Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5086–5090, June 1993
Biochemistry

The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase

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Communicated by Nicholas R. Cozzarelli, March 5, 1993

ABSTRACT **For efficient DNA replication of papillomaviruses, only two viral-encoded proteins, E1 and E2, are required. Other proteins and factors are provided by the host cell. E2 is an enhancer of both transcription and replication and is known to help E1 bind cooperatively to the origin of DNA replication. E1 is sufficient for replication in extracts prepared from permissive cells, but the activity is enhanced by E2. Here we show that purified E1 can act as an ATP-dependent DNA helicase. To measure this activity, we have used strand displacement, unwinding of topologically constrained DNA, denaturation of duplex fragments, and electron microscopy. The ability of E1 to unwind circular DNA is found to be independent of origin-specific viral DNA sequences under a variety of experimental conditions. In unfractionated cellular extracts, E1-dependent viral DNA replication is origin-dependent, but at elevated E1 concentrations, replication can occur on non-origin-containing DNA templates. This conversion from an origin-dependent replication system to a nonspecific initiator system is discussed in the context of the current understanding of the initiation of chromosomal DNA replication.**

Helicases are critical enzymes in the semiconservative replication of DNA (1). Although some polymerases can melt duplex DNA and progressively catalyze strand displacement ahead of the growing polynucleotide chain, this melting is usually inefficient and requires the aid of a helicase. Helicases also work in conjunction with proteins having a strong affinity for single-stranded (ss) DNA, which stabilize the melted duplex as the helicase catalyzes processive unwinding. Often a helicase is brought to the site on DNA where replication initiates by interaction with specific DNA binding proteins that preassemble at the origin of DNA replication. For *Escherichia coli* or its λ phages, the DnaB helicase is efficiently loaded onto the respective origin sites by DnaA and DnaC (2) or the λ-encoded O and P proteins (3). In eukaryotes, little is known as to how cellular helicases may become associated with replication complexes, although, for some of the well-studied animal viruses such as the polyomaviruses and the herpesviruses, viral-encoded helicases are also equipped to be site-specific DNA binding proteins that can recognize the start site for replication. Other auxiliary factors may, therefore, not be required for loading. The simian virus 40 large tumor antigen is such an origin-recognizing helicase (4) that initiates replication by unwinding the origin site as the complex cellular polymerizing machinery assembles (5, 6). Herpes simplex virus 1 (HSV-1) encodes two proteins that can catalyze DNA strand displacement on helicase substrates, and one of these proteins binds specifically to the duplex HSV-1 origin of replication, although extensive duplex unwinding has not been detected (7, 8).

We have described an *in vitro* replication system for bovine papilloma virus 1 (BPV-1) DNA (9, 10) that may provide additional insight into the interactions between helicases and ancillary replication proteins. For efficient replication, this cell-free system requires two virally encoded proteins, E1 and E2, and host cell extracts. A cis-acting origin of DNA replication has been mapped, and the cell-free cis and trans requirements are similar to those for viral DNA replication within the living cell (11, 12). Important differences do exist between the two DNA replication assays. In the cell, the E2 protein and DNA sequences that make specific contacts with E2 are absolutely required, whereas in the biochemical assay E1 alone is sufficient and replication is enhanced by the E2 protein, even in the absence of an E2 site. Considerable evidence supports the hypothesis that E2 stimulates replication in part by aiding the E1 protein to bind viral DNA (refs. 9, 10, and 13 and A. Stenlund, personal communication). This cooperativity is believed to be a function of protein–protein and protein–DNA interactions (10). Thus, at sufficiently high E1 concentrations, with naked DNA as a template for replication, E1 binding may not be absolutely rate determining, and *in vitro* replication can be qualitatively independent of E2. E1, a 68-kDa phosphoprotein, is known to be a site-specific DNA binding protein (9, 10, 12, 14) that binds and hydrolyzes ATP (15, 16). We therefore asked whether E1 has helicase and duplex unwinding activities. Here we show that the protein is an ATP-dependent helicase, but in the absence of other factors, it can unwind circular duplex DNA promiscuously in an origin-sequence-independent manner. Furthermore, at high levels of E1, origin-independent DNA replication can be detected in the cell-free system.

