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ANALYZING THE REGULATION OF HPV REPLICATION AND TRANSCRIPTION IN ITS NATURAL HOST, THE KERATINOCYTE

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ABSTRACT

Each papillomavirus is species specific and replicates persistently in a specific type of cutaneous or mucosal epithelium. The keratinocytes of the basal layer of the epithelium harbor a reservoir of replicating viral genomes. The processes of the HPV life cycle depend on elements in the viral genome that are regulated by host cell specific factors and therefore, ideally, analyses of viral transcription and replication should be carried out in primary keratinocytes. However, these studies are limited by the current cell culture and transfection methods. Improvements in the transfection and culture of keratinocytes will enable more sensitive, accurate and earlier analyses of viral replication and transcription. To test this we developed an optimized keratinocyte culture system using a Rho-Kinase (ROCK) inhibitor, Y-27632, that greatly improved the growth rate of primary keratinocytes and immortalized these cells. Further analysis revealed that the immortalized keratinocytes appeared to retain important characteristic of primary keratinocytes important for HPV research. Transient HPV replication analysis was enhanced in Y-27632-treated keratinocytes, presumably due to improved transfection efficiency and survival of transfected cells. Thus, improvements in primary cell culture and survival after transfection vastly improve the study of the establishment of HPV gene expression and replication. This allowed us to develop a complementation assay to study elements important for viral DNA replication. Using the complementation assay and the Y-27632 treated cells we were able to confirm the significance of the three E2 binding sites most proximal to the E6 promoter for transient replication. Finally, we designed immortalization assays using complete HPV genomes with targeted mutations to confirm that the information amassed from reporter assays holds up in more physiologically relevant systems. We were able to demonstrate that the TATA box, the Sp1 site, and the Ap1 site proximal to the E6 promoter, but not the distal Ap1 site, were dispensable for E6 and E7 expression. Collectively, these studies shed light on the significance

of previously identified regulatory elements in the HPV life cycle as well as offer new strategies for future HPV life cycle studies.

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ABBREVIATIONS

BP- Base Pair

BPV- Bovine Papillomavirus

CAT- Chloramphenicol Acetyltransferase

CRPV-Cottontail Rabbit Papillomavirus

DMEM- Dulbecco's Modified Eagle's Medium

DNA- Deoxyribonucleic Acid

E6-AP- E6 Associated Protein

EDTA- Ethylenediaminetetraacetic Acid

EGF- Epidermal Growth Factor

EV- Epidermodysplasia Verruciformis

FBS- Fetal Bovine Serum

GDP- Guanonsine Diphosphate

GPCR- G-Protein-Coupled Receptor

GTP- Guanosine Triphosphate

HCK- Human Cervical Keratinocyte

HCl- Hydrochloric Acid

HFK- Human Foreskin Keratinocyte

HPV- Human Papillomavirus

HVK- Human Vaginal Keratinocyte

JCV- JC virus

KOAc- Potassium Acetate

MME- Minichromosome Maintenance Element

M1- Mortality Stage 1

M2- Mortality Stage 2

NaOH- Sodium Hydroxide

NCS- Newborn Calf Serum

NMSC- Nonmelanoma Skin Cancer

OD- Optical Density

O/N- Overnight

PBS- Phosphate Buffer Solution

PCR- Polymerase Chain Reaction

PD- Population Doubling

PML- Progressive Multifocal Leukoencephalopathy

pRb- Phosphorylated (active) Retinoblastoma

PV- Papillomavirus

QRT-PCR- Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA- Ribonucleic Acid

ROCK- Rho-associated Coiled-Coil Domain Containing Protein Kinase

RT-PCR- Reverse Transcriptase Polymerase Chain Reaction

SCC- Squamous Cell Carcinoma

SDS- Sodium Dodecyl Sulfate

SSPE- Saline-Sodium Phosphate-EDTA

ssDNA- Salmon Sperm DNA

STI- Sexually Transmitted Infection

STR- Short Tandem Repeat

SV40- Simian Virus 40

TERT- Telomerase Catalytic Component (human- unless otherwise specified)

URR- Upstream Regulatory Region

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Chapter 1

Introduction

Immortalization

Biogerontology, the study of the biological processes giving rise to old age, is critical for developing our understanding of disease processes as well as normal cell behavior. For the first half of the 20th century, when cell culture was just getting underway, popular belief maintained that aging was the result of extracellular causes. Therefore, it was reasoned that cells isolated from the organism and cultured *in vitro* are free from these limitations and are inherently immortal. Dr. Alexis Carrel is considered to be the father of this theory because of his success in culturing fibroblasts derived from chicken heart tissue for 34 years (1). Dr. Carrel argued that the failure of others to reproduce his results was due to inferior culturing techniques. Later it was suggested that Carrel's 'superior' culturing technique involved feeding the cells with media supplemented with an extract of chick embryo tissue that was prepared daily and is likely to have contained freshly isolated cells(1).

For half a century no one challenged Carrel's theory of cellular immortality, despite the fact that no one could reproduce his results. But finally, in 1961, Leonard Hayflick and Paul Moorehead successfully discredited this theory when they published their work demonstrating that media inadequacies alone are not responsible for the limited life span of cells in culture (2). They did this by co-culturing old and young cells together in the same media. They found that the young cells retained the ability to divide while the old cells stopped dividing (2). From this work they concluded that primary cells derived from normal tissue and cultured *in vitro* have an intrinsic limitation on their growth as normal diploid cells. Graphically, they represented the

natural growth of cells in culture using a sigmoidal pattern where the cells have three distinct growth phases. Initially, primary cells lag in growth upon transfer to culture, followed by an exponential growth phase, and finally a third phase where the cells senescence and stop growing (3). The inverse of the logic that normal cells are mortal, that immortal cells are abnormal, was another important conclusion of Hayflick's discoveries. In a follow-up paper, Hayflick distinguished cells in cultures as normal, diploid mortal cells, which he called cell strains, from immortal, heteroploid, and abnormal cells, which he called cell lines (4). Furthermore, Hayflick proposed that cells have a predetermined, quantifiable number of cell divisions which they can undergo before reaching senescence. He reasoned that there must be an internal cellular meter that measures every cell division, which he named the replicometer (1).

Last year, Elizabth H. Blackburn, Carol W. Greider, and Jack W. Szostak were awarded the Nobel Prize in Physiology or Medicine 2009, for their work unveiling the identity of Hayflick's replicometer. Collectively, their work and the work of others demonstrates how each round of replication is recorded by cells as the result of the design of the cellular replication machinery. In the 1970s researchers discovered that DNA polymerase is unable to replicate the 3' ends of linear duplex DNA (1;5-7). Research over the next two decades revealed that critical genetic material is protected from progressive degradation during replication by a stretch of innocuous, repetitive DNA sequences at the ends of chromosomes now known as telomeres (8). This provided the molecular support for Hayflick's limit and for the cellular basis of aging. Additionally, the discovery of telomerase, an enzyme which, when expressed, is capable of stabilizing telomere ends, provided an explanation for immortality (8).

A decade before Hayflick turned the prevailing cell culture theory of immortality on its head, cells grown from a biopsy of a patient who died of cervical cancer began their infinite life in culture. These cells, named HeLa after their donor, Henrietta Lacks, became the first immortal human cell line to be propagated in tissue culture (9). A small sample of the tissue from Mrs.

Lacks' biopsy was sent to Dr. George Otto Gey, at the Tissue Culture Laboratory in the Department of Surgery at The Johns Hopkins Hospital, for research purposes (10). Dr. Gey had been trying for years to isolate an immortal cancer cell line from patient samples. Dr. Howard Jones, from John Hopkins, described the lesion from Mrs. Lacks as "unlike any other cancer he had ever seen", according to a television interview he gave in 1997 (9). As Henrietta Lacks' condition worsened, Dr. George Gey was already realizing the robust growth potential of her cells in culture. Unfortunately, the prolific growth of her cells *in vitro* matched the rapid progression of her disease *in vivo*, which ultimately took her life only 8 months after her initial biopsy and diagnosis. At the time, little was known about the cause of cervical cancer; as a matter of fact Henrietta Lacks' cause of death was even misdiagnosed as epidermoid carcinoma. It wasn't until 20 years later, when the original slides were re-examined, that the sample was properly identified as originating from an adenocarcinoma of the cervix (11). HeLa cells have become the most widely used cell line in medical research and have contributed to countless medical breakthroughs, including the first production of the polio vaccine in 1952 and the discovery that the enzyme telomerase is re-activated in cancer cells (9;12).

The presence of human papillomavirus (HPV) in HeLa cells wasn't identified until the mid-1980s (13). It was around this time that many investigators started to uncover the association between HPV and cervical cancer, with many reports of HPV being present in precancerous or cervical cancer biopsies (14-17). Throughout the rest of the 1980s and the following decade, researchers began to establish a better understanding the oncogenic nature of HPV, and in 1995 the HPV types 16 and 18 were declared to be human carcinogens (18). Other high-risk papillomavirus types were added to the list later (19). Epidemiological data have identified HPV in nearly all cases of cervical cancer (20), as well as in some head and neck cancers (21). The viral source of this cancer has led to the development of vaccines that protect aginst infection with the 2 most common types of HPV found in cervical cancers (22-24).

HPV types and infection

zur Hausen, who won the 2008 Nobel Prize in Physiology or Medicine for his pioneering efforts in discovering the connection between HPV and cervical cancer, began uncovering the relationship between HPV and cancer in the mid 1970s (25-27). Papillomavirus (PV) types were first identified by hybridization analysis using purified DNA from tissue samples. Now over 100 types of papillomavirus have been fully sequenced and classified into groups based on sequence similarities (Figure 1-1) (28). Different types of papillomaviruses are defined as having greater than 10% differences in the L1 nucleotide sequence. Groups of closely related types form species and groups of closely related species form genera (28). The basic genomic organization of each papillomavirus type has remained stable for a period of greater than 100 million years, and comparative sequence analysis suggests that these viruses have evolved over time with their hosts.

PV infections cause benign lesions in cutaneous or mucosal epithelial tissue. Each PV type has a preferred host and site of infection (28). Of the twelve genera of PVs, five are HPVs. Lesions caused by a subset of the alpha genera of the HPVs, known as "high-risk" types, are at a greater risk of malignant progression (28;29). Many of these HPV types are found to be ubiquitously distributed around the globe. HPV type 16 is responsible for the greatest percentage of cervical cancer cases per year followed by types 18, 45, 31, and 33 (see Figure 1-2) (30). These data were used to support the rationale behind the development of vaccines to offer protection against the two most common types HPV16 and 18. However, together these types only account for approximately 70% of cervical cancer, and there are slight differences in the relative predominance of the most common types in different geographical regions around the world (31).

Figure 1-1. Phylogenetic organization of PV types.
Image adapted from de Villier *et al*. (2004) (28)

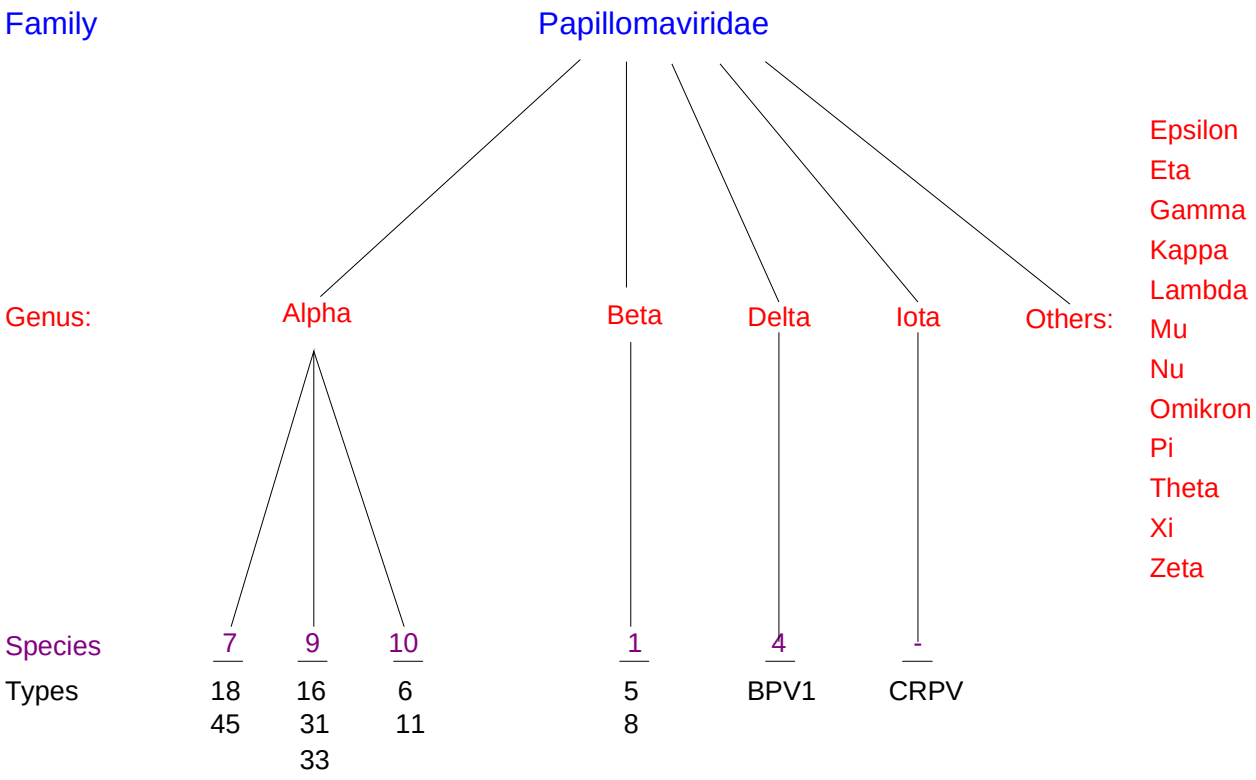
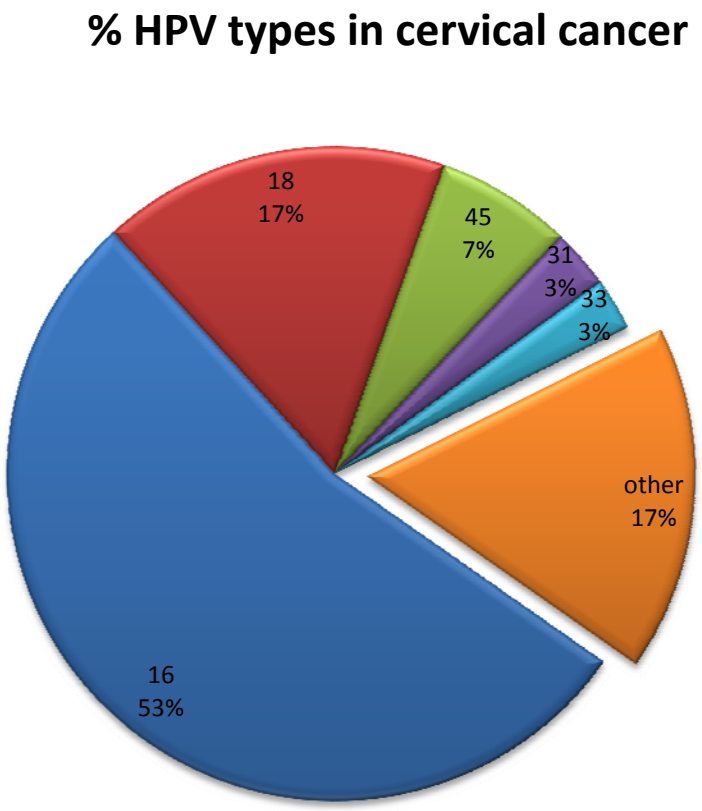

Family
Papillomaviridae
Genus:
Alpha
Beta
Delta
Iota
Others:
Epsilon
Eta
Gamma
Kappa
Lambda
Mu
Nu
Omikron
Pi
Theta
Xi
Zeta
Species
7
9
10
1
4
-
Types
18
45
16
31
33
6
11
5
8
BPV1
CRPV

Figure 1-2. Frequency of HPV types.

Data from Munoz et al. (2004) (29). Type-specific frequency of cervical cancer cases worldwide.


% HPV types in cervical cancer
18
17%
45
7%
31
3%
33
3%
other
17%
16
53%

According to the Center for Disease Control and Prevention, sexually active American women have a lifetime risk of greater than 80% of contracting at least one strain of genital HPV by the age of 50, making HPV the most common sexually transmitted infection (STI) (32). Fortunately, in follow up studies it is found that the majority of women who have tested positive for an HPV infection are cleared of the infection within a couple years (33). However, for a small percentage of women, the HPV infection is not cleared and viral DNA can persist as an autonomously replicating episome in benign lesions. The continued presence of the oncogenic viral proteins E6 and E7 can lead to genetic instability and malignant progression. The idea that malignant progression is a consequence of a long-term persistent infection and is a critical component of the cancer equation is supported by the fact that the peak rate of cancer incidence occurs years after the peak in infections (33).

Cervical cancer is not the only cancer associated with HPV infections. Figure 1-3 shows the number of cases of other cancers that have been associated with HPV infections and the total world-wide number of these cancers, demonstrating the relative burden of HPV-induced cancer in each of these cancers (34). In addition, cancer is not the only known disease associated with HPV infections; a summary of the most common and best characterized HPV-associated diseases is listed in Table 1-1. Warts were the first identified clinical manifestation of HPV infection and there are many different types of warts caused by different types of HPV types (Table 1-1) (35).

Figure 1-3. HPV-related cancers.

Total annual worldwide number of cancer cases (light gray bars). Annual Worldwide number of HPV-associated cases (dark grey bars). Data adapted from Parkin (2006) (34)

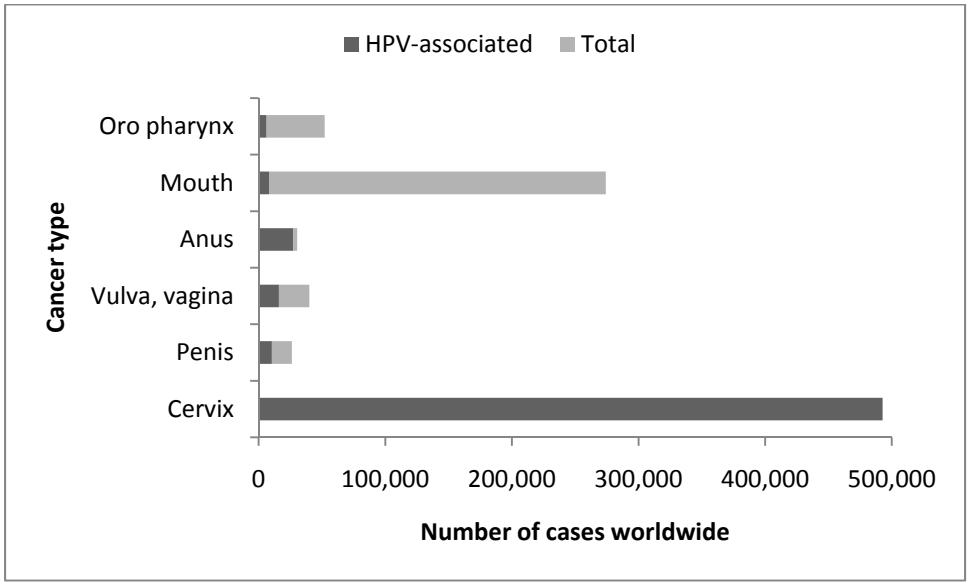

HPV-associated
Total
Oro pharynx
Mouth
Anus
Vulva, vagina
Penis
Cervix
Cancer type
0
100,000
200,000
300,000
400,000
500,000
Number of cases worldwide

Table 1-1. HPV-associated disease

Data from Hoory *et al*. (2008) (35). Types shown are the most common types found associated with each clinical disease. EV- Epidermodysplasia Verruciformis; SCC- Squamous Cell Carcinoma; NMSC- Nonmelanoma Skin Cancer.

HPV-associated Cancers	**Types Associated**	**HPV-associated disease**	**Types associated**
Genital		Warts	
Cervical	16,18,31,45	Common	1,2,4,27 57
Vaginal	16	Plantar	1,2,4,27 57
Vulvar	16	Flat	3,10,28,41
Penile	16	Anogenital	6,11
Anal	16	EV	5,8
Head and Neck SSC	16	Recurrent Respiratory Papillomatosis	6 and 11
NMSC	5,8		

Because of the strict species specificity of papillomaviruses, no animal model exists to study HPVs. Bovine papillomaviruses (BPV), particularly BPV1, and cotton-tail rabbit papillomaviruses (CRPV) have become popular models to study the basic principles of papillomavirus infections *in vivo*. CRPV, or Shope papillomavirus, was one of the first papillomaviruses discovered and infects the skin of rabbits. Like the high-risk HPVs it also causes cancer (36;37). BPV1, the most extensively studied of the six BPV types, causes benign lesions in fibroblasts resulting in fibropapillomas in cattle (38). BPV1 has also been used extensively in many *in vitro* studies after it was discovered that BPV1 could transform rodent cells in culture (38-40). BPV1 research has shed light on many important PV biological processes such as replication, transcription, chromosomal segregation, neoplasia, the host-immune response, and finally the efficacy of anti-papillomavirus vaccines (36;41). However, of course, important differences have also been found between BPV1 and HPV, such as the organization of the cis-elements in the Upstream Regulatory Region (URR) and the mechanism

of viral genome segregation (42). This has highlighted the importance of verifying the results found with BPV1 in HPV.

Experimentally, the promitogenic properties of the E6 and E7 proteins of the high-risk HPV types enable primary human foreskin keratinocytes (HFKs) in culture to bypass senescence and become immortalized (43-46;46). On the other hand, the low-risk types, which are not usually associated with cancer, do not immortalize cells in culture. The E6 and E7 proteins of the low-risk HPV types do not have the same ability to reprogram the cell cycle as the oncoproteins of the high-risk types (47-52).

HPV Replication and Life Cycle

Papillomaviruses establish infections in the basal cells of mucosal or cutaneous epithelial tissue where their genomes can persist as extrachromasomally replicating elements for many years. PVs demonstrate a strict epithelial tropism and will only replicate in keratinocytes. In addition, the complete life cycle of HPV depends on the differentiation program of the host tissue and infectious particles can only be produced in stratified epithelium. The PV life cycle has three distinct stages of replication. In the first stage, establishment, the viral genome is initially replicated to equip the cell with a reservoir of infected cells. After the virus has established an infection in the basal cells of the epithelial tissue, the viral DNA must be duplicated and partitioned to daughter cells along with the chromosomal DNA during cell division in order to maintain a persistent infection. This is known as the maintenance stage. As the infected cells differentiate, the virus completes its viral cycle in the upper differentiated tissue where the viral genome is amplified for encapsidation by the late capsid proteins L1 and L2, which are expressed

in the upper layers. This produces a supply of newly formed infectious particles that are then shed with the dead cells of the surface layer of the skin (see McBride (2008) for a review (42)).

Cell-free replication assays revealed that HPV replication is similar to SV40 replication in that they both 'borrow' many of the same host cellular replication factors (53-55). The role of the essential viral replication protein, T-antigen, in SV40 replication is fulfilled by the HPV viral proteins E1 and E2 (56-58). E2 serves as the origin of replication recognition factor and E1 functions as a replicative DNA helicase. As the replicative DNA helicase, E1 expression is absolutely essential for viral replication. However, in most cases the E1 binding affinity for its cognate sequence is very weak. Therefore, E1 depends on recruitment by E2, which has a higher binding affinity for the origin. Studies have found that E1 overexpression can overcome the challenge in binding and when present at high levels is sufficient for replication initiation (59). However, since high expression levels of the E1 protein are not found in a normal infection, this is not applicable information and is merely experimental evidence. In fact, Stubenrauch *et al*. reported that E1 is the limiting factor in HPV replication (60).

The cis-elements of the viral genome required for HPV replication have been analyzed using transient replication assays. In these assays a plasmid containing the origin of replication is co-transfected along with expression vectors that provide the essential replication proteins. After an incubation period of a few days, the plasmids containing the origin of replication are analyzed for efficient replication. Alternatively, entire genomes can be transfected into cells capable of supporting HPV replication. In these assays, the essential replication proteins E1 and E2 are provided by the transfected genome. The advantage of this system is the ability to study the full viral genome in its natural context and in the presence of basal expression levels of the replication proteins. The disadvantage of this system is the reliance of expression of the essential viral replication proteins on a construct that may be lost overtime. This complicates the ability to distinguish effects of changes made in the URR on replication from the effects of these changes

on transcription which controls expression of the essential viral replication proteins. Nevertheless, transient studies using both systems have identified a distinct origin of replication in the URR that is essential for viral replication (57;58;61-63).

Finally, because HPVs are able to establish persistent infections, long-term replication assays are important to study the elements that are required for genome maintenance over time. In BPV1, the minimal origin of replication was not sufficient for long-term replication. The addition of a sequence within the URR called the minichromosome maintenance element (MME) which contains multiple E2 binding sites, was found to be necessary and sufficient for long-term genome maintenance over several generations (64). This observation was instrumental in identifying the additional role of the E2 protein in viral genome maintenance. It was subsequently shown that the E2 protein partitions and maintains the viral genomes by attaching them to mitotic chromosomes in complex with the cellular bromodomain-containing protein, Brd4 (65;66). In BPV1, at least 8 of the 11 E2 binding sites located in the URR are required for genome maintenance (64). The role of the E2 binding sites in regards to genome maintenance in the high-risk HPVs is unknown. Because at least some of these sites have been found to be essential for transient replication, it is difficult to study their role in long-term replication. Since there are only 4 E2 sites, it is interesting to speculate whether they alone would be sufficient as BPV1 requires at least 8. It is possible that there are additional alternative cis-elements involved in segregation of the high-risk HPVs. Tan *et al*. have proposed that HPV genomes achieve copy control by associating with the nuclear matrix (67). This study identified the presence of five distinct regions in the HPV16 genome that the authors called matrix attachment regions (MARS) and which associate with the nuclear matrix. However, this study did not establish whether these elements impart stable genome maintenance. MARS are sequences that anchor chromatin fibers to the nuclear matrix and generate transcriptionally active domains. They control the

compartmentalization of the nucleus and include enzymes suggesting that they may have a function apart from their structural role (67).

HPV gene expression

HPVs infect epithelial tissue and express proteins that disrupt the host cell cycle program. The HPV genome is approximately 8000 base pairs and encodes 5 early proteins (E1, E2, E5, E6, and E7) and 3 late proteins (E4, L1 and L2). Early studies identified the E6 and E7 genes as the oncogenic agents in HPV infections. The HPV genomes recovered from malignant tumors are often found integrated into the host chromosomal DNA. It is proposed that this event leads to a disruption in the regulation of the oncogenic E6 and E7 proteins and results in unstable cell cycle regulation which can lead to genetic instability.

As mentioned, the HPV life cycle is tightly linked to the differentiation program of the epithelial host tissue. Presumably the dynamic expression of host regulatory genes responsible for the differentiation program of the host tissue also enables the ordered sequence of the events in the HPV life cycle. Transcription occurs uni-directionally from one or several promoters. These transcripts undergo differential splicing to produce numerous poly-cistronic mRNAs representing the seven to nine PV genes (68). Expression of the early and late genes is controlled by independent, distinct promoters (69). There has been great interest in understanding how the early promoter is regulated because it is responsible for the expression of the oncogenes, E6 and E7.

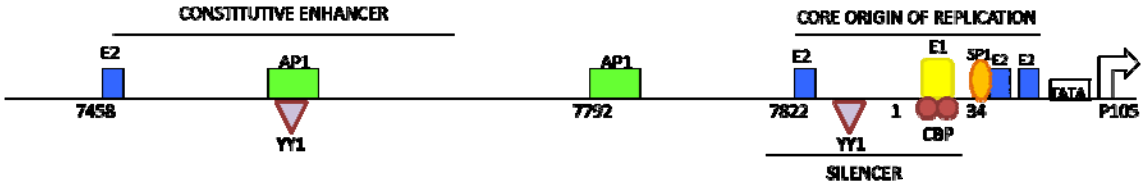
A sequence of DNA approximately 800 base pairs long, located upstream of the first open reading frame, does not encode for any genes. This stretch of sequence contains many elements that control replication and transcription and is therefore known as the upstream regulatory region or URR. The original studies investigating the transcriptional control by this

region used DNA footprinting analysis and chloramphenicol acetyltransferase (CAT) or luciferase reporter assays to identify which transcription factors are important in the complex regulation of HPV gene expression. In general, these studies led to the conclusion that the early promoter is primarily controlled by the Sp1 and the TATA box binding sites and negatively regulated by high expression levels of the viral E2 protein (68).

These studies also identified additional regions within the URR that regulate viral transcription and replication. An enhancer region is located approximately 400 base pairs upstream of the E6 promoter and is thought to play a role in the epitheliotropic specificity of PV transcriptional regulation (Figure 1-4) (68;70;71). Another region approximately 100 base pairs in length, which overlaps the origin of replication, was found to have a negative impact on transcriptional activity and is therefore termed a silencer region (68;72). The purpose of having opposing activating and repressive elements in the URR provides a mechanism to vary the activity of the promoters in different contexts. The primary cis-elements found in the HPV silencer are binding sites for the transcription factors YY1 and CDP (68;71-73). The primary element(s) found in the enhancer region is one or more Ap1 binding sites. The expression of the family members of all of these transcription factors changes upon differentiation and, as a result, the binding profile of these elements changes. The relative abundance of the various members of the Ap1 family changes throughout the course of differentiation (74;75). In addition, the expression of YY1 and CDP decreases upon differentiation, relieving the repression of the early promoter (71;76;76).

The viral E2 protein plays an essential role in the complex regulation of the early promoter. Initial characterization of the integration site of viral genomes in cervical tumors revealed that integration often occurs in the downstream portion of the early gene region (77-79). This suggested that the loss of downstream early genes, E1 and E2, might play a role in the HPV cancer equation. Further analysis identified that the viral E2 protein could suppress activity from

Figure 1-4. Organization of cis-responsive elements in the HPV18 URR


CONSTITUTIVE ENHANCER
CORE ORIGIN OF REPLICATION
E2
AP1
AP1
E2
E1
SP1
E2
E2
TATA
7458
YY1
7792
7822
YY1
1
CBP
34
P105
SILENCER

the early promoter (80-83). However, E2 has also been found to activate early promoter transcription, as is the case for the BPV1 early promoter (84). The fate of E2 transcriptional regulation appears to depend on a variety of factors including; relative expression levels of the E2 protein, the promoter type, the cell type, and the number and location of the E2 binding sites located in the URR (85-87).

In the high-risk HPV types, the number and location of the E2 binding sites are highly conserved (Figure 1.4). There are four E2 binding sites and three are located within the origin of replication (63). The two binding sites that are most proximal to the early promoter are sandwiched between the Sp1 and TATA box binding sites, which are considered to be the most critical transcription factors for the activation of the early promoter (68). All these factors, the Sp1, E2 and TATA box binding protein, compete for binding to these cis-elements due to the close proximity of the binding sites for these factors (88;89). As a result, the fate of the HPV early promoter transcription depends on antagonistic effects of these factors as determined by the competition among these transcription factors for binding to this tight region in the URR. However, more recent data suggests that E2 repression is not simply due to its binding in this region. E2 recruits components such as Brd4 and other repressor complexes to repress transcription (90;91).

HPV Induced Immortalization

HPV-induced immortalization is an unintentional consequence that results from the pro-mitogenic signals that the virus uses to enable viral replication in differentiated cells which have normally exited the cell cycle. The pro-mitogenic signals of HPV are primarily attributed to the viral oncogenes E6 and E7 because these proteins together are sufficient to induce immortalization (reviewed in Munger and Howley (2002) (44). How these viral proteins

accomplish this has been a primary focus of the past 30 years of HPV research and there are new oncogenic properties of these proteins currently under investigation. In general, E7 acts to suppress apoptotic signals and allows survival of severely damaged cells. To complement these activities, E6 primarily functions to drive replication and cell growth (52). The best characterized properties of the E6 and E7 proteins are their ability to interfere with the functions of the major tumor suppressor genes, p53 and pRb, respectively.

As mentioned above, possibly the most potent and certainly the best characterized property of the viral E6 protein is its ability to target the major tumor suppressor gene, p53, for degradation (92). This is accomplished by the binding of E6 to the cellular E6 Associated Protein (E6-AP), which is a ubiquitin protein ligase. This complex binds p53 and targets it for degradation, resulting in almost undetectable levels of the p53 protein in HPV-infected cells (92). However, a mutation in the E6 protein that is defective in its ability to degrade p53 is still capable of immortalizing embryonic cells (93;94). This led to the discovery of p53-independent mechanisms of E6-mediated immortalization and the identification of other oncogenic properties of E6 (for a review see Thomas et al (1999) (92)). Of note is the ability of the E6-E6-AP complex to cooperate with c-Myc to activate telomerase expression (95-100).

The primary transforming role of the E7 protein is its ability to bind the active form of the retinoblastoma protein, pRB. This association effectively prevents the binding of pRb to E2F, a transcription factor that drives the S phase of the cell cycle via cyclin A and cyclin E (101). However, like the viral E6 protein, E7 is multifunctional in its oncogenic capacity. E7 also inactivates the cyclin inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, thus relieving the negative influence of this pathway on cell growth (102). E7 has been shown to directly induce genomic instability by inducing centriole amplification (103). In addition, recently, E7 has also been suggested to reinforce E6-mediated upregulation and maintenance of telomerase expression (97).

While the E6 and E7 proteins together are sufficient for cellular transformation, oncogenic properties of E5 have also been identified and these may augment E6- and E7-mediated oncogenesis. In BPV1, the E5 protein is actually the most potent transforming factor (104-109). While the HPV-16 E5 protein does not possess the same transforming activity, it does stimulate proliferation in keratinocytes via the Epidermal Growth Factor (EGF) signal transduction pathway (110-112).

HPV Research: Model Cell Lines and Culturing Techniques

The wide distribution and popularity of HeLa cells has led to a very thorough characterization of the molecular processes of these cells, whether they represent normal cellular features or abnormal carcinogenic properties. These cells, as well as other cell lines derived from cervical cancers, have helped to identify the mechanisms and characteristics of HPV-induced cancer such as viral genome integration (77;113-115). The CaSki and SiHa cell lines both have integrated copies of HPV16. However, the SiHa cell line was found to harbor a single integrated copy of the viral genome, while the CaSki cell line was found to contain approximately 500 copies (79;116). HeLa cells also have multiple copies of the HPV18 genomes integrated in 5 different sites in the cellular DNA (117;118).

In order to study the complete viral life cycle, however, it is necessary to examine cells that maintain extrachromosomal copies of the viral genome (119). There are a few established cell lines isolated from low-grade cervical lesions that represent early stages of infection. The CIN-612 cell line represents a mixed population of HPV-31b infected cells isolated from a low-grade cervical lesion by George Wilbanks and Lou Laimins (120;121). Two different clones were isolated from this cell line; the 6E subclone that contains integrated copies of the viral genome and the 9E subclone which stably maintains extrachromosomal copies of the viral DNA

(121;122). The 20863 subclone of the W12 cell line maintains extrachromosomal copies of HPV16. This clone was isolated by Paul Lambert from a population of cells isolated from a low-grade cervical lesion originally collected by Margaret Stanley (123;124). However, the extrachromosomal state of the viral DNA in these cells is relatively unstable and has been shown to be lost overtime as populations of cells with integrated DNA take over the culture (125;125;126).

Often it is desirable to use uninfected cells to examine individual viral proteins or to follow the course of infection from establishment and episomal replication through malignant progression to non-productive infections with integrated viral DNA. For these purposes various cell types, including non-keratinocyte cell lines, have been used. For the most part, the use of keratinocytes are preferable because of the strict cell-type specificity of HPVs and because these cells are the natural host of the virus. There are a handful of immortalized keratinocyte cell lines that are HPV-negative: the HaCaT (127), SCC-13 (128), NIKS (129), N-Tert (130), and C-33a (131) cell lines. The NIKS and HaCaT cell lines are spontaneously immortalized human keratinocytes that retain the ability to differentiate, an important property of normal keratinocytes that enables the analysis of the full viral life-cycle in these cells. However, these cells have genetic abnormalities that are the consequence of the events that led to their spontaneous immortalization; the NIKS have trisomy of chromosome 8 (129), and the HaCaTs have a large number of translocations and deletions by passage 50 (132). The SCC13 cell line was derived from an HPV-free squamous cell carcinoma (128). C33-A cells are a rare example of an HPV-negative cervical cell line. While widely used for HPV-research, there are data to suggest that these cells have abnormal properties critical for their application in HPV studies. Discrepancies have been reported in the transcriptional regulation of the HPV URR in these cells compared to other keratinocyte lines (133;134). The N-tert line is a primary keratinocyte line transduced with the hTERT gene and grown from a subpopulation of cells that spontaneously bypassed

senescence due to a mutation in the $p16^{INK4a}$ gene (130;135). The immortalization of this line resulted from a more controlled process and theoretically has fewer unknown variables. However, all of these cell lines have disruptions in major tumor suppressor pathways such as p53 and p16 that allow these cells to bypass this normal checkpoint. For this reason, they do not accurately represent primary cells that have functional tumor suppressor pathways intact. This can be problematic for the study of oncogenic viruses such as HPV.

Primary keratinocytes represent the ideal model that best exemplifies the environment that HPV encounters upon infection. Culturing conditions for primary keratinocytes were first established by researchers Rheinwald and Green, who developed a co-culture system with mitotically inactivated fibroblast feeder cells in serum-containing media (136). Later, a feeder-free system was established to grow keratinocytes in a serum-free medium (137). However, extrachromosomal HPV genomes are only maintained efficiently in the presence of fibroblast feeder cells and, therefore, this system may represent the superior model for HPV research (122;138). Cell lines that contain custom-made, high-risk HPV genomes can be generated *in vitro* by transfecting primary keratinocytes with cloned DNA and selecting for immortalization (43;45;139). This is a very powerful tool for HPV research that allows the analysis of specific mutations in the high-risk HPV genomes in their natural host.

Keratinocytes are unique in their ability to differentiate. The organotypic raft culture is a method that allows keratinocytes to differentiate over the course of a few days, the amount of time it takes for basal cells to stratify completely. The keratinocytes are seeded onto a collagen matrix that is lifted onto a grid to establish an air-liquid interface which signals the keratinocytes to differentiate (140;141). Growing HPV positive keratinocytes in the organotypic raft culture system is the only way to produce authentic, infectious virions in culture (142;143). Therefore, this is a very useful system that enables the study of the complete HPV life cycle.

