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DNA-Binding Activity of Simian Virus 40 Large T Antigen Correlates with a Distinct Phosphorylation State

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The state of phosphorylation and the relationship of various subclasses of simian virus 40 large T antigen (large T) differing in DNA-binding activity, degree of oligomerization, age, and subcellular distribution were investigated. Young large T (continuously labeled for 4 h late in infection) comprised about 20% of the total cellular large T. It was phosphorylated to a low degree and existed primarily in a monomeric form, sedimenting at 5S. More than 50% of this fraction bound to simian virus 40 DNA, preferentially to origin-containing sequences. Old large T (continuously labeled for 17 h, followed by a 4-h chase) represented the majority of the population. It was highly phosphorylated and predominantly in an oligomeric form, sedimenting at 15S to 23S. Only 10 to 20% of this fraction bound to simian virus 40 DNA. Another subclass of large T which was extracted from nuclei with 0.5 M salt resembled newly synthesized molecules in all properties tested; it was phosphorylated to a low degree, sedimented at 5S, and bound to viral DNA with high efficiency (>70%). Two-dimensional phosphopeptide analysis of the individual subclasses revealed two distinct phosphorylation patterns, one characteristic for young, monomeric, and DNA-binding large T, the other for old, oligomeric, and non-DNA-binding large T. All sites previously identified in unfractionated large T (K. H. Scheidtmann et al., J. Virol. **44**:116–133, 1982) were also phosphorylated in the various subclasses, but to different degrees. Peptide maps of the DNA-binding fraction, the 5S form, and the nuclear high-salt fraction showed two prominent phosphopeptides not previously characterized. Both peptides were derived from the amino-terminal region of large T, presumably involved in origin binding, and probably represent partially phosphorylated intermediates of known phosphopeptides. Our data show that the DNA-binding activity, age, and oligomerization of large T correlate with distinct states of phosphorylation. We propose that differential phosphorylation might play a role in the interaction of large T with DNA.

Simian virus 40 (SV40) large T antigen (large T) is a multifunctional protein of approximately 82,000 daltons encoded by two noncontinuous segments of the early region of the SV40 genome. It is involved in viral and cellular DNA replication, viral and cellular transcription, and neoplastic transformation. In addition, it provides adenovirus helper function and specifies a tumor-specific transplantation antigen presumably located on the surface of SV40-transformed cells (for a review, see references 59 and 61). In vitro, large T binds to DNA, specifically to the origin of replication on the viral genome (20, 48, 54, 58), and has ATPase activity, which may be an intrinsic property of the molecule (60). It exists in monomeric, dimeric, and tetrameric forms (4, 11, 39) and associates with a cellular 53,000-dalton (53K) protein (11, 18). It is not understood how large T performs several distinct functions. It may consist of several domains, each with a distinct function; in fact, a series of mutants was used recently to assign some properties of large T to separate domains of the polypeptide (6, 7, 9, 15, 37, 51, 53). Alternatively, large T could assume different functional states which may be generated and converted by modification reactions, such as phosphorylation (56, 64), acylation (21), or ADP ribosylation (16).

Large T is phosphorylated at eight or more sites clustered in two separate regions of the polypeptide (43, 62), one of which is close to the region involved in DNA binding (32, 38, 50). It appears likely that the introduction of negative charges into the large T molecule will affect its properties, in particular DNA binding. Published studies indicate certain correlations between phosphorylation, sedimentation, and DNA binding. It has been reported that oligomeric forms of large T are more phosphorylated than are monomers (11, 18), that binding of large T to cellular DNA correlates with a high degree of phosphorylation (18, 30), and, consistent with these findings, that only oligomeric (di- and tetrameric) forms bind to DNA (4, 28). On the other hand, it was demonstrated that the 5S to 7S form (which is phosphorylated to a low degree) has higher DNA-binding activity (12, 14), and more recently it was found that dephosphorylation of large T in vitro does not affect its binding to the origin of DNA replication (49). The reasons for some of the discrepancies are not known.

In the present investigation we asked how different subclasses of large T are related to each other and whether they differ in their phosphorylation patterns. Special attention was given to the relationship between phosphorylation state and DNA-binding properties. We found that newly synthesized large T in monomeric form was in a low phosphorylation state and highly active in DNA binding. A fraction of these molecules was tightly associated with nuclear structures. Older large T, found predominantly in oligomeric form, was phosphorylated to a higher degree and bound poorly to DNA. Both these subclasses exhibited distinct phosphorylation patterns, as shown by two-dimensional peptide mapping.

MATERIALS AND METHODS

Cell line and virus. The propagation of cells (TC7 line of African green monkey kidney cells) and virus (SV40 large-

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plaque strain, originally obtained from P. Tegtmeyer) and infection and metabolic labeling of cells were carried out as described previously (43) unless otherwise indicated. In double-labeling experiments, 1 mCi of $^{32}P_i$ and 0.2 mCi of [^{3}H]leucine (Amersham Corp.) per 9-cm plate were applied for 4 h in Eagle medium lacking phosphate and leucine; in long labeling experiments, $^{32}P_i$, [^{3}H]leucine, or both were used at 0.1 and 0.5 mCi, respectively, in complete medium.

Isolation of large T. Cytoplasmic extracts from infected and radiolabeled cells were prepared essentially as described before (43); briefly, cells were lysed in Nonidet P-40 containing isotonic buffer, pH 8 (pH 8 lysis buffer), and nuclei were removed by low-speed centrifugation. Under these conditions, about 70% of large T was in the cytoplasmic supernatant. To isolate the residual large T, nuclei were washed, resuspended in pH 8 lysis buffer, and further extracted by adding 5 M NaCl to a final concentration of 0.5 M; nuclei were sedimented at 13,000 × *g* for 5 min, and the resulting nuclear high-salt extract was immediately diluted to 0.14 M NaCl. Isolation of large T by immunoprecipitation with hamster antitumor serum and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (22, 24, 26, 44).

DNA-binding experiments. Binding of large T to SV40 DNA was performed in two ways. (i) To separate the DNA-binding and nonbinding fractions of large T, extracts from infected, radiolabeled cells were passed over SV40 DNA-cellulose columns as described by Oren et al. (34), with the following modifications. To reduce nonspecific binding, pH 7.4 and 0.14 M NaCl rather than pH 6 and 0.1 M salt were used in the initial binding step. Extracts prepared with pH 8 lysis buffer had a reproducible pH of 7.4 and were passed over SV40 DNA-cellulose without further adjustments; nuclear high-salt extracts were adjusted to pH 7.4 and 0.14 M salt. To avoid possible denaturation of the large T, all experiments were carried out immediately after preparation and processing of the extracts. Generally, 1 ml of extract (ca. 2×10^6 cells) was passed over 1.2-ml SV40 DNA-cellulose columns (ca. 300 μg of SV40 DNA) which had been preequilibrated with equilibration buffer [10 mM $NaPO_4$ (pH 7.5), 0.14 M NaCl, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid, 1 mM dithiothreitol, 10% glycerol, 0.5% Nonidet P-40, 1 μg of aprotinin per ml]. The columns were washed with 8 volumes of equilibration buffer, and the bound proteins were eluted with elution buffer (equilibration buffer adjusted to pH 8.5 and 1 M NaCl). Large T was then purified by immunoprecipitation and SDS-PAGE.

