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Differentiation-Induced Changes in Promoter Usage for Transcripts Encoding the Human Papillomavirus Type 31 Replication Protein E1

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The life cycle of human papillomaviruses (HPVs) is tied to keratinocyte differentiation. One key event in the viral life cycle is the differentiation-dependent increase in viral replication. This increase in replication activity results in an amplification of the HPV genome from approximately 50 copies per cell in basal keratinocytes to thousands of copies of the viral genome per cell in suprabasal keratinocytes. To characterize the events associated with this differentiation-dependent increase in HPV replication, we have initiated studies of mRNAs encoding the HPV replication protein E1 during the differentiation of cell lines that stably maintain episomal HPV DNA. Differentiation induced the expression of several transcripts that hybridized to an E1-specific probe. One of these messages, a 3.7-kb transcript, did not hybridize to a probe specific for the early promoter upstream of the E6 open reading frame. RNase protection analysis confirmed an induction of unspliced messages derived from the differentiation-dependent promoter at nucleotide 742 in the middle of the E7 open reading frame. These observations demonstrate a differentiation-induced increase in E1 mRNAs derived from the viral late promoter and suggest a role for increased E1 expression during amplification of the HPV genome.

INTRODUCTION

Human papillomaviruses (HPVs) cause hyperproliferative lesions in differentiating epithelia. Of the more than 70 different types of HPV characterized to date, approximately one-third are specific for anogenital epithelia. A subset of the genital-specific viruses are referred to as "high risk," which induce lesions of the cervical epithelium and are the etiologic agents of cervical cancer (zur Hausen and Schneider, 1987).

The HPV life cycle is tied to the keratinocyte differentiation program (Laimins, 1993). Virions are thought to infect the basal layer of the epithelium via microscopic lesions. In the basal cells, the HPV genome is established at approximately 50 episomal copies per cell. HPV establishment and maintenance are associated with expression of early HPV RNAs, and these RNAs encode the oncoproteins E6 and E7 as well as the replication proteins E1 and E2. These early messages are transcribed from the early promoter, which is designated P97 in HPV31 (see Fig. 1). As the infected basal keratinocytes divide, daughter cells then begin to differentiate, and the late HPV functions are induced, which culminate in the formation of HPV virions. Among these late events is the expression of late mRNAs, which variously encode the E1^E4, E5, L1, and L2 open reading frames (ORFs) and which are induced from the differentiation-specific promoter located in the E7 ORF (Hummel *et al.,* 1992). Furthermore, the HPV genome is amplified during differentiation to thousands of copies per cell. Although each of these events has been characterized during differentiation, the temporal relationship between these late events in the HPV life cycle is not understood.

Replication of the HPV genome requires the E1 and E2 proteins. E1 and E2 can support replication of HPV11 in a cell-free system (Liu *et al.,* 1995), and both are also required for transient replication of minimal origins of several HPV types as well as bovine papillomavirus (for review, see Laimins (1998)). Finally, the stable maintenance of BPV-1 and HPV31 requires intact E2 binding sites (Piirsoo *et al.,* 1996; Stubenrauch *et al.,* 1998). It is believed that E2 protein serves as a tether and recruits E1 to the origin. Once bound to the origin, E1 protein likely facilitates DNA unwinding by virtue of its helicase and ATPase activities (Bream *et al.,* 1993; Hughes and Romanos, 1993).

In previous studies, we demonstrated that most HPV31 transcripts are spliced, polycistronic RNAs. Many of these transcripts utilize the major splice donor within E1 (nt 877) in conjunction with the major splice acceptor within E4/E2 (nt 3295; Fig. 1). Studies using monolayer cultures of HPV31b-immortalized 612 cells have shown that the transcript encoding the complete E1 open reading frame is approximately 4.2 kb (Hummel *et al.,* 1992). This mRNA is thought to originate from the early promoter, P97, and terminate at the early polyadenylation site. It is not clear whether this 4.2-kb message includes

