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Restoration of Telomeres in Human Papillomavirus-Immortalized Human Anogenital Epithelial Cells

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Loss of telomeres has been hypothesized to be important in cellular senescence and may play a role in carcinogenesis. In this study, we have measured telomere length in association with the immortalization and transformation of human cervical and foreskin epithelial cells by the human papillomavirus type 16 or 18 E6 and E7 open reading frames. By using a telomeric TTAGGG repeat probe, it was shown that the telomeres of precrisis normal and E6-, E7-, and E6/E7-expressing cells gradually shortened with passaging (30 to 100 bp per population doubling). Cells that expressed both E6 and E7 went through a crisis period and gave rise to immortalized lines. In contrast to precrisis cells, E6/E7-immortalized cells generally showed an increase in telomere length as they were passaged in culture, with some later passage lines having telomeres that were similar to or longer than the earliest-passage precrisis cells examined. No consistent association could be made between telomere length and tumorigenicity of cells in nude mice. However, of the three cell lines that grew in vivo, two had long telomeres, thus arguing against the hypothesis that cancer cells favor shortened telomeres. Our results indicate that arrest of telomere shortening may be important in human papillomavirus-associated immortalization and that restoration of telomere length may be advantageous to cells with regard to their ability to proliferate.

Normal human cells have a limited lifespan in culture. It is widely believed that progression of cells to malignancy requires an overriding of the natural program of cellular senescence (i.e., immortalization) (9, 32, 45). Much of what is known about the mechanisms of immortalization comes from studies of small DNA viruses that transform human cells. For example, it has been shown that the human papillomavirus (HPV) E6 and E7 proteins bind to and inactivate the p53 and retinoblastoma (Rb) tumor suppressor gene products, respectively (10, 21, 41, 51). Simian virus 40 large T antigen and adenovirus E1A and E1B have similar properties (11, 28, 42, 52). Mutations in these viral proteins that abrogate p53 and Rb binding also affect immortalizing activity (21, 42, 43), indicating that inactivation of the p53 and Rb pathways may be essential for small DNA virus-induced immortalization. Many human cancers that are not associated with viruses contain mutations in p53 and/or Rb (36), suggesting that inactivation of p53 and Rb also plays a role in non-virus-associated transformation. There is evidence, however, that factors other than p53 and Rb are important in the immortalization process. For example, cells transformed by viruses that inactivate p53 and Rb generally go through a crisis before immortalized cell lines are established (45), suggesting that further events are necessary to achieve immortalization.

The hypothesis that telomere loss plays a role in cellular senescence has recently received much attention (12, 17, 37, 53). In all vertebrates, 5 to 15 kb of telomeres, mostly in the form of TTAGGG DNA repeats, are found at the ends of chromosomes (1, 12, 35). Telomeres have been implicated as being involved in DNA replication, nuclear anchoring, and chromosome positioning (12, 55). It has been shown that as normal human cells are cultured in vitro, there is a progressive shortening of telomere sequences (18, 29). Telomeres also shorten as a function of age in cells from normal human blood, skin, and colonic mucosa (2, 19, 30). This shortening occurs because DNA polymerase is unable to completely replicate ends of double-stranded DNA in somatic cells (29, 37). Thus, the telomeres shorten with each round of replication. As a result of this shortening, it is thought that critical genes at the ends of chromosomes either become deleted or are inactivated, thus leading to cell death (29, 37, 53). Alternatively, silent senescence genes could become activated by removal of telomere heterochromatic regions (53). Shortened telomeres may also result in genetic instability. Prior to cell senescence, there is an increase in frequency of chromosome aberrations such as telomere fusion (3, 38). This damaged DNA may cause cells to go into growth arrest and to senesce. Unicellular eukaryotes compensate for telomere loss (13, 27, 44), and it is believed that this is due to the action of telomerase, an enzyme that has the ability to add telomeric sequences de novo (reviewed in reference 4). *Tetrahymena* telomerase has been characterized and is a ribonucleoprotein (13, 14). Some mutations in the RNA component of *Tetrahymena* telomerase result in shortening of telomeres and cell senescence much like is observed for human somatic cells (54). It has been hypothesized that normal human somatic cells do not contain active telomerase, whereas germ line and immortalized cells have activated telomerase and therefore are not subject to telomere shortening (7, 34). For example, human embryonic kidney cells that have been immortalized by simian virus 40 or adenovirus have short but stabilized telomeres, suggesting that telomerase is functioning in these cells (7). Although many cancer cells have shortened telomeres (8, 19, 46), the role of telomere shortening in carcinogenesis remains unclear.

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As part of a study on the genetic instability associated with HPV immortalization, we have measured telomere length in pre- and postcrisis HPV 16 or 18 E6- and E7-expressing human anogenital epithelial cells. Human anogenital epithelial cells are natural hosts of HPV, and HPV 16 and 18 types are both strongly associated with anogenital cancers (20). Our data suggest that immortalization of anogenital epithelial cells by HPV involves activation of a mechanism to elongate telomeres. We have found that HPV-immortalized cells tend to restore their telomeres with passaging in culture, consistent with the hypothesis that longer telomeres are advantageous to proliferating cells.

