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

CATHERINE BONNE-ANDRÉA,[1]† FRANÇOISE TILLIER,[1] GINA D. McSHAN,[2] VAN G. WILSON,[2] AND PHILIPPE CLERTANT[1]*

INSERM U470, Centre de Biochimie, University of Nice, Nice, France,[1] and Department of Medical Microbiology & Immunology, Texas A&M University, College Station, Texas[2]

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We previously devised cell-free conditions supporting efficient replication of bovine papillomavirus type 1 (BPV1) DNA (C. Bonne-Andréa, S. Santucci, and P. Clertant, 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 *ars*1), 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

* Corresponding author. Mailing address: Centre de Biochimie, Campus Valrose, 06108 Nice Cedex 2, France. Phone: (33) 4 92 07 64 19. Fax: (33) 4 92 07 64 05. E-mail: clertant@naxos.unice.fr.
† Present address: CNRS ERS 155, 34033 Montpellier, France.

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 heterologous 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, the bovine papillomavirus type 1 (BPV1) *ori* contains a mandatory E2 binding site (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 invariable.

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 Sf9 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 *Eco*RV site of the pBluescript SK$^+$ vector (Stratagene) a 160-bp BPV1 DNA fragment (nucleotides [nt] 7855 to 81 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 [26]). The replication-defective derivative pSKori$^-$ was obtained by inserting a 12-bp *Xho*I linker into the *Hpa*I 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 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.

Synthetic origins of replication 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′-TGGCAGAAGCCAC-5′ for the E2 binding site (E2BS-10, [26]), 5′-ATTGTTGTTAACAATAAT-5′ and 3′-TACAACAATTGTTATTA-5′ for the 18-bp E1 binding palindrome (IR) (22), and 5′-GATCTAAGTAAAGACTATGTATTTTTT-3′ and 3′-ATTCATT TCTGATACATAAAAAAGATC-5′ for the AT-rich region. Both the E2BS-10 and IR cassettes were blunt-ended, while the AT-rich cassette had *Bam*HI 5′ overhanging ends. Initially, the 18-bp IR cassette was cloned into the *Sma*I site of pUC18 by standard procedures to generate pE1. Subsequently, the E2BS-10 cassette was cloned into the *Ecl*136II site of pE1. During screening, clones were identified with one (pE1E2), two (pE1E2D), or three (pE1E2T) copies of the E2BS-10 cassette inserted. Finally, the AT-rich cassette was added to pE1, pE1E2, and pE1E2T by being cloned into the *Bam*HI site of these DNAs to generate pBATFE1, pBATFE1E2, and pBATFE1E2T, respectively. This resulted in a spacing of 7 bp between the AT-rich element and the 18-bp IR and a spacing of 11 bp between the 18-bp IR and E2BS-10. All clones were confirmed by sequencing. Their ability to function as specific templates for in vivo BPV1 DNA replication was examined as described previously (19) by a transient assay performed into CHO cells electroporated with each construct together with plasmids pCG*Eag* and pCGE2 (52); these experiments will be described elsewhere (32).

DNA preparations used as templates for in vitro replication were obtained (by a standard alkaline lysis procedure and density equilibrium centrifugation) from *E. coli dam*$^+$ 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 *Dpn*I enzyme and fully resistant to its isoschizomer *Mbo*I.

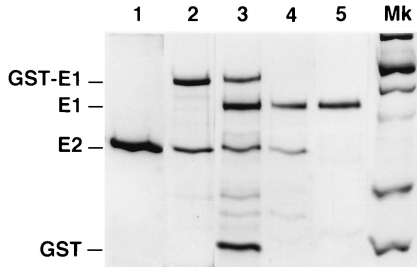


FIG. 1. Purified E1, E2, and E1-E2 complex. Proteins were expressed in Sf9 cells by infection with the appropriate baculovirus vector(s), purified by affinity chromatography as described in Materials and Methods, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie 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 by two recombinants expressing 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.

