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Induction of Human Papillomavirus Type 18 Late Gene Expression and Genomic Amplification in Organotypic Cultures from Transfected DNA Templates

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The genetic analysis of human papillomavirus (HPV) functions during the vegetative viral life cycle is dependent upon the ability to generate human keratinocyte cell lines which maintain episomal copies of transfected viral genomes. We have previously demonstrated that lipofection of normal human foreskin keratinocytes with recircularized cloned HPV-31 genomic sequences resulted in a high frequency of cell lines which maintained viral genomes as extrachromosomal elements (M. G. Frattini, H. Lim, and L. A. Laimins, Proc. Natl. Acad. Sci. USA 93:3062–3067, 1996). Following the growth of these cell lines in organotypic (raft) cultures, the differentiation-dependent expression of viral late genes, the amplification of viral genomes, and virion biosynthesis were observed. In the present study, we demonstrate that these methodologies are not restricted to HPV-31 but are applicable to other HPV types, including the oncogenic HPV-18. HPV-18 genomes were purified from bacterial vector sequences, religated, and transfected into normal human foreskin keratinocytes together with a neomycin-selectable marker. Following drug selection, resistant cells were expanded and examined for the state of the viral DNA. All cell lines examined were found to contain approximately 100 to 200 episomal copies of HPV-18 DNA per cell. Growth of these cell lines in raft cultures resulted in the differentiation-dependent expression of the E1^E4 and L1 capsid genes. In addition, viral genome amplification was observed in suprabasal cells following DNA in situ hybridization analysis of differentiated raft cultures. The induction of these late viral functions has previously been shown to be directly associated with differentiation-dependent virion biosynthesis. Our studies indicate the ability to perform a detailed genetic analysis of the various phases of the viral life cycle, including control of the differentiation-dependent late viral functions, using a second oncogenic HPV type.

The life cycle of human papillomaviruses (HPVs) is directly linked to epithelial cell differentiation (18, 21). Papillomaviruses infect basal epithelial cells and establish their genomes as extrachromosomal elements, which replicate in synchrony with cellular DNA replication. Following cell division, infected daughter cells migrate from the basal region and begin the process of differentiation. Upon terminal differentiation in the granulosa and cornified layers of infected epithelia, vegetative viral DNA replication, or amplification, is induced, which is followed by the activation of viral late gene expression and the assembly of infectious virions (18, 21). Attempts to propagate human papillomaviruses in tissue culture have only recently been successful and were facilitated by the utilization of organotypic (raft) cultures to achieve the terminal differentiation of epithelial cells (15, 22). Using human foreskin explants (xenografts) which had been infected with HPV-11 and grown under the renal capsule of nude mice for several months, Dollard et al. observed the continued synthesis of HPV-11 following transfer to organotypic cultures (8). In other studies, Meyers et al. induced the synthesis of HPV-31 virions in organotypic cultures by using a latently infected cell line, CIN 612, which was derived from a patient biopsy, and stably maintained episomal copies of HPV-31 DNA (22). The latter studies suggested that if normal human keratinocyte cell lines which maintained transfected viral DNA sequences as episomes could be isolated, then it should be possible to induce vegetative viral replication and virion biosynthesis from these lines following their terminal differentiation in raft cultures.

Several reports have described an inability to isolate cell lines which maintained episomal copies of transfected HPV-16 DNA sequences (4). This inability could be due to either different methodologies or the use of HPV-16 viral isolates which had been cloned from cervical tumors. In these tumors, viral DNA is usually found integrated into host genomic sequences and it may contain mutations which could preclude episomal maintenance. Since success had been achieved in inducing virion biosynthesis with latently infected biopsy-derived cell lines containing HPV-31, we initially examined the ability of transfected cloned HPV-31 genomes to be stably maintained as episomes following lipofection of normal human keratinocytes, the natural host cell (15). Normal human foreskin keratinocytes were cotransfected with recircularized HPV-31 DNA sequences and a plasmid expressing the neomycin resistance gene. Following a brief selection period, drug-resistant colonies were expanded and examined by Southern blot analyses for the state of the viral DNA. All of the resultant cell lines examined were found to contain primarily extrachromosomal copies of HPV-31 DNA at approximately 50 to 100 copies per cell. When these cell lines were grown in organotypic cultures, differentiation-dependent viral late gene expression, genome amplification, and virion biosynthesis were observed. These procedures have been used to initiate a genetic analysis of

