The Human Papillomavirus Type 16 E6 and E7 Oncoproteins Dissociate Cellular Telomerase Activity from the Maintenance of Telomere Length*

Hubert Stöpppler‡, Dan-Paul Hartmann‡, Levana Sherman§, and Richard Schlegel¶

From the ‡Molecular Pathology Program, Department of Pathology, Georgetown University Medical Center, Washington, DC 20007 and §Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, 69978 Israel

The “high risk” subgroup of human papillomaviruses (e.g. HPV-16 and HPV-18) infect and induce tumors of mucosal epithelium. These neoplasms, which can progress to malignancy, retain and express the papillomavirus E6 and E7 oncogenes. In vitro, the E6 and E7 proteins associate with the cellular p53 and Rb proteins and interfere with their normal growth-regulatory functions. We report here that primary human keratinocytes transduced with the HPV-16 E6 gene, but not the E7 gene, express significant telomerase activity. However, despite this detectable enzymatic activity, E6-transduced cells continue to shorten their telomeres during in vitro passaging similar to control cells and to cells expressing the E7 and E6+E7 genes. At late passages, however, E7-transduced cells partially restore telomere length, although they lack detectable telomerase activity, demonstrating that E6-independent, telomerase-independent events mediate this change.

The human papillomaviruses (HPVs)1 associated with cervical cancer are designated as the “high risk” subgroup of HPVs (e.g. HPV-16 and 18) (1) and encode two viral oncoproteins, E6 and E7, which exhibit immortalizing and transforming activities in various rodent and human cell types (2–14; reviewed in Refs. 15 and 16). The oncogenic potential of these proteins is due, at least in part, to their ability to interfere with the function of two cellular tumor suppressor proteins, p53 and the retinoblastoma susceptibility gene product, Rb. The E6 protein binds to the cellular p53 protein and promotes its ubiquitin-dependent degradation (17, 18). E7 protein associates with Rb and interferes with its binding to E2F, resulting in impaired Rb cell cycle control functions (19–21). The natural host cell of these viruses, the keratinocyte, is immortalized by the efficient expression of E7 protein (22). Although the E6 protein cannot independently immortalize these cells, it greatly augments the biological activity of the E7 protein (23), induces resistance to signals for terminal differentiation (24), and prolongs precrisis life span (25). Recently, it has been demonstrated that the expression of E6 in primary human keratinocytes leads to an activation of telomerase (26), an enzyme capable of preventing the shortening of telomeres during DNA replication (27–31).

Telomeres, the ends of human eucaryotic chromosomes, are shortened progressively during cell aging in vivo and in vitro (29, 30, 32–34). The telomeric ends of chromosomes consist of a stretch of tandemly repeated sequences complexed with DNA-binding proteins that position chromosomes within the nucleus, protect chromosomal ends, and prevent chromosomal fusion that can occur in late-passage or senescent cells (27, 28, 31). Germ-cell telomeres are longer than somatic cell telomeres and are maintained with age (35–37), probably due to the activated ribonprotein telomerase in these cells (38). In contrast to primary somatic cells, immortalized cells generally retain telomere length during in vitro propagation. The DNA polymerase telomerase, a ribonucleoprotein complex, is capable of elongating the 3' lagging DNA strand by adding tandemly repeated sequences to this DNA strand. The ribonucleoprotein complex uses parts of its RNA molecule as a template for the polymerization of telomeric tandem repeats, counteracting the shortening of telomeres during the replication of DNA by DNA polymerase α.

We observed that early-passage keratinocytes contained detectable telomerase activity that was lost at later passage numbers (between passages 2 and 6). Because both E6 and E7 genes can enhance and prolong human primary keratinocyte growth in vitro, we evaluated whether they might also induce alterations in chromosomal processing and maintain telomeres in a lengthened stage. The analysis of primary human foreskin keratinocytes expressing the HPV-16 E6, E7, or E6+E7 (E6/7) genes revealed that E6- and E6/E7-expressing cells were telomerase-positive, independent of their passage number. E7-expressing cells, like vector-infected control cells, demonstrated an activated telomerase at early-passage numbers that was rapidly lost during cell passaging. The detectable telomerase activity in precrisis E6- and E6/E7-expressing cells, however, did not result in the maintenance of telomere length. All cells, independent of their expression of HPV oncogenes and telomerase, equally shortened their telomeres. However, E7-expressing cells demonstrated partial restoration of telomeric length without the concomitant activation of telomerase during their extended life span.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary human keratinocytes were derived from neonatal foreskins as described (10) and grown in KSF medium (Life Technologies, Inc.) supplemented with gentamycin. The primary cells were infected with derivatives of the amphotrophic LXSN retrovirus expressing various HPV-16 open reading frames (E6, E7, and E6+E7). The retroviruses were generated as described (39) using existing recombinant vectors (24). Retrovirus-infected cells were selected in G418 (100 µg/ml medium) for 10 days. G418-resistant colonies were pooled from each transduction and passed every 3–4 days (ratio of 1:5).

