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The role of the human papillomavirus type 18 E7 oncoprotein during the complete viral life cycle

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Abstract

The role of the human papillomavirus oncoprotein E7 in carcinogenesis has been extensively studied. While the role of HPV E7 in the viral life cycle has also been studied, certain disparities exist, indicating that genotype differences may influence the role that E7 plays in the viral life cycle. In this study, we investigated the role of HPV18 E7 in the viral life cycle in order to gain a further understanding of this issue. To determine the role that HPV18 E7 plays in the viral life cycle, a translation termination substitution mutant of E7 in the context of the full HPV18 genome was created. We introduced linearized HPV18 E7-deficient genomic DNA into primary keratinocytes, where it recircularized and was maintained episomally at a range of five to several hundred copies of HPV genomic DNA. The mutant genomes failed to amplify following epithelial stratification and differentiation in organotypic culture. Moreover, virion morphogenesis did not occur. We found that the expression of HPV16 or HPV18 E7 in *trans* was able to rescue the amplification defect but not the defect in virion morphogenesis. These studies indicate that HPV18 E7 plays a critical role in the productive stage of the viral life cycle. In addition, these studies add further proof to the hypothesis that genotype differences exist for the role of E7 during the viral life cycle.

Keywords: Human papillomavirus; Viral life cycle; E7

Introduction

Human papillomaviruses (HPV) are small, double-stranded DNA tumor viruses that infect the squamous epithelium and induce papillomas. The epitheliotropic HPVs can be divided into subsets based on their ability to infect specific anatomic regions. One such subset of HPVs infects the anogenital region and can be further divided into low- and high-risk HPVs based on the frequency with which they induce viral-associated tumors. Low-risk HPVs are rarely found in malignant tumors but induce benign lesions, while high-risk HPVs are frequently found associated with cervical and other anogenital carcinomas. The causal relationship between HPV infection and cervical cancer is primarily due to the ability of the viral oncoproteins, E6 and E7, to abrogate the cell cycle (Huibregtse and Beaudenon, 1996; Jones and Munger, 1996). Perhaps, the most extensively studied interactions between E6 and E7 and cellular targets are the ability of E6 to bind to and degrade p53 (Scheffner et al., 1992) and the ability of E7 to bind to pRB (Jones and Munger, 1997). While numerous studies have been performed to understand the role that these oncoproteins play in carcinogenesis, their role in the complete viral life cycle remains unclear, as marked differences appear to exist between the roles of the oncoproteins from different high-risk HPV types.

The complete life cycle of HPV is tightly linked to the differentiation program of the host epithelium (Howley, 1996; Meyers et al., 1992, 1997). HPV gains entry to the mitotically active basal cells through microabrasions in the epithelium. It is in this layer that the initial phase of the viral

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life cycle occurs, during which the viral genome is established as a low copy number nuclear plasmid. As the infected cells begin the normal differentiation program, the basal cells divide and a daughter cell leaves the basement membrane to stratify and differentiate. This leads to the start of the productive viral life cycle, during which the viral genome is amplified, late genes are expressed, and virions are produced. HPVs are dependent on the host DNA replication machinery to synthesize viral DNA. Uninfected daughter cells typically exit the cell cycle as they begin to differentiate. In order to replicate their genomes to high levels prior to encapsidation, HPV-infected cells do not exit the cell cycle in suprabasal cells. This cell cycle exit block is believed to be due to the functions of the viral oncoprotein E7 (Cheng et al., 1995; Demeter et al., 1994).

The organotypic (raft) epithelial culture system has been used to develop an in vitro system capable of reproducing the entire viral life cycle (Meyers et al., 1992, 1997). This system has been used to study the complete life cycle of HPV31b (Frattini et al., 1996; Meyers et al., 1992; Ozbun and Meyers, 1997, 1998a, 1998b, 1999a, 1999b), and infectious stocks of HPV16, HPV18, HPV31b, HPV39, HPV45, and chimeric HPV18/16 have also been produced using this system (McLaughlin-Drubin and Meyers, 2004; McLaughlin-Drubin et al., 2003, 2004; Meyers et al., 1992, 1997, 2002). In this study, we have used this system to investigate the role that the HPV18 E7 oncoprotein plays in the complete viral life cycle. We show that HPV18 E7 is required for amplification of the viral genome upon differentiation of the host tissue and that expression of both HPV16 and HPV18 E7 in *trans* is sufficient to rescue amplification but not infectious virus production.

