The E8′E2 Gene Product of Human Papillomavirus Type 16 Represses Early Transcription and Replication but Is Dispensable for Viral Plasmid Persistence in Keratinocytes

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A conserved E8′E2 spliced mRNA is detected in keratinocytes transfected with human papillomavirus type 16 (HPV-16) plasmid DNA. Expression of HPV-16 E8′E2 (16-E8′E2) is independent of the major early promoter, P97, and is modulated by both specific splicing events and conserved cis elements in the upstream regulatory region in a manner that differs from transcriptional regulation of other early viral genes. Mutations that disrupt the predicted 16-E8′E2 message also increase initial HPV-16 plasmid amplification 8- to 15-fold and major early gene (P97) transcription 4- to 5-fold over those of the wild type (wt). Expressing the 16-E8′E2 gene product from the cytomegalovirus (CMV) promoter represses HPV-16 early gene transcription from P97 in a dose-dependent manner, as detected by RNase protection assays. When expressed from the CMV promoter, 16-E8′E2 also inhibits the amplification of an HPV-16 plasmid and a heterologous simian virus 40 (SV40) ori plasmid that contains E2 binding sites in cis. In contrast, cotransfections with HPV-16 wt genomes that express physiologic levels of 16-E8′E2 are sufficient to repress HPV-16 plasmid amplification but are limiting and insufficient for the repression of SV40 amplification. 16-E8′E2-dependent repression of HPV-16 E1 expression is sufficient to account for this observed inhibition of initial HPV-16 plasmid amplification. Unlike with other papillomaviruses, primary human keratinocytes immortalized by the HPV-16 E8 mutant genome contain more than eightfold-higher levels of unintegrated plasmid than the wt, demonstrating that 16-E8′E2 limits the viral copy number but is not required for plasmid persistence and maintenance.

High-risk (HR) oncogenic mucosal human papillomavirus (HPV) types are the major cause of most carcinomas of the uterine cervix, as well as many other anogenital tumors, and are found in 20 to 30% of cancers of the head and neck (HNC) (36). While many HR HPVs share genomic organization and conserved sequence homologies, they vary significantly in their prevalences in vivo. HPV-16 is the most prevalent HR HPV, as it is present in nearly 50% of cervical and anogenital carcinomas and in more than 90% of HPV-associated HNC (15, 46). In contrast, the sequence-related HPV-31 is significantly less prevalent than HPV-16 in cervical carcinomas and is rarely detected in HNC. Mechanisms determining HPV type-specific variations in viral persistence and malignant progression, however, are poorly understood.

Papillomaviruses (PVs) replicate as extrachromosomal double-stranded DNA plasmids after infection of the basal keratinocyte host, stably persisting in low copy numbers (10, 19, 25). Early PV gene expression and plasmid amplification in the initial stages of HPV infection appear to be tightly regulated (18, 21, 30) through both transcriptional and posttranscriptional mechanisms (reviewed in references 43 and 60). Both the E1 and E2 proteins are required for PV replication (6, 14, 18), and as shown for HPV-31, transcripts encoding these factors from the plasmid genome are detected early after infection, followed by the production of mRNA encoding other early viral gene products (34). This temporal, regulated expression of limiting levels of these transcription and replication modulators early in HPV infection suggests that these viral gene products are critical to the establishment phase of the viral life cycle. The precise mechanisms that limit HPV early gene expression or initial plasmid amplification and modulate the establishment of a stable viral copy number, however, have not been completely defined.

The full-length E2 and spliced E8′E2 isoforms are conserved in PVs, with E8′E2 transcripts identified in bovine papillomavirus type 1 (BPV-1) (26, 27), HPV-11 (40), cotton-tail rabbit papillomavirus (CRPV) (23), HPV-31 (52), and HPV-16 (11, 45). As in BPV (1, 13, 16, 58), E2 gene products expressed from a variety of HPV5s modulate early gene transcription (5, 47, 48, 53, 56) and initial plasmid amplification (4, 21, 29, 35, 37) by distinct E2 structural domains, as shown in HPV-16 (41) and HPV-31 (51). The HPV E2 protein isoforms exert their transcriptional and replication effects by interacting with defined cis binding sites which are also conserved in mucosal and cutaneous HPVs (42) as well as in animal PVs. E8′E2 interferes with E2-dependent transcriptional activation by the full-length E2 proteins via competitive binding to conserved E2 binding sites in BPV-1 (T. Haugen, unpublished data) and HPV-31 (54). Similarly, HPV E8′E2 products can also inhibit plasmid replication (2, 61).

This study uses a newly developed complementation assay for HPV-16 replication to define the structure of the HPV-16...
E8’E2 (16-E8’E2) citron, its regulation by cellular and viral trans-acting factors, and the role of the 16-E8’E2 gene product in modulating critical early events in HPV-16 infection. These results demonstrate that 16-E8’E2 inhibits initial HPV-16 plasmid amplification primarily by repressing E1 replicase expression. Furthermore, in contrast to E8’E2 function in HPV-31, 16-E8’E2 is not required for plasmid persistence and maintenance.

