Human Papillomavirus Type 31b E1 and E2 Transcript Expression Correlates with Vegetative Viral Genome Amplification

Michelle A. Ozbun1 and Craig Meyers2

Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

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Human papillomavirus (HPV) genome replication is dependent on the expression of E1 and E2 proteins. The organotypic (raft) culture system was used to investigate changes in viral early gene expression and vegetative genome replication during the complete life cycle of HPV type 31b (HPV31b). We have previously shown the synthesis of HPV31b viral particles as early as 10 days of growth of CIN-612 9E raft tissues (Ozbun, M. A., and Meyers, C. (1997). J. Virol. 71, 5161–5172). In the present study, we investigated the structures and temporal expression levels of HPV31b E1 and E2 transcripts, as well as the replication of the viral genome during the viral life cycle. The amplification state of the HPV31b genome was maximal at 10 days of raft tissue growth. Furthermore, the expression levels of E1 and E2 RNAs correlated with vegetative viral DNA replication. Levels of E1- and E2-specific transcripts were dissimilar throughout the viral life cycle. E2 RNA levels remained relatively constant, whereas E1 RNA levels were upregulated during the maximal amplification of viral genomes and the biosynthesis of virions. These data indicate that E1 may be the major regulator of viral genome amplification in preparation for DNA packaging and virion morphogenesis. © 1998 Academic Press

INTRODUCTION

Human papillomaviruses (HPVs) are small DNA viruses that infect surface epithelia and cause both benign and malignant lesions (Broker and Botchan, 1986; zur Hausen, 1991). The subgroup of HPVs that produce lesions with a high probability of progression to malignancy are termed high-risk viruses; HPVs that rarely progress to invasive cancer are known as low-risk viruses. The high-risk HPV types that give rise to cervical cancers include types 16, 18, 31, 33, and 45; HPV types 6 and 11 are examples of low-risk viruses (Lorincz et al., 1992). The capacity of the viruses to cause tumors (or warts) can be partially attributed to their ability to establish a persistent infection characterized by the maintenance of episomal viral genomes in their host. However, progression to malignancy is generally associated with the integration of the viral genome into the host cell DNA (Cullen et al., 1991).

The complete HPV replication cycle resulting in the production of virions (i.e., infectious progeny) is tightly linked to the differentiation state of the infected cells (Taichman and LaPorta, 1987; Meyers et al., 1992, 1997). According to a current model, the establishment of a persistent papillomavirus infection occurs when the virus enters a basal cell through a microabrasion in the epithelial architecture (White et al., 1963; Broker and Botchan, 1986). Viral persistence is set up through the maintenance of extrachromosomal viral genomes at 50–200 copies per infected cell on cellular division (Broker and Botchan, 1986). During epithelial differentiation, HPV genomes are amplified to levels of 1000±10,000 copies per cell (Taichman and LaPorta, 1987; Bedell et al., 1991). In the upper, differentiated layers of the epithelium, late gene products are expressed, and together, late proteins and amplified viral DNA (vDNA) presumably lead to the morphogenesis of progeny virions (Meyers et al., 1992, 1997; Frattini et al., 1996).

The majority of data on papillomavirus genome replication are derived from transient assays using bovine papillomavirus type 1 (BPV1) (reviewed by Lambert, 1991). A number of recent studies have shown that HPVs share many of the BPV1 replication activities. The origin of HPV DNA replication is located in the upstream regulatory region (URR) of viral genomes just 5′ to the E6 open reading frame (ORF) (Del Vecchio et al., 1992). Jointly, the HPV E1 and E2 viral gene products are necessary for the replication of vDNA (Chiang et al., 1992; Del Vecchio et al., 1992; Lu et al., 1993; Frattini and Laimins, 1994b). HPV E1 is a nuclear phosphoprotein that binds ATP and has DNA helicase activity (Bream et al., 1993; Hughes and Romanos, 1993). The E1 protein binds to a conserved A/T-rich sequence within the minimal origin of HPV DNA replication (Chiang et al., 1992; Del Vecchio et al., 1992; Frattini and Laimins, 1994b). E2 is also a nuclear protein and can activate transcription by...
binding as a dimer to the E2 binding site (E2BS), a conserved sequence of ACCGN_4CGGT (Androphy et al., 1987; McBride et al., 1989; Bream et al., 1993). Four consensus E2BSs are located in the URR of HPV genomes; one E2BS lies just 3' to the A/T-rich E1 binding site in the viral replication origin. HPV E2 complexes with E1 and can stimulate E1-mediated replication (Bream et al., 1993; Frattini and Laimins, 1994a; Frattini and Laimins, 1994b). The affinity of E1 binding to the replication origin is increased by E2 proteins and requires an adjacent E2BS (Frattini and Laimins, 1994a). In addition, alterations in E1:E2 ratios affect the pattern of replication factors binding to the viral origin in vitro (Frattini and Laimins, 1994a). However, the mechanisms underlying the regulation of E1 and E2 expression and vegetative vDNA replication during the viral life cycle are poorly understood.

