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Award Number: DAMD17-97-1-7125

TITLE: Design of a Ribozyme to Inactive Telomerase Activity in Breast Tumors

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (30 Sep 98 - 29 Sep 99)

4. TITLE AND SUBTITLE
Design of a Ribozyme to Inactive Telomerase Activity in Breast Tumors

5. FUNDING NUMBERS
DAMD17-97-1-7125

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***
Telomerase expression has been associated with the immortality and metastasis of malignant breast cancer cells. Telomerase activity has been detected in several human cancers including breast carcinomas. However, telomerase activity is either very low or not detectable in normal cells of the body. These observations suggest the development of anti-telomerase strategies may prevent cancer cell division without adverse effects on normal cell populations. This investigation describes the development of a novel therapeutic approach directed against the telomerase complex required for cancer cell division. Catalytic RNA sequences, called ribozymes, will be expressed in breast cancer cell lines, to specifically recognize, cleave and eliminate the telomerase complex. This ribozyme therapy is designed to prevent tumor cell division and eliminate the metastatic potential of breast cancers. Retroviral vectors will deliver therapeutic genes encoding for the anti-telomerase ribozyme to breast cancer cells. These cells will be evaluated for anti-cancer ribozyme activity and for changes in cancer cell division to provide the basis for further development of this therapeutic approach in primary tumors from patients with malignant breast cancer. Gene therapy using ribozymes to eliminate cancer-causing molecules offers a promising strategy for the treatment of malignant breast cancer.

14. SUBJECT TERMS
Breast Cancer, Postdoc Award

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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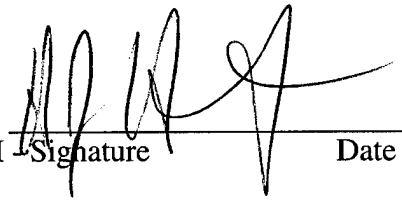

PI Signature 10/28/99 Date

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Introduction

Breast cancer is the most common malignancy in U.S. women and the second leading cause of cancer death, exceeded only by lung cancer in the United States (1). This fact has lead to intense clinical and biological research efforts to determine the cause of breast cancer and the molecular events essential to the development of breast cancer. A number of factors including over expression of the ERBB-2 gene, over expression of the epidermal growth factor receptor, DNA aneuploidy, estrogen and progesterone receptor status and the diminished expression of BRCA-1, appear to be involved in breast cancer development (2, 3, 4, 5, 6). An understanding of the role these factors play in normal cell function and the development of cancer may provide the insight required for the design of novel strategies to treat a variety of tumors.

Telomerase activity has also been associated with the development of breast cancer (7). Telomeres are specialized DNA-protein structures containing unique $(TTAGGG)_n$ repeats at the ends of eukaryotic chromosomes that are important for the protection and replication of chromosomes during cell division (8). The ends of linear DNA cannot be replicated by the conventional DNA polymerase complex, which requires a labile RNA primer to initiate DNA synthesis. In the absence of a mechanism to overcome this "end replication" problem, chromosome stability is compromised and cells would not be able to pass their complete genetic complement from generation to generation, thereby jeopardizing further growth. Therefore, most eukaryotic species, including humans, utilize a specialized enzyme, telomerase, to regenerate telomeric DNA at the end of chromosomes (9). Telomerase is a ribonucleoprotein with RNA and a protein (hTERT) components. The RNA component of telomerase contains a short region [the template domain = $(CCCUAA)_n$] complementary to the one or more repeats of the G-rich telomeric DNA. The mechanism of action of telomerase is a recurring copying of the template domain, involving an elongation phase where deoxyribonucleotides are sequentially added to the 3' end of the telomere, followed by a slower translocation phase, in which the relative position of the telomerase and the telomere advance one repeat, thus positioning the enzyme for another elongation phase. In somatic cells, telomerase activity decreases, possibly due to suppression by secondary genes (10, 11), as the number of cell divisions increases during development, reaching a non-detectable level in mature somatic cells.

Following its original discovery in the ciliate *Tetrahymena* and subsequently in Hela cells, telomerase was observed to be active in immortal cell populations and human tumors in vivo, but undetectable in normal somatic cells in vitro or in vivo (12, 13). Furthermore, a sensitive PCR-based assay has detected low levels of telomerase activity in the human testes and higher levels in advanced tumors, with no activity detected in normal somatic tissues and benign tumors (14). Observations of the chromosomes in somatic cells reveal that telomeres appear to undergo progressive erosion as the chromosomes of these cells lose their terminal TTAGGG repeats with each cell division (10). This loss of telomere length coincides with a reduction in telomerase activity. In contrast, in cell lines and tumors, the shortening of telomeres is retarded and telomerase remains active. Counter et al. have suggested that telomerase activation is an important step in the immortalization of human cancer cells and tumor development (15). Although telomerase activation may be insufficient for cells to proliferate indefinitely, enzyme expression and the stabilization of telomeres appears to be associated with the achievement of "immortality" in cancer cells and may be required to maintain tumor growth (16). Indeed, in a landmark set of experiments, scientists at the Massachusetts Institute of Technology determined the minimal key elements required for the transformation of normal somatic cells to cancer cells. In addition to two oncogenes (SV40 large T-oncoprotein and the oncogenic allele of H-ras), expression of the telomerase catalytic subunit (hTERT) was required for the direct tumorigenic conversion of normal human epithelial and fibroblast cells (24). These observations led to the hypothesis that the development of anti-telomerase strategies may elicit therapeutic effects on malignant cancer cells and tumors and prevent further cancer cell division without adverse effects on normal somatic cell populations.

