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HPV Episomal Copy Number Closely Correlates with Cell Size in Keratinocyte Monolayer Cultures

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W12E keratinocytes maintaining episomal copies of HPV DNA were separated according to size by centrifugal elutriation. HPV DNA copy number was greatly increased in the largest, most differentiated cells of the population. The large cells with the highest HPV copy number also showed evidence of endoreduplication of host cell DNA. Other cell lines maintaining episomal copies of HPV18 and HPV31 were also tested with all lines showing similar results. The results demonstrate that increase in HPV DNA copy number correlates well with increased cell size, a fundamental marker of keratinocyte differentiation. The results also indicate that simple monolayer cultures may be useful for studying the relationship between differentiation, HPV DNA replication, and cell-cycle events.

Key Words: HPV; papillomavirus; endoreduplication; cell cycle; checkpoint; keratinocyte; episome; differentiation; cell growth.

INTRODUCTION

The human papillomavirus (HPV) family is composed of more than 100 small DNA viruses that infect stratified squamous epithelia. The HPV lifecycle is intimately linked to the host cell program of differentiation. The virus initially infects basal cells, the proliferative compartment of keratinizing epithelia, where it is maintained and replicated as an episome along with the host cell DNA (Chow and Broker, 1994). HPV life cycle events including viral genome amplification, late gene expression, and packaging occur in the differentiated keratinocytes of the host epithelium (Howley, 1996). Infectious virions are then shed within the dead, cornified epithelial cells.

The availability of keratinocytes maintaining HPV episomes holds the promise of allowing genetic studies that were previously unfeasible. Cell lines carrying replicating HPV episomes have been isolated by tissue culture from cervical biopsies (Sterling *et al.,* 1990; Hummel *et al.,* 1992; Jeon *et al.,* 1995) and created in the laboratory following transfection of keratinocytes with cloned, viral DNA (Frattini *et al.,* 1996; Meyers *et al.,* 1997; Flores *et al.,* 1999; Thomas *et al.,* 1999). HPV-positive cells created by either means maintain viral episomes over time in culture, amplify viral DNA following differentiation, and support late gene expression in complex culture systems. They also produce infectious virions or virus-like particles under culture conditions that support the final stages of keratinocyte differentiation (Sterling *et al.,* 1990; Braun *et al.,* 1993; Meyers *et al.,* 1992, 1997; Flores *et al.,* 1999). However, progress in the study of HPV *in vitro* has been slow due to a lack of simple culture systems supporting viral amplification and other functions dependent upon differentiation.

Keratinocyte cultures are heterogeneous with regard to proliferative potential and differentiated state. Cycling cells are continuously arresting and differentiating according to numerous factors that include state of confluence (cell–cell interactions), and growth factor and calcium ion concentration (Fisher, 1994; Dotto, 1999). The dependence of HPV amplification and other functions upon keratinocyte differentiation prompted us to investigate the correlation between HPV copy number, cell-cycle position, and cell size in keratinocyte lines that maintain episomal copies of HPV. Significant heterogeneity with regard to HPV copy number within cell monolayers was found following separation of cells by centrifugal elutriation. The differences in HPV copies per cell correlates with the size of the cell, with the largest cells in the population maintaining the highest copy number. The large, high copy cell populations also showed evidence of endoreduplication of host cell DNA. Our data indicate that simple keratinocyte monolayers maintaining HPV episomes support aspects of the viral life cycle that include cell size and differentiation-associated increases in viral DNA copy number.

RESULTS

Quantitative PCR of HPV DNA

Taqman PCR of cloned HPV16, HPV18, and HPV31 templates was linear over a range of 10 to 10^8 copies per

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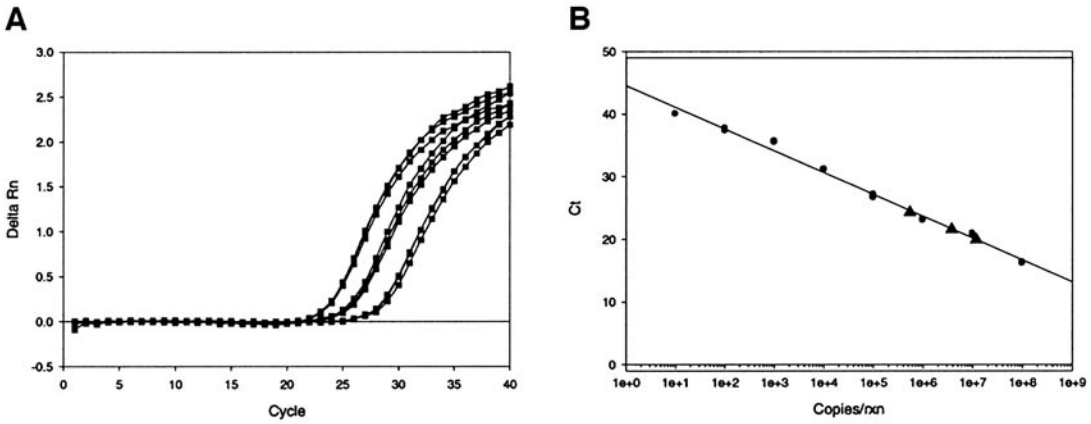

