
Telomerase Suppression by Chromosome 6 in a Human Papillomavirus Type 16-Immortalized Keratinocyte Cell Line and in a Cervical Cancer Cell Line

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Background: High-risk human papillomavirus (HPV) types play a major role in the development of cervical cancer *in vivo* and can induce immortalization of primary human keratinocytes *in vitro*. Activation of the telomere-lengthening enzyme telomerase constitutes a key event in both processes. Because losses of alleles from chromosome 6 and increased telomerase activity have been observed in high-grade premalignant cervical lesions, we analyzed whether human chromosome 6 harbors a putative telomerase repressor locus that may be involved in HPV-mediated immortalization. **Methods:** Microcell-mediated chromosome transfer was used to introduce chromosomes 6 and 11 to the *in vitro* generated HPV type 16 (HPV16)-immortalized keratinocyte cell line FK16A and to the *in vivo* derived HPV16-containing cervical cancer cell line SiHa. Hybrid clones were analyzed for growth characteristics, telomerase activity, human telomerase reverse transcriptase (hTERT) and HPV16 E6 expression, and telomere length. FK16A hybrid clones were also transduced with an hTERT-containing retrovirus to examine the effect of ectopic hTERT expression on growth. Statistical tests were two-sided. **Results:** Introduction of human chromosome 6 but not of chromosome 11 to both cell lines yielded hybrid cells that demonstrated crisis-like features (i.e., enlarged and flattened morphology, vacuolation, and multinucleation) and underwent growth arrest after a marked lag period. In the chromosome 6 hybrid clones analyzed, telomerase activity and hTERT messenger RNA (mRNA)

expression were statistically significantly reduced compared with those in the chromosome 11 hybrid clones (for telomerase activity, $P = .004$ for the FK16A hybrids and $P = .039$ for the SiHa hybrids; for hTERT mRNA expression, $P = .003$ for the FK16A hybrids). The observed growth arrest was associated with telomeric shortening. Ectopic expression of hTERT in FK16A cells could prevent the telomeric shortening-based growth arrest induced by chromosome 6. **Conclusions:** Chromosome 6 may harbor a repressor of hTERT transcription, the loss of which may be involved in HPV-mediated immortalization. [J Natl Cancer Inst 2001;93:865–72]

Infection with high-risk human papillomavirus (HPV) types has been implicated as the most important risk factor for the development of cervical cancer (1). Moreover, high-risk HPV types can induce immortalization of primary human epithelial cells *in vitro*, via a multistep process (2,3). In the first step, the oncoproteins E6 and E7 encoded by high-risk HPV types inactivate p53 and Rb, respectively, resulting in an extended, although still limited, lifespan. Eventually, these cells enter a so-called crisis period, from which immortal cells emerge at a low frequency (4), a phenomenon referred to as bypass of crisis. There is strong evidence that a recessive alteration(s) within the host-cell genome underlies the bypass of crisis (5).

A potentially important key event in the immortalization process is the arrest of telomeric shortening, a process that is thought to act as a molecular clock that controls the replicative lifespan of normal

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somatic cells. One mechanism of telomere stabilization in immortal cells is the activation of the telomere-lengthening enzyme telomerase (6,7). Studies (8–11) have shown that messenger RNA (mRNA) expression of the telomerase catalytic subunit (i.e., human telomerase reverse transcriptase [hTERT]) is the rate-limiting determinant of telomerase activity. In addition, ectopic expression of hTERT has been found to avert crisis in precrisis HPV-transformed keratinocytes [(12); Steenbergen RD: unpublished observations]. This finding indicates that deregulation of hTERT mRNA expression is sufficient for epithelial cells harboring transcriptionally active high-risk HPV to acquire an immortal phenotype.

The importance of telomerase activation in HPV-mediated carcinogenesis is underlined by the observation that hTERT mRNA expression is increased and telomerase activity is detectable in the majority of cervical carcinomas and in a subset of high-grade premalignant cervical lesions (13,14). Because allelic losses of chromosomes 3p and 6 are the most common genetic alterations observed in high-grade premalignant lesions of the cervix (15–17), these chromosomes may harbor candidate hTERT suppressor loci. *In vitro* studies (18,19) have mapped a candidate telomerase repressor gene(s) to chromosome 3p in a renal cell carcinoma cell line and in a breast carcinoma cell line. However, analysis of premalignant cervical lesions revealed no exclusive association between allelic loss at 3p and telomerase activity (van Duin M, Walboomers JM, Steenbergen RD, Meijer CJ, Snijders PJ: unpublished observations). Thus, there may exist alternative mechanisms, independent of 3p, for telomerase deregulation in (pre)malignant cervical lesions.

In this study, we set out to analyze the other chromosome that is frequently affected in (pre)malignant cervical lesions (i.e., chromosome 6) for potential telomerase-suppressive effects. To this end, we subjected both the well-characterized HPV type 16 (HPV16)-immortalized keratinocyte cell line FK16A (7) and the HPV16-containing cervical cancer cell line SiHa to microcell-mediated chromosome transfer. We introduced chromosome 6 and chromosome 11 in both cell lines and analyzed telomerase activity and growth arrest. We also examined whether growth arrest could be precluded by ectopic expression of hTERT complementary DNA (cDNA).

