API1 Enhances Polyomavirus DNA Replication by Promoting T-Antigen-Mediated Unwinding of DNA

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Received 16 October 1995/Accepted 24 April 1996

An early step in the initiation of polyomavirus DNA replication is viral large-T-antigen-mediated unwinding of the origin. We report that components of the API transcription factor, Fos and Jun, interact with T antigen in vitro to enhance unwinding of the viral origin. This provides a biochemical basis for the capacity of API1 to activate viral DNA replication in vivo.

Elucidation of the components of the replication complexes of polyomavirus and simian virus 40 has provided the means to determine how the replication of these viruses is regulated. This knowledge is also being applied to studies of the replication of their eukaryotic hosts. From such studies, it is becoming apparent that many eukaryotic origins of replication rely upon transcriptional activators for their function (5, 12, 14, 24, 25, 32, 36, 38, 47, 56). Such proteins have been suggested to affect DNA replication by altering chromatin structure, by interacting with essential cellular replication proteins so as to regulate assembly and/or function of initiation complexes, by altering the structure of the template, or by promoting transcription through the origin (9, 10, 19, 21, 23, 28–30, 38, 49). Elaboration of these mechanisms will provide greater understanding of the control of eukaryotic DNA replication.

The importance of transcription activators in DNA replication was first established for murine polyomavirus, whose enhancer contains a PEA1 site bound by API (composed of Fos and Jun proteins) and other transcription factors (14, 18, 31, 33, 40, 45, 51, 54, 55). Mutation of this site in the viral enhancer profoundly reduces DNA replication in vivo. Alternatively, two or more copies of the PEA1 site can substitute for the viral enhancer to activate DNA replication (20, 33, 41, 51, 55). Additional evidence for the involvement of Fos and Jun in viral DNA replication is provided by the observations that ectopic expression of Fos and Jun (or v-Jun) in murine F9 embryonal carcinoma cells (which contain low levels of these proteins) stimulates viral DNA replication and that ectopic expression of the adenovirus E1A protein (which blocks binding of API to DNA) in mouse fibroblasts represses viral DNA replication (13, 22, 39, 44, 60).

In this report we provide evidence that Fos and Jun interact with polyomavirus large T antigen and promote its capacity to unwind the viral origin. This provides a biochemical basis for the capacity of the PEA1 site in the viral enhancer to activate DNA replication in vivo.

MATERIALS AND METHODS

DNAs. p314d DNA contains the polyomavirus A3 wild-type genome cloned at the BamHI site of pBR322. pG314d and B1 DNAs contain the polyomavirus A3 and B1 mutant (51) genomes, respectively, cloned at the EcoRI site of pGL101. pMKSO11 DNA contains the polyomavirus genome with the mutant SO11 origin (52) cloned at the BamHI site of pMK16. pAd175-BglII E- DNA contains the polyomavirus Ad175 genome (17) lacking the viral enhancer between nucleotide (nt) 5027 (BeII) and nt 5929 (PvuII) cloned at the EcoRI site of pGL101. pUC-BD DNA contains the viral enhancer-origin-early promoter sequences (nt 5027 [BeII] to nt 1790 [DdeI]) excised from M13mp9-D8 (51) cloned in pUC18. pBR3E-DNA contains three copies of the viral enhancer (nt 5027 [BeII] to 5929 [PvuII]) cloned into pBR322 and was kindly provided by Mark Martin, University of Missouri—Columbia.

DNA replication assays. Cultures of mouse 3T6 cells were transfected with double-stranded test DNAs, with or without helper DNAs or competitor DNAs, together with carrier salmon sperm DNAs, and were introduced into cells by calcium-phosphate coprecipitation essentially as previously described (51–53). At 48 h posttransfection, low-molecular-weight DNAs were isolated and digested with pancreatic RNase and proteinase K and then subjected to phenol-chloroform extraction and ethanol precipitation. The purified DNAs were digested with DpnI and EcoRI and fractionated through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with radiolabeled polyomavirus DNA. To avoid the complication of variable expression of T antigen, the test DNAs and the competitor DNAs were constructed so as to be unable to produce T antigen, leaving its expression confined to the helper DNAs.