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HPV functions during the viral life cycle by using a cloned HPV-31 genome containing a frameshift mutation in the E1 open reading frame (ORF) as a template. The E1 ORF encodes the viral initiator protein, which contains origin-binding, ATPase, and helicase activities and is required for transient replication of HPV DNA (5, 13, 14, 26, 27). Mutation of the HPV-31 E1 ORF resulted in integration of the viral genomes into host genomic sequences as well as a failure to activate significant levels of viral late gene expression, despite evidence of terminal differentiation in organotypic cultures (15). These studies indicated that HPV genomes must be maintained as episomes for the induction of differentiation-dependent late viral functions.

To determine if this methodology for the generation of episome-containing cell lines was restricted to HPV-31 or whether it was applicable to additional HPV types, we transfected normal human foreskin keratinocytes with recircularized HPV-18 DNA sequences. Unit length HPV-18 genomes (approximately 8 kb) were excised from the plasmid p18-1.5 (2), which contains one and one-half permuted HPV-18 genomes, by restriction with *Eco*RI. The 8-kb HPV-18 genome was purified away from both plasmid sequences as well as the remaining one-half genome by agarose gel electrophoresis. The viral DNA was then eluted, recircularized with T4 DNA ligase, and precipitated with ethanol. Following resuspension in 1× Tris-EDTA, HPV-18 genomes (5 μg) were transfected along with 2 μg of pSV_2neo (15), which expresses the neomycin resistance gene, into second-passage normal human foreskin keratinocytes (Clonetics Corp.). For these experiments, a total of three different foreskin donors were used. Transfections were done in 60-mm-diameter plates with the LipofectAce lipofection reagent (GIBCO) and serum-free medium (KGM; Clonetics Corp.) according to the instructions provided by the manufacturer. Twenty-four hours posttransfection, cells were trypsinized and plated into serum-containing medium (15) with 100 μg of G418 (GIBCO)/ml. Drug selection was performed until all cells on the mock control transfection plate had sloughed off, which typically occurred at 6 to 10 days if the medium was changed every other day. Following drug selection, colonies were pooled or individual colonies were cloned and expanded. Using this technology, we generated approximately 25 to 40 colonies per transfection. A total of four pooled cultures and six clonal lines were established from four separate experiments. A typical Southern blot analysis for one pooled culture and three clonal lines, designated L18P or L18-1, -2, or -3, respectively, is shown in Fig. 1. All cell lines examined were found to contain primarily episomal genomes at approximately 100 to 200 copies per cell. Each established cell line was also found to stably maintain viral DNA extrachromosomally for over 10 passages in culture and after cryopreservation (data not shown).

To determine if the L18 cell lines could activate late viral functions upon terminal differentiation, we examined these lines following their growth in organotypic cultures (15). A typical hematoxylin- and eosin-stained cross section of an L18P raft culture is shown in Fig. 2A. As seen in panel A, nuclei were found to be retained throughout all layers of the epithelium and were present even in the keratinized suprabasal region. When cross sections of stratified rafts were stained with an antibody to filaggrin, strong staining was observed in the terminally differentiated cornified cell layer (Fig. 2C). Filaggrin is an intermediate-filament-associated protein and a marker of terminal differentiation whose synthesis closely correlates with the expression of HPV late genes in raft cultures (1, 15, 22). Consistent with the detection of filaggrin expression, the synthesis of E1^E4, a late protein expressed only in terminally