MATERIALS AND METHODS

DNA Templates. pLYSX (or SX; 5433 bp) was constructed by inserting the *Xba* I–*Sma* I (nt 6132–945) origin-containing fragment of BPV-1 into pUC19. This fragment contains the entire upstream regulatory region. pLYIII (5170 bp) contains the late-region *Bam*HI–*Hin*dIII (nt 4450–6958) fragment of BPV-1. pKS and pKSO were as described (9).

DNA Helicase Assays. A standard DNA unwinding reaction mixture (20 μl) contains 30 mM Hepes (pH 7.5), 7 mM $MgCl_2$, 45 mM potassium glutamate, 4 mM ATP, 100–200 ng of relaxed circular DNA ($3–6 \times 10^{-14}$ mol), 40 mM phosphocreatine, creatine phosphokinase (100 μg/ml), bovine serum albumin (50 μg/ml), *E. coli* single-stranded DNA binding protein (SSB; Pharmacia; 30 μg/ml), polyglutamic acid (150 μg/ml), and 10 units of HeLa topo I. Additional experiments showed that removal of polyglutamic acid from the reaction mixture did not alter the properties of E1 reported. Reaction mixtures were titrated in the range of 1.4×10^{-12} to 7.4×10^{-12} mol (100–500 ng) of E1. After incubation at 32°C for 2 h, reactions were terminated by adding 20 μl of stop solution,

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Abbreviations: BPV-1, bovine papilloma virus 1; topo, topoisomerase; 2D, two dimensional; ss, single stranded; SSB, ssDNA binding protein; RPA, replication protein A; WT, wild type.

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to reach final concentrations of 1% SDS, 12 mM EDTA, and proteinase K at 60 μg/ml and then incubated for 30 min. The mixtures were extracted with phenol and chloroform. The DNA products were fractionated in a 0.8% agarose gel and analyzed by Southern blot hybridization. The strand-displacement assay was performed as described by Mohr *et al.* (17). A 42-base oligonucleotide was labeled by a kinase reaction and annealed to an M13 ss template, and the substrate was purified by Sephadex chromatography. The reaction mixtures contained E1 as indicated and ≈10 ng of labeled substrate, 30 mM Hepes (pH 8.0), 4 mM ATP, 7 mM $MgCl_2$, and 0.5 mM dithiothreitol and were incubated at 37°C for 1 h. The reactions were terminated as described (17). The products were then analyzed by PAGE in an 8% gel and autoradiography. For the helicase assay utilizing small duplex DNA, the ori$^+$ and ori$^-$ fragments were prepared by digesting pKSO with *Hin*dIII, labeling the DNA with all four [^{32}P]dNTPs and the Klenow fragment of DNA polymerase I, and then digesting the mixture of DNAs with *Pvu* II. This generated a 406-bp ori$^+$ fragment and a 258-bp ori$^-$ DNA fragment. These two fragments were purified by PAGE and used as substrates. The reaction conditions were identical to those used in the unwinding assay, except that the topoisomerase (topo 1) was omitted and that the total DNA substrates were estimated to be 0.5 ng.

Replication and Two-Dimensional (2D) Gel Assay. *In vitro* DNA replication assays were done with modifications of published conditions (9). Each reaction mixture (25 μl) contains 30 mM Hepes (pH 7.5), 7 mM $MgCl_2$, 45 mM potassium glutamate, 4 mM ATP, 200 ng of DNA, 40 mM phosphocreatine, creatine phosphokinase (100 μg/ml), polyglutamic acid (150 μg/ml), 10 μl of cell extract (8 mg/ml), and E1 and DNA-affinity-purified E2 as indicated. Other components are as described (9) or in the text. Reactions were incubated at 37°C for 2 h and DNA was processed as described (9). To analyze replication intermediates, a 120-μl reaction mixture was assembled, scaling up the components from the standard reaction. Six samples were taken at various times through a 2-h reaction and then pooled before analysis. For the origin-specific template, 500 ng of E1 and 60 ng of E2 were in the mixture, and for the ori$^-$ reaction, 3 μg of E1 and no E2 was employed. For 2D gel analysis, 8 μl of DNA replication products was digested with corresponding restriction enzymes and then used for the analysis as described (18).

