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Two E2 Binding Sites (E2BS) Alone or One E2BS Plus an A/T-Rich Region Are Minimal Requirements for the Replication of the Human Papillomavirus Type 11 Origin

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Human papillomaviruses (HPVs) cannot be propagated in vitro, but the DNA can be replicated transiently in an assay in the presence of two *trans*-acting viral proteins, E1 and E2. Using this assay, we have defined the minimal *cis*-acting elements of the origin of replication of HPV type 11. Most HPV genomes are conserved at the origin of replication, and the core contains three E2 binding sites (E2BS) surrounding an A/T-rich spacer region. The present results show that the minimal requirement for replication is either two E2BS alone or the A/T-rich region plus one E2BS; in the latter case the relative position of the E2BS is important. In all the studies, the presence of both E1 and E2 proteins was essential for replication, yet only the E2BS was required at the origin. We have shown that E1, E2, and the origin of replication containing an E2BS from a complex in vitro, and our data are consistent with a model in which E2 acts to target E1 to the HPV type 11 replication origin.

Human papillomaviruses (HPVs) are an important component of the etiology of benign and malignant disease of the lower genital tract (4, 10, 17). For example, HPV types 6 and 11 (HPV-6 and -11) commonly cause benign lesions, such as genital warts (12, 18), while HPV-16 and -18 cause malignant disease, in particular carcinoma of the cervix (4, 10, 17). Studies of the viral life cycle have been hampered by the lack of an in vitro system to propagate the virus. Replication of HPV viral genomes with subsequent production of infectious virus particles has been achieved with only limited success in in vitro cell systems that do not allow genetic studies (9, 20). However, recently studies of the transitory replication of the DNA genome from HPVs (6, 8) and animal papillomaviruses (34–36) have indicated that two proteins are essential for replication and have delineated a region of the genome as the origin of replication.

The major development in the transitory replication assay was the high level of transfection of cells (10 to 20%) achieved by using electroporation. With this technique, Ustav et al. (34–36) showed that the early proteins E1 and E2 of bovine papillomavirus type 1 (BPV-1) were essential for replication of the BPV-1 genome. They mapped the origin of replication to a 58-bp region of the genome located between bases 7914 and 27. While this region contains an E1 binding site and an E2 binding site (E2BS), mutations in the E2BS, which should abrogate E2 binding, did not prevent replication in the presence of both E1 and E2. Since it has been shown that E1 and E2 can form a complex (2, 21, 27), these results suggest that the activity of E2 can be mediated through its interaction with E1 at the origin of replication. Recently, more-detailed mutational analysis has shown that E2 binding at the origin is required along with the A/T-rich region incorporating the E1 binding site (36). However, it appears that while the binding of E1 or E1 and E2 to the origin is necessary, it is not sufficient since some mutated origin regions, while able to bind E1, are unable to replicate (30).

The transitory replication of the genome of HPVs has been obtained recently, and both E1 and E2 proteins were again found to be essential (6, 8). The E2 protein is a transactivator-repressor protein that binds to DNA at the consensus sequence $ACCN_6GGT$ (1, 16). Multiple copies of the E2BS are found within the upstream regulatory region of all papillomaviruses, and their major function in HPVs appears to be the down regulation of the major early promoter, which is located just upstream of the first open reading frame (ORF) coding for E6 (reviewed in reference 29). There appear to be three possible proteins encoded by the HPV-11 E2 ORF, a full length 43-kDa protein (6), a protein coded for by the C-terminal part of the ORF (E2-C), and an E1M^E2C protein, which contains part of the amino terminus of E1 spliced to the C terminus of E2 (5, 25). The full-length E2 is necessary for the replication of plasmids containing the origin of replication of either animal papillomaviruses or HPVs (6).

Most of our knowledge of the E1 protein comes from work with the BPV-1 E1 protein. E1 is a 68-kDa DNA binding phosphoprotein with helicase and ATPase activities (2, 27, 28, 32, 37, 40). It binds specifically to BPV-1 DNA at the origin region, around the *Hpa*I site (at nucleotide 1), bounded by two inverted repeats (35, 36). However, other sequences in this general area may also be important, since a region outside the repeats, when removed by digestion with *Hph*I, destroys E1 binding (36). In the same study, it was found that binding itself may not be sufficient, as mutations at the origin, which do not affect E1 binding, do prevent replication. This is consistent with the results of others (30). Recently, E1 at high concentrations, has been shown in an in vitro replication assay to replicate DNA without a BPV-1 origin (39). BPV-1 E1 and E2 can be found in a complex when expressed in insect cells as baculovirus recombinants (2, 21). E1 binds preferentially to non- or underphosphorylated E2, and the C-terminal region appears to be important, since point mutations or deletions in the C-terminal region abrogate the ability of E1 to bind to E2

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(15). While E1 and E2 bind to the origin region independently, E2 enhances the binding of E1 to the origin (27) and has recently been shown to bind to the replication protein A (14), which is an important component of the replication complex in yeast and mammalian cells (11, 38).

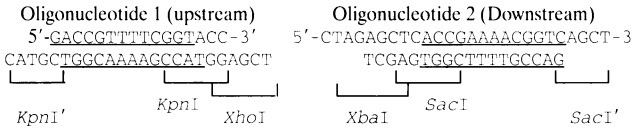
The HPV-11 minimal origin of replication has been mapped to a region that corresponds to the BPV-1 origin and is composed of an 80-bp region, spanning nucleotide 1, which contains three E2BS bounding an A/T-rich spacer element (6). Similar regions have been observed as origins in HPV-16 and -18 (8, 22). A common finding is the need for an E2 site for replication of HPV-11 (6), HPV-18 (22), and BPV-1 (34) origin region plasmids; however, little is known other than the requirement for the A/T-rich region. In the present study, we have mapped the *cis* elements important in transitory replication of the HPV-11 origin and show that unlike BPV-1, HPV-11 does not require an E1 binding site and E1 can bind to the origin through interaction with E2. In the absence of an E1 binding site, two E2BS constitute a minimal functional HPV-11 origin. In the presence of the E1 binding site, one E2BS is apparently still necessary for DNA replication, and its position relative to the spacer region is important.

