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Murine Polyomavirus and Simian Virus 40 Large T Antigens Produce Different Structural Alterations in Viral Origin DNA

SUBARNA BHATTACHARYYA, HEATHER E. LORIMER, AND CAROL PRIVES*

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

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Murine polyomavirus (Py) and simian virus 40 (SV40) encode homologous large T antigens (T Ags) and also have comparable sequence motifs in their core replication origins. While the ability of SV40 T Ag to produce specific distortions within the SV40 core replication origin (ori) in a nucleotide-dependent fashion has been well documented, little is known about related effects of Py T Ag on Py ori DNA. Therefore, we have examined viral origin DNA binding in the presence of nucleotide and the resulting structural changes induced by Py and SV40 T Ags by DNase I footprinting and $KMnO_4$ modification assays. The structural changes in the Py ori induced by Py T Ag included sites within both the A/T and early side of the core origin region, consistent with what has been shown for SV40. Interestingly, however, Py T Ag also produced sites of distortion within the center of the origin palindrome and at several sites within both the early and late regions that flank the core ori. Thus, Py T Ag produces a more extensive and substantially different pattern of $KMnO_4$ modification sites than does SV40 T Ag. We also observed that both T Ags incompletely protected and distorted the reciprocal ori region. Therefore, significant differences in the interactions of Py and SV40 T Ags with ori DNA may account for the failure of each T Ag to support replication of the reciprocal ori DNA in permissive cell extracts.

In general, initiation of DNA replication involves recognition of the origin of replication by a site-specific DNA-binding protein, which then, in the presence of nucleotide, induces sufficient structural distortions in the DNA to help facilitate the entry of the replication machinery (see references 3, 7, and 19 for reviews). Murine polyomavirus (Py) and simian virus 40 (SV40) are closely related papovaviruses whose genomes are similar in size and organization. In each case, only one viral protein, the large T antigen (T Ag), is required for viral replication; the rest of the replication factors are supplied by the host cell. SV40 T Ag, far more extensively studied than its Py counterpart, has been the subject of several excellent reviews, most recently and exhaustively by Fanning and Knippers (20). Nevertheless, it is now clear that the two T Ags display common biochemical properties that come into play during many steps from initiation through the process of bidirectional DNA replication. These include sequence-specific DNA-binding activity (9, 10, 18, 26), nucleotide-induced ori DNA binding (4, 16, 31) and hexamer formation (13, 36, 47, 61), DNA-unwinding activity (12, 24, 62, 63), DNA helicase activity (12, 53, 55, 62), and ATPase activity (10, 24). Moreover, both T Ags require and undergo specific interactions with the appropriate DNA polymerase α primase (37, 39, 40, 50).

Consistent with the similarities between the two T Ags are several features common to both viral replication oris. Mutagenic studies have defined the core oris of both Py and SV40 in vivo (2, 27, 32, 38, 46) and in vitro (18, 30, 46, 56). The T Ags bind specifically to regions containing two or more copies of the pentanucleotide 5′-G(A/G)GGC-3′ (11, 18, 26, 44). SV40 DNA has strong binding sites for its T Ag: site I flanking the early border of and site II within the center of the core ori. The SV40 core ori comprises a minimal 64-bp region which is essential and sufficient for its DNA replication. It may be divided into three essential domains (14, 17). In the center of the core ori there is a 27-bp perfect inverted repeat (T Ag binding site II) containing four copies of the pentanucleotide sequences aligned as two-head-to-two-tail pairs on opposite strands of the DNA. Site II is flanked by an imperfect inverted repeat of 15 bp on the early side of the origin, called the early palindrome (EP). On the late side, there is a 17-bp A/T-rich tract, forming the third required component of the core ori. The A/T region contains sequences that contribute to DNA bending (15, 25).

The regulatory region within Py viral DNA has three high-affinity binding sites, A, B, and C, containing three, two, and four pentanucleotides, respectively, on the early side of the core ori. The center of the Py core ori contains a 34-bp inverted repeat with four pentanucleotides aligned similarly to those in the SV40 ori. Additionally, there is an A/T-rich tract on the late side of the ori. Therefore, a central T Ag-binding site-containing palindrome and a late-side A/T-rich region are common features of SV40 and Py core oris. Although there are no sequences homologous to the SV40 EP on the early side of the Py ori central palindrome, there is a region that is highly pyrimidine rich on one strand (the R/Y region) that is likely to fulfill a function similar to that of the SV40 EP. Additionally, the early border of the core ori contains two pentanucleotides that are part of site A; they are aligned similarly to the two pentanucleotides of SV40 site I, although some base substitutions in these pentanucleotides do not inhibit replication (57).

The use of chemical modification assays has allowed the determination of specific sequences within the AT and EP regions that are distorted by SV40 T Ag in the presence of nucleotide (5, 54). Since the distorted A/T region is not sensitive to S1 nuclease digestion, it is therefore "untwisted" but not completely unwound. However, S1 nuclease sensitivity indicates that the SV40 EP is actually melted by T Ag. Interestingly, high concentrations of SV40 T Ag produce $KMnO_4$-sensitive sites in the EP even in the absence of the other two core domains (42).