(ii) To assess the specificity of the DNA-binding reaction, the assay described by McKay (27) was employed, using the same conditions (salt concentration and pH) used for the binding experiments with DNA-cellulose. [^{35}S]methionine-labeled extracts from SV40-infected cells were incubated with ^{32}P-labeled *Ava*II fragments of SV40 DNA at 25°C for 15 min. Subsequently, large T was immunoprecipitated under standard conditions. DNA fragments bound to the immune complex were eluted in buffer containing 10 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, and 1% SDS and electrophoresed on 1.5 or 2% agarose gels in buffer containing 40 mM Tris acetate (pH 7.8), 5 mM sodium acetate, and 1 mM EDTA. The gel was fixed with ethanol, dried, and autoradiographed.

Preparation of SV40 DNA, DNA-cellulose, and ^{32}P-labeled SV40 DNA restriction fragments. SV40 DNA (form I) was isolated from infected cells by Hirt extraction (19) and further purified by equilibrium centrifugation in ethidium bromide-CsCl density gradients. Coupling of DNA to cellulose (Whatman CF11) was carried out by the method of Alberts et al. (1). Calf thymus DNA-cellulose used for control experiments was obtained from Sigma Chemical Co. Cleavage with restriction endonuclease *Ava*II (Bethesda Research Laboratories) was done by the manufacturer's specifications. The mixture of *Ava*II fragments was labeled by nick-translation (41).

Sedimentation in sucrose gradients. Cell extracts were prepared by lysing cells in 0.25 ml of pH 8 lysis buffer per plate. Extracts were cleared by centrifugation at 13,000 × *g* at 4°C for 10 min and layered onto 5 to 30% sucrose gradients containing 10 mM HEPES [2-(4-[2-hydroxyethyl]-piperazinyl-[1])-ethanesulfonic acid] (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. Centrifugation was performed in a Beckman SW55 rotor as indicated in the figure legends. Large T was isolated from gradient fractions and processed as described above for the DNA-binding experiments.

Determination of the phosphorylation state of large T. After purification of large T by immunoprecipitation and SDS-PAGE, it was localized on the unfixed dried gel by autoradiography. It was then either eluted in Protosol (New England Nuclear Corp.) to determine the amount of [^{3}H]leucine and ^{32}P or processed for two-dimensional phosphopeptide analysis as described previously (13, 43, 44). Briefly, large T was extracted from the gel, precipitated with 20% trichloroacetic acid, oxidized with 3% performic acid, and digested sequentially with trypsin and pronase E. The digestion mixture was analyzed on cellulose thin-layer plates by electrophoresis at pH 1.9 (6% [vol/vol] formic acid, 1.25% [vol/vol] acetic acid, 0.25% [vol/vol] pyridine) at 1,300 V for 20 or 25 min and by ascending chromatography in isobutyric acid buffer (isobutyric acid-pyridine-acetic acid-butanol-water, 65:5:3:2:29) for 5 h (43). The phosphopeptides were visualized by autoradiography with intensifying screens.

RESULTS

Characterization of the DNA-binding behavior of SV40 large T. To separate the subclasses of large T with different DNA-binding properties, we used DNA-cellulose chromatography as described by Oren et al. (34). Extracts from SV40-infected, radiolabeled cells were passed over DNA-cellulose columns, and bound proteins were eluted with buffers of increasing pH and salt concentration. Large T was purified from the different subfractions by immunoprecipitation and SDS-PAGE. Initial binding conditions were pH 6 and 0.1 M NaCl and elution conditions were pH 8.5 and 0.14 or 1 M NaCl (34). Under these conditions 80% of the total large T present in the extract bound to DNA. From 70 to 80% of the bound material was eluted with low salt (low-affinity class), and 20 to 30% was eluted with high salt (high-affinity class), confirming the results of Oren et al. (34). However, these experiments did not reveal any specificity in binding. There was no difference in the amount of large T which bound to SV40 or calf thymus DNA. Furthermore, the degree of phosphorylation of DNA-binding and non-DNA-binding large T, as determined by double-labeling with ^{32}P and [^{3}H]leucine, was almost identical (data not shown). We reasoned that the binding conditions used (low pH and low salt) did not allow discrimination between unspecific and specific binding because of competition between low- and high-affinity molecules for the same binding sites.

Increasing the pH to 7.0, as described by Oren et al. (34), was still insufficient to exclude unspecific binding to cellular DNA (not shown). To work out conditions favoring specific

TABLE 1. Specificity of DNA binding of SV40 large T

Chromatography column	Fraction	Amt of label in large T[a]				
		^3H		^{32}P		
		cpm	% of total	cpm	% of total	$^{32}P/^3H$ ratio
SV40 DNA-cellulose	Nonbinding	380	53	7,408	89	19
	Binding	345	47	946	11	2.7
Calf thymus DNA-cellulose	Nonbinding	705	94	7,980	91	11
	Binding	40	6	760	9	19
Cellulose	Nonbinding	690	94	9,275	95	13.5
	Binding	45	6	535	5	12

[a] Labeling was performed synchronously for 4 h. Values are from one representative experiment of three independent determinations.

binding of large T to SV40 DNA, the binding reaction was done at pH 7.4 and increasing salt concentrations (0.14, 0.23, and 0.32 M NaCl). When binding was carried out in 0.14 M NaCl, about 50% of ^3H-labeled but only 11% of ^{32}P-labeled large T molecules were retained by the column (Table 1). Thus, the DNA-binding fraction of large T was considerably less phosphorylated than the nonbinding fraction. The degree of binding of large T to calf thymus DNA was reduced to background levels under these conditions; it was as low as the degree of binding to plain cellulose (Table 1). Higher salt concentrations (0.23 or 0.32 M) completely prevented the binding of large T to SV40 DNA (not shown). Therefore, in all subsequent experiments binding was performed at pH 7.4 and 0.14 M NaCl.

To test whether, under these conditions (pH 7.4 and 0.14 M NaCl), binding of large T to viral DNA occurred specifically at the origin of replication, the DNA immunoprecipitation assay described by McKay (27) was employed. Extract from infected cells was incubated with ^{32}P-labeled *Ava*II restriction fragments of SV40 DNA, and the large T-DNA complex was immunoprecipitated. DNA fragments bound to large T were analyzed on agarose gels as described above. Of the six DNA fragments generated by *Ava*II, only fragment D, containing the origin of replication (49), was bound by large T (not shown). From this experiment we conclude that, under the stringent conditions used for DNA-cellulose chromatography, large T binds preferentially to origin-containing sequences.

Binding of purified large T to DNA. Since the DNA-binding properties of large T might be modified by cellular proteins in the extract, an experiment with purified large T was conducted. Purification was performed by immunoaffinity chromatography with antibodies raised against a synthetic peptide corresponding to the carboxy-terminal 11 amino acid residues of large T (65). The antibodies were coupled to Sepharose and used as an immunosorbent to isolate large T from the infected-cell extract. Bound large T was released from the antibodies with an excess of synthetic peptide under mild conditions (pH 7.5, 20 mM NaCl). The degree of purification was assessed by quantitative densitometry of gel tracks of [^{35}S]methionine-labeled proteins before and after immunoaffinity chromatography (not shown). The large T preparation thus obtained was 600-fold enriched over the crude extract and about 40% pure (Fig. 1, inset, lane a).

