JOURNAL OF VIROLOGY, May 1984, p. 636–640
0022-538X/84/050636-05$02.00/0

Vol. 50, No. 2

Differential Phosphorylation of Cytoplasmic and Nuclear Variants of Simian Virus 40 Large T Antigen Encoded by Simian Virus 40-Adenovirus 7 Hybrid Viruses

KARL H. SCHEIDTMANN,[1]* JÖRG SCHICKEDANZ,[1] GERNOT WALTER,[1]† ROBERT E. LANFORD,[2] AND JANET S. BUTEL[2]

Institut für Immunbiologie der Universität Freiburg, D-7800 Freiburg, Federal Republic of Germany,[1] and Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77030[2]

Received 1 August 1983/Accepted 25 January 1984

The phosphorylation patterns of cytoplasmic and nuclear forms of simian virus 40 large T antigen encoded by simian virus 40-adenovirus 7 hybrid viruses were analyzed by two-dimensional peptide mapping. The PARA(cT) mutant which encodes a large T antigen defective for nuclear transport was used as source for cytoplasmic large T antigen. The data suggest that the large T antigen is phosphorylated in a sequential manner at a subset of sites in the cytoplasm and at additional sites in the nucleus.

Simian virus 40 (SV40) large T antigen (large T), a phosphoprotein of 82,000 daltons, performs various functions, such as initiation of viral DNA replication and regulation of transcription or stimulation of cellular DNA and RNA synthesis (18, 26, 27). The bulk of large T is located in the nucleus (25), and a small fraction is found in the plasma membrane (3–5, 12, 19, 24). Posttranslational modification might play a role in directing large T to its appropriate cellular compartment. Acylation appears to be specific for membrane-associated large T (10). Phosphorylation might be involved in nuclear transport (14), in the interaction of large T with DNA (17, 21), or in oligomerization (1, 7, 9, 16). Large T is phosphorylated in a reversible manner (6, 23, 29), at eight or more sites that are clustered in two separate regions of the molecule (11, 20, 28). In this study we asked in which cellular compartment large T becomes phosphorylated with parental [PARA(nT)] and mutant [PARA(cT)] strains of SV40-adenovirus 7 hybrid virus as tools. PARA(cT)-encoded large T is not transported to the nucleus and was shown to be less phosphorylated than nuclear large T (13, 14). The mutation is dominant since coinfection with wild-type (wt) and mutant virus inhibits nuclear transport of wt large T (13). In the present paper we demonstrate (i) that distinct subsets of sites are phosphorylated in the cytoplasm and in the nucleus and (ii) that some of the cytoplasmic sites are characteristic of newly synthesized molecules of wt large T.

TC7 cells (African green monkey kidney) were infected with PARA(nT) or PARA(cT) (13) and labeled with $^{32}P_i$ from 20 to 24 h postinfection. Cells were extracted with lysis buffer (pH 8) containing 0.5% Nonidet P-40; large T was purified by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15, 20); it was digested with trypsin and pronase and subjected to two-dimensional peptide mapping as described in the legend to Fig. 1. For comparison, large T from SV40-infected cells was analyzed in the same way. The two large T variants differed markedly in their phosphorylation patterns (Fig. 1). The pattern of the nuclear variant, PARA-nT [large T encoded by PARA(nT)], was similar to that of wt SV40 large T, indicating that PARA-nT and wt large T are phosphorylated at identical sites (Table 1; reference 20). The pattern of the mutant, PARA-cT [large T encoded by PARA(cT)], was quite different. Peptides 3, 5, 9, 10, and 11 were completely absent and peptides 1, 2, and 7 were strongly reduced; peptide 4 seemed to lack a component(s) present in the corresponding peptide of wt large T, thus it was designated 4′. On the other hand, peptides 6 and 12, which represent only minor components in wt large T, were rather strongly labeled in the mutant. Using a different peptide mapping system, Jarvis et al. found PARA-cT to lack at least one prominent phosphopeptide (D. L. Jarvis, R. E. Lanford, and J. S. Butel, Virology, in press). These data are in agreement with previous results of Lanford and Butel (14), who showed by double-labeling experiments that the phosphate content of the cytoplasmic variant is about half that of nuclear large T.