MATERIALS AND METHODS

Cell Lines

The cell line FK16A was established by transfection of primary human foreskin keratinocytes (EK94–2) with the entire HPV16 genome. Immortalization of these cells was found previously to be associated with an arrest of telomeric shortening and activation of telomerase (7). The cells were initially grown in serum-free keratinocyte growth medium (Life Technologies, Inc., Breda, The Netherlands) supplemented with bovine pituitary extract (50 µg/mL), epidermal growth factor (5 ng/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) (all from Life Technologies, Inc.) and were then adapted to growth in Dulbecco's modified Eagle medium (DMEM)/Ham's F12 medium (3:1) (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), and hydrocortisone (0.4 µg/mL) (all from Life Technologies, Inc.). The cervical carcinoma cell line SiHa was obtained from the American Type Culture Collection (Manassas, VA) and was cultured in DMEM supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM).

Cells were harvested from subconfluent cultures by trypsinization and washed with phosphate-buffered saline (PBS), and cell pellets were collected by centrifugation at 400g for 5 minutes at room temperature. DNA was isolated by use of the Puregene DNA isolation kit (Biozym, Landgraaf, The Netherlands), RNA was isolated by RNAzol B (Tel-Test Inc., Friendswood, TX), and protein was extracted for telomeric repeat amplification protocol (TRAP) analysis as described previously (7).

Microcell-Mediated Chromosome Transfer

Microcell-mediated chromosome transfer was performed essentially as described by Killary and Fournier (20), with some modifications. Mouse A9 donor cells containing one copy of human chromosome 6 or chromosome 11 tagged with a hygromycin B resistance gene fused to a retroviral thymidine kinase gene (A9-Hytk6 and A9-Hytk11) were used as donor cells (21). Microcells were generated by treating mouse A9 donor cells (9×10^6 cells) with 0.075 µg/mL of Colcemid (Sigma Chemical Co., St. Louis, MO) for 48 hours followed by an incubation with 5 µg/mL of cytochalasin B (Sigma Chemical Co.) for 30 minutes at 37°C. The microcells were filtered once through 8-µm filters and twice through 5-µm filters, pelleted at 4500 rpm for 5 minutes at room temperature, and resuspended in 3-mL of DMEM containing 100 µg/mL of phytohemagglutinin. Recipient cells (2×10^6 cells) were washed three times with DMEM and incubated for 25 minutes at 37°C with the microcell suspension. Unattached microcells were then removed by aspiration, and the recipient cells were incubated for 1 minute with a 47% polyethylene glycol-1500 solution and then washed three times with DMEM. Microcell hybrids were selected 48 hours after fusion by culturing in the presence of 150 and 300 µg/mL hygromycin B (Roche Diagnostics, Almere, The Netherlands) for FK16A and SiHa, respectively. After 2–3 weeks of hygromycin selection, colonies

were picked by use of cloning cylinders. The isolated hybrid clones were grown continuously in the presence of hygromycin B. Individual clones were designated as MCF (microcell fusion) followed by experiment number and clone number, e.g., MCF 15–1.

Microsatellite Polymerase Chain Reaction

Polymerase chain reaction (PCR) for polymorphic microsatellite loci was performed as described previously (22) by use of the informative markers D6S105 (located at 6p22) for analysis of chromosome 6 hybrids and D11S860 (located at 11p15) for analysis of chromosome 11 hybrids. Primer sequences were obtained from the Genome Database (The Johns Hopkins University, Baltimore, MD).

PCR products were combined with a commercial-size standard (GS350 Rox; Applied Biosystems [ABI]–Perkin-Elmer Corp., Foster City, CA), run on a 6% polyacrylamide gel, and analyzed by use of an ABI 373 XL sequencer and Genescan 3.1 software (Applied Biosystems–Perkin-Elmer Corp.).

Reverse Transcription–PCR

Reverse transcription–PCR (RT–PCR) was performed as described previously (14) by use of primers specific for hTERT (14) and primers specific for the HPV16 E6 gene (7). The latter primers amplify HPV16 E6*1, a spliced form of E6, in addition to full-length E6. To ensure the quality of the target RNA, we carried out RT–PCR by use of primers specific for the U1 small nuclear ribonucleoprotein-specific A protein (snRNP U1A). This target also served as a reference for the semiquantitative assessment of hTERT and HPV16 E6 mRNA levels. Semiquantitative assessment of mRNA levels was performed by RT–PCR for 28 cycles on 50 ng of RNA as described previously (14). RT of all three targets was performed in a single reaction, followed by separate PCR reactions for each target, as described previously (14).

To avoid amplification of residual genomic DNA, primers flanking splice sites within the genes were used and reactions without RT added during cDNA synthesis were included. Moreover, because the presence of hTERT cDNA in the hTERT-transduced cells can interfere with the quantification of hTERT expression, the purified RNA was pretreated with RQ1DNase (Promega Corp., Leiden, The Netherlands) to remove residual DNA.

To quantify RNA expression, RT–PCR products were separated on a 1.5% agarose gel and transferred to nylon filters (Genescreen; Du Pont NEN, Boston, MA), and the filters were hybridized with an hTERT-specific oligonucleotide probe (14), an HPV16 oligonucleotide probe (5'-GTATTAAGTGTCAAAAGCCACTGTGTCCTG-3'), or an snRNP U1A-specific oligonucleotide probe (14). Signals were quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). mRNA expression levels in the hybrid clones were normalized to the levels measured in parental cells, i.e., FK16A and SiHa, respectively, according to the following formula: intensity ratio (hTERT or E6/snRNP U1A) of hybrid clones/intensity ratio (hTERT or E6/snRNP U1A) of parental cells \times 100%.