MATERIALS AND METHODS

Plasmids. (i) E1- and E2-expressing plasmids. HPV-11 plasmids pCHE1 and pCHE2 contain the HPV-11 E1-coding region (bp 832 to 2779) and E2-coding region (bp 2723 to 3824), respectively, cloned downstream of the cytomegalovirus immediate-early promoter. E1 and E2 ORFs were amplified by polymerase chain reaction (PCR) with a *Bam*HI site introduced at bp 812 for E1 and at bp 2680 for E2 and an additional *Kpn*I site introduced at bp 4300 at the 3′ end of E2. The E1 fragment between the *Bam*HI site (bp 812) and the *Spe*I site (bp 3167) and the E2 fragment between the *Bam*HI site (bp 2680) and the *Kpn*I site (bp 4300) were cloned into the pCG vector (33) between the *Xba*I and *Spe*I sites (by using pCG-BPV-E2 [35]) and the *Xba*I and *Kpn*I sites, respectively, with the *Xba*I and *Bam*HI sites blunt ended. Both pCHE1 and pCHE2 were sequenced prior to use.

(ii) HPV-11 origin series of plasmids. The plasmid pHPV-11ori.1 contains the region of HPV-11 between the *Bam*HI (bp 7092) and *Nsi*I (bp 236) sites, cloned into pSK+ at the *Bam*HI site. This plasmid, along with origin plasmids from HPV-6b (pBSH6bOri, fragment between nucleotides 6493 [*Pst*I] and 257 [*Ppu*MI]) and BPV-1 (pUC/BPVPst [35], *Pst*I fragment from nucleotide 7092 to 236), was used in the initial experiments shown in Fig. 1. To construct plasmids with different portions of HPV-11 origin core, synthetic oligonucleotides containing a *Bam*HI site were used to PCR amplify the specific fragment, which was then cloned at the *Bam*HI site of pSK+. Plasmids were amplified between the following base pairs: N9 between bp 7886 and 61, M12 between bp 7904 and 61, M8 between bp 7886 and 53, M4 between bp 7886 and 34, no. 46 between bp 7904 and 53, and no. 54 between bp 7904 and 34. All constructs were confirmed by DNA sequencing and are shown diagrammatically in Fig. 2.

(iii) pEBS$_{X-Y}$ series of plasmids. A number of plasmids containing an artificial spacer, between E2BS 1 and E2BS 2, were then made with various numbers of E2BS. This was carried out by using homologous synthetic oligonucleotides with restriction sites at either end, which when they hybridize create E2BS. Different restriction sites were used at the 5′ end, or upstream of the spacer (*Kpn*I and *Xho*I sites), i.e., at E2BS 1, and at the 3′ end, or downstream of the spacer (*Sac*I and *Xba*I sites), i.e., at the EBS 2. Their sequences are



where *Kpn*I′ and *Sac*I′ have compatible sticky ends with *Kpn*I and *Sac*I, respectively, but do not recreate the restriction sites because of single base changes. The E2BS are underlined. After oligonucleotide 1 is cloned into pSK+ between the *Kpn*I and *Xho*I sites, a new *Kpn*I site is brought in by the oligonucleotide in order to allow tandem cloning of the same oligonucleotide. Similarly, for oligonucleotide 2, another *Sac*I site in the oligonucleotide allows the cloning of additional E2 sites. pEBS$_{X-Y}$ plasmids, containing X copies of oligonucleotide 1 (containing E2BS) upstream and Y copies of oligonucleotide 2 downstream of the spacer, were created by adding on the oligonucleotides by using the enzymes *Kpn*I and *Xho*I and *Sac*I and *Xba*I for oligonucleotides 1 and 2, respectively. All constructs were sequenced and are shown in Fig. 4A.

(iv) pOri series of plasmids. The pOri series of plasmids contains both the natural spacer and the artifical spacer to investigate whether the overall size of the sequences between the E2BS was important. The HPV-11 spacer, isolated from M4 by *Bam*HI digestion, was cloned into pEBS$_{1-2}$ linearized with *Bam*HI (see Fig. 6A). This allows the cloning of both spacers in tandem. The natural spacer was cloned in two orientations with respect to E2BS 1. Plasmids pOriIIS and pOriIIA (see Fig. 6A) were created by digesting pOriIS and pOriIA with *Hinc*II and *Bam*HI and removing the artificial spacer except for 16 bp at the 5′ end and 20 bp at the 3′ end (see Fig. 6A). This recreated the natural spacer in both orientations.

All the constructs described in "HPV-11 origin series of plasmids," "pEBS$_{X-Y}$ series of plasmids," and "pOri series of plasmids" have the same pSK+ bacterial plasmid background, and therefore differences should be due to the DNA content of the inserts.

Transient transfection. For the majority of experiments, the following transfection protocol was used. Log-phase cultures of 293 cells were trypsinized and resuspended in growth medium (Dulbecco modified Eagle medium with 10% calf serum) at density of 10^7 cells per ml. The plasmids pCHE1 and pCHE2 were added so that a 0.25-ml cell suspension would contain 3 μg of each plus 50 μg of single-stranded salmon sperm DNA and 2.5 μl of 0.5 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.2. After the cell suspension was aliquoted into 0.25-ml portions, 0.5 μg of the test plasmid DNA containing various origin region constructs was added, and the mixture was electroporated with a Bio-Rad Gene Pulser at 170 V and 960 μF. After standing at room temperature for 10 min, cells were washed with 6 ml of complete medium and then seeded onto a 100-mm plate. Typically, transfected cells were harvested at 72 h after electroporation. When only E1 or E2 was used, the protocol was the same as described above with the omission of one plasmid. For the time course experiments, cells were mixed with the plasmids and then aliquoted into 0.25-ml amounts, and electroporation was carried out. Since the number of cells will increase over the time period, before the samples were loaded onto the gel, the amount of DNA was standardized to the number of cells present at 4 h.

Extraction and DNA analysis. At times after transfection, low-molecular-weight DNA was extracted by the method of Hirt, as previously described (35), with slight alterations. Briefly, 0.6 ml of solution II (0.67% sodium dodecyl sulfate

[SDS], 0.13 N NaOH) was added to the tissue culture plates after cells were rinsed twice with phosphate-buffered saline, lysates were scraped into Eppendorf tubes and mixed with 0.3 ml of solution III (3 M potassium acetate), and the DNA was precipitated with isopropanol. LiCl (5 M) was then used to remove high-molecular-weight nucleic acids. One-eighth of the isolated DNA was used for digestion with *Dpn*I and either *Eco*RI or *Bam*HI (as specified in the figure legends), run on an 0.8% agarose gel, and blotted by the method of Southern, and the resulting filters were hybridized with [^{32}P]dCTP-labeled plasmid (pSK+) DNA probe. Filters were exposed to X-ray film for periods of 3 to 5 h. To quantify the relative replication abilities of different plasmids, we used a Molecular Dynamics laser scanner and PDI Quantity One software to scan the replicated bands (*Dpn*I resistant), and we calibrated against unreplicated bands (*Dpn*I sensitive). Scanning was carried out in the linear range of the X-ray film and scanner. X-rays shown here are not those used in quantitation. In some experiments, scintillation counting of radioactivity in bands cut from nitrocellulose was used to confirm the results of scanning.