Electron Microscopy. Unwinding reactions using pLYSX as a template were incubated for 2 h at 32°C and then placed on ice. The DNA concentration was adjusted to 0.64 μg/ml with 10 mM $MgCl_2$ and 10 mM Hepes (pH 7.5), and cytochrome *c* was added to a final concentration of 10 μg/ml. Samples were spread by the aqueous drop method (19).

RESULTS

E1 Is a Helicase. To determine whether the E1 protein has intrinsic helicase activity, the wild-type (WT) protein and a mutant form (PS434) were expressed from baculovirus vectors and purified to homogeneity (>95%) as described (9). The PS434 allele with a Pro → Ser codon change renders E1 incapable of binding ATP and is defective for *in vivo* replication (15). A helicase substrate was made by annealing a labeled oligonucleotide to a ssDNA circle (17). As shown in Fig. 1 *A* and *B*, the WT E1 was capable of displacing the linear DNA from the circle. However, the mutant PS434 had only 10–20% of the WT activity. As anticipated, the PS434 mutant was defective for *in vitro* DNA replication (data not shown). This helicase activity was dependent on ATP at all concentrations of E1 tested (Fig. 1 *C* and *D*).

E1 Can Initiate Unwinding from Within Duplex DNA. A helicase assay more pertinent to substrates involved in DNA replication uses topologically constrained duplex DNA. Covalently closed circular DNA was relaxed with topo I and then incubated with E1, ATP, SSB, and topo I. A highly unwound (melted) duplex structure called form U can be created by a helicase under such conditions, and after extraction of all proteins, the original population shows new supercoiled forms when analyzed by agarose gel electrophoresis. Fig. 2*A* indicates that WT E1 could create such forms on both ori$^+$ DNA and an Ori$^-$ DNA template of similar size. Again, the mutant PS434 was defective in this assay. No quantitative difference in apparent form U with SX (Ori$^+$) or LYIII (Ori$^-$) DNA was detected at any given level of E1 tested. Furthermore, titration of DNA templates from 0.1 to 1.0 μg of DNA with constant E1 (500 ng) showed no quantitative difference between Ori$^+$ and Ori$^-$ DNA. A similar lack of origin specificity for unwinding in this type of assay has been demonstrated for simian virus 40 large tumor antigen by Wold *et al.* (20). However, for tumor antigen, the nonspecific reaction was very sensitive to salt concentration. Presumably, the higher salt concentration increased the requirement for stronger DNA–protein interactions. At various levels of NaCl, Ori$^+$ and Ori$^-$ DNA were equivalent substrates for the unwinding catalyzed by E1 (data not shown). Fig. 2 shows that neither *E. coli* SSB nor human replication protein A (RPA; also an SSB) could create the unwound DNA species. However, since E1 at 500 ng without an SSB could produce the U form, E1 itself probably had high affinity for ssDNA (Fig. 2, compare lane 3 with lanes 4–6). In other experiments (not shown here), we observed that addition of mammalian RPA to the reaction did not change or eliminate the Ori-independent nature of unwinding. The reaction needed a nucleotide, but this requirement could be supplied by all four NTPs or dATP. The poorly hydrolyzable adenosine 5′-[γ-thio]triphosphate was not an effective substitute (Fig. 2*C*). The K_m for ATP hydrolysis was 200 μM; therefore, at lower nucleotide concentrations, preferences for a particular nucleotide may indeed be observed.

The assay described above indicates that a highly unwound species can be created by E1, but an extensive toroidal

