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Control of Human Papillomavirus Type 11 Origin of Replication by the E2 Family of Transcription Regulatory Proteins

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Replication of human papillomavirus type 11 (HPV-11) DNA requires the full-length viral E1 and E2 proteins (C.-M. Chiang, M. Ustav, A. Stenlund, T. F. Ho, T. R. Broker, and L. T. Chow, Proc. Natl. Acad. Sci. USA 89:5799–5803, 1992). Using transient transfection of subgenomic HPV DNA into hamster CHO and human 293 cells, we have localized an origin of replication (*ori*) to an 80-bp segment in the upstream regulatory region spanning nucleotide 1. It overlaps the E6 promoter region and contains a short A+T-rich segment and a sequence which is homologous to the binding site of the bovine papillomavirus type 1 (BPV-1) E1 protein in the BPV-1 *ori*. However, unlike the BPV-1 *ori*, for which half an E2-responsive sequence (E2-RS) or binding site suffices, an intact binding site is essential for the HPV-11 *ori*. Replication was more efficient when additional E2-RSs were present. The intact HPV-11 genome also replicated in both cell lines when supplied with E1 and E2 proteins. Expression vectors of transcription repressor proteins that lack the N-terminal domain essential for E2 transcriptional *trans* activation did not support replication in collaboration with the E1 expression vector. Rather, cotransfection with the repressor expression vectors inhibited *ori* replication by the E1 and E2 proteins. These results demonstrate the importance of the N-terminal domain of the E2 protein in DNA replication and indicate that the family of E2 proteins positively and negatively regulates both viral DNA replication and E6 promoter transcription.

Initiation of viral DNA replication involves multiple *cis*-control sequences and *trans*-acting factors. The most important *cis* element is the origin of replication (*ori*), which consists of a core region and an auxiliary component (for reviews, see references 15 and 49). The core region specifies the initiation site and is required under all conditions. The auxiliary sequences encompass enhancer elements and determine the efficiency of replication but are dispensable under certain conditions. *trans*-acting factors essential for initiation of viral DNA replication include *ori*-specific viral proteins and the general host replication machinery (for reviews, see references 5 and 44). For simian virus 40 and polyomavirus, the large T antigen is the only viral protein required (16). For bovine papillomavirus type 1 (BPV-1) and human papillomaviruses (HPVs), the full-length products of the viral E1 and E2 genes are mandatory both in vivo and in a cell-free replication system (9, 50, 54; also see below).

The full-length BPV-1 E1 protein is a 68-kDa phosphoprotein and contains an ATP-binding domain near its carboxyl terminus (4, 39, 46). It associates specifically with the BPV-1 *ori*, which spans nucleotide position 1 (52–54). The HPV type 11 (HPV-11) full-length E1 protein has a predicted molecular mass of 73.5 kDa but migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as an 82-kDa protein (9). The full-length E2 proteins of animal papillomaviruses and HPVs are also phosphoproteins of approximately 43 to 49 kDa (2, 22, 31). Depending on the promoter or enhancer sequence context, the E2 protein either activates or represses the homologous viral promoter (10, 36, 43, 48) by binding as a dimer to a 12-bp palindrome, $ACCN_6GGT$ or a variation, $ACCN_6GTT$, called the E2-responsive sequence (E2-RS) or E2-binding site (1, 21, 29; for reviews, see references 20, 32, and 42). Multiple copies of the E2-RS are located in the viral upstream regulatory region (URR) or long control region of all papillomaviruses.

The BPV-1 E2 open reading frame encodes two additional proteins that have a truncated or alternative amino terminus (E2-tr and E8/E2) but the same carboxyl terminus, which serves as the protein dimerization and DNA-binding domain (12, 22, 25, 27). The HPV-11 E2 open reading frame encodes three proteins as well, a full-length E2 protein of 43 kDa (9), an E2-C protein (38), which is equivalent to E8/E2, and a 42-kDa E1M^E2C protein, which contains 147 amino acids derived from the amino terminus of the E1 protein fused to the DNA-binding domain of the E2 protein (7, 8). All E2 proteins devoid of the *trans*-activating domain at the amino terminus of the full-length E2 protein function as binding-site-specific transcription repressors (7, 11, 12, 14, 27, 48), and the transcription repressor protein of BPV-1 is unable to support replication (50, 54). A mutation in the initiation codon of the BPV-1 E2-tr increases the transformation efficiency and extrachromosomal copy number of viral DNA (26, 35). However, mutations eliminating both E2-tr and E8/E2 result in opposite phenotypes (26). Conversely, autonomous replication of BPV-1 DNA is inhibited in cell lines transduced with an expression vector of the HPV-11 E1M^E2C cDNA (7). Clearly the roles of the family of E2 proteins on viral transcription and episomal replication are complex and remain to be delineated.