Recently, a highly sensitive polymerase chain reaction-based telomerase assay called TRAP (Telomeric Repeat Amplification Protocol) was used to examine telomerase activity in a variety of breast cancer and non-cancerous breast tissues (7, 14). Telomerase activity was detected in greater than 90% of breast cancer tissues with negligible levels observed in only 4% of non-cancerous breast tissues. Furthermore, the telomeric length in the noncancerous tissues ranged from 8 to 15 kilobases while in contrast the telomeres in the various breast cancers ranged from 3.4 to 27 kilobases. These findings suggest that

telomerase activity may be associated with the development of malignant breast tumors and that the development of anti-telomerase or pro-senescence therapies may prevent cancer cell division. Proposed anti-telomerase therapies may include pharmacological inhibition (nucleoside analogs), transcriptional repression or genetic intervention (17). Because telomerase belongs to a class of reverse transcriptases (RNA-directed DNA polymerases), nucleoside analogs such as those used against HIV reverse transcriptase may be a useful anti-telomerase therapy. However, as with other forms of chemotherapy, these analogs are likely to have poor selectivity and tumors may develop resistance to these drugs (18). Ideally, an anti-telomerase therapy would be selective for cancer cells and induce cancer cell senescence.

The following proposal describes the preliminary development of one possible therapeutic strategy directed against telomerase expression in breast cancer cells using gene therapy (19). The following investigation utilizes the expression of catalytic RNA sequences (20), called ribozymes (21, 22, 23), to specifically recognize and cleave the mRNA encoding for the protein component of the telomerase complex in breast cancer cells, thereby preventing telomeric extension of cancer cell chromosomes. By abolishing telomerase activity and preventing telomere extension, breast cancer cells transduced with the anti-telomerase ribozyme may become senescent. An amphotrophic murine retroviral vector will be used to package and deliver the gene encoding for the anti-telomerase ribozyme. Retroviral vectors offer the advantage of requiring actively dividing cell populations for gene expression. Thus, the anti-telomerase ribozyme will only be expressed and active in retrovirally transduced cell populations, such as breast cancer cells, that are involved in rapid cell proliferation. For future therapeutic applications *in vivo*, targeting ligands or chimeric proteins can be designed and incorporated into the retroviral envelope to target specific cell populations and avoid normal cells of the body that undergo cell proliferation such as the gastrointestinal lining and cells of the immune system. The expression and efficacy of the anti-telomerase ribozyme for abolishing telomerase activity and preventing breast cancer cell division will be the main focus of the proposed investigation.

Body

I. Construction and Packaging of Retroviral Vectors Containing Multiple Copies of the Anti-Telomerase Ribozyme Candidates

We last reported that the use of ribozymes to target the RNA component of telomerase were ineffective and use of the truncated form of the Nerve Growth Factor Receptor (ΔNGFR) was not a suitable selectable marker for these studies. As reported we identified several target sites for ribozyme therapy in the sequence of the mRNA encoding for the protein component of the telomerase complex (Table-1). Each of these ribozyme candidates have been cloned into retroviral vectors and will be evaluated for anti-telomerase activity in breast cancer cell lines. Vector construction was confirmed by PCR, restriction enzyme digestion and DNA sequencing. As described previously, Moloney based retroviral vectors have been constructed and will be used to deliver each therapeutic ribozyme gene to the target cancer cells. This amphotropic virus permits the delivery of genes to any cell type, followed by long term expression of the therapeutic ribozyme for evaluation *in vitro* or *in vivo* models for breast cancer.

Table-1: Target sequences in the mRNA of the protein component (hTERT) of the telomerase complex and their corresponding ribozyme sequence.