For the DNA-binding experiments, large T was labeled in infected cells with [^3H]leucine and ^{32}P for 4 h and purified as described above. Bovine serum albumin (final concentration, 0.01%) was added to the purified large T. It was then passed over an SV40 DNA-cellulose column under the same conditions used for the crude extract. Binding and elution of large T were monitored by Cerencov counting and SDS-PAGE (Fig. 1). The ^3H ^{32}P content of the binding and nonbinding fractions was determined from gel-purified large T; 44% of ^3H- and 17% of ^{32}P-labeled large T bound to the DNA (Table 2). The DNA-binding fraction had a lower phosphate content than the nonbinding fraction. This result indicates that the DNA-binding properties of large T are not greatly influenced by cellular proteins.

Relationship between DNA-binding, phosphorylation, and age of large T. Oren et al. (34) have shown that the DNA-binding activity of large T depends on its age, young large T showing a higher binding activity than old large T. It was therefore of interest to investigate a possible relationship between these properties and the phosphorylation state of large T. SV40-infected cells were double-labeled with [^3H]leucine and ^{32}P. To distinguish between young and old large T, [^3H]leucine was applied for 1 or 4 h (newly synthesized or young large T) or for 17 h followed by a 4-h chase (old large T) (Table 3). Because the phosphate residues on

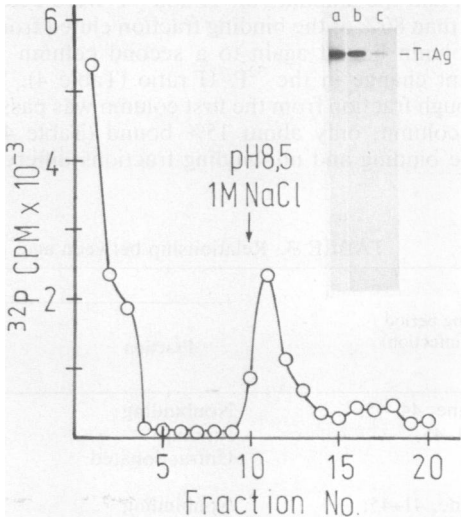

FIG. 1. Binding of immunoaffinity-purified large T to SV40 DNA. Large T was purified from SV40-infected, double-labeled cells by immunoaffinity chromatography; 0.01% bovine serum albumin and 1 volume of 2× DNA equilibration buffer were added, and large T was subjected to DNA-cellulose chromatography. Inset: large T (T-ag) after purification by immunoaffinity chromatography (a); large T from the nonbinding (b) and DNA-binding (c) fractions.

TABLE 2. Binding to SV40 DNA of large T purified by immunoaffinity chromatography[a]

Large T fraction	^3H		^{32}P		$^{32}P/^3H$ ratio
	cpm	% of total	cpm	% of total	
Nonbinding	3,040	56	480	83	0.16
Binding	2,355	44	100	17	0.04

[a] Values are means of two independent determinations.

large T have high turnover rate (10, 45, 63), ^{32}P labeling does not distinguish between young and old molecules. Therefore, ^{32}P was always applied for 4 h before harvesting. Binding to SV40 DNA-cellulose and isolation of large T were carried out as described above. The fraction of ^3H-labeled large T which bound to DNA varied with the labeling time (Table 3). Newly synthesized or young large T molecules bound with high efficiency (70 and 50%, respectively), whereas only 20% of old large T was retained by the column. Of the ^{32}P-labeled large T, 20 to 30% bound to DNA whether young or old. The same values were obtained when ^{32}P was applied for 20 h (not shown). We conclude that (i) newly synthesized large T molecules have higher DNA-binding activity than older ones, confirming previous results (34) and (ii) the high DNA-binding activity of young large T correlates with a low degree of phosphorylation. In contrast, no correlation between DNA binding and phosphorylation was apparent with old large T.

Rebinding experiments showed that the low phosphate content of the DNA-binding fraction and the high phosphate content of the nonbinding fraction were intrinsic properties of these fractions. Labeled cell extracts were subjected to DNA-cellulose chromatography. About ⅓ of the flow-through and the binding fractions was immunoprecipitated directly to isolate large T. The remaining ⅔ was passed over a second SV40 DNA-cellulose column; the eluate from the first column was adjusted to pH 7.4 and 0.14 M NaCl before the second binding reaction. Large T was isolated from each fraction, and the amounts of ^3H and ^{32}P were determined.

More than 80% of the binding fraction eluted from the first DNA column bound again to a second column without a significant change in the $^{32}P/^3H$ ratio (Table 4). When the flowthrough fraction from the first column was passed over a second column, only about 15% bound (Table 4). In this case, the binding and nonbinding fractions differed in their relative ^{32}P content, so that the binding fraction had again a lower $^{32}P/^3H$ ratio than the nonbinding fraction. This result indicates that not all molecules with binding potential were retained on the first column.

Separation of large T subclasses with different degrees of oligomerization. Fanning et al. (11) and Greenspan and Carroll (18) reported that monomeric large T is less phosphorylated than its oligomeric forms. On the other hand, Prives et al. (39) and Gidoni et al. (14) found no difference in the relative phosphate content of the various sedimentation forms. To investigate the relationship between oligomerization and phosphorylation, SV40-infected cells were double-labeled as before with [^3H]leucine for 4 or 17 h and with ^{32}P for 4 h. Extracts were prepared and sedimented through 5 to 30% sucrose gradients as described above. The distribution of large T was determined by immunoprecipitaion and SDS-PAGE. Figure 2a shows the overall distribution of ^{32}P-labeled large T over the gradient. The bulk of large T (about 60%) sedimented at 15S, corresponding to tetramers (4, 39); a minor fraction of about 20% sedimented in the 5S region, representing monomeric large T (4, 39). A considerable fraction of large T sedimented faster than 15S, possibly as a complex with the cellular 53K protein (11, 18).

To determine the sedimentation behavior and the degree of phosphorylation of young and old large T, the distribution of ^3H and ^{32}P over the gradients was determined. About 80% of young large T (labeled with [^3H]leucine for 4 h) sedimented at 5S, and only a small portion sedimented in the 15S region (Fig. 2b). On the other hand, ^{32}P-labeled large T showed a distribution similar to that shown in the autoradiograph (Fig. 2a). Consequently, young large T in the monomeric form had a lower phosphate content than it did in the oligomeric form. This result is in agreement with previous data (11, 18). In contrast, the sedimentation profile of old large T (labeled with [^3H] leucine for 17 h) paralleled that of ^{32}P-labeled large T, with a major peak at 15S and a smaller one at 5S (Fig. 2c). Thus, the differently sedimenting forms of old large T showed no difference in phosphate content and in this respect resembled the DNA-binding and nonbinding fractions of old large T (cf. Table 3). These results can be best explained by assuming that because of the turnover of the phosphate residues, ^{32}P is predominantly incorporated into preexisting molecules, representing the majority of the large T population and being mainly in oligomeric form, and that newly synthesized molecules, which are mainly in monomeric form, are phosphorylated only to a small extent.