The Rho Pathway

Rho was named to reflect its homology with the Ras family of oncogenes (Ras-homologous) and was therefore also predicted to possess similar oncogenic properties as the Ras proteins. However, interestingly, no mutations in the Rho genes are known to be associated with human tumors (144). The primary target of the Rho pathway is the actin filaments of the cytoskeleton. Initial characterization of the Rho protein identified its effect on actin stress fibers in fibroblasts (145). Since this effect was similar to the stress fiber formation induced by serum-treatment it was suggestive that growth factors play a role in cytoskeletal organization (146).

The Rho pathway is stimulated by extracellular signals binding to membrane bound G-protein-coupled receptors which signal the activation of the Rho GTPases through guanine nucleotide exchange factors (GEFs) as shown in Figure 1-5 (147). In total, there are 20 known mammalian GTPases in the Rho family belonging to three main classes: Rho, Rac, and Cdc42 (148;149). The Rho class is comprised of three isoforms, RhoA, RhoB, and RhoC. The first effectors of the Rho class of GTPases to be identified were the Rho Kinases (ROCKs). There are two known isoforms of the ROCK proteins, ROCK I and ROCK II (150).

In addition to the cellular functions mediated through the actin cytoskeleton, there are many other cellular behaviors linked to the Rho pathway as a consequence of the large amount of effector proteins regulated by the Rho GTPases. Notably, with regard to the scope of this thesis, is the reported effect of the changes in the Rho pathway on cell growth (151). However, the exact effect of the Rho pathway on proliferation remains unclear because of the complexity involved in the factors influencing cell growth. It seems that a number of factors all contribute to the effect of the Rho pathway on the fate of cell growth including the regulation between proliferation and differentiation, regulation of apoptosis, cell type, cell contacts, and the type of stimulus used to

Figure 1-5. The Rho pathway

Image adapted from Ridley et al (2003) (147). GPCR: G-protein-coupled receptors; GDP: Guanonsine Diphosphate; GTP: Guanosine Triphosphate.

activate or inhibit the Rho pathway (152;153). This complexity has led to conflicting reports about the effect of the Rho pathway on cell growth.

In keratinocytes, two different groups reported opposite effects of the Rho pathway on differentiation. In 2003, McMullen *et al*. (2003) concluded that inhibiting the Rho Pathway prevents differentiation and therefore the Rho pathway naturally acts to induce differentiation in mouse keratinocytes (154). However later in 2006, the authors of Grossi et al (2006), who used mouse and human keratinocytes, reported the opposite conclusion; that inhibiting the Rho pathway promotes differentiation and therefore must naturally promote proliferation in lieu of differentiation (155). The authors of McMullan *et al.* (2003) had used both C3 ADP-ribosyltransferase (C3), from *Clostridium botulinum*, and Y-27632 as inhibitors of ROCK. They measured keratinocyte growth using BrdU-incorporation assays and by measuring colony forming efficiency of the treated and untreated keratinocytes. In contrast, the authors of Grossi *et al.* (2006), had only examined differentiation and only used C3 to inhibit ROCK. It is possible that the Rho pathway has different roles under proliferative or differentiating conditions. The Rho pathway may suppress proliferation when induced to differentiate, but may suppress proliferation in normal culture conditions. It is also possible that C3 and Y-27632 affect additional pathways and that either one may have a more pleiotropic effect than assumed.

The small molecule, Y-27632, has become the most commonly used ROCK inhibitor (147;156). However, this compound has also been shown to bind and inhibit other protein kinases including protein kinase C-related protein kinase 2 (PRK2), mitogen- and stress-activated protein kinase 1 (MSK1), mitogen-activated protein kinase-activated protein kinase 1b (MAPKAP-K1b) Phosphorylase kinase (PHK), and citron kinase (157;158). The concentration of Y-27632 needed for inhibition of ROCK-II and PRK2 are very similar, while the other kinases are inhibited at much lower concentrations (158).

Chapter 2

Materials and Methods

Plasmids

Specific mutations in the cis-element binding sites were introduced into the full-length HPV18 genome, cloned in the pBR322 plasmid, using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). Primers (Integrated DNA Technologies, Inc.) used for mutagenesis are listed in Table 2-1. Mutations were verified in isolated clones by sequencing. 5µg of genomic DNA was digested from the vector sequence and then electroporated into $1x10^6$ HFKs.

HPV18 and 31 subgenomic replicons were constructed by deleting large fragments of the full length genome as illustrated in Figure 5-6. The HPV18 replicons were generated using the following restriction enzymes in the pBR322HPV18 plasmid; NcoI (6233) and BlpI (823) for the 18ΔURR replicon, Blp1 (824) and AleI (1945) for the 18ΔER replicon, and KpnI (4795-6273) for the 18ΔLR replicon. The HPV31 replicons were generated using the following restriction enzymes in the pBRminHPV31 plasmid; BanII (815) and BlpI (6271) for the 31ΔURR replicon, BanII (816) and EcorV (2143) for the 31ΔER-S replicon, HpaI (218) and EcorV (2143)for the 31ΔER-L replicon, and BlpI (6270) and XbaI (4999) for the 31ΔLR replicon.

Table 2-1. Primers used for Site-directed mutagenesis

Mutated nucleotides are indicated in red

Primer Site		Primer Sequence
E2BS1	Forward	5' GGAGTAACCGAAAACGGTCGGGATTGAAAACGGTGTATATAAAAG 3'
	Reverse	5' CTTTTATATACACCGTTTTCAATCCCGACCGTTTTCGGTTACTCC 3'
E2BS1(2)	Forward	5' GGAGTAACCGAAAACGGTCGGGACCGAAAACAGTGTATATAAAAG 3'
	Reverse	5' CTTTTATATACACTGTTTTCGGTCCCGACCGTTTTCGGTTACTCC 3'
E2BS1(3)	Forward	5' GGAGTAACCGAAAACGGTCGGGTTCGAAAACCCAGTATATAAAAG 3'
	Reverse	5' CTTTTATATACTGGGTTTTCGAACCCGACCGTTTTCGGTTACTCC 3'
E2BS2	Forward	5' GTATATAAAAAAGGGAGTAACCGAAAACAGTCGGGACCGAAAACG 3'
	Reverse	5' CGTTTTCGGTCCCGACTGTTTTCGGTTACTCCCTTTTTTATATAC 3'
E2BS2(2)	Forward	5' GTATATAAAAAAGGGAGTAATTGAAAACGGTCGGGACCGAAAACG 3'
	Reverse	5' CGTTTTCGGTCCCGACCGTTTTCAATTACTCCCTTTTTTATATAC 3'
E2BS2(3)	Forward	5' GTATATAAAAAAGGGAGTACACGAAAACACTCGGGACCGAAAACG 3'
	Reverse	5' CGTTTTCGGTCCCGAGTGTTTTCGTGTACTCCCTTTTTTATATAC 3'
E2BS1/2	Forward	5' GGAGTAACCGAAAACAGTCGGGATTGAAAACGGTGTATATAAAAG 3'
	Reverse	5' CTTTTATATACACCGTTTTCAATCCCGACTGTTTTCGGTTACTCC 3'
E2BS3	Forward	5' GCTGTGCATACATAGTTTATGCAATTGAAATAGGTTGGGCAGCAC3'
	Reverse	5' GTGCTGCCCAACCTATTTCAATTGCATAAACTATGTATGCACAGC 3'
E2BS4	Forward	5' CCTCCATTTTGCTGTGCAATTGATTTCGGTTGCCTTTGGCTTATG 3'
	Reverse	5' CATAAGCCAAAGGCAACCGAAATCAATTGCACAGCAAAATGGAGG 3'
Sp1	Forward	5' GTAGTATATAAAAAAGTTAGTAACCGAAAACGGTCGGGACCG 3'
	Reverse	5' CGGTCCCGACCGTTTTCGGTTACTAACTTTTTTATATACTAC 3'
Ap1pr	Forward	5' CCCTTAACATGAACTATAATATAAGTAAGCTGTGCATACATAG 3'
	Reverse	5' CTATGTATGCACAGCTTACTTATATTATAGTTCATGTTAAGGG 3'
Ap1d	Forward	5' GGCGCATATAAGGCGCACCTGGTATTACTTATTTCCTGTCCAGG 3'
	Reverse	5' CCTGGACAGGAAATAAGTAATACCAGGTGCGCCTTATATGCGCC 3'
Tatabox	Forward	5' CGGTCGGGACCGAAAACGGTGTATATTTATGATGTGAGAAACAC 3'
	Reverse	5' GTGTTTCTCACATCATAAATATACACCGTTTTCGGTCCCGACCG 3'
TataboxG/C	Forward	5' CGGTCGGGACCGAAAACGGTGTAGCCGACAGATGTGAGAAACAC 3'
	Reverse	5' GTGTTTCTCACATCTGTCGGCTACACCGTTTTCGGTCCCGACCG 3'

Cell Culture

3T3 J2 feeder cells

3T3 J2 feeder cells (136) were grown in liquid Dulbecco's modified Eagle's medium (DMEM), 4500mg/L D-glucose and L-glutamine, no sodium pyruvate, supplemented with 10% heat inactivated Newborn Calf Serum and 25 µg/mL gentamycin or 100U Penicillin and 100 µg/ml streptomycin in a 37°C 5% CO_2 humidified incubator and fed every 2-3 days. Feeders were not used beyond passage 20.

3T3 J2 feeder cells were growth arrested by one of two methods: (1) gamma-irradiation or (2) mitomycin-C treatment.

1. Feeder cells were trypsinized and collected in a 15 ml falcon tube on ice. Cells were irradiated in a Gamma-Cell 1000 Nordian at 6000 Rads and seeded onto 10 cm tissue culture dishes in feeder media at approximately 30-40% confluence. Cells were incubated at least 2 hours before seeding with keratinocytes.
2. Mitomycin C was added to feeder cells maintained in 10 ml feeder media in a 10 cm plate at a final concentration of 8 μg/mL and incubated for 2-4 hours. Media containing the mitomycin C was removed and cells were washed 3 times with PBS. Then feeders were trypsinized and seeded in E-media at 30-40% confluence.

Keratinocytes

HFKs were isolated from neonatal foreskins and maintained in medium 154 (Cascade Biologics) ((159), (143)). In the absence of feeders, keratinocytes were grown in 154 medium supplemented with Human Keratinocyte Growth Supplement and Gentamicin/Amphotericin (Invitrogen) or in Keratinocyte Growth Media (KGM) (Invitrogen). An alternative method was used in the case of the keratinocytes grown in Dick Schlegel's lab. Human cervical keratinocytes (HCKs) and human vaginal keratinocytes (HVKs) isolated from adult hysterectomy tissue specimens were provided by Craig Meyers (160). All human tissues were collected with institutional approval.

The epidermis was separated from the dermis of the foreskin tissue. This was done by one of two methods:

1. By physical separation: Foreskin tissue was collected and washed in 1XPBS containing 50 μg/ml gentamycin sulfate (Gibco BRL) and 1X nystatin (Sigma Chemical Co.). The dermis was separated from the epidermis using a scalpel to remove the reddish-pink tissue (dermis) from the skin colored epidermis. The

detached epidermis was minced and incubated in 20 ml 0.5% Trypsin-EDTA (Gibco-BRL) at 37°C in a spinner flask to obtain a single cell solution. The trypsin solution was poured into 20mls of E-medium, containing 5% FCS (Hyclone), taking care not to include any undigested tissue. On average, 7 foreskin samples were pooled together for each sample preparation. HFKs were pelleted at 300 rpm, resuspended in 154 medium and cultured at 37°C in a 5% CO_2 humidified incubator and fed every other day with 154 medium.

2. By incubation in dispase: At circumcision, foreskins were provided as a gift from John Vogel. Upon isolation, foreskin tissues was washed with 1X PBS, cut in half and placed with the epidermal side up in 5-10 mls dispase (25 U/ml dispase in Hank's balanced salt solution; 1:1) O/N at 4°C. The next day the epidermal layer was peeled off the pink dermis tissue and placed in 2 ml 0.05% Trypsin-EDTA (Invitrogen) for 10-12 minutes at 37°C with gentle shaking every few minutes to obtain a single cell suspension. The trypsin solution was strained through a Netwell (Corning) into a new tube containing 10 ml Dulbecco's Modified Eagle Medium (DMEM) /10% Fetal Bovine Serum (FBS). On average, 7 foreskin samples were pooled together for each sample preparation. HFKs were pelleted at 300 rpm. resuspended in 154 medium and cultured at 37°C in a 5% CO_2 humidified incubator and fed every other day with 154 medium.

In the presence of growth arrested 3T3 J2 feeder cells, HFKs were grown either in F medium or E-medium.

1. F-Media: [3:1 (v/v) F-12 (Ham)(Invitrogen)-DMEM(Invitrogen), 5% FBS (Gemini Bio-Products), 0.4µg/ml hydrocortisone (Sigma), 5 µg/ml insulin (Sigma), 8.4 ng/ml cholera toxin (Calbiochem), 10 ng/ml EGF, 24 µg/ml adenine(Sigma), 100U Penicillin-100 µg/ml streptomycin (Invitrogen)] (161) (123).

2. E-media [3:1 (v/v) DMEM - F-12 (Ham), 10% FCS (Hyclone), 0.4 µg/ml hydrocortisone (Calbiochem), 0.1 nM cholera toxin (Schwarz-Mann), 5 µg/ml transferrin (Sigma), 2 nM 3,3'-5-triodo-L-thyronine (Sigma)] supplemented with 5 ng/ml EGF and 5 µg/ml insulin (Sigma) (62;162).

Cells were grown in the presence or absence of 10 µM Y-27632 (Alexis Biochemicals), as indicated. A 5 mM stock solution of Y-27632 was prepared by dissolving 25 mg in 14.79ml of sterile double-distilled H_2O. When the cells reached approximately 80% confluence, cells were subcultured by removing the fibroblast feeder cells with 1X versene (0.2 g/L EDTA•4Na in phosphate-buffered saline) (Invitrogen) and collecting keratinocytes by trypsinization. In general, primary or HPV-transfected keratinocytes were split at a 1:10 ratio and Y-27632-treated keratinocytes were split at a 1:20 ratio onto a 10 cm plate with growth arrested J2 3T3 feeder cells. Population doubling was calculated as: PD=3.32 (log(# cells harvested/# cells seeded)).

The CIN612 9E clone that harbors episomal copies of HPV31b was originally isolated from a mixed population of cells, the CIN-612 line which was from a low-grade cervical lesion established by George Wilbanks and Lou Laimins (120;121). The 20863 subclone of the W12 cell line that maintains episomal copies of HPV16 was cloned by Paul Lambert and was derived from a low-grade cervical lesion originally collected by Margaret Stanley (123;124).

Organotypic Raft Culture

Organtotypic rafts were grown one of two ways:

1. Organotypic raft cultures were generated as described previously(163), with modifications.
    a. First, a collagen dermal equivalent was generated by mixing the following components at the indicated ratio: 1/10 10X DMEM, 1/10 reconstitution

buffer [2.2% $NaHCO_3$, 0.05N NaOH, 200 mM Hepes], 8/10 rat tail collagen. 1-2x10^6 fibroblasts were added to 8mls of collagen mix. 0.75 ml of mixture was added to each well of a 24 well dish and allowed to solidify O/N in a 37°C 5% CO^2 humidified incubator.

b. The next day, 1X10^5 keratinocytes were seeded onto the rat tail type 1 collagen dermal equivalent containing the J2 3T3 feeder cells.

c. The next day, a sterile spatula was run along the edges of the rafts in the wells to separate the collagen from the plastic walls. The rafts were incubated O/N to shrink the layer of keratinocytes on the raft surface.

d. The next day, rafts were lifted onto stainless steel grids and were fed by diffusion from below with raft medium [3:1 (v/v) DMEM:F-12; 10% FBS; 0.4 μg/ml hydrocortisone; 0.1 nM cholera toxin; and 5 μg/ml transferrin].

e. Fully stratified epithelial tissue was collected after 10-17 days and fixed in Formalin (30% Formaledehyde) for 4 hours at room temperature. The tissues were then stored in 70% EtOH at 4°C.

f. Tissue samples were sent to American Histolabs for paraffin embedding and sectioning onto positive-charged slides. Some slides were selected for H&E staining, which was also performed by American Histolabs.

g. Images of the H&E slides were acquired using a Leica DM IRBE microscope captured with a Zeiss AxioCam camera. Fluorescent images were acquired using a Leica TCS-SP5 laser scanning confocal imaging system.

2. Organotypic raft cultures were grown as described (143).

a. First the collagen dermal equivalent was formed by aliquoting 2.5 ml of the following collagen mixture [10% 10X reconstitution buffer (2.2 g sodium bicarbonate, 4.7 g HEPES, and 75 ml of 0.062N NaOH in 100 mls), 10%

10X DMEM, 80% rat-tail type I collagen (BD Bioscience Discovery, Cat No. 356236), $2.5x10^6$ J2 3T3 fibroblasts per ml of the final volume, and 2.4 µL 10N NaOH per ml of the final volume] in a 6 well tissue culture dish and the collagen matrix was allowed to solidify in a 37°C incubator for at least 2 hours before seeding keratinocytes on top.

b. After the collagen matrix had solidified, $1x10^6$ keratinocytes were seeded on top onto the collagen in 3-4 ml of E-medium and allowed to attach overnight in a 37°C incubator.

c. The next day, the medium was removed from the wells, and the rafts were lifted out of the dish using a sterile lab spoon to separate the edges of the collagen from the walls of the well and underneath the raft. Rafts were placed onto a raised wire mesh grid placed inside of a 10 cm tissue culture dish keeping the keratinocyte side up.

d. Raft cultures were fed every other day with E-medium containing 5% FBS, 100 U/ml nystatin, and 10 µM C8 for 10 days, ensuring that the top of the rafts were kept dry.

e. Tissues were harvested by scraping the epithelium from the dermal equivalent and frozen at -70°C for use in infectivity assay.

f. Tissue samples including the dermal equivalent were harvested for histology, placed into dampened tissue embedding cassettes, and fixed in 10% neutral buffered formalin. Embedding and staining were performed by Lynn Budgeon of the Department of Pathology. Microphotography of the H&E slides was performed using a Nikon Labphot-2 microscope mounted with a Nikon N6000 camera.

Detection of Proteins in Paraffin-Embedded Tissue Sections

Unstained slides were stained by immunoflourescence as described by Pei *et al.* (164)

1. First the slides were incubated in Xylene (1X10 minutes, 1X 5minutes) to deparaffinize the tissue sections followed by a series of graded ethanol washes to rehydrate the tissue (100%, 80%, 50%, 30%, 2X2 minutes; ddH_20, 2X5 minutes).
2. For antigen retrieval, the slides were first pre-soaked in antigen retrieval buffer [10 m*M* citrate buffer, pH 6.0 (approximately 43 mL of 0.1 *M* sodium citrate solution, 7 mL of 0.1 *M* citric acid solution in 500 mL buffer)] in a glass container ensuring that the slides are completely submerged. The container was placed in the microwave set at 50% power level (approximately 650 Watts) for 12–15 min (3 × 5 min, with 1 min intervals), and allowed to cool for 15–20 min. Once the slides cooled, they were washed in PBS for 5 minutes.
3. To block nonspecific binding proteins, slides were placed tissue side down on top of a drop of 100 µl 10% normal donkey serum/PBS per slide placed on a strip of parafilm for one hour at room temperature.
4. Next, slides were incubated in 100 µl antibody solution (5% normal donkey serum/PBS). For diluted antibodies, 5 µl normal donkey serum was added to 95 µl of the diluted antibody and applied to the tissue-side of the slide. Undiluted antibodies were diluted according to suggested or optimized dilution concentrations in 5% normal donkey serum/PBS solution and applied to slides. Slides were incubated face-side down on a parafilm strip for 1 hour at room temperature. Slides were then washed in PBS 3 times for 5 minutes

5. Secondary antibodies were also diluted in 5% normal goat serum/PBS and incubated with slides on parafilm for 1 hour at room temperature. Slides were washed in PBS 3 times for 5 minutes.
6. Steps 4 and 5 were repeated for multiple staining.
7. Anitbodies used were: pre-diluted mouse mAb anti-keratin 14 (Ab-1; Thermo Fisher Scientific); pAbs used were: goat antiserum against filaggrin (N-20) at 1:50 dilution, and rabbit antiserum against involucrin (H-120) at 1:50 dilution from Santa Cruz Biotechnology.

Transfections

All HPV-transfected cell lines used were created in the lab by introducing the full-length HPV genomes into primary HFKs using two alternative methods of electroporation.

I. Standard electroporation of linearized HPV genomes were performed as previously described (165)
    1. 30 µg wild-type or mutant genomes were released from vector sequences by digestion with the restriction enzyme used for cloning. DNA was phenol-chloroform extracted from the digestion buffer, ethanol precipitated and resuspended in 25µl 1X TE.
    2. A total of 10 µl of the 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.
    3. A total of $5x10^6$ keratinocytes in a volume of 250 µl of E medium containing 10% FBS and 5 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) pH 7.2 was added to the DNA mixture.

4. The DNA and keratinocyte solution was transferred to an electroporation cuvette and electroporated using a Gene Pulser (Bio-Rad Laboratories) set at 210 V and 960 µFd.
5. The electroporated cell suspension was layered onto 10 ml of E medium containing 10% FBS and centrifuged at 300 rpm for 10 min.
6. The medium was removed and the cell pellets were resuspended in E medium containing 10% FBS.
7. The cells were added to 10-cm tissue culture plates containing mitomycin-C-treated J2 3T3 feeder cells.
8. A total of 5 ng/ml of EGF was added to the culture medium the next day.
9. The cultures were fed every other day for 7 days with E medium containing 10% FBS and 5 ng/ml EGF.
10. After the 7-day period, the cells were fed with E medium containing 5% FBS and 5 ng/ml EGF until the keratinocytes grew to confluence.
11. Confluent cultures were split at a ratio of 1:10 and fed every other day with 5% FBS E-medium. Cultures were passed until they were considered immortal (the time at which they outgrew the negative control of cells electroporated with only ssDNA).

II. Amaxa electroporation of re-circularized HPV genomes and subgenomic replicons

1. 50 µg of wild-type or mutant genomes were released from vector sequences by digestion with the restriction enzyme used for cloning in 300 µl digestion volume followed by phenol/chloroform extraction and ethanol precipitation.
2. Linearized genomes were re-ligated in 10 mls of 1X ligation buffer [20 mM Tris pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP] to favor re-

circularization of the HPV genomes rather than re-insertion of the vector. This was followed by phenol/chloroform extraction and ethanol precipitation.

3. Electroporations were performed using the using the AMAXA electroporation system according to the manufacturer's instructions (Lonza).
4. 2 μg of re-circularized HPV genomes were mixed with 4.25 μl of 10mg/ml sonicated salmon sperm DNA for each electroporation.
5. $2x10^6$ or $5x10^6$ primary HFKs/electroporation were resuspended in 100 μl nucleofector solution (Amaxa Keratinocyte Kit), added to the DNA mix, and transferred to an Amaxa electroporation cuvette. The cell-DNA solutions were electroporated using the T-09 program defined by the manufacturer for enhanced keratinocyte survival.
6. The cell suspension was removed from the cuvette using the Amaxa pipette and transferred to 10cm tissue culture dishes containing irradiated 3T3 J2 fibroblasts and pre-warmed F-media (no EGF) and placed in a 37°C 5% CO_2 incubator.
7. The next day, the medium was replaced with EGF-containing F-media. The cells were fed every 2-3 days and maintained in EGF-containing F-media at 37°C in a 5% CO_2 incubator. New feeders were added as needed until cells reached approximately 80% confluence at which time they were split at a 1:10 ratio, and passed until they were considered immortal (the time at which they outgrew the negative control of cells electroporated with only ssDNA).

DNA extraction from keratinocytes

DNA was extracted from cells by one of two methods:

1. Low-molecular weight DNA was extracted from the cells by HIRT extraction:
    a. Keratinocytes were trypsinized, counted, and pelleted when cultures were approximately 80% confluent and after versene removal of feeder cells.
    b. Cells were resuspended in 200 µl Solution I [50 mM glucose, 25 mM Tris-Cl (pH8), and 10 mM EDTA (pH8)].
    c. Cells were lysed by adding 400 µl Solution II [1%SDS, 0.2N NaOH] made fresh at each extraction. Samples were incubated on ice for 5 minutes.
    d. Lysed samples were neutralized by adding 300 µl Solution III [3M KOAc, 11.5% glacial acetic acid], followed by incubation on ice for 10 minutes.
    e. Samples were centrifuged at 4°C for 10 minutes.
    f. The nucleic acids were collected and precipitated with 0.6 volumes of isopropanol and incubated at -20°C at least O/N.
    g. The precipitated DNA was centrifuged and resuspended in 200 µl HIRT digestion buffer (20 mM Tris (pH8), 100 mM NaCl, 10 mM EDTA, 0.2% SDS, and 200 mg/ml proteinase K (fresh)) and incubated for 30 minutes at 37ºC, then at 50°C for 30 minutes.
    h. Samples were purified by a phenol/chloroform extraction, followed by a chloroform/isoamyl alcohol extraction, using Phase Lock Gel tubes (Eppendorf-Brinkmann) and precipitated with 0.3M sodium acetate and 2.5 volumes ethanol.
    i. Pelleted DNA was resuspended in 200 µl TE and reprecipitated with 0.3M sodium acetate and 2.5 volumes ethanol.
    j. Pellets were washed in 70% ethanol, resuspended in 50 µl TE containing 20 µg/ml RNAse A, and incubated for 20 minutes at 68°C.
2. Total cell DNA extraction or Raft DNA extraction

a. Frozen cell pellets or raft tissues were thawed and resuspended in DNA Extraction buffer (400 mM NaCl; 10 mM Tris-Cl, pH 7.4; 10 mM EDTA).
b. RNase A was added to a final concentration of 50 µg/ml and SDS to a final concentration of 0.2%.
c. HIRT solution was incubated for 37ºC O/N on a rotating belt.
d. Proteinase K was added to a final concentration of 50 µg/ml.
e. Solution was incubated at 37ºC for 1-4 hours on rotating belt.
f. DNA was sheared by passing through an 18-gauge needle approximately 10 times
g. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol solution (25:24:1) 2X, and finally 1X with an equal volume of chloroform.
h. DNA was precipitated using 0.1 volume of 3M NaOAc and 2 volumes of 100% EtOH, and pelleted by centrifugation.
i. DNA pellet was washed in 70% EtOH and dried at RT and resuspended in 100 µl of 1X TE Buffer.

RNA preparation

RNA was extracted from cells at approximately 80% confluence grown on 10 cm dishes. Fibroblast feeder cells were removed by versene treatment. 3mls of Trizol (Invitrogen) was used to lyse cells and cells were collected by scraping. RNA extraction was performed according manufacturer's instructions. RNA integrity was confirmed by using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA).

Southern Analysis

DNA samples were separated by 0.8% agarose gel electrophoresis and transferred onto a Nytran SPC membrane (Whatman) using the TurboBlotter system, according to manufacturer's instructions, for alkaline transfer, and cross-linked with a Stratalinker UV Crosslinker (Stratagene) under the auto cross-link setting, 1200μJ/cm^2 x100. The probe was prepared by digesting the full length HPV genome with the appropriate enzymes to release the complete HPV genome from the cloning vector, and purifying the viral sequences by agarose gel electrophoresis and purification using QIAquick Gel Extraction Kit. Genomic probes were prepared by digesting plasmid DNA from the vector sequence (pBR322HPV18, pBRminHPV31, pML2dHPV11, and pBR322HPV6b were digested with cloning digestion enzymes EcoRI, HindIII, BamHI, and BamHI respectively). The genome was purified from plasmid sequences by agarose gel electrophoresis separation and purified using the Qiaquick Gel Extraction Kit (Qiagen). Genomic sequences, purified from plasmid DNA, were then radiolabeled with ^{32}P- dCTP [3000 Ci/mmol] using the Random Primed DNA labeling Kit (Roche) according to manufacturer's instructions followed by removal of unincorporated nucleotides using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare) according to manufacturer's instructions. Membranes were incubated in a pre-hybridization buffer [3X SSC, 2% SDS, 5X Denhardts, and 200 μg/ml ssDNA] at 68ºC for at least 1 hour before hybridization. $1x10^6$ counts/ml of the corresponding HPV type radiolabeled genomic DNA was added to the hybridization buffer [3X SSC, 2% SDS, 5X Denhardts, and 200 μg/ml ssDNA] and incubated in a hybridization oven at 68°C O/N. The next day, labeled membranes were washed in 0.1% SDS and 0.1X SSC until no background signal was detected and exposed to MS film (Kodak) or a Phosphor imager screen and analyzed using Typhoon (Molecular Dynamics).

Alternate Southern Protocol:

Total cellular DNA was isolated as described in DNA extraction section. A total of 5 μg of total cellular DNA was digested with EcoRI, which linearizes the HPV18 genome at nucleotide 2400 or loaded directly into gel without digestion. The samples were then separated by 0.8% agarose gel electrophoresis and transferred onto a GeneScreen Plus membrane (PerkinElmer Life Sciences, Inc. Boston, MA) using an O/N upward transfer with 0.4N NaOH. The next day, the membrane was baked for 2 hours at 80ºC. Genomic probe was prepared by digesting plasmid DNA from the vector sequence (pBR322HPV18 with cloning digestion enzyme EcoRI). The digested genomes were purified from plasmid sequences by agarose gel electrophoresis separation and Gene Clean (Bio101). Membranes were first pre-hybridized in pre-hybridization buffer [50% Formamide, 4X SSPE, 5X Denhardts, 1% SDS, 0.5 mg/ml ssDNA] for 1-2 hours at 42ºC. The radioactive labeling of DNA sequences and the hybridizations were carried out using DNA sequences labeled with [α-32P]dCTP (3,000 Ci/mmol; DuPont NEN), using a Random Primed DNA labeling kit (Boehringer Mannheim Corp.) according to the manufacturer's instructions. Labeled probe was separated from unincorporated nucleotides by centrifugation through a Sephadex G-50 column (Boehringer Mannheim Corp.). Hybridization was carried out with 1 x 10^6 cpm per ml of appropriate probe in hybridization buffer [50% Formamide, 4X SSPE, 5X Denhardts, 1% SDS, 10% Dextran sulfate, 0.5 mg/ml ssDNA]. Membranes were washed to remove nonspecific hybridization and then exposed to film.

Western Analysis

Proteins were extracted in 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% glycerol supplemented with inhibitors Complete and PhosphoSTOP (Roche). Protein samples were quantitated using the BCA Protein Assay Kit (Thermo Scientific). Protein amounts loaded onto

gels were determined by equal-loading based on the lowest concentration sample for each set. Samples were resolved on gradient (4-12%), 10%, or 12% NuPage gels, electrotransferred to Immobilon-P membrane (Millipore), and probed with the relevant antibodies before detection by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). mAbs were against p53 at a dilution of 1:1,000 (DO-1; Santa Cruz Biotechnology) and α-tubulin at a dilution of 1:10,000 (B-5-1-2; Sigma-Aldrich). pAbs against Myc at a dilution of 1:1,000 (N-262), p21 at a dilution of 1:1,000 (C-19), and p16 at a dilution of 1:1,000 (C-20) were from Santa Cruz Biotechnology.

Real-Time qRT-PCR

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for detection of p16, Myc and GAPDH mRNA, was performed by Xuefeng Liu and Yuhai Dai (Georgetown University) as previously described (97;138;166).

qRT-PCR for detection of hTERT mRNA was performed using an ABI 7900HT Sequence Detection System (Applied-Biosystems) and the TaqMan Gene Expression Assay for hTERT, Assay ID Hs00972646_m1 and RPO as a endogenous control, #4326314E (Applied-Biosystems) . qRT-PCR was carried out according to manufacturers instructions for the TaqMan Gene Expression Assays using cDNA generated from 400ng total RNA using the SuperScript III First-Strand Synthesis Kit (Invitrogen) for RT-PCR and oligo(dT)20, according to manufacturer's instructions. All reactions were run in triplicate. Standard curves were generated using a pooled mix of the samples with high hTERT expression. The data were analyzed with SDS 2.1 software (Applied Biosystems). For each sample, the amounts of the target and RPO transcripts were determined from the appropriate standard curve. Values were adjusted for transfection efficiency by measuring the level of RPO gene expression and adjusting the values appropriately. Values

were converted to copy number and standardized across experiments. Thus, transcript copy numbers can be directly compared among the experiments presented.

Telomere Length Assay

Telomere length assays were performed by Xuefeng Liu and Yuhai Dai. Genomic DNA was extracted from cells and the average telomere length was assessed by a modified method of the RT- PCR–based telomere assay described previously (167). Briefly, the ratio (T/S) of telomere repeat copy number to single copy gene (HBG1) number was determined using a Bio-Rad IQ5 thermocycler. Five ng genomic DNA was amplified and detected with SYBR Green Super Mix (Bio-Rad). The primers for telomeres were: Tel-1 5' CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3, and Tel-2 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'; and for HBG1 5'-TGTGCTGGCCCATCACTTTG, and HBG2 5'- ACCAGCCACCACTTTCTGATAGG-3'. Reaction conditions were: 1 cycle, 95°C, 5m; 41 cycles, 95°C, 15s; 1 cycle, 60°C for 45s. All reactions were carried out in triplicate and compared to a standard curve of 0, 0.2, 1, 5, 25, 125 ng genomic DNA (telomere length 10.4 kb) from Telo-kit (Roche). The T/S ratio (dCt) for each sample was calculated by normalizing the average HBG Ct value to the average telomere Ct value.

Genetic Analysis

Karyotype analysis was conducted by Molecular Diagnostic Services, Inc. San Diego, CA. Metaphase spreads were prepared and stained to observe chromosomal G bands. Twenty

metaphase spreads were analyzed and five complete karyotypes were prepared from each cell line.

Short Tandem Repeat (STR) analysis on cellular DNAs was performed using the PowerPlex 1.2 STR genotyping kit (Promega) by Molecular Diagnostic Services, Inc., San Diego, CA. Loci used for analysis were D5S818, D13S317, D7S820, D16S539, vWA, TH01, Amelogenin, TPOX, and CSF1PO.

Viral Infectivity and Titering

Virions were isolated from the epithelial tissue grown in the organotypic rafts. Epithelial tissue was removed from the dermal equivalent following 10-12 days of stratification. The tissue was ground in a 7.5 ml dounce homogenizer using 600 µl buffer 1 (1 M NaCl, 0.05 M Na_2HPO_4 [pH 8.0]). The homogenizer was washed twice with another 200 µl buffer 1 and the pooled homogenized solution was centrifuged at 10,500 x g for 10 minutes at 4 °C. The supernatants, viral stocks, were stored at −20 °C.

The HPV18 infectivity studies were based on an *in vitro* system described by Smith *et al*. (168). HaCaT cells, an immortalized human keratinocyte cell line (kindly provided by Norbert Fusenig), were grown to confluence in 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 25 µg/ml gentamicin. The HPV18 virus stock was sonicated for 30 seconds on ice and then serially diluted (1:20, 1:100, 1:500. 1:1,000, 1:5,000, 1:10,000, 1:20,000, 1:50,000, 1:75,000, 1:100,000, 1:1,000,000) with cell culture medium. The medium was aspirated from the HaCaTs 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 hours at 37°C and another 0.5 ml of medium was added at the 24 hour time point.

The ability of the HPV18 virus to infect the HaCaT cells after 48 hours of incubation was determined by the presence of the spliced HPV18 E1^E4 mRNA species (168). mRNA was purified from the infected cells using the mRNA capture kit (Roche Molecular Biochemicals). Briefly, the medium was aspirated from the cells and the cells were washed two times with 0.5 ml ice-cold 1XPBS. 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 minutes in a cup horn sonicator on ice. A total of 2 µl of 1:4 diluted biotinylated oligo dT was added to each lysate. The samples were incubated for 10 min at 42°C. A total of 50 µl of the lysate was transferred to a streptavidin-coated PCR tube and incubated for 3 minutes at 37°C. The RNA captured in the tubes was washed three times with 200 µl of wash buffer and subsequently used in a reverse transcription 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 were 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. Ten percent 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' TCCCACGTGTCCAGGTCGTGT 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' CCACACAGAGTACTTGCGCTCAGG 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 (PE Biosystems). The temperature profile for the outer PCR reaction was 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, with a final 10-minute extension at 72°C. The temperature profile for the nested PCR reaction was 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, with a final 10-minute extension at 72°C. All PCR products were visualized by electrophoresis in a 2% agarose-ethidium bromide gel. The virus titer was determined to be the last dilution at which the spliced transcript could be detected.

Primer Extension

RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions, followed by treatment with DNase I. RNA integrity was analyzed using the Eukaryote Total RNA Nano Kit with the 2100 bioanalyzer. Primer Extension analysis was carried out using the Primer Extension System- AMV Reverse Transcriptase Kit (Promega) according to the manufacturer's instructions using 10-30 μg isolated RNA. The primer 5'CAGTTCCGTGCACAGATCAGGTAGC3' (Integrated DNA Technologies, Inc.), located in the E6 gene at nucleotide 166, was labeled using γ-^{32}ATP (3000 Ci/mmol). Labeled RNA was resolved on a 6%TBE-Urea gel (Invitrogen) or an 8% acrylamide (19:1 acrylamide:bis), 7M urea gel.

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Human Keratinocytes are Efficiently Immortalized by a Rho Kinase Inhibitor

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Contributions: I wrote the preliminary manuscript and performed all the experiments presented in it, with the exception of the following: the duplicate keratinocyte growth in the absence of feeders as well as the RT-PCR analysis for p16, c-Myc, and GAPDH were performed by Xuefeng Liu and Yuhai Dai (Georgetwon University); the fibroblast growth experiment was performed by Alison McBride.
Alsion McBride helped to revise the manuscript.

Chapter 3

Human Keratinocytes are Efficiently Immortalized by a Rho Kinase Inhibitor

Introduction

Somatic cells have a limited lifespan and gradually slow in growth and stop dividing, a process known as cellular senescence. This process is thought to limit the vulnerability of aging cells to disease. Human keratinocytes are invaluable for the study of skin biology and the pathogenesis of skin-related diseases, but their short lifespan in culture is a limitation. Different conditions have been developed to optimize the culture of keratinocytes; for example, the presence of fibroblast feeder cells increases the proliferative capacity of primary keratinocytes from approximately 20 to 40-60 population doublings (169;170). Spontaneously immortalized keratinocyte lines, for example HaCaT (127) and NIKS (129), have been used for skin-related research. However, these cell lines have genetic abnormalities such as mutations in p53 (171) or isochromosomes (129).