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Passage	Ct	Copies/rxn	DNA dilution	Copies/culture	Cells/culture	Copies/cell
16	20.93	1.91×10^6	1/100	1.91×10^8	1.5×10^5	1273
23	22.57	6.92×10^5	1/100	6.92×10^7	2.025×10^5	342
30	25.29	1.24×10^5	1/100	1.24×10^7	2.75×10^5	45

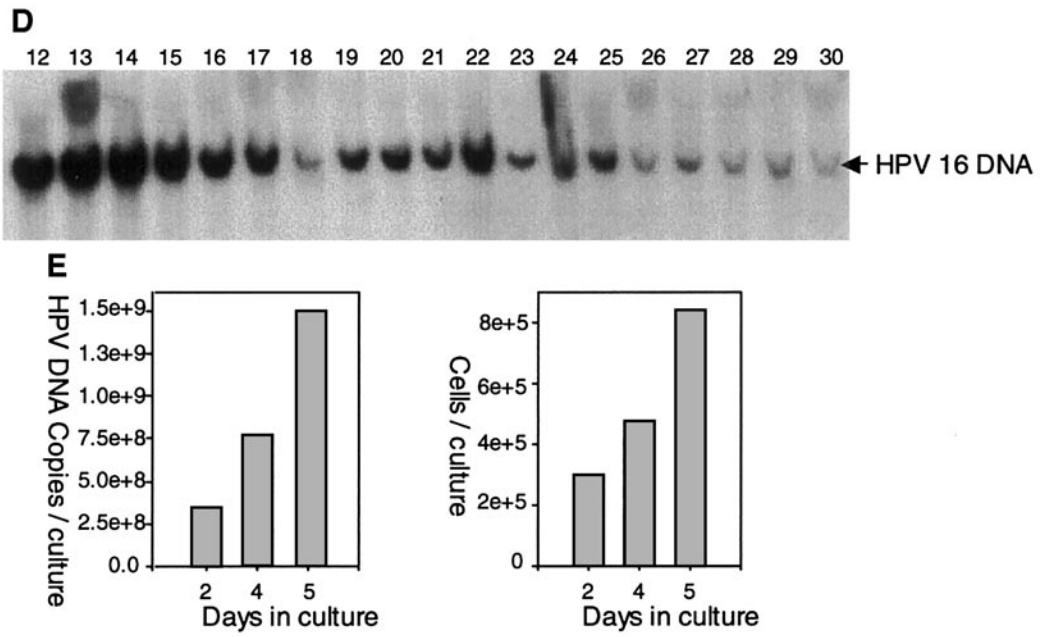

FIG. 1. Quantification of viral DNA copy number in W12E cells by Taqman PCR. Hirt DNA prepared from a known number of W12E cells from various passages was quantified by plotting threshold cycle (Ct) on a standard curve prepared with cloned HPV16 DNA. (A) Change in fluorescence (ΔRn) plotted against the PCR cycle of three Hirt DNA samples (each in triplicate) from passage 16, 23, and 30 cells identifies the threshold cycle (Ct) at which fluorescence, measured in real-time, first appears above background. (B) Standard curve is generated with a known quantity of cloned HPV16 DNA (circles) and unknown samples (A) are plotted on this curve to identify copies of HPV16 in each Hirt DNA sample. (C) Table expressing the Ct and copies per reaction generated in A and B as a function of Hirt DNA dilution and number of cells from which DNA samples were prepared. Using this approach, the number of copies of HPV16 per cell is accurately calculated. The number of copies per cell decreases substantially with passage in culture. (D) Southern blot of 50 μg total DNA digested with *Bam*HI and probed with full-length HPV16 probe confirms decrease of HPV16 DNA with increasing passage number. Numbers above blot indicate passage number of cells. (E) Replicating W12E cells were followed over time in culture. Total copies of HPV16 DNA increased with increase in total cell number over time, demonstrating the replication and maintenance of HPV DNA levels within a growing population of cells.