TABLE 3. Relationship between age, phosphorylation state, and DNA-binding behavior of SV40 large T

Labeling period (h postinfection)	Fraction	Amt of label in large T[a]				
		^3H		^{32}P		$^{32}P/^3H$ ratio
		cpm	% of total	cpm	% of total	
[^3H]leucine, 44–45; $^{32}P_i$, 41–45	Nonbinding	3,020	29	5,520	68	1.83
	Binding	7,590	71	2,680	32	0.35
	Unfractionated	830		640		0.77
[^3H]leucine, 41–45; $^{32}P_i$, 41–45	Nonbinding	2,310	49	6,780	81	2.94
	Binding	2,420	51	1,590	19	0.66
	Unfractionated	520		835		1.61
[^3H]leucine, 24–41; $^{32}P_i$, 41–45	Nonbinding	13,090	80	11,898	80	0.91
	Binding	3,240	20	3,000	20	0.93
	Unfractionated	1,443		1,408		0.98

[a] Data are representative for four individual experiments.

TABLE 4. Rebinding to SV40 DNA of DNA-binding and nonbinding large T subclasses

Step	Fraction applied to column	Fractions obtained	Amt of label in large T[a]				
			^3H		^{32}P		$^{32}P/^3H$ ratio
			cpm	% of total	cpm	% of total	
1	Cell extract	Flowthrough 1	1,160	44	6,240	63	5.4
		DNA-binding 1	1,480	56	3,650	37	2.4
2	Flowthrough 1	Flowthrough 2	1,040	85	10,700	93	10.3
		DNA-binding 2	180	15	720	7	4.0
3	DNA-binding 1	Flowthrough 3	240	12	920	15	3.8
		DNA-binding 3	1,900	88	5,270	85	2.8

[a] Data are from two individual experiments.

This interpretation is further supported by an experiment in which ^{32}P was administered in the presence of cycloheximide. Under these conditions, the incorporation of ^{32}P into large T was reduced by only 25%, but the overall distribution of ^{32}P-labeled large T was comparable to that found in the other experiments (Fig. 2d).

Relationship between oligomerization and DNA binding. There is some controversy as to whether large T binds to DNA in monomeric or oligomeric form (4, 12, 14, 28). To determine the DNA-binding behavior of the different sedimentation forms of large T, SV40-infected cells were labeled with [^{35}S]methionine for 2 h late during infection, and the cell extract was sedimented through a 5 to 25% sucrose gradient at 42,000 rpm for 13 h, conditions allowing the separation of monomeric (5S), dimeric (7S), and tetrameric (15S) subclasses. One half of the individual gradient fractions was immunoprecipitated directly to determine the distribution of large T, and the other half was subjected to the DNA immunoprecipitation assay, using ^{32}P-labeled *Ava*II restriction fragments, as described above, in 0.5 ml of equilibration buffer at 25°C for 15 min. The 5S form of large T showed the highest DNA-binding activity (Fig. 3). No particular peak of DNA-binding activity was noticed at the 7S position of the gradient. A minor peak occurred in the 15S region. The profile of DNA-binding activity paralleled exactly that of the distribution of newly synthesized large T, with a major peak at 5S and smaller one at 15S (cf. Fig. 2b). Similar results were obtained with double-labeled extract by DNA-cellulose chromatography (not shown). These data confirm again that newly synthesized large T molecules bind better to DNA than do older ones; since the former are mainly in a monomeric form, this form represents the DNA-binding form. It should be mentioned that under less stringent conditions (80 mM NaCl), old large T in the 15S form bound to DNA as efficiently as young large T (not shown). Also, under the conditions used, all forms of large T bound preferentially to the origin-containing *Ava*II D fragment (Fig. 3).

Characterization of a new subclass of large T tightly associated with nuclear structures. In the experiments described above, cell extracts were prepared with a low salt- and detergent-containing buffer, yielding mainly soluble nuclear (nucleoplasmic) large T (52). A fraction of large T in this extract was capable of binding to DNA in vitro. Once bound, it could be eluted only with high salt (>0.5 M). If we assume that large T binds to chromatin in vivo with similar strength, then chromatin-associated large T might be retained in the nuclei during our regular extraction procedure. To test this possibility, infected cells were double-labeled with [^3H]leu-

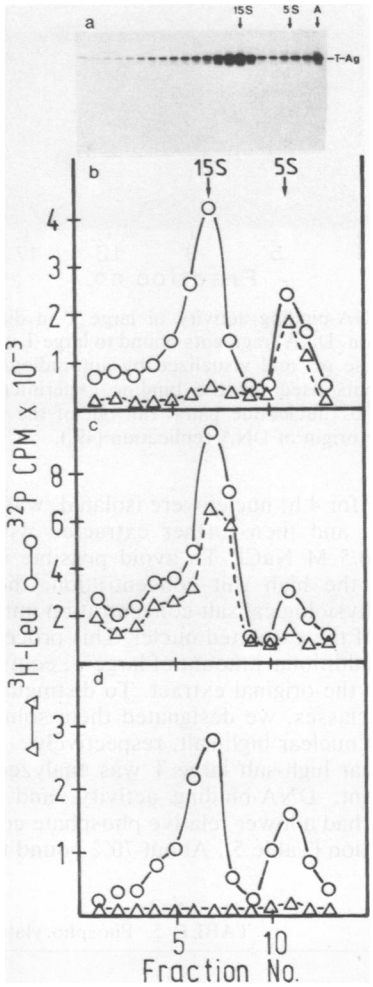

FIG. 2. Relationship between oligomerization and phosphorylation state. SV40-infected cells were labeled (a and b) synchronously with [^3H]leucine and $^{32}P_i$ for 4 h (young large T), (c) sequentially with [^3H]leucine for 17 h and then with $^{32}P_i$ for 4 h without [^3H]leucine (old large T), or (d) with [^3H]leucine and $^{32}P_i$ for 4 h in the presence of cycloheximide (10 μg/ml). Extracts were sedimented in a Beckman SW55 rotor at 1°C and 48,000 rpm for 3.5 (a) or 4 (b, c, and d) h. Sedimentation constants (arrows) were determined from ^{14}C-labeled eucaryotic rRNA sedimented in a parallel gradient. (a) Autoradiograph showing the distribution of ^{32}P-labeled large T over the gradient; A, large T isolated from an unfractionated extract. (b, c, and d) Distribution of ^3H and ^{32}P associated with gel-purified large T.