Continuous replication of primary human cells is blocked by two separate events: mortality stage 1 (M1; replicative senescence) and mortality stage 2 (M2; crisis). At M1, signaling by shortened telomeres results in activation of the p53 and pRb pathways. M2 represents a critical period of genomic instability, with extremely eroded telomeres resulting in chromosomal fusions and translocations. In retinal pigment epithelial cells and foreskin fibroblasts (which have decreased expression of components of the p16/Rb pathway), telomerase expression is sufficient to bypass both M1 and M2 and stabilize and elongate chromosome ends

(172). However, telomerase expression is not sufficient for immortalization of keratinocytes and $p16^{INK4a}$ function must also be disrupted (130;173).

Keratinocytes expressing the E6 and E7 proteins encoded by "high-risk" HPV types bypass both M1 and M2 blocks and become immortal (reviewed in (174)). E7 inactivates and degrades the pRb retinoblastoma tumor suppressor protein to induce G1/S phase progression of the cell cycle. This process increases the levels of $p16^{INK4a}$, but inactivation of the pRb pathway renders it ineffectual (173). The E6 protein degrades p53, which is activated in response to the usurpation of the pRb pathway. E6 also induces expression of telomerase and allows the cells to proliferate beyond senescence and evade crisis. Thus, the oncoproteins of the high-risk HPV types target the $p16^{INK4a}$ and telomerase pathways that are important for keratinocyte immortalization.

Rho GTPases are a subfamily of the Ras superfamily of proteins that play essential roles in a cell adhesion, cytokinesis and cell migration (reviewed in (175)). Rho kinase (ROCK) is a downstream effector of the Rho pathway. Mammalian cells encode two Rho kinases, ROCK 1 and 2, which phosphorylate a wide range of substrates on serine or threonine residues. These substrates are involved in processes such as stress fiber formation, actin stabilization, the development of focal adhesions, and apoptosis (reviewed in Riento and Ridley (2003)) (147). ROCK inhibitors such as Y-27632 and fasudil have been found to have diverse and profound effects on cell behavior and have great therapeutic promise in many areas of disease (176).

Inhibition of ROCK greatly increases the cloning efficiency of human embryonic stem cells (177) and human keratinocytes (178). In the current study, we demonstrate that treatment with Y-27632 greatly increases long term proliferation of primary human keratinocytes and, unexpectedly, enables them to efficiently bypass senescence and become immortal without detectable cell crisis. This is the first example of a defined chemical compound mediating

efficient cell immortalization and we anticipate that the resulting immortalized keratinocytes will be invaluable for the research of many aspects of keratinocyte biology and may have significant therapeutic and diagnostic potential.

Results

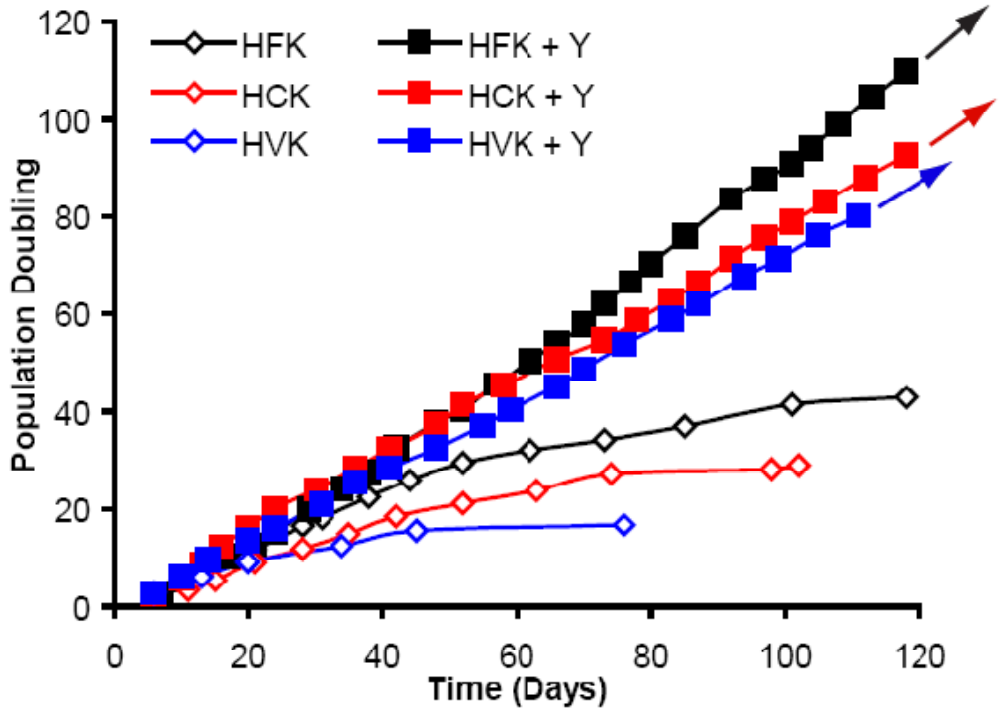
Y-27632 immortalizes primary human keratinocytes

Rho kinase inhibition has been reported to affect keratinocyte proliferation and differentiation (154;178). To further explore the effect of Rho kinase inhibition on the long term growth of keratinocytes, human neonatal foreskin keratinocytes and adult vaginal and ectocervical keratinocytes were cultured in the presence or absence of 10μM Y-27632, a well-characterized inhibitor of the Rho-associated kinase, ROCK (179). As shown in Figure 3.1A, in the absence of Y-27632, the growth rate of all three keratinocyte types slowed with time and senescence was observed at approximately population doubling 20-40, depending on the specific cell type. However, in the presence of Y-27632, a dramatic increase in cellular proliferation of all three types of keratinocytes was observed within days and continued indefinitely. Y-27632 treated cells had a steady growth rate, as indicated by the constant slope of population doubling versus time. All three keratinocyte types efficiently bypassed senescence with no observed decline in growth rate. As shown in Table 3-1, efficient keratinocyte immortalization was observed at least eight times with three different donor pools of foreskin keratinocytes (strains a, b, and c) and twice each with ectocervical and vaginal keratinocytes. Foreskin keratinocytes have been cultured for up to 150 passages for a period of 500 days in media supplemented with Y-27632 and can be considered immortal (see Figure 3-1B). Rare spontaneously immortalized cells

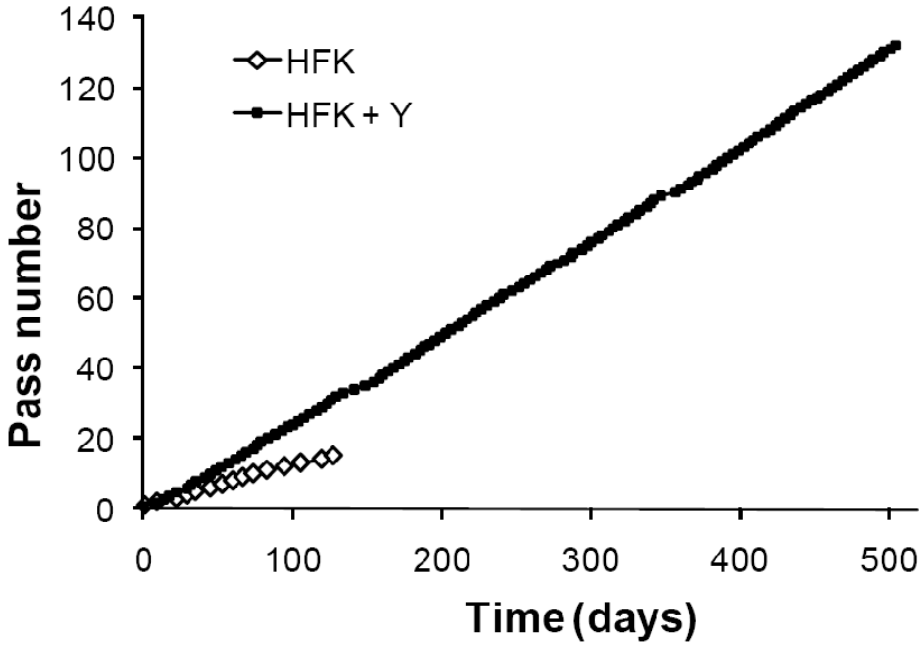
Figure 3-1. Growth of HFKs cultures in Y-27632

A. Growth rate of human keratinocytes from foreskin (HFK strain c; black) ectocervix (HCK; red) and vaginal tissue (HVK; blue) cultured in the presence (solid squares) or absence (open diamonds) of 10 μM Y-27634. The arrows indicate that these cells lines continued to divide indefinitely. Growth rate is measured as population doubling/day. B. Human foreskin keratinocytes (HFK:a strain) were cultured in the presence (solid squares) or absence (open diamonds) of 10 μM Y-27632. Cells were passed at a ratio 1:10 at the times shown, with a few exceptions when cells were split 1:20.

A


HFK
HCK
HVK
HFK + Y
HCK + Y
HVK + Y
Population Doubling
Time (Days)
0
20
40
60
80
100
120

B


HFK
HFK + Y
Pass number
Time (days)
0
20
40
60
80
100
120
140
0
100
200
300
400
500

would occasionally grow out from quiescent cells that were close to senescence in the absence of Y-27632. However, this only occurred after a long lag period suggesting that individual cells had picked up rare mutations allowing them to escape senescence. In contrast, Y-27632-treated cells grew steadily at all times. Genetic analysis was carried out on the immortalized foreskin keratinocyte HFK:a strain, at passage 94, to ensure that it was identical to the original donor cells. Short tandem repeat analysis, a method used to distinguish individuals based on the highly polymorphic nature of certain regions of chromosomes, showed that the immortalized cells were genetically indistinguishable from the original keratinocytes, eliminating the possibility of contamination by an immortalized cell line (Table 3-1).

Table 3-1. Life span of keratinocytes cultured with Y-27632

[1] Cells were cultured to the population doubling shown and were considered to be immortal.

[2] Cells were determined to be senescent at the population doubling shown. Senescence was defined as growth rate (population doubling/day) ≤ 0.2 within the time period of one month.

HFK: human foreskin keratinocyte; HCK: human cervical keratinocytes; HVK: human vaginal keratinocyte.

Keratinocyte strain		**+Y-27632[1]**	**-Y-27632[2]**
HFK	a	PD195 PD193 PD177 PD183 PD145	PD62 PD51 PD42 PD29 PD29
	b	PD199	PD34
	c	PD150 PD110	PD69 PD43
HCK		PD93 PD72	PD28 PD22
HVK		PD80 PD66	PD17 PD15

Immortalization by Y-27632 is dependent on co-culture with fibroblasts

Culturing keratinocytes in the presence of fibroblast feeder cells increases the lifespan of keratinocytes (135;138) and could contribute to the observed immortalization by Y-27632. Therefore, we analyzed the effect of Y-27632 on foreskin keratinocytes cultured on plastic in two different types of serum-free medium, in the absence of fibroblast feeder cells. Y-27632 treatment resulted in somewhat increased proliferation but this was not as pronounced as in the presence of feeders. Furthermore, in repeated experiments, these cells did not efficiently bypass senescence (Figure 3-2). Therefore, co-culture with feeder fibroblasts is required in concert with Y-27632 treatment to immortalize keratinocytes.

Morphology of Y-27632 immortalized keratinocytes resembles early passage, basal-like keratinocytes

At early passages, primary keratinocytes are actively dividing and are small, cuboidal and homogeneous in shape (Figure 3-3). When cultured with fibroblasts, they grow in tightly packed colonies and resemble basal keratinocytes. As they approach senescence, their morphology changes and they become flat and heterogeneous with an enlarged cytoplasmic volume. The morphology of all three types of Y-27632 immortalized keratinocytes was similar to early passage, actively dividing cells.

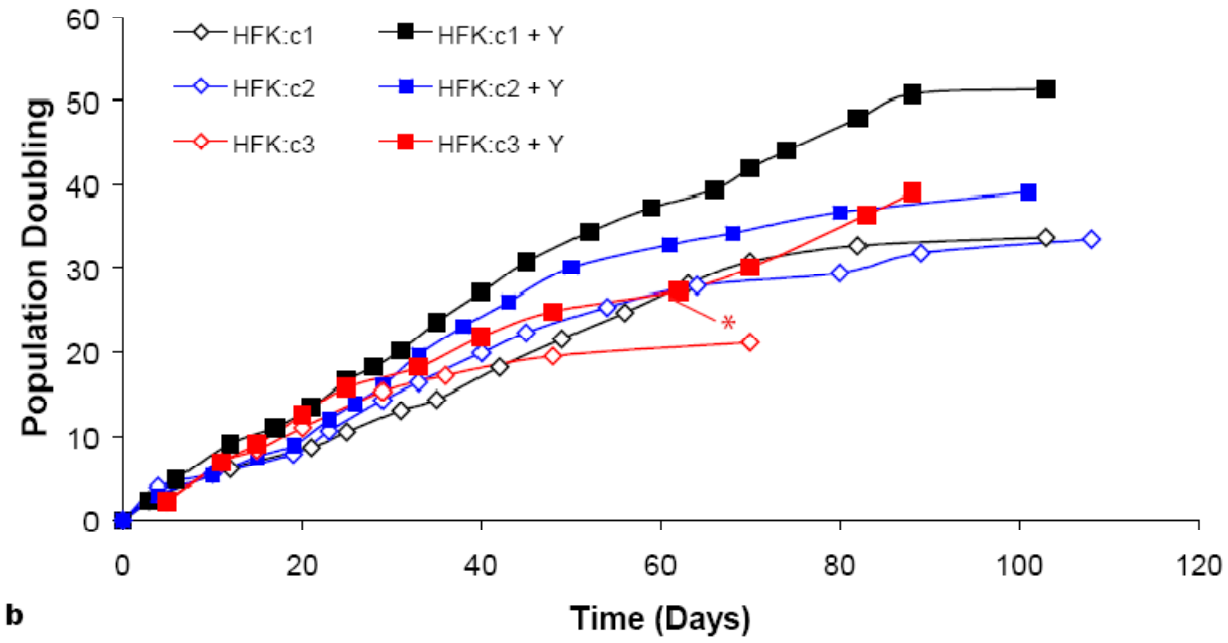
The karyotype of Y-27632 immortalized cells is normal

Immortalization of primary human keratinocytes is rare and the resulting cells have genetic changes and abnormal karyotypes. To evaluate whether similar genetic alterations occurred during Y-27632-mediated cell immortalization, we analyzed the karyotype for the foreskin

Figure 3-2. Growth of HFKs in the absence of feeder fibroblasts

HFKs were passed on five separate occasions on plastic in (a) serum-free 154 medium or in (b) KGM media in the presence or absence of (b) 5 μM or (a)10 μM Y-27632. All cells became senescent and ceased growing at the times indicated, except for HFK:c3 +Y, where cells grew out after crisis (*).

a


HFK:c1
HFK:c1 + Y
HFK:c2
HFK:c2 + Y
HFK:c3
HFK:c3 + Y
Population Doubling
Time (Days)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
*

b

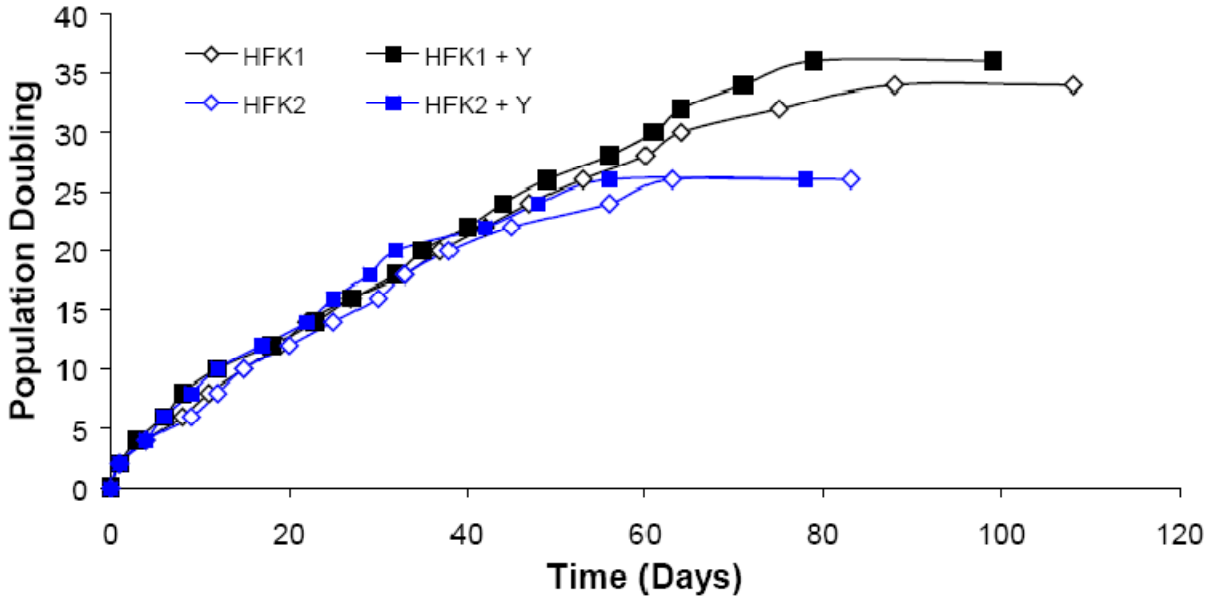
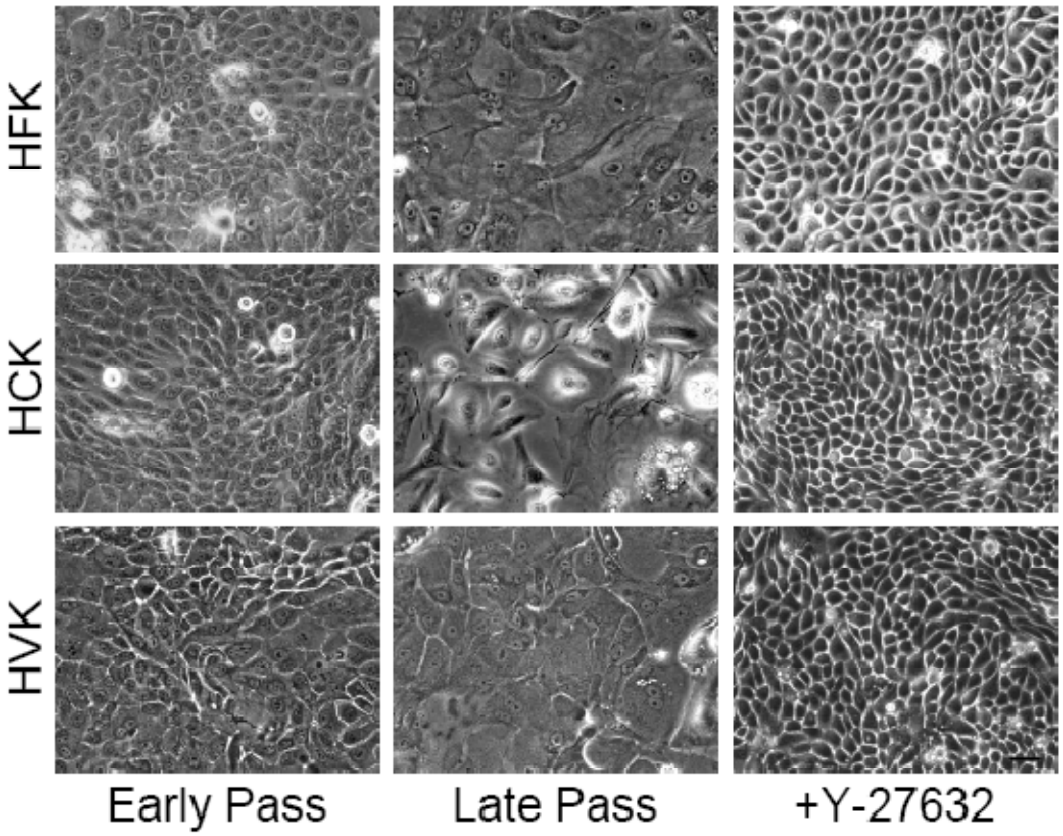

HFK1
HFK1 + Y
HFK2
HFK2 + Y
Population Doubling
Time (Days)
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100
120

Figure 3-3. Morphology of Y-27632-immortalized cells

Left column: Images of human foreskin (HFK), ectocervical (HCK), and vaginal keratinocytes (HVK) at pass P1. Centre column: keratinocytes near senescence (HFK P15, HCK P9, HVK P5). Right column: keratinocytes immortalized by 10 μM Y-27632 (HFK P100, HCK P29, HVK P26). Scale bar: 10μm.


HFK
HCK
HVK
Early Pass
Late Pass
+Y-27632

keratinocyte lines strain a, which had been cultured in the presence of Y-27632 for 95 passages. The karyotype of the immortalized cells was identical to that of the donor cells, with no apparent abnormalities (Figure 3-4).

Telomerase is upregulated in Y-27632-immortalized cells

hTERT is the active subunit of telomerase, which maintains the telomere caps throughout the multiple cell divisions of development. hTERT expression is turned off in most somatic cells and so the telomere ends become progressively shorter over multiple cell divisions. Replicative senescence is triggered as these protective ends shorten (180). To overcome this constraint, most tumor-derived or immortal cell lines have reactivated hTERT expression to maintain the telomere ends. Quantitative RT-PCR analysis showed that the level of hTERT mRNA increased with passage of foreskin keratinocytes in the presence of Y-27632 (see Figure 3-5A). As a comparison, hTERT mRNA levels were also determined in keratinocytes immortalized with HPV18. The "high-risk" HPV E6 protein directly upregulates hTERT transcription as part of the immortalization process (98). By passage 34 in Y-27632, hTERT mRNA levels were comparable to those in HPV18-immortalized keratinocytes. A similar induction of hTERT mRNA was observed in vaginal and cervical keratinocytes immortalized by Y-27632, as well as in another strain of foreskin keratinocytes (Figure 3-6A).

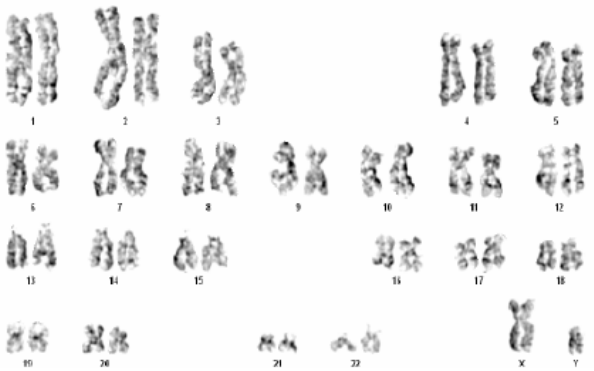
The lengths of telomeres shorten, but are stabilized, in keratinocytes immortalized by Y-27632

In HPV immortalized cells, telomere ends erode despite telomerase induction, but the shortened length becomes stable (181). We observe a similar phenomenon in Y-27632 immortalized cells. The relative length of telomeres was measured using a quantitative PCR assay. Despite increased

Figure 3-4. Genetic analysis of control of Y-27632-treated HFKs

Karyotype of the HFK:a strain at passage 5 and after 94 passages in Y-27632.

HFK:a, P5



HFK:a + Y, P94

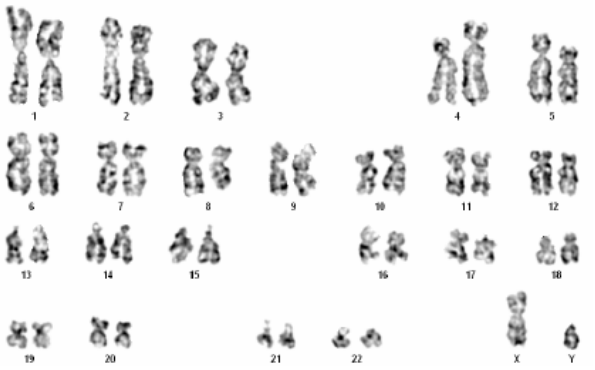


Figure 3-5. Telomerase expression and telomere length in Y-27632-immortalized HFKs

A. Relative levels of hTERT mRNA from HFK strain a, cultured in the absence or presence of 10 μM Y-27632, at the pass indicated, as quantitated by real-time PCR.B. Relative length of telomeres in HFK strain a cultured in the absence or presence of 10 μM Y-27632, at the pass indicated, as quantitated by real-time PCR.

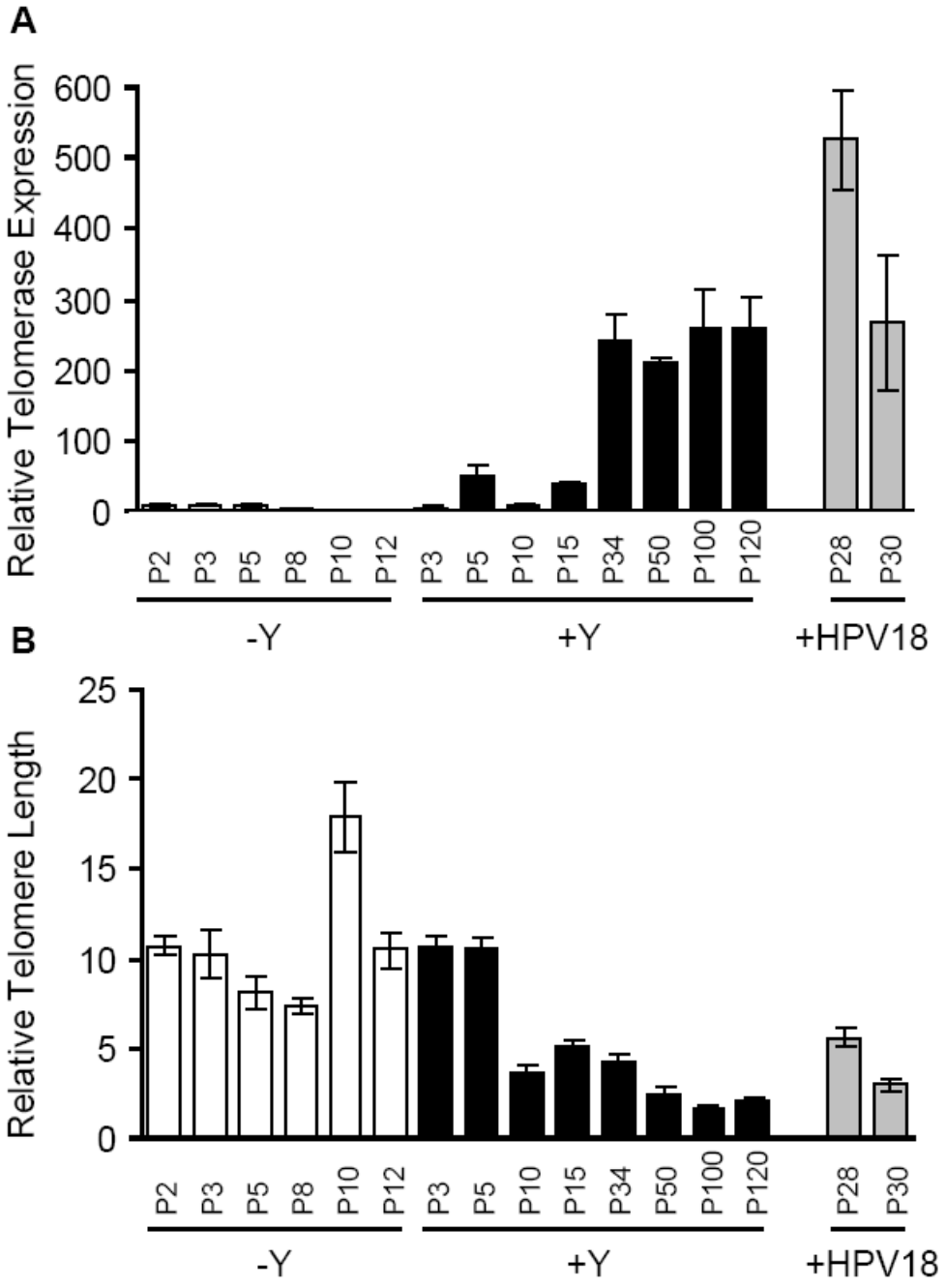
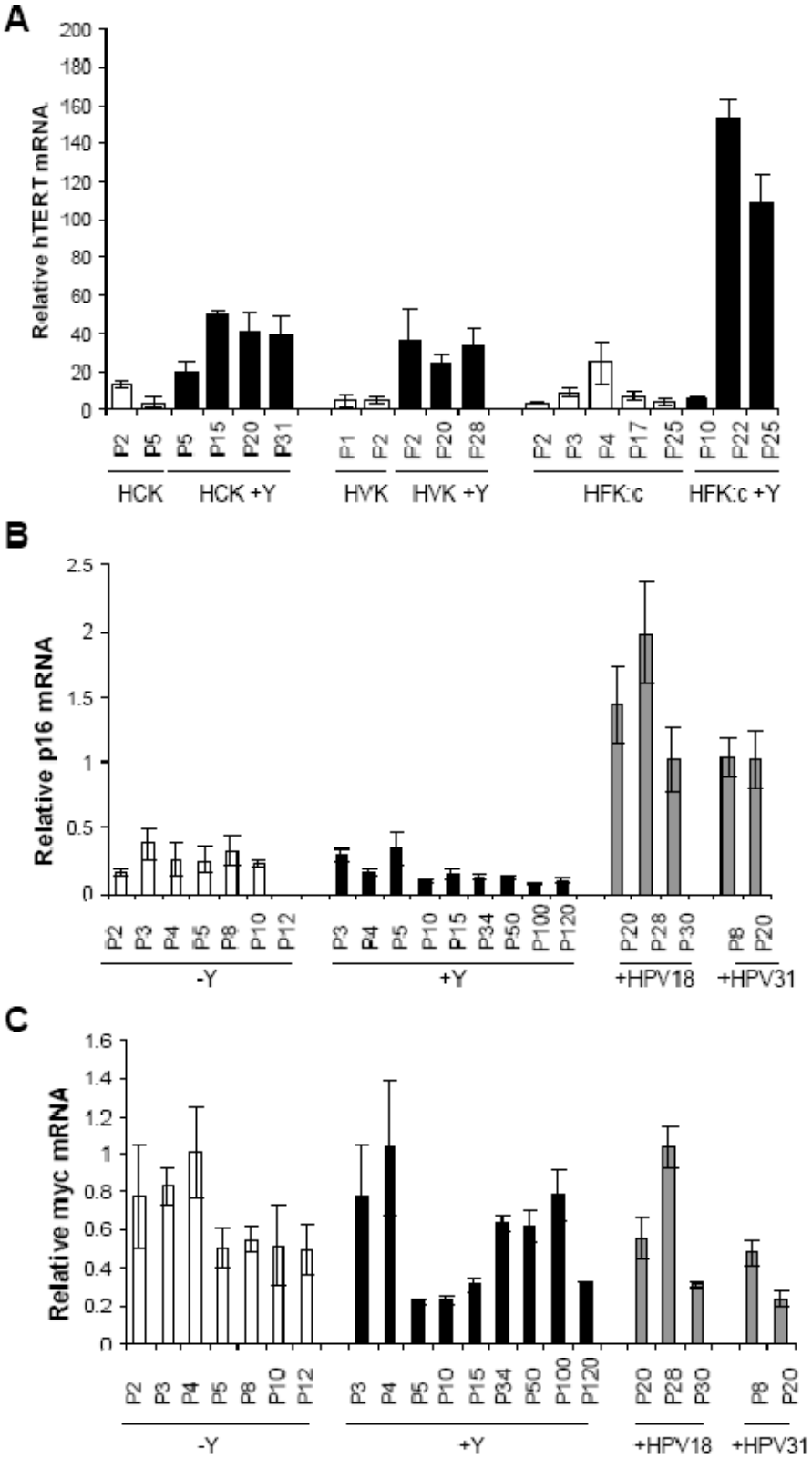

A
Relative Telomerase Expression
600
500
400
300
200
100
0
P2
P3
P5
P8
P10
P12
P3
P5
P10
P15
P34
P50
P100
P120
P28
P30
-Y
+Y
+HPV18
B
Relative Telomere Length
25
20
15
10
5
0
P2
P3
P5
P8
P10
P12
P3
P5
P10
P15
P34
P50
P100
P120
P28
P30
-Y
+Y
+HPV18

Figure 3-6. Relative mRNAlevels of hTERT, p16 and Myc in Y-27632-treated cells

A. Levels of hTERT mRNA were determined in HFK:c strain, HCK and HVK cells cultured in the absence or presence of 10 μM Y-27632 by QRT-PCR. B. Levels of myc mRNA were determined in HFK:a strain cultured in the absence or presence of Y-27632, HPV18 immortalized keratinocytes and HPV31 containing CIN-612 cells by QRT-PCR. C. Levels of p16 mRNA were determined in HFK:a strain cultured in the absence or presence of Y-27632, HPV18 immortalized keratinocytes and HPV31 containing CIN-612 cells by QRT-PCR.


A
Relative hTERT mRNA
200
180
160
140
120
100
80
60
40
20
0
P2 P5 P5 P15 P20 P31 P1 P2 P2 P20 P28 P2 P3 P4 P17 P25 P10 P22 P25
HCK HCK +Y HVK HVK +Y HFK:c HFK:c +Y
B
Relative p16 mRNA
2.5
2
1.5
1
0.5
0
P2 P3 P4 P5 P8 P10 P12 P3 P4 P5 P10 P15 P34 P50 P100 P120 P20 P28 P30 P8 P20
-Y +Y +HPV18 +HPV31
C
Relative myc mRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P2 P3 P4 P5 P8 P10 P12 P3 P4 P5 P10 P15 P34 P50 P100 P120 P20 P28 P30 P8 P20
-Y +Y +HPV18 +HPV31

levels of telomerase expression, the length of the telomeres in cells cultured with Y-27632 became progressively shorter with passage (see Figure 3-5B). However, the length became stable from passage 50 to 120 and was similar to the length of telomeres in HPV18 immortalized cells.

P16^{INK4a} is expressed in Y-27632-immortalized cells

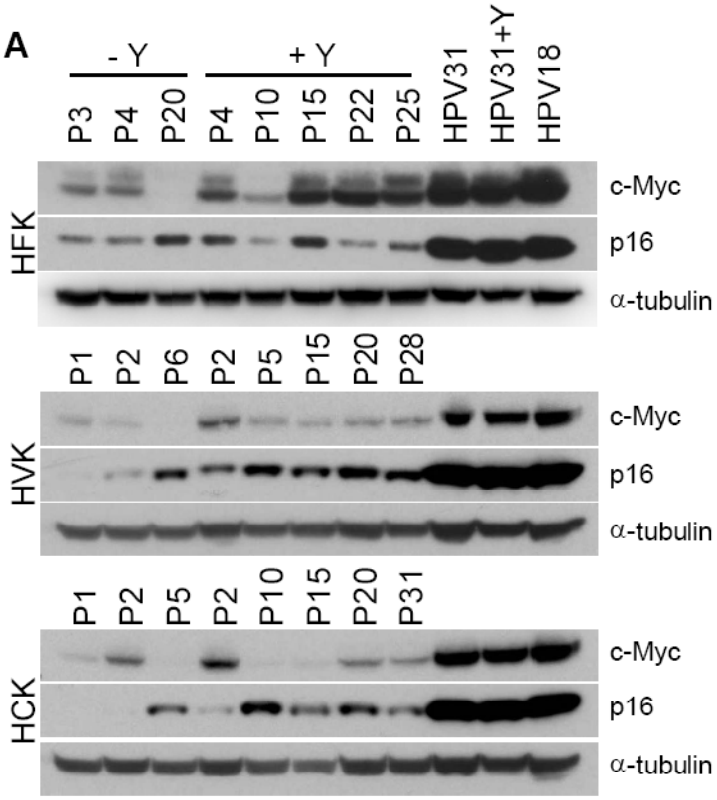
Telomerase expression is not sufficient for immortalization of human keratinocytes and the pRB/p16^{INK4a} pathway must also be inactivated (130). We examined p16^{INK4a} mRNA and protein levels in keratinocytes during long term culture with Y-27632. p16^{INK4a} protein expression (see Figure 3-7A) and mRNA (Figure 3-6B) were still observed, albeit at a low level, after long-term culture with Y-27632. However, at this point we do not know whether the observed p16^{INK4a} is functional. In contrast, the level of p16^{INK4a} in HPV immortalized cells is very high, but non-functional because of inactivation of the pRb pathway (173). Y-27632 treatment had no effect on the p16^{INK4a} levels in these HPV-immortalized cells.

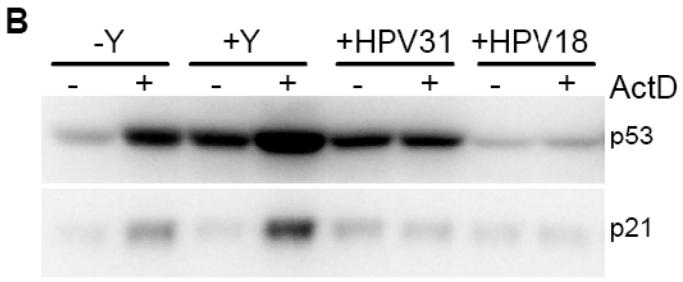
c- Myc is upregulated in Y-27632-immortalized cells

The Myc protein binds to the E-boxes of the hTERT promoter to induce transcription (182) and HPV E6 requires Myc for cellular immortalization (166). As shown in Figure 3-7A, Y-27632 has both short term and long term effects on Myc expression in all three keratinocyte types. Myc protein levels are induced transiently immediately after culture with Y-27632 (compare p4 for HFK and P2s for HCK and HVK). After this initial induction there is a general decrease in Myc protein levels which gradually increase again over time in all three cell types. A preliminary experiment showing the corresponding Myc mRNA expression levels is shown in Figure 3.7C. At very late passages (p107), the level of Myc protein is equivalent to that in an HPV31 containing

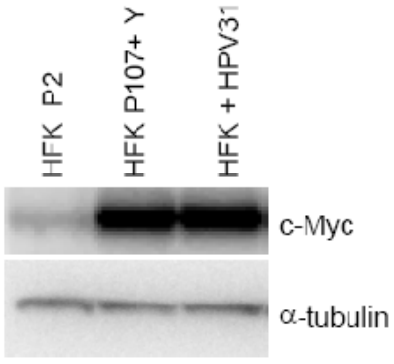
Figure 3-7. Expression of p16, p53, p21 and Myc proteins in cells cultured with Y-27632

A. Immunoblot analysis of Myc and p16 proteins in HFK strain c, HVK, and HCK cells cultured in the absence or presence of 10 μM Y-27632, and collected at the pass indicated. Cells containing oncogenic HPV31 and HPV18 viruses are included as controls. α-tubulin is included as a loading control. B. DNA damage was induced by treatment of cells with actinomycin D. The response was measured by immunoblot analysis of p53 protein levels and its downstream target p21. HFKs grown without Y-27632 were assayed at P4, and those cultured in 10 μM Y-27632 were assayed at P122.


