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Removal of Serine Phosphates from Simian Virus 40 Large T Antigen Increases Its Ability To Stimulate DNA Replication In Vitro but Has No Effect on ATPase and DNA Binding

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The effect of phosphorylation on the ability of simian virus 40 large T antigen to stimulate DNA synthesis in vitro was tested. Treatment of affinity-purified large T antigen with calf intestinal alkaline phosphatase resulted in the removal of 70 to 80% of the phosphate residues. Only serine-bound phosphate residues were affected. Phosphatase-treated large T antigen stimulated in vitro DNA synthesis fourfold over the untreated control. The stimulation was strongest at early times of DNA replication. At later times, DNA replication proceeded at equal rates with dephosphorylated and untreated large T antigen. The ATPase activity of large T antigen was not affected by phosphatase treatment. The origin-binding activity of large T antigen was tested over a wide range of large T antigen to DNA ratios, including DNA excess, and in the presence and absence of carrier DNA. Under no condition was an effect of dephosphorylation of large T antigen on its DNA-binding activity observed. These findings might indicate that phosphorylation at serine residues modulates the interaction of large T antigen with cellular factors. During DNA synthesis large T antigen was substantially rephosphorylated by kinases in the HeLa cell extract. As shown by two-dimensional peptide mapping, this phosphorylation occurred at all known in vivo sites. No phosphatase and protease activities were detectable in the HeLa cell extract.

Simian virus 40 (SV40) large T antigen is phosphorylated at serine and threonine residues clustered in two regions of the polypeptide chain. One region is located between Ser-106 and Thr-124 in the amino-terminal part of the molecule; the other is located between Ser-639 and Thr-701 toward the carboxy terminus (17, 33). The phosphate residues are added to large T antigen in a stepwise fashion, some in the cytoplasm and others after its transport to the nucleus (19, 25). They differ markedly in turnover rates; serine phosphates turn over faster than threonine phosphates (16, 34). It appears that serine phosphates are attached to large T antigen by cytoplasmic kinases and that threonine phosphates are attached by nuclear protein kinases.

The amino-terminal phosphate residues are not located within the DNA-binding domain, which maps between amino acids 139 and 223 on the polypeptide (15, 24). However, phosphorylation might exert an indirect effect on DNA binding. The role of phosphorylation in the binding of large T antigen to the origin of DNA replication has been studied by several investigators, but the results from these studies are conflicting. Whereas Shaw and Tegtmeyer (22) found that removal of serine phosphates with alkaline phosphatase had no effect on binding, Simmons et al. (26) reported a 1.5- to 2-fold increase in origin-binding activity after removal of phosphates with the same enzyme. Baumann, on the other hand, observed no effect of alkaline phosphatase but found a significant decrease in origin-specific binding after removal of serine and threonine phosphates with potato acid phosphatase (1). These studies support the previous notion that phosphorylation at Thr-124 might be a prerequisite for DNA binding as measured by the McKay assay (13, 18). Genetic studies do not support the idea that phosphorylation is important in controlling origin binding of large T antigen. Removal of the amino-terminal serine and threonine residues by site-directed mutagenesis had no effect on DNA binding in vitro, although it severely hampered DNA replication in vivo (7).

There is no evidence that phosphorylation at serine or threonine residues plays a role in the ATPase activity of large T antigen (1), and its transforming properties are not affected by the phosphorylation of Ser-111, Ser-112, Ser-123, and Thr-124, as shown by site-directed mutagenesis of these potential phosphorylation sites. However, a decreased transformation activity was observed after removal of the phosphorylation site Ser-106 (7).

The recent development of a cell-free system for SV40 DNA replication made it possible to search for new functions of large T antigen and to study the potential role of phosphorylation in such functions (9, 10, 29, 35). Stillman and collaborators isolated a mutant form of large T antigen which behaved like the wild type with respect to ATPase and origin-binding activity but failed to support DNA replication in vitro (30). These findings indicated that an additional function besides ATPase and origin-binding activity is required for replication. Smale and Tjian reported that in vitro SV40 DNA replication was inhibited by certain monoclonal antibodies against large T antigen which had no effect on ATPase and origin binding (27). However, these antibodies prevented the formation of a complex between large T antigen and DNA polymerase α, suggesting that the ability of large T antigen to interact with polymerase α might be essential for the initiation of SV40 DNA replication.

We studied the effect of dephosphorylation of large T antigen on its ability to support DNA replication in vitro. We found that dephosphorylation of large T antigen with alkaline phosphatase, which removed phosphate from serine but not threonine residues, had a pronounced stimulatory effect on DNA replication, whereas ATPase and origin-binding activity were not altered by this treatment. The effect was strongest at early times of the replication reaction and

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seemed to shorten the lag that precedes maximal DNA synthesis.

MATERIALS AND METHODS

Cell lines and viruses. Cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Irvine Scientific). Large T antigen was prepared from confluent layers of 293 cells infected with a recombinant adenovirus type 5-SV40 hybrid virus (AdSVR111) supplied by Y. Gluzman, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. In the AdSVR111 virus, the early (E1) region of the adenovirus type 5 was replaced by the early region of SV40 virus fused to the adenovirus type 5 major late promoter (5). Confluent monolayers of 293 cells were infected at a multiplicity of about 5 PFU per cell. The PAb419 cell line produces monoclonal antibodies against an epitope in the N-terminal region of SV40 large T antigen (6).