Results

Extension of HFK lifespan by E7 mutant HPV18

In order to investigate the role of the full length E7 oncoprotein in the life cycle of HPV18 a mutant HPV18 genome was constructed in the context of pBSHPV18. Early-passage HFKs were transfected with either wild-type or E7 mutant HPV18. Cells were selected in culture for extended growth/immortalization. During this time, the cultures went through crisis, and following the crisis, the cell lines were expanded. 100% of the twenty each HPV18 wild-type and E7 mutant electroporations displayed an extended lifespan as compared to the non-transfected control cells, which started to die approximately 1 month after electroporation (data not shown). Thus, HPV18 E7 is not necessary for extension of the lifespan of HFKs. Two of the HPV18E7 null lines, HPV18E7Δ:3 and HPV18E7Δ:4, were chosen for further growth in culture to determine the extent of the lifespan extension caused by the HPV18 E7 mutant genome. Both lines have been growing for more than a year and a half in culture and continue to grow well. Thus, we conclude that HPV18E7 is not necessary for immortalization of HFKs.

Episomal maintenance of E7 mutant HPV18 genomes

We next investigated whether E7 was necessary for stable maintenance and replication of the HPV18 genome during the non-productive stage of the viral life cycle. Total DNA was isolated from the HPV18 wild-type and E7 mutant cell lines after several weeks of growth in culture. While the presence of the wild-type HPV18 genome was confirmed by Southern blot, the HPV18 E7 mutant genomes were absent, even though we are able to detect as little as one HPV genome copy per cell (data not shown). The HPV18 E7 mutant lines were allowed to grow for approximately 70 days total in culture (corresponding to passage four in culture) before they were re-analyzed by Southern blot. Total DNA was isolated and digested with *Hind*III, which does not digest the HPV18 genome, and *Eco*RI, which linearizes the genome. The digested DNA was Southern blotted and probed with an HPV18 genomic probe (Fig. 1). Forms II and I DNA are present in the 0× lane, indicating that the genome is episomal. Additionally, we were unable to detect the presence of integrated DNA, which would have been manifested as a band migrating above the form II DNA near the agarose gel well on the Southern blot. A comparison of the linear DNA in the 1× lane to the HPV18 copy number controls reveals that the genome in this representative line is being maintained at greater than 100 copies/cell (Fig. 1). Overall, the mutant genome was maintained at approximately five to several hundred copies per cell in the lines studied (data not shown).

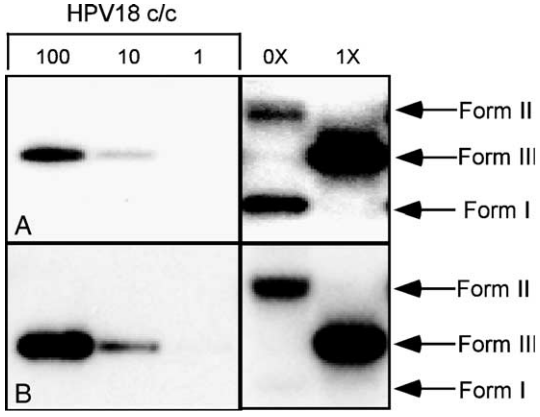

Fig. 1. Southern (DNA) blot hybridization of representative HPV18 wild-type and HPV18 E7Δ: 4 cell lines grown in monolayer culture. Cellular DNA was isolated from cultures approximately 70 days after electroporation (passage 4). Five micrograms of total cellular DNA was separated by electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labeled HPV18 genomic probe. (A) HPV18 wild-type DNA. (B) HPV18 E7Δ null DNA. Samples in the 0× lanes were digested with *Hind*III, which does not digest the HPV18 genome, and samples in the 1× lanes were digested with *Eco*RI, which linearizes HPV18. HPV18 copy number standards are shown. Arrows indicate Form II DNA (nicked), Form III DNA (linear), and Form I DNA (supercoiled).