* This research was supported in part by Grant R01CA53371 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Telomerase Shortening in Cells Possessing Telomerase Activity

Telomeric Repeat Amplification Protocol—A modified telomeric amplification protocol (TRAP) was performed as described by Kim et al. (38). Cells were harvested by trypsinization followed by a wash in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum to inactivate trypsin. The cells were washed a second time in phosphate-buffered saline and frozen at −70 °C. Cell pellets (5 × 10^6) were lysed in 800 μl of lysis buffer (0.5% 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate, 10 mM Tris-Cl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 5 mM β-mercaptoethanol, and 10% glycerol) for 30 min on ice. The lysates were then centrifuged for 2 min at 14,000 × g (4 °C), and 600 μl of the supernatant were removed. The protein concentration of the supernatant was determined using a DC Protein Assay kit (Bio-Rad). RNase inhibitor (RNasin from Promega Corp.) was added afterward to a final concentration of 10 units/ml. The remaining nuclear pellet was digested with proteinase K and treated with phenol; chromosomal high molecular weight DNA was isolated by standard techniques for Southern blot analysis. The TRAP assay was performed in a 0.2-ml MicroAmp reaction tube (Perkin Elmer) containing 98 μl of a reaction mixture composed of 1 × Taq polymerase buffer (10 mM Tris-HCl, pH 8.8, 1 mM KCl, and 0.02% Tween 20), 1.5 mM MgCl2, 100 ng of TS primer (5′-AATCCGTCGACGAGTT-3′), 200 μM of each deoxyxynucleoside triphosphate, and 0.05–6 μg of protein extract. The reaction mixture was incubated for 20 min at 25 °C. The samples were then heated to 60 °C, and 1 μl (100 ng/μl) of CX primer (3′-AATCCCATCTCCAGCCATTTCCTCC-5′) and 0.5 μl of Taq polymerase (Promega) DNA polymerase, 6 units/μl (Perkin Elmer) were added before the samples were heated to 94 °C for 90 s. The polymerase chain reaction was performed for 28 cycles under the following conditions: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s. Ten % of the polymerase chain reaction product was separated on 8% SDS-polyacrylamide gel (1 × TBE), and the gel was stained with a Gelcode color silver stain kit (Pierce). To demonstrate telomerase specificity, control samples were either digested for 20 min at 37 °C with 1 μg of RNase A and 1 μg of RNase H or the samples were heated for 10 min to 100 °C prior to the telomerase reaction.

Southern Blot—Three or 10 μg of chromosomal high molecular weight DNA were restricted with the indicated restriction endonucleases for Southern blots (protocols as before). The restriction endonucleases used were HinfI, BglII, and RsaI restriction enzyme (New England Biolabs). Southern blot analysis of telomere length was determined using 3 μg of each deoxyxynucleoside triphosphate and 6 units/μl of Taq polymerase (Promega) DNA polymerase; 6 units/μl (Perkin Elmer) were added before the samples were heated to 94 °C for 90 s. The polymerase chain reaction product was separated on 8% SDS-polyacrylamide gel (1 × TBE), and the gel was stained with a Gelcode color silver stain kit (Pierce). To demonstrate telomerase specificity, control samples were either digested for 20 min at 37 °C with 1 μg of RNase A and 1 μg of RNase H or the samples were heated for 10 min to 100 °C prior to the telomerase reaction.

RESULTS

The E6 and E7 Genes Independently Extend the Life Span of Neonatal Foreskin Keratinocytes—Primary human foreskin keratinocytes were infected with amphotropic retroviruses expressing either the HPV-16 E6, E7, E6/7 genes or the neomycin resistance gene (control). Cells were selected in serum-free keratinocyte medium containing G418 as described under “Experimental Procedures” and passaged at a ratio of 1:5. Keratinocytes infected with control retrovirus ceased proliferation at passages 10–12, whereas those transduced with E6/7 established into cell lines. Keratinocytes expressing either E6 or E7 alone exhibited extended life span and continued to proliferate beyond passage 26.