A
- Y
+ Y
P3
P4
P20
P4
P10
P15
P22
P25
HPV31
HPV31+Y
HPV18
HFK
c-Myc
p16
α-tubulin
P1
P2
P6
P2
P5
P15
P20
P28
HVK
c-Myc
p16
α-tubulin
P1
P2
P5
P2
P10
P15
P20
P31
HCK
c-Myc
p16
α-tubulin


B
-Y
+Y
+HPV31
+HPV18
- + - + - + - +
ActD
p53
p21

C


HFK P2
HFK P107+ Y
HFK + HPV31
c-Myc
α-tubulin

cell line (Figure 3-7C). The long term increase in Myc levels is consistent with the increase in hTERT expression indicating that increased telomerase expression could be due to Myc induction.

The tumor suppressor gene p53 is expressed in Y-27632 immortalized cells and can mediate a normal DNA damage response

The tumor suppressor gene, p53, prevents aberrant proliferation and arrests the growth of cells that have sustained genetic damage. In most cancer-derived or immortalized cell lines the p53 pathway is either mutated or suppressed to allow cells to proliferate in conditions of aberrant growth regulation. As shown in Figure 3-7B, p53 protein levels are increased in keratinocytes cultured with Y-27632 but this does not appear to be inhibitory to cell growth and the downstream effector, p21 is not induced. To test whether the p53 pathway was functional in the Y-27632 immortalized cells, we analyzed the response of the cells to p53-induced growth arrest mediated by DNA damage. Normal cells exhibit growth arrest when exposed to a mutagen but this arrest is abrogated in cells immortalized by the HPV E6 and E7 oncoproteins (183;184). Keratinocytes were treated with 0.5nM actinomycin D, which induces DNA strand breaks and induces a p53-mediated growth arrest. Early passage keratinocytes and Y-27632 treated keratinocytes exhibited a normal DNA damage response; both p53 and the p53-responsive protein, p21, were upregulated (see Figure 3-7B). In contrast, the HPV31 containing cell line CIN612 and HPV18 immortalized keratinocytes did not induce p53 levels, or the p53 pathway, in response to DNA damage. Therefore, Y27632 immortalized keratinocytes retain a normal DNA damage response.

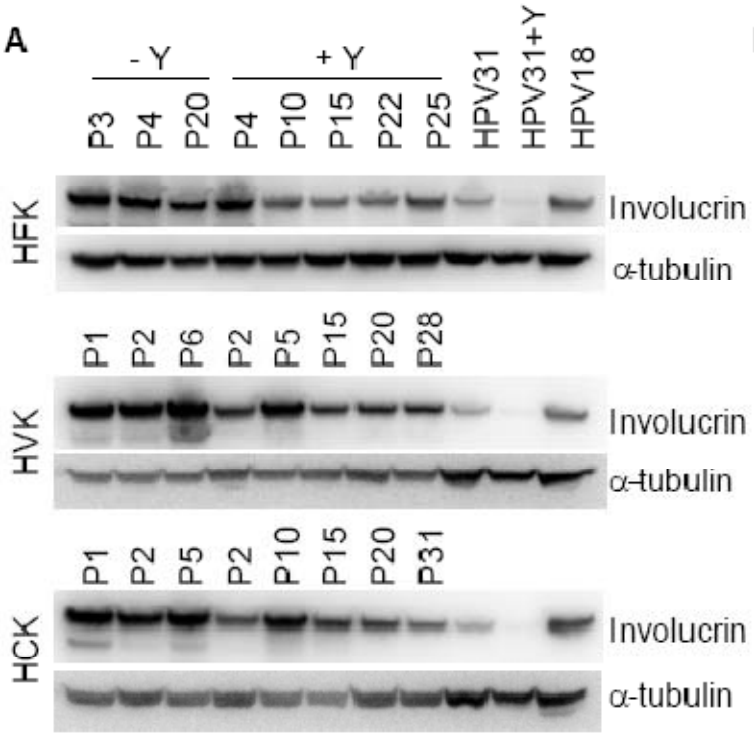
Y-27632-treated cells retain the capacity to differentiate

McMullan *et al.* have shown that suspension-induced differentiation of human keratinocytes is inhibited by blocking ROCK function (154)**.** In monolayer culture in the presence of fibroblast feeders, a fraction of keratinocytes undergo differentiation (185). Therefore, Y-27632 might inhibit differentiation and promote proliferation under these conditions. To examine whether culture with Y-27632 inhibits differentiation in monolayer culture, we assayed for the presence of involucrin in cells cultured in the presence of 10 μM Y-27632. The levels of involucrin decreased with continued passage in the presence of the ROCK inhibitor (Figure 3-8A) indicating that Y-27632 did reduce the propensity for differentiation in these cultures. We also tested the effect of Y-27632 on the ability of keratinocytes to differentiate into a stratified tissue in organotypic raft culture (Figure 3-8B). Early passage HFKs were seeded onto a collagen matrix containing fibroblasts and cultured as a "raft" either with or without Y-27632 in the raft media. However, no differentiation or stratification was observed when Y-27632 was present in the raft culture medium, confirming the findings of McMullan *et al.* in a different differentiation system. However, one very important characteristic of normal keratinocytes is their ability to differentiate and stratify into a tissue equivalent. To determine whether keratinocytes that had been cultured in Y-27632 for many passages retained their differentiation potential if the ROCK inhibitor was removed, we assayed their ability to form a stratified epithelium in organotypic raft culture. Early passage HFKs and HFKs that had been cultured up to this point in Y-27632 containing medium were seeded onto a collagen matrix containing fibroblasts and cultured as a "raft" at the liquid-air interphase for 17 days without Y-27632 in the raft media. As can be seen in Figure 3-9A panel b, keratinocytes that had been previously cultured in the presence of Y-27632 for 18 passages could produce a stratified epithelium in organotypic culture.

To further demonstrate that the stratified epithelial tissue grown from Y-27632 immortalized cells expressed appropriate differentiation markers, we analyzed the expression of keratin 14 (expressed in the basal layer), involucrin (upper spinous layer) and filaggrin (granular/cornified layer) by immunofluorescence on fixed tissue sections. As shown in Figure 3-9B, raft tissue grown from either untreated or Y-27632 treated cells expressed these differentiation markers in the appropriate layer (186). Thus, keratinocytes that had been previously cultured with the ROCK inhibitor retained their capacity to differentiate normally into a stratified epithelial tissue.

Figure 3-8. Effect of Y-27632 on differentiation

A. Immunoblot analysis of Involucrin proteins in HFK strain c, HVK, and HCK cells in the absence or presence of 10 μM Y-27632, collected at the pass indicated. Cells containing oncogenic HPV31 and HPV18 viruses are included as controls. HPV31 +Y was grown in 10 μM Y-27632 for 15 passes. B. HFK P1 keratinocytes grown in organotypic raft culture for 14 days in raft media (a) without or (b) with 10 μM Y-27632 .


A
- Y
+ Y
P3
P4
P20
P4
P10
P15
P22
P25
HPV31
HPV31+Y
HPV18
HFK
Involucrin
α-tubulin
P1
P2
P6
P2
P5
P15
P20
P28
HVK
Involucrin
α-tubulin
P1
P2
P5
P2
P10
P15
P20
P31
HCK
Involucrin
α-tubulin

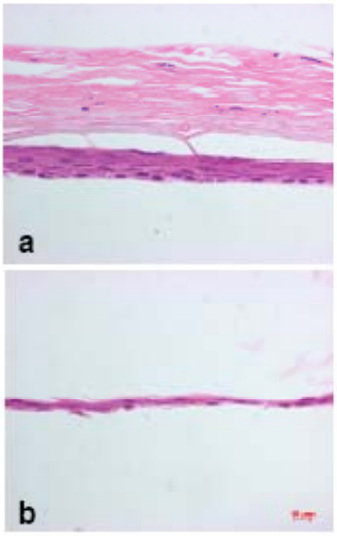
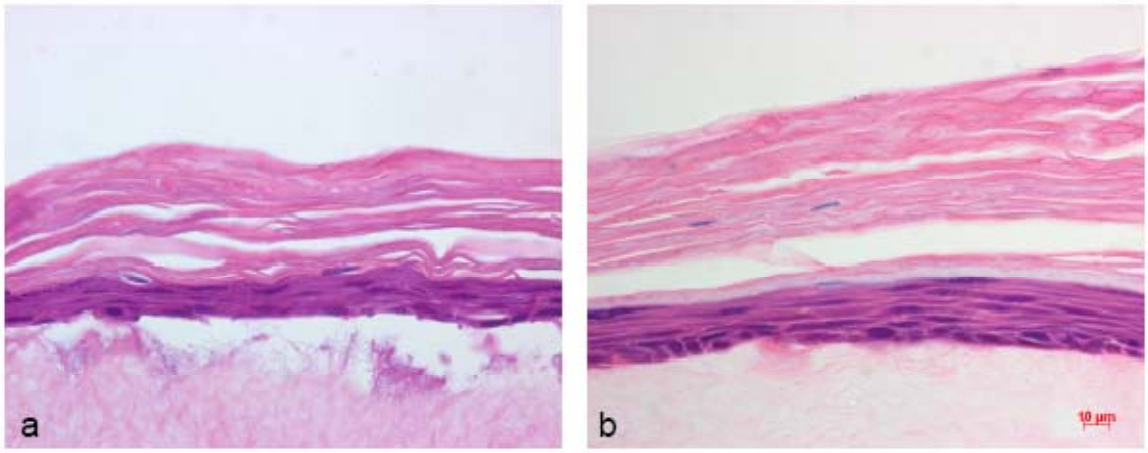

B
a
b

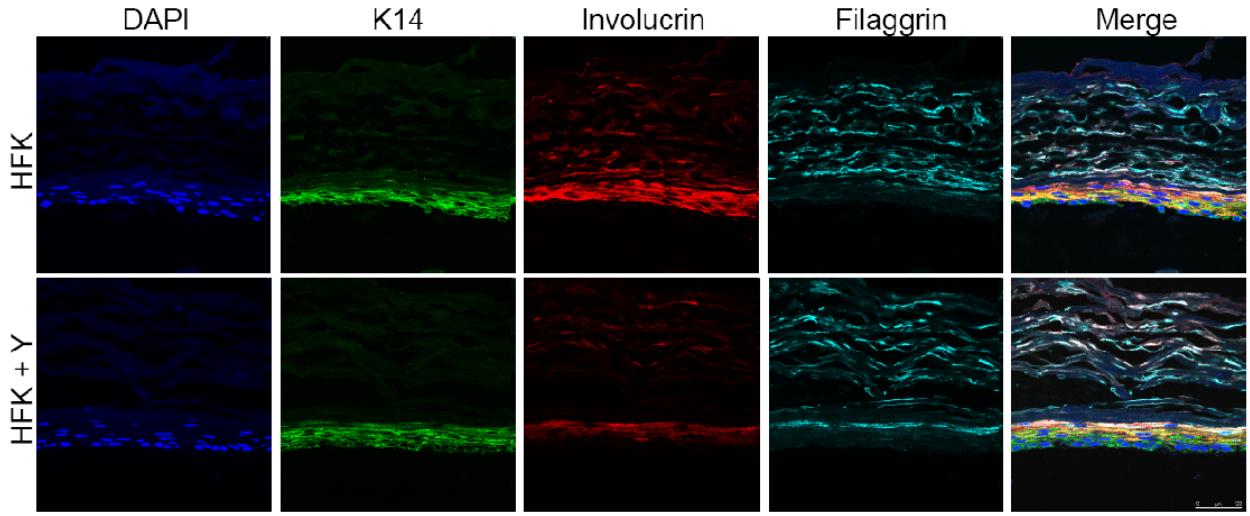
Figure 3-9. Ability of Y-27632-immortalized HFKs to differentiate

A. H&E stained histological sections of primary keratinocytes at (a) P1 or (b) after 18 passes in 10 μM Y-27632, cultured in organotypic raft culture for 17 days. Scale bar is 10 μM. B. 17 day organotypic raft tissue sections from normal primary keratinocytes at (a) P1 or (b) after 18 passes in 10 μM Y-27632 were stained for DNA (DAPI; blue); keratin K14 (green; basal layer), involucrin (red; spinous layer), and fillaggrin (cyan; granular and cornifed layers). Scale bar is 50 μM.

A



B



Discussion

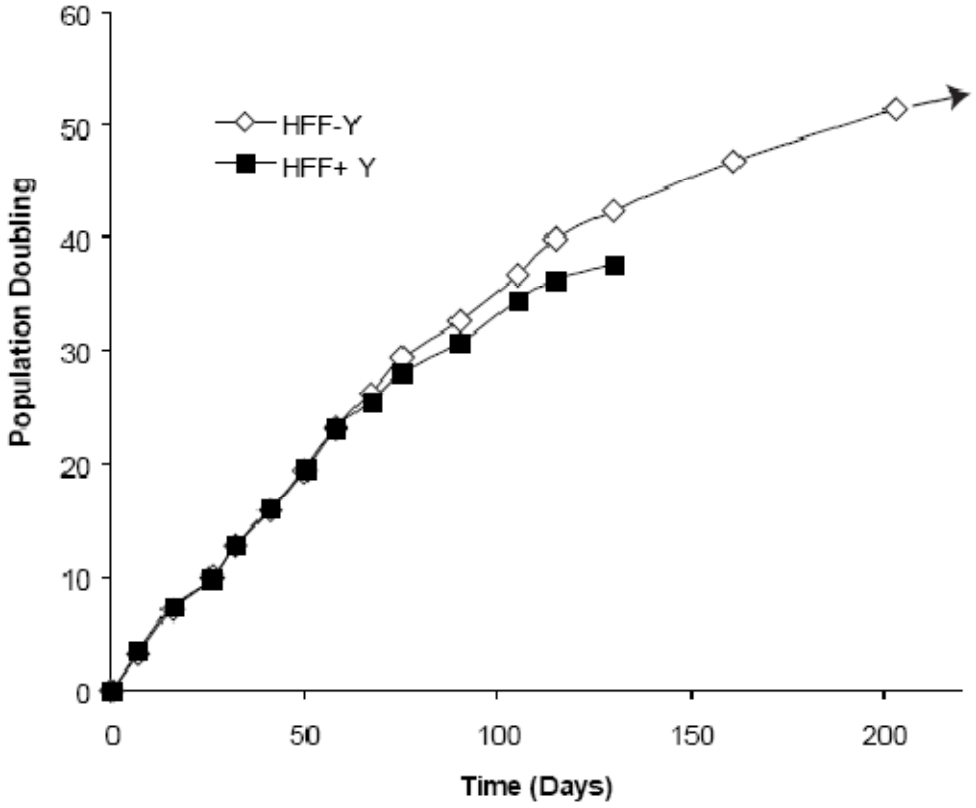
This report describes the effect of a ROCK inhibitor, Y-27632, on keratinocyte proliferation, immortalization and differentiation. Y-27632 treatment was shown to result in the bypass of senescence and immortalization of different types of keratinocytes from human foreskin, vaginal and cervical epithelium. Preliminary experiments also indicate that human foreskin fibroblasts treated with Y-27632 did not show enhanced proliferation, or bypass senescence (see Figure 10) suggesting that this is a keratinocyte specific phenomenon. These observations have far-reaching implications for the study and treatment of various skin diseases; improved culture and extended lifespan of keratinocytes will prove invaluable for both research and therapeutic purposes.

To date, our results show that Y-27632 immortalized cells are functionally equivalent to normal cells. They have a normal karyotype, an intact DNA damage response and are able to form a stratified epithelium in organotypic culture. The immortalized keratinocytes demonstrate upregulated telomerase mRNA levels and telomeres that have shortened, but remain at a stable length. Myc protein levels increased with continued passage and this may be responsible for upregulation of hTERT mRNA expression. Honma *et al.* have shown that Y-27632 rapidly induces Myc mRNA expression in human keratinocytes (187) and this might explain the initial induction of Myc that we observe in the first passage with Y-27632.

Previous studies have shown that telomerase expression is not sufficient for keratinocyte immortalization and inactivation of $p16^{INK4a}$ may also be required to bypass senescence in keratinocytes (130;135;173). Y-27632 immortalized keratinocytes express low levels of the $p16^{INK4a}$ protein but we assume that the cells are either resistant to its anti-proliferative effect or protein levels are too low to mediate an inhibitory response. Rhea *et al.* also observed $p16^{INK4a}$ expression in spontaneously immortalized keratinocytes cell lines (188).

Figure 3-10. Growth of Y-26732-treated fibroblasts

Human foreskin fibroblasts were cultured in the presence (solid squares) or absence (open diamonds) of 10 μM Y-276T32. Cells were passed when confluent at the times shown. Arrow indicates continued growth.


60
50
40
30
20
10
0
Population Doubling
HFF-Y
HFF+ Y
0
50
100
150
200
Time (Days)

We have identified two factors in this study to be important for keratinocyte immortalization: (1) culture with feeder fibroblasts; and (2) exposure to a ROCK kinase inhibitor. We have also observed that Myc is consistently upregulated during immortalization. These factors regulate the equilibrium between keratinocyte proliferation and differentiation. Prior studies have shown that culture of keratinocytes with feeder cells enhances lifespan, possibly in part by inducing telomerase (135;138). However, culture of keratinocytes with feeders also results in the expression of differentiation markers in a subset of cells when compared to culture on plastic (185). As shown in Figure 3-8, Y-27632 reduces this tendency in monolayer culture. Moreover, Honma *et al.* propose that the Rho-GTPase pathway may control Myc activity (187). Myc has a positive role in keratinocyte proliferation but also can promote differentiation of epidermal stem cells (189). We postulate that the interaction of these pathways is important for Y-27632 mediated immortalization.

Future studies will address the mechanism of Y-27632 mediated immortalization of keratinocytes. ROCK can phosphorylate a number of different downstream targets and we do not yet know exactly which of these pathways are important for keratinocyte immortalization. Y-27632 can also inhibit other Rho effector kinases such as citron kinase and protein kinase PKN, but its affinity for ROCK kinase is 20 to 30 times higher (158). Some of the observed effect of Y-27632 on keratinocytes could also be mediated by effects of ROCK inhibitor on the feeder fibroblasts, since efficient immortalization is only observed in their presence.

The current study shows many parallels between Y-27632-induced immortalization and HPV-mediated immortalization. In both cases telomerase is induced and the cells efficiently bypass senescence. Another parallel is the finding by Charette and McCance that both HPV16 E7 protein and Y-27632 treatment of human foreskin keratinocytes resulted in increased cell migration (190). However, Y-27632 immortalized cells have a normal DNA damage response

and karyotype compared to HPV-immortalized cells and are therefore preferable for many studies.

Keratinocytes are the natural host cells for all HPVs, but non-oncogenic HPVs are difficult to study because they do not immortalize these cells. Thus, Y-27632-treated keratinocytes will be very useful for long-term analysis of non-oncogenic HPV-related studies. Although not associated with cancer, these viruses are responsible for a great burden of recalcitrant disease such as genital warts, respiratory papillomatosis and cutaneous warts. These lesions can be especially problematic in individuals who are immunocompromised by HIV infection or organ transplantation. Y-27632 immortalized keratinocytes that can differentiate and support the entire viral life cycle will allow testing of anti-viral therapies in a system that closely reflects the *in vivo* situation.

Human foreskin keratinocytes are most often used for HPV studies because of the availability of this tissue from routine circumcision and difficulties in obtaining sufficient numbers of cells from other tissues. However, these keratinocytes might not be the best host for papillomavirus studies as HPV infection of the foreskin is often clinically inapparent. A more appropriate cell type for the study of the many HPVs is that of the uterine cervix. We show here that Y-27632 can immortalize vaginal and cervical keratinocytes, making such cells much more amenable to study. Different HPVs have a very specific tropism for different regions of epithelia. Y-27632 treatment and expansion of small numbers of keratinocytes derived from different types of epithelia could greatly increase our understanding and treatment of HPV infection.

The ability to greatly expand and derive tissue from different types of primary human keratinocytes is of great research and therapeutic benefit. In an era where personalized medicine is becoming increasingly important, patient-derived keratinocytes or tissue equivalents could be used to test therapies specific for the donor. Isolation and rapid expansion of keratinocytes from a small tissue biopsy by culture with Y-27632 could greatly increase the efficiency and time frame

of this process. For example, small number of keratinocytes can be isolated, expanded in monolayer culture and developed into tissue sheets for autologous epidermal replacement of regions with extensive burns or ulcers (191). Keratinocyte-mediated gene therapy is another intensively studied area of research whereby therapeutic genes can be introduced into autologous keratinocytes and regrafted onto the host (192).

Transplantation of immortalized human keratinocytes onto human hosts raises concerns of potential tumorigenic conversion. However, our studies have shown that ROCK immortalized cells have a normal p53-mediated growth arrest response and have no gross genetic abnormalities. Furthermore, there is evidence that ROCK inhibitors prevent rather than promote tumor progression and metastasis in human and animal models (193;194). The Rho/ROCK pathway has been shown to function in the cardiovascular system, central nervous system, cancers, and embryonic development (195). This pathway is an important therapeutic target and one ROCK inhibitor (fasudil) is already marketed for cerebral vasospasm after surgery (196) and is currently being tested for the treatment of angina pectoris, acute cerebral thrombosis and other vascular diseases. Our study demonstrates that ROCK inhibitors may also be useful for generating immortal but “normal” tissue equivalents and cell lines that may be employed in a variety of medical and research applications.

Chapter 4

Effect of Y-27632 on HPV Replication

Introduction

The anogenital HPV types are divided into "high-risk" or "low-risk" categories based on their association with cancer (29). "High-risk" HPV types can be detected in more than 95% of cervical cancers, and in 1992 it was determined by Munoz and Bosch that there is "compelling evidence in favor of a causal interpretation of the association between HPV and cervical cancer" (19;197). The five most common types of HPV found in cervical cancer biopsies are HPV types 16, 18, 45, 31, and 33. HPV16 is the most common type in all regions of the world while the prevalence of the other types varies by region (31). Clinically, infections by HPV type 18 tend to be much more aggressive than infections with other types and progress to malignant stages more rapidly (198-201). HPV31, which is in the same species group as HPV16, has a very long incubation period until neoplasia develops and is infrequently associated with integration (202). As for the non-cancerous lesions, HPV types 6 and 11 are responsible for approximately 90% of cases of genital warts (203;204).

There are many studies that have investigated the differences between the features of clinical lesions from infections by the different HPV types, but only few studies have examined the different viral genome-host interactions in cell lines transfected with the different HPV genomes *in vitro*. One study by Lace *et al*. (2009) suggests a difference in the replication of keratinocytes transfected by different HPV types (205). The authors reported that there were notable differences in the replication efficiency of different HPV types transfected into keratinocytes (205). In transient assays using SCC13 cells, they found that HPV11 replication

levels were 10 times greater than HPV18 and HPV31 and 30 times greater than HPV16. The authors also found that HPV18 was the most effective at establishing persistent infections with the most potent growth altering potential in cervical, foreskin, and tonsillar keratinocytes (205). This experimental result seems to support the clinical findings that HPV18 infections tend to be more aggressive and have a shorter time in developing into malignant lesions than the other common HPV types (201;206).

The keratinocytes of the basal layer of the epithelium are the natural host of an HPV infection and are therefore the ideal model for understanding how this virus establishes a persistent infection. These viruses infect epithelial tissue and express proteins that disrupt the host cell cycle program. Cell lines that maintain HPV genomes episomally can be generated *in vitro* by transfecting cloned DNA into primary keratinocytes (44;119;207-209). Stable maintenance and long-term studies analyzing the life-cycle of the high-risk HPV types have been possible using primary keratinocytes due to the pro-mitogenic properties of the oncoproteins E6 and E7 expressed from these viruses (44). However, the low-risk HPV types do not immortalize primary keratinocytes because the E6 and E7 proteins expressed from these viruses are not able to transform infected cells (52;205;210-212). This has limited stable maintenance studies for the low-risk HPV types to be carried out in immortalized cell lines such as the N-terts (213;214). Nevertheless, there have been a handful of studies that have examined HPV11 replication in transfected primary keratinocytes which were limited to lifespan of the cells (211;215). Mungal *et al*. (1992) reported that robust replication is seen for only about two passages following transfection after which time the copy number per cell declines (215). However, Thomas *et al*. (2001) argue that the genomes are maintained episomally until the keratinocytes reach senescence (211). Mungal *et al*. argued that the establishment of replicating HPV11 genomes required co-transfection and selection with a drug-selectable marker (215).

Because HPV has different modes of replication, it is often important to examine both transient and long-term replication. Genomes that are able to replicate transiently, may not be maintained over time in culture (64). Additionally, because of the difficulties associated with long-term propagation of the low risk HPV viruses *in vitro,* there is little experimental evidence that these types can be maintained for long periods in culture; and the previous work in these types has mainly focused on transient replication. As with infections by the high-risk HPV types, most lesions caused by the low-risk HPV types spontaneously regress, but in a few cases can persist for years (35). Therefore, these viruses, like the high-risk HPV types, are able to establish persistent infections. Because of the social and economical burden of the clinical manifestation of these infections, it would be advantageous to learn how these viruses can establish persistent infections.

HPV replication can be studied using cell lines transfected with HPV DNA or cell lines that stably maintain episomal viral genomes and which have been derived from low-grade cervical lesions (119;121;123;208). There are a few established cell lines isolated from low-grade cervical lesions that represent early stages of infection. The CIN-612 cell line represents a mixed population of HPV-31b infected cells isolated from a low-grade cervical lesion by George Wilbanks and Lou Laimins (120;121). The 9E subclone stably maintains extrachromosomal copies of the viral DNA (121;122). The 20863 subclone of the W12 cell line that maintains extrachromosomal copies of HPV16 was cloned by Paul Lambert and was originally derived from a low-grade cervical lesion collected by Margaret Stanley (123;124). However, the extrachromosomal state of the viral DNA in W12 cells is relatively unstable and has been shown to be lost overtime when cells with the viral genome integrated into the cellular DNA overtake the culture (125;126).

In the previous chapter, we demonstrated that treatment of primary keratinocytes with the ROCK inhibitor, Y-27632, dramatically improves the growth potential of the cells. In the studies

presented in this chapter we investigated how this improved culturing technique could benefit HPV research since keratinocytes represent the ideal model in which to study HPV biological processes. We found that treatment with Y-27632 improved the overall transfection efficiency and survival of HFKs. In addition, we found that the improved transfection conditions enhanced detection of transient replication in HPV-transfected HFKs. However, it does not seem that Y-27632 treatment directly affects HPV replication because we did not see a difference of replication levels of the episomally maintained HPV31b genome in the 9E cell line. Finally, under the conditions used in these experiments, we were not able to establish persistent infections with either low-risk or high-risk genomes in the drug-treated cells. We postulate that this is due to the fact that the untransfected cells outgrow the transfected cells.

Results

HFKs grown in +Y-27632 have a significantly higher apparent transfection efficiency

The ability to transfect HFKs with HPV DNA has been a very valuable tool that allows advanced genetic analysis of cloned HPV genomes in a physiologically relevant context (165;211;213;216). However, one of the disadvantages in using primary keratinocytes for HPV transfection is their poor transfection efficiency and their poor recovery and survival after transfection (163;215;217;218). First we wanted to determine whether or not Y-27632 treatment affects the transfection efficiency of HFKs. To test this, we electroporated primary HFKs grown in the absence or presence of 10 μM Y-27632 with 0.5 μg/μl pmaxGFP using the Amaxa neonatal keratinocyte nucleotransfection kit on the T-07 setting (Lonza). We found that the percentage of GFP positive HFKs grown in the presence of Y-27632 following transfection was

statistically significantly ($P < 0.0001$) greater than the percentage of GFP positive HFKs grown in the absence of Y-27632 (Figure 4-1).

Y-27632 treatment greatly enhances detection of transient HPV replication in transfected cells

As discussed in the previous chapter, we found that treatment of primary keratinocytes with the Rho Kinase inhibitor, Y-27632, greatly enhances the proliferative capacity of these cells. To determine whether treatment with Y-27632 has any effect on viral replication in HPV-transfected HFKs, we analyzed levels of transient viral replication in HPV-transfected HFKs grown in the absence or presence of 10 μM Y-27632. Viral genomes from the high-risk HPV types 18 and 31 were transfected using nucleofection into early passage primary foreskin keratinocytes grown in either F-media or F-media supplemented with 10 μM Y-27632, for at least one pass. The Y-27632-treated cells continued to be cultured in the drug post-transfection while the control cells continued to be cultured in F-media without the drug. Low-molecular weight DNA was isolated at two and five days post-transfection to determine the levels of transient HPV replication in the cells. The isolated DNA was cleaved with EcoRI to linearize the HPV genome and with DpnI to digest input DNA. Using this method we can determine the level of replicated genomes in each HFK cell line at various time points. We found that the control HFKs grown in the absence of Y-27632 and transfected with HPV18 or HPV31 had undetectable levels of viral replication two days post-transfection (Figure 4-2A). In contrast, HPV replication was clearly evident in the Y-27632-treated cells collected at the same time. By five days post-transfection, detection of viral genomes extracted from the total cells could be seen in the control HFKs. However, the copy number present in the Y-27632-treated HFKs was much higher. Replication levels were quantified based on the intensity of the bands in the Southern blots (Figure 4-2B).

Figure 4-1. Transfection Efficiency of HFKs grown in the absence or presence of Y-27632

Percentage of GFP positive HFKS grown in the absence (HFK) or presence of Y-27632 (HFK+Y) following transfection using the Amaxa nucleofector. Cells were electroporated using the Amaxa neonatal keratinocyte nucleotransfection kit on the T-07 setting. Data represents 7 samples of electroporated cells generated in 4 independent experiements. A students two-tailed unpaired t test indicated that the difference in the data sets were statistically significant. $P < 0.0001$.

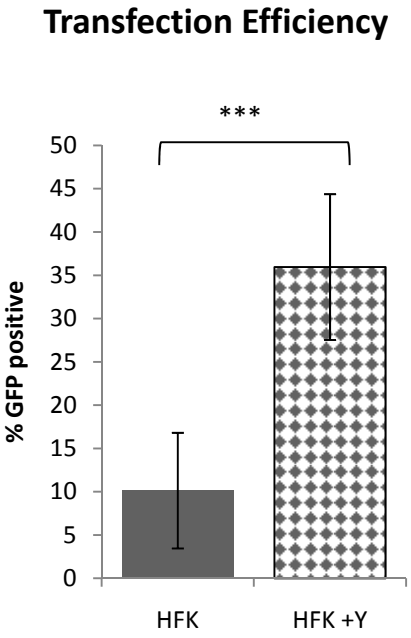
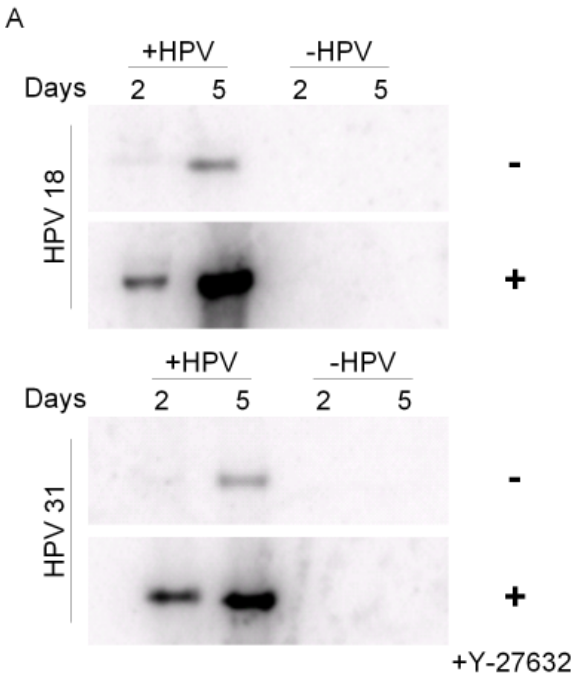
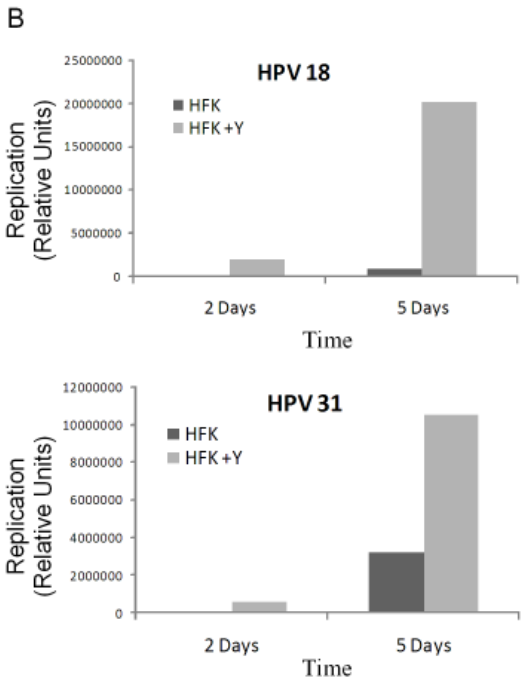

Transfection Efficiency
***
% GFP positive
50
45
40
35
30
25
20
15
10
5
0
HFK
HFK +Y

Figure 4-2. Effect of Y-27632-treatment on replication of HPV in transfected HFKs

A. Low molecular weight DNA from HFKs at passage 2 transfected with either the indicated HPV type (+HPV DNA) or with carrier DNA alone (-HPV DNA), grown in the absence or presence of 10μM Y-27632 was analyzed by Southern blotting. Cells were analyzed at 2 and 5 days post-transfection. HIRT extracts were digested with a restriction enzyme to linearize the DNA (EcoRI for HPV18 transfected cells, and HindIII for HPV31 transfected cells) and Dpn I to distinguish newly replicated DNA. B. Quanification of the intensity of the bands from the Southerns in (A).


A
+HPV
-HPV
Days
2
5
2
5
HPV 18
-
+
+HPV
-HPV
Days
2
5
2
5
HPV 31
-
+
+Y-27632


B
HPV 18
HFK
HFK +Y
Replication (Relative Units)
25000000
20000000
15000000
10000000
5000000
0
2 Days
5 Days
Time
HPV 31
HFK
HFK +Y
Replication (Relative Units)
12000000
10000000
8000000
6000000
4000000
2000000
0
2 Days
5 Days
Time

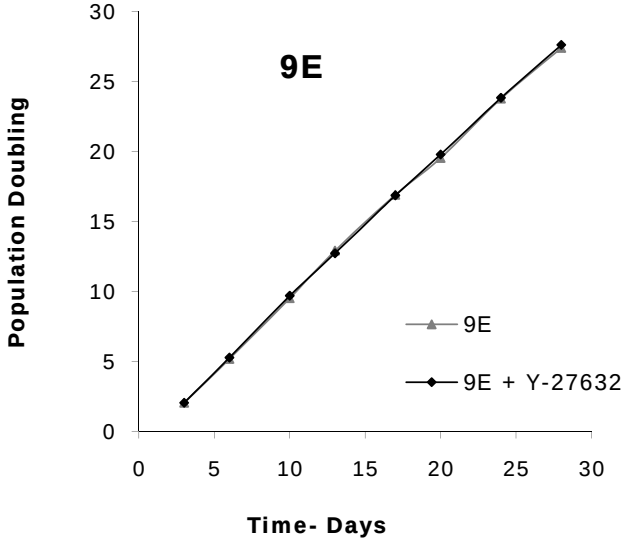
Based on this data, the HPV DNA present in the control HFKS was on average approximately 3.6% of the amount present in the HFKs grown in Y-27632. These samples represent the total amount of cells that were collected at two and five days. Because of the improved growth rate of the drug-treated HFKs, the +Y DNA samples represent much larger number of cells. Therefore, the increase we observed may simply reflect an increased amount of viral DNA because of a greater number of cells represented in these samples and not necessarily a greater copy number/cell. In addition, since we also found that Y-27632-treatment increased the transfection efficiency, it is also likely that the +Y samples had a greater percentage of HPV-positive cells in the population of HFKs collected for analysis.

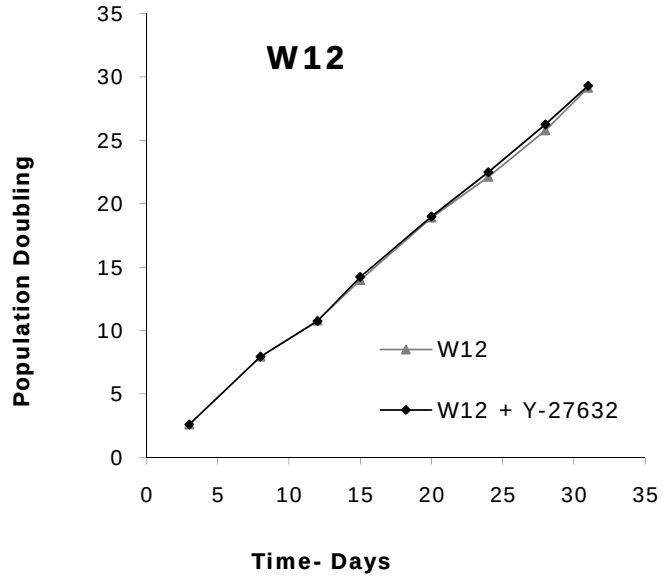
Y-27632-treatment does not affect growth rate of 9E and W12 cell lines

The W12 20863 clone and the CIN-612 9E clone were both derived from low grade cervical lesions and harbor episomal copies of HPV16 and HPV type 31b, respectively (121;124). These cell lines grow at a steady and constant rate because of the presence of the HPV oncogenes E6 and E7. Therefore we reasoned that Y-27632 would have no effect on the growth rate of these cells, which already grow at approximately 1PD per day. To test this, we grew the 9E and the W12 cell lines and split them into cultures maintained in F-media with or without 10 μM Y-27632 and measured the growth rates of each culture. As can be seen in Figure 4-3 we found no significant difference in growth rate in either cell line cultured in the presence or absence of Y-27632.