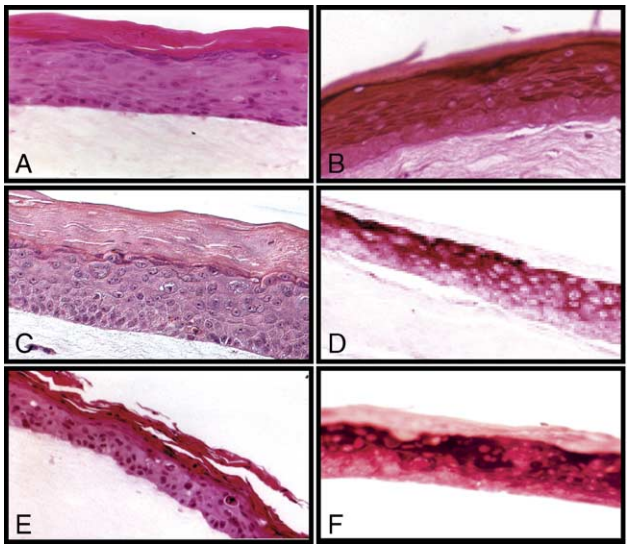

Fig. 2. Hematoxylin and eosin and involucrin staining of HFK, HPV18 wild-type and HPV18 E7Δ:4 raft tissue. (A–B) HFK raft tissue. (C–D) HPV18 wild-type raft tissue. (E–F) HPV18 E7 null raft tissue (from passage 12 cells). (A, C, E) Hematoxylin and eosin staining. (B, D, F) Involucrin staining (20× magnification).

This range is similar to what our laboratory observes with wild-type HPV18 (data not shown). These results demonstrate that E7 is not needed for HPV18 DNA synthesis in the non-productive stage of the viral life cycle, however, HPV18 E7 null genomes begin replicating their genomes considerably slower than wild-type HPV18.

Morphology of HPV18E7 null cell lines grown in raft cultures

We were interested in examining the effect of the loss of HPV18 E7 on tissue differentiation and morphology, as the life cycle of HPV is tightly linked to the differentiation program of the host tissue. While HPVs do affect the expression of keratinocytes differentiation markers, we do not see an evidence of this at the early stages, similar to our previous studies (McLaughlin-Drubin et al., 2003). Several HPV18 E7 null lines were allowed to stratify and differentiate in the raft culture system, and tissue sections were stained with hematoxylin and eosin to observe tissue morphology. Representative data from the HPV18E7Δ:4 line can be seen in Fig. 2. Occasional koilocytes and periodic parakeratotic bodies, and a normal looking cornified layer could be observed (Fig. 2). Overall, the mutant raft cultures differentiated similarly to wild-type HPV18 raft cultures (Fig. 2) indicating that the loss of HPV18 E7 did not affect the ability of the tissue to differentiate.

Amplification of HPV18 E7 mutant genomes upon growth in raft culture

We next analyzed the ability of the HPV18 E7 null cell lines to complete the viral life cycle. Prior to encapsidation, the HPV genome is amplified in differentiating squamous epithelium. To investigate DNA amplification, HPV18 E7 null raft cultures were grown and total DNA was harvested. DNA samples were digested with either *Hind*III, which does not digest the HPV18 genome, or *Eco*RI, which linearizes the genome, and analyzed by Southern blot. Fig. 3 shows that the viral genome decreased in amount upon growth in raft culture. Thus, HPV18E7 is essential for HPV18 DNA amplification.

Infectious HPV18 E7 mutant biosynthesis

Amplification of the viral genome is a necessary step in the viral life cycle that must occur prior to infectious virus production. We suspected that infectious HPV18 E7 mutant virus is not produced, as amplification of the HPV18 E7 mutant genome does not occur upon differentiation of the raft culture tissue. HPV18E7Δ:3 and HPV18E7Δ:4 raft cultures were grown and putative viral stocks were prepared as described previously (McLaughlin-Drubin et al., 2003, 2004; Meyers et al., 1997, 2002). The HPV18 infectivity studies were based on an in vitro system described by Smith et al (McLaughlin-Drubin et al., 2003, 2004; Smith et al., 1995). HaCaT cells were infected with a 1:100 dilution of the putative viral stock and were harvested 48 h post-infection. The HPV18 spliced E1^E4 transcript represented by a nested PCR product of approximately 226 bp can be seen in the HPV18 wild type sample. The absence of the HPV18 spliced E1^E4 transcript in the HPV18 E7 mutant samples indicates that the putative HPV18 E7 mutant viral

