Phosphorylation and Active ATP Hydrolysis Are Not Required for SV40 T Antigen Hexamer Formation*

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Inga Reynisdóttir†, Heather E. Lorimer‡, Paula N. Friedman, Edith H. Wang, and Carol Prives§

From the Department of Biological Sciences, Columbia University, New York, New York 10027

ATP induces structural alterations in SV40 large T antigen and promotes changes in its interaction with the viral replication origin. We have analyzed nucleotide-induced changes in T antigen structure in the absence of origin DNA. Most preparations of immunopurified T antigen contain several discrete species ranging in size from monomers through oligomers larger than hexamers. The predominant species consist of monomers and dimers. Incubation of T antigen with ATP or dATP leads to a dramatic and rapid increase in the appearance of T antigen hexamers. Weakly and nonhydrolyzable analogs of ATP are effective as well, indicating that hexamer formation does not require active ATP hydrolysis. After incubation of T antigen with [γ-32P]ATP, stable association of the labeled nucleotide with all detectable forms occurs. Removal of greater than 80% of the T antigen phosphate residues does not significantly affect the formation of T antigen hexamers, although changes in the distribution and mobility of the other species of T antigen are apparent. Furthermore, T antigen synthesized in and purified from Escherichia coli and, therefore, presumably un- or underphosphorylated, is capable of forming hexamers. Nucleotide-induced T antigen hexamer formation thus appears to require neither protein phosphorylation nor active ATP hydrolysis.

SV40 large T antigen is a multifunctional protein whose DNA replication functions have been well characterized (for review see Chalberg and Kelly, 1989; Stillman, 1989; Borowiec et al., 1990, Fanning and Knippers, 1992). Several of T antigen's biochemical activities are required for initiation of replication at the SV40 origin. In particular, T antigen interacts specifically with binding sites within the core origin (Delucia et al., 1983; Jones and Tjian, 1984; Deb et al., 1987; Myers and Tjian, 1980). This protein-DNA interaction leads to distortions in the structure of origin DNA such that localized regions are melted (Borowiec and Hurwitz, 1988b; Parsons et al., 1990; Borowiec et al., 1991). This localized melting is then followed by a more extensive bidirectional unwinding of DNA by T antigen, a process dependent upon the presence of single-stranded DNA-binding protein (Dean et al., 1987; Dodson et al., 1987; Wold and Kelly, 1988; Dean and Hurwitz, 1991). T antigen also binds to DNA polymerase α-primase (Dornreiter et al., 1990; Gannon and Lane, 1987; Smale and Tjian, 1986) and, in the presence of single-stranded DNA binding protein, stimulates primase activity (Collins and Kelly, 1991; Dornreiter et al., 1992). The unwind origin then serves as a template for synthesis of primed DNA by DNA polymerase α-primase on both early and late strands.

The initiation functions of T antigen show striking similarities to the proteins that are involved in initiating DNA replication from the Escherichia coli origin, oriC. Indeed, T antigen appears to embody many of the properties of the E. coli dnaA, dnaB, and dnaC gene products (reviewed in Bramhill and Kornberg, 1988; Marians, 1992). However, a major difference is that, unlike the prokaryotic proteins, the replication functions of T antigen are regulated by its state of phosphorylation (reviewed in Prives, 1991). It is, therefore, of interest to ask whether the quaternary structure of T antigen is regulated by ATP and whether this regulation is influenced by the state of phosphorylation of T antigen.

The interaction of T antigen with ATP and other nucleotides is central to all of these activities. T antigen both binds and hydrolyzes ATP (Giacherio and Hager, 1979; Tjian and Robbins, 1979; Griffin et al., 1980). Tryptic peptide mapping and binding to ATP analogs localized the ATP binding domain of T antigen to the region between amino acids 418 and 526 (Clerc and Cuzin, 1982; Clerc et al., 1984; Bradley et al., 1984; Bradley, 1990). In keeping with this function, the region also contains a mononucleotide binding fold motif (Bradley et al., 1987). Both unwinding and high affinity T antigen binding to the origin DNA require ATP or nonhydrolyzable ATP analogs (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988a, 1988b; Parsons et al., 1990; Borowiec et al., 1991). The DNA helicase activity of T antigen, which is inherent in its ability to fully unwind the origin, requires the hydrolysis of ATP or other nucleotides (Goetz et al., 1988; Wiekowski et al., 1988; Scheffner et al., 1989). The requirements for hydrolyzable or nonhydrolyzable forms of ATP for these activities strongly suggest that the structure of T antigen changes as a result of its interaction with nucleotides such as ATP. Indeed, using techniques of electron microscopy (Dean et al., 1988), glycerol gradient sedimentation analysis (Dean et al., 1992), and nondenaturing gel electrophoresis (Parsons et al., 1991, Dean et al., 1992, Reynisdóttir and Prives, 1992), it was shown that addition of ATP to T antigen causes the assembly of smaller forms of T antigen into larger oligomers. These aggregates were demonstrated to be hexamers, with two such T antigen hexamers binding to the SV40 core origin (Dean et al., 1987; Mastrangelo et al., 1989; Parsons and Tegtmeyer, 1992; Wessel et al., 1992; Reynisdóttir and Prives, 1992; Dean et al., 1992).