McKay immunoprecipitation DNA binding assay. DNA binding was performed according to the method of McKay (19) with some modifications. The crude soluble extracts of E1 and E2 proteins in buffer A (20 mM Tris-HCl [pH 9], 0.3 M NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg [each] of leupeptin and pepstatin per ml) were obtained from *Spodoptera frugiperda* (Sf-9) cells 3 days after infection with baculovirus recombinants. The extracts were neutralized with a half volume of buffer B (100 mM Tris-HCl [pH 6.8], 0.3 M NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, and the same protease inhibitors as described above). Crude extracts (25 to 50 μl) were incubated at 4°C for 2 h with 2 μl of anti-E1 or anti-E2 polyclonal antibodies in 200 μl of buffer C (50 mM Tris-HCl [pH 7.2], 0.5% Nonidet P-40, 2 mM dithiothreitol, and the same protease inhibitors as described above) supplemented with 300 mM NaCl. Fifty microliters of 50% protein A-Sepharose equilibrated in the same buffer was added and incubated for 1 h, and the whole immunocomplex was collected by centrifugation for 2 min at 5,000 rpm in an Eppendorf centrifuge. The beads containing the immunocomplex were washed three times with 0.5 ml of buffer C containing 150 mM NaCl and resuspended in 200 μl of buffer C with 150 mM NaCl and 200 μg of salmon sperm DNA per ml. End-labeled DNA fragments were added, and the mixture was incubated for 1 to 2 h at 4°C. The beads were collected by centrifugation as described above, washed four times with buffer A containing 150 mM NaCl, incubated for 15 min at 65°C in dissociation buffer (50 mM EDTA [pH 8], 1% SDS), phenol-chloroform extracted, and ethanol precipitated. The dissociated DNA fragments were separated on a 2.5% agarose gel or a 3.5 to 12% gradient nondenaturing polyacrylamide gel. The gel was dried and exposed to X-ray film.

Construction of HPV-11 E1 and E2 baculovirus transfer vectors. The HPV-11 E1 and E2 coding sequences were cloned by PCR amplification of pKS-HPV11. An HPV-11 full-length genomic DNA clone has been described previously (24), as has the oligonucleotide primer set (P1/P2 [3]) used for amplification of the entire E1 ORF (3,212 bp). We introduced *Bam*HI and *Kpn*I sites by primer-mediated mutagenesis at the 5′ and 3′ ends, respectively, to clone E1 in pVL-1393 (obtained from M. D. Summers, Texas A&M University, College Station). This transfer vector construct was designated pVL-11E1. The E2 forward primer sequence (nucleotide coordinates 2697 to 2716) was 5′-CAT TGA GGA TCC AGA GGA CG-3′ (sequence according to Dartmann et al. [7]), and the E2 reverse primer (complementary to nucleotide coordinates 4356 to 4385) had the sequence 5′-TAA AAA AGC TTT ACT CTA GTT CAT GTA CAC-3′ (the restriction enzyme recognition sequences introduced by PCR are underlined). After amplification, the 1,689-bp E2 PCR product was digested with *Bam*HI and *Hin*dIII and subcloned into pBluescript KS−. The E2-coding sequence was then transferred to pVL-1393 via the *Bam*HI and *Kpn*I sites. This construct was named pVL-11E2.

Construction and purification of Ac11E1 and Ac11E2 recombinant baculoviruses. pVL-11E1 and pVL-11E2 were separately cotransfected into Sf-9 cells with *Autographa californica* nuclear polyhedrosis virus (AcNPV) genomic DNA. Methods used for cotransfection, selection, purification, and immunologic screening of recombinant viral stocks have been described previously (23, 24, 31).

Production of HPV-11 E1 and E2 rabbit polyclonal antisera. Polyclonal rabbit antisera were raised against the products of the HPV-11 E1- and E2-coding sequences, which were expressed as β-galactosidase fusion proteins in *Escherichia coli* by previously described methods (3, 24).

RESULTS

Time course of replication of papillomavirus origins. It has previously been shown that plasmids containing a papillomavirus origin will replicate in a transitory assay over a 96-h period, with a maximum level seen at 72 to 96 h. To determine the optimum time point for measuring plasmid replication, we carried out a time course of replication of plasmids containing the origins of replication of HPV-6b, HPV-11, and BPV-1 from 4 to 72 h after electroporation (Fig. 1A). Plasmid replication was first observed at 12 h and then increased over the next 24 h, with a maximum signal observed at either 48 or 72 h. The bands at the bottom of the gel (Fig. 1A, I) represent input, *Dpn*I-sensitive plasmid DNA. Separate long-term assays revealed that replication decreased gradually after 96 h and was undetectable by the 11th day (data not shown). In subsequent experiments cells were harvested 72 h after electroporation.

HPV-11 and HPV-6b are closely related and share over 80% homology at the DNA level, while BPV-1 is more distantly related. We tested the abilities of E1 and E2 from HPV-11 and HPV-6 to replicate the origins of HPV-6 and -11, respectively. Only the results for the HPV-11 origin are shown (Fig. 1B), although similar results were seen with the HPV-6 origin plasmid. There appeared to be complete reciprocity between the proteins of HPV-6b and -11, even when the E1 from one type was used with the E2 from another. However, although E1 and E2 from BPV-1 were able to replicate the HPV-11 origin when either of the BPV proteins was cotransfected with a corresponding HPV protein, very little replication was observed (Fig. 1B). These results indicated that protein-protein interactions are important at the origin of replication; therefore, the rest of this paper concentrates on the replication of HPV-11 in the presence of HPV-11 E1 and E2.