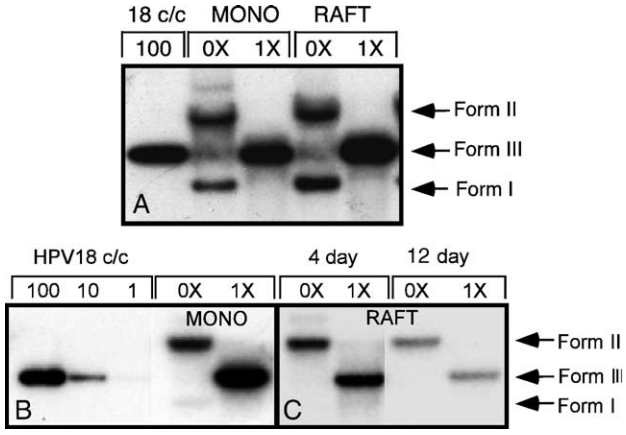

Fig. 3. Southern (DNA) blot hybridization of HPV18 E7Δ:4 cell line grown in monolayer and raft culture. Five micrograms of total cellular DNA was separated by electrophoresis on an 0.8% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labeled HPV18 genomic probe. (A) HPV18 wild-type DNA from monolayer (mono) and 12-day raft cultures. (B) HPV18 E7 null DNA from passage 12 monolayer cultures (mono) and 4- and 12-day raft cultures (also from passage 12 cells). Samples in the 0× lanes were digested with *Hind*III, which does not digest the HPV18 genome, and samples in the 1× lanes were digested with *Eco*RI, which linearizes HPV18. HPV18 copy number standards are shown. Arrows indicate Form II DNA (nicked), Form III DNA (linear), and Form I (supercoiled).

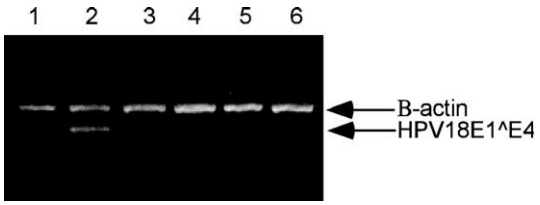

Fig. 4. Infectious titer of HPV18 cell lines. Shown is a 2% agarose gel of nested RT-PCR-amplified HPV18 E1^E4 and β-actin. Lane 1, negative control (no virus). Lane 2, positive control (HPV18WT). Lane 3, HPV18E7Δ. Lane 4, HPV18E7Δ infected with lxsn retroviral control. Lane 5, HPV18E7Δ infected with HPV16E7 retrovirus. Lane 6, HPV18E7Δ infected with HPV18E7 retrovirus. β-actin and HPV18 E1^E4 are indicated in the right and molecular size markers are indicated in the left. All mutant putative viral stocks were obtained from raft cultures made from passage 12 cells.

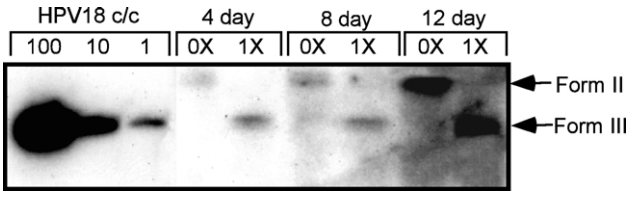

Fig. 6. Southern (DNA) blot hybridization of HPV18 E7 retrovirus-infected HPV18 E7Δ:4 cell line grown in monolayer and raft culture. Five micrograms of total cellular DNA from 4-, 8-, and 12-day raft cultures was separated by electrophoresis on an 0.8% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labeled HPV18 genomic probe. Samples in the 0× lanes were digested with *Hind*III, which does not digest the HPV18 genome, and samples in the 1× lanes were digested with *Eco*RI, which linearizes HPV18. HPV18 copy number standards are shown. Arrows indicate Form II DNA (nicked) and Form II DNA (linear). All mutant raft cultures were made with passage 12 cells.

stocks are not infectious, indicating that the mutant failed to make infectious virus (Fig. 4).

trans complementation of the HPV18 E7 null amplification defect by HPV16 E7 and HPV18 E7

