Bovine Papillomavirus Type 1 DNA Replication: the Transcriptional Activator E2 Acts In Vitro as a Specificity Factor

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Received 8 January 1997/Accepted 3 June 1997

We previously devised cell-free conditions supporting efficient replication of bovine papillomavirus type 1 (BPV1) DNA (C. Bonne-Andréa, S. Santucci, and P. Cler tant, J. Virol. 69:3201–3205, 1995): the use of highly active preparations of viral initiator protein E1, together with extract from a particular cell source, allowed the synthesis of complete DNA circles through successive rounds of replication; this occurred in the absence of the viral transcriptional activator E2, required in vivo for the replication of viral genomes. We now report that adding E2 to cell-free assays produced only slight effects both on the yield of E1-dependent DNA synthesis and on the quality of newly made DNA molecules when a template carrying a wild-type BPV1 replication origin (ori) was used. The performance of mouse cell extracts, unable to sustain efficient BPV1 DNA replication in the presence of E1 only, was likewise not improved by the addition of E2. In a proper in vitro environment, E1 is thus fully capable of efficiently initiating viral DNA synthesis by itself, an activity which is not enhanced by interaction with E2. An important effect, however, was detected: E2 totally suppressed the nonspecific replication of ori-defective DNA templates, otherwise observed in high E1 concentrations. We examined the requirements for building a minimal DNA sequence behaving in vitro as a specific ori sequence under stringent recognition conditions, i.e., in the presence of both E1 and E2. Only two elements, the 18-bp E1 binding palindrome and an AT-rich sequence, were required in cis to allow specific cell-free DNA replication; there seemed to be no need for an E2 binding site to ensure discrimination between specific ori templates and other DNA molecules, even in the presence of E2. This suggests that during the initiation of BPV1 DNA replication, at least in vitro, E2 acts as a specificity factor restricting the action of E1 to a defined ori sequence; this function, likely not demanding the direct binding of E2 to cognate DNA sites, might primarily involve protein-protein interactions.

In eukaryotic cells, the initiation of DNA synthesis appears to involve, in addition to specific mechanisms aimed at unwinding the DNA double helix at defined locations along the genome, an auxiliary role for transcription factors (see reference 11 for a review). This has been evidenced by genetic analyses of the elements controlling in cis the start of DNA synthesis at specific origins of replication (ori), like those of small oncogenic DNA viruses infecting mammalian cells (polyomavirus or simian virus 40 [SV40]) or the ars elements of baker’s yeast. In all cases, the replication origins are made of (i) a central core, containing both a defined sequence recognized by a specific initiator protein (large T antigen or ORC complex) and a less defined sequence supposed to allow unwinding of the DNA duplex by DNA helicases (an AT-rich sequence or duplex unwinding element [21]), and (ii) one or several adjacent enhancer-like elements. The presence of auxiliary sequences either greatly enhances the efficiency of firing up DNA synthesis on the nearby core origin (for SV40) or is even required for the occurrence of this process (for polyomavirus). ori auxiliary sequences contain binding sites for transcription factors (AP-1 for polyomavirus, SP1 for SV40, and ABF1 for ars1), whose expression is needed for their replicative function. To a certain extent, they can be replaced by other sequences, which bind other transcription factors of similar properties (12, 17). Notably enough, these enhancer elements are totally dispensable for performing in vitro replication with naked templates in the cases of SV40 and polyomavirus DNA. Consequently two mechanisms have been invoked for explaining their role, with experimental evidence supporting each of them: prevention of the repression of DNA replication by a chromatin structure in keeping the ori region accessible to initiator proteins (7, 8) and/or tethering of proteins of the replication machinery for cooperation with initiators already bound to the origin (18).

Papillomaviruses (PV) keep to this pattern with one important peculiarity. Two viral proteins, E1 and E2, are normally required for replicating PV DNA (54). E1 is similar to another well-studied viral initiator protein, SV40-polyomavirus large T antigen (10): E1 is a nuclear phosphoprotein (42, 51) with DNA helicase and ATPase activities (30, 44, 46, 59) that is able to recognize and unwind an 18-bp palindrome within the viral replication origin (47, 49, 55, 56, 59). PV origins are short sequences (less than 100 bp) comprising mainly an E1 binding site and important flanking elements (see, for instance, references 14 and 53) such as AT-rich sequences and one or several binding sites for the second viral protein, transactivator E2. The E2 open reading frame was initially discovered controlling viral transcription, and it is expressed mainly in two forms (see reference 31 for a review): (i) short variants with DNA binding activity only, acting as repressors, and (ii) a larger protein, possessing an additional domain for transcriptional activation. Both forms exist as dimers and bind the same palindromic...
sequences dispersed at several places along the PV genome. Only the larger form is needed for the replication of viral DNA (54), so we will refer to it as protein E2 in this paper (the E2 repressors, which may play a role in modulating DNA replication, are likely not involved in the initiation process since they are not needed for replication). At first sight, then, PV protein E2 appears to be a viral counterpart to the host transcription factors involved in helping the viral initiator of polyomavirus and SV40. This statement is an oversimplification, however, for two reasons. First, contrary to polyomavirus large T antigen and cell transcription factors, which do not interact directly, the E2 protein enters into stable molecular association with the initiator E1 (2, 36), at least when properly modified by phosphorylation (28). Making a complex with E2 indeed greatly modifies the binding of E1 to the ori region by both improving affinity and lengthening the protected sequence (29, 45, 47, 58). Second, while proteins E1 and E2 can be exchanged between different papillomaviruses to ensure the replication of heterogeneous origins (9, 14), the structure of the PV replication origin varies notably depending on the virus type: in addition to an E1 binding site and an AT-rich sequence (53); that of human papillomavirus type 11 (HPV11) and HPV18 can be built of only two E2 sites, while requiring proteins E1 and E2 for replication (41, 52). Finally, the replication of HPV1a DNA requires only the expression of E1, and accordingly the HPV1a ori can be reduced to an E1 binding site and an AT-rich sequence only (14). The evolution of the PV family thus appears to have resulted in different scenarios, albeit with the same casting of two conserved protein partners, whose interactions with each other as well as with DNA targets have remained invariant.

