The DNA-Binding Properties of Polyomavirus Large T Antigen Are Altered by ATP and Other Nucleotides

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Received 16 July 1990/Accepted 15 October 1990

We have examined the influence of ATP on the DNA-binding properties of polyomavirus large T antigen (Py TAG). Utilizing nitrocellulose filter binding, DNase I footprinting, and gel mobility shift assays, we observed that ATP increased Py TAG binding to DNA fragments containing either all Py TAG-binding sites (whole origin) or those sites within (core origin) or adjacent to (early) the origin of replication. Even nonspecific binding to DNA fragments lacking Py TAG-binding sites was increased somewhat by ATP. Binding to the core origin was increased to a greater extent than binding to other DNA fragments tested. Gel band mobility shift assays revealed that ATP increased the production of core origin-specific Py TAG-DNA complexes of high molecular weight. ATP stimulation depended on the presence of $MgCl_2$. Other nucleotides and nonhydrolyzable ATP analogs also increased Py TAG binding to the core origin but to various degrees: ATP, dATP, 5'-adenylyl imidodiphosphate (AMPPNP) > 5'-adenylyl methylenediphosphate (AMPPCP) > dCTP > UTP > TTP. GTP and dGTP did not increase DNA binding by Py TAG. The rates of association and disassociation of Py TAG with all the DNA fragments were altered by the presence of ATP. DNase I footprinting showed that ATP extensively extended the region protected within the core origin and also produced a distinctive DNase I-hypersensitive site on the late strand at nucleotides 5255 to 5262 (TTACTATG).

Polyomavirus large T antigen (Py TAG) is the major viral regulatory protein that is required for replication of the viral genome (reviewed in references 19 and 23). Although not as well characterized as the corresponding TAG encoded by simian virus 40 (SV40), several Py TAG biochemical properties have been described. Thus it is now established that Py TAG, like SV40 TAG, is a DNA-binding protein which interacts specifically with DNA sites that contain multiple copies of the consensus TAG pentanucleotide 5'-GA/GGGC-3' (11, 12, 42, 47). Such sites have been identified within and adjacent to the Py core origin of replication. The core origin, defined both *in vivo* (13, 28, 29, 32, 35, 53) and *in vitro* (43) by using deletion and base substitution mutants, contains sequences extending from nucleotide (nt) 5265 to nt 57. It lies between enhancer elements on the late side (7, 20, 27, 31, 35, 51, 54, 55) and Py TAG-binding sites B and C (11, 21) on the early side. This is similar to the SV40 regulatory region, which also has transcriptional regulatory elements on the late side and a high-affinity TAG-binding region on the early side of the SV40 core origin (reviewed in references 19 and 23). Within the Py core origin region are a GC-rich palindrome that contains two pairs of head-to-tail pentanucleotides in a head-to-head orientation on opposite strands and site A (11, 21) on the early side, which contains three pentanucleotides in head-to-tail array. Py TAG-binding sites B and C contain two and four copies of the pentanucleotide, respectively, also in head-to-tail orientation, with the pentanucleotides in site C on the opposite strand from those in sites A or B. It was reported previously that Py TAG binds with high affinity to sites A, B, and C (11, 12, 21, 47). Three additional weak Py TAG binding sites, 1, 2, and 3, were identified by DNase I footprinting (11). These required 10-fold-greater quantities of Py TAG in order to obtain detectable binding than did sites A, B, and C. Py TAG-binding sites 1 and 2 overlap the origin palindrome and A/T

regions, respectively, and were defined as discrete sites because of a DNase I-sensitive site identified between them. They are separated by only 1 or 2 nt, however, and as site 2 does not itself contain any copies of the consensus pentanucleotide, they may be parts of a single domain of interaction. Site 3 is directly adjacent to the late side of the core origin and is within the B enhancer element. It has one perfect and one imperfect pentanucleotide and is separated from sites 1 and 2 by at least 20 nucleotides.

The replication functions of Py and SV40 TAGs have common features. In addition to their comparable interactions with DNA, both Py (24, 49) and SV40 (8) TAGs display ATPase activity. Additionally, Py TAG (49, 56a), like SV40 TAG (14, 50), is a DNA helicase that unwinds double-stranded DNA containing the viral replication origin. These similarities are reflected in the significant homologies of regions within Py TAG that are involved in its origin-binding (10, 37, 52), putative zinc finger (1, 2), and nucleotide-binding (6, 9) functions to those regions involved in the analogous activities of SV40 TAG. It is therefore likely that Py and SV40 TAGs play similar roles in the initiation of viral replication. However, there are several biological and biochemical characteristics that are unique to each of these proteins. Py and SV40 display species specificity for DNA replication both *in vivo* (reviewed in reference 19) and *in vitro* (36), such that Py origin-containing DNA (ori-DNA) replicates only in some rodent cells, including those of murine origin, while primate cells exclusively support the replication of SV40 ori-DNA. Furthermore, although Py and SV40 TAGs can bind specifically to each other's ori-DNA (42, 47), they cannot initiate replication from the reciprocal origin *in vitro* (see reference 36 and references therein). While both TAGs can immortalize primary cells, only SV40 TAG can oncogenically transform established cell lines (reviewed in references 22 and 23). Potentially related to this difference is the observation that unlike SV40 TAG, Py TAG does not bind to the product of the p53 tumor suppressor gene (57). Since the p53-binding region on SV40 (see refer-

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ence 41 and references therein) overlaps sequences that display significant homology in SV40 and Py TAG and that were predicted to be involved in nucleotide binding (6), it is possible that there are significant differences in the structure and function of this general region. Such differences might in turn be reflected in the way that the two proteins function in initiating DNA replication.