It is, therefore, of interest to ask how the quaternary structure of T antigen is regulated by ATP and whether this regulation is influenced by the state of phosphorylation of T antigen. In this way we have characterized the response of immunopurified T antigen to nucleotides by zonal velocity gradient centrifugation and nondenaturing polyacrylamide gel electrophoresis.
ATP Induces Hexamerization of SV40 T Antigen

EXPERIMENTAL PROCEDURES

Purification of T Antigen—SV40 T antigen was purified from SF21 insect cells that had been infected with the recombinant baculovirus virus expressing the virus gene, pETV55SVT (O’Reilly and Miller, 1988) by immunoaffinity procedures as described previously (Wang et al., 1989). For experiments using T antigen synthesized in bacteria, an E. coli strain, BL21 p17 TAg, that contained the SV40 large T DNA linked to the bacteriophage T7 promoter (Mohan et al., 1989) was kindly provided by Y. Gluzman. T antigen was immunopurified from extracts of bacterial cell cultures (500 ml) by the same procedure as for baculovirus-expressed T antigen. Yields of T antigen isolated from 500-ml cultures of E. coli were generally only 1/10 of those obtained from 10 150-mm plates of baculovirus-infected insect cells.

Nondenaturing Polyacrylamide Gels—Nondenaturing gels were prepared and used essentially as described by Wang and Prives (1991b). Acrylamide stocks containing acrylamide: N,N-bisacrylamide (80:1) were used to prepare either 4.4-10% or 4.4-20% gradient gels in TG buffer (50 mM Tris-HCl and 50 mM glycine (pH 8.8)) to which ammonium persulfate and TEMED (pH 10) were added to final concentrations of 0.3 and 0.03%, respectively. No stacking gels were used. Gels were run for 3 h at 170 V in TG buffer. A solution containing glycerol (98%), bromphenol blue (1%), and xylene cyanol (1%) was added to a final concentration of 10% to each sample prior to loading onto gels. Molecular weight marker proteins, 1 μg each, including thyroglobulin (M, 670,000), phosphorylase (M, 94,000), and albumin (M, 66,000), purchased from Bio-Rad were added to gels to aid in determination of the molecular weight of the T antigen forms. After electrophoresis gels were electrotransferred to nitrocellulose which was briefly stained with Ponceau Red to locate marker proteins prior to immunostaining of T antigens with PAb 416 (Harlow et al., 1981).

T Antigen Hexamer Formation Assays—Reaction mixtures for hexamerization assays (20 μl) containing 40 mM creatine phosphate (di-Tris salt (pH 7.7)), 0.5 mM dithiothreitol, 7 mM MgCl2, and T antigen in the presence or absence of the indicated quantity of nucleotide were incubated for 15 min at 37°C.

Sucrose Gradient Centrifugation—Reaction mixtures (500 μl) containing T antigen and the other components in the presence or absence of 4 mM ATP were incubated at 37°C for 30 min and then loaded onto 5-ml linear gradients of 5-20% sucrose in phosphate-buffered saline and centrifuged at 25,000 rpm for 16 h at 4°C. Fractions containing equal volumes (approximately 300 μl) were collected from the bottom of the gradient, and 40-μl aliquots from each fraction were loaded directly onto nondenaturing gels followed by electrotransferring and immunostaining as above.

ATP Binding—Increasing concentrations of T antigen, p53, bovine serum albumin, and thyroglobulin were incubated with 1 μCi of [γ-32P]ATP at 25°C in a 50-μl volume containing 40 mM creatine phosphate (di-Tris salt (pH 7.7)), 7 mM MgCl2, and 0.5 mM dithiothreitol for 10 min. Mixtures were then filtered through 25-mm nitrocellulose filters (Schleicher and Schuell) that had been presoaked in 25 mM HEPES (pH 7.0). Filters were then washed three times with 5 ml of the HEPES buffer, dried, and counted by liquid scintillation. To determine the molar ratio of ATP bound to T antigen, reaction mixtures contained a constant amount of T antigen (0.25 μg), 2 μCi (2 pmol) of [γ-32P]ATP, and increasing amounts of nonradioactive ATP-S. The amount of [γ-32P]ATP retained by T antigen was measured by filtration as above. To analyze which oligomeric forms of T antigen had bound nucleotide after incubation with buffer containing [γ-32P]ATP, the reaction mixtures were separated on nondenaturing gels followed by electrotransferring to nitrocellulose, immunostaining, and exposure of blot to x-ray film.

