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Bovine Papillomavirus E1 Protein Can, by Itself, Efficiently Drive Multiple Rounds of DNA Synthesis In Vitro

CATHERINE BONNE-ANDREA,* SABINE SANTUCCI, AND PHILIPPE CLERTANT

Centre de Biochimie, Unité 273 de l'Institut National de la Santé et de la Recherche Médicale, 06108 Nice Cedex 2, France

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Bovine papillomavirus E1 protein was found to be as efficient as the simian virus 40 large T antigen in initiating DNA synthesis in a cell-free system derived from COS1 cells. Multiple rounds of DNA synthesis occur, initiated at the bovine papillomavirus type 1 origin. Therefore, E1 functions in vitro as a lytic virus initiator.

Bovine papillomavirus type 1 (BPV1) can persist latently in virally transformed cells where the viral genome is maintained as stable nuclear plasmids at about 50 to 200 copies per cell, replicating in synchrony with the host DNA (4, 8, 9, 13). In contrast to BPV1, simian virus 40 (SV40) exhibits uncontrolled, high-level replication in permissive cells, accumulating to over 10,000 copies per lytically infected cell (5, 17). Since both viruses rely entirely upon the host cell replication machinery for their duplication, it is likely that the differences between BPV1 and SV40 lie with the proteins involved in the initiation of replication. A single viral protein, the large T antigen (TAg), is required for SV40 replication (3, 7, 29). In the case of BPV1, two virus-encoded proteins, E1 and E2, are necessary for replication in vivo (27). E1 has been clearly established as the BPV1 origin recognition factor (6, 28, 30, 32), sharing a number of biochemical activities with TAg (18–22, 25, 33). However, in previous studies, E1 alone could not initiate DNA synthesis efficiently in in vitro replication assays. The transcriptional factor E2 was absolutely required at low levels of E1 and markedly stimulated the replication reaction at higher E1 levels (32).

In soluble cell-free systems, plasmid DNA molecules containing the SV40 origin are subjected to multiple rounds of DNA replication (24, 31). On the other hand, reinitiation of DNA replication was not observed in an in vitro BPV replication system, suggesting that the BPV origin may be prevented from reinitiating replication in vitro (32). In an attempt to characterize the viral and cellular elements that control BPV DNA replication, we decided to study this process in vitro. We recently described a new procedure for E1 isolation from Sf9 cells infected with the baculovirus recombinant AcNPV-E1, which optimizes recovery of E1 activity (1). However, although the replication activity of E1 was significantly higher than the activities reported in previous studies (21, 32), the rate of in vitro BPV replication was markedly dependent on the origin of the cell extract. In all the mouse cell extracts tested, the replication products mostly consisted of replicative intermediates, whereas primate cell extracts (monkey CVI and COS cells or human 293 cells) allowed a high rate of BPV DNA replication, supporting efficient production of mature circular DNA molecules (1). In this study, we further analyzed the functional properties of E1 by directly comparing its activity with the activity of SV40 TAg in a soluble cell-free system derived from monkey cells (COS1) in which both initiators could be studied under identical experimental conditions.

Requirement of E1 for BPV DNA replication in vitro. In vitro DNA replication assays were performed as previously described (1). The pSV-BPV+ plasmid (BPV-ori+) used as a template for replication (see Fig. 4B) contains the SV40 replication origin and a BPV origin-containing fragment (i.e., the 3.1-kb *Hin*dIII-*Eco*RI fragment of the BPV genome). The pSV-BPV plasmid containing an origin mutation (BPV-ori−) was generated by a linker insertion into the palindromic motif for E1 binding, a mutation known to drastically abolish BPV replication in vivo (23, 28). E1 protein was added to reaction mixtures containing a cytoplasmic COS cell extract (10) and either BPV-ori+ or BPV-ori− plasmid DNA. Very little DNA synthesis was observed in the absence of E1, but addition of increasing amounts of the protein stimulated incorporation of radioactive precursor with both templates, albeit at a lower level with the BPV-ori− DNA. Levels of DNA synthesis were extremely high by comparison with previously reported in vitro systems for BPV DNA replication (21, 32, 33). As shown in Fig. 1, with BPV-ori+ plasmid, DNA synthesis was already observed with 50 ng of E1 in a 100-μl reaction mixture and increased with the amount of E1, yielding an incorporation of over 80 pmol of dCTP. ori-independent DNA synthesis became detectable with 150 ng of E1 and reached almost 50% of the ori-dependent DNA synthesis. These results showed that the system exhibits a strict requirement for the BPV origin of replication only at very low levels of E1 and confirmed that E1 can open up DNA sequences with a less stringent sequence specificity to use them as the replication origin (1, 14, 33). Figure 2A shows a time course analysis comparing ori+ and ori− templates in the presence of equal amounts of E1 protein (250 ng), at which ori-independent replication was detected. When ori+ DNA was used as a template, DNA synthesis increased linearly for at least 60 min, whereas a lag of approximately 30 min was observed with the ori− template.