Figure 4-3. Effect of Y-27632-treatment on growth of 9E and W12 cell lines

Growth rate of 9E and W12 cell lines cultured in the presence (gray triangles) or absence (black diamonds) of 10μM Y-27634 for 8 passages. Population doubling was calculated as: PD=3.32 (log(# cells harvested/# cells seeded)).


9E
Population Doubling
Time- Days
9E
9E + Y-27632
0
5
10
15
20
25
30


W12
Population Doubling
Time- Days
W12
W12 + Y-27632
0
5
10
15
20
25
30
35

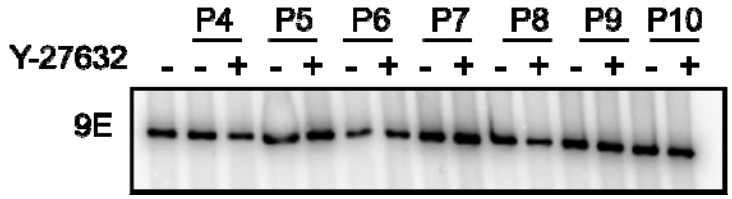
Episomal genome copy number is not affected by presence of Y-27632 in 9E cell line

Since we found that the 9E cell line had no significant growth advantage when treated with Y-27632, this cell line could be used to determine whether treatment with Y-27632 directly affects HPV replication or whether the increased copy number that we observed for the high-risk-transfected genomes grown in the presence of Y-27632 simply reflects the increased number of transfected cells. The CIN-612 9E cell line has been previously shown to stably maintain episomal copies of HPV31 over passage in culture(122). We extracted the low-molecular weight DNA from the 9E cell line at various passes after culture in F-media with or without 10 μM Y-27632. The number of cells present at the time of collection for each sample varied and therefore in an attempt to compare the levels of HPV replication in an equal amount of cells, the amount of sample digested was calculated based on the OD at A260 of each sample. The vast majority of the DNA in the samples is contaminating high molecular weight cellular DNA and circular mitochondrial DNA and these can be used as an internal control. As can be seen in Figure 4-4A, there is no significant difference in the levels of replication of HPV31 in the 9E grown in the absence or presence of Y-27632. Figure 4-4A shows one of the Southern blots demonstrating the levels of HPV31 maintained in this cell line grown in the absence or presence of the drug over time. The bar chart in Figure 4-4B shows the quantification of the levels of replication relative to the initial starting level averaged in two experiments. The differences seen between the control and the drug-treated pair were found not to be statistically significant using a two-tailed, paired students t-test ($P= 0.348935$, $P= 0.205143$ and $P= 0.483679$, $P= 0.493575$ for passages 5, 6, 9 and 10 respectively). This data suggests that Y-27632 does not directly enhance HPV replication and that the higher levels of HPV replication we saw previously in the transfected cell lines were likely due to the greater number of cells represented in the +Y samples (Figure 4-2).

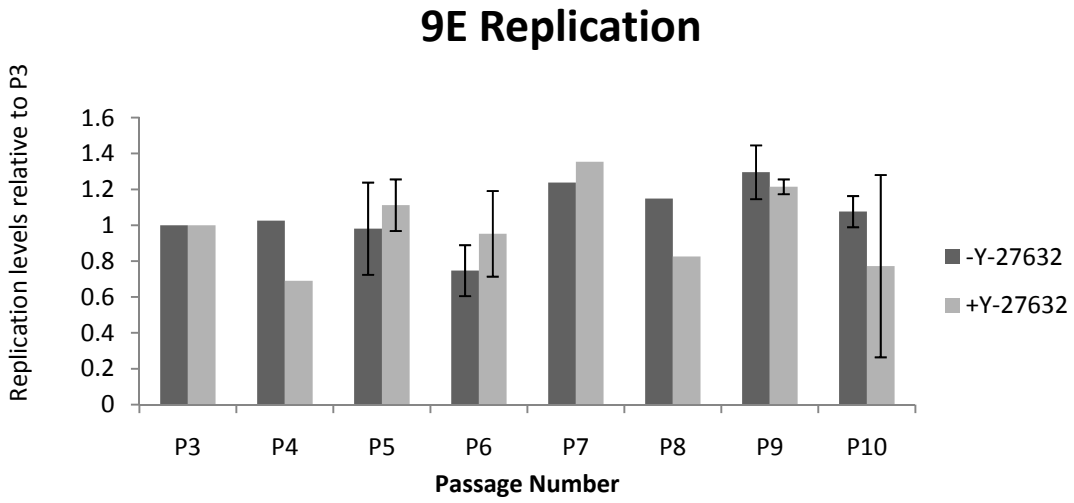
Figure 4-4. Effect of Y-27632-treatment on replication of HPV genomes in 9E cell line

A. Low molecular weight DNA from 9E cells grown in the absence or presence of 10μM Y-27632 was analyzed by Southern blotting. Cells were analyzed at the passages indicated. HIRT extracts were digested with HindIII, a single-cutter in HPV31, to linearize the genomic DNA. B. Quanification of intensity of genomic bands represented in Southern blots. X-axis indicates passage number. Y-axis represents replication levels relative to initial passage, P3 before drug was added. Data for duplicate or triplicate experiments are represented in passages 3, 4, 5, 6, 9, and 10. Error bars represent the standard deviation for these values.

A


P4
P5
P6
P7
P8
P9
P10
Y-27632
- - + - + - + - + - + - + - +
9E

B


9E Replication
Replication levels relative to P3
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P3
P4
P5
P6
P7
P8
P9
P10
Passage Number
-Y-27632
+Y-27632

Alternatively, the difference could be attributed to an effect on transient replication, as observed in the transfected HFKs, but not in maintenance replication, as seen in the 9E cell line.

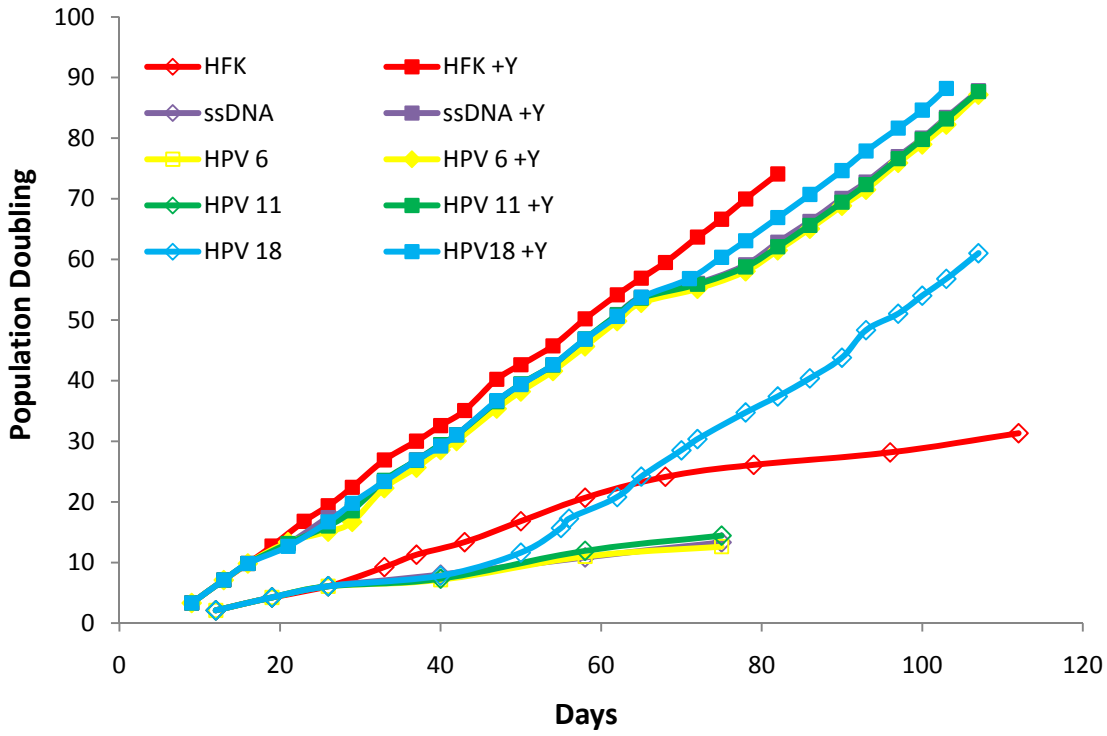
Y-27632 treatment improves growth of transfected HFKs

Lace *et al*. (2009) had observed differences in the growth characteristics of HFKs transfected with the common HPV types 11, 16, 18, and 31. While the E6 and E7 proteins of the low-risk HPV genomes can extend the life-span of transfected HFKs, they are not able to overcome senescence like the high-risk genomes. To test if treatment with Y-27632 would improve the overall growth of HFKs transfected with the low risk genomes, we transfected human foreskin keratinocytes with HPV types 6, 11, and 18 grown in the absence or presence of 10 μM Y-27632. Transfection was performed at passage 3 for both the control HFKs and the HFKs grown in Y-27632. This corresponds to 26 days in culture for the control HFKs and 16 days for the Y-27632-treated HFKs. In Figure 4-5A we can see how electroporation greatly affects the growth of the control HFKs. However, we found that the growth was affected much less in the Y-27632-treated HFKs. The growth rate of the control HFKs was 0.45PD/day following electroporation as opposed to 0.95PD/day for the Y-27632 HFKs. This growth advantage also applies to the cells transfected with both the low-risk and high-risk HPV genomes. Moreover, we find that the control HFKs transfected with the low-risk genomes senesced at the same time as the mock-transfected HFK's. In contrast, the drug-treated HFKs transfected by all DNAs continued to grow indefinitely. Therefore, treatment with the drug improves the survival and growth of transfected HFKs.

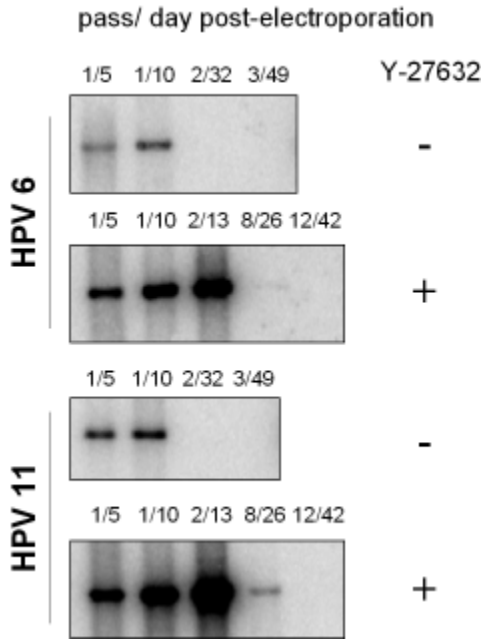
Figure 4-5. Effect of Y-27632-treatment on long-term replication of low-risk HPV transfected HFKs

A. Growth curves of primary HFKs (red) grown in the absence (squares) or presence (diamonds) of 10μM Y-27632 (+Y). HFKs mock transfected with ssDNA (purple) or transfected with HPV6 (yellow), HPV11 (green), or HPV18 (aqua) were also grown in the absence (squares) of presence (diamonds) of 10μM Y-27632 (+Y). Population doubling was calculated as: PD=3.32 (log(# cells harvested/# cells seeded)). B. Low molecular weight DNA from HFKs transfected with the indicated HPV type grown in either the absence or presence of 10μM Y-27632 was analyzed by Southern blotting. Cells were analyzed either at 5 days post-transfection, 10 days post-transfection, or at late passages as indicated. HIRT extracts were digested with a restriction enzyme to linearize the viral DNA (EcoRI for HPV18 transfected cells, and BamHI for HPV6 transfected cells) and Dpn I to distinguish newly replicated DNA. Each lane is labeled by the pass number at which the sample was collected and the number of days post transfection at which the samples were collected.

A


Population Doubling
Days
HFK
HFK +Y
ssDNA
ssDNA +Y
HPV 6
HPV 6 +Y
HPV 11
HPV 11 +Y
HPV 18
HPV18 +Y
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120

B


pass/ day post-electroporation
Y-27632
HPV 6
1/5 1/10 2/32 3/49
-
1/5 1/10 2/13 8/26 12/42
+
HPV 11
1/5 1/10 2/32 3/49
-
1/5 1/10 2/13 8/26 12/42
+

Y-27632 treatment also enhances detection of low-risk HPV replication in transfected cells

After finding that the treatment with Y-27632 was able to extend the growth of HFKs transfected with the low-risk HPV genomes, we wanted to determine whether the viral genomes were maintained in these cells. To answer this, we analyzed the low molecular weight DNA extracted from the transfected HFKs for the presence of HPV genomes using Southern blot analysis. Treatment of Y-27632 improved the apparent levels of replication detected in low-risk HPV transfected HFKs immediately following electroporation (Figure 4-5B). However, after passage in culture, the levels of viral DNA declined in both the control HFKs and the HFKs grown in the +Y-27632 media. In both cell types it seems that the low risk viral genomes are lost over time in culture.

Long term maintenance of high-risk HPV genomes is gradually lost over-time in Y-27632-treated HFKs

Unlike the low-risk types, the high-risk HPV types have a selective growth advantage in transfected primary HFKs (216;219;220). This feature has been useful for HPV-research since the high-risk genomes immortalize cells harboring the virus and, thus, enabled the use of primary keratinocytes for these studies. Interestingly, when we examined the DNA extracted from HFKs transfected with the high-risk genome HPV18 and grown in Y-27632-supplemented media, we found the same gradual loss of transfected genomes that we saw for the low-risk types (Figure 4-6). After day 10, replication was in fact higher in the control HFKs grown without the inhibitor. By passage 12, the viral genomes were undetectable in the drug-treated HFKs. The high-risk HPVs immortalize primary cells due to their expression of the viral oncogenes E6 and E7. This allows the cells that harbor the genomes to be selected for against the cells that would enter crisis and senesce without the viral oncogenes present. This explains the stable maintenance of the

HPV genomes transfected into the control cells. Clearly, it seems that this property of the high-risk HPV genomes is not an advantage to these cells and is not necessary for their prolonged growth in culture. The proliferation induced by Y-27632 negates the growth advantage offered by the high-risk E6 and E7 and enables HPV-negative cells to outgrow cells positive for HPV.

Episomal genome copy number decreases with time in both drug-treated and non-drug treated cultures of the W12 cell line

In contrast to the CIN-612 9E cell line, episomal maintenance of the HPV16 genome harbored in the W12 cell lines has been shown been shown to be lost over time as cells with integrated genomes gradually take over the culture (125). To determine whether Y-27632 affects plasmid maintenance of the HPV episomal genomes in the W12 cell line, we grew these cells in the absence or presence of 10 μM Y-27632 for ten passes. As previously reported, we found a slight decline in the levels of episomally maintained HPV16 genome copies in the control cells after several passes in culture (Figure 4-7A). This also appears to be the case for the Y-27632-treated cells as well. Quantification of the genome copies in these samples from triplicate experiments are shown in the bar graph chart in Figure 4-7B. However, we did notice that the epsiomal copies of HPV16 were maintained at a slightly higher level in the Y-27632-treated cells compared to the control W12 cells at the same pass. This difference was not statistically significant (P= 0.609292587, P=0.127028616 and P=0.524149419 for passages 5, 6 and 10 respectively in a 2-tailed students T-test) and therefore only represents a slight observational difference. These data suggest that the presence of Y-27632 might possibly support episomal maintenance by weakening the selective advantage of cells with integrated genomes and deserves further investigation.

Figure 4-6. Effect of Y-27632-treatment on long-term replication of high-risk HPV transfected HFKs

Low molecular DNA from the HFKs transfected with HPV type 18 grown in the absence or presence of 10μM Y-27632 were analyzed by Southern blotting. Cells were analyzed either at 5 days post-transfection, 10 days post-transfection, or at late passages, as indicated. HIRT extracts were digested with EcoRI to linearize the viral DNA and Dpn I to distinguish newly replicated DNA. Each lane is labeled by the pass number at which the sample was collected and the number of days post transfection at which the samples were collected.

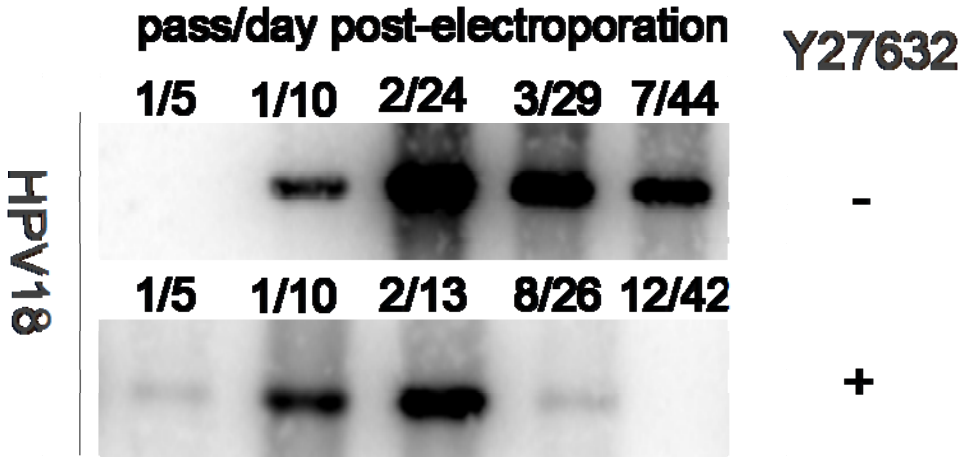
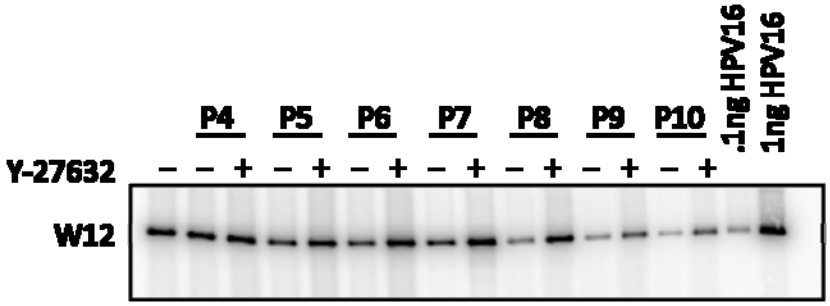

pass/day post-electroporation
Y27632
1/5
1/10
2/24
3/29
7/44
-
HPV18
1/5
1/10
2/13
8/26
12/42
+

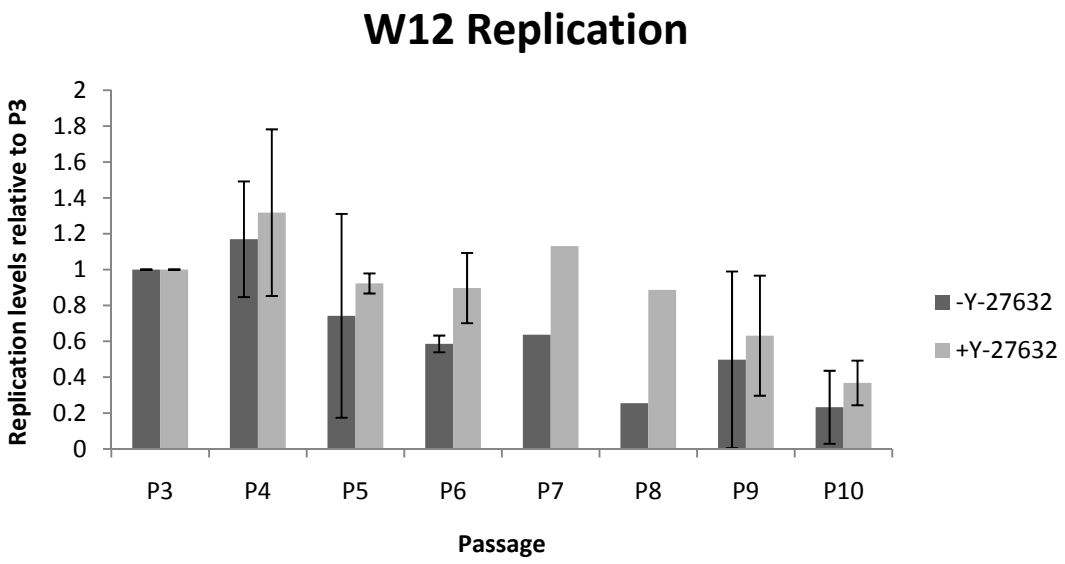
Figure 4-7. Long term episomal maintenance of HPV16 genome in W12 cells grown in the absence or presence of 10μM Y-27632

A. Low molecular weight DNA from W12 cells grown in the absence or presence of 10μM Y-27632 were analyzed by Southern blotting. Cells were analyzed at the passages indicated. HIRT extracts were digested with BamHI to linearize the genomic DNA. On the right-hand side of the image, controls were included to represent 0.1ng and 1.0ng of linearized HPV16 genomic DNA. B. Quanification of intensity of genomic bands represented in Southern blots. X-axis indicates passage number. Y-axis represents replication levels relative to initial passage, P3 before drug was added. Data for duplicate experiments are represented in passages 1, 5, 6, 9, and 10. Error bars represent the standard deviation for these values.

A


P4
P5
P6
P7
P8
P9
P10
1ng HPV16
1ng HPV16
Y-27632
– – + – + – + – + – + – + – +
W12

B


W12 Replication
Replication levels relative to P3
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P3
P4
P5
P6
P7
P8
P9
P10
Passage
-Y-27632
+Y-27632

Discussion

In this study we found that Y-27632 treatment improves the analysis of transient replication in primary keratinocytes. The transfection efficiency of primary HFKs is dramatically improved when the cells are cultured in Y-27632. We also found greater amounts of replicated viral genomes, for both the low-risk and high-risk HPVs, in the DNA isolated from cells grown in the drug compared to the control HFKs in transient assays. However, our data suggest that treatment with Y-27632 does not affect HPV replication directly, but rather the treatment simply improves the growth of the transfected HFKs. We saw that treatment with Y-27632 had no noticeable effect on the replication of the HPV31 genomes maintained in the 9E cell line. When the cell growth rate is not affected by the drug-treatment, as was the case for the 9E cell line, we saw no appreciable difference in viral replication levels, thus supporting the hypothesis that the drug-treatment is not directly affecting viral replication.

While transient replication of the both the low-risk and high-risk HPV genomes was improved in the transient assays, we found that we could not establish long-term persistent infections for either types of HPV in the drug-treated HFKs. While this is not necessarily a new phenomenon for the low-risk genomes, this is a new and interesting behavior of the high-risk genomes, which normally immortalize the transfected HFKs and can be maintained in the HFKs indefinitely. Once the high-risk genomes in the control HFKs have passed crisis, the surviving infected cells are able to maintain the transfected genomes indefinitely due to the ability of HPV genomes to establish persistent infections. Because the HFKs grown in the drug do not need the growth advantage offered by the transfected HPV genomes, there is no selective growth advantage for cells successfully transfected with HPV genomes.

The mechanism explaining the loss of the viral genomes is unclear from our data. If the infected cells had a survival capacity equal to the uninfected cells, and the growth of both types

were equal in the presence of the drug, we would expect that the ratio of infected vs. uninfected cells would remain constant over time and that both cell types would survive equally. Since we observed the loss of infected cells, the possibility that Y-27632 may negatively affect the long-term maintenance of the HPV genomes cannot be ruled out. Human cells have developed a mechanism to get rid of foreign DNA to prevent damage (221). Therefore it is likely that the HFKs grown in the presence of Y-27632 are actively eliminating the transfected HPV genomes as a defense mechanism against foreign DNA. Experiments applying Y-27632 to a uniform population of HPV-positive HFK's is currently in progress to test the maintenance of HPV-positive genomes in the presence of the drug. This will help to clarify whether the loss of the viral genomes over time is the result of the Y-27632-treatment or the cells own defense mechanisms. Since we observed the loss of the low risk type in the untreated HFKs it is likely that the loss we observe in both types is the consequence of the cellular response to rid the cell of foreign DNA.

The initial characterization of Y-27632 revealed that this drug has a high specificity for the Rho kinases (ROCK) (179;222). ROCK-II is the predominant isoform of the Rho kinases present in keratinocytes (154). Tanaka *et al*. (2006) have shown that ROCK-II is equally distributed between cytoplasmic and nuclear fractions of the cell (223). Moreover, they report that ROCK-II is associated with components of the nuclear matrix (223). Because Tan *et al*. (1998) have proposed that HPV genome maintenance is mediated through matrix attachment regions, it is possible that Y-27632s interaction with the nuclear matrix may disrupt this association and prohibit long-term maintenance of HPV genomes (67). Since we have not identified the precise mechanism that results in Y-27632-induced immortalization of primary keratinocytes it is difficult to know what processes may be affected and how this might affect the HPV life cycle. Therefore, as with other immortalized cell lines, use of Y-27632-treated cells for HPV-research must be approached with caution and an awareness of the potential limitations of

this model system. Further analyses, such as the ability of HPV-transfected HFKs treated with Y-27632 to produce infectious virus in raft tissue cultures, will be helpful to determine whether the complete viral life cycle is affected by treatment with this drug.

Previous studies have introduced HPV16 into immortalized cell lines, but they have reported conflicting conclusions (214;217). First, Brune and Durst (1995) electroporated the HaCaT cell line with three different isolates of HPV16 (214). The authors reported that while they did find replication of HPV11 in the HaCaTs, they did not observe replication using three different isolates of HPV16. The authors took care in the selection of the HPV16 genomes used to ensure that they did not include the E1 frameshift mutation discovered in the original HPV16 prototype (214). Later, Flores *et al*. (1999) reported that they were able to establish stably replicating HPV16 genomes transfected into the NIKS cell line, although they do not specify a length of time (217). A possible explanation for the differences seen in these two studies may stem from the different immortalized cell types used in each study. It could be argued that the NIKS cell line represents a more authentic model of primary keratinocytes because these cells only contain one major genetic abnormality (trisomy of chromosome 8), they more accurately mimic stratified epithelial tissue when induced to differentiate using organotypic raft cultures, and unlike the HaCaT cells, they are still dependent on feeders and EGF for their growth (129;217). In both of these studies, HPV-transfected cells were selected using co-transfection with the drug-selectable marker G418. Therefore, it will be important to use a selection method to establish a uniform population of transfected cells before we can conclude that HPV replication is lost in the presence of Y-27632.

Thomas *et al*. (2001) had found that HFKs transfected with HPV11 improved the growth of primary HFKs (211). We did not notice any appreciable difference in the growth of the HFKs transfected with carrier DNA or either of the low risk HPV6 or 11 genomes. One explanation for the difference in our findings could be the method of transfection used in our experiments.

Thomas *et al*. (2001) had used lipofectamine to transfect the HFKs in their studies, while we used electroporation in our experiments. Electroporation induces a shock to the cells and results in higher cell death following transfection (163). This might thwart the slightly enhanced growth seen by lipofection, which is not as toxic to the primary cells. Therefore, it is possible that this less damaging method used by Thomas *et al*. (2001) allowed them to observe more subtle effects of the transfected genome on cell growth immediately following transfection.

Unlike the 9E cell line, we saw a gradual decline in episomally maintained genomes in the W12 cells grown either in the presence or absence of the drug. However, after prolonged culture of the W12 cells in the drug, we found that there was a slightly higher level of episomally replicated genomes in the cells grown in the presence of Y-27632. Because Y-27632 improves the growth of the keratinocytes, it is possible that the drug helps to alleviate the selective pressure that favors the growth advantage of cells that harbor integrated genomes. It seems likely that before treatment with the drug, the original stock of W12 cells that were used in this study represented a mixed population of cells containing both episomal and integrated viral genomes. Normally, growth of cells harboring integrated genomes is favored because they have unregulated expression of E6 and E7 and therefore have a greater potential to interfere with cell cycle controls (123). We postulate that the drug alleviates this pressure by providing another means of growth support. Since the difference in episomal maintenance between the control cells and the drug-treated cells was not significant, it would be useful to repeat this experiment using conditions that might accentuate the effect of the drug. Extrachromosomal genomes are not efficiently maintained in HFKs cultured in the absence of fibroblast feeder cells in serum-free medium (122;138). Therefore, we could exploit this condition to force integration and determine the ability of the drug to preserve episomal maintenance compared to the control cells.

Chapter 5

Effect of mutations in the E2 binding sites on HPV18 transcription and replication

Introduction

Integration of the HPV genome into cellular chromosomes is an important factor in the progression from benign to malignant infections (13;77;131). The integration sites from high-grade cervical cancer lesions very frequently occurrs in the middle of the E2 Open Reading Frame (ORF) resulting in the loss of E2 expression (80;224). Because of the association between the loss of the E2 protein and malignant progression, the role of the E2 protein in HPV infections has been a prominent topic of study. This correlation was substantiated when it was discovered that E2 acts as a transcriptional repressor of the promoter controlling the expression of the viral oncogenes E6 and E7 (83;88;89). Therefore, loss of E2 results in unregulated expression of E6 and E7. However, to date, no single model has provided a clear relationship for the process of integration and the loss of E2 and malignant progression. In addition, the role of E2 in transcriptional control is very complex and depends on the isoform and type of the E2 protein, the abundance of E2, the promoter type and the cell type in which analysis is performed (85-87;225). This complexity is compounded by the fact that E2 is multifunctional and plays many roles in the viral life cycle, including transcription, replication, and genome maintenance which complicates the analysis of E2 regulation.

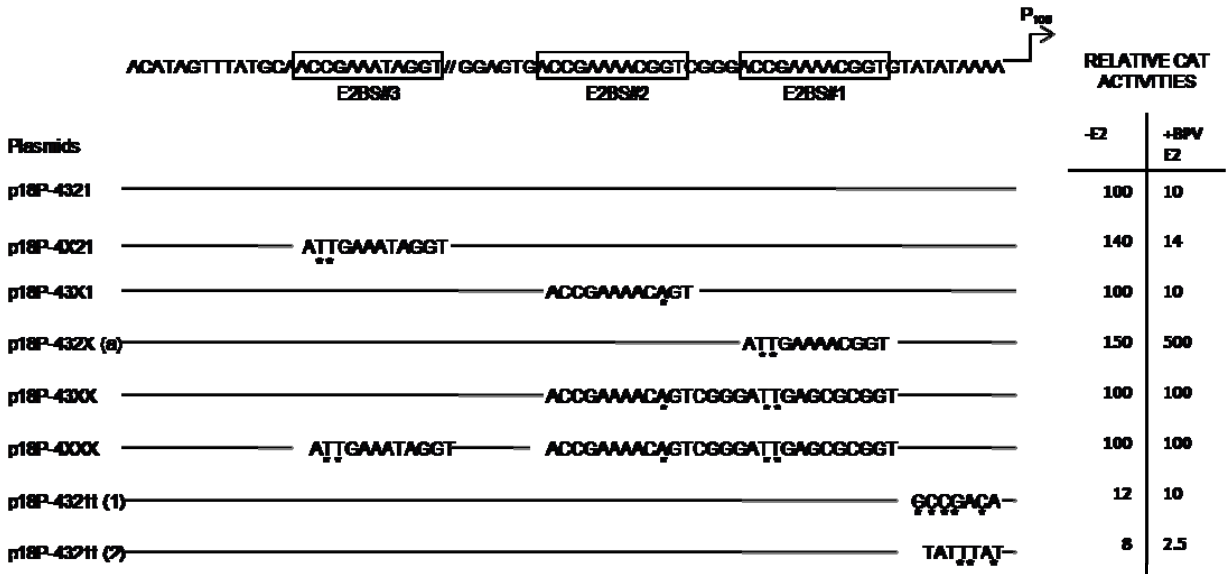
The E2 protein has a C-terminal DNA-binding domain that recognizes and binds to the palindromic sequence, $ACCGN_4CGGT$, as a homodimer. The transactivation domain at the N-terminus of E2 is essential for transcriptional regulation as well as for initiating replication by

binding E1 and therefore recruiting E1 to the origin of replication (70). The E2 N-terminal domain also binds cellular proteins and it has been shown that BPV1 E2 binds to Brd4 and this interaction links the viral genome to cellular chromosomes (66;226;227). In BPV1, it has been determined that at least 8 of the 11 E2 binding sites located in the untranslated regulatory region are required for genome maintenance (64;228).

There are four conserved E2 binding sites in the high-risk HPVs, which have been shown to play a role in replication and transcription. However, analysis of mutations in the E2 binding sites is complicated since these sites are multifunctional and thought to be involved in replication, transcription, and potentially genome segregation. Because of their role in transcription, mutations in these sites affect early gene expression, including that of E1 and E2, which are essential for replication. To study the impact of these cis-elements specifically on replication it is necessary to ensure that expression of the essential replication factors E1 and E2 is intact.

Previous studies have examined the effect of the E2 binding sites on the HPV18 early promoter using reporter assays (82;86;89;229;230). Thierry *et al*. (1991) mutated three of the four E2 binding sites either individually or in various combinations (Figure 5-1). In this study the E2 binding sites were numbered counting from the promoter, with binding site #1 being closest to the promoter and #4 being the most distal from the promoter. These mutations in the E2 binding sites were designed to minimize changes to the basal level of the promoter activity. The CAT activity of each mutant plasmid was measured relative to the wild-type URR plasmid, either in the absence or presence of the BPV1 E2 expression plasmid, in C33a cells. The authors found that the E2 binding site, #1, closest to the promoter, has a dominant role relative to the other E2 binding sites and seems to be single-handedly responsible for E2-mediated repression on promoter activity. However, the E2 binding site at position #2 is antagonistic to site #1 because the loss of both binding sites #1 and #2, abrogated the increase found in the mutated E2 binding site #1 plasmid (230).

Figure 5-1. Promoter activity of URR constructs with mutations in the E2 binding sites Modified from Thierry and Howley (1991) (230). The 200 nucleotide sequence of the wild-type HPV18 URR sequence is represented at the top of the figure. The sequences for the three binding sites for the viral E2 protein are boxed. The mutated promoter plasmids are listed on the left hand of the figure. The mutated residues are indicated by asterisks. The relative CAT activities are indicated on the right (expressed as a percentage relative to the wild-type activity). The CAT activities were obtained by transfection of the cervical carcinoma cell line C33 in the absence (-E2) or the presence (+E2) of the BPV1-E2 expression plasmid pC59.


ACATAGTTTATGCACCGAAATAGGTTGGAGTGACCGAAAACGGTCGGGACCGAAAACGGTGTATATAAAA
E2BS#3
E2BS#2
E2BS#1
RELATIVE CAT ACTIVITIES
Plasmids
-E2
+BPV E2
p18P-4321 100 10
p18P-4021 ATTGAAATAGGT 140 14
p18P-43X1 ACCGAAAACAGT 100 10
p18P-432X (a) ATTGAAAACGGT 150 500
p18P-430X ACCGAAAACAGTCGGGATTGAGCGCGGT 100 100
p18P-400X ATTGAAATAGGT ACCGAAAACAGTCGGGATTGAGCGCGGT 100 100
p18P-4321t (1) GCCGACA 12 10
p18P-4321t (7) TATTTAT 8 2.5

However, these studies examined the URR isolated from the full genome context. There is evidence to suggest that the results from these types of analyses may not necessarily apply to the virus when the elements function in the complete viral genome (231). The reporter assays use E2 expression vectors and likely do not represent the physiological levels of E2 in a natural infection. Transcriptional control by E2 depends on the relative levels of E2 expression; very high and very low levels of E2 can have dramatically different results (85;86;232). Therefore, it is important to verify whether these elements have the same effect in the full context of the genome when E2 is expressed at basal levels.

Stubenrauch *et al*. (1998) examined the role of the E2 binding sites with regard to replication using the full-length genome (60). The authors reported that none of the individual E2 binding sites was essential for transient replication. They saw an increase in the transient replication of the genome with a mutation in the E2 binding site most proximal to the promoter. This site has been shown to be responsible for the transcriptional repression role of E2 (81;82;86;89;229;230). Therefore, it seems likely that the increase in replication they observed was due to the loss of the transcriptional repression role of this binding site, resulting in greater amounts of E1 and E2, and not due to a direct effect on replication. This example demonstrates the complications involved in the analysis of these binding sites when they are examined in the full length genome and rely on viral functions that are inherently dependent on each other.

To verify the role of the E2 binding sites in the complete HPV 18 genome, we transfected primary keratinocytes with HPV genomes mutated in the four E2 binding sites either individually or in various combinations. We modeled the mutations after those used in Thierry and Howley, 1991, as shown in Figure 5-1. We found that mutation of any of the E2 binding sites individually did not disrupt the immortalization capability of these mutant viruses indicating that the viruses remained transcriptionally active. Analysis of the mutated viral genomes present in the immortalized cell lines indicated that the viral genomes were maintained at very low copy levels

and were most likely integrated into the host chromosomes. We also further analyzed the role of the E2 binding sites in replication using a complementation system that allows the analysis of these sites while ensuring wild-type expression levels of the replication proteins. From these studies, we found that only E2 binding site #4 was nonessential for transient replication.