Notwithstanding the HPV1a exception, as a general rule the E2 transactivator is required for viral DNA replication. Genetic analysis has localized determinants for replicative function within the N-terminal part of the BPV1 E2 molecule, a domain that is also required both for E1 binding and for transcriptional activation (1, 5, 13, 15, 16, 57). Targeted mutagenesis has allowed us to clearly distinguish between these three functions, the binding of E1 being a likely prerequisite to the replicative function of E2. It is generally considered that the binding of E2 to its DNA targets is also involved in BPV1 DNA replication; two E2 mutations have, however, been described as abolishing both DNA binding and transcriptional activation but retaining dimerization and E1 binding capacities, with the result that the replicative function of the E2 protein has remained intact (15, 57). Knowing whether the role played by E2 in viral DNA replication is mediated or not through direct binding to a cognate site within the origin thus deserves further attention.

Cell-free systems allowing PV DNA replication helped to clarify the role of E2 to a certain extent. BPV1 DNA synthesis in vitro was reported to occur in soluble extracts from mouse cells in the presence of protein E1 (37, 45–47, 58, 59). It is well established that, contrary to the in vivo situation, the cell-free replication of polyomavirus and SV40 DNA does not depend on either transcription factors or ori auxiliary sequences; however, the addition of E2 largely improved in vitro BPV1 DNA synthesis by factors of from 4 to 20 (though the increase was much lower than that observed in parallel for the binding of E1 to the ori sequence). Notably, even under optimal conditions (i.e., in the presence of E2), limited yields of DNA synthesis were obtained, amounting to only 5 to 20% of the levels usually obtained for SV40 DNA replication in vitro (23, 50). Finally, in the rare occurrences in which in vitro DNA synthesis was characterized as being semiconservative, it was apparently limited to only one round of replication. Cell-free systems were sufficient, however, to show that E2 could be replaced by other acidic transcriptional activators, helping to tether the replication protein A to the origin of replication (24); their use also helped illustrate how prior binding of E2 to ori DNA could prevent the inhibition of replication exerted by the establishment of nucleosomal structures in vitro (25). We recently reported conditions sustaining much higher levels of cell-free BPV1 DNA synthesis, satisfying both the criteria of semiconservation and bidirectionality on either side of the origin (3, 4). Under these conditions BPV1 DNA synthesis is as efficient as that of SV40 DNA in vitro and proceeds through successive rounds of replication. As previously reported by others (47, 59), however, synthesis is not limited to specific ori DNA templates when high concentrations of protein E1 are used. These findings indicated that E1 has a full capacity to initiate runaway replication by itself, as do initiators of lytic viruses like SV40 large T antigen. Here we report that under the conditions allowing highly efficient BPV1 DNA synthesis, the E2 protein exerts only marginal effects on the replication of specific ori DNA templates, while it totally suppresses that of nonspecific DNA; moreover, in playing the role of a specificity factor, E2 does not seem to require the presence of a cognate binding site within the ori sequence.

MATERIALS AND METHODS

Biological materials. The cell lines employed for preparing soluble extracts for cell-free replication, the recombinant baculoviruses used for expressing the BPV1 proteins into S9 cells, and the antibodies able to recognize these proteins were described previously (4, 42, 44).

Plasmid DNAs used as templates for replication. The pSV-BPV1+ composite plasmid was described previously (3). The pSKori™ plasmid was built by inserting within the EcoRV site of the pBluescript SK+ vector (Stratagene) a 160-bp BPV1 DNA fragment (nucleotides [nt] 7855 to 801 in the viral genomic sequence [6]) carrying what was defined as a minimal origin for BPV1 DNA replication (55) together with an adjacent E2 binding site (E2BS-11 [20]). The replication-defective derivative pSKori™ was obtained by inserting a 12-bp XhoI linker into the Hpal site of the pSKori™ DNA, thus interrupting the palindromic 18-bp E1 binding site within the BPV1 ori sequence (19, 34, 53, 58). The capacity of these constructs to behave as carrying a functional PV BPV1 replication origin was examined by assaying for transient replication after transfection into human 293 cells expressing constitutively viral proteins E1 and E2, as will be described elsewhere (40); as expected, the replication of pSKori™ DNA was very efficient (more than 500 copies/cell 72 h after transfection), while that of pSKori™ was undetectable. Synthesis of replicons were constructed by sequential cloning of double-stranded oligonucleotide cassettes into the pUC18 multiple cloning sequence (see Fig. 7). Complementary single-stranded oligonucleotides were treated by T4 polynucleotide kinase in the presence of 1 mM ATP, heated to 70°C for 5 min, and slowly cooled to room temperature. The specific complementary pairs were 5′-ACCGTCTTCGGTG-3′ and 3′-GGCCGAGAACCC-5′ for the E1 binding site (E2BS-10, 20), 5′-ATGTGTTATCAATAAT-5′ and 3′-TACAACAATTGTTATTA-5′ for the 18-bp E1 binding palindromic (IR) (22), and 5′-GATCTAAGTAAAGACTATGTATTTTT-3′ and 3′-ATTICATTTCGTCATACATAAAAAAGACGC-5′ for the E1 origin site (E2BS-11, 20). DNA preparations used as templates for in vitro replication were obtained (by sonication of exponentially growing cultures so as to be fully methylated on both strands on every DNA strand) by using the EcoRI enzyme and fully resistant to its isoschizomer MboI. DNA preparations used as templates for in vitro replication were obtained (by sonication of exponentially growing cultures so as to be fully methylated on both strands on every GATC site). Accordingly they were found to be both fully sensitive to the DpnI enzyme and fully resistant to its isoschizomer MboI.
Expression and purification of viral proteins. BPV1 E2 and the glutathione S-transferase–E1 fusion protein (GST-E1) were expressed by infecting Sf9 cells with the appropriate recombinant baculoviruses, as reported previously (43). In order to purify the E1 protein, GST-E1-infected cells were lysed by hypotonic shock and the nuclear fraction was extracted in a selective manner by a combination of a high concentration of salt, a high pH, and a high concentration of Mg, as described previously (3). Fusion protein was isolated by binding to glutathione-Sepharose beads (Pharmacia), and the beads were washed and finally submitted to specific proteolysis by incubation in the presence of coagulation factor Xa at 4°C, as described in reference 4. The supernatant produced contained mostly soluble E1 (at least 80% pure [Fig. 1, lane 5]); it was always used immediately (i.e., within the next 2 h) for replication assays, because of the marked instability of E1 replicative activity. E1 content was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in parallel to, or even after, the replication assays.