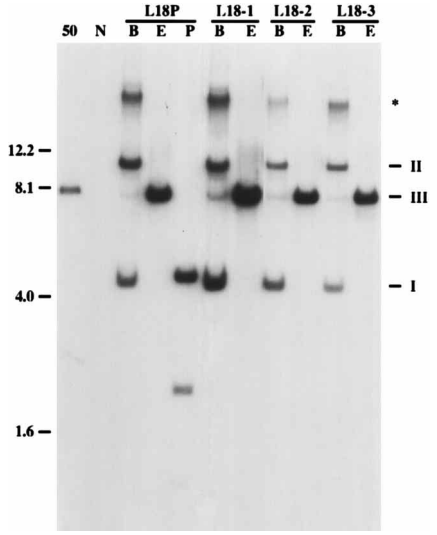

FIG. 1. Southern blot analysis of L18P, L18-1, L18-2, and L18-3 monolayer cultures demonstrating episomal maintenance of the transfected HPV-18 genomes. Total genomic DNA (10 μg) was restricted with either *Bgl*II (B), which does not cut the HPV-18 genome and indicates episomal maintenance of the viral genomes, *Eco*RI (E), which linearizes the 8.0-kb HPV-18 genome, or *Pvu*II (P), which cuts the HPV-18 genome three times, generating 4.4-, 2.2-, and 1.2-kb fragments. Due to its size, the 1.2-kb fragment is only faintly visible on this exposure. Southern blot analyses were performed as described previously (15). Lane 1 (50) is a genome copy number control representing 50 copies per cell and lane 2 (N) is an untransfected normal human foreskin keratinocyte negative control. Molecular size markers are indicated in kilobases. I, II, and III represent supercoiled, relaxed circular, and linear forms of viral DNA, respectively. The asterisk indicates slower-migrating concatenated viral DNA species.

differentiated epithelial cells, was detected in the granulosa and cornified layers of L18P raft cultures (Fig. 2E). The E1^E4 protein has been shown to induce collapse of the cytokeratin network when expressed in certain cell types in vitro, and it may play a role in vegetative viral DNA replication or in facilitating virion release during a productive infection in vivo (3, 9, 10, 24, 25). The E1^E4 staining pattern in raft cultures of HPV-31 transfected and biopsy-derived cell lines has previously been shown to be cytoplasmic (15, 24). A similar staining profile can be seen in L18P raft cultures, with positive cells exhibiting cytoplasmic staining in the absence of nuclear staining (Fig. 2E). Since no HPV-18 E1^E4 antiserum was available, we used the cross-reactivity of a recently developed Fab fragment to HPV-16 E1^E4 proteins (TVG405), which was isolated from a synthetic immunoglobulin repertoire displayed on the fd bacteriophage (11, 11a, 17). TVG405 has an affinity for HPV-16 E1^E4 protein in the nanomolar range and contains an *myc* gene epitope tag recognized by the commercially available 9E10 antibody (Boehringer Mannheim) to allow immunodetection (11, 11a). The observed HPV-18 E1^E4 staining was specific, as no staining was seen with the 9E10 antibody alone as a control (Fig. 2F). We next examined cross sections by immunohistochemistry for the synthesis of the HPV-18 capsid protein L1. Using an antibody generated against HPV-18 L1 virus-like particles (H18.J4 [7]), we observed nuclear staining in a subset of terminally differentiated cells in the L18P rafts (Fig. 2D). This pattern of staining is similar to that observed with the other established L18 cell lines, both pooled cultures and clonal lines (data not shown).

To examine the late messages expressed in the L18 cell lines, Northern blot analyses were performed on mRNA from L18 rafts with a DNA probe from the L1 gene, and a representative