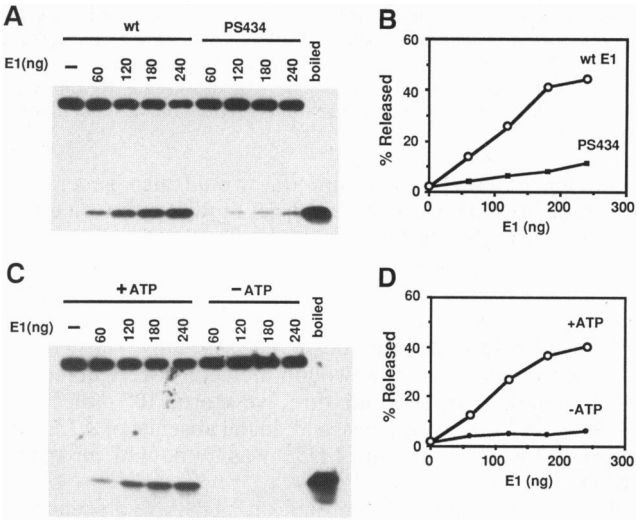

FIG. 1. E1 is a DNA helicase. (*A*) E1 has DNA helicase activity. The upper band is the starting substrate. The lower band represents the displaced oligonucleotide as a result of helicase activity or boiling of the substrates. The amount of E1 protein (WT or mutant PS434, in ng) used in the reactions is indicated at the top of the figure. (*B*) Quantitation of displaced oligonucleotide shown in *A*. The intensity of each band was measured by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). (*C*) ATP is required for the strand-displacement activity. The helicase assay was carried out as described above except that ATP was omitted where indicated. (*D*) Quantitation of the helicase activity shown in *C*.

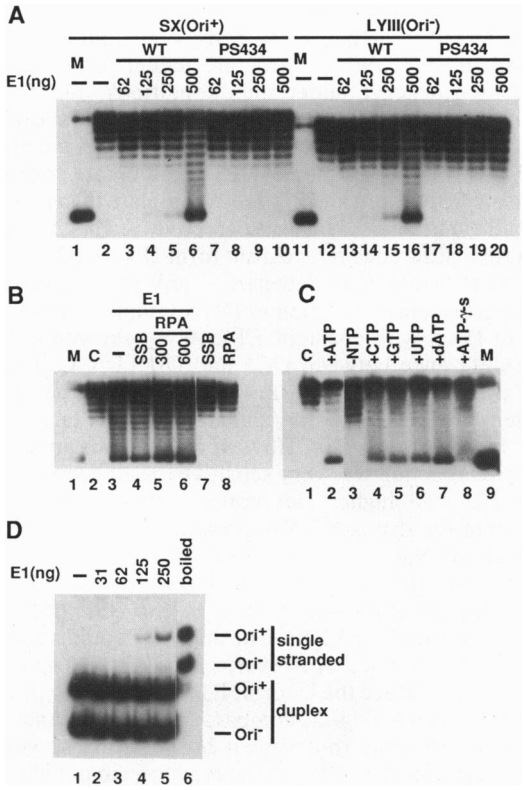

FIG. 2. E1 can initiate unwinding from within duplex DNA. (*A*) Unwinding activity of WT E1 vs. mutant PS434 E1. In each set of titrations, E1 protein concentrations are indicated in ng. M, DNA marker. Lanes: 2 and 12, no E1 was added. (*B*) Effects of SSB and RPA on unwinding. Lanes: 4 and 7, 600 ng of *E. coli* SSB; 5, 6, and 8, 300, 600, and 600 ng of RPA, respectively; 2, 7, and 8, no E1. (*C*) Effects of NTPs on unwinding. Lanes: 3, no NTPs; 2, 4–8, 4 mM ATP, 4 mM CTP, 4 mM GTP, 4 mM UTP, 4 mM dATP, and 4 mM adenosine 5′-[γ-thio]triphosphate (ATP-γ-S), respectively; 1, no E1. (*D*) Fragment unwinding assay. Equal molarity of an ori$^+$ DNA fragment (406 bp) and an ori$^-$ fragment (258 bp) were mixed and used as substrates. The lane labeled "boiled" provides a marker for ssDNA positions. A band corresponding to the denatured ori$^+$ fragment is clearly visible, and one corresponding to the ori$^-$ fragment with 10% of the intensity is detectable at the highest concentrations of E1.

wrapping of the DNA around E1 would also generate a supercoiled molecule with rapid gel mobility. To ascertain that the faster-migrating species created by E1 did indeed represent unwound molecules, the reaction products were examined by electron microscopy. Under these conditions, the protein was not extracted from the templates, so simple θ-shaped molecules were observed. As shown in Fig. 3, images characteristic of unwound molecules were detected. In the complete reaction mixture, we scored 107 molecules, 23 (21%) of which were unwound; in the absence of ATP, 100 molecules were scored and 1 (1%) was found with an apparent bubble.