The BPV-1 minimal *ori* has been mapped recently to a 60-bp DNA fragment which is highly homologous among different animal papillomaviruses and HPVs (52, 54). There are three elements in the BPV-1 *ori*: an E1-binding site spanning nucleotide position 1, an A+T-rich region, and, unexpectedly, only half of the palindromic E2-RS, despite an absolute requirement for the full-length E2 protein in transfected cells (52). In a cell-free replication system, the BPV-1

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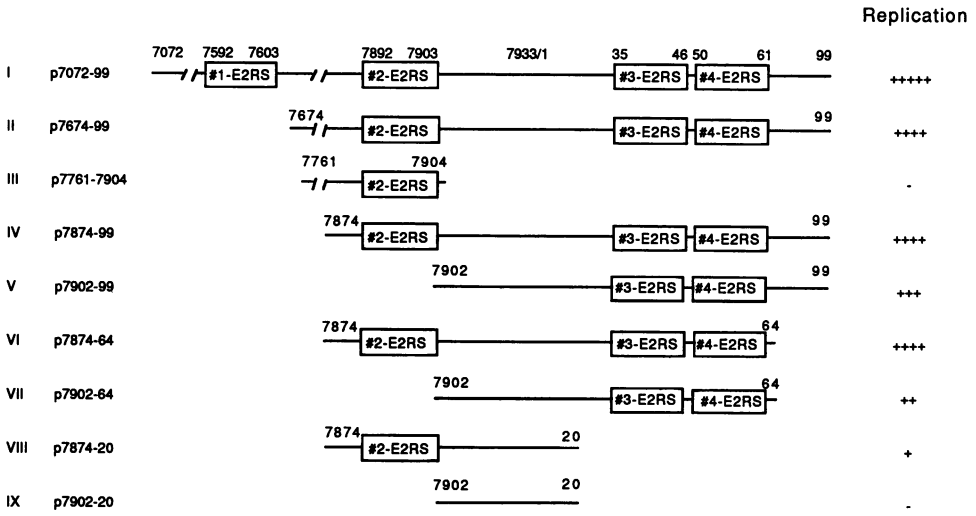

FIG. 1. HPV-11 URR and deletion subclones. Boxes represent the E2-RSs, with nucleotide numbers indicating the boundaries of the E2-RS and HPV-11 DNA subgenomic sequences. The number of plus signs reflects qualitative estimations of *ori* strength on the basis of replicating signal intensity; the minus sign denotes negative results even after long exposures of autoradiograms.

E1 protein expressed from a baculovirus vector alone can initiate replication but the efficiency is greatly increased when purified full-length E2 protein is added (54).

We have recently shown that homologous regions of the URR of six distinct HPV types and of Shope cottontail rabbit papillomavirus contain an origin of replication. Transient replication of these URR-containing plasmids and of the BPV-1 *ori* in many mammalian cell lines was supported by full-length E1 and E2 proteins encoded by either HPV-11 or BPV-1 (9). In this report, we localized an HPV-11 *ori* to an 80-bp DNA segment by using deletion and site-directed mutations and transient-replication assays in transfected cells. We demonstrate a significant difference from the BPV-1 *ori*. Moreover, the E2-RS-specific viral transcription repressor proteins E2-C and E1M^E2C have the ability to suppress *ori* function.

MATERIALS AND METHODS

Plasmid constructions. Plasmid p7674-99 (with numbers referring to nucleotide positions of the subgenomic fragment boundaries) was constructed by transferring the HPV-11 URR fragment (nucleotides [nt] 7674–7933/1–99) flanked by *Hin*dIII sites from 24-N (10) into the *Bam*HI site of pML2d (40) after blunt-end ligation. p7874-99 was generated by digesting pUR23-3 (11) with *Bsu*36I and *Hin*dIII. The URR fragment was then cloned into the *Bam*HI site of pML2d by blunt-end ligation. p7874-64 and p7874-20 were created by cleaving pUR27 and pUR26 (21), respectively, with *Bsu*36I and *Bam*HI; the fragments were then similarly cloned into pML2d. p7902-99, p7902-64, and p7902-20 were cloned as just described except that *Bst*EII was used instead of *Bsu*36I to generate the 5′ endpoint. p7761-7904 and pSV*ori* were prepared by transferring the URR fragment (nt 7761 to 7904) and simian virus 40 *ori* sequence, separately, each flanked by *Bam*HI and *Hin*dIII sites, from 8-N (21) to the *Bam*HI site of pML2d by blunt-end ligation. p7072-99 containing the HPV-11 URR fragment spanning nt 7072–7933/1–99 in pML2d has been described previously (9). The orientations of each insertion were identified by DNA sequencing. Except for p7902-99, p7902-64, and p7902-20, the URR inserts in all the plasmids were in the sense orientation relative to the interrupted and truncated Tet^r gene between the *Cla*I and *Sal*I sites of the pML2d vector, i.e., *Cla*I–5′ URR insert 3′–*Sal*I. The HPV-11 plasmids are shown in Fig. 1.