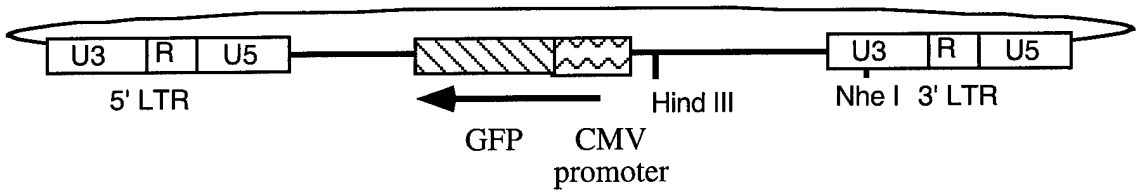
mRNA TARGET SEQUENCE OF TELOMERASE PROTEIN (hTERT)	RELATIVE POSITION	RIBOZYME SEQUENCE
5′ GCTGCGTCCTGCTGCG 3′	6-21	5′ CGACaagaGACGACGC 3′
5′ CAGGTGTCCTGCCTGA 3′	272-287	5′ GTCCaagaGACGGACT 3′
5′ CAGGGGTCCTGGGCCC 3′	812-827	5′ GTCCaagaGACCCGGG 3′
5′ CCGGGGTCCCCCTGGG 3′	684-699	5′ GGCCaagaGGGGACCC 3′
5′ GAGGTGTCCCTGAGTA 3′	2721-2736	5′ GAGGTGTCCCTGAGTA 3′
5′ TGAGTGTCCGGCTGAG 3′	3618-3633	5′ ACTCaagaGCCGACTC 3′
5′ CGAGTGTCCAGCCAAG 3′	3621-3656	5′ GCTCaagaGTCGGTTC 3′
5′ TGAGTGTCCAGCACAC 3′	3660-3675	5′ ACTCaagaGTCGTGTG 3′
5′ TGGGGGTCCCTGTGGG 3′	3933-3948	5′ ACCCaagaGGACACCC 3′

Each LNL-6 based murine retroviral vector construct contains multiple copies of the gene encoding for an anti-telomerase ribozyme candidate driven by pol III t-RNA^{val} promoter as well as the selectable marker (Figures 1-3). Following packaging and transduction of target cells, retroviral vector containing three copies of a therapeutic ribozyme has been observed to improve the expression of the therapeutic gene by 50 to 100 fold(25). Green Fluorescent Protein (GFP) or neomycin resistance (Neo) was used as the selectable marker in these constructs. Following construction of these vectors containing multiple copies of the therapeutic ribozyme candidate or an inactivated anti-telomerase ribozyme control, these vectors were packaged into retroviral particles for *in vitro* evaluation of anti-telomerase activity in breast cancer cells.

1.1 Construction of LNL-6 Based Vectors with the Anti-Telomerase Ribozyme

Moloney based retroviral vectors have been constructed and will be used to deliver the therapeutic ribozyme gene or inactivated ribozyme control to the target cancer cells. For each ribozyme candidate, a LNL-6 based murine retroviral vector was constructed to contain multiple copies of the gene encoding for the therapeutic or inactivated anti-telomerase ribozyme driven by pol III t-RNA^{val} promoter as well as the selectable marker (GFP or Neo) driven by the CMV promoter (Figure-2). The basic LNL-6 retroviral plasmid containing the GFP or Neo selectable markers driven by the CMV promoter is called LC-GFP (Figure-1). There are several possible target sequences located in the mRNA encoding for the protein component of telomerase for ribozyme targeting and cleavage, and all have been constructed and cloned into retroviral vectors (Table-1).

Figure-1: Illustration of the LC-GFP plasmid containing the selectable marker (GFP or neomycin resistance) driven by the CMV promoter.



In order to produce a triple copy vector, the ribozyme cassette is cloned into two sites located in the basic LC-GFP retroviral plasmid. The first site is located between the retroviral long terminal repeats (LTR) and the second is directly in the 3' LTR (pLNL-6TC). To produce a triple copy vector, the plasmid DNA containing the two copies of the anti-telomerase cassette must first be transfected into a retroviral packaging cell line (GP+Am12 or PA317). Supernatants of a cellular clone containing the double copy retroviral vector and negative for RCR will be used to transduce each of the breast cancer cell lines. Once the pLNL-6TC vector is packaged and undergoes reverse transcription in the cytoplasm of transduced breast cancer cells, it will produce a third copy of the anti-telomerase ribozyme in the U3 region of the 5' LTR of the integrated proviral DNA (LNL-6TC: Figure-2). For each therapeutic ribozyme candidate, inactivated anti-telomerase ribozymes were synthesized which included the specific hybridization regions to control for anti-sense effect but did not possess the cleavage sequence, rendering them inactive (Figure-3).

Figure-2: Illustration of the murine retroviral vector producing multiple copies of each anti-telomerase ribozyme (Rz) candidate driven by the tRNA promoter. A second ribozyme cassette is inserted between the LTRs in the plasmid DNA . Following reverse transcription and integration into the breast cancer cell genome, this vector DNA produces a third copy of the anti-telomerase ribozyme. The selectable marker is Neomycin resistance driven by the CMV promoter.