In several assays, E1 can bind ori$^+$ DNA in a sequence-dependent manner. We were therefore surprised that it showed no specificity in the unwinding experiments described above. We reasoned that E1 may bind to nonspecific DNA with some affinity, and thus with a plasmid-sized substrate, the difference in loading E1 between ori$^+$ and ori$^-$ DNA may be too small for one to observe in a form U assay. Alternatively, lack of specificity could be the result of a special sequence in plasmid LYIII that could easily be unwound (the late region of BPV-1 has several patches of A-T-rich runs). We therefore asked whether specificity in unwinding could be observed in an assay using small restriction fragments of the BPV-1 URR. As shown in Fig. 2*D*, a 406-bp ori$^+$ fragment was the preferred fragment denatured by E1. In this assay, E1 showed a 5- to 10-fold specificity for the ori$^+$ fragment, and at the concentration of E1 used, the reaction was dependent upon a ssDNA binding protein and ATP (data not shown). E2 interfered with the activity of E1 in this fragment unwinding assay and in the form U experiments. Clearly, this effect requires further exploration, as it has important implications for the fate of E2 after initiation.

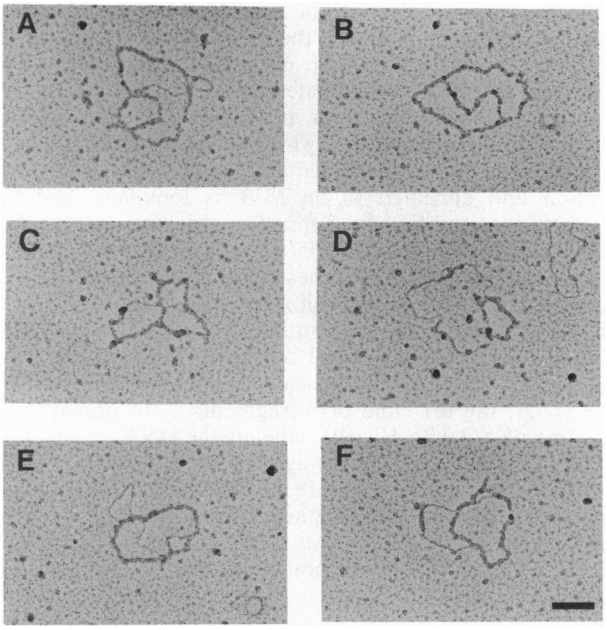

FIG. 3. Electron microscope images of circular DNA unwound by the E1 protein. Six representative pictures of unwound molecules are shown. After unwinding reactions, the DNA–protein complexes were stained with cytochrome *c* and examined as described. (Bar in *F* = 100 nm.) The cytochrome *c* concentration was such that in many examples a clear distinction between duplex DNA and ssDNA was clear. For example, in *D* the duplex DNA is not coated with SSB (or perhaps E1) and is thus thin, whereas the denatured loops are coated and appear thick. In *B* the distinction is lost presumably because of excessive cytochrome *c* staining. Such images were not detected in the spreads from samples not containing E1.

The Origin Requirement for *in Vitro* Replication Is E1-Level-Dependent. It is difficult to compare the specific activity of E1 in a purified system to that of E1 in a crude extract. For example, the protein may bind nonspecifically to other proteins or be activated by specific associations with cofactors. Nevertheless, the above results, which showed that, in the absence of competitor DNA, E1 could effectively unwind Ori$^-$ plasmid DNA, led us to reexamine the absolute origin sequence requirements for *in vitro* BPV-1 DNA replication; it is known that unwound nonspecific DNA can effectively serve as a template for most polymerase/primase systems (21). In Fig. 4, such a titration of E1 with Ori$^+$ or Ori$^-$ templates for *in vitro* replication is shown. With 85 ng of E1, almost no replication was detected on either template; however, when supplemented with E2, a robust reaction was observed with Ori$^+$ DNA as reported (9). With increasing E1 concentration, E2 had less effect. Significantly, with 500 ng of E1, replication of pKS (Ori$^-$) DNA was readily detected, albeit at much lower levels than was the Ori$^+$ DNA. In contrast to the specific replication, this BPV-1 Ori-independent replication was inhibited by E2 (Fig. 4, compare lanes 15 with 19).

