JOURNAL OF VIROLOGY, Dec. 1995, p. 7682–7687 Vol. 69, No. 12
0022-538X/95/$04.00+0

Replication Efficiency of Bovine Papillomavirus Type 1 DNA Depends on *cis*-Acting Sequences Distinct from the Replication Origin

VALÉRIE PIERREFITE AND FRANÇOIS CUZIN*

INSERM U273, Université de Nice, 06108 Nice Cedex 2, France

Received 19 April 1995/Accepted 5 September 1995

The viral elements required for the initiation of replication of bovine papillomavirus type 1 DNA include the origin region and two *trans*-acting factors, the E1 and E2 proteins. We now report that the replication efficiency of a DNA molecule which contains these three elements is modulated by other viral sequences. By measuring the extent of replication of deleted viral genomes in transfected mouse cells, we identified sequences required for maximal efficiency. Addition of these sequences to a construct carrying only the minimal origin region increased its replication. Among these *cis*-active elements, we identified a 69-bp fragment (nucleotides 4921 to 4990) which contains at least two binding sites for cellular proteins. One of them is the murine protein termed CDEBP, which recognizes the octameric motif ATCACGTG, identical to the yeast CDEI element. Either deletions affecting this CDEI box or a point mutation which impairs binding of CDEBP markedly decreased the extent of viral DNA replication. They had no detectable effect on viral transcription.

Bovine papillomavirus type 1 (BPV-1) DNA, which replicates as an extrachromosomal multicopy plasmid in transformed rodent cells (8), is one of the few model replicons for the analysis of DNA replication in higher eukaryotes. Transient assays led to the identification of three elements, which together are sufficient for initiating replication in transfected mouse cells: a core origin sequence (*ori*) and two *trans*-acting viral proteins, E1 and E2 (19, 20). The minimal *ori* sequence (nucleotides [nt] 7914 to 27) contains an AT-rich region and binding sites for the E1 and E2 proteins. On the other hand, the large *Hin*dIII-*Bam*HI subgenomic fragment designated 69T, originally defined as the minimum fragment required for transforming activity (10), contains the *ori* region and expresses the E1 and E2 proteins. It was nevertheless not maintained as a stable episome in transformed cells (8, 17) except when selection was applied for a linked resistance gene (13). The region of the viral genome corresponding to the small *Hin*dIII-*Bam*HI fragment, in addition to its known function of coding for the late viral proteins, therefore includes sequences that are required for the stable maintenance of viral episomes.

We investigated whether a *cis* effect of viral sequences outside the *ori* region could be evidenced by measuring the replicative ability of subgenomic fragments transfected in mouse cells. We observed that several regions modulate the replication efficiency of the viral genome and analyzed in more detail a short sequence which contains several binding sites for cellular proteins of unknown functions.

MATERIALS AND METHODS

Plasmids and mutants. Plasmid pV142-6 contains the full-length BPV-1 genome cloned at the unique *Bam*HI site of vector pVV2 (2). pH4 plasmid also contains the complete viral genome but inserted at the *Hin*dIII site of pML2 (1). Plasmid pM69 consists of the 69T BPV-1 DNA fragment cloned in the *Hin*dIII-*Bam*HI fragment of pML2. Plasmids pVd259 and pH4d259 were generated from pV142-6 and pH4, respectively, by excision of the small *Pvu*II-*Pvu*II fragment (nt 4786 to 5045) and recircularization. Plasmid pMB2, used to generate a 3.6-kb fragment containing the *ori*, consists of the complete viral genome cloned at the *Bam*HI site of pML2 (12).

For the construction of plasmids pH4d124 and pH4d55, two BPV-1 DNA fragments were amplified by PCR with the same upstream primer covering the *Bam*HI site (P1; nt 4450 to 4468; 5′-CGGGATCCAGAGCTGTAACAG-3′ [nonviral sequence underlined]). Downstream primers were P2 (nt 4905 to 4921; 5′-CTGCAATACTGCGGGGCGTG-3′) for pH4d124 and P3 (nt 4974 to 4990; 5′-CTGGATCTTCCGTGGGCACC-3′) for pH4d55. Both P2 and P3 oligonucleotides contain a half *Pvu*II site at their 5′ ends (underlined). After *Bam*HI digestion, each fragment was inserted into the large *Bam*HI-*Pvu*II fragment of pH4.

Plasmid pH4/7A was generated to introduce an A-to-T change at position 4935 of the BPV-1 genome in plasmid pH4. Site-directed mutagenesis was achieved by overlap extension using PCR as described previously (7) with primers P4 (5′-GCCTCTAAATCACGAGGCAT-3′; nt 4921 to 4940), P5 (5′-ATGCCTCGT GATTTAGAGGC-3′; nt 4921 to 4940), P6 (5′-GAACATGGTCTACT GGAAGG-3′; nt 4358 to 4377), and P7 (5′-TACCTGACATGTAGCTGTGG -3′; and nt 5134 to 5153).