* Corresponding author. Mailing address: Department of Biological Sciences, 816 Fairchild Building, Columbia University, New York, NY 10027. Phone: (212) 854-2557. Fax: (212) 865-8246.

In light of the fact that Py T Ag shares many biochemical properties with SV40 T Ag, it is likely that the initiation of Py DNA replication also involves structural alterations at the core origin. Indeed, Py T Ag is very efficient at unwinding SV40 origin-containing DNA, although SV40 T Ag is not equally proficient in unwinding Py DNA (62). However, despite its ability to unwind SV40 origin-containing DNA, Py T Ag cannot mediate its replication in vivo or in vitro (39, 40, 62). In order to gain further insight into the dynamics of these interactions, we have examined the interactions of both Py and SV40 T Ags on their own as well as reciprocal origins by DNase I footprinting and $KMnO_4$ modification assays.

MATERIALS AND METHODS

In vitro ori DNA replication. The reaction conditions and methods used for the preparation of mouse FM3A extracts and human HeLa cell extracts and the purification of Py and SV40 large T Ags have been described previously (39, 46, 60). Py and SV40 T Ags were purified from Sf27 insect cells infected with recombinant baculovirus vEV55PyT (62) or vEV55SVT (41), respectively. Reaction mixtures (50 μl) contained 40 mM creatine phosphate di-Tris (pH 7.7); 7 mM $MgCl_2$; 0.5 mM dithiothreitol [DTT]; 4 mM ATP; 200 μM each CTP, UTP, and GTP; 100 μM each dATP, dCTP, and dGTP; 20 μM [α-^{32}P]TTP (2×10^4 cpm/pmol); 100 μg of creatine kinase per ml; FM3A or HeLa cell extract (0.2 to 0.4 mg of protein); replication origin-containing plasmid DNA; and purified T Ags at the indicated concentrations. Reaction mixtures were incubated at 33°C for 3 h. Aliquots (5 μl) of reaction mixtures were then subjected either to acid precipitation and scintillation counting or to product analysis, whereby the DNA was purified from reaction mixtures and linearized in the presence or absence of *Dpn*I, followed by 0.8% agarose gel electrophoresis. The Py origin DNA used (pBE102) contained the A2 strain of polyomavirus nucleotides (nt) 5022 to 1562 inserted into a pML vector (28). The SV40 origin-containing DNA pATSVO contains nt 5171 (*Hin*dIII) to 294 (*Kpn*I mutated to *Eco*RI) of the SV40 origin inserted into pAT153 at the *Hin*dIII and *Eco*RI sites (48).

DNase I footprinting assay. DNA fragments were prepared by 3′-end labeling with [α-^{32}P]TTP and the Klenow fragment of *Escherichia coli* DNA polymerase I, cleaving with a second restriction endonuclease, and purifying the resulting fragments from a polyacrylamide gel. The SV40 ori DNA fragment was from pATSVO, end labeled at the *Hin*dIII site at nt 5171 and then cut at nt 294 with *Eco*RI. Py ori DNA was from pA373.A2 (58), end labeled at the *Dde*I site at nt 187 and then cut at nt 5075 with *Hin*fI. Various amounts of T Ag were incubated for 15 min at 33°C in R buffer (40 mM creatine phosphate di-Tris salt [pH 7.7], 4 mM ATP, 7 mM $MgCl_2$, 0.5 mM DTT, 10 μg of bovine serum albumin, 0.1 μg of nonspecific pAT153 plasmid DNA, 5 fmol of ori DNA). DNase I digestion was carried out with 6 ng of DNase I diluted in 20 mM $CaCl_2$ for 2 min at 33°C. Reactions were stopped by adding 50 μl of DNase I stop solution (2 M ammonium acetate, 0.2% sodium dodecyl sulfate [SDS], 100 mM EDTA, 100 μg of salmon sperm DNA per ml). The DNA was then purified by extraction with phenol and chloroform, followed by ethanol precipitation. Samples were resuspended in 90% deionized formamide in 1× Tris-borate-EDTA and analyzed on 8% acrylamide–8 M urea gels, which were then dried and exposed to X-ray film. The locations of areas of DNase I protection were determined by comparison to adjacent sequencing reactions.

DNA-binding assays were also performed with supercoiled plasmid DNA, adapting a previously described protocol (42). Reaction mixtures (30 μl) containing T Ag and plasmid DNA were incubated in binding buffer (40 mM creatine phosphate di-Tris salt [pH 7.7], 7 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 10 μg of bovine serum albumin) for 15 min at 33°C. The DNase I digestion conditions were similar to those used for fragment footprinting. The reactions were stopped with 10 μl of stop solution (2 M ammonium acetate, 100 mM EDTA, 100 μg of salmon sperm DNA, 0.2% SDS), followed by addition of 50 μl of phenol, and the samples were heated at 80°C for 2 min. The DNA was extracted and purified on G-50 Sephadex spin columns, and the DNA eluate was made up to 70 μl. To half of the sample was added 4 μl of 50 mM NaOH and 1 ng of primer (5′ labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase). The primers used to probe Py DNA were 5′-GCTCAGAACTCTATCCATGA-3′, which is complementary to the early strand at nt 192, and 5′-GGTTTTGCAAG AGGAAGCAA-3′, complementary to the late strand at nt 5194. For SV40 DNA, the *Hin*dIII pBR322 sequencing primer (Promega), 5′-GCAATTTAACT GTGAT-3′ (counterclockwise), was used to probe the late strand. The mixtures were heated at 80°C for 3 min and then fast-cooled on ice. The annealing reaction was carried out at 45°C for 3 min in 10× annealing buffer (500 mM Tris [pH 7.2], 100 mM $MgSO_4$, 2 mM DTT). Samples were primer extended in the presence of all four deoxyribonucleotides (to a final concentration of 500 μM) with 1 U of Klenow polymerase for 10 min at 52°C. The reactions were quenched with 17 μl of stop solution (4 M ammonium acetate, 20 mM EDTA). The DNA samples were precipitated, resuspended in 10 μl of deionized formamide, and analyzed on a 8% polyacrylamide–8 M urea gel. Sequencing reactions were run in parallel to help locate the areas of protection.