Treatment of T Antigen with Alkaline Phosphatase—T antigen, immunopurified from SF21 insect cells (1010 cells) that had been labeled for 4 h with 0.5 mCi [32P]orthophosphate in 3 ml of phosphate-free TC-100 media (Speciality Media), was incubated with either 0 or 1 unit of calf intestinal alkaline phosphatase (Boehringer Mannheim) in 10 μl Tris-HCl (pH 8.5), 1 mM MgCl2, and 1 mM ZnCl2 for 20 min at 37°C. Mixtures were then adjusted to contain 40 mM creatine phosphate (di-Tris salt (pH 7.7)) and 0.5 mM dithiothreitol and, where indicated, 7 mM MgCl2 and 4 mM ATP were added and the incubation continued at 37°C for 15 min. After separation on 4.4-20% nondenaturing gels, electrophoretic transfer and immunostaining with antibody PAb 416, the blots were exposed to x-ray film.

The abbreviations used are: TEMED, N,N,N’,N’-tetramethylethylenediamine; ATP-S, adenosine 5’-O-thiotriphosphate; AMP-PNP, 5’-adenyl imidodiphosphate; AMP-PCP, 5’-adenyl methylidenephosphate.

RESULTS

Nondenaturing Polyacrylamide Gels Reveal Multiple Forms of SV40 T Antigen, Whose Distribution Is Altered by ATP and MgCl2—We used nondenaturing polyacrylamide gels to characterize the forms of T antigen and their response to nucleotides. The T antigen preparations were over 80% pure as determined by silver staining and were active in mediating the replication of SV40 ori-DNA in HeLa cell extracts (data not shown). Such preparations were incubated at 37°C for 15 min either without ATP and MgCl2 or with each of these separately or both together prior to loading onto nondenaturing gels (Fig. 1). T antigen that had been incubated in the absence of ATP and MgCl2 consisted of an extensive series of forms that ranged from monomers to a series of less abundant oligomers extending to decamers and even larger oligomers that could not be resolved in these gels. The relative ratio of monomers to dimers and higher forms varied among different preparations and between experiments, but generally monomers were the predominant form with dimers less abundant and additional forms still less plentiful. Monomers were identified as such based on several criteria including the following: 1) they were the fastest migrating form on the gel, 2) their migration relative to established purified molecular weight markers was consistent with a protein in the size range of monomeric T antigen, and 3) T antigen that had been boiled for 10 min in standard SDS-gel electrophoresis sample buffer, displayed a rather smeared appearance but essentially comigrated with the fastest migrating nondenatured T antigen species (data not shown). In contrast to the other forms of T antigen, the monomers frequently appeared to be heterogeneous, displaying significantly broader bands that were frequently resolved into at least two closely migrating species (see Fig. 2). It should be noted here that it is likely that purification of T antigen using alternate protocols can give rise to preparations of T antigen that are more highly enriched in very large aggregated forms (Parsons et al., 1991).

We determined that ATP and MgCl2 together, but not separately, caused the dramatic appearance of a species of T antigen that was observed to have a molecular mass of approximately 670,000 daltons (Fig. 1, lane a). The effect of ATP and MgCl2 was very similar to that observed for ATP alone in the absence of MgCl2 (Fig. 1, lane b). The concentrations of ATP and MgCl2 used were 4 and 7 mM in all cases, respectively. The protein products were resolved on a 4.4-20% gradient nondenaturing polyacrylamide gel, transferred to nitrocellulose, and probed with monoclonal antibody PAb 416. The positions of the molecular mass standards (in kilodaltons), and the multimeric forms of T antigen are indicated on the left and right, respectively.
that migrated in the close vicinity of, but not always identical
with, the hexameric oligomer seen in the absence of ATP and
MgCl₂. This was accompanied by significant changes in the
amount, sharpness, and relative migration of the other T an-
tagant species, suggesting that these forms had been altered in a
manner leading to formation of the ATP hexamers. Not only
were there fewer forms that were smaller than hexamers, but
there were also far less of the more highly oligomeric species.
This suggests that ATP and MgCl₂ caused not only the coales-
cing of other presumably smaller forms of T antigen into
hexamers, but also the dissociation of the more aggregated T
antigen species. Although incubation of T antigen in the pre-
ence of ATP but without MgCl₂ had little effect (Fig. 1, compare
lanes a and c), pretreatment with MgCl₂ caused a marked reduc-
tion in the appearance of forms more complex than dimers. Whether MgCl₂ prevented their formation during the preincubation period or caused their disaggregation once formed could not be determined in these experiments, although alternate experimental protocols using glycerol gradients sug-
gest that it is the latter (Dean et al., 1992). That ATP requires
MgCl₂ for its interaction with T antigen is consistent with the
well established fact that most reactions requiring ATP func-
tion efficiently only with added MgCl₂ and in some cases with
MnCl₂ as well. We have not yet tried MnCl₂ in this series of
experiments, but in related studies we found that only MgCl₂,
and not MnCl₂, supports the altered binding of SV40 and poly-
oma T antigens to their respective origin in the presence of ATP
(Lorimer et al., 1991). Thus, in describing all subsequent ex-
periments in this study, ATP refers to ATP/MgCl₂.