To investigate the regulation of vDNA replication by the E1 and E2 gene products, we used the organotypic, or raft, culture system, which supports the complete HPV life cycle (Meyers et al., 1992, 1997). Using the cervical intraepithelial neoplasia (CIN)-612 9E cell line, which maintains ~50 copies of HPV31b per cell, we characterized transcripts potentially encoding the E1 and E2 proteins. In addition, we correlated the expression of these transcripts with the amplification of vDNA during the process of epithelial differentiation and virion biosynthesis in raft tissue cultures.

RESULTS

Temporal and spatial amplification of HPV31b DNA during stratification of CIN-612 9E epithelium in the organotypic culture system

The CIN-612 9E cell line in monolayer culture maintains the HPV31b genome episomal at an average of ~50 copies per cell (Bedell et al., 1991; Hummel et al., 1992). To determine the onset and extent of HPV31b viral DNA (vDNA) amplification in the raft tissue culture system, CIN-612 9E epithelial cells were grown as raft tissues as described in Materials and Methods. Because protein kinase C (PKC) induction has been shown to increase the differentiation of HPV-infected tissues in the raft system (Meyers et al., 1992, 1997; Ozbun and Meyers, 1996, 1997, 1998), the rafts were treated with the synthetic diacylglycerol 1,2-diocanoyl-sn-glycerol (C8:0) every other day to induce the PKC pathway. Rafts were harvested every second day for 16 days (days 2±16). Total DNA was harvested from the raft tissues, digested with restriction enzymes, and analyzed by agarose gel electrophoresis and Southern (DNA) hybridization for HPV31b vDNA. A comparison with DNA copy number standards indicated that the 2-day rafts contained an average of ~200 copies of HPV31b DNA per cell (data not shown). Viral DNA (vDNA) amplification continued to increase at day 4, peaked at about day 10, and then began to decrease (Fig. 1). The peak in vDNA at day 10 coincides with our previous data showing the presence of intranuclear HPV31b viral particles as early as day 10 in CIN-612 9E raft tissues (Ozbun and Meyers, 1997). We found it curious that the levels of vDNA declined after day 10; however, these results were reproduced in at least four separate experiments using similar extraction conditions. We considered the possibility that as the rafts continued to grow and stratify, they contained more cell layers with a larger fraction of cells no longer supporting vDNA replication. We reasoned that the analysis of DNA from a set number of cells (i.e., a whole raft or a fraction thereof) might alleviate problems with increasing cell numbers during the stratification of the raft tissues. However, the amounts of total DNAs obtained from raft tissues were remarkably equivalent from day 4 to day 16. Thus, adjustment of the amounts of DNA analyzed by Southern blotting accordingly did not significantly alter the results compared with those shown in Figure 1.

To investigate the spatial and temporal vegetative replication of HPV genomes during the viral life cycle, in situ hybridizations were performed on tissue sections of CIN-612 9E rafts harvested at various time points. We previously reported that CIN-612 9E raft tissues reach maximal stratification and differentiation by day 12 in the organotypic tissue culture system (Ozbun and Meyers, 1997). In situ hybridization for HPV31b vDNA in CIN-612 9E tissues harvested at day 4 gave moderate hybridization signals in a few nuclei, whereas most nuclei showed weak or no hybridization (Fig. 2E). As the raft tissues continued stratification and differentiation to day 8, more nuclei were found to show strong hybridization signals with the HPV31 probe (Fig. 2F). Moreover, the strong hybridization signals indicating amplification of HPV31b genomes were observed throughout the tissue and in all strata of the epithelia. By days 12 and 16, the CIN-612 9E tissue
tissues maintained strong HPV31 hybridization signals in many nuclei (Figs. 2G and 2H). However, unlike the 8-day raft tissues, which showed hybridization in most cells, the 12- and 16-day raft tissues showed increasing areas with no hybridization signal. To more effectively illustrate that the number of cells supporting viral DNA amplification diminished after 8 days, the total number of positive cells within a defined, equivalent area of each cross section was counted. Areas were counted on eight different sections of each time point, and the data are presented in Table 1. Thus, the in situ hybridization data are in agreement with the Southern blot hybridization data showing an increase in vDNA content in the tissues through day 10 in the raft system and then decreasing levels of vDNA thereafter. In situ hybridization of the HPV31 probe to CIN-612 9E monolayer cells gave weak-to-moderate signals in most all of the nuclei (Fig. 2L). 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than 4.2 kb was also observed in 12- and 16-day rafts using both probes (Fig. 3, lanes 4 and 5). In addition to the 4.2-kb transcript, the E2 probe detected a 2.4-kb RNA species in all of the CIN-612 9E samples (Fig. 3B). In the CIN-612 9E raft tissues, the 2.4-kb E2 RNA species followed the same general expression pattern as did the 4.2-kb E2 RNA species. The highest levels of the 2.4-kb E2 ORF-containing RNAs were observed in CIN-612 9E monolayer cells and in rafts harvested at day 12 (Fig. 3B, lanes 1 and 4, respectively). The E1-specific probe also detected a transcript of 2.4 kb in rafts harvested at 8, 12, and 16 days (Fig. 3A, lanes 3±5, respectively) but not in monolayer cells or 4-day raft tissues (Fig. 3A, lanes 1 and 2, respectively).