In order to verify that the null mutation was responsible for the phenotypes observed, we next wished to reintroduce the wild-type E7 gene and to test if the reintroduction reverses the effects observed with the null mutant. In order to investigate this, we wished to determine if the expression of either HPV18 E7 or HPV16 E7 could complement the amplification defect in *trans*. In addition to verifying that the amplification defect was due to the loss of E7, these studies would address the type-specific nature of the role of E7 in the HPV18 viral life cycle. The HPV18E7Δ:4 line was infected with 10 cfu/ml of HPV18E7 or HPV16E7 retrovirus. Following selection with G418 to ensure that the cells contained the E7 retrovirus, the cells were allowed to stratify and differentiate in the raft culture system, and tissue sections were stained with hematoxylin and eosin to observe tissue morphology. Overall, infection with the HPV16 and HPV18 E7 retroviruses did not affect the ability of the tissue to stratify and differentiate (Fig. 5).

We next analyzed the ability of HPV16 and HPV18 E7 to rescue the amplification defect seen in the HPV18 E7 null cell lines. Uninfected, HPV16 E7-, and HPV18 E7-infected HPV18 E7 null raft cultures were grown and total DNA was harvested. DNA samples were digested with either *Hind*III, which does not digest the HPV18 genome, or *Eco*RI, which linearizes the genome, and analyzed by Southern blot (Fig. 6). The uninfected HPV18 E7 null genome did not amplify while the retrovirus-infected HPV18 E7 null genome did amplify (Fig. 6). Thus, the amplification defect seen in the HPV18E7 null lines is, in fact, due to the loss of E7, and this defect can also be complemented in *trans* by HPV16 E7 (data not shown).

We next investigated the effect of expressing HPV16 E7 and HPV18 E7 in *trans* on the ability of the mutant genomes to produce infectious virus. Unlike its effect on amplification, the *trans* expression of HPV16 E7 and HPV18 E7 did not rescue the infectivity defect seen in the HPV18 E7 mutant genomes (Fig. 4).

Discussion

In this study, we have examined the role of the HPV18 oncoprotein E7 in the complete viral life cycle. Although HPV18 E7 mutant genomes were not detectable by Southern blot as early as were wild type HPV18 genomes, the introduction of HPV18 E7 mutant genomes did extend the

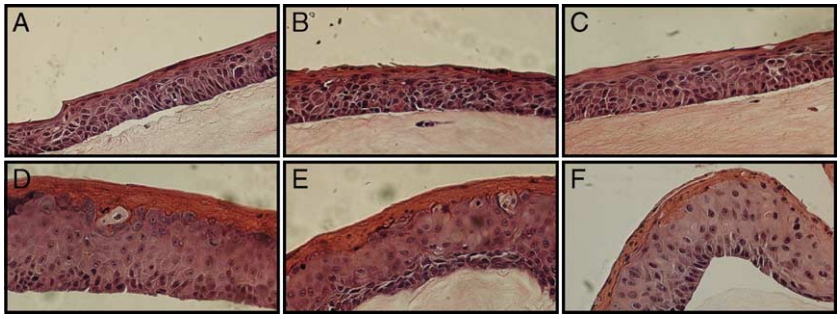

Fig. 5. Hematoxylin and eosin staining of HPV18E7Δ:4 raft tissues. Panels A–C, 5-day raft tissues. Panels D–F, 10-day raft tissues. Panels A and D, HPV18 E7 null raft tissues. Panels B and E, HPV18 E7 null + HPV16 E7 retrovirus raft tissues. Panels C and F, HPV18 E7 null + HPV18 E7 retrovirus raft tissues. All mutant raft cultures were made with passage 12 cells.

lifespan of HFKs at equal rates as wild-type HPV18. Moreover, the genomes in these cells were maintained episomally, indicating that HPV18 E7 does not play a major role in the non-productive stage of the viral life cycle. In contrast, we determined that HPV18 E7 plays a key role in the productive stage of the HPV18 life cycle. Although the morphology of raft cultures of HFKs containing HPV18 E7 mutant genomes appeared similar to raft cultures containing wild type genomes, genomes lacking E7 were unable to amplify upon differentiation of the host tissue and to produce infectious virus. Finally, by expressing both HPV16 and HPV18 E7 in *trans*, we were able to rescue the defect in amplification but were unable to rescue the defect in virus production.