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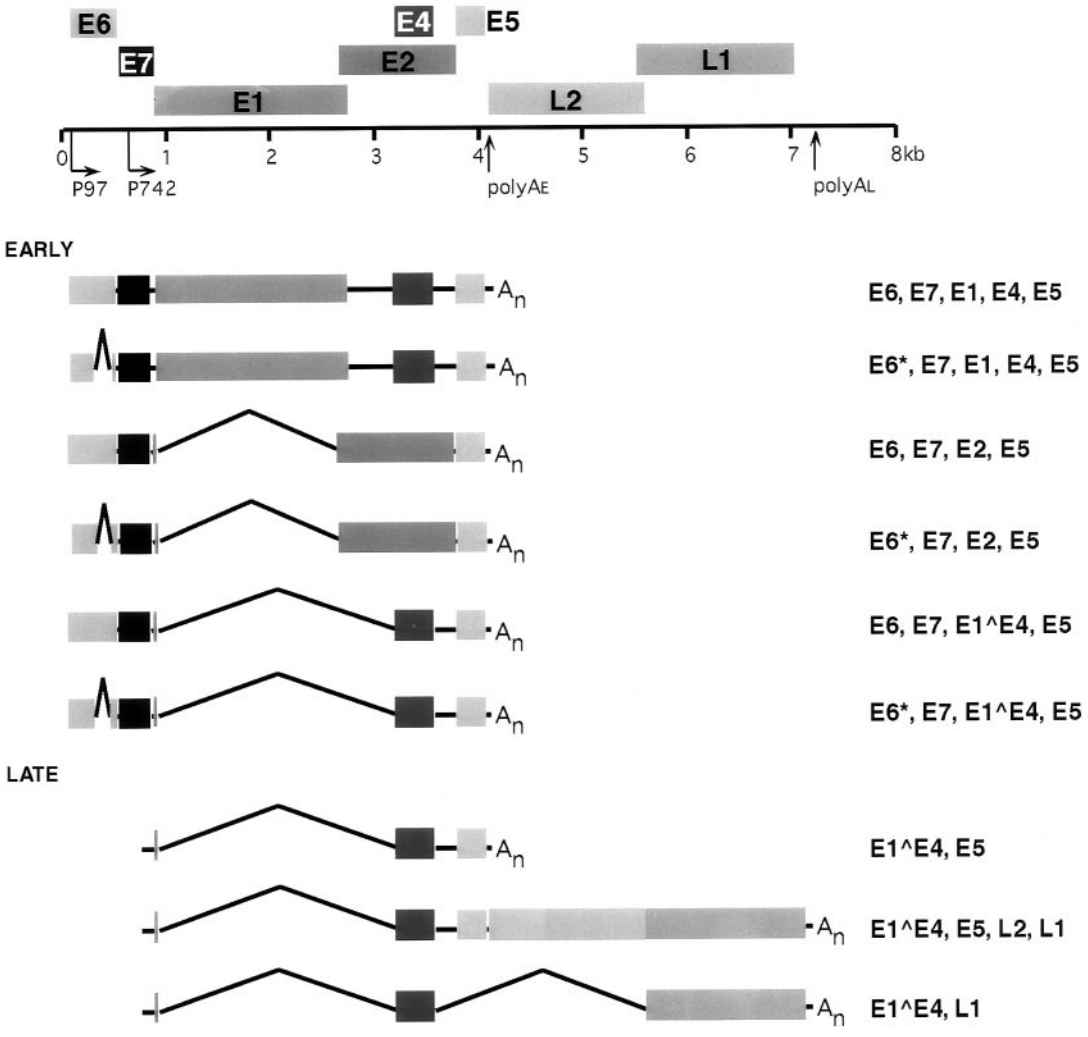

FIG. 1. Previously characterized transcripts expressed in HPV31b-immortalized CIN 612 cells. Transcripts were previously characterized (Hummel *et al.,* 1992, 1995) from CIN 612 cells maintained in monolayer cultures (Early) or induced to differentiate during growth in raft cultures (Late).

an intact E6 ORF or is spliced to form a truncated E6 ORF (E6*). It has been suggested that some E1 messages initiate directly upstream of E1, but if such messages exist, they represent a minority of the E1 transcripts in monolayer cultures (Hummel *et al.,* 1992, 1995). Still, little is known about E1 messages and how their expression may vary during differentiation.

To examine the messages that encode HPV31 E1 during differentiation, we have characterized the transcripts expressed in monolayer cultures and in cultures suspended in semisolid medium. Suspension of HPV-immortalized keratinocytes in semisolid medium has recently been shown to rapidly induce late HPV31 functions including activation of P742 (the differentiation-induced promoter of HPV31), E1^E4 protein accumulation, L1 mRNA accumulation, and genome amplification (Ruesch *et al.,* 1998). Many studies of the HPV life cycle have been performed in organotypic raft cultures that contain both undifferentiated and differentiated layers that complicate analysis of HPV late functions. In contrast, suspension in methylcellulose leads to the coordinate differentiation of large numbers of keratinocytes and, thus, presents a simplified approach to specifically study induction of late functions.