Minimal E2BS requirements. The cores of the replication origin from different papillomaviruses are composed of (i) a poly(A/T) region 30 to 40 bp in length and a 4- to 6-bp poly(A) tract, which we have termed the spacer region, and (ii) three E2BS surrounding the spacer, with one at the upstream side (5′ to the spacer) and two downstream of the spacer (3′) (Fig. 2). The total length of the origin cores is 100 bp for the HPVs. Transitory replication assays localized the activity to this core for BPV-1 (35), HPV-11 (6), and HPV-18 (22). To investigate systematically the role in replication of each part of the core, i.e., the HPV-11 spacer and the E2BS on both sides, we

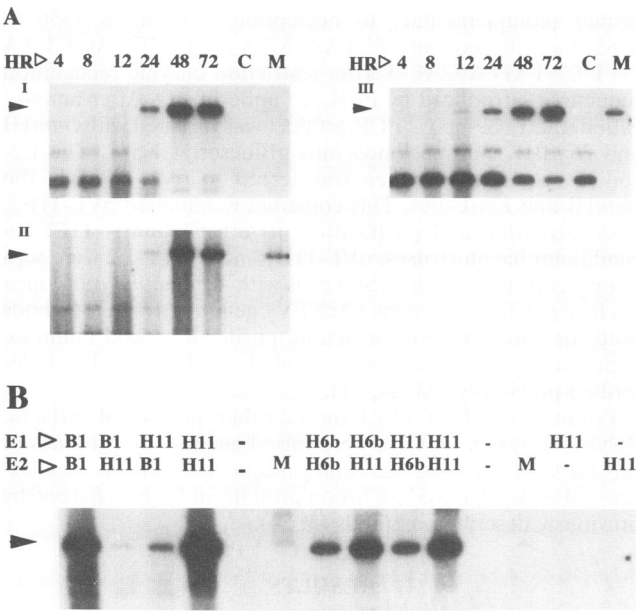


FIG. 1. Replication assays of origin-containing plasmids of papillomaviruses. (A) Time course experiment on origin replication of HPV-11, BPV-1, and HPV-6b. I, BPV-1 origin plasmid (pUC/BPVPst [35]) digested with *Bam*HI and *Dpn*I and hybridized with a ^{32}P-labeled DNA probe consisting of the *Pst*I fragment from the BPV-1 genome (bp 4387 to 1999). II, HPV-11 origin plasmid pHPV-11ori.1 digested with *Eco*RI and *Dpn*I and hybridized with a ^{32}P-labeled probe (*Bam*HI-*Nsi*I fragment, bp 7092 to 236). III, HPV-6b origin plasmid pBSH6Ori digested with *Eco*RI and *Dpn*I and hybridized with a ^{32}P-labeled DNA probe of the full HPV-6 insert. (B) Replication assay of HPV-11 origin plasmid pHPV-11Ori.1, using different combinations of E1 and E2 plasmids from BPV-1 and HPV-11 and from HPV-6b and HPV-11. All the extracted DNA was digested with *Bam*HI and *Dpn*I. The dashes above some lanes indicate that no E1 or E2 of HPV-11 was used for the transfections. C and M are negative controls (origin plasmids alone without corresponding E1 and E2 plasmids for the transfections) and marker DNAs (10 pg) of the corresponding plasmids linearized with the same linearizing enzymes as specified above, respectively. The arrowheads indicate the positions of the replicated origin plasmids.

constructed a complete series of plasmids containing different portions of the HPV-11 origin core (Fig. 2).

We first investigated the necessity for E2BS surrounding the spacer. The plasmids used (Fig. 2) were amplified by PCR and cloned into pSK+ (Stratagene, La Jolla, Calif.), at the *Bam*HI site. The orientation was checked by sequencing. The core plasmid, N9, is the positive control and was found to replicate as well as the HPV-11ori.1 plasmid used in the experiments shown in Fig. 1, which contained other HPV-11 sequences around the origin of replication. Plasmid 54, which contains the 64-bp spacer of HPV-11 origin core, was negative in the replication assay (Fig. 3A). When one E2BS was present on either side of the spacer, a low level of replication was seen, with plasmid 46 (E2BS on the 3′ end) showing a 10-fold-stronger signal than M4 (E2BS on the 5′ end; Fig. 3A; also see Fig. 5). Surprisingly, two E2BS with the spacer exhibited very high replication activities, comparable to that of the intact core as confirmed by scanning the X-ray film (Fig. 3A). To verify that the activities were truly due to replication of the transfected plasmid and not to a small amount of input DNA remaining after *Dpn*I digestion, the extracted DNA was digested with *Mbo*I, which cuts only unmethylated nucleic acids. The bands representing the replicated form of DNA were completely removed by digestion with *Mbo*I and *Dpn*I (Fig. 3B). The marker was undigested.

Requirement for the spacer region between the E2BS. To investigate the necessity of the spacer, which contains a conserved poly(A/T)-rich region and a poly(A) tract, we replaced the 64-bp spacer with a fragment of the same size from the polylinker of Bluescript pSK+ (Stratagene). The origin sequence plasmid $pEBS_{1\text{-}2}$ is arranged in a manner equivalent to the wild-type origin N9, which is shown on the same film (Fig. 4C); however, it consistently replicated at a lower level than N9 (a 10- to 20-fold lower [Fig. 4C and 5]), indicating a role of the normal spacer in optimal replication. The negative control, pSK+ with no E2BS ($pEBS_{0\text{-}0}$) and containing the polylinker used to substitute for the real spacer, was unable to replicate (Fig. 4B and C). In addition, plasmids with one E2BS at the 5 or 3′ end of the polylinker did not replicate to a detectable level (Fig. 4B and C).

Replication and the number of E2BS. Since one E2BS allowed the replication of the plasmid containing the normal spacer region of HPV-11 but did not permit the replication of a plasmid with an artificial spacer, we decided to investigate the dependence of replication on the number of E2 sites when an artificial spacer was present. Addition of two E2BS greatly increased the efficiency of replication (plasmids $pEBS_{0\text{-}2}$ and $pEBS_{1\text{-}1}$, Fig. 4B and C), and three and four E2BS increased replication 10- and 20-fold, respectively, above that seen for two E2BS (Fig. 4B and C). However, the addition of up to four E2BS, either all at the 3′ end or two at each end of the polylinker (Fig. 4B and C), did not result in replication to the same level as with plasmids containing the normal HPV-11 spacer (compare plasmid N9 with the others in Fig. 4B and C). Therefore, a plasmid containing two E2BS, as the only HPV-11 origin core elements, is able to replicate in the presence of both E1 and E2 proteins.

Position of E2BS with respect to the spacer. To investigate the effect on replication of the position of the E2BS relative to the spacer, we compared the replication activities of plasmids containing a number of E2BS located at different positions around the normal HPV-11 spacer and the artificial spacer from the polylinker of pSK+. The most striking result was the effect of the relative position of a single E2BS around the normal spacer (Fig. 3A and 5, plasmids 46 and M4). Plasmid 46 with the E2BS at the 3′ end of the spacer always replicated more efficiently (20-fold) than plasmid M4 (Fig. 3A and 5). When two E2BS were present, then the relative positions with regard to the spacer did not seem to matter (Fig. 3A and 5, plasmids M8 and M12).