The E2 protein was extracted as described previously (44): after lysis of infected Sf9 cells by hypotonic shock at pH 6.2, the nuclear pellet was extracted with 0.5 M NaCl at pH 7.0. E2 was purified from the clarified lysate by affinity chromatography, with the help of monoclonal antibody B202 (39) coupled to protein A-Sepharose (Pharmacia) in the same way as that devised for SV40 large-T-antigen purification (48). A column made with 0.5 mg of pure immunoglobulin G/1 ml of packed beads allowed us to purify as much as 150 μg of the E2 protein (extracted from 2 × 10^6 cells). Fractions were eluted by triethylamine (pH 11) buffer, neutralized immediately by adding a 1/10 volume of 1 M HEPES (pH 7), brought to 50% glycerol, and stored at −70°C. The eluate (Fig. 1, lane 1) consisted of 90% pure E2 protein, with minor contaminants being electrofocused E1 (at least 80% pure [Fig. 1, lane 5]); it was always used immediately (i.e., within the next 2 h) for replication assays, because of the marked instability of E1 replicative activity. E1 content was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in parallel to, or even after, the replication assays.

We purified the E1-E2 molecular complex (2, 36, 43) from Sf9 cells coinfected with both E2 and GST-E1 viruses (44). From previous observations we knew that the E1-E2 complex could be extracted from Sf9 isolated nuclei in the same way as free E2 protein, i.e., by moderate ionic strength at neutral pH (43), while free E1 required a much higher pH to be extracted quantitatively (42). A similar procedure; lane MK, molecular size markers.

FIG. 1. Purified E1, E2, and E1-E2 complex. Proteins were expressed in Sf9 cells by infection with the appropriate baculovirus vector(s), purified by affinity chromatography as described in Materials and Methods, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining. Lane 1, E2 obtained by eluting a B202 monoclonal antibody column; lanes 2 to 4, successive steps of glutathione-Sepharose chromatography used to purify the E1-E2 complex (lane 2, proteins bound to affinity beads from an extract of insect cells coinfected with both E2 and the GST-E1 fusion protein; lane 3, the same material incubated for 4 h with factor Xa; lane 4, supernatant obtained from the same digestion); lane 5, E1 protein purified by a similar procedure; lane MK, molecular size markers.

RESULTS

E2 does not greatly affect E1-dependent cell-free BPV1 DNA replication. We initially obtained efficient cell-free DNA replication by using extracts of COS1 SV40-transformed monkey cells and a composite DNA template (pSV-BPV+) carrying the SV40 and BPV1 origins of replication (3, 4) together with viral protein preparations. This in vitro system allowed us to directly compare the replication processes driven by either BPV1 protein E1 or SV40 large T antigen; in both cases, similar levels of in vitro DNA synthesis were reached through the succession of several rounds of bona fide replication. Other cell types were found to provide soluble extract of similar properties: extract from human 293 cells sustained cell-free BPV1 DNA replication with the same characteristics and the same yield, amounting to 0.5 daughter molecules/template molecule for a ratio of only 10 to 15 E1 molecules/DNA template molecule. Results were further extended to another DNA template, pSKori7, carrying a short BPV1 sequence of only 160 bp surrounding the replication origin. As shown in Fig. 2, a succession of replicative rounds took place during the 90 min of incubation, as detected by the susceptibility of the newly synthesized DNA to the MboI enzyme, whose cleavage is restricted to nonmethylated GATC sites (present only on daughter molecules resulting from at least two rounds of synthesis); MboI digestion resulted not only in a shift from slow-migrating replicative intermediates to faster forms but also in the appearance of the full set of pSKori7 fragments.

Adding purified E2 protein to the in vitro assays performed with the pSKori7 template with a constant amount of baculovirus-expressed E1 protein in the presence of 293 cell extract (Fig. 2) did not greatly modify either the level of synthesis or the susceptibility of the newly-made DNA products to MboI digestion. A dual effect was reproducibly found for E2: low amounts of E2 produced a small increase in the overall extent of replication, in any case larger than 1.5-fold under our conditions, with optimal replication observed for an apparent stoichiometry lower than 1 E2 molecule/2 E1 molecules (considered monomers), while higher amounts of E2 resulted in partial inhibition, the extent of which depended especially on the template (see below and Fig. 8). E2 exerted the same dual effect upon second rounds of DNA synthesis. Low E2 amounts enhanced the labeling of MboI fragments somewhat more than they did for total synthesis; this might be explained in a simple way by hypothesizing that E2 should help stabilize active E1 molecules, an effect expected to be particularly marked for delayed events, as are the second rounds of synthesis.