It was shown that although the origin-binding domain of SV40 TAG is discrete from the ATPase region, the interaction of SV40 TAG with its viral origin is both altered and augmented by the presence of ATP and other nucleotides (3, 18). Since it was found that Py TAG binds very weakly to sequences within the origin palindrome in binding studies performed under conditions not compatible for DNA replication, we have reexamined the DNA-binding properties of Py TAG. In light of the fact that Py TAG differs from SV40 TAG in amino acid sequences that are adjacent to the nucleotide-binding region, it was particularly of interest to determine whether, as was shown for SV40 TAG, the DNA-binding properties of Py TAG are influenced by nucleotides. Using Py TAG that was active in mediating replication of Py ori-DNA in murine cell extracts, we have demonstrated that several but not all nucleotides stimulate the binding of Py TAG both specifically and nonspecifically to DNA. The most dramatic effects of such nucleotides, leading to changed and greatly increased interactions of Py TAG with DNA, were in the region of the core replication origin.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* insect cells (Sf27 cells) and the Py TAG-producing recombinant baculovirus vEV51LT (44) were kindly provided by L. Miller. Sf27 cells were grown at 27°C in TC-100 medium (GIBCO) containing 10% heat-inactivated fetal calf serum (HyClone) and 0.25% tryptose broth. F-4 monoclonal-producing hybridoma cells (39) were a gift from D. Pallas and E. Harlow. F-4 cells were grown in 40% Dulbecco modified Eagle medium–40% F-10 medium (GIBCO)–20% fetal calf serum.

Purification of Py TAG. Sf27 insect cells were infected with vEV51LT. After 48 h, the cells were lysed and Py TAG was immunoaffinity purified according to previously published methods (57). Generally, 10⁹ infected insect cells produced approximately 1 mg of Py TAG after immunoaffinity purification.

Replication of Py ori-DNA in vitro. Reaction conditions and methods used for the preparation of mouse FM3A cell extracts have been previously described (36). Reaction mixtures were incubated at 33°C and subsequently analyzed by either acid precipitation or agarose gel electrophoresis following *DpnI* digestion. Py origin-containing plasmid DNA (pBE102) contains nt 5022 to 1562 of the A2 strain of Py inserted into a pML vector (30).

Preparation of end-labeled Py ori-DNA fragments. p37.3 A2 (54), which is Py A2 inserted into the *Bam*HI site of pAT153, was cut with *DdeI* and *Hin*FI to produce a 408-bp DNA fragment (whole origin) containing the entire regulatory region of Py extending from the *Hin*FI site at nt 5075 to the *DdeI* site at nt 187. This fragment was in turn cut with *Bst*NI at nt 8 to produce a 179-bp fragment containing all the Py TAG-binding sites (A, B, and C) on the early side of the origin (early). p76-4 (43), obtained from W. Folk, which contains Py sequences with restriction enzyme linkers flanking the core origin—a *XhoI* linker inserted at nt 5269 and a *Sall* linker at nt 64—was cut with *Sall* and *XhoI* to produce a 94-bp fragment extending from nt 5269 to nt 67 and

containing the minimal sequences necessary for efficient in vitro replication (core origin). Nonspecific fragments were from pAT153 cut with *Hin*FI. All these fragments were 3' end labeled with α -³²P-labeled deoxynucleoside triphosphates and were purified from 6% polyacrylamide in 0.5 × TBE (1 × is 89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA).

Nitrocellulose filter binding of Py TAG-DNA complexes. The nitrocellulose filter assay was modified from a previously published filter binding method (3). α -³²P-3'-end-labeled DNA fragments (5 fmol) were preincubated at 37°C in 50 μ l of R buffer (40 mM creatine phosphate di-Tris salt [pH 7.8], 0.2 mg of bovine serum albumin per ml, 7 mM MgCl₂, 200 ng of nonspecific plasmid DNA pAT153 [except when examining nonspecific DNA binding] per ml, 0.5 mM dithiothreitol) with either 4 mM ATP (pH 7.8) or 18 mM NaCl (to compensate for the total sodium present in the ATP solution) for 5 min. Py TAG (500 fmol) was then added, and incubation continued for 20 min. The mixtures were then filtered under suction through 0.45- μ m-pore-size nitrocellulose filters (Schleicher & Schuell) that had been presoaked in 25 mM HEPES (Sigma) buffer (pH 7.0). The filters were then washed three times with 5 ml of the HEPES buffer, dried, and counted by liquid scintillation.