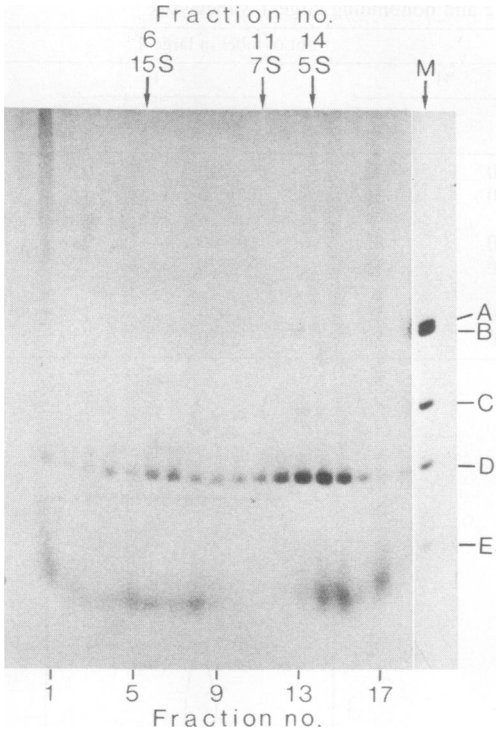

FIG. 3. DNA-binding activity of large T in different states of oligomerization. DNA fragments bound to large T were analyzed on a 1.5% agarose gel and visualized by autoradiography. Lane M, DNA fragments used for the binding experiment (fragment F, consisting of 31 nucleotide pairs, ran out of the gel; fragment D contained the origin of DNA replication [49]).

cine and ^{32}P for 4 h; nuclei were isolated, washed with pH 8 lysis buffer, and then further extracted with lysis buffer containing 0.5 M NaCl. To avoid possible inactivation of large T by the high salt concentration, the extract was diluted to physiological salt concentration immediately after separation of the extracted nuclei. This procedure yielded a substantial additional amount of large T, equivalent to about ¼ of that in the original extract. To distinguish the various large T subclasses, we designated them soluble or nucleoplasmic and nuclear high-salt, respectively.

The nuclear high-salt large T was analyzed for its phosphate content, DNA-binding activity, and sedimentation behavior. It had a lower relative phosphate content than the soluble fraction (Table 5). About 70% bound to SV40 DNA. The $^{32}P/^{3}H$ ratio of the DNA-binding fraction was similar to that of total nuclear high-salt large T, whereas that of the nonbinding fraction was somewhat higher (Table 5). Sedimentation analysis revealed that the nuclear high-salt large T was mainly in the 5S form. As a control, nucleoplasmic large T was incubated in 0.5 M NaCl for the same time period as was required for the preparation of the nuclear high-salt extract; no disaggregation of oligomeric large T in the soluble fraction was observed (data not shown). Thus, the nuclear high-salt fraction of large T had all the characteristics of newly made molecules, i.e., low phosphate content, monomeric form, and high DNA-binding activity.

To see whether the nuclear high-salt fraction consisted in fact of newly synthesized large T, a pulse-chase experiment was performed. SV40-infected cells were labeled with [^{35}S]methionine for 1 h and chased for 1, 3, 6, or 16 h. After the pulse, the nuclear high-salt fraction comprised about 20% of total labeled large T. This amount decreased during the first 3 h of the chase, reaching a constant level of about 10% (not shown). Thus, about one-half of the nuclear high-salt large T fraction is transiently and the other half is more stably associated with nuclear structures.

Phosphorylation patterns of different subclasses of large T. The various subclasses of large T described above were isolated and analyzed by two-dimensional peptide mapping for differences in their phosphorylation patterns. As shown previously, the phosphorylation sites of large T can be almost completely separated on two-dimensional fingerprints after sequential digestion with trypsin and pronase (43). When DNA-binding and nonbinding subclasses were analyzed this way, the patterns shown in Fig. 4 (top) were obtained. The phosphorylated residues of individual phosphopeptides are listed in Table 6. The pattern of the nonbinding fraction was very similar to that of unfractionated (nucleoplasmic) large T (not shown). In contrast, the phosphorylation pattern of the DNA-binding fraction differed from that of the nonbinding fraction in the relative intensity of individual phosphopeptides. With peptides 2 and 4, which had similar intensities on both peptide maps, used as references, peptides 3, 8, and 13 were more strongly labeled in DNA-binding than in nonbinding large T; peptides 5, 6, and 12, which were prominent in the binding fraction, were almost absent in the nonbinding fraction (although they were detectable after longer exposures).

Similar differences were observed when the various sedimentation forms of large T were analyzed (Fig. 4, center). The pattern of the monomeric 5S form resembled that of the DNA-binding fraction. On the other hand, the oligomeric 15S and 23S forms gave rise to peptide maps similar to that

TABLE 5. Phosphorylation state and DNA-binding activity of nuclear high-salt large T

Fraction	Amt of label in large T[a]				
	^{3}H		^{32}P		
	cpm	% of total	cpm	% of total	$^{32}P/^{3}H$ ratio
Soluble extract (unfractionated)[b]	1,800		15,600		8.6
High-salt extract					
Unfractionated[b]	840		3,320		3.9
Flowthrough	220	33	1,400	46	6.3
Binding	455	67	1,625	54	3.6

[a] Labeling was performed synchronously for 4 h. Data are taken from one representative experiment out of three individual determinations.
[b] These values were determined from portions and do not reflect the actual distribution of large T between the soluble and high-salt extracts.