cell (Figs. 1A and 1B). Using standard curves generated in this assay, HPV16 DNA levels were quantified in Hirt DNA preparations from numerous passages of W12E cells. The cells carried an average of 1200 copies per cell of HPV16 in early passages, but copy number progressively decreased over time in culture up to passage

30, when an average of 50 copies per cell was reached (Fig. 1C). Similar results were obtained with HCK18:1B cells (data not shown). Southern blots of W12E cell total DNA correlated well with the Taqman PCR results and confirmed that HPV16 was present in an extrachromosomal state (Fig. 1D). HPV DNA copy number was lost over numerous passages and weeks in culture, but short-term studies of HPV copy number clearly showed that viral DNA was replicated, and cellular copy number maintained, in log growing cultures. For instance, over 2–5 days in culture HPV16 DNA levels increase in direct proportion to the number of cells in the culture (Fig. 1E). These results were highly reproducible in all cell lines tested; growing cell cultures always maintain a similar HPV copy number per cell within any given passage.

Isolation of W12E cells by centrifugal elutriation

Centrifugal elutriation of passage 16 W12E cells and subsequent Taqman PCR analysis of the separated cell populations demonstrated that HPV16 DNA copy number dramatically increased with cell size. The relative numbers of cells in the different fractions were 65% small (16–28 ml/min), 27% intermediate (30–36 ml/min), and 8% large (38–46 ml/min). HPV DNA copy number correlated well with cell size (Fig. 2A). The asynchronous (unseparated) cell population maintains an average of approximately 1300 HPV16 copies per cell, but a large difference exists in HPV16 copy number between the smallest cells (eluting at the lowest flow rates) and largest cells (eluting at highest flow rates). The smallest cells had the fewest copies of HPV16, averaging approximately 100 copies per cell (Fig. 2A). With increasing cell size, a steady accumulation of HPV16 episomes was noted (Fig. 2A). The largest cells, isolated at flow rates of 36 ml per minute and above, contained greater than 3500 copies of HPV16 per cell. This result, repeated numerous times, consistently demonstrated an amplification of at least 15-fold over the smallest cells in the population.

Cell size and HPV16 copy number also correlated well with expression of markers of keratinocyte differentiation. The smallest cell population expressed little or no detectable keratin 10 (K10) or involucrin, while increases in cell size was accompanied by an increase in expression of both proteins (Fig. 2B). Expression of K10 correlated particularly well with accumulation of high HPV16 copy number (Figs. 2A and 2B). Involucrin was detectable in smaller cells relative to K10, although it too increased in direct correlation with cell size.

Flow cytometry showed that the smallest cells in the population (isolated at flow rates of 16–20 ml per minute) were significantly enriched for cells in G1 of the cell cycle (Fig. 2C). Higher flow rates (22 to 26 ml per minute) yielded cells entering S-phase as well as a significant G1 component, while cells isolated at 28 to 32 ml per minute were enriched for cells in S and G2/M. Thus, W12E cells