Telomerase Assay

Proteins extracted from each clone at early passage (i.e., within 15 population doublings after

microcell fusion, when cells were still proliferating) were used for telomerase assays. Telomerase activity was analyzed on 500 ng of protein extract by use of the TRAP-eze telomerase detection kit (Intergen, Purchase, NY). This kit includes a 36-base-pair (bp) internal DNA standard for assessment of PCR inhibitors, and the internal DNA standard serves as a reference for quantitation of telomerase activity. To determine whether the obtained signals were specific for telomerase, telomerase activity was also assayed after heat inactivation of the protein extract for 10 minutes at 70 °C. No signals were obtained from heat-inactivated protein. For the majority of samples, reactions were performed in duplicate or triplicate, and the mean values were taken to quantify telomerase activity.

Telomerase activity was quantified by measuring signal intensities of telomerase products by use of a PhosphorImager (Molecular Dynamics). Signal intensities obtained with the parental cells were set to 100%. The percentage of telomerase activity in hybrid clones was calculated as follows: intensity ratio (telomerase/internal standard) of hybrid clone/intensity ratio (telomerase/internal standard) of parental cells \times 100%.

Southern Blot Analysis of Telomere Length

To determine telomere length, Southern blot analysis was performed as described previously (23), with minor modifications. Purified DNA (2.5 μ g) was digested with *RsaI* and *HinfI* (Roche Diagnostics) and separated on a 0.7% agarose gel. DNA was transferred to a nylon filter (Genescreen; Du Pont NEN), and the filter was hybridized to a radiolabeled telomeric repeat-specific oligonucleotide probe (TTAGGG)₄.

Staining for Stress-Associated β -Galactosidase Expression

Cells were stained for stress-associated β -galactosidase (SA β -gal) expression according to the protocol of Dimri et al. (24). Cells were stained at one or two time points within 15 population doublings following microcell fusion. In some cases, cells were stained directly on the tissue culture plate; in other cases, 50–500 cells of the hybrid clones were kept aside during passaging and cultured overnight on a glass slide. The cells were washed with PBS, fixed for 5 minutes at room temperature with 2% formaldehyde/0.2% glutaraldehyde, washed again in PBS, and incubated overnight at 37 °C (in the absence of CO₂) with fresh SA β -gal stain solution (i.e., 1 mg/mL of X-gal [5-bromo-4-chloro-3-indolyl β -D-galactoside] in 40 mM citric acid/sodium phosphate at pH 6.0, with 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride). The cells were then washed with PBS and embedded in aquamount. Hybrid clones were scored as being SA β -gal positive when 5% or more of the cells stained for SA β -gal. This cutoff level was chosen because SA β -gal-positive cells were occasionally seen in the parental cells but always at a proportion of less than 5%.

Construction of Retroviral hTERT Vector and Retroviral Transductions

The retroviral hTERT construct (LZRS-hTERT-IRES [internal ribosomal entry site]-GFP [green

fluorescence protein]) and empty retroviral vector (LZRS-linker-IRES-GFP) have been described previously (25). Expression of these vectors was monitored by expression analysis of the GFP, which is cloned downstream of an IRES. Helper-free retrovirus supernatants were produced after CaPO₄-mediated transfection of vectors in the amphotropic producer cell line Phoenix-Ampho [(26); from Dr. G. P. Nolan, Stanford University School of Medicine, CA]. The hTERT construct was competent in activating telomerase in primary human keratinocytes and in HPV-transformed precancerous keratinocytes (Steenbergen RD: unpublished observations). In addition, this construct induces immortalization of T cells (25).

Both FK16A and FK16A/Hyg 6 cells were transduced overnight with the retrovirus supernatants in the presence of 10 μ g/mL of Dotap (Boehringer Mannheim GmbH, Mannheim, Germany). Average transduction efficiencies were approximately 50%. GFP-positive (i.e., transduced) cells were isolated by fluorescence-activated cell sorting.

Statistical Analysis

Fisher's exact test (two-sided) was used to assess the significance of differences in phenotypic alterations induced by chromosome 6 versus chromosome 11 and by hTERT versus vector-only transductants. Differences were considered to be statistically significant at $P < .05$.

RESULTS

Effects of Chromosomes 6 and 11 on Growth and Crisis-Like Features in Immortal FK16A Cells

With the use of a microcell-mediated chromosome transfer, hygromycin-tagged human chromosomes 6 and 11 were introduced separately into the HPV16-immortalized keratinocyte cell line FK16A, which was shown previously to express functional telomerase (7). Chromosome 11 was used as a control based on the observation that introduction of chromosome 11 in cervical cancer cells suppressed tumorigenicity but did not affect the immortal phenotype (27,28).

Two independent rounds of chromosome 6 transfer resulted in 56 hygromycin-resistant colonies, designated FK16A/Hyg6, 49 of which were picked. After transfer of chromosome 11, seven hybrid clones, designated FK16A/Hyg11, were picked. In 27 of 28 FK16A/Hyg6 and in two of two FK16A/Hyg11 hybrid clones analyzed, an additional allele derived from the introduced chromosome could be detected by use of microsatellite PCR.

A summary of all data obtained from the hybrid clones is shown in Table 1. Twenty-four (49%) of 49 FK16A/Hyg6 clones showed a complete growth arrest on passaging compared with only one (14%) of seven FK16A/Hyg11 clones

($P = .116$). All clones showing a complete growth arrest had the morphologic characteristics of cells in crisis, such as enlarged and flattened morphology, vacuolation, and multinucleation. Ten of the 24 FK16A/Hyg6 clones that ceased growing proliferated for five to 15 population doublings before the onset of a growth arrest; the remaining 14 clones underwent growth arrest after 20–30 population doublings. The median number of population doublings before complete growth arrest was 21 (interquartile range, 11–26 population doublings). The single FK16A/Hyg11 clone that showed a growth arrest underwent only five population doublings before growth retardation became manifest.