$KMnO_4$ modification assay. The procedure used for $KMnO_4$ modification was adapted from methods described previously (5, 6, 54). Reaction mixtures were essentially the same as for DNase I protection assays, although 4 mM 5′-adenylyl imidophosphate (AMPPNP) was used instead of ATP and bovine serum albumin was omitted from the buffer. After incubation for 15 min at 33°C, $KMnO_4$ was added to a final concentration of 6 mM, and the reactions proceeded for an additional 4 min prior to addition of β-mercaptoethanol to a final concentration of 1 M. The DNA was extracted once with phenol and chloroform and spun on a G-50 Sephadex spin column. The conditions for primer extension of purified DNA were essentially the same as described for DNase I protection assays. The samples were run in an 8% polyacrylamide–8 M urea gel next to DNA sequencing lanes.

RESULTS

Py and SV40 T antigens cannot support replication of reciprocal ori-containing DNA in permissive cell extracts. SV40 and Py replicons show marked host species specificity in vivo and in vitro (references 39 and 40 and references therein). Thus, SV40 T Ag cannot mediate replication of SV40 ori DNA in murine cells or cell extracts, and similarly, Py cannot support Py ori DNA synthesis in human or monkey cells or cell extracts. The ability of both the T Ags to negotiate replication exclusively in permissive cell extracts was shown to be the result of species-specific interactions between the T Ags and host cell DNA polymerase α (37, 39, 40, 50). Both the T Ags bind specifically to the same pentanucleotides and can interact with SV40 and Py origin DNA (44, 49), and these elements are arranged in a very similar (but not identical) manner within the viral core origins. Considering these facts together, it remained possible that each T Ag might be able to support replication of either origin in a permissive cell extract. To test this, similar quantities of immunopurified Py and SV40 T Ags (Fig. 1A) were compared for their ability to support replication of both viral ori DNAs in murine FM3A and human HeLa cell extracts, respectively (Fig. 1B and C). While each T Ag was capable of mediating substantial amounts of replicated products from its related viral ori DNA, neither was capable of supporting replication from the reciprocal origin. It was therefore of considerable interest to examine more closely, under replication conditions (i.e., in the presence of nucleotides and at 33°C), the interactions of the T Ags with the two viral origins.

Py and SV40 T Ags protect their cognate core ori DNA but show an incomplete pattern of protection from DNase I digestion on reciprocal ori-containing DNA. The abilities of Py and SV40 T Ags to protect either a fragment (Fig. 2A) or a supercoiled plasmid (Fig. 2B) from DNase I digestion were compared. In either condition, the results were similar: Py T Ag exhibited much more extensive protection of the Py core ori region than did SV40 T Ag. In the latter case, although SV40 T Ag protection of the core ori was weak, there were numerous DNase I-hypercutting sites. Both T Ags showed some protection within Py sites B and C, although DNase I hypercutting was more extensive with SV40 T Ag. Examination of binding of SV40 and Py T Ags on SV40 ori DNA similarly showed that SV40 T Ag more completely protected its own origin-containing site II and particularly the neighboring site I region (Fig. 2C). Our data thus show that there are marked differences in the way that the two T Ags bind to the two regions in the presence of nucleotide.

Py T and SV40 T Ags cause virus-specific alterations of origin DNA. $KMnO_4$ oxidizes nucleotides (primarily T residues) that have become exposed by alteration in the structure of the double helix (6). These oxidized residues cause chain termination by certain DNA polymerases (and thus bands on gels), the positions of which can be determined by comparison with DNA sequencing reactions and thus can be used to identify the sites at which DNA is melted or untwisted. SV40 T Ag