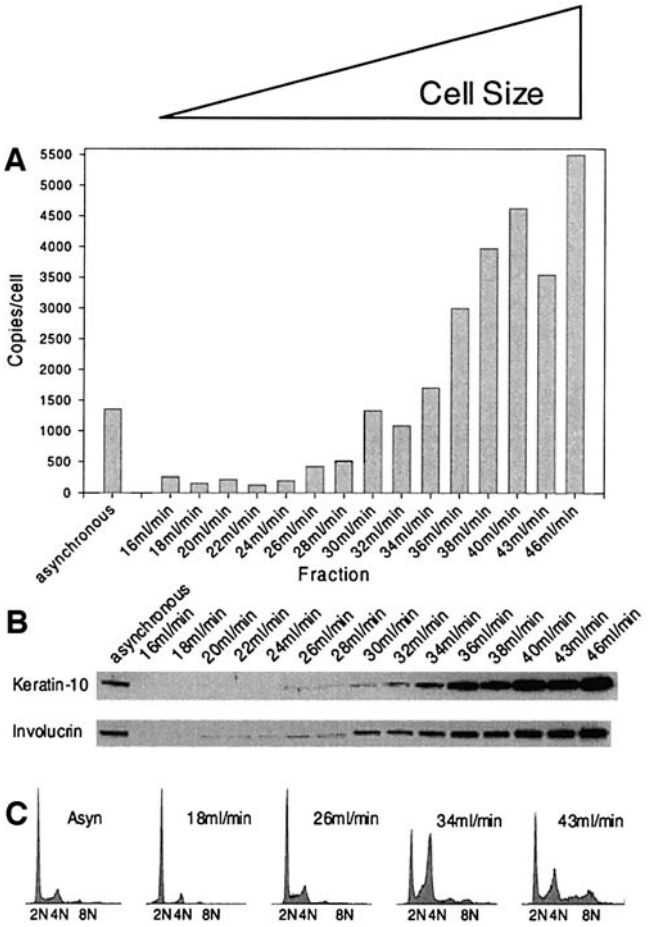

FIG. 2. Passage 16 W12E cells were separated according to size by elutriation and analyzed by Taqman PCR (A), Western blot (B), and flow cytometry (C). Results show an excellent correlation between HPV16 copy number and differentiated state of the cells indicating that HPV16 amplification occurs spontaneously in cell monolayers. (A) HPV16 copy number in passage 16 W12E cells. Unseparated (asynchronous) W12E cells, as well as W12E cells separated according to size by centrifugal elutriation, were analyzed by Taqman PCR and the average copies of HPV16 DNA calculated. With increasing cell size an increase in HPV16 DNA copies was noted. (B) Western blotting of the elutriated cells for keratin-10 (K10) and involucrin demonstrates a tight correlation between cell size and differentiated state. The smallest population of cells shows little or no evidence of differentiation with progressive accumulation of differentiation markers with increasing cell size. The expression of both proteins correlates well with the amplification of HPV16 DNA (A). (C) Sample flow cytometry profiles of DNA content of elutriated cell populations showing that smallest cells are enriched for cells in G1 of cell cycle, while progressively larger cells are enriched in cells in S-phase and G2/M. The largest cells in the population contain cells from all phases of the cell cycle with a large G2/M representation, as well as a significant peak of cells with an 8N complement of DNA.

entering G2/M showed a small increase in HPV16 copy number relative to G1 cells (Fig. 2C). The largest cells having the highest HPV DNA levels, isolated at flow rates above 34 ml per minute, contained cells in all phases of the cell cycle. Among these cells was a significant population with an 8N complement of DNA (Fig. 2C).

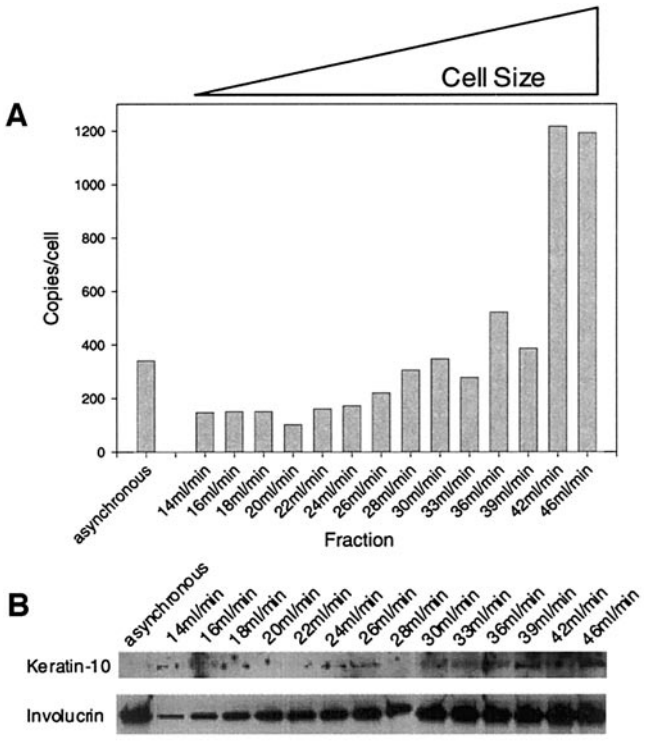

FIG. 3. Elutriated HCK18:1B cells show properties similar to W12E cells with an excellent correlation between cell size and differentiated state. (A) A strong correlation again existed between cell size and HPV18 copy number with the smallest cells having the lowest HPV18 DNA copy number, and the largest cells maintaining greater HPV18 DNA copy number (Fig. 3A). (B) Western blotting for keratin-10 (K10) and involucrin demonstrates that HCK18:1B cells express little or no K10, while significant levels of involucrin are detected in cells from all elutriation fractions. The smallest cells contain the lowest levels of involucrin with a progressive accumulation of involucrin with increasing cell size. (C) Flow cytometry reveals that the smallest cells are enriched for G1 cells while the largest cells contain cells from all cell-cycle phases and a significant DNA content >4N with an 8N component.

