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13. ABSTRACT *(Maximum 200 words)*

The goal of this research is to test two new strategies to treat breast cancer, based on altering telomerase action. The ribonucleoprotein telomerase synthesizes telomeric DNA by copying a template RNA sequence within the essential RNA of telomerase. Without telomerase, as cells divide, their telomeres shrink and eventually the cells cease dividing. Telomerase activity is commonly found in human breast cancer cells but not in many normal cells. First, inhibitors of telomerase will be used to prevent telomerase from maintaining telomeres. We previously found that we can inhibit telomerase from breast cancer cell lines with reverse transcriptase inhibitors. The second novel (gene therapy) strategy will direct telomerase to synthesize "toxic" telomeric DNA sequences in breast cancer cells, therby quickly causing them to cease dividing. In this second strategy, the telomerase RNA template is altered. Progress to date: 1) we tested 139 compounds and found three new telomerase-specific inhibitors. 2) We have introduced engineered telomerase RNA genes that direct synthesis of mutated telomeres into breast cancer cells in culture. We determined that these telomerase RNA gene sequences caused deleterious telomeric DNA synthesis, thereby interfering with cell proliferation.

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Elizabeth W Brackett August 6, 1999

PI - Signature Date

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INTRODUCTION

The overall aim of the research funded by this grant is to manipulate telomerase activity in human breast cancer cells or pre-cancerous breast epithelial cells, in order to block cancer progression. Telomerase, a cellular ribonucleoprotein enzyme discovered several years ago in my laboratory (Greider and Blackburn, 1985, 1989), has the function of synthesizing one strand of the essential telomeric DNA by copying a short template sequence within the RNA moiety of telomerase into telomeric DNA. Telomerase adds this telomeric DNA to the ends of chromosomes to make up for the inability of other DNA polymerases in the cell to replicate these ends completely. We recently also showed that telomerase has a telomere capping function that stabilizes chromosomes even without net elongation of telomeres (Prescott and Blackburn 1997; Zhu et al., 1999). We and others have shown that telomerase is often activated in immortalized human breast cancer cell lines, and in breast tumors (Kim et al., 1994; Avilion et al., 1996; Strahl and Blackburn, unpublished work). It is much less active, or not active at all, in normal breast epithelium. This proposal was to explore two related approaches, one completely novel, for the treatment of breast cancer and/or prevention of breast cancer progression. Both approaches consist of interfering with telomere maintenance by telomerase, and each represents a new potential avenue for chemotherapy of human breast and other cancers. The long-term goal is to develop the information from these experiments into therapies for clinical use in the treatment and/or prevention of breast cancer.

Toward the first approach, we had previously shown that we can shorten the telomeres of immortalized human lymphoid cells in culture using a telomerase inhibitor (Strahl and Blackburn, 1996). Inhibition of telomerase in cells would potentially block telomere maintenance, and hence potentially block cancer cell growth. We have now tested 139 compounds for their possible action as telomerase inhibitors. One long-term goal is to determine whether telomerase inhibitors will be useful clinically in the prevention and/or treatment of breast cancer. Such inhibitors have not been tested clinically or even in breast cancer model systems.

The second approach is a novel gene therapy approach, exploiting the activation of telomerase commonly seen in breast cancer cells. The strategy is to engineer the telomerase RNA in breast cancer cells, causing the synthesis of "toxic" telomeres that block cancer cell multiplication. This second strategy is novel in concept and nature, because instead of attempting to inhibit telomerase action in cancer cells, it turns that action itself against cancer cells. The design of mutated template sequences was based on those we have used for the *Tetrahymena, K. lactis* and *S. cerevisiae* telomerase RNA templates (Yu et al., 1990; Gilley et al., 1995; McEachern and Blackburn, 1995; Kirk et al., 1997; Smith and Blackburn, 1999). Such mutations caused synthesis of telomeric DNA at the ends of the chromosomes that led to blocked cell division.

Technical objectives

Specific aims for the 24 month funding period:

1) Determine the effects of inhibiting telomerase activity on growth of immortalized breast cancer cells in culture.
2) Determine telomerase activity levels in breast epithelial cancer cells grown in primary short-term cultures directly from patient tumors, and the effects of inhibiting telomerase activity on the growth of these cells.
3) Introduce mutated telomerase RNA genes, engineered so that they direct synthesis of mutated telomeres, into breast cancer cells in culture, to determine which telomerase RNA gene sequences cause deleterious telomeric DNA sequences to be synthesized, thereby preventing cell division. The long-term goal is to determine whether the tumor growth of these telomerase template-engineered cells is altered after xenografting them into the nude mouse tumor model system.