Extraction, purification, and metabolic labeling of large T antigen. Cell extracts from 293 cells infected with the AdSVR111 virus were prepared 24 to 28 h postinfection. Approximately 10^9 cells were lysed with 20 ml of lysis buffer containing 20 mM Na_2HPO_4, (pH 9.0), 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 50 μM Leupeptin (Sigma Chemical Co.), and 10 μg of phenylmethylsulfonyl fluoride per ml. Large T antigen from 10^9 cells was bound to 0.5 ml of protein A-Sepharose (Pharmacia Fine Chemicals) that contained covalently bound PAb419 (21). The protein A-Sepharose containing bound PAb419 is referred to herein as PAb419 resin. The PAb419 resin was washed with buffers of increasing ionic strength and pH (3, 4, 23). Large T antigen was eluted at pH 10.7 and 750 mM NaCl. The eluate was neutralized with 0.1 M glycine hydrochloride (pH 2.4) and concentrated in an Amicon ultrafiltration unit to a protein concentration of 0.4 to 1.2 mg/ml. Protein concentrations were determined by the method of Bradford with bovine serum albumin (Sigma) as a standard (2). The final storage buffer consisted of 20 mM Tris hydrochloride (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 50% glycerol. The preparations of large T antigen were usually >95% pure as judged from Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels (8) but contained various amounts of an N-terminal fragment of approximately 19 to 20 kilodaltons. This fragment was presumably generated through incorrect splicing and premature termination of translation of the large-T-antigen mRNA (14). Samples of 2×10^7 293 cells infected with the AdSVR111 virus were metabolically labeled 24 to 28 h postinfection with 1 mCi of $H_3{}^{32}PO_4$ (ICN Pharmaceuticals Inc.) in 3 ml of phosphate-free Dulbecco modified Eagle medium supplemented with 2% phosphate-free fetal calf serum.

Dephosphorylation of large T antigen with calf intestinal alkaline phosphatase. Dephosphorylation was carried out on large T antigen bound to PAb419 resin. A 0.5-ml sample of PAb419 resin was washed with phosphatase buffer containing 20 mM Tris hydrochloride (pH 8.5), 100 mM NaCl, and 5 mM $MgCl_2$. The reaction was started by the addition of 0.5 ml of phosphatase buffer containing 500 U of calf intestinal alkaline phosphatase (Sigma) followed by a 15-min incubation at 37°C under agitation of the reaction vessel. The reaction was stopped by the addition of 10 ml of ice-cold lysis buffer. After several washes with lysis buffer, large T antigen was eluted from the PAb419 resin and concentrated as described above. To determine the extent of dephosphorylation, in vivo ^{32}P-labeled large T antigen was treated with calf intestinal alkaline phosphatase as described and analyzed on SDS-polyacrylamide gels. Large T antigen not treated with the enzyme was used as a control. The removal of radioactive label was determined by liquid scintillation spectrometry of the excised and solubilized gel bands corresponding to large T antigen. The dephosphorylation of specific phosphorylation sites of large T antigen was determined by two-dimensional phosphopeptide analysis of metabolically ^{32}P-labeled large T antigen.

Two-dimensional phosphopeptide analysis. Phosphopeptide analysis was carried out as described previously (18). Briefly, immunopurified large T antigen was analyzed on 12.5 or 15% SDS–polyacrylamide gels and eluted from the unfixed, dried gels with a buffer containing 50 mM NH_4HCO_3 and 0.1% SDS. After trichloroacetic acid precipitation and oxidation with 3% performic acid, large T antigen was sequentially digested with trypsin and pronase E. The digestion mixture was applied to the middle of thin-layer cellulose plates (Merck & Co., Inc.) and analyzed 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 25 min. Ascending chromatography was in isobutyric acid buffer (isobutyric acid-pyridine-acetic acid-butanol-water, 65:5:3:2:29) for 6 h. The phosphopeptides were visualized by autoradiography with intensifying screens. To determine the content of phosphoserine and phosphothreonine of dephosphorylated large T antigen, the above digestion mixture was incubated with 6 N HCl at 110°C for 90 min. The products were lyophilized over NaOH pellets and analyzed by electrophoresis on thin-layer cellulose plates in pH 1.9 buffer for 40 min at 1,000 V. The phosphoamino acids were visualized by autoradiography.

ATPase activity. ATPase assays were carried out in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2)–100 mM NaCl–5 mM $MgCl_2$–0.01% Nonidet P-40–20 μM [α-^{32}P]ATP (2 to 5 μCi; ICN) at 37°C. Conversion of ATP to ADP was determined by spotting 1-μl samples of the reaction mix on phosphoethyleneimine-cellulose plates (Merck) that were developed in 0.75 M KH_2PO_4 (pH 3.5). The reaction products were visualized by autoradiography, and the amount of ATP and ADP was determined by liquid scintillation spectrometry. The specific activity was expressed as conversion of nanomoles of ATP per hour per milligram of large T antigen.