pMT2-E1 and pMT2-E2, which express the HPV-11 E1 and E2 proteins, respectively, have been described previously (9). pMT2-E2C expressing the HPV-11 E2-C protein from a modified cDNA pRSE2-C(3-11) (11) was constructed as follows. The *Bam*HI-*Pst*I fragment spanning HPV-11 cDNA nt 812–847^3325–3900 preceded by an ATG in the linker in the E2 reading frame was isolated and cloned into pcDNA1 (Invitrogen Corp.) to generate pCMV-E2C. After addition of *Eco*RI linkers, the *Hin*dIII-*Xba*I fragment containing the E2-C insert was cloned into the *Eco*RI site of pMT2 (23) to generate pMT2-E2C. pRS-CM4 contains the double-spliced HPV-11 E2-C insert spanning nt 812–847^1256–1272^3377–4402 driven by the Rous sarcoma virus promoter. This transcript was cloned as a derivative cDNA from C-33A cells transfected with the E1M^E2C cDNA expression vector pRS-CM (nt 812–1272^3377–4402) (7, 8). The insert from pRS-CM4 was isolated after double digestion with *Bam*HI and *Hin*dIII and similarly cloned into pMT2 to generate pMT2-CM4. The pRS-CM-M849 plasmid expressing the HPV-11 E1M^E2C protein is identical to pRS-CM (7) except the nucleotide T at nt 849 was replaced by C by using site-directed mutagenesis (24) as described below with the oligonucleotide 5′-GACGATTCAGGCACA GAA-3′ (with the mutation underlined), which is complementary to HPV-11 nt 3′-838 to 855-5′. This mutation does not change the encoded amino acid of the E1M^E2C protein; however, it inactivates the splice donor site at nt 847 which functions at low frequency in the wild-type E1M^E2C cDNA to generate the CM4 transcript in transfected cells. This mutated cDNA isolated from pRS-CM-M849 by *Bam*HI and *Hin*dIII digestions was then cloned into pMT2 as described above to generate pMT2-CM-M849. In immunoprecipitations of extracts of pMT2-CM-M849-transfected COS cells, only E1M^E2C, but not CM4, protein was detected (8).

Mutagenesis. p7674-99(M234) was constructed by transferring the URR fragment spanning nt 7674 to 99 flanked by *Hin*dIII sites from 24-N(M234) to the *Bam*HI site of pML2d after blunt-end ligation. 24-N(M234) contains mutations in

one half-site of each of the three copies of E2-RS (Fig. 4A) and was constructed by site-directed mutagenesis (24) in two stages as follows. The *Hin*dIII fragment from 24-N (nt 7674 to 99) was cloned into pBluescript(+) (Stratagene) to generate pBS24-N, which was then used to generate single-stranded M13 phage DNA in *Escherichia coli* RZ1032 after infection with M13 helper phage R408. The single-stranded phage DNA served as template for second-strand DNA synthesis by using the primer oligonucleotide containing mutations to create pBS24-N(M34), which had mutations introduced into E2-RS 3 and 4. pBS24-N(M234) was then constructed by digesting pBS24-N(M34) at *Bsu*36I and *Bst*EII sites flanking copy 2 of the E2-binding site and replacing it with a synthetic double-stranded DNA fragment, 5′-TAAGGTCACACACCTGCAGATCTTTTCG-3′ containing (underlined) mutations for ACCGG in E2-RS 2. The *Hin*dIII fragment of pBS24-N(M234) was then swapped with the corresponding fragment of 24-N to generate 24-N(M234). The mutations were confirmed by DNA sequence analysis.

Transient-replication assays. CHO cells were maintained in F-12 medium supplemented with 10% fetal bovine serum. Human C-33A and 293 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 10% calf serum, respectively. Replication assays with CHO, 293, and C-33A cells were conducted as previously described (9, 50). Briefly, 50 μg of sheared single-stranded salmon sperm DNA, 0.5 μg of supercoiled replication reporter plasmid, and 5 μg each of pMT2-E1 and pMT2-E2 were electroporated with 5×10^6 cells in 250 μl of growth medium containing 10% fetal bovine serum and 5 mM BES (*N,N*′-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffer (pH 7.2) at 960 μF and 230 V for CHO cells, 170 V for 293 cells, and 180 V for C-33A cells by using a Bio-Rad Gene Pulser. Under these conditions, the transfection efficiency was generally 50% or higher in CHO cells and approximately 25% in 293 and C-33A cells as judged by the percentage of cells positive for β-galactosidase after transfection of pCMV-βgal (8, 51). One-quarter of the cell suspension was plated onto each of three 100-mm plates. One-third of the low-molecular-weight DNA isolated from each time point (approximately 36, 60 and 84 h after electroporation) was digested with 10 U each of *Pst*I and *Dpn*I for 6 h or longer. *Dpn*I digested (methylated) input DNA, while *Pst*I linearized the newly replicated plasmids except for p7674-99, which was cleaved into two fragments. Linear p7072-99 DNA was used as the hybridization probe, which was labeled with [^{32}P]dCTP by random hexamer priming. For experiments involving negative factors, various amounts of the repressor-expressing plasmids were mixed with pMT2 DNA to a total of 5 μg and then added to the rest of the DNA mixture for electroporation. For replication of the whole genomic HPV-11 DNA, cloned DNA was digested with *Bam*HI to excise the HPV-11 genome from the pSVO10 vector (7). After phenol-chloroform extraction and ethanol precipitation, 3 μg of the digested DNA was mixed with 50 μg of carrier DNA, with or without 5 μg each of pMT2-E1 and pMT2-E2, and used for electroporation. The replication assays were performed at least twice.

