Telomerase Repressor Sequences on Chromosome 3 and Induction of Permanent Growth Arrest in Human Breast Cancer Cells

Andrew P. Cuthbert, Jacqueyn Bond, Deborah A. Trott, Sandra Gill, Jessica Broni, Alison Marriott, Guennadi Khoudoli, E. Kenneth Parkinson, Colin S. Cooper, Robert F. Newbold

Background: Activation of the enzyme telomerase, which has been associated with cellular immortality, may constitute a key step in the development of human cancer. Telomerase is repressed in most normal human somatic cells. This study was conducted, using a genetic complementation approach, with the aim of identifying and mapping the genes responsible for repressing telomerase and, simultaneously, to establish the effect of experimentally induced telomerase repression on human tumor cell growth. Methods: Individual human chromosomes isolated from normal diploid cells and tagged with bacterial antibiotic resistance genes (for later selection) were introduced into cells of the human breast carcinoma cell line 21NT by means of microcell transfer. Selected hybrid clones were screened for telomerase activity by use of the polymerase chain reaction-based telomere repeat amplification protocol (TRAP) assay, and the proliferative fate of the hybrid clones was determined. Regions of the introduced chromosomes associated with telomerase repression were mapped using segregant hybrids and a deletion analysis that employed microsatellite DNA markers. Results: Strong repression of telomerase was observed following transfer of human chromosome 3 into 21NT cells but not after transfer of chromosomes 8, 12, or 20. The vast majority of hybrid clones with repressed telomerase entered permanent growth arrest after 10–18 population doublings. Deletion analysis of nonrepressed segregant monocromosome 3 hybrids indicated two regions on the short arm of chromosome 3 (3p21.3-p22 and 3p12-21.1) where telomerase regulator genes may be located. Conclusions: Telomerase in human breast cancer cells is efficiently repressed by a gene or genes on normal human chromosome 3p, and this repression is associated with permanent growth arrest of the tumor cells. [J Natl Cancer Inst 1999;91:37–45]

The concept that cellular immortality is an essential phenotype in malignant tumor cell populations and is a prerequisite for the clonal evolution of cancer has been debated for more than two decades (1). The evidence in support of this notion has stemmed mainly from cell culture studies. For example, when certain human carcinomas are explanted in vitro under optimal growth conditions [e.g., see (2)], immortal cells frequently dominate the cultures. In marked contrast, normal human somatic cells have a finite proliferative potential in culture, entering a phase of irreversible growth arrest, termed replicative senescence, after a limited number of divisions (3,4). The induction of an immortal phenotype appears to be a necessary step in the process of malignant transformation of mammalian somatic cells in culture, even when powerful chemical carcinogens and oncogenes are used as inducing agents (1,5). Immortalization of normal human diploid cells in vitro is extremely rare in comparison with rodent counterparts (5,6). Consequently, the idea that replicative senescence provides human cells with a robust antiproliferative mechanism for tumor suppression has attracted much support (7,8).

Recent comparisons of the activity of the enzyme telomerase in normal and immortal human cells have led to the formulation of an attractive molecular model to explain replicative cellular senescence and immortalization (9). Telomerase, an RNA-dependent DNA polymerase, was first identified in primitive eukaryotes (10). Telomerase compensates for telomere loss due to the “end replication problem” (11), exonuclease attack (12), and possibly oxidative damage (13). Most normal human cells, other than germ cells, do not have detectable telomerase activity and, as a result, telomeres shorten at rates ranging from 40 to 200 base pairs (bp) per cell division depending on the cell type (14). In contrast, the vast majority of human cancers possess active telomerase (9) and thus are able to maintain their telomeres. Based on these findings, it has been proposed (15) that the progressive loss of telomeres provides a “counting” mechanism for determining cellular replicative potential. In this model, the induction of senescence is ultimately triggered in cells with critically shortened telomeres via the activation of some, as yet unknown, cell cycle inhibitory pathway.