The fact that the difference in the frequency of FK16/Hyg6 and FK16/Hyg11 clones showing growth arrest was not statistically significant most likely reflects the outgrowth of immortal revertants in growth-retarded populations of FK16A/Hyg6 cells. In 23 (92%) of the 25 FK16A/Hyg6 clones in which no complete growth arrest was seen, crisis-like characteristics were initially observed, but later passages showed an outgrowth of immortal cells. By contrast, no crisis-like characteristics were observed in the six FK16A/Hyg11 clones that failed to show a complete growth arrest. Thus, altogether 96% (47 of 49) of FK16A/Hyg6 clones versus 14% (one of seven) of FK16A/Hyg11 clones showed crisis-like characteristics ($P < .001$).

Expression of the SA β -gal marker can also provide an indication of cells in crisis that undergo growth arrest. Forty-three FK16A/Hyg6 hybrid clones and seven FK16A/Hyg11 clones were analyzed for the SA β -gal marker; 21 (49%) and one (14%), respectively, were scored as positive for this marker ($P = .117$). The lack of a statistically significant difference may reflect the fact that cells were stained for SA β -gal expression at only one or two time points within 15 doublings following microcell fusion. Indeed, an additional 47% (20 of 43) of the FK16A/Hyg6 clones that were SA β -gal negative at the time of staining showed crisis-like characteristics at later passages. Therefore, 95% (41 of 43) of the FK16A/Hyg6 clones versus 14% (one of seven) of the FK16A/Hyg11 clones that were analyzed for both parameters showed crisis-like features and/or SA β -gal staining ($P < .001$).

These data indicate that, in contrast to chromosome 11, chromosome 6 induces

Table 1. Summary of results* on FK16A and SiHa hybrid clones† containing chromosome 6 or 11

Parameter analyzed	FK16A/Hyg6	FK16A/Hyg11	<i>P</i>	FK16A/vector/Hyg6	FK16A/hTERT/Hyg6	<i>P</i>
No. of clones picked	49	7		12	14	
Complete growth arrest	49% (24 of 49)	14% (1 of 7)	.116	58% (7 of 12)	0% (0 of 14)	.001
Crisis-like features	96% (47 of 49)	14% (1 of 7)	<.001	83% (10 of 12)	0% (0 of 14)	<.001
SA β-gal-positive staining‡	49% (21 of 43)	14% (1 of 7)	.117	75% (6 of 8)	0% (0 of 12)	.001
Reduction in telomerase activity§	81% (26 of 32)	0% (0 of 4)	.004	100% (3 of 3)	17% (2 of 12)	
Reduction in hTERT mRNA expression	86% (18 of 21)	0% (0 of 4)	.003	100% (3 of 3)	0% (0 of 5)	
Telomere length decline¶	90% (9 of 10)	ND		ND	0% (0 of 3)	
No reduction in HPV16 E6 mRNA	100% (21 of 21)	100% (4 of 4)		100% (3 of 3)	100% (5 of 5)	
Microsatellite PCR (additional chromosome 6 or 11)≠	96% (27 of 28)	100% (2 of 2)		100% (3 of 3)	100% (7 of 7)	
	FK16A/Hyg6/vector	FK16A/Hyg6/HTERT	<i>P</i>	SiHa/Hyg6	SiHa/Hyg11	<i>P</i>
No. of clones picked	1	1		34	20	
Complete growth arrest	100% (1 of 1)	0% (0 of 1)	.500	25% (7 of 28)	0% (0 of 20)	.032
Crisis-like features	100% (1 of 1)	0% (0 of 1)	.500	61% (17 of 28)	10% (2 of 20)	<.001
SA β-gal-positive staining‡	100% (1 of 1)	0% (0 of 1)	.500	50% (12 of 24)	ND	
Reduction in telomerase activity§	100% (1 of 1)	0% (0 of 1)	.500	60% (12 of 20)	0% (0 of 5)	.039
Reduction in hTERT mRNA expression	ND	ND		60% (3 of 5)	ND	
Telomere length decline¶	ND	0% (0 of 1)		ND	ND	
No reduction in HPV16 E6 mRNA	ND	ND		80% (4 of 5)	ND	
Microsatellite PCR (additional chromosome 6 or 11)≠	ND	ND		100% (20 of 20)	95% (19 of 20)	

*Results are given in percentages with the number of clones showing the parameter/number of clones analyzed; *P* values are based on Fisher's exact test.

†Clones are as follows: FK16A/Hyg6, FK16A cells fused with human chromosome 6; FK16A/Hyg11, FK16A cells fused with human chromosome 11; FK16A/vector/Hyg6, FK16A cells transduced with LZRS-linker-IRES-GFP vector and then fused with chromosome 6; FK16A/hTERT/Hyg6, FK16A cells transduced with LZRS-hTERT-IRES-GFP vector and then fused with chromosome 6; FK16A/Hyg6/vector, FK16A cells fused with chromosome 6 and then transduced with LZRS-linker-IRES-GFP; FK16A/Hyg6/hTERT, FK16A cells fused with chromosome 6 and then transduced with LZRS-hTERT-IRES-GFP; SiHa/Hyg6, SiHa cells fused with chromosome 6; and SiHa/Hyg11, SiHa cells fused with chromosome 11. ND = not determined; mRNA = messenger RNA; IRES = internal ribosomal entry site; GFP = green fluorescence protein.

‡Defined as stress-associated β-galactosidase (SA β-gal) staining in ≥5% of the cells.

§Defined as a reduction of at least 70% and 60% relative to levels in parental FK16A or SiHa cells, respectively (except for FK16A/hTERT/Hyg6 cells, which were compared with FK16A/hTERT cells).

||Defined as a reduction of at least 80% relative to levels in parental FK16A cells (except for FK16A/hTERT/Hyg6 cells, which were compared with FK16A/hTERT cells).