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 (4, 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 by 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 2×10^8 cells). Fractions were eluted by triethanolamine (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 mostly proteolytic products as judged by B202 recognition on Western blots; DNA binding activity, examined by gel shift assays using a specific oligonucleotide (a 20-mer spanning E2BS-10), was found to be consistent with stoichiometric association (1 nmol of E2BS-10/nmol of E2 dimer [data not shown]).

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 GST-E1-E2 complex, existing in the cells coinfected by the corresponding viruses (4), was quantitatively extracted in the same way as was E2, leaving free GST-E1 in the remaining nuclear pellet. The GST-E1-E2 complex was further purified by affinity chromatography with glutathione-Sepharose. Proteolysis by factor Xa, performed in the same way as was done for preparing E1, led to preparations consisting mostly of E1-E2 complex (Fig. 1, lanes 2 through 4), which were found to bind the E2 responsive element in the same way as did E2 alone (not shown).

In vitro assays for DNA replication. Soluble extracts for cell-free replication were prepared as described previously (23). Assays were performed as already described (3, 4) by incubating cell extract of various sources (200 μg of total proteins) and template DNA (either 0.15 μg of pSKori- and pUC-derived constructs or 0.25 μg of pSV-BPV+ DNA) together with viral proteins (usually 50 ng of E2 and 150 ng of E1) in a volume of 50 μl under standard conditions (50) at 37°C for the indicated times (60 min or more). The extent of DNA synthesis was monitored by counting the incorporation of [^{32}P]dCMP into acid-insoluble material. DNA products were further purified and analyzed as described previously (4); autoradiograms were scanned with the help of an LKB Ultroscan densitometer.

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, pSKori$^+$, 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 *Mbo*I enzyme, whose cleavage is restricted to nonmethylated GATC sites (present only on daughter molecules resulting from at least two rounds of synthesis); *Mbo*I 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 pSKori *Mbo*I fragments.

Adding purified E2 protein to the in vitro assays performed with the pSKori$^+$ 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 *Mbo*I 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 *Mbo*I 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 pSKori$^+$ 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 *Mbo*I or *Dpn*I restriction enzyme (data not shown). Thus, under in vitro conditions