The reduced phosphorylation of PARA-cT could be due to alterations in phosphorylation sites or be a consequence of the transport defect, i.e., if large T does not enter the nucleus it cannot be phosphorylated by nucleus-specific kinases. This question was approached by double infection with PARA(cT) and SV40, in which most wt large T is retained in the cytoplasm due to the dominant effect of the mutation, but which provides all potential phosphorylation sites. In the first case, wt large T would show its usual phosphorylation pattern; in the second, it would show a pattern similar to that of PARA-cT. Large T was isolated from double-infected and ^{32}P-labeled cells and analyzed by peptide mapping. As shown in Fig. 1 (mixed infection), the phosphorylation pattern of this mixed large T population was similar to that of the cytoplasmic variant alone. Minor differences were noticed for peptides 4 and 5. Peptide 4 seemed to contain a second component (as in wt SV40 large T and PARA-nT), and peptide 5 was present only in large T from the mixed infection but not in PARA-cT.

To rule out the possibility that synthesis of wt large T was inhibited by the co-infecting PARA(cT) virus, cells were double infected with a constant amount of PARA(cT) and varying amounts of SV40. The yield of large T from these infections increased proportional to the amount of SV40 (Fig. 2a, Table 2). The subcellular distribution of large T in the double-infected cells was investigated by cell fraction-

* Corresponding author.
† Present address: Department of Pathology, School of Medicine, University of California at San Diego, La Jolla, CA 92093.