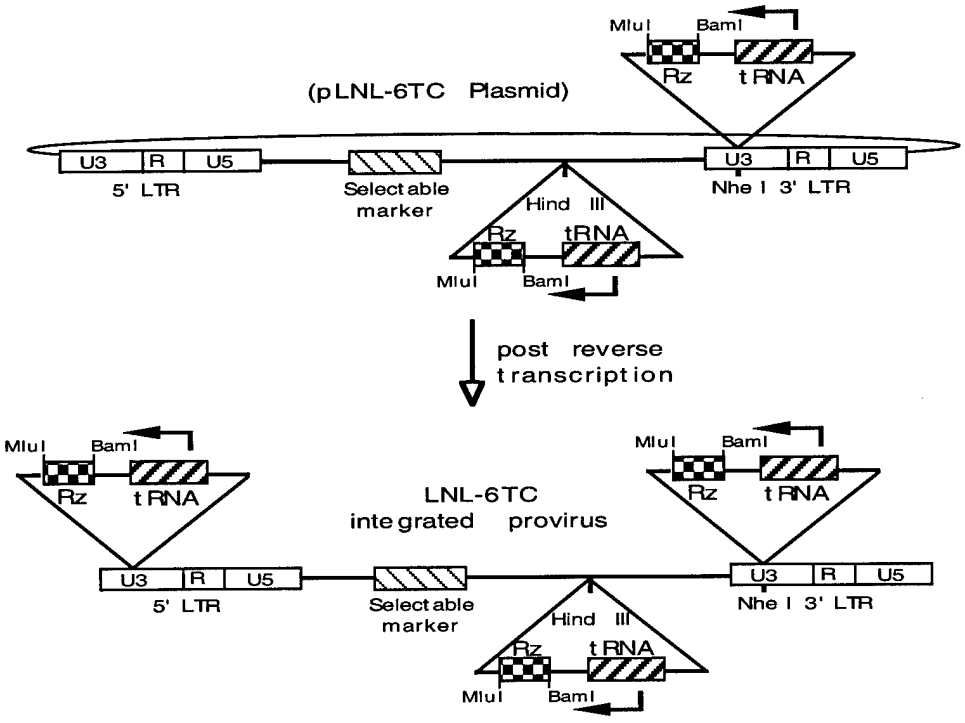
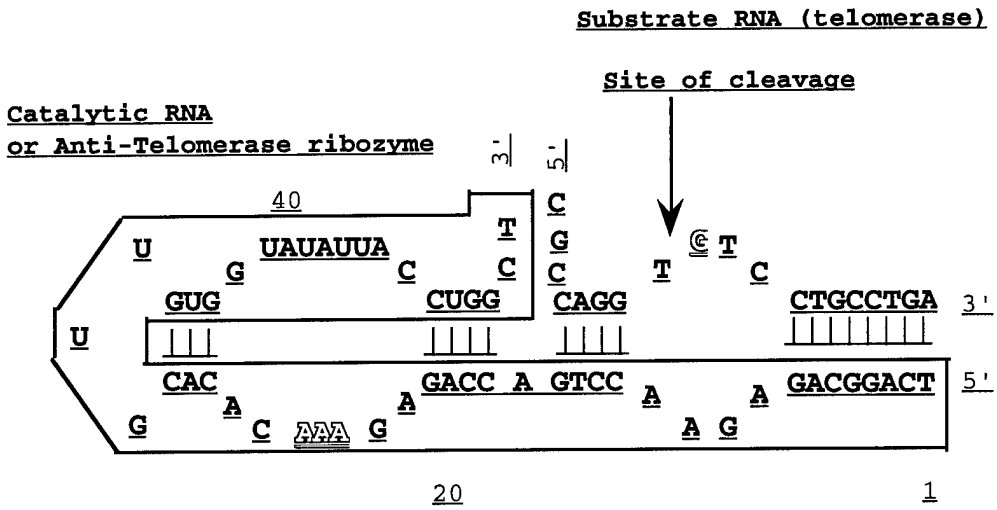


Figure-3: A diagram of an anti-Telomerase Hairpin Ribozyme Candidate



1.2 Packaging of the Retroviral Vectors containing the Anti-Telomerase Ribozyme Candidates

Following construction of the retroviral vectors with therapeutic or inactivated forms of the anti-telomerase ribozyme candidates, each vector was packaged using retroviral particle producing cell lines(GP+Am12 or PA317). Viral particles produced by these cell lines can be used to transduce breast cancer cell lines in the proposed experiments. The GP+Am12 cell line produces moderate to high titers of retroviral particles but is less likely than other producer cell lines to generate retroviral competent retrovirus (RCR), a strategic problem for retroviral-based gene therapies. PA317 cells produce very high titers of retroviral particles. Although uncommon, PA317 are more likely than GP+Am12 cells to generate RCR. Because we are interested in evaluating the anti-telomerase ribozyme candidates in breast cancer cells *in vitro*, we initially packaged the retroviral vectors in PA317 cells to obtain high titers for transduction of breast cancer cell lines. GP+Am12 cells would be used for the production of viral particles for primary breast cancer cells or *in vivo* applications in the future.

PA317 cells were transfected with each of the retroviral plasmids containing multiple copies of the anti-telomerase ribozyme candidates or inactivated controls. Transfected cells were selected and clones for each ribozyme were titered using a basic cos cell system. Briefly, cos cells were transduced with supernatants (3 ml) from each producer cell clone for a period of three days. Following transduction, transduced cos cells were selected using G418. Cos cell colonies were counted to determine transduction efficiency and producer cell titers. Furthermore, supernatants from selected cos cells were collected and added to a second set of non-transduced cos cells to test for the generation of Replication Competent Retrovirus (RCR). For all of the anti-telomerase ribozyme constructs and controls, producer cell clones were selected that generated high-titer, replication incompetent, retroviral particles with transduction efficiencies ranging from 90 to 95%. Supernatants from each of these producer cell clones corresponding to an anti-telomerase ribozyme candidate or inactivated control are being used to transduce breast cancer cell lines.