DNA binding. DNA-binding experiments were carried out by the method of McKay (13). SV40 DNA was a gift from Ben Tseng. A 1-μg sample of SV40 DNA was digested with 8 U of restriction endonuclease *Ava*II (Boehringer Mannheim Biochemicals) in a volume of 30 μl for 4 h at 37°C as recommended by the supplier. The fragments were end labeled with 5 U of the Klenow fragment of DNA polymerase (New England BioLabs, Inc.) and 10 μCi of [α-^{32}P]dGTP as described by Maniatis et al. (12). Before the DNA binding, 0.7 μg of purified large T antigen was dephosphorylated with 3 U of calf intestinal alkaline phosphatase for 15 min at 37°C in 50 μl of phosphatase buffer. As a control, the enzyme was omitted from the reaction. Various amounts of untreated or dephosphorylated large T antigen were added to the DNA-binding assays. The binding buffer contained 20 mM NaH_2PO_4 (pH 7.0), 0.1 mM EDTA, 2 mM dithiothreitol, 0.05% Nonidet P-40, 0.01% bovine serum albumin, 4 ng of ^{32}P-labeled SV40 fragments, and 800 ng of sheared salmon sperm DNA, when present. The binding reactions were allowed to proceed at room temperature for 60 min. The large T antigen-DNA complex was removed from the mixture by sequential addition of 20 μl of PAb419 and 40 μl of

10% (wt/vol) formaldehyde-fixed *Staphylococcus aureus* (Calbiochem-Behring). The immunocomplexes were washed twice with 10 mM Tris hydrochloride (pH 8.0) containing 150 mM NaCl and 0.5% Nonidet P-40. The DNA was released from the bacteria with 50 mM Tris hydrochloride (pH 6.8)–5 mM EDTA–2.5% SDS–10 mM dithiothreitol–20% β-mercaptoethanol–5% glycerol, heated at 65°C for 15 min, and analyzed on 1.5% agarose gels as described previously (18). The amount of origin fragment was determined by scintillation spectrometry of the excised gel bands.

DNA replication. HeLa cell extract and plasmid pKHSO were generously provided to us by Joachim Li and Thomas Kelly, Jr. Plasmid pKHSO contains the complete SV40 origin of DNA replication cloned into plasmid pKP55, originally designed by Keith Peden (11). The standard reaction mixture contained 30 mM HEPES (pH 7.5), 7 mM $MgCl_2$, 4 mM ATP, 40 mM phosphocreatine, 0.1 mg of creatine kinase (Boehringer) per ml, 15 mM Na_2HPO_4 (pH 7.7), 25 μM [α-^{32}P]dCTP (2.5 μCi; ICN), 100 μM (each) ribonucleoside and deoxyribonucleoside triphosphates, 30 ng of plasmid pKHSO, 75 μg of HeLa cell extract, and various amounts of untreated or dephosphorylated large T antigen in a total volume of 25 μl. The reactions were started by adding large T antigen and raising the temperature to 37°C. The specific incorporation of deoxyribonucleoside triphosphates into acid-precipitable material was determined by liquid scintillation spectrometry. The reactions were stopped at various times by the addition of SDS, EDTA, and proteinase K (Boehringer) to final concentrations of 0.4%, 15 mM, and 0.2 mg/ml, respectively. The purified DNA was analyzed on 1.4% agarose gels and visualized by autoradiography of the dried gels.

Rephosphorylation of large T antigen during DNA replication. Rephosphorylation of large T antigen during the replication reaction was analyzed by addition of [γ-^{32}P]ATP (5×10^4 cpm/pmol; Amersham Corp.) to the standard replication assay containing 0.8 μg of dephosphorylated large T antigen. Since the in vitro system contained a high concentration of ATP, it was necessary to add a large amount of radioactive ATP to get large T antigen sufficiently labeled for peptide mapping. The reaction was stopped after 2 h by the addition of 0.5 ml of lysis buffer. Large T antigen was reisolated by immunoprecipitation and subjected to phosphopeptide analysis. The half-life of γ-^{32}P-labeled ATP in the replication mixture containing 10 μCi of [γ-^{32}P]ATP per 25 μl was determined by spotting 1-μl samples of the mixture at various times on phosphoethyleneimine-cellulose plates. Analysis of the reaction products was as described above. To test for the presence of phosphatase activity in the replication mixture, large T antigen metabolically labeled with ^{32}P was bound to PAb419 resin and incubated for 2 h with the replication mixture. As a control, HeLa cell extract was omitted from the reaction. Large T antigen was released from the Sepharose by boiling with gel buffer and analyzed on a 15% SDS–polyacrylamide gel. The amount of radioactive label present in the bands corresponding to large T antigen was determined as described above.

RESULTS

Removal of phosphate from serine residues in SV40 large T antigen has no effect on its ATPase and DNA-binding activity. The primary goal of this investigation was to study the possible role of phosphorylation of SV40 large T antigen in SV40 DNA replication. This was done by treating large T antigen with alkaline phosphatase and assaying it in an in vitro replication system. In parallel, the effect of phosphatase treatment on ATPase and origin-binding activity was also measured. The dephosphorylation reaction was carried out with T antigen bound to monoclonal antibodies covalently attached to protein A-Sepharose. The ATPase and DNA replication experiments were conducted with large T antigen released from the monoclonal antibodies by high pH treatment as described in Materials and Methods. Figure 1 shows a two-dimensional phosphopeptide map of alkaline phosphatase-treated large T antigen isolated from ^{32}P-labeled cells infected with the adenovirus type 5-SV40 hybrid virus. Only three phosphopeptides, designated with numbers 12, 12a, and 13, were resistant to phosphatase (Fig. 1b). The map of untreated large T antigen is shown in Fig. 1a. As demonstrated previously (19), the phosphatase-resistant peptides 12 and 13 contain Thr-124 and Thr-701, respectively. We assume that 12 and 12a represent overlapping peptides both containing phosphothreonine 124. Peptide 12a is more intense relative to 12 in the map of untreated