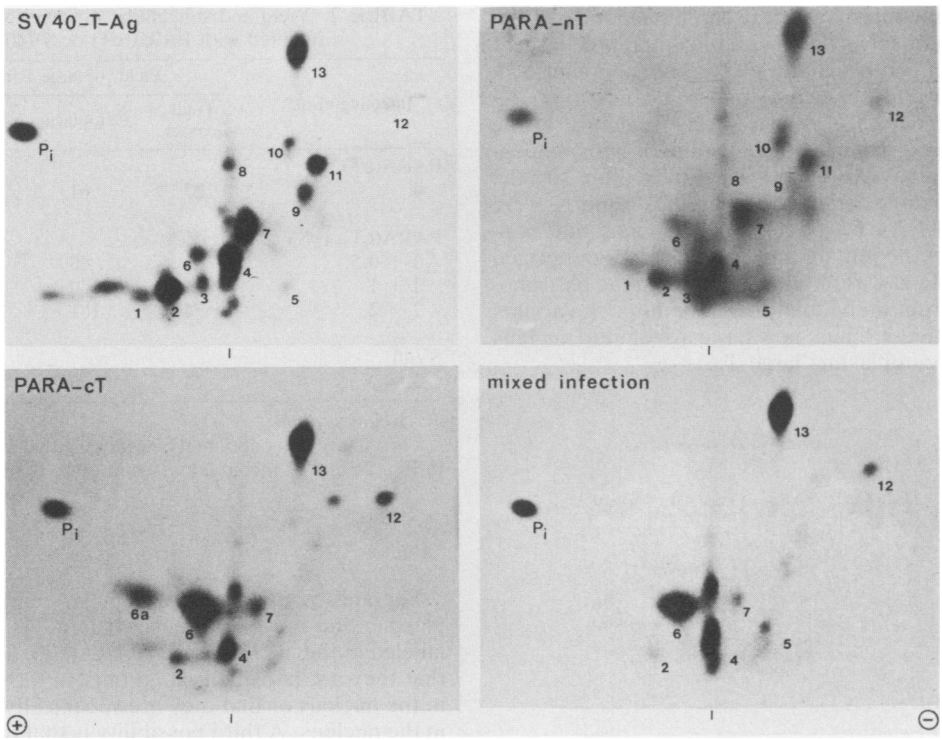

FIG. 1. Phosphopeptide analysis of nuclear and cytoplasmic large T. TC7 cells were infected with SV40-adenovirus 7 parental (PARA-nT) or mutant (PARA-cT) strains, or double-infected with PARA(cT) and wt SV40 (mixed infection). Cells were labeled with 1 mCi of $^{32}P_i$ from 20 to 24 h postinfection and then extracted with lysis buffer at pH 8 (10 mM $NaPO_4$, 140 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1 μg of Aprotinin per ml). Large T was purified by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15, 20); after elution from the gel, it was digested with trypsin and pronase E as previously described (20). The resulting digestion mixtures were applied in the middle of thin-layer cellulose plates (vertical dash) and electrophoresed at pH 1.9 (6% formic acid, 1.25% acetic acid, 0.25% pyridine) at 1,300 V for 20 min (horizontal direction, cathode on the right). Ascending chromatography was performed in isobutyric acid buffer (isobutyric acid:pyridine:acetic acid:butanol:water, 65:5:3:2:29) for 5 h (20). For comparison, large T from SV40-infected cells (SV40-T-Ag) was analyzed in the same way. The phosphopeptides were visualized by autoradiography with intensifying screens. Numbering of the peptides is according to Scheidtmann et al. (21).

ation experiments. When PARA-cT was present in excess of or in amounts similar to wt large T, nuclear transport of wt large T was largely inhibited. On the other hand, when more wt large T was present than PARA-cT, a considerable fraction of the former migrated into the nucleus. (Fig. 2b, Table 2). From the above considerations one would expect that the fraction of wt large T migrating into the nucleus becomes phosphorylated at additional, nucleus-specific sites. The peptide map shown in Fig. 2c confirmed this prediction. These results demonstrate that neither synthesis nor phosphorylation of wt large T was inhibited by the co-infecting PARA(cT) virus. We conclude that the reduced phosphorylation occurring in PARA-cT (and in wt large T during co-infection) was due to its cytoplasmic localization rather than alterations of phosphorylation sites. In the meantime, the mutation in PARA(cT) has been identified by marker rescue and sequencing as an exchange of Lys-128 to Asn (R. E. Lanford, submitted for publication), thus providing further evidence for our conclusion. In summary, these data indicate that phosphorylation of large T takes place at a distinct subset of sites in the cytoplasm and at additional sites in the nucleus. It should be considered that the accumulation of large T in the cytoplasm of mutant-infected cells represents an artificial situation and cytoplasmic phosphorylation might occur to a lesser extent under normal conditions, when large T is rapidly transported to the nucleus.

TABLE 1. Phosphorylation sites of large T

Phosphorylated residues[a]	Peptides[b]	Phosphorylated in:	
		Cytoplasm	Nucleus
Ser-106	1		+
Ser-111	4, 6	+	
Ser-112[c]	4	+	+
?[d]	5	+	
Ser-123	7, 11		+
Thr-124	12, (7, 11)[e]	+	
Ser-639	2, (4)[f]		+
Ser-676, Ser-677, Ser-679	8, 9, 10		+
Thr-701	13	+	+

[a] From reference 20.
[b] Numbering is according to Scheidtmann et al. (21) and differs from that in reference 20.
[c] Not definitely identified.
[d] Phosphorylated residue not identified.
[e] Thr-124 is also present in peptides 7 and 11.
[f] Ser-639 is also present in peptide 4 since peptide 4 appears to be a mixture of two or three peptides of similar composition.