Cloning and sequencing of E1 and E2 gene transcripts expressed during the HPV31b life cycle

The structures of the HPV31b early gene transcripts were determined by RNA PCR amplification and sequence analysis. Total RNAs were extracted from CIN-612 9E monolayers and rafts, were treated with DNAse I, and were subjected to RNA PCR using primers specific for the early region of HPV31b (Fig. 4). After PCR amplification, cDNA products were cloned and sequenced. Three novel cDNAs corresponding to spliced transcripts containing the early gene ORFs of HPV31b were identified; one product corresponding to an unspliced transcript was observed. The use of primers E6 5’ and E1 3’

![Diagram](image-url)

FIG. 4. cDNA-derived HPV31b sequences from PKC-induced CIN-612 9E tissues. Total or poly(A) RNA was extracted from CIN-612 9E raft tissues at day 12 and monolayer cultures. RNA (1 μg) was subjected to reverse transcription, and the cDNAs were amplified by PCR with the primer pairs indicated (Table 1). (Top) Results of dideoxy sequencing of cloned PCR-amplified cDNA products. Below the sequence data is a schematic of the HPV31b genome arbitrarily linearized following the late polyadenylation signal (A+). HPV31b nt numbering is based on the sequence of HPV31 (Goldsborough et al. 1989). The major ORFs are shown as open boxes, and the upstream regulatory region (URR) is denoted. Bent arrows indicate the constitutively expressed promoters P77, P99, and P3320, and the differentiation-specific promoter P342 (Hummel et al., 1992; Ozbun and Meyers, 1998). The early and late polyadenylation sites (A+) at nt 4138±4143 and 7227±7231, respectively, are shown. Restriction sites important for cloning (HincII at nt 1003) and for genome linearization (XbaI at nt 4998) are given. Placement of oligonucleotide primers used for PCR analyses are shown by arrows. (A±D) The transcripts are predicted to initiate at both P77 and P99 promoters (Ozbun and Meyers, 1998) and to end at the early poly(A)+ site (A+). The thin, dotted lines mark regions removed by splicing; open and stippled boxes represent ORFs; and thick lines are noncoding regions. (Right) The ORF coding potential of the transcript. (A) RNA PCR using primers E6 5’ and E1 3’ gave a 595-bp partial cDNA containing the 36* splice and the E1*I splice (top). The construct containing this cDNA was designated pCR31b-E69E. (B) Primers E6 5’ and E1 3’ gave rise to an RNA-PCR product of ~800 bp corresponding to a partial cDNA containing E6,E7,E1*I,E2 ORFs. (C) RNA PCR using primers E6 5’ and E1±2 3’ resulted in a 2080-bp partial cDNA containing the ORFs E6* and E7 and the 5’ region of E1. The construct containing this cDNA was designated pCR31b-E6E1±2b. (D) Primers E6 5’ and E1±2 3’ gave an RNA PCR product of ~2300 bp corresponding to a partial cDNA containing the unspliced E6,E7,E1 ORFs.
resulted in the amplification of a product of 595 bp (Fig. 4A). Sequencing revealed this partial cDNA to be made up of the E6* ORF, the E7 ORF, and a splice from the E1 donor at nucleotides (nt) 877 to nt 2646 upstream of the E2 start codon at nt 2693. The latter splice results in the truncation of the E1 ORF to a 10-amino-acid fusion peptide designated E1*I. We recently reported the structure of this transcript as one that initiates from the P77 promoter (Ozbun and Meyers, 1998). RNA PCR amplification on total and polyadenylation-selected [poly(A)+] RNA preparations from CIN-612 9E cells reproducibly resulted in a product of ∼800 bp corresponding in size to transcript B in Figure 4, a potential transcript containing the ORFs of E6*, E7, and the 5′ region of E1. RNA PCR amplification using the same primers on total and poly(A)+ RNA preparations from HPV-negative epithelial raft tissues of SCC-13 cells yielded no products (data not shown). Transcripts A and B (Fig. 4) are predicted to initiate at either or both of the P77 and P99 promoters (Ozbun and Meyers, 1998). Continuation of these spliced RNAs through to the HPV31b early poly(A)+ site at nt 4138±4143 would yield transcripts ∼2.4 kb in length depending on the extent of mRNA polyadenylation. This is in agreement with the Northern blot analyses showing an E2 ORF-specific transcript of ∼2.4 kb (Fig. 3B). The use of primers E6 5′ and E1±2′ resulted in the amplification of a product of 2080 bp (Fig. 4C). Sequencing revealed this partial cDNA to contain the ORFs of E6*, E7, and the 5′ region of E1. RNA-PCR amplification using either total or poly(A)+ RNA preparations from CIN-612 9E cells reproducibly yielded a product corresponding in size to transcript D in Figure 4. This transcript is predicted to contain the E6,E7,E1 ORFs. Because this product is predicted not to contain any spliced sequences, we cannot rule out that amplification was from contaminating vDNA and not from viral RNA. Again, RNA PCR amplification using primers E6 5′ and E1±2′ on total and poly(A)+ RNA preparations from SCC-13 cells failed to yield products of similar sizes (data not shown). Viral RNAs depicted in Figures 4C and 4D are also predicted to initiate at either or both of the P77 and P99 promoters (Ozbun and Meyers, 1998), and continuation through to the early poly(A)+ site would yield polyadenylated transcripts of ∼4.2 kb. These RNAs would also contain the E2 ORF, and the data concur with the Northern blot analyses showing E1 and E2 ORF-specific transcripts of ∼4.2 kb (Fig. 3). Using the primer E1 5′ and various downstream primers shown in Figure 4, we found no evidence for additional E1 ORF-containing transcripts that might correspond to the ∼2.4-kb E1 transcript detected by Northern blot analysis (Fig. 3A).