As shown above, no detectable replication of the plasmid occurred when one E2BS was present along with the artificial spacer. Plasmids $pEBS_{0\text{-}1}$ and $pEBS_{1\text{-}0}$ consistently did not replicate to a detectable level (Fig. 4 and 5), while plasmids containing two E2BS, $pEBS_{0\text{-}2}$ and $pEBS_{1\text{-}1}$, showed low levels of replication activity. Plasmids with either three or four E2BS replicated more efficiently (10- and 20-fold, respectively), but the relative positions did not significantly affect the level of replication (Fig. 5).

Effect of spacer length on replication. The length (64 to 69 bp) and sequence of the spacer are conserved between HPV types (Fig. 2). To assess the effects of changing the length of the spacer on replication, we increased the size by a factor of two. This was done by cloning the natural HPV-11 spacer into the *Bam*HI site of $pEBS_{1\text{-}2}$, which has the same number and positional pattern of E2BS as the HPV-11 origin core, to form plasmids pOriIS and pOriIA, where S and A stand for the sense and antisense orientations, respectively, with regard to

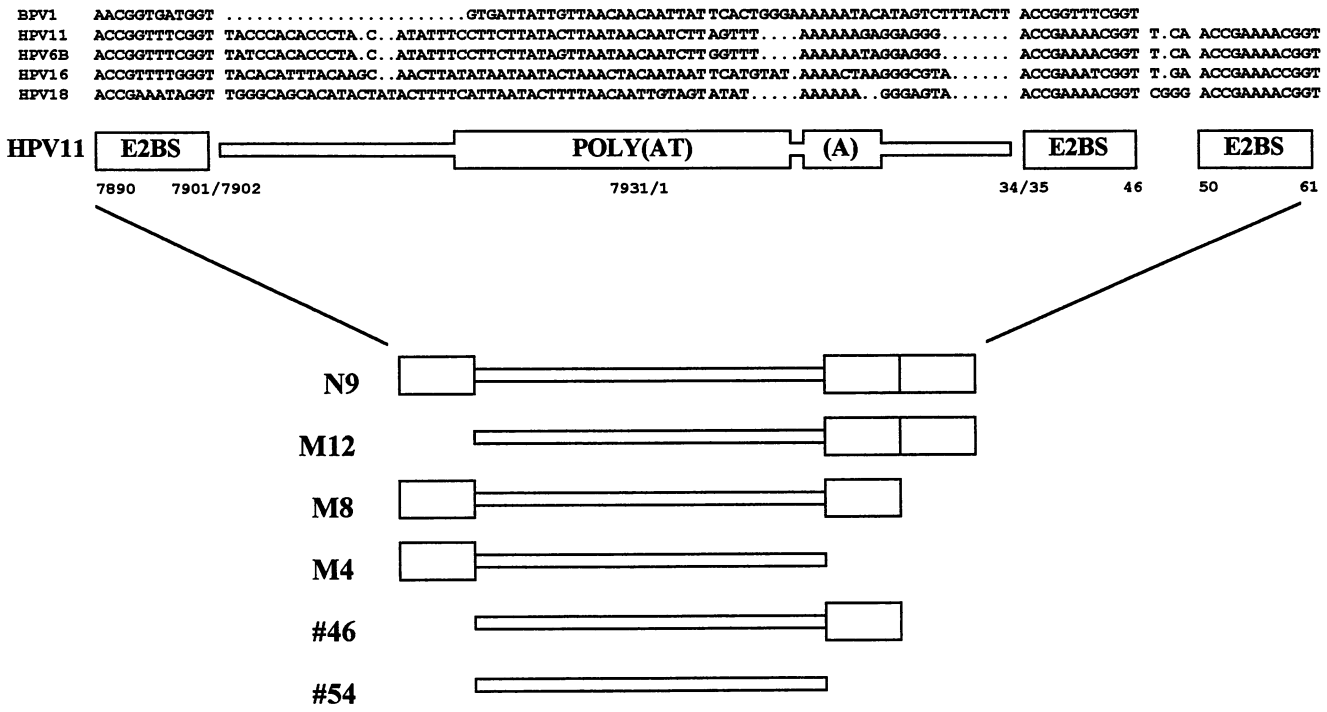


FIG. 2. Sequences of the core regions of the origins of BPV-1, HPV-11, HPV-6b, HPV-16, and HPV-18. The diagrams below the sequences represent the conserved elements in the core region and the origin series of plasmids. The double lines represent the HPV-11 origin spacer spanning bp 7902 to 34 and the boxes at either end represent the E2BS. The full HPV-11 origin core region, as represented by plasmid N9, spans nucleotides 7890 to 61.

E2BS 1, 2, and 3 (Fig. 6A). Plasmids pOriIIS and pOriIIA were derived by deleting the pSK+ polylinker between the *Sma*I and *Hinc*II sites (Fig. 6B). They have the same organization as the natural HPV-11 origin core, except that there are extra sequences at the junctions between the spacer region and the E2BS and that the spacer region is in both orientations (Fig. 6A).

Increasing the spacer region twofold decreased the efficiency of replication fourfold (Fig. 6B, plasmids pOriIS and pOriIA compared with N9). Deleting the artificial spacer from these two plasmids but leaving 20 and 16 bp, respectively, at the 5′ and 3′-prime ends of the normal HPV-11 spacer again caused a similar decrease in the replicative ability of the plasmids (Fig. 6B). Therefore, increasing the distance of the A/T-rich region from the 3′-positioned E2BS did not decrease replication to the level observed when the polylinker replaced the natural spacer.

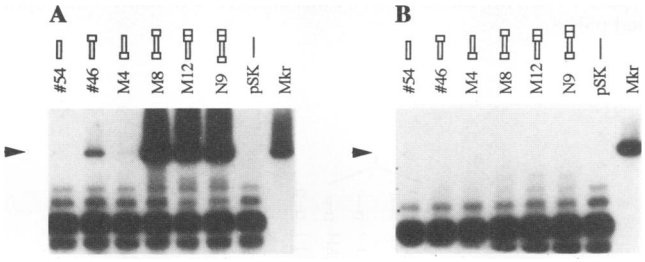


FIG. 3. Replication assays of plasmids containing different components of the HPV-11 origin core as illustrated in Fig. 2. Each lane is marked with the name and diagram of the plasmid. The double lines represents the spacer of the HPV-11 origin core, the single line represents the pSK+ linker, and open squares represent the E2BS of the HPV-11 origin core. pSK is included as a negative control, and Mkr represents 20 pg of *Bam*HI-linearized pSK+. The arrowheads indicate the position of the replicated plasmids. (A) Hirt extracts were digested with *Bam*HI and *Dpn*I and hybridized with ^{32}P-labeled pSK+ plasmid. (B) Same panel A except that the Hirt extracts were digested with *Mbo*I, which cuts only unmethylated DNA, in addition to *Bam*HI and *Dpn*I. The marker was undigested in both panels.