Recently, we found that subclasses of newly synthesized and "old" (wt) large T subclasses differ in their extent of phosphorylation and exhibit distinct phosphorylation patterns (21). It was therefore of interest to compare the patterns of cytoplasmic (PARA-cT) and newly synthesized wt large T. New large T is in a low-phosphorylation state and

exists mainly in monomeric form; it can be separated from the bulk of highly phosphorylated and oligomeric old large T by sucrose gradient centrifugation (7, 9). The monomeric 5S form was isolated and subjected to peptide mapping. As shown in Fig. 3 (SV40, 5S), a characteristic feature of the pattern of new large T is the strong labeling of phosphopeptides 5, 6, and 12 relative to the other peptides (compare the pattern of wt SV40 large T in Fig. 1). Peptides 6 and 12 were also typical for PARA-cT (see above). Evidence that peptides 6 and 12 from the mutant and from newly synthesized wt large T are identical was obtained by mixing the phosphopeptides derived from individually isolated large T variants (Fig. 3; mixed peptides). Thus, new large T from the nucleus closely resembles cytoplasmic large T.

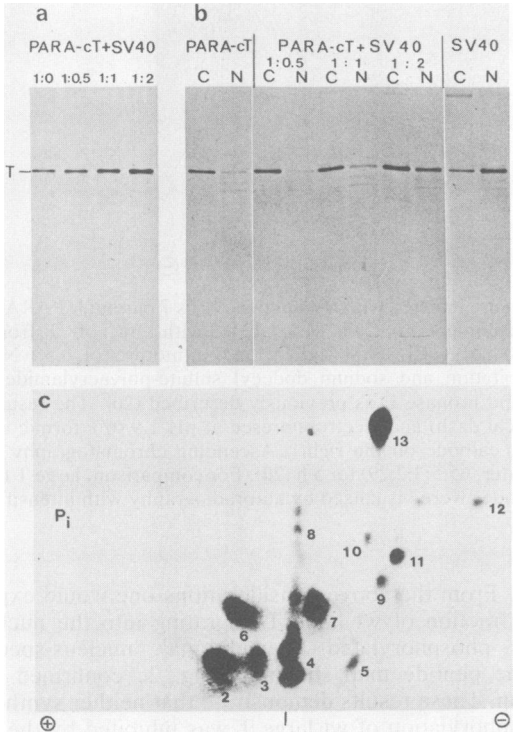

FIG. 2. Yield, subcellular distribution, and phosphopeptide analysis of large T from cells co-infected with PARA(cT) and SV40. (a, b) Cells were infected either with PARA(cT) or SV40 or double-infected with a constant amount of PARA(cT) (ca. 5 PFU per cell) plus SV40 at ratios of 1:0.5, 1:1, or 1:2. Cells were labeled with 0.1 mCi of [^{35}S]methionine from 26.5 to 27 h postinfection. Cytoplasmic and nuclear extracts were obtained by sequential extraction in buffer C (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.4], 0.3 M sucrose, 50 mM NaCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 μg of Aprotinin per ml, 0.5% Triton X-100) at 0°C for 1 min and in buffer N (0.1 M Tris-hydrochloride [pH 9.0], 0.1 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 μg of Aprotinin per ml) for 15 min, respectively (modified from Ben-Ze'ev et al. [2] and Deppert and Pates [4]). Large T was isolated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by fluorography (15); the relative amounts of large T were determined by scanning the autoradiographs (data are shown in Table 2). (a) Large T from aliquots of total extracts; (b) large T from aliquots of the corresponding cytoplasmic (C) and nuclear (N) extracts. (c) Cells were double-infected with PARA(cT) and SV40 at a ratio of 1:3; cells were labeled with ^{32}P, and large T was isolated and analyzed by peptide mapping as described in the legend to Fig. 1.

TABLE 2. Yield and subcellular distribution of large T in cells infected with PARA(cT) or SV40, or both

Infecting virus[a]	Yield[b] of large T in:			Ratio (cytoplasm/nucleus)
	Total extract	Cytoplasm	Nucleus	
PARA(cT)				
1	27	61	7	8.7
PARA(cT) + SV40				
1 + 0.5	35	88	10	8.8
1 + 1	54	140	23	6.1
1 + 2	74	161	44	3.7
SV40				
1	24	26	76	0.32

[a] Relative units.
[b] Arbitrary units obtained by scanning the autoradiographs shown in Fig. 2a and b; mean values from three determinations.