II. Retroviral Transduction and Selection of Breast Cancer Cells Expressing the Anti-Telomerase Ribozyme Candidates

Four human breast cancer cell lines (MCF-7, MDA-453, SK-BR-3 and BT-474) are known to have various levels of telomerase activity. Each of these breast cancer cell lines will be transduced with supernatants harvested from the PA317 producer clones containing retroviral particles carrying each anti-telomerase ribozyme candidate or inactivated controls. Because of the number of anti-telomerase ribozyme candidates with controls, the first set of experiments focused on the transduction of the MCF-7 breast cancer cell line which is known to have a moderate to high level of telomerase expression and

four anti-telomerase ribozyme candidates, 272, 812, 2721 and 3621. MCF-7 cells were seeded in a six well plate (10^6 cell per ml). When the breast cancer cells were 60% confluent, they were transduced with supernatants from each of the corresponding producer cell lines for a period of three days. As a control for transduction efficiency, cos cells were transduced using similar samples of the same supernatants. Transduced MCF-7 and cos cells were selected using G418 (500-750 μg/ml of media). To date, only MCF-7 and cos cells transduced with the 2721 and 3621 anti-telomerase ribozymes have generated viable cell colonies. The PA317 producer clones for 272 and 812 ribozymes are currently being re-evaluated for viral particle production. The results would indicate that the producer clones for these ribozymes have stopped generating retroviral particles as neither MCF-7 or cos cells incubated with supernatants formed colonies upon selection. Alternatively, 272 and 812 anti-telomerase ribozymes could target mRNA encoding for "housekeeping genes" common to both MCF-7 and cos cells, resulting in cell death. This scenario is less likely than loss of retroviral particle production. The 272 and 812 ribozymes will be repackaged in PA317 cells and evaluated in breast cancer cell lines with the other anti-telomerase ribozyme candidates. Selected MCF-7 and cos cell clones transduced with the 2721 and 3621 anti-telomerase ribozymes were isolated for further analysis of telomerase activity.

III. Assessment of Breast Cancer Cell Lines Transduced with Anti-Telomerase Ribozymes

Breast cancer cell lines transduced with anti-telomerase ribozymes and controls will be evaluated for ribozyme and target mRNA expression using real-time RT-PCR, telomerase expression using Western Blot Analysis, changes in cell division using cell proliferation assays, and telomerase activity using a commercial TRAP assay. Due to advancement in technologies and knowledge of telomerase, some of these assays have been modified from the original proposal as they are more sensitive and provide a comprehensive evaluation the anti-telomerase ribozyme candidates in these breast cancer cell lines. Currently, selected MCF-7 cells transduced with the 2721 and 3621 anti-telomerase ribozymes and non-transduced MCF-7 cells have been used to standardize these assays for the proposed experiments. The results to date are outlined below.

3.1 Morphological Assessment of MCF-7 Cells Transduced with Anti-telomerase Ribozymes

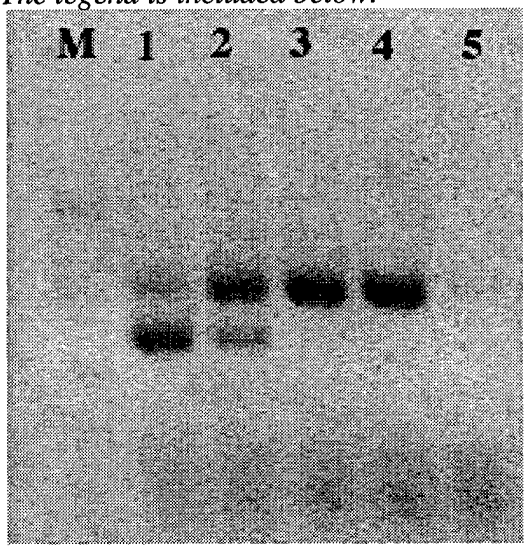
MCF-7 and cos cells transduced with 2721 and 3621 anti-telomerase ribozymes did form G418 resistant colonies. As expected, cos cells transduced with retroviral particles expressing each ribozyme candidate continued to grow normally in G418 supplemented media. Anti-telomerase ribozymes are not expected to have any effect on the cell growth or proliferation on this primate cell line. If there are changes in cos cell proliferation or growth, this may indicate the inadvertent targeting of mRNA encoding for a "housekeeping gene" or other crucial messages by these ribozymes. In cos cells transduced with retroviral vectors containing 2721 and 3621 anti-telomerase ribozymes, no adverse effects were observed following selection and normal growth continued uninhibited with no production of RCR. However, growth was inhibited in MCF-7 cells transduced with these anti-telomerase ribozyme candidates. After 30 days post-selection, MCF-7 cells transduced with the 2721 anti-telomerase ribozyme remained sparse and could not be grown or passaged. Currently, MCF-7 cells are being re-transduced with retroviral particles containing the 2721 anti-telomerase ribozyme to see if these results can be duplicated. MCF-7 breast cancer cells transduced with the 3621 anti-telomerase ribozyme were also observed to have retarded growth compared to non-transduced MCF-7 cells (Appendix A). At 20 days post-transduction and selection, MCF-7 cells transduced with the 3621 anti-telomerase ribozyme formed 7 to 10 very small colonies (50 to 170 cells per colony) that did not grow or expand by 45 days post-selection. Once again, the lack of cell proliferation could be attributed to anti-telomerase ribozyme activity. Colonies did form indicating an initial period of cell division. However, cell proliferation has ceased in these cells which must be evaluated for ribozyme, expression, target mRNA cleavage, and telomerase activity. The approximated number of transduced cells in the well was no more than 1200 cells and this low number of cells poses a problem for ribozyme evaluation. The sparse number of viable MCF-7 cells transduced with the 3621 anti-telomerase ribozyme posed a problem for RT-PCR and Western Blot Analysis but would still permit the detection of telomerase activity. These techniques are described and discussed below as they pertain to this study.