E6, but not E7, Generates Keratinocyte Cultures with Telomerase Activity—We observed, in three independent experiments, that E6 retroviruses generated human keratinocyte cultures that expressed the active telomerase.
Telomerase Activity Is Maintained in E6-transduced Keratinocytes—We also compared the telomerase activity of retrovirus-infected keratinocytes at passage 26, far past the time when the HPV-negative control cells had undergone crisis and lost their proliferative potential (passage 10). The TRAP assay depicted in Fig. 2A demonstrates that E6-transduced cells continued to display telomerase activity throughout passaging, whereas the vector- and E7-transduced cells were negative.

During Extended Life Span, Telomere Length Is Partially Restored in E7-transduced, but not E6-transduced, Keratinocytes—The expression of the E6 or E7 protein leads to an extended keratinocyte life-span in vitro in comparison to non-infected or vector-infected control cells (25). In one of our experiments, E6-transduced cells proliferated beyond passage 26. Despite the continued presence of telomerase activity in these late-passage cells (Fig. 2A), we observed a further shortening of the mean telomere length to ~5 kb (decreased from ~8.5 kb at passage 10) (Fig. 2B). E6-transduced cells lost an estimated 60–80 base pairs per population doubling. The corresponding E7-expressing cells also demonstrated a similar loss of telomeric length between passages 10 and 21 (Fig. 2B). However, from approximately passage numbers 21 to 26, the E7-transduced keratinocytes showed an increase in telomere length (Fig. 2B), although there was no detectable telomerase activity expression of the E6/7 genes gave similar results. Although low levels of telomerase were observed in control keratinocytes and in E7-expressing cells at early passages (passages 4 and 6), this activity was absent at later passages (passages 8 and 10), presumably reflecting the loss of basal epithelial "stem" cells in the neonatal human foreskin keratinocyte population. The detection of telomerase activity in normal keratinocytes was dependent upon the isolation procedures and the number of cell divisions needed to establish the primary culture and was normally detected up to passages 2–3 but never after passage 6.

Mixing experiments (Fig. 1B) using a telomerase-negative E7 cell extract (Fig. 1A; E7 passage 8) and telomerase-positive cell extracts (Fig. 1A; E6, E6/7 passage 8, and a cell extract from an HPV-16 immortalized keratinocyte line) demonstrated that the loss of TRAP assay activity in E7 extracts was not due to the presence of an inhibitor of telomerase or polymerase activity. Thus, the gradual passage-dependent loss of telomerase activity in E7-expressing cells represents a true loss of enzymatic activity.

Telomere Length Decreases during Early Passages of All Transduced Keratinocytes—To determine whether the observed activation of telomerase in E6-transduced keratinocytes (Fig. 1A) was accompanied by altered processing of the chromosome telomeres, we harvested nuclei from keratinocytes at the indicated passages and performed Southern blotting on isolated cellular DNA with a telomeric probe to evaluate average telomere length (Fig. 1C). Cells were evaluated until passage 10 when the HPV-negative control cells reached crisis. Southern blot analysis demonstrated that regardless of the HPV genes transfected, all cells exhibited shortened telomeres similar to control-transduced cells. During the first 10 passages, the average telomere length decreased from approximately 10 to 8.5 kb, which represents an average loss of 60–80 base pairs per population doubling. This is in agreement with previous studies in primary human fibroblasts (32, 34) and human embryonic kidney cells. The correlation between the loss of keratinocyte growth potential (at 30–40 population doublings) and the shortening of telomeres by 1.5–2.0 kb also resembles the differences in telomere length seen in vivo between fibroblasts from human embryos and late-age individuals (30, 33).