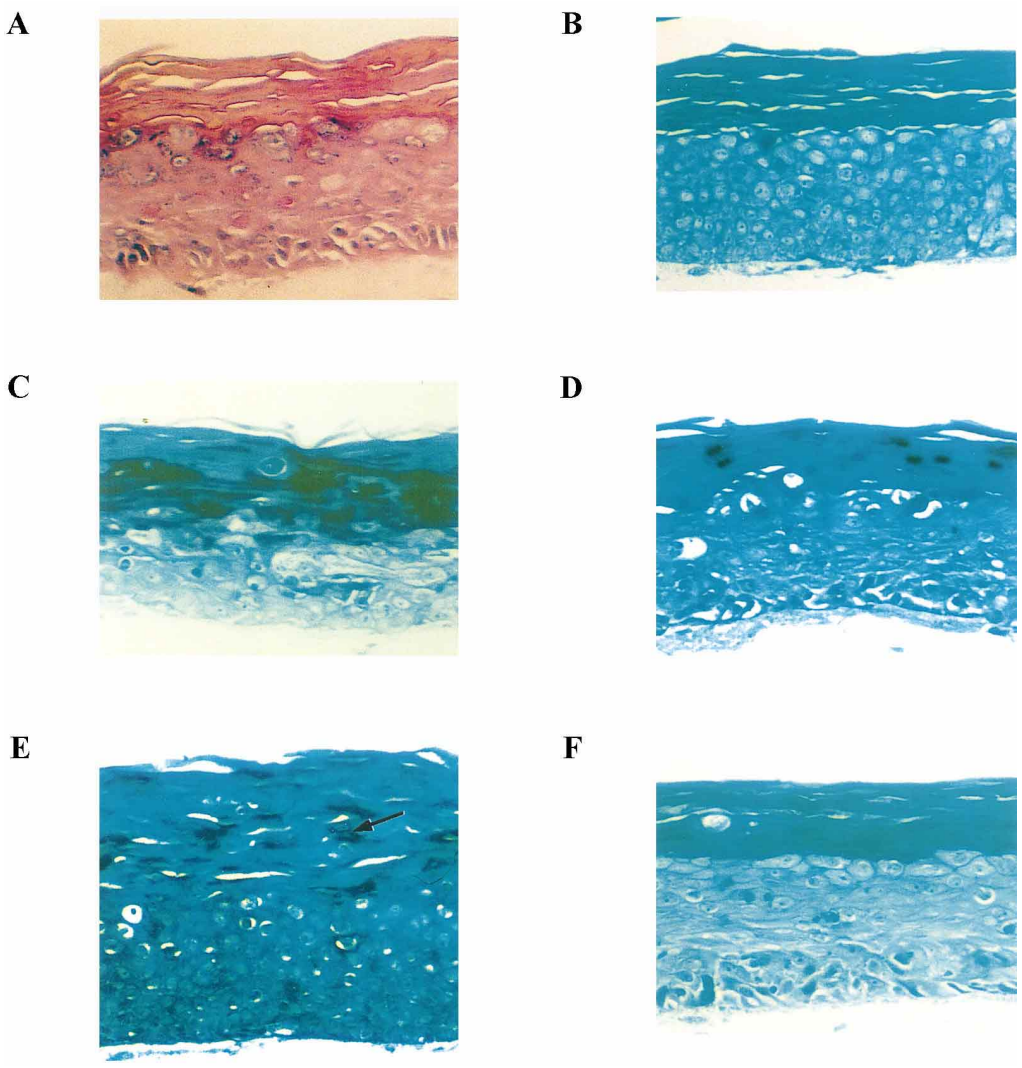

FIG. 2. Immunohistochemistry of cross sections of L18P raft cultures was performed as described previously (15, 22) using the Vectastain Elite ABC kit (Vector Laboratories). (A) Hematoxylin and eosin staining of an L18P raft culture. (B to F) Immunostaining of L18P raft cultures with a negative control antibody (B); a filaggrin-specific monoclonal antibody (Biomedical Technologies, Inc.) (C); H18.J4, a monoclonal antibody specific for HPV-18 L1 virus-like particles (7) (D); TVG405, an HPV-16 E1^E4-specific Fab fragment, and 9E10 (Boehringer Mannheim), an anti-*myc* monoclonal antibody which is directed against an epitope tag on TVG405 (E); and 9E10 alone (F). TVG405 was isolated from a synthetic immunoglobulin phage display library (11, 11a). The arrow in panel E indicates two positively stained cells.