A

Fig. 2. Analysis of T antigen oligomers separated by zonal
velocity gradient centrifugation. Fractions from sucrose gradients af-
after centrifugation of mixtures containing T antigen (10 μg) incubated in
the absence (A) or presence (B) of ATP were collected (no. 1 is bottom of
the gradient) and aliquots subjected to nondenaturing gel electropho-
resis and Western blotting using PAb 416 as probe. The migration of
marker proteins (molecular mass in kilodaltons) are shown on the left.
Markings in lane 13 in the region of the monomer are an artifact of the
Western blot.

Density Gradient Sedimentation Analysis of T Antigen

Several Nucleotides Induce T Antigen Hexamer Formation

We determined which nucleotides were capable of inducing hexamerization by T antigen (Table I). We chose two concentra-
tions, the one that is commonly used for ATP during repli-
cation reactions, i.e. 4 mM, and one that was expected to be

| Nucleotide | Concentration | Ratio (H/M) |
|------------|---------------|-------------|
| ATP        | 40**          | 0.29        |
| dATP       | 4000**        | 15.30       |
| ATP-S      | 4000          | 13.60       |
| AMP-PCP    | 4000          | 34.70       |
| AMP-PNP    | 4000          | 0.90        |
| ADP        | 4000          | 0.38        |
| AMP        | 4000          | 1.40        |
| AMP        | 4000          | 0.002       |
| CTP        | 4000          | 0.003       |
| dCTP       | 4000          | 0.134       |
| UTP        | 4000          | 0.398       |
| TTP        | 4000          | 0.001       |
| GTP        | 4000          | 1.540       |
| dGTP       | 4000          | 0.001       |
|            |               | 0.002       |

During the course of other experiments, confirming studies published by Dean et al. (1992), we have determined that in-
cubation of purified T antigen with ATP causes a shift in its
sedimentation from more slowly to more rapidly sedimenting
forms. It was of interest to analyze fractions of T antigen col-
clected after similar sucrose gradient centrifugation analysis on
nondenaturing gels. Accordingly, T antigen was incubated ei-
ther without or with ATP and subjected to centrifugation through 5–20% sucrose gradients (Fig. 2). Aliquots of the col-
lected gradient fractions were loaded directly onto nondenatur-
ing gels and analyzed as in Fig. 1. As expected, the fractions
containing more slowly sedimenting material were enriched in
monomers and dimers, whereas the more sucrose-dense frac-
tions contained very little of either. These gradient conditions
did not permit any separation of monomers from dimers. In this
particular experiment the monomer form itself appeared
clearly as a doublet. There was a progressive increase in the
size and complexity of the oligomers as a function of sediment-
rate. Incubation with ATP again caused a marked con-
version of T antigen forms to the hexamer species. The
hexamer peak (no. 9) sedimented at approximately the same
position as the hexamers formed in the absence of nucleotide,
indicating that there was not a significant difference in shape
between hexamers formed in the presence or absence of ATP
that was discernible using these gradients. Note that in this
experiment as in Fig. 1, ATP also appeared to cause a reduction
in the amount of forms of T antigen that were larger than
hexamers. Thus, sucrose gradients confirmed that T antigen
displays a marked alteration in its quaternary structure as a
function of its interaction with ATP.

The indicated nucleotides were used at either 40 μM (•) or 4 mM (••). The formation of oligomers was analyzed on nondenaturing gels, trans-
fused to nitrocellulose that was subsequently probed with PAb 416. The
estimated ratio of hexamers to monomers (H/M) was determined by
scanning Western blots and quantitating the appropriate bands using a
Bio-Image Visage 110 densitometer system (Millipore).