RESULTS

16-E8’E2 spliced message encodes a repressor of early gene transcription. As in other cell lines containing extrachromosomal replicating plasmids of BPV-1 (7) and HPV-31 (34), a conserved 16-E8’E2 transcript has been detected in an explant-derived, HPV-16-positive, cervical carcinoma cell line, W12E (11). We tested for the presence of a 16-E8’E2 transcript in our model system, primary keratinocytes stably transfected with the HPV-16 W12E (11) plasmid. Total RNA was isolated from a clonal cell line using Trizol (Invitrogen) and subjected to capillary gel electrophoresis on a 1.0% agarose gel. Total RNA was isolated from 10^6 primary HFK cells per 100-mm dish, transfected with 5 μg HPV DNA and 1 μg of RSV-neo. The cells were transferred via serial dilution onto three replicate plates containing irradiated J2 feeder cells. Cells were plated at 100 to 200 μg G418/ml E medium for 5 days, and allowed to grow for another 10 to 20 days without selection. After the total colony numbers (ranging from 20 to 60 colonies per transfection) from replicate plates were determined, individual colonies were isolated using cloning cylinders and expanded from dishes with <40 colonies. Total DNA was harvested (as described above) from clonal cultures to determine HPV DNA status and plasmid copy number.

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A purified amplification product, which revealed the G(1302)-to-C(3358) splice junction. A similar transcript was detected in SCC13 cells transiently transfected with HPV-16 plasmid (data not shown).

Since the 16-E8’E2 gene product contains the C-terminal E2 dimerization and DBD, it would be expected that it could interfere with E2-dependent activation of early gene expression by direct competition for binding to E2 sites and/or by

FIG. 1. A 16-E8’E2 spliced message encodes a transcriptional repressor. (A) Organization of the HPV-16 genome; the E8 and E2 coding regions are shown in black. (B) Total mRNA was isolated from HFKs that were stably transfected with HPV-16 DNA. The reverse-transcribed mRNA was amplified by reverse transcription-PCR with primers located within the E2 coding regions and upstream of the E8 coding regions (see Results). (C) Expression vectors used in this study. (D) 16-E8’E2 interferes with E2 transactivation. HeLa cells were cotransfected with pCG (16)-E2 (10 ng), E2x2-Sp1x2 tk (-38) cat (3 μg), and the indicated quantities of pCG (16)E8’E2 plasmid. Enzymatic CAT activities are expressed as a percentage of the baseline and represent averages of two or three independent experiments. (E to G) 16-E8’E2 interferes with enhancer function. Cells were transfected with tk promoter cat constructions containing E2 binding sites upstream or downstream of the SV40 enhancer or a control (G) without E2 binding sites. (H) 16-E8’E2 represses enhancer-dependent P97 transcription. HeLa cells were transfected with p(16)-6153-506 cat, the indicated amounts of pCG (16)E8’E2, and pSVNΔ13cat as an internal transfection control. HPV-16 E2 binding sites 4 through 1 are represented by black boxes; Enh, keratinocyte-dependent enhancer. Total RNA was analyzed by RNase protection. (C to G) The total amount of pCG vector was kept constant by the addition of reciprocal amounts of the plasmid pCG-neo.
forming inactive heterodimers with the full-length E2 transactivator protein (E2-TAD) (57). The cDNA sequence encompassing spliced 16-E8-E2 was ligated into a pCMV-driven pCG vector backbone to form pCG (16)E8-E2 (Fig. 1C). In contrast to early gene expression from the native HPV promoters, constitutive 16-E8-E2 expression from the cytomegalovirus (CMV) promoter construct is not subject to feedback inhibition from E2 isoforms. Therefore, this CMV construct permits the examination of dose-dependent 16-E8-E2 effects in our transcription and replication assays.

In transiently transfected HeLa cells, pCG (16)E8-E2 inhibited the transcription from a heterologous, E2-dependent CAT expression construct in a dose-dependent manner (Fig. 1D). To evaluate the ability of 16-E8-E2 to interfere with enhancer function, we used SV40 enhancer-driven cat constructions. When E2 binding sites were adjacent to the SV40 enhancer (either upstream or downstream), CAT expression was repressed with increasing amounts of pCG (16)E8-E2 (Fig. 1E and 1F). In contrast, a construct without E2 binding sites was not inhibited (Fig. 1G). These results demonstrate that 16-E8-E2 represses transcription in a dose-dependent manner presumably through a specific interaction with its cognate E2 binding site.

The HPV-16 upstream regulatory region (URR) contains a cell type-dependent enhancer required for the activation of the P97 promoter by cellular transcription factors (8). As seen with SV40 constructs, the addition of pCG (16)E8-E2 repressed the transcription from the native P97 promoter (Fig. 1H) in RNase protection assays, presumably via 16-E8-E2 binding to conserved E2 binding sites within the HPV-16 URR. These results show that 16-E8-E2 can inhibit both the native, enhancer-driven HPV-16 P97 promoter and heterologous enhancer promoters, provided that E2 binding sites are present in cis.