BODY
ACCOMPLISHMENT OF EACH TASK IN STATEMENT OF WORK

Overview: In light of the results obtained with the ddG resistance of human breast cancer (MCF-7) cells in culture (see under Task 1), and the results obtained with dominant telomerase RNA template mutants in yeast (see under Task 16), after carrying out Task 1, we decided to focus on the "toxic telomeres" gene therapy approach (i.e., Tasks 15 and 16 in Technical Aim #3), instead of the originally planned Tasks 7-14. The same strategies that were outlined in Tasks 7-14, originally for the investigation of effects of putative telomerase inhibitors, were accordingly adapted in testing the effects of expressing mutant telomerase RNA genes in MCF-7 cells.

Technical objective #1: Determine the effects of inhibiting telomerase activity on telomere maintenance and growth of immortalized breast cancer cells in culture.

Task 1: Titrate inhibitor ddG [causes reproducible and striking telomere shortening in cultured lymphoid cells (Strahl and Blackburn, 1996)] to determine its concentration cytotoxic to MCF-7, MCF-7 M1, MDA435, MDA435-M1, UACC-812, UACC-812-M1 and MCF10A human breast cancer cells (cell lines described in Methods) after short-term (1 week) growth, using concentrations up to 1 mM.
Accomplishments: The effect of ddG on telomere maintenance in breast cancer cells (MCF-7 cells) in culture was tested.
Rationale: Based on our earlier work on a protozoan telomerase (Strahl and Blackburn, 1994), we reported previously that two nucleoside analog inhibitors of reverse transcriptases, ddGTP and AZT-TP, inhibit human telomerase activity *in vitro*, that ddG in the cell growth medium causes reproducible and striking telomere shortening in B- and T-cells in culture, and that AZT caused shortening in some cultures (Strahl and Blackburn, 1994, 1996). We also proposed that both telomerase and telomerase-independent mechanisms can determine telomere lengths in these T- and B-cell lines (Strahl and Blackburn, 1996).

Problems encountered: In short-term culture (1 to 2 weeks) MCF-7 cells were completely resistant to cytotoxicity of ddG at concentrations in the medium of even up to 1 millimolar. This unprecedentedly high level of resistance raised the question of whether this nucleoside analog was being taken up by these cells.

Task 2: Determine whether prolonged passaging of these cell lines in subtoxic concentration of ddG causes decreased cell growth rates or viabilities, using previously published methods to monitor for lymphoid cell lines (Strahl and Blackburn, 1996).

Task 3: Determine whether telomeres shorten during prolonged passaging of these cell lines in subtoxic concentration of ddG, using Southern blotting analysis (Strahl and Blackburn, 1996).

Task 4: Titrate other inhibitors including but not limited to Ara-G, ddI, ddA, d4T, and Foscarnet to determine concentrations cytotoxic to the same set of human breast cancer cells used in Tasks 1 -3.

Because of the results obtained in Task 1, Tasks 2, 3, 4 and 6 were not done; instead, Task 5 and Tasks 13-16 were the focus of the work.

Task 5: For inhibitors available in triphosphate form, use TRAP assays as described previously (Strahl and Blackburn, 1996) to test that telomerase activity from each cell line is sensitive to inhibitor. Test non-nucleoside analogs (e.g. Foscarnet) directly.

Rationale
The goal was to test whether telomerase inhibitors can prevent telomerase from maintaining telomeres. Telomeric tracts are several thousand base pairs long in normal somatic cells, but are often shorter in cancerous cells. In addition, cancer cells divide more rapidly than typical normal somatic cells. Therefore, even if telomerase activity is needed over the long-term for some normal cell types, inhibiting

telomerase over the short-term may be a way of targeting cancerous cells for chemotherapy without severe effects on normal cells. Toward that goal, we first optimized the telomerase assay, then tested 139 different compounds for effects on telomerase activity *in vitro*.

Accomplishments:

Optimization of telomerase assays: The TRAP assay for measurement of *in vitro* telomerase activity levels was modified to deal with issues of signal specificity that we had uncovered (C. Strahl, and EHB, in collaboration with T. Tlsty's group, UCSF; unpublished observations). Telomerase was assayed using preparations from MCF-7 breast cancer cell lines and also, for comparative purposes, Jurkat T-cells (a T-cell leukemia line). The extracts were partially purified by a method we had previously developed and optimized (Strahl and Blackburn, 1996 and unpublished work). We defined the range of cell or protein amounts (determined by micro-Bradford assays) within which the assay showed linearity. The assays were tested to be in the linear range by titrating the amounts of extract used and the amounts of each extract that gave linear quantitative activity levels. We found that this is especially critical for breast epithelial cells. In addition we identified artifactual positive signals in the TRAP assay, and devised controls to ensure signals are attributable to telomerase action itself.