Table I

The induction of T antigen hexamer formation by various nucleotides

—We determined which nucleotides were capable of inducing hexamerization by T antigen (Table I). We chose two concentra-
tions, the one that is commonly used for ATP during repli-
cation reactions, i.e. 4 mM, and one that was expected to be
suboptimal, i.e., 40 μM. Efficiency of hexamer formation was assessed from the ratio of hexamers to monomers (H:M) formed at 40 μM and 4 mM ATP, dATP, and ATPγS were clearly the most effective nucleotides in inducing the formation of hexamers. AMP-PNP was also capable of inducing hexamers, although much less efficiently, whereas AMP-PCP did so poorly. Hexamers were also formed in the presence of ADP, but neither AMP nor cyclic AMP (not shown) had any significant effect on T antigen. The ability of ATPγS and AMP-PNP to induce the formation of hexamers is consistent with the observation that both nucleotides cause altered binding to (Borowiec and Hurwitz, 1988a) and melting of the origin (Borowiec and Hurwitz, 1988b) by T antigen. This suggests that these weakly or non-hydrolyzable nucleotides induce a conformational alteration in T antigen that affects its initial interactions with the origin in a manner that does not require active hydrolysis. Although the next most effective hydrolyzable nucleotide was TTP, it was still an order of magnitude less effective than the adenine-based nucleotides, and furthermore, it did not induce any detectable hexamers at a concentration of 40 μM. The other common nucleoside triphosphates and deoxynucleoside triphosphates were only marginally effective at causing the formation of hexamers, and GTP and dGTP appeared to be completely inert.

The Binding of [γ-35S]ATP to T Antigen—To further understand the interaction of T antigen with ATP and to determine which forms of T antigen are bound to the nucleotide, we attempted to establish conditions for nitrocellulose filter binding assays in which the binding of radiolabeled [α-32P]ATP to T antigen could be followed. We were unable to detect any labeled ATP bound to T antigen (data not shown). However, when similar experiments were attempted with a labeled derivative of ATP that is hydrolyzed extremely slowly, i.e., [γ-35S]ATP, it was possible to demonstrate the binding of T antigen to this nucleotide in a concentration-dependent manner (Fig. 3 A). The specificity of binding by T antigen to [γ-35S]ATP is supported by the fact that three additional purified proteins, tested at similar concentrations, did not retain the labeled nucleotide on nitrocellulose filters. The nitrocellulose filter binding assay allowed us to estimate the molar ratio of ATPγS bound to T antigen. By varying the amount of nucleotide added to binding reactions, we determined that it was possible to bind up to 0.15 mol of nucleotide/mol of T antigen (Fig. 3 B). It is likely that at the saturation point for binding of ATPγS that of the T antigen had been converted to hexamers, since we had observed that this nucleotide is extremely efficient in inducing hexamerization. That approximately one-sixth of the T antigen was maximally bound to nucleotide suggested the possibility that only hexamers were capable of retaining bound ATPγS. In order to determine which forms of T antigen are bound to nucleotide, [γ-35S]ATP was added to reaction mixtures along with a concentration of unlabeled ATP sufficiently low to permit detection of the labeled nucleotide but high enough to induce hexamer formation. Mixtures were loaded onto nondenaturing gels, and, after electrophoresis, subjected to Western blotting using PAb 416 to identify the different species of T antigen (Fig. 3 C).

Fig. 3. T Antigen binding of ATPγS. A, increasing concentrations of T antigen (0.2 μg/μl), p53 (0.1 μg/μl), bovine serum albumin (BSA, 0.5 μg/μl), or thyroglobulin (0.5 μg/μl) were incubated at 25 °C for 10 min in a 50-μl volume with 1 μCi of [γ-35S]ATP (1174 Ci/mM). The bound ATP was measured by nitrocellulose filter binding and scintillation counting. B, mixtures (50 μl) containing 0.25 μg of T antigen (2.5 pmol) were incubated as above with 2 μCi of [γ-35S]ATP and increasing concentrations of ATP-γS as shown. Binding was measured by nitrocellulose filtration and scintillation counting. The results are graphed as pmol of ATPγS bound by 1 pmol of T antigen (percent of total counts bound × pmol ATPγS in reaction × 2/5) as a function of ATPγS added. C, T antigen (1.0 μg) was incubated as in A with 10 μCi of [γ-35S]ATP and 100 μM unlabeled ATP followed by nondenaturing gel electrophoresis. The gel was electrotransferred to nitrocellulose and T antigen forms detected by immunostaining with PAb 416 as probe (W) and autoradiography (A). The markers on left indicate the oligomeric forms of T antigen.
ATP Induces Hexamerization of SV40 T Antigen

FIG. 4. T antigen hexamers form rapidly. T antigen (1.0 μg) was incubated at 37 °C for the indicated times in the presence (+) or absence (−) of 4 mM ATP. The reaction mixtures were analyzed on a nondenaturing gradient gel, transferred to nitrocellulose, and probed with monoclonal antibody PAb 416. The positions of standard protein markers (molecular mass in kilodaltons) are indicated on the left and the T antigen multimeric forms on the right. The blot was then exposed to x-ray film. Since very similar patterns of T antigen forms were identified by Western blotting and autoradiography, this indicated strongly that all detectable forms of T antigen that can be identified bind to [γ-32P]ATP. Although it is likely that this is also the case with ATP itself, it was not possible to confirm this possibility using these experimental protocols.