3.2. Assessment of Anti-Telomerase Ribozyme Expression and Changes in Levels of Target mRNA Using RT-PCR

Real-time, quantitative RT-PCR will be used to assess the expression of the therapeutic and inactivated anti-telomerase ribozymes in each of the cancer cell lines at various time points following transduction and selection. In addition, a similar quantitative approach using the Sybr Green I one step RNA amplification Kit (Roche Molecular Biochemicals, Indianapolis, In) will also be used to assess changes in the levels of the target hTERT mRNA transcript in these breast cancer cell lines. For both sets of experiments, RNA must first be isolated from each of the cell lines prior to RT-PCR, usually with a minimum of 5000 cells. The isolation of total RNA from the cancer cells in culture is carried out via the RNAzol method(Tel-Test "B", Inc.). These total RNA preparations will be used for the detection of ribozyme expression and target mRNA using RT-PCR.
For RT-PCR, the 2 μL eluted total RNA preparation is combined with the reactants from the One Step Light Cycler-RNA amplification Sybr Green I Kit and the respective ribozyme or telomerase (hTERT) specific oligonucleotides. The reaction undergoes 30 minutes of reverse transcription for 30 minutes followed by 30 cycles of PCR (94°C 1 sec, 52°C 5 sec, 72°C 20 seconds) in the Light Cycler. The Light Cycler offers the advantage over conventional PCR techniques as it is faster, more sensitive and is quantitative. The PCR products are loaded onto a 2.5% agarose gel for electrophoresis and examined for ribozyme expression and hTERT mRNA levels.

Figure-4: hTERT mRNA detection in MCF-7 breast cancer cells using quantitative RT-PCR using the Light Cycler. The concentration of mRNA encoding for full length hTERT is kept constant (1ng/μL) and a truncated form of hTERT (Δ hTERT = lower band) is used as competitor RNA in decreasing concentrations (internal control) to evaluate levels of hTERT mRNA from cancer cells. As the concentration of competitor mRNA is increased, the amount of detectable hTERT mRNA is decreased. Products were electrophoresed on a 2.5% agarose gel. The legend is included below.

Lane	Description
M	Marker
1	500pg of Δ hTERT mRNA
2	50pg of Δ hTERT mRNA
3	5pg of Δ hTERT mRNA
4	0.5pg of Δ hTERT mRNA
5	Water (No Template)



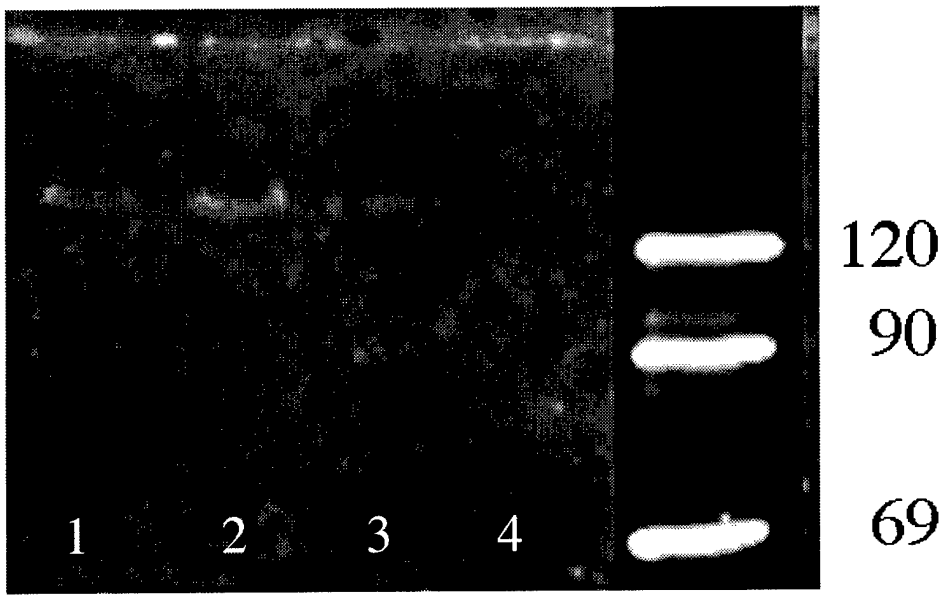
For the current investigation, the major advantage of using the Roche Light Cycler System is sensitivity. As observed with the 3621 anti-telomerase ribozyme, the number of viable cancer cells following transduction and selection may be severely limited if the ribozyme is effective. Thus, the amount of RNA isolated from a very few number of cells (less than 5000) may not be adequate for conventional RT-PCR. However, the Light Cycler and the Sybr Green I Kit are 100-fold more sensitive than ethidium bromide. We have completed initial standardization assays using total RNA from MCF-7 cells and oligonucleotides specific for the target hTERT mRNA (Figure-4). A known concentration of competitor RNA corresponding to a truncated form of the hTERT was used in each sample. As expected, when the concentration of competitor