The methodology used in this study to introduce HPV genomes into keratinocytes utilizes immortalization as selection rather than the addition of a selection marker. The reason that HPV18 E7 mutant genomes were not detectable by Southern blot as early as HPV18 wild type genomes is therefore unlikely due to differences in selective advantage for growth over control cells, as cells that are not immortalized will die when the control cells die. Although there is no selective advantage for growth, E7 does affect genome replication. We have previously shown that a 3-week lag period exists between electroporation of HPV18 genomes into keratinocytes and subsequent recircularization and detection by Southern blot (McLaughlin-Drubin and Meyers). This lag period is almost double for HPV31, 39, and 45 (McLaughlin-Drubin and Meyers, 2004). Thus, we hypothesize that E7 plays a role in the kinetics of recircularization and establishment of the HPV genome, and that differences in the speed at which this occurs exist between HPV types.

The observation that HPV18 E7 mutant genomes extended the lifespan of HFKs is in sharp contrast to previous findings in the literature. It has been shown that HPV16 E7 and HPV31a E7 mutant genomes were incapable of extending the lifespan of primary HFKS (Flores et al., 2000; Thomas et al., 1999). One explanation for this finding could be differences in the protocols used in our studies as compared to those used in previous studies. We performed experiments with HPV31 E7 mutant genomes in a manner similar to those presented in this paper and obtained results consistent with the previously published results (Thomas et al., 1999). Therefore, we hypothesize that the differences observed are due to genotype-specific differences in the requirement for E7 in the extension of the lifespan of HFKs. A comparison of these and previous studies indicate that other genotype-specific differences in the requirement for E7 in the viral life cycle also exist. While HPV31 E7 mutant genomes are unable to replicate as stable episomes in primary HFKs (Thomas et al., 1999), studies on HPV16 E7 mutant genomes in the immortalized HFK cell line, BC-1-Ep/SL, showed that HPV16 E7 mutant genomes are able to replicate as stable episomes (Flores et al., 2000). This finding is similar to our finding that HPV18 E7 mutant genomes are able to replicate as stable episomes in primary HFKs. A closer look at both the phylogenetic relationship between the virus types and their correlation to disease risk may shed light on these differences. Interestingly, both HPV16- and HPV31 E7-deficient genomes, which are unable to extend the lifespan of HFKs (Flores et al., 2000; Thomas et al., 1999), belong to phylogenetic group A. On the other hand, HPV18 E7-deficient genomes, which belong to group C, are able to extend the lifespan of HFKs. We speculate that another portion of the HPV18 genome is capable of extending the lifespan of HFKS and this particular function may be missing in HPV16 and HPV31. It would be interesting to perform similar studies with HPV39- and HPV45-deficient genomes, as these virus types also belong to group C. In addition to a possible link between E7 function and phylogenetic relationship, a possible link between E7 function in the viral life cycle and disease risk also exists. HPV31 is typically considered a mid-risk HPV and HPV31 E7-deficient genomes are not maintained episomally (Thomas et al.), while the high-risk HPV16 (Flores et al., 2000) and HPV18 E7-deficient genomes are maintained episomally. Support for this hypothesis lies in studies of the role of the low-risk HPV11 E7 during the viral life cycle, which showed that HPV11 E7-deficient genomes cannot be maintained episomally after passaging of the cells (Oh et al., 2004). Therefore, it appears that the role of E7 in maintaining viral episomes may be linked to disease correlation.

Previous studies have shown that the E6–E7 region of HPV18 was capable of immortalizing HFKs (Hudson et al., 1990). In addition, while E7 alone was capable of immortalizing the cells at a low frequency, E6 alone had no effect (Hudson et al., 1990). Thus, we were surprised to find that our HPV18 E7 null cell lines, which contained E6, were capable of continued growth in culture, indicating that the cells were indeed immortalized. It must be noted, however, that the earlier experiments on the immortalization capabilities of E6 and E7 were done by overexpressing E6 and E7 in isolation (Hudson et al., 1990), providing only a limited understanding of the function of the oncoproteins. Our studies were performed using the entire HPV genome containing a mutation in the E7 ORF, providing a much clearer picture of the role of the oncoproteins in the complete viral life cycle.