RESULTS

Differentiation induces HPV transcripts. To characterize the transcripts encoding the HPV31 E1 ORF induced during differentiation, we used two cell lines, CIN 612 cells and L31 cells. CIN 612 cells were derived from a cervical lesion and have previously been shown to maintain episomal HPV31b genomes (Bedell *et al.,* 1991; Rader *et al.,* 1990). We also generated an HPV31-immortalized keratinocyte line *in vitro,* L31, by transfection of cloned, circular genomes into human primary foreskin keratinocytes as previously described (Frattini *et al.,* 1996). To confirm the presence of stable viral episomes in CIN 612 and L31 cells, we performed Southern analysis. Southern analysis confirmed the stable mainte-

nance of episomal copies of HPV in L31, similar to those in 612 cells, as shown by the presence of supercoiled HPV DNA (Fig. 2). The digestion of L31 DNA with *Xba*I, however, gave rise to multiple bands. These likely represent either partial digestion with *Xba*I or the presence of some cells in the mass culture that contained integrated copies of HPV31 DNA. Nonetheless, the majority of HPV 31 DNA in the L31 cell line exists as viral episomes.

Northern analysis of total RNA was then used to characterize the HPV31 E1 transcripts expressed in keratinocytes during monolayer culture and following suspension culture in semisolid medium (Fig. 3). Suspension in semisolid medium for 16 to 24 h has been shown to induce differentiation-dependent late gene expression (Ruesch *et al.,* 1998). A probe derived from the E4 and E5 open reading frames that detects known HPV messages derived from the early region was used (nts 3278–4101; Klumpp *et al.,* 1997). This analysis of RNAs isolated from monolayer cultures of CIN 612 or L31 cells detected three major transcripts of approximately 1.3, 1.7, and 4.2 kb in size. These results were consistent with previous studies that characterized similar transcripts in monolayer cultures and raft cultures (Hummel *et al.,* 1992). The most abundant transcript expressed in monolayer cultures is the 1.7-kb message that initiates at the viral early promoter (P97) and encodes E6, E7, E1^E4, E5 ORF or E6*, E7, E1^E4, and E5 (band B). The 1.3-kb transcript (band C) encodes the E1^E4 and E5 ORFs and originates from the differentiation-specific promoter, P742; its appearance in monolayer cultures may correspond to low-level activity of P742 due to spontaneous differentiation within a subset of monolayer cells. The 4.2-kb transcript (band A) is thought to represent unspliced P97 messages encoding all early genes including a complete E1 ORF.

Following 20 h of suspension culture in methylcellulose medium, two major changes were detected in HPV transcripts (Fig. 3). As previously observed in both raft cultures (Hummel *et al.,* 1992) and growth in semisolid medium (Ruesch *et al.,* 1998), a 1.3-kb transcript was induced in both 612 and L31 cells. Growth in methylcellulose also induced a second change in HPV messages: differentiation-dependent transcripts of 3.7 and 3.4 kb were induced in CIN 612 RNA, and similar RNAs were detected from methylcellulose cultures of L31 cells. The 3.7-kb RNA was induced to levels significantly greater than the 4.2-kb message expressed in monolayer cultures (see below).

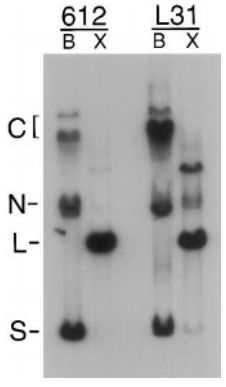

FIG. 2. CIN 612 cells and L31 cells maintain episomal HPV DNA. Southern analysis was performed on 10-μg samples of total keratinocyte DNA. DNAs were digested with *Bam*HI (B; does not cut within HPV31) or *Xba*I (X; cuts once within HPV31), and the blot was hybridized to a probe generated from the complete HPV31 genome. Bands corresponding to supercoiled (S), full-length linear (L), nicked open circular (N), and concatameric (C) HPV DNA are indicated.

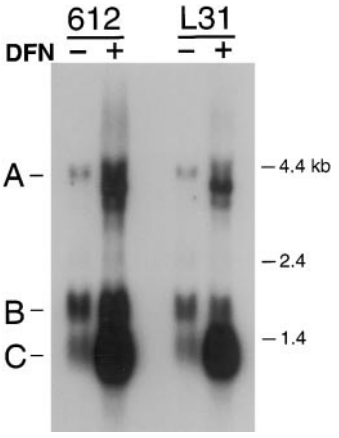

FIG. 3. Differentiation induces multiple HPV transcripts. CIN 612 and L31 total RNAs were isolated from monolayer cultures (−DFN) or cultures induced to differentiate (+DFN) by culture in semisolid medium for approximately 20 h. Northern analysis was performed with an E4/E5 probe using 10 μg of each RNA sample. Bands corresponding to transcripts of approximately 4.2 kb (A; unspliced E1), 1.7 kb (B; E6,E7,E1^E4,E5), and 1.3 kb (C; E1^E4,E5) are indicated.