Isolation of HCK 18:1B and 31NHFK cells by centrifugal elutriation

The HCK18:1B cell line was next examined to determine whether other HPV-positive cell lines exhibited similar characteristics. HCK 18:1B cells carry transfected HPV18 DNA that is also maintained in an episomal state (Meyers *et al.,* 1997). Passage 14 HCK18:1B cells, separated by centrifugal elutriation (Fig. 3), give a profile similar to W12E cells. HCK18:1B cells have 85% of cells in the small fractions (14–26 ml/min), 10% in the intermediate fractions (28–33 ml/min), and 5% in the large fractions (36–46 ml/min). Taqman PCR quantification of HPV18 DNA demonstrates that the unseparated population of cells maintains an average of 350 copies per cell. The smallest cells in the population contain approximately 100 copies per cell while the largest cells harbor greater than 1000 copies per cell.

Only low levels of K10 were detected in the largest population of HCK 18:1B cells, while involucrin was more abundant in these cells than in W12E cells (Fig. 3B). Similar to W12E cells, involucrin levels steadily increased with increasing HCK18:1B cell size. DNA flow cytometry profiles of elutriated HCK 18:1B cells were also similar to those of W12E cells (Fig. 3C). The largest cells had DNA levels exceeding 4N, again including a significant population of cells with an 8N DNA content.

We then examined a cell line carrying HPV31 DNA derived in our lab by transfecting normal human foreskin keratinocytes (NHFK). These cells (31NHFK-10), examined at passage 6, also gave similar results, with the largest cells exhibiting the highest HPV episomal copy number as well as a significant 8N population (Fig. 4). To date we have examined a total of seven cell lines carrying episomal copies of high-risk HPV. In all cases, a significant increase in HPV DNA copy number was ob-

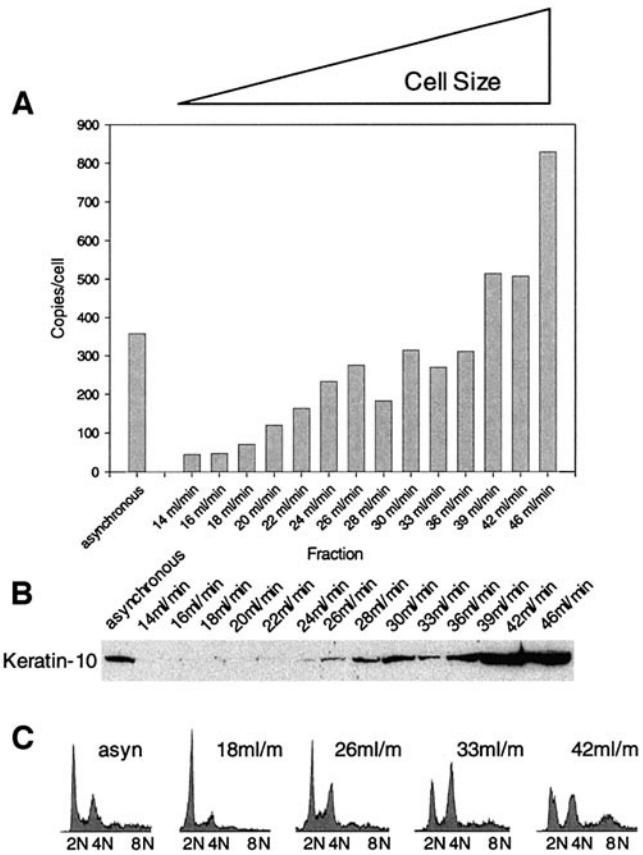

FIG. 4. Elutriated 31NHFKs were analyzed for HPV31 DNA content and by flow cytometry. (A) Taqman PCR of elutriated 31NHFKs shows an increase in HPV31 episome copies per cell that correlates with increasing cell size. (B) Flow cytometry of elutriated cell populations shows an 8N peak of cells in the largest cells of the population carrying the highest HPV copy number.