Telomere Shortening in Cells Possessing Telomerase Activity—Telomerase activity was normally detected up to passages 2–3 but never after passage 6. We also compared the telomerase activity of retrovirus-infected keratinocytes with that of primary human fibroblasts (32, 34) and human embryonic kidney cells. The correlation between the loss of keratinocyte growth potential (at 30–40 population doublings) and the shortening of telomeres by 1.5–2.0 kb also resembles the differences in telomere length seen in vivo between fibroblasts from human embryos and late-age individuals (30, 33).

strains with significant telomerase activity at early passage (e.g. passages 4 and 6, Fig. 1A), similar to the recently published results of Klingelhutz et al. (26). The concomitant enzymatic activity in these late-passage cells (Fig. 2A), we observed a further shortening of the mean telomere length to ~5 kb (decreased from ~8.5 kb at passage 10) (Fig. 2B). E6-transduced cells lost an estimated 60–80 base pairs per population doubling. The corresponding E7-expressing cells also demonstrated a similar loss of telomeric length between passages 10 and 21 (Fig. 2B). However, from approximately passage numbers 21 to 26, the E7-transduced keratinocytes showed an increase in telomere length (Fig. 2B), although there was no detectable telomerase activity expression of the E6/7 genes gave similar results. Although low levels of telomerase were observed in control keratinocytes and in E7-expressing cells at early passages (passages 4 and 6), this activity was absent at later passages (passages 8 and 10), presumably reflecting the loss of basal epithelial "stem" cells in the neonatal human foreskin keratinocyte population. The detection of telomerase activity in normal keratinocytes was dependent upon the isolation procedures and the number of cell divisions needed to establish the primary culture and was normally detected up to passages 2–3 but never after passage 6.

Mixing experiments (Fig. 1B) using a telomerase-negative E7 cell extract (Fig. 1A; E7 passage 8) and telomerase-positive cell extracts (Fig. 1A; E6, E6/7 passage 8, and a cell extract from an HPV-16 immortalized keratinocyte line) demonstrated that the loss of TRAP assay activity in E7 extracts was not due to the presence of an inhibitor of telomerase or polymerase activity. Thus, the gradual passage-dependent loss of telomerase activity in E7-expressing cells represents a true loss of enzymatic activity.

Telomere Length Decreases during Early Passages of All Transduced Keratinocytes—To determine whether the observed activation of telomerase in E6-transduced keratinocytes (Fig. 1A) was accompanied by altered processing of the chromosome telomeres, we harvested nuclei from keratinocytes at the indicated passages and performed Southern blotting on isolated cellular DNA with a telomeric probe to evaluate average telomere length (Fig. 1C). Cells were evaluated until passage 10 when the HPV-negative control cells reached crisis. Southern blot analysis demonstrated that regardless of the HPV genes transfected, all cells exhibited shortened telomeres similar to control-transduced cells. During the first 10 passages, the average telomere length decreased from approximately 10 to 8.5 kb, which represents an average loss of 60–80 base pairs per population doubling. This is in agreement with previous studies in primary human fibroblasts (32, 34) and human embryonic kidney cells. The correlation between the loss of keratinocyte growth potential (at 30–40 population doublings) and the shortening of telomeres by 1.5–2.0 kb also resembles the differences in telomere length seen in vivo between fibroblasts from human embryos and late-age individuals (30, 33).

Telomere Shortening in Cells Possessing Telomerase Activity—Telomerase activity was normally detected up to passages 2–3 but never after passage 6. We also compared the telomerase activity of retrovirus-infected keratinocytes with that of primary human fibroblasts (32, 34) and human embryonic kidney cells. The correlation between the loss of keratinocyte growth potential (at 30–40 population doublings) and the shortening of telomeres by 1.5–2.0 kb also resembles the differences in telomere length seen in vivo between fibroblasts from human embryos and late-age individuals (30, 33).

Telomerase Activity Is Maintained in E6-transduced Keratinocytes after E6- or E7-induced “extended life span.” In A, telomerase activity is maintained in E6-transduced extended life span keratinocytes. Keratinocytes expressing E6 or E7 were analyzed at passages 10 and 26 for telomerase activity as described above. At passage 26, keratinocytes expressing E6 remained positive for telomerase, and E7-transduced cells remained negative. Vector-transduced keratinocytes did not proliferate beyond passages 10–12 and could not be evaluated for enzymatic activity. In B, late passage, E7-transduced keratinocytes lengthen telomeric DNA. Keratinocytes expressing E6 or E7 were analyzed for telomere length at passages 21 and 26 as described above. E6-transduced cells continued to lose telomeric DNA until the average telomere length was approximately 5.0 kb. At passage 26, the E7-transduced cells gained telomeric DNA, which was also more heterogeneous in size. The vector-transduced keratinocytes failed to proliferate beyond passage 10, despite containing telomeres (~8.5-kb size; Fig. 1C) that were longer than E6- or E7-transduced cells at passage 21 (~5.0-kb size).