The mechanism by which telomerase is repressed in normal human cells appears to operate at the level of transcriptional regulation of the catalytic subunit (i.e., the protein component) of the telomerase ribonucleoprotein (16). In very recent studies (17,18), reconstitution of telomerase activity in normal human cells by ectopic expression of the cloned (16,19–21) gene en-
coding the catalytic subunit resulted in cells with a substantially increased proliferative capacity. Furthermore, in a previous investigation, Feng et al. (22) showed that expression of antisense sequences directed to the RNA component of human telomerase produced telomere shortening and growth crisis in a subclone of the immortal HeLa cell line. However, despite such strong evidence supporting a causal relationship between telomerase and immortalization in vitro, direct proof that the telomerase activity seen in human cancer cells is the result of a distinct genetic event in the process of carcinogenesis (i.e., resulting in re-expression of the telomerase catalytic subunit) is still lacking. It remains formally possible that the targets for malignant transformation in vivo are rare telomerase-positive cells [e.g., stem cells or progenitor cells; see (23)] and, therefore, that telomerase activity in cancer results from clonal selection rather than induction (24, 25).

To resolve this and other important questions concerning the role of telomerase in cell senescence and cancer, we have adopted a somatic cell genetic approach based on microcell-mediated monochromosome transfer to identify genes in the normal human genome that induce replicative senescence and/or telomerase repression when introduced into human tumor cells. For this purpose, we have developed a unique panel of highly stable monochromosome human:mouse ‘‘donor’’ hybrid cell lines representing the complete normal human chromosome complement (26) and have shown that these can be used effectively in the functional identification and accurate subchromosomal localization of novel antiproliferative genes (27). Our recent screen of these chromosomes (28) using a human head and neck carcinoma cell line as a recipient for monochromosome transfer revealed strong telomerase repressive activity specifically associated with the introduction of a single, cyogenetically intact copy of human chromosome 3. In the present study, we used monochromosome transfer to investigate in detail the effects of human chromosome 3 on telomerase activity in an early-passage human breast cancer cell line derived from a primary intraductal carcinoma and attempted to relate the extent of telomerase repression to the timing of growth arrest (replicative senescence) induced in these cells by the new chromosome. In addition, we undertook a detailed analysis of the structural integrity of the introduced chromosome 3 in nonresponsive (i.e., immortal) hybrid segregants to identify regions on the short arm of chromosome 3 (frequently deleted in human cancers) where the candidate telomerase regulator gene(s) may reside.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions**

A newly established breast cancer cell line, 21NT (provided by R. Sager and V. Band, Dana-Farber Cancer Institute, Boston, MA) (29), was used as the recipient for monochromosome transfer studies. This cell line was derived from a primary intraductal carcinoma of the breast and is one of a series of well-characterized lines from the primary tumor and metastatic deposits of a single 36-year-old patient. Early-passage cells were cultured in modified Eagle medium alpha supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 50 μg/mL gentamicin, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 1.0 μg/mL insulin, 2.8 μM hydrocortisone, and 12.5 ng/mL epidermal growth factor as described by Band et al. (30). Mouse(A9):human monochromosome hybrid donor cell lines A9-Hytk3, A9-Hytk8, and A9-Hytk20 carrying a selectable fusion gene marker, Hytk (Hy, bacterial hygromycin phosphotransferase; tk, herpes simplex virus thymidine kinase) tagged on normal human chromosome 3 (Hytg-3), 8 (Hytg-8), and 20 (Hytg-20), respectively, derived by us previously (26), were maintained in Dulbecco’s modified Eagle medium containing 10% FBS and 400 U/mL hygromycin B (Calbiochem Corp., San Diego, CA). Monochromosome hybrid donors A9+3 (30) and A9+12 (31) carrying a neo (neo = bacterial neomycin-resistance gene)-tagged human chromosome 3 (Neo-3) and 12 (Neo-12), respectively, were obtained from Coriell Cell Repositories (Camden, NJ) and were maintained in 400 μg/mL geneticin (G418) (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD).