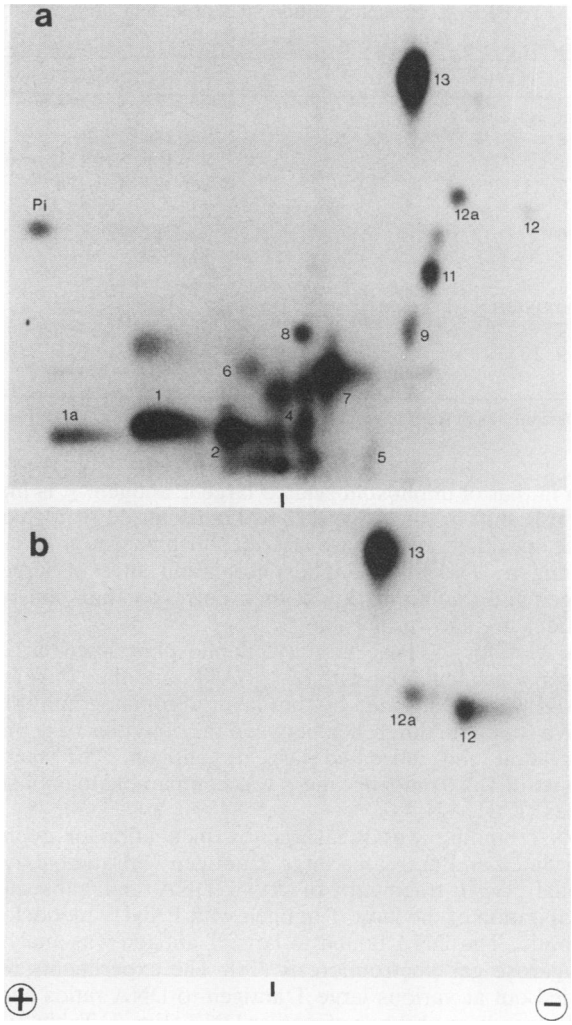

FIG. 1. Phosphopeptide analysis of dephosphorylated large T antigen. Large T antigen, metabolically labeled with ^{32}P, was dephosphorylated with calf intestinal alkaline phosphatase and analyzed by two-dimensional peptide mapping as described in Materials and Methods. Samples were applied to the middle of the thin-layer cellulose plates as indicated by the vertical dashes. (a) Untreated; (b) dephosphorylated.

TABLE 1. Phosphorylation sites of large T antigen 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 reference 18.

than in that of dephosphorylated large T antigen. It is likely that this shift in the ratio of 12 to 12a is caused by a change in the specificity of pronase after dephosphorylation of large T antigen. The in vivo phosphorylation sites of large T antigen and the numbering of their corresponding phosphopeptides are shown in Table 1.

To test for ATPase activity, dephosphorylated large T antigen was incubated with [α-^{32}P]ATP, and the conversion to ADP was determined by thin-layer chromatography (Fig. 2). We found no difference between the activities of dephosphorylated and untreated large T antigen. The specific activity of 1,200 nmol h^{-1} mg^{-1} was comparable to published values (1, 31, 32).

DNA binding was measured by incubation of dephosphorylated and untreated large T antigen with radioactively labeled *Ava*II fragments of SV40 DNA and subsequent precipitation of the large T antigen with PAb419 monoclonal antibody. The DNA bound to large T antigen was analyzed by agarose gel electrophoresis (18). The experiments were carried out at various large T antigen-to-DNA ratios and in the presence or absence of carrier DNA (Fig. 3, Table 2). At a large T antigen-to-DNA ratio of 30:1 (wt/wt) in the presence of carrier DNA, about 50% of the origin fragment was

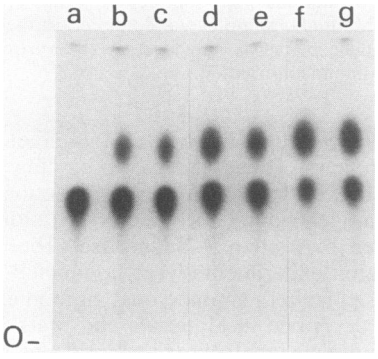

FIG. 2. ATPase activity of dephosphorylated large T antigen. Samples (0.8 μg) of dephosphorylated and untreated large T antigen were assayed for their ability to hydrolyze ATP as described in Materials and Methods. Samples (1 μl) of the reaction mixtures were spotted on phosphoethyleneimine-cellulose plates at various times of the reaction. The plates were developed in 0.5 M KH_2PO_4 (pH 3.5). Reaction products were visualized by autoradiography. Lanes: a, control (no large T antigen); b, d, and f, hydrolysis by dephosphorylated large T antigen for 15, 30, and 60 min, respectively; c, e, and g, hydrolysis by untreated large T antigen for 15, 30, and 60 min, respectively. O, Origin.