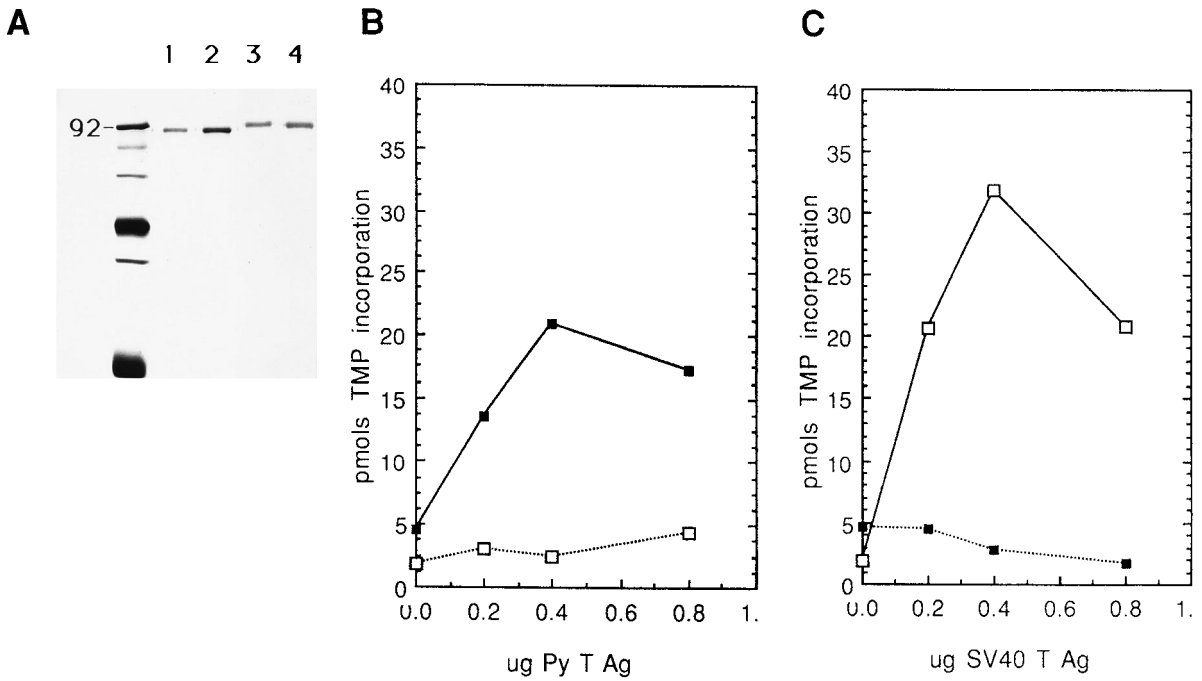

FIG. 1. Replication of Py and SV40 ori DNA mediated by immunopurified Py and SV40 T Ags. (A) Silverstained SDS-polyacrylamide gel showing 0.4 and 0.8 μg, respectively, of Py T Ag (lanes 1 and 2) and SV40 T Ag (lanes 3 and 4). The 92-kDa marker (left lane) is indicated. (B and C) Replication reactions contained either Py (■) or SV40 (□) origin-containing DNA with FM3A mouse cell extracts and the indicated quantities of Py T Ag (B) or with HeLa cell extracts and the indicated quantities of SV40 T Ag (C).

has been shown to alter the structure of SV40 DNA in a way that allows strong $KMnO_4$ modification of bases within the A/T and the EP regions of the SV40 core origin. We examined Py T Ag's ability to cause structural distortions in Py ori DNA as well as in comparison to SV40 T Ag and also compared the effect of each T Ag on the SV40 core ori. Supercoiled plasmid ori DNA was incubated with Py or SV40 T Ag, modified by $KMnO_4$, and analyzed according to the procedures in Materials and Methods. With Py T Ag, we were unable to detect any $KMnO_4$ modification sites when we used ATP. However a nonhydrolyzable derivative, AMPPNP, allowed the identification of sites (Fig. 3). Since a nonhydrolyzable ATP analog was also previously shown to increase the interactions of both T Ags with ori DNA and also to induce each T Ag to form hexamers (4, 5, 31, 47, 61), we consider the use of AMPPNP valid for analysis of Py T Ag-induced structural alterations in viral DNA. Possible explanations for the failure to detect modifications when hydrolyzable ATP was used are presented in the Discussion section below.

When the changes in Py ori DNA produced by Py and SV40 T Ags were compared, both similarities and differences were observed (Fig. 3A, B, and C). On the early strand, Py T Ag caused many discrete sites of distortion within and flanking the core origin (Fig. 3A and B, arrows). Additional weaker sites within the central palindrome were also noticeable (Fig. 3, brackets). Moreover, in contrast to SV40 T Ag, which caused DNA distortions that were restricted to the SV40 and Py core origins, Py T Ag also induced distortions at many distal sites within regions flanking the core ori on the early and the late sides. SV40 T Ag strongly induced untwisting at the A/T site on the early strand of Py ori DNA (Fig. 3A). Therefore, on the early strand of Py DNA, there was strong A/T region distortion by both Py and SV40 T Ags, but Py T Ag was unique in inducing numerous additional $KMnO_4$-sensitive sites. On the late strand, differences and similarities between Py T Ag (Fig. 3B and C) and SV40 T Ag (Fig. 3C) were also discerned. Py T Ag induced several sites throughout the Py core origin. The most distinctive ones were within and close to the R/Y region. Additionally, there was increased $KMnO_4$ modification of sites within the central palindrome and the A/T region. Although the sites detected with SV40 were qualitatively similar, there was one quantitative difference: one site in the R/Y region was actually made significantly more amenable to $KMnO_4$ modification by the SV40 T Ag.