E2 appeared thus to have only a marginal effect on cell-free BPV1 DNA replication. This was discrepant with results reported by others (37, 47, 58, 59), who observed large stimulation factors (at least fourfold) with overall cell-free DNA synthesis much less active than ours, by using extracts from mouse FM3A cells. Reasoning that we might have left undetected a larger E2 effect by using concentrations of active E1 protein that were too high, we performed another set of in vitro assays in which we varied the amounts of E1 while keeping E2 constant; the experiment (Fig. 2c) confirmed our previous results: no effect of E2 on DNA synthesis was detected at any concentration of E1. In the same way, we purified the E1-E2 complex, formed in insect cells before extraction (see Materials and Methods and Fig. 1), for which we found the same capacity to sustain pSKori7 DNA synthesis in vitro as for E1 protein alone; similar amounts of protein yielded almost identical levels of synthesis (see Fig. 5). Moreover, the DNA products exhibited the same susceptibility to the MboI or DpnI restriction enzyme (data not shown). Thus, under in vitro conditions...
allowing high replicative activity for the E1 protein alone, the E2 protein fails to stimulate the BPV1 DNA replication process significantly.

**E2 does not complement defective mouse cell extracts.** As previously reported (4), in the presence of E1 only, the mouse cell extracts employed in most other studies for supporting BPV1 DNA replication in vitro were found to be significantly less active than extracts from monkey COS1 or human 293 cells: dCMP incorporation values for the mouse cell extracts were lower by a factor of 5 to 10 (Fig. 3), and, more importantly, DNA synthesis did not result in semiconservative formation of mature DNA molecules as assessed by resistance to the DpnI enzyme. Since the stimulation by E2 of BPV1 DNA synthesis had been observed in experiments performed with mouse cell extracts, we reasoned that an E2-like factor, capable of exerting the same effect as the E2 protein on E1-driven replication processes, might be present in COS1 or 293 cells but missing from mouse cells. We thus compared the effects of E2 on BPV1 DNA replication in in vitro assays using extracts from various species. As visible in Fig. 3, E2 produced much greater stimulation of dCMP incorporation with extract from mouse ts85 cells (a cell line derived from FM3A cells) than with 293 cell extract; as ts85 extract allowed much weaker synthesis in the absence of E2, the maximal levels of incorporation were the same for both extracts. Moreover, the analysis by gel electrophoresis of the newly made DNAs indicated a clear difference in the replicative intermediates (RI): the use of mouse extract resulted in products migrating close to form II DNA, presumably replicated on very short distances, while the RIs produced with the help of 293 cell extract migrated much more slowly. Furthermore, in the latter case the labeling of form I molecules was stronger.

Figure 4 shows the results of another experiment, performed in order to determine whether E2 would improve the capacity of mouse cell extract to sustain semiconservative synthesis of full-sized DNA molecules and second rounds of replication. While extracts from both COS1 and 293 cells allowed production of slow-migrating RIs, DpnI-resistant form I molecules, and MboI-susceptible new DNA molecules, this was clearly not the case for the extract from mouse ts85 cells. These results indicated that the stimulatory effect of E2 on dCMP incorporation observed with cell extract from mouse origin did not reflect a bona fide process of DNA replication but rather some irrelevant phenomenon of either inoperative or repair-like DNA synthesis.

**E2 suppresses E1-dependent nonspecific replication of ori-defective DNAs.** An important effect exerted by E2 on cell-free DNA synthesis was revealed by using template molecules devoid of a functional origin of replication. As already reported (3), high concentrations of protein E1 sufficed to sustain reasonable levels of DNA synthesis with even nonspecific templates like pUC18 DNA (see below). When usual assay mixtures (containing E1, 293 cell extract, and DNA) were complemented by E2 in an amount which would result in maximal synthesis in the case of specific templates (as for pSKori+ DNA), the replication of nonspecific DNA templates was almost totally inhibited, down to the background level observed in control incubations devoid of E1 (data not shown; also see Fig. 6 and 7). The same effect was observed when we compared the activities of the E1 protein and the E1-E2-preformed complex (Fig. 5) on two DNA templates carrying either the wild-type or a mutated BPV1 origin of replication: in the case of the ori+ template, comparable levels of DNA synthesis were obtained for similar amounts of either protein preparation; in the case of ori-defective DNA, remarkably, only back-ground levels of incorporation were measured for any E1-E2 amount, while efficient DNA synthesis took place with E1, at least for high protein concentrations. This effect was further examined by assessing the susceptibility to the DpnI enzyme of the products of cell-free synthesis obtained with ori-defective DNA (Fig. 6). With pUC18 DNA as a template with 293 cell extract, parallel incubations containing comparable amounts of a mixture of either E1 and E2, the E1-E2 complex, or E1 only were run for various times at 37°C; while some labeling is
visible in DpnI fragments (even for the assays containing E2 proteins), form I DNA molecules resistant to the enzyme, indicative of complete semiconservative synthesis, were obtained for the late incubation time but only in the absence of E2. E1 is thus able to sustain bona fide replication of nonspecific DNA molecules in vitro, although to a lower degree than for the true ori template. This is possible, however, only in the absence of the E2 protein, which acts in vitro as a specificity factor to restrict the action of E1 to proper origin sequences.