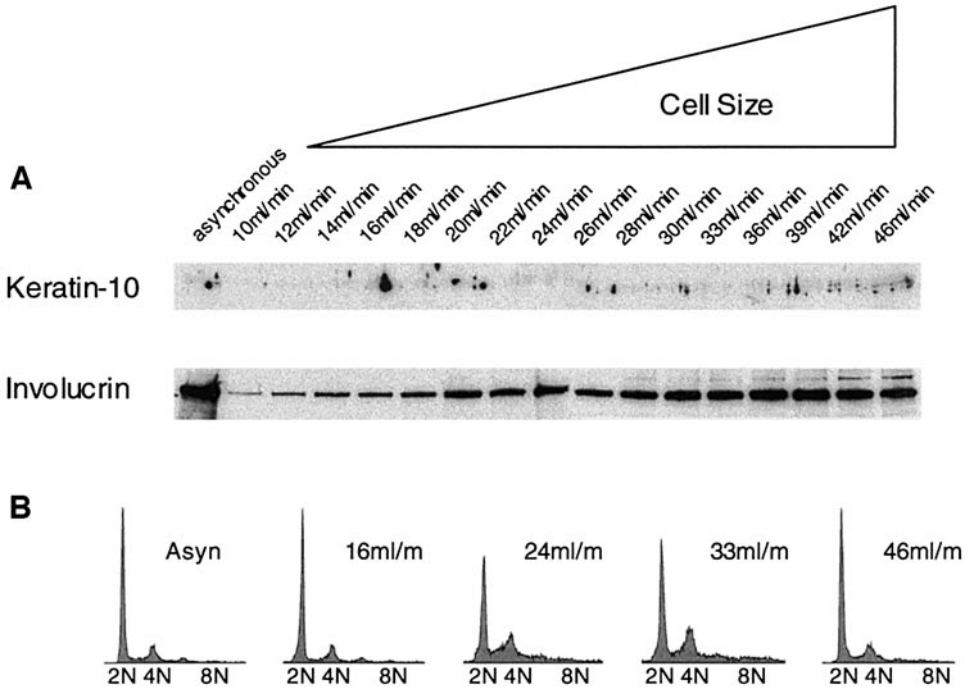

FIG. 5. Elutriated normal human foreskin keratinocytes (NHFKs; passage 3) were analyzed by Western blotting and flow cytometry. (A) Western blots show little evidence of K10 expression, while involucrin expression correlates closely with increasing cell size. (B) Flow cytometry shows that NHFKs that do not carry HPV DNA do not show evidence of an 8N cell population.

served between the small and large cell populations, accompanied by evidence of endoreduplication of host cell DNA.

Normal NHFKs isolated by centrifugal elutriation

Passage 3 NHFKs were separated by centrifugal elutriation and analyzed by Western blotting and flow cytometry (Fig. 5). Little K10 expression was detected, even in the largest cells of the population, while involucrin expression clearly correlated with cell size (Fig. 5A). Flow cytometry demonstrated that, unlike cells harboring HPV DNA, the largest cells did not contain a population with 8N DNA content (Fig. 5B).

DISCUSSION

The approach reported here provides a simplified method for studying increases in HPV episomal copies associated with increased keratinocyte cell size and differentiated state. Keratinocytes differentiate in culture in response to numerous environmental factors that include cell density and cell–cell contact, and growth factor and calcium ion concentration (Fisher, 1994; Dotto, 1999). Differentiation is accompanied by an increase in cell size, differential expression of genes, and alterations in cell morphology and structure. In the present study, cultured keratinocytes maintaining episomal HPV DNA were separated by size using centrifugal elutriation, and real-time PCR was used to measure viral DNA levels with a high degree of precision. Numerous HPV-positive cell lines showed clear evidence of increases in viral DNA copy number associated with increased cell size in monolayer cultures. This size-associated viral DNA increase was an important contributor to total HPV DNA levels in all HPV-positive cell lines examined. Both clonal (W12E, 31NHFK) and nonclonal (HCK18:1B) cells exhibited these properties.

Immunoblotting clearly showed that expression of differentiation markers such as K10 and involucrin was associated with larger cell size and correlated with increased episomal copy number. Interestingly, nonclonal populations of cells (normal human keratinocytes and HCK18:1B) examined by Western blotting expressed low levels of K10, while the clonal cell populations (W12E and 31NHFKs) showed robust K10 expression in the largest cells. These results indicate that the clonality of the culture may contribute to ability to differentiate, and thus indirectly to HPV DNA levels. At present it is not clear if the increases in viral DNA copy number that we observe are analogous to viral DNA amplification associated with keratinocyte differentiation (Howley, 1996). Studies of intact (warts) or reconstituted (organotypic cultures) HPV-positive epithelia have shown that HPV DNA amplification occurs within the viable, suprabasal cell layers, but not in conjunction with expression of a particular marker of differentiation (Stoler *et al.,* 1986, 1992; Beckmann *et al.,* 1985; Ruesch *et al.,* 1998; Jian *et al.,* 1998; Flores *et al.,* 2000). Our results are consistent with these observa-

tions. Expression of different markers of differentiation was variable among the HPV-positive cell lines we examined, and so, not necessarily correlated with increases in HPV episome copy number. On the other hand, our results indicate that cell size correlates very well with increases in HPV DNA episomes.

The state of confluency is known to influence keratinocyte differentiation. Our own studies have shown that confluent cultures exhibit an overall increase in copies of HPV DNA over subconfluent cultures. For this reason, we chose to examine HPV-positive cells harvested from 70% confluent cultures. Within these cultures are spontaneously differentiating keratinocytes that exhibit characteristics of amplifying cells. It is likely that as the state of culture confluence increases that the proportion of differentiating cells, as well as HPV copies per cell, will show an associated increase.