DNA unwinding assays. A 450-bp DNA template containing the polyomavirus origin-enhancer sequences between nt 5027 and 190 was excised from pUC-BD with HindIII and EcoRI, gel purified, and labeled to a specific activity of 2 × 106 dpm/μg with α-32P-labeled deoxynucleoside triphosphate by using Klenow fragment. Polyomavirus T antigen was immunopurified from infected SF-9 cells as described by Wang and Prives (57) by using a baculovirus which expresses T antigen provided by B. Schaffhausen (Tufts University, Boston, Mass.) and the anti-monomonal antibody from hybridoma cells provided by D. Pallas (Massachusetts General Hospital, Boston). His-tagged Fos and Jun proteins were prepared from bacteria and purified over Ni-chelate columns as described by Abate et al. (2). The purified T antigen and Fos and Jun proteins were more than 90% pure as analyzed by Coomassie brilliant blue staining after polyacrylamide gel electrophoresis.

T-antigen-mediated unwinding was measured essentially as previously described (27) except with a higher dithiothreitol (DTT) concentration so as to maintain Jun DNA binding activity (59). To detect stimulation of T-antigen-mediated unwinding by Fos and Jun, the T-antigen-to-DNA ratio was reduced to a level just below that required to cause detectable unwinding of the origin DNA (usually 0.25 μg of T antigen to 1 ng of labeled origin DNA with 0.2 μg of competitor pBR322 DNA). Fos and Jun were used at a severalfold excess relative to the T-antigen concentration. The labeled DNA substrate (approximately 1 ng) was preincubated with Fos and Jun in 80 mM Tris buffer (pH 7.8) containing 5 mM EDTA. Proteins were digested with 2 μg of proteinase K per ml, and then single-stranded DNA was separated from duplex DNA by 5% polyacrylamide gel electrophoresis.

Detection of T-antigen binding to Jun. His-tagged Jun or Fos proteins prepared from bacteria as described by Abate et al. (2) were fractionated through polyacrylamide gels and blotted to nitrocellulose. Following blocking with 5% nonfat dry milk in phosphate-buffered saline solution for 1.5 h, the filter was incubated with (or, in one experiment, without) T antigen (15 to 30 μg) in blocking solution for 3 h and then washed with phosphate-buffered saline solution. Jun complexes with T antigen was detected by reaction with a rat monoclonal antibody to Jun. The immune complexes were detected by reaction with a peroxidase-labeled secondary antibody. The reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate and the color was read at 490 nm.
and high-affinity T-antigen binding sites (pUC-BD; Fig. 1, compare lanes 7 and 8), but replication was not blocked by two other competitor DNAs lacking B and C high-affinity T-antigen binding sites in the viral regulatory region (Fig. 1, lanes 9 to 12).

That the SO11 noncompetitive helper DNA promotes replication of the B1 mutant with a defective PEA1 site in its enhancer, whereas the wild-type helper DNA does not, suggests that the PEA1 site in the viral enhancer is required for polyomavirus DNA replication when T antigen is limiting. This, in turn, implies that API binding to the PEA1 site in the enhancer might stimulate DNA replication by promoting the function of T antigen in DNA replication; if this is the case, one might suspect that T antigen interacts with Fos and/or Jun, the components of API.

**RESULTS**

**A conditional requirement for the PEA1 site in the polyomavirus enhancer to activate viral DNA replication.** Our previous work and that of others have documented the importance of the PEA1 site in the viral enhancer for viral DNA replication (33, 44, 51). For example, the impaired DNA replication of polyomavirus mutant B1 was attributed to the mutant PEA1 site in its enhancer, because a point mutation at the PEA1 site diminished AP1 binding and DNA replication (33, 51). Thus, in the experiments reported here, a test DNA containing the viral origin linked to the B1 mutant enhancer did not replicate when cotransfected into mouse fibroblasts together with a replication-competent polyomavirus wild-type helper DNA which expressed large T antigen. (Fig. 1, compare lane 3 with lane 4; also see data in reference 51).

We and others have also observed that replication-competent wild-type helpers compete for replication of test DNAs, indicating that one or more factors limit replication in vivo (41, 52). Indirect evidence suggests that the limiting factor in such replication assays is the viral T antigen (data not shown). To reduce competition for T antigen by the helper DNA, we constructed a noncompetitive helper (SO11) which could not replicate because of several point mutations in the origin but whose early promoter was fully functional (52). When a test DNA with the viral origin linked to the B1 enhancer was cotransfected into mouse fibroblasts with the noncompetitive, replication-defective SO11 helper DNA, the test DNA with the B1 enhancer replicated efficiently (Fig. 1, compare lane 5 with lane 6). In agreement with the notion that the limiting factor in these assays is free T antigen, we found that the stimulatory effect of the SO11 helper upon replication of the B1 mutant was blocked by cotransfection of a competitor DNA which contains the polyomavirus regulatory region, including the B and C high-affinity T-antigen binding sites (pUC-BD; Fig. 1, compare lanes 7 and 8), but replication was not blocked by two other competitor DNAs lacking B and C high-affinity T-antigen binding sites in the viral regulatory region (Fig. 1, lanes 9 to 12).