Specific origins of BPV1 DNA replication in vitro do not need to carry an E2 binding site. We wanted to identify the cis-acting elements present within the origin of replication that specifically allow E1-driven DNA synthesis in the presence of E2 to start. A set of constructs was made by successively inserting into the multiple cloning site of pUC18 DNA modular elements corresponding to three functionally distinct sequences forming the 62-bp ori DNA sufficient for BPV1 replication in vivo (Fig. 7): the 18-bp palindromic E1 binding site, an AT-rich 22-bp sequence, and an E2 binding site, aligned in the same order as in the BPV1 genome. This resulted in synthetic putative origins, containing or lacking either the AT-rich sequence or the E2 binding site. These DNA constructs were assayed for the capacity to replicate transiently in transfected cells expressing in trans both the E1 and E2 proteins. The results, to be published in detail elsewhere (32), are summarized in Fig. 7; they clearly verified that the presence of each one of the three ori components is required in vivo for replicating viral DNA, thus evidencing the modular structure of the BPV1 replication origin. Although the constructs differed from the wild-type origin both by the spacing between the three elements and by the strength of the E2 binding site, a situation which might explain lower replication rates, their replication in vivo was found to require at least one E2 binding site; moreover, increasing the number of E2 sites noticeably improved replication levels.

We used these constructs as templates in cell-free replication assays. As E1 or E1-E2 preparations vary somewhat in replicative efficiency and have to be used immediately, all constructs were assayed in parallel with the same protein preparations to allow direct comparison between templates. The incorporation of radioactive dCMP into DNA was measured and is presented in Fig. 7; in addition, DNA products were...
FIG. 6. The replication of an ori-defective template DNA, supported to some extent by the presence of E1 alone, is suppressed by the addition of E2. Replication assays were performed by using pUC18 DNA as a template in the presence of E1 (150 ng) with or without E2 (50 ng) or the E1-E2 complex (corresponding to 150 ng of E1), as indicated. Aliquots were taken at increasing incubation times (15, 30, 60, and 90 min), and the DNA was purified, submitted to exhaustive digestion with the DpnI restriction enzyme, and analyzed by agarose gel electrophoresis.

purified and analyzed by DpnI digestion in order to assess the semiconservation of synthesis. E1 concentration was kept high enough to allow replication of both specific (pSKori1) and nonspecific (pUC18) templates in the absence of E2; this may explain some variability within the same experiment (ca. 20%). Two types of ori constructs might be distinguished in the assays performed with E1 only: (i) those replicating with low efficiency (less than 25% of that found for the wild-type ori template) and lacking either a functional E1 binding site (pSKori2) or an AT-rich element (pE1 and pE1E2, etc.), and (ii) those replicating at levels close to that of the wild type, for which an E2 binding site was dispensable, a result that was not surprising since the assays were performed in the absence of E2. Although within each class of constructs some variation can be seen, which might simply reflect variability in the assays, the differences between the two classes were marked enough to be considered significant. Only two ori elements, therefore, likely provide for efficient DNA replication in vitro in the absence of E2: the E1 binding site, allowing recognition of DNA templates by the initiator protein, and the AT-rich sequence, likely helping to easily unwind the origin DNA.

In the presence of E2, the same elements were indeed required and the constructs could be classified according to their ability to replicate in vitro in an order confirmed by similar results obtained with the E1-E2 complex. When AT-rich or E1 binding sequences were lacking in synthetic ori (as in pSKori1 and pE1E2, for instance), precursor incorporation dropped dramatically to background levels, and DpnI-resistant form I DNA was no longer detectably made in vitro. In the opposite way, an origin reconstituted from its three elements (pBATFE1E2) was replicated to lower levels than wild-type pSKori3 but with detectable production of DpnI-resistant circular molecules. Surprisingly enough, the same construct lacking an E2 binding site (pBATFE1), which was unable to support replication in vivo, still provided higher levels of synthesis in vitro. As was also the case for transient replication in transfected cells in vitro, in the presence of E2 the replication levels were always lower for synthetic templates than for wild-type ori DNA; this is likely due to either improper spacing between individual elements (e.g., the E1 binding site and AT-rich sequence), the use of a high-affinity E2 binding site (as discussed further below), or both. The results obtained both in vivo and in vitro for pBATFE1E2, pBATFE1, pE1, and pE1E2 can, however, be directly compared, allowing simple conclusions. In vitro, E2 clearly helps E1 to distinguish specific templates which possess both an E1 binding site and an AT-rich element from nonspecific DNAs; moreover, discrimination may occur in the absence of an E2 binding site on the template molecule. This suggests that in PV DNA replication, E2 has the capacity to play the role of a specificity factor, a function that, under our conditions, is likely not mediated by direct recognition of DNA. Cell-free experiments cannot tell us whether this new property is biologically important for the virus or not; it appears, however, to be distinct from the function that E2 has to play in vivo, which requires the presence of an E2 binding site within the origin (55) (Fig. 7).

E2 inhibits the cell-free replication of DNA templates carrying numerous or high-affinity E2 binding sites. Another unexpected finding is visible in Fig. 7: increasing the number of E2 binding sites in the ori constructs decreased their efficiency to function in vitro as templates for replication in the presence of both the E1 and E2 proteins. The level of in vitro synthesis varied inversely with the number of E2 binding sites in the origin (compare pBATFE1, pBATFE1E2, and pBATFE1E2T). The behavior of the construct carrying three E2 binding sites was striking: it replicated best in vivo; in vitro, its behavior in the absence of E2 was found to be very similar to that of the wild-type origin template. However, in the presence of E2, we could not detect any significant semiconservative replication (i.e., formation of DpnI-resistant circles). This result can be interpreted in several ways; it must be noted, however, that the E2 binding sequence used here for constructing synthetic origins (E2BS-10) is recognized with high affinity, while the site normally present in the wild-type origin (E2BS-12) binds E2 poorly (26). It appears likely that replacing the low-affinity E2 binding site adjacent to the E1 palindrome by a high-affinity one has a negative effect on the efficiency of cell-free replication performed in the presence of protein E2. This is also visible (Fig. 7) by comparing pBATFE1 (no E2 binding site) to pBATFE1E2 (one E2 binding site). High-affinity binding of protein E2 by DNA targets, especially when numerous on the DNA template molecule, seems to inhibit in vitro the process of initiating replication triggered by the BPV1 E1 protein.