Reinitiation of DNA replication in vitro. To test for authentic replication promoted by the E1 protein in COS cell extracts, the DNA samples were cut with *Dpn*I. This restriction endonuclease cleaves only DNA methylated on both strands. In vitro-replicated DNA, becoming hemimethylated after one round of DNA synthesis, is thus completely resistant to digestion (16). As shown in Fig. 2B, a *Dpn*I-resistant band migrating to the position of a supercoiled monomer circle (form Io) was observed when ori+ DNA was used in the reaction. During the

* Corresponding author. Mailing address: Unité 273 de l'INSERM, Centre de Biochimie, Parc Valrose, 06108 Nice Cedex 2, France. Phone: (33) 93529945. Fax: (33) 93529917.

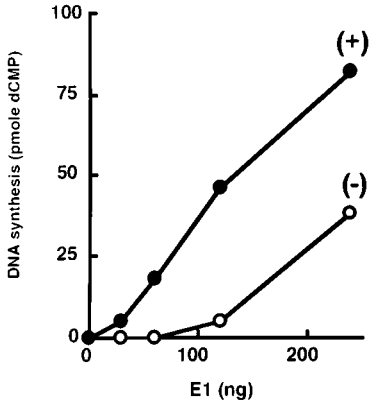

FIG. 1. Stimulation of DNA synthesis by increasing amounts of E1 protein. Standard 100-μl reaction mixtures containing 450 μg of COS cell cytoplasmic extract and 0.5 μg of either pSV-BPV+ (+) or pSV-BPV− DNA (−) were incubated for 2 h at 37°C. The absolute amount of [α-^{32}P]dCMP incorporation was measured by scintillation counting and converted to picomoles of dCMP incorporated in DNA synthesis.

first 90 min of incubation, accumulation of monomer circles occurred in parallel with replicating intermediates (RI) completely resistant to *Dpn*I digestion. With longer incubations, form Io slightly decreased whereas RI increased in intensity. The appearance of *Dpn*I-resistant RI suggested that reinitiation occurred in the same plasmid molecules during in vitro replication. This hypothesis was confirmed by digesting reaction products with the restriction endonuclease *Mbo*I, which recognizes the same site as *Dpn*I, but cuts only the unmethylated DNA that may result from two or more rounds of replication. Labeled *Mbo*I fragments were already observed after 45 min of incubation, and they accumulated with longer times (Fig. 2B). At 90 min, *Dpn*I-resistant form Io DNAs were for the most part *Mbo*I sensitive. Furthermore, *Dpn*I-resistant form Io, which was less intense at 150 min than at 90 min, almost completely disappeared at 150 min in the *Mbo*I-treated samples, suggesting that the majority of the ori+ plasmids had undergone multiple rounds of DNA replication. By comparison, DNA products formed with ori− DNA in the presence of an equal amount of E1 (250 ng) showed no detectable form Io resistant to *Dpn*I digestion. However, the detection of DNA fragments migrating slower than the largest *Dpn*I fragment suggested that initiation events did occur on BPV-ori− plasmids and that initiated replication forks progressed around the template, consistent with the time course presented in Fig. 2A. Detection of both *Dpn*I-resistant RI and *Mbo*I digestion fragments, albeit at a very low level compared with BPV-ori+ DNA, suggested that some molecules were subjected to more than one initiation event.

The activity of E1 in the COS cell extract was directly compared with the activity of SV40 TAg. This comparison was carried out at an E1 concentration that does not allow detection of ori-independent DNA synthesis (Fig. 1). pSV-BPV+ plasmid was used as the template in reaction mixtures containing either E1 (120 ng) or TAg (800 ng). In both cases, DNA synthesis increased linearly for at least 60 min and incorporated over 60 pmol of dCTP. As shown in Fig. 3, the products of the E1 reaction were almost the same as those obtained with TAg, demonstrating the high efficiency of E1 within a COS cell extract to produce completely replicated molecules after multiple rounds of replication.