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**Fos and Jun interact with T antigen.** We searched for interactions between T antigen and Fos or Jun by incubating Fos and Jun (each immobilized on a membrane) with T antigen; after extensive washing, T antigen complexed with Fos or Jun was detected with polyclonal anti-T-antigen sera. Baculovirus-expressed, purified polyomavirus T antigen formed a complex with immobilized bacterially expressed and purified full-length Jun as well as with several Jun proteins lacking amino-terminal sequences up to residue 186 (Fig. 2A, lanes 1 to 4). However, no interaction was detected with a Jun protein truncated to residue 224 (Fig. 2A, lane 5). Much weaker interactions between T antigen and Fos were detected (Fig. 2A, lane 7, and data not shown), and no interaction was detected with a truncated Fos containing only residues 118 to 211 (Fig. 2A, lane 8).

That these interactions were specific was indicated by the analysis whose results are shown in Fig. 2B: no anti-T-antigen reactivity was detected with Jun when incubation with T antigen was omitted (lane 1), compared with results obtained when T antigen was allowed to complex with Jun (lane 3).

**Fos and Jun stimulate T-antigen-mediated DNA unwinding.** T antigen helps assemble the viral replication complex, and a key step is the T-antigen-mediated unwinding of DNA containing the origin of DNA replication (16, 43, 46). Unwinding of origin DNA in vitro is dependent upon the concentration of T antigen (57) (Fig. 3), and the conditional requirement for a PEA1 site for DNA replication in vivo suggests that API is required primarily when T antigen is limiting. Therefore, to search for an effect of Fos or Jun upon DNA unwinding, we employed a concentration of T antigen just below the threshold required to cause detectable unwinding of a fragment of DNA containing the origin under our assay conditions. Consonant with the notion that API might stimulate T-antigen unwinding, with levels of T antigen which caused undetectable unwinding in our assay (0.5 ng or below), significant stimulation of T-antigen-mediated unwinding by Fos and Jun was observed (Fig. 4, compare lane 4 with lanes 5 to 7 and lane 11 with lane 13). In repeated assays, the stimulation by Fos and Jun was somewhat variable, for it depended upon the ratio of Fos and Jun to T antigen, the ratio of T antigen to the DNA template, and the quality of these proteins. (For example, repeated freezing and thawing inactivated the capacity of Fos and Jun to stimulate unwinding as well as the capacity of T antigen to unwind DNA.) Nevertheless, the stimulatory effect of Fos and Jun was repeatedly observed to cause a 2- to 10-fold-increased quantity of unwound DNA after 60 min of incubation with T antigen. This stimulation required T antigen (Fig. 4, compare lanes 1 to 3 with lanes 4 to 7) and was not caused by a nonspecific protein such as bovine serum albumin (Fig. 4, lane 14) nor by prior incubation of T antigen with cdc2 kinase and ATP, which is known to stimulate simian virus 40 large-T-antigen unwinding activity (Fig. 4, lane 12) (34, 35, 37).

Stimulation of T-antigen-mediated unwinding correlated with the capacity of Fos and Jun to interact with T antigen.
alone was capable of stimulating unwinding (Fig. 4, lane 6, and Fig. 5, lane 5), and Fos had a much smaller effect (Fig. 4, lane 5, and Fig. 5, lane 4), which paralleled its weaker binding (Fig. 2A, lanes 1 and 6). Furthermore, the truncated form of Jun (residues 224 to 234) which failed to interact with T antigen (Fig. 2A, lane 5) did not stimulate unwinding (Fig. 5, lane 6).