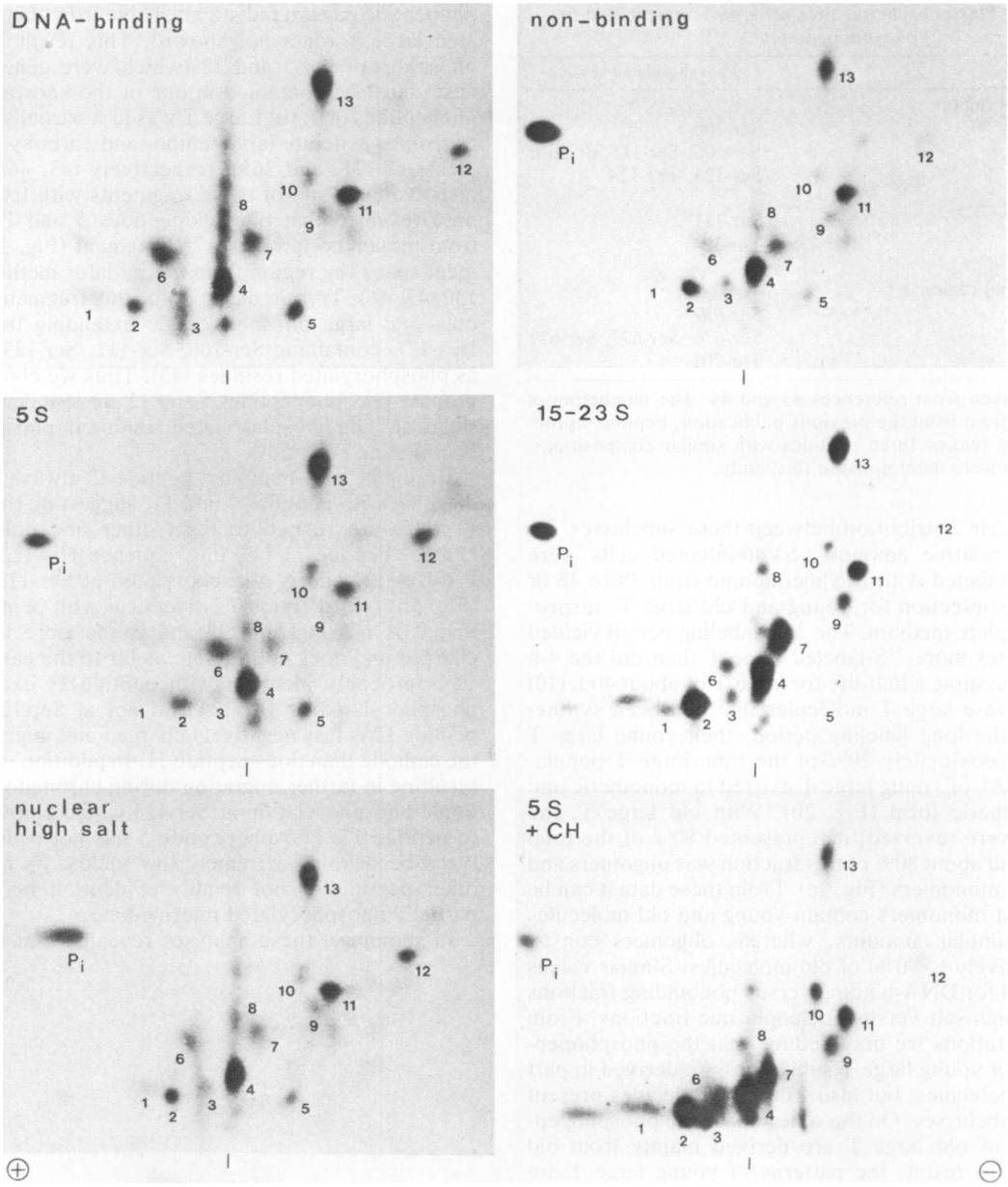

FIG. 4. Phosphopeptide analysis of subclasses of large T. Infected cells were labeled with $^{32}P_i$ for 4 h. Soluble and nuclear high-salt extracts were prepared. Large T was processed for two-dimensional peptide mapping as described in the text. Samples were applied to the center of thin-layer cellulose plates (vertical dash below each panel) and electrophoresed for 25 min (DNA-binding fraction) or 20 min (all other samples). Ascending chromatography was performed in isobutyric acid buffer. In one set of experiments, infected cells were treated with cycloheximide (CH; 10 μg/ml) for 2 h before and during the labeling period, and the 5S fraction of large T was isolated and analyzed. Exposure times: DNA-binding and 5S fractions, 2 weeks; nonbinding and 15S to 23S fractions, 1 and 3 days, respectively; nuclear high-salt fraction, 1 week.

of nonbinding large T. Finally, the nuclear high-salt large T fraction (Fig. 4, bottom, left) revealed a phosphorylation pattern similar to those of the DNA-binding and 5S fractions. Thus, all subclasses with a high proportion of young large T molecules and high DNA-binding activity showed a common phosphorylation pattern; this pattern was distinct from that of old and nonbinding large T subclasses.

From the double-labeling experiments described above, we concluded that young large T molecules are less phosphorylated than old ones. The peptide maps (Fig. 4) might give the opposite impression, since the phosphopeptide pattern of young large T appears to be more complex, showing a larger number of strongly labeled phosphopeptides than does that of old large T. However, it should be noticed that the peptide maps of young large T were exposed 7 to 14 times longer than were those of old large T. Moreover, it must be considered that the subclasses typical for young large T contained a substantial fraction of old molecules.

The extent of cross-contamination between the different subclasses, for example between mono- and oligomers, can be estimated from the relative amounts of young and old

TABLE 6. Phosphorylation sites of large T represented in phosphopeptides[a]

Peptide	Phosphorylated residue
Amino-terminal region	
1	Ser-106
3	Ser-106, Ser-111, or both
4, 7, 11	Ser-123, Thr-124
5	?
4, 6	Ser-111
12	Thr-124
Carboxy-terminal region	
2, 4	Ser-639
8, 9, 10	Ser-676, Ser-677, Ser-679
13	Thr-701

[a] Data are taken from references 43 and 44. The numbering of peptides is different from the previous publication. Peptide 4 probably consists of two or three peptides with similar compositions. Peptide 12 data were determined in this study.

large T and their distribution between these subclasses. To estimate the relative amounts, SV40-infected cells were continuously labeled with [^{35}S]methionine from 38 to 42 or 20 to 42 h postinfection for young and old large T, respectively, in complete medium. The 22-h labeling period yielded about five times more ^{35}S-labeled large T than did the 4-h period. If we assume a half-life for large T of about 40 h (10) and neglect those large T molecules that had been synthesized before the long labeling period, then young large T comprised approximately 20% of the total large T population. About 80% of young large T existed in monomeric and 20% in oligomeric form (Fig. 2b). With old large T, the percentages were reversed; it represented 80% of the total population, and about 80% of this fraction was oligomers and only 20% was monomers (Fig. 2c). From these data it can be calculated that monomers contain young and old molecules in about equimolar amounts, whereas oligomers consist almost exclusively (>90%) of old molecules. Similar values were obtained for DNA-binding versus nonbinding fractions and nuclear high-salt versus nucleoplasmic fractions. From these considerations we may deduce that the phosphopeptide patterns of young large T subclasses are derived in part from young molecules, but also from old molecules present in the same subclasses. On the other hand, the phosphopeptide patterns of old large T are derived mainly from old molecules. As a result, the patterns of young large T are more complex than those of old large T.

Further support for this assumption was obtained from an experiment in which infected cells were labeled with ^{32}P in the presence of cycloheximide and the 5S fraction of large T was isolated and analyzed by peptide mapping. In this case, the 5S fraction gave rise to the typical phosphorylation pattern of old rather than young large T, i.e., the levels of phosphopeptides 5, 6, and 12 were greatly reduced (Fig. 4, bottom, right). Similar results were obtained for the nuclear high-salt fraction (not shown).

Origin of phosphopeptides 5 and 12. The major differences between the phosphorylation patterns of young and old large T subclasses occurred in phosphopeptides 5, 6, and 12. Peptide 6 is phosphorylated at Ser-111 in the amino-terminal cluster of phosphorylation sites (43). Peptides 5 and 12 have not been characterized previously because they are hardly detectable on peptide maps of unfractionated large T. To determine their origin, the 5S form of large T was isolated and analyzed in two ways. (i) Digestion with trypsin alone (without subsequent cleavage with pronase) revealed a phosphopeptide pattern indistinguishable from that of unfractionated large T (data not shown). This result indicated that phosphopeptides 5 and 12 (which were generated by pronase) must be contained in one of the known tryptic phosphopeptides (43). (ii) Large T was first partially digested with trypsin to generate large amino- and carboxy-terminal fragments of 17K and 26K, respectively (43, 46). Further exhaustive digestion of these fragments with trypsin and pronase revealed that phosphopeptides 5 and 12 are derived from the amino-terminal 17K fragment (Fig. 5a). This fragment spans the region from the initiator methionine to Arg-130 (43, 46). Tryptic digestion of this fragment gives rise to only one large phosphopeptide extending from Tyr-68 to Lys-127, containing Ser-106, Ser-111, Ser-123, and Thr-124 as phosphorylated residues (43). Thus we conclude that the pronase phosphopeptides 5 and 12 are also derived from this segment. The phosphorylated residue in phosphopeptide 12 is threonine (Fig. 5b).