Rapid Formation of T Antigen Hexamers—Determining the time required for hexamer formation, samples of T antigen, in the presence or absence of ATP, were incubated for various time intervals prior to loading onto gels and immediate application of electrical current. We found that hexamer formation was rapid and occurred within 2 min (Fig. 4). The time required to induce hexamers was heavily dependent on the concentration of T antigen. More concentrated preparations frequently formed hexamers so rapidly that a small amount of hexamers could be detected even at the “zero” time point (where zero is defined as the time interval between adding T antigen to the other reaction mixture components and addition of completed mixture to the gel), whereas more dilute preparations required longer time periods to assemble into the maximum quantity of hexamers (data not shown). Addition of glutaraldehyde to the reactions did not appreciably affect the results of time course experiments (data not shown). It should be noted that incubation of T antigen for periods of up to 1 h prior to electrophoresis did not result in a complete conversion of smaller forms into hexamers; there always remained substantial quantities of detectable amounts of monomers and dimers, even with highly concentrated preparations. Whether this reflects a situation of dynamic exchange between hexamers and other forms or the inability of some T antigen molecules within the nonhexamer forms to assemble into hexamers is not yet known.

Does Phosphorylation Play a Role in the Formation of T Antigen Hexamers?—The replication functions of T antigen were shown previously to be both negatively and positively regulated by phosphorylation (reviewed in Prives, 1991). T antigen possesses at least two repressing phosphates that down-regulate its ability to unwind the replication origin (Virshup et al., 1989, 1992). In addition, phosphorylation of a single residue, Thr184, is required for its efficient binding to the replication origin (McVey et al., 1989). T antigen isolated from baculovirus-infected insect cells was shown to be phosphorylated at sites similar to those found on T antigen isolated from monkey cells, although the ratio of phosphates at different sites varied somewhat (Höss et al., 1990). It is, therefore, of considerable relevance to determine whether either the distribution of T antigen quaternary forms or its ability to form hexamers was regulated by its state of phosphorylation.

It was shown previously that treatment of SV40 T antigen with calf intestinal alkaline phosphatase removes approximately 80% of the phosphate residues from T antigen (Grissler et al., 1987; Mohr et al., 1987). Such treatment was shown to cause a striking increase in the ability of T antigen to mediate ori-DNA replication in vitro. T antigen was isolated from insect cells that had been labeled with [32P]orthophosphate for 4 h prior to extraction and immunoprecipitation purification. We confirmed that treatment of the labeled T antigen with increasing quantities of calf intestinal alkaline phosphatase did not lead to removal of more than 75–80% of the labeled phosphate. Calf intestinal alkaline phosphatase-treated and untreated T antigen preparations were incubated with and without ATP prior to electrophoresis on nondenaturing gels followed by Western blotting as in the previous experiments (Fig. 5). The blots were then exposed to x-ray film. When the treated and untreated T antigens were compared by immunoblotting and autoradiography, several observations were made. First, comparing the autoradiogram to the blots, it appeared that the relative distribution of phosphate in the non-calf intestinal alkaline phosphatase-treated T antigen forms was approximately proportional to the overall distribution of immunoreactive T antigen forms, although the ATP-induced hexamers appeared to be somewhat more highly phosphorylated. Second, treatment of T antigen with calf intestinal alkaline phosphatase had little effect on its ability to assemble into hexamers as evidenced by the fact that although there was a marked reduction in the amount of labeled hexamer after calf intestinal alkaline phosphatase treatment, there was no significant effect on the amount of total immunoreactive hexameric T antigen as shown by the Western blot. Finally, comparing the patterns of calf intestinal alkaline phosphatase-treated and untreated T antigen on the developed Western blot and on the autoradiogram, there were significant reductions in the migrations of the monomers, dimers, and even trimers. Although no obvious change in the migration of tetramers, pentamers, and hexamers was evident, this may be due to poor resolution (i.e. compression of bands) in

2 F. Dean, personal communication.

FIG. 5. Removal of phosphates with calf intestinal alkaline phosphatase does not alter the formation of T antigen hexamers. 32P-Labeled T antigen (2.0 μg) was preincubated with the indicated amount of calf intestinal alkaline phosphatase for 20 min at 33 °C. The reaction mixtures were then adjusted to conditions favorable for hexamer formation in the absence (−) or presence (+) of 4 mM ATP. After an additional 15 min at 37 °C, the samples were resolved on nondenaturing polyacrylamide gradient gels and analyzed by Western blotting and autoradiography. The positions of standard protein markers (molecular mass in kilodaltons), and the multimeric forms of SV40 T antigen are indicated on the left and right, respectively.
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Fig. 6. Unphosphorylated T antigen forms hexamers. T antigen (0.5 µg) produced in insect cells (lanes a-c) and T antigen (1.0 µg) produced in E. coli (lanes a’-c’) were either loaded directly onto the gel (a) or incubated at room temperature for 10 min in the absence (b) or presence (c) of 4 mM ATP. Samples were analyzed on nondenaturing gradient gels, transferred to nitrocellulose, and probed with monoclonal antibody 416. Native molecular masses of protein standards (in kilodaltons) run in parallel lanes are indicated at left.