blot is shown in Fig. 3A. Two species of late messages were detected in differentiated L18 rafts (Fig. 3A, lane 4), which are similar in size to those previously seen for HPV-31 late messages following growth in organotypic culture (15, 20). Based on our studies with HPV-31, the larger of the two messages, which corresponds to a size of approximately 4.7 kb, is likely the E1^E4, E5, L2, and L1 transcript. The smaller 2.3-kb message likely encodes the E1^E4 and L1 ORFs. When the blot was stripped and reprobed with an E4-specific DNA probe, an induction of E4 transcription was seen in raft cultures (Fig. 3B, lane 4), which likely represents an E1^E4 and E5 transcript (19). This result is consistent with a differentiation-dependent late promoter being located within the E7 ORF. A similar promoter, located within the E7 ORF, has previously been identified in both HPV-16 and -31 (16, 20). The 1.7-kb transcript seen in both monolayer (Fig. 3B, lanes 2 and 3) and raft (Fig. 3B, lane 4) cultures likely represents the E6* or E6, E7, E1^E4, and E5 transcript (19). Figure 3C shows the same Northern blot after it was reprobed with E6 DNA sequences and demonstrates similar levels of a 1.7-kb transcript in both monolayer (Fig. 3C, lanes 2 and 3) and raft (Fig. 3C, lane 4) cultures. This transcript most likely represents the same 1.7-kb message shown in Fig. 3B, the E6* or E6, E7, E1^E4, and E5 transcript. Interestingly, there appears to be a monolayer-specific 1.0-kb transcript (Fig. 3C, lanes 2 and 3) which contains E6 but not E4 sequences (compare Fig. 3B, lanes 2 and 3). This 1.0-kb message was not detected in raft culture mRNA even after a longer exposure (data not shown). Previous studies with a cell line containing episomal copies of HPV-16 DNA (W12) has demonstrated the presence of a 1.0-kb transcript in monolayer cultures (12). This message has the potential to encode the E6* and E5 proteins of HPV-16. Whether the 1.0-kb monolayer-specific transcript shown in Fig. 3C has the same coding potential in HPV-18 is currently under investigation. When the Northern blot shown in Fig. 3 was reprobed with the glyceraldehyde-3-phosphate dehydrogenase

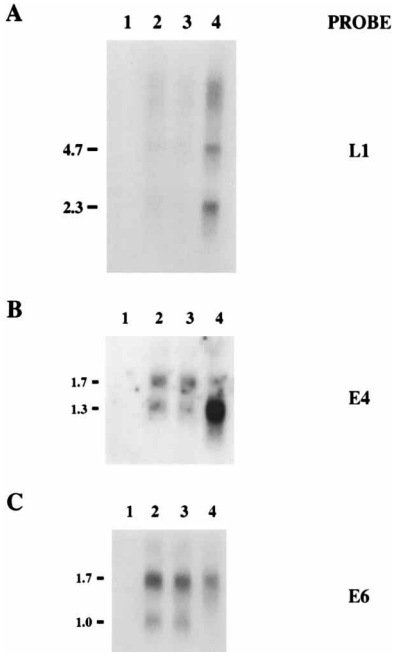

FIG. 3. Northern blot analysis of mRNA from monolayer cultures of normal human foreskin keratinocytes (lanes 1), L18P monolayers (lanes 2), L18-1 monolayers (lanes 3), and L18P raft cultures (lanes 4). mRNA was isolated and Northern blot analyses were performed as described previously (15). The sizes of the hybridizing transcripts are indicated in kilobases. DNA probes are from the L1 (A), E4 (B), and E6 (C) genes of HPV-18 as indicated.

gene, similar amounts of mRNA were detected in each lane (data not shown). The above-mentioned Northern blot analyses are in general agreement with previously published studies from HPV-11, -16, and -31 transcription analyses (6, 16, 19, 20).

A second differentiation-dependent late viral function is the process of DNA amplification (18, 21). We investigated whether the L18 cell lines were amplifying HPV-18 genomes in raft cultures by DNA in situ hybridization with an HPV-18-specific DNA probe (Enzo Diagnostics) as described by the manufacturer. As shown in Fig. 4B, a significant increase in HPV-18 copy numbers is seen in the differentiated suprabasal cells, which is consistent with vegetative genome amplification. No such signal was observed with a negative control hybridization with a DNA probe specific for pBR322 plasmid sequences (Fig. 4C). Figure 4A represents a positive control hybridization with a DNA probe specific for human genomic sequences and demonstrates nuclear staining in all cells.

In previous studies, the synthesis of L1 proteins and genome amplification were directly associated with the biosynthesis of HPV virions, as determined by electron microscopy (8, 15, 22). In those studies, the synthesis of virions was consistently observed in the presence of L1 protein synthesis. Given these previous observations with HPV-11 and -31, we anticipate that the cell lines containing HPV-18 are synthesizing virions when grown in organotypic cultures. A more stringent test would be to determine if the HPV-18 virions are infectious.