To measure the time course of association of Py TAG with DNA, filter-binding mixtures were prepared as described above with the following modifications. R buffer was prepared with or without 4 mM ATP and without DNA. Aliquots (50 μ l) of this buffer were preincubated with either 1 pmol of Py TAG or 10 fmol of labeled DNA fragment and 20 ng of pAT153. The Py TAG mixture was then added to the DNA-containing mixture, and after various times, the combined reaction mixtures were passed through nitrocellulose filters.

Py TAG disassociation studies were performed by incubating filter-binding mixtures with or without 4 mM ATP first in the absence of Py TAG for 5 min and then with added Py TAG for 5 min. A 100-fold molar excess of Py origin-containing plasmid DNA was then added. The mixtures were filtered and counted as described above at the appropriate time.

Gel shift assay. 3'-End-labeled core origin (94-bp) and nonspecific (75-bp) DNA fragments were preincubated in R buffer at 37°C for 5 min. Py TAG was then added to produce a final volume of 20 μ l, and incubation continued for 5 min. Glutaraldehyde was added to 0.1%, and after incubation for an additional 5 min, the samples were analyzed on a 4% polyacrylamide gel in 0.25 × TBE.

DNase I footprinting. Py TAG was incubated in R buffer with Py ori-DNA that had been 3' end labeled with α -³²P-deoxynucleoside triphosphates (dNTPs) at the *DdeI* site at nt 187 under the conditions used in filter binding in the presence of 100 ng of nonspecific DNA. After 5 min of incubation with Py TAG at 37°C, 1 ng of DNase I was added in a volume of 10 μ l containing 100 mM MgCl₂, 10 mM CaCl₂, 500 mM NaCl, and 1 μ g of sheared salmon sperm DNA. The DNase I reaction proceeded for 1 min at 37°C and was then terminated with 50 μ l of DNase stop solution (100 mM EDTA, 2 M ammonium acetate, 0.2% sodium lauryl sulfate, and 100 μ g of sheared salmon sperm DNA). The samples were then extracted with 1:1 (vol/vol) phenol-chloroform and precipitated with ethanol. The pellets were washed twice with 80% ethanol, dried, dissolved in 90% deionized formamide in TBE, and electrophoresed on an 8% polyacrylamide–8 M urea sequencing gel in TBE which was then dried and autoradiographed.

Materials. Anti-mouse immunoglobulin G agarose was purchased from HyClone. Restriction enzymes were ac-

FIG. 1. Py TAG purified from insect cells mediates the replication of Py ori-DNA plasmid in vitro. (A) Two levels (10 μ l in lanes 1 and 3, 5 μ l in lanes 2 and 4) of two fractions of immunoaffinity-purified Py TAG were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, silver stained, and compared with a marker (lane M) containing 1 μ g of protein per band. The sizes of the

quired from both Boehringer Mannheim and New England BioLabs. The Klenow fragment of DNA polymerase I was purchased from New England BioLabs. [α - ^{32} P]dNTPs were obtained from New England Nuclear (Du Pont), and DNase I was from Worthington Biochemical Corp.

RESULTS

Py TAG purified from recombinant baculovirus-infected insect cells replicates Py ori-DNA in vitro. Py TAG was purified by immunoaffinity procedures from insect cells infected with recombinant baculovirus vEV51LT and was the predominant protein visualized by silver staining on polyacrylamide gels (Fig. 1A). To assess the quantity of Py TAG, both colorimetric analysis and visualization of bands on silver-stained gels with comparison to protein standards were used. The purity and activity of immunoaffinity-purified Py TAG varied somewhat between preparations, and therefore comparisons are more accurate within experimental groups than between them. In a typical experiment, however, 1 pmol of immunoaffinity-purified TAG mediated the incorporation of 10 pmol of TTP into replication products of Py ori-DNA (pBE102) in mouse FM3A cell extracts. The generation of fully replicated, *DpnI*-resistant, progeny DNA molecules was completely dependent on the presence of Py TAG (Fig. 1B). In addition, with increasing quantities of TAG, the level of DNA synthesis, as determined by acid precipitation, increased sigmoidally (Fig. 1C), consistent with results previously observed with Py TAG immunoaffinity purified from recombinant adenovirus-infected monkey cells (36). In one case, when the amount of plasmid ori-DNA was varied with 1.5 pmol of Py TAG, the level of incorporation increased until a maximum of 18 pmol of TTP incorporated was reached in the presence of 45 fmol of template ori-DNA (Fig. 1D). Generally, 50:1 ratios of Py TAG to ori-DNA were required before replication products were easily detected, and ratios of 100:1 were optimal. Whenever possible, experiments examining the DNA-binding properties of Py TAG used a 100:1 ratio of Py TAG to DNA.