When the abilities of SV40 and Py T Ags to distort SV40 ori DNA (late strand) were compared, the results were striking (Fig. 4). SV40 produced the characteristic alterations in SV40 A/T and EP regions that had been described previously. By contrast, Py T Ag was virtually ineffective at inducing any significant changes in SV40 ori DNA that allow $KMnO_4$ modification. This result was repeatedly obtained with several preparations of Py T Ag that were highly competent to mediate replication and also to distort Py ori DNA. Although it remains possible that examination of the interaction of Py T Ag with the early strand of SV40 DNA would yield sites of modification, the inability of Py T Ag to cause detectable $KMnO_4$ modification sites on the late strand leads to the conclusion that Py and SV40 T Ags interact very differently with the two viral origins.

DISCUSSION

In this study, we compared the ability of preparations of SV40 and Py T Ags that were carefully matched for purity and replication function to bind and distort both viral oris as well as to mediate replication from ori DNA in vitro. Little or no DNA synthesis was observed in reactions containing the reciprocal viral oris, confirming previous results in vivo (1). Thus,

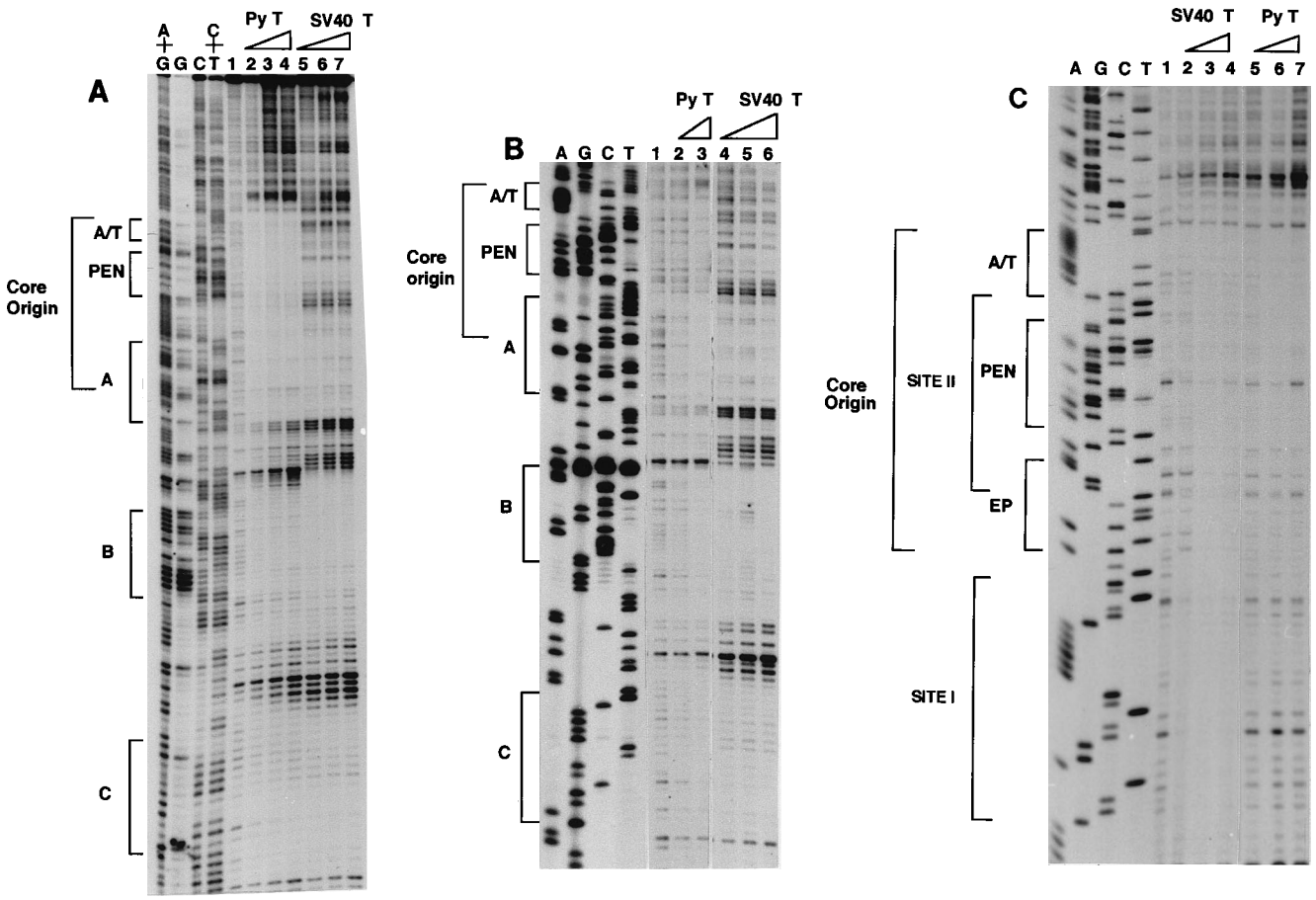

FIG. 2. DNase I footprint analysis of Py and SV40 T Ags on Py and SV40 ori DNA. (A) Protection of Py origin-containing linear DNA fragment from DNase I by increasing quantities of Py (0.2, 0.4, and 0.6 μg) or of SV40 (0.5, 1, and 1.5 μg) T Ag. (B) A similar DNase I footprint was performed on Py origin-containing supercoiled plasmid DNA. In this case, increasing amounts of Py (0.15 and 0.375 μg) and SV40 T Ag (0.25, 0.5, and 0.75 μg) were added to 0.15 μg of plasmid DNA. (C) DNase I footprint on SV40 origin-containing supercoiled plasmid DNA (0.35 μg) with SV40 (0.2, 0.4, and 0.6 μg) or Py (0.2, 0.4, and 0.6 μg) T Ag. Lanes 1, no protein added. Sequencing lanes are marked at top left, and ori DNA landmarks are marked to the left of each panel. PEN, T Ag binding pentanucleotides: G(A/G)GGC.