DNase I footprinting. DNase I footprinting was performed with either plasmid 14-O (10), which contained the wild-type DNA spanning nt 7730 to 99, or plasmid 14-O(M234), which contained mutations in E2-RS copies 2, 3, 4 and was constructed by swapping the corresponding *Sph*I and *Bsu*36I fragments between 14-O and 24-N(M234). HPV-11 E2-C protein was expressed from the lambda p_L promoter in the pEV-vrf vector after heat inactivation of the lambda repressor (11). The induced or uninduced bacterial control lysate was incubated with the *Hin*dIII fragment from either plasmid 14-O or 14-O(M234) 3′ end labeled with ^{32}P at nt 99. DNase I footprinting was performed as previously described (11).

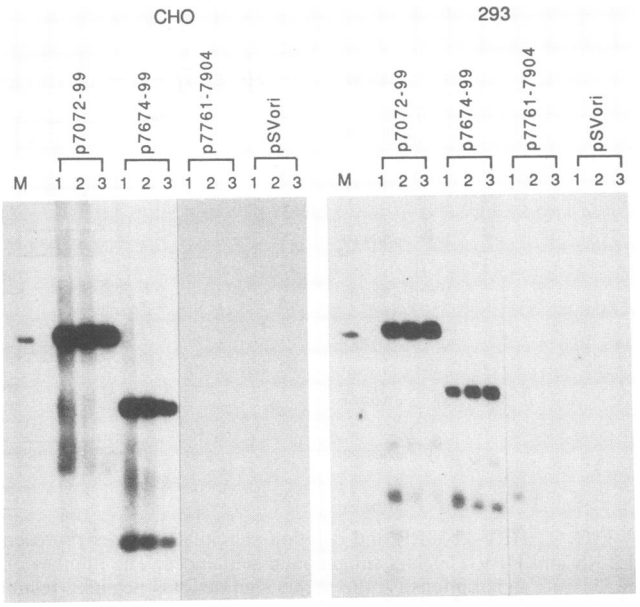

FIG. 2. Replication of HPV-11 URR plasmids in CHO and 293 cells. Replication assays were performed as described previously (9, 50; see also Materials and Methods). In this and subsequent figures, the mobility marker (M) consists of 20 pg of linear p7072-99 DNA and 1, 2, and 3 are the time points when low-molecular-weight DNA was harvested approximately 36, 60, and 84 h after electroporation. The *Pst*I restriction enzyme used to digest the plasmids prior to Southern blotting created linear monomers of all plasmids except p7674-99, which was cleaved into two fragments. The autoradiograms were exposed for 6 h for CHO cells and 18 h for 293 cells at −70°C with intensifying screens.

RESULTS

Delineation of the HPV-11 origin of replication. An HPV-11 URR plasmid, p7072-99 (with numbers referring to nucleotide boundaries of the subgenomic fragment), spanning nt 7072–7933/1–99 (Fig. 1, line I) replicates in the presence of mixed or matched combinations of E1 and E2 proteins specified by HPV-11 and BPV-1 in a variety of mammalian cell lines of both epithelial and fibroblastic lineage (9). To localize this HPV-11 origin of DNA replication, plasmids p7674-99 and p7761-7904 (Fig. 1, lines II and III, respectively) were constructed and tested in CHO cells and in the adenovirus-transformed human kidney epithelial cell line 293 by cotransfection with the HPV-11 E1 and E2 expression plasmids, pMT2-E1 and pMT2-E2 (9), driven by the adenovirus major late promoter (23). Only p7674-99 replicated efficiently in both cell lines (Fig. 2). p7761-7904, which failed to replicate, contains an intact E2-binding site known to respond to E2 transcriptional *trans*-activation in conjunction with other enhancer elements (21). Thus, additional essential sequence elements lie downstream of nt 7904. Substitution of the simian virus 40 *ori* sequence for the HPV-11 URR did not confer on the plasmid an ability to replicate (Fig. 2), indicating that interactions between *cis* elements and *trans*-acting factors are virus specific.