Nucleotides increase Py TAG binding to origin region DNA fragments. Increasing amounts of Py TAG were incubated with ^{32} P-end-labeled whole, core, and early DNA fragments (as shown at the bottom of Fig. 2) in the presence and absence of ATP, and then the binding mixtures were filtered through nitrocellulose. In the absence of ATP, Py TAG bound to the whole origin much more effectively than to either early or core origin DNA fragments (Fig. 2A, B, and C). The viral protein exhibited slightly higher affinity for early DNA than for core origin DNA. The addition of 4 mM ATP changed the DNA-binding properties of Py TAG mark-

protein marker bands (in kilodaltons) are indicated on the left. (B) Reaction mixtures as described in Materials and Methods containing 200 ng (37 fmol) of Py ori-DNA (pBE102) and 0 or 100 ng (1 pmol) of Py TAG were incubated for 3 h at 33°C. Progeny DNA was purified from the reactions and linearized with *PvuI* in the presence or absence of *DpnI*. DNA samples were electrophoresed in 1% agarose and visualized by autoradiography. (C) Reaction mixtures contained the indicated amounts of pBE102 DNA and Py TAG. After 3 h of incubation at 33°C, a 5- μ l aliquot of each reaction was acid precipitated and counted by liquid scintillation. (D) Reaction mixtures containing the indicated amounts of Py ori-DNA and 1.5 pmol of Py TAG were incubated for 3 h at 33°C, after which the amount of acid-insoluble radioactivity was determined by liquid scintillation.

FIG. 2. ATP increases Py TAg binding to origin region DNA fragments. Reaction mixtures containing or lacking 4 mM ATP were incubated with increasing amounts of Py TAg as indicated and ^{32} P-end-labeled Py whole origin (A), core origin (B), or early (C) DNA fragments (D).

edly. Binding to the whole origin (Fig. 2A) was increased by as much as fourfold at low concentrations (0.4 pmol) of Py TAg and by less than twofold at higher concentrations (1.4 pmol). ATP increased the binding of Py TAg to the core origin even more dramatically, by up to 10-fold throughout the entire concentration range of the protein tested (Fig. 2B). In contrast, binding to the early fragment was increased only approximately twofold by ATP (Fig. 2C).

A ratio of Py TAg to DNA (100:1) which is nonsaturating for ori-DNA binding and is also optimal for detecting progeny ori-DNA in *in vitro* DNA replication reactions was chosen for examining the effects of increasing concentrations of ATP on Py TAg-DNA interactions. Ori-DNA fragments were preincubated for 5 min at 37°C with increasing

concentrations of ATP, after which Py TAg was added and the incubation continued for 20 min prior to filtration through nitrocellulose. In the absence of ATP, Py TAg bound over 40% of the whole origin fragment, 25% of the early fragment, and 10% of the core origin fragment (Fig. 3A). With increasing levels of ATP, i.e., from 0.05 to 4 mM, Py TAg initially bound more DNA in all cases. Binding to the whole origin was maximal between 0.5 and 1.0 mM ATP, with 70% of the input DNA bound. The early fragment was bound maximally at the same ATP concentration as was the whole origin, but the binding increased only to 40%. Although core origin binding was increased to over 40% by 0.5 mM ATP, maximum binding was not reached until 4 mM ATP had been added, at which point 70% of the fragment was bound.

FIG. 3. Nucleotides affect Py TAG binding to DNA. Py TAG and ^{32}P -labeled Py origin region DNA fragments were incubated in replication buffer at 37°C with various amounts of nucleotides as indicated. The samples were assayed by nitrocellulose filter binding. (A) Increasing concentrations of ATP were added to reaction mixtures containing Py TAG and origin region DNA fragments. The effects of ribonucleotides (B), deoxyribonucleotides (C), and nonhydrolyzable ATP analogs (D) on binding of Py TAG to core origin DNA are also shown.

All four ribonucleotides, the four deoxyribonucleotides, and two nonhydrolyzable ATP analogs were tested for their abilities to stimulate Py TAG binding to the Py core origin (Fig. 3B, C, and D). The same conditions were used as for the ATP concentration curve described above. Under these conditions at all the nucleotide concentrations tested, only ATP, dATP, 5'-adenylyl imidodiphosphate (AMPPNP), and 5'-adenylyl methylenediphosphate (AMPPCP) stimulated complex formation three- to sevenfold over a wide concentration range. A few of the other nucleotides stimulated core origin binding to a lesser extent (dCTP > UTP > TTP), but higher concentrations were required and the maximal effect was less (two- to fourfold) than for the ATP-derived compounds. CTP, GTP, and dGTP had no discernible effect on core origin binding.

ATP stimulation of Py TAG core origin binding is affected by divalent cations. Most ATP-dependent DNA-protein interac-

tions require divalent cations, frequently Mg^{2+} but sometimes Mn^{2+} , Zn^{2+} , or Ca^{2+} (see reference 38 and references therein). Py TAG also contains a putative zinc finger at amino acids 452 to 472 (1, 2). Accordingly, the effects of increasing concentrations of MgCl_2 and other divalent cations on the binding of Py TAG to core origin DNA fragments in the presence and absence of 4 mM ATP were tested. In the absence of ATP, increasing concentrations of MgCl_2 inhibited binding such that Py TAG-DNA interactions were virtually abolished by a 50 mM concentration (Fig. 4). However, in the presence of 4 mM ATP when MgCl_2 was absent, less complex was formed than in the absence of both MgCl_2 and ATP. Upon the addition of increasing amounts of MgCl_2 to reactions containing ATP, core origin binding increased, reaching an optimum at 7 mM MgCl_2 . At 20 mM MgCl_2 , binding was equivalent to that seen in the absence of MgCl_2 , and by 50 mM, it was inhibited further, although it was still