¶Defined as a reduction in median telomere length compared with that in parental FK16A cells at passages 86 and 89.

≠Defined as the presence of an additional chromosome 6 or chromosome 11 in the FK16A and SiHa Hyg6 and Hyg11 clones, respectively, as determined by microsatellite polymerase chain reaction.

growth arrest at high frequency after a substantial lag period, a phenomenon that is accompanied by the appearance of a crisis-like morphology and the expression of SA β-gal.

Effects of Chromosomes 6 and 11 on Telomerase Activity, hTERT Transcription, and Telomere Shortening

We next examined the effect of the added chromosome on telomerase activity. A total of 32 FK16A/Hyg6 clones and four FK16A/Hyg11 clones were assayed. Telomerase activity was reduced in 81% (26 of 32) of FK16A/Hyg6 clones and in 0% (none of four) of FK16A/Hyg11 clones (*P* = .004; Fig. 1, A and B). Whenever a clear reduction in telomerase activity was observed, activity was always reduced by 70% or more compared with that in parental FK16A cells. To assess whether the reduced activity of telomerase resulted from the reduced expression of its catalytic subunit, hTERT

mRNA expression was analyzed. Eighteen (86%) of 21 FK16A/Hyg6 clones analyzed versus none of four FK16A/Hyg11 clones showed a reduction in hTERT mRNA expression (*P* = .003; Fig. 2, A). The reduction in hTERT mRNA expression was always 80% or more compared with parental FK16A cells. In none of the clones analyzed was HPV16 E6 mRNA expression reduced (Fig. 2, A), which makes it unlikely that the observed growth arrest resulted from a reduction in viral oncogene expression.

Southern blot analysis by use of a telomeric repeat-specific probe showed a reduction in telomere length relative to that in parental cells in 90% (nine of 10) of FK16A/Hyg6 clones analyzed. These clones had a median telomere length of 2 kilobases (kb) (interquartile range, 1.9–2.1 kb) after approximately 25 population doublings, whereas the median telomere length of parental cells was 3.5 kb (interquartile range, 3.45–3.55 kb) (Fig. 3, A). This telomere length decline of about 1.5

kb is in line with the estimated telomere reduction of 65–100 bp per cell division (6) and with previous observations on telomere shortening in preimmortal HPV-containing foreskin keratinocytes (7).

Effect of Ectopic Expression of hTERT in FK16A/Hyg6 Hybrids on Growth Arrest

To test whether the growth arrest seen in FK16A/Hyg6 hybrid clones resulted from the repression of hTERT expression, cells were transduced with an hTERT expression vector before microcell-mediated chromosome transfer. FK16A cells were transduced with either an empty vector (LZRS-linker-IRES-GFP) or the hTERT expression vector (LZRS-hTERT-IRES-GFP). Chromosome 6 was subsequently introduced to GFP-positive cells, resulting in 12 FK16A/vector/Hyg6 and 14 FK16A/hTERT/Hyg6 hybrid clones. By microsatellite PCR, an additional chromosome 6 allele could be identified in all hybrid clones analyzed.