Differentiation induces E1 messages. The size of the 3.7-kb transcript suggested that this message may retain an intact E1 open reading frame. To characterize the differentiation-induced transcripts of 612 cells, we performed Northern analyses with a probe specific for HPV messages initiated at P97 as well as with a probe specific for the E1 ORF (Fig. 4). As noted above, the most abundant transcript in monolayer cultures of HPV-immortalized cells is 1.7 kb in size, and this message is generated by utilizing the major splice donor at nt 877 and the major acceptor at nt 3295 (Hummel *et al.,* 1992). Messages encoding E1 necessarily retain this intron for the integrity of the complete E1 ORF. We designed an E1 probe (nts 1282–2616) such that it hybridized to the intron sequences and would not hybridize to known E2 messages (see Fig. 1). In monolayer CIN 612 cells, only the 4.2-kb transcript hybridized to this E1-specific probe (Fig. 4B). In 612 cells cultured in methylcellulose, however, we detected a family of bands with the E1 probe: the major transcript detected was approximately 3.7 kb, and less abundant transcripts of 4.2, 3.4, and 2.5 kb were also observed. Similar transcripts were also observed in L31 cells cultured in semisolid medium (not shown).

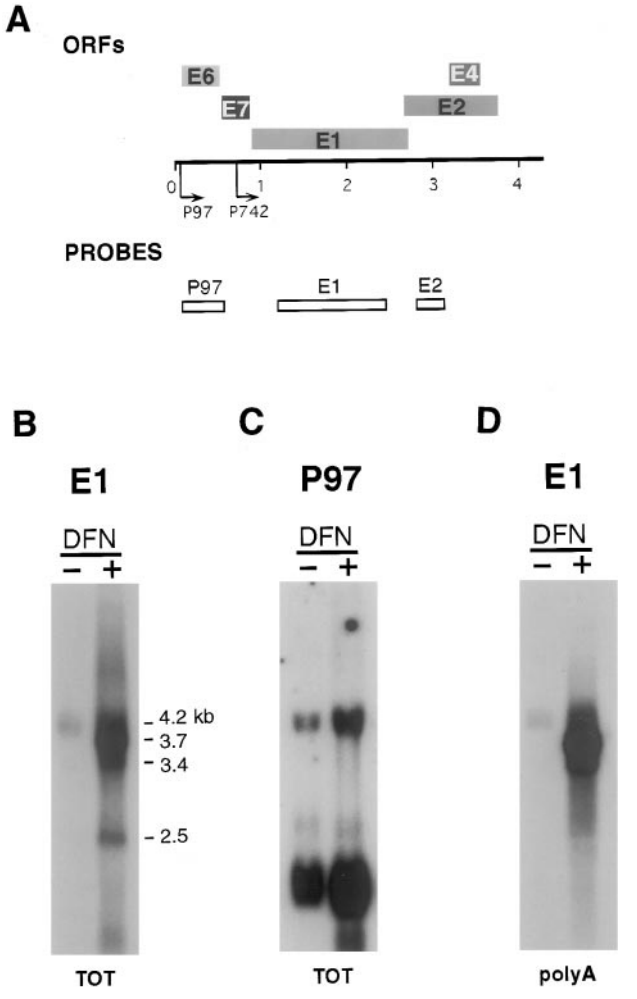

FIG. 4. E1 mRNAs are induced by differentiation. Northern blots of CIN 612 RNA were analyzed with probes (A) specific for the E1 ORF (B) or a P97-specific probe (C). A blot of poly(A)$^+$ RNA was probed with the E1 probe used in A (D). RNA samples purified from monolayer or methycellulose suspension cultures are indicated as −DNF or +DFN, respectively. Lanes correspond to 10 μg of total RNA (B and C) or 3 μg of poly(A)$^+$ RNA (D).

To characterize the E1 transcripts induced during culture of CIN 612 cells in semisolid medium, we next analyzed the same Northern blot with an E6E7 probe that was designed to detect only transcripts originating from the early promoter, P97 (nts 99–664; Fig. 4C). The E6E7 probe hybridized to the 1.7- and 4.2-kb bands, indicating that these mRNAs were initiated from P97, but the E6E7 probe failed to bind the 3.7-, 3.4-, or 2.5-kb messages. Therefore, the 3.7-, 3.4-, and 2.5-kb RNAs induced during differentiation lack the 5′ sequences characteristic of RNAs transcribed from P97. Instead, the 3.7-, 3.4-, and 2.5-kb E1 RNAs were transcribed from a promoter further downstream, which was likely to be the differentiation-specific late promoter, P742.