FIG. 4. ATP-mediated effects on Py TAG-DNA binding require $MgCl_2$. Py TAG was incubated with ^{32}P -end-labeled core origin DNA in the presence of increasing concentrations of $MgCl_2$ and in the presence or absence of 4 mM ATP as indicated. Samples were assayed by nitrocellulose filter binding.

considerably higher than when ATP was absent at this concentration of $MgCl_2$. Similar results were obtained when the relative binding of SV40 TAG to SV40 core origin DNA as a function of increasing concentrations of $MgCl_2$ was compared in the presence and absence of 4 mM ATP (data not shown). None of the other divalent cations tested ($MnCl_2$, $CaCl_2$, and $ZnCl_2$) substituted for $MgCl_2$ in medi-

ating the ATP enhancement of core origin binding, although they did alter Py TAG binding to the core origin to various extents (data not shown).

ATP specifically increases the binding of Py TAG to a DNA fragment containing the core origin. To examine the specificity of the ATP-induced changes in Py TAG-DNA binding as well as changes in the amount of the complexes formed, we incubated end-labeled core origin and nonspecific DNA fragments with Py TAG. After being cross-linked with glutaraldehyde, free and TAG-complexed DNA were analyzed on 4% polyacrylamide gels (Fig. 5A). In the absence of ATP, only the highest concentration of Py TAG tested, representing a TAG/DNA ratio of 1,000:1, produced a detectably shifted DNA band. In the presence of ATP, a shifted DNA band was clearly visible at a TAG/DNA ratio of 350:1, and at the highest Py TAG concentration tested, the quantity of unbound 94-bp core origin band was greatly reduced, while the unbound nonspecific fragment was not detectably affected. Quantitation of the two DNA fragments by densitometry revealed that 100% of the nonspecific fragment remained unbound, while in the presence of ATP at the highest concentration of Py TAG tested, only 50% of the specific fragment was unbound. Furthermore, in all lanes in which the shifted band was detectable, the amount of DNA fragment shifted was equivalent to the amount depleted from the unbound core origin. The complex in all cases migrated slower than the 3.6-kb DNA marker, indicating a very large complex whose size was difficult to estimate under these conditions.

As an alternative measure of the specificity of Py TAG for the core origin, we performed a competition experiment in which Py TAG was bound to either ^{32}P -labeled Py core origin or a mixture of nonspecific plasmid DNA fragments in the presence of increasing concentrations of unlabeled nonspecific plasmid DNA. This experiment revealed an ATP-

FIG. 5. ATP increases the amount of core origin DNA specifically bound by Py TAG. (A) Py TAG (0 to 5 μ l; 700 fmol/ μ l) was incubated with 2.5 fmol each of ^{32}P -3'-end-labeled 94-bp core origin DNA and a pAT153-derived 75-bp nonspecific fragment at 37°C with or without ATP as indicated. The samples were fixed with glutaraldehyde and analyzed on a polyacrylamide gel. The positions and sizes (in base pairs) of DNA markers are shown on the left. (B) Py TAG (1 pmol) was incubated with 1 ng of ^{32}P -labeled core origin or of ^{32}P -labeled nonspecific *Hinf*I-digested pAT153 DNA and increasing amounts of unlabeled pAT153 plasmid DNA in the presence or absence of ATP. DNA binding was measured by nitrocellulose filtration. Symbols: ●, core origin with ATP; ○, core origin without ATP; ◇, nonspecific DNA with ATP; ×, nonspecific DNA without ATP.

dependent increase in origin-binding specificity (Fig. 5B). In the absence of ATP, a 5-fold excess (5 ng) of unlabeled nonspecific DNA reduced the amount of labeled nonspecific DNA bound by 50%, while a 20-fold excess was required to reduce core origin binding by 50%. In the presence of ATP, a 20-fold excess of competitor DNA was required to reduce the nonspecific binding to 50%, but under these conditions, a 1,000-fold excess of nonspecific DNA reduced the core origin bound only to 66%. Thus ATP increased the binding affinity of Py TAG for the core origin from 4-fold to over 50-fold relative to the nonspecific DNA fragments.

ATP influences rate of association of Py TAG to DNA. To further characterize the nature of the ATP-induced change in the DNA-binding properties of Py TAG, we examined the rate at which Py TAG bound to the three origin region DNA fragments or a nonspecific DNA fragment that did not contain pentanucleotides. The time course of formation of protein-DNA complexes was measured by using a modification of the filter-binding assay that permitted determination of binding over intervals of 5 s (see Materials and Methods). When Py TAG-ori-DNA complex formation was measured, it was clear that the greatest difference between reactions containing or lacking ATP was within the first 5 s, the earliest interval measured. ATP thus effected an initial rapid increase in the amount of DNA bound that was followed by decreased rates of binding in subsequent intervals until plateau levels of bound DNA were reached. Maximum levels of binding were reached by 60 s when whole origin (Fig. 6A) or early (Fig. 6C) fragments were used, while binding to the core origin DNA fragment (Fig. 6B) continued to increase over 5 min, albeit at a progressively slower rate. In the absence of ATP, the initial rates of binding were dramatically slower than in its presence in all cases. The plateau levels of DNA bound at this TAG concentration were somewhat greater for the whole origin than for either the core or early fragments, both in the presence and in the absence of ATP. This may be relevant to previously reported results showing that site B or C or both increase the efficiency of replication from the core Py origin *in vivo* (58).