**Mapping the 16-E8-E2 cistron.** To further define the cistron generating the 16-E8-E2 product, we used the same strategy recently applied to mapping the structure and defining the regulation of the HPV-16 E1 cistron (24a). Since the E8-E2 product in other HPVs has been described as an inhibitor of plasmid replication, we used 16-E8-E2-dependent inhibition of initial HPV-16 plasmid amplification (also referred to as “transient replication”) as a measure of 16-E8-E2 expression. SCC13 cells were transfected with full-length religated E8– mutant or wt HPV-16 plasmids (Fig. 2A). The amplification of wt HPV-16 is readily detected by Southern blotting DpnI/BamHI-digested total DNA harvested 5 days after infection (Fig. 2A, lane 1). Mutations in the E1 ORF or E2 ORF (either in the transactivation domain [TAD] or DBD), as depicted in Fig. 2B, abolished detectable replication; thus, expression of HPV-16 E1 and E2 gene products is necessary for efficient initial plasmid amplification (Fig. 2A, lanes 3 to 5). The deletion of the nt 863-to-1220 segment (within the 5′ E1 ORF) also prevented plasmid amplification (Fig. 2A, lane 7). A termination codon inserted into the E8 ORF (E8−), however, increased HPV-16 amplification 10-fold (Fig. 2A, lane 2). The disruption of the 16-E8-E2 splice donor site at nt 1302 (SD 1302−) also relieved amplification inhibition (Fig. 2A, lane 6). These results demonstrate that the initial HPV-16 plasmid amplification is repressed by a spliced viral gene product containing the E8 ORF.

In a parallel study (24a), the replication of an otherwise replication-defective plasmid, for example, one incapable of expressing E1, is rescued by adding a second plasmid species that expresses the missing replication factor in trans (e.g., the E1 gene product). The complementation of E1, which is essential for initial plasmid amplification, resulted in comparable amplifications of both plasmid species. Similarly, the transfection of an equimolar mixture of the wt HPV-16 plasmid expressing the E8 ORF (plasmid B) and the E8− mutant genome (defective for E8 expression; plasmid A) lowers the total replication of both plasmid species to levels similar to those produced by the wt alone (Fig. 2A, lane 9). This observed repression via cotransfection of E8-expressing and E8-defective plasmid species constitutes our complementation assay that tracks E8-dependent inhibition of initial HPV-16 plasmid amplification (Fig. 2A).

Co-transfection of the E8− plasmid with the E2 TAD− plasmid also resulted in amplification levels similar to those of the
wt plasmid. This indicates that the E2 TAD—construct is still capable of expressing 16-E8/E2 in trans (Fig. 2A, lane 10). Since transfection with the DBD—mutant along with the E8—mutant permits increased plasmid amplification, it suggests that this negative factor is eliminated by a mutation in the DBD of E2 (Fig. 2A, lane 11). Simultaneous digestion with XbaI (which is a unique site within the DBD— or TAD—constructs) yielded a different size for the DBD—fragment on Southern blots and showed that both the E8— and the E2 DBD—HPV-16 DNAs amplified to equivalent levels (data not shown). These results demonstrate that both the E8 and E2 DBD ORFs are required to inhibit initial HPV-16 plasmid amplification and are consistent with the expression of a 16-E8/E2 product.

In a cotransfection of the E8—plasmid with a plasmid containing a deletion of the major early gene promoter and adjacent ORFs (nt 7466 to 863), 16-E8/E2 still inhibited plasmid amplification (Fig. 2A, lane 12). This result was confirmed with additional deletions in the HPV-16 major early cistrons, for example, in the keratinocyte-dependent enhancer (with nt 7466 to 56 deleted); the P97, E6/E7 promoter, and E6 ORF (nt 56 to 500 deleted); and the E6 and E7 ORFs (nt 500 to 863 deleted). None of these deletions disrupted 16-E8/E2-dependent activity (data not shown). In contrast, cotransfection with a plasmid containing a deletion (nt 863 to 1220) upstream of the E8 ORF relieved 16-E8/E2-dependent inhibition of plasmid amplification (Fig. 2A, lane 13). This result suggested that cis elements necessary for efficient 16-E8/E2 expression are located in this region. Taken together, these findings are consistent with the expression of a spliced 16-E8/E2 gene product that requires cis elements immediately upstream of the E8 ORF and suppresses HPV-16 plasmid amplification in trans.

Although 16-E8/E2 can repress transcription when ectopically expressed from a heterologous promoter, we sought to determine whether endogenous levels of 16-E8/E2 expressed from a native promoter(s) could modulate transcription from the HPV-16 major early promoter, P97. HPV-16 plasmid genomes containing mutations in the E8 and E2 ORFs were transfected into SCC13 cells, and the total RNA was harvested after 40 h—before detectable plasmid replication (data not shown). Plasmids containing mutations within the 16-E8/E2 ORF displayed three- to fourfold greater transcription than that of the wt in RNase protection assays (Fig. 3, compare lane 1 to lanes 3, 4, 5, and 7). The increase in transcription, however, was not due to plasmid replication, as demonstrated by the E1-, E8-, and E2 DBD—constructs which are replication defective (Fig. 2, lanes 3 to 5). Taken together, these data demonstrate that the defined 16-E8/E2 cistron generates an efficient repressor of early viral gene transcription that is independent of its inhibition of plasmid replication.