Peptides 5, 6, and 12, which are characteristic for cytoplasmic and newly synthesized large T, were only weakly labeled in the nuclear form (PARA-nT). This suggests either that they are phosphorylated only in the cytoplasm and not in the nucleus or that they are specifically dephosphorylated in the nucleus. A third possibility is that they contain several phosphorylation sites, only some of which are phosphorylated in the cytoplasm; additional phosphorylation in the nucleus would result in conversion to other peptides. Peptides 12 and 11 are probably related in this way; both peptides have the same sequence (His-122-Ser-Thr-Pro-Pro-Lys-127), but peptide 12 is phosphorylated only at Thr-124 and peptide 11 at Ser-123 and Thr-124 (20, 21). Peptides 4 and 13 appeared with similar intensities in cytoplasmic and nuclear large T, indicating that they are phosphorylated in both compartments. Phosphopeptides 1, 2, 3, and 7 through 11, which are exclusively found in nuclear large T, are phosphorylated during entry into the nucleus or within the nuclear compartment. This may reflect the subcellular distribution of the kinases involved in phosphorylation of large T. These data are summarized in Table 1.

It has been suggested that phosphorylation might play a role in converting large T from a monomeric to an oligomeric form (1, 7, 9, 16, 21). From this suggestion, one would expect that cytoplasmic large T does not form oligomers because of its low phosphorylation state. When large T from PARA(cT)-infected cells was analyzed by sucrose gradient centrifugation, the majority (60 to 70%) of the molecules sedimented with 5 to 7S (monomers, dimers, or both), and the rest sedimented with 15S (tetramers) (data not shown). This latter result suggests that oligomerization does not solely depend on but might be facilitated by phosphorylation. There might be an equilibrium between mono- and oligomers determined by the state of phosphorylation.

The *trans* effect of the PARA(cT) mutation is not understood. Three possible mechanisms have been suggested (13). (i) A PARA-cT-associated protein kinase activity might cause aberrant phosphorylation of both PARA-cT and wt large T. The data presented here do not support this idea. (ii) Transport of large T to the nucleus might be facilitated by a cellular factor. If the mutant large T bound to this factor irreversibly, it would no longer be accessible for wt large T. (iii) It is possible that PARA-cT and wt large T form complexes which cannot enter the nucleus, whereas in single