DNA replication starts from the BPV origin at each round of synthesis. BPV DNA replication using a mouse cell extract

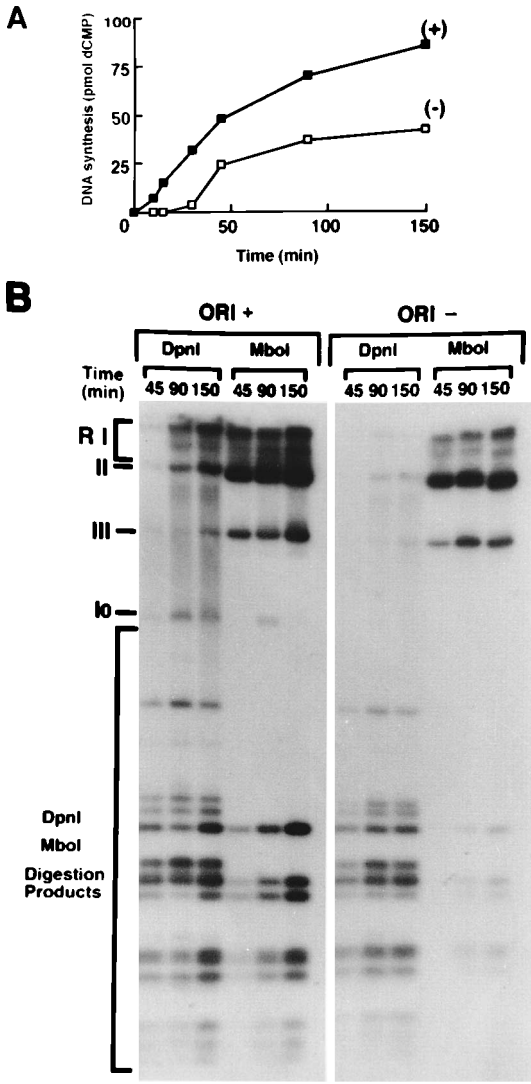

FIG. 2. DNA products of plasmid replication and reinitiation of replication in vitro. pSV-BPV+ (+) or pSV-BPV− (−) (500 ng) were incubated in a COS cell extract (450 μg) supplemented with 250 ng of E1 protein and incubated at 37°C. Samples were withdrawn at the indicated times and subjected to acid precipitation and product analysis. (A) Kinetics of DNA synthesis with BPV-ori+ and BPV-ori− DNA as template. (B) Analysis of the replication products with restriction endonucleases *Dpn*I and *Mbo*I. Replication products from the time points were digested with *Dpn*I or *Mbo*I and then fractionated by electrophoresis in 1.2% agarose in the presence of ethidium bromide (1 μg/ml), so as to observe relaxed, covalently closed DNA circles migrating at the position of supercoiled DNA. Equal volumes of the reaction mixture (10 μl) were added to each lane. RI, Io, II, and III designate the migration positions of replicative intermediate DNA and pSV-BPV form Io (supercoiled monomer circle), form II (nicked monomer circle), and form III (linear monomer DNA), based on DNA standards electrophoresed in parallel and visualized with ethidium bromide. The bracket on the left indicates the position of *Dpn*I- or *Mbo*I-generated DNA fragments of pSV-BPV.

supplemented with E1 and E2 proteins was shown to proceed in both directions from the in vivo-defined origin of replication (32). However, in this study, the level of DNA synthesis was extremely low (6 pmol of dNMP incorporated in 2 h at 37°C) and E2 was required in the replication assay. To examine further the capacity of E1 to initiate DNA synthesis at the BPV origin and to determine whether bidirectional synthesis was maintained with E1 alone, pSV-BPV+ was labeled in vitro for short periods in reaction mixtures containing either E1 (120

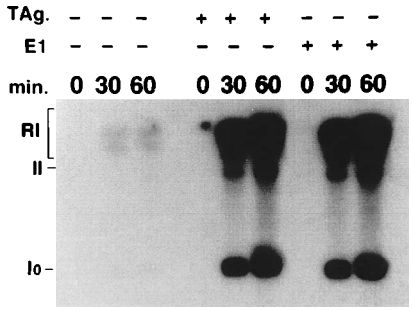

FIG. 3. Analysis of DNA products synthesized in the presence of either BPV E1 or SV40 TAg. pSV-BPV+ (0.5 μg) was incubated with 400 μg of COS cell extract alone or supplemented with either 120 ng of E1 protein or 800 ng of immunopurified TAg at 37°C. The protein added (+) to the reaction and the time of incubation are indicated above each lane (−, not added). DNA products were purified and fractionated directly by electrophoresis in 0.8% agarose.