Plasmid pSKO, a gift of P. Clertant, consists of a 204-bp fragment of BPV-1 DNA (nt 7841 to 100) including the minimal *ori*, cloned at the *Eco*RV site of pBluescript. Plasmid pSKOBX was obtained by ligating the *Bam*HI-*Xba*I BPV-1 fragment (nt 4451 to 6132) to a *Bam*HI-*Xba*I fragment of pSKO. Plasmid pSKOP contains the *Pvu*II-*Pvu*II fragment (nt 4786 to 5045) inserted at the *Sma*I site of pSKO. Plasmids pCGMlu and pCGE2 (20) were used to overexpress the E1 and E2 proteins, respectively, from the immediate-early promoter of cytomegalovirus. Plasmid pCMVβ-gal, expressing *Escherichia coli* β-galactosidase under the control of the immediate-early promoter of cytomegalovirus, is a gift of P. Brulet. Plasmid pIL7, used to control the recovery of low-molecular-weight DNA, contains a cDNA fragment from the rat COII mitochondrial gene (5).

Cells and transfections. C127 mouse cells (11) were maintained in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum (ATGC). Transient replication assays were carried out after electroporation of 1 μg of BPV-1 DNA as described by Ustav and Stenlund (19). In the experiments in which the E1 and E2 expression vectors were cotransfected, 5 μg of each plasmid was used. For each electroporation, plasmid pCMVβ-gal was used to check that the transfection efficiencies were similar for the different viral DNAs.

Transient replication assays. At various times after electroporation, low-molecular-weight DNA was extracted by the alkaline lysis procedure (15) and digested with a restriction enzyme linearizing BPV-1 molecules and with *Dpn*I, which cleaves unreplicated material. Samples were electrophoresed on 0.8% agarose gels and transferred onto a nitrocellulose membrane. Southern blots were hybridized with ^{32}P-labeled BPV-1 and pIL7 DNAs. Quantitation of newly replicated viral DNA was performed by densitometric analysis, using different autoradiographic exposures to take into account different degrees of saturation. The results were normalized relative to the hybridization signal generated by the pIL7 mitochondrial probe.

Northern (RNA) blot analysis. Total RNA was extracted with an RNA-Now kit (Biogentex, Inc.). Northern blot hybridization was performed with a BPV-1 DNA probe according to established procedures (15).

Gel retardation assays. Gel retardation assays were performed with either a 16-mer double-stranded synthetic oligonucleotide including the CDEI motif (underlined) and corresponding to the viral DNA sequences from nt 4925 to

* Corresponding author. Phone: (33) 93 52 99 21. Fax: (33) 93 52 98 96. Electronic mail address: cuzin@unice.fr.

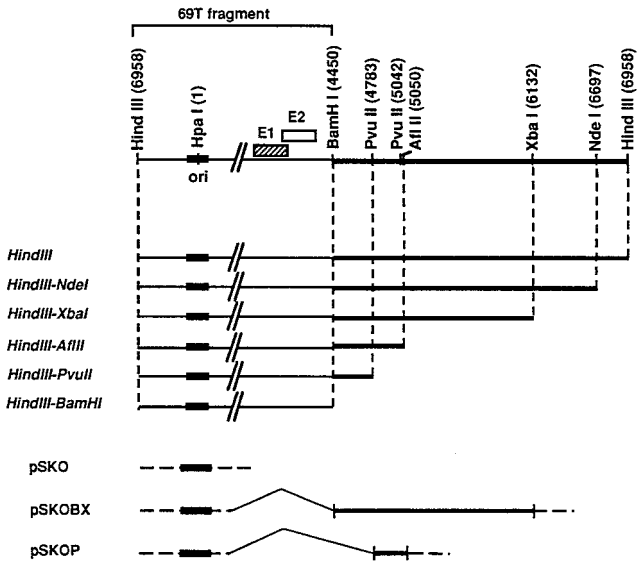

FIG. 1. Genomic and subgenomic BPV-1 DNA molecules analyzed in transient replication assays. The BPV-1 DNA fragments expected to replicate after digestion of plasmid pH4 with the indicated restriction enzymes are represented. All of them contain the *ori* region (closed box). The thick line corresponds to the late coding sequences. Plasmid pSKO contains a 204-bp fragment of BPV-1 DNA (nt 7841 to 100) covering the *ori* region, cloned in a bacterial vector (dashed line). Plasmids pSKOBX and pSKOP plasmids were generated by inserting, respectively, the *Bam*HI-*Xba*I (nt 4451 to 6132) and *Pvu*II-*Pvu*II (nt 4786 to 5045) BPV-1 DNA fragments (thick line) in pSKO.