Quantification of E1 and E2 RNAs expressed during the viral life cycle

To quantify the relative changes in temporal E1 and E2 mRNA expression among CIN-612 9E untreated monolayer cultures and PKC-induced rafts harvested at various time points, samples were analyzed by ribonuclease (RNase) protection assays (RPAs) using antisense RNA probes specific to internal regions of the E1 and E2 ORFs (Fig. 5C). The E1-specific riboprobe protected the expected 337-nt fragment of RNA (Fig. 5A), whereas the E2-specific riboprobe protected a 260-nt fragment, as predicted (Fig. 5B). For both probes, the RPAs gave patterns of temporal RNA levels consistent with the results of the Northern analyses. Monolayer cultures of CIN-612 9E cells were found to express both E1 and E2 RNAs (Figs. 5A and 5B, lane 1). Furthermore, E1 and E2 transcript levels peaked at day 12 in PKC-induced CIN-612 9E rafts (Figs. 5A and 5B, lane 4). The relative changes in the levels of the E1 and E2 transcripts were determined by densitometric scanning (Fig. 5D). The results indicated that the levels of E1 and E2 RNAs were similar in undifferentiated monolayer cells and in partially differentiated 4-day raft tissues. However, E1 transcripts showed a stronger peak in expression over that of the E2 RNAs at 12 days in differentiating raft tissues supporting the HPV31b life cycle. These RPA data were representative of several analyses. The bands of ∼200 and ∼120 nt seen in the sample lanes appeared to be due to E1 probe degradation because they were visible in the probe lane on longer exposure of the gel (Fig. 5A).

RPAs using cDNA-derived probes were performed to determine the temporal expression pattern of specific spliced viral mRNAs during PKC-induced differentiation of the raft epithelial tissues. Total RNAs from PKC-induced rafts were harvested at intervals after lifting to the air-liquid interface; representative results of multiple experiments are shown in Fig. 6. The cDNA clones obtained from RNA PCR and illustrated in Figures 4A and 4C served as templates for antisense RNA probes for RPAs. The results verified that the temporal expression patterns of the spliced E1 and E2 RNA species were similar to the results obtained by Northern analysis (Fig. 3) and to the results obtained by RPA using probes internal to the E1 and E2 ORFs (Fig. 5). The antisense riboprobe of 705 nt made from the E6*,E7,E1 template (Fig. 6C) had the potential to detect and quantify both species of E1 ORF-containing transcripts shown in Figure 4 (transcripts C and D). Specific protection of the E6*,E7,E1 transcript showed a protection of a 682-nt fragment as expected (Fig. 6A). This indicated that the E6*,E7,E1 transcript was expressed in a temporal fashion similar to that observed by Northern and internal RPA analyses (Figs. 3A and 5A, respectively). The protection of an unspliced E6,E7,E1 transcript by the same probe was expected to be 591 nt in length. However, no protection of this size was seen, indicating that the E6,E7,E1 RNA species was not a major constituent of E1 ORF-containing transcripts (Fig. 6A). As expected, the E6*,E7,E1 riboprobe detected two versions of transcripts using the E1 splice donor: E6*,E7,E1* and E6,E7,E1* (Fig. 6A). These sequences are present on the 5′ ends of many spliced HPV31b transcripts,
including those containing the E2 ORF (Figs. 4A and 4B) (Ozbun and Meyers, 1998), those containing the E1–E4 fusion ORF (Hummel et al., 1992; Ozbun and Meyers, 1997), and numerous late gene RNAs (Ozbun and Meyers, 1997). Thus, it is not surprising that these protected fragments were detected in greater quantities than the E6*,E7,E1 transcripts. An E7,E1*I,E2 antisense riboprobe of 276 nt (Fig. 6C) was created to detect and quantify the spliced species of E2 ORF-containing transcripts shown in Figure 4 (transcripts A and B). Specific protection of the E7,E1*I,E2 transcript produced a 169-nt protection as expected (Fig. 6B). Temporal expression followed the same pattern observed for E2 transcripts by Northern analysis (Fig. 3B) and by internal E2 ORF RPA (Fig. 5B). As anticipated, strong 131-nt protections corresponding to transcripts containing E7,E1^ were observed. Densitometry scanning of the protected fragments of the spliced E1 and E2 transcripts gave results similar to those obtained from the internal E1- and E2-ORF RPAs shown in Figure 5D (data not shown).