A series of URR 5′ and 3′ deletion plasmids was then

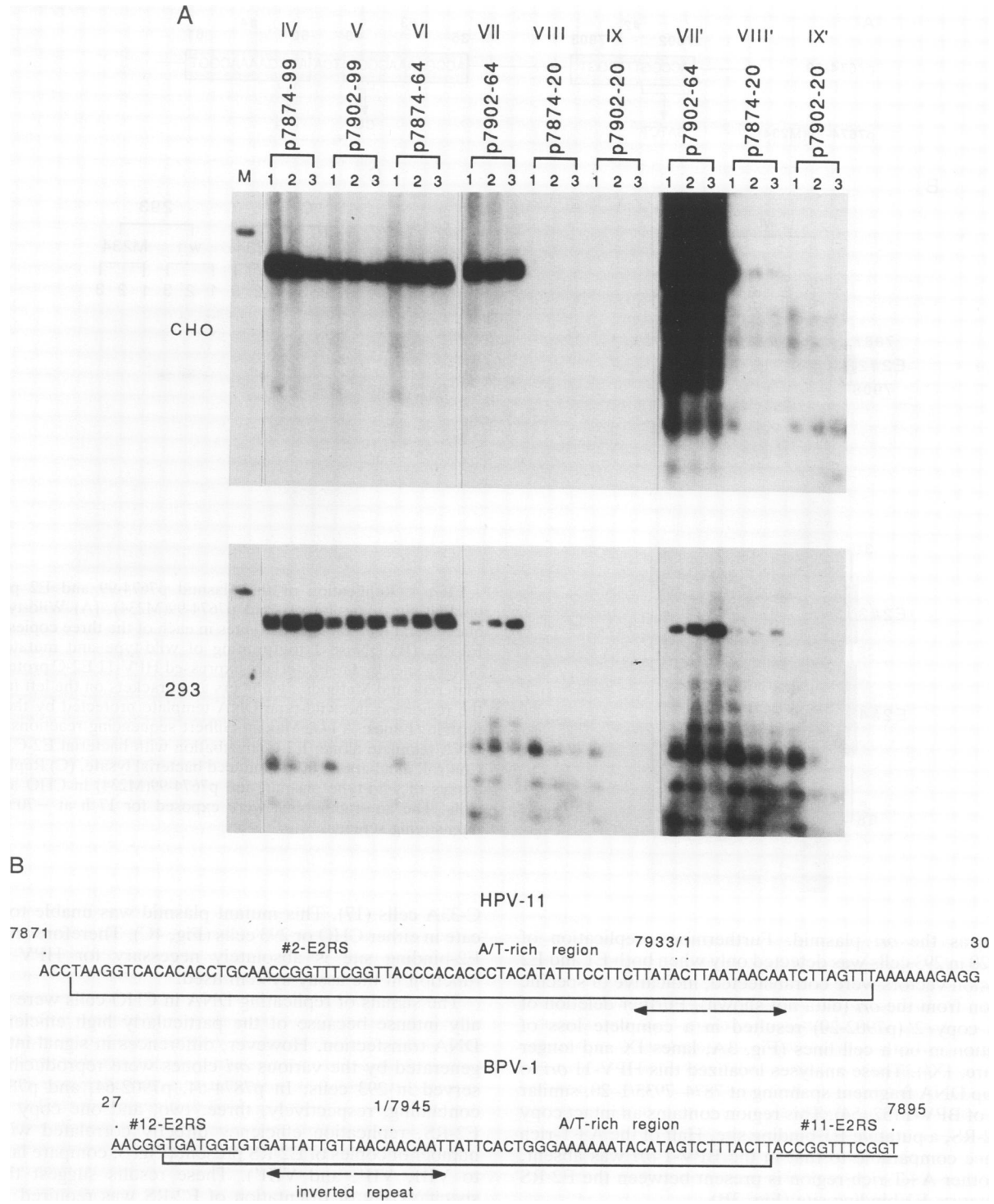

FIG. 3. Localization of the HPV-11 origin of replication. (A) Replication assays of various URR deletion plasmids. Roman numerals correspond to the plasmids described in Fig. 1. The autoradiograms (IV to IX) were exposed for 6 h for CHO cells and 18 h for 293 cells at −70°C with intensifying screens. VII′, VIII′, and IX′ are 48-h exposures of lanes VII to IX, respectively, to reveal the replication signal of plasmid p7874-20. (B) HPV-11 *ori* sequences (bracketed). Flanking sequences are also shown. The partial inverted repetition (arrows) has homology to the BPV-1 E1-binding site, marked as an inverted repetition in the BPV-1 *ori*, which is also shown for comparison.

constructed (Fig. 1, lines IV to IX). Efficient replication persisted to p7902-64 in both cell lines (Fig. 3A, lanes IV to VII). Plasmid p7874-20 replicated weakly but was clearly detected (Fig. 3A, lanes VII and VIII and, at longer exposure, lanes VII′ and VIII′). The interpretation that these faint bands represent true replication is supported by the absence of bands specific for the E1 and E2 expression vectors, which were each electroporated at 10 times the