Active E1 molecules are capable in theory of building up molecular complexes with E2, and we know that this was indeed the case in cell-free conditions, as shown by the specificity effect by which E2 helped to recognize proper ori sequences. As a likely explanation for the negative cis effect exerted by the presence of numerous and/or high-affinity E2 sites on template DNA, we reasoned that these sites might engage E1-E2 complexes into some form of nonproductive DNA binding. E2-mediated binding to these sites would block E1 in the wrong position or conformation, rendering it unable to bind further or to unwind the ori DNA duplex. This hypothesis was reinforced by the following experiment (Fig. 8): pSV-BPV1, which carries in addition to the 160-bp ori sequence present in pSKori2 2.7 kb of flanking BPV1 DNA with 12 additional E2 binding sites (most of them recognized with high affinity), was used in vitro as a template in parallel assays containing increasing amounts of E2 protein. As a result, DNA synthesis de-

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increased strikingly in a dose-dependent manner, down to background incorporation levels (obtained for E2 amounts which otherwise would have resulted in optimal synthesis if the short ori construct pSKori\(^2\) were used).

**DISCUSSION**

Under cell-free conditions allowing replication of BPV1 DNA with high efficiency with the help of viral protein E1 (3, 4), we observed that the presence of viral protein E2 produced only marginal effects, both on the synthesis level and on the frequency by which second rounds of replication were reinitiated (less than 1.5- and 3-fold stimulation at best, respectively [Fig. 2]). These results were obtained by using a DNA template (pSKori\(^+\)) carrying only a short BPV genomic fragment, which precisely covers the minimal sequence previously defined as being the origin of viral DNA replication (19, 34, 53, 55, 56). This is at variance with most previous studies on cell-free BPV1 DNA replication (37, 47, 58, 59) performed by using a comparable template (pKSO) which reported significant stimulation by E2 of DNA synthesis; enhancement by E2, varying considerably but being always greater than fourfold, never resulted in levels of synthesis as high as those we report here. This discrepancy with our present results might likely be explained by the use of inadequate cell extracts (a point to be discussed later) and of partially inactive E1 preparations. As already noted (4), active E1 preparations, able to sustain cell-free DNA synthesis as efficiently as SV40 large T antigen, were found to be especially unstable, with replicative capacity dropping dramatically through successive chromatographic steps or upon storage at 4°C (as performed in most other studies). We also noted that the extraction procedures used in other studies resulted in only partial solubilization of protein E1 (43) and might thus have selected E1 subspecies of peculiar characteristics.

We are confident in the significance of our results, showing only marginal stimulation by E2 protein of cell-free BPV1 DNA synthesis, for three reasons. First, the lack of significant stimulation is unlikely to result from artifactual inactivity of E2 preparations, because these were checked for proper DNA binding. Also of note, we prepared E2 from baculovirus-infected insect cells by a technique widely used by others, who...
reported stimulatory effects (58, 59); our results can thus hardly be explained by the lack of proper posttranslational modifications of the E2 protein. Second, similar results were obtained when titrating a constant amount of E1 with various amounts of E2 and vice versa (Fig. 2a and c). Last, we did not find significant differences in cell-free replication of specific DNA templates when using either E1 protein only, a mixture of E1 and E2, or, significantly, preparations of the in vivo-made complex between the two proteins (Fig. 5 and 7). As likely explanations for the significant stimulation by E2 observed in previous studies, we can imagine either some kind of stabilization (or partial reactivation) of protein E1 provided by interaction with E2 or a more specific effect occurring for a molecular subspecies of E1, whose conformation or posttranslational modification may differ from that of the E1 protein responsible in our assays for high DNA synthesis. In agreement with the first hypothesis, small E2 amounts appeared to preferentially stimulate second rounds of replication (Fig. 2).

Consistent with the second hypothesis, Melendy et al. recently reported that the same soluble extract from 293 cells failed to support DNA synthesis in the presence of bacterially expressed E1 protein (33). Replacing the extract by partially purified protein fractions from the same source, however, sustained vigorous BPV1 DNA replication at levels comparable to those we report here; notably also, only low stimulation by E2 was observed (less than threefold), occurring only for low E1 concentrations. It is worth noting that none of the purified proteins sufficing for in vitro reconstitution of the whole process of SV40 DNA replication were able to replace the unknown factor(s) missing in the 293 cell extract. We have verified that, while having normal ATPase and DNA-binding activities (44), bacterial E1 was not able to sustain any detectable DNA synthesis in the presence of 293 cell extract (data not shown), as seen by Melendy et al. (33). Comparing both sets of results suggests that the E1 protein produced in bacteria might lack critical posttranslational modification, possibly phosphorylation, since in mammalian cells E1 is phosphorylated on both N- and C-terminal sites (42, 51). Sequence similarities between E1 and SV40 large T antigen (10, 38), especially for putative phosphorylation motifs, together with the fact that SV40 DNA replication depends on the T-antigen phosphorylation state (20, 35), may support this hypothesis.