We tested whether telomerase inhibitors acted similarly *in vitro* on telomerase activity from human breast epithelial cells and lymphoid cells. For example, it is unknown whether telomerase in different human cell types has different post-translational modifications, or is composed of gene products of different protein gene family members, that might alter its enzymatic properties in different tissues. ddGTP and AZT-TP had the same profiles of inhibition of telomerase activity from both the immortalized human breast epithelial cell line MCF-7 and an immortalized T-cell lymphoid cell line, using TRAP assays. Therefore, from the limited data so far, there is no indication that telomerase activities from two very different cell types (lymphoid versus breast epithelial) have different enzymatic properties.

Terminal deoxynucleotidyl transferase (TdT); a mimic of telomerase in the TRAP assay that potentially produces false positives for telomerase activity.

In collaboration with Thea Tlsty's group, Department of Pathology, University of California San Francisco, we found that TdT could give a false positive reaction for telomerase activity. Using a set of primers (TS and CS primers; Kim et al., 1995) we found that commercially available purified TdT produced the same 6 base ladder pattern of bands as authentic telomerase. These bands had electrophoretic mobilities that were indistinguishable from those produced by authentic telomerase activity (even in high resolution sequencing gels). At high RNase concentrations (which have often been used as controls for human telomerase activity) this false positive reaction was inhibited. Thus such a false positive reaction might be misinterpreted as telomerase. The high concentrations of RNase also unspecifically inhibited the PCR amplification step in the TRAP telomerase assay. However, the RNAse concentration needed to block the TdT false positive reaction was much higher than that needed to completely inhibit telomerase activity. Therefore, RNase controls for telomerase activity were done using a low RNase concentration that we have determined is specific for inhibition of telomerase.

Identification of novel telomerase inhibitors *in vitro*

The bases for choosing these compounds were:

(i) Nucleoside analogs that mimic the normal substrates for telomerase, and thus may compete or bind to sites that affect activity. We have shown such effects for AZT and ddG with human lymphoid cell and breast cancer cell telomerase, which act (at least in part) by competing for the nucleoside triphosphate substrate binding site of telomerase (Strahl and Blackburn, 1996). In addition, high dGTP concentrations and G-rich primers stimulate telomerase by binding elsewhere on the telomerase ribonucleoprotein (Lee and Blackburn, 1993). Accordingly, we tested a large number of purine (base) analogs made by combinatorial chemistry techniques for inhibitory or other effects that may be exerted through that mechanism.

(ii) A set of compounds derived from the compound Calcomine Orange, that were designed from structural studies to bind into the HIV reverse transcriptase active site.

Accomplishments:
Established breast cancer cell lines were used for direct comparison of conventional and TRAP *in vitro* telomerase assays, with which we have extensive experience (Strahl and Blackburn, 1996; E. Orr and EHB, in preparation)). The 1-tube TRAP *in vitro* telomerase assay was adopted as the best method for screening of inhibitors. We tested 139 compounds to determine whether they act as telomerase inhibitors *in vitro*.

Additional controls (besides the minus-RNase and no-extract controls standardly used in telomerase activity assays) included adding the solvent DMSO that was used to dissolve a subgroup of the inhibitors, at the highest concentration used. To distinguish inhibition of telomerase from effects on the Taq polymerase used for the PCR step of the TRAP assay, each assay consisted of a pair of reactions: one (the experimental) in which the inhibitor was added at the beginning of the telomerase elongation step, and the other (the control) in which the inhibitor was added after the elongation step and before the PCR product amplification step. Each inhibitor was tested at three concentrations—1, 5 and 10 micromolar. Products were analyzed by DNA sequencing gel electrophoresis. This type of gel produced the highest resolution of products.

The 139 different compounds screened for their ability to act as inhibitors of telomerase *in vitro* were divided into the two classes of compounds i and ii described above:

(i) Purine analogs
Three compounds at 5 micromolar concentration inhibited telomerase, although they also inhibited the PCR step: 6-(4-methoxy)purine, Compound SJK-III-37 and Compound 5-2.

Another group of test compounds (5 guanine derivatives) appeared to stimulate telomerase activity at 10 micromolar concentration.