4940 (DS1; 5′-CTAAATCACGTGGCAT-3′) or a 92-bp BPV1 DNA fragment from nt 4898 to 4990. The latter was generated by PCR amplification using a 20-mer single-stranded oligonucleotide from nt 4898 to 4917 (5′ to 3′) as the upstream primer and the P3 oligonucleotide as the downstream primer. For competition experiments, a double-stranded synthetic oligonucleotide containing a T-to-A point mutation in the CDEI motif (DS2; 5′-GCCTCTAAATCACGaG GCAT-3′) was used. Reactions (30 μl) were performed in 10 mM Tris-HCl (pH 7.5)–40 mM NaCl–1 mM EDTA–1 mM β-mercaptoethanol–4% glycerol in the presence of 5 μg of poly(dI-dC) heteropolymer and 0.5 ng (25,000 cpm) of probe end labeled with ^{32}P. After addition of 5 to 10 μl of total cellular extracts (3), the mixture was incubated for 20 min on ice. For supershift assays, two distinct antibodies recognizing the CDEBP protein were used (4). PS7 was obtained after purification by affinity binding of polyclonal antibodies against the CDEBP protein made in *E. coli*. Antiserum Ab61 was raised against a peptide corresponding to residues 205 to 219 of the CDEBP sequence. Five microliters of either PS7 (0.1 μg/μl) or Ab61 (0.07 μg/μl) was added to the cellular extracts before incubation with the probe. One microliter of the preimmune serum (10 μg/μl) was used as a negative control. Samples were loaded onto a 4% polyacrylamide gel in Tris-EDTA buffer and electrophoresed at 12.5 V/cm at 4°C. Gels were fixed for 15 min in 10% acetic acid–10% ethanol, dried at 80°C, and exposed on X-ray film (Kodak XAR-5).

RESULTS

Viral sequences located outside the 69T fragment are required for efficient replication of BPV-1 DNA in transient assays. We assayed the replicative ability of genomic and subgenomic BPV-1 molecules by analyzing the *Dpn*I-resistant material obtained after electroporation of viral DNA in mouse C127 cells essentially as described by Ustav and Stenlund (19). Each time point corresponds to the content of one plate, the total number of cells increasing with time. Efficiencies of transfection, monitored by the cotransfer of an indicator plasmid (pCMVβ-gal), were comparable in all of these experiments. Hybridization with a probe (pIL7) for a mitochondrial gene was used to monitor the recovery of low-molecular-weight DNA. Figure 1 shows the different fragments of BPV-1 DNA tested. All of them were obtained by enzymatic digestion of plasmid pH4. Each fragment contains the *ori* and the promoter region as well as the open reading frames encoding the E1 and E2 proteins. Figure 2 shows an example of a replication assay for these subgenomic viral DNAs. While the *Hin*dIII-*Nde*I (nt 6958 to 6697) and *Hin*dIII-*Xba*I (nt 6958 to 6132) fragments replicated essentially like the wild-type (WT) BPV-1 genome, both the *Hin*dIII-*Afl*II (nt 6958 to 5050) and *Hin*dIII-*Pvu*II (nt 6958 to 4783) fragments showed at 144 h after electroporation a much (more than 95%) reduced efficiency. In addition to the *Hin*dIII-*Afl*II, *Hin*dIII-*Pvu*II, and *Hin*dIII-*Bam*HI subgenomic BPV-1 DNAs, we could also detect in most experiments a faint signal at the position of full-length BPV-1 DNA, resulting from an incomplete digestion of plasmid pH4. In the case of the 69T fragment (nt 6958 to 4450), newly synthesized viral DNA was nearly undetectable. These results indicate that DNA sequences in the late region (between nt 6132 and 4450) are required for the efficient transient replication of a BPV-1 DNA fragment including the minimal replicon (*ori*, E1, E2, and promoter region).

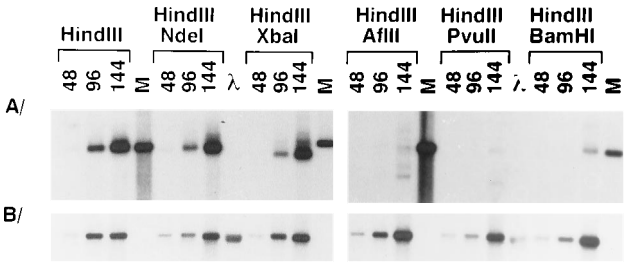

FIG. 2. The *Bam*HI-*Xba*I fragment (nt 4450 to 6132) is required for efficient transient replication of BPV-1 DNA. C127 cells were electroporated with restriction fragment mixtures from various digests of plasmid pH4. Transfection efficiencies, determined after cotransfection with plasmid pCMVβ-gal were quite similar with the different DNAs. At the indicated time point (48, 96, or 144 h), low-molecular-weight DNA was isolated and digested with *Dpn*I and *Eco*RI. Samples were analyzed by Southern blotting and hybridization with a BPV-1 DNA probe (A) and the pIL7 mitochondrial DNA probe (B). Lanes λ, molecular weight DNA marker (cross-hybridization with the pIL7 probe); lanes M, linear viral DNA.

In these transient replication assays, the E1 and E2 proteins were expressed from the transfected BPV-1 molecule. We therefore examined whether the inefficient replication of subgenomic BPV-1 DNA fragments could result from a poor expression of these two viral proteins.

