Ag [36]) but incapable of replicating viral DNA (7, 11, 23). A correlation between the presence of super T-Ag and anchorage-independent growth by cells (2, 10) was confirmed by our data. A small fraction of the total super T-Ag in the cell appears to be associated with the nuclear matrix (35); we found that super T-Ag is also transported to the cell surface.

Super T-Ag may be generated as a result of internal in-phase duplications in the coding region of the T-Ag gene (20–22), with the duplicated sequence corresponding to the region of the SV40 genome to which many of the *tsA* mutations have been mapped (17), or by differential splicing between two integrated partial copies of the viral genome (19). Rearrangements of integrated viral sequences can occur in SV40-transformed cells after the initial integration event (1, 2, 8, 12, 14, 25, 26) or soon after infection, before viral DNA integration (19, 24). Some such rearrangements result in the capacity to generate super T-Ag (if a functional SV40 origin of replication is present [9]). Presumably, simi-

lar rearrangements of integrated viral sequences are responsible for the generation of super T-Ag in transformed mammary epithelial cells. One unexpected observation was the reappearance of WT T-Ag in tumors induced by the parental line that synthesized only a super T-Ag. Others also have noted that viral sequences encoding a super T-Ag may be converted to a gene encoding WT-size T-Ag, either before transfected genes become integrated (24) or during subcloning of a parental transformant (8). In our study, this occurred during tumor formation in nude mice.

One important contrast with previous studies of super T-Ag is that this study characterized cells of documented epithelial origin. Because the transformation of epithelial cells is not analogous in all respects to the transformation of fibroblasts (3, 4), factors affecting cell growth and phenotypic transformation must be delineated for epithelial cells. Although it is difficult to conclude what function(s), if any, super T-Ag contribute to determining the phenotype of transformed cells, their relative ubiquity suggests that they may provide the cell with some selective growth advantage. However, there was clearly no selectivity for super T-Ag during tumor development in nude mice. It may be that super T-Ag is most important as a marker to reflect genomic rearrangements by the resident viral genes in the transformed cells.

This study was supported in part by Public Health Service grant CA25215 from the National Cancer Institute.

The assistance of Nora L. Walker is gratefully acknowledged.