(ii) Calcomine Orange derivatives
3 out of 20 tested had an apparently specific inhibitory effect on telomerase, and not the PCR step, at 5 micromolar concentration. These 3 are therefore lead compounds for further investigation. Of the remaining 17, 10 inhibited the telomerase elongation step but also the PCR reaction at 5 micromolar concentration, and 7 others had no effect on telomerase or the PCR step at 1 micromolar concentration.

Task 6:. Determine effects on cell growth and viability of prolonged passaging of each cell line in subcytotoxic level of each inhibitor as described previously (Strahl and Blackburn, 1996).

See comments under Task 1.

Task 7: Determine whether telomere shortening occurs in these cell lines in subtoxic concentration of each inhibitor, using previously published methods (Strahl and Blackburn, 1996).

Instead, done for hTER template mutant transformant lines.

Task 8: If any inhibitors begin to cause telomere shortening or slow cell growth, test combinations of inhibitors, as described previously, for augmented or synergistic effects (Strahl and Blackburn, 1996).

Task 9: Test that telomerase activity has remained sensitive to inhibitor after prolonged passaging, as described previously (Strahl and Blackburn, 1996).

Technical objective #2: Determine telomerase activity levels in breast epithelial cancer cells grown directly from patient tumors in primary short-term cultures (tumor explant cultures), and effects of inhibiting telomerase activity.

Task 10: Use telomerase TRAP assays at different passages (see Preliminary Results), during growth of tumor explant cultures as described (Dairkee et al., 1995).

Task 11: Using the same methods, measure telomerase levels in the "normal" peripheral duct epithelial cells from the same individuals, grown out in separate short-term cultures. Compare with tumor cell telomerase levels determined in Task 10. Analyze results statistically.

Task 12: Use *in vitro* TRAP assays to test whether any inhibitors found in Technical Objective #1 also inhibit telomerase activity from these primary epithelial tumor cell cultures.

Tasks 8-12—see comments under Task 1

Task 13: Perform short-term cytotoxicity titrations of each inhibitor using miniaturized cultures grown in 96-well micro-titer dishes. Monitor cell growth microscopically.

Instead, adapted for hTER template mutant transformant lines—see under Task 16.

Task 14: Test whether cessation of cell growth of tumor explant cultures can be hastened by growth in the inhibitor.

Instead, adapted for hTER template mutant transformant lines—see under Task 16.

Technical objective #3: Synthesis of "toxic" telomeres:
Introduce mutated telomerase RNA genes, engineered so that they direct synthesis of mutated telomeres, into breast cancer cells in culture, to determine which telomerase RNA gene sequences cause deleterious telomeric DNA sequences to be synthesized, thereby preventing cell division.

Rationale

About 99% of cells in the adult human body are not proliferating, and are therefore unlikely to be affected by altering telomerase. Therefore, we initially have used an inducible promoter that is not tissue specific to express the mutated telomerase RNA genes, in order to determine, first, whether "toxic" telomere effects can be found in cells in culture. The design of the mutated telomerase RNA templates was based on those that we have shown produce "toxic" telomeres in *Tetrahymena, K. lactis* and *S. cerevisiae* .

Task 15: Months 1-5. Perform site-directed mutagenesis of subcloned telomerase RNA template bases, as described by us previously (e.g. Gilley et al., 1995). Insert into suitable vector. Check sequences by resequencing.

This was completed.

Detailed description of accomplishment of Task 15:
Cloning of telomerase RNA genes containing specifically mutated template regions: We sub-cloned a 598 bp segment containing the 451 base human telomerase RNA gene (hTER gene) plus ~147 bp of downstream region (Feng et al., 1995). The gene was cloned by PCR from the genome of a human cell line with highly active telomerase (the Jurkat T cell lymphocyte cancer cell line) in order to have a telomerase RNA allele that was known to be functional.

The cloning required optimization of the PCR conditions for this particular gene sequence. What initially appeared to be a problem was that, upon obtaining sequence, the sequence did not match that in the published database at several positions in the coding region of the hTER gene. However, that original database sequence was subsequently been shown to be in error at those positions. We found a sequence that did match the correct sequence that was published subsequently. To ensure that we had

obtained the correct sequence, 6 independently obtained cloned PCR products were sequenced. Half (3/6) had a novel base change (at position 137 in the coding sequence of the RNA) that differed from that in the database and was not a PCR artifact. (One cloned sequence also had a PCR -generated error). Cloning was also accomplished from normal (non-cancer cell) human genomic DNA. 5 independent clones were sequenced and these results confirmed the sequencing results obtained with the Jurkat cell clones, and also confirmed the published corrected sequence and another allelic variant (a single base change outside the mature RNA sequence at position 514) (Bryan et al. 1997).