The same fragments were cotransfected with expression vectors providing in *trans* the E1 and E2 proteins. As a control, a 3.6-kb fragment of BPV-1 that contains the *ori* region was added to the transfection mixtures. This fragment (nt 4780 to 471), generated by *Pst*I digestion of plasmid pMB2, replicates efficiently provided that the E1 and E2 proteins are supplied (20). Results are presented in Fig. 3. An unexpected but reproducible observation was that the *Pst*I-*Pst*I fragment replicated to a lesser extent when transfected alone or in combination with the 69T fragment than in the other cotransfections. It was, however, always replicated, indicating that the expression of E1 and E2 was not rate limiting. Deletions of the BPV-1 sequences from nt 6958 (*Hin*dIII) to 6132 (*Xba*I) had little effect on viral replication, while further extension to nt 5050 (*Afl*II) or 4783 (*Pvu*II) induced 96 and 99% reductions, respectively, in the amount of *Dpn*I-resistant fragments. Again, the replication of 69T was nearly undetectable except at the latest time points. An additional internal control was provided in some experiments by the small amounts of residual full-length viral DNA. Although not detectable prior to transfection (lanes M in Fig. 3), it replicated more efficiently than the subgenomic fragments (*Hin*dIII-*Afl*III digest). Expression of

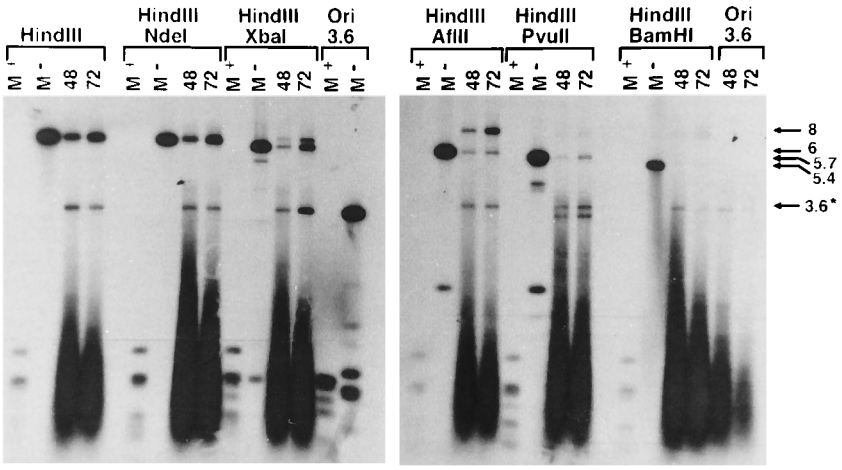

FIG. 3. Transient replication assay of subgenomic fragments in the presence of E1 and E2 expression vectors. These fragments were obtained by digestion of plasmid pH4 with various restriction enzymes except for the *Hin*dIII-*Bam*HI digest, in which case plasmid pM69 was used to generate the 69T fragment. BPV-1 DNAs were electroporated in C127 cells with *Pst*I-digested pMB2 DNA and the expression vectors encoding the E1 and E2 proteins. At the indicated time point (48 or 72 h), low-molecular-weight DNA was isolated and digested with *Dpn*I and *Hpa*I. The Southern blot was hybridized with a BPV-1 DNA probe. Lanes M^-, restriction fragments used for transfection; lanes M^+, same fragments after digestion with *Dpn*I endonuclease. Arrows indicate the sizes (in kilobases) of the different replicated BPV-1 DNAs. The 3.4-kb fragment seen in the *Hin*dIII-*Pvu*II lanes is generated by digestion of the 3.6-kb fragment, as a result of incomplete inactivation of the *Pvu*II endonuclease.

E1 and E2 proteins does not therefore appear to be a rate-limiting factor in these experiments. Northern hybridization data reported below (see Fig. 7) further support this conclusion for the sequences between nt 4786 and 5045. On the other hand, as previously shown by others (20) and confirmed by experiments described below, inefficient recircularization of restriction fragments whose ends are not compatible does not appear to be a likely explanation. We therefore conclude that sequences within the *Bam*HI-*Xba*I region affect the replication of BPV-1 DNA.

Addition of sequences from the late region increases the replication efficiency of a bacterial vector carrying only the *ori* region. Deletion of the sequences between the *Bam*HI and *Xba*I sites decrease the replication efficiency of the viral genome. To determine whether these sequences were sufficient to exert a helper effect of replication, we tested constructs in which they had been added to a plasmid carrying only a short viral sequence covering the replication origin (Fig. 1). Plasmid pSKO contains the fragment of BPV-1 from nt 7841 to 100. As expected, it replicates when cotransfected with the E1 and E2 expression vectors but to a limited extent comparable with that previously reported for similar *ori*-only constructs (19). Experiments were then performed with two other plasmids, pSKOBX and pSKOP, carrying an added viral fragment with either a large part of the late coding region (pSKOBX) or only the short *Pvu*II-*Pvu*II fragment (pSKOP; see Materials and Methods). Equimolecular amounts of the three plasmids (10, 10.5, and 15 μg for pSKO, pSKOP, and pSKOBX, respectively) were transfected either in parallel or together. Results shown in Fig. 4 show a clear enhancing effect of the added sequences on the replication of the constructs, measured 72 h after electroporation.