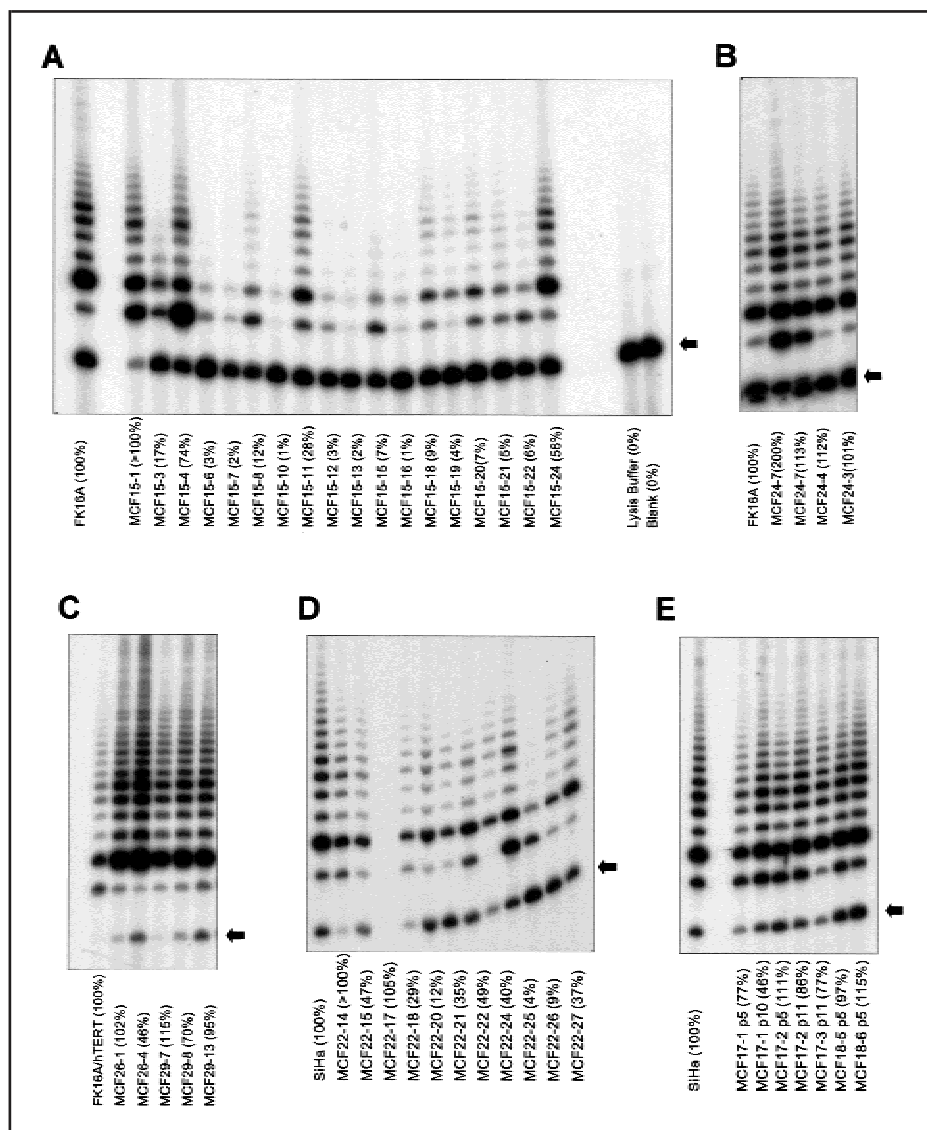


Fig. 1. Telomerase activity detected by telomeric repeat amplification protocol analysis. **Panel A:** FK16A parental cells and FK16A/Hyg6 hybrid clones (MCF15 clones -1, -3, -4, -6, -7, -8, -10, -11, -12, -13, -15, -16, -18, -19, -20, -21, -22, and -24). **Panel B:** FK16A/Hyg11 hybrid clones (MCF24 clones -3, -4, and -7). **Panel C:** FK16A/hTERT/Hyg6 hybrid clones (MCF26 clones -1 and -4 and MCF29 clones -7, -8, and -13). **Panel D:** SiHa/Hyg6 hybrid clones (MCF22 clones -14, -15, -17, -18, -20, -21, -22, -24, -25, -26, and -27). **Panel E:** SiHa/Hyg11 hybrid clones (MCF17 clones -1, -2, and -3 and MCF18 clones -5 and -6). The relative telomerase activity in hybrid clones compared with parental cells and standardized against the internal control is given in **parentheses**. **Arrow** in each panel points to the internal control. **Panel A** includes two negative controls: blank (no protein added) and lysis (lysis buffer added).

As Table 1 indicates, seven (58%) of the 12 FK16A/vector/Hyg6 hybrids showed a complete growth arrest after approximately 25 population doublings, whereas none of the 14 FK16A/hTERT/Hyg6 clones revealed any growth retardation after 40 population doublings ($P = .001$). Crisis-like features were seen in 83% (10 of 12) of the FK16A/vector/Hyg6 clones but in none of the 14 FK16A/hTERT/Hyg6 clones ($P < .001$). Moreover, 75% (six of eight) of the FK16A/vector/Hyg6 clones analyzed and none of the 12 FK16A/hTERT/Hyg6

clones analyzed expressed SA β -gal ($P = .001$).

Compared with FK16A parental cells, telomerase activity was reduced in all three FK16A/vector/Hyg6 hybrids analyzed (Table 1). Because ectopic expression of hTERT in FK16A cells resulted in an increased telomerase activity compared with parental FK16A cells, telomerase expression in FK16A/hTERT/Hyg6 clones was compared with that in FK16A/hTERT cells. Only two (17%) of 12 FK16A/hTERT/Hyg6 hybrids showed a reduction in telomerase activity of more

than 70% compared with FK16A/hTERT cells (Fig. 1, C). Similarly, all (three of three) FK16A/vector/Hyg6 clones analyzed but none of the five FK16A/hTERT/Hyg6 clones analyzed showed reduced hTERT mRNA expression levels compared with parental FK16A and FK16A/hTERT cells, respectively (Fig. 2, B). No reduction in HPV E6 expression was seen in any of the clones analyzed (Fig. 2, B). Southern blot analysis showed a dramatic increase in telomere length in all (three of three) FK16A/hTERT/Hyg6 hybrids analyzed (Fig. 3, A).

To determine whether ectopic expression of hTERT could also preclude the induction of a growth arrest after telomerase suppression by chromosome 6, early passage cells of a FK16A/Hyg6 hybrid clone that underwent a complete growth arrest after 25 population doublings were transduced separately with the control and hTERT vector. Whereas the control cells (FK16A/Hyg6/vector) and the untransduced FK16A/Hyg6 cells lacked telomerase activity and showed a growth arrest after 25 doublings, the hTERT-transduced FK16A/Hyg6 cells (FK16A/Hyg6/hTERT) expressed telomerase and could be cultured continuously (Table 1). Similar to the FK16A cells that were transduced with hTERT before microcell fusion, a strong increase in telomere length was seen in these hTERT-transduced cells.

Effects of Chromosomes 6 and 11 on Telomerase Activity in SiHa Cervical Cancer Cells

To determine whether chromosome 6 can also suppress telomerase in *in vivo*-derived HPV-containing cervical cancer cells, chromosomes 6 and 11 were introduced to SiHa cervical cancer cells. After transfer of chromosome 6 into SiHa cells, 34 hygromycin-resistant clones, designated SiHa/Hyg6, were picked. In addition, 20 clones were isolated after transfer of chromosome 11 (SiHa/Hyg11). In all of the 20 SiHa/Hyg6 clones and in 19 of the 20 SiHa/Hyg11 clones analyzed, the introduced chromosome could be detected by microsatellite PCR. Six of the SiHa/Hyg6 clones were lost during passaging. Of the remaining 28 SiHa/Hyg6 clones, seven (25%) showed a complete growth arrest within approximately five to six population doublings, whereas no growth arrest was observed in the SiHa/Hyg11 clones ($P = .032$). However, 61% (17 of 28) of the SiHa/Hyg6 clones showed crisis-like features after passag-

ing, whereas only 10% (two of 20) of the SiHa/Hyg11 clones did ($P < .001$). This apparent disparity between complete growth arrest and crisis-like features is likely to be due to the outgrowth of immortal segregants in SiHa/Hyg6 clones.