During peptide mapping, peptide 12 always migrated on a diagonal with peptides 7 and 11, suggesting that these three peptides are related to each other and differ mainly by charge. Peptide 11 has the sequence His-122-Ser-Thr-Pro-Pro-Lys-127 and is phosphorylated at Ser-123 and Thr-124 (Fig. 5b) (43). Peptide 7 is identical with peptide 11 except that it is missing His-122 and is therefore less positively charged and does not migrate as far to the cathode. Peptide 12 is probably identical with peptide 11 except that it is phosphorylated at Thr-124 and not at Ser-123. Therefore, peptide 12 is less negatively charged and migrates farther to the cathode than does peptide 11. In addition, it is less polar, resulting in farther migration during chromatography. Additional phosphorylation at Ser-123 would convert peptide 12 to peptide 11. Phosphopeptide 5 has not been further analyzed because of extremely low yields. Its relationship to other peptides is not readily evident; it may represent a partially phosphorylated intermediate.

In summary, these analyses revealed that the phosphor-

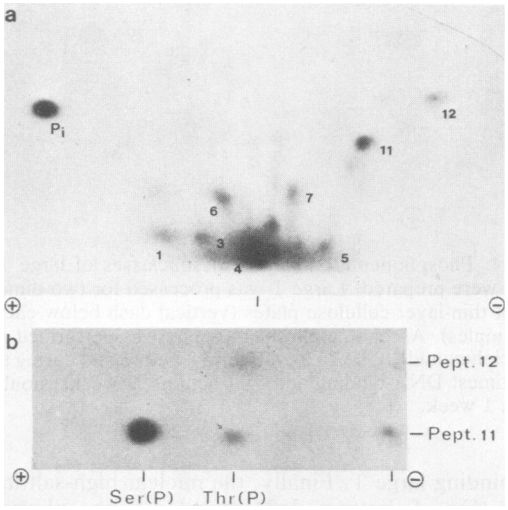

FIG. 5. Origin of phosphopeptides 5 and 12. (a) Phosphopeptide map of the amino-terminal 17K fragment of large T. Phosphopeptides missing on this map are derived from the carboxy-terminal region of large T (43). (b) Phosphopeptide 12 was eluted from the thin-layer plate and hydrolyzed in 5.6 N HCl (110°C, 2 h); the hydrolysate was mixed with unlabeled serine phosphate and threonine phosphate [Ser(P) and Thr(P)] and analyzed by electrophoresis at pH 1.9 (64). For comparison, peptide 11 was analyzed in the same way. The autoradiographs were exposed for 3 weeks. The low yield of phosphopeptide 5 did not allow a similar analysis to be made.

ylation patterns of the various large T subclasses differ mainly within the amino-terminal sites. The prominent peptides characteristic for young large T, particularly peptide 12, appear to be partially phosphorylated intermediates which may be converted to their fully phosphorylated counterparts later on.

DISCUSSION

Relationship between different subclasses of large T. To elucidate the relationship between phosphorylation and various properties of large T, particularly its DNA-binding activity, we isolated subclasses of large T, defined by their DNA-binding properties, sedimentation behavior, and subcellular localization, and analyzed their phosphorylation states. Young and old (preexisting) molecules were distinguished by radioactive labeling for various times. DNA-binding experiments were performed under stringent but physiological conditions to separate molecules with high and low affinities for origin sequences (termed DNA-binding and nonbinding fractions, respectively). Our study revealed the following relationships. (i) Subclasses with a high DNA-binding activity contained a large proportion of young large T molecules; they were in monomeric form and phosphorylated to a low degree. (ii) Nonbinding subclasses consisted mainly of old molecules; they were in oligomeric form and phosphorylated to a higher degree.

However, the separation of the individual subclasses was not complete. Not all young large T molecules were in the DNA-binding fraction or in monomeric form, and not all old molecules were in the nonbinding fraction or in oligomeric form. There are probably several reasons for this cross-contamination. (i) Young molecules were defined by a 4-h label with [^{3}H]leucine. A fraction of these molecules might have been converted to old molecules during the labeling period. This assumption is consistent with the finding that DNA binding was more efficient with molecules labeled for 1 h instead of 4 h (70 versus 50%). The relatively long 4-h labeling period was chosen to obtain sufficient amounts of radiolabeled material. It was short enough, however, to reveal the differences between young and old large T molecules. On the other hand, the 4-h chase after the 17-h labeling period might have been too short to convert all the young molecules to old ones. This would explain why 20% of the old molecules bound to DNA. In fact, after longer chases of 6 to 10 h, the DNA-binding fraction decreased to 5% of the total (unpublished data). (ii) The relatively large fraction of young large T in the nonbinding subclass may in part be the result of inactivation. When extracts were kept at 0°C overnight or at −20°C for 1 week, the binding activity of young large T was almost completely lost, whereas a fraction of the old molecules was still capable of binding (unpublished observation). (iii) Part of the cross-contamination may reflect turnover and interconversion between different forms of large T (see below).

The phosphopeptide maps of the various subclasses revealed two important results. (i) They clearly show the relationship between different subclasses, i.e., the DNA-binding, monomeric nuclear high-salt fractions on one hand and the nonbinding, oligomeric fractions on the other; (ii) they demonstrate how young and old large T molecules differ, suggesting that their different phosphorylation states are the basis of their different properties, particularly their DNA-binding behavior. The greater complexity of the phosphopeptide patterns of young large T subclasses probably derives from superimposition of the patterns of young and old large T molecules, which are both present in about equimolar amounts in socalled young large T, as was pointed out above. This interpretation is supported by the finding that in the presence of cycloheximide, the 5S, or nuclear high-salt, fractions gave rise to the typical phosphorylation pattern of old rather than young large T. Furthermore, in a cytoplasmic variant of large T encoded by adenovirus 7-SV40 hybrid virus PARA(cT) (23), only phosphopeptides 4, 6, 7, 12, and 13 were found to be phosphorylated (K. H. Scheidtmann et al. submitted for publication). It appears that some of these "cytoplasmic sites" (phosphopeptides 6, 12, and 13) are specifically enriched on peptide maps of young large T. Phosphorylation at additional sites (in the nucleus) then gives rise to the typical pattern of old large T. There may be intermediate phosphorylation stages which presently cannot be distinguished.

Relationship between phosphorylation and oligomerization. A causal relationship between phosphorylation and oligomerization is suggested by previously published findings. The oligomeric forms of large T can be dissociated to monomers and reassembled, whereas newly synthesized monomers cannot form oligomers in vitro (31). This finding implies that newly synthesized monomers are qualitatively different from monomers derived from oligomers in vitro. Since modifications such as ADP ribosylation and acylation can only account for a minute subfraction of large T (16, 21), phosphorylation seems more likely to be involved in the conversion of monomers to oligomers. Baumann and Hand (3) reported aggregation of the large T-related D2 protein after in vitro phosphorylation. The finding that a small fraction of old molecules always appeared as monomers, even when pulse-labeling was followed by a 10-h chase period (unpublished result), might reflect a recycling process in which monomers are regenerated from oligomers by specific dephosphorylation. Alternatively, there might be an equilibrium between mono- and oligomers, even in the fully phosphorylated state.