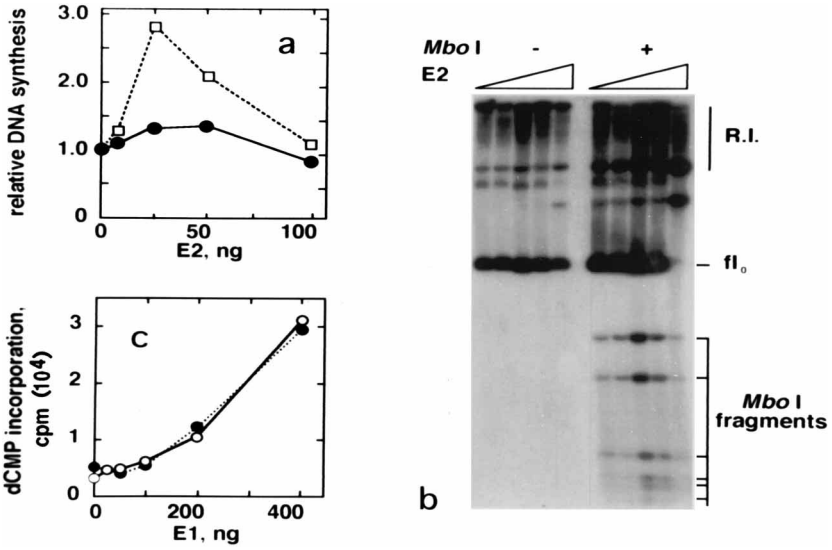


FIG. 2. Effect of E2 on E1-dependent BPV1 DNA replication in vitro. Assays were performed as described in Materials and Methods by incubating pSKori$^+$ template DNA in the presence of 293 cell extract and purified E1 and E2 proteins as indicated for 90 min at 37°C. (a) Relative values for DNA synthesis obtained by using a fixed amount of protein E1 (150 ng) and various amounts of protein E2, as indicated. Closed circles, incorporation of radioactive dCMP into trichloroacetic acid-precipitable material; open squares, densitometric estimate of the labeling of the *Mbo*I fragments in the gel shown in panel b. (b) The labeled DNA products were purified and analyzed by agarose gel electrophoresis either directly (30% of every sample) or after exhaustive cleavage by the *Mbo*I restriction enzyme (60% of every sample), as indicated on the top of the autoradiogram; in the margin, marked positions indicate RI and closed circular molecules (fI_0). (c) Total synthesis as described for panel a but with various amounts of E1 in the absence (open circles) or with a constant amount (50 ng; closed circles) of the E2 protein.

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 *Dpn*I 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, *Dpn*I-resistant form I molecules, and *Mbo*I-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 incomplete 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 background 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 *Dpn*I 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

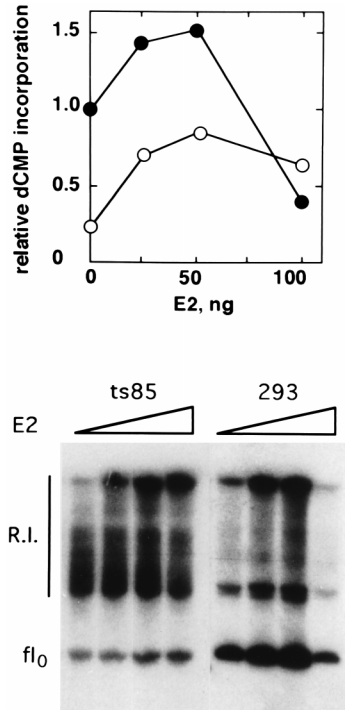


FIG. 3. Comparison of BPV1 DNA synthesis obtained in vitro by using extracts from either mouse ts85 cells (open circles) or human 293 cells (closed circles). Similar amounts of soluble extracts were incubated for 2 h in the presence of 0.15 μg of pSKori$^+$ DNA and 170 ng of protein E1 with various amounts of E2 as indicated. Upper panel, relative levels of dCMP incorporation normalized to that observed for E1 alone in the presence of 293 cell extract. Lower panel, analysis by agarose gel electrophoresis of the labeled products from the same experiment. fI_0, closed circular DNA molecules.

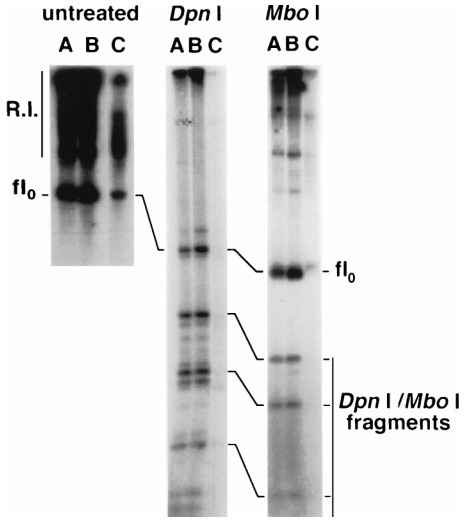


FIG. 4. BPV1 DNA synthesis sustained in vitro by various cell extracts in the presence of the E1-E2 complex. Incubations (90 min at 37°C) were performed in the presence of purified E1-E2 complex (corresponding to ca. 150 ng of protein E1) and similar amounts of extracts from 293, COS1, or ts85 cells by using pSKori$^+$ DNA as a template. Purified DNA products were then submitted to exhaustive digestion with either the *Dpn*I or *Mbo*I restriction enzymes (isoschizomers sensitive to adenine methylation in opposite ways, as discussed in the text) and analyzed by agarose gel electrophoresis as indicated. Lanes A, B, and C correspond to extracts from COS1, 293, and ts85 cells, respectively. fI_0, closed circular DNA molecules.