We then sub-cloned each mutant-template and the control wild type hTER gene into a vector with a selectable marker. Mutated template sequences were made by standard methods. The sequences of the mutagenized RNAs were confirmed by DNA sequencing. Constructs were made with a vector containing a Tetracycline-inducible promoter (commercially available, Clontech) to control transcription of the telomerase RNA gene.

Task 16: Months 6-24. Transfect mutated (or wild type control) telomerase RNA gene in vector construct into breast cancer cell lines. Identify transformant lines. Where appropriate, induce expression of mutated telomerase RNA gene construct. Monitor growth properties of (induced) transformants.

This was completed.

Detailed description of accomplishment of Task 16:
New cell lines generated: Two breast cancer cell lines were chosen for use as the parent lines: MDA231 (supplied by Dr. Chris Benz, UCSF) and MCF-7-Tet-Off (purchased from Clontech). MDA231 cells were transfected with the Tet-on Plasmid and to date stable transfectants have been identified and frozen for further strain construction. In the future, quality control for inducibility with tetracycline will be performed.

The MCF-7 parent cell line is derived from a breast adenocarcinoma pleural effusion. The MCF-7-Tet-Off line is stably transformed with a Tet-Off plasmid (pUHD15-1neo, commercially available from Clontech). MCF-7-Tet-Off cells were transfected with each of the 5 different mutant telomerase RNA gene constructs, or with the wild type telomerase RNA gene construct as a control. The 5 mutant template sequences are listed in Table 1-99. Each hTER gene was placed under the control of an upstream modified doxycycline-repressable CMV promoter, plus a downstream SV40 polyA site, in the vector, which carried a hygromycin-resistance marker. Stable transfectant lines were obtained (Table 1-99).

Expression of mutant-template telomerase RNA (hTER) genes in MCF7 breast cancer cells: We have successfully achieved the objective of expression of mutant telomerase RNA (hTER) genes in MCF-7 breast cancer cells. We performed assays for telomerase for the MCF-7-Tet -Off cell line, found conditions that were suitable for the assay for this cell line, and showed the MCF-7 Tet-Off line is telomerase-positive. Thus this cell line was predicted to be competent to assemble and utilize the hTER transcript from an introduced hTER gene construct.

Cells were grown in selection medium to obtain stably transfected lines. The medium for cell culture was 90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, Tetracycline-free, 2 mM L- glutamine, and 100 micrograms/ml each of sodium penicillin G, streptomycin sulfate and hygromycin. Doxycycline was also present to keep expression of the hTER construct uninduced during the selection. Once cell lines had been selected, stocks were frozen and thawed as needed for experiments.

TABLE 1-99

Transfection of breast cancer cell lines with telomerase RNA template mutants*

Mutant*	WT	1	2	3	4	5
Number of stable transfectant lines obtained:	25	34	42	31	28	26
Number of cell lines induced and telomerase RNA prepared:	18	31	27	26	12	20
Number of cell lines tested by RT-PCR and Northern blotting and confirmed to date to express the introduced telomerase RNA (in progress):	4	12	11	5	5	4

Footnotes to Table 1-99. *Mutant designations. Telomerase RNA base(s) mutated are indicated by the residue number in the telomerase RNA coding sequence, numbered from the 5' end of the RNA, and the base to which the residue was changed indicated by G (guanine) or A (adenine). +AA: two additional A residues were introduced. WT: wild -type. See Figure 1.

WT	wild-type
mutant 1	46, 47, 50, 51G
mutant 2	46, 47, 50-53G
mutant 3	47A, 50G
mutant 4	47A, 50G, 53A
mutant 5	49+AA, 49A

Each cell line listed in the Table 1-99 was an independent transformant clonal line.

Expression of the introduced telomerase RNA genes: Expression of the mutant-template or wild type introduced telomerase RNA gene constructs has been monitored for several of the cell lines to date by RT-PCR, using oligonucleotide primers specific for the altered template region sequences (see Table 1-99), and by Northern blotting.

For all 5 mutants we used RT-PCR, and for selected mutants Northern blotting, to assess mutant telomerase RNA levels in induced and non-induced cells at different time points after induction. Where complete, the Northern blotting data has confirmed in all cases the RT PCR results. In addition, the Northern blotting showed that the introduced telomerase RNA gene produced the expected longer hTER transcript carrying extra nucleotides at the 5' end, as has been described for hTER expressed from the CMV promoter used in this work (Mitchell et al. 1999).