Regulation of 16-E8/E2 expression. As noted for many HR HPVs, conserved cis elements within the noncoding URR are critical modulators of early gene expression (8, 22, 44, 59). Although our deletion analysis showed that the major early promoter and adjacent ORFs were dispensable for 16-E8/E2-dependent inhibition of plasmid amplification (Fig. 2A), we wanted to determine whether conserved cis elements within the URR, which are critical for major early promoter regulation, contributed to the regulation of 16-E8/E2 expression. We modified our complementation assay (as depicted in Fig. 2) to determine whether discrete cis mutations in the HPV-16 coding and noncoding sequences influenced 16-E8/E2-dependent repression of initial plasmid amplification, independent of their effects on E1 or E2 expression, in transfected SCC13 cells by Southern blotting (Fig. 4). As shown in Fig. 2, the mutation of the 16-E8 ORF relieves the repression of HPV-16 plasmid amplification (Fig. 4, plasmid A, lane 2), resulting in elevated plasmid levels compared to those of the wt (Fig. 4, lane 1). The cotransfection of the E8-defective construct (plasmid A) with equimolar quantities of a second plasmid expressing 16-E8/E2, but not E1 or E2 (plasmid B, or E1-/E2 TAD-), results in restoration of 16-E8/E2-dependent repression, generating replication levels similar to those of the HPV-16 wt plasmid (Fig. 4, compare lane 3 to lane 1). In contrast to our initial genetic complementations (Fig. 2), cis mutations introduced into the E8/E2-expressing plasmid B in these refined complementation assays allowed us to gauge their effects on 16-E8/E2 expression by tracking 16-E8/E2-dependent repression of initial plasmid amplification, independent of any cis effects these mutations might also have on E1 or E2 expression.

Consistent with our deletion analysis of the 16-E8/E2 cistron (Fig. 2, lane 12), mutations in cis elements critical for E1 and E6/E7 expression did not abrogate 16-E8/E2-dependent activity. The mutation of the TATAA box at nt 65, T(65), which drives expression of the P97 major early promoter, had no effect on 16-E8/E2-dependent repression of plasmid amplification (Fig. 4, lane 5). Similarly, the mutation of the splice acceptor site at nt 409, SA(409), which is required for E7 and E1 expression, had no significant effect on E8/E2-dependent inhibition (Fig. 4, lane 4).

The mutation of the keratinocyte-dependent enhancer (Fig. 4, lane 6), the proximal E2 sites (sites 1 and 2) (Fig. 4, lane 7),
or the distal E2 sites (sites 3 and 4) (Fig. 4, lane 8), however, partially relieved 16-E8/E2-dependent inhibition of amplification, resulting in 5- to 6-fold repression compared to 11- to 15-fold repression with the unaltered E8 expressor construct (Fig. 4, lane 3). Taken together, these results demonstrate that 16-E8/E2 expression is partially supported by the E2 binding sites, presumably through the binding of the full-length E2 transactivator (E2-TA). 16-E8 expression may also be supported by the keratinocyte-dependent enhancer via binding of E2 and E2 TAD- indicate mutations in the respective genes, while SA409 refers to the indicated splice acceptor signal at nt 409. T(65) refers to mutation of the TATAA box at nt 65, enh− refers to the mutated keratinocyte-dependent enhancer, and E2#1+2− and E2#3+4− indicate mutations in the respective E2 binding sites. A linearized HPV-16 genome (30 pg) was included as a positive blotting control.

We were unable to detect 16-E8/E2 transcripts using RNase protection assays (data not shown). This indicates that 16-E8/E2 mRNA levels are lower than the comparatively abundant levels of major early E6/E7 mRNA. As no promoter has been clearly defined for E8 or E2 expression, we engineered a target plasmid that contained three E2 cistrons (E2x3 SVE cat or E2-SV40) to quantify the ability of 16-E8/E2 to directly repress the replication of a heterologous ori driven by the large Tag of SV40. The consensus E2 binding site affinities in the SV40 construct are similar to the E2 binding site affinities in HPV-16 (57). Increasing amounts of pCG (16)E8/E2 repressed the replication of E2-SV40 (Fig. 6B, lanes 2 to 5). In contrast, the replication of the SV40 ori plasmid lacking E2 sites was unaffected by the addition of 16-E8/E2 (Fig. 6B, lanes 7 to 10). A construct expressing only

FIG. 4. cis elements regulate 16-E8/E2 expression. Equimolar quantities of HPV-16 E8-defective (plasmid A) and E8-expressing (plasmid B) plasmid species were cotransfected into SCC13 cells as illustrated in the complementation scheme. Total DNA was harvested, and the total replication from both plasmid A and B species was monitored by Southern blotting after appropriate digestion. E1−, E8−, and E2 TAD− indicate mutations in the respective genes, while SA409 refers to the indicated splice acceptor signal at nt 409. T(65) refers to mutation of the TATAA box at nt 65, enh− refers to the mutated keratinocyte-dependent enhancer, and E2#1+2− and E2#3+4− indicate mutations in the respective E2 binding sites. A linearized HPV-16 genome (30 pg) was included as a positive blotting control.