MATERIALS AND METHODS

Cells and cell culture. Normal human ectocervix (cx) cells from surgical specimens were established in a monolayer culture as described previously (5). A recombinant retroviral system was used to transfect the different HPV genes into the cx cells. The retroviral constructs and the retroviral infection protocol have been described (15, 16, 33). Briefly, the HPV16 E6, E7, or E6/E7 open reading frames were cloned into a murine-based retroviral vector, LXSN, which contains the neomycin resistance gene. The constructs were transfected into the packaging cell line PA317, and recombinant retroviruses in the supernatant were collected. Early-passage cx cells were infected at a high titer with the different retroviral constructs, plated at various densities, selected in G418, and then passaged as pools or ring cloned. Eight ring clones were isolated for each group. Cells were passaged 1:5 when confluent (usually every 4 or 5 days except around crisis, when intervals between passaging were longer). Pre- and postcrisis cells were collected at different passages for DNA extraction. Several of the HPV-immortalized cell lines analyzed in this study have been described previously (24–26). The 18-5, 1811, and FEH cell lines were derived by transfection of human foreskin keratinocytes with the complete HPV 18 genome (24, 25). The FEP-EIL cell line was made by transfection of human foreskin keratinocytes with the HPV 16 genome (26). Uterine smooth muscle cells infected by LXSN 16 E6/E7 and smooth muscle cell culturing conditions have been previously described (39). All epithelial cells were grown in GIBCO keratinocyte serum-free media (GIBCO/BRL, Gaithersburg, Md.).

Tumorigenicity studies and cytogenetic and FACS analyses. Tumorigenicity data for the 1811, FEH, FEP-EIL, and 18-5 cell lines has been described previously (22, 24). Cells were injected subcutaneously into athymic male nude mice at 3×10^6 to 5×10^6 cells per site (one site per mouse). Mice were kept until they formed tumors or until 6 months after injection. Cytogenetic analysis was performed by a slide culture method on G-banded chromosomes as described previously (47). For aneuploidy studies, 50 to 100 spreads were counted. Approximately 10 spreads were examined for more detailed studies. Fluorescence-activated cell sorting (FACS) was performed as previously described (49), with minor modifications, by using a protocol for propidium iodide staining of cell nuclei. Fluorescence of individual nuclei was monitored with a FACScan flow cytometer equipped with an argon-ion laser at 488 nm and 250 mW light output and a Lysis II software (Becton Dickinson Immunocytometry Systems). A total of 10,000 events were collected, and the data were analyzed by Reproman Software (Fine-Facts Software, Seattle, Wash.). Gating was used to exclude the presence of doublets.

DNA extraction and Southern blotting. Genomic DNA was isolated by previously described methods (40). DNA was digested with the appropriate restriction enzyme (GIBCO/BRL) according to the manufacturer's protocol, run on agarose gels, and blotted onto Hybond N+ membranes (Amersham, Arlington Heights, Ill.) as previously described (6). Probes were [^{32}P]CTP random primed by using a random priming kit (Boehringer Mannheim, Indianapolis, Ind.). Blots were hybridized and washed as described previously (6). Hybridized blots were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and also analyzed by phosphoimaging (23).

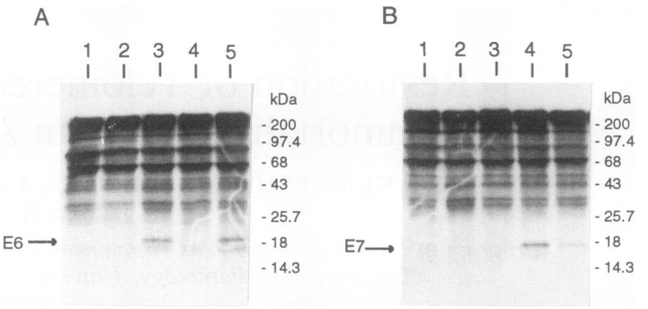

FIG. 1. RIP analysis of HPV 16 E6 and E7 expression in retrovirally transfected cx cells. (A) HPV 16 E6 RIP; (B) HPV 16 E7 RIP. Lanes: 1, normal cx (passage 3); 2, LXSN cx (passage 5); 3, LXSN 16 E6 cx (passage 6); 4, LXSN 16 E7 cx (passage 6); 5, LXSN 16 E6/E7 cx (passage 6). Molecular mass standards are indicated to the right of each panel.

Vectors and probes. The telomeric TTAGGG repeat probe (50) was kindly provided by T. de Lange (University of California, San Francisco). This probe has been used previously by others to measure telomere length (1, 8, 18, 19, 46). The LXSN retroviral vector (33) was obtained from A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, Wash.), and the HPV LXSN constructs (15, 16) were provided by D. Galloway (Fred Hutchinson Cancer Research Center).

RIP. Radioimmunoprecipitations (RIPs) to detect HPV 16 E6 or E7 proteins were done according to described protocols (15, 16) by using rabbit polyclonal antibodies provided by D. Galloway.