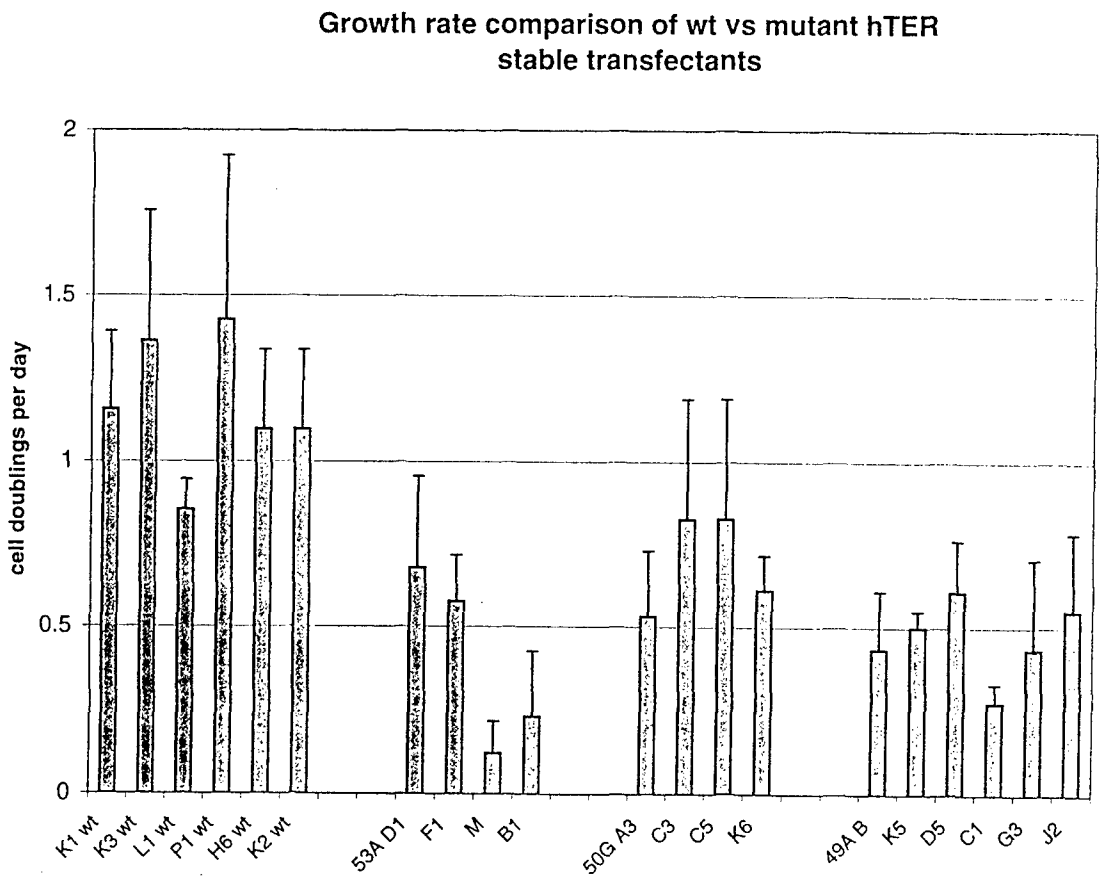
Induction had different properties from those expected based on the manufacturer's description of the properties of the MCF-7 Tet-Off cell line used: 1) for all cell lines that showed any detectable

expression of the introduced hTER gene construct, even the uninduced cells showed significant levels of transcription of the introduced hTER gene; i.e., repression was leaky 2) the manufacturer's description of the MCF-7 Tet-Off line used in these experiments was that induction would reach a peak 12- 24 hr after removal of doxycycline from the medium. However, no, or only low levels of, induction were noted after 1 and 2 days of growth in inducing conditions. Induced levels then increased several fold over the subsequent 2 – 6 weeks of growth under inducing conditions.

Cell growth properties and growth rates: Cell growth rates were monitored as follows: after selection for the marker carried on the plasmid as follows: cells were grown in medium containing 0 (induced) or 2 microg/ml (uninduced) doxycycline. We kept track of growth rate of each independent transformant clonal cell line by allowing cells to grow to confluence, then splitting them at appropriate ratios. Cells were passaged as soon as they reached confluence. Slow-growing cells were passaged at intervals no longer than 2 weeks, even if they had not reached confluence. Thus, cells were passaged based on whichever occurred first - confluence or 2 weeks.