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<th>Plasmid A: HPV-16 E8-</th>
<th>Plasmid B: HPV-16 E1/E2 TAD-</th>
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<td>P-E8</td>
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<td>EB ORF</td>
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Primers (with 5′ ends, as in Fig. 5A at nt 1071, nt 1120, and nt 1142) amplified fragments from the competimer control (Fig. 6B, lanes 2, 7, and 11) with approximate mobilities of 540, 492, and 465 bp, respectively. Amplification of the cDNA alone generated fragments spanning the 1302-3358 splice junction with approximate mobilities of 422, 374, and 347 bp (Fig. 6B, lanes 4, 9, and 13). The ratio of the cDNA-competimer mixtures (Fig. 6B, lanes 3, 8, and 12) was quantified by scanning densitometry. The greater the ratio, the more cDNA was present in the original mixture. Since the cDNA aliquots were from the same mixture, the ratios of PCR products derived from one set of primers can be compared to those of another. The primer at nt 1126 to 1142 amplified 20-fold more cDNA than the primer at nt 1099 to 1029 and >100-fold more than the primer at nt 1051 to 1071 (Fig. 5B, compare lane 12 with lanes 3 and 8). These data show that the 16-E8/E2 spliced product extends upstream to just before nt 1126, indicating that there is a potential promoter or, alternatively, a splice acceptor, in this region.

Taken together, our cistron-mapping data suggest that a transcript encoding the 16-E8/E2 gene product is expressed independently from the major early gene promoter from a putative start site upstream of the E8 ORF and that limiting levels of this message are present in keratinocytes harboring persistently replicating HPV-16 plasmids.

HPV-16 E8′E2 represses initial plasmid amplification. To confirm that 16-E8′E2 represses initial HPV-16 plasmid amplification, we transfected the pCG (16)E8′E2 expressor together with intact HPV-16 wt or E8− mutant plasmid DNA. Increasing quantities of transfected pCG (16)E8′E2 repressed HPV-16 DNA amplification of either the wt or E8− mutant plasmids in a dose-dependent manner (Fig. 6A, lanes 1 to 4 and 5 to 8, respectively). Although 16-E8′E2 can interfere with early transcription factor synthesis by interfering with HPV-16 P97 transcription (Fig. 1H), the E8′E2 of HPV-31 has also been shown to repress the replication of plasmids with heterologous origins when E2 binding sites are present in cis. We sought to determine whether 16-E8′E2 could similarly modulate viral replication by the direct repression of ori function, independent of its transcription repression function.

We engineered a target plasmid that contained three E2 binding sites adjacent to the SV40 origin of replication (ori) (E2x3 SVE cat or E2-SV40) to quantify the ability of 16-E8′E2 to directly repress the replication of a heterologous ori driven by the large Tag of SV40. The consensus E2 binding site affinities in the SV40 construct are similar to the E2 binding site affinities in HPV-16 (57). Increasing amounts of pCG (16)E8′E2 repressed the replication of E2-SV40 (Fig. 6B, lanes 2 to 5). In contrast, the replication of the SV40 ori plasmid lacking E2 sites was unaffected by the addition of 16-E8′E2 (Fig. 6B, lanes 7 to 10). A construct expressing only
the DBD of E2 [pCG (16)E2 DBD], however, was not effective over the same concentration range as 16-E8∧E2, demonstrating that the E8 domain is required for the efficient inhibition of ori function (data not shown). These results show that, similarly to HPV-31, constitutively expressed HPV-16 E8∧E2 can repress the replication of a heterologous plasmid when E2 motifs are adjacent to the ori.

The pCG (16)E8∧E2 expression vector appears to efficiently produce 16-E8∧E2 in transiently transfected cells. However, constitutively expressed levels of 16-E8∧E2 in these cultures may be much higher than in cells containing replicating HPV-16 plasmid genomes expressing endogenous levels of 16-E8∧E2 from its native promoter(s). We therefore measured the SV40 Tag-dependent replication of the E2-SV40 plasmid in the presence of a replicating HPV-16 wt genome that expresses endogenous levels of 16-E8∧E2. The use of an E8-mutant in HPV-16 served as a no-16-E8∧E2 control. In cotransfections where wt HPV-16 is 95% repressed by endogenous levels of 16-E8∧E2, no repression of the E2-SV40 plasmid is observed (Fig. 6C, lane 4). This result demonstrates that while physiologic levels of 16-E8∧E2 can effectively repress initial HPV-16 plasmid amplification, they are not sufficient to repress E2-SV40 plasmid amplification. Since inhibition of replication from both plasmid species requires 16-E8∧E2 expression and cis E2 sites (Fig. 6A and B), this suggests that the interactions of the 16-E8∧E2 product with these sites are incomplete under these conditions. We conclude that 16-E8∧E2-dependent repression of the SV40 replication occurs via a mechanism that is distinct from 16-E8∧E2 repression of HPV-16 replication.