RESULTS

Normal cx cells have a short lifespan of approximately 22 to 25 population doublings (PD). In order to analyze telomere length changes in cells that expressed HPV proteins at an early passage, a highly efficient amphotropic retroviral infection system was utilized. By using this system, HPV16 E6, E7, and E6/E7 open reading frames and vector (LXSN) alone were stably transfected into early-passage cx cells. All groups (normal, LXSN, LXSN 16 E6, LXSN 16 E7, and LXSN 16 E6/E7) were analyzed by RIPs early after infection and were found to express the appropriate HPV E6 or E7 proteins (Fig. 1). The different groups of pooled cells senesced or went through crisis at different times. Normal and vector control cells senesced between passages 10 and 11 (22 to 25 PD). HPV16 E6 and E7 alone extended the lifespan of the cells to passage 20 to 23 (45 to 52 PD) and passage 15 to 16 (34 to 36 PD), respectively, but did not immortalize them. LXSN 16 E6/E7 cx cells also had an extended lifespan but went through crisis between passages 18 and 22 (41 to 50 PD). No ring clones from any of the groups became immor-

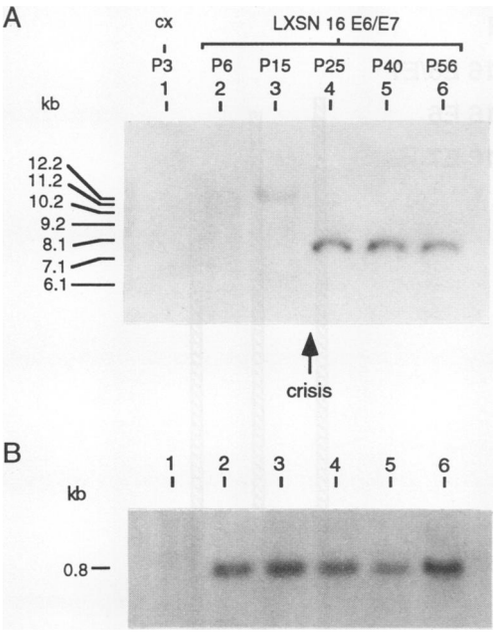

FIG. 2. Analysis of retroviral integration sites in LXSN 16 E6/E7-transfected cx cells. (A) DNA was cut with *Bam*HI, an enzyme which cuts once within the retroviral construct. Digested DNA was run on a 0.8% gel, Southern blotted, and hybridized with a ^{32}P-labelled *Eco*RI-*Bam*HI 0.8-kb E6/E7 fragment of LXSN 16 E6/E7. Lanes: 1, normal cx (passage [P] 3); 2 to 6, LXSN 16 E6/E7 cx cells at the passages indicated. The 16 E6/E7 cells went through crisis between passages 18 and 22. (B) The same DNAs as in panel A were cut with *Eco*RI and *Bam*HI, which cuts twice within the LXSN 16 E6/E7 construct. Digested DNA was analyzed as for panel A. Note that the precrisis E6/E7 signal is similar to the late-passage postcrisis signal, indicating that precrisis cells had on the average one copy of the retroviral construct per cell. Molecular size standards are indicated on the left.

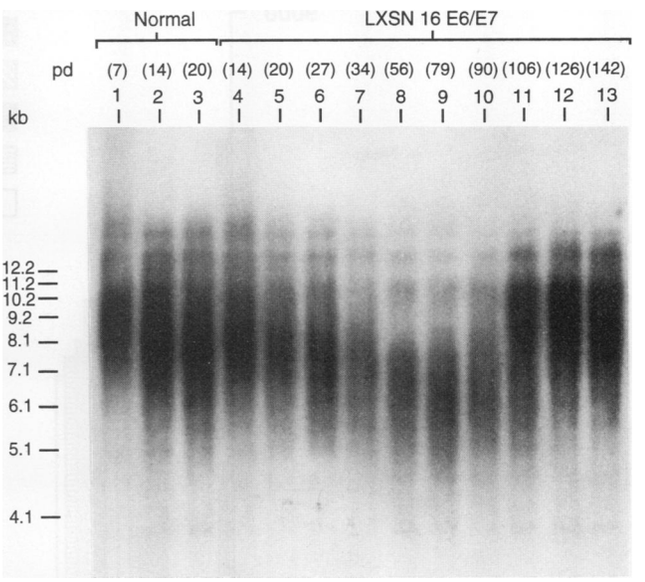

FIG. 3. Telomere length analysis of normal cx and HPV 16 E6/E7-expressing cells at progressive PD. DNAs were digested with *Bgl*II, run on a 0.7% agarose gel, Southern blotted, and hybridized with a ^{32}P-labelled telomeric TTAGGG repeat probe. Lanes: 1 to 3, normal cx cells at the PD indicated; 4 to 13, LXSN 16 E6/E7 cx cells at the PD indicated. LXSN 16 E6/E7 cells went through crisis between 41 and 50 PD (passage 18 to 22). Note that telomere lengths decreased until near crisis, stabilized, and then increased after crisis. Molecular size standards are indicated on the left.

talized, although LXSN 16 E6/E7 clones had extended lifespans of 5 to 15 PD compared with those of clones from other groups. Only the pooled population of LXSN 16 E6/E7 cells gave rise to immortal cells. These grew out from a small number of colonies after crisis. Examination of retroviral integration sites showed that early-passage pooled precrisis HPV16 E6/E7 cells were a mixed population, as indicated by a diffuse signal on the autoradiogram (Fig. 2A, lane 2). A dominant clone began to emerge in later-passage precrisis cells (Fig. 2A, lane 3), but this was not the same clone that survived crisis (Fig. 2A, lanes 4 to 6), suggesting a selective pressure for the latter clone during crisis.