A previous study of HPV31-positive LKP-31-1 cell monolayers identified a small population of the cells that were positive by FISH for HPV31; the number of these HPV-positive cells increased following differentiation of the cells in methylcellulose (Ruesch *et al.,* 1998). Our own FISH experiments have identified a similar, small population of cells in several cell lines (data not shown). However, given that all cells in our cultures are HPV-positive, FISH was not sensitive enough to detect HPV DNA in the vast majority of cells. For this reason, FISH is likely to be greatly underestimating the total number of cells that are increasing HPV copy number in the total culture. The development of more sensitive DNA *in situ* hybridization techniques, coupled with the real-time PCR approaches reported here, should lead to better estimates in the future.

As HPV-positive keratinocytes divide in culture, HPV DNA levels are maintained by replicating the viral DNA. For this reason we always observe viral DNA levels increasing in parallel with cell number for any given passage of cells. These same cells lose episomes over multiple passages, spanning multiple weeks in culture, but the fold gain between small and large cells for both early and late passage cells remains the same. Therefore, we do not believe that loss of copy number in late passage cells is due to loss of keratinocyte differentiation and accompanying HPV amplification. Previous reports have shown that a similar loss of HPV levels correlates with integration of viral DNA in W12E cells (Jeon *et al.,* 1995), and this remains a possibility in our system. Another possibility is that cell culture merely selects for low copy number cells so that loss of HPV DNA occurs in high passage cells.

This study shows that cell size, perhaps the oldest and best known characteristic of the differentiated keratinocyte (Green, 1980), is an excellent predictor of HPV DNA copy number in cell culture. Increases of more than 15-fold in HPV DNA copy number are routinely measured between the smallest and largest cells of the cultures. Evidence for endoreduplication in those cells carrying the highest HPV DNA copy numbers suggests a connection between cell-cycle checkpoint control and HPV replication in these cells. Our findings provide a simplified approach that can be used to study the relationship between HPV viral DNA replication and keratinocyte cell differentiation.

MATERIALS AND METHODS

Cells and cell culture

W12E cell clone 20863 (Jeon *et al.,* 1995) was grown on mitomycin C-treated J2 3T3 cells in media containing three parts Dulbecco's modified Eagle medium (DMEM) and one part F12 media according to a modification of the methods of Rheinwald and Green (1975). Media was supplemented with 0.4 μg/ml hydrocortisone, 10 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenine, 5 μg/ml transferrin, 5 μg/ml 3,3′,5-triiodo-thyronine (T_3), 10 ng/ml epidermal growth factor (EGF), 1% penicillin/streptomycin, and 5% fetal bovine serum (FBS). HCK18:1B cells, a nonclonal, heterogeneous population of HPV18-positive keratinocytes (Meyers *et al.,* 1997), were cultured identically to the W12E cells, but with 10% FBS. All cells were passaged at 70% confluency at a split ratio of 1:5.

An HPV31 episomal cell line (31NHFK) was created according to variations on previously published procedures (Frattini *et al.,* 1997). Passage 2 NHFKs were transfected with full-length HPV31 DNA (ATCC, Rockville, MD) that had been liberated from its pBR322 vector with *Eco*RI, recircularized with T4 DNA ligase (Gibco-BRL), phenol-chloroform extracted, and concentrated by ethanol precipitation. The HPV31 DNA was transfected with LipofectAMINE (Gibco-BRL) at a 5:1 ratio by weight with pSV-neo. After 48 h the transfected cells were passaged and selected for 7 days in 200 μg/ml G418 (Sigma) in antibiotic-free growth media containing 10% FBS. HPV-positive cells were then pooled and cloned, and a HPV31-positive clone (31NHFK-10) containing ~400 episomes per cell was selected for further study.

Elutriation

All cells were harvested at 70% confluency. Approximately 4×10^7 cells were detached with Trypsin–EDTA (0.05% trypsin, 0.53% mM EDTA; Gibco-BRL) and resuspended in 5 ml Ca^{+2} and Mg^{+2}-free phosphate buffered saline (PBS) with 0.1% bovine serum albumin. Cells were loaded into a JE-5.0 elutriation system from Beckman (Palo Alto, CA) at a centrifuge speed of 1500–2000 rpm, and a flow rate of 12 ml/min using 16-gauge silicone tubing with a MasterFlex pump (Cole-Parmer Co., Chicago, IL). Fractions (100 ml) were collected at each increase in flow rate between 16 and 46 ml/min using 2 ml/min incremental increases. Cells were collected by centrifugation, counted, and analyzed by immunoblotting,

flow cytometry, and Taqman PCR. Cell size was routinely monitored by phase contrast microscopy.