We then examined the effects of 16-E8∧E2 expression from its native promoter in trans on E1-dependent plasmid amplification in our refined complementation assays. As described in Fig. 4, cotransfection of two plasmid constructs, which individ-
ually fail to replicate, results in limited amplification of both plasmid species (Fig. 6D, lane 2). Endogenous expression of 16-E8∧E2 (plasmid A) is sufficient to inhibit the amplification of both the E8-defective (plasmid B) and the E8-expressing (plasmid A) species (Fig. 6D, lane 2). Mutation of the E8 ORF is sufficient to derepress plasmid amplification from both plasmids in this system (Fig. 6D, compare lanes 2 to 5). The mutation of the proximal E2 binding sites (sites 1 and 2) in plasmid B (the source of E1) results in an increase in the amplification of plasmid A due to the increased expression of E1 in trans (Fig. 6D, compare lanes 2 and 3). As we have shown in a parallel study (24a), mutation of the proximal E2 sites also impairs origin function in plasmid B (note the absence of the respective Dpn-resistant 5.4-kb band in Fig. 6D, lane 3). As a control, the simultaneous mutation of the E1 ORF totally abolishes replication, confirming that our complementation assay monitors E1-dependent activity (Fig. 6D, lane 4). In the absence of 16-E8∧E2, mutation of the proximal E2 binding sites (which abrogate E2 binding in vitro [57]) results in an even greater derepression (Fig. 6D, compare lanes 5 and 6). This suggests that the E2-TA may also repress E1 expression. We conclude that physiologic levels of 16-E8∧E2 are sufficient to repress expression of limiting levels of E1, resulting in inhibition of E1-dependent plasmid amplification.

16-E8∧E2 is not necessary for plasmid persistence and maintenance. We quantitatively compared the capacities of HPV-16 and HPV-31 wt and E8 mutant constructs to amplify initially after transfection into SCC13 cells (Fig. 7A). Both the HPV-16 E8− and HPV-31 E8− constructs amplified 7.5- to 19-fold more efficiently than their respective wt constructs (Fig. 6D, lane 4). In the absence of 16-E8∧E2, mutation of the proximal E2 binding sites (which abrogate E2 binding in vitro [57]) results in an even greater derepression (Fig. 6D, compare lanes 5 and 6). This suggests that the E2-TA may also repress E1 expression. We conclude that physiologic levels of 16-E8∧E2 are sufficient to repress expression of limiting levels of E1, resulting in inhibition of E1-dependent plasmid amplification.
FIG. 7. The HPV-16 E8 gene product modulates plasmid copy number. (A) SCC13 cells were transfected with HPV-31 and HPV-16 (wt and E8\(^{-}\) mutant) plasmids. Total DNA was harvested after 5 days and analyzed by Southern blotting. Replication activities from three HPV-16 transfections and a representative experiment with HPV-31 E8\(^{-}\) were quantified by scanning densitometry and normalized to the respective wt levels. Linearized HPV-16 or HPV-31 genomes were included as positive blotting controls. (B) HPV-16 or HPV-31 (wt and E8\(^{-}\) mutant) constructs were cotransfected with an RSV-neo plasmid into primary HFKs. Colony-forming efficiencies are expressed as increases above mock transfection levels with RSV-neo alone and represent averages of three independent transfections. (C) Total cellular DNA from clonal HPV-16 and HPV-31 HFK cultures was harvested, digested with “single-cutter” or “no-cutter” enzymes, and analyzed by Southern blotting. DNA forms: C, concatameric; OC, open circle; L, linearized; and S, supercoiled.
7A, lanes 3 and 8, respectively). The HPV-31 and HPV-16 E1– constructs were included as replication-defective, negative controls (Fig. 7A, lanes 2 and 7), while titrations of linearized HPV-16 and HPV-31 genomic fragments were used as quantitative controls (Fig. 7A, lanes 4, 5, 9, and 10, respectively). These data demonstrate that the initial amplifications of both HPV-16 and HPV-31 (as previously reported [61]) are similarly repressed by an E8 gene product.

We then analyzed the capacities of the HPV-16 and HPV-31 wt and E8– mutant constructs to immortalize primary HFKs in three independent long-term transfections, using two distinct donor foreskins (Fig. 7B). Interestingly, the HPV-16 E8– construct initially formed colonies after transfection into HFKs with an efficiency comparable to that of the HPV-16 wt genome. The HPV-31 E8– construct, however, was nearly threefold less efficient at initial HFK colony formation than the HPV-31 wt and, similar to mock (neo)-transfected keratinocytes, did not generate clonal cultures with extended life spans. Clonal cultures derived from primary HFKs transfected with either HPV-16 wt or E8– plasmids exhibited extended life spans (>30 population doublings) and stably replicating plasmids. The HPV-16 E8– cultures, however, were found to contain elevated extrachromosomal plasmid levels, with an average of 91 copies/cell versus 12 copies/cell with keratinocytes immortalized with the HPV-16 wt plasmid (Table 1), as measured by Southern blotting (Fig. 7C). This shows that 16-E8/E2 negatively modulates the HPV-16 copy number but is not required for plasmid maintenance in HPV-16-immortalized keratinocytes.

Growth rates, expressed as population doublings in days, of the HPV-16 E8–/HFK clonal cultures (2.4 days) were distinct from those observed in HPV-16 wt/HFK clonal cultures (2.0 days) or uninfected primary keratinocytes (3.0 days) (Table 1). These data confirm that, while both HPV-16 and HPV-31 wt and E8– mutant plasmids could initially amplify in transient-transfection assays, not all constructs were capable of successfully immortalizing primary keratinocytes. An HPV-31 plasmid with a similar mutation in the E8 ORF (in agreement with previous results [52]) was not able to persist and extend the life spans of keratinocytes, as no persisting cultures harboring this plasmid construct were obtained. The HPV-16 E8 mutant genome, however, was able to persist as an extrachromosomal plasmid.