The *Pvu*II-*Pvu*II region of BPV-1 DNA (nt 4786 to 5045) contains elements required for a maximal replication efficiency. The two types of experiments reported above point to a role of sequences in the *Bam*HI-*Xba*I region, with a marked effect of the short *Pvu*II-*Pvu*II region (nt 4786 to 5045). We further investigated a possible role of this 259-bp sequence by first generating a deletion mutant (d259). Figure 5 shows the level of replication over a 5-day period of the d259 and WT genomes in C127 cells. Deletion of the *Pvu*II-*Pvu*II fragment led to a 75 to 90% reduction of the replication efficiency. Two smaller deletions were then generated within this region and assayed for replication. The d124 and d55 viral genomes lack 124 bp (nt 4921 to 5045) and 55 bp (nt 4990 to 5045, respectively), of the *Pvu*II-*Pvu*II region. Figure 6 shows that at 120 h after electroporation, the d124 mutant genome replicates as poorly as the d259 BPV-1 DNA (in the experiment shown, a decrease of 80% compared with WT DNA). In contrast, the d55 viral genome replicates better than the two other deletion mutants, its replication efficiency being only slightly less than that of the complete genome.

Altogether, these results suggest that critical elements for maximal replication efficiency are localized between nt 4921 and 5045. Furthermore, the efficient replication of the d55 viral DNA suggests a role of the 69-bp sequence between nt 4921 and 4990, which is absent in d124 and present in d55.

The *Pvu*II-*Pvu*II sequence is not required for maximal expression of the early region. As indicated above, the decreased replication efficiency of the viral subgenomic fragments was not changed upon cotransfer of constructs expressing the early proteins E1 and E2. We concluded that it was unlikely that the

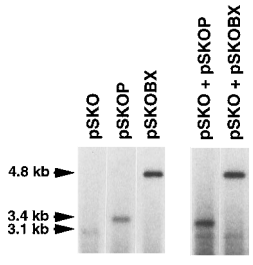

FIG. 4. Fragments of the late region increase the replication efficiency of a construct with only a minimal *ori* region. Plasmids pSKOP (3.4 kb) and pSKOBX (4.8 kb) were transfected in C127 cells along with the E1 and E2 expression vectors, either alone or with pSKO (3.1 kb); 72 h later, the low-molecular-weight DNA was extracted and digested with *Hpa*I and *Dpn*I, and the Southern blot was hybridized with a BPV-1 probe.

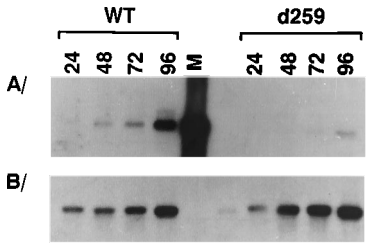

FIG. 5. Inefficient replication of the d259 BPV-1 DNA. Plasmids pV142-6 and pVd259 were digested with *Bam*HI, and the viral sequences were purified. After electroporation of C127 cells, low-molecular-weight DNA was extracted at the indicated time points (24, 48, 72, and 96 h). Samples were digested with *Eco*RI and *Dpn*I and analyzed by Southern blotting with a BPV-1 DNA probe (A) and a mitochondrial DNA probe (B). Lane M, linearized BPV-1 DNA.

observed effect could result from a *cis* effect leading to a reduced rate of expression of the early viral genes that could secondarily affect replication. To confirm this point, we analyzed comparatively the viral RNAs synthesized after transfection of either the WT or the d259 BPV-1 DNA. As shown in Fig. 7, neither quantitative nor qualitative differences in viral transcription could be detected.

The 69-bp sequence defined by deletion analysis contains at least two binding sites for cellular factors, one of which is the CDEBP protein. To further clarify the role of the region defined as critical by deletion analysis, we first examined if it contains binding sites for cellular proteins. A 92-bp fragment of BPV-1 DNA (nt 4898 to 4990) generated by PCR amplification was used as a probe for gel retardation assays. As shown in Fig. 8, two retarded complexes are observed with C127 total cellular extracts. The addition of a 50- to 100-fold excess of the unlabeled 92-bp fragment significantly reduced complex formation, while both complexes were unaffected by a 1,000-fold excess of nonspecific DNA competitor. Examination of the nucleotide sequence within the 69-bp region revealed the presence of the octameric motif ATCACGTG, identical to the yeast centromeric element CDEI. A sequence which also fits the CDEI consensus was previously observed to bind a nuclear

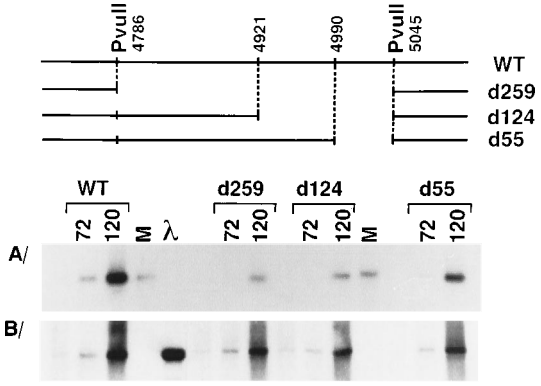

FIG. 6. Transient replication analysis of deletion mutants in the *Pvu*II-*Pvu*II region. At the top are indicated the deletions that were generated in plasmid pH4. pH4d259 lacks the complete *Pvu*II-*Pvu*II fragment. Plasmids pH4d124 and pH4d55 have deletions of 124 and 55 bp, respectively. Prior to electroporation, the different plasmids were digested with *Hin*dIII to separate the bacterial and the viral sequences. At the indicated time points (72 and 120 h), low-molecular-weight DNA was extracted and digested with *Eco*RI and *Dpn*I. Samples were analyzed by Southern blotting with a BPV-1 DNA probe (A) and a mitochondrial DNA probe (B). Lane λ, molecular weight DNA marker (cross-hybridization with the pIL7 probe); lane M, linearized BPV-1 DNA.