Introduction of chromosome 6 to SiHa cells also affected other characteristics of the cells. Twelve (50%) of the 24 SiHa/Hyg6 clones analyzed expressed SA β -gal. Sixty percent (12 of 20) of the SiHa/Hyg6 clones analyzed and none of the five SiHa/Hyg11 clones analyzed showed a reduction in telomerase activity at early passage ($P = .039$; Fig. 1, D and E). When a clear reduction in telomerase was observed, activity was always reduced by 60% or more compared with parental SiHa cells. In addition, a reduction in hTERT mRNA expression relative to that in parental SiHa cells was seen at early passage in three (60%) of the five SiHa/Hyg6 clones analyzed, while HPV16 E6 mRNA expression remained unaltered in four (80%) of five of these clones (Table 1). The single clone showing reduced HPV16 E6 expression showed no reduction in hTERT expression.

To find an explanation for the observed difference in lag period between chromosome transfer and induction of growth arrest in FK16A and SiHa cells (i.e., a median of 21 doublings in FK16A cells versus five to six doublings in SiHa cells), telomere length was measured in parental SiHa cells. By Southern blot analysis, the median telomere length in SiHa cells was found to be 2.5 kb (Fig. 3, B), 1 kb shorter than the median length in parental FK16A cells. This result confirms that a growth arrest based on telomeric shortening will be reached earlier in the SiHa/Hyg6 clones than in the FK16A/Hyg6 clones.

DISCUSSION

In this study, we have provided evidence that an added human chromosome 6 is able to repress hTERT mRNA expression in immortalized FK16A and SiHa cells, resulting in a reduction of telomerase activity. This observation suggests that chromosome 6 harbors either a repressor of hTERT expression or an inducer of an hTERT repressor. Moreover, introduction of chromosome 6 resulted in the appearance of crisis-like features and growth arrest after a lag pe-

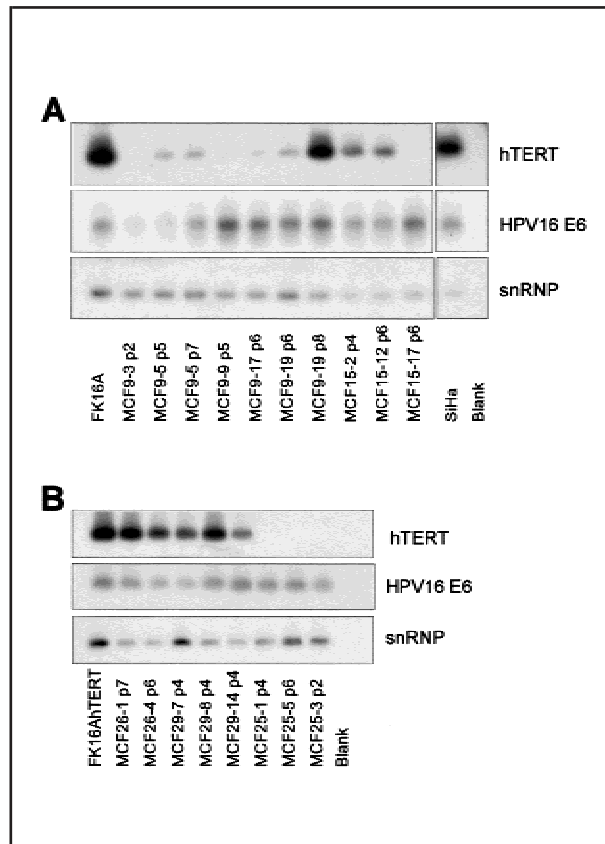


Fig. 2. hTERT, human papillomavirus type 16 (HPV16) E6, and snRNP U1A messenger RNA expression detected by reverse transcription-polymerase chain reaction (PCR) followed by Southern blot hybridization of the PCR products. SiHa RNA was included as a positive control, and blank (no RNA added) was included as the negative control. **Panel A:** FK16A/Hyg6 hybrid clones (MCF9 clones -3, -5, -9, -17, and -19 and MCF15 clones -12 and -17). The increase in hTERT expression at passage 8 in clone MCF9-19 was associated with the outgrowth of revertant cells. **Panel B:** FK16A/hTERT/Hyg6 hybrid clones (MCF26 clones -1 and -4 and MCF29 clones -7, -8, and -14) and FK16A/vector/Hyg6 hybrid clones (MCF25 clones -1, -3, and -5). Because of the high hTERT expression in the hTERT-transduced cells, the hTERT blot in **panel B** was exposed for less time than the hTERT blot in **panel A**, p indicates passage number; one passage equals three to four population doublings.