**Discussion**

This study has defined the 16-E8’E2 gene product as a critical negative modulator of early gene expression and initial plasmid amplification in HPV-16. 16-E8’E2 is transcribed independently of the major early E6/E7 promoter, P97. We have not, however, ruled out the possibility of 16-E8’E2 expression from alternate promoters producing alternately spliced mRNAs and potentially employing cryptic splice sites, which could be differentially regulated during the viral life cycle. The levels of 16-E8’E2 mRNA appear to be limiting in comparison to the major early E6/E7 transcripts which originate from P97, as measured by RNase protection assays (data not shown). Using complementation assays, we examined the effect of cis mutations on 16-E8’E2-dependent repression of initial plasmid amplification. Modulation of 16-E8’E2 expression, however, varies from regulatory mechanisms controlling expression of E6/E7 (22) or E1 (24a). Limiting levels of 16-E8’E2 expression are partially supported by conserved E2 binding sites and the keratinocyte-dependent enhancer, which have been shown to repress or activate P97 transcription and to modulate plasmid amplification.

**16-E8’E2-dependent repression of early viral gene transcription.** We observed 16-E8’E2-dependent repression of early gene transcription from E2-dependent reporter plasmids and an extrachromosomal HPV-16 plasmid. In the context of the intact HPV-16 genome expressing the full complement of viral gene products at endogenous levels, we noted 16-E8’E2-dependent transcriptional repression of the major early promoter P97 in a transiently transfected keratinocyte cell line.
(SCC13). These results demonstrated that 16-E8’E2 repressed early gene transcription, independent of its inhibition of HPV replication. The precise mechanism of this transcriptional repression, however, remains to be defined.

In BPV, E2 repressor isoforms inhibit the full-length E2-TA by forming E2 repressor/E2-TA heterodimers capable of displacing the E2-TA homodimers at their cognate binding sites (reviewed in reference 25). Any of the BPV E2 isoforms may also displace cellular transactivators bound to overlapping motifs (49). Similarly, the 16-E8’E2 gene product could conceivably compete with the full-length HPV-16 E2-TA gene product at the HPV-16 E2 sites, or cellular transactivators binding to overlapping motifs (3, 56), and thus inhibit E2-dependent transactivation of early gene expression via displacement. Alternatively, 16-E8’E2 bound to its cognate sites could interfere with cellular trans-acting factors via quenching. These results support a model of early viral gene expression where cellular trans-acting factors binding to the HPV-16 URR could drive immediate-early expression of HPV gene products, like E1 and E2, which are critical for initial plasmid amplification, and E6 and E7, which are critical for extended growth of the infected keratinocyte host. As the initial amplification of the HPV plasmid progresses, sufficient levels of 16-E8’E2 would be expressed to form a feedback loop, reducing subsequent viral gene expression and plasmid replication as viral persistence is established.

16-E8’E2-dependent repression of initial plasmid amplification. Using complementing mutant HPV-16 genomes, we have shown that expression of a viral trans-acting factor from the defined 16-E8’E2 cistron represses initial plasmid amplification. Since constitutive 16-E8’E2 expression similarly repressed the replication of HPV-16 and an SV40 origin plasmid only if it was linked in cis to E2 binding sites, 16-E8’E2 appeared to directly inhibit the replication machinery via interaction with the E2 binding sites in cis. This mechanism of direct interference with cellular replication factors at the HPV-16 origin by 16-E8’E2 appears to be similar to the repression of an Epstein-Barr virus ori by HPV-31 E8’E2 (61) or E2F-dependent repression of an SV40 ori (50).

The full-length E2 isoform (E2-TA) interacts with cellular replication factors to modulate HPV replisome assembly and function (4, 32, 33). 16-E8’E2 could inhibit initial plasmid amplification by disrupting the initial assembly of the replicating complex and recruitment of necessary cellular replication factors by forming dysfunctional heterodimers with the full-length E2 protein. Alternatively, a recent study indicates that the HPV-31 E8’E2 is capable of modulating transcription and replication by interacting with cellular corepressors (2).

In this study, however, when physiologic levels of 16-E8’E2 are expressed from a cotransfected wt HPV plasmid, inhibition of the SV40 ori construct is not observed while HPV-16 plasmid amplification is fully inhibited. If the target of replication inhibition were a common cellular replication factor, similar repression levels of both replicating plasmid targets should be observed. This suggests that the endogenous 16-E8’E2 levels may be too low to adequately occupy the E2 sites on the cotransfected SV40 plasmid. Since the E2 sites introduced into the SV40 plasmid are similar in E2 binding activity to the HPV-16 E2 sites, this suggests that low endogenous levels of 16-E8’E2 during initial amplification are also insufficient to directly interfere with the replication machinery.

In a parallel study, we have shown that E2 gene products can inhibit expression of limiting levels of the E1 replicase, consequently resulting in the inhibition of initial HPV-16 plasmid amplification (24a). However, increased expression of E1 was able to restore higher plasmid amplification levels in the presence of endogenous levels of 16-E8’E2; this suggests that the primary mechanism of 16-E8’E2 repression of HPV-16 replication is a result of 16-E8’E2 inhibition of E1 expression. This is consistent with a model of modulation of HPV replication via the regulated expression of limiting levels of critical viral replication factors (e.g., E1) in the early stages of infection, as proposed for HPV-31 (21).