FIG. 2. Telomerase activity and telomere length in keratinocytes after E6- or E7-induced “extended life span.” In A, telomerase activity is maintained in E6-transduced extended life span keratinocytes. Keratinocytes expressing E6 or E7 were analyzed at passages 10 and 26 for telomerase activity as described above. At passage 26, keratinocytes expressing E6 remained positive for telomerase, and E7-transduced cells remained negative. Vector-transduced keratinocytes did not proliferate beyond passages 10–12 and could not be evaluated for enzymatic activity. In B, late passage, E7-transduced keratinocytes lengthen telomeric DNA. Keratinocytes expressing E6 or E7 were analyzed for telomere length at passages 21 and 26 as described above. E6-transduced cells continued to lose telomeric DNA until the average telomere length was approximately 5.0 kb. At passage 26, the E7-transduced cells gained telomeric DNA, which was also more heterogeneous in size. The vector-transduced keratinocytes failed to proliferate beyond passage 10, despite containing telomeres (~8.5-kb size; Fig. 1C) that were longer than E6- or E7-transduced cells at passage 21 (~5.0-kb size).
in these cells (Fig. 2A). In contrast, E6-transduced cells at passage 26 continued to shorten their telomeres. The increase in telomere length of E7-expressing cells might indicate, as observed in other tissue culture systems (40), that the cells have reached immortalization crisis.

The Transduced Keratinocyte Cultures Contain, on Average, at Least One Copy of the HPV Gene Per Cell and Express Telomerase at a Level Equivalent to Clonally Derived, HPV-immortalized Cell Lines—Although all keratinocyte strains were isolated following G418 selection and should consist only of cells transduced with HPV genes, we performed Southern blot analysis of chromosomal DNA from the indicated keratinocyte strains to verify the presence of viral genes. Keratinocytes transduced with E6, E7, or E6/7 were assayed at passages 4 and 10 to determine the average copy number of HPV genes in the cell population. Fig. 3 demonstrates that all keratinocyte strains contained more than one copy of the indicated HPV oncogene per cell.

Telomerase at a Level Equivalent to Clonally Derived, HPV-immortalized Cell Lines—Although all keratinocyte strains were isolated following G418 selection and should consist only of cells transduced with HPV genes, we performed Southern blot analysis of chromosomal DNA from the indicated keratinocyte strains to verify the presence of viral genes. Keratinocytes transduced with E6, E7, or E6/7 were assayed at passages 4 and 10 to determine the average copy number of HPV genes in the cell population. Fig. 3A demonstrates that all keratinocyte strains contained more than one copy of the transduced HPV gene per cell at both early and late passages. This result suggests that the observed shortening of telomeres in E6-transduced cells is not the consequence of inefficient gene transduction or HPV gene instability.

Evidence that most or all of the E6-transduced keratinocytes are expressing telomerase is derived from the comparative analysis of telomerase activity in transduced cells and clonally...
derived cell lines immortalized by either HPV-16 or HPV-18 DNA or by SV40 virus (Fig. 3B). The telomerase activities of the tested HPV-immortalized cell lines were equivalent to the telomerase activity of HeLa cells (data not shown). The extracts of nonimmortalized, transduced cell strains exhibited telomerase activity that was similar to that observed in several independent clonal cell lines, suggesting that the transduced keratinocyte population (like the clonal cell lines) consists mostly of cells expressing telomerase.

**Specificity and Sensitivity of the TRAP Assay**—To demonstrate that the polymerase chain reaction-based TRAP assay was indeed measuring telomerase activity rather than the contamination of cytoplasmic extracts with chromosomal telomeric DNA, we treated the cell extracts with RNase A and H for 20 min at 37 °C or with 100 °C exposure for 10 min. In both cases, telomerase activity was completely abolished, indicating that the riboprotein telomerase was responsible for the observed results (Fig. 4A).

Serial dilution of cell extracts demonstrated that E6-transduced cells had telomerase activity similar to E6/7-expressing cells and that the sensitivity of the TRAP assay was sufficient to detect activity in approximately 100 cells (Fig. 4B). This is consistent with the sensitivity of the assay using established cell lines (e.g. HeLa cells), further supporting our previous findings that the E6-transduced keratinocyte population was composed predominantly of cells expressing telomerase (Fig. 3B).

