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Replication of Human Papillomavirus (HPV) DNAs Supported by the HPV Type 18 E1 and E2 Proteins

FRANCIS SVERDRUP AND SALEEM A. KHAN*

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Received 28 July 1993/Accepted 22 September 1993

Transient replication of human papillomavirus (HPV) type 18 DNA was shown to require the viral E1 and E2 proteins. A 108-bp sequence within the long control region (nucleotides 12 to 119) was sufficient to function as the origin, but maximal replication required a region of 177 bp from positions 7800 to 7857 and 1 to 119 of HPV-18. The E1 and E2 proteins of HPV-18 also supported transient replication of plasmids containing the origins of HPV-1a and bovine papillomavirus type 1 to low levels. Interestingly, the level of replication observed with the HPV-6b origin was higher than that obtained with the homologous HPV-18 origin.

Papillomaviruses contain circular double-stranded DNA genomes that usually replicate autonomously at a relatively fixed copy number (2, 7, 14). They provide useful model systems with which to study regulated DNA replication in mammalian cells. Many human papillomaviruses (HPVs) are associated with anogenital lesions (32). HPV type 16 (HPV-16) and HPV-18 have been implicated in the causation of cervical carcinomas (17, 32). The DNA of HPV-16 and HPV-18 is present in an extrachromosomal form in premalignant lesions but is usually found integrated in the chromosomes of malignant cells (25, 32). Recent studies with bovine papillomavirus type 1 (BPV-1) and various HPVs have shown that their replication requires an origin of replication (ori) present within the long control region (LCR) and two virally encoded, *trans*-acting replication proteins, E1 and E2 (3, 4, 6, 14, 20, 23, 29–31). The E1 and E2 proteins of BPV-1 and HPVs are quite homologous, and recent studies have suggested a lack of specificity of these proteins in replicating plasmids containing the ori sequences of other papillomaviruses (4, 6). The E1 protein of BPV-1 has ATPase, DNA helicase, and ori-specific DNA binding and unwinding activities (27, 28). The E2 protein is a transcriptional activator protein that binds with high affinity to palindromic $ACCGN_4CGGT$ sequences (8, 10, 11, 16, 19, 22). The E2 protein forms a specific complex with the E1 protein and enhances its binding to the ori, which contains the binding sites for both the E1 and E2 proteins (15, 18, 26). We have used a transient replication assay to identify the *cis*- and *trans*-acting sequences required for the replication of HPV-18 DNA.

Transient replication of plasmids containing the HPV-18 LCR in various cell lines. Transient replication analysis of plasmids containing the complete genome of HPV-18 in human 293 and HeLa cells revealed that these DNAs replicated very poorly (data not shown). To determine whether this was due to low-level expression of the E1 and E2 genes, plasmids expressing the E1 and E2 proteins from the heterologous simian virus 40 early promoter were generated. All recombinant DNA procedures were carried out as described previously (24). A 3.8-kb *Pvu*II-*Nae*I fragment of HPV-18 that includes nucleotides (nt) 853 to 4595 of HPV-18 (5) and contains the E1 and E2 open reading frames (ORFs) was inserted into the *Hin*cII site of pUC7 to generate p18E1E2. The E1 ORF was then isolated as a 2,151-bp *Bam*HI-*Eco*RII fragment (the *Eco*RII site was filled in with the Klenow fragment of DNA polymerase I before digestion with *Bam*HI). The expression vector pSG5 (Stratagene) contains the simian virus 40 early promoter and unique restriction sites downstream from this promoter (9). Plasmid pSG5 was first linearized with *Bgl*II, the 5′ overhangs were filled in, and the plasmid was then digested with *Bam*HI. The E1-containing fragment was then inserted into pSG5 to generate pSGE1 (6,223 bp). Plasmid pSGE1 contains HPV-18 nt 854 to 2999, which include the E1 ORF. An E2-expressing plasmid was generated by isolating a 1,136-bp *Asp*I-*Nde*I fragment of HPV-18, filling in the ends with the Klenow fragment, and inserting the fragment into the *Hin*cII site of pUC7 to generate pUCE2. The E2 ORF was then isolated from this plasmid as a *Bam*HI cassette and cloned into the *Bam*HI site of the expression vector pSG5 to generate pSGE2 (5,226 bp). Plasmid pSGE2 contains HPV-18 nt 2783 to 3920. On the basis of the DNA sequence of HPV-18, pSGE1 is expected to encode a 73-kDa E1 protein, while pSGE2 should encode a 41-kDa E2 protein. Plasmid pUCLCR-18 was generated by inserting an LCR-containing, 1,048-bp *Bam*HI fragment of HPV-18 (nt 6929 to 7857/1 to 119) into the *Bam*HI site of pUC19. Transient replication assays were done with 2×10^5 to 4×10^5 cells in 60-mm-diameter plates. Transfection of various cell lines with plasmid DNA was done by calcium phosphate coprecipitation (1). Transfection efficiencies typically ranged from 40 to 50% for C-33A cells, 15 to 25% for HeLa cells, and 15 to 30% for 293 cells (data not shown).