All of the experiments described above were also carried out in the presence of E1 or E2 alone (data not shown), and no replication was detectable for any of the constructs, indicating an absolute requirement for both viral proteins in these transitory assays.

Binding of E1 and E2 proteins at the origin. McKay assays (19) were carried out with recombinant baculoviruses expressing E1 and E2 proteins and a mixture of DNA fragments incorporating various origin region constructs. E1 bound weakly to the fragment containing the A/T-rich spacer (Fig. 7A, lane 2) and not to any of the other segments, including a fragment containing the artificial spacer, while the preimmune serum was negative for all fragments (Fig. 7A, lane 1). E2, as expected, bound to any fragment that contained an E2BS (Fig. 7B, lanes 7, 9, and 10). When both E1 and E2 were added to the mixture of DNA fragments, anti-E1 antibodies immunoprecipitated those fragments that contained an E2BS (Fig. 7B, lane 6). The 288-bp fragment immunoprecipitated by anti-E1 antibodies contains two E2BS and the artificial spacer and replicates in the presence of E1 and E2 proteins ($pEBS_{0\text{-}2}$ [Fig. 4B and 5]). Therefore, E1 and E2 are contained within the same complex, which binds to the origin through E2BS. The DNA fragment to which E1 bound had additional nucleotides on either side of the origin core. To confirm that E1 bound to

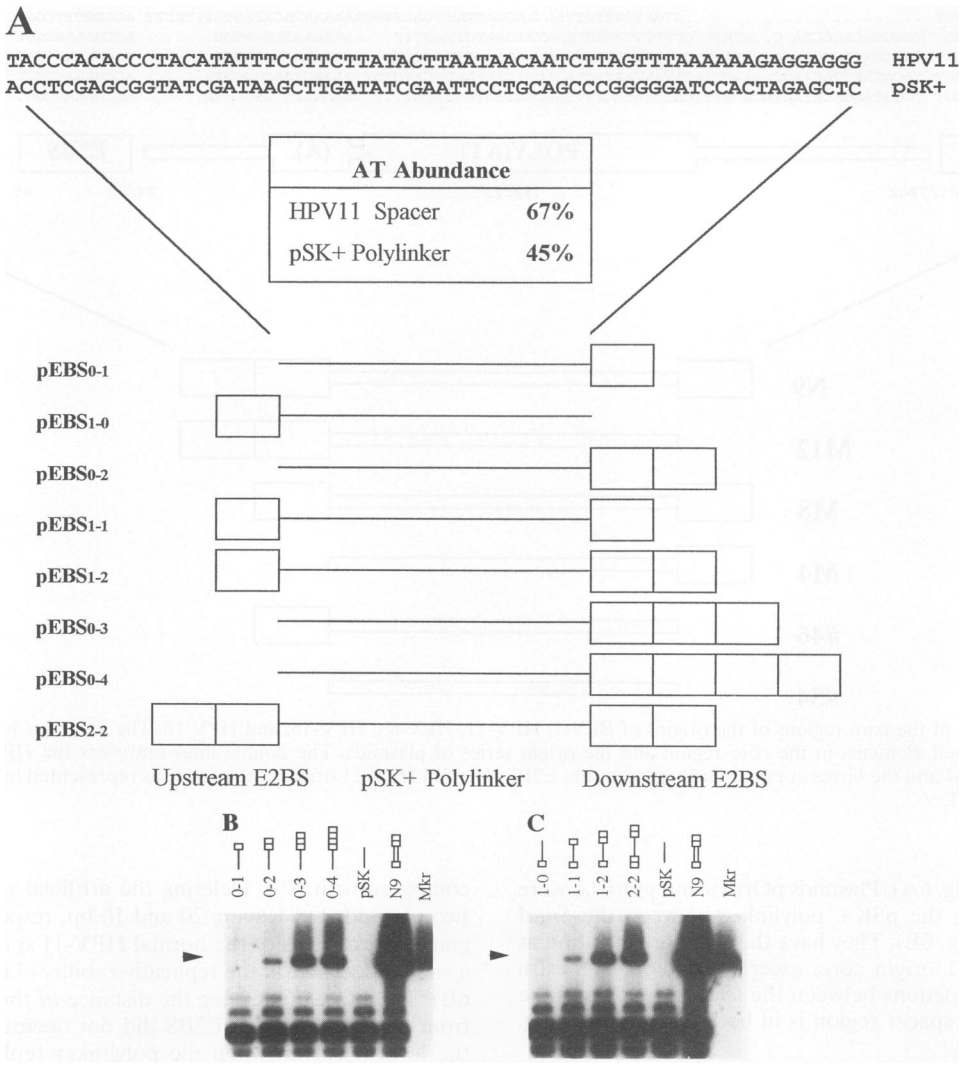


FIG. 4. Diagrams of the $pEBS_{X-Y}$ series of plasmids and the replication assays. (A) Sequence comparison between the HPV-11 spacer and pSK+ polylinker, with their A/T contents listed below. The lower part illustrates the pEBS series of plasmids. The single line represents the pSK+ polylinker, and the boxes represent the E2BS, with 6 nucleotides between each E2BS. (B and C) Replication assays of the $pEBS_{X-Y}$ series of plasmids, where X and Y represent the number of E2BS at the 5′ and 3′ ends of the spacer, respectively. Open squares, E2BS; double line, HPV-11 spacer; single line, the 64-bp pSK+ polylinker used to replace the HPV-11 spacer. The Hirt-extracted DNA was digested with *Bam*HI and *Dpn*I and hybridized as for Fig. 3. The marker (Mkr) is 20 pg of *Bam*HI-linearized pSK+.

the origin core, the core was purified from the plasmid N9 by *Bam*HI and the 104-bp fragment was used in a McKay assay. Once more E1 bound very weakly to the core fragment and is in fact not visible with an overnight exposure (16 h; Fig. 8, lane 2). However, binding was observed after a 5-day exposure (data not shown). The larger 1.0-kbp fragment bound E1 more efficiently (Fig. 8, lane 3), which indicates that perhaps other sequences surrounding the core have an effect on binding or that E1 may need nonspecific flanking sequences for optimal binding. Efficient E1 binding to the 104-bp replication core was evident, either in the presence of E2 (Fig. 8, lane 5) or when fivefold more E1 was used for the binding assay (Fig. 8, lane 7).