To measure telomere length, cells from the different groups were collected for DNA every three passages before crisis and then at various times for the HPV16 E6/E7 cells that survived crisis. The DNA from the different passages was Southern blotted and probed with labelled TTAGGG repeat telomeric probe. As an example, a blot of normal and LXSN 16 E6/E7 cx cells at different passages is shown (Fig. 3). The telomere signal is a diffuse band which is due to heterogeneity in telomere terminal restriction fragment length resulting from variation in the number of TTAGGG repeats (1). Signals at a higher molecular weight are more intense because there are more copies of the repeats. The constant bands represent nontelomeric DNA which hybridize to the TTAGGG probe (7). It is apparent from the blot that there are differences in average telomere length in cells of different passages. In order to quantitate telomere length, blots were read by phosphoimaging analysis. The resulting peaks (minus the constant band signals) were approximately Gaussian in distribution, and the midpoint of each peak was used as a weighted average of telomere size. The molecular weights were estimated by comparison to standards, and the results from the different cells were plotted (Fig. 4). Telomere loss occurred in all cells as they approached senescence or crisis. This was true regardless of which HPV oncogenes were being expressed and continued in those cells with extended lifespan until crisis. Telomere lengths decreased at approximately 30 to 100 *bp*/PD, which is a rate that is similar to that estimated from other studies on telomere loss in cultured cells (7, 17). Presenescent HPV16 E6/E7 ring clones also showed a similar pattern of telomere length decrease (data not shown). Telomere lengths were not measured during crisis, because there was a substantial decrease in cell numbers during this period. However, 16 E6/E7 cells immediately after crisis had shorter telomeres than 16 E6/E7 cells before crisis, suggesting that further loss occurred during crisis. Telomere length in the immortalized cells showed some stabilization shortly after crisis (Fig. 3 and 4). Interestingly, however, the postcrisis cells of later passage showed an increase in average telomere length. In fact, the average telomere length in the 16 E6/E7 cells at the latest passages examined was similar to that observed in normal cx cells at the earliest passage examined and was relatively stable with passaging (Fig. 4).

To determine whether telomere restoration was a general phenomenon for HPV E6/E7-containing cells, we examined several other HPV-immortalized human keratinocyte cell lines that had been developed in the laboratory. These cells were derived by transfection of the whole HPV 16 or 18