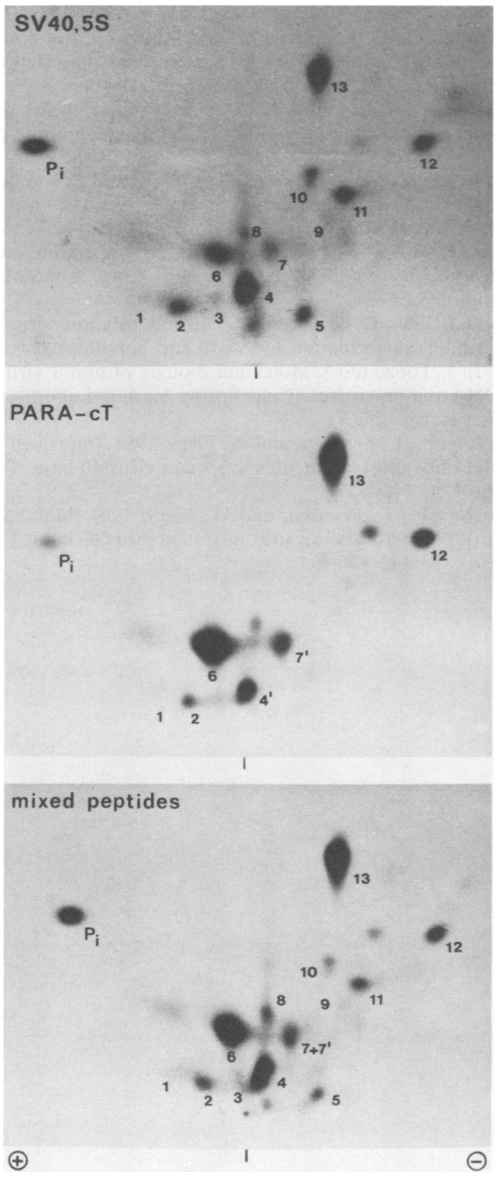

FIG. 3. Comparison of phosphopeptides from newly synthesized wt large T and cytoplasmic large T. TC7 cells were infected either with SV40 or with PARA(cT) and labeled with $^{32}P_i$ for 4 h. The extract from PARA(cT)-infected cells was processed as described in the legend to Fig. 1. The SV40-infected cell extract was sedimented through a 5 to 30% sucrose gradient in buffer containing 10 mM HEPES (pH 7.8), 5 mM $NaPO_4$, 5 mM KCl, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, and 1 μg of Aprotinin per ml in a Beckman SW55 rotor at 1°C at 48,000 rpm for 4 h. Large T was purified from individual gradient fractions by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 5S fraction containing the majority of newly synthesized large T (7, 9) was further processed for peptide mapping (SV40, 5S). To assess the identity of phosphopeptides 6 and 12 deriving from newly synthesized wt large T or PARA-cT, the phosphopeptides from both individually isolated forms of large T were mixed and analyzed on the same peptide map (mixed peptides).

infections newly synthesized large T in the cytoplasm is in monomeric form and enters the nucleus in this state (J. Schickedanz and K. H. Scheidtmann, manuscript in preparation). Consistent with this latter possibility is the finding that excess wt large T is readily translocated into the nucleus (see above). We are presently testing this possibility.

In conclusion, our data suggest that large T is first phosphorylated in the cytoplasm at a distinct subset of sites (Ser-111, Thr-124, Thr-701, and perhaps two additional residues in peptides 4 and 5) and enters the nucleus in this state. Recent data suggest that large T binds to DNA in a low phosphorylation state (8, 17, 21). Additional phosphorylation might modulate the interaction with DNA and lead to oligomerization.

The excellent technical assistance of Birgit Echle is gratefully acknowledged.

This work was supported in part by the Deutsche Forschungsgemeinschaft, SFB 31, and in part by research grants CA 22555 and CA 09197 from the National Cancer Institute and by Public Health Service grant RR-05425 from the National Institutes of Health.