Two observations suggested that the Fos- and Jun-stimulated T-antigen-mediated unwinding of the polyomavirus origin depends upon the ability of Fos and Jun to bind DNA; however, additional work is required to confirm this. Deletion of the viral enhancer (and, concomitantly, the PEA1 site) in the DNA substrate utilized to measure unwinding somewhat reduced the Fos- and Jun-stimulated T-antigen-mediated unwinding (data not shown). Also, Fos and Jun stimulation of T-antigen-mediated unwinding was enhanced fourfold by inclusion of 5 mM DTT in the assay, in place of 0.5 mM DTT, and was threefold enhanced by the Jun activator protein Ref-1 (61) in the presence of 0.5 mM DTT (data not shown). Thus, stimulation of unwinding paralleled conditions which promote Jun binding to DNA (3, 4, 59). One technical problem we encountered in these experiments is that under the conditions used in the unwinding assay, Fos and Jun bound to DNA templates lacking bona fide PEA1 sites (as detected by gel shift experiments). Strict dependence of the stimulation of T-antigen-mediated unwinding upon the PEA1 site present in the enhancer, as is observed with DNA replication in vivo, may not be readily demonstrable with the concentrations of T antigen and Fos and Jun employed in this assay (which exceed the concentrations inside the infected cell), or it may require nucleosome-assembled templates. A precedent for the latter suggestion arises from studies of the stimulation of simian virus 40 and bovine papillomavirus DNA replication in vitro by transactivators (9, 10, 29).

FIG. 2. Detection of interactions between T antigen and Fos and Jun. Lanes display equivalent amounts of bacterially expressed, His-tagged Jun or Fos proteins or their truncated forms (2) fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and incubated with T antigen, washed extensively, and incubated with anti-T-antigen sera. (A) Lane 1, Jun; lane 2, truncated Jun from amino acids (a.a.) 30 to 334 (Jun 30–334); lane 3, Jun 57 to 334; lane 4, Jun 186 to 334; lane 5, Jun 224 to 334; lane 6, T antigen; lane 7, Fos; lane 8, Fos 118 to 211. M, molecular mass. (B) Lanes 1 and 3, Jun; lanes 2 and 4, T-antigen (Tag). The filter with lanes 1 and 2 was not incubated with T-antigen prior to incubation with anti-T serum.

FIG. 3. Unwinding of polyomavirus (PyV) origin DNA by T antigen (Tag). The quantity of T antigen (in micrograms) used in each assay is given below each lane. ss, single-stranded; ds, double-stranded.
and Jun, how such interactions promote unwinding, and how other effectors of viral DNA replication (such as proteins binding to the PEA3 site in the viral enhancer) synergize with Fos and Jun (33, 58).

A precedent for the stimulation of initiator protein-mediated unwinding of a viral origin occurs in the enhancement of bovine papillomavirus replication by the viral E2 protein (19, 28–30, 49, 62). Our work provides an analogous explanation for how Fos and Jun help regulate polyomavirus DNA replication. However, it is noteworthy that polyomavirus employs for this purpose cellular transcription factors (Fos and Jun) which also activate transcription of numerous cellular and viral genes. Polyomavirus DNA replication in vivo is stimulated by phorbolester (tetradecanoylphorbol acetate) and by the viral middle T and small T antigens (6, 8, 42), which increase the intracellular concentrations of Jun and Fos and cause their posttranslational modification (26, 48, 50, 59). The polyomavirus replication system provides an excellent opportunity to study how such regulatory events influence DNA replication.

Similar transcription factors have been implicated in regulating DNA replication of other viruses and of the cell (12, 15, 24, 32, 47, 56). By altering the concentration and the activity of such transcription factors, these viruses (and the cell) may modulate early steps in the process of DNA replication. Defining the role of these transcription factors in polyomavirus DNA replication should provide useful insights into mechanisms used by these other viruses and by cells.

ACKNOWLEDGMENTS

We thank Sarah Scanlon, Xian Wang, and Li Li for advice, Mary Ellen Roth for help in preparing the manuscript, Tom Curran for suggestions, B. Schaffhausen for the baculovirus expressing large T antigen, David Pallas for the hybridoma expressing F-4 antibody, and Mark Martin for pBR3E-DNA.

Support was provided by Public Health Service grant CA38538.

REFERENCES


FIG. 4. Unwinding of origin DNA by T antigen (Tag). Lanes 1 to 3 and 11 contain no T antigen; lanes 4 to 7 contain 0.5 µg of T antigen; lane 8 contain 1.0 µg of T antigen; lanes 11 to 14 contain 0.25 µg of T antigen (from a different preparation). ss, single-stranded; ds, double-stranded; BSA, bovine serum albumin.

FIG. 5. Unwinding of origin DNA by T antigen (Tag). Lanes 2 to 6 contain 0.25 µg of T antigen. Jun-Δ contains sequences truncated up to residues 224 and 334 (2). ss, single-stranded; ds, double-stranded.
19. Gilette, T. G., M. Lusky, and J. A. Borowiec. 1987. Two different factors bind to the alpha domain of the polyomavirus enhancer, one of which also interacts with the SV40 and c-fos enhancers. EMBO J. 6:1331–1337.