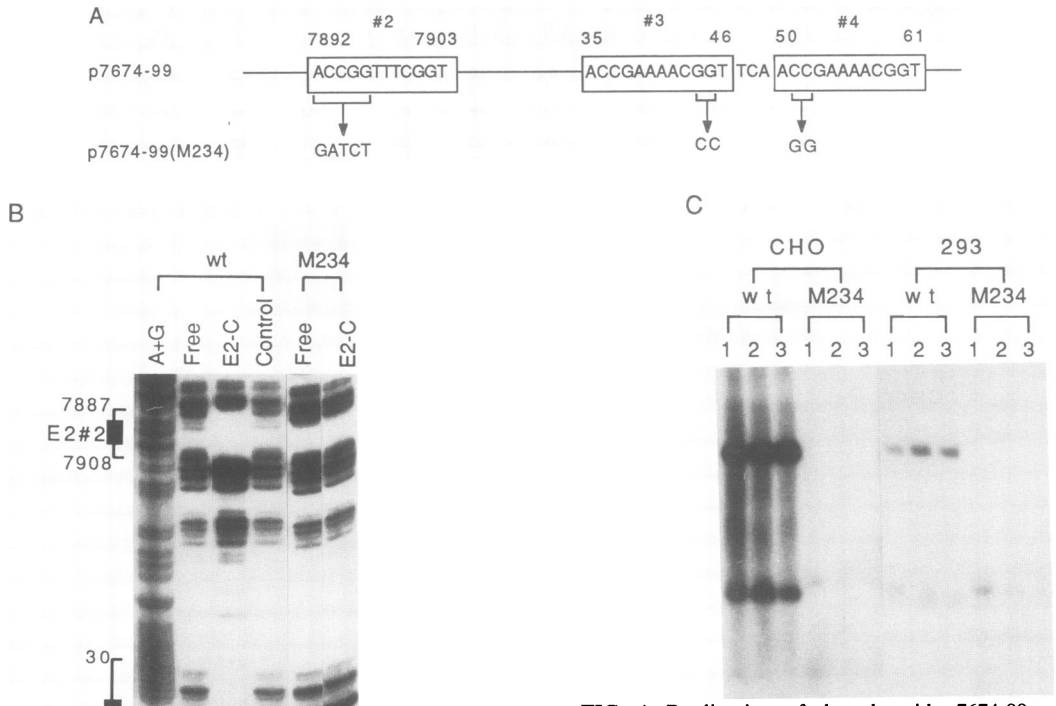

FIG. 4. Replication of the plasmid p7674-99 and E2 protein-binding site mutation plasmid p7674-99(M234). (A) Wild-type sequence and mutations at half-sites in each of the three copies of the E2-RS. (B) DNase I footprinting of wild-type and mutated E2-binding sites by the bacterially expressed HPV-11 E2-C protein (see Materials and Methods). Numbers and brackets on the left indicate the region on the wild-type DNA template protected by the E2-C protein. Lanes: A+G, Maxam-Gilbert sequencing reactions; Free, DNA template alone; E2-C, incubation with bacterial E2-C lysate; Control, incubation with uninduced bacterial lysate. (C) Replication assays of wild-type plasmid and p7674-99(M234) in CHO and 293 cells. The autoradiograms were exposed for 17 h at −70°C with intensifying screens.

amount as the *ori* plasmid. Furthermore, replication of p7874-20 in 293 cells was detected only when both E1 and E2 expression vectors were cotransfected, indicative of specific initiation from the *ori* (data not shown). Further deletion of E2-RS copy 2 (p7902-20) resulted in a complete loss of replication in both cell lines (Fig. 3A, lanes IX and longer exposure, IX′). These analyses localized this HPV-11 *ori* to an 80-bp DNA fragment spanning nt 7874–7933/1–20, similar to that of BPV-1 (52, 54). This region contains an intact copy 2 of E2-RS, a putative E1-binding site. Half of the A+T-rich sequence comparable to that in the BPV-1 *ori* was absent, but another A+T-rich region is present between the E2-RS and putative E1-binding site (Fig. 3B).

Requirement for an intact E2 protein-binding site. To determine whether only half a dyad of the E2-RS palindrome is sufficient to support *ori* function, as is the case for the BPV-1 minimal *ori*, we mutated half-sites of all three E2-binding sites (copies 2, 3, and 4) in p7674-99 by site-directed mutagenesis (Fig. 4A). These mutations efficiently eliminated the binding of bacterially expressed E2-C protein (Fig. 4B), which has an identical DNase I footprint to the full-length E2 protein (11, 21). The E6 promoter in p7674-99(M234) is no longer repressed by either BPV-1 E2 or HPV-11 E2-C in transfected human cervical carcinoma C-33A cells (17). This mutant plasmid was unable to replicate in either CHO or 293 cells (Fig. 4C). Therefore an intact E2-binding site is absolutely necessary for HPV-11 *ori* function in the assay system used.

The signals of replicating DNA in CHO cells were generally intense because of the particularly high efficiency of DNA transfection. However, differences in signal intensity generated by the various *ori* clones were reproducibly observed in 293 cells. In p7874-64, p7902-64, and p7874-20, containing, respectively, three, two, and one copy of the E2-RS, replication efficiency directly correlated with the number of copies of E2-RS present (Fig. 3, compare lanes VI to VIII, VII′, and VIII′). These results suggest that no specific copy or orientation of E2-RS was required for *ori* function, since either E2-RS 2 in one orientation or E2-RS 3 and 4, both in the opposite orientation, were capable of supporting replication in both cell lines.