**DISCUSSION**

The ability of the E6 protein to extend the precritical life span of human keratinocytes and to activate telomerase is in agreement with the recent independent finding of Klingelhütz et al. (26). However, by simultaneously measuring telomere length and telomerase activity during sequential passages, we have demonstrated that the E6-dependent activation of telomerase has no detectable effect on the normal shortening of telomeres during in vitro cell passaging. In addition, we have also demonstrated that E7-transduced cells lengthen telomeres after prolonged passaging (potentially at cellular immortalization) and that this telomere elongation occurred independent of detectable telomerase activity. The finding that E7-transduced cells elongate telomeres without detectable telomerase activity is in agreement with a study of Bryan et al. (41) describing immortal human cell lines without detectable telomerase activity. Although the elongation of telomeres during and after cell crisis normally coincides with a high degree of chromosomal aberrations, keratinocytes immortalized by E6/7 typically display few or limited chromosomal abnormalities (42, 43).

The current study demonstrates that the E6-dependent increase in telomerase activity is rapid and occurs by the time the retrovirus-infected keratinocytes have been selected in G418. However, it is unclear whether this increase in enzymatic activity represents the induction of telomerase, the activation of telomerase, or possibly the selection of telomerase-positive stem cells. Thus, it remains a possibility that, following the infection of primary keratinocytes by the E6 retrovirus, the E6 oncogene selectively augments the growth of telomerase-positive cells. This selection would result in the amplification of a keratinocyte population containing high telomerase activity. It is also possible that all of the primary keratinocytes express telomerase activity and that E6 functions to maintain this phenotype. However, it is believed that telomerase activity is, in general, not present in somatic cells but is restricted to germ-line cells and tumor cells (30, 38).

The telomerase activity detected in E6-transduced keratinocytes is presumed to represent the activity of all cells in the population rather than a minor subpopulation for two reasons: (a) Southern blot analysis indicates that, on the average, each keratinocyte contains at least one copy of the transduced HPV gene(s). Thus, it is likely that most of the transduced cells are expressing E6 and are, therefore, altered in telomerase activity. Unfortunately, however, there are no in situ techniques to evaluate whether E6 protein (or telomerase) is expressed in each cell; and (b) the level of telomerase detected in the G418-selected, E6-transduced keratinocyte population is equivalent to the telomerase level observed in a clonal keratinocyte cell line that was immortalized by E6/7 (Fig. 3).

Although only the E6 protein increases cellular telomerase activity, both E6 and E7 are able to extend the keratinocyte life span in vitro, suggesting that prolongation of keratinocyte proliferation is not necessarily dependent upon telomerase activity. In addition, our findings indicate that telomere length is not the critical determinant for the cessation of keratinocyte proliferation in vitro because E6- and E7-transduced cells have shorter telomeres than control (vector-transduced) cells. Finally, our results demonstrate that increased or sustained telomerase activity is insufficient to induce cellular immortalization because E6-transduced genital keratinocytes do not establish into cell lines.

The experimental finding that E7-transduced cells elongate telomeres after prolonged passaging indicates that there must be E6-independent, telomerase-independent mechanisms for the elongation of telomeres in E7-transduced cells. Our results, therefore, support the findings of Bryan et al. (41), who postulate that a mechanism exists for the elongation of telomeres that is not accompanied by detectable levels of telomerase. It is interesting that the observed lengthening of telomeres in E7-expressing cells appears to coincide with the onset of cell crisis, suggesting a potential role in cell immortalization.