We transfected plasmid pUCLCR-18 (0.5 μg), containing the HPV-18 LCR, into C-33A and 293 cells along with 5 μg each of plasmids pSGE1 and pSGE2, expressing the E1 and E2 proteins of HPV-18. Two to four days posttransfection, low-molecular-weight DNA was isolated by the Hirt extraction procedure (12). The samples were first digested with *Eco*RI. This treatment linearized the various plasmids except pSGE1, which was cleaved into two fragments, one of which contains vector sequences complementary to the probe used. To distinguish between replicated and unreplicated DNA, one half of each sample was treated with an excess of *Dpn*I to remove the unreplicated, input methylated DNA (21). The DNA samples were analyzed by electrophoresis on 0.7% agarose gels, using

* Corresponding author. Mailing address: Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Room East 1240 Biomedical Science Tower, Pittsburgh, PA 15261. Phone: (412) 648-9025. Fax: (412) 624-1401. Electronic mail address: Khan@med.pitt.edu

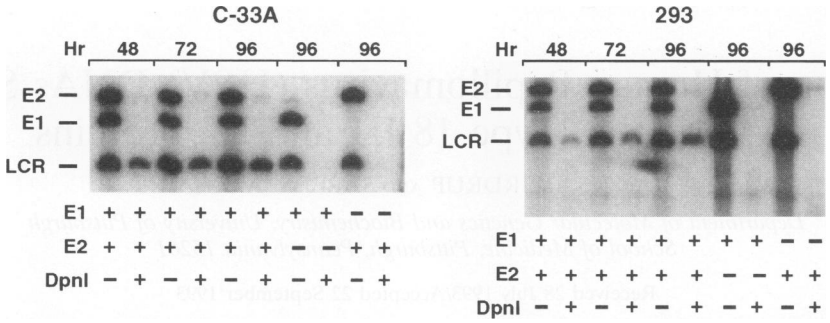

FIG. 1. Replication of plasmid pUCLCR-18 in C-33A and 293 cells. C-33A and 293 cells were transfected with 0.5 μg of pUCLCR-18 and 5 μg each of pSGE1 and pSGE2, and the Hirt fractions were analyzed at 48, 72, and 96 h after transfection. The positions of pUCLCR-18 (LCR), pSGE1 (E1), and pSGE2 (E2) are indicated. The autoradiograms were exposed for 6 h for C-33A cells and 16 h for 293 cells.

Tris-borate-EDTA buffer. The DNA was transferred to GeneScreen, and the membranes were hybridized (24) to ^{32}P-labeled pUC19 probe generated by using a random primer labeling kit (Amersham). The specific activity of the probes typically ranged from 5×10^8 to 1×10^9 cpm/μg of the DNA. Blots were subjected to autoradiography at −70°C with intensifying screens. No replication of plasmid pUCLCR-18 was observed when this plasmid was transfected alone or cotransfected with either pSGE1 or pSGE2 alone (Fig. 1). However, pUCLCR-18 replicated efficiently, as evidenced by *Dpn*I resistance of the pUCLCR-18 band when the cells were cotransfected with both pSGE1 and pSGE2 (Fig. 1). This conclusion is further supported by the absence of *Dpn*I-resistant bands corresponding to the E1 and E2 expression plasmids, which were transfected at 10-fold-higher amounts than the LCR-containing plasmid (Fig. 1). The *Dpn*I-untreated lanes also served as controls for the comparable transfection efficiencies in various samples. The vector pUC19 showed no replication under any condition (data not shown). Replication of plasmid pUCLCR-18 was detected at 48 h after transfection and increased up to 96 h posttransfection. The replication efficiency was higher in C-33A cells than in 293 cells (Fig. 1). However, this could be due to the higher transfection efficiency of C-33A cells (40 to 50%) than of 293 cells (15 to 30%). These results showed that the E1 and E2 proteins are both necessary and sufficient for the transient replication of plasmids containing the LCR of HPV-18. These results are consistent with recent results obtained with BPV-1 and HPV systems (3, 4, 6, 23, 29, 30). It is likely that low-level replication observed with the HPV-18 genomic DNA (data not shown) was due to poor expression of the E1 and/or E2 proteins from their natural promoter. In a separate experiment, various amounts of pUCLCR-18 were cotransfected into C-33A cells along with 5 μg each of pSGE1 and pSGE2. Replication of the ori plasmid increased as the amount of transfected DNA was increased, and a saturation was reached at 0.5 μg (data not shown). In subsequent experiments, 0.5 μg of plasmid pUCLCR-18 was therefore used.