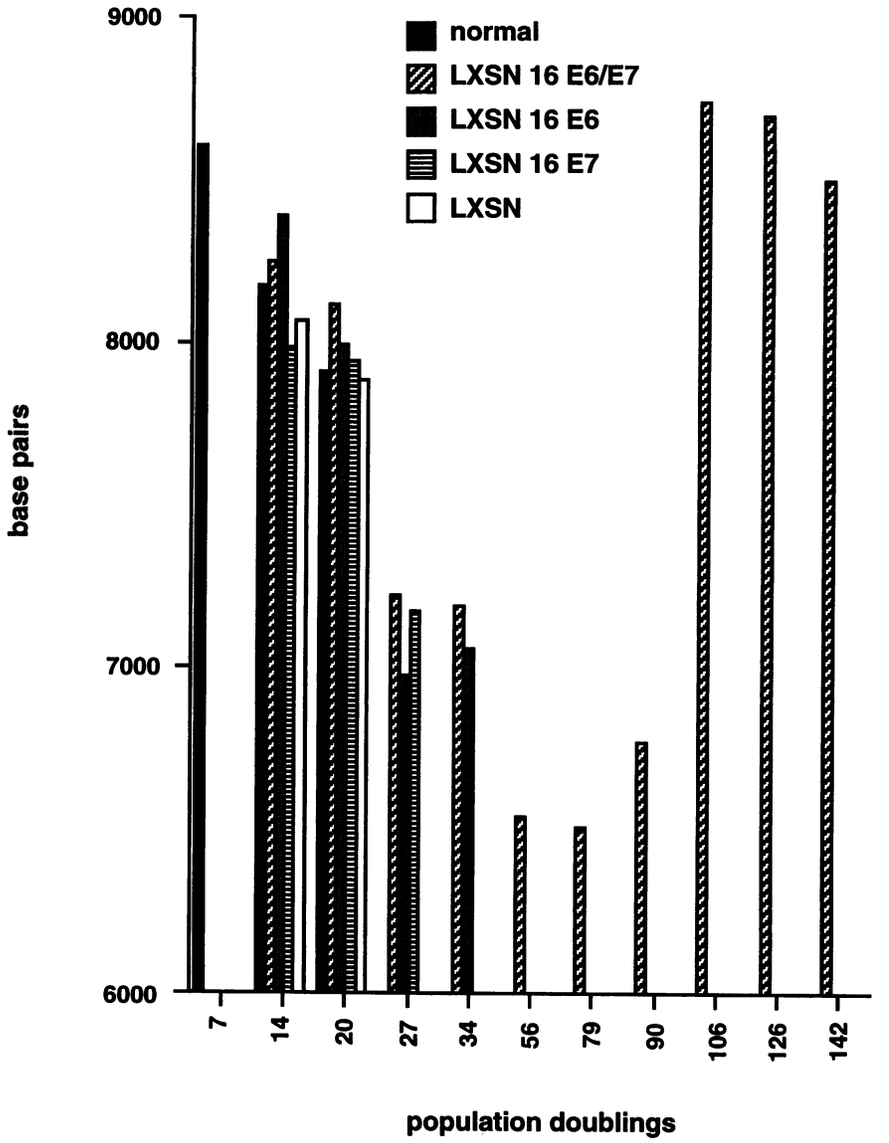

FIG. 4. Determination of average telomere length in normal cx and retrovirally transfected cx cells. Blots of the different groups of cells at progressive PD were hybridized with ^{32}P-labelled TTAGGG probe for in Fig. 3 and were read by a phosphoimaging machine. The average molecular length of the telomeres was determined as described in the text. Cell types are: cx, normal cx cells; LXSN, cx cells infected with vector control; LXSN 16 E6, cx cells infected with a construct containing HPV 16 E6 ORF; LXSN 16 E7, cx cells infected with a construct containing HPV 16 E7 ORF; LXSN 16 E6/E7, cx cells infected with HPV 16 E6 and E7 ORFs. Note that all of the different groups show telomere loss until crisis. Only HPV 16 E6/E7-expressing cells survived crisis and went on to restore telomeres.

genome (24–26). Precrisis cells were not available for these analyses. However, we were able to analyze DNA from early- and late-passage postcrisis cells. All of the cell lines that had short telomeres at early passage showed at least some increase in average telomere length at late passage (Fig. 5 and 6). In two of the cell lines (1811 and FEP-EIL), the net gain in telomere length was relatively large (2 to 4 kb), whereas the 18-5 cell line showed a small increase (0.5 kb). In one cell line (FEH), the telomeres were already apparently quite long at the earliest passage examined, but the intensity of the telomere band was greater at later passage, indicating more copies of telomere repeats (Fig. 5). One possible explanation for this result is that the telomeres of different chromosomes in early-passage FEH may be heterogeneous in length, with most chromosomes having very short telomeres, and therefore giving little signal, and a small subset having telomeres that are long. Both the FEH and the 1811 cell lines had telomere lengths and signal intensities at late passage that were similar to those of early-passage normal cells. Thus, all of the later-passage cell lines showed an increase in telomere length and/or an increase in telomere repeat copy number, suggesting that there is a selective advantage in restoring telomeres. We did not notice a significant change in in vitro growth between early- and late-passage cells, so any advantage from having longer telomeres is likely to be subtle, which may explain why telomere lengthening was not observed until later passages. No correlation could be made between telomere length and in vivo growth (Table 1). However, of the three cell lines that grew in nude mice, two (late-passage 1811 and