DISCUSSION

The origins of replication of HPV-11, -16, and -18 have been mapped to a region around bp 1, and all contain three E2BS surrounding an A/T region (Fig. 2). Two virus-coded proteins,

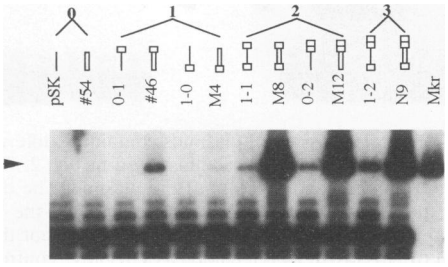


FIG. 5. Comparison between plasmids containing same number of E2BS but different spacer sequences. The filter was hybridized as for Fig. 3. The plasmid constructs are described in Fig. 2 and 4. The numbers above the brackets are the numbers of E2BS in each construct. The arrowhead indicates the replicating plasmid DNA. The marker (Mkr) is 20 pg of *Bam*HI-linearized pSK+.

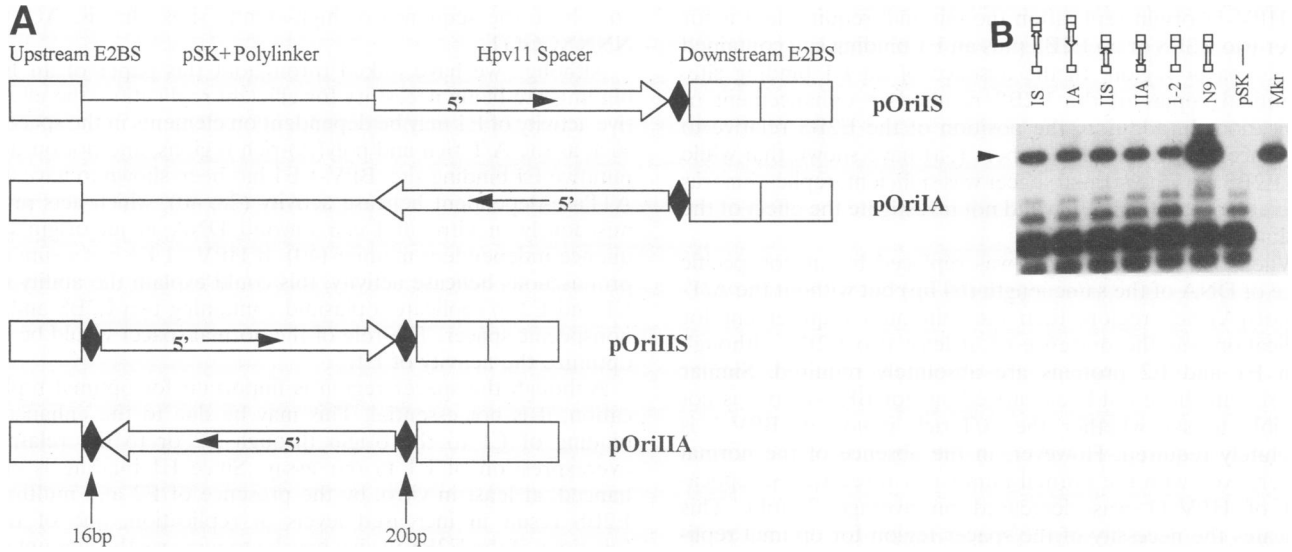


FIG. 6. Replication assay with the pOri series of plasmids. (A) Diagrams of the pOri series of plasmids. The double line represents the HPV-11 origin spacer with its orientation indicated by the arrow, the single line represents the pSK+ polylinker, boxes represent the E2BS, and diamonds represent the extra bases resulting from cloning. (B) Replication assays of pOri series plasmids. In the diagrams the open box represents E2BS, the solid line represents the pSK+ polylinker, and the double line represents the HPV-11 origin core spacer with orientation given by the arrow. The plasmid DNA was digested with *Bam*HI and *Dpn*I and filter hybridized as for Fig. 3.

E1 and E2, are both essential for the replication of plasmids containing the origin-of-replication sequences. The present study investigates the roles of two important adjacent elements in the origin-of-replication region of HPV-11, namely, the E2BS and the A/T-rich sequences lying between them. The literature suggests differences between BPV-1 and HPV-11 DNAs in the requirements for *cis* elements in transitory replication assays: only one E1 site is essential in vivo (36) and in vitro (40) in the case of BPV-1. However, recent reports show that for BPV-1 an E2BS can substitute for the lack of an E1 binding site in vivo (30), and, with further mapping, an E2BS was shown to be essential (34). In this study we show that

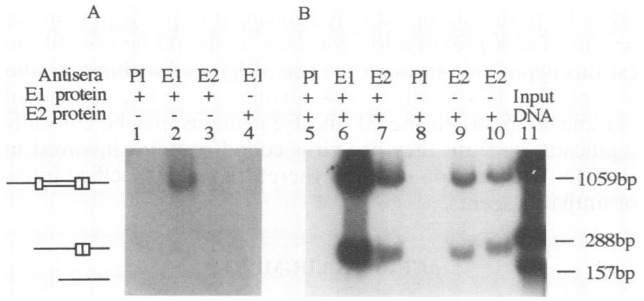


FIG. 7. Immunoprecipitation of DNA binding proteins E1 and E2 by McKay assays (19) with origin region fragments. Proteins were immunoprecipitated from recombinant baculovirus cell extracts, added to mixtures of DNA fragments, and run on a 3.5 to 12% gradient SDS-polyacrylamide gel electrophoresis gel. Panels A and B are two parts of the same filter exposed for different periods of time. Proteins and antisera added to the assay are indicated at the top of each lane (PI, preimmune serum) (A) Binding of HPV-11 E1 to HPV-11 DNA fragments. The 1,095-bp fragment is from bp 7072 to 236 and contains the origin core with three E2BS (boxes) and spacer (double lines), the 288-bp fragment is from the plasmid $pEBS_{0\text{-}2}$ and contains two E2BS (boxes) and the polylinker spacer (single line), and the 157-bp fragment is part of pSK+ from bp 532 to 689 and represents a negative control. The film was exposed for 48 h. (B.) Binding of E1, E2, and mixtures of each to the DNA fragments described for panel A. The proteins and antisera added are indicated above each lane. The input DNA fragments are shown in lane 11. The film was exposed for 10 h.