Replication of the HPV-18 ori plasmid is dependent on the relative levels of E1 and E2 proteins. Experiments were done to test whether the replication efficiency of the HPV-18 ori plasmid is affected by the relative amounts of the cotransfected E1- and E2-expressing plasmids. In one series of experiments, 0.5 μg of pUCLCR-18 and 5 μg of pSGE2 were cotransfected into C-33A cells along with various amounts of pSGE1. No replication was observed when 0, 0.2, and 0.5 μg of pSGE1 were used, as evidenced by the lack of a *Dpn*I-resistant pUCLCR-18 band (Fig. 2). Replication was detected when 1 μg of pSGE1 was used, and maximal replication was observed when 5 μg of this plasmid was transfected (Fig. 2). In another

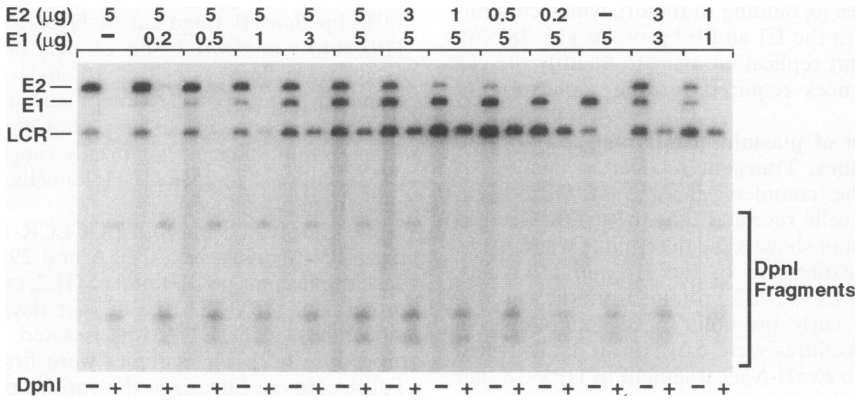

FIG. 2. Replication of an HPV-18 LCR-containing plasmid in the presence of various amounts of the HPV-18 E1- and E2-expressing plasmids. One-half microgram of plasmid pUCLCR-18 was cotransfected with the indicated amounts of the E1- and E2-expressing plasmids into C-33A cells. Four days after transfection, low-molecular-weight DNA was harvested and linearized with *Eco*RI. One half of each sample was digested with *Dpn*I to remove the unreplicated DNA. The Southern blot was probed with ^{32}P-labeled pUC19 DNA. Positions of the bands specific for pUCLCR-18, pSGE1, and pSGE2 are indicated. The *Dpn*I-sensitive unreplicated DNA is present as several faster-migrating bands. The autoradiogram was exposed for 1 h.

