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Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins

(eukaryotic DNA replication/human papillomaviruses/replication factors)

CHENG-MING CHIANG*†, MART USTAV‡, ARNE STENLUND‡, THAU F. HO*, THOMAS R. BROKER*, AND LOUISE T. CHOW*§

*Department of Biochemistry, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, NY 14642; and ‡Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724

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ABSTRACT **We have shown that E1 and E2 proteins of human papillomavirus type 11 (HPV-11) were essential to support the replication of the homologous viral origin (*ori*) in a transient replication assay, similar to reports on bovine papillomavirus type 1 (BPV-1). Unexpectedly, matched or even mixed combinations of E1 and E2 proteins from HPV-11 or BPV-1 replicated either *ori* in human, monkey, and rodent cell lines of epithelial or fibroblastic lineage, albeit with varied efficiencies. Either set of viral proteins was also able to initiate replication of *ori*-containing plasmids from many other human and animal papillomaviruses. Thus the interactions among the cis elements and trans factors of papillomaviruses are more conserved than expected from the other members of the papovavirus family, simian virus 40 and polyomavirus, for which large tumor antigen does not replicate a heterologous *ori* in either permissive or nonpermissive cells. We infer that the stringent species and tissue specificities observed for papillomaviruses *in vivo* are not entirely due to direct restrictions on viral DNA replication. Rather, transcriptional control of viral gene expression must play a dominant role.**

The initiation of DNA replication occurs at precisely defined origins (*ori*) to which specific control proteins bind. Simian virus 40 (SV40) and polyomavirus have long been used as models for investigations of mammalian DNA replication. Initiation of SV40 or polyomavirus replication requires the homologous large tumor antigen, is species specific, and occurs only in a permissive cell environment (1–4). However, the uncontrolled replication of these lytic viruses does not reflect the precise regulation of cellular DNA replication, which takes place once per cell cycle in the S phase. In contrast, papillomaviruses have regulated and uncontrolled replication phases during their life cycle and therefore offer a unique opportunity to study eukaryotic DNA replication.

Human papillomavirus (HPV) types trophic for mucosal epithelia, including HPV-11, are highly infectious pathogens that cause genital warts and laryngeal papillomatosis. A subset of the genital types, including HPV-16 and -18, is also associated with the development of neoplasia and progression to cancer. In subclinical or benign infections, viral DNA persists as low-copy-number plasmids in the nuclei of the undifferentiated basal stem cells. Productive replication takes place only in the more differentiated upper strata of the epithelium. Although a culture system has recently been developed in which preinfected tissues produce progeny HPV-11 virions (25), propagation of HPVs has not been achieved *in vitro* starting from virions or transfected DNA. In transfected or infected cells, HPV DNA either integrates into host chromosomes or is lost. Therefore it has not been possible, until now, to perform molecular studies of autonomous HPV DNA replication in cell culture.

In contrast, bovine papillomavirus type 1 (BPV-1) in transformed rodent cell lines replicates extrachromosomally at a constant copy number (5) in synchrony with chromosomal DNA replication (6, 7). Replication of BPV-1 DNA in cultured cells or in cell-free systems depends on two virus-encoded proteins, the full-length products of the E1 and E2 open reading frames (ORFs) (8, 9). The minimal BPV-1 *ori* sequence is located at the 3′ end of the upstream regulatory region (URR) within a 60-base-pair (bp) DNA fragment including an (A + T)-rich region, a consensus sequence to which all papillomaviral E2 proteins bind, and an E1 protein binding site spanning nucleotide (nt) 1 (9, 10). The E1 binding site is partially conserved among several papillomaviruses compared (9, 10). The roles of the viral proteins in DNA replication are still under investigation. The BPV-1 E1 and E2 proteins form a complex when overproduced in a baculovirus expression system (11–13). *In vitro* the E2 protein enhances E1 binding to the *ori* (9).

As a first step in dissecting the requirements for HPV DNA replication, we expressed the HPV-11 E1 and E2 proteins from the adenovirus major late promoter and tested their abilities to support extrachromosomal replication of a HPV-11 URR-containing plasmid in different cell lines. The BPV-1 E1 and E2 expression plasmids were also used to test their abilities to support HPV DNA replication. Unexpectedly, mixed as well as matched combinations of these two sets of viral replication proteins were capable of replicating either viral *ori*-containing plasmid, with varied efficiencies in most of the cell lines tested. We also demonstrated the generality of *ori* recognition of many heterologous papillomaviruses by either set of viral replication proteins.