Results

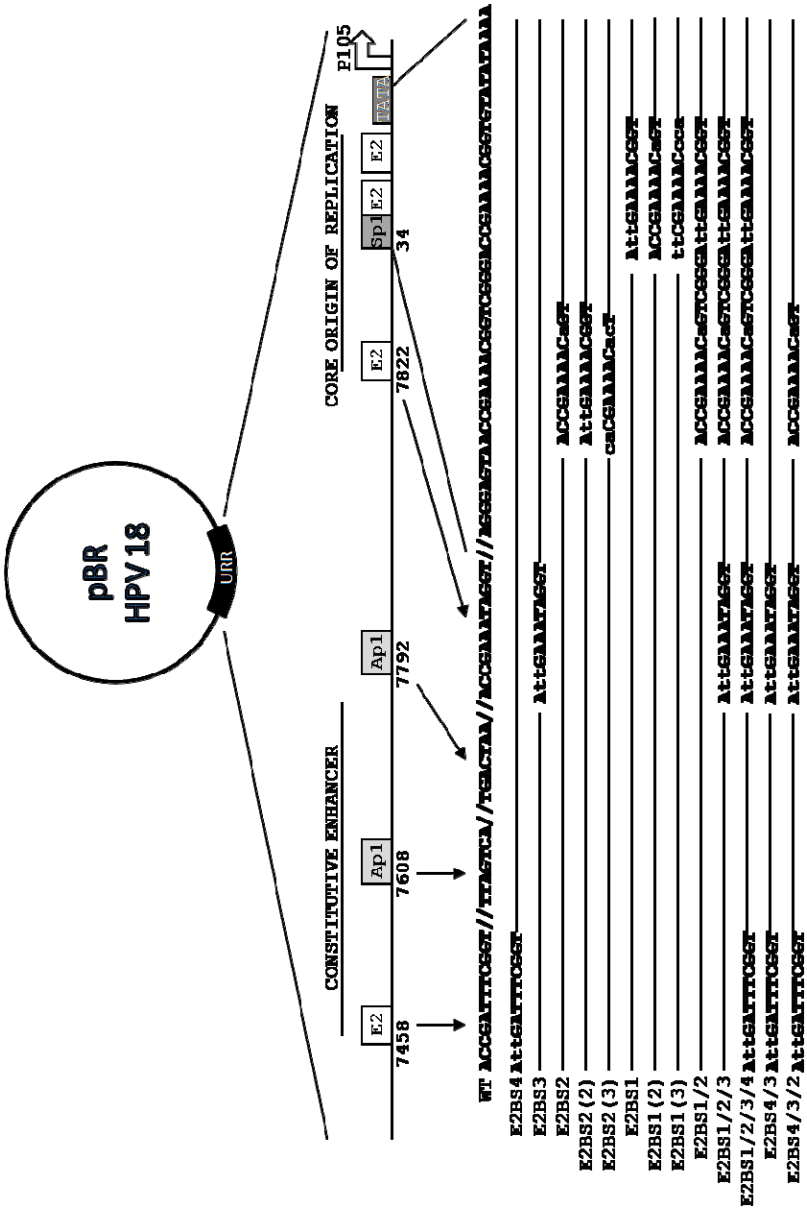
Mutations in the E2 binding sites in the HPV18 URR

Reporter assays measuring the effect of mutations in the E2 binding sites on the activity of the early promoter in HPV showed that the E2 binding sites are involved in transcriptional regulation of the URR (74;86;230;232). In these studies, the entire URR was removed from the coding regions of the genome and placed upstream of the CAT gene. Mutational analysis indicated that the viral E2 binding sites played an important role in regulating the activity of the early promoter in these reporter assays. Of the four binding sites, the E2 binding site most proximal to the promoter was found to be the site responsible for the transcriptional repression on the early promoter (82;229;230).

To validate the significance of these findings in the context of the genome, we introduced the same binding sites mutations into the full-length genome. Using site-directed mutagenesis, we created mutant HPV18 genomes as shown in Figure 5-2. Two additional mutations were added for the E2 binding sites at positions 1 and 2. The second set, E2BS1(2) and E2BS2(2) were included to match each other since the mutations in these binding sites were different. The third set, E2BS1(3) and E2BS2(3) were included to mimic the mutations made in HPV31 by Stubenrauch *et al*. (60). Because the original study had not included the most distal E2 site, #4,

Figure 5-2. E2 binding site mutations in the HPV18 URR

Point mutations were introduced into the full-length HPV18 genome. The location of each binding site is shown in the schematic at the top of the figure. The wild-type sequence of the binding sites is shown below the schematic. The mutated residues in the plasmids listed on the left are indicated by lower case letters.


pBR
HPV 18
URR
CONSTITUTIVE ENHANCER
CORE ORIGIN OF REPLICATION
E2
Ap1
Sp1
TATA
P105
7458
7608
7792
7822
34
WT
E2BS4
E2BS3
E2BS2
E2BS2 (2)
E2BS2 (3)
E2BS1
E2BS1 (2)
E2BS1 (3)
E2BS1/2
E2BS1/2/3
E2BS1/2/3/4
E2BS4/3
E2BS4/3/2

we also included plasmids mutated in this site for a more comprehensive analysis of all the E2 binding sites. Three additional plasmids were added, E2BS1/2/3/4, E2BS3/4, and E2BS2/3/4, which represent new combinations of the E2 binding sites not used by Thierry *et al*. (1991). Mutated genomes were sequenced to verify the presence of the mutations and at least 3 independent plasmids with the correct mutations were selected.

HPV genomes with mutations in E2 binding sites can immortalize cells.

To test the significance of the E2 binding sites on early promoter activity in the context of the full genome, we transfected keratinocytes with the mutated full-length linearized genomes and determined whether or not the transfected mutant genomes could immortalize the cells. For these studies we used normal, primary human foreskin keratinocytes, which have a limited lifespan and will slow in their growth in culture and eventually senesce. Only cells that express the viral oncogenes, E6 and E7, will bypass senescence and continue to divide indefinitely. Therefore, the ability of primary HFKs to bypass senescence is a measure of early promoter activity driving E6 and E7 expression. Furthermore, the use of primary HFKs allows us to study the mutated viral genomes in a cell type that closely models the natural host for HPV infections. Immortalization was defined as the ability to outgrow the negative control HFKs that were mock-transfected with only the carrier DNA. Each mutant genome was electroporated into HFKs on at least three separate occasions. The resulting cell lines were named to indicate the specific plasmid used for the transfection (i.e. E2BS1:2, E2BS1:6, etc.) followed by a letter to indicate each unique cell line (i.e. E2BS1:2a, E2BS1:2b; E2BS1:6a, E2BS1:6b, etc.). The results of the immortalization experiments are summarized in Table 5.1.

Table 5-1. Immortalization assays results of E2 binding site mutations

Mutations	# of transfections	# of immortalized cell lines
HPV18	5	5
No HPV	14	0
E2BS4	4	4
E2BS3	4	4
E2BS2	4	4
E2BS2(2)	4	4
E2BS2(3)	4	4
E2BS1	4	4
E2BS1(2)	4	4
E2BS1(3)	5	4
E2BS1/2	3	3
E2BS1/2/3	4	4
E2BS1/2/3/4	6	3
E2BS4/3	4	4
E2BS4/3/2	4	4

Genomes with mutations in each individual E2 binding site, and in most combinations, were able to efficiently drive proliferation of the transfected HFKs beyond normal senescence. We only observed inefficient immortalization of the HFKs transfected with the HPV18 genome with mutations in all 4 E2 binding sites. The HFKs transfected with these genomes immortalized in only 50% of the experiments performed. A fragment of the URR from the transfected immortalized cell lines was amplified by PCR and sequenced to verify the conservation of the mutations.

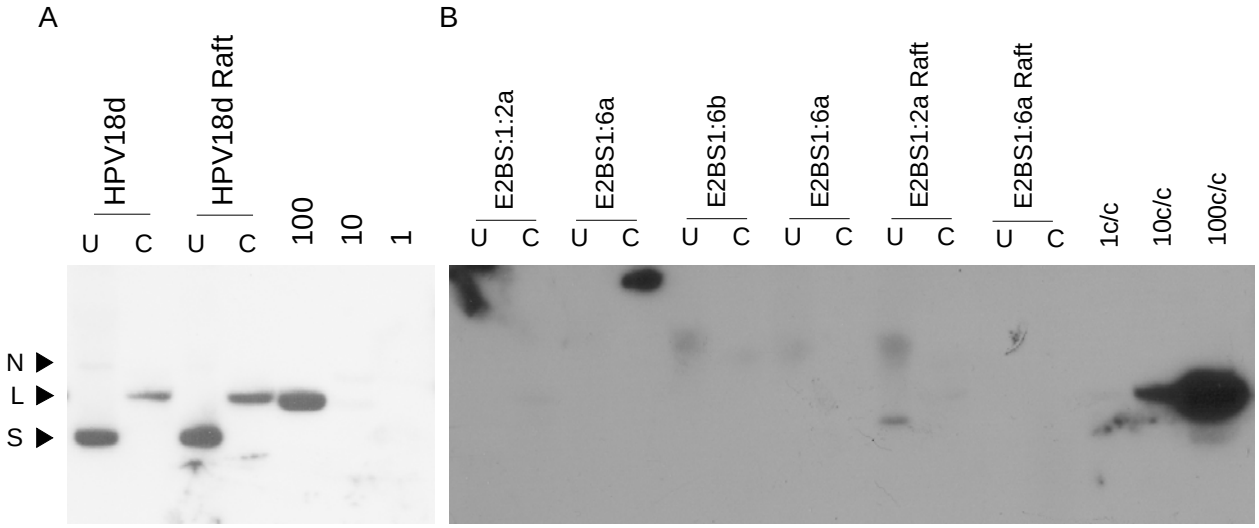
E2 binding site mutations are maintained at very low levels

As mentioned earlier, HFKs transfected with HPV18 genomes mutated individually in each of the four E2 binding sites all became immortalized. Episomal genomes can be maintained

for years in the host basal cells of persistent papillomavirus infections. The existence of stably replicating extrachromosomal genomes has been shown in both cell lines isolated from clinical lesions (122;124) as well as from cell lines established in the laboratory by transfection with HPV genomes (119;208;217). However, high-risk HPV genomes can also become integrated into the host chromosome over time and this step is a major hallmark of malignant progression of HPV infections. One way to test the integration state of the transfected genomes is to analyze the migration patterns on Southern blots. Genomes that are maintained episomally migrate as supercoiled and nicked open circular molecules when undigested total DNA samples are separated by agarose gel electrophoresis and as a linear band when digested with an enzyme that cuts once in the HPV genome. This can be seen in the HFKs transfected with the wild-type HPV18 genome in Figure 5-3A. This method also allows us to measure the viral copy number per cell by comparing the relative levels of the linear genomes in each sample to copy number controls calculated to represent 1, 10, or 100 copies per cell. To test the state of viral genomes in the cell lines with mutations in the E2 binding sites, we performed Southern blot analysis on the total extracted DNA from each of the cell lines. We examined the genomic DNA either immediately after the cell lines had bypassed the growth of the non-HPV control cells, at later passes (around passage 9), or both. The Southern blots revealed that the viral DNA in the samples extracted from the HFKs transfected with wild-type HPV18 replicate at approximately 50-100 copies per cell (see Figure 5-3A). In some cases we also analyzed the DNA extracted from organotypic rafts to see if the viral genomes were amplified in this culture system. Because the viral genomes are normally amplified in the stratified layers of differentiated epithelial tissue, we would expect to see a greater copy number from the DNA samples extracted from the raft tissue than in from the monolayer cells. We observed a slight amplification of the HPV18 genome in the DNA samples extracted from the raft tissue compared to the DNA extracted from the monolayer cell culture (see Figure 5-3A). However, this amplification was not dramatic, and

Figure 5-3. Replication of HPV genomes with mutations in the E2 binding sites

(A) Southern (DNA) blot hybridization of DNA extracted from HPV18 transfected HFKs cultured as monolayers or an organotypic raft. “Uncut” lanes were loaded with 5 µg of total cell undigested DNA. “Cut” lanes contain 5µg of total cellular DNA digested with EcoRI. HPV18 100, 10, and 1 copy number standards are shown on the right. Arrows on the left indicate nicked viral DNA (N), linear viral DNA (L), and supercoiled viral genomic DNA (S). (B) Southern (DNA) blot hybridization of DNA extracted from HFKs transfected with HPV18 genomes with mutations in the E2 binding sites, as indicated, cultured as monolayers or an organotypic raft. “Uncut” lanes contain 5 µg of total cell undigested DNA. “Cut” lanes contain 5µg of total cellular DNA digested with EcoRI. HPV18 100, 10, and 1 copy number standards are shown on the right. Arrows on the left indicate nicked viral DNA (N), linear viral DNA (L), and viral genomic DNA (S).


A
HPV18d
HPV18d Raft
U C U C
100
10
1
N
L
S
B
E2BS:1:2a
E2BS1:6a
E2BS1:6b
E2BS1:6a
E2BS1:2a Raft
E2BS1:6a Raft
U C U C U C U C U C U C
1c/c
10c/c
100c/c

not consistent in other lines created with wild-type HPV18 DNA on separate occasions. Often, the viral genome was found to be present at similar levels when comparing the monolayer and raft samples.

An example of a typical Southern blot for cell lines transfected with the genomes mutated in the E2 binding sites is shown in Figure 5-3B. We found that the mutated viral genomes were often undetectable in the DNA samples from the immortalized cells when tested by Southern blotting using the traditional downward transfer protocol. Initially the cell lines were tested immediately after the cells bypassed senescence (as determined by their ability to grow beyond the time that the negative controls reached senescence) which was generally around passage 5. However, after we struggled to detect the presence of the transfected genomes at this time point, we began collecting DNA at a later pass to determine whether this would enable better establishment of the transfected genomes. However, in the lines we tested at a later pass, we did not notice a significant difference and often the viral genomes were still undetectable. The mutated viral genomes were undetectable in the Southern blots for the transfected cell lines tested, suggesting that they are present at very low levels, presumably at approximately 1 copy per cell. Moreover, we did not see significant amplification of the mutated viral genomes in any of the cell lines grown as raft tissue. These results suggest that the viral genomes are integrated in these cell lines. A summary of the lines tested and the results can be found in Table 5.2.

Table 5-2. Southern blot analysis of transfected mutant genomes.

Legend: ND=Not done; c/c= copy per cell

Mutant	Early Pass (4-6)	Late Pass (9 or 10)	Raft
HPV18d	ND	~25c/c	~50c/c
E2BS1:2a	undetectable	ND	1c/c, episomal
E2BS1:6a	undetectable	1c/c, not episomal	undetectable
E2BS1:6b	1c/c, not episomal	1c/c, not episomal	1c/c, not episomal
E2BS2:1a	undetectable	undetectable	undetectable
E2BS2:2a	undetectable	undetectable	undetectable
E2BS2:2b	undetectable	ND	ND
E2BS3:1b	1c/c, not episomal	ND	ND
E2BS3:2a	1c/c, not episomal	ND	undetectable
E2BS3:2b	undetectable	ND	ND
E2BS4:1b	undetectable	ND	ND
E2BS4:2b	undetectable	ND	ND
E2BS1/2/3:3a	undetectable	ND	ND
E2BS1/2/3:8a	undetectable	ND	ND
E2BS1/2/3:8c	ND	undetectable	undetectable
E2BS3/4:1a	ND	1c/c, not episomal	1c/c, not episomal
E2BS3/4:2a	ND	1c/c, not episomal	1c/c, not episomal
E2BS2/3/4:3a	ND	undetectable	undetectable
E2BS2/3/4:4a	ND	undetectable	undetectable

By using a more sensitive Southern transfer method, the Whatman turbo blotter, we were able to reanalyze the samples to determine the state of the genomes present in these cell lines. One representative line was used for each mutated genome. The Southern blots of the cell lines transfected with HPV genome constructs with mutations in only one individual E2 binding site are shown in Figure 5-4A. Interestingly, one line, E2BS1:2a, which had undetectable viral DNA when the sample from passage 4 was tested by the traditional Southern method, had a very high copy number at passage 10. The original mutation for this genome was confirmed to be present and intact in this cell line at this pass. Moreover, the migration pattern of the cut DNA is consistent with the linearized genome size, as indicated by the yellow arrow. Also, the uncut DNA reveals the presence of a supercoiled band that migrates at the same size as the wild-type supercoiled band as indicated by the red arrows. This suggests that this mutated viral genome is

Figure 5-4. Replication of HPV genomes with mutations in the E2 binding sites

A&C. Southern (DNA) blot hybridization of total cellular DNA extracted from monolayers of HPV18 transfected HFKs. “Uncut” lanes contain 5 µg of total cell undigested DNA. “Cut” lanes contain 5µg of total cellular DNA digested with EcoRI. HPV18 100, 10, and 1 copy number standards are shown on the right. Yellow arrows indicate linear viral DNA and red arrows indicate supercoiled viral genomic DNA. B&D. Ethidium Bromide stained 0.8% agarose gel of total cellular DNA extracted from monolayers of HPV18 transfected HFKs. “Uncut” lanes contain 5 µg of total cell undigested DNA. “Cut” lanes contain 5µg of total cellular DNA digested with EcoRI. HPV18 100, 10, and 1 copy number standards are shown on the right.

A
U C | E2BS 1:2a
U C | E2BS 1:6b
U C | E2BS 2:1a
U C | E2BS 2:2a
U C | E2BS 3:1b
U C | E2BS 3:2a
U C | E2BS 4:1b
U C | E2BS 4:2b
C C | HPV18b
1c/c
10c/c
100c/c
B
U C | E2BS 1:2a
U C | E2BS 1:6b
U C | E2BS 2:1a
U C | E2BS 2:2a
U C | E2BS 3:1b
U C | E2BS 3:2a
U C | E2BS 4:1b
U C | E2BS 4:2b
C C | HPV18b
1c/c
10c/c
100c/c
C
U C | E2BS 1/2:1b
U C | E2BS 1/2:5b
U C | E2BS 3/4:1a
U C | E2BS 3/4:2a
U C | E2BS 2/3/4:3a
U C | E2BS 2/3/4:4a
U C | E2BS 1/2/3/4:7c
U C | E2BS 1/2/3/4:8c
U C | E2BS 1/2/3:8a
U C | E2BS 1/2/3:3a
C C | HPV18d
C C | HPV18e
1c/c
10c/c
100c/c
D
U C | E2BS 1/2:1b
U C | E2BS 1/2:5b
U C | E2BS 3/4:1a
U C | E2BS 3/4:2a
U C | E2BS 2/3/4:3a
U C | E2BS 2/3/4:4a
U C | E2BS 1/2/3/4:7c
U C | E2BS 1/2/3/4:8c
U C | E2BS 1/2/3:8a
U C | E2BS 1/2/3:3a
C C | HPV18d
C C | HPV18e
1c/c
10c/c
100c/c

maintained episomally in this line. However, this does not appear to be consistent in the E2BS1:6b line, which contains the same mutation. There are also faint bands migrating at the linear size in the cut samples for a few other samples: E2BS3:1b, E2BS4:1b, and E2BS4:2b. However, these bands are very faint and there is no evidence of supercoiled DNA in the uncut samples. It is possible that the genomes have multiple genomes integrated in tandem in the host chromosomal DNA, thus generating a linear fragment from between the tandem copies upon digestion. Therefore, without the presence of the supercoiled viral DNA it cannot be concluded that the linear bands represent episomally maintained genomes in these samples. In addition, there is also a smear of viral DNA that migrates very slowly at the same position as the cellular DNA when comparing the Southern to the ethidium bromide stained gels (Figure 5-4B). This genome migration pattern is similar to that observed for the 6E cell line, which was determined to be integrated (121). Therefore, it is likely that the mutated viral DNAs are integrated into the host cellular DNA in these cell lines.

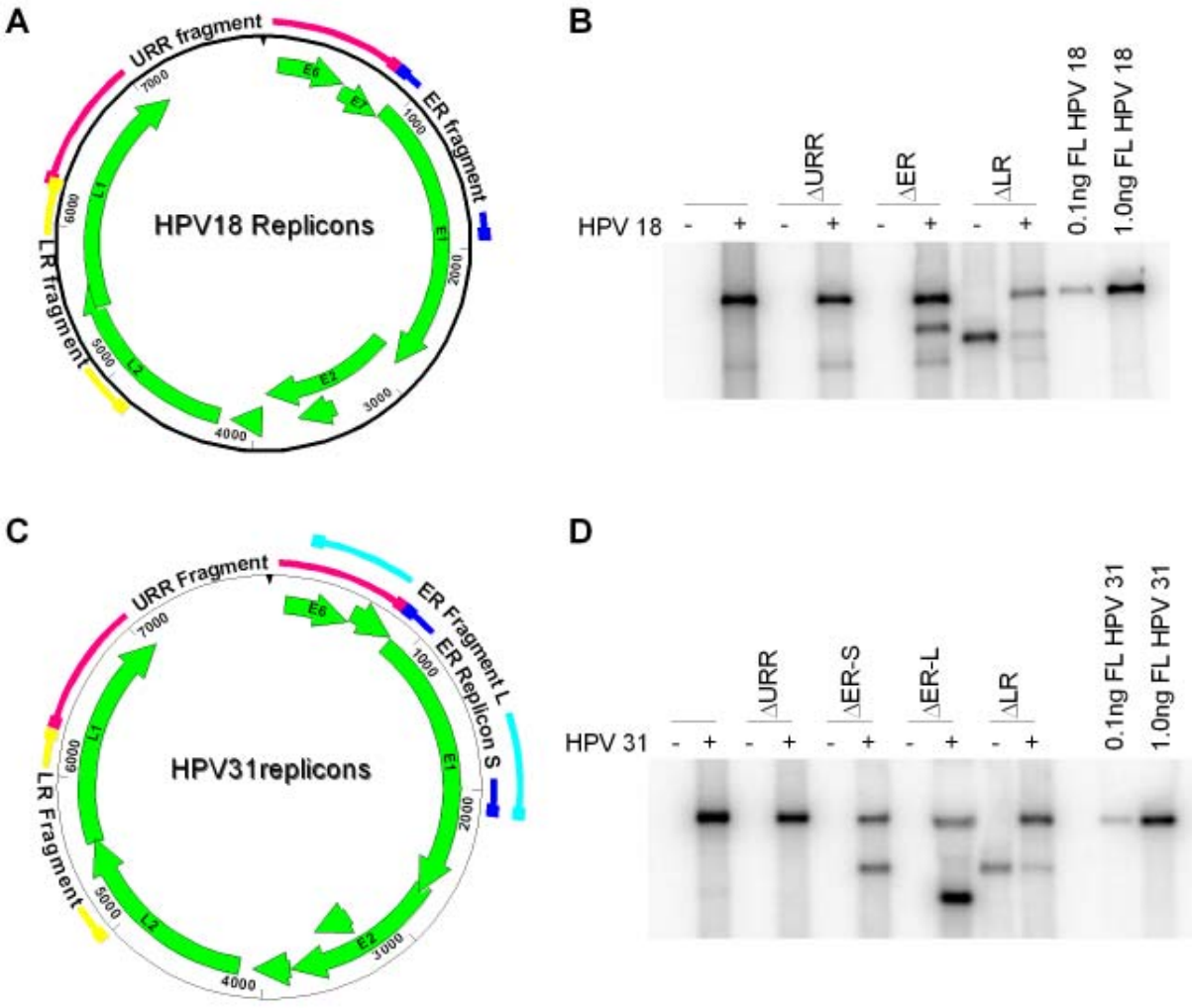
The cell lines transfected with the multiple E2 binding site mutant HPV18 genomes are shown in Figure 5-4C. There are three samples, from the cell lines E2BS1/2:5b, E2BS3/4:1a, and E2BS2/3/4:4a, that have bands in the digested DNA samples migrating at the same size of the linearized plasmid copy controls. But, once again, there is no evidence of supercoiled DNA in the uncut samples. Also there is a smear of viral DNA that migrates at the same position as the cellular DNA when comparing the Southern to the ethidium bromide stained gels (Figure 5-4D). Collectively these results suggest that the cell lines transfected with any of these combinations of E2 binding site mutations, are not efficiently maintained extrachromasomally.

Establishment of a complementation system to elucidate the role of the E2 binding sites on viral DNA replication

The results of the immortalization assays can only tell us whether or not the mutated cis-elements are essential for E6 and E7 expression driven by the early promoter. It is very difficult to identify the role of the E2 binding sites with regards to a specific process of the viral life cycle, such as replication, because the viral E2 protein is multifunctional and involved in both transcription and replication. Because the E2 protein regulates transcription from the early promoter, it also controls its own production as well as the expression of the other early genes. Changes in the level of E2 would affect its role in replication as well as the role of E1. This was the exact problem encountered in the studies by Stubenrauch *et al.* (1998) (60). In order to study the role of the E2 binding sites specifically with regard to replication, it is important to ensure that expression of the E2 protein is not affected by mutations in the binding sites that would affect early promoter transcription. For this purpose, we developed a complementation assay that would allow us to study the effect of mutations in the E2 binding sites on replication of a replicon, while ensuring wild-type expression of E2 in trans by co-transfection of a wild-type genome. The replicon is a truncated version of the wild-type genome with large deletions of selected regions. By deleting large fragments we are able to distinguish replication of the replicon from the wild-type genome by difference in the electrophoretic mobility of the linearized products. To test the theory and execution of the complementation assay, we began by generating three different control replicons by deleting large regions of the HPV18 and 31 genomes as shown in Figure 5-5A and C. The ΔURR replicons, 18ΔURR or 31ΔURR, have a deletion spanning the entire URR from nucleotides 6271-815 in HPV31 and 6233-823 in HPV18. The LR replicons, 18ΔLR and 31ΔLR, contain a deletion disrupting both late proteins L1 and L2, from nucleotides 4999-6270 in HPV31 and 4795-6273 in HPV18. The deletion in the HPV18 ER replicon, 18ΔER, starts in the E7 open reading frame at nucleotide 824 and extends to 1942,

Figure 5-5. Southern blot analysis of complementation

A&C. Illustrations of the position of deleted fragments in theHPV18 (A) and HPV31 (C) replicons. B&D. Southern blot analysis of HIRT DNA extracted from HFKs either transfected with the replicons indicated (or control plasmid as a negative control) alone or co-transfected with the replicons indicated and the full-length wild-type HPV genomes 10 days after electroporation. Samples were digested with an enzyme to linearize the replicons and the full-length HPV DNA. HPV18 complementation assay is represented in B and HPV31 complementation assay is represented in D. 0.1ng and 1.0ng genomic DNA was linearized and run as a size marker to the right of the gels.


A
URR fragment
ER fragment
LR fragment
HPV18 Replicons
E6
E7
E1
E2
L1
L2
7000
1000
2000
3000
4000
5000
6000
B
HPV 18
- + - + - + - + 0.1ng FL HPV 18 1.0ng FL HPV 18
ΔURR
ΔER
ΔLR
C
URR Fragment
ER Fragment L
ER Replicon S
LR Fragment
HPV31replicons
D
HPV 31
- + - + - + - + 0.1ng FL HPV 31 1.0ng FL HPV 31
ΔURR
ΔER-S
ΔER-L
ΔLR

eliminating most of the E1 open reading frame. Two deletion plasmids were designed for the HPV31 ER region; a large deletion, 31ΔER-L, starts in the E6 open reading frame at 218 and continues through most of the E1 reading frame to nucleotide 2143, and a smaller deletion fragment, 31ΔER-S, that mimics the 18ΔER and omits nucleotide 218-2143. The 31ΔER-L was designed to eliminate both the E6 and E7 genes entirely while the 31ΔER-S was included to mimic the 18ΔER replicon.

Since the URR contains the origin of replication, this region is essential for replication and the replicons with a deletion in this region, 18ΔURR and 31ΔURR, act as a negative control because without the origin of replication they should not be able to replicate under any condition. The ΔER replicons that contain the URR, but have a deletion in the early region abrogating E1 expression, are only able to replicate in the presence of the wild-type genome, which provides the replication proteins in trans. The ΔLR replicons act as a positive control because the deleted region in these replicons does not code for any essential replication proteins and therefore should be able to replicate on their own whether or not they are co-transfected with a full-length wild-type genome.

Efficient complementation of a replicon with a deletion in the ER by the wild type genome

To test the design of the complementation assay, HFKs were transfected with subgenomic replicons in the presence or absence of a full-length wild-type HPV genome to complement the replicons. DNA samples collected from cells transfected with each replicon, with or without a full-length WT genome, were digested with DpnI and a linearizing enzyme EcoRI and HindIII for HPV18 and HPV31, respectively. DpnI was used to digest input DNA to small fragments leaving intact only newly replicated DNA. At ten days post-transfection, we could clearly identify replicated products. As seen in Figure 5-5B and D, we did not detect

replication of the ΔURR replicon in the presence or absence of the full-length wild-type genomes in either HPV18 or 31, as expected. The ER encodes the essential replication proteins E1 and E2. Therefore, this replicon was not able to replicate by itself because it does not produce the essential replication proteins. However, co-transfection with a wild-type genome provided the essential replication proteins in trans and allowed the replicon with a deletion in the ER, ΔER, to replicate only when in the presence of the full-length wild-type genome. Both 31ΔER replicons were able to successfully replicate in the co-transfection samples. However, it appears that the 31ΔER-L replicon replicated at higher levels. As expected the replicon ΔLR is able to replicate on its own. However, interestingly, this replicon replicates poorly in the presence of the wild-type genome. This unexpected result is intriguing and determining the possible cause of this result could be explored in follow-up experiments. It seems that there are higher levels of replication of the wild-type, full-length genome than the replicon and this might suggest that there are elements contained in the L1 and L2 region that enhance replication. Since the wild-type genome has these elements intact, it could be out-competing the ΔLR replicon for replication or maintenance factors.

Complementation assay allows the analysis of the role of the E2 binding sites in replication

Based on the successful complementation of the ΔER replicons shown in the previous experiment, we could use this assay to test transient replication of replicons with mutations in the E2 binding sites. To generate ΔER replicons with mutated E2 binding sites, we subjected the genomes with mutations in the E2 binding sites to the same digestions as those used to generate the wild type ΔER replicon. We used the same mutated E2 binding site genomes used in the immortalization assays. We renamed these mutant plasmids to distinguish the full-length E2 binding site mutation constructs from the 18ΔER replicons with mutations in the E2 binding sites.

The 18ΔER E2 binding site mutated replicons were named with an "X" followed by the number of the E2 binding site in which the mutation resides. For example, the 18ΔER replicon with a mutation in the most proximal E2 binding site was labeled X1.

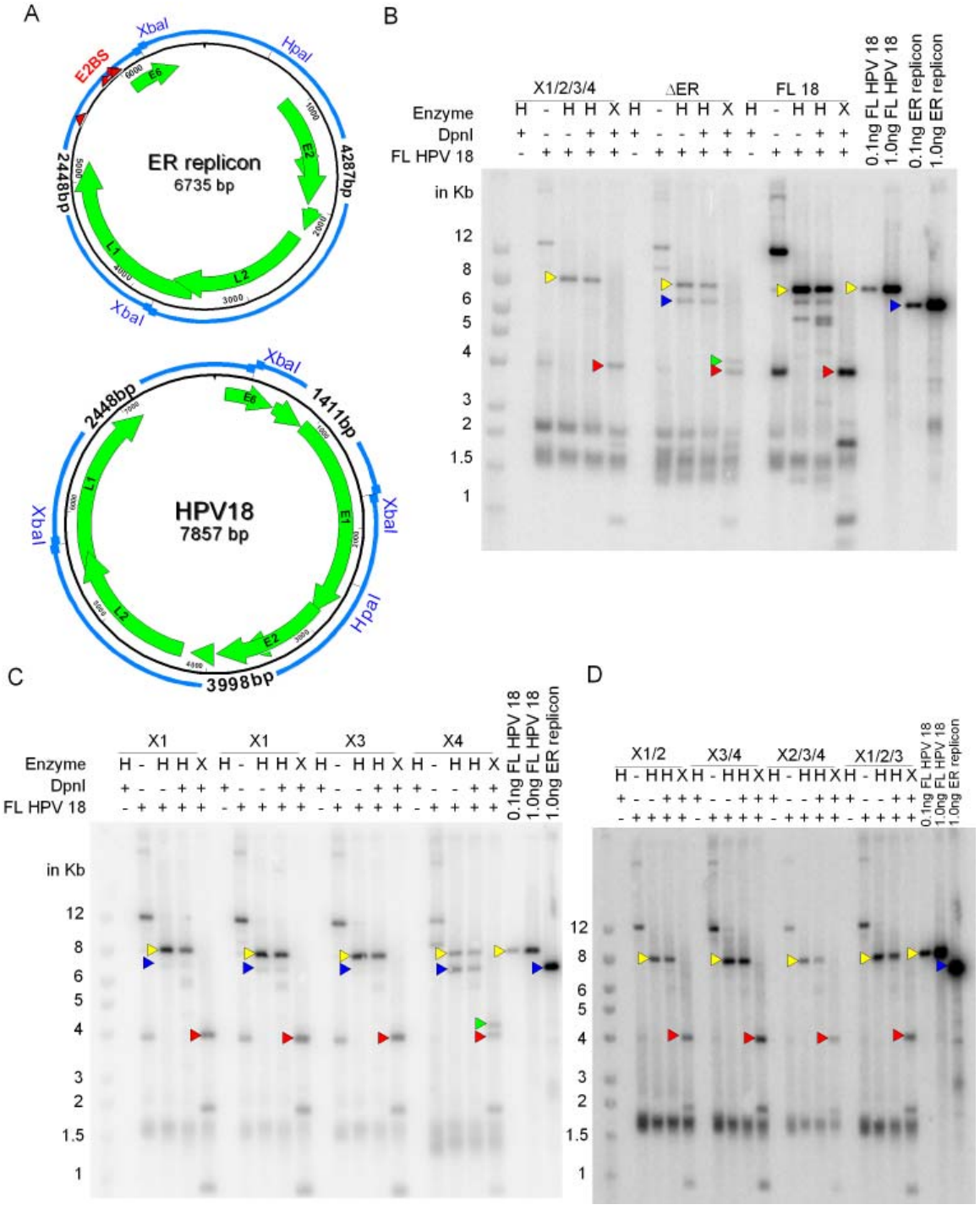
Low-molecular weight DNA was extracted from the transfected cells seven days post-transfection. This time multiple digestions were performed to better distinguish the full-length wild-type genomic replication and that of the replicon. A diagram of the restriction map of the enzymes used for both the replicon and the full-length HPV DNA is shown in Figure 5-6A. HpaI has one digestion site in the E1 open reading frame, outside of the ER deletion fragment, and therefore linearizes both the full-length genome and the 18ΔERreplicon. XbaI has three digestion sites in the full length HPV18 genome, but only 2 in the 18ΔER replicon because one of the sites is contained within the deleted fragment. Therefore, digestion with this enzyme can help to further distinguish the full length genomic DNA from the replicons.

The linearized wild-type replicon, with no mutations in any of the E2 binding sites, is approximately 6.7kb in size and is included as a positive control for the complementation assay in Figure 5-6B. We can identify replication of the replicon in this sample by the presence of the 6.7kb band in the HpaI digested samples (indicated by the blue arrow in Figure 5-6B) and by the presence of the 4.3kb band (indicated by the green arrow in Figure 5-6B). In the samples on the right that were transfected with full length HPV18 alone, we don't see bands of these sizes. Also shown in this blot are samples from HFKs co-transfected with the full-length HPV18 only as a control and the 18ΔER replicon with all E2 binding sites mutated. No bands are present at the sizes represented by the 18ΔER replicon with all E2 binding sites mutated, and therefore it appears that at least some of the E2 binding sites are necessary for transient replication.

To identify precisely which binding sites are essential for replication, we conducted the complementation assay using the 18ΔER replicon backbone with mutations in each individual E2 binding site, as can be seen in Figure 5-6C. Preliminary results from this assay suggest that only

Figure 5-6. Replication of the E2 binding site mutant replicons

A. Schematic of the restriction sites in both the ΔER replicons as well as the full-length wild-type HPV18 genome. Sites for the single HpaI site and the multiple XbaI sites are shown. In addition, the lengths of the fragments resulting from the XbaI digestions are shown. B-D. Southern blot analysis of digested HIRT DNA extracted from co-transfected HFKs, seven days post-transfection. DNA samples were either undigested or digested with HpaI (H) or XbaI (X) and DpnI as indicated. Yellow arrows= linearized HPV18 genome, Blue arrows= linearized ΔER Replicons, Green arrows= 4,287bp XbaI digest fragment from ΔER Replicons, Red arrows= 3,998bp XbaI digest from HPV18 genomes.


A
XbaI
HpaI
E2BS
E6
ER replicon
6735 bp
4287bp
2448bp
E7
E2
L1
L2
XbaI
XbaI
2448bp
1411bp
HPV18
7857 bp
XbaI
HpaI
3998bp
B
X1/2/3/4
ΔER
FL 18
Enzyme
DpnI
FL HPV 18
H - H H X H - H H X H - H H X
+ - - + + + - - + + + - - + +
- + + + + - + + + + - + + + +
0.1ng FL HPV 18
1.0ng FL HPV 18
0.1ng ER replicon
1.0ng ER replicon
in Kb
12
8
6
5
4
3
2
1.5
1
C
X1
X1
X3
X4
Enzyme
DpnI
FL HPV 18
H - H H X H - H H X H - H H X H - H H X
+ - - + + + - - + + + - - + + + - - + +
- + + + + - + + + + - + + + + - + + + +
0.1ng FL HPV 18
1.0ng FL HPV 18
1.0ng ER replicon
in Kb
12
8
6
5
4
3
2
1.5
1
D
X1/2
X3/4
X2/3/4
X1/2/3
H - H H X H - H H X H - H H X H - H H X
+ - - + + + - - + + + - - + + + - - + +
- + + + + - + + + + - + + + + - + + + +
0.1ng FL HPV 18
1.0ng FL HPV 18
1.0ng ER replicon
12
8
6
5
4
3
2
1.5
1

the replicon which has a mutation in the most distal E2 binding site, X4, is able to replicate efficiently as demonstrated by a strong band at the position of the linearized replicon, as well as a strong signal at 4.3kb in the XbaI digested sample. There is a very faint band that can be seen at approximately 6.7kb in the linearized samples for all three of the other E2 binding site mutant-transfected cells. However, the 4.3kb band in the XbaI digested samples is barely visible. It is possible that these replicons are able to replicate at very low levels much less efficiently than the wild-type replicon and that the bands seen represent a low level of replication in these cell lines. It is possible that the remaining E2 binding sites are able to compensate weakly for the loss of the individual sites.

To test if mutations in multiple E2 binding sites are able to replicate in this system, we co-transfected replicons with mutations in multiple E2 binding sites with the full-length HPV18 genome (5-6D). We could not detect bands representative of the 18ΔER replicon in any of the combination constructs. Therefore, it appears that efficient replication requires the three most proximal binding sites. The two E2 binding sites that are most proximal to the promoter are not sufficient to support efficient replication, because the replicon with mutations in the two most distal binding sites, X3/4, does not demonstrate efficient transient replication. Referring back to the schematic of the HPV18 URR in Figure 5-2, all three proximal E2 binding sites are located within the origin of replication and were previously found to be essential for transient replication of HPV18 using an alternative replication assay (63).

Discussion

In this chapter we examined the role of the E2 binding sites on transcription and replication. We found that genomes with mutations in any of the E2 binding sites were still able to efficiently immortalize transfected HFKs. The one exception was the genome that had mutations in all 4 E2 binding sites. However, Southern blot analysis revealed that the viral genomes were maintained at very low levels and were most likely integrated into the host genome. The complementation assay designed in this study enabled the direct analysis of the E2 binding sites on replication without the complications that previous studies addressing this issue have encountered. Using this assay we found that three of the four binding sites are essential for short-term replication of transfected replicons.