In this study, we have demonstrated that HPV differentiation-dependent viral functions can be induced following transfection of HPV-18 genomes. The transfection of recircularized HPV-18 genomes resulted in a high frequency of normal human foreskin keratinocyte cell lines which stably maintained episomal copies of HPV-18 DNA, and it is similar to that seen with cloned HPV-31 sequences (15). These studies indicate

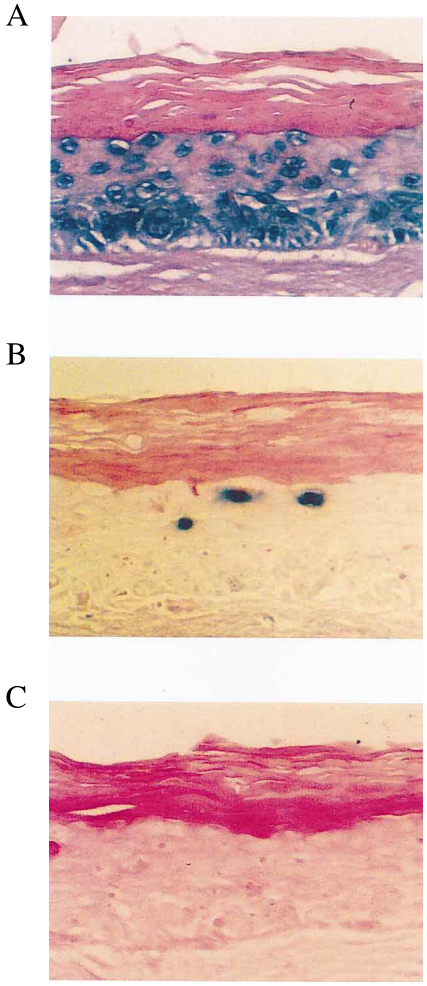

FIG. 4. DNA in situ hybridization analyses of L18P raft cultures were performed as described by the manufacturer (Enzo Diagnostics). Sections were probed with either a positive control DNA probe specific for total human genomic sequences (A), an HPV-16- and -18-specific DNA probe (B), or a negative control DNA probe specific for pBR322 plasmid sequences (C).

that the methods we have described to generate cell lines which are capable of inducing vegetative viral replication in organotypic cultures are applicable to additional HPV types. These methods may be more amenable to the study of the oncogenic HPV types, since they encode genes which are able to immortalize keratinocytes (18, 21). However, we believe that similar results can be achieved with the nononcogenic types, such as HPV-11, but it will require rapid analysis in raft cultures before cellular senescence. This would be consistent with observations by Mungal et al., who reported the ability to maintain transfected copies of HPV-11 as extrachromosomal elements following transfection of human keratinocytes for only one to three passages prior to senescence (23).

Our studies with HPV-18 underscore the close association of the viral pathogenesis process with epithelial differentiation, a concept that has been previously documented with HPV-11 and -31 (8, 15, 22). This methodology will now provide the opportunity to initiate not only a detailed analysis of HPV-18 gene expression during cellular differentiation in vitro but also genetic studies on HPV-18 functions during the vegetative viral life cycle. Comparisons of the HPV-18 and HPV-31 transfected keratinocyte lines demonstrate a large number of sim-

ilarities in the viral life cycles. Both viral types were found to maintain viral DNA episomally at approximately 50 to 200 copies per cell. In addition, both types induced similar levels of viral genome amplification and late gene expression in raft cultures. The patterns and sizes of the late transcripts were also similar, suggesting that the L1 and L1-L2 messages previously demonstrated for the transfected HPV-31 cell lines have similar counterparts in the HPV-18 lines. In vivo, however, one major difference between the two viral types lies in the specific type of malignancy with which they are associated. HPV-31, like the closely related type 16, induces squamous cell carcinomas, while HPV-18 is associated at a high frequency with aggressive adenocarcinomas (18, 21). This suggests that specific differences exist within the respective life cycles, and it will therefore be important to determine the source of these variations in order to better understand the process of progression from an infected epithelium to malignancy. These types of analyses should provide important information on the normal functions of viral proteins, whose activities are altered upon malignant conversion, as well as possibly identifying new type-specific targets for antiviral therapy.

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