LITERATURE CITED

1. **Baumann, E. A., and R. Hand.** 1982. Phosphorylation and dephosphorylation alter the structure of D2 hybrid T antigen. J. Virol. **44**:78–87.
2. **Ben-Ze'ev, A., A. Duerr, F. Salomon, and S. Penman.** 1979. The outer boundary of the cytoskeleton: a lamina derived from plasma membrane proteins. Cell **17**:859–865.
3. **Deppert, W., and R. Henning.** 1979. SV40 T-antigen related molecules on the surface of adenovirus 2-SV40 hybrid virus infected HeLa cells and on SV40-transformed cells. Cold Spring Harbor Symp. Quant. Biol. **44**:225–234.
4. **Deppert, W., and R. Pates.** 1979. Cell surface location of simian virus 40-specific proteins on HeLa cells infected with adenovirus type 2-simian virus 40 hybrid viruses $Ad2^+ND1$ and $Ad2^+ND2$. J. Virol. **31**:522–536.
5. **Deppert, W., and G. Walter.** 1982. Domains of simian virus 40 large T-antigen exposed on the cell surface. Virology **122**:56–70.
6. **Edwards, C. A. F., G. Khoury, and R. G. Martin.** 1979. Phosphorylation of T-antigen and control of T-antigen expression in cells transformed by wild-type and *tsA* mutants of simian virus 40. J. Virol. **29**:753–762.
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. **Fanning, E., K.-H. Westphal, D. Brauer, and D. Cörlin.** 1982. Subclasses of simian virus 40 large T antigen: differential binding of two subclasses of T antigen from productively infected cells to viral and cellular DNA. EMBO J. **1**:1023–1028.
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. U.S.A. **78**:105–109.
10. **Klockmann, U., and W. Deppert.** 1983. Acylation: a new post-translational modification specific for plasma membrane-associated simian virus 40 large T-antigen. FEBS Lett. **151**:257–259.
11. **Kress, M., M. Resche-Rigon, and J. Feunteun.** 1982. Phosphorylation pattern of large T antigens in mouse cells infected by simian virus 40 wild type or deletion mutants. J. Virol. **43**:761–771.
12. **Lanford, R. E., and J. S. Butel.** 1979. Antigenic relationship of SV40 early proteins to purified large T polypeptide. Virology **97**:295–306.
13. **Lanford, R. E., and J. S. Butel.** 1980. Inhibition of nuclear migration of wild-type SV40 tumor antigen by a transport-defective mutant of SV40-adenovirus 7 hybrid virus. Virology **105**:303–313.
14. **Lanford, R. E., and J. S. Butel.** 1980. Biochemical characterization of nuclear and cytoplasmic forms of SV40 tumor antigens encoded by parental and transport-defective mutant SV40-adenovirus 7 hybrid viruses. Virology **105**:314–327.
15. **Mann, K., T. Hunter, G. Walter, and H. Linke.** 1977. Evidence for simian virus 40 (SV40) coding of SV40 T-antigen and the SV40-specific proteins in HeLa cells infected with nondefective adenovirus type 2-SV40 hybrid viruses. J. Virol. **24**:151–169.

16. **Montenarh, M., and R. Henning.** 1983. Self-assembly of simian virus 40 large T antigen oligomers by divalent cations. J. Virol. **45**:531–538.
17. **Morrison, B., M. Kress, G. Khoury, and G. Jay.** 1983. Simian virus 40 tumor antigen: isolation of the origin-specific DNA-binding domain. J. Virol. **47**:106–114.
18. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. **31**:360–369.
19. **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.
20. **Scheidtmann, K.-H., B. Echle, and G. Walter.** 1982. Simian virus 40 large T antigen is phosphorylated at multiple sites clustered in two separate regions. J. Virol. **44**:116–133.
21. **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.
22. **Scheidtmann, K.-H., A. Kaiser, A. Carbone, and G. Walter.** 1981. Phosphorylation of threonine in the proline-rich carboxy-terminal region of simian virus 40 large T antigen. J. Virol. **38**:59–69.
23. **Scheidtmann, K. H., A. Kaiser, and G. Walter.** 1981. Phosphorylation sites in SV40 large T antigen, p. 1273–1279. *In* O. M. Rosen and E. G. Krebs (ed.), Cold Spring Harbor conferences on cell proliferation, vol. 8: protein phosphorylation. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Soule, H. R., R. E. Lanford, and J. S. Butel.** 1980. Antigenic and immunogenic characteristics of nuclear and membrane-associated simian virus 40 tumor antigen. J. Virol. **33**:887–901.
25. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene A. J. Virol. **16**:168–178.
26. **Tjian, R.** 1981. Regulation of viral transcription and DNA replication by SV40 large T antigen. Curr. Top. Microbiol. Immunol. **93**:5–24.
27. **Tooze, J.** 1980. Lytic cycle of SV40 and polyoma virus, p. 125–204, and Transformation by SV40 and polyoma virus, p. 205–296. *In* J. Tooze (ed.), Molecular biology of tumor viruses, part 2: DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Van Roy, F., L. Fransen, and W. Fiers.** 1983. Improved localization of phosphorylation sites in simian virus 40 large T antigen. J. Virol. **45**:315–331.
29. **Van Roy, F., L. Fransen, and W. Fiers.** 1983. Metabolic turnover of phosphorylation sites in simian virus 40 large T antigen. J. Virol. **45**:442–446.