To confirm that these differentiation-induced E1 RNAs initiated at P742 constituted functional messages, we isolated the poly(A)$^+$ RNAs expressed in CIN 612 cell cultures and examined them for transcripts encoding the E1 ORF (Fig. 4D). The poly(A)-containing E1 RNAs detected in methylcellulose cultures included the 4.2-, 3.7-, and 3.4-kb P742 transcripts. However, the 2.5-kb E1 RNA initiated at P742 that was observed in total RNA was not detected in poly(A)$^+$ RNA (compare Figs. 4B and 4D). This suggests that the 2.5-kb band is not a functional message and may correspond to improperly processed transcripts or, possibly, the intron excised from normally processed E1^E4, E5 messages. On the other hand, the 3.4-, 3.7-, and 4.2-kb bands appear to represent functional mRNAs. Furthermore, the 3.7- and 3.4-kb RNAs detected upon differentiation appear to encode novel E1 messages.

Our Northern analysis indicated that the 3.7-kb transcript was approximately 0.5 kb smaller than the unspliced E1 message initiated at P97 and is consistent with an unspliced E1 message initiated at P742. The 3.4-kb mRNA may represent a variant of the 3.7-kb message that is spliced, initiated at a downstream promoter, or terminated upstream of the early polyadenylation site (nt 4138). Both the 3.4- and the 3.7-kb messages were significantly induced during differentiation relative to the 4.2-kb E1 message. Quantitation of the bands in Fig. 4D by PhosporImage analysis was used to determine relative expression levels, although this analysis provided only an estimate of expression levels due to the limited resolution of the gel system. Despite these limitations, significant differences in induction were detected. Quantitation revealed that the 3.7-kb message expressed in semisolid cultures was increased 27-fold over the levels of the 4.2-kb message expressed in monolayer cultures, and the 3.4-kb message was induced 7.8-fold. The 4.2-kb transcript was induced 6-fold upon differentiation. The similar levels of induction observed for both the 4.2- and the 3.4-kb messages suggests that both RNAs may be initiated at P97. Alternatively, the 3.4-kb RNA may be initiated from the same promoter as the 3.7-kb message but may exhibit reduced RNA stability relative to the 3.7-kb RNA.

Differentiation-induced E1 mRNAs initiate at P742. In order to confirm the initiation site of the differentiation-induced E1 messages, RNase protection analysis was performed (Fig. 5). A probe that could discriminate between spliced and unspliced messages originating from either P97 or P742 was used (Klumpp *et al.,* 1997; Stubenrauch *et al.,* 1998); this probe spanned both P742 and the splice donor at nt 877 (see Fig. 5A, bottom). If transcripts encoding the complete E1 ORF were induced at P742, we expected to observe an increase in unspliced RNAs from that promoter. Differentiation of CIN 612 cells by culture in semisolid medium induced two classes of transcripts that originated from P742. The shorter class of transcripts utilize the splice donor at nt 877, whereas the longer transcripts are unspliced and represent messages encoding a complete E1 ORF.

The appearance of a series of protection products is consistent with the recent characterization of a family of closely spaced initiation sites near nt 742 (Ozbun and Meyers, 1997). By comparing the relative intensities of bands representing the spliced and unspliced P742 bands, we note that the apparent expression levels differ among RNAs initiated at the multiple transcriptional start sites. Since total RNA fractions were used for the RNase protection assay, this may reflect differential stability of RNAs that were not polyadenylated. Quantitation of the P742 unspliced protection products by PhosporImage analysis indicated that these messages were induced 6.8-fold following suspension culture. This moderate level of induction, compared with the approximately 27-fold increase observed by Northern analysis (see Fig. 4D), may also reflect the presence of nonpolyadenylated HPV messages in the total RNA fractions.

E2 messages are induced during differentiation. Since E1 binding to the replication origin is thought to be mediated by E2, E2 messages might be coordinately regulated with E1 transcripts during differentiation. To examine this possibility, we characterized the E2 messages expressed in monolayer and suspension cultures of CIN 612 cells (Fig. 6). A probe corresponding to nts 2708–3200 (see Fig. 4A) was used to detect E2 transcripts on Northern blots of both total and polyadenylated RNA pools. This probe necessarily cross-hybridized to E1 messages but was designed so as not to hybridize to messages containing a spliced E1^E4 ORF (see Fig. 1). The E2 probe detected a 2.5-kb transcript in RNA purified from semisolid cultures. A minor species of approximately 2.0 kb was also evident. The 2.5-kb transcript is consistent with the previously characterized E2 message of CIN 612 cells originating from P97 (Hummel *et al.,* 1992), whereas the 2.0-kb transcript may represent a novel, differentiation-induced E2 message possibly initiated at P742. Quantitation indicated that the 2.5-kb RNA was increased 6.5-fold in methylcellulose cultures. Therefore, E2 messages are also induced during differentiation but to a lesser extent than E1 mRNAs.