The immortalization assays performed in these studies were meant to validate the findings of previous transcription studies using a more physiologically relevant system. Initial studies to identify important regulatory elements often used simplified systems that altered the context of the promoter in the viral genome by isolating the URR from the entire genome and sometimes even placing the regulatory elements in front of a heterologous promoter. While these assays were very valuable in terms of providing insight about what factors might be involved, the results of these studies must be verified in the natural context of the entire genome.

In a study by Soeda *et al*. (2006), a chimeric extrachromosomal vector containing the entire HPV16 URR, early genes and independent replication machinery were transfected into primary keratinocytes (161). The design of the chimeric plasmid was intended to enable the analysis of the effects of the regulatory elements without affecting the replication stability of the plasmid. The authors examined the effect of mutations in the E2 binding sites and confirmed that the E2 binding sites #1 and #2 were both important for E2-mediated transcriptional repression. These results are in agreement with previous transcriptional analyses. However, unlike previous

work, they did not find a significant contribution to transcriptional regulation by the E2 binding site #4. This study effectively addressed many of the limitations of the previous studies by using a physiologically relevant system. However, this study only followed the activity of the promoter two days post-transfection, and therefore could only identify the short-term effects of the E2 binding sites on transcription. This study did not evaluate the long-term effect of the E2 binding sites with regard to immortalization. Moreover, when the authors examined the effect of the E2 binding sites using only the endogenous E2 generated from the chimeric plasmid, they noticed a very mild effect of the mutations on transcription from the early promoter. Since any effect on the transcription levels of the early promoter also affects the expression levels of E2, this could have been minimizing the effects of the mutations on transcription.

Our immortalization assay has the same complications encountered in the study by Soeda *et al*. (2006) and the study by Stubenrauch *et al*. (1998). The immortalization assays presented in this chapter were designed to use conditions that best represent a natural infection. The ability of all the HPV genomes with mutations in the E2 binding sites to immortalize the transfected HFKs shows us that each of the individual sites is not essential in driving transcription in a system that is the best mimic of a natural infection to date. However, examining mutations in the complete genome during the entire course of infection generates results that are difficult to interpret. Because the E2 binding sites are involved in transcriptional control of the early promoter, mutations in the E2 binding sites, would in turn affect the levels of E2 available for regulation. Since E2 can both activate and repress transcription depending on a number of factors, including the position of the binding site and the levels of E2 present, expression of E1 and E2 may either increase or decrease depending on these factors. It would be important to measure the expression levels of these proteins throughout the growth of the transfected HFKs to have a better idea of the precise effects of these mutations and how they have affected transcription. This would help us

understand whether the results we observe are due to the transcriptional control by the E2 binding sites, or an effect on viral replication.

Our findings suggest that while none of the E2 binding sites are essential for immortalization, they all significantly affect the extrachromosomal state of the viral genome. There was evidence that most of the mutated viral genomes had integrated into the cellular DNA. Integration of HPV31 genomes with mutations in the E2 binding sites was also observed in stable replication assays performed in primary keratinocytes by Stubenrauch *et al*. (1998). The authors reported finding integration in three of the four E2 binding site mutant genomes with the exception of the binding site at position #3 (they referred to this site as #2 because they numbered the sites in a reverse order).

Integration is not a natural part of the viral life cycle, and is not expected with transfection of wild-type high risk HPV genomes. Normally the expression levels of E6 and E7 from episomally maintained genomes is sufficient to immortalize transfected cells, as we see in the wild-type controls. Therefore, it seems that the integrated genomes found in the cell lines with mutated E2 binding sites were a consequence of abnormal regulation of E6 and E7 in these cells. If the expression levels of E6 and E7 were too low in these cells, then cells with integrated genomes may have a growth advantage. If the viral genomes have integrated in a manner similar to the integrated genomes found in cervical cancer lesions, and disrupt the E2 open reading frame, then regulation by the E2 binding sites is a mute point. These lines would have lost the regulation by the E2 binding sites. Additionally, it cannot be ruled out that the genomes have integrated in regions of the cellular chromosome where there may be cellular cis-acting sequences that affect the transcription of the viral early promoter (202).

Because this system does not allow for the distinction between the effects of the regulation by the E2 binding sites on transcription and replication, it is also possible that the loss of the E2 binding sites caused the viral genomes to integrate. If the viral genomes were not able

to replicate on their own due to the loss of these sites, then the only way they would survive is to integrate. However, in this situation, it seems that integration would be a random event and would not be seen in all cell lines. It seems likely that the loss of the E2 binding sites somehow drives integration. It is possible that the loss of the E2 repressional regulation led to an increase in E6/E7 expression that led to genomic instability and this, in turn, caused viral integration.

We designed the complementation assay in an attempt to examine the role of the E2 binding sites specifically on viral replication in the genomic backbone without affecting expression of the viral replication proteins. The finding that the three binding sites most proximal to the promoter were essential for transient replication is in agreement with the previously identified location of the origin of replication (63;70;233). However, these results are not in agreement with the results reported in Stubenrauch *et al*. (1998) who found that none of the E2 binding sites were essential for transient replication. While this study also used full-length genomes harboring mutations in the E2 binding sites, the authors had conducted their transient assays in the immortalized cell line SCC-13 cells. In addition, in the transient assay they used, the results are complicated by the transcriptional effects of the E2 binding sites since there was no exogenous expression of the viral replication proteins. Because we have avoided these problems in our complementation assay, we can have more confidence that our results represent the true effect of the E2 binding sites on replication.

Since we found that three of the four E2 binding sites were essential for transient replication, we can postulate that the low level of viral genomes present in the immortalized cells was due to their inability to replicate efficiently. The E2 binding site #4, however, was able to replicate efficiently in the transient complementation assay and we would expect that these genomes would behave more like the wild-type genomes in the immortalization assays. However, we found that these genomes were also maintained at very low levels. Therefore, it seems that these mutant genomes may not be maintained efficiently. This suggests that either this

site may have a role in the long-term maintenance of the viral genomes or impacts the transcription of the replication proteins E1 and its own production.

A study by Lace *et al.* (2008) also used a complementation assay to examine the role of E2 in replication (234). However, this study used a very different approach in the design of their complementation assay. In their assay, mutations in the E1 and E2 open reading frames were introduced into separate HPV16 genomes so that expression of E1 and E2 was controlled by only one of the two transfected viral genomes. The mutated genomes were therefore unable to replicate on their own but would be to replicate when co-transfected due to the expression of the replication proteins provided in trans by each of the genomes. The authors also used their complementation assay to study the effect of the E2 binding sites on replication and transcription. The design of their complementation assay allowed them to distinguish the effects of mutations in the E2 binding site on replication or on transcription. Their results confirmed that the E2 binding site most proximal to the promoter represses transcription, while the other three binding sites activate transcription of the early promoter as found in previous studies. As far as the role of the E2 binding sites with regards to replication, they also found that all three binding sites proximal to the early promoter were all required for efficient replication. These results are in agreement with the results of the role of the E2 binding sites with regards to replication that we found in our complementation assays. The different approach in the design of these assays helps to validate these findings using two different approaches. The complementation assay used in this study is very useful for examining the effect of cis-elements on both transcription and replication. However, unlike the complementation assay used in our analysis, the assay designed by Lace *et al.* (2008) cannot be used for long-term maintenance studies since the expression of either the essential replication proteins would be lost over time in a replication-deficient genome.

The replication products for the complementation assays were analyzed seven days post-transfection. This time point extends beyond the four days that is typically used to measured

transient replication (57;61;235;236). However, stable replication was found up to six days in Ustav and Stenlund, 1991 (56). Therefore, our analysis at day seven should be a good indication of whether or not transient replication with the replicons is possible.

The results with our complementation assay indicate that this system will be very useful to determine which elements are important for long-term genome maintenance. However, since we found that three of the four E2 binding site mutants are not capable of efficient transient replication, we would not be able to use these constructs for long-term replication to determine whether these sites are important for genome maintenance. To study which cis-elements are important for genome maintenance, new replicons must be designed using a different approach to see if elements exist that are not essential for transient replication but are essential for stable maintenance. Hubert *et al*. (1999) had examined the effects of mutations in functionally distinct regions of the HPV31 URR in transient and stable replication assays (62). The authors found that deletion of the 5' URR domain in fact led to an increase in transient and stable replication levels. These assays examined the effect of mutations in the URR in the background of the full-length genome, but did not provide any exogenous source of E1 and E2. Therefore, it is difficult to conclude from this study whether the observed effects of the mutations are directly affecting replication or are the indirect result of the loss of E1 and E2 due to altered transcriptional activity. Our complementation assay would enable us to make this distinction and to verify whether or not the 5' URR region upstream of the origin of replication is directly involved in modulating replication.

Chapter 6

Ap1, Sp1, and TATA box binding sites not essential for HPV-induced immortalization of primary HFKs

Introduction

The life cycle of HPV is tightly linked to the differentiation program of the epithelial host tissue. Presumably the dynamic expression of host regulatory genes responsible for the differentiation program of the host tissue is also responsible for the ordered sequence of events observed in the HPV life cycle. The expression of the early and late genes is controlled by independent, distinct promoters. There has been a great interest in understanding how the early promoter is regulated due to the fact that it is responsible for the expression of the oncogenes, E6 and E7.

A stretch of DNA, approximately 800 base pairs long, is located upstream from the first open reading frame and does not encode for any genes. This sequence contains many elements that control replication and transcription and is therefore called the Upstream Regulatory Region (URR). Previous studies using reporter assays and DNA footprinting analysis have identified elements, that when mutated, affect promoter activity. These assays identified a number of cellular transcription factor as playing important roles in the regulation of the papillomavirus URR, including Ap1, NF-1, Sp1, YY1, Oct1, and the TATA box protein (237-239).

Of these transcription factor binding sites, those that were determined to be critical for early promoter transcription are Sp1, Ap1 and the TATA box (68;70). Sp1 is a general cellular transcription factor that activates the early promoter based on reporter assays (239). The Sp1 binding site is situated directly upstream of the E2 binding site #2 (second-most proximal to the

promoter). Part of the complex regulation of the early promoter stems from the competition for binding to this site between Sp1, an activator of transcription, and E2, a transcriptional repressor that was reviewed in the previous chapter (229). Moreover, Sp1 has been shown to contribute to the epithelial-specific transcriptional regulation of the early promoter (240).

Similar to Sp1, Ap1 is also a transcriptional activator of the early promoter and is responsible for the cell-type specific control of HPV transcription. The Ap1 transcription factor is a dimer pair of Jun or Jun/Fos family members (241;242). The heterogeneity of the dimerization possibilities of this transcription factor is thought to play a role in the tight control of the viral life cycle when coupled to the host pattern of differentiation. In the lower undifferentiated layers of epithelial tissue cFos is predominantly expressed, as opposed to the upper layers where JunB expression is upregulated. Therefore, the Ap1 transcription factor switches from a cFos/JunD heterodimer pair to a JunB/JunD heterodimer upon differentiation (74;75).

Thierry *et al*. (1987) first identified p105 as the major promoter for the early transcripts of HPV18. Thierry and Howley (1991) mutated multiple cis-elements believed to affect transcription, including the TATA box at nucleotide p73, and analyzed the impact of these mutations using reporter assays. The first TATA box mutation, designed to resemble the TATA box found in the SV40 promoter, was included to determine whether an alternative TATA box could substitute for promoter activity. The design of the second TATA box mutation was intended to completely abolish activity by substituting guanines (G) and cytosines (C) for the thymines (T) and adenines (A) (243). Thierry and Howley (1991) observed an almost complete loss of promoter activity with each of the CAT expression plasmids containing both of the TATA box mutations. These results seemed to highlight the significance of the p105 promoter.

HPV18 has been used as a model in many of these studies and therefore the regulatory elements in the URR of this virus type have been well characterized using footprinting and

reporter assays (74;230;237;239;244). However, because these assays analyzed the role of the early promoter in an artificial context, it is important to verify whether these elements behave the same way in a model that more closely mimics a natural infection; one that uses a primary keratinocytes and the full-length genome. One such study that examined the effect of various elements in the HPV31 URR found that the Ap1 and Sp1 binding sites reduced the replication efficiency of the full viral genome in transfected HFKs (62). This was likely due to decreased expression of E1 and E2 that resulted from the loss of these transactivators of the early promoter. The HPV31 URR has four Ap1 binding sites whereas the HPV18 URR only has two. Analysis of the genomes present in the stably transfected cell lines was performed at passage three and the authors found that the genomes with mutations in each of the four Ap1 binding sites and the Ap1 binding site were integrated (62).

To determine the impact of these mutations on transcription in the context of the entire HPV18 genome, we tested the ability of HPV18 genomes mutated in the key regulatory elements of the major promoter to immortalize transfected primary HFKs. We found that cells transfected with HPV genomes mutated in all binding sites for Ap1, Sp1, and the TATA box were able to bypass senescence suggesting that the mutant genomes in these cells are still capable of driving E6 and E7 expression from the early promoter. Analysis of the mutated viral genomes present in the immortalized cell lines revealed that the integration status of the infected genomes varied. The few cell lines that did maintain episomal copies of the viral genome were able to produce infectious virus when grown in fully differentiated epithelial tissue suggesting that these mutated viral genomes were still capable of completing the entire HPV life cycle.

Results

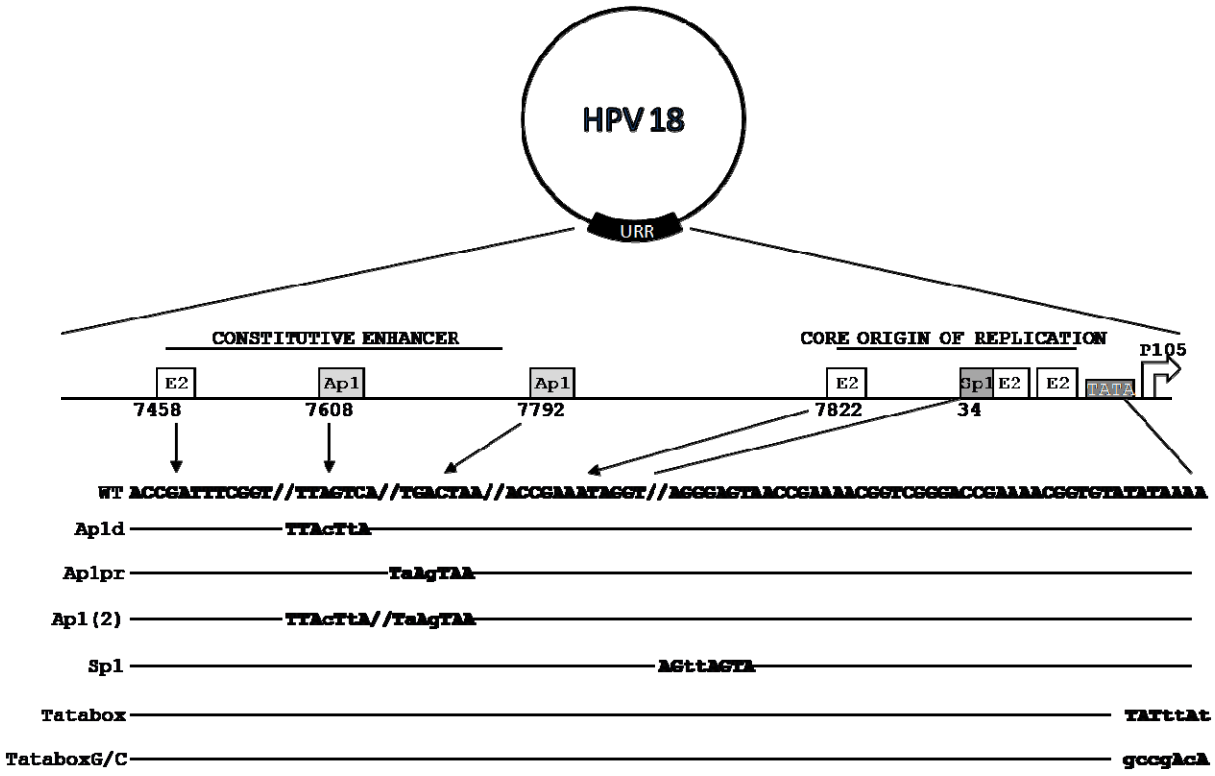
Mutations in cis-elements in the HPV18 URR

Reporter assays measuring the effect of mutations in various transcription factor binding sites on the activity of the early promoter in the HPV18 Upstream Regulatory Region (URR) had previously identified elements critical for regulation of transcription (74;230) (see Figure 6-1). In these studies, the entire URR was removed from the coding regions of the genome and placed upstream of the CAT reporter. Mutational analysis of transcription factor binding sites in these reporter assays suggested that the Ap1, Sp1, and the viral E2 binding sites play an essential role in regulating the activity of the early promoter (74;229;230). In the previous chapter, we further examined the role of the E2 binding sites in the full length HPV18 genome. In this chapter we looked at the role of the Ap1, Sp1 and TATA box cis elements in the full-length HPV18 genome. Using site-directed mutagenesis, we created the mutant HPV18 genomes that are shown in Figure 6-1 and which were modeled after the mutations used in reporter assays (74;229;230). Two different TATA box mutations were used by Thierry *et al*. (1991); a slightly altered TATA box that is identical to the SV40 TATA box (Tatabox) and a conversion which should completely ablate binding of the TATA box binding protein (TataboxG/C) (230) (see Figure 5-1). Genomic clones were sequenced to verify the presence of the mutations and at least three independent plasmids with the correct mutations were selected for analysis.

HPV genomes with mutations in critical regulatory binding sites are still able to immortalize primary keratinocytes.

To test the significance of the selected transcription factor binding sites on early promoter activity in the context of the full genome, we electroporated cells with the mutated full-length

Figure 6-1. Point mutations in Ap1, Sp1, and TATA box binding sites in the HPV18 URR Mutations were modeled after those used by Thierry *et al*. (1992) and Demeret, Yaniv, and Thierry (1994) and Thierry and Howley (1991) were introduced into the full-length HPV18 genome cloned into the EcoRI site of the pBR vector (74;229;230). The location of each binding site is shown in the schematic at the top of the figure. The wild-type sequence of the binding sites is shown below the schematic. The mutated residues in the plasmids listed on the left are indicated by lower case letters.


HPV 18
URR
CONSTITUTIVE ENHANCER
CORE ORIGIN OF REPLICATION
P105
E2
Ap1
Ap1
E2
Sp1
E2
E2
TATA
7458
7608
7792
7822
34
WT ACCGATTTCGGT//TTAGTCA//TGACTAA//ACCGAAATAGGT//AGGGAGTAACCGAAAACGGTCGGGACCGAAAACGGTGTATATAAAA
Ap1d TTAcTTA
Ap1pr TaAgTAA
Ap1(2) TTAcTTA//TaAgTAA
Sp1 AGttAGTA
Tatabox TATttAt
TataboxG/C gccgAcA

linearized genomes to determine whether or not they could immortalize the cells. For these studies we used normal, primary human foreskin keratinocytes, which normally have a limited lifespan and will slow in their growth in culture and eventually senesce. Only cells that express the viral oncogenes, E6 and E7, will bypass senescence and continue to divide indefinitely (44;139). Therefore, the ability of the primary HFKs to bypass senescence is a measure of early promoter activity driving E6 and E7 expression. Furthermore, the use of primary HFKs allows us to study the mutated viral genomes in a cell type which closely models the natural host for HPV infections. Immortalization was defined as the ability to outgrow the negative control HFKs which were electroporated with only carrier DNA. To account for the possibility that different batches of HFKs may possess different features, we created more than one cell line from different batches of HFKs for each mutant. Genomes with each cis element mutation were electroporated into HFKs on at least three separate occasions using different batches of HFKs. The resulting cell lines were named according to the mutated genome used for the transfection (i.e. Ap1d:3, Ap1d:5, Ap1pr:2, Ap1pr:4, etc.) followed by a letter, indicating a unique cell line, since the electroporations were repeated to generate multiple lines from each construct (i.e. Ap1d:3b, Ap1d:3c, Sp1:3c, Sp1:3d, etc.). The results of immortalization are summarized in Table 6.1.

Table 6-1. Immortalization assays results of Ap1, Sp1, TATA box binding site mutations

Mutations	# of transfections	# of immortalized cell lines
HPV18	5	5
No HPV	14	0
Ap1d	6	4
Ap1pr	3	3
Ap1d/pr	3	1
Sp1	3	3
TATA box	4	4
TATA box G/C	4	4

All HFKs transfected with mutations in the proximal Ap1 binding site alone and in the Sp1 site became immortalized. However, the genomes that had mutations in the Ap1 site distal to the promoter, including a construct that had mutations in both the proximal and distal Ap1 site were not 100% effective in immortalizing the infected HFKs. Interestingly, all of the HFKs transfected with genomes containing the two types of TATA box mutations were able to immortalize the cells very efficiently. Of the cell lines included in these results, a few were selected for sequencing of the viral genome. Primers designed for the URR around the cis elements were used to amplify the URR of the HPV DNA present in the total DNA extracts of the immortalized cell lines. In all samples tested and used in this report, the mutations were still intact in the infected cell lines (Table 6.2).

DNA migration patterns of genomes mutated in the Ap1 and Sp1 binding sites in immortalized HFKs

The immortalization assays revealed that the Sp1 and the proximal Ap1 binding site are not essential for early promoter activity. However, we wanted to see if other viral functions were affected by the loss of these sites. Another important feature of the HPV life cycle is the episomal integrity of the viral genome (119). To test whether the transfected genomes in the immortalized cell lines are extrachromosomal elements or whether they have integrated into the host genome, we analyzed the migration patterns of the mutant genomes on Southern blots. Genomes that are maintained episomally migrate as supercoiled and nicked bands when undigested total DNA samples are separated by agarose gel electrophoresis and as a linear band when digested with an enzyme that cuts once in the HPV genome, as seen in the HFKs transfected with the wild-type HPV18 genome (Figure 5-2). This method also allows us to measure the viral copy number per cell by comparing the relative levels of the linear genomes in

each sample with the prepared copy controls calculated to represent 1, 10, or 100 copies per cell. Thus, we performed Southern blot analysis on the total extracted DNA from each of the cell lines grown in monolayer culture. An example of the Sp1 cell lines transfected with the mutated genomes is shown in Figure 6-2A. The Sp1:3 construct was used to generate three independent cell lines Sp1:3a, Sp1:3c, and Sp1:3d. All of these cell lines display a different genomic profile. In the Sp1:3a cell line, it appears that the viral genome is integrated because we do not see a linearized episomal band in the digested sample and we do not see any evidence of a supercoiled band in the undigested sample. In contrast, the genomes in both the Sp1:3c and the Sp1:3d lines appear to be maintained episomally, however at different levels, approximately 5 and 10 copies per cell, respectively. These lines were sequenced and the mutations were confirmed in all of these cell lines.

Examples of the Ap1 cell lines are shown in Figures 6-2B and C. The state of the viral genomes in these cells seems similar to what we saw in the cell lines with the E2 binding site mutations in the previous chapter. These mutated viral genomes are almost undetectable in the Southern blots indicating that they are maintained at very low copy numbers. In the few samples in which we can see faint bands, it appears that the bands migrate at a high molecular weight. These results suggest that these mutant genomes are integrated into the cellular DNA. This data suggests that the Ap1 binding sites are essential for episomal maintenance of the transfected genomes. A summary for the results of all the Sp1 and Ap1 cell lines can be found in Table 6.2. Amplification of these genomes was not apparent when comparing the extracted raft DNA to the monolayer DNA.

Figure 6-2. Replication of HPV genomes with mutations in the Sp1 and Ap1 binding sites Southern (DNA) blot hybridization of DNA extracted from HFKs transfected with HPV18 genomes with mutations in the Sp1 binding sites (A) and the Ap1 binding sites (B&C) cultured as monolayers or an organotypic raft. “Uncut” lanes contain 5 µg of undigested total cell DNA. Cut lanes contain 5µg of total cellular DNA digested with EcoRI. HPV18 100, 10, and 1 copy number standards are shown on the right. Samples were run on the same gel but the lanes were noncontiguous, as indicated by the break in the image.

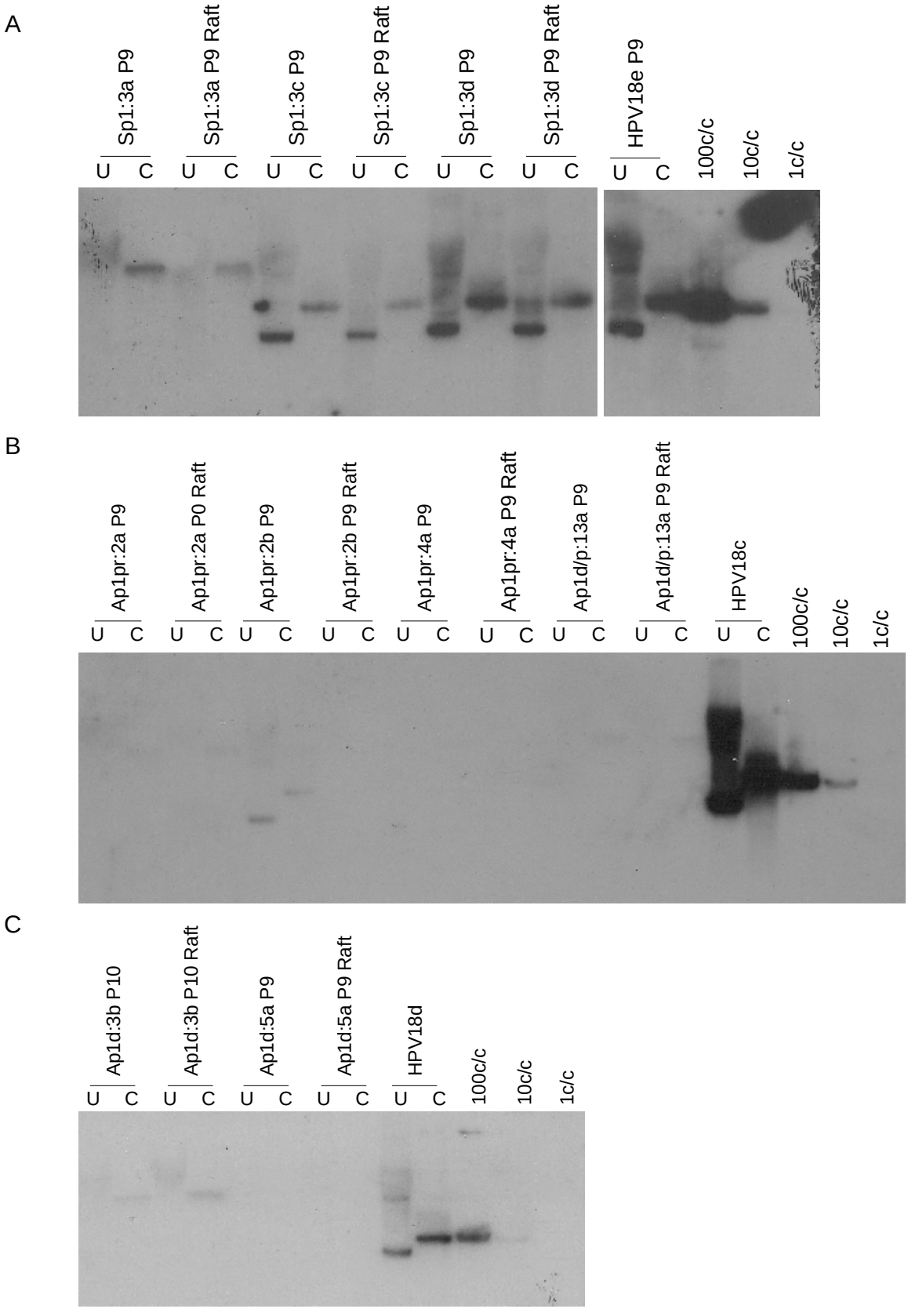

A
U
C
Sp1:3a P9
Sp1:3a P9 Raft
Sp1:3c P9
Sp1:3c P9 Raft
Sp1:3d P9
Sp1:3d P9 Raft
HPV18e P9
100c/c
10c/c
1c/c
B
Ap1pr:2a P9
Ap1pr:2a P0 Raft
Ap1pr:2b P9
Ap1pr:2b P9 Raft
Ap1pr:4a P9
Ap1pr:4a P9 Raft
Ap1d/p:13a P9
Ap1d/p:13a P9 Raft
HPV18c
100c/c
10c/c
1c/c
C
Ap1d:3b P10
Ap1d:3b P10 Raft
Ap1d:5a P9
Ap1d:5a P9 Raft
HPV18d
100c/c
10c/c
1c/c

Table 6-2. Southern blot analysis of transfected mutant genomes

NT=Not tested

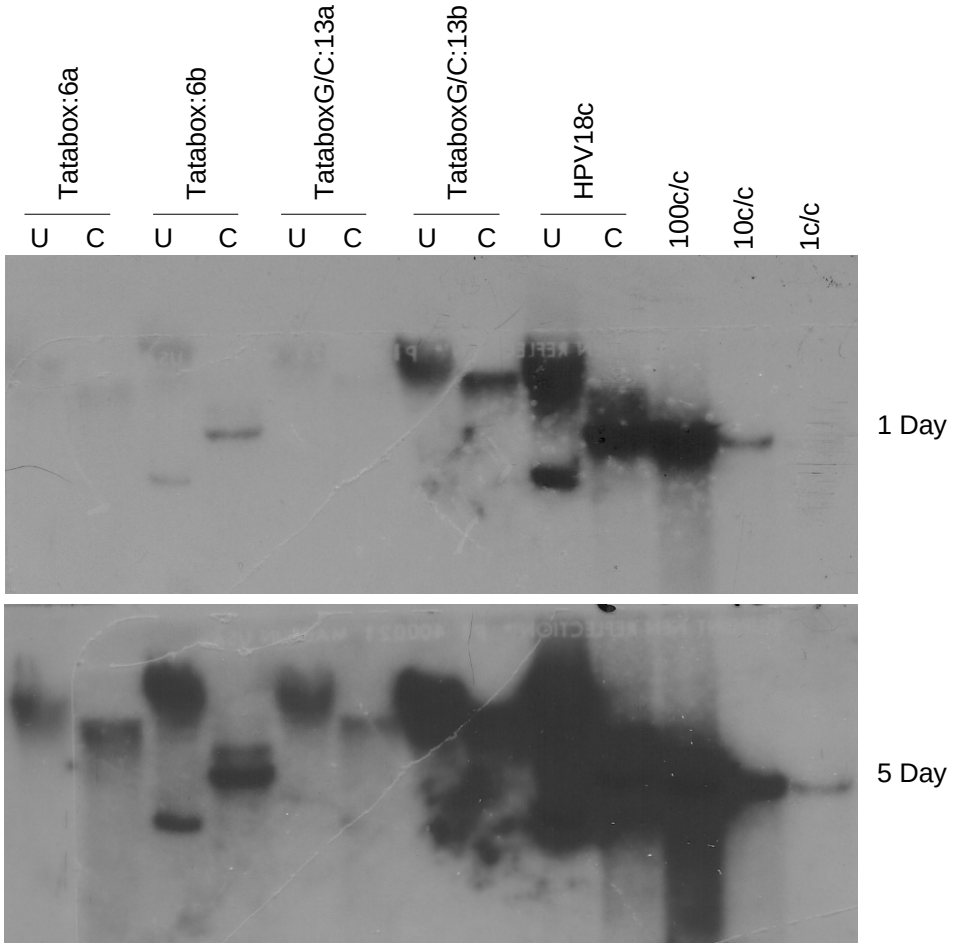
	Sequence	copy #	episomal band	supercoiled band
HPV18b	wild-type	>1	+	+
HPV18c	NT	>100	+	+
HPV18d	NT	50-100	+	+
HPC18e	NT	25	+	+
Ap1d:3b	mutant	1	-	-
Ap1d:5a	NT	undetectable	unknown	unknown
Ap1pr:2a	mutant	undetectable	unknown	unknown
Ap1pr:2b	mutant	1	+	+
Ap1pr:4a	mutant	undetectable	unknown	unknown
Ap1d/p:13a	mutant	1	-	-
Sp1:3a	mutant	5	-	-
Sp1:3c	mutant	5	+	+
Sp1:3d	mutant	10	+	+

The HPV genomes in the cell lines transfected with the TATA box mutations are also expressed at low levels and possess characteristics of integrated lines.

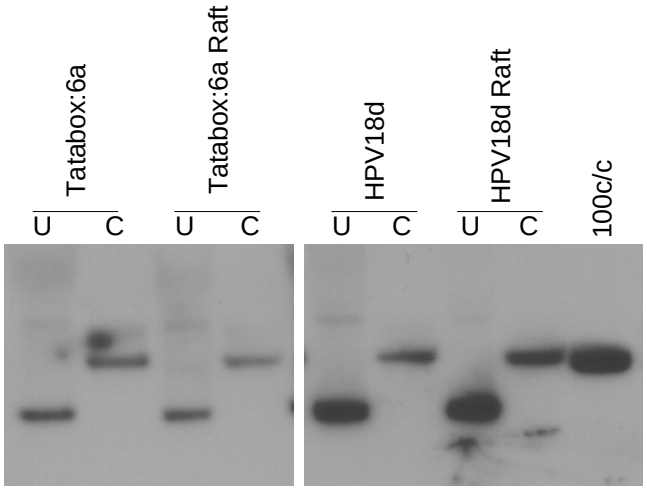
Perhaps the most interesting finding from the immortalization experiment was the ability of the constructs with mutations in the TATA box to immortalize the cells. To further characterize the TATA box mutant cell lines, Southern blot analysis was also performed on the DNA isolated from these cell lines to verify the state of the viral genomes and to establish the copy numbers maintained in each cell line. As can be seen in Figure 6-3A, each of the mutated genomes in the Tatabox cell lines replicate the viral DNA to a different degree and with different profiles. The linearized genomes from the Tatabox:6a,TataboxG/C:13a, and TataboxG/C:13b cell lines all produce high-molecular-weight hybridization signals. The absence of a band migrating at the supercoiled position in the undigested samples suggests that these genomes are integrated. In contrast, the linearized genome from the Tatabox:6b cell line appears to be

Figure 6-3. Replication of HPV genomes with mutations in the TATA box binding sites (A)Southern (DNA) blot hybridization of DNA extracted from monolayer cultures of wild-type or mutant HPV transfected HFKs. “Uncut” lanes contain 5 µg of total cell undigested DNA. “Cut” lanes contain 5µg of total cellular DNA digested with EcoRI. HPV18 100, 10, and 1 copy number standards are shown on the right. The top image is a film exposed overnight and the bottom of a film exposed for 5 days. (B) Southern (DNA) blot hybridization of DNA extracted from HPV transfected HFKs cultures as a monolayer or an organotypic raft. “Uncut” lanes contain 5 µg of total cell undigested DNA. “Cut” lanes contain 5µg of total cellular DNA digested with EcoRI. HPV18 100, 10, and 1 copy number standards are shown on the right.

A


Tatabox:6a
Tatabox:6b
TataboxG/C:13a
TataboxG/C:13b
HPV18c
U
C
100c/c
10c/c
1c/c
1 Day
5 Day

B


Tatabox:6a
Tatabox:6a Raft
HPV18d
HPV18d Raft
U
C
100c/c

migrating at approximately 8kb and at the same position as the wild-type HPV18c control. Moreover, in the uncut samples there is evidence of a band likely to be the supercoiled episomal genomic DNA and that also migrates at the same position in the gel as the supercoiled DNA present in the wild-type cell lines. These data suggests that the viral genome Tatabox:6b is maintained episomally. The copy number of genomes present in each line appears to vary among the cell lines. However, all of the mutant cell lines contain significantly fewer copies of the genome than the cell line infected with wild-type HPV18. The results for all the Tatabox cell lines are summarized in Table 6.3.

Table 6-3. Southern blot analysis of transfected mutant genomes

	Sequence	copy #	episomal band	supercoiled band
Tatabox:6a	mutant	10	wrong size	-
Tatabox:6b	mutant	20	+	+
Tatabox:14a	mutant	1	+	-
Tatabox:14b	mutant	1	wrong size	-
Tatabox G/C:13a	mutant	Undetectable	wrong size	-
Tatabox G/C:13b	mutant	10	wrong size	-
Tatabox G/C:15a	mutant	<1	-	-
Tatabox G/C:15b	mutant	1	+	-

Viral replication does not appear to amplify in mutant or wild-type upon differentiation in rafts

To determine whether the Tatabox:6b cell line that appears to contain episomally maintained genomes could complete the viral cycle, the cell lines were also seeded on organotypic rafts and grown for ten days to test for amplification. Total cell DNA was extracted from the raft tissue and analyzed by Southern blotting to determine whether the HPV genome is amplified upon differentiation (Figure 6-3B). We saw no evidence of amplification in the DNA extracted from the raft tissue when compared to the monolayer cells. However, we also did not

observe significant amplification in the DNA extracted from the raft tissue grown from wild-type HPV-transfected cell lines. Therefore this does not necessarily indicate that these genomes are defective in this function of the viral life cycle.

Histochemical analysis of Raft Tissue

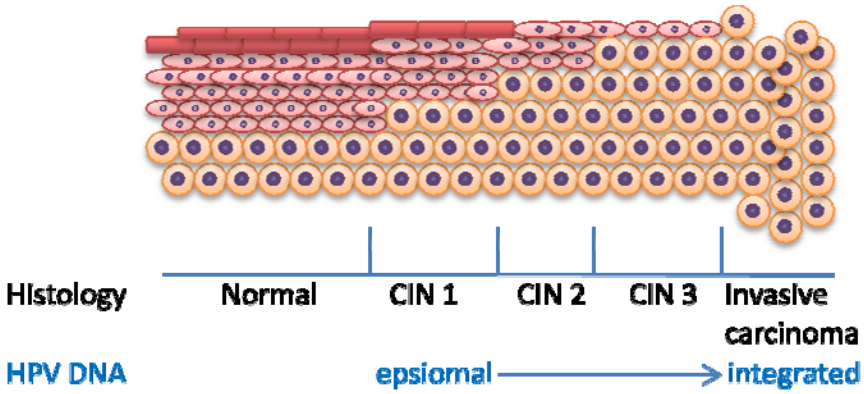
Cells with persistent papillomavirus infections are capable of maintaining episomal genomes for years in the host basal cells. The existence of stably replicating extrachromosomal genomes has been shown in both cell lines isolated from clinical lesions (122;124) as well as from cell lines established in the laboratory by transfection with HPV genomes (119;208;217). However, we know that the high-risk HPV genomes are susceptible to integration into the host chromosome over time and this step is a major hallmark of malignant progression of HPV infections. HPV genomes defective in driving expression of the early promoter produce insufficient amounts of E1 and E2 and do not have the necessary factors to replicate independently. As a result, these genomes may be more prone to integrate into the host chromosome, which would allow them to replicate along with the cellular DNA.