Nonspecific DNA fragments that lacked pentanucleotides were also examined by using this protocol. As the amount of unlabeled pAT153 DNA used as a carrier for specific ori-DNA fragment-binding assays markedly reduced Py TAG-nonspecific-DNA complex formation (e.g., see Fig. 5B), unlabeled carrier was omitted from the binding reactions. Under these conditions it was clear that, as had been observed in the competition experiment shown in Fig. 5B, ATP resulted in a marked increase in the DNA bound by Py TAG (Fig. 6D). Again the effects of ATP at the very earliest times were the most marked, and the overall effect of the nucleotide was a rapid increase in binding, with half-maximal association reached by 5 s, while in the absence of ATP, complexes formed more slowly over the time observed, with half-maximal binding requiring at least 3 min.

Disassociation rate of Py TAG from DNA is affected by ATP. To analyze the disassociation of Py TAG from bound DNA, TAG was incubated with DNA fragments for 5 min and then a large molar excess of cold DNA was added. The samples were filtered through nitrocellulose at intervals extending to 45 min. Py TAG disassociated slowly from the whole origin in both the presence and the absence of ATP, remaining stably bound after 45 min, which is consistent with the large number of binding sites per DNA molecule (Fig. 7A). In the presence of ATP, approximately 50% disassociation was reached at 45 min, while in the absence of ATP, the majority (65%) of the DNA originally bound was still in complex after

45 min. Py TAG disassociated from the core origin somewhat more quickly. In the absence of ATP, 50% disassociation was reached by 15 min, and only 26% of the original complex remained after 45 min (Fig. 7B). In the presence of ATP, Py TAG disassociated from the core origin slightly more slowly, such that after 20 min, 50% of the complex had been released, and at the end of 45 min, 38% of the fragment still remained bound. Py TAG disassociation from the early DNA fragment followed a very different pattern (Fig. 7C). In the absence of ATP, the initially bound DNA remained stably associated with the protein over the entire 45 min. In the presence of ATP, however, disassociation from this fragment followed a pattern similar to that observed with the core origin fragment, with 50% disassociated after 20 min. Within 10 min, as a result of this phenomenon, more molecules of DNA remained bound in the absence of ATP than in its presence. Disassociation from the whole origin thus appears to represent a combination of the effects seen with the core plus the early DNA fragments. The disassociation rate of Py TAG from a nonspecific DNA fragment was initially accelerated by ATP, but a substantial fraction of the complex was stable over the course of the 45-min assay, and 50% release did not occur during this time (Fig. 7D).

DNase I footprinting reveals a change in protection of ori-DNA by Py TAG in the presence of ATP. Previously published DNase I footprint analyses of Py TAG interactions with DNA containing the Py replication origin were performed at 20 to 25°C in the absence of ATP (11, 12, 21). As our data indicated that ATP dramatically alters Py TAG binding affinities for DNA fragments within the origin region, we examined DNase I footprint patterns to determine if the presence of ATP altered the sequences protected. The whole Py ori-DNA fragment was labeled on the late strand at the *DdeI* site at nt 187, and 5 to 10 ng per reaction were incubated with increasing quantities of Py TAG in the presence or absence of 4 mM ATP. After DNase I digestion, the resulting DNA fragments were analyzed on a 6% polyacrylamide denaturing gel (Fig. 8A).

In the absence of ATP and with increasing quantities of Py TAG, sites A and B (as well as site C, for which the data are not shown), on the early side of the origin palindrome, were protected at significantly lower concentrations of Py TAG than was the origin palindrome itself, confirming previous studies (11, 12, 21). However, in our experiments, we were not able to show the previously assigned sites 1 and 2 as distinct entities or to identify protected sequences corresponding to site 3. Whether this is a result of differences in the sources of Py TAGs used or in the assay conditions remains to be determined.

In the presence of 4 mM ATP, protection of the core origin region was markedly increased, such that at the highest concentration of Py TAG (1.5 µg; TAG/DNA ratio of 600:1), the entire core origin region was protected and the borders between sites within the core origin that are visible in the absence of ATP were virtually eliminated. The presence of ATP also produced a Py TAG-dependent DNase I-hypersensitive site at nt 5255 to 5262, a region immediately upstream of the previously defined site 2 on the late side of the core origin. To determine the concentration of ATP required to induce this altered protection pattern of the origin region, a constant level of Py TAG (0.75 µg; TAG/DNA ratio of 300:1) was incubated with 3'-end-labeled ori-DNA in the presence of increasing amounts of ATP. In the footprint shown in Fig. 8B, in the absence of ATP, protection of the origin palindrome and of sites A, B, and part of C could be detected. At the lowest concentration of ATP (0.01 mM), DNase I pro-

FIG. 6. ATP affects association rates of Py TAG binding to DNA fragments. Py TAG (1.0 pmol) and 0.01 pmol of whole origin (A), core origin (B), early (C), or nonspecific ^{32}P -labeled (D) DNA were incubated for the times indicated in replication buffer at 37°C in the presence or absence of ATP. The DNA was then added to the TAG, and the mixtures were filtered through nitrocellulose filters immediately after the times indicated.