**Microcell-Mediated Monochromosome Transfer and Cytogenetic Analysis of Hybrids**

Microcell-mediated chromosome transfer of normal human chromosomes into 21NT cells was carried out as described by us (26, 32) previously. Microcell hybrids were selected from postfusion cells in medium supplemented with either hygromycin B (400 U/mL) for hyg (hyg = hygromycin-resistance gene)-tagged chromosomes or G418 (800 μg/mL) for neo (neo = neomycin resistance gene)-tagged chromosomes. Preliminary cytogenetic characterization of the subset of 21NT hybrids that did not undergo growth arrest was performed by chromosome fluorescence in situ hybridization (FISH) by use of digoxigenin-labeled chromosome-specific probes (Oncor, Inc., Gaithersburg, MD). Fluorescein isothiocyanate fluorescence was observed using an Olympus epifluorescence microscope attached to an uncooled CCD camera system (Sony; Cologne, Germany).

**Assay of Telomerase Activity in 21NT Cells and Monochromosome Hybrids**

Telomerase activity was assayed using the standard telomere repeat amplification protocol (TRAP) (33) and the TRAP-eze telomerase detection kit (Oncor, Inc.). 21NT colonies and monochromosome hybrid derivatives were lysed in situ in 200 μL of ice-cold lysis buffer. Protein concentrations were determined with a Coomassie blue protein assay kit (Pierce Chemical Co., Rockford, IL). Reaction mixtures for the standard TRAP included 200 ng of protein, 5 attomols of an internal standard—either a 150-bp myogenin complementary DNA (cDNA) (33) or, in the case of the TRAP-eze assay, the shorter (36-bp) internal DNA standard included with the kit—to facilitate quantitative comparisons of telomerase activity and to control for Taq polymerase inhibitors. 25 μM deoxynucleotide triphosphates, and 4 μCi each of 10 nCi/mL [α-32P]deoxyctydine triphosphate and [α-32P]deoxythymidine triphosphate. Reaction mixtures were incubated for 1 hour at room temperature prior to polymerase chain reaction (PCR) amplification of telomerase extension products. PCR conditions were one cycle of 90 °C/90 seconds, 94 °C/35 seconds followed by 35 cycles of 94 °C/30 seconds, 50 °C/30 seconds, and 72 °C/45 seconds. The final extension step was 72 °C/60 seconds. PCR products were resolved in 10% non-denaturing polyacrylamide gels and visualized by use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Signal intensities of telomerase products were standardized against the internal control and subjected to semiquantitative evaluation using ImageQuant™ software (Molecular Dynamics).

**Subchromosomal Localization of Candidate Telomerase Repressor Gene(s)**

A total of 44 polymorphic dinucleotide markers, mapped to the short arm of chromosome 3, were selected from the Genethon Human Genetic Linkage Map (34) and the Human Genome Database (Johns Hopkins University, Baltimore, MD). The relative order of these markers was established from data available in the Genetic Location Database (35). Cytogenetic information was also taken into consideration in cases where linkage data were weaker. Markers were assessed for informativeness by defining the allelotype of both chromosome 3 donor hybrids (A9-Hytk3 and A9+3) and the 21NT (recipient) cell line. DNA was isolated from hybrid and parental cells (control) by proteinase K digestion and phenol/chloroform extraction (36). DNA samples were diluted to 6 ng/μL and 30 ng was added to 15-μL aliquots of PCR mix containing 200 nM of each primer (Advanced Biotechnologies, Epsom, Surrey, U.K.). One of each primer pair was labeled with one of three fluorescent tags: HEX, TET, or 6-FAM. PCR was carried out using conditions that yielded a single product of the appropriate size. PCR amplification products were mixed and separated by electrophoresis on an ABI 377 DNA sequencer (Applied Biosystems Inc. [ABI], Foster City, CA).