this region of the gel. The previous noted complexity of the monomeric form was also reduced after calf intestinal alkaline phosphatase treatment, indicating that this complexity is related to heterogeneity of phosphorylated forms of T antigen. We conclude that although calf intestinal alkaline phosphatase treatment does affect the migration of some T antigen forms, it does not alter significantly the ability of T antigen to form hexamers in the presence of ATP. Calf intestinal alkaline phosphatase treatment of this level of T antigen caused a 5–10-fold stimulation of its ability to mediate ori-DNA replication in vitro, suggesting that the removal of repressing phosphates from T antigen was not related to its ability to form hexamers.

Calf intestinal alkaline phosphatase does not remove all of the phosphates from T antigen, and calf intestinal alkaline phosphatase-treated T antigen is active in ori-DNA replication. What of the other phosphates on T antigen? In particular, is Thr194 necessary for the ability of T antigen to form hexamers? Removal of the calf intestinal alkaline phosphatase-resistant phosphates from T antigen can be accomplished by acid phosphatase (Klausing et al., 1988). However, our batches of potato acid phosphatase caused considerable degradation of T antigen. Therefore, we turned to another approach, namely to purify T antigen from bacteria. Such T antigen preparations were shown previously to be either un- or greatly underphosphorylated and concomitantly to display very poor ori-DNA replication function (Mohr et al., 1989). However, they responded with a dramatic increase in their ori binding and ori replication functions after phosphorylation of Thr194 with purifiedcdc2 kinase (McVey et al., 1989). To determine whether their defective interactions with the origin is related to an inability to form hexamers, T antigen was purified from E. coli by immunoadfinity procedures. Although such T antigen preparations were somewhat unstable and generally yielded less T antigen than was generated from baculovirus-infected insect cells, it was nevertheless possible to obtain sufficient and comparable quantities of T antigen isolated from bacteria and insect cells to evaluate their relative properties on nondenaturing gels (Fig. 6). Bacterial and baculovirus expressed T antigens were applied directly to gels or after incubation in the absence or presence of ATP. There were marked differences in the display of monomers through trimers between the two preparations. In particular, the bacterial T antigen forms were much less well resolved and showed essentially a smear of immunoreactive material in this region of the Western blot. Within this region dimers could be well discerned. However, it is clear from these experiments that incubation of the T antigen from bacteria with ATP caused the appearance of substantial quantities of hexamers; indeed, the ratio of hexamers to monomers was fairly similar to that seen with the baculovirus derived T antigen. Thus, it is unlikely that phosphorylation plays any role in the ability of T antigen to assemble into hexamers.

DISCUSSION

We have characterized the effect of ATP and other nucleotides in inducing the assembly of SV40 T antigen hexamers. Our results confirm and extend those of others who have examined the role(s) of nucleotide in T antigen oligomerization and DNA replication function. They also address the fact that extracts of both SV40-infected and -transformed cells contain several discrete forms of T antigen as detected by zonal velocity density gradient centrifugation (reviewed in Fanning and Knippers, 1992).

The experiments herein show that several nucleotides, in addition to ATP, will support hexamer formation. During the course of these studies we noted, while repeating our determinations of the various nucleotides required for hexamer formation, that the results differed quantitatively from one experiment to another. We believe that the major cause for the variations observed is related to the concentration of the T antigen preparation used. More concentrated preparations formed hexamers more readily with lower concentrations of nucleotides. Despite these quantitative variations, a clear qualitative hierarchy of nucleotide response was apparent. The most effective nucleotide, as defined by inducing maximal formation of hexamers at the lowest nucleotide concentration, was dATP with the other nucleotides ordered as follows: ATP >> TTP > UTP, CTP, dCTP >> GTP > dGTP. It should be noted that concentrations of dATP as low as 4 µM and of ATP as low as 10 µM induce hexamer formation with some preparations of T antigen. Thus, as a cautionary note, we suggest that it is possible that batches of other nucleotides contaminated with even a small amount of adenine nucleotides might provide deceptively positive results. Nonetheless, these results were consistent with experiments comparing the effectiveness of different nucleotides in supporting T antigen DNA helicase and duplex DNA unwinding activities (Goetz et al., 1988; Wiekowski et al., 1988; Wang and Prives, 1991a), although our observation that dATP is more effective than ATP for hexamerization is at variance with published studies examining these activities. However, our observation that dATP is more effective than ATP in promoting hexamer formation is more consistent with requirements for enhanced DNA binding (Borowiec and Hurwitz, 1988a) than with the requirements for helicase and unwinding.