the failure of replication must be due to the inability of either the T Ag or a cellular factor to interact correctly with the appropriate DNA. The synthesis of fully replicated DNA in vitro is dependent upon the presence of the intact viral core ori (30, 46, 56). It has been shown that auxiliary viral sequences flanking the late side of the core ori in SV40 and Py are not responsible for this specificity, indicating that the crucial species-specific interaction is within the core ori (1).

While the interactions of SV40 T Ag with the SV40 ori have been examined in extraordinary detail (references 3 and 20 and references therein), there has been considerably less insight into its murine Py counterpart. The two T Ags, however, share a number of features that lead to the prediction that they should be similar in both structure and function. Both have DNA-binding domains that bind specifically to sites containing multiple copies of the same consensus pentanucleotide and both bind to and hydrolyze ATP. In each case, interaction with nucleotide leads to hexamerization and increased interactions with the core viral origin. Indeed, the amino acid sequences of the DNA- and ATP-binding domains of the T antigens are highly homologous. Furthermore, DNA binding by SV40 (reviewed in references 20 and 45) and Py (59) T Ags are both positively and negatively regulated by phosphorylation as well. These similarities predict that, like SV40 T Ag, Py T Ag should also be able to introduce specific distortions in the viral origin that can be detected as sites of $KMnO_4$ modification. Indeed, it would also be reasonable to assume that the corresponding regions that are melted or untwisted in the SV40 origin would be similarly affected in the Py origin.

However, our experiments indicate that Py T Ag possesses both similar and unique interactions with Py ori DNA compared with its SV40 counterpart. These are summarized in Fig. 5. As with SV40 T Ag, Py T Ag introduces discrete $KMnO_4$ modification sites within the late (A/T) and early (R/Y) elements of the core origin. Several bases are modified by $KMnO_4$ within the R/Y region and within the poly(A/T) tract on the late border of the core ori. However, unlike with SV40 T Ag, several other sites within the core origin are also apparent. These are located within the central palindrome region but exclude the pentanucleotides that are contacted by T Ag. In this way, the action of Py T Ag more closely resembles the melting of the bovine papillomavirus (BPV) origin introduced by the BPV E1 protein, in which more extensive structural changes over the region of BPV that was protected by the viral initiation protein (23) were identified. Furthermore, while the modification sites in Py DNA required the presence of AMP-PNP (unpublished), we have been unable to successfully identify sites of deformation when ATP (i.e., hydrolyzable nucleotide) was used. By contrast, the ability of SV40 to melt and untwist the SV40 core origin can be observed in the presence