tection actually appeared to decrease slightly in all sites, but at 0.1 mM ATP, the pattern was altered such that the DNase I-hypersensitive site, noted above, appeared and the entire core origin was protected. The third pentanucleotide of site A was somewhat less protected at the lower concentrations of ATP than it was with either 0 or 4 mM ATP, which is consistent with a previous report that this pentanucleotide is not necessary for *in vitro* replication (36). At longer exposures of these footprints, it was observed that sites B and C were also slightly less protected at intermediate concentrations of ATP than they were in its absence (data not shown). At 4 mM ATP, the entire origin was protected and regions of DNA between sites A and B and between sites B and C were somewhat less sensitive to DNase cleavage as well.

Thus DNase I footprint experiments showed that ATP not only increased binding of Py TAG to the origin but also effected significant alterations in the sequences protected in this region. The changes we observed were consistent with the previously established sequence requirements for Py core origin function.

DISCUSSION

Our experiments revealed that Py TAG binding to DNA containing the core origin was strongly stimulated by ATP under conditions suitable for *in vitro* DNA synthesis. This augmentation was not dependent on nucleotide hydrolysis, as nonhydrolyzable ATP analogs were also efficient in

FIG. 7. ATP alters the disassociation of Py TAG from DNA fragments. Py TAG was preincubated with whole origin (A), core origin (B), early (C), and nonspecific ^{32}P -labeled (D) DNA fragments at 37°C in replication buffer in the presence or absence of ATP. A 100-fold molar excess of unlabeled Py ori-DNA was then added. The mixtures were filtered through nitrocellulose at the times indicated.

activating DNA binding. All the adenosine-derived NTPs could substitute effectively for ATP, a finding similar to previously reported results with SV40 TAG binding to the SV40 core origin (3). However, the relative degree to which the other nucleotides stimulated binding differed from the situation with SV40. Most notably, CTP, which was reported to stimulate SV40 TAG-core origin binding, had no effect on Py TAG. Furthermore, those nucleotides which enhanced Py TAG binding to a lesser extent (dCTP, UTP, and TTP) not only required higher concentrations to stimulate origin binding but also had significantly reduced effects.

Previous articles have reported variously that ATP inhib-

its SV40 TAG binding to viral DNA (56) or that it stimulates this binding (3, 18). Our data suggest an explanation for this discrepancy. In experiments reporting inhibition by ATP, binding was performed in the absence of Mg^{2+} , whereas when ATP was reported to stimulate binding, Mg^{2+} was present. Our experimental results indicate that the increase in DNA binding by Py TAG mediated by ATP was dependent on the concentration of MgCl_2 present. In the absence of MgCl_2 , concentrations of ATP under 0.5 mM had no effect on core origin binding and concentrations of ATP over 1 mM became increasingly inhibitory; i.e., binding was reduced 50% at 10 mM ATP (data not shown). We have obtained

FIG. 8. DNase I footprinting reveals increased protection of the Py core origin by Py TAg. (A) Increasing amounts, as indicated, of Py TAg (shown as duplicate samples) were incubated with 7 ng of Py ori-DNA ^{32} P 3' end labeled on the late strand with or without 4 mM ATP as indicated. One of the 0- μ g Py TAg duplicate lanes was distorted and was removed for purposes of clarity. (B) Py TAg (750 ng) was incubated in duplicate with increasing amounts of ATP as indicated and with Py ori-DNA as in panel A. After DNase I digestion, products were analyzed by electrophoresis and autoradiography. The Py TAg-binding sites are shown in brackets. Arrows show the location of pentanucleotides contacted by TAg. The sequence of the DNase I hypercutting induced by Py TAg and ATP is shown on the right of panel B.

essentially similar results in comparable experiments with SV40 TAG and SV40 ori-DNA fragments (data not shown). Thus, although additional technical variations were described in previously published reports, our experiments may suggest a way to reconcile the differences.

It is known that ATP, as well as other NTPs, will associate with a Mg^{2+} ion, which binds to two adjacent phosphate oxygens (38). Magnesium is required for the function of a number of kinases and ATPases, perhaps because it alters the configuration of the phosphate groups on ATP and thus facilitates hydrolysis or, alternatively, because it allows enzyme binding to DNA by neutralizing the negative charge of the phosphate groups. However, in a previous report it was hypothesized that the apparent stimulation of SV40 TAG by ATP and $MgCl_2$ was due merely to ATP chelation of the Mg^{2+} ion, which is by itself inhibitory (48). While we have observed that both ATP and $MgCl_2$ were individually inhibitory, together they greatly stimulated core origin DNA binding significantly above binding in their absence, both for Py TAG and for SV40 TAG on their respective DNAs (SV40 data not shown). Importantly, very low concentrations (0.1 mM) of ATP were sufficient to significantly increase binding in the presence of 7 mM $MgCl_2$ for both TAGs (SV40 data not shown). Thus the concentration of ATP or dATP that stimulated binding was significantly lower than that required to quantitatively chelate Mg^{2+} at its optimal concentration, and therefore it seems unlikely that the effect of ATP is due merely to $MgCl_2$ chelation. The effect of $MgCl_2$ may instead be due to its ability to change the conformation and/or charge of ATP so as to make it a more suitable substrate for TAG.