HPV types 6 and 11 use a differentiation-dependent promoter in the E7 ORF to initiate a subset of E1 ORF-containing transcripts (Chow et al., 1987; DiLorenzo and Steinberg, 1995). The analogous promoter in HPV31b is P74 (Hummel et al., 1992; Ozbun and Meyers, 1997). Using primer E1±4 3′ (Table 1, Fig. 4) in primer extension assays, we detected a minor population of E1-specific transcripts initiating at P74 in RNA samples from differentiating raft tissues but not RNAs from monolayer cells (data not shown). Nucleoside S1 and exonuclease VII protection assays confirmed these observations but showed that >90% of the E1 RNAs initiated at the P77/P99 promoters (data not shown). An E1 transcript initiating at P74 and continuing through the E1 ORF to the early poly(A) site would be predicted to be ∼3.4 kb in length. E1 and E2 RNAs of this approximate size can be seen below the arrow, indicating the 4.2-kb transcripts in the Northern blot samples of 12- and 16-day rafts (Figs. 3A and 3B, lanes 4 and 5, respectively). Primer extension analyses using primers E7 3′, E1±4 3′, and E4 3′ (Table 1, Fig. 4) suggested the presence of a 5′ RNA end at HPV31b nt 834. However, nuclease protection assays using RNases, nucleoside S1, and exo-nuclease VII provided no supportive data for an RNA start site in this area (data not shown).

Correlation of E1 and E2 transcript levels with vDNA amplification

The increase in E1 and E2 mRNA synthesis between 8 and 12 days coincided with the peak in vDNA amplification.
tion in the differentiating raft tissues (Fig. 1). The correlation between E1 and E2 transcript levels and vDNA amplification during the viral life cycle was investigated. The data were compared from densitometry scanning of the autoradiogram of a Southern blot of linearized vDNA from rafts harvested at various time points (as in Fig. 1; data not shown) and from the autoradiograms of the quantitative RPAs shown in Figures 5 and 6. As the ratio of E1 transcripts to E2 RNAs increased, vDNA was found to be amplified (Fig. 7). The ratio of E1 to E2 transcripts was greatest between days 8 and 12, corresponding to the peak in vDNA replication observed at day 10 in the raft tissues.

DISCUSSION

We investigated the amplification of HPV31b vDNA and characterized the structures and temporal expression patterns of E1 and E2 transcripts during the complete viral life cycle using the organotypic (raft) tissue culture system. We found the raft tissue culture system faithfully mimics important morphological and biochemical aspects of epithelial differentiation (Ozbun and Meyers, 1996, 1997) and references therein). Our careful optimization and maintenance of tissue growth in the raft system result in reproducible histological and quantitative molecular data. Moreover, we are consistently able to purify high levels of total RNA from 4–16-day rafts (≥100 µg/raft). PKC-induced CIN-612 9E tissues undergo a program of stratification and differentiation by day 12 in the raft system, morphologically resembling the in vivo tissue from which the cells were originally derived (Ozbun and Meyers, 1997). Furthermore, by day 12, the CIN-612 9E raft tissues are fully able to support the complete HPV life cycle as assayed by the ability to detect vDNA amplification, late gene transcripts, capsid proteins, and viral particles (Bedell et al., 1991; Meyers et al., 1992; Ozbun and Meyers, 1997, 1998). Using a cell line that maintains episomal copies of HPV18, we recently demonstrated the purification of infectious viral particles from raft tissues harvested at day 12 (Meyers et al., 1997). We analyzed monolayer cultures in parallel with raft tissues as a point of reference. However, our previous analyses of temporal raft epithelial differentiation and HPV31b late gene transcripts indicated that monolayer cells are not analogous to basal cells either morphologically or biochemically (Ozbun and Meyers, 1997). In addition, we point out that 2–4-day raft tissues with their dermal equivalent (i.e., the fibroblast–collagen matrix) and three-dimensional growth are structurally and morphologically akin to basal cells. This is the first investigation of temporal HPV genome amplification and E1 and E2 transcript expression during the differentiation-dependent viral life cycle. We report that amplification of vDNA peaks near day 10 as the ratio of E1 to E2 transcripts is greatest during the in vitro propagation of HPV31b.