Data were collected using the Genescan Collection and Analysis Software (ABI version 1.2.1). The resultant files were transferred to Genotypier (ABI version 1.1.1) for analysis and allele calling.
Telomerase Activity in 21NT Cells and Microcell Hybrid Colonies

Telomerase activity present in 21NT breast cancer cells and in monochromosomal hybrid derivative clones (constructed using normal human chromosomes 3, 8, or 20) was determined using the TRAP assay (33). Hybrid colonies were lysed in situ to permit analysis of telomerase in cells during their normal division phase prior to reduction in growth rate and senescence. Strong repression of telomerase activity was observed (Table 2) in 36 (78%) of 46 Hyg-3/21NT hybrids and in 20 (67%) of 30 Neo-3/21NT hybrids. In contrast, TRAP assays performed on Hyg-8/21NT and Hyg-20/21NT hybrid clones at an equivalent stage of development revealed low levels of telomerase activity in only eight (11%) of 73 and two (8%) of 24 clones, respectively (Table 2); these proportions could be wholly accounted for by the interclonal variability in telomerase activity in the parental 21NT cell line (13% of subclone extracts possessed activity 10% or lower than that of a mass culture extract).

Telomerase activity was judged as being repressed if TRAP ladder intensities were less than 10% of the signal generated by an extract of a representative colony of 21NT parental cells (quantified relative to the internal standard following phosphorimaging). In practice, telomerase activity in the majority of monochromosomal 3 hybrids was less than 2% of the control. Fig. 1 shows typical results (TRAP gels and ImageQuantTM analysis of TRAP ladder signal intensities) obtained with five Hyg-3/21NT hybrids (four of which were telomerase repressed), five Neo-3/21NT hybrids (three of which were repressed) plus, as controls, 10 Hyg-8/21NT hybrids and the parent 21NT cell line. The 21NT extract was derived from a colony of similar size to the hybrids and a 10-fold dilution of the 21NT sample gave a corresponding reduction in the signal intensity of the TRAP ladder. The range of telomerase activities observed in the Hyg-8/21NT hybrids was similar to that detected in randomly isolated 21NT colonies. Failure to generate TRAP ladders after known positive extracts were treated with ribonuclease confirmed the specificity of the assay for the ribonucleoprotein telomerase. Reproducible standard internal control (SIC) signal intensities eliminated the possibility that the weak TRAP ladders seen with a number of extracts were caused by Taq polymerase inhibitors. In addition, mixing weakly positive or negative samples with positive samples produced no evidence for the presence of enzyme inhibitors in the former extracts (data not shown).

Mean telomere restriction fragment (TRF) lengths in the 21NT parental cells were determined by Southern blotting (39) with a (TTAGGG)4 oligonucleotide probe. Results indicated that 21NT cells possess short telomeres, since mean TRFs were approximately 3 kilobases (kb) in length (data not shown). The cell numbers in senescing hybrids were too small to allow TRF measurement and because of this we were unable to prove that telomere attrition took place prior to growth arrest. However, the shortness of TRFs in the parent cells was consistent with the low

Table 1. Characterization of division potential of 21NT microcell hybrids

<table>
<thead>
<tr>
<th>Chromosome transferred</th>
<th>No. of microcell fusions</th>
<th>No. of hybrids recovered (per 10^6 cells)</th>
<th>No. of senescence hybrids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyg-3</td>
<td>6</td>
<td>257 (5.4)</td>
<td>231* (90)</td>
</tr>
<tr>
<td>Neo-3</td>
<td>2</td>
<td>111 (6.9)</td>
<td>100* (90)</td>
</tr>
<tr>
<td>Hyg-8</td>
<td>1</td>
<td>22 (2.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Neo-12</td>
<td>1</td>
<td>25 (3.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hyg-20</td>
<td>1</td>
<td>44 (5.5)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Of these hybrids, 24 Hyg-3 and 13 Neo-3 clones produced immortal variants after cells had passed through growth crisis; the remainder entered permanent growth arrest. (Note: the transferred chromosomes were tagged with bacterial resistance genes for hygromycin B or neomycin.)