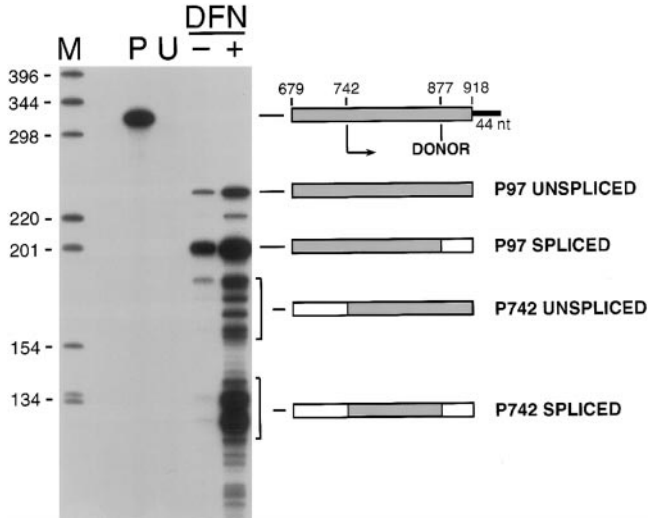

FIG. 5. Induction of E1 mRNAs detected by RNase protection. 10 μg of total CIN 612 RNA purified from monolayer (−DFN) or methylcellulose cultures (+DFN) was analyzed by RNase protection using a probe spanning both P742 and the major splice donor at nt 877. P indicates undigested probe, and U indicates digestion of an unprotected probe. Protection products corresponding to specific messages are indicated. 44 nt indicated in the probe structure corresponds to heterologous, vector-derived sequences.

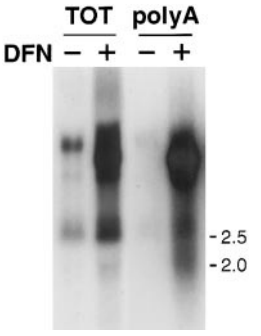

FIG. 6. Differentiation induces E2 transcripts. A Northern blot of CIN 612 RNA was analyzed with a probe specific for the E2 ORF (see Fig. 4A). RNA samples purified from monolayer or methylcellulose suspension cultures are indicated as −DNF or +DFN, respectively. Lanes correspond to 10 μg of total RNA or 3 μg of poly(A)$^+$ RNA. Autoradiograms of total and poly(A)$^+$ lanes were exposed differentially to aid interpretation.

DISCUSSION

In this study, we characterized the mRNAs encoding the HPV31 replication proteins E1 and E2 following suspension culture in semisolid medium. The transcription of HVP genes is dependent upon epithelial differentiation, and suspension culture of HPV cell lines has recently been shown to induce many key differentiation-dependent events in the HPV life cycle including induction of late gene expression and genome amplification (Ruesch *et al.,* 1998). In undifferentiated cells the primary mRNA encoding E1 is 4.2 kb in size and originates from the early promoter, P97. We found that epithelial differentiation induces the accumulation of a second E1-specific transcript of 3.7 kb that originated from the differentiation-specific promoter, P742.

While the 3.7-kb transcript is the major E1 message induced by culture in semisolid medium, multiple E1 RNAs were evident in Northern blots (see Fig. 3). The 3.4-, 3.7-, and 4.2-kb E1 messages indicate that HPV31 cell lines express a family of E1 RNAs. The 3.7- and 3.4-kb messages have not been reported previously, and our ability to detect them is presumably due to our use of the suspension culture technique. Several previous studies of HPV functions during differentiation have relied upon the raft culture system. Raft culture can induce HPV late functions including viriogenesis (Frattini *et al.,* 1997; Meyers *et al.,* 1992, 1997). However, only a minority of