PKC-induced CIN-612 9E raft tissues were analyzed at various times during growth in the raft system. Initially, we found it surprising that vDNA levels increased up to days 10±12 and then began to drop. We expected the genome levels to either keep increasing from day 2 to day 16 or to peak and remain constant as viral particles assembled and accumulated in the keratinized tissues. We increased the detergent or reducing agent concentrations, or both, in the DNA extraction buffer with the goal of releasing vDNA from particles potentially trapped in the keratin bundles of the suprabasal cells. However, this approach failed to yield an increase in vDNA extraction (data not shown). In situ hybridization was used to
analyze the levels of HPV31b genomes in CIN-612 9E monolayers and raft tissues. The copy number average of ~50 genomes per cell was readily detected in monolayer cells. Spatial analysis by in situ hybridization indicated that nearly every nuclei gave evidence of vDNA amplification in CIN-612 9E 8-day raft tissues. Conversely, raft tissues harvested after 8 days showed increasing areas of cells that lacked vDNA amplification in their nuclei, with many nuclei showing no evidence of the basal number of ~50 copies per cell. The focal amplification of vDNA in certain cells with other groups of cells showing no vDNA was also observed in 14-day rafts from another clonal cell line containing episomal copies of HPV31b (CIN-612±5) (Bedell et al., 1991). These investigators suggested that there might be preferred regions of viral amplification. Our data showing amplification of vDNA in most nuclei of CIN-612 9E raft tissues at day 8 suggests that the high levels of vDNA once present in most cells are lost, suppressed, or otherwise inaccessible to the techniques used. The phenomenon of decreasing HPV activities in raft tissues after days 8±12 seems to be a general one, as we also have observed decreased levels of early gene transcripts (present report), late gene transcripts (Ozbun and Meyers, 1997), and expression from viral promoters (Ozbun and Meyers, 1998). Thus, our findings suggest the general decrease in vDNA amplification in the raft tissues after day 10 may be a function of gradual and subtle viral breakdown in the raft system and not necessarily one of viral mandate. There appears to be some dependence of vDNA amplification on cellular differentiation as monolayer and 4-day raft tissues do not fully support vDNA amplification (present study; Bedell et al., 1991). Nonetheless, we and others have shown that undifferentiated basal epithelial cells support vDNA amplification (present study; Bedell et al., 1991; Frattini et al., 1996), indicating that vegetative viral DNA amplification is not completely differentiation dependent. Although there are clinical lesions that show no detectable HPV vDNA in the basal or lower suprabasal cells, there are also cases in which HPV vDNA was readily detected in these lower strata and throughout the epithelial strata (Gruendorf and zur Hausen, 1979; Koss, 1987; Schneider et al., 1987; Valles et al., 1987; Tase et al., 1989; Cooper et al., 1991). Therefore, it appears more likely that virion assembly is differentiation dependent because the viral genomes are packaged by late proteins, which are expressed only in the differentiated suprabasal layers of the epithelium (Meyers et al., 1992; Frattini et al., 1996).

Levels of both E1 and E2 transcripts were maximally expressed at day 12 in the raft tissues, albeit the E1 RNAs were induced to a greater extent than the E2 RNAs. These analyses give no information on the spatial expression patterns of E1 and E2 RNAs throughout the epidermal tissues. However, in situ hybridization techniques localized HPV11 E1 and E2 transcripts to the nuclei of basal and suprabasal cells with a greater concentration of the RNAs in the upper, differentiated layers of the epithelium (Stoler et al., 1989; Dollard et al., 1992). Furthermore, HPV11 E1 transcript levels by in situ hybridization appeared to be higher than E2 RNA levels in raft tissues (Dollard et al., 1992).

Structural analyses of the high-risk HPV31b transcripts provided a number of similarities as well as many differences with low-risk HPVs. We detected four transcripts with the potential of encoding the E2 ORF and two transcripts containing the E1 ORF. The HPV31b transcripts illustrated in Figure 4 are structurally similar to HPV11 E1 and E2 ORF-containing transcripts (Chow et al., 1987; Rotenberg et al., 1989), with a distinction being that low-risk HPV types do not use a splice within the E6 ORF that yields the E6* ORF (Smotkin et al., 1989). We found no evidence for splice junctions analogous to those reported in the HPV11 E1M*E2C or E1M,E2C transcripts (Chiang et al., 1991). Based on RNA PCR and Northern blot analyses, we conclude that the HPV31b transcripts initiate at the P77 or P99 promoters, or both, and are polyadenylated at the early poly(A)+ site (Ozbun and Meyers, 1998). Low-risk HPVs use a differentiation-dependent promoter in the E7 ORF to initiate a subset of E1 ORF-containing transcripts (Chow et al., 1987; DiLorenzo and Steinberg, 1995). However, we found only a very minor subset (<10%) of HPV31b E1 ORF-contain-
might account for such an E1 transcript. This species. However, we characterized no cDNAs that we do not believe these to be the same transcript scripts (E6,E7,E1*I,E2 and E6*,E7,E1*I,E2) do not contain the E1 probe failed to recognize this in both the raft tissues and the monolayers. Because the E1 probe failed to recognize this ~2.4-kb transcript in the monolayers and because the ~2.4-kb E2 transcripts (E6,E7,E1*I,E2 and E6*,E7,E1*I,E2) do not contain any of the sequences recognized by the E1 probe, we do not believe these to be the same transcript species. However, we characterized no cDNAs that might account for such an E1 transcript. This ~2.4-kb RNA species likely contributes substantially to the increase in total E1 ORF-containing transcripts found in the differentiating raft tissues and also may be important for E1 protein synthesis. The short E1*I ORF terminates 30 bp upstream of a good translational consensus sequence preceding the E2 ORF. Analyses of HPV11 transcripts indicated that functional E2 proteins were efficiently synthesized from E6,E7,E1*I,E2 (also called E6,E7,E2) but not from E6,E7,E1,E2 RNAs in which the E1 ORF overlaps the E2 start codon (Rotenberg et al., 1989).