Table 2. Determination of telomerase activity in 21NT microcell hybrid colonies

<table>
<thead>
<tr>
<th>Hybrid (No. of experiments)</th>
<th>No. of hybrids assayed*</th>
<th>No. of hybrids with telomerase repressed† (%)</th>
<th>No. of hybrids with normal telomerase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyg-3/21NT (5)</td>
<td>46</td>
<td>36 (78)</td>
<td>10 (22)</td>
</tr>
<tr>
<td>Neo-3/21NT (1)</td>
<td>30</td>
<td>20 (67)</td>
<td>10 (33)</td>
</tr>
<tr>
<td>Hyg-8/21NT (5)</td>
<td>73</td>
<td>8 (11)</td>
<td>65 (89)</td>
</tr>
<tr>
<td>Hyg-20/21NT (1)</td>
<td>24</td>
<td>2 (8)</td>
<td>22 (92)</td>
</tr>
</tbody>
</table>

*Telomerase assay was performed on actively proliferating hygromycin B (hyg)-resistant or neomycin (neo or G418)-resistant colonies of greater than 1000 cells.
†Colonies were classified as negative at greater than 10-fold repression of telomerase activity in comparison with the telomerase activity produced by parent 21NT cells.
division potential of those monochromosome 3:21NT hybrids that exhibited strongly repressed telomerase.

**PCR and FISH Analysis of Microcell Hybrids for the Presence of Transferred Chromosome 3 Material**

Preliminary evidence of successful monochromosome 3 transfer into 21NT cells was obtained by PCR using primers directed at the selectable marker (neo or Hytk). Amplification of the expected sequence occurred in all of the DNA samples from 20 randomly selected clones tested (data not shown). Insufficient division potential in hybrids destined to undergo delayed growth arrest (i.e., the majority of hybrids) precluded metaphase FISH analysis. In parental 21NT metaphase spreads (modal chromosome number 45), two large submetacentric chromosomes were fully painted (Fig. 2). Chromosome 3 material was also detected in single discrete regions of three other 21NT chromosomes (two interstitial in location and one a translocation). Of six immortal (i.e., revertant or segregant) Hyg-3/21NT hybrids tested, four carried an additional submetacentric chromosome 3 (Fig. 2). The remaining two hybrids possessed novel subchromosomal fragments but were without an additional intact chromosome 3 indicating that, in these clones, the introduced copy of chromosome 3 had undergone breakage and translocation of fragments to 21NT chromosomes.

**Reappearance of Telomerase Activity in Monochromosome 3/21NT Hybrids Escaping Crisis**

Around one in 11 chromosome 3/21NT hybrids escaped the induced growth arrest after a prolonged growth crisis. A total of 36 of these revertant hybrids was established from 14 experiments. To investigate further the dependence of 21NT cell immortality on the presence of active telomerase, extracts from these hybrids were subjected to TRAP immediately following their emergence from crisis. Telomerase activity in all samples was comparable to that observed in the parental cell line. Similarly, all of 48 immortal Hyg-3/21NT hybrid clones that proliferated continuously in the absence of crisis proved strongly telomerase positive. Thus, in all cases, hybrid immortality was strongly linked to the presence of functional telomerase.

To obtain evidence of a causal link between telomerase repression and delayed growth arrest in 21NT/monochromosome 3 hybrids, actively proliferating Hyg-3/21NT and Neo-3/21NT hybrid colonies of unknown fate were isolated by the use of cloning cylinders and tested simultaneously for telomerase activity and division potential. Ten Hyg-20/21NT hybrid colonies were included in the study as controls. One half of each colony was lysed for TRAP analysis; the remaining cells were successfully returned to culture with the exception of clone 16.4 that failed to re-establish. The results are shown in Fig. 3. Seven of 10 monochromosome 3 hybrids underwent growth arrest and TRAP analysis of proliferating cell lysates revealed telomerase activity.
to be repressed in all of these clones. Interestingly, one initially repressed hybrid (clone 16.1) entered growth arrest but reverted to immortality after crisis. This coincided precisely with restoration of telomerase activity (data not shown). Functional telomerase activity detected in two immortal clones (15.2 and 16.5) that displayed no evidence of crisis was 35% and 120% of parental cell levels, respectively. All 10 Hyg-20/21NT control hybrids were immortal and nine of these had high levels of telomerase activity detected in two immortal clones (15.2 and 16.5) that displayed no evidence of crisis was 35% and 120% of parental cell levels, respectively. All 10 Hyg-20/21NT control hybrids were immortal and nine of these had high levels of telomerase.