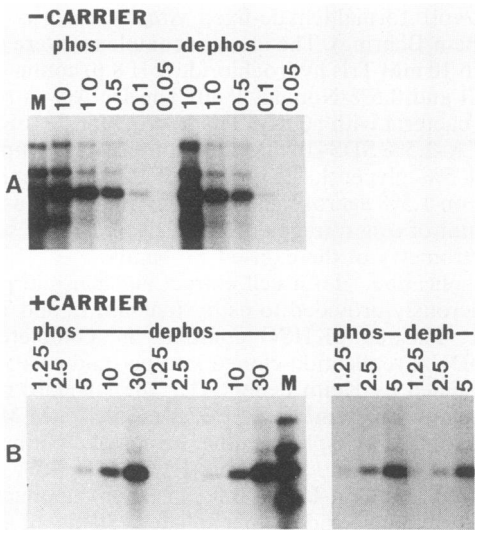

FIG. 3. DNA binding of large T antigen dephosphorylated with calf intestinal alkaline phosphatase. Samples (4 ng) of ^{32}P-labeled *Ava*II-cleaved SV40 DNA were incubated with various amounts of purified large T antigen. For example, the number 30 indicates a ratio of 120 ng of large T antigen to 4 ng of labeled SV40 DNA fragments. Carrier DNA (800 ng) was added as indicated. The samples on the right in panel B represent a longer exposure of the corresponding samples on the left. M is the marker track representing one-half of the labeled fragments added to each assay.

bound to large T antigen. When the ratio was lowered to 1.25:1, the amount of bound origin fragment dropped to 0.5%, indicating that at this ratio the amount of origin fragment was in large excess over that of large T antigen. At all large T antigen-to-SV40 DNA ratios tested in the pres-

TABLE 2. Origin binding by untreated and dephosphorylated large T antigen[a]

T/DNA ratio[b]	Phosphatase	Carrier[c]	Bound origin fragment (%)[d]
30	−	+	49
30	+	+	46
10	−	+	17
10	+	+	19
5	−	+	5
5	+	+	4
2.5	−	+	2
2.5	+	+	1
1.25	−	+	0.5
1.25	+	+	0.5
10	−	−	64
10	+	−	76
1	−	−	44
1	+	−	36
0.5	−	−	28
0.5	+	−	28
0.1	−	−	6
0.1	+	−	3
0.05	−	−	1.5
0.05	+	−	1.5

[a] Data were obtained from the experiment shown in Fig. 3. The amounts of ori fragment were determined by scintillation spectrometry of the excised gel bands and by scanning of the autoradiogram.

[b] DNA was constant at 4 ng of ^{32}P-labeled *Ava*II-cleaved SV40 DNA, and the amount of large T antigen was varied.

[c] Carrier was 800 ng of salmon sperm DNA.

[d] Percent bound of ^{32}P-labeled origin fragment in the assay.

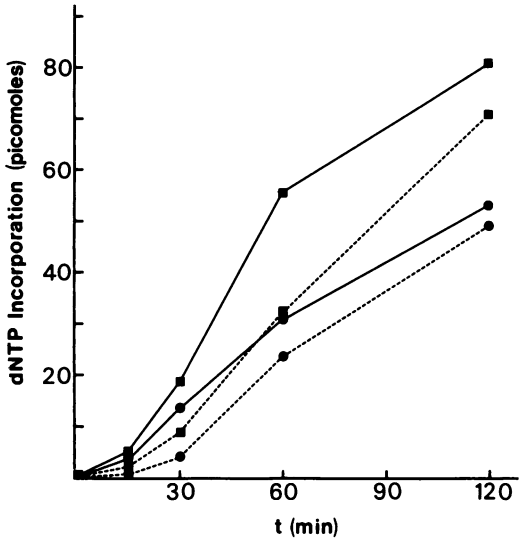

FIG. 4. Stimulation of in vitro DNA replication by dephosphorylation of SV40 large T antigen. In vitro DNA replication was started by the addition of various amounts of dephosphorylated or untreated large T antigen. Incorporation of deoxynucleotide triphosphates was determined by liquid scintillation spectrometry of acid-insoluble material collected at the times indicated. Symbols: (■——■) 0.7 μg of dephosphorylated large T antigen, (●——●) 0.3 μg of dephosphorylated large T antigen, (■---■) 0.7 μg of untreated large T antigen, (●---●) 0.3 μg of untreated large T antigen.

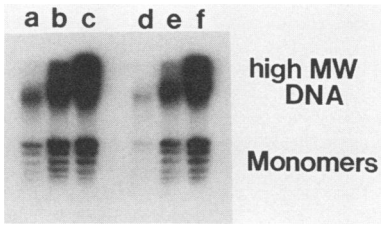

FIG. 5. Product analysis of DNA replication stimulated by dephosphorylation of SV40 large T antigen. Purified dephosphorylated or untreated large T antigen was used in the in vitro replication assay. DNA synthesis was terminated after various times of incubation, and the reaction products were analyzed on a 1.4% agarose gel. The ladder of bands corresponds to monomeric plasmid molecules with different degrees of supercoiling. The high-molecular-weight DNAs represent undissolved, catenated molecules. Lanes: a, b, and c, reaction products with dephosphorylated large T antigen at 30, 60, and 120 min, respectively; d, e, and f, controls at 30, 60, and 120 min, respectively.