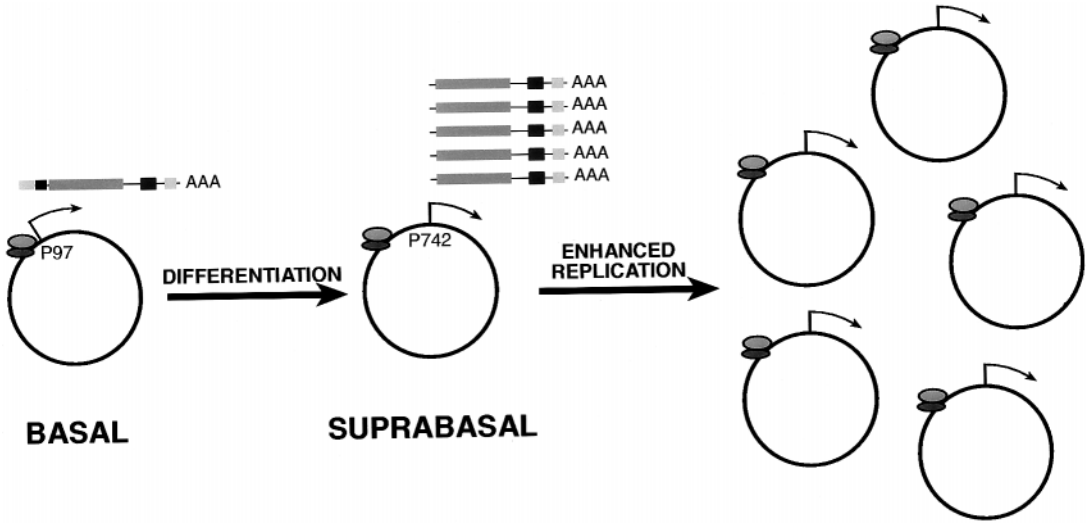

FIG. 7. A model for the temporal relationship of events contributing to genome amplification. Differentiation induces basal cells or monolayer cultures to initiate E1 messages from P742. The increased levels of E1 mRNA lead to an increase in E1 protein with a subsequent enhancement of HPV replication in suprabasal cells or methylcellulose cultures. The increased viral copy number thereby provides more templates for further E1 induction, leading to amplification.

cells in a raft culture—those within a specific stratum of the emerging epithelium—will activate viral late functions. Thus, any specific differentiation-dependent HPV event may be obscured by the population of cells that either have not yet differentiated or have proceeded past a particular point in differentiation. Recently, raft culture has been used to characterize E1 and E2 messages during differentiation of CIN 612 cells (Ozbun and Meyers, 1998). Raft culture also induced expression of E1 (roughly sixfold) and E2 messages, and only E1 messages initiated at P97 were observed. In our studies, we believe that a greater number of cells differentiate, and this likely facilitated the identification of multiple E1-specific transcripts.

The differentiation-dependent induction of the 3.7-kb E1 mRNA by 27-fold suggests that a shift in promoter usage occurs for the expression of E1 messages during differentiation of cells harboring HPV31. Although E1 messages from P97 continue to accumulate following suspension culture, they are not induced to a level similar to the 3.7-kb mRNAs. One possible advantage to the virus of a promoter shift is that the 3.7-kb messages could allow for more efficient translation of the E1 ORF (the first ORF on this message) than from the 4.2-kb RNAs that contain the E6 and E7 ORFs upstream of E1. Furthermore, we have hypothesized that E2 down-regulates its own expression (and potentially that of E1) by binding to sites adjacent to the P97 promoter (Stubenrauch *et al.,* 1998). A shift in promoter usage upon differentiation would remove this autoregulation that may otherwise restrict the expression of transcripts encoding replication factors in basal cells. The up-regulation of E1 messages, which are also likely to be more readily translated, may permit for a rapid and differentiation-dependent increase in E1 protein levels. This suggests one potential model for the temporal relationship of events involved in HPV genome amplification: differentiation induces transcription of P742, E1 messages accumulate, and the resultant increase in E1 protein facilitates enhanced replication of the HPV genome (see Fig. 7).

We found that differentiation induced E1 mRNA accumulation to a much greater extent than E2 messages. This differential induction suggests possible differences in replication protein requirements for genome amplification, yet studies to test these models are complicated by the low cellular concentrations of HPV E1 and E2 proteins present in cells. In one model, E1 protein could be the limiting factor with E2 protein present in sufficient amounts to support the enhanced viral replication. Alternatively, E1 protein may be induced at the level of transcription while E2 activity is increased by a posttranscriptional mechanism. Current models of E1 function at the replication origin suggest that differences in stoichiometric requirements may contribute to the differential induction of E1 and E2. While E2 is thought to function as a homodimer (Frattini and Laimins, 1994), the helicase activity of E1 is induced upon formation of an E1 hexamer around single-stranded DNA (Sedman and Stenlund, 1998). Therefore, enhanced HPV replication may require a greater induction of E1 protein relative to E2 for the support of viral genome amplification. A thorough analysis of these models requires examination of E1 and E2 at the protein level. Although the low level of E1 and E2 proteins may complicate these analyses, we are currently developing antibodies to facilitate such studies. In summary, our studies demonstrate the induction of E1 messages during differentiation by a promoter shift that leads to the accumulation of a 3.7-kb E1 mRNA initiated

at the differentiation-dependent promoter. These results suggest that differentiation-dependent changes in the expression of HPV replication proteins occur during viriogenesis.