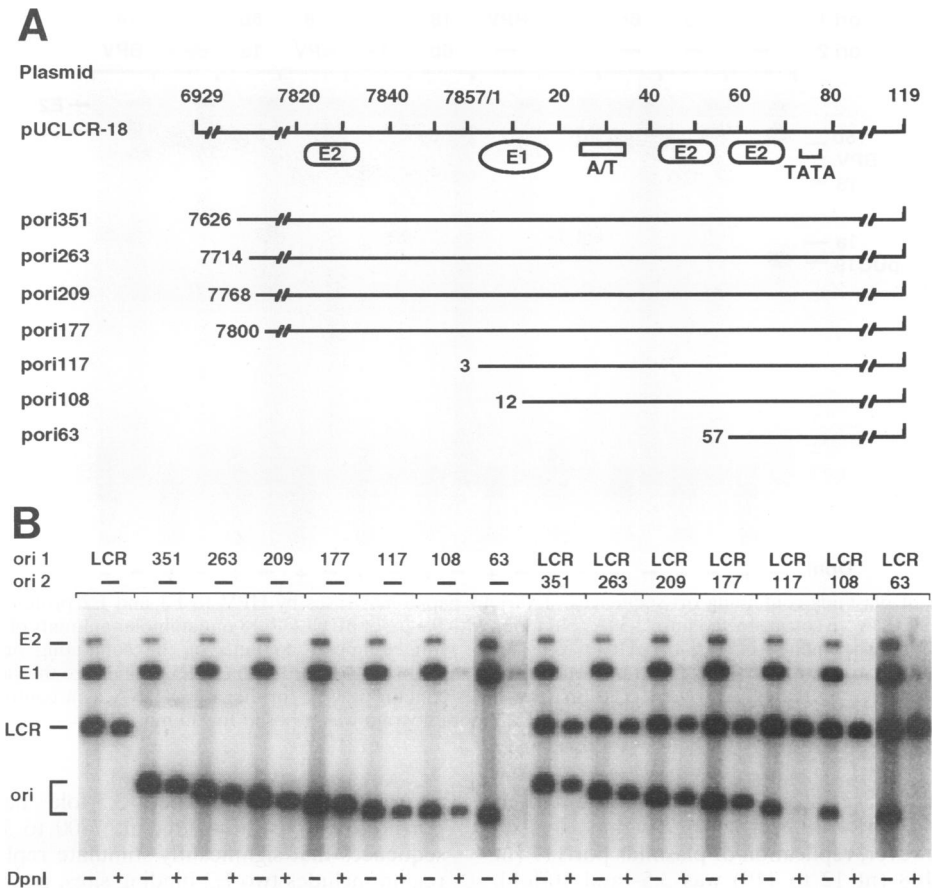

FIG. 3. Localization of the HPV-18 ori. (A) Boundaries of the various ori deletion constructs used in transient replication analyses. Locations of the E2 binding sites, the putative E1 binding site, the TATA box, and the A/T-rich region are indicated. Numbers correspond to the published sequence of HPV-18 (5). (B) Transient replication analyses of the various ori plasmids. One-half microgram of pUCLCR-18 and equimolar amounts of the various ori plasmids were cotransfected into C-33A cells along with 5 μg of pSGE1 and 0.5 μg of pSGE2. In competition experiments, 0.5 μg of pUCLCR-18 along with equimolar amounts of the various ori plasmids were cotransfected. Four days after transfection, low-molecular-weight DNA was isolated and linearized with *Eco*RI. One half of each sample was treated with *Dpn*I, and the samples were analyzed as described in the text. The positions of pUCLCR-18 and other ori-specific bands are indicated. The autoradiogram was exposed for 1.5 h.

series of experiments, 0.5 μg of pUCLCR-18 and 5 μg of pSGE1 were cotransfected along with various amounts of pSGE2. Efficient replication was observed in the presence of 0.2 μg of pSGE2, and maximal replication was obtained in the presence of 0.5 and 1 μg of E2 (Fig. 2). The replication of plasmid pUCLCR-18 was inhibited when 3 and 5 μg of plasmid pSGE2 were used. Densitometric analysis of the ori-specific bands in lanes treated and untreated with *Dpn*I showed that about 55 to 60% of the ori DNA had undergone replication under optimal conditions. These results showed that replication of the ori DNA was highest when a 10:1 or 5:1 ratio of plasmids pSGE1 and pSGE2 was used. In another experiment, no replication of plasmid pUCLCR-18 was observed in HeLa cells in the presence of 5 μg each of plasmids pSGE1 and pSGE2 (data not shown). However, replication of the ori plasmid was observed when lower amounts of pSGE2 were used (not shown). These results suggest that the E2 protein may regulate replication of HPV DNA when present in high levels. However, other possibilities for an indirect role of E2 in the inhibition of replication cannot be ruled out. Recently, the BPV-1 E2 protein has been shown to inhibit the proliferation of several cervical carcinoma cell lines by blocking the progression from the G_1 to the S phase of the cell cycle (13). It is possible that the E2 protein inhibits HPV replication at high levels by affecting cell growth.