Taken together, these results show that 16-E8’E2 can repress early viral gene expression, independent of its effects on the replication machinery. Conversely, 16-E8’E2 can directly inhibit plasmid replication, independent of its ability to repress transcription. However, in a setting where physiologic levels of 16-E8’E2 can inhibit both transcription and replication, it is 16-E8’E2-dependent repression of E1 expression that determines initial HPV-16 plasmid amplification levels. As HPV-16 infection progresses from an establishment to a maintenance (or viral persistence) phase, 16-E8’E2 could similarly repress expression of the viral replication factors E1 and/or E2 contributing to subsequent stabilization of the extrachromosomal viral load in HPV-immortalized host cells. Integration of the HPV-16 genome frequently disrupts the E2 ORF and is thought to deregulate expression of the E6 and E7 viral transforming genes. Such an integration pattern would also disrupt expression of the E8’E2 gene product and its repression of viral gene expression.

16-E8’E2 modulation of plasmid copy number. 16-E8’E2 also plays a role in the maintenance phase of the viral life cycle by limiting the levels of persistently replicating extrachromosomal HPV-16 plasmids. The E8’- mutant of HPV-16 replicates at elevated levels compared to the HPV-16 wt, indicating loss of a replication inhibitor, but it reaches a stable plasmid copy number as opposed to the “runaway replication” observed in SV40 infection. Thus, complex mechanisms regulating persistent HPV replication appear to be distinct from those modulating the replication of other DNA tumor viruses (28, 55). This HPV replication phenotype could reflect the exhaustion of limiting viral or cellular factors. Alternatively, HPV-16 replication could be modulated by an accumulation of other viral gene products which could antagonize replisome assembly of, for example, an E2-TA product or additional undefined HPV E2 isoforms. However, in contrast to BPV, where the robust expression of a truncated E2 transcription repressor (E2-TR) plays a critical role in viral transcription and replication, no evidence of transcripts capable of encoding an equivalent HPV-16 E2-TR isoform has been documented in the HPV-16 genome. In addition, mutation of putative ATG start sites within the E2 ORF, capable of encoding a potential 16-E2-TR gene product, resulted in no measurable effect on initial HPV-16 plasmid amplification (data not shown).

The phenotypes of PV-immortalized cell lines containing mutations within the E8’E2 ORFs vary among different PV types that have been investigated to date. In contrast to HPV-16, BPV plasmids containing a mutation in the E8 ORF gen-
erated C127 cell lines with plasmid copy levels similar to those observed with the BPV wt construct. Mutations in the E2-TR, a truncated isoform of E2, exhibited 10- to 20-fold-higher copy numbers of BPV-1 plasmids in derived C127 cell lines (26, 39) than the wt. The E9-E2 protein of CRPV is a homologue of E8-E2 in mucosal HPVs. Jeckel et al. reported that ORF mutants in the CRPV E9 were able to form tumors with replicating CRPV at the same rate and copy number as the wt DNA, suggesting that E9-E2 is not required for persistent replication (23). HPV-31, while sharing sequence homologies with HPV-16, also displays a different phenotype than does HPV-16 when its E8 ORF is mutated. As in previous reports, we noted that HPV-31 E8-mutant genomes displayed increased initial plasmid amplification in transiently transfected cells but were unable to persistently replicate in long-term cultures (52). Although we cannot formally rule out the possibility of leaky 16-E8′E2 expression from the HPV-16 E8-construct, the nucleotide substitution within the 16-E8 ORF is identical to that described for the homologous HPV-31 E8 ORF mutation (61), rendering this explanation inadequate to account for the disparate and reproducible phenotypes observed.

Why does the HPV-16 E8-mutant generate clonal, immortalized HFK cultures with persistently replicating plasmids while the E8-mutant of HPV-31 does not? Perhaps it is due to the threefold-greater baseline plasmid amplification observed with the HPV-31 wt than with the HPV-16 wt (Fig. 7A). The higher replication level of the HPV-31 E8-mutant plasmid may be toxic to host cells and might exceed a critical threshold in the initial stages of the HPV life cycle, triggering potential host defenses to elevated viral DNA or an accumulation of viral polypeptides. The elevated HPV-16 E8 ′copy number observed in our clonal cultures, however, may not exceed this critical threshold, as it is stabilized at its new higher level, indicating that further increases in viral load are limited by an undetermined mechanism. Therefore, HPV type-specific modulation of viral load in the establishment phase of infection could play a role in the greater prevalence of HPV-16 in both asymptomatic infections and HPV-associated cancers.

There may also be additional, as yet unidentified, differences between the replication machineries of HPV-16 and HPV-31 that further modulate plasmid persistence. The disparity between E8′E2 functions in HPV-16 and HPV-31 in the early stages of the HPV life cycle is consistent with observations that HR HPV types have evolved as unique viruses, with diverse properties determining their distinct pathogenicities and prevalences in HPV infection. These results underscore the need to independently examine the viral strategies of establishment and persistence of various HPV types.

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