The conditions of the helicase and unwinding reactions are such that hexamers, which most likely are the functional units of helicase, are formed. However, guanine-based nucleotides are virtually ineffective in supporting T antigen duplex DNA unwinding (Goetz et al., 1988; Wang and Prives 1991a) but can support T antigen helicase activity with about 1/10 the efficiency of ATP (Wiekowski et al., 1988; Wang and Prives, 1991a). Our measurement of the efficiency of T antigen hexamer formation with GTP is at least 3 orders of magnitude lower than with ATP. Therefore, it is possible that GTP-T antigen hexamers are not stable enough for DNA unwinding or analysis by gel electrophoresis but have sufficient stability and activity for helicase function. An interesting alternative hypothesis is that monomers of T antigen might contribute to helicase activity. The fact that all forms of T antigen can bind to nucleotide...
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Two general (and not necessarily exclusive) speculations can be offered. First, the presence of inactive T antigen hexamers may be yet another means to regulate the amount of functional T antigen in infected cells. Dean et al. (1992) have shown that hexamers assembled in the absence of DNA are inactive in ATP-dependent DNA binding, untwisting, and unwinding and, therefore, in supporting replication of SV40 ori-DNA. However, T antigen autoregulates in at least two modes through transcription. It is not known what form of T antigen is involved in this regulation, and there has been copious speculation as to the necessity for maintaining the precise balance of T antigen and other viral components throughout the cell cycle. Second, it is now established that T antigen binds to several proteins that are involved in regulating cell growth and cycling. Perhaps T antigen hexamers are the preferred species for binding to such proteins as p53, pRB, or p107. Determining the form(s) of T antigen that bind one or more of these tumor suppressor proteins is of interest and is currently underway.

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Our data show that certain weakly or nonhydrolyzable nucleotides are also capable of supporting hexamer formation. ATP·S was as efficient as ATP in this regard and was useful for nucleotide binding studies, allowing us to determine that the nucleotide was found associated with all species of T antigen. It also supported our observations with AMP-PNP showing that active nucleotide hydrolysis is not required for hexamerization. It is not understood why AMP-PNP supported hexamerization, albeit weakly, whereas AMP-PCP was entirely ineffective. According to a model proposed by Bradley et al. (1987), there are contact points between the T antigen amino acids within the nucleotide binding fold and the sugar, phosphate, and magnesium. cAMP probably does not fit into the pocket because of its cyclical conformation, and AMP may lack sufficient phosphates for stable contacts. ADP showed a limited effectiveness in inducing hexamers, indicating that three phosphates are not a prerequisite for detecting the consequences of interactions with T antigen. The observation that hydrolysis is not essential. Nucleotide binding may serve the dual function of providing energy for helicase activity as well as inducing the conformational change that results in hexamer formation. The fact that [γ-32P]ATP is detectably bound to T antigen, whereas [α-32P]ATP is not, suggests that ATP hydrolysis results in a rapid release of ATP from T antigen. As ATP hydrolysis is required for helicase and unwinding, it is likely that the release of ATP is a necessary component of the energy transduction and turnover aspect of these T antigen functions. That we detect ATP·S bound to all forms of T antigen is consistent with the fact that ATP is associated with T antigen dimers (Bradley, 1990) and that smaller forms are the intermediates in the formation of hexamers (Dean et al., 1992). Our calculations suggest that the stoichiometry of ATP·S bound to T antigen is roughly 1:6. Thus, it is tempting to speculate that one molecule of nucleotide is found associated with each hexamer. As all forms of T antigen retain this nucleotide analog, this hypothesis can be extended by a supposition that an ATP bound T antigen is capable of serving as a seed undergoing a conformational shift that allows it to bind most readily to a total of five more monomers. However, this theory is contradicted by binding studies which using the ATP analog fluorosulfonylbenezoyl 5'-adenosine derived a ratio of nucleotide to T antigen of one to one (Bradley, 1990). Whether the difference between these data and ours is due to the source and preparation of T antigen or to the fact that different adenine-based derivatives were employed remains to be determined.

T antigen hexamers are apparently assembled around origin DNA in the presence of ATP (Dean et al., 1992). It has been hypothesized that this may require or be affected by the phosphorylation state of T antigen (Virshup et al., 1989, 1992). Our results show that the incubation of phosphorylated or bacterially expressed T antigen with ATP lead to normal levels of hexamer formation. Thus, phosphorylation is not necessary for hexamer formation. It is possible that phosphorylation is necessary for hexamer formation around origin DNA or for separation of the two hexamers once bound to DNA. What then is the significance of the observation that hexamers can assemble efficiently in the absence of DNA and that substantial quantities of DNA-free hexamers accumulate in infected cells? Two general (and not necessarily exclusive) speculations can be offered.
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