To ask whether these replication products represent true *de novo* DNA synthesis or simply repair synthesis, Brewer and Fangman 2D gel analysis (18) was used. In this analysis,

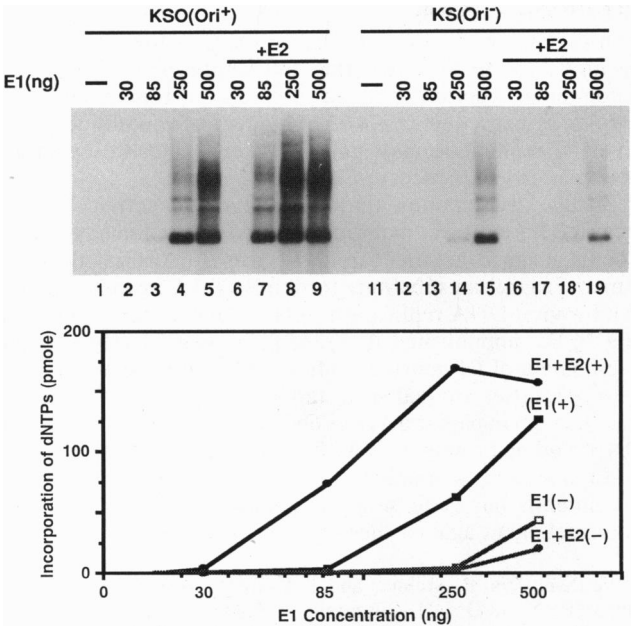

FIG. 4. Replication of Ori$^+$ and Ori$^-$ templates in the cell-free system. (*Upper*) Titration of E1 protein for replication activity with either Ori$^+$ or Ori$^-$ DNA templates. The amount of E1 added is indicated. E2 (20 ng) was added to the reaction mixtures whose products are shown in the lanes labeled +E2. Thus lane 3 represents the low level of replication detected with 85 ng of E1 in the reaction mixture and lane 7 shows the products with 85 ng of E1 and 20 ng of E2. Replication products were isolated and fractionated by agarose gel electrophoresis. An autoradiogram is shown. (*Lower*) The absolute amount of dNTP incorporation was measured by scintillation counting and converted to pmol of synthesis.

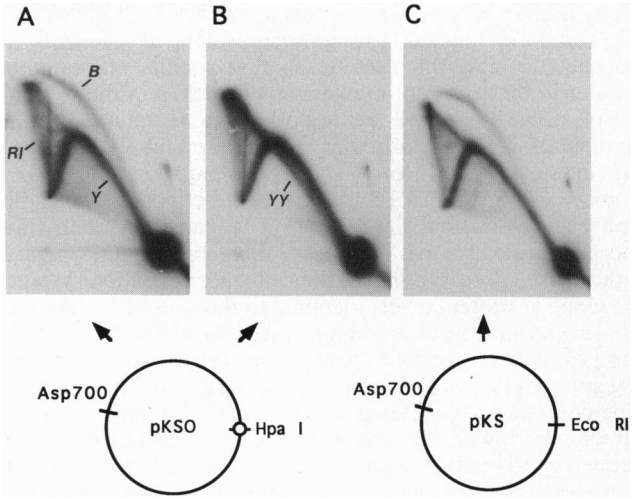

FIG. 5. 2D gel analysis of DNA products from the *in vitro* replication. *In vitro* DNA replication and the 2D gel analysis were performed. (*A*) Replication products of pKSO were digested with *Asp*700. (*B*) pKSO was digested with *Hpa* I. (*C*) pKS was digested with *Eco*RI. When pKSO was used as a template, both E1 and E2 were added to the reaction mixtures. When pKS was the template, E1 was the only viral protein included. Autoradiograms are shown. The patterns labeled B include the family of replication intermediates with an internal bubble, where the bubble grows from the centers toward the ends. The Y locates the family of intermediates with a Y shape where the fork grows toward one end. Similarly, the YY denotes the family of double Y forms. RI locates putative recombination intermediates (21). The location of the origin in the pKSO plasmid is shown below the autoradiogram by an open circle.