Relationship between oligomerization and DNA binding. Recently it was reported that the oligomeric (especially dimeric) but not the monomeric form of large T binds to SV40 DNA, suggesting that oligomerization is a prerequisite for DNA binding (4). Previous DNA-binding studies have shown that young large T in the 5S to 7S form binds better and more specifically to viral DNA than do older molecules in the 15S form (12, 13, 39). A distinction between monomers (5S) and dimers (7S) was not made in these studies. We found that the monomeric form had the highest DNA-binding activity. Fanning and co-workers have obtained similar results (E. Fanning, personal communication). The discrepancy with the aforementioned findings could be attributed in part to different assay conditions. At lower pH or salt concentration, oligomers bind to viral DNA as well as monomers do (12; unpublished data). In addition, we observed that the DNA-binding activity of young large T rapidly fades as the extract ages, whereas the binding activity of oligomers appeared to be much more stable. This could explain the failure of other investigators to detect binding of monomers from partially purified large T preparations. Our data do not imply that large T oligomers do not bind in general. As pointed out above, the term nonbinding applies only for the stringent conditions under which binding was strongly reduced, indicating that they have a lower affinity. This may be of functional significance (see below).

Relationship between phosphorylation and DNA-binding activity. Our studies demonstrate that the low phosphorylation state of newly synthesized large T correlates with its

high ability to bind to viral DNA. This is in contrast to an earlier report claiming that highly phosphorylated large T has a higher affinity for DNA than does large T in a low phosphorylation state (30). We could not confirm this finding under any conditions; we always observed a lower phosphate content for the DNA-binding fraction. However, this correlation was less pronounced or even negligible when binding experiments were performed at a lower pH or salt concentration (see above) or with DNA from calf thymus (M. Hardung, M.D. thesis, Universität Freiburg, Freiburg, Federal Republic of Germany, 1983). Possibly phosphorylation has different effects on large T depending on whether it interacts with viral or cellular DNA. The amino-terminal phosphorylation sites are encoded between 0.508 and 0.520 map units (43) in a region required for origin-specific binding (32, 38, 50), whereas sequences further downstream encoded between 0.39 and 0.44 map units are involved in unspecific binding to cellular DNA (29, 38). Thus, it is conceivable that phosphorylation at the amino-terminal sites affects the interaction of large T with the origin but not with other DNA sequences. Indeed, the differences in the phosphorylation patterns of the DNA-binding and nonbinding fractions occurred mainly in the amino-terminal sites.

DNA contact regions have been studied extensively in procaryotic DNA-binding proteins, i.e., repressor proteins of bacteriophage and *Escherichia coli* (33, 42). These studies revealed several characteristic features of DNA-binding regions. (i) They are in a typical helix-turn-helix conformation, facilitating binding to the major groove of DNA. (ii) Binding to the phosphate backbone is usually unspecific. (iii) Sequence specificity of binding is provided mainly by hydrogen bonding between the side chains of hydrophilic amino acids and purine and pyrimidine bases. (iv) Interestingly, serine and threonine residues (besides glutamine, histidine, arginine, and lysine) are often involved in specific binding. Phosphorylation of these residues would certainly alter the protein-DNA interaction. (v) There seems to be a consensus sequence, Ser-Gln-X-Ser-Arg, in the contact regions of several repressor proteins. Large T contains a similar sequence, Ser-120-Gln-His-Ser-Thr-124, which is partly present in phosphopeptides 7, 11, and 12; it contains Thr-124 in peptide 12 and, in addition, Ser-123 in peptides 7 and 11 as phosphorylated residues. It is conceivable that this region participates in the specific interaction of large T with the origin of replication and that sequential phosphorylation of Thr-124 and Ser-123 alters this interaction. This would also explain the pH dependence of the DNA-binding reaction, since above pH 7 the phosphate residues acquire an additional negative charge.

Significance of the nuclear high-salt fraction of large T. The fraction of large T extracted from nuclei with high salt had the highest DNA-binding activity, showed a phosphorylation pattern characteristic of young large T, and was in monomeric form. This subclass could be associated with viral (or cellular) chromatin (25, 36, 40, 47) or with the nuclear matrix (5, 52). Staufenbiel and Deppert (52) demonstrated that chromatin-associated large T has a relatively short half-life of about 2 h, whereas the nuclear matrix association seems to be rather stable. In our pulse-chase experiment we also found chasable and stable fractions which might correspond to chromatin- and matrix-bound large T, respectively. A preliminary analysis of large T associated with viral nucleoprotein complexes revealed a phosphopeptide pattern similar to that of the DNA-binding fraction of soluble large T, suggesting a correlation between phosphorylation state and functional state.

DNA binding and functions of large T. Binding of large T to the origin region mediates the initiation of DNA replication (50), repression of early gene transcription late in infection (2, 57), and stimulation of late gene transcription (8, 35). These functions might be performed by different subclasses. Viral DNA replication depends on the continuous synthesis of new large T molecules (66). Thus, initiation of DNA replication is probably mediated by newly synthesized monomers in a low phosphorylation state. Their tight binding to DNA might serve to direct the cellular replication machinery to the replication origin. Tegtmeyer et al. (55) argued that tight binding would abolish replication and that large T must either be released or become part of the replication complex to allow chain elongation. Both these processes could be accomplished by phosphorylation. Perhaps phosphorylation in situ leads to oligomerization and complex formation, thereby allowing reversible interaction with DNA. Since phosphorylation itself is reversible (10, 45, 63), specific dephosphorylation might restore the original functional state.

Oligomeric large T might play a role in regulating early transcription. Phosphorylation and subsequent oligomerization appear to have two effects. (i) They lead to stabilization and accumulation of large T; temperature-sensitive large T does not form oligomers and has a high turnover at the restrictive temperature (11, 57). (ii) They reduce its affinity to viral DNA (12, 14, 32; this study). Accumulation of large T molecules late during infection leads first to a shift in the initiation site of early mRNA and then to repression of early transcription (2, 17, 57). However, a certain level of transcription must be maintained to allow the production of new large T molecules for DNA replication (see above). This could be achieved by the weaker, reversible binding of highly phosphorylated tetramers. Temperature shift experiments with *tsA*-infected cells have shown that autoregulation of early transcription ceases at about the same rate as oligomers dissociate (2, 11). Based on these considerations, we suggest that phosphorylation and oligomerization of large T do not simply lead to inactivation, but play a role in a well-balanced replication system.

Based on our data, the following life cycle for large T could be envisioned. After synthesis, large T is phosphorylated at certain sites in the cytoplasm, e.g., Ser-111, Thr-124, and Thr-701. It is then transported into the nucleus. Since it is produced in large quantities, only a fraction of the molecules participates in viral DNA replication; these bind to viral DNA as monomers while still in a low phosphorylation state. Subsequent phosphorylation at additional sites, e.g., Ser-123, might then modulate the interaction with DNA or with enzymes involved in replication. This may lead to aggregation and dissociation from the DNA. After dephosphorylation and disaggregation, these molecules could enter a new functional cycle. The majority of the molecules might be directly phosphorylated and converted to oligomers. They might play a role in regulating early and late gene expression.

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