mRNA is increased, the amount of detectable hTERT mRNA is decreased. Furthermore, as little as 0.5 to 1.0 pg of RNA can be detected using the Light Cycler, which may prove to be a major advantage for detecting levels of anti-telomerase ribozyme and target hTERT mRNA in a limited number of breast cancer cells following retroviral transduction and selection. This standardized method for RNA detection will be used for all breast cancer cell lines transduced with the anti-telomerase ribozyme candidates to assess ribozyme expression and determine levels of target hTERT mRNA.

3.3. Evaluation of Telomerase hTERT protein expression in Breast Cancer Cell Lines using Western Blot Analysis

We proposed that expression of the hTERT protein in the breast cancer cell lines could be assessed using Western Blot Analysis with the polyclonal antibody (NB100-141, Novus Biologicals, Littleton, CO) raised against the human hTERT component of the telomerase complex. To evaluate this protein detection system, non-transduced MCF-7 breast cancer cells and Hela cells ($1x10^6$ cells) were lysed with cell lysis buffer. Protein concentrations were determined for each of the cell lysates and 10, 25, 50 and 100 μg of protein were be loaded on a 10% SDS-PAGE gel. Electrophoresed proteins were transferred overnight onto PVDF membrane and subsequently blotted for hTERT using the polyclonal antibody and peroxidase labeled sheep anti-rabbit IgG. Proteins could be visualized and quantified using a chemiluminescent peroxidase sensitive substrate(SuperSignal, Pierce, Rockford,IL) and a multiimager (Fluor-S MAX, BioRad, Hercules, CA). With the Fluor-S MAX, levels of hTERT expressed in these cell samples could be quantified and compared between transduced (ribozyme and inactivated ribozyme) and non-transduced breast cancer cells as well as across the various cancer cell lines that are known to express different levels of the telomerase protein. As evident from Figure-5, this method is a viable option for the detection of the hTERT component of telomerase but needs to be optimized. The polyclonal antibody NB100-141 is supposed to detect the hTERT protein of the telomerase complex at approximately 120 to 130 kD. We have completed this Western Blotting experiment several times using the manufacture's protocol and our own established protocols with mixed results on the detection of the 100 kD protein. The polyclonal antibody does detect the hTERT protein of the telomerase construct. We will continue to search for an alternative commercial monoclonal antibody that recognizes the hTERT protein of telomerase.

Figure-5: Western Blot using the anti-hTERT polyclonal antibody NB100-141 to detect hTERT protein expression in MCF-7 and hela cells. The polyclonal antibody recognized a protein at approximately 125 to 130 kD corresponding to the hTERT of the telomerase complex. Lanes 1 and 2 are MCF-7 cell lysates at 10 and 50 μg of protein, respectively. Lane 3 is Hela cell lysate at 10μg of protein. Lane 4 is background staining. The final lane is the molecular weight marker with the bands demarcated in kD.

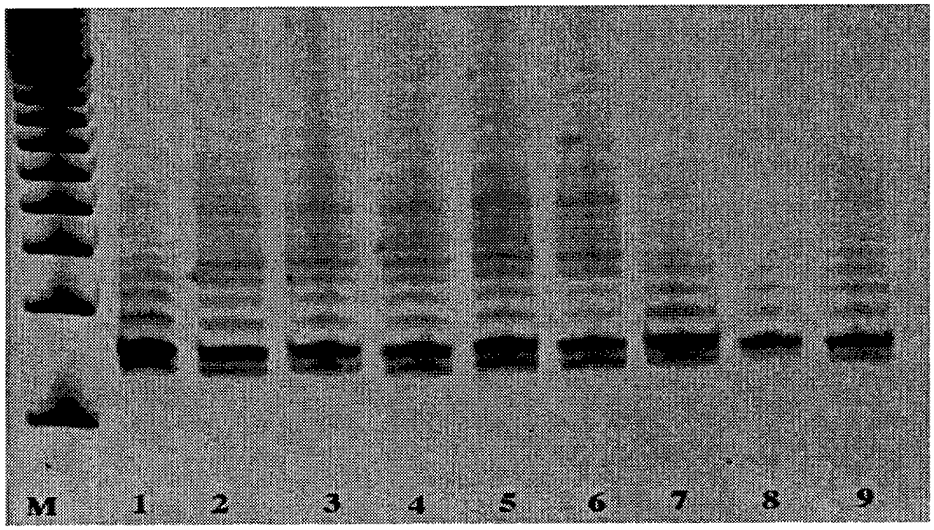


3.4. Evaluation of Telomerase Activity in Breast Cancer Cell Lines Following Retroviral Transduction with the Anti-Telomerase Ribozymes