In all WT transformant lines (with one exception described below), cells always reached confluence within 10 days and were passaged by splitting them 1:7 or 1: 15 with fresh medium. For example, a confluent flask of WT transformant cells was split 1: 7, grown until it reached confluence again after 10 days of growth, when it was again split 1: 5 and allowed to grow. The exception was one WT line, which grew slowly for 1 - 2 passages after thawing, then underwent an increase in growth rate.

In the case of all the mutant template lines tested, no line grew as fast as the WT lines. The mutant lines were split often as little as 1: 3, despite which they frequently failed to reach confluence after 2 weeks' growth. As noted above, these mutant cells were split no less frequently than once per 2 weeks regardless of progress to confluence.



For each independent transformant cell line, the histogram corrects for the split ratio used at each passage, and the number of days between successive passages, to show the calculated average cell division rate for that cell line. The calculated rate shown is based on all cells reaching confluence when split, which was the case for all WT hTER transformed lines. However, it was not the case for mutant hTER cell lines in a typical passage, because these cell lines all grew relatively slowly. Hence, all the cell growth rates plotted for the three types of mutant lines (53A, 50G and 49A) represent underestimates of the actual cell division rates, while the WT rates are the actual rates. .

Problems encountered: To obtain stable transformants with integrated hTER constructs, cells were grown initially in selective medium which also contained doxycycline to keep the introduced genes repressed. 10 nanograms/ml doxycycline, based on the manufacturer's protocol manual supplied with the MCF-7-Tet-Off cell line specifying 5 nanograms/ml doxycycline for uninduced conditions this cell line. Upon enquiry, the manufacturer stated that the 5 nanogram/ml concentration listed in the manual was incorrect and should have been 2 micrograms/per ml. Thus the lines were initially selected under conditions in which minimally partial induction of the hTER gene was expected. If the hTER allele produces a growth defect (as our results suggest is the case for the 3 mutant hTER gene constructs - see above), this may have affected the profile of cell lines obtained, because the selected colonies might be expected to be those with lower expression of the hTER template mutant allele. The correct doxycycline concentration was then used for continued growth of the cells in the "uninduced" state (but see notes above).

The transfection system resulted in lower expression of the hTER gene than expected, despite being on an expression plasmid with a strong promoter.
To optimize expression of the hTER gene, we performed a time course to determine the course of induction. Maximal induction of expression was not reached at 24 hrs as published, but rather was reached only after at least 2- 4 weeks after cells were put into inducing growth conditions.

Currently underway/ in progress:

1. To optimize expression of the hTER gene, we are currently trying an amplification protocol (based on the method originally used with methotrexate selection for increasing the DHFR gene copy number). This protocol involves selecting subpopulations of the MCF-7 cells carrying the mutant hTER gene in gradually stepped up concentrations of the drugs used for selection of inserted genes hygromycin and G418 in this case. These experiments have been initiated for a subclone of the MCF-7-Tet-Off cell line carrying the mutant 53A D1. It is currently being grown in 400microg/ml hygromycin and G418 (each).

2. New expression vectors: a. pLNCx plasmid - viral construct
The inducible retroTET system (which we have obtained from Helen Blau's lab, Stanford) will be used to generate new lines overexpressing the mutated hTER genes under the natural hTER promoter and/or the 3' processing region of the hTER gene. By performing site-directed mutagenesis, we have made a mutant 53A hTER gene (see footnote to Table 1-99), sequenced it to check it has the correct sequence, and constructed in two versions : its full length genomic version, and a version containing the endogenous 3' downstream region and a CMV promoter. These have been cloned into the pLNCx vector. We have transfected the retroviral packaging line with a GFP reporter gene, then infected it with different titers of the vector alone and are currently selecting stably transfected lines in selection medium.

Cell growth properties are still being monitored for the two remaining hTER template mutants. This work is in progress.

Preliminary results on telomere length have been obtained: after 6 weeks of growth under inducing conditions, telomeres in the 53A and 49A mutant lines are much shorter than in the control WT hTER transformed line. Further telomere blotting analyses are in progress

Summary of results for Technical Objective #3: We have successfully achieved the objective of expression of mutant telomerase RNAs in MCF-7 breast cancer cells. These initial studies used an inducible promoter. To date we have sufficient data sets on 3 mutants to assess the effects on growth and telomere length: we have analyzed 6 independent wild type hTER transformant lines (the control lines), 4 independent 53A mutant lines, 4 independent 50G mutant lines and 6 independent 49A mutant lines. Based on this one data set available to date, there appears to be a significance difference in growth rates of WT and mutant stable transfectants.