The binding of SV40 TAG to the SV40 replication origin has been shown to be altered both qualitatively and quantitatively by ATP. In the presence of ATP, up to 10- to 15-fold-more complexes between SV40 TAG and core origin DNA were formed (3, 18). Examination of these complexes by electron microscopy revealed that ATP induced formation at the origin of large bilobed protein complexes that apparently consist of double hexamers of SV40 TAG (15, 33), whereas in the absence of ATP, the largest polymers are trimers and tetramers (34). In the absence of DNA, ATP causes SV40 TAG to oligomerize (16), although oligomeric forms of TAG are much less active than monomers in ori-DNA binding, DNA helicase, and unwinding (25, 46, 50).

While neither we nor others have reported the effects of nucleotides on Py TAG protein-protein interactions, our data suggest that ATP may indeed affect the oligomerization state of Py TAG. In analyzing the rates of association of Py TAG with different DNA fragments, we noted that Py TAG required a significantly longer time to bind maximally to the core origin DNA fragment, suggesting that more complicated interactions may be occurring in this region than with other DNA sequences. Furthermore, our DNase I footprint data revealed a greatly extended region of protection in the region of the core origin when ATP was present. Indeed, we have recently been able to show directly that ATP induces the conversion of Py TAG monomers to hexamers (56a). These data are all consistent with the formation at the Py core origin of a larger protein complex analogous to the highly specialized nucleoprotein structures formed by SV40 TAG at the SV40 origin.

The dissociation constant K_d has been determined for several DNA-binding proteins, including the *lac* repressor (45) and SV40 TAG (26, 48). The *lac* repressor is particularly amenable to mathematical analysis, as one repressor tetramer binds one operator (5). SV40 TAG, however, exists in a

number of oligomeric states, from monomers through hexamers, and the SV40 origin contains three distinct binding sites within which are a total of seven perfect pentanucleotides. Nonetheless, by analyzing the SV40 system in the absence of ATP as if it were bimolecular, treating each DNA molecule as one binding site, and assuming the TAG molecules to be tetramers, quantifications of this complicated system have been generated (26). Py protein-DNA interactions are likely to be similarly complex. The Py origin contains four to six separate binding sites, with a total of 14 perfect and several imperfect pentanucleotides. Using assumptions similar to those used in the case of SV40, we attempted to determine reaction constants for the binding of Py TAG to DNA. These were not constant when determined by standard bimolecular equations, and accordingly the Scatchard plots of our data were not linear (data not shown). Furthermore, if Py TAG follows the oligomerization pattern of SV40 TAG in the presence of ATP, which our data suggest it does, then the stoichiometry of Py TAG binding to DNA would change in the presence of ATP and any mathematical analysis of the reaction kinetics would have to use different parameters according to the presence or absence of ATP. Despite these caveats, our calculations of the dissociation equilibrium constant K_d showed a 10- to 50-fold increase in Py TAG affinity for Py ori-DNA in the presence of ATP (data not shown).

SV40 TAG has been shown to require three domains in the SV40 core origin to accomplish the DNA binding and unwinding necessary to initiate replication (40). The domains of the SV40 core origin consist of a 17-bp imperfect inverted repeat (5'-CACTACTTCTGGAATAG-3') on the early side, a four-pentanucleotide origin palindrome at the center, and a 17-bp A/T-rich region on the late side. Since extended A/T regions are less stable than other base pair groups and since this particular A/T region includes a region of DNA "bending" (17), it was initially predicted that SV40 TAG would bind at the origin palindrome and then unwind the A/T region. Surprisingly, the early palindrome was shown to untwist before the A/T region, as detected by potassium permanganate oxidation, dimethyl sulfate methylation, and deletion mutagenesis (4, 40). It was also shown that high concentrations of SV40 TAG can unwind the early palindrome in the absence of other ori-DNA domains (40).

The sequences on the early side of the Py origin palindrome, unlike those in SV40, do not themselves form an additional palindrome. In fact, although the distance between the origin palindrome and the first early side pentanucleotides (site 1 in SV40, site A in Py) are similar, there is very little sequence homology in this region. The Py core origin does have a four-pentanucleotide origin palindrome similar to that of SV40 and a 16-bp A/T-rich region (with one G/C base pair) which contains an 8-bp potential bending site. Interestingly, our footprinting results revealed a strong DNase I hypercutting region on the late border of the core origin 10 bp upstream of the A/T-rich region. It may well be that Py TAG, unlike SV40 TAG, unwinds the A/T-rich region on the late side of the origin first. Experiments are in progress to further analyze the mode by which ATP affects the ability of Py TAG to initiate unwinding of the Py origin of replication.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA26905 from the National Institutes of Health.

We thank Ella Freulich and David Perlstein for excellent technical assistance. W. Folk is thanked for helpful comments during the

course of these experiments. We are also very grateful for the critical reading and advice of John Reinitz and Cyrus Levinthal.

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