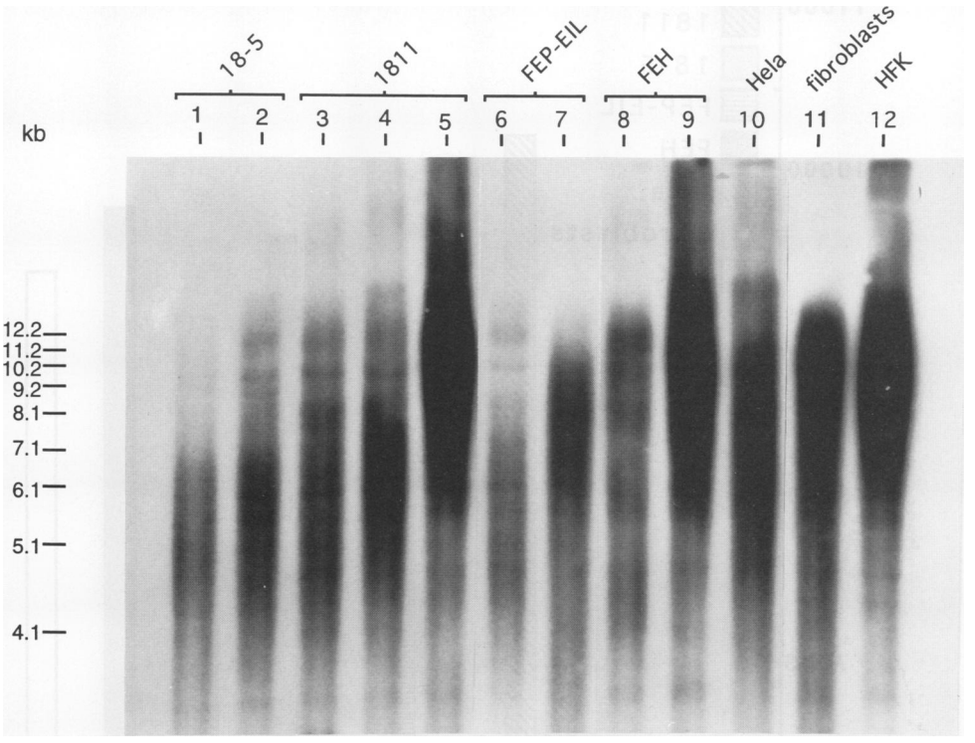

FIG. 5. Telomere length analysis of HPV 16- and 18-immortalized keratinocyte lines at different passages. Precrisis 1811 cells were not available for these analyses, and the indicated passages represent passages after crisis. DNA was analyzed as described in the legend to Fig. 3 and in the text. Lanes: 1, 18-5 (passage 29); 2, 18-5 (passage 81); 3, 1811 (passage 15); 4, 1811 (passage 44); 5, 1811 (passage 104); 6, FEP-EIL (passage 12); 7, FEP-EIL (passage 40); 8, FEH (passage 15); 9, FEH (passage 103); 10, HeLa cells; 11, normal fibroblasts (passage 2); 12, normal human foreskin keratinocytes (HFK) (passage 4). Molecular size standards are indicated on the left.

LXSN 16 E6/E7 cx) had relatively long telomeres, indicating that shortened telomeres are not required for tumorigenic growth.

Because telomere shortening has been hypothesized to play a role in causing genetic instability, we examined precrisis cells from each retrovirally transfected group and early- and late-passage postcrisis LXSN 16 E6/E7 cx cells for chromosome abnormalities. Early-passage precrisis cells from all groups had normal karyotypes with little aneuploidy (8 to 12% for control and experimental groups). FACS analysis verified these findings, showing a relatively normal DNA content profile in E6/E7-expressing cells before crisis (Fig. 7A to C). Cells that expressed E6 or E7 alone or E6 and E7 together began to show aneuploidy near crisis, and early-passage postcrisis E6/E7 cells had a dramatic increase in DNA content (Fig. 7D), with 70 to 80% aneuploidy. Interestingly, later-passage postcrisis cells were also 70 to 80% aneuploid, with a DNA content that was similar to that of early-passage postcrisis cells (Fig. 7E). Thus, there was apparently much genetic instability during crisis but not as much so thereafter.

DISCUSSION

In this report, we have shown that telomeres shorten in normal cervical and HPV 16 E6- and E7-expressing cells as they age in vitro. Expression of E6 or E7 alone extended the lifespan of the cells but did not immortalize them. Cells that expressed both E6 and E7 went through crisis and gave rise to a clonal, immortal cell line with short telomeres. These immortalized cells showed a progressive gain in telomere length with passaging. Other HPV-immortalized human keratinocyte lines also showed lengthening of telomeres at later passages. Our results suggest a model in which several events are necessary for the immortalization of human anogenital epithelial cells. In this model, expression of HPV E6 and E7 proteins, and hence inactivation of both p53 and Rb, respectively, extends the lifespan of cells past a first critical period when normal cells senesce. As evidenced by the proliferative crisis in the E6/E7-expressing cells, at least one more event is required for immortalization. The switch from telomere shortening to telomere elongation in postcrisis cells suggests that activation of a mechanism to restore telomeres is this necessary event. Furthermore, our observation that postcrisis cells of later passage tend to have telomeres longer than those of earlier-passage cells may indicate that restoration of telomeres is favored for in vitro growth.

The results of our studies, using a relevant human cell transformation system, are consistent with the results of Counter et al. (7), in which human embryonic kidney cells immortalized by simian virus 40 or adenovirus type 5 were shown to exhibit a shortening of telomeres before crisis and a stabilization of telomere length after crisis. However, in that study, restoration of telomeres was not observed. In our studies, the 18-5 cell line also did not show much lengthening of telomeres, even after long-term passaging. We have also observed that human uterine smooth muscle cells infected by LXSN 16 E6/E7 exhibited telomere loss before crisis and then stabilization of telomere length at a partially restored