The morphology of HPV infected tissue depends on the stage of the infection. Clinically, cervical lesions have been categorized into three stages of cervical intraepithelial neoplasia (CIN): CIN 1, 2, or 3 (see Figure 6-4A). In low grade CIN 1 lesions, the tissue still demonstrates differentiation and stratification, although there is a more pronounced layer of actively dividing basal cells. This is the vegetative stage of HPV infections when the virus is able to complete its full life cycle and produce infectious particles. As long as the viral genome is episomally maintained the virus is able to complete its full life cycle (119). However, as lesions progress to more malignant stages of an HPV infection, evidence of stratified epithelium is lost. The nucleated basal cells become predominant throughout the span of the tissue. Over time, the viral

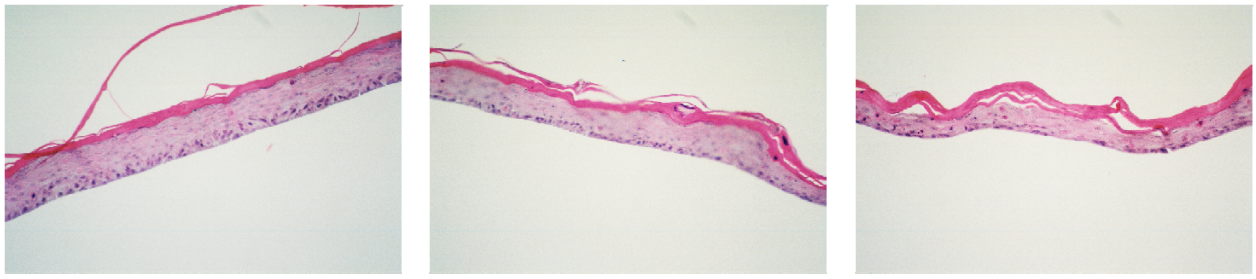
Figure 6-4. Histochemical analysis of HPV-infected tissue

A. Data from Lowy and Schiller (2006) (245). Model representing the progression of epithelial tissue from normal, uninfected tissue to an invasive carcinoma stage. The histological description of the stage represented is described below in black indicates. Shown in blue is the status of the HPV genomic DNA at the relative stages of the infection. B. H&E stained tissue sections from organotypic rafts grown from either wild-type transfected HFKs (HPV18) or the HFKs transfected with the TATA box mutations. The exact cell line (Tatabox:6a and TataboxG/C: 13a) is indicated below the image.

A



B



HPV 18 TATAbox:6b TATAboxG/C:13a

genome is often found integrated in the high- grade lesions at CIN 3 and infectious viral particles are no longer produced (245). Histochemical analysis of organotypic raft tissue grown from cell lines infected with integrated HPV genomes, such as from SiHa cells, demonstrates a morphology similar to high grade CIN 3 lesions, with nucleated cells present throughout the depth of the tissue and no clear distinction of distinct layers of the tissue (246).

Several studies have shown that the pathology of organotypic raft tissue grown from HPV-transfected cell lines created in the lab resembles dysplastic tissue from early stage cervical lesions(119;142;207;208). To examine pathology of the tissue grown from the lines transfected with the mutated HPV genomes, organotypic rafts were grown from the cell lines, preserved in paraffin, and sectioned for H&E staining. As can be seen in Figure 6-4B the tissue grown from the HFKs transfected with wild-type HPV DNA resembles a low grade CIN 1 lesion, since there is still evidence of fully stratified layers of the epithelium. We concluded earlier, in the Southern analysis, that the wild-type genomes harbored in these cells were maintained episomally. The Tatabox:6b cell line, which we also determined to be maintained episomally by Southern analysis, also resembles a low- grade CIN 1 lesion. However, interestingly, the TataboxG/C:13b cell line, which appeared to harbor integrated viral genomes by Southern analysis, also appears to have stratified layers and more closely resembles a low-grade lesions than high-grade lesions with no distinct layers.

All but one of the mutant HPVs cell lines are able to produce infectious virus.

Because integration represents an abortive infection, another way to measure the stage of the infection is to test whether or not infectious virus is produced in the organotypic raft cultures (247). Once the cell lines passed crisis we were able to grow them on organotypic rafts for 12 days and analyze the viral lysate extracted from the raft tissue for its ability to infect HaCaT cells.

The viral lysate was prepared by dounce homogenizing three rafts per preparation. To establish the relative viral load of each cell line, we prepared serial dilutions of the viral lysate to determine which dilution no longer contained infectious virus. We prepared RNA from infected HaCaT cells and assayed for the spliced transcript E1^E4 to distinguish active viral transcription from incoming viral DNA. The infectivity assays revealed that the lysates from the Tatabox:6a and TataboxG/C:13a cell lines were not infectious at any dilution. However, we found that the viral lysates prepared from the cell lines Tatabox:6b and TataboxG/C:13b were both infectious up to dilutions of 1:100 in the TataboxG/C:13b cell line and up to a dilution of 1:5,000 in the Tatabox:6b cell line. However, lysates extracted from rafts grown from the same cell lines but thawed from frozen stocks at a later time had lower infectivity titers. The wild-type line also varies in infectivity titers between the original cell line and the cells thawed from the frozen stock. These results are summarized in Table 6.4.

Table 6-4. Viral titers from infectivity assays

	Original	Duplicate
HPV18	1:1,000,000+	Infectious at 10,000 and 1,000,000 but not 100,000
Tatabox:6a	0	0
Tatabox:6b	1:5,000	1:1,000
TataboxG/C:13a	0	0
TataboxG/C:13b	1:100	0

Different transcription initiation start sites for different TATA box mutations.

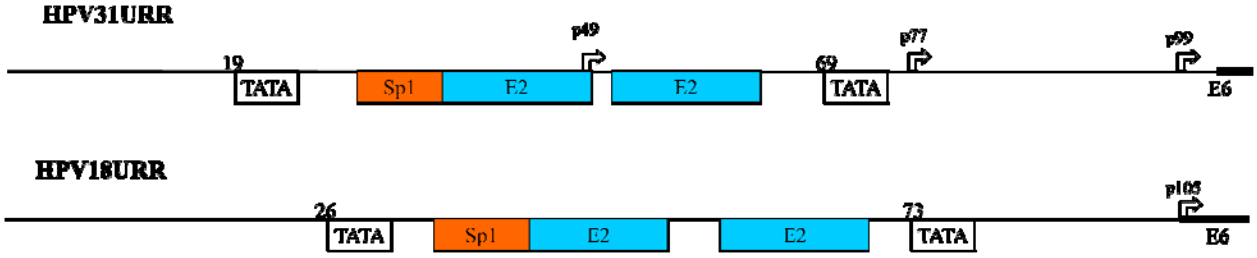
To understand how the genomes with mutations in the major TATA box were able to initiate transcription, we examined the mRNA transcripts to identify the transcription start site. The majority of the transcripts from wild-type HPV18 initiate at p105, as previously demonstrated by Thierry *et al*. (1987) (248). However, promoter analysis from HPV31 mRNA transcripts has identified less abundant transcriptional start sites upstream from the major P99 start site (249). A schematic of the HPV31 URR with the mapped promoter sites and the HPV18 URR is shown in Figure 6-5A. We found an upstream TATA box that starts at nucleotide 19 in HPV31 and 26 in HPV18. Schneider-Gadicke and Schwarz first identified the two TATA box elements located in the first 100 base pair sequence of HPV18 (78). However, they concluded that "No evidence could be obtained from the primer extension analysis indicating that the second TATA box sequence located at position 19 also directs initiation of transcription." It was noted that transcription start sites were found upstream but this data was not shown and remained unexplored (78). From this point forward, the upstream TATA box was, for the most part, ignored and forgotten about and studies have mainly focused on the major promoter at position 105 in HPV18 using the TATA box at position 73 in HPV18.

To identify the start site of the transcripts in the two different mutated Tatabox cell lines, we extracted the total cellular RNA and performed primer extension analysis on the transcripts. We found that the majority of the early transcripts in the Tatabox:6 cell lines, which harbor the genomes with the TATA box mutation that mimics the SV40 TATA box, initiate at the p105 promoter (see Figure 6-5B). However, the majority of the early transcripts from the TataboxG/C:13 cell lines, which have a complete disruption in the TATA box at position 73, initiate at an upstream promoter, p56. Sequencing analysis confirmed that the exact location of this start site.

Figure 6-5. Upstream TATA box and promoters in the HPV URR

A. Schematic of the HPV31 and HPV18 URR. Arrows indicate identified promoters. Nucleotide positions for the start site of each identified TATA box sequence are shown at the upper left hand corner of the TATA box. B. Primer extension gel is shown for the indicated cells lines. On the left is a diagram of the transcript products and how the length of the products translates to the start sites in the schematic representation of the HPV188 URR in the upper right hand of the image.

A


HPV31URR
19
TATA
Sp1
E2
p49
E2
69
TATA
p77
p99
E6
HPV18URR
26
TATA
Sp1
E2
E2
73
TATA
p105
E6

B

Discussion

In this chapter we examined the ability of full-length HPV18 genomes with mutations in cis-elements to immortalize transfected primary keratinocytes. HPV genomes mutated in the Ap1 binding site proximal to the promoter, the Sp1 binding site, and both of the TATA box binding sites were able to efficiently immortalize the transfected primary HFKs. We found that only one genome, with a mutation in the Ap1 site distal to the early promoter, was ineffecien at producing immortalized HFK cell lines. This indicates that the proximal Ap1, the Sp1 and the TATA box binding sites are not essential to drive E6 and E7 expression contrary to what was previously concluded based on the reporter assays. Further analysis on the cell lines immortalized by the genomes mutated in the TATA box binding site at nucleotide 73 suggest that viral transcripts are able to initiate from p105 start site using a TATA box that resembles the SV40 TATA box sequence. This indicates that this slight alteration can substitute for the wild-type sequence. In the case of the TATA box mutation that dramatically altered the consensus sequence, transcripts appear to initiate upstream using a TATA box at position 26. The viral genomes appeared to be integrated in the majority of the cell lines immortalized by the HPV18 genomes mutated in the TATA box site. However, we found that two of the Tatabox cell lines were still capable of producing infectious virus suggesting that at least some of the cells in these lines harbor episomal genomes and retain the ability to complete the viral life cycle.

The base pair substitutions used to generate the mutated cis-elements used in this study were modeled after the mutations created in earlier studies (74;229;230). Upon close examination, it appears that there was an error in the original HPV18 sequence because the sequence published in these papers as shown in Figure 5-1 does not match the wild-type HPV18 sequence stored in GenBank (NCBI Reference Sequence: NC_001357.1) (250). There is a base pair substitution at nucleotide 41 which is the last nucleotide in the Sp1 binding site, where a “G”

was used in these studies in contrast to the "A" which is reported in the GenBank wild-type HPV18 sequence. There must have been a major misunderstanding of the HPV18 wild-type sequence in the original studies using this HPV type because multiple reports from more than one group also had the same error in the wild-type HPV18 sequence used in these studies (74;229;230;239;244;248). In the initial report that identified binding of the Sp1 transcription factor in the HPV18 URR, the correct sequence was documented(251). Gloss and Bernard (1990) noted that the HPV18 sequence bound by Sp1 deviated from the consensus sequence, GGGCGG (251;252). These authors also showed a sequence alignment of the Sp1 binding site between the major high-risk and low-risk HPV types. It is interesting to note that some HPV types, HPV-6b, HPV-11 and HPV31, all have a "G" at the 41 position, while the others, HPV-16, HPV-18 and HPV-33 have an "A" at this site. It makes sense that 6 and 11 would have the same sequence since these are the low risk types. It is interesting to speculate that there is a functional consequence of the fact that HPV31 has the same sequence as the low risk types since clinical lesions with HPV31 have been associated with a very low frequency of integration as well as a very long incubation period until cancer diagnosis (202). While the mutations used in this study were modeled after the base pair substitutions used in these studies, the wild-type sequence used in our studies matched the GenBank sequence as can be seen in Figures 5-2 and 6-1. While this may, in part, explain the differences between our results, most likely, the different systems used to analyze these sites play a bigger role in influencing the conflicting results.

The distal Ap1 binding site was the only binding site that we found to be critical for immortalization since we found that these mutated genomes were only able to immortalize the HFKs in half of the transfections. This binding site is located in a region that was previously identified as the constitutive enhancer region of the HPV18 URR (248). In the few cases in which the cells transfected with these mutant genomes did immortalize, the viral genomes were barely detectable in Southern blots indicating that these genomes were present at a very low copy

number in these cell lines. However, sequencing analysis of the viral URR amplified by PCR from total cellular DNA isolated from the cells did verify that the mutated genomes were in fact present in these immortalized cell lines. In the few cell lines that did immortalize it is possible that the integration event may have resulted in a change in transcriptional regulation so that the Ap1 site was no longer necessary to drive expression of the early genes E6 and E7. Therefore, it is possible that in the case of the cells that had not immortalized, the viral genomes either did not integrate or did integrate in a transcription non-permissive manner and were not able to drive E6/E7 expression without the Ap1 site present.

In the infectivity assays with the Tatabox cell lines, we found that the infectious titers of the two cell lines that still produced infectious virus decreased when we re-tested the thawed cells from the same cell lines. It is possible that these cell lines represent a mixed population of cells that maintain episomal HPV genomes and cells that have integrated genomes. Cells infected with integrated genomes have a selective growth advantage and therefore it is possible that the cultures are slowly being overtaken by the cells with integrated genomes, which is why we observe a gradual decline in infectivity (123;125). It is likely that if we carried these experiments out and tested these cell lines at later passages that they would no longer be infectious over time as the population of cells with integrated genomes take over the culture.

There are strong correlations between HPV genome integration and unregulated E6 and E7 expression with invasive cervical cancers but not with non-invasive CIN2/3 lesions. Integration and E6/E7 deregulation is a chicken and the egg mystery begging the question whether integration precedes E6 and E7 deregulation or whether E6 and E7 deregulation cause viral integration. The early discovery that the site of integration frequently results in the loss of the viral transcriptional repressor E2 led many to believe that integration precedes E6/E7 mediated genomic instability. However, it is possible that prolonged E6 and E7 expression results in the genetic instability that promotes viral integration. In support of this, more recently

studies that have investigated the levels of E6/E7 expression in different staged cervical lesions find that increased E6 and E7 expression does not correlate with integration (205;253). The previous studies that had found a positive correlation between high E6/E7 expression and integration were either based on experimental evidence or simply categorized the expression of E6/E7 in all cervical cancer tissues irrespective of the viral genome status (254;255). No one had looked at the relationship between E6 and E7 expression levels and integration directly from biopsy tissue. We did not directly measure E6 and E7 expression levels in our studies. In the case of the Tatabox cell lines that appear to have integrated viral genomes, the simple fact that these cell lines immortalized does not necessarily indicate that there are higher expression levels of E6 and E7 in these cells. Therefore, it will be interesting to determine the levels of E6 and E7 maintained in these cells.

During the initial characterization of viral transcription from the early promoter, the presence of transcripts that initiate upstream of the 'major' promoter were identified (248;256). Promoter analysis of the HPV31 early promoter identified two additional minor transcription initiation start sites using primer extension analysis on mRNA transcripts. Close examination of the sequence upstream of the early promoter reveals two identical TATAAAA sequence in both HPV31 and HPV18. In HPV31, the duplicate TATA box is located approximately 24 nucleotides upstream of the p49 initiation site found in the earlier primer analysis studies, which is the typical distance found between a functional TATA box and the promoter (257). This supports the possibility that this TATA box is indeed functional. Likewise, the position of the upstream TATA box in the HPV18 URR is also exactly 24 upstream of the initiation start site we identified in our primer extension analysis (as shown in Figure 6-5).

The existence of the second, upstream TATA box probably serves a purpose other than simply functioning as a back-up for the major TATA box at position 73. The studies presented here do not address what the natural role of this cis-element may be in the wild-type genome. It

is likely that this cis-element provides yet another mechanism to finely tune transcriptional regulation in different settings. It is possible that the upstream TATA box may produce transcripts that have different splicing signals and therefore might possibly generate early proteins that are under different regulatory controls. The fact that the level of transcripts initiated from this promoter are normally very low makes it difficult to study the effect of these transcripts, but it does not necessarily imply that they are insignificant with regard to their purpose in the HPV life cycle. Sequence alignment of the major low-risk and high-risk types has revealed that only the high-risk genomes contain this upstream TATA box element as seen in Figure 6-6. This suggests that the presence of this second TATA box may contribute to the oncogenic properties of these viruses.

A study by Lace *et al*. (2008) also identified transcripts that initiated at an upstream promoter, p14, in HPV16 (258). These authors concluded that E1 transcripts that are initiated from this upstream promoter are responsible for limiting the amplification of HPV16 genomes upon initial infection of the keratinocytes of the basal layer (259). This study suggests a functional role for the upstream TATA box.

The presence or absence of either TATA box may affect the oncogenicity of different variants. The regulatory region of the human papovavirus JC virus (JCV) also contains duplicate TATA box elements (260). JCV is associated with progressive multifocal leukoencephalopathy (PML). Further analysis revealed that differences exist in the regulatory region of the JCV promoter between JC viruses isolated from healthy individuals versus the viruses isolated from PML patients. A study by Pfister *et al*. (2001) found that the sequences isolated from the PML patients contain tandem repeats resulting in two TATA box sequences. However, the viruses isolated from healthy patients only have one copy of this sequence containing the TATA box. The authors concluded that the presence of the tandem repeats correlates with a poor clinical outcome of PML patients (260). These findings seem to resonate with the fact that the presence

Figure 6-6. Sequence alignment of the URR for HPV types 6b, 11, 16, 18 and 31

TATA box sequences are highlighted. The consensus sequence of all types is shown above.

```
Majority --TXATAAT---AACAATCTTAGTATAAAAAXAXAGGGXGTGACCGAAAACGGTTGA-ACCGAAAACGGTTXGTATATAAAXCAGXCXXA

HPV-6b   --GT-TAAT---AACAATCTTGGTTTAAAAA-ATAGGAGG-GACCGAAAACGGTTCA-ACCGAAAACGGTTG-TATATAAACCAGCCCTA 80

HPV 11   --CT-TAAT---AACAATCTTAGTTTAAAAA-AGAGGAGG-GACCGAAAACGGTTCA-ACCGAAAACGGTTA-TATATAAACCAGCCCAA 80

HPV16    ----ACTAC---AATAATTCATG□ATAAAACTA-AGGGCGTAACCGAAATCGGTTGA-ACCGAAACCGGTTAGTATA-AAAGCAGACATT 80

HPV18    ATTAATACTTTTAACAATTGTAGTATA□AAAAA-AGGGAGTAACCGAAAACGGTCGGGACCGAAAACGGT--GTATATAAAAGATGT-GA 87

HPV31    --TAATAAT---AATAATCTTAG□ATAAAAAAGTAGGGAGTGACCGAAAGTGGT-GA-ACCGAAAACGGTTGGTATATAAAGCACATAGT 73
```

of two TATA box elements correlates with the pathogenicity of these viruses since the duplicate TATA box in HPV is only found in the high risk types.

As mentioned in earlier sections of this thesis, E2 plays a role in transcriptional repression of the HPV early promoter in part by competing for the binding site with the nearby TATA box cis element(89;261). In addition, studies have found that E2s repressive activity is primarily determined by the E2 binding site most proximal to the promoter(230;261). Therefore, it is likely that the presence of the upstream TATA box in the high-risk HPV types allow transcription of the oncogenes E6 and E7 in circumstances when binding of the downstream TATA box is blocked by E2 binding to this site. Since the low-risk HPV types do not have the upstream TATA box, they do not have this alternative mechanism to activate the oncogenes. This could serve as a possible explanation for the correlation between the presence of the upstream TATA box and the oncogenicity of the high risk types.

Chapter 7

Discussion

Keratinocyte immortalization: friend or foe? In the first half of this thesis, a useful method of keratinocyte immortalization was described. This novel method of immortalization will hopefully provide a new beneficial tool in keratinocyte research and possibly even result in clinical therapeutic applications. In the second half of this thesis, the elements of the virus required for expression of the oncogenes responsible for HPV-induced immortalization were examined. The clinical goal in this regard is to target and disrupt the feature of the virus that enables immortalization.

There are some interesting comparisons to be made regarding HPV vs. Y-27632- induced immortalization. In Chapter 3 co-culture with fibroblast feeder cells was found to be a necessary condition of Y-27632-mediated immortalization of primary HFKs. In contrast, the high-risk HPV genomes can induce immortalization in primary keratinocytes grown with or without feeder cells. However, co-culture with feeders is required for episomal maintenance of HPV genomes (138;262). In normal epithelial tissue fibroblasts are present in the dermal layer of the skin. Therefore the cells normally in direct contact with the fibroblasts are the keratinocytes of the basal layer of the epithelial tissue, those that are actively dividing. It is believed that fibroblasts secrete growth factors that help to maintain the cells of the basal layer in an actively dividing state (263;264). This could explain why keratinocytes cultured in the presence of fibroblasts have a longer lifespan. This would also support why the fibroblasts feeder layer is a necessary co-factor for Y-27632-induced immortalization. It is also possible that this growth advantage helps to prevent integration of the HPV genomes because it relieves the selective pressure for

integration. It seems as if the feeders are able to complement the functions of the E6/E7 genes to maintain a natural balance that allows the cells in culture to accurately model a normal infection where the genomes are maintained episomally. The feeders plus the viral oncogenes enable immortalization without succumbing to the potent oncogenic properties of viral integration. Immortalized HFKs that maintain extrachromosomal HPV genomes are more comparable to Y-27632-immortalized HFKs than cells with integrated viral genomes because they retain some of the proper regulation of normal keratinocytes. We saw how keratinocytes immortalized by episomally maintained HPV genomes as well as the Y-27632- immortalized keratinocytes are still able to differentiate in the organotypic raft culture and produced fully stratified tissue. Viral genomes are normally maintained episomally in the natural state of a productive HPV infection and integration is an aberrant event that is a dead end for the viral life cycle. Therefore, it is possible that the feeders may provide some of the same functions for the viral oncogenes as it provides for the drug. As we learn more about the role the feeders play in augmenting Y-27632-induced immortalization, this may reveal new insights about the contributions of the feeders to enable episomal maintenance of the viral genomes in a natural productive infection.

In the studies presented here there is no way of distinguishing whether Y-27632 is acting on the keratinocytes directly to promote proliferation or causing some effect in the co-cultured fibroblasts that indirectly affects the growth of the keratinocytes. Future studies to address this might include using alternative methods of targeted ROCK inhibition so that we can block the Rho pathway in either the keratinocytes or the fibroblasts to determine if the effect on proliferation is cause by the block in the Rho pathway in the keratinocytes themselves or whether it's actually some effect on the fibroblasts. McMullan *et al.* (2003) generated HFKs transfected with retroviral vector that encodes a inducible constitutively active ROCK-II (154). We could use this technique to generate fibroblast lines that constitutively express ROCK-II. By using these fibroblasts in the co-culture with keratinocytes we could determine whether or not the

inactivation of the Rho pathway in the fibroblast feeder cells plays a role in the Y-27632 induced immortalization of the keratinocytes.

Y-27632 effectively inhibits both ROCKI and ROCK-II (158). McMullan *et al.* (2003) report that the predominant form of ROCK in keratinocytes is ROCK-II (154). Moreover, they show that specific activation of ROCK-II causes cell ycle arrest and an increased involucrin expression. This data is consinsent with our results that show that ROCK inhibition releases a block in proliferation. However, because we have only used Y-27632 as a ROCK inhibitor it is difficult to conclude with certainty that the results we observe in our assays is necessarily due to ROCK. Y-27632 is known to bind to and inhibit other kinases such as protein kinase C-related kinase 2 and citron kinase, albeit with much lower affinities. To further confirm that the immortalized phenotype of the Y-27632-treated cells is in fact due to ROCK inhibition, analyzing the effects of an alternative method of ROCK-II inhibition would be helpful to rule out the possibility that the phenotype is the result of any other pathway affected by Y-27632. Vishnubhotla et al. (2007) demonstrate effective knockdown of ROCK-II using a targeted siRNA (265). HFKs transfected with an expression vector encoding siRNA to selectively block ROCK-II could serve as an alternative method to target ROCK. Because of the results reported by McMullan et al. (2003) showing that constitutively-activated ROCK-II effectively blocks proliferation, it is likely that siRNA knock-down will release the cell-cycle inhibition by ROCK and mimic the phenotype seen upon Y-27632 treatment.

The Rho pathway may play a role in the HPV life cycle. A study by Holthusen *et al*. (2009) found that the viral protein L2 interacts with Vav2 (266). One of the downstream targets of Vav2 is the GTPase, RhoA (267). The authors found that L2 expression reduces the levels of active RhoA. The authors propose that the purpose of the L2-Vav2 interaction may serve two functions. They argue that the L2 mediated down-regulation of the Rho pathway augments the effects of E6 and E7 to maintain the cells in the upper differentiated layers in a replication-

permissive environment. These results as well as the results reported by McMullan *et al*. (2003) support our findings that down regulation of the Rho-pathway results in increased keratinocyte proliferation. L2 is the minor capsid protein and is necessary for HPV infection (154). The L2 protein has been implicated in a wide variety of roles including escorting the viral DNA to the nucleus, replication, endosomal egress, and interacting with a number of cytoskeletal elements (268-272). Holthusen *et al*. (2009) postulate that another role of the Vav2-L2 interaction is to mediate endocytosis of viral particles. Based on these hypotheses it would seem that Y-27632-treatment would create a favorable environment for HPV infection.

Another study by Charette and McCance (2007) also identified a role of the Rho pathway in cervical cancer metastasis via the Akt/p27 pathway (190). The authors found that E7 increases cytoplasmic p27 which binds to RhoA and inhibits downstream events in the Rho pathway, including the activation of the downstream effectors ROCKI and II (273). Therefore the authors propose that cervical cancer metastasis can be induced by inhibition of the Rho pathway through the relocation of p27 by E7. However, in direct opposition to this conclusion, a recent paper by Amine *et al*. (2009) reports that inhibition of the Rho pathway using Y-27632 and Cidofivir prevents metastasis in HPV-positive tumor cells (274). In any case, like Holthusen *et al*. (2009), Charotte and McCance (2007) also find that HPV-infection leads to inhibition of the Rho pathway, suggesting a common mechanism shared by these immortalizing agents.

In addition to applying lessons learned about Y-27632-induced immortalization, the cells themselves may serve as a useful tool to HPV research. From our results, it appears that the Y-27632-immortalized cells represent a superior model to the available immortalized keratinocyte cell lines because they are free from any major genetic abnormalities and appear to retain important features of normal keratinocytes. It is possible that these cells could provide a solution for some of the current limitations in HPV research. The results presented in Chapter 4 demonstrated that Y-27632-treatment significantly improved the transfection efficiency of

primary keratinocytes. It is possible that the drug treatment will have even more benefits that have not yet been identified. For instance, as proposed in Chapter 4, primary keratinocytes immortalized by Y-27632 may be used to study the long-term maintenance of the low-risk HPV genomes in a physiologically relevant model.

It is possible that the Y-27632-immortalized cells might have other potential benefits for HPV research. The viral E2 expression is toxic to most cell lines and this has restricted its analysis in most cell culture systems (275). Parish *et al.* (2006) found that there are two separate and distinct pathways by which E2 induces apoptosis: one in which is dependent on the presence of an HPV genome, and one that is independent. The HPV-independent mechanism of E2-induced apoptosis is mediated through p53 activation (275). In Chapter 3, we show that p53 expression is functional but elevated in the Y-27632-immortalized cells. Therefore, it seems that these cells are unaffected by high levels of functional p53 and it is possible that the Y-27632-treated cells could possibly be resistant to E2's toxic effect. This would enable stable studies of the E2 protein in transfected HFKs or other cell lines which is currently very limited due to the toxic effect of E2.

Y-27632 may also be used to improve the culturing capacity of rare and precious primary keratinocytes that are normally difficult to come by or to propagate in culture. For example, in work not included in this thesis, we have found that HPV 5 and 8, which are the HPV types commonly associated with Epidermodysplasia verruciformis (EV), do not replicate in primary HFKs. The inability of EV-associated HPV types to replicate in cultured cells has also been reported in Tsumori *et al*. (1982) (276). Since these viruses only cause disease in patients with EV, it is possible that these viruses may only be able to replicate in keratinocytes isolated from EV patients. The data presented in Chapter 3 revealed that Y-27632-induced immortalization applies to a wide variety of keratinocyte types including cervical, vaginal, and foreskin keratinocytes. Therefore, it is possible that Y-27632 treatment may also extend the lifespan of

keratinocytes isolated from EV patients. This may enable the analysis of the replication of the EV-associated HPV types *in vitro*.

The experiments included in this thesis explored the multifunctional role of E2 in the HPV life cycle. The design of the complementation assay allowed us to examine the role of the E2 binding sites in transient replication. However, the design of this assay also lends itself to similar analysis of cis-elements in long-term replication and genome maintenance. One of the important features of E2 not examined in this thesis is the alleged role of E2 in genome maintenance. Because we found that the E2 binding sites were required for transient replication, these sites must remain intact to examine long-term maintenance as well. Instead of asking what sites are required for stable maintenance, this assay could be used to determine what elements are not required for maintenance. To do this, we could mutate regions outside of the origin of replication and follow genome maintenance over time. This would eliminate the possibility that other cis-elements are involved.

Mutational analysis of the E6 and E7 oncoproteins has indicated that these viral proteins may also play a role in genome maintenance (220;277;278). Because E6 and E7 are required for the immortalized state of primary keratinocytes, their expression is necessary to study maintenance in prolonged cultures of primary keratinocytes. However, use of the Y-27632-immortalized HFKs would circumvent this requirement of E6 and E7. Replicons designed with mutations in the E6 and E7 open reading frames in a manner that would not disrupt expression of E1 and E2 could be used to confirm whether the loss of E6 and E7 also affects episomal maintenance of the transfected genomes in primary keratinocytes. Thomas *et al*. (1999) had found loss of episomal viral genomes in HFKs transfected with E6 and E7 mutant viral genomes after just one month. However, because these cells rely on the selective advantage of E6 and E7 for optimal growth, it is possible that their results simply reflect the selective pressure for integration on growth rather than a direct role in genome maintenance. Studies with Y-27632-

treated cells would help to clarify this since there would be no pressure towards integration. However, since we observed loss of transfected genomes maintained in Y-27632 in Chapter 4, we would need to determine whether stable maintenance of transfected HPV genomes is even possible in the presence of Y-27632 using a drug-selection method as discussed in the Chapter 4 discussion.

Data from Chapter 3 revealed that the Y-27632-immortalized keratinocytes and the HPV-immortalized keratinocytes share similar features such as shortened telomeres and upregulation of telomerase and c-Myc. However, we also found differences such as the response to DNA damage signals, and slight differences in the morphology of the raft tissues grown from the different immortalized cells. In regards to these features, the Y-27632-immortalized keratinocytes better reflect what we see with normal primary keratinocytes. The HPV-immortalized keratinocytes behave more like transformed cells because of their abnormal response to DNA damage signals and slightly abnormal tissue morphology. When analyzing the phenotypes of these immortalized cell lines, it seems there is a spectrum of immortalized states from that which reflects the properties of normal stem cells to the oncogenic state of cells transformed by HPV.

It is still unclear what drives HPV infections from an immortalized state to a transformed state when infected cells become tumorigenic. Many studies have suggested that the critical determinant of the fate of HPV-infected cells involves the integration of the viral genome into the cellular DNA (77;115;279). However, as Hafner *et al*. (2008) argue, it is also possible that disregulation of E6 and E7 precedes viral integration and causes the genetic instability that causes integration (253). In the studies presented in this thesis we often found that loss of various different cis-elements in the HPV URR resulted in integration but not in the loss of E6 and E7 expression. We did not directly measure E6 and E7 expression levels and cannot tell whether the level of expression of the oncogenes changed in our experiments. It is possible that the mutated

cis-elements in our studies, such as the E2 binding sites, affected the ability of the genomes to replicate and this drove integration. In future studies, it would be useful to repeat these studies and examine the expression levels of E6 and E7 as well as the state of the viral genome at frequent time points throughout the immortalization process to determine the sequence of events that took place in these cells transfected with the mutant genomes.

Carcinogenesis is known to be a multistep process involving the stepwise inactivation of the many pathways designed to prevent cellular transformation. Cervical cancer is a very good example of this fact as it generally takes many years for an HPV infection to become malignant. The time involved in malignant progression and the fact that many infections do not progress to cancer has led many to speculate about what co-factors might be involved in the carcinogenic process. Epidemiologic studies have exposed a number of risk-factors associated with cervical cancer including the use of oral contraceptives, smoking, parity, and co-infection with either HIV or HSV-2 (33). Many of these could simply serve as indicators of individuals actively engaged in sexual activity and could therefore merely indirectly reflect individuals whose behavior puts them at a higher risk of infection rather than a direct causal relationship.

The immortalization potential of the high-risk HPV types has been demonstrated in the studies in this thesis as well as in numerous previous reports in foreskin and cervical keratinocytes (43;280-282). However, many of these studies point out that the HPV-immortalized cells are not tumorigenic (43;280-283). The viral oncogenes E6 and E7 have been used to immortalize human mesenchymal stem cells and human marrow stromal cell lines without displaying transformed phenotypes (284;285). These cells still retain the ability to differentiate upon stimulation. It is possible that the studies that find that HPV-induced immortalization is not tumorigenic are not exposed to the confounding co-factors that transform HPV-infected cells *in vivo*. Alternatively, it is possible that the transformation process occurs over a longer time period than the length of time that the studies were conducted.

The regeneration capacity of stem cells represents a healthy role for immortalization in normal tissue homeostasis. Our results suggests that the Y-27632-immortalized cells may possess desirable stem-cell like qualities such as the capacity to indefinitely regenerate, while the HPV-immortalized cells possess pathogenic features of immortalization not yet fully understood. Distinguishing the differences of the immortalization processes in these two cell lines could help us understand the difference between a healthy capacity to divide versus a dangerous susceptibility to a pathogenic state.

In this thesis we examined the role of the cis-elements in the HPV life cycle with regards to transcription and replication with a particular focus on the viral E2 binding sites. The original work identifying the roles of the E2 protein began 20 years ago. In that time, much of the rudimentary knowledge about this protein was established. However, there are still major questions left unanswered about how this protein behaves in a natural infection and during the process of carcinogenesis. Often new assays designed to resolve the flaws of previous studies end up generating new limitations. The compact design of the HPV genome has demanded double-duty out of many of the viral proteins and even of the regulatory region. While this complicates its analysis, this is also serves as an intriguing model that represents a finely tuned instrument capable of achieving so much with so little. We and others have attempted to design assays that can single out each role of E2, however, as before, these assays themselves have created their own set of challenges and shortcomings. We found that while the complementation assay proved to be very useful for validating the role of the E2 binding sites in transient replication, the assay could not be used to examine the effect of mutations in the E2 binding sites on long-term genome maintenance. As for the transcription analysis of the E2 binding sites, many questions remain about the order of events in the immortalization process as well as the distinction between the effect of the mutations on transcription and replication. Progress in this

field will demand further fine-tuning of these assays to target each distinct function of E2 constantly followed by validation in physiologically relevant systems.

HPV and Y-27632 represent powerful instruments that will hopefully aid in achieving a better understanding of normal and pathogenic biological processes. The study of HPV and other oncogenic viruses have led to many important discoveries that extend beyond just the field of virology and have helped develop our understanding of fundamental cellular processes such as replication and transcription. With the events leading up to the recent advent of the HPV vaccine, a lot of attention has been placed on the HPV capsid studies and immunology. However, the vaccine only serves as a prophylactic defense against HPV infection and there will still be a need to find a treatment for those who are already infected. Now may be an appropriate time to shift the focus back to the viral processes that can be targeted for cures such as viral replication and transcription. Disrupting elements essential for genome maintenance would prevent persistent infections. Since we know that time is such a critical component of malignant progression in cervical cancer preventing the persistence of existing infections would avert malignant transformation. Alternatively, we could envision therapeutics targeted to disrupt expression of the oncogenes E6 and E7. Many studies have shown that E2 expression in HPV-transformed cells causes cell death. Therefore, it seems reasonable to develop strategies to deliver E2 to cells with integrated HPV genomes.

In contrast to the sluggish state of HPV replication and transcription research, studies with Y-27632 are the latest rage. A report in the journal *The Scientist* commented on the fact that shortly after a publication by Watanabe *et al*. (2007), which described the ability of Y-27632 to improve the growth of embryonic stem cells, supplies of Y-27632 sold out with certain manufacturers (177;286). Y-27632 shows promise of being equally advantageous, as HPV has been in the past, to our understanding of fundamental processes important for medical advances. When reviewing the literature surrounding this compound, it seems as if Y-27632 is the newest

and latest wonder drug. Multiple therapeutics have been proposed using Y-27632 and are currently under investigation including the use of Y-27632 in the treatment of various cardiovascular diseases, such as hypertension and arteriosclerosis, bronchial asthma, cancer and Alzheimer's disease (194;287-290). In this era where the importance of personalized medicine is realized and transitioning from a concept to a reality, tools that allow for the propagation of embryonic and adult stem cells serve as a valuable source of renewable healthy tissue with which to replace diseased or damaged tissue. However, because there are so many similarities between cancer and stem cells, great care must be taken in the design of medical therapeutics using these new tools and agents. It seems to me that Y-27632 dials back the clock on keratinocytes to the adult stem cell stage but no further, since the cells retain keratinocyte features and do not de-differentiate into omnipotent stem cells. This is an ideal balance that hints that this drug does not induce cells to cross the dangerous line to a cancer-causing state. Therefore, the great interest in this drug seems justified and hopefully it will live up to the hype.

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PATENT

McBride and Chapman, Use of ROCK Inhibitor, Y-27632, to Sustain Primary Human Keratinocytes in a Proliferative State
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