ence of carrier DNA, no significant difference in origin binding between untreated and dephosphorylated large T antigen was observed. In the absence of carrier DNA, the protein-to-DNA ratio was varied from 10:1 to 0.05:1. Again, there was no difference between untreated and dephosphorylated large T antigen in the ability to bind origin fragment. The level of origin binding was generally higher in the absence than in the presence of carrier. For example, at a ratio of 10:1, about 65% of the origin fragment was bound without carrier and only 17% was bound with added carrier DNA. This result indicates that carrier DNA competes with the origin for binding to large T antigen. We also noticed that in the absence of carrier and at high protein-to-DNA ratios, high amounts of the nonorigin fragments were bound to large T antigen (Fig. 3), and that decreasing the amount of large T antigen resulted in more specific binding of the origin fragment. This result may be explained by assuming that the nonorigin fragments compete with the origin fragment in binding to large T antigen.

Enhanced initiation of in vitro SV40 DNA replication by dephosphorylated large T antigen. After finding that the origin binding and ATPase activities of large T antigen were independent of its phosphorylation state, we asked if its ability to support DNA replication in the in vitro replication system described by Li and Kelly (9) was affected by phosphatase treatment. It seemed possible that an interaction of large T antigen with some cellular factors or enzymes required for SV40 DNA replication was influenced by phosphorylation (27). A time course of DNA replication with two concentrations of dephosphorylated and untreated large T antigen is shown in Fig. 4. The data clearly demonstrate a stimulatory effect of dephosphorylation which was two- to fourfold after 15- and 30-min reactions and was higher at the lower concentration of large T antigen. At later times, the rates of DNA synthesis were similar with dephosphorylated and untreated large T antigen, as illustrated by the nearly parallel time courses. The stimulation of DNA replication at early times was also apparent when the reaction products were analyzed on a 1.4% agarose gel (Fig. 5). The dependence of the stimulatory effect on the concentration of large T antigen was further demonstrated by the results shown in Fig. 6. When the concentration of large T antigen was decreased from 0.8 to 0.1 μg, the stimulation of replication caused by the dephosphorylation increased from twofold to fourfold, respectively.

Phosphorylation of large T antigen by HeLa cell extract. One would have expected that dephosphorylated large T antigen might have an advantage at each initiation event throughout the entire reaction. This should have led to a relatively higher amount of replicated DNA molecules

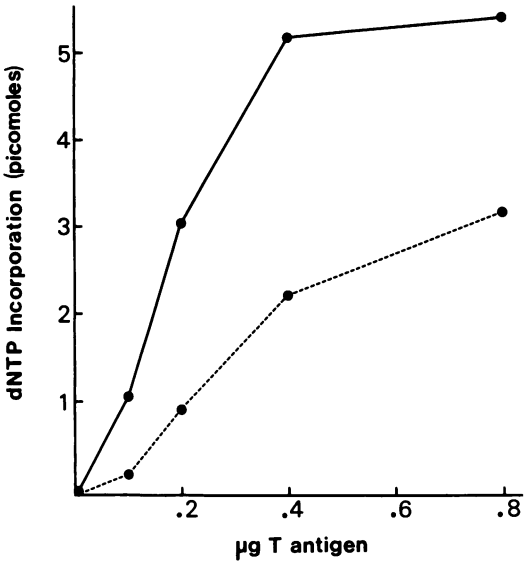

FIG. 6. Difference in stimulation of in vitro DNA replication between dephosphorylated and untreated SV40 large T antigen as a function of large-T-antigen concentration. Various amounts of untreated or dephosphorylated large T antigen were added to the in vitro DNA replication reaction. Incorporation of deoxynucleotides into acid-insoluble material after a 15-min reaction was determined by liquid scintillation spectrometry. Symbols: (●——●) dephosphorylated large T antigen, (●---●) control.