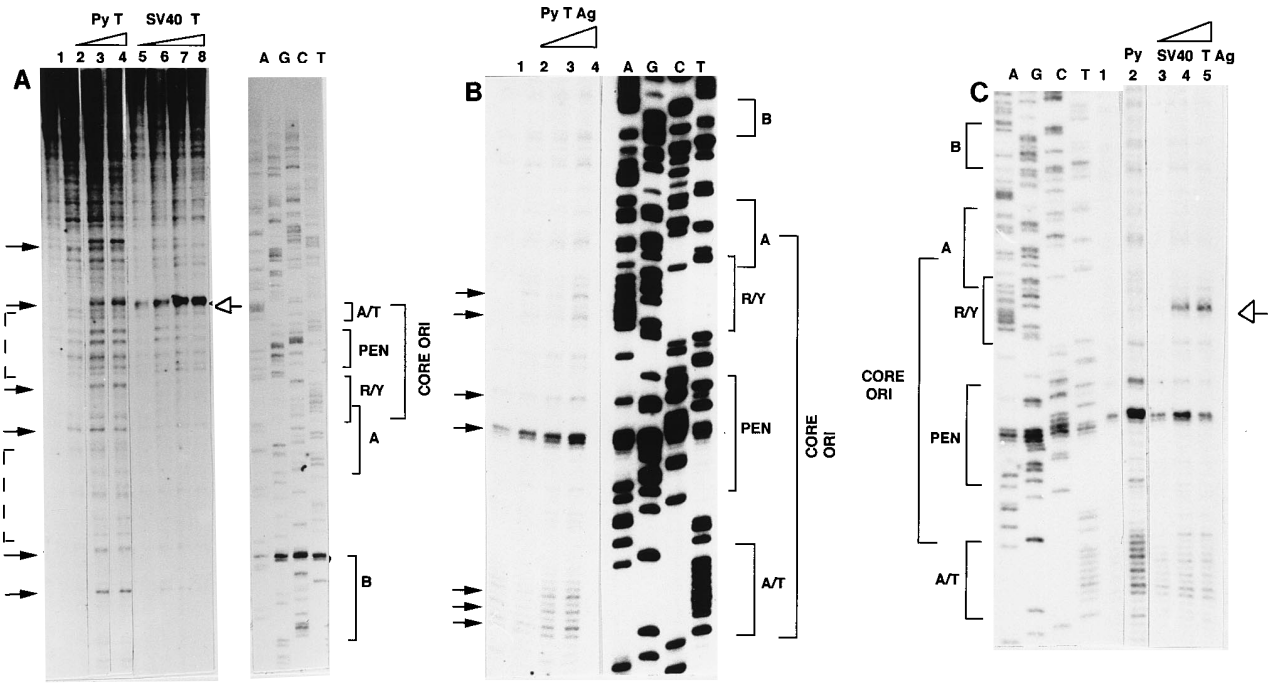

FIG. 3. $KMnO_4$ modifications of Py ori DNA in the presence of Py and SV40 T Ags. (A) Early coding strand modifications. Py ori-containing plasmid DNA (0.5 μg) was incubated with an increasing amount of Py (0.025, 0.075, and 0.2 μg) or SV40 (0.125, 0.25, 0.5, and 0.75 μg) T Ag or no protein (lane 1), in the presence of 4 mM AMPPNP. (B) Late strand modifications. Increasing amounts of Py T Ag (0.125, 0.25, and 0.3 μg) or no protein (lane 1) were incubated with Py ori DNA (0.2 μg). After treatment with $KMnO_4$ and purification, DNA samples were annealed to primers complementary to the early or late coding strand of Py DNA and then extended with polymerase as described in Materials and Methods. Sequencing lanes are marked with ori DNA landmarks. (C) Increasing amounts of SV40 T Ag (0.05, 0.15, and 0.25 μg) were incubated with Py ori DNA. Filled arrows indicate Py T Ag strong modification site. Open arrow shows strong SV40 T Ag site. Brackets indicate additional weaker sites modified by Py T Ag.

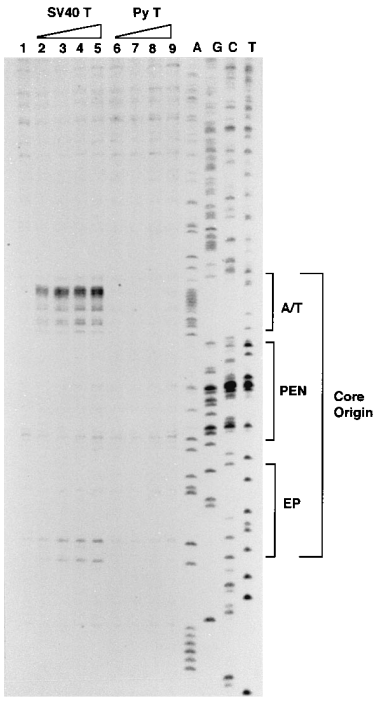

FIG. 4. $KMnO_4$ modification of SV40 ori DNA by SV40 and Py T Ags. Increasing amounts of SV40 (0.125, 0.25, 0.5, and 0.75 μg) or Py (0.05, 0.1, 0.2, and 0.3 μg) T Ag or no protein (lane 1) were incubated with SV40 ori DNA (0.5 μg). Samples were treated with $KMnO_4$, and the DNA was analyzed by primer extension using ^{32}P-end-labeled primer complementary to the late coding strand of the ori DNA.

of both hydrolyzable and nonhydrolyzable nucleotides. One possible reason for this difference is that in the presence of ATP, Py T Ag unwinds the origin so rapidly and extensively that no discrete $KMnO_4$ sites could be identified. Indeed, in a previous study, when the ability of Py and SV40 to unwind origin-containing DNA fragments was compared, there was a marked difference in the efficiency and specificity with which the two T Ags functioned (62). Py T Ag was capable of efficiently unwinding duplex fragments containing either the Py or the SV40 origin or even lacking any origin fragments whatever. Only at very high levels of competitor (carrier) DNA was it possible to discern a difference between the unwinding of Py ori DNA and nonspecific DNA fragments. Nevertheless, despite its ability to unwind many sources of DNA very effectively, from both previous studies and our data described herein, it is clear that the ability of Py T Ag to distort SV40 DNA is very defective.