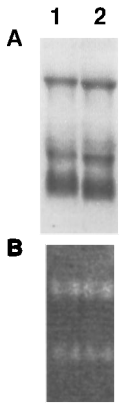

FIG. 7. Viral transcripts in d259- and WT BPV-1-transfected cells. C127 cells were transfected with either the WT (lane 1) or d259 (lane 2) viral genome. Total RNA was analyzed by Northern hybridization with a BPV-1 DNA probe (A). The amount of total RNA loaded in each lane is shown after ethidium bromide staining of the gel before transfer (B).

protein, CDEBP, subsequently characterized by cDNA cloning and immunochemical analysis (3, 4, 6). The protein, which belongs to a family defined by sequence similarities with the Alzheimer precursor protein, has been subsequently described by others under the name APLP2 (16). The 16-bp double-stranded oligonucleotide DS1 encompassing this motif efficiently competed with the formation of the faster-migrating complex (Fig. 8). Accordingly, using DS1 directly as a probe in the gel retardation assay, we observed the formation of at least one retarded complex (Fig. 9A). To determine if the complex includes the CDEBP protein, we performed the same experiment after preincubation of cellular extracts with the anti-CDEBP antibodies PS7 and Ab61 (4). They both induced a noticeable supershift of the retarded complex (Fig. 9B). The 69-bp region required for efficient replication thus contains several binding sites for cellular factors, one of which is the CDEBP protein.

A point mutation impairing protein binding to the CDEI motif decreases transient replication efficiency of BPV-1 DNA. To assess the function of the CDEBP-CDEI interaction in BPV-1 replication, we introduced a point mutation previously

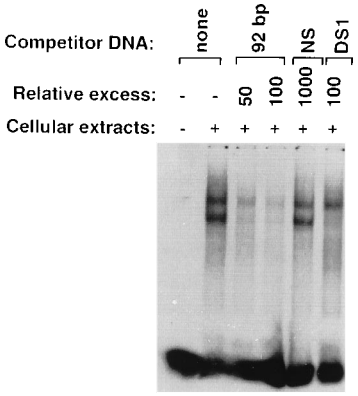

FIG. 8. Viral sequences required for efficient replication contain several binding sites for cellular proteins. The probe is a 92-bp BPV-1 DNA fragment (nt 4898 to 4990). Two retarded complexes are visible in the presence of C127 total cellular extracts. At the top are indicated the relative excesses of the different unlabeled competitors. The double-stranded oligonucleotide DS1 contains the CDEI motif (nt 4925 to 4940). NS, nonspecific competitor.

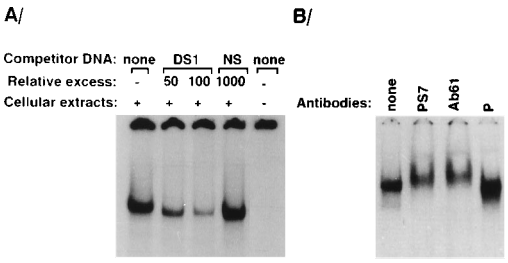

FIG. 9. The CDEI motif of BPV-1 binds the CDEBP protein. The probe is the DS1 oligonucleotide, containing the CDEI box. (A) A retarded complex is observed in the presence of C127 cellular extracts. The relative excesses of the different unlabeled competitor DNAs are indicated at the top. DS1, unlabeled DS1 oligonucleotide; NS, nonspecific competitor (pBR322) DNA. (B) The protein-DNA complex is supershifted in the presence of anti-CDEBP antibodies. P, preimmune serum. In these experiments, electrophoresis was performed for longer times to improve the resolution of the various complexes; and so the free probe was excluded from the gel.

observed to affect CDEBP binding to the CDEI element (3). We first checked the effect on protein binding of the mutation, which replaces the octamer ATCACGTG with ATCACGAG. The oligonucleotide DS2, which contains the mutated CDEI site, was tested as a competitor in a gel retardation assay with a DS1 probe (WT CDEI). As expected, the mutation resulted in a marked decrease in binding of CDEBP (Fig. 10a). Its effect on the replication of an otherwise intact BPV-1 genome is shown in Fig. 10b. At 168 h after electroporation, the amounts of newly replicated 7A and d259 DNAs were decreased by 65 and 75%, respectively, compared with WT DNA. This result suggests that the CDEI motif may be, via its interaction with the CDEBP protein, a critical element for the efficient replication of viral DNA.