**Fine-Structure Deletion Mapping of Candidate Telomerase Repressor Gene(s)**

Based on our previous experience in mapping human cell growth arrest genes (27), immortal 21NT hybrids that arose during this investigation either (a) by continuous exponential proliferation of hybrid clones or (b) from a rare event during crisis (see above) were regarded as potential segregants that may have suffered loss of a critical telomerase repressor gene (or genes) on chromosome 3. Our recent analysis (28) of the structural integrity of a transferred normal chromosome 3 in rare, telomerase-positive, head and neck carcinoma (BICR31) hybrids revealed no evidence of deletions on 3q, the long arm of chromosome 3, but did show nonrandom deletions in the 3p21 region of the short arm. Therefore, in this study, deletion mapping of 21NT segregants was focused on the short arm of chromosome 3.

Allelotypes were obtained from 125 segregants recovered from 14 independent experiments, in which both the Hyg-3 and the Neo-3 chromosomes had been used to demonstrate telomerase repression and delayed growth arrest in 21NT microcell hybrids. Thirty-eight informative polymorphic markers have been cryopreserved for further fine-structure mapping. Twelve clones contained single contiguous deletions and their analysis identified two common regions of loss (Fig. 5). Deletions in six of these clones defined a discrete region at 3p21.3-3p22, with two clones containing deletions in only a single marker (D3S3623). Retention of informative adjacent markers in clone Hyg-3/10.7 defined the smallest commonly deleted region (SCDR, SCDR-1 of less than or equal to 2.1 cM (centiMorgans) (35). Two clones were deleted at D3S3623 and at two informative adjacent distal markers (D3S1277 and D3S1619). Overlapping deletions in a region defined by D3S1619-D3S1260 were also observed in 28 of 46 hybrids in which deletions were more extensive or complex.

A second distinct region of deletion between D3S3697 and D3S3722 at 3p12-p21.1, defined by clone Hyg-3/9.6 (SCDR-2 = 14.7 cM), was identified in six other hybrids (Fig. 5). Deletions in these clones appeared less heterogeneous than deletions involving the distal region (3p21.3-p22) because of the lower density of informative markers used at the proximal boundary of 3p.

**DISCUSSION**

The presence of active telomerase in the vast majority of human cancers, but not in surrounding normal tissue, has led to the suggestion that telomerase is derepressed at some point in the process of tumor development and that this event endows a
Fig. 4. Analysis of (AC)n microsatellite sequence-tagged sites in chromosome 3/21NT microcell hybrids: examples of Genescan software determined alleotypes (see “Materials and Methods” section) for informative D3S3521, D3S1263, D3S3623, and D3S3697 sites, showing allele discrimination. Key to scans (top to bottom): (i) hybrid with the donor chromosome 3 allele retained along with endogenous 21NT allele(s); (ii) hybrid with donor chromosome 3 allele deleted; (iii) 21NT parent cell line; (iv) unrelated standard positive control cell line (originally derived from a chronic lymphocytic leukemia [CLL]); and (v) A9-Hyk3 or A9+3 donor hybrid. Individual allele peaks identified in each scan are assigned a number (boxed) by Genescan. D3S3521: 21NT, 9; transferred allele, 7; CLL, 3,4. D3S1263: 21NT, 5,9; transferred allele, 3; CLL, 3,9. D3S3623: 21NT, 6; transferred allele, 8; CLL, 4,5. D3S3697: 21NT, 9,15; transferred allele, 22; CLL, 18,20. Peak height is a relative measure of the intensity of the fluorescent polymerase chain reaction product.