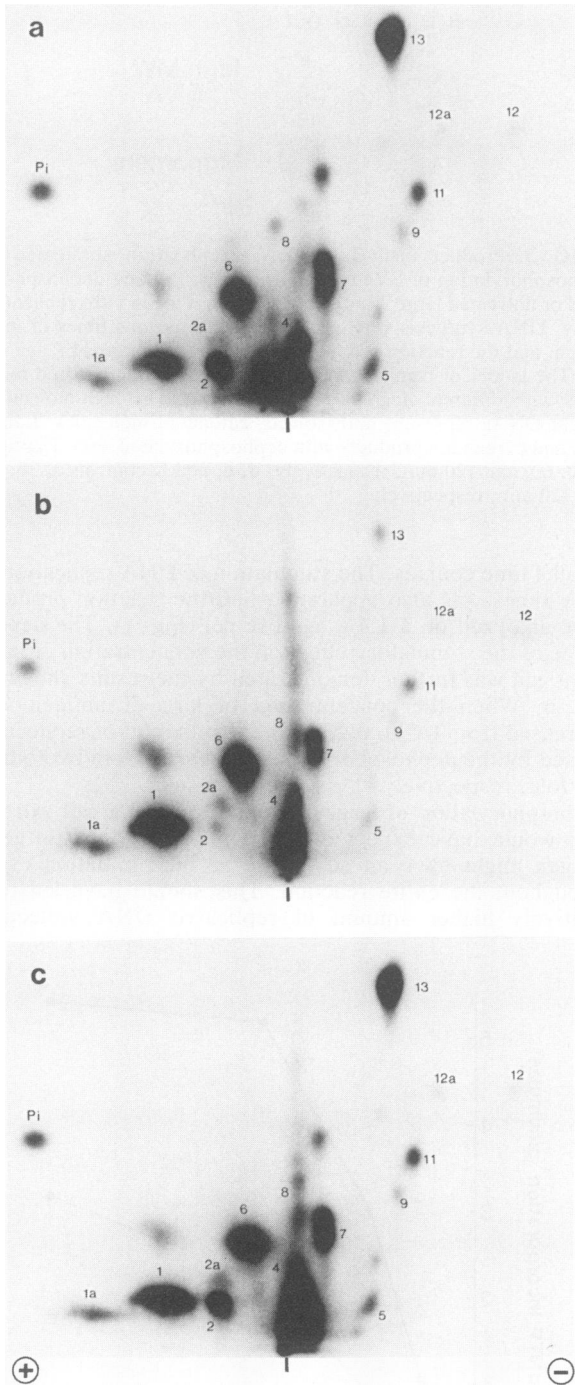

FIG. 7. Rephosphorylation of large T antigen by HeLa cell extract. Dephosphorylated large T antigen was added to the replication reaction supplemented with [γ-^{32}P]ATP. Large T antigen was reisolated after 2 h and analyzed by two-dimensional phosphopeptide mapping as described in Materials and Methods. In vivo-^{32}P-labeled large T antigen was used for a mixing experiment. Samples were applied to the thin-layer chromatography plates as indicated by the vertical dashes. (a) In vivo-^{32}P-labeled large T antigen; (b) in vitro-phosphorylated large T antigen; (c) mixture of a and b.

toward the end of the replication reaction in the presence of dephosphorylated large T antigen. It was surprising that after an initial lag phase, dephosphorylation had no further effect on DNA replication. However, it seemed possible that dephosphorylated large T antigen became quickly modified, that is, rephosphorylated by kinases in the HeLa cell extract and, therefore, lost the ability to initiate replication faster than untreated large T antigen. In preliminary experiments we found, indeed, that large T antigen became quickly rephosphorylated in vitro. Dephosphorylated large T antigen was incubated for 2 h with HeLa cell extract under the same conditions used for DNA replication, except that [γ-^{32}P]ATP was added. After the reaction, large T antigen was isolated by immunoprecipitation and subjected to peptide mapping. Figure 7b shows a phosphopeptide map of the in vitro-labeled large T antigen. The pattern is similar to the map of the in vivo-labeled protein (Fig. 7a). All the phosphopeptides found in vivo were present in the map of the in vitro-phosphorylated T antigen, although the incorporation into specific sites differed considerably. Ser-106 (peptides 1 and 1a), Ser-111 (peptides 4 and 6), and Ser-123 (peptide 7) were the major phosphate acceptors. Thr-124 (peptides 11, 12, and 12a), Thr-701 (peptide 13), Ser-639 (peptide 2), and Ser-676, -677, and -679 (peptides 8 and 9) were phosphorylated to a small extent. One reason for the low phosphorylation of Thr-124 and Thr-701 could be that the phosphate bound to these residues was not removed by the alkaline phosphatase (Fig. 1). We calculated that the amount of phosphate incorporated during the in vitro reaction was approximately 0.4 mol/mol of large T antigen. This may be an underestimation of the total amount incorporated, since the radioactive ATP became quickly degraded. The [γ-^{32}P]ATP added to the extract had a half-life of about 8 min (data not shown). If the higher activity of dephosphorylated large T antigen was lost because it became rephosphorylated during the lag phase of the in vitro replication reaction, then incubation of dephosphorylated large T antigen with HeLa cell extract before the start of DNA replication should reverse the effect of dephosphorylation. However, alkaline phosphatase-treated large T antigen retained its higher activity after preincubation with HeLa cell extract (data not shown). The reason might be that the extent of rephosphorylation of Ser-123 was not sufficient. The studies by Scheidtmann et al. (18, 19) and Baumann (1) indicated that phosphorylation at Ser-123 and Thr-124 might be crucial for the interaction of large T antigen with DNA. Although phosphorylation at Thr-124 could be the prerequisite for DNA binding, additional phosphorylation at Ser-123 (and possibly other residues) could downregulate DNA-binding activity in the presence of cellular factors (1, 18).

It was of interest to find out whether the HeLa cell extract contained active phosphatases in addition to protein kinases. This was determined by incubating in vivo-^{32}P-labeled large T antigen with HeLa cell extract for 2 h and measuring the removal of ^{32}P after isolation of the large T antigen by immunoprecipitation. There was no phosphatase activity in the extract under the conditions of DNA replication. Identical amounts of radioactivity were recovered from large T antigen incubated with extract and from the control sample that was not incubated with extract (data not shown). Since no peptide maps were determined for the ^{32}P-labeled large T antigen after the incubation with HeLa extract, we cannot exclude a small amount of phosphatase activity.

DISCUSSION

Our studies demonstrate that the removal of serine-bound phosphates from SV40 large T antigen enhances its ability to stimulate in vitro SV40 DNA replication but has no effect on its ATPase and origin-specific DNA-binding activities.