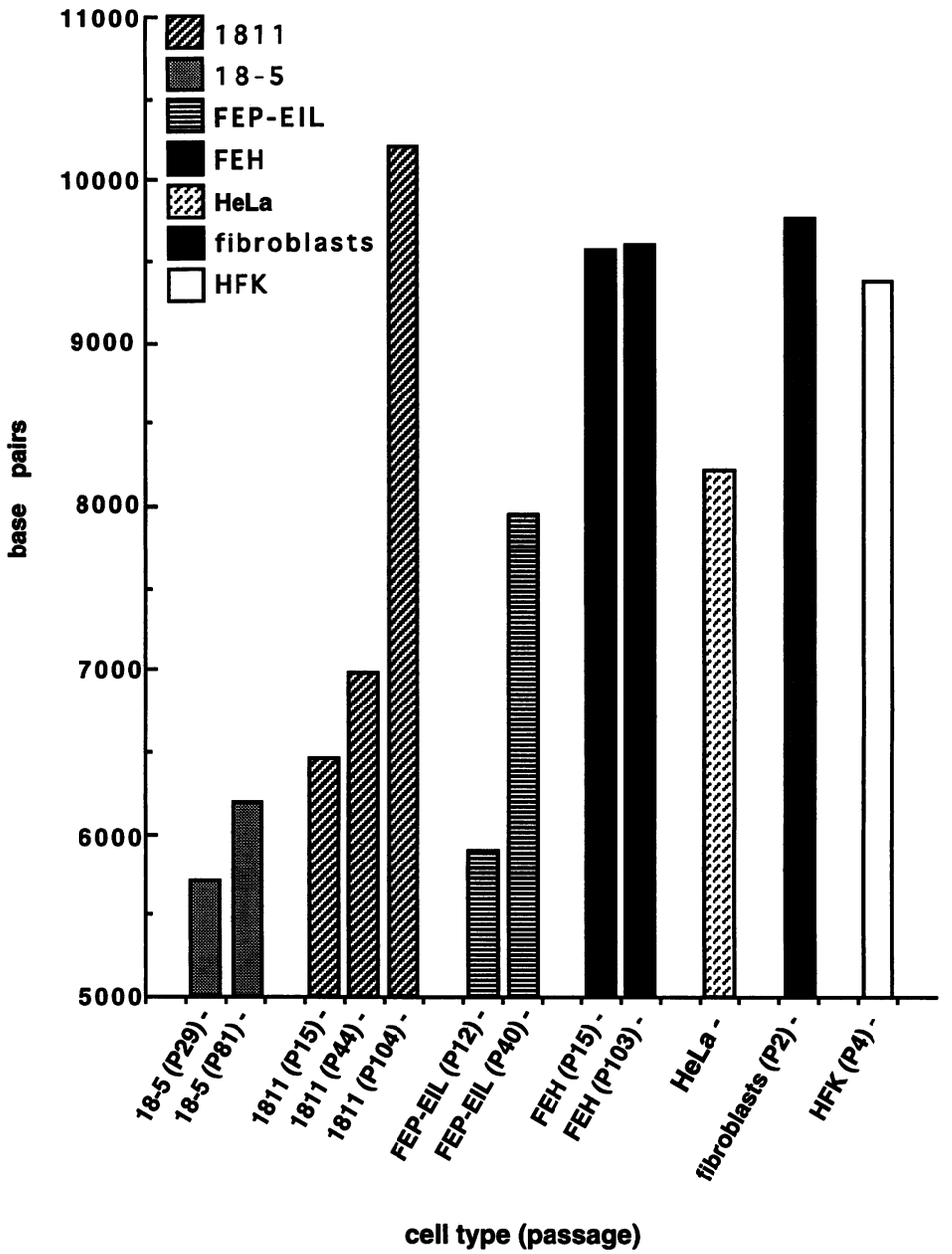

FIG. 6. Telomere length in postcrisis HPV 16- or 18-immortalized human keratinocytes. Average telomere lengths were determined as described in the text. Except for normal cells, passage (P) numbers represent passages after crisis. Cell types: 18-5, HPV 18-immortalized keratinocytes; 1811, HPV 18-immortalized keratinocytes; FEP-EIL, HPV 16-immortalized keratinocytes; FEH, HPV 18-immortalized keratinocytes; HeLa, HPV 18-containing cervical cancer line; normal fibroblasts; normal foreskin keratinocytes.

state (unpublished data). The HeLa cell line, which contains HPV 18, has relatively long telomeres, but some groups have reported variants of HeLa with shorter telomeres (8). Therefore, full telomere restoration does not appear to be necessary for long-term growth but may be favored when it occurs and under certain conditions. Because cells isolated from cancers often show shorter telomeres than normal cells, it is possible that short telomeres are favored for in vivo growth. However, our results would argue against this hypothesis. Although there was no correlation between telomere length and tumorigenicity, two of the three cell lines that were able to grow in vivo had long telomeres. Furthermore, a cell line established from an 1811 tumor also had elongated telomeres (data not shown). One explanation for shorter telomeres in some cancers may be that a mechanism to restore telomeres is only partially active. Alternatively, certain tumors (e.g., benign proliferations) may have not yet activated a mechanism to restore telomeres.

An argument could be made that the longer telomere lengths that we observed in later-passage cells had little to do with selection for cells with longer telomeres but was instead a function of population dynamics. If this were the case, the longer telomeres observed in later-passage cells could be the result of a small population of early-passage postcrisis cells

TABLE 1. Tumorigenicity of early- and late-passage postcrisis HPV-immortalized cell lines

Cell type	Early passage[a]		Late passage[a]	
	Telomere length[b] (bp)	Tumors/ site	Telomere length[b] (bp)	Tumors/ site[c]
LXSN 16 E6/E7 cx	6,540	0/3	8,710	3/3
FEP-EIL	5,860	0/3	8,020	0/3
1811	6,420	0/10	10,400	12/12
18-5	5,660	0/3	6,160	3/3
FEH	9,740	0/3	9,760	0/3

[a] Tumorigenicity data for FEP-EIL, 1811, 18-5, and FEH have been published (22, 24, 26). These cell lines were tested for tumorigenicity within five passages of the earliest and latest passages listed in Fig. 5. LXSN 16 E6/E7 cx was tested at passage 25 (PD 56) and passage 60 (PD 135).

[b] Passages of telomere measurement are within five passages of tumorigenicity testing.