ng) or TAg (800 ng) for comparison. Aliquots were removed at the time points indicated in Fig. 4, and the DNA was isolated and digested with either *Sau*3AI or *Mbo*I (*Sau*3AI cuts the same site as *Dpn*I and *Mbo*I but is not affected by the methylation state of the DNA). The resulting largest fragments, varying in length from 485 to 1,085 bp, were subjected to agarose gel electrophoresis (Fig. 4A). As expected, when the replication was dependent on SV40 TAg, the first *Sau*3AI fragments to be labeled at 10 min were the 485-bp SV40 origin fragment (F) and to a lesser extent fragment C. As previously reported, during the first 10 min of incubation, very little elongation of nascent DNA chains occurs, but after this lag, subsequent time points revealed progressive labeling of restriction fragments bidirectionally from the SV40 origin (Fig. 4A). On the other hand, at the first time point of the E1-dependent DNA synthesis, enhanced preferential labeling of the *Sau*3AI-A fragment containing the BPV origin was already observed along

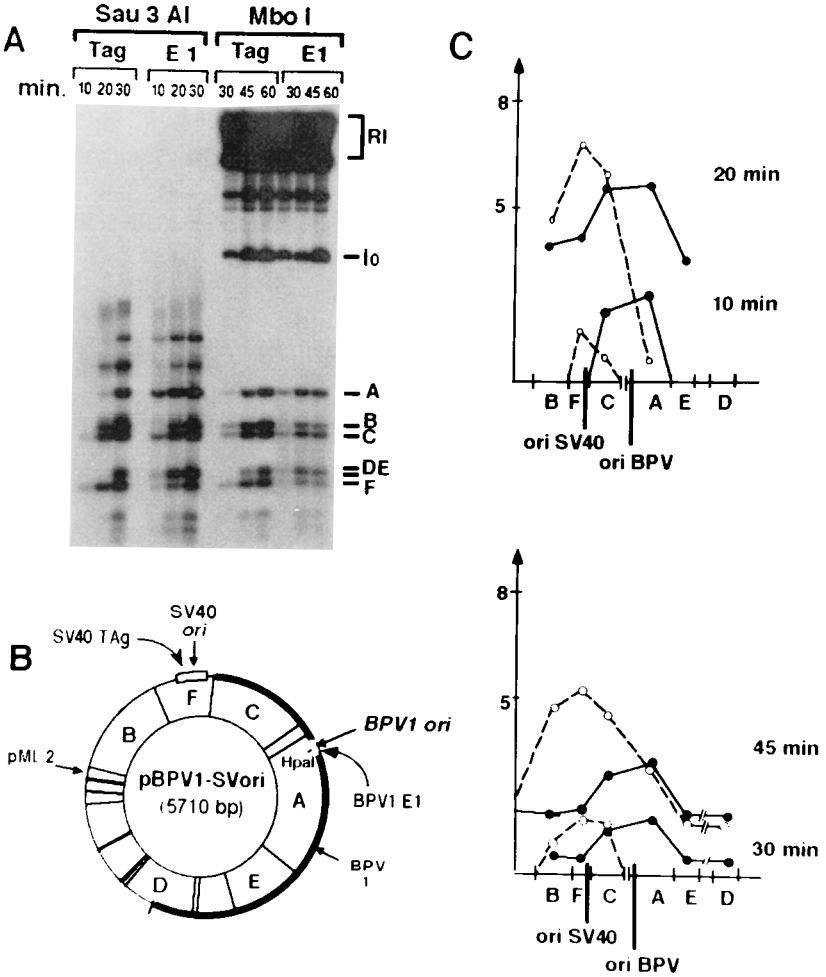

FIG. 4. Replication proceeds symmetrically from the BPV origin at each round of synthesis. Replication of pSV-BPV+ was carried out in COS cell extract with either BPV E1 (120 ng) or SV40 TAg (800 ng) as described in the legend to Fig. 3. At each indicated time point, 15 μl of reaction sample was taken and then the DNA products were purified and digested with either *Sau*3AI (at 10, 20, and 30 min) or with *Mbo*I (at 30, 45, and 60 min) as indicated. The resulting fragments were separated by electrophoresis through a 1.3% agarose gel. The gel was fixed, dried, and exposed to film. The autoradiogram is shown in panel A. The six largest *Mbo*I fragments are indicated by the letters to the right of the gel, namely, A (1,085 bp), B (824 bp), C (770 bp), D (560 bp), E (538 bp), and F (485 bp) (two bands of larger molecular size were always found resistant to digestion with *Sau*3AI). (B) A restriction map of pSV-BPV is presented, showing SV40 and BPV replication origins and *Sau*3AI/*Dpn*I/*Mbo*I fragment sites. (C) The relative amount of label in each fragment was quantitated by densitometry of the X-ray film (several film exposures per gel were analyzed to ensure a linear range in film response). The area under each peak, corresponding to the amount of label in each fragment, was corrected for the number of bases present in each fragment. The graphs show the relative level of labeling for the different samples when the F fragment was given an arbitrary value of 1. Fragments containing SV40 ori and BPV ori are indicated. The relative amounts of labeling for the 10- and 20-min samples digested with *Sau*3AI (top) and for the 30- and 45-min samples digested with *Mbo*I (bottom) are shown. Synthesis in the presence of TAg (open circles) and E1 (closed circles) is shown.

with labeling of fragment C and weak labeling of fragments E and F. Thus, DNA replication in the presence of E1 was bidirectional and started from the BPV origin sequences. Because some elongation of nascent DNA chains was already detected at 10 min, labeling of the most origin-distal fragments (B and D) also occurred at an earlier time point (20 min) than in the TAg-dependent synthesis. Analysis of the first labeled *Mbo*I fragments demonstrated that second rounds of DNA replication had already started at 30 min in both replication reactions, but principally that new rounds of synthesis were still specifically initiated at the viral origin in the E1 as in the TAg replication assay. The results of this analysis are shown in Fig. 4C. Replication appeared to proceed in both directions from the BPV origin