The introduction of HPV18 E7 rescued the amplification defect seen in the HPV18 E7-deficient cell lines, indicating that the amplification defect was indeed due to the lack of E7. Intriguingly, the introduction of HPV16 E7 also rescued this defect. On the other hand, the introduction of either HPV16 E7 or HPV18 E7 did not rescue the ability to produce infectious virus. It is possible that E7 is required early during the production of cells lines to establish a cellular environment that will be capable of infectious virus production. In addition, it is also possible that the mutation in E7 may affect *cis* elements involved in splicing or late gene expression via

promoter activity. Further studies utilizing additional E7 null mutations will be necessary to address this issue. These data shed light on the genotype-specific nature of the requirement for E7 during the life cycle of HPV. In addition, the ability to infect the HPV18 E7 null cell line with E7 expressing retroviruses lays the foundation for a powerful system to study the role of E7 in the viral life cycle. Specifically, E7 retroviruses containing mutations in particular functions of E7 can be used to dissect exactly which features of the oncoprotein are needed during the productive stage of the viral life cycle.

Materials and methods

Plasmids and constructs

pBSHPV18 (a generous gift from H. zur Hausen) contains the HPV18 genome inserted into the *Eco*RI site of pBS. A translation termination mutant of E7 of HPV18 was constructed via the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Three stop codons (ochre) were engineered at amino acids nine through eleven.

Keratinocyte and organotypic raft cultures

Primary human foreskin keratinocytes (HFK) were isolated from newborn circumcision as described previously (McLaughlin-Drubin et al., 2003). Keratinocytes were grown in 154 medium (Cascade Biologics, Inc., Portland, OR) supplemented with Human Keratinocyte Growth Supplement Kit (Cascade Biologics, Inc.). Keratinocyte lines stably maintaining HPV DNA following electroporation were grown in monolayer culture using E medium in the presence of mitomycin C treated J2 3T3 feeder cells (Meyers, 1996; Meyers et al., 1992, 1997).

Organotypic (raft) cultures were grown as previously described (Meyers, 1996; Meyers et al., 1992, 1997). Briefly, cell lines were seeded onto rat tail type 1 collagen matrices containing J2 3T3 feeder cells. Following cell attachment and growth to confluence, the matrices were lifted onto stainless steel grids. Once at the air–liquid interface the raft cultures were fed by diffusion from below with E medium supplemented with 10 μM 1,2-dioctanoyl-*sn*-glycerol (C8:O; Sigma Chemical Co.). Raft cultures were allowed to stratify and differentiate for 10 days, as viral gene expression has been shown to peak between 10 and 12 days in the raft system (Ozbun and Meyers, 1997).

Electroporation of primary keratinocytes

Primary human foreskin keratinocytes were electroporated as previously described (Meyers et al., 1997, 2002). Briefly, pBSHPV18 and pBSHPV18E7Δ plasmid DNA were digested with *Eco*RI, linearizing the viral DNA in E1 and separating it from vector sequences. 10 μl of the *Eco*RI-digested DNA (1 μg/μl) was mixed with 4.25 μl of sonicated and denatured salmon sperm DNA (10 μg/μl) in a 1.5-ml Eppendorf tube. 5×10^6 keratinocytes in a volume of 250 μl of E medium containing 10% fetal bovine serum (FBS) and 5 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid were added to the DNA mixture. The DNA and keratinocyte solution was transferred to an electroporation cuvette and electroporated using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA) set at 210 V and 960 μF. The electroporated cell solution was then layered into 10 ml of E medium containing 10% FBS and centrifuged at 25 × *g* for 10 min. The medium was removed and the cell pellets were resuspended in E medium containing 10% FBS. The suspensions were then added to 10-cm tissue culture plates containing mitomycin C-treated J2 3T3 feeder cells. 5 ng of EGF/ml was added to the culture medium the next day. The cultures were fed every other day for 7 days with E medium containing 10% FBS and 5 ng EGF/ml. After the 7-day period, the cells were fed with E medium containing 5% FBS and 5 ng EGF/ml until the keratinocytes grew to confluence, at which time the EGF was omitted from the medium.

Southern (DNA) blot hybridization

Total cellular DNA was isolated as previously described (Meyers et al., 1992; Ozbun and Meyers, 1998a). 5 μg of total cellular DNA was digested with *Hind*III, which does not digest the HPV18 genome or *Eco*RI, which linearizes the genome. The samples were then separated by 0.8% agarose gel electrophoresis and transferred onto GeneScreen Plus membrane (New England Nuclear Research Products, Boston, MA) as previously described (Ozbun and Meyers, 1998a). Hybridization of the Southern blot was performed as previously described (Meyers et al., 1992; Ozbun and Meyers, 1998a), probing with an HPV18 complete genomic probe.