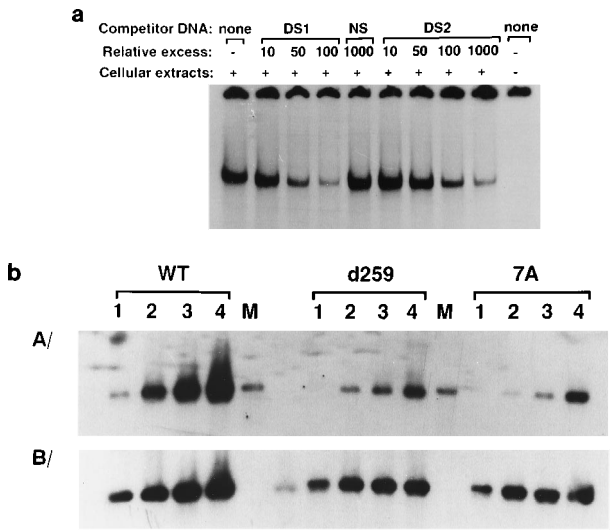

FIG. 10. A point mutation in the CDEI element decreases both binding of the CDEBP protein and the replication efficiency of BPV-1 DNA. (a) Gel retardation assay, using as a probe radioactively labeled oligonucleotide DS1 (BPV-1 nt 4925 to 4940, encompassing the CDEI box). Increasing concentrations of either unlabeled DS1 or DS2 oligonucleotide were added as indicated. NS, nonspecific competitor (pBR322) DNA. (b) C127 cells were electroporated with purified WT, d259, or 7A viral genome. At days 2 (lane 1), 5 (lane 2), 6 (lane 3), and 7 (lane 4) after electroporation, low-molecular-weight DNA was extracted and digested with *Eco*RI and *Dpn*I restriction enzymes. The Southern blot was hybridized with labeled BPV-1 (A) and mitochondrial DNA probes (B). Lanes M, linearized BPV-1 DNA.

DISCUSSION

In addition to the *ori* region and the open reading frame encoding the E1 and E2 proteins, viral sequences within the late region appear to be necessary for sustained replication of BPV-1 DNA, both in the context of the complete viral genome and in constructs with only a minimal *ori*. Although it is not the only sequence element that plays a role in modulating replication efficiency, one critical region appears to extend between nt 4921 and 4990. This short sequence exerts a *cis* effect that cannot be explained by an enhancing effect on the transcription of the early genes, an explanation that was unlikely in any case considering that the transcriptional control elements of BPV-1 have been the subject of exhaustive studies (see reference 18 for review).

Among the numerous questions that remain to be solved concerning the role of these sequences in BPV-1 replication, one could ask whether all of the cells replicate the deleted viral DNA at a lower rate than the complete viral genome, the alternative possibility being that only a fraction of the cells replicate the plasmid at a WT level. In situ hybridization analysis may answer this question and may provide important information as to the mechanism of the observed effects. These effects could result either from variations in the rate of replication or from the irregular segregation of the replicated copies. Since these transient replication assays extend over several cell generations, a defect in the distribution of copies between sister cells could result in a decrease in the final number of replicated molecules. Another relevant question is whether the reduced replication efficiency measured in short-term assays is paralleled by a corresponding effect on the maintenance of the mutated genomes in stably transformed clones. Studies on established cell lines demonstrating a strong effect of the d259 deletion under a specific set of conditions will be described in a separate report (13a).

One of the most immediate benefits of these studies could be to provide a way to identify nuclear proteins involved in the control of replication and in the maintenance of the genomes. The 69-bp fragment contains several binding sites for cellular proteins, and one has been already identified. Although its exact function at the molecular level remains to be determined, several lines of evidence suggest that the CDEBP-CDEI interaction is involved in the stabilization of genetic material. The protein is expressed in every cell type and species that has been tested (4, 16). It has been found to be essential during the early development of the mouse embryo, as demonstrated by the toxic effects of microinjected DNA fragments carrying the CDEI motif (3) and of anti-CDEBP antisense oligonucleotides added to the culture medium (4). In both cases, development was arrested before the blastocyst stage, with nuclei showing highly abnormal DNA contents. Furthermore, a fragment of mouse DNA designated p12B1, which was found hereditarily maintained as an autonomous genetic element in transgenic mouse families, contains a CDEI site, the integrity of which is required for establishment of the episome (9, 14).

Although the present study was focused on the region extending from nt 4921 to 4990, other sequences appear to play a role in modulating the extent of replication of subgenomic DNA fragments. The *Afl*II-*Xba*I DNA fragment (nt 5050 to 6132) seems to contain positively *cis*-acting sequences, as demonstrated by the reduced ability of the *Hin*dIII-*Afl*II fragment to replicate in transient experiments. It is also of interest that although the *Hin*dIII-*Afl*II and *Hin*dIII-*Pvu*II subgenomic fragments replicate poorly compared with the complete genome, the 3.6-kb control fragment replicates efficiently. This observation suggests that the BPV-1 subgenomic molecules

may contain negative control elements which are absent from the *ori*-containing control fragment. When these elements are present in a BPV-1 DNA fragment containing the minimal origin, the late region appears to provide a positive, perhaps compensatory, function for efficient replication of viral DNA. An additional degree of complexity is indicated by the unexpected but reproducible observation that the 3.6-kb *Pst*I-*Pst*I fragment replicates better when it is cotransfected with a complete viral genome than when it is either transfected alone or cotransfected with the 69T fragment (Fig. 3). This effect may result from complex competitive interferences. This possibility was not investigated further in the present series of experiments, in which the replication of the 3.6-kb fragment was used only as a control for the availability of E1 and E2 in sufficient amounts. Taken together, these observations indicate that the minimal sequences required for transient episomal replication (E1, E2, and *ori*) are part of a more complex system involving a balanced interplay between negative and positive *cis*-acting control elements.