visible in *Dpn*I 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

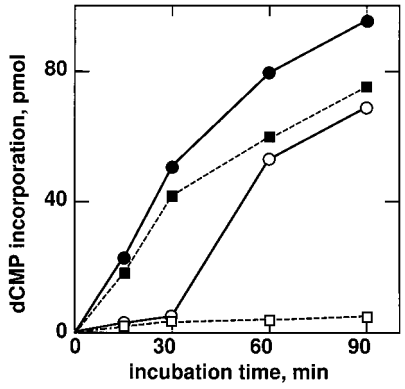


FIG. 5. DNA replication performed in vitro in the presence of the E1-E2 complex is restricted to templates carrying a specific origin of replication. Parallel incubations were performed in the presence of 293 cell extract with the addition of either purified protein E1 (closed symbols) or the E1-E2 complex (open symbols, corresponding to similar amounts of protein E1, i.e., 150 ng) with either pSKori$^+$ (circles) or pSKori$^-$ (squares) DNA template. At the times indicated, aliquots were taken, submitted to trichloroacetic acid precipitation, and counted.

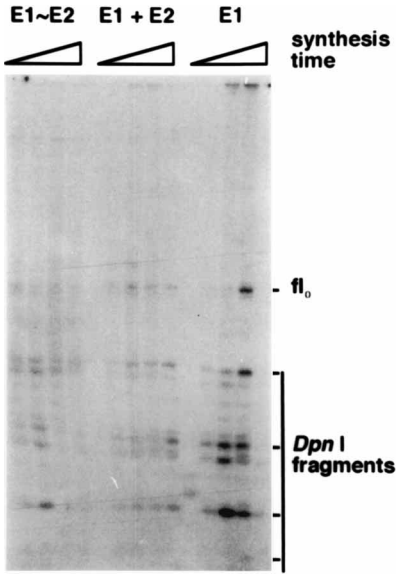


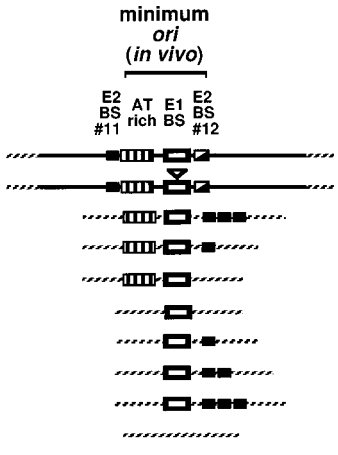
FIG. 6. The replication of an *ori*-defective template DNA, supported to some extent by the presence of E1 alone, is suppressed by the addition of E2. Replication assays were performed by using pUC18 DNA as a template in the presence of E1 (150 ng) with or without E2 (50 ng) or the E1-E2 complex (corresponding 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 *Dpn*I restriction enzyme, and analyzed by agarose gel electrophoresis.

purified and analyzed by *Dpn*I digestion in order to assess the semiconservation of synthesis. E1 concentration was kept high enough to allow replication of both specific (pSKori$^+$) 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 (pSKori$^-$) 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 pSKori$^-$ and pE1E2, for instance), precursor incorporation dropped dramatically to background levels, and *Dpn*I-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 pSKori$^+$ but with detectable production of *Dpn*I-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 *Dpn*I-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-BPV+, which carries in addition to the 160-bp *ori* sequence present in pSKori$^+$ 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-