[c] The 18-5 and LXSN 16 E6/E7 cx cell lines gave rise to cysts, whereas 1811 gave rise to aggressive carcinomas.

with long telomeres becoming dominant with long-term passaging in a random fashion, rather than actual restoration in individual cells. However, all five HPV-immortalized epithelial cell lines used in this study were shown to be clonal early after crisis by examination of viral integration sites (48). Furthermore, if telomere length is simply a matter of population dynamics, one would expect some of the cell lines to have, after crisis, long telomeres that get shorter with passaging. This never occurred. All of the later-passage HPV-immortalized cells that we examined had some degree of telomere restoration, with three of five late-passage lines having telomere lengths that were similar to those observed in early-passage precrisis cells. Thus, in our studies, telomere restoration appears to be common for HPV-immortalized epithelial cells and suggests a selective advantage for telomere restoration.

The mechanism of telomere elongation in human cells remains unclear, although HeLa cell extracts have been shown to contain an activity that adds TTAGGG repeats to oligonucleotide primers (34), suggesting that a telomerase-like enzyme is active in HeLa cells. Once a putative human telomerase is cloned, it will be of interest to determine whether and, if so, how the gene is activated in immortalized cells. Furthermore, a functional telomerase gene could be transfected into cells that express HPV E6 and E7 to determine the significance of telomerase activation in immortalization and progression of cells to malignancy.

It has been proposed that shortening of telomeres may actually play a role in causing the genetic instability that leads to genetic alterations that cause cancer (17, 19). On the one hand, telomere shortening could act as a safeguard against too much proliferation by causing cell senescence. On the other hand, telomere shortening could actually assist the transformation process by causing genetic instability. In our studies, chromosome abnormalities occurred near or at crisis, when telomeres were at their shortest length. Late-passage postcrisis cells were not significantly more aneuploid than early-passage postcrisis cells, suggesting that some stability had been attained in cells that had the ability to restore telomeres. It is possible that cells go into crisis because of the gross genetic damage associated with telomere loss and that the appearance of aneuploidy after crisis is the result of rare aneuploid variants that overcome senescence by activating a mechanism to restore telomeres. Recently, loss of p53 function has been implicated as being important in allowing cells with DNA damage to continue through the cell cycle (31). The binding and degradation of

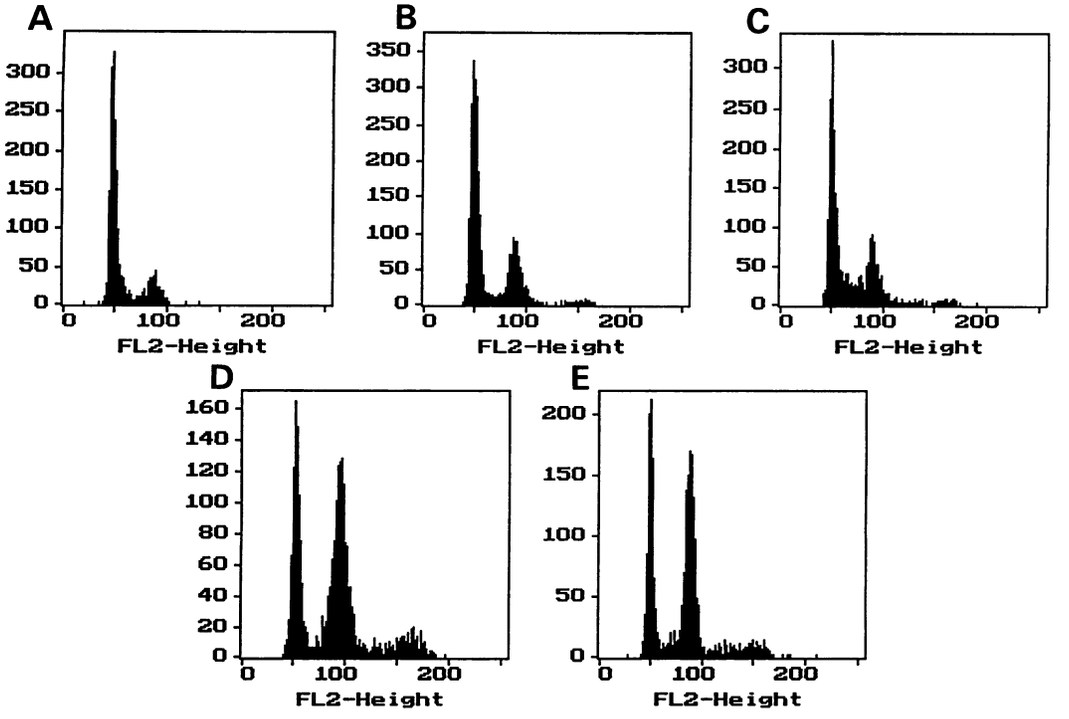

FIG. 7. FACS analysis of propidium iodide-stained nuclei of HPV 16 E6/E7 expression human cervical cells. (A) LXSN vector control, PD 14; (B) precrisis 16 E6/E7, PD 14; (C) precrisis 16 E6/E7, PD 27; (D) postcrisis 16 E6/E7, PD 60; (E) postcrisis 16 E6/E7, PD 126.

p53 by HPV E6 might play a similar role. Further studies are needed to determine the roles of telomere loss and of other factors, such as p53 inactivation, in genetic instability and cellular senescence.

In conclusion, we have shown that telomere restoration may be important in the HPV-induced immortalization of human anogenital epithelial cells. The HPV immortalization system described in these studies may be of further use in understanding the role of telomere loss and restoration in the development of HPV-associated human cancers.

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