ACKNOWLEDGMENTS

We are indebted to K. Marcu for critical reading of the manuscript, to P. Brulet for the gift of pCMVβgal, to P. Clertant for the gift of pSKO, and to A. Stenlund for the E1 and E2 expression plasmids and for helpful advice concerning the transient replication assay. The expert technical assistance of P. Abbe, M. Cutajar, and C. Minghelli is gratefully acknowledged.

This work was made possible by grants from Association pour la Recherche sur le Cancer.

REFERENCES

1. **Binétruy, B., G. Meneguzzi, R. Breathnach, and F. Cuzin.** 1982. Recombinant DNA molecules comprising bovine papilloma virus type 1 DNA linked to plasmid DNA are maintained in a plasmidial state both in rodent fibroblasts and in bacterial cells. EMBO J. **1:**621–628.
2. **Binétruy, B., J. Schiller, D. Lowy, C. Cerni, and F. Cuzin.** 1990. Nonselective analysis of the transformation of FR3T3 rat cells by bovine papillomavirus type 1: regulations of viral transcription associated with phenotypic transformation. Oncogene **5:**1645–1651.
3. **Blangy, A., P. Léopold, F. Vidal, M. Rassoulzadegan, and F. Cuzin.** 1991. Recognition of the CDEI motif GTCACATG by mouse nuclear proteins and interference with the early development of the mouse embryo. Nucleic Acids Res. **19:**7243–7250.
4. **Blangy, A., F. Vidal, M. Rassoulzadegan, and F. Cuzin.** 1995. CDEBP, a site-specific DNA-binding protein of the "APP-like" family, is required during the early development of the mouse. J. Cell Sci. **108:**675–683.
5. **Glaichenhaus, N., and F. Cuzin.** 1987. A role for ID repetitive sequence in growth- and transformation-dependent regulation of gene expression in rats fibroblasts. Cell **50:**1081–1090.
6. **Hanes, J., H. von der Kammer, G. I. Kristjansson, and K. H. Scheit.** 1993. The complete cDNA coding sequence for the mouse CDEI binding protein. Biochim. Biophys. Acta **1216:**154–156.
7. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
8. **Law, M. F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papilloma virus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA **78:**2727–2731.
9. **Léopold, P., J. Vailly, F. Cuzin, and M. Rassoulzadegan.** 1987. Germ line maintenance of plasmids in transgenic mice. Cell **51:**885–886.
10. **Lowy, D. R., I. Dvoretsky, R. Shobert, M. F. Law, L. Engel, and P. M. Howley.** 1980. In vitro tumorigenic transformation by a defined sub-genomic fragment of BPV DNA. Nature (London) **287:**72–74.
11. **Lowy, D. R., E. Rands, and E. M. Scolnick.** 1978. Herpes-independent transformation by unintegrated Harvey sarcoma virus DNA. J. Virol. **26:** 291–298.
12. **Lusky, M., and M. R. Botchan.** 1985. Genetic analysis of bovine papillomavirus type 1 *trans*-acting replication factors. J. Virol. **53:**955–965.
13. **Meneguzzi, G., B. Binétruy, M. Grisoni, and F. Cuzin.** 1984. Plasmidial maintenance in rodent fibroblasts of a BPV1-pBR322 shuttle vector without immediately apparent oncogenic transformation of the recipient cells. EMBO J. **3:**365–371.

13a. **Pierrefite, V., B. Binétruy, M. Rassoulzadegan, and F. Cuzin.** Submitted for publication.

14. **Rassoulzadegan, M., P. Léopold, J. Vailly, and F. Cuzin.** 1986. Germ line transmission of autonomous genetic elements in transgenic mouse strains. Cell **46:**513–519.
15. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Sandbrink, R., C. L. Masters, and K. Beyreuther.** 1994. Complete nucleotide and deduced amino acid sequence of rat amyloid protein precursor-like protein 2 (APLP2/APPH): two amino acids length difference to human and murine homologues. Biochim. Biophys. Acta **1219:**167–170.
17. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of a bovine papillomavirus/pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA **79:**7147–7151.
18. **Sousa, R., N. Dostatni, and M. Yaniv.** 1990. Control of papillomavirus gene expression. Biochim. Biophys. Acta **1032:**19–37.
19. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10:**449–457.
20. **Ustav, M., E. Ustav, P. Szymanski, and A. Stenlund.** 1991. Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. EMBO J. **10:**4321–4329.