MATERIALS AND METHODS

Plasmid Construction. The HPV-11 URR-containing plasmid, p7072-99, was constructed by transferring the URR fragment (nt 7072–7933/1–99) flanked by *Hin*dIII and *Xba* I sites from pUR23-3 (14) into the *Bam*HI site of pML2d (15) by blunt-end ligation. pMT2-E1 and pMT2-E2 expressing HPV-11 E1 and E2 proteins from the adenovirus major late promoter were constructed as follows. The E1 coding fragment between *Bam*HI (nt 812) and *Nsi* I (nt 2817) was isolated from pRS/11E1A$^+$ (16) and cloned into the cytomegalovirus immediate early promoter expression vector pcDNA1 (In-

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Abbreviations: SV40, simian virus 40; HPV, human papillomavirus; BPV, bovine papillomavirus; CRPV, cottontail rabbit papillomavirus; URR, upstream regulatory region; nt, nucleotide(s); ORF, open reading frame; CHO, Chinese hamster ovary.

†Present address: Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021.
§To whom reprint requests should be addressed.

vitrogen, San Diego) to generate pCMV-E1. It was then transferred, after addition of *Eco*RI linkers, into the *Eco*RI site of pMT2 vector (17) to generate pMT2-E1. The E2 cDNA fragment spanning nt 812–847^2622–3900 was isolated from pRS/11E1$_{1-2}$A$^+$ (16) after *Bam*HI and partial *Pst* I digestion. It was similarly cloned to generate first pCMV-E2 and then pMT2-E2. Plasmid pUC/PstI (nt 4780–7945/1–471 in the pUC19 vector) containing the BPV-1 *ori* and pCGEag and pCGE2 expressing the BPV-1 E1 and E2 protein have been described previously (8, 10).

The TrpE–E1N fusion protein expression plasmid pATH2-CM3 contains a HPV-11 sequence spanning nt 812–1392 plus 3866–4402 cloned into the *Bam*HI and *Hin*dIII sites of pATH2 (18) and encodes the amino-terminal portion (nt 812–1392) of the E1 ORF (nt 832–2779) and four additional amino acids before termination. pATH1-E2 containing the HPV-11 DNA fragment (nt 2756–4556) that spans the bulk of the E2 ORF (nt 2723–3824) was similarly cloned and expressed as a TrpE fusion protein.

Preparation of HPV-11 E1 and E2 Antibodies. Expression of TrpE–E1N and E2 fusion proteins was induced with indoleacrylic acid in bacteria DH5α and HB101, respectively. The fusion proteins were isolated from polyacrylamide gels and used as immunogens in 4-month-old female Dutchland rabbits. The MPL+TDM emulsion (Ribi Immunochem) was used as an adjuvant in generating E1 antibodies, whereas Freund's adjuvant was used to elicit E2 antibodies. The specificities of the antisera were confirmed by Western blots of bacterially expressed proteins.

Western Blotting. Five micrograms of pMT2-E1 and pMT2-E2 was electroporated with a Gene Pulser (Bio-Rad) at 180 V and 960 μF separately into COS-7 cells with 50 μg of sheared single-stranded salmon sperm DNA. Cells were harvested from a 35-mm plate in 200 μl of 1× Laemmli buffer 48 hr after electroporation. One-tenth-volume samples were loaded onto an SDS/10% polyacrylamide gel. Western blotting was performed as described (19) using 1000-fold dilutions of anti-TrpE–E1N or anti-TrpE–E2 polyclonal antibodies. Color detection was conducted utilizing the alkaline phosphatase system (19).