Flow cytometry

Flow cytometry was performed as previously described (Garner-Hamrick and Fisher, 1998). Cells were harvested, pelleted, and fixed by resuspension in 90% ethanol. The cells were stained with propidium iodide solution (50 μg/ml propidium iodide, 0.2 mg/ml RNase A) and 10^4 cells analyzed for DNA content on a FACscan flow cytometer (Becton Dickinson, La Jolla, CA).

DNA isolation

Total DNA was isolated by the lysis of cells in DNAzol (Life Technologies, Gaithersburg, MD) followed by ethanol precipitation. Low molecular weight DNA was isolated by the Hirt method (Hirt, 1967). Briefly, cell pellets were lysed in 0.6% SDS with 10 mM EDTA, and NaCl added to a final concentration of 1 M. The samples were stored for 18 h at 4°C and centrifuged, and low molecular weight DNA precipitated from the supernatant by the addition of isopropanol.

DNA blotting

Total W12E cell DNA (50 μg) was digested with *Bam*HI and run on a 0.7% agarose gel. After transfer to Nytran (Schleicher & Scheull, Keene, NH), the DNA was probed with gel-purified full-length HPV16 that had been liberated from pUC19 with *Bam*HI and random primed (Random Primers DNA Labeling Kit, Life Technologies) in the presence of [α-^{32}P]dCTP.

Immunoblotting

Cells were lysed in Laemmli 2× sample buffer (Laemmli, 1970) at 5×10^3 cells/μl. Lysates of 2×10^4 cells were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and electrophoretically transferred to nitrocellulose membranes (NitroPure, Micron Separations, Westborough, MA). Membranes were blocked with Blotto (8% nonfat milk in 20 mM Tris pH 7.5 and 150 mM NaCl) and incubated with either anti-keratin10 (1:1000) or anti-involucrin (1:500) (both from NeoMarkers, Union City, CA) in Blotto for 1 h. The membranes were then rinsed with TENT buffer (50 mM Tris, pH 7.5, 3 mM EDTA, 250 mM NaCl, and 0.2% Tween 20) and incubated with horseradish peroxidase linked anti-mouse IgG (Pierce; 1:10000) in Blotto. Following several washes with TENT buffer, the membranes were developed using ECL reagents (Amersham, Arlington Heights, IL).

Taqman (Real-Time) PCR

Quantitative PCR was performed using real-time PCR technology on the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). All primers were designed using Primer Express 1.0 (Applied Biosystems) software. The probes were labeled with the 5′ reporter dye FAM (6-carboxyfluorescein) and the 3′ quencher dye TAMRA (6-carboxytetramethylrhodamine) (Oligos, Etc., Wilsonville, OR). All PCR primers and probes were designed within the L1 gene:

HPV16:
sense 5′-TGGAGGACTGGAATTTTGGTCTA
antisense 5′-CAATTGCCTGGGTTACAAACC
probe 5′-TCCCCCAGGAGGCACACTAGAAGATACTT

HPV18:
sense 5′-TTTGGTTCAGGCTGGATTGC
antisense 5′-GCAGATGGAGCAGAACGTTTG
probe 5′-TCGCAAGCCCACCATAGGCCC

HPV31:
sense 5′-CCTGCTATTTTGGAAGATTGGAAT
antisense 5′-GGCCTGTGAGGTGACAAACC
probe 5′-CAAGACTATGTGTTAGATTTGCAACCTGAGGCA

A standard curve was generated using 10^8 to 10 copies of purified HPV16/pUC19, HPV18/pBR322, or HPV31/pBR322 DNA per reaction. The following formula was used to calculate total copies of DNA in stock samples: $(1.82 \times 10^{15})(\mu g/\mu l \text{ stock DNA})/(\text{length in base pairs})(2) =$ copies/μl stock DNA. PCR reactions contained a final concentration of 1× Universal Master Mix (Applied Biosystems), 300 nM of each primer (Oligos, Etc.), and 200 nM probe (Applied Biosystems) in a reaction volume of 50 μl. Hirt DNA samples from a known number of cells were analyzed for HPV DNA content in triplicate reactions. The number of HPV DNA copies/cell was determined by multiplying the HPV copies/PCR reaction (determined by the standard curve) by the dilution factor for DNA from the original Hirt prep to get total copies HPV DNA/original sample. The copies/sample were then divided by the number of cells in the sample (counted prior to DNA isolation) to obtain the HPV copies/cell.

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