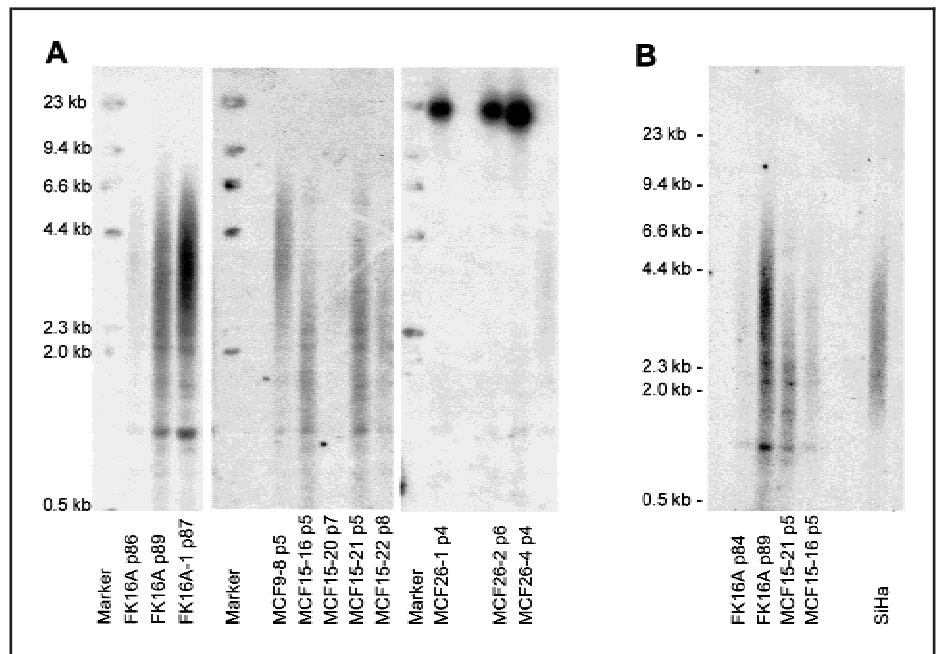


Fig. 3. Telomere length analyzed by Southern blot hybridization of the telomeric probe (TTAGGG)₄ to *RsaI* and *HinfI*-digested genomic DNA. **Panel A:** parental FK16A cells (at passages 86 and 89) and a monoclonal FK16A subclone (FK16A-1 passage 87), five different FK16A/Hyg6 hybrid clones (MCF9-8 and MCF15 clones -16, -20, -21, and -22), and three different FK16A/hTERT/Hyg6 hybrid clones (MCF26 clones -1, -2, and -4). **Panel B:** parental FK16A cells (at passages 84 and 89), two FK16A/Hyg6 clones (MCF15 clones -21 and -16), and parental SiHa cells. Lambda DNA digested with *HindIII* is used as a size marker. Four FK16A/Hyg6 clones (MCF15-16, MCF15-20, MCF15-21, and MCF15-22) show a reduction in mean telomere length compared with that in FK16A cells. No telomere reduction is seen in one immortal revertant clone of FK16A/Hyg6 (MCF9-8), and a marked increase in telomere length is seen in the three FK16A/hTERT/Hyg6 clones (MCF26-1, MCF26-2, and MCF26-4). SiHa cells had shorter telomeres than FK16A cells. p indicates passage number; one passage equals three to four population doublings. kb = kilobase.

riod, phenomena that could be blocked by ectopic expression of hTERT.

Together, these findings suggest that the growth arrest induced by chromosome 6 is dependent on telomeric shortening. This conclusion is supported by two further observations. First, FK16A and SiHa cells showed differences in the lag time between chromosome transfer and manifestation of growth arrest. The SiHa cells, whose median telomere length was 1 kb less than that of FK16A cells, generally showed growth arrest about 15 population doublings earlier than FK16A cells. This difference is in line with the assumption that telomeres shorten about 65–100 bp per cell division in mortal cells and that a critically short telomere length would trigger growth arrest (6). Second, no alterations were found in HPV16 E6 mRNA expression in the chromosome 6 hybrid clones. Consequently, it is unlikely that the induced growth arrest resulted from a reduction in viral oncogene expression. Indeed, a recent study (29) has shown that interference with viral oncogene expression restores the senescence program and that the restoration process occurs rapidly and is not based on telomere shortening.

Both FK16A/Hyg6 and SiHa/Hyg6 hybrids gave rise to revertant clones, but the frequency of such revertants was higher in SiHa hybrids. The relatively high frequency of revertant clones in SiHa/Hyg6 hybrids might be explained by the fact that SiHa cells contain, on average, eight hTERT gene copies per cell (30). Consequently, the introduction of a single hTERT repressor on chromosome 6 might not be sufficient to suppress all copies in the different clones.

Transfer of chromosome 6 has been associated previously with an induction of senescence in other human cell lines (31–33). However, it is unknown whether the observed growth arrest in these cell lines was telomeric shortening based. Moreover, the putative senescence loci involved in the different cell lines have been mapped to different regions on the long arm of chromosome 6, i.e., 6q14–21, 6q25–26, and 6q26–27 (32–34). These data suggest that multiple tumor suppressor genes located on chromosome 6 may be involved in the regulation of senescence in different, possibly cell type-specific, manners.

Previously, the repression of telomerase activity by the introduction of a single human chromosome has also been dem-

onstrated for chromosomes 3, 7, and 10 (18,19,35,36). Thus, there may exist alternative mechanisms, possibly but not necessarily cell type dependent, for deregulation of telomerase activity. At the moment, it is unclear whether the chromosome 6 suppression observed in this study is strictly HPV related. It also remains to be determined whether chromosome 6-dependent telomerase deregulation represents a common or rare mechanism during HPV-mediated cervical carcinogenesis and to what extent other chromosomes may be involved as well.

Nevertheless, the present demonstration of a telomeric shortening-based growth arrest by chromosome 6 in two HPV-containing cell lines indicates that dysregulation of a gene on this chromosome is involved in immortalization of at least a subset of cells transformed by HPV *in vitro* and *in vivo*. Identification of this gene may yield clues to telomerase deregulation in cervical carcinomas and potentially in other types of cancer. The identification of this gene may not only offer possibilities for the development of novel anticancer therapies but also provide a molecular progression marker for premalignant cervical lesions.

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NOTES

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