These findings are in agreement with a report from Shaw and Tegtmeyer, who showed by DNA footprinting and DNase protection experiments that alkaline phosphatase treatment of large T antigen had no effect on DNA binding (22). Similarly, Baumann found that DNA binding was unaltered by alkaline phosphatase (1). On the other hand, Simmons et al. reported a 1.5- to 2-fold stimulation of binding to the purified SV40 origin-containing fragment caused by alkaline phosphatase (26). They suggested that dephosphorylation produced more DNA-binding sites on large T antigen and also increased the rate of DNA binding. These authors carried out the DNA binding under DNA excess. Our DNA-binding studies were conducted under a variety of different conditions, including considerable variation of the large T antigen-to-SV40 DNA ratio (over 200-fold), and in the absence and presence of carrier DNA. We did not observe an effect of dephosphorylation on binding of large T antigen to the origin fragment under any conditions.

While our studies were in progress, we were informed of the recently published results of Mohr et al. (14). These authors found no effect of dephosphorylation of large T antigen with alkaline phosphatase on ATPase activity and only a "slight" stimulatory effect on DNA binding when using origin fragments containing large-T-antigen binding sites I and II. Interestingly, dephosphorylation caused a large increase in DNA binding to the isolated binding site II but not to the isolated binding site I. As in the present study, Mohr et al. found a significant stimulation of in vitro DNA replication resulting from dephosphorylation of large T antigen.

In a previous report by Scheidtmann et al. it was found that the DNA-binding activity of large T antigen correlated with a distinct phosphorylation state (18). Newly synthesized large T antigen was phosphorylated to a low degree and existed primarily in a monomeric form sedimenting with 5S. It had a high affinity for SV40 DNA as measured by binding to SV40 DNA cellulose. By contrast, "old" large T antigen was highly phosphorylated and predominantly existed in an oligomeric form with a lower affinity for SV40 DNA. Two-dimensional phosphopeptide analysis revealed distinct phosphopeptide patterns for new and old large T antigen. Based on these studies the following scheme for the life cycle of large T antigen was proposed: large T antigen becomes phosphorylated in the cytoplasm at certain sites (Ser-111, Ser-112, Thr-124, Thr-701). It is transported to the nucleus, where it binds to SV40 DNA while still in a low phosphorylation state. Subsequent phosphorylation at additional sites (Ser-123, Ser-106) then modulates the interaction with DNA, resulting in dissociation and oligomerization. According to this model, phosphorylation at Thr-124 might be a prerequisite for DNA binding (18, 20). Furthermore, removal of phosphate from Ser-106 and Ser-123 with alkaline phosphatase might activate the DNA-binding activity of large T antigen. To reconcile the findings of Scheidtmann et al. with the present data, one should consider the possibilities that SV40 large T antigen interacts with cellular factors which enhance its DNA-binding ability and that phosphorylation at Ser-123 and/or Ser-106 might interfere with the binding of these factors. In this case, removal of serine phosphates with alkaline phosphatase would have no effect on DNA binding in the absence of these factors. This might explain why we and others did not observe an effect of alkaline phosphatase on DNA binding since purified large T antigen was used in the experiments (1, 22). On the other hand, the DNA-binding studies of Scheidtmann et al. were carried out with crude extracts containing cellular factors, and this may be the reason why in this case the DNA-binding activity was dependent on the state of phosphorylation.

Stillmann et al. described a mutant of large T antigen (Lys-224 to Glu) which has wild-type levels of ATPase activity and binds to the replication origin but fails to support SV40 DNA replication in vitro (30). They suggested that the binding of large T antigen to a host factor is an essential step in the initiation of DNA replication, and that this mutant is defective in carrying out this interaction. Smale and Tjian reported that large T antigen binds to DNA polymerase α and that monoclonal antibodies that inhibit this binding also inhibit in vitro DNA replication (27). They suggested that this interaction might precede the binding of large T antigen to the origin of replication. It is possible that phosphorylation of large T antigen inhibits binding to polymerase α.

A large number of mutations have been introduced into the gene of large T antigen to localize regions in the polypeptide responsible for DNA replication and DNA binding. Interestingly, mutants affecting the phosphorylation sites Ser-106, Ser-123, and Thr-124 were defective for SV40 DNA replication in vivo. However, the large T antigens encoded by these mutants showed normal origin binding activity in vitro (7). These findings suggest that phosphorylation at these sites does not directly influence DNA binding but interferes with another property of large T antigen such as interaction with host factors. This interpretation is compatible with the finding that the DNA-binding domain on the large-T-antigen polypeptide maps between amino acids 139 and 223, that is, downstream of the amino-terminal phosphorylation sites.

Recently, Wold et al. studied the events which take place during the lag phase preceding maximal DNA synthesis in the in vitro DNA replication system (36). They found that the lag phase could be almost completely eliminated by preincubating large T antigen with SV40 origin DNA and a cellular factor, presumably a single-stranded DNA-binding protein, in the presence of ATP. The findings suggested that the helicase activity of large T antigen mediates unwinding and melting of the SV40 origin (28). Again, it is conceivable that phosphorylation interferes with the interaction between large T antigen and the cellular factor.

The elucidation of the role of phosphorylation in the function of large T antigen is hampered by the finding that HeLa cell extract contains high protein kinase activity. In the future, experiments with a DNA replication system consisting only of purified components will be carried out. Hopefully, these experiments will allow us to obtain a better understanding of the role of phosphorylation in DNA replication in vitro and in vivo.

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