Repression of HPV-11 *ori* replication by multiple forms of E2 transcription repressors. In addition to E2-C and E1M^E2C, we recently recovered from transfected cells a double-spliced transcript (CM4) which encodes a protein with a different amino terminus but is otherwise identical to E2-C (see Materials and Methods). Both the CM4 protein and the E1M^E2C protein were produced more efficiently than E2-C (11) in an in vitro transcription and translation

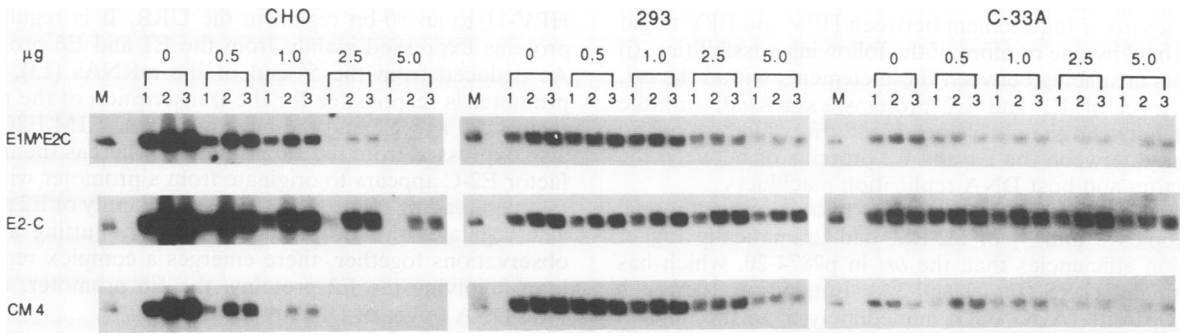

FIG. 5. Repression of *ori* replication by negative viral factors in different cell lines. E1M^E2C, E2-C, and CM4 were expressed individually from pMT2-CM-M849, pMT2-E2C, and pMT2-CM4, respectively (see Materials and Methods). The indicated amounts of plasmid DNA expressing negative factors were mixed with pMT2 DNA to a total of 5 μg in each electroporation mixture, which also contained 0.5 μg of p7072-99, 5 μg each of E1 and E2 expression plasmids, and 50 μg of carrier DNA. The autoradiogram was exposed for 3.5 h at −70°C with intensifying screens.

analysis (data not shown). Cotransfection with a plasmid expressing any of these three forms of E2-C proteins resulted in suppression of the E2-independent E6 promoter activity (7, 8, 10, 11, 17). Repression is more effective with E1M^E2C and CM4 than E2-C (7, 8) and is probably due to occlusion of the formation of transcription initiation complexes around the TATA motif or the binding of transcription factor Sp1, as reported previously (18, 47).

To determine whether the transcription repressor proteins support or inhibit *ori* function in transient-replication assays, we expressed in separate clones the three transcriptional repressor proteins from the vector plasmid pMT2 (23). Protein accumulation was confirmed in transfected COS-7 cells by Western blotting with rabbit polyclonal antibodies raised against bacterially expressed *trpE*-E2 fusion protein (7, 8). Cotransfection of the full-length E1 expression vector together with either the E1M^E2C or the CM4 vector failed to support transient replication of p7072-99 in CHO, 293, or C-33A cells (data not shown). In contrast, cotransfection with any of the three transcription repressor expression vectors with the full-length E1 and E2 expression vectors strongly inhibited replication of p7072-99 in CHO cells (Fig. 5). The extent of repression increased in a dose-responsive manner with the amount of the expression vector cotransfected. Repression in human 293 cells was less severe (Fig. 5). The efficacies of CM4 and E1M^E2C were comparable, and both were higher than that of E2-C, probably because of more efficient protein expression. To rule out potential complications that might have been introduced by the endogenous adenovirus E1A proteins constitutively expressed in 293 cells, we tested C-33A cervical carcinoma cells which contain no known viral DNA. Similar results were obtained at the highest input of the repressor vectors (Fig. 5).

Replication of whole genomic HPV-11 DNA. To test the ability of whole genomic HPV-11 DNA to replicate transiently in transfected cells, we excised it from the vector and used the linear DNA mixture for electroporation. Replication was observed only when both E1 and E2 protein expression vectors were cotransfected (Fig. 6). These results indicate that the expression of the replication proteins from the whole genomic DNA was insufficient in these cells to support episomal replication, in keeping with the strict species specificity of the human virus and the epithelial differentiation-dependent viral gene expression and DNA replication observed in vivo (45). Interestingly, replication was more efficient in 293 cells than in CHO cells, in which it is weak but reproducible, a behavior similar to that observed with the shortest *ori* plasmid, p7874-20 (Fig. 3A).

DISCUSSION

We have constructed a series of HPV-11 URR deletion mutations and tested them for their ability to replicate as plasmids in transiently transfected mammalian cells. The results are summarized in Fig. 1. An *ori* sequence has been localized to an 80-bp DNA fragment spanning nucleotides 7874–7933/1–20, which is comparable to that of the BPV-1 *ori* (Fig. 3B). However, unlike the BPV-1 *ori*, an intact E2 protein-binding site is absolutely essential, since an *ori* plasmid in which half-sites of each of the three copies of the E2-RS were mutated is unable to replicate (Fig. 4C). These results stress the importance of comparative studies of various papillomaviruses. Experiments are in progress to determine whether there are additional distinctions. Whether this sequence also serves as an *ori* in the whole genomic context remains to be determined. In any case, the