In this study, we found that maximal vDNA amplification in the raft system occurred when the ratio of E1 to E2 transcripts was increased by ~3:1. Sverdrup and Khan (1994) showed that optimal replication of HPV18 origins occurred at a 5±1:1 ratio of E1 to E2 expression plasmids using transient assays. These data do not address the issue of E1 and E2 protein levels in the cells. However, results from footprinting assays on the minimal HPV31b replication origin indicate that E1 binds to nt 7905±24, a region with a 10-bp inverted repeat 5’ to a high-affinity E2BS at nt 38±49 (Frattini and Laimins, 1994b). The size of the E1-protected region suggests that multiple E1 proteins might bind as a complex to the sequence, whereas E1/E2 complexes bind to E2BSs (Frattini and Laimins, 1994b,1994a). In addition, transfection of E1 expression vectors into CIN-612 9E monolayer cells (which we showed to contain similar levels of E1 and E2 RNAs; see Fig. 5D) was found to increase viral genomic copy number ~3-fold (Frattini and Laimins, 1994a). Together, these findings are in accord with a model in which vegetative HPV vDNA amplification occurs as the protein ratio of E1 increases over that of E2 during the later stages of the viral life cycle. Interestingly, it was recently demonstrated that there is a switch from the semiconservative theta structure mode of HPV16 viral genome replication to the rolling circle mode of viral replication during epithelial differentiation of WI2-E cells in the raft system (Flores and Lambert, 1997). It is tempting to speculate that the increased ratio of E1 to E2 proteins may contribute to this mechanistic switch.

MATERIALS AND METHODS

Cell and rat tissue culture

The CIN-612 9E cell line is a clonal line established from a cervical intraepithelial neoplasia grade I biopsy (Bedell et al., 1991) that maintains ~50 episomal copies of HPV31b per cell (Hummel et al., 1992). The HCK181Bj cell line is a clonal line that maintains episomal copies of HPV18 at ~100 copies per cell (Meyers et al., 1997). The SCC-13 cell line was established from a squamous cell carcinoma of the facial epidermis and is HPV negative (Rheinwald and Beckett, 1981). Epithelial cells were cultured in E medium with mitomycin C-treated murine J2 3T3 fibroblast feeder cells (Meyers, 1996). Organotypic (raft) tissue cultures for in vitro differentiation were maintained as previously described (Meyers, 1996; Ozbun and Meyers, 1996, 1997). Epithelial cells were seeded onto collagen matrices containing J2 3T3 fibroblasts submerged under E medium. When the epithelial cells had grown to confluence, the media were removed, and the collagen matrices were lifted onto stainless steel grids. Subsequent feeding of the epithelium was via diffusion of E medium from below the matrix. Epithelial tissues were allowed to stratify and differentiate at the air-liquid interface over a 16-day period. Rafts were treated with 10 μM 1,2-dioctanoyl-sn-glycerol (C8:0; Sigma Chemical Co., St. Louis, Missouri) in E medium every other day. Raft tissues were harvested at various time points beginning with the second day after being lifted to the air-liquid interface (day 2) and extending to day 16 after being lifted.

Nucleic acid extractions

Total cellular DNA was harvested by incubating raft tissues for 3 h at 55°C in 10 mM Tris±Cl, pH 7.5, 25 mM EDTA, 0.2% SDS, 100 mg/ml proteinase K, and 50 mg/ml RNase A. The DNAs were sheared by passage 10 times through an 18-gauge needle. The solution was extracted twice with an equal volume of phenol±chloroform±isoamyl alcohol (25:24:1) and then extracted once with an equal volume of chloroform±isoamyl alcohol (24:1). The DNAs were ethanol precipitated using 0.3 M sodium acetate. Total RNAs were extracted from rafts, and monolayer cells were extracted with TRizol Reagent (GIBCO BRL, Bethesda, Maryland). RNA samples were treated with deoxyribonuclease (DNase) I to remove copurifying viral and cellular DNA (Ozbun and Meyers, 1997, 1998).
RNA concentrations were based on optical densities; concentrations were verified by electrophoresis through agarose gels and staining with ethidium bromide.

Southern (DNA) and Northern (RNA) blotting and hybridization

Total cellular DNA samples (5 μg) were either digested with BamHI, which does not cut in the HPV31b genome, or with XbaI, which linearizes the HPV31b genome at nt 4998. Total cellular DNA samples were separated on 0.8% agarose gels; total RNA samples (20 μg) were separated on 1% agarose±0.66 M formaldehyde gels as described (Ausubel et al., 1995). The nucleic acids were transferred to GeneScreen Plus membranes (New England Nuclear Research Products, Boston, Massachusetts), which were handled according to the manufacturer's instructions. pBS-HPV31 was digested with EcoRI to release the complete HPV31 genome, and the HPV31 sequences were purified from plasmid sequences by agarose gel electrophoresis and gene cleaning (Bio101, Vista, California). The E1 ORF probe was made from plasmid pCR31b-E1 (see below) and includes HPV31b nt 1290±2400. The E2 ORF-specific probe was synthesized from pCR31-E2 and contains HPV31 nt 2880±3279. The radioactive labeling of DNA sequences and the hybridizations were carried out as previously described (Ozbun and Meyers, 1997). Membranes were washed to remove nonspecific hybridization and then exposed to Reflection film with intensifying screens (DuPont NEN).