	In vitro DNA synthesis				*In vivo* replication
	E1 only		E1 + E2	E1~E2	E1 + E2
pSKori$^+$	100	+++	92 ++	87	++
pSKori$^-$	23	±	3 -	5	-
pBATFE1E2T	80	+	7 -	11	++
pBATFE1E2	92	+++	18 +	13	+
pBATFE1	63	+	37 +	25	-
pE1	25	++	11 ±	0	-
pE1E2	19	±	5 -	12	-
pE1E2D	25	±	6 ±	6	-
pE1E2T	20	+	0 -	3	-
pUC18	13	±	0 -	ND	-

FIG. 7. Replication of DNA templates carrying variously reconstructed replication origins. In addition to pSKori$^+$ (which contains 160 bp of BPV1 DNA sequence, indicated by a continuous black line, spanning the 62-bp minimal *ori* sequence and carrying the E2 binding site E2BS-12, making up part of the *ori* sequence, and an additional site for E2, E2BS-11) and pSKori$^-$ (obtained by disrupting the E1 binding site by linker insertion), various constructs were made by inserting within the polylinker of the pUC18 bacterial vector (hatched line) three oligonucleotides from the BPV1 sequence: the 18-bp E1 binding site (E1BS) (open rectangle), the AT-rich element (striped rectangle), and an E2 binding site (E2BS-10, nt 7781 to 7792) (small black rectangle) (see Materials and Methods). These constructs were used as templates in parallel assays for in vitro DNA synthesis performed by using 293 cell extract with the aid of either protein E1 (alone or with addition of E2) or the E1-E2 complex (90-min incubation at 37°C with the same protein amounts as given in the legend to Fig. 6). DNA synthesis was evaluated by measuring the incorporation of radioactive dCMP into trichloroacetic acid-precipitable material and presented here relative to the value obtained for pSKori$^+$ in the presence of E1 alone (taken as 100%, with all values being first subtracted for the background incorporation measured in a control assay in the presence of extract alone). In the experiments performed with separately purified E1 and E2 proteins, labeled DNA products were also purified, submitted to extensive digestion with the *Dpn*I restriction enzyme, and analyzed by gel electrophoresis; the production of labeled full-sized circular DNA molecules entirely resistant to *Dpn*I being indicative of semiconservative replication, the presence and intensity of a corresponding band is marked here by "+," "−," and "±" symbols on the right side of each column. Experiments were performed in parallel to evaluate the capacity of the DNA constructs to be used as templates for replication in vivo by transfecting them into mouse C127 cells (together with plasmid constructs helping to express both the E1 and E2 proteins) and examining them 48 h later for the presence of *Dpn*I-resistant full-sized circular molecules by Southern blot analysis, as shown in the right-hand column (to be detailed elsewhere [32]).

creased 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$^+$ 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

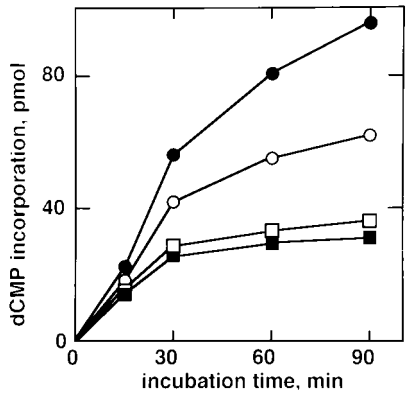


FIG. 8. Inhibition by E2 of the in vitro replication of a template carrying a 3-kb BPV1 DNA fragment, which contains 13 binding sites for E2 in addition to the minimal *ori* sequence. Replication assays were performed by using 0.25 μg of pSV-BPV+ DNA (see text) as a template in the presence of 180 ng of protein E1 and various amounts of protein E2 (closed circles, open circles, open squares, and closed squares represent 0, 25, 50, and 100 ng of E2, respectively). Aliquots (10 μl) were taken at various times of incubation, submitted to trichloroacetic acid precipitation, and counted.

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 *Dpn*I 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 *Dpn*I-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, 58). 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 artifactual 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 pBATFE1E2 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 *Dpn*I 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, poly(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 pBATFE1 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.

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