Localization of the HPV-18 ori. To localize the HPV-18 ori, the following plasmids were generated (Fig. 3A) by cloning restriction fragments internal to the LCR (after fill-in with the Klenow fragment of DNA polymerase I where necessary) into pUC19 cleaved with *Hin*cII and *Bam*HI: pori63, containing a 63-bp *Ava*II-*Bam*HI fragment (nt 57 to 119); pori108, containing a 108-bp *Mse*I-*Bam*HI fragment (nt 12 to 119); pori117, containing a 117-bp *Ase*I-*Bam*HI fragment (nt 3 to 119); pori177, containing a 177-bp *Alu*I-*Bam*HI fragment (nt 7800 to 7857/1 to 119); pori209, containing a 209-bp *Acc*I-*Bam*HI fragment (nt 7768 to 7857/1 to 119); pori263, containing a 263-bp *Afl*II-*Bam*HI fragment (nt 7714 to 7857/1 to 119); and pori351, containing a 351-bp *Mva*I-*Bam*HI fragment (nt 7626 to 7857/1 to 119). The 3′ end of each of these ori plasmids extended up to the *Bam*HI site located at nt 119 of HPV-18. These plasmids were tested for the ability to replicate in C-33A cells in the presence of the E1- and E2-expressing plasmids (Fig. 3B). Densitometric analysis of the autoradiogram showed that plasmids pori351 (HPV-18 nt 7626 to 7857/1 to 119), pori263 (nt 7714 to 119), pori209 (nt 7768 to 119), and pori177 (nt 7800 to 119) replicated to approximately the same levels

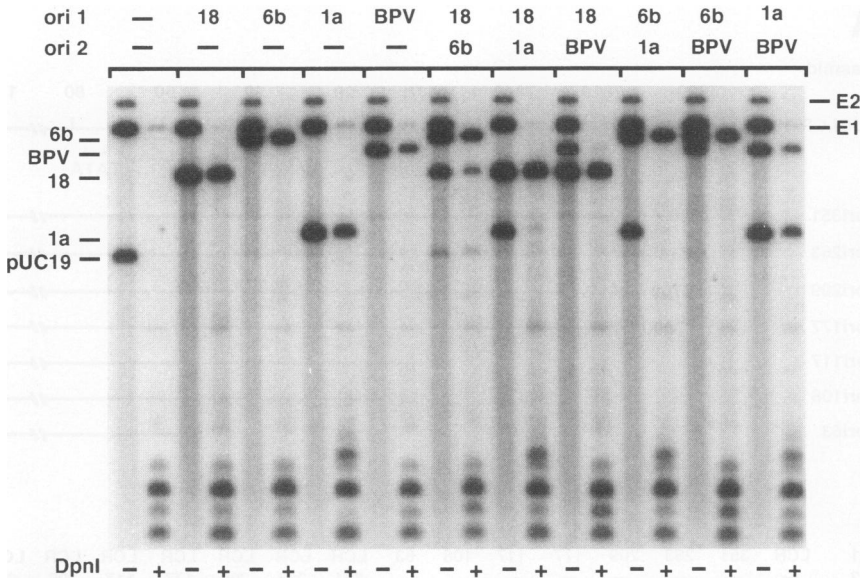

FIG. 4. Replication of plasmids containing ori sequences of various papillomaviruses by HPV-18 E1 and E2 proteins. Five micrograms of pSGE1 and 0.5 μg of pSGE2 were cotransfected into C-33A cells along with 0.5 μg of pUCLCR-18 or equimolar amounts of other LCR-containing plasmids. In competition experiments, 0.5 μg of pUCLCR-18 along with equimolar amounts of the competing ori plasmids was used. Low-molecular-weight DNA was extracted 72 h after transfection. The samples were digested with *Eco*RI to linearize the various DNAs. One half of each sample was subsequently treated with *Dpn*I. The first two lanes contain pUC19 (ori-negative) DNA as a control. The *Dpn*I-sensitive unreplicated DNA is present as several faster-migrating bands. The autoradiogram was exposed for 30 min.

(less than 1.2-fold difference) as pUCLCR-18, indicating that the complete functional HPV-18 ori is located within a 177-bp region (Fig. 3B). However, replication of plasmids pori117 (nt 3 to 119) and pori108 (nt 12 to 119) was 3.5- and 10-fold, respectively, lower than replication of pUCLCR-18 (Fig. 3B). Plasmid pori63 (nt 57 to 119) failed to replicate (Fig. 3B).