In situ hybridizations

Harvested rafts were fixed in 10% buffered Formalin and embedded in paraffin, and 4-μm cross sections were prepared. Thin sections on slides were incubated at 55°C for 1 h followed by two 3-min rinses in xylene to remove residual paraffin. The sections were rehydrated in graded ethanol and then incubated in phosphate-buffered saline (PBS). Monolayer cells were grown in slide chambers, and subconfluent cells were fixed in 10% buffered Formalin for 10 min; the slides were subsequently processed identically to the tissue sections. The tissues were denatured in 0.02 M HCl for 10 min, rinsed in 0.01% Triton X-100 in PBS for 90 s, and washed twice in PBS for 3 min each. The sections were digested with 100 μg/ml of proteinase K in TE buffer for 10 min at 37°C and then neutralized in a solution of 2 mg/ml glycine in PBS for 5 min. Control sections were pretreated with either 550 units/ml of RNase-free DNase I or 10 mg/ml RNase A. The slides were washed in cold 20% acetic acid for 15 s and then allowed to air dry for 15 min. Fluorescein-labeled probes were synthesized, and in situ hybridizations were performed using the DNA color kit (Amersham Life Sciences, Buckinghamshire, UK) with minor modifications. Probes of HPV31, HindIII-digested lambda DNA, or total CIN-612 9E DNA were mixed with hybridization buffer, and 40-μl aliquots were applied over the tissue sections. The probe solutions and sections were covered with polyethylene coverslips and were denatured on a dry block surface at 100°C for 10 min. The samples were incubated in a humidified box at 42°C for 16±20 h. Stringency washes and detection of specific hybridizations were performed as recommended by the manufacturer. The tissue sections were counterstained with eosin Y and visualized by light microscopy.

RNA PCR analyses, cloning, and sequencing

DNase I-treated total RNAs were reverse transcribed using oligo(dT) 16 or random hexamer primers, and PCR was performed using the GeneAmp RNA PCR kit according to the manufacturer's directions (Perkin-Elmer, Branchburg, New Jersey). All PCR primers (Table 1) were synthesized by Operon Technologies (San Diego, California) and were used at 0.5 μM. The thermocycling profile was as follows: 4-min time delay at 94°C; 35 cycles including 94°C for 30 s, 58±60°C for 1 min, and 72°C for 2 min; and a 15-min extension at 72°C. PCR products were cloned using the TA cloning kit (Invitrogen, San Diego, California), pCR31b-E1 contains a 1111-bp fragment of the HPV31b E1 ORF generated by PCR using primers E1±2 5’ and E1±2 3’ on cDNA sequences from CIN-612 9E tissues, and it contains HPV31b E6±E7±E1±I sequences (Fig. 4A). pCR31b-E79E was produced by cloning of the 272-bp PCR product generated with primers E7 5’ and E1 3’ on cDNA from CIN-612 9E tissues, and it contains HPV31b E7±E1±I sequences. The samples were incubated in a humidified box at 42°C for 16±20 h. Stringency washes and detection of specific hybridizations were performed as recommended by the manufacturer. The tissue sections were counterstained with eosin Y and visualized by light microscopy.
Nuclease protection assays and primer extension analyses

Radioactive antisense RNA probes were synthesized using the MAXI script/RPA II kit (Ambion Inc., Austin, Texas) as previously described (Ozbun and Meyers, 1996, 1997). pCR31b-E1 was digested with StyI, which yielded a 416-nt antisense probe predicted to protect 337 nt of the E1 ORF. pCR31b-E2 was digested with Dral, which gave a 339-nt riboprobe expected to protect 260 nt specific to the E2 ORF. pCR31b-E79E was digested with AccI, giving rise to a 248-nt riboprobe predicted to protect 169 nt of the E7/E1/E2 transcript and 132 nt corresponding to the E7/E1 region. EcoRI digestion of pCR31b-E6*E1 yielded a 705-nt antisense probe. This riboprobe was predicted to protect 682 nt from the E6/E7/E1 transcript, 592 nt from an unspliced E6,E7,E1 transcript, 556 nt from the E6*,E7*,E1* region, and 465 nt of transcripts containing the region E6,E7,E1*. Probes were purified and hybridizations were performed as previously reported (Ozbun and Meyers, 1996, 1997). Samples were analyzed by electrophoresis through 5% polyacrylamide:7 M urea gels followed by autoradiography. RNA Century standards were prepared as per the manufacturer's recommendations (Ambion Inc.). The intensity of protected fragments was measured by scanning laser densitometry. Nuclease S1 and exonuclease VII protection assays and primer extension analyses were performed as previously described (Ozbun and Meyers, 1997, 1998).

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