The second point at variance with most previous works is the source of cell extract we used for performing the in vitro assays. Extracts prepared from several mouse cell lines, especially the FM3a cells used by others for in vitro studies of BPV1 DNA replication as well as an FM3a mutant (ts85), did not support E1-dependent DNA synthesis as efficiently as did 293 or COS1 cell extracts (4); dCMP incorporation values were in the range of those measured by others, i.e., less than 20% of those provided by “permissive” 293 cell extract. Moreover, although DNA synthesis strongly depended on E1, it did not proceed through semiconservative replication up to complete molecules (Fig. 4). While adding E2 to assays performed with mouse cell extract enhanced dCMP incorporation, it did not improve the quality of DNA synthesis; as shown by its electrophoretic migration and susceptibility to the DpnI restriction enzyme, newly made DNA might have resulted either from abortive replication starting from accurate initiation events or even from irrelevant repair-like replacement synthesis. The process by which DNA synthesis was supported by mouse cell extract thus could hardly be considered genuine replication, that is, semiconservative synthesis of complete DNA molecules, even in the presence of E2.

We found, however, that E2 was able to exert a specific effect upon BPV1 cell-free DNA synthesis by suppressing the nonspecific replication of ori-defective templates. As already seen by others (47, 59), nonspecific DNA was also found to be a template for cell-free DNA synthesis in the presence of high E1 concentrations (4) (Fig. 5 to 7); the process can be considered genuine DNA replication, at least in part since it was producing DpnI-resistant form I molecules (Fig. 6). It was completely abolished by replacing E1 with the E1-E2 complex (Fig. 5) or by adding E2 to the in vitro assays (Fig. 6). This observation defines a new role for E2, that of being a specificity factor for BPV1 DNA replication, as already suggested by others (22). By using the same 293 cell extract for replicating HPV11 DNA, Kuo et al. (22) noted also that the presence of both BPV1 E1 and E2 proteins did not allow cell-free replication of pUC19 DNA. At discrepancy with our findings, however, their data were consistent with a need for E2 in order to replicate ori DNA. The difference might be explained by the use of distinct experimental conditions: Kuo et al. used long preincubation steps, relying on kinetic peculiarities of the HPV11 system that we know not to apply to BPV1 (see reference 3). Such steps might be unsuited for keeping E1 in the active form needed to provide high-efficiency BPV1 DNA replication, allowing only the weak DNA synthesis observed in most previous works; this might explain, as a corollary, the large stimulatory effect of E2 recently reported by these authors (27).

In addition to this specificity effect, we also observed that high E2 concentrations might exert a negative effect on ori DNA replication (Fig. 2 and 3) similar to that previously noted by others studying cell-free synthesis of BPV1 DNA under conditions of low efficiency (29). Comparably, we observed strong inhibition by E2 of cell-free DNA replication for DNA templates carrying either a second high-affinity E2 binding site near the origin or multiple E2 binding sites located at a distance (Fig. 7 and 8). This effect might represent an artificial phenomenon peculiar to cell-free replication resulting from trapping initiator protein E1 into nonproductive complexes with E2; such complexes, once bound to irrelevant E2 sites through their E2 moiety, would prevent further initiation by E1, since the proper DNA context (i.e., an E1 binding site and AT-rich element) for triggering DNA synthesis would not exist. On the other hand, this negative effect might well correspond to a physiological mechanism by which E2 would help to limit the extent of viral DNA replication. This hypothesis, however, is rendered unlikely by the opposite behaviors observed for synthetic ori’s like pBATFE1E2 and pBATFE1E2T in vitro and in vivo (Fig. 7): adding two more E2 binding sites to pBATFE1E2 DNA improved replication in vivo, while it depressed in vitro synthesis. Definitive conclusions on the nature of the inhibitory effect exerted by E2 on cell-free BPV1 DNA synthesis would require however a more detailed examination, especially by using other ori constructs.

Under the cell-free conditions allowing high levels of BPV1 DNA synthesis, we found that only two sequence elements were both required and sufficient for defining a specific origin of replication (Figure 7): an E1 binding site and an AT-rich element. As expected from previous results, E2 increased the stringency of ori recognition by suppressing the replication of template molecules devoid of either element; it did not appear to qualitatively change the sequence requirements, however. Strikingly, the absence of an E2 binding site did not seem to affect the capacity of ori DNA to be replicated in vitro in the presence of E2, while the lack of an AT-rich sequence abolished it. The latter element might be more important than yet suspected: for relaxed initiation of DNA synthesis (i.e., in the absence of E2), the lack of an AT-rich element in synthetic ori templates had the same negative effect as mutating or remov-
ing the E1 binding site (compare the pSKori+ and pSKori- and the pBATTE1E2 and pE1E2 pairs). In this respect, the uneven labeling distribution along ori-negative templates might also be significant. For pUC18 DNA (Fig. 6), preferential labeling occurred in a particular DpnI fragment (third from the top, nt 1662 to 2003, numbered as in reference 60); this fragment is the nearest to the most AT-rich sequence in pUC18 (nt 1563 to 1593, with 28 AT pairs contained in a very short fragment excluded from the gel). This may suggest that in the absence of E2, E1 is able to initiate bona fide replication on any naked DNA molecule by taking advantage of AT-rich stretches of nucleotides; nonspecific initiation events occur at low frequency, however, being detected only for high E1 concentrations. In the presence of E2, the action of E1 is restricted to specific ori DNA by binding the 18-bp ori palindrome and requires an adjacent AT-rich element for initiating DNA synthesis. This second element likely helps for proper DNA unwinding, but this point needs to be addressed in detail. E2 binding sites are not required for and even do not seem to improve the initiation of viral DNA synthesis by E1 under our cell-free conditions. In the same manner, E2 does not seem to limit the action of E1 to the sole ori sequences carrying E2 binding sites.