Histochemical analyses

Raft cultures were grown for 12 days, harvested, fixed in 10% neutral-buffered formalin, and embedded in paraffin. 4 μm sections were cut and stained with hematoxylin and eosin as previously described (Meyers et al., 1992).

Retroviral propagation, tittering, and infection

Recombinant retroviruses were generated using the pCL vector system (Naviaux et al., 1996). Recombinant retroviral stocks were generated in RAT-1 cells as described (Naviaux et al., 1996). HPV18 E7 null cell lines were infected with 10 cfu/ml of recombinant retrovirus for 2 h. Cells were selected with 500 μg of G418 per ml beginning 48 h after infection. Selection lasted 3 to 4 days and was

stopped when the cells began to detach from the plate. The cells were grown to confluence and seeded onto collagen matrices for growth in raft culture.

Virus isolation and in vitro infectivity assay

Virions were isolated as previously described (Meyers et al., 1997, 2002). The HPV18 infectivity studies were based on an in vitro system described by Smith et al (Smith et al., 1995). HaCaT cells, an immortalized human keratinocyte cell line (kindly provided by Norbert Fusenig), were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin in 24 well plates. The wild type and mutant HPV18 virus stocks were sonicated for 30 s on ice and then serially diluted with cell culture medium. The medium was aspirated from the HaCaT cells and 0.5 ml of each dilution of virus stock was added per well. One well on each plate received 0.5 ml of medium without virus as a control. The cells were incubated with the virus for 48 h at 37 °C.

The ability of the wild type and mutant HPV18 viruses to infect the HaCaT cells after 48 h of incubation was determined by the presence of the spliced HPV18 E1^E4 mRNA species (Smith et al., 1995). mRNA was purified from the infected cells using the mRNA capture kit (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, the medium was aspirated from the cells and the cells were washed two times with 0.5-ml ice cold 1× PBS. The final PBS wash was aspirated from the cells and 0.25-ml lysis buffer was added to each well. The cell lysates were removed from the wells and sonicated for 2 min in a cup horn sonicator on ice. 4 μl of 1:20 diluted biotinylated oligo dT was added to each lysate. The samples were incubated for 10 min at 42 °C. 50 μl of the lysate was transferred to a streptavidin-coated PCR tube and incubated for three min at 37 °C. The RNA captured in the tubes was washed three times with 200 ml of wash buffer and subsequently used in a RT reaction utilizing reagents from the First Strand cDNA kit (Roche Molecular Biochemicals). The cDNA was then used for nested PCR to detect the HPV18 E1^E4 cDNA. Forty cycles of PCR was performed on the cDNA using 5′ GTTGTGTATGTGTTGTAAGTGTGA 3′ as the forward primer (located at nucleotide position 772–795 in the HPV18 genome) and 5′ GTCCACAATGCTGCTTCTCCG 3′ (located at nucleotide 3580–3600 in the HPV18 genome) as the reverse primer. 10% of the first PCR mixture was used as template for 40 cycles of nested amplification utilizing 5′ GAATTGGCTAGTAGTAGAAAGCT 3′ (located at nucleotide position 801–824 in the HPV18 genome) as the forward nested primer and 5′ TCCC-ACGTGTCCAGGTCGTGT 3′ (located at nucleotide position 3555–3575 in the HPV18 genome) as the reverse nested primer. An additional set of primers specific for β-actin was included in the PCR mixture as a control for mRNA detection. The forward primer for the first reaction was 5′ GAACCCCAAGGCCAACCGCGA 3′ and the reverse primer was 5′ CCACACAGAGTACTTGCGCT-CAGG 3′. The forward primer for the nested reaction was 5′ GATGACCCAGATCATGTTTG 3′ and the reverse primer was 5′ GGAGCATGATCTTGATCTTC 3′. All PCR reactions contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 125 ng of each forward and reverse primer, and 2.5 units of Taq polymerase (Fisher Scientific). The temperature profile for the first reaction was 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min with a final 10 min extension at 72 °C. The temperature profile for the second reaction was 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s with a final 10 min extension at 72 °C. All PCR products were visualized by electrophoresis in a 2% agarose-ethidium bromide gel.

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