various families of replication intermediates were detected by characteristic arcs; such arcs cannot be generated by simple gap-filling mechanism or nick translation. When replicated, pKSO DNA was cleaved with *Asp*700 and analyzed; a bubble arc indicative of θ forms (labeled B in Fig. 5*A*) was detected among the replication intermediates. *Asp*700 cleaves the template plasmid roughly 180° from the specific origin site at an *Hpa* I site, and therefore, a family of simple bubble intermediates is predicted. A family of simple Y forms was also predominant in this and in all 2D gels analyzed, probably because of nuclease activity in the extract. We also note that a conspicuous recombination pattern (labeled RI) was also present in these patterns. As anticipated, when the replication intermediates of pKSO (Ori$^+$) were digested with *Hpa* I (which cleaves at the center of the origin), a double Y arc was detected and the bubble arc was lost (Fig. 5*B*). The products synthesized on the Ori$^-$ DNA showed that the late double Y arc and the bubble arc were simultaneously detected, irrespective of what enzyme cleaved the DNA. Fig. 5*C* shows the 2D gel pattern of the replication products of pKS digested with *Eco*RI; an identical pattern was obtained when digested with *Asp*700 (data not shown). These results show that initiation on Ori$^-$ templates does occur and that simple repair synthesis does not account for the replication.

DISCUSSION

The E1 protein of BPV-1 is an ATP-dependent DNA helicase, and a point mutation in the ATP binding domain of the protein renders the activity defective. As E1 is a site-specific DNA binding protein that binds to the origin, it seems reasonable to propose that the unwinding reaction plays a critical role in the initiation reaction. In preliminary data on E1 helicase activity (9), we relied primarily upon strand displacement assays, which are less sensitive to low levels of contaminating nuclease activity than are the unwinding reactions using circular DNA. Improved yields of E1 allow us to perform the assays on covalently closed circles. We have not been able to find conditions in which the unwinding reaction on topologically constrained DNA is absolutely dependent upon origin-specific sequences. Various titrations including substrate DNA concentrations, salt conditions, and protein levels did not alter the specificity of the reaction. Unanswered questions relate to the ratio of specific to nonspecific DNA binding constants for E1. In particular, we do not know how broad a range of duplex sequences E1 can bind to and what DNA sequence parameters determine the ease with which the protein can initiate unwinding. Under stringent conditions of DNase protein protection assays (9, 10, 12) or in a DNA–protein immunoprecipitation assay (14), E1 binds in a site-specific manner. However, in assays utilizing glutaraldehyde fixation, E1 can be cross-linked nonspecifically to ori$^-$ DNA (unpublished observations). These results suggest that for E1-mediated unwinding in the absence of cofactors (or inhibitors), it is sufficient for the protein to make relatively nonspecific contacts with the template.

Given these concerns about the ability of E1 to bind duplex DNA nonspecifically, we asked whether the addition of competitor DNA could shift the specificity of unwinding circles toward origin-containing DNA. In titrations of such competitor λ DNA, even in a 10-fold excess of carrier, little difference was observed between pLYIII (ori$^-$) and pSX (ori$^+$) DNA (data not shown). Nevertheless, we believe that in the *in vitro* replication reactions, E1 is involved in unwinding circular DNA in an origin-sequence-dependent manner. Significantly, utilizing small DNA fragments, E1 shows preference for melting the origin-containing fragment. These experiments show that under certain conditions E1 can initiate unwinding from within duplex DNA in a site-directed manner. These fragment assays are different from the form U

assay and are independent of topos. Moreover, if the size of a specific binding site is roughly equivalent to the size of a nonspecific site, the specific to nonspecific site ratio is greater in the fragment unwinding assay than in the U form assay. Clearly, it will be important to understand what sequences, if any in particular, are required for E1-mediated unwinding of pLYIII (ori$^-$) DNA. The fragment assay does, however, indicate that E1 may be an effective initiator if the nonspecific reaction is reduced or if E1 can be made to bind more specifically. E2 may play some role in this regard although we point out that in the cell-free replication system, E1 alone at protein levels identical to those used in the form U assays can lead to origin-specific replication. Ultimately, the proper template that most mimics *in vivo* conditions for the study of BPV-1 DNA replication is a chromatin complex. The core histones, when associated with the template, may effectively lower the concentration of nonspecific DNA sequences in the late region of the viral genome and allow for a more specific assembly of the replication apparatus at the origin.