**Acknowledgment**—We thank Dr. Melissa Conrad Stöppler for discussion and critical reading of the manuscript.

**REFERENCES**

1. zur Hausen, H. (1991) Virology 184, 9–13
2. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 463–467
3. Band, V., DeCaprio, J. A., Delmolino, L., Kulesa, V., and Sager, R. (1991) J. Virol. 65, 6671–6676
4. Bedell, M., Jones, K., and Laimins, L. (1987) J. Virol. 61, 3635–3640
5. Chester, P. M., Vousden, K. H., Edmonds, C., and McCance, D. J. (1990) J. Gen. Virol. 71, 449–453
6. Durst, M., Darlievka-Petrusevska, R. T., Boukamp, P., Fusseneg, N. E., and Gissmann, L. (1987) Oncogene 1, 251–256
7. Hawley-Nelson, P., Vousden, K. H., Hubbert, N. L., Lowy, D. R., and Schiller, J. T. (1989) EMBO J. 8, 3905–3910
8. Phelps, W. C., Yee, C. L., Müller, K., and Howley, P. M. (1988) Cell 53, 539–547
9. Pirisi, L., Yasumoto, S., Feller, M., Doniger, J., and DiPaolo, J. A. (1987) J. Virol. 61, 1061–1066
10. Schlegel, R., Phelps, W. C., Zhang, Y. L., and Barbosa, M. (1988) EMBO J. 7, 3181–3187
11. Storey, A., and Banks, L. (1993) Oncogene 8, 919–924
12. Vousden, K. H., Doniger, J., DiPaolo, J. A., and Lowy, D. R. (1988) Oncogene 3, 167–175
13. Yasumoto, S., Burkhartt, A., Doniger, J., and DiPaolo, J. A. (1986) J. Virol. 57, 572–577
14. Kanda, T., Watanabe, S., and Yoshikie, K. (1988) Virology 165, 321–325
15. Mansur, C. P., and Androphy, E. J. (1993) Biochem. Biophys. Acta 1155, 323–345
16. Stöppler, H., Conrad Stöppler, M., and Schlegel, R. (1994) Intervirology 37, 168–179
17. Scheffner, M., Werness, B. A., Huijbregtse, J. M., Levine, A. J., and Howley, P. M. (1990) Cell 63, 1129–1136
18. Werness, B. A., Levine, A. J., and Howley, P. M. (1990) Science 249, 76–79
19. Heck, D. V., Yee, C. L., Howley, P. M., and Munger, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4442–4446
20. Dyson, N., Howley, P. M., Munger, K., and Harlow, E. (1989) Science 243, 934–937
21. Dyson, N., Guida, P., Knüfer, M., and Harlow, E. (1992) J. Virol. 66, 6592–6602
22. Halbert, C. L., Demers, G. W., and Galloway, D. A. (1991) J. Virol. 65, 473–478
23. Barbosa, M. S., and Schlegel, R. (1989) Oncogene 4, 1529–1532
24. Sherman, L., and Schlegel, R. (1996) J. Virol. 70, 3269–3279
25. Klingelhütz, A. J., Barber, S. A., Smith, P. P., Dyer, K., and McDougall, J. K.
26. Klingelhoitz, A. J., Foster, S. A., and McDougall, J. K. (1996) Nature 380, 79–82
27. Blackburn, E. H., and Szostak, J. W. (1984) Annu. Rev. Biochem. 53, 163–194
28. Blackburn, E. H. (1991) Nature 350, 569–573
29. Lindsey, J., McGill, N. I., Lindsey, L. A., Green, D. K., and Cooke, H. J. (1991) Mutat. Res. 256, 45–48
30. Harley, C. B. (1991) Mutat. Res. 256, 271–282
31. Zakian, V. A. (1989) Annu. Rev. Genet. 23, 579–604
32. Harley, C. B., Futcher, A. B., and Greider, C. W. (1990) Nature 345, 458–460
33. Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K., and Allshire, R. C. (1990) Nature 346, 866–868
34. Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10114–10118
35. Allshere, R. C., Dempster, M., and Hastie, N. D. (1989) Nucleic Acids Res. 17, 4611–4627
36. Cross, S. H., Allshire, R. C., McKay, S. J., McGill, N. I., and Cooke, H. J. (1988) Nature 338, 771–774
37. de Lange, T., Shiue, L., Myers, R. M., Cox, D. R., Naylor, S. L., Killery, A. M., and Varmus, H. E. (1990) Mol. Cell. Biol. 10, 518–527
38. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Hu, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. J. (1994) Science 266, 2011–2015
39. Miller, A. D., and Rosman, G. J. (1989) BioTechniques 7, 980–990
40. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. (1992) EMBO J. 11, 1921–1929
41. Bryan, T. M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R. R. (1995) EMBO J. 14, 4240–4248
42. Pei, X. F., Qin, N. G., Meck, J. M., and Schlegel, R. (1994) Pathobiology 62, 43–52
43. Popescu, N., and DiPaolo, J. A. (1990) Cancer Res. 50, 1316–1323