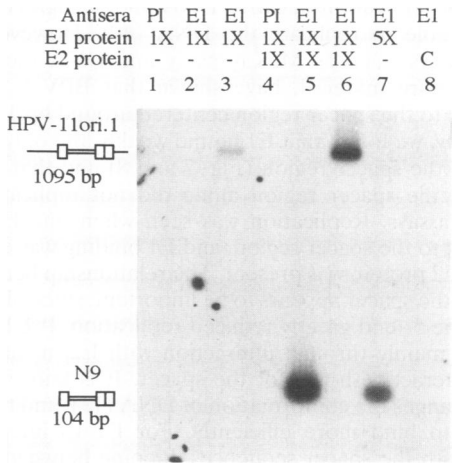


FIG. 8. Immunoprecipitation of DNA binding proteins E1 and E2. Baculovirus-derived E1 and E2 proteins were immunoprecipitated and mixed with DNA fragments by the method of McKay (19), run on a 2.5% agarose gel, dried, and exposed for 10 h to X-ray film. The proteins and antisera added are indicated at the top of each lane (PI, preimmune serum), with 1X indicating the same concentration as for Fig. 7, while 5X indicates a fivefold increase in the amount of E1 protein added. The DNA fragments were a 1,095-bp fragment from the pHPV-11ori.1 plasmid as for Fig. 7, which was added to lanes 1, 3, 4, 6, and 8, and a 104-bp fragment of only the core region of plasmid N9, which was added to lanes 1, 2, 4, 5, 7, and 8. The squares represent E2BS, and the double lines represent the A/T-rich spacer. Lane 8 (control) contains extracts from recombinant baculovirus containing only the plasmid immunoprecipitated with postimmune E1 and mixed with both DNA fragments.

for HPV-11 origin replication the minimal requirement is for either two E2BS or an E2BS plus an E1 binding site contained in the spacer region. In the absence of an E1 binding site, additional copies of the E2BS resulted in enhancement of replication. In addition, the position of the E2BS relative to the spacer was important. Others (5a) have shown that while one E2BS plus the normal spacer was sufficient, replication was at a very low level, and they did not investigate the effect of the binding site position.

When the HPV-11 spacer was replaced by an nonspecific piece of DNA of the same length (64 bp) but without the A/T- or poly(A)-rich region, then the minimum requirement for replication was the presence of at least two E2BS, although both E1 and E2 proteins are absolutely required. Similar experiments have not been carried out for BPV-1, so it is not possible to say whether the A/T-rich region of BPV-1 is absolutely required. However, in the absence of the normal spacer, even with the normal number of three E2BS, replication of HPV-11 was decreased on average 20-fold. This indicates the necessity of the spacer region for optimal replication of the origin. The length of the spacer between the E2BS was of marginal significance, since when the length was doubled by the addition of 64 bp from the polylinker of pSK+ or increased by up 36 bp, there was only a fourfold reduction in replication.

The crystal structure of the E2 C-terminal region has shown that two E2 units, symmetrically paired, bind to a single E2BS (13). The E1 protein of BPV-1 binds via its C terminus to E2, with preference for underphosphorylated E2 (15, 21), and the E2BS sites at the origin may target E1 to the origin (21). The direct binding of E1 to DNA is more problematic in that E1 appears to bind both specifically and nonspecifically. In in vitro replication assays using purified components, BPV-1 E1 bound to origin-of-replication-negative DNA and at high concentrations was able to replicate the DNA (40). However, DNA binding assays, such as McKay assay and DNA footprinting using less pure material, have shown that BPV-1 E1 binds specifically to the spacer region centered around bp 1 (30, 36). In our study, we found that E1 bound weakly and only to DNA containing the spacer region (Fig. 7 and 8) and that plasmids containing the spacer region alone did not replicate in the transitory assays. Replication was seen when one E2BS was added next to the spacer region, and E1 binding was enhanced when the E2 protein was present. The relationship between the E2BS and the spacer appears to be important, since placing the E2BS at the 5′ end greatly reduced replication. If E1 binds at the origin mainly through interaction with E2, it may subsequently interact with part of the spacer. It is known that E2 binding changes the conformation of DNA (13), and this might allow E1 to bind more efficiently. For E1 to interact at a distance with the spacer sequences, looping between E2 sites may be required, a phenomenon known to occur at some origins of replication and particularly between transcription factors (for a review, see reference 26). The fact that addition of 20 and 16 bp, respectively, at the 3′ and 5′ ends of the spacer had only a marginal effect on replication would support some form of looping. However, even plasmids with two E2BS at the same end of the spacer replicated as well as plasmids with E2BS at each end. For BPV-1, increasing the distance between E2BS and the E1 binding site, in an area similar to that used for additions in this study, by 3 to 10 bases significantly decreased the replicative ability of the plasmids (34). The authors (34) suggested that either affinity of E2 for DNA or multiple E2 sites might overcome distance constraints. It should be noted that the E2BS in HPV-11 around the origin core have the sequence of high-affinity sites, that is, ACCG NNNNCGGT.

Although we have talked throughout this paper of an E1 binding site being necessary for efficient replication, the effective activity of E1 may be dependent on elements in the spacer, such as the A/T-rich and poly(A)-rich regions, and not on the putative E1 binding site. BPV-1 E1 has been shown to have an ATPase-dependent helicase activity (28, 40), which acts promiscuously in vitro and can unwind DNA in an origin-sequence-independent manner (40). If HPV-11 E1 has a similar promiscuous helicase activity, this could explain the ability of E1 and E2 to replicate a plasmid containing two E2BS and a nonspecific spacer. The role of the normal spacer would be to optimize the activity of E1.

Although the spacer region is important for optimal replication, it is not essential. This may be due to the enhanced binding of E1 to the origin through E2 or to the relative overexpression of E1 in our assay. Since E1 binding is enhanced, at least in vitro, by the presence of E2 and multiple E2BS result in increased levels of replication, one of the functions of the E2BS in vivo may be to increase the amount of E1 at the origin to a level that will function efficiently. This role may be important because so far the only transcript found in HPV-infected tissue which will code for E1 is a polycistronic mRNA composed of a complete readthrough of the early region. The E1 ORF is the third in line and will presumably be translated very inefficiently. Therefore, in the early stages of replication there may be a need to concentrate limited replication factors, especially as the virus is attempting to initiate replication in differentiating cells, which presumably will also have low levels of the cell's replicative machinery.

In conclusion, it appears that either the A/T-rich spacer region and one E2BS or two E2BS alone can constitute a minimal HPV-11 origin element. Furthermore, the positioning of the E2BS relative to the spacer is important. In all cases replication is dependent on the presence of both E1 and E2 proteins, indicating the importance of the protein-protein interactions at the origin. In the light of these data, it seems reasonable to speculate that the function of E2 may be to tether E1 to the origin, thereby allowing it to participate in the formation of a preinitiation complex. It should be possible to test this hypothesis through the use of E1–Gal-4 chimeras and by replacing E2BS with Gal-4 binding sites. The functional relationship between the E1 and E2 proteins may be clinically significant, because they are virus-coded proteins involved in viral DNA replication and may therefore make specific targets for antiviral agents.

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