MATERIALS AND METHODS

Cell lines. CIN 612 cells have been described previously (Bedell *et al.,* 1991; Rader *et al.,* 1990). Primary human foreskin keratinocytes were purchased from Clonetics (San Diego, CA) or were obtained by dissociation of human foreskin. L31 cells were generated by transfection (see below).

Cell culture. Primary human foreskin keratinocytes were maintained in KGM (Clonetics) for fewer than six passages. HPV-immortalized keratinocytes were plated onto mitomycin C-treated NIH 3T3 feeders and were maintained in E medium supplemented with 5 ng/ml EGF as previously described (Rader *et al.,* 1990). Primary keratinocytes were transfected with 5 μg ligated HPV DNA and 2 μg pSV2neo using Lipofectamine (Gibco) in KGM, replated onto feeders the following day, and selected for 5–10 days with 200–50 μg/ml G418 (Frattini *et al.,* 1996).

Keratinocytes were cultured in semisolid medium as described (Ruesch *et al.,* 1998). Briefly, cocultures of keratinocytes were treated with Versene to remove feeder fibroblasts, trypsinized, and pelleted. The pelleted keratinocytes were then resuspended in 1 ml E medium and mixed into 10 ml E medium containing 1.5% methylcellulose in 60-mm petri dishes. The resulting semisolid cultures were grown at 37°C for 18–24 h. Cells were then recovered by scraping the semisolid medium into conical tubes, diluting with PBS, and pelleting followed by another PBS wash.

Hybridization analysis. For Southern analysis, total genomic keratinocyte DNA was isolated from a confluent 10-cm dish by proteinase K digestion followed by phenol extraction (Klumpp *et al.,* 1997). Keratinocyte DNA (5–10 μg) was digested with the appropriate restriction enzyme, electrophoresed through 0.7% agarose, and transferred to ZetaProbe membrane (Bio-Rad) in 0.4 M NaOH. A probe was labeled with [α-^{32}P]dCTP by random-hexamer primed synthesis using the complete HPV31 genome as a template. Prehybridization and hybridization were performed at 65°C in 0.5 M sodium phosphate, pH 7.2, containing 7% SDS, 1 mM EDTA, and 10 × 6 cpm/ml probe. Washes were performed at a final stringency of 0.2× SSC, 1% SDS at 55°C.

For Northern analysis, total cellular RNA was purified from a subconfluent 10-cm dish of keratinocytes (or from tissue derived from raft cultures or methycellulose cultures) using Trizol reagent (Gibco) according to the manufacturer's protocol. For some experiments, polyadenylated RNA was purified from the total RNA using the PolyATtract system from Promega (Madison, WI). Ten micrograms of total RNA was electrophoresed through 0.8% agarose containing 2.2 M formaldehyde in Mops buffer, transferred to ZetaProbe in 10× SSC, and fixed to the membrane by UV-crosslinking. Prehybridization, hybridization, and washes were performed as described above for Southern analysis. Templates specific for E4/E5 (nts 3278–4101), E6/E7 (nts 99–664), E1 (nts 1282–2616), and E2 (nts 2709–3200) probes were generated by PCR.

RNase protection assays. RNase protection assays were performed on 6–10 μg of total RNA. Precipitated RNA pellets were hybridized to 250,000 cpm antisense ^{32}P riboprobe in 10 μl of 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide overnight at 37°C. RNase digestion was performed by the addition of 300 μl (ice-cold) of 10 mM Tris–HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA containing 10 μg/ml RNase A and 40 U/ml RNase T1; digestion proceeded for 60 min at 37°C. The RNase reaction was stopped by the addition of 3.5 μl of 20% SDS and 12.5 μl of 10 mg/ml proteinase K followed by digestion for 15 min at 37°C. Samples were then extracted with phenol:chloroform and precipitated with EtOH prior to resuspension in formamide loading buffer and resolution on a 6% sequencing gel. The riboprobe for examination of P742 was generated by digesting pRP-P742 with *Eco*RI and transcribing with SP6 polymerase (Klumpp *et al.,* 1997); the probe corresponded to nts 679–918 of HPV 31 sequence as well as 44 nt of vector-derived sequences at the 3′ end.

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