The observation that no E2 binding site was needed in order to build an in vitro functional BPV1 origin of replication was discrepant with another recent cell-free study (45) showing that origin recognition occurred through concomitant binding of E1 and E2, interacting as a molecular complex, to adjacent E1 and E2 binding sites and that both sites were required (with proper strength and spacing) for defining a functional origin. As already discussed, different conclusions might reflect different experimental conditions, likely to correspond to distinct replication modes. In order to carefully reconstitute in vitro DNA synthesis with the same requirements as those shown by in vivo replication of viral plasmids, Sedman and Stenlund (45) used both bacterial E1 and mouse FM3A cell extract, with the result of only very low levels of DNA synthesis. Moreover, in order to suppress nonspecific initiations by E1, their assays were performed in the presence of competitor DNA, p(dA-dT), that we found to inhibit the high-efficiency cell-free DNA replication supported by 293 cell extract (not shown). It is thus unlikely that we were examining the same process as did Sedman and Stenlund. On the other hand, the properties of the E1-E2 complex might vary depending on E1 conformation or modification; in this respect, as we used especially active E1 preparations, it is tempting to speculate that the corresponding E1 form might interact with E2 in a special way, forming a complex capable of stringent recognition of ori sequences in the absence of an E2 binding site. This hypothesis would also apply for the E1-E2 complex of similar properties made in insect cells. This question requires further examination. Another point raised by the work of Sedman and Stenlund (45) is the importance of proper spacing and/or sequence context for allowing E1-E2 cooperative action. By using synthetic origins not entirely fulfilling this requirement, we might have left undetected an important contribution of a properly located E2 site to the process of ori recognition; this also would require a more detailed examination by using another set of ori constructs made of the same elements properly spaced and having the conserved spacer sequences in between. The imperfect structure of our synthetic origins raised a more general question, that of the biological relevance of the observations made with their use. This is a limitation common to all in vitro systems reproducing a cell molecular process, but cell-free studies have the important virtue of clearly revealing the biochemical capacities of the various participants to the process.

In this respect, our results strongly suggest that, by virtue of interacting with active E1 molecules in a proper molecular context for sustaining highly efficient BPV1 DNA synthesis (293 cell extract), E2 has the capacity to ensure specific recognition of replication origins, even in the absence of a cognate binding site at the origin.

How could E1-E2 interactions result in more stringent recognition of the BPV1 replication origin in the absence of any E2 binding site within the origin? The evidence suggests that the molecular association between both proteins may reduce E1 capacity, either to be stably bound to or to unwind nonspecific DNA. If so, DNA binding by E2 might represent a dispensable function in the process of viral DNA synthesis, a proposal consistent with the properties of two E2 mutants devoid of DNA binding activity but able to support, together with E1, viral DNA replication (15, 57). Direct binding of E2 to a cognate site near the origin, however, is critical for allowing efficient replication in vivo, as shown by the pBATTE1 construct, devoid of an E2 binding site, found to be an efficient template for replication in cell-free assays but not in vivo in transiently transfected cells (Fig. 7). In addition to being a specificity factor ensuring accurate recognition of defined sequences as viral origins (the only function we have discovered in vitro by working on naked DNA templates), E2 has indeed to play another role in vivo. This second, mandatory function is likely similar to that ensured by host transcription factors in the case of polyomavirus DNA replication, as documented previously (24, 25).

To reconcile all data obtained to date by in vitro studies of BPV1 DNA synthesis, we propose, as a simple working model, that E1 might sustain cell-free replication in two ways. (i) In a low-efficiency mode, apparently limited to only one round of replication and mostly producing incomplete molecules, synthesis is enhanced by the presence of E2, an effect which requires an E2 binding site as a component of the replication origin. (ii) In a high-efficiency mode of DNA replication, proceeding through successive rounds of synthesis up to complete circular molecules, the presence of E2 has only a marginal effect, as shown here. A critical parameter for determining which sort of cell-free synthesis could be driven by E1 is the cell type used for preparing soluble extract. Another parameter is E1 itself, whose full activity would require a proper molecular state, as yet undefined but likely to involve posttranslational modification and specific conformation. In both modes, DNA synthesis is not limited to specific ori templates. In this respect, however, our results also show that E2 restricts the initiation events to specific origins; they further suggest that this property might not require the presence of an E2 binding site at the replication origin, as it is likely mediated primarily by interactions between proteins E1 and E2 (at least for the high-efficiency mode). The biological relevance of the two modes of cell-free BPV1 DNA synthesis remains to be examined. They do, however, mimic the in vivo situation: during the differentiation of infected keratinocytes within viral neoplasms, the PV genomes undergo two successive modes of replication, the first one being limited to the maintenance of viral plasmids and the second one corresponding to endless runaway synthesis. Characterizing the critical parameters defining the mode of cell-free DNA synthesis, that is, identifying the proteins present in mouse or 293 cell extracts which may act as positive or negative regulators of E1 function, might shed light on the mechanism of the shift between the two modes of PV DNA replication.

ACKNOWLEDGMENTS

We thank Elliot Androphy for the precious gift of B202 hybridoma cells and Laimonis Laimins for providing the baculovirus E2 expres-
sion vector. The skilful technical help of Daniel Puel in plasmid cloning and of Bernard Chabanne in preparing photographs is also gratefully acknowledged.

This work was rendered possible by support from the Association pour la Recherche contre le Cancer (grant 6112 to P.C.) and the American Cancer Society (grant VM-183 to V.W.).

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