Fig. 5. Summary of allelotype analysis in 13 chromosome 3/21NT hybrids carrying simple contiguous deletions. The smallest commonly deleted regions (SCDRs) identified in the exogenous chromosome 3 at 3p21.3-p22 (SCDR-1) and 3p12-p21.1 (SCDR-2) are indicated by solid bars to the right, and their defining markers are in bold. N3: hybrids constructed with Neo-3 chromosome (chromosome 3 tagged with a bacterial neomycin resistance gene); H3: hybrids constructed with Hyg-3 chromosome (chromosome 3 tagged with a bacterial hygromycin B resistance gene).
clonally evolving neoplastic cell lineage with immortality (40,41). Telomerase, therefore, may represent an attractive molecular target against which novel therapeutic approaches could be developed. There are two key unanswered questions that may have an important bearing on whether antitelomerase therapies are likely to be effective: 1) Is the appearance of telomerase in the common human cancers the result of selection (24) or induction (25) (and therefore what is the likely consequence to normal tissues of inhibiting telomerase in vivo)? and 2) To what extent do human cancer cells uniquely depend on telomerase for their continued proliferation?

In this study, we have shown that telomerase can be strongly repressed in a well-characterized breast carcinoma cell line by the experimental introduction of a normal copy of human chromosome 3 and that repression is tightly associated with the induction of delayed growth arrest of the tumor cells. Additionally, by fine mapping the structural integrity of the introduced chromosome in the subset of monochromosome 3 hybrids that retained normal levels of telomerase activity, we have obtained strong evidence supporting the existence of a gene or genes that negatively regulate telomerase in normal cells. This analysis has defined two candidate regions on the short arm of chromosome 3 where telomerase repressor gene(s) may be located.

The definitive identification, and subsequent molecular cloning, of gene(s) that repress telomerase in human cells will have important consequences in relation to providing definitive answers to the two questions posed above. For example, the availability of cloned telomerase repressor sequences as probes should permit the mechanism of inactivation (i.e., mutational or epigenetic) of such genes in a variety of human cancers to be investigated directly and the timing of inactivation to be determined. In addition, the availability of vectors expressing telomerase repressor gene cDNA(s) will enable the efficacy of induced telomerase repression at arresting the growth of various human cancer cells to be properly evaluated and the rate at which immortal variants emerge in mass cultures of stably repressed transfectants to be accurately quantified.

The division potential of our monochromosome 3/21NT hybrids (10–18 population doublings) was consistent with the short mean TRF length we observed in the parent cells (around 3 kb). In a previous study, Ohmura et al. (42) also associated chromosome 3-induced growth arrest in RCC23 renal carcinoma cells with telomerase repression and telomere shortening, although the fate of only three clones was followed to terminal division. In their experiments, the greater division potential of these three RCC23 hybrids (23–43 population doublings) is not unexpected given the longer TRFs (around 6 kb) observed in the parent tumor cell line. Work to date, therefore, strongly points to the presence of one or more telomerase repressor genes, located on chromosome 3, which restore the program of replicative senescence in fully malignant human carcinoma cells.