It would be most reasonable to assume that the failure of Py T Ag to induce $KMnO_4$ modification within the SV40 origin is related to its relatively weak and incomplete protection of the SV40 core origin. However, although we also observed that the binding of SV40 T antigen to the Py origin was clearly deficient and different from the far more complete protection by Py T Ag, nevertheless SV40 was capable of introducing distortions in both the Py A/T region and the R/Y region that corresponds to the SV40 core origin EP element. These data suggest that the Py origin may require more than distortion in these regions and thus may highlight the importance of the additional modification sites induced by Py T Ag both within and flanking the core origin. Thus, the two T Ags may interact in a very different way with the two core origins that may not be exclusively a result of simple DNA binding but may relate to their interactions with nucleotide or to protein-protein interactions in the

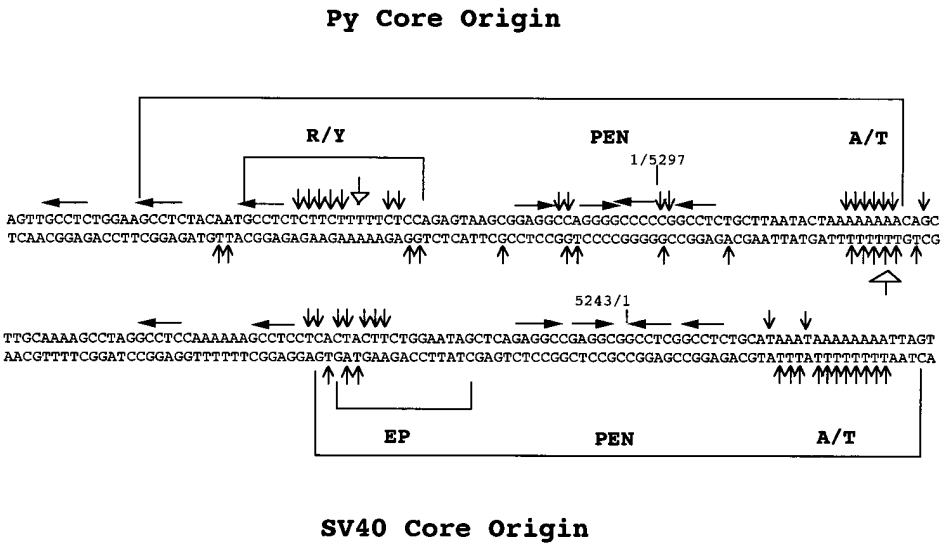

FIG. 5. Comparison of structural modifications of Py ori DNA by Py T Ag and of SV40 ori DNA by SV40 T Ag. Ori sequences are aligned according to the center of origin palindromes. Horizontal arrows represent G(A/G)GGC pentanucleotide sequences. Vertical filled arrows indicate bases modified by $KMnO_4$ in the presence of Py T (top) or SV40 T (bottom). Open arrow indicates bases modified by SV40 T Ag on Py DNA.

two hexamers when bound to the central palindromes of the two origins.

Both T Ags altered sequences near the early border of their respective core origins. SV40 T Ag also distorted a few sites on the early side of the Py DNA. This region in SV40 DNA is part of an imperfect palindrome, although only the eight distal bases of this EP palindrome are melted (5). Base substitutions in the distal half generally abolished replication, while those in the proximal half of the EP generally resulted in replication efficiencies greater than 50% of those of wild-type origins (14). The equivalent region in Py DNA has no palindromic sequences but is notable for being centered in an 18-bp polypyrimidine/polypurine (R/Y) tract. Furthermore, some replication-deficient mutations are localized to this R/Y element and not to the G(A/G)GGC pentanucleotide at its early border (29, 33).

These data raise interesting possibilities about the nature and specificity of T Ag binding and unwinding properties. For instance, is the actual sequence in the EP or R/Y of primary importance, or is the spacing most important, with the actual sequence being secondary? Unfortunately, less detailed mutagenesis has been performed on the Py origin than on the SV40 origin. Nonetheless, several nonessential bases have been found between the Py central palindrome and the R/Y sequence (33, 57), and several mutations which diminish but do not abolish replication are actually in the R/Y element but mutate G residues to A, maintaining the polypurine/polypyrimidine structure (33, 57). Furthermore, a linker insertion in the R/Y abolished Py replication in vivo and in vitro (28, 46). This insertion is located in the center of the region that we observed to be structurally altered by Py T Ag, thus modifying the active sequences as well as the location. In the case of SV40 DNA, an insertion or deletion in the proximal half of the EP abolishes SV40 replication in vivo and in vitro (14), while base substitutions in this region decrease replication by less than 50%. Most single base substitutions in the distal half of the SV40 EP abolish replication, although several of these mutations changed AT base pairs to CG base pairs, both altering the total composition of the base pair and switching the positions of pyrimidines and purines. These are not conservative mutations, and the resulting defects in replication may not be due to an absolute sequence requirement.

Examining the DNA sequences of the EP and R/Y regions of several papovaviruses reveals that SV40, the related human BK and JC viruses, and bovine Py (which does not show much homology in its central palindrome) all contain the ACTACTT (ACT) sequence, as well as homologous A/T regions and central palindromes (21, 51, 52), which is unwound by SV40 T Ag on SV40 DNA. Furthermore, SV40 T Ag is capable of mediating the replication of both BK and JC viral origin-containing DNA even though the sequences proximal to the ACT box are not conserved and are not palindromic (34, 35). Another primate papovavirus, simian lymphotropic papovavirus, contains a sequence that is nearly identical to the Py R/Y sequence as well as a very similar central palindrome, in terms of both base composition and spacing (43). Although this is a primate-specific virus that does not replicate in mouse cells, the early half of its origin can be substituted for the early half of the Py origin, and the resulting chimeric origin is replicated in vivo and in vitro in the presence of Py T Ag (30a). Thus, the ACT box or the R/Y sequence may contribute to papovavirus-T Ag specificity.

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