The *in vitro* DNA replication system shows similar specificity for the cis and trans components expected from experiments performed within the cell. Thus, over a broad range of E1 concentrations, it is origin-dependent and responds to E2. The lack of specificity in the unwinding assay led us to ask whether we could find sufficiently high concentrations of E1 to promote origin-independent replication. Although this origin-independent replication is 5-fold lower than that detected with ori$^+$ DNA, the 2D gel analysis suggests that the synthesis is replicative and not repair. Under origin-dependent conditions, previous measurements of incorporation of radiolabel into restriction fragments showed that the bulk of the actual initiation in the cell-free replication system occurs near the origin. Thus we attribute the predominant Y arcs in Fig. 5 *A* and *B* to the breakage of the intermediates by shear force or nuclease activity. However, we cannot exclude the possibility that even under low E1 concentrations, where the origin sequence is required, some initiation occurs outside the origin site.

Further study of the origin-sequence-independent replication is warranted for it may be relevant to *in vivo* replication of nonviral DNA in a variety of systems. Although specific regions of initiation for DNA replication have been defined at certain loci in mammalian cells, it is only in budding yeast where definitive evidence shows a genetic requirement for specific origin sequences (21). It is conceivable that replication occurs reproducibly at certain loci without a true genetic requirement for those specific sequences. In studies of DNA replication in *Xenopus laevis* eggs (22, 23), *Paramecium tetraurelia* (24), nematodes (25), and human cells (26), it appears that transforming DNA can replicate and be maintained without a strict requirement for sequence. The permissive state of these systems may reflect an accessibility of the DNA template to an abundant helicase that can unwind DNA with a relaxed sequence specificity. Such helicases may have affinity for the polymerase α–primase complex. Tethering the polymerase to such an unwound locus may lead to an initiation site. We know that E1 does have such affinity for this polymerase (unpublished data), and the switch from an origin-dependent system to an independent one in the cell-free system seems to be a function of E1 levels. In this regard it is intriguing that Burnett *et al.* (27) identified by electron microscopy a large number of apparently randomly dispersed eye forms in the BPV-1 DNA extracted from bovine lesions. Though these eye forms were not shown by direct methods to be replication initiation sites, it was suggested that BPV-1 replicates at this stage in the viral life cycle from a multitude of start sites. It is possible that in vegetative BPV-1 replication, E1 levels are considerably higher than those reached in latently infected cells, where a unique origin sequence appears necessary for plasmid replication. Furthermore, it will be important to learn whether overexpression of E1 from recombinant vectors can lead to a relaxed requirement for specific origin sequences *in vivo*. Under such conditions, low levels of replication may start from other sites (or severely divergent ori sequences) in the viral genome.

Finally, we mention that the enzymatic activity of E1 reported here may be helpful in a variety of clinical studies. The E1 genes from other papillomaviruses, including those of human origin, can substitute for the BPV-1 E1 product in the cell for viral DNA replication (refs. 28 and 29 and J. Russell and M.B., unpublished data). It thus seems likely that all papillomaviral E1 genes encode an ATP-dependent helicase with properties equivalent to the BPV-1 product. We know that the human papillomavirus 6b E1 product is a site-specific DNA binding protein with ATPase activity (L.Y., J. Russell, R. Li, and M.B., unpublished data). Therefore, a single drug that inhibits this helicase activity may serve as an effective universal agent against these human pathogens.

We thank Drs. B. Stillman and T. Kelly for generous gifts of the human RPA and Drs. N. Cozzarelli, R. Kanaar, and J. Hurwitz for stimulating conversations. This work was supported by grants from the U.S. Public Health Service (CA42414 and 30490).

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