with equal efficiency at each round of DNA synthesis. However, these data do not exclude the possibility that once initiated, replication proceeds by a random unidirectional fork movement on each DNA molecule. Bidirectional fork movement on individual replicating molecules could be confirmed only by electron microscopy analysis. On the basis of the kinetics presented in Fig. 4, it appears also that the time required for E1 to initiate DNA synthesis at the BPV origin is shorter than for TAg at the SV40 origin. This result may indicate that the requirements for the formation of an initiator-origin complex at the BPV origin are not as stringent as in the SV40 system.

We have shown in this study that the BPV E1 protein functions as an initiator of DNA replication that is at least as efficient as SV40 TAg. Previous work on the requirements for in vitro BPV replication have led to the conclusion that the transactivator E2 helps to deliver E1 to the BPV origin of replication, because at low levels of E1, almost no replication was observed and addition of E2 was needed to stimulate the replication reaction (32, 33). In contrast, our results show that E1, even at very low levels, is sufficient to drive extensive DNA synthesis from the BPV origin, demonstrating that E1 behaves in vitro in the same manner as SV40 TAg does. As recently reported (1), E1 is a rather unstable protein, and when its purification time is kept to a minimum, its activity, as measured by the replication assay, is extremely high but drops quickly with time to reach a low level of activity comparable to the activity reported in previous studies (21, 32, 33). We found also that within the E1-E2 complex, E1 activity is somehow stabilized. We believe that, in binding to E2, E1 might retain a conformation more suitable for recognizing its binding site within the origin. This hypothesis could explain why an E2 binding site adjacent to the BPV origin of replication was not necessary in the in vitro system established in Botchan's laboratory (11, 12, 32–34), but the significance of E2 action in vitro remains to be determined.

Our data raise new possibilities for the regulation of BPV DNA synthesis. BPV replication in latently infected cells does not continue after reaching a certain number of copies per cell, which implies that E1-mediated DNA replication may be negatively controlled in dividing cells. A first question concerns the role of the transcriptional factor E2, since E2 is absolutely required for BPV replication in vivo. It was recently suggested that E2 might perform functions similar to those hypothesized for other transcriptional activators in DNA replication (12). However, different observations suggest that E2 might have other functions. A specific requirement for E2 is indeed observed for in vivo BPV replication (26), whereas the identity of a transcriptional activator is not so important in the case of SV40 or polyomavirus (2). E2 was found to stimulate the polyomavirus origin when linked to E2 DNA binding sites (15), but no other transcription factor has yet been found that can substitute for E2 to allow BPV replication in vivo (26, 28). By specifically requiring its own transactivator in the DNA replication process, BPV may have a way to control its mode of replication. With such an hypothesis, in contrast to the one originally proposed, E2 may have a dual function in the DNA replication process, acting as a transactivator at the origin and modulating E1 activity via specific protein-protein interactions. Further studies using this in vitro system should enable us to investigate this new hypothesis.

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