If we find that cell proliferation can be stopped by synthesis of "toxic" telomeres, then one long-term goal will be to minimize potentially deleterious expression of mutant telomerase RNA in those normal cells with active telomerase. To that end, the Her2 gene promoter (reviewed in Hung et al., 1995), which is specific for breast tissue cells, will be used to target expression of the altered telomerase RNA gene to breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of new class of inhibitors of human telomerase.
- Expression in breast cancer cells of novel versions of human telomerase RNA (hTER) genes carrying template mutations made by site-directed mutagenesis.
- Discovery that growth of human cancer cells in culture is slowed by co-expression of hTER genes carrying specific template mutations, even in the presence of the expressed endogenous wild type telomerase RNA.

REPORTABLE ACCOMPLISHMENTS

Presentations

Presented at the weekly Bay Area UCSF Cancer Center Breast Oncology Program Research Meetings and the UCSF Cancer Center Seminar Series, 1999.

Publications

1. Effects of Calcomine Orange and purine derivatives on human telomerase activity *in vitro.* Erica Orr, Nathaniel Grey, Peter Schultz, Karl Maurer, George Kenyon, EHB, In preparation.

2. Cell growth and telomere effects of introduced human telomerase RNA mutant-template genes in human MCF-7 breast cancer cells. Inna Botchkina, Melissa Rivera, EHB, In preparation,

Patents

None beyond initial instigation with UCSF institutional officers.

Degrees

In progress – advanced to Candidacy for Ph.D. degree: Melissa Rivera

Employment Opportunities Generated by This Work

Employment of Erica Orr, Staff Research Associate II
Employment of Inna Botchkina, Staff Research Associate III

Prostate Cancer Research Opportunities Generated by this Work

A spin off of this research has been to use the same strategy described under Technical Objective #3 in research using human prostate cancer cells (Moses Kim, Ph.D. student).

New Collaborations Generated by this Work

1. Collaboration in determining telomerase activity levels in breast tumors and breast cancer cells, and improving the telomerase assay *in vitro* to overcome artifactual signals: Drs. Helen Smith, Shanaz Dairkee, Gail Colbern, California Pacific Medical Center, San Francisco, and Dr. Thea Tlsty, Department of Pathology, UCSF.

2. Collaboration in analyses of effects of novel potential inhibitors of telomerase, based on a rational drug design approach: Drs. Karl Maurer and George Kenyon, School of Pharmacy, UCSF (now at the University of Michigan, Ann Arbor, MI).

3. Collaboration in analyses of effects of novel potential inhibitors of telomerase, based on our previous work demonstrating special effects of dG residues on telomerase action: Drs. Nathaniel Grey and Peter Schultz, Department of Chemistry, University of California, Berkeley.

4. Mouse xenograft experiments. Dr. Refaat Shalaby, California Pacific Medical Center, San Francisco.

Development of cell lines

MCF-7-Tet -Off cells (purchased from Clontech) were transfected with each of the 5 different mutant telomerase RNA gene constructs, or with the wild type telomerase RNA gene construct as a control. The 5 mutant template sequences are listed in the footnote to Table 1-99. Cell lines stably expressing hTER gene constructs with mutated and wild-type template sequences have been generated as described in Table 1-99.

Funding applied for based on work supported by this award

(i) UCSF Cancer Center Breast Oncology Program Funding, July 1, 1999 to June 20, 2000
Project: The effects of expressing template mutant telomerase RNA genes in breast cancer cells

(ii) UCSF Prostate Cancer Center Award, January 1, 1999 to December 31, 1999
Project: Interference with Telomeres to Treat Prostate Cancer

CONCLUSIONS:

We have preliminary evidence that a telomerase RNA (hTER) template mutation, even in the presence of the endogenous wild type hTER gene, has a co-dominant deleterious effect on breast cancer cell growth in culture. This makes a gene therapy approach based on such a mutant gene more feasible than had been thought previously.

Inhibitors of telomerase, and expression of mutant-template telomerase RNA genes, can now be tested for their effects in tumor growth in a mouse model system. One can also predict that it may be possible to identify small molecules that, as with the *Tetrahymena* telomerase RNA C48U mutation (Gilley et al., 1995), could turn human telomerase into an enzyme that synthesizes the wrong sequence that could target breast cancer cells, as an alternative to the gene therapy approach.

REFERENCES

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