Our experiments reported here have established a clear-cut relationship between telomerase repression and the induction of permanent growth arrest in a large number of independently derived monochromosome 3 hybrids. All hybrid clones with repressed telomerase either senesced or entered a pronounced growth crisis. No telomerase-negative clones were observed that retained immortality, and all clones that reverted to immortality via a rare event (after crisis) were telomerase positive. Alternative pathways of human telomere maintenance (possibly recombinational) have been implicated in a number of immortal cell lines, including a subset of simian virus 40 (SV40) large T-antigen-immortalized diploid fibroblasts (43) and a small proportion of human tumor cell lines (44). These cell lines generally have low or undetectable levels of telomerase activity and hypervariable telomere lengths. Clearly, from a therapeutic perspective, it is important to know, for a representative panel of tumor cell populations, whether and at what frequency such variants emerge under conditions of stringent telomerase repression. In this study, we observed no evidence of reversion to immortality via an alternative (nontelomerase-related) pathway in nearly $10^7$ cumulative hybrid cell divisions. It should be stressed that our experimentally induced (and, in many cases, strong) repression of telomerase activity was achieved by only a single normal copy of chromosome 3. Therefore, in normal diploid somatic cells, it is reasonable to expect that both alleles of a telomerase repressor gene would need to be functionally deleted for telomerase to be activated. Further work with a telomerase repressor gene cDNA vector (with which multiple-copy transfection could be readily achieved) or with powerful chemical inhibitors of telomerase will facilitate quantification of events (e.g., leading to activation of an alternative pathway) occurring at rates below $10^{-7}$ per cell division.

The two nonrandom genetic losses (at 3p21.3–p22 and 3p12–21.1) identified in this investigation, by allelotype analysis of rare immortal revertants, involve regions that commonly suffer loss of heterozygosity (LOH) and homozygous deletion in human cancers. The large (proximal) deletion (SCDR-2) at 3p12–p21.1 defined in six hybrids spans breast cancer-specific allele losses and several nonrandom homozygous deletions identified in breast, cervix, colon, lung, and renal carcinomas (45). The putative tumor suppressor gene FHIT (fragile histidine triad) has been mapped to 3p14.2 (46) and therefore could in theory be considered as a candidate, although the role of FHIT as a tumor suppressor gene is unclear (47). Interestingly, a deletion involving 3p13–p14.2, associated with human papillomavirus E6- and E7-immortalization of human uroepithelial cells, also overlaps this region (48). Similarly, Fusenig and Boukamp (49) have associated this region of chromosome 3p with immortalization in the minimally transformed human skin keratinocyte cell line HaCaT (50).

The distal deletion (SCDR-1) at 3p21.3-p22 defined by six other hybrid segregants is also of possible relevance to human cancer. Two clones were exclusively deleted at D3S3623 and the informativeness of adjacent markers in one of these indicated a minimal region that overlaps discrete homozygous deletions identified in lung cancer cell lines and tumors (51–53). This region is distal to a cluster of overlapping lung cancer-specific deletions at 3p21.3 that has also been associated with tumor suppressive activity in mouse A9 cell hybrids carrying a fragment of human chromosome 3 (54). Homozygous deletions at 3p21.3-p22 have not been reported in breast cancers. However, allelotyping studies have identified nonrandom LOHs involving this region (55) that were associated with tumor aneuploidy and poor patient prognosis.

Allelotype analysis of the head and neck carcinoma cell line, BICR31, in which we demonstrated chromosome 3-mediated repression of telomerase (28), revealed that most 3p microsatellites had undergone LOH (56). Although the absence of matched normal DNA precluded definitive allelotyping of the 21NT parent cell line, the great majority of markers were found to be heterozygous, even though G banding (29) and FISH
analysis indicated that extensive rearrangement of chromosome 3 had occurred. However, many tumor types, including breast cancer, have shown allelic losses in both regions of deletion identified in our chromosome 3/21NT hybrid segregants.

Our observation that two contiguous interstitial 3p deletions (rather than a single nonrandom genetic loss) were associated with immortality and functional telomerase was somewhat unexpected. The results could indicate the presence of two genes necessary for repressing telomerase, either of which may be deleted to cause derepression, and which encode elements of a single telomerase regulatory pathway. Alternatively, only one (possibly 3p21.3-p22) of the regions may represent the subchromosomal location of a single telomerase repressor, particularly since 3p14 has been well documented as a fragile site (47). This notwithstanding, we believe that the mapping information we have obtained to date provides sufficient grounds to justify the initiation of a positional cloning program to isolate the gene(s).

To this end, we are currently screening (by spheroplast transfer) yeast artificial chromosomes (57) from contigs mapping to the two regions of interest for telomerase repressive function in 21NT cells.

REFERENCES


NOTES

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