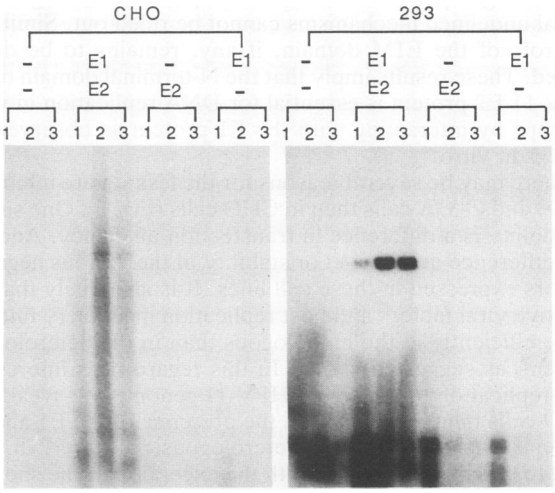

FIG. 6. Replication of whole genomic HPV-11 DNA in CHO and 293 cells. A mixture of linear HPV-11 DNA, the cloning vector, and carrier DNA was electroporated into CHO or 293 cells in the presence or absence of HPV-11 E1 or E2 expression plasmids. The autoradiograms were exposed for 17 h at −70°C with intensifying screens.

differences in *ori* requirement between HPV and BPV might be explained by one or more of the following possibilities: (i) variations in spacing between these elements within the *ori*, (ii) the level of E1 and E2 proteins expressed, (iii) the affinities between the *cis* elements and *trans* factors, or (iv) the affinity between the E1 and E2 proteins or between the viral factors and host DNA replication machinery.

p7874-64 and p7902-64, containing two or one additional copy (copies 3 and 4) of E2-RS, had dramatically higher replication efficiencies than the *ori* in p7874-20, which has only a single E2-RS (Fig. 1 and 3A). In addition, there is a variant Sp1 site (AGGAGG) immediately 5′ to the tandem E2-RS in the first two plasmids (19). E2 proteins bound to the extra E2-binding sites may trap E1 protein and increase its effective local concentration. Alternatively, the additional E2 site or the Sp1-binding site may function as enhancers; the bound proteins might interact with another host transcriptional factor(s) and prevent nucleosome formation in the region surrounding *ori*, as suggested for the role of the enhancers on the simian virus 40 and polyomavirus origin of replication (6, 28, 34). It is interesting that, unlike the other plasmids, the smallest *ori* in p7874-20 replicated more efficiently in 293 cells than in CHO cells (Fig. 3A) despite a considerably lower transfection efficiency in 293 cells (8, 51). This observation might indicate that, in the absence of enhancer-cognate proteins, the interactions among viral *ori*, viral replication proteins, and host replication machinery are more stable and efficacious in the homologous human cells than in the heterologous rodent cells.

The E1 and E2 proteins form a complex when overexpressed from baculovirus vectors (3, 30, 33), and the presence of the E2 protein can enhance the binding of the E1 protein to its target sequence in vitro (54). However, the existence of the E1-E2 complex in BPV-1-transformed cells has not been detected. We demonstrated that transcription repressors without the *trans*-activating amino-terminal domain of the full-length E2 protein did not support replication but instead negatively regulated *ori* replication (Fig. 5; data not shown). The negative viral factors might compete with E2 for binding sites located in the *ori* or by forming heterodimers with the E2 protein (4) which were incapable of supporting replication. However, repression through additional undefined mechanisms cannot be ruled out. Similarly, the role of the E1M domain, if any, remains to be determined. These results imply that the N-terminal domain of the HPV-11 E2 protein is essential for DNA replication in vivo, perhaps by interacting with the E1 protein as observed for BPV-1 in vitro.

There may be several reasons for the less severe inhibition in 293 and C-33A cells than in CHO cells (Fig. 5). One simple possibility is a difference in transfection efficiency. Another is a difference in the level or stability of the various negative factors expressed in these cell lines. It is also likely that the positive viral factors and host replication machinery interact more efficiently in the homologous than in the heterologous system, as suggested above. In this regard, it is interesting that replication of the whole HPV-11 genome was weaker in CHO cells than in 293 cells in the presence of the E1 and E2 proteins expressed from a heterologous promoter, in contrast to other *ori* plasmids (with the exception of the shortest *ori* plasmid, p7874-20) (compare Fig. 2, 3A, and 6). This phenomenon might be attributed to the more efficient repression of *ori* function in CHO cells by one or more of the negative viral proteins generated from the genomic DNA. This possibility remains to be investigated.

In summary, we have localized an origin of replication of HPV-11 to an 80-bp region in the URR. It is regulated by proteins expressed mainly from the E1 and E6 promoters. As deduced from the 5′ end of the mRNAs (13), the E1 promoter is responsible for the transcription of the positive factors E1 and E2 and the negative factor E1M^E2C; E2 is also expressed from the E6 promoter, whereas the negative factor E2-C appears to originate from a promoter within the E1 open reading frame (13, 37, 41). The family of E2 proteins also regulates the E6 promoter activity. Putting all these observations together, there emerges a complex regulatory loop involving the E2 proteins, the E6 promoter, and the viral DNA origin of DNA replication.

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