To rigorously investigate the efficiency of replication of the various ori plasmids, 0.5 μg of pUCLCR-18 and equimolar amounts of the various ori plasmids were cotransfected into C-33A cells along with pSGE1 and pSGE2. Under these conditions, the test ori plasmids are expected to compete with the control plasmid pUCLCR-18 for various replication proteins. The level of replication of the various ori plasmids compared with that of pUCLCR-18 indicates their relative efficiency of replication. As seen in Fig. 3B, pori351, pori263, pori209, and pori177 replicated as efficiently as pUCLCR-18. However, pori117 replicated very poorly, and pori108 failed to replicate. Taken together, these experiments demonstrate that a 108-bp region (nt 12 to 119) is sufficient for replication, whereas a maximum of 177 bp (nt 7800 to 7857/1 to 119) is required for full ori activity. These results are consistent with those recently reported for HPV-18 showing that the ori is located between positions 7767 and 119 (23). Our results further demonstrate that nt 7767 to 7800 are dispensable for HPV-18 replication. Densitometric analysis indicated that pori117 (nt 3 to 119) replicated approximately threefold better than pori108. pori117 contains an additional 9 nt at its 5′ end compared with pori108. While pori108 contains one-half of the putative E1 binding site centered around the *Hpa*I-like sequence, pori117 includes the complete site (Fig. 3A). These results demonstrate that plasmids containing one-half of the putative E1 binding site can replicate, although the presence of the complete site enhances replication. pori63, which contains a single E2 binding site but lacks other 5′ sequences, did not replicate, confirming that replication requires at least a part of the E1 binding site. Densitometric analysis showed that the plasmid pori177 replicated 3.5-fold better than plasmid pori117, demonstrating that nt 7800 to 7857/1 to 3 contain sequences that significantly stimulate replication. The 117-bp region includes two E2 binding sites, an A/T-rich region, and the putative E1 binding site, whereas the 177-bp region includes these sequences and an additional E2 binding site (Fig. 3A). These results suggest that the presence of an additional E2 site enhances the replication efficiency in a cooperative manner. It is likely that the presence of an additional E2 binding site stimulates replication by significantly increasing the recruitment of the E1 protein to the ori. The requirement of one or more E2 binding sites, an E1 binding site, and an A/T-rich region for HPV-18 replication is similar to those reported for HPV-11 and BPV-1 (3, 23, 29, 30).

HPV-18 E1 and E2 proteins support replication of plasmids containing ori sequences from other papillomaviruses. Recent studies have shown that the E1 and E2 proteins of HPV-11 and BPV-1 can support the replication of plasmids containing heterologous ori sequences (4, 6). To determine the specificity of the HPV-18 E1 and E2 proteins in replication, plasmids containing the LCRs of various papillomaviruses were tested in transient replication assays. Plasmid pUCLCR-6b was generated by ligating the 1,639-bp *Nde*I fragment from HPV-6b (nt 6499 to 7902/1 to 236) into the *Nde*I site of pUC19. Plasmid pUCLCR-1a was generated by inserting a 1,260-bp *Dpn*I fragment of HPV-1a (nt 6781 to 7815/1 to 227) into the *Bam*HI site of pUC19. Plasmid pUCLCR-BPV was obtained by inserting a 1,462-bp *Hin*dIII-*Pst*I fragment of BPV-1 (nt 6958 to 7945/1 to 475) into the corresponding sites of pUC19. These plasmids were cotransfected into C-33A cells along with the pSGE1 and pSGE2 (Fig. 4). The relative levels of replication were determined by measuring the radioactivity in the origin bands in filter strips by liquid scintillation counting. These data showed that while pUCLCR-6b replicated to the same extent as pUCLCR-18, replication of pUCLCR-1a and pUCLCR-

BPV was two- and fourfold, respectively, lower than that of pUCLCR-18 (Fig. 4). Experiments were also carried out to determine the ability of the various ori sequences to compete with the homologous HPV-18 ori. Interestingly, pUCLCR-6b replicated about fourfold better than pUCLCR-18 in competition experiments (Fig. 4). Plasmids pUCLCR-1a and pUCLCR-BPV did not show any detectable replication in the presence of pUCLCR-18 or pUCLCR-6b (Fig. 4). These results were obtained consistently in many different experiments. The ori regions of the various papillomaviruses have significant homology (4). These results demonstrate that the E1 and E2 proteins of HPV-18 can cross-react weakly with the ori sequences of HPV-1a and BPV-1. Since the HPV-18 E1 and E2 proteins replicated a plasmid containing the HPV-6b ori better than the one containing the HPV-18 ori in competition experiments, their interaction with the HPV-6b ori is presumably stronger. Whether the E1 or the E2 protein or both determine the specificity and extent of replication from the papillomavirus oris remains to be determined.

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