A Telomeric Repeat Amplification Protocol (TRAP) assay is used to quantitatively determine changes in telomerase activity in transduced breast cancer cells (Roche Biochemicals, Indianapolis, IN). In the TRAP assay, telomerase from breast cancer cells synthesizes extension products, which then serve as templates for amplification by PCR, generating a ladder of products with 6 base pair increments starting at 50 nucleotides. A positive signal in a TRAP assay requires hTERT from cancer cell extracts capable of extending an oligonucleotide with three or more TTAGGG repeats, thereby validating the assay for specific detection of telomerase activity. This 6 base pair extension or "ladder" may be visualized using a 12% non-denaturing acrylamide gel. In a secondary quantitative step, the Roche ELISA system which utilizes digoxigenin-labeled probes for detection, changes in telomerase activity can be observed and quantitated at a wavelength of 450nm. Since the TRAP assay is based on the RT activity of telomerase, we are currently developing a TRAP assay for the sensitive Roche Light Cycler that performs real-time RT-PCR and can directly quantify telomerase activity. The premise of these experiments suggests that if the ribozyme candidates are effective in preventing or reducing expression of the telomerase protein component, amplification will be impeded.

Figure-6: TRAP assay results from a breast cancer cell line (MCF-7) visualized using a 12% non-denaturing acrylamide gel. Telomerase activity produces a 6 base pair extension of telomeres resulting in a "ladder" effect. If anti-telomerase ribozymes are effective in preventing hTERT expression, the ladder will not be produced. The legend is located below.

Lane	Description
M	20 bp marker
1	10 cells
2	60 cells
3	120 cells
4	250 cells
5	500 cells
6	1000 cells
7	heat inactivated
8	buffer control
9	positive control



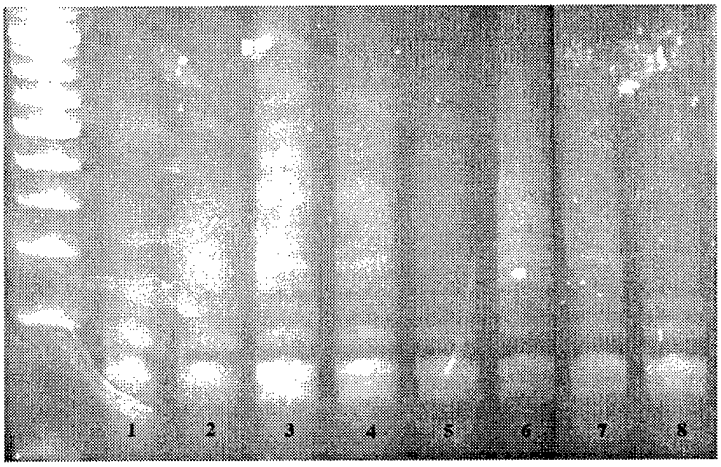
Initial experiments were completed using non-transduced MCF-7 breast cancer cells to standardize the TRAP assay and determine the sensitivity for our project (Figure-6). The TRAP assay can detect telomerase activity in as little as 10 cells, indicating the sensitivity of the assay is very high. In the heat-inactivated and buffer only controls, the primers involved in the PCR amplification are observed to produce primer dimers and form the early products of the 6 base pair extension or "telomerase ladder". The ladder is interrupted in these negative control samples, but the early products could prove to be problematic for the ELISA portion of the Roche TRAP Assay. These early products can bind digoxigenin-labeled probes and contribute to the background of the ELISA and decrease sensitivity. These problems can be overcome by using the Light Cycler to complete the TRAP assay as this machine can detect oligonucleotide dimers and subsequent optimization of the system can eliminate these artifacts.

MCF-7 cells transduced with the 3621 anti-telomerase ribozyme candidate were evaluated for telomerase activity using a conventional TRAP Assay (Figure-7). With the conventional TRAP Assay (Figure-7), differences in telomerase activity based on cancer cell lysate concentrations are very difficult to visualize using a 12% non-denaturing acrylamide gel and the BioRad Gel Doc 2000 system. To improve sensitivity, acrylamide gels were stained with Sybr Green I rather than ethidium bromide. Through comparison of intensity using the gel imaging system, at concentrations of 500 and 50 cells,

telomerase amplification is reduced by a mean of 34% in MCF-7 cells transduced with the 3621 anti-telomerase ribozyme. However, this difference is not detectable at lower cell numbers (5 cells) with this method of quantitation. This method of quantitation using the gel imaging system is sufficient for preliminary evaluation of changes in telomerase activity in transduced breast cancer cells and control cells, but is inconsistent and may vary with each TRAP assay. Furthermore, we have observed that due to oligonucleotide dimerization and PCR amplification of longer than 33 cycles, the 6 base pair ladder can be amplified to levels that appear similar despite starting the reaction with 100 fold less cells. Thus, it is imperative to develop a more accurate system for analysis of TRAP assays.