LITERATURE CITED

1. **Bender, M. A., and W. W. Brockman.** 1981. Rearrangement of integrated viral DNA sequences in mouse cells transformed by simian virus 40. J. Virol. **38**:872–879.
2. **Blanck, G., S. Chen, and R. Pollack.** 1983. Integration, loss, and reacquisition of defective viral DNA in SV40-transformed mouse cell lines. Virology **126**:413–428.
3. **Butel, J. S., J. P. Dudley, and C. A. Noonan.** 1980. Transformed mammary epithelial cells: correlation of *in vitro* properties with tumorigenicity, p. 317–345. *In* C. McGrath, M. J. Brennan, and M. A. Rich (ed.), Cell biology of breast cancer. Academic Press, Inc., New York.
4. **Butel, J. S., C. Wong, and D. Medina.** 1984. Transformation of mouse mammary epithelial cells by papovavirus SV40. Exp. Mol. Pathol. **40**:79–108.
5. **Chandrasekaran, K., D. J. Winterbourne, S. W. Luborsky, and P. T. Mora.** 1981. Surface proteins of simian-virus-40-transformed cells. Int. J. Cancer **27**:397–407.
6. **Chang, C., D. T. Simmons, M. A. Martin, and P. T. Mora.** 1979. Identification and partial characterization of new antigens from simian virus 40-transformed mouse cells. J. Virol. **31**:463–471.
7. **Chaudry, F., G. J. Belsham, and A. E. Smith.** 1983. Biochemical properties of the 145,000-dalton super-T antigen from simian virus 40-transformed BALB/c 3T3 clone 20 cells. J. Virol. **45**:1098–1106.
8. **Chen, S., G. Blanck, and R. Pollack.** 1984. Reacquisition of a functional early region by a mouse transformant containing only defective simian virus 40 DNA. Mol. Cell. Biol. **4**:666–670.
9. **Chen, S., D. S. Grass, G. Blanck, N. Hoganson, J. L. Manley, and R. E. Pollack.** 1983. A functional simian virus 40 origin of replication is required for the generation of a super T antigen with a molecular weight of 100,000 in transformed mouse cells. J. Virol. **48**:492–502.
10. **Chen, S., M. Verderame, A. Lo, and R. Pollack.** 1981. Nonlytic simian virus 40-specific 100K phosphoprotein is associated with anchorage-independent growth in simian virus 40-transformed and revertant mouse cell lines. Mol. Cell. Biol. **1**:994–1006.
11. **Clayton, C. E., M. Lovett, and P. W. J. Rigby.** 1982. Functional analysis of a simian virus 40 super T-antigen. J. Virol. **44**:974–982.
12. **Clayton, C. E., and P. W. J. Rigby.** 1981. Cloning and characterization of the integrated viral DNA from three lines of SV40-transformed mouse cells. Cell **25**:547–559.
13. **Deppert, W., K. Hanke, and R. Henning.** 1980. Simian virus 40 T-antigen-related cell surface antigen: serological demonstration on simian virus 40-transformed monolayer cells in situ. J. Virol. **35**:505–518.
14. **Hiscott, J., D. Murphy, and V. Defendi.** 1980. Amplification and rearrangement of integrated SV40 DNA sequences accompany the selection of anchorage-independent transformed mouse cells. Cell **22**:535–543.
15. **Kit, S., T. Kurimura, and D. R. Dubbs.** 1969. Transplantable mouse tumor line induced by injection of SV40-transformed mouse kidney cells. Int. J. Cancer **4**:384–392.
16. **Kress, M., E. May, R. Cassingena, and P. May.** 1979. Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. J. Virol. **31**:472–483.
17. **Lai, C. J., and D. Nathans.** 1975. A map of temperature-sensitive mutants of simian virus 40. Virology **66**:70–81.
18. **Lanford, R. E., and J. S. Butel.** 1979. Antigenic relationship of SV40 early proteins to purified large T polypeptide. Virology **97**:295–306.
19. **Levitt, A., S. Chen, G. Blanck, D. George, and R. E. Pollack.** 1985. Two integrated partial repeats of simian virus 40 together code for a super-T antigen. Mol. Cell. Biol. **5**:742–750.
20. **Lovett, M., C. E. Clayton, D. Murphy, P. W. J. Rigby, A. E. Smith, and F. Chaudry.** 1982. Structure and synthesis of a simian virus 40 super T-antigen. J. Virol. **44**:963–973.
21. **May, E., J.-M. Jeltsch, and F. Gannon.** 1981. Characterization of a gene encoding a 115 K super T antigen expressed by a SV40-transformed rat cell line. Nucleic Acids Res. **9**:4111–4128.
22. **May, E., M. Kress, L. Daya-Grosjean, R. Monier, and P. May.** 1981. Mapping of the viral mRNA encoding a super-T antigen of 115,000 daltons expressed in simian virus 40-transformed rat cell lines. J. Virol. **37**:24–35.
23. **May, E., C. Lasne, C. Prives, J. Borde, and P. May.** 1983. Study of the functional activities concomitantly retained by the 115,000 M_r super T antigen, an evolutionary variant of simian virus 40 large T antigen expressed in transformed rat cells. J. Virol. **45**:901–913.
24. **May, P., M. Resche-Rigon, J. Borde, C. Breugnot, and E. May.** 1984. Conversion through homologous recombination of the gene encoding simian virus 40 115,000-molecular-weight super T antigen to a gene encoding a normal-size large T antigen variant. Mol. Cell. Biol. **4**:1141–1151.
25. **Mounts, P., and T. J. Kelly, Jr.** 1984. Rearrangements of host and viral DNA in mouse cells transformed by simian virus 40. J. Mol. Biol. **177**:431–460.
26. **Sager, R., A. Anisowicz, and N. Howell.** 1981. Genomic rearrangements in a mouse cell line containing integrated SV40 DNA. Cell **23**:41–50.
27. **Santos, M., and J. S. Butel.** 1982. Association of SV40 large tumor antigen and cellular proteins on the surface of SV40-transformed mouse cells. Virology **120**:1–17.
28. **Santos, M., and J. S. Butel.** 1984. Antigenic structure of simian virus 40 large tumor antigen and association with cellular protein p53 on the surfaces of simian virus 40-infected and -transformed cells. J. Virol. **51**:376–383.
29. **Santos, M., and J. S. Butel.** 1985. Surface T-antigen expression in simian virus 40-transformed mouse cells: correlation with cell growth rate. Mol. Cell. Biol. **5**:1051–1057.
30. **Slagle, B. L., R. E. Lanford, D. Medina, and J. S. Butel.** 1984. Expression of mammary tumor virus proteins in preneoplastic outgrowth lines and mammary tumors of BALB/cV mice. Cancer Res. **44**:2155–2162.
31. **Smith, A. E., R. Smith, and E. Paucha.** 1979. Characterization of different tumor antigens present in cells transformed by simian virus 40. Cell **18**:335–346.
32. **Soule, H. R., and J. S. Butel.** 1979. Subcellular localization of simian virus 40 large tumor antigen. J. Virol. **30**:523–532.

33. **Soule, H. R., R. E. Lanford, and J. S. Butel.** 1982. Detection of simian virus 40 surface-associated large tumor antigen by enzyme-catalyzed radioiodination. Int. J. Cancer **29**:337–344.
34. **Tevethia, S. S.** 1980. Immunology of simian virus 40, p. 581–601. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
35. **Verderame, M. F., D. S. Kohtz, and R. E. Pollack.** 1983. 94,000- and 100,000-molecular-weight simian virus 40 T-antigens are associated with the nuclear matrix in transformed and revertant mouse cells. J. Virol. **46**:575–583.
36. **Wilson, V. G., and R. L. Williams.** 1985. Origin binding by a 100,000-dalton super T-antigen from SVT2 cells. J. Virol. **56**:102–109.