Alterations in \( p53 \) and \( p16^{INK4} \) Expression and Telomere Length during Spontaneous Immortalization of Li-Fraumeni Syndrome Fibroblasts

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Normal cells have a strictly limited growth potential and senesce after a defined number of population doublings (PDs). In contrast, tumor cells often exhibit an apparently unlimited proliferative potential and are termed immortalized. Although spontaneous immortalization of normal human cells in vitro is an extremely rare event, we observed this in fibroblasts from an affected member of a Li-Fraumeni syndrome kindred. The fibroblasts were heterozygous for a \( p53 \) mutation and underwent senescence as expected at PD 40. In four separate senescent cultures (A to D), there were cells that eventually recommenced proliferation. This was associated with aneuploidy in all four cultures and either loss (cultures A, C, and D) or mutation (culture B) of the wild-type (wt) \( p53 \) allele. Loss of wt \( p53 \) function was insufficient for immortalization, since cultures A, B, and D subsequently entered crisis from which they did not escape. Culture C has continued proliferating beyond 400 PDs and thus appears to be immortalized. In contrast to the other cultures, the immortalized cells have no detectable \( p16^{INK4} \) protein. A culture that had a limited extension of proliferative potential exhibited a progressive decrease in telomere length with increasing PD. In the culture that subsequently became immortalized, the same trend occurred until PD 73, after which there was a significant increase in the amount of telomeric DNA, despite the absence of telomerase activity. Immortalization of these cells thus appears to be associated with loss of wt \( p53 \) and \( p16^{INK4} \) expression and a novel mechanism for the elongation of telomeres.

When cultured in vitro, normal somatic cells have a strictly limited proliferative potential and undergo senescence after a maximum of about 70 population doublings (PDs) (21). In contrast, many populations of tumor cells exhibit an immortal phenotype. The mechanisms of immortalization are not yet understood at a genetic level, but some insights have been obtained by studying the immortalization of normal human cells in vitro by the transforming genes of DNA tumor viruses such as the simian virus 40 (SV40) large-T-antigen gene. These genes induce immortalization indirectly via a two-stage process. The first stage is a brief extension of life span associated with inactivation of the protein products of negative regulatory genes, including the \( p53 \) and retinoblastoma susceptibility (\( RB-1 \)) genes, after which the cells cease dividing and enter “culture crisis.” The second stage is the escape of rare clones from crisis, presumably as a result of one or more genetic changes (reviewed in reference 6).

These genetic changes often appear to result in the acquisition of telomerase activity. It has been proposed that the progressive telomeric shortening characteristic of normal somatic cells is an important component of senescence (42) and that the immortalization of normal human cells results from the reactivation of the enzyme telomerase (17). Telomerase adds TTAGGG repeats onto mammalian telomeres, preventing their shortening (37), and it is proposed that telomerase is normally active in germ cells but not somatic cells (19). SV40-transformed primary human embryonic kidney cells exhibited progressive telomere shortening and lacked telomerase activity up until the time at which the culture entered crisis. In an immortal clone arising from this crisis culture, however, telomerase activity was present and the telomere length was stabilized (8). Similar results have been obtained for human papillomavirus (HPV)-transformed human epithelial cells and human B lymphocytes immortalized by Epstein-Barr virus (9, 26). Furthermore, established immortal cell lines do not exhibit telomere shortening, and telomerase activity is present in most immortal human and murine cell lines examined to date (8, 9, 25, 26, 37). Telomerase activity has also recently been demonstrated in metastatic cells of a human ovarian carcinoma (10) and malignant human hematopoietic cells (39).

The concept that immortalization involves multiple events which may include inactivation of the \( p53 \) protein and the \( p110^{RB-1} \) product of \( RB-1 \) together with unknown genetic or epigenetic changes that result in expression of telomerase activity has thus arisen. The involvement of \( p53 \) and \( p110^{RB-1} \) is supported by studies in which mutant DNA tumor virus proteins that are unable to bind one or the other of these proteins were found to have a reduced capacity for extending cellular proliferative potential (reviewed in reference 6). It must be noted, however, that the DNA tumor virus proteins that induce immortalization have many functions other than inactivation of \( p53 \) and \( p110^{RB-1} \), and mutations often affect more than one of these functions. For example, the region of SV40 large T antigen that binds \( p110^{RB-1} \) also binds to the negative regulatory proteins \( p107 \) and \( p130 \) (13, 15). The roles of the \( p53 \) and \( RB-1 \) genes are therefore still unclear. The involvement of the \( p53 \) gene in control of proliferative potential is supported by the...
observation that cells from p53-null mice have a high rate of spontaneous immortalization (54). However, there appear to be substantial differences in the immortalization of murine and human cells; for example, spontaneous immortalization occurs commonly in murine cells and very rarely in human cells. Thus, the relevance of the results with p53-null mouse cells to the mechanisms of human cell immortalization requires clarification. An antisense oligomer against p53 did not alter the proliferative potential of human fibroblasts, although an antisense oligomer against RB-1 resulted in an extension of their life span by about 10 PDs (16). In contrast, injection of near-senescent human diploid fibroblasts with a retrovirus encoding a dominant-negative mutant of p53 extended their proliferative life span by 17 PDs (4).

Li-Fraumeni syndrome (LFS) is a familial cancer syndrome associated with inheritance of a mutant p53 gene (30, 31, 35, 53). We have been using heterozygous p53 wild-type (wt)/mutant fibroblasts from an individual with LFS as a model system for studying the role of p53 in immortalization (34). Spontaneous immortalization of LFS fibroblasts has been reported previously: dermal fibroblasts from seven individuals with LFS continued proliferating beyond the point at which normal controls underwent senescence, and cells from two of these individuals became immortalized (3). However, this result has not been replicated in experiments using the same dermal fibroblasts (32) or fibroblasts from LFS breast stroma (51).

In this study, we found that during continuous passaging of LFS fibroblasts, three of four separate cultures developed loss of heterozygosity (LOH) for p53; i.e., the wt p53 allele was lost. The culture which did not develop LOH for p53 developed a point mutation in the wt allele of p53. This loss or mutation was associated with chromosomal aberrations and a temporary escape from senescence, similar to that seen when SV40 genes are introduced into normal cells. We conclude that loss of wt p53 expression is causally related to extension of proliferative potential but is not sufficient for immortalization. All four cultures remained wt for p16 expression with undetectable p16 protein, which is consistent with recent evidence indicating that p16 has limited immortalizing potential but is not sufficient for immortalization (49). All four cultures exhibited no period of crisis but continued proliferating and became immortalized.

During the period of limited life span extension, telomere shortening continued to occur, but in the immortalized cells, there was a progressive increase in the amount of telomeric DNA. This occurred in the absence of any detectable telomerase activity, thus indicating the existence in human cells of a novel mechanism for telomere lengthening.

MATERIALS AND METHODS

Cells. LFS fibroblasts (III-CF cells) were derived from an Ablant cell line of a primary breast cancer from an affected LFS family member (III-C). III-CF cells have been described previously (34, 56). They have an normal diploid karyotype and fibroblastic morphology. The sequence of the p53 intron 4 spliced donor site (15, 25, 51). Cell lysates were prepared from 10^5 cells, and the immunoprecipitates were separated by electrophoresis on 12% SDS-PAGE gels and transferred electrophoretically to nitrocellulose paper in 10% methanol–10% acetic acid for 1 h at room temperature. The nitrocellulose papers were incubated with 5% nonfat dry milk in phosphate-buffered saline for 1 h and then incubated with the appropriate antibody overnight. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibodies and visualized by chemiluminescence.

Protein analysis. The presence of p110^Rb and p16^INK4a was detected by in vivo metabolic labeling with [3S]-labeled methionine followed by immunoprecipitation with monoclonal p110^Rb (Novocastra Laboratories Ltd.) or polyclonal p16^INK4a antibodies (Oncogene). The presence of the p19^Arf protein was detected by immunoprecipitation with monoclonal (Oncogene) or polyclonal p19^Arf antibodies (Oncogene). The presence of the p16^INK4a and p14^Arf proteins was detected by Western blot analysis with monoclonal p16^INK4a and p14^Arf antibodies (Oncogene) or polyclonal p16^INK4a antibodies (Oncogene).

Telomerase activity was measured using the TRAP assay (60). The TRAP assay is a assay for telomerase activity that is based on the principle that telomerase extends telomeric DNA by adding the telomeric repeat (TTAGGG) n times. The assay involves the use of a telomeric repeat priming PCR reaction that includes a telomerase-specific primer and a reverse primer that is complementary to the telomeric repeat. The PCR reaction is performed without the addition of any nucleotides, and the products are detected by electrophoresis on an agarose gel. The amount of telomeric DNA that is amplified by the telomerase reaction is proportional to the amount of telomerase activity in the sample. The amount of telomeric DNA that is amplified by the telomerase reaction is proportional to the amount of telomerase activity in the sample. The amount of telomeric DNA that is amplified by the telomerase reaction is proportional to the amount of telomerase activity in the sample.

DNA sequencing. LOH for p53 was determined by DNA sequencing. PCR was used to amplify a 320-bp fragment of the p53 gene across the exon 4/ intron 4 junction. PCR primers were as follows: sense (within exon 4), 5′-AAGGTGTAATCAGCTGTCCCGT-3′; antisense (within exon 3), 5′-ATCCCAGAGTTGCTTT-3′. Genomic DNA was extracted by using a DNA extraction kit (Stratagene). PCR was performed using genomic DNA from cells and the equivalent of 10^5 cells, and the amplified fragments were electrophoresed on agarose gels and stained with ethidium bromide. The bands were visualized under ultraviolet light and were excised and ethanol precipitated. The DNA was resuspended in water and used as a template for DNA sequencing. Sequences were determined by using the Sequenase version 2.0 DNA sequencing kit and the M13 sequencing primer (Amersham).

Animals. LFS fibroblasts (III-CF cells) were derived from an explant culture of a human breast cancer from a patient with LFS (3). The III-CF cells were maintained in RPMI 1640 medium with 10% fetal bovine serum and gentamicin at a concentration of 100 ng/ml. Cells were passaged as required by trypsinization at a ratio of 1:2 to 1:3 at 1 to 2 days. The cells were then cultured in 100-mm culture dishes at 37°C in a humidified atmosphere of 5% CO2 in air. The culture medium was changed every 2 to 3 days. The cells were detached from the dishes by trypsinization at a ratio of 1:2 to 1:3. The cells were then harvested by centrifugation at 1,000 rpm for 5 min.

DNA synthesis. DNA synthesis was determined by the BrdU labeling index. The BrdU labeling index was determined by incubating the cells with BrdU for 48 h. The BrdU was then removed, and the cells were washed with 0.1% trypsin in phosphate-buffered saline and fixed in 70% ethanol. The cells were then incubated with a 1:100 dilution of fluorescein isothiocyanate-conjugated anti-BrdU antibody (1:100 dilution; Immunotech). The cells were then washed and resuspended in 1.5 ml of PBS. The DNA was then stained with 1.0 μg/ml of propidium iodide (Sigma). The cells were then analyzed by flow cytometry.

Cell cycle analysis. Cells were harvested by trypsinization and counted. The cells were then washed with PBS and fixed in 70% ethanol. The cells were then washed and resuspended in 1.5 ml of PBS. The DNA was then stained with 1.0 μg/ml of propidium iodide (Sigma). The cells were then analyzed by flow cytometry.

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into the subscapular regions of BALB/c nu/nu mice. One site per mouse was injected in 10 mice (5 female and 5 male). Injection sites were monitored for tumor development two to three times per week.

RESULTS

III-CF cells undergo senescence in culture. Four separate mass cultures of III-CF cells (designated A, B, C, and D) were passaged as described above, commencing at PD 40. All four cultures senesced almost immediately after the start of the experiment (Fig. 1; senescence was defined as a failure of the population to double within a period of 30 days, accompanied by characteristic morphological changes such as enlargement and flattening). All four mass cultures had undergone only one PD after 60 days in culture and remained senescent for approximately 85 days.

Immortalization of III-CF cells. After the period of senescence, all four cultures recommenced proliferating at variable rates (Fig. 1). Cultures A, B, and D proliferated for a further 36, 32, and 32 PDs, respectively (i.e., total PDs in culture of 76, 72, and 72), after which they again ceased proliferation, failing to achieve one additional PD during a minimum of 150 days. We refer to this second nonproliferative period as culture crisis by analogy to the behavior of SV40-transformed cells, which also usually cease proliferation after a temporary escape from senescence (14). The III-CF cells in culture crisis had morphological changes similar to those seen in senescent cells. In contrast, culture C continued to proliferate at an increased rate and without any period of crisis and has now exceeded 400 PDs.

Loss of wt p53 correlates with temporary escape from senescence in III-CF cells. Mass cultures A to D were analyzed for LOH for p53 by DNA sequencing, after escape from senescence, at PD 63 to 74 (Fig. 2a). Presenescent III-CF cells are heterozygous for p53 at the last base of exon 4; i.e., both cytosine and thymine bases are present at this locus on sequencing (antisense). Cultures A, C, and D demonstrated LOH for p53, as evidenced by loss of the wt (cytosine) p53 allele. Culture B remained heterozygous for p53. Sequencing of both strands of the coding region of the p53 gene in culture B at PD 63 revealed a point mutation in exon 5 that results in a valine-to-glycine substitution in codon 157 (Fig. 2b). Both alleles of the p53 gene from this culture at PD 63 were then cloned separately and sequenced to determine which allele had sustained the new mutation. The allele which contained the LFS splice site mutation was wt in exon 5, but the other (previously wt) allele had a T-to-G point mutation at the second base of codon 157 (data not shown). Thus, culture B no longer contained wt p53. The p53 gene was also sequenced in late passage culture C cells (PD 145) to ascertain whether there had been loss of the mutant p53 allele. The mutant allele was still present in these cells (Fig. 2a).

p110Rb and p16INK4a status in III-CF cells. The p110Rb and p16INK4a status of the four subcultures of III-CF cells was determined by protein immunoprecipitation at a time point after they had escaped from senescence (PD 59 to 64) and also in the immortalized culture C cells at PD 200 (Fig. 3). p110Rb was wt in all cultures on the basis of a protein of the expected size. To date, all RB-1 mutations have been shown to confer an abnormal mobility or phosphorylation status on the resultant protein. Slight smearing of the bands on the autoradiograph indicates that the protein is phosphorylated normally. p16INK4a was detected at low levels in cultures A, B, and D but not in culture C either immediately after escape from senescence or when immortalized (PD 200). Although WI-38 cells appear to express more p16INK4a than cultures A, B, and D in this experiment, in repeat experiments, the amount of p16INK4a detected in these cultures was comparable to that in normal fibroblasts.

Morphological changes accompany immortalization of III-CF cells. Proliferating cells in cultures A, B, and D retained a distinctly fibroblastic phenotype (Fig. 4a): small, spindle-shaped cells which did not overgrow the monolayer. Senescent

FIG. 1. Growth kinetics of III-CF cells. Four separate cultures of III-CF cells (A, B, C, and D) were subcultured continuously in vitro commencing at PD 40. Cumulative PD values were calculated at each passage.

FIG. 2. LOH for p53 in III-CF cells. Genomic DNA was extracted from cells of cultures A to D at the PD indicated. PCR-amplified fragments of the p53 gene were sequenced, and the gels were loaded in the following order from left to right: G, A, T, and C. (a) The noncoding strand at the p53 exon 4/intron 4 junction is shown. As has previously been shown for affected members of this LFS family, presenescent III-CF (PD 18) and culture B cells are heterozygous for a C-to-T transition at the last base of exon 4. The other cultures have lost the wt p53 allele. (b) The coding strand of part of p53 exon 5 is shown. Culture B cells contain a novel T-to-G transversion at the second base of codon 157.
and crisis cells exhibited profound morphological changes (Fig. 4b), i.e., gross enlargement, flattening, and development of granular cytoplasmic inclusions and processes. In contrast, the immortalized culture C cells were smaller, rounded, and generally more epithelioid in appearance (Fig. 4c). They demonstrated progressively decreased contact inhibition and an ability to overgrow the monolayer (data not shown).

**Origin of immortalized III-CF cells and III-CF cells in crisis.** Single-locus DNA fingerprinting using a highly polymorphic probe, MS-1 (12), confirmed that all of the III-CF cultures described had the same MS-1 alleles as the parent III-CF cells from which they were derived (data not shown). The possibility that immortalization of III-CF culture C cells was the result of SV40 contamination was excluded by immunocytochemistry analysis using a monoclonal antibody directed against SV40 T antigens (data not shown). To confirm that the immortalized cells were fibroblasts and not epithelial, immunocytochemical staining was performed with a monoclonal antibody directed against the epithelial cell-specific protein keratin-18. Staining was observed in the epithelial control cells (BEAS-2B [45]) but not in III-CF culture C cells (data not shown). When the cells were stained with an antibody directed against the fibroblast-specific enzyme prolyl hydroxylase, the III-CF culture C cells were positive and the BEAS-2B epithelial cells were not (data not shown).

**Telomere length progressively decreased in nonimmortalized cells but increased in the immortalized culture.** Analyses of the telomere lengths of precrisis (culture A) and immortalized (culture C) III-CF cells are shown in Fig. 5 and 6. The frequently cutting restriction enzymes HindIII and RsaI digest genomic DNA at points outside of, but not within, the TTAGGG repeats present at the ends of human chromosomes. As a result, probing the digested DNA with a (TTAGGG)₃ probe detects TRFs that include all of the terminal repeats and a subtelomeric region. The TRFs appear as a smear on a gel (Fig. 5) because both the size of the subtelomeric region and the number of TTAGGG repeats vary on different chromosomes, and the mean TRF length varies between cells within a population. The mean TRF length decreased in nonimmortal III-CF cells (culture A) from PD 44 to 76 at a rate of about 54 bp per PD (Fig. 6a). This rate of mean TRF shortening is within the range seen in studies of normal cells (1, 18, 20, 29). The total intensity of the telomeric signal also decreased (Fig. 6b), indicating that the decrease in mean TRF length was due to a reduction in the number of TTAGGG repeats and not due to rearrangements or loss of only the subtelomeric portion of the TRF.

III-CF cells that became immortalized (culture C) also showed a reduction in mean TRF length and amount of telomeric DNA from PD 44 to 73, at a rate of 43 bp per PD (Fig. 6). Between PD 73 and 77, the pattern of the telomeric hybridization signal changed dramatically (Fig. 5b). The discrete smear at around 9 kb disappeared and was replaced with a more diffuse signal that extended over much of the lane. A discrete band at about 4.5 kb that was insensitive to Bal 31 exonuclease digestion (data not shown) appeared, indicating that it is not a telomeric fragment. This band presumably represents nontelomeric DNA sequences that hybridize to the TTAGGG probe, as have been observed by others (18, 20).
The changes in telomere pattern at the PD 73 to 77 transition point were reproducible: when culture C cells that had been cryopreserved at PD 73 were thawed and passaged for a further 10 PDs, the telomere changes observed were virtually indistinguishable from those shown in Fig. 5b. This result presumably indicates that a subpopulation of cells characterized by a discrete 4.5-kb band together with a diffuse telomeric smear was already present prior to PD 73 and that their overgrowth of the culture is a reproducible event.

It was not possible to accurately calculate the mean TRF length for the immortalized culture C cells beyond PD 77, since the telomeric smear is more diffuse, and much of the signal lies outside the resolution of the gel (i.e., >23 kb). However, it is clear that there was a gradual increase in heterogeneity of TRF length. By PD 222, TTAGGG-hybridizing fragments extended from the wells almost the entire length of the gel. While this finding indicates that the TRFs on some chromosomes had become shorter, there was also a gradual increase in intensity of TRFs at the limit of resolution of the gel (about 23 kb). The concomitant gradual increase in total signal intensity after PD 90 (Fig. 6b) indicates that the amount of telomeric DNA was increasing in these cells.

We considered the possibility that the increase in signal at limiting mobility on the gel shown in Fig. 5b was due to chromosomal rearrangements that resulted in very large interstitial telomere-like sequences. However, Bal 31 digestion of culture C DNA at PD 222 resulted in a decrease in the intensity of this signal (5), indicating that the very large TTAGGG-hybridizing fragments are telomeric.

**Telomerase activity was not detected in immortalized III-CF cells.** A recently developed PCR-based assay (25, 51) was used to measure telomerase activity in culture A and culture C cells. Although the assay was able to detect activity in the equivalent of 10 HeLa cells (data not shown), no activity was detected in the equivalent of 1,000 culture C cells (Fig. 7) which were reproducibly telomerase negative up to 287 PD (data not shown). Culture A also had no detectable telomerase activity (data not shown).

The PCR-based telomerase assay was also carried out with a 100-fold range of protein concentrations (0.1, 1, and 10 μg of total protein) for lysates of HeLa cells and III-CF culture C cells at PD 222. No telomerase activity was detected in the culture C cells at any protein concentration, while the HeLa extract remained telomerase positive at all protein concentrations (data not shown). To determine whether the culture C extracts contain inhibitors of the telomerase assay, a mixing experiment was performed. Extract amounts representing 1 μg each of culture C and HeLa cells were mixed, allowed to stand at room temperature for 30 min, and then used in the telomerase assay. The telomerase activity of the HeLa extracts was...
not diminished (data not shown). These experiments indicate that the lack of detectable telomerase activity in the III-CF culture C cells is unlikely to be due either to lack of sensitivity of the assay or to inhibitors of the assay.

**Karyotypic alterations accompany LOH for p53.** Karyotypic analyses were performed on nonimmortal III-CF cultures A to D at PD 60, 57, 68, and 67, respectively, and also on the immortalized culture C (PD 180). In contrast to early-passage III-CF cells, which were previously shown to have a normal diploid karyotype (data not shown), a range of abnormalities was observed in each culture. Ploidy varied in the nonimmortal cultures from mainly hypodiploid to mixed populations including hypertetraploid cells (Table 1). Nonimmortal culture C cells had a mixed population with respect to ploidy, and 50% of cells were hypotetraploid. Immortalized culture C cells were predominantly hypotetraploid, suggesting that it was the hypotetraploid subpopulation of cells in the earlier culture that contained the immortalization-competent clone(s). A range of chromosomal aberrations was present within each culture (Table 2). A large number of marker chromosomes (8 to 29 per culture) were identified. Several of these markers, e.g., del(9) (p12) and 21p+, were common to all of the cultures, indicating that some karyotypic changes had occurred before the four cultures were separated. Other markers, e.g., 16qter>16p13::?, in culture A were unique to individual cultures, showing the independent origins of the extended life span cells. There were eight markers unique to the immortalized culture C cells: del(2) (p11), del(2) (pter>p10), 2q+, del(3) (q13), del(3) (qter>q10), 18q+, 13q+, and (9q+::20p). Some of these changes were present in every karyotype examined, indicating the clonal origin of these immortalized cells.

**Tumorigenicity.** Immortalized III-CF cells were assayed for tumorigenicity by subcutaneous injection into athymic nude mice. No evidence of tumor development was observed in any of 10 injected mice over a period of 1 year.

**DISCUSSION**

Our data provide the first clear evidence that loss of wt p53 expression results in a finite increase in the in vitro proliferative potential of human fibroblasts. III-CF cells, which contain only one wt p53 allele (56), senesced at about 40 PDs, as expected for normal fibroblasts from a donor of this age (1). Loss of wt p53 by either mutation or deletion was clearly insufficient for immortalization but was associated with limited life span extension in three of four cultures. Life span extension in LFS fibroblasts has been observed previously: cultures of dermal fibroblasts derived from seven individuals with LFS became senescent at the same time as control cultures but subsequently, and temporally in most cases, were able to continue proliferation; however, the p53 status of these cultures was not determined (3). Our results here are in agreement with those of our previous study of the effects of wt and mutant SV40 large T antigens on normal and LFS fibroblasts, which showed that inactivation of wt p53 was the major determinant of proliferative life span extension (34). These data are supported by the finding that fibroblasts from p53-deficient mice proliferated continuously in vitro without senescence (54). It was recently shown that retrovirus-mediated transfer of a dominant-negative mutant p53 gene was able to extend the proliferative potential of human fibroblasts; the authors were not able to conclude whether this effect was due solely to loss of wt p53 function or whether transforming properties of the mutant p53 gene also played a role (4). Although we cannot exclude the possibility of additional genetic changes, the emergence of cells with an extended proliferative potential in four of four separate III-CF cultures strongly suggests that loss of wt p53 function is sufficient for this effect. Our results appear to conflict with those of Hara et al., who found that an antisense

![TABLE 1. Ploidy of nonimmortal and immortal III-CF cultures](http://mcb.asm.org/)

<table>
<thead>
<tr>
<th>Culture</th>
<th>PD</th>
<th>Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>60</td>
<td>Hypotetraploid (minor population in diploid range)</td>
</tr>
<tr>
<td>B</td>
<td>57</td>
<td>25% hypodiploid, 50% hypotetraploid, 25% hypertetraploid</td>
</tr>
<tr>
<td>C</td>
<td>68</td>
<td>25% hypodiploid, 50% hypertetraploid, 25% hypertetraploid</td>
</tr>
<tr>
<td>C</td>
<td>180</td>
<td>Hypotetraploid</td>
</tr>
<tr>
<td>D</td>
<td>67</td>
<td>Hypotetraploid</td>
</tr>
</tbody>
</table>

* Chromosome counts were performed on 30 to 34 metaphase spreads of cells from cultures A to D at the PD indicated.

![TABLE 2. Chromosomal aberrations detected in III-CF cultures](http://mcb.asm.org/)

<table>
<thead>
<tr>
<th>Chromosomal aberration</th>
<th>No./30 metaphases examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>(60)</td>
</tr>
<tr>
<td>Chromosome gaps</td>
<td>2</td>
</tr>
<tr>
<td>Dicentric chromosomes</td>
<td>22</td>
</tr>
<tr>
<td>Acentric fragments</td>
<td>3</td>
</tr>
<tr>
<td>Ring chromosomes</td>
<td>2</td>
</tr>
<tr>
<td>Chromatid breaks</td>
<td>2</td>
</tr>
<tr>
<td>Tricentric chromosomes</td>
<td>3</td>
</tr>
<tr>
<td>Minutes</td>
<td>5</td>
</tr>
<tr>
<td>Double minutes</td>
<td>6</td>
</tr>
<tr>
<td>No. of different marker chromosomes in 6–8 metaphases examined</td>
<td>24</td>
</tr>
</tbody>
</table>

* PD level at which karyotypes were analyzed.
oligomer against p53 had no effect on the proliferative potential of human fibroblasts (16); a possible explanation for this discrepancy is that the decrease in p53 expression induced by the antisense oligomer was insufficient for an effect on proliferative life span to be observed.

Since the DNA tumor viruses that are able to induce a finite extension in the proliferative life span of human fibroblasts encode proteins that bind to p110\(^{RB}\) as well as to p53, we analyzed p110\(^{RB}\) in the four cultures of LFS fibroblasts that exhibited spontaneous escape from senescence. In each case, the p110\(^{RB}\) was of normal size and phosphorylation pattern, indicating the presence of wt RB-1. In view of evidence from several recent studies that there is an inverse correlation between the expression of wt p110\(^{RB}\) and p16\(^{INK4}\) in non-virally immortalized cell lines, we also examined expression of p16\(^{INK4}\) protein and found that it was readily detectable in the three cultures in which escape from senescence was only temporary. Since the majority of alterations of the p16\(^{INK4}\) gene involve deletion of the entire gene (24, 40, 41), it is likely that the detection of a protein product is reflective of a wt gene sequence. In culture C, which became immortalized, p16\(^{INK4}\) expression was not detected; PCR analysis of genomic DNA from these cells showed that the loss of expression was due to homozygous deletion of the p16\(^{INK4}\) gene (46). Although the loss of wt p53 and p16\(^{INK4}\) expression presumably contributed to the immortalization of these cells, we have evidence that these changes are insufficient for immortalization: in other experiments, we have obtained a clone of III-CF cells that has lost both wt p53 and p16\(^{INK4}\) expression but still has a finite proliferative potential (46). Therefore, additional changes must be required for immortalization to occur, even though the culture C cells became immortalized without any overt period of crisis. It has also been observed that SV40-induced immortalization sometimes occurs without any obvious crisis (2, 23, 45). Presumably the stochastic immortalizing event(s) occurred sufficiently early for the immortalized cells to have overgrown the culture by the time the rest of the cells ceased proliferation.

The additional events may be facilitated by the karyotypic destabilization that occurred following loss of wt p53. Cytogenetic analyses revealed the acquisition of a number of karyotypic abnormalities in III-CF cells: aneuploidy and marker chromosomes were evident in all cultures (Tables 1 and 2). That several markers were common to all cultures indicated that some chromosomal abnormalities had occurred in the cultures prior to the time at which they were separated (PD 40). Unique markers in individual cultures indicated that abnormalities had arisen independently in each culture after PD 40, and in the case of immortalized culture C cells, there were additional unique markers indicative of clonality. The immortalized cell line was hypotetraploid and presumably arose from the hypotetraploid subpopulation evident in culture C at PD 68. The presence of ring and dicentric chromosomes is said to indicate chromosome end-to-end fusions. This is consistent with a model in which telomere shortening, such as that observed in III-CF cells, facilitates such chromosomal associations. Similar abnormalities are observed in many human tumors (22, 48).

The extensive karyotypic derangements exhibited by the III-CF cells are consistent with those observed previously in spontaneously immortalized LFS fibroblasts (3) and are also seen in SV40-immortalized cells (47). Given that wt p53 has been lost or inactivated in the III-CF cells and is inactivated by large T antigen in SV40-immortalized cultures, this finding strongly suggests that the karyotypic abnormalities observed in both are related specifically to the loss of wt p53. p53 inactivation has been previously linked to genomic instability; cells containing mutant p53 have been shown to exhibit DNA amplification and inappropriate DNA replication (38). p53 inactivations have been shown to occur immediately prior to the emergence of aneuploid subclones during the development of colorectal carcinoma (7).

In our study, one of four III-CF cultures (culture C) became immortalized spontaneously. We have also observed immortalization in one of two III-CF cultures that had been transfected with a control expression vector (46); although it is possible that insertional mutagenesis occurred following transfection of this plasmid, it is most likely that this finding also represents spontaneous immortalization. DNA and protein analyses show that this independently immortalized culture has undergone the same molecular events as the culture C cells; it expresses wt p110\(^{RB}\) but not p16\(^{INK4}\), has lost the wt p53 allele and has homozygous deletion of the p16\(^{INK4}\) gene, and has no telomerase activity (5, 46). In a previous study, spontaneous immortalization was also seen in two of seven LFS fibroblast cultures (3). However, in at least two other studies, immortalization of LFS fibroblasts was not seen (32, 51). The simplest explanation of the different outcomes of these studies is that the putative genetic changes resulting in immortalization are stochastic and that the likelihood of observing immortalization is dependent on the number of cells maintained in culture.

After this study was completed, we learned of a study in which LFS breast epithelial cells infected with a retrovirus became immortalized; although retrovirus-induced mutagenesis cannot be excluded, it seems likely that this finding also represents spontaneous immortalization (51). In this study, breast stromal fibroblasts became immortalized following infection with a retrovirus encoding HPV type 16 E7 protein (which binds to cellular proteins including p110\(^{RB}\) but does not become immortalized spontaneously. For reasons discussed above, inactivation of p110\(^{RB}\) by E7 in these cells may be equivalent to the loss of p16\(^{INK4}\) expression observed in our III-CF culture C cells. An explanation for the occurrence of spontaneous immortalization in breast epithelial cells but not fibroblasts in the study of Shay et al. (51) may therefore be that inactivation of both p53 and p110\(^{RB}\) (or an equivalent event) is required for immortalization of fibroblasts, whereas inactivation of p53 but not p100\(^{RB}\) is required for immortalization of the breast epithelial cells. This seems likely in view of an earlier study by Shay et al. (52) in which immortalization could be induced in breast epithelial cells containing wt p53 by the introduction of HPV E6 alone, whereas immortalization of fibroblasts with wt p53 required both HPV E6 and E7.

Our data show for the first time a clear link between loss of wt p53 and the continuing telomere shortening characteristic of temporary escape from senescence. Continued reduction of telomere length below that seen in senescent cells has previously been documented in cells containing the transforming genes of SV40 or HPV (8, 26), but these genes encode proteins with many functions in addition to the inactivation of p53. Our data are consistent with p53 being involved in the normal process whereby telomere shortening beyond a critical point results in the proliferative arrest that defines senescence. The mechanism of the growth arrest characteristic of crisis is unknown but clearly does not involve p53 since it occurred in cultures that had lost wt p53 function.

We observed an increase in the amount of telomeric DNA following spontaneous immortalization of human fibroblasts. However, before this increase in telomere length occurred, the cells had undergone a sudden alteration in telomere size and organization; the amount of telomeric DNA decreased, and a novel nontelomeric TTAGGG-related signal appeared. This occurred reproducibly in culture C between PD 73 and 77, at

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approximately the same PD at which the other three cultures were entering crisis. It is therefore likely that this sudden alteration in the structure of the telomeres was associated with immortalization. It is possible that the cells which went on to become immortal were derived from the clonal selection of a single cell. The altered telomeric signal observed may merely reflect the telomere arrangement that this subclone of cells happened to possess, since subclones within an immortal population may have different mean TRF sizes (5, 38). Alternatively, it may be that a cell possessing very short telomeres had a greater number of rearranged chromosomes and hence a greater probability of rearrangements involving putative senescence genes. In either case, it is clear that the amount of telomeric DNA increased after immortalization of these cells.

The increase in telomeric DNA in the immortalized III-CF cells was not associated with telomerase activity. Three telomerase-negative SV40-immortalized cell lines have been reported previously (25, 38), and we have identified 14 other telomerase-negative cell lines, all of which show very long and heterogeneous telomeres similar to those in culture C at PD 222 (5).

One possible explanation for these observations is that the apparently telomerase-negative cells elongate their telomeres as a result of a temporary burst of telomerase activity which is then shut off. Our data demonstrate, however, that this is not the case for the immortalized III-CF culture C cells since they were consistently negative during the period when telomere lengthening was occurring. These cells must therefore have an alternative mechanism for telomere lengthening which could possibly involve recombination analogous to that seen in yeast cells (33, 55) or terminal transposition of retrotransposons as possibly involved in immortalization of Epstein-Barr virus-transformed human B lymphocytes. J. Virol. 68:3410–3414.


36. Mann, G. Personal communication.