Transient Replication Assays in Transfected Cells. Chinese hamster ovary (CHO) cells were maintained in F-12 medium supplemented with 10% fetal bovine serum. NIH 3T3 and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum. COS-7, C-33A, HeLa, and SiHa cells were all maintained in DMEM with 10% fetal bovine serum. Cells were ≈80% confluent when harvested for electroporation. Each electroporation mixture contained 0.5 μg of supercoiled *ori*-containing plasmid, 5 μg of each protein expression plasmid, and 50 μg of carrier DNA. Electroporation was performed with 5 million cells in 250 μl of growth medium containing 10% fetal bovine serum and 5 mM Bes buffer (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, pH 7.2) at 960 μF and 230 V for CHO cells, 170 V for 293 cells, 200 V for NIH 3T3 and HeLa cells, and 180 V for COS-7, C-33A, and SiHa cells. Transient replication assays were conducted after *Dpn* I digestion of input DNA as described (8), except that *Pst* I and *Eco*RI were used to linearize HPV-11 and BPV-1 *ori* plasmids, respectively, in lysates of low-molecular-weight DNA. One-third of each lysate was used in Southern blot analysis. Hybridization probes, as specified in the figure legends, were labeled with [^{32}P]dCTP by random hexamer priming. Samples at time points 1, 2, and 3 were collected approximately 36, 60, and 84 hr after electroporation. The length of exposure was given in each figure legend. The autoradiograms from selected lanes were traced with an LKB Ultroscan XL densitometer to estimate the largest fold differences in representative experiments.

RESULTS

Expression of HPV-11 E1 and E2 Proteins. Autonomous replication of BPV-1 DNA requires the viral E1 and E2 proteins (8, 9). We wished to determine whether analogous viral proteins are required for transient replication of HPV DNA. Accordingly, HPV-11 E1 and E2 proteins were expressed from the human adenovirus major late promoter in pMT2 expression vectors (Fig. 1*a*). When the expression plasmids were individually electroporated into COS-7 cells (SV40-transformed monkey kidney CV-1 cells), an 82-kDa E1 protein and a 43-kDa E2 protein were detected by Western blotting when compared to control cells, using polyclonal antibodies raised against bacterially expressed TrpE fusion proteins (Fig. 1*b*). Migration of the E1 protein was somewhat slower than the anticipated size of 73.5 kDa, whereas the E2 protein was similar to the expected size.

Replication of HPV-11 and BPV-1 *ori* Plasmids by Various Combinations of Viral E1 and E2 Proteins. Plasmid p7072-99 contains HPV-11 URR sequences from nt 7072–7933/1–99 and, by analogy to BPV-1, should include the putative *ori*. Replication was detected only when HPV-11 E1 and E2 expression plasmids were cotransfected (Fig. 2*a*, groups I–IV) in human 293 cells (adenovirus 5-transformed human kidney epithelial cells) and CHO cells, in which BPV-1 *ori* plasmids also replicate in the presence of homologous viral replication proteins (10). The HPV-11 E1 and E2 expression plasmids did not replicate (data not shown), suggesting

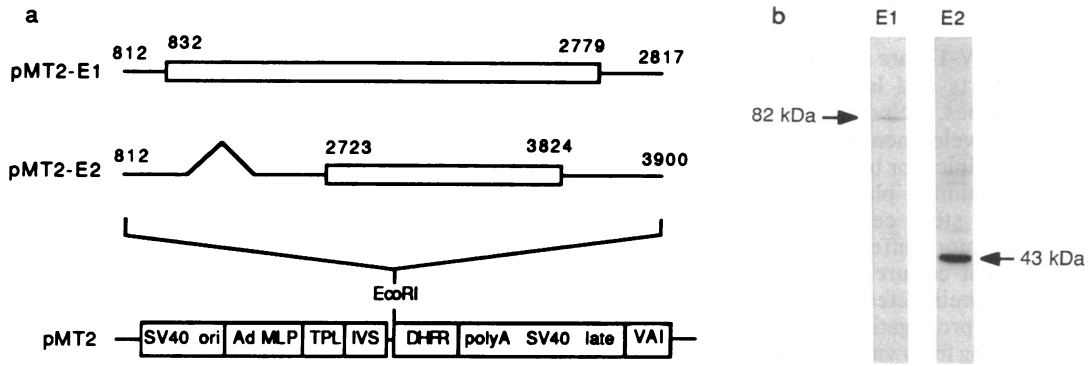

FIG. 1. Expression of HPV-11 E1 and E2 proteins. (*a*) E1 and E2 expression plasmids. pMT2-E1 and pMT2-E2 express HPV-11 E1 and E2 proteins from the pMT2 vector (see text). The key components of pMT2 include the SV40 *ori* and enhancer, the human adenovirus major late promoter (Ad MLP), tripartite mRNA leader sequence (TPL) and adenovirus-associated small RNA I (VAI) gene, the dihydrofolate reductase (DHFR) gene, and the SV40 late poly(A) signal and addition site (17). IVS, intervening sequence. (*b*) Detection of E1 and E2 proteins in electroporated COS-7 cells by Western blotting using polyclonal antibodies raised against TrpE-E1N and TrpE-E2 fusion proteins. Arrows point to protein bands not present in the control cells.

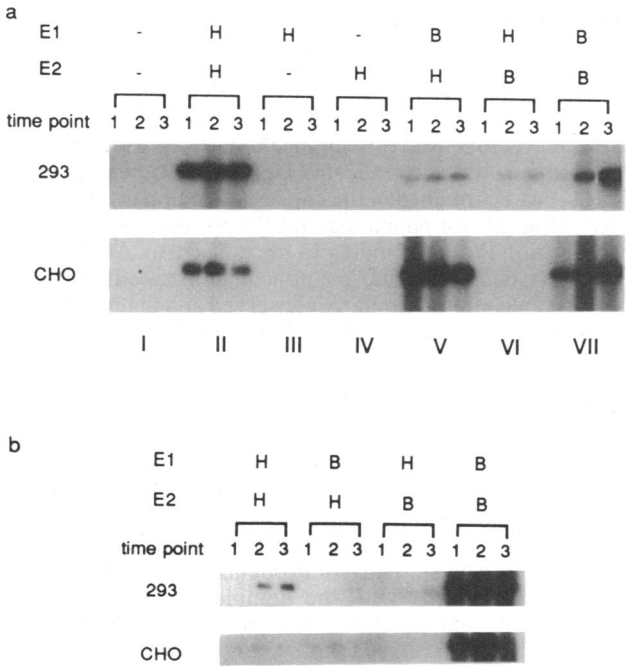

FIG. 2. Replication of HPV-11 and BPV-1 *ori* DNA by combinations of HPV and BPV E1 and E2 proteins in 293 and CHO cells. (*a*) Replication of the HPV-11 plasmid p7072-99 by E1 and E2 proteins specified by either HPV-11 (H) or BPV-1 (B). HPV-11 and BPV-1 E1 and E2 proteins were expressed from pMT2-E1, pMT2-E2, pCGEag, and pCGE2, respectively. (*b*) Replication of the BPV-1 *ori*-containing plasmid pUC/PstI by matched or mixed replication proteins. Linearized p7072-99 DNA was ^{32}P-labeled and used as a probe for both. Time points 1, 2, and 3 were collected at 36, 60, and 84 hr after electroporation. Exposure was for 5 hr (*a*) or 16 hr (*b*) at −70°C with intensifying screens.

specific cis elements—i.e., an *ori* must be present in p7072-99. We also tested the ability of the 72-kDa E1 and 48-kDa E2 proteins of BPV-1 expressed from the cytomegalovirus promoter of the pCG vector (8) to function at the HPV-11 *ori*. The expression of the BPV-1 E1 and E2 proteins has previously been demonstrated and confirmed by Western blotting and immunoprecipitation using specific antisera in some of the cell lines used in this study (refs. 8 and 10; data not shown). In light of the specificity of *ori* recognition by large tumor antigens of SV40 and polyomavirus, one might expect that the papillomaviral E1 protein would confer similar restricted *ori* recognition. Surprisingly, the heterologous BPV-1 proteins were able to replicate p7072-99 in these two cell lines with efficiencies comparable to those achieved by the homologous HPV-11 proteins (Fig. 2*a*, group VII). Moreover, both sets of viral replication proteins were able to function in 293 cells on the whole HPV-11 genomic DNA excised from a cloning vector (C.-M.C., G. Dong, T.R.B., and L.T.C., unpublished results). In CHO cells, the combination of BPV-1 E1 protein plus HPV-11 E2 also replicated p7072-99 effectively, whereas HPV-11 E1 plus BPV-1 E2 functioned very weakly, if at all. The mixed combinations of the two sets of viral proteins weakly supported replication in 293 cells (Fig. 2*a*, groups V and VI).

We also examined the abilities of matched or mixed combinations of the viral proteins to replicate the BPV-1 *ori* plasmid pUC/PstI (10). In agreement with previous reports, the homologous BPV-1 proteins were proficient in supporting BPV-1 *ori* replication (Fig. 2*b*). With the exception of the combination of HPV E1 plus BPV E2, which failed to support replication in CHO cells, all other combinations replicated the BPV-1 *ori* weakly in CHO and 293 cells (Fig. 2*b*).

Although the quantitation of the BPV-1 or HPV-11 replication proteins was not evaluated in these and subsequent replication assays, the fact that matched combinations in almost all cases functioned well with at least one viral *ori* in each of the cell lines suggests that inefficient replication by mixed combinations did not result from poor viral protein expression. Rather, deficiencies probably reflected less effective interactions among the three essential components, the *ori*, viral E1 and E2 proteins, and the host replication machinery.

Replication of the HPV-11 *ori* Plasmid in Different Cell Lines. To examine whether replication of the HPV-11 *ori* plasmid could occur in other cell types in response to replication proteins specified by the two viruses, we tested murine fibroblasts (NIH 3T3), monkey COS-7 cells, and human cervical carcinoma cells (C-33A, HeLa, SiHa). As in 293 and CHO cells, no replication could be detected when E1 and E2 expression vectors were absent (Fig. 3, group I). The specific requirement for the HPV-11 *ori* was again evident because the HPV-11 protein expression vectors failed to replicate in any of the cell lines except for COS-7 (see below). Plasmid p7072-99 replication was observed in most combinations of E1, E2, and host cells. The BPV-1 proteins replicated p7072-99 most efficiently in NIH 3T3 cells, whereas HPV-11 proteins functioned best in COS-7 cells (Fig. 3, groups II and V). This latter result can be attributed at least in part to the efficient translation (17) of E1 and E2 mRNAs expressed from the SV40 *ori*-containing vectors that replicated to high copy numbers in COS cells. Replication by either matched set of proteins was robust in C-33A cells, a cervical carcinoma cell line that contains no known endogenous HPV DNA (20), but was weak in HeLa and SiHa cells that contain integrated HPV-18 and HPV-16 DNA, respectively (Fig. 3, group II). In contrast, the combination of HPV E1 plus BPV E2 supported little or no replication in NIH 3T3, HeLa, or SiHa cells (Fig. 3, group III). Similarly, only marginal replication by BPV-1 E1 plus HPV-11 E2 was detected in SiHa cells (Fig. 3, group IV). Replication in mouse mammary C127, monkey kidney CV-1, and cervical carcinoma CaSki cells that contain integrated HPV-16 was detected upon long exposure of the autoradiograms (data not shown).

Viral E1 and E2 Proteins Also Support Replication of *ori*-Containing Plasmids from Many Other Human and Animal

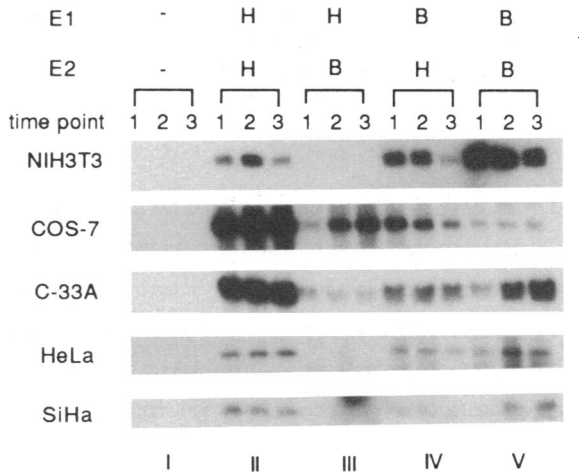

FIG. 3. Replication of the HPV-11 *ori*-containing plasmid p7072-99 in different cell lines. *Pst* I-linearized p7072-99 was used as the hybridization probe except for the experiment in COS-7, for which the HPV-11 URR fragment isolated from pUR23-3 was used. Exposure was for 3 hr for COS-7 cells, 6 hr for C-33A cells, and 46 hr for NIH 3T3, HeLa, and SiHa cells.

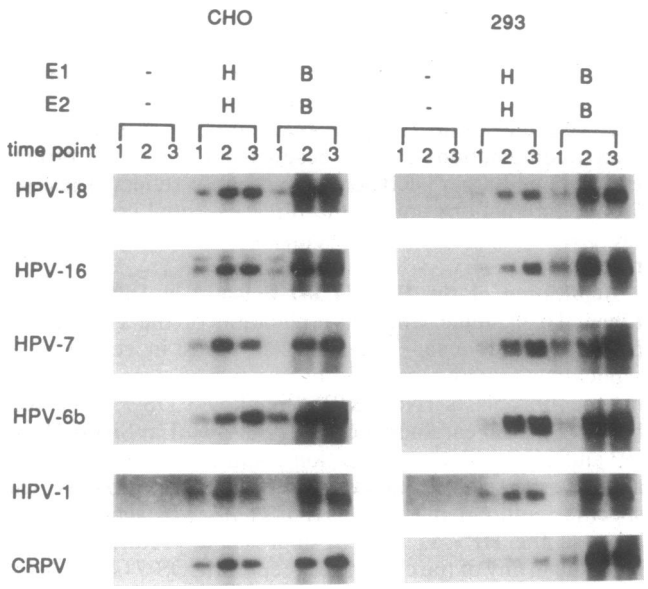

FIG. 4. Replication of different papillomavirus *ori*-containing plasmids by HPV-11 and BPV-1 E1 and E2 proteins in CHO and 293 cells. Plasmids pUR71, pUR11, pUR51, pUR81, and pUR41 contain URRs from CRPV, HPV-1, HPV-6b, HPV-7, and HPV-16, respectively (21). pUR32 contains the HPV-18 URR in the opposite orientation to that in pUR31 (21). The ^{32}P-labeled cloning vector pCAT-A (21), common to these pUR plasmids, was used as the hybridization probe. Exposure was for 6 hr.

Papillomaviruses. The reciprocal substitution of the E1 and E2 proteins specified by HPV-11 and BPV-1 is indicative of functionally conserved viral proteins and mechanisms for initiation of papillomavirus DNA replication. To test their generality, in similar replication studies we used the cloned URRs (21) from HPV-1 and HPV-7 (both of which infect cutaneous skin), HPV-6b, HPV-16, and HPV-18 (all trophic for mucosal epithelia), and cottontail rabbit papillomavirus (CRPV, with trophism to cutaneous skin). Each plasmid was able to replicate in 293 and CHO cells in the presence of either set of HPV-11 or BPV-1 replication proteins (Fig. 4), whereas the cloning vector without URR sequences did not (data not shown). DNA sequence alignment among these and several other HPVs revealed a partially conserved region spanning nt 1 (Fig. 5), which corresponds to the *ori* of BPV-1 (9, 10) and HPV-11 (C.-M.C., G. Dong, T.R.B., and L.T.C., unpublished results). We infer that this conserved region represents the replication origin of these viruses.

DISCUSSION

We report the autonomous replication of HPV URR-containing plasmids in transiently transfected cells. As with BPV-1 (8, 9), replication required viral E1 and E2 proteins. Since the vectors used for cloning the URR or for expressing viral proteins did not replicate, we infer that the papillomavirus *ori* was contained in the conserved region in each of the URRs (Fig. 5). All papillomaviral E2 proteins regulate viral transcription through binding to the consensus $ACCN_6GGT$ E2-responsive sequences. We now demonstrate that its role in DNA replication is similarly conserved and that *ori*-binding E1 proteins also appear to function analogously.

Quite unexpected was our observation that the *ori* of each of the eight papillomaviruses tested was replicated by either HPV-11 or BPV-1 replication proteins (Figs. 2 and 4). In addition, the HPV-11 and BPV-1 *ori* sequences were recognized by mixed combinations of the viral E1 and E2 proteins. Overall, the *ori* sequences of HPVs appeared to be promiscuous and were able to replicate well in response to either HPV-11 or BPV-1 replication proteins in rodent and mammalian cell lines of epithelial or fibroblast lineage. The BPV-1 *ori* functioned better with the homologous than with the heterologous viral proteins. Whether this preference extended to cell lines other than 293 and CHO cells was not tested. Nevertheless, BPV-1 replication proteins function in human and rodent cells on the homologous *ori* (refs. 8 and 10; this study).

Densitometric tracing of autoradiograms indicated that within one experiment in a given cell line, the efficiencies of replication of different papillomaviral *ori* plasmids in response to different combinations of viral proteins were within 35-fold of one another (data not shown). However, transient replication assays emphasize qualitative rather than quantitative results because factors affecting the overall efficiency of replication are many, including the relative and absolute amounts of the viral proteins expressed and the interactions among *ori* sequences, viral proteins, and host proteins. In addition, transfection efficiency becomes a factor when different cell lines are used. Despite this uncertainty about replication efficiencies, it is obvious that the interactions between the cis elements and trans factors for papillomaviruses are more conserved than for the other members of the papovavirus family, SV40 and polyomavirus, that do not replicate in response to such mixed combinations. The replication specificities of SV40 and polyomavirus rely on the species-specific interaction between large tumor antigen and cellular DNA polymerase α/primase complex and the single-stranded DNA binding protein (replication factor A) (1, 2, 22–24). On the basis of our results, it is likely that a similar

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                                                .1                                                                  60.
HPV-7                                           ATGTTTAATAATTGT   ACAGTTACTAAAG          GGCGTA ACCGAAAACGGT CCGACCGAAAACGGT
                .7774                                  7815..1                                                      64.
HPV-1   ACCGAATTCGGTCGTAAAAA TCTAAAGTGAT GATTG TTGTTGTTAACTACCATCAT   TCA TTATTCTAGTTAC      AACAAG AACCTAGGAGTT ATATGCCAGAAGTAA
                .7861                                  7902..1                                                      61.
HPV-6b  ACCGGTTTCGGTTATCCACACCCTACATATTT CCTTC TTATAGTTAATAACAATCTT   GGTTTAAAAAATA          GGAGGG ACCGAAAACGGT TCAACCGAAAACGGT
                .7892                                  7933..1                                                      61.
HPV-11  ACCGGTTTCGGTTACCCACACCCTACATATTT CCTTC TTATACTTAATAACAATCTT   AGTTTAAAAAAGA          GGAGGG ACCGAAAACGGT TCAACCGAAAACGGT
                .7859                                  7901..1                                                      61.
HPV-16  ACCGTTTTGGGTTACACATTTACAAGCAACTTATATAA TAATACTAAACTACAATAAT   TCATGTATAAAACTAAG      GGCGTA ACCGAAATCGGT TGAACCGAAACCGGT
                .7822                          7857..1                                                              69.
HPV-18  ACCGAAATAGGTTGGGCAGCACATACTATACTTTCAT TAATACTTT  TAACAATTGT   AGTATATAAAAAAG         GGAGTA ACCGAAAACGGT CGGGACCGAAAACGGT
                .47                                    1..7868                                          7827.
CRPV    ACCGATATCGGTTTCTGG CCATTACATGTTT CTTAA TTATTGTTAGCAACAACCAT    CATTAAA    TCAAAGTG CATAAC ACCGCATGCGGT
                .27                                    1..7945                                          7895.
BPV-1   AACGGTGATGGT                          GTGA TTATTGTTAACAACAAT TATTCACTGGGAAAAAATACATAGTCTTTACTT ACCGGTTTCGGT
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FIG. 5. Alignment of the putative papillomavirus origin sequences. Conserved sequences around nt 1 are in bold type. An (A + T)-rich region is present to the 5′ side of nt 1 in BPV-1 but to the 3′ side on the sense strand in all the other papillomaviruses. The consensus E2 binding sequences are underlined. The minimal BPV-1 *ori* (9, 10) is bracketed. The HPV-7 sequence upstream of nt 1 also exhibited extensive homology (H. Delius, personal communication). Dots denote nucleotide coordinates for which the genomic positions are given. Gaps were introduced to achieve maximal homology. DNA sequences were obtained from published data. Alignments of the *ori* sequences of many other papillomaviral types are available upon request.

restriction, but not as strict, also exists for papillomaviruses. It would be interesting to determine whether various combinations of E1 and E2 proteins show differential affinities for such cellular replication protein components.

Another surprising observation was that HPV replication was observed in cells of epithelial and fibroblast lineage from a wide range of mammalian cells (Figs. 2–4), in contrast to the strict species and tissue tropisms these viruses exhibit *in vivo*. Our results demonstrate that the *in vivo* specificities are not entirely due to direct restrictions on viral replication. Host transcription factors required to activate expression of the viral replication genes would appear to play a dominant role.

It is worth noting that the one cervical carcinoma cell line that contains no HPV DNA, C-33A, supported robust HPV-11 DNA replication, whereas all three HPV-containing cervical carcinoma cell lines tested, HeLa, SiHa, and CaSki, supported HPV-11 *ori* replication poorly, if at all. We speculate that a deficiency in supporting viral DNA replication might have led to viral integration in these carcinoma cells in the first place. The development of a HPV DNA replication assay system should make it possible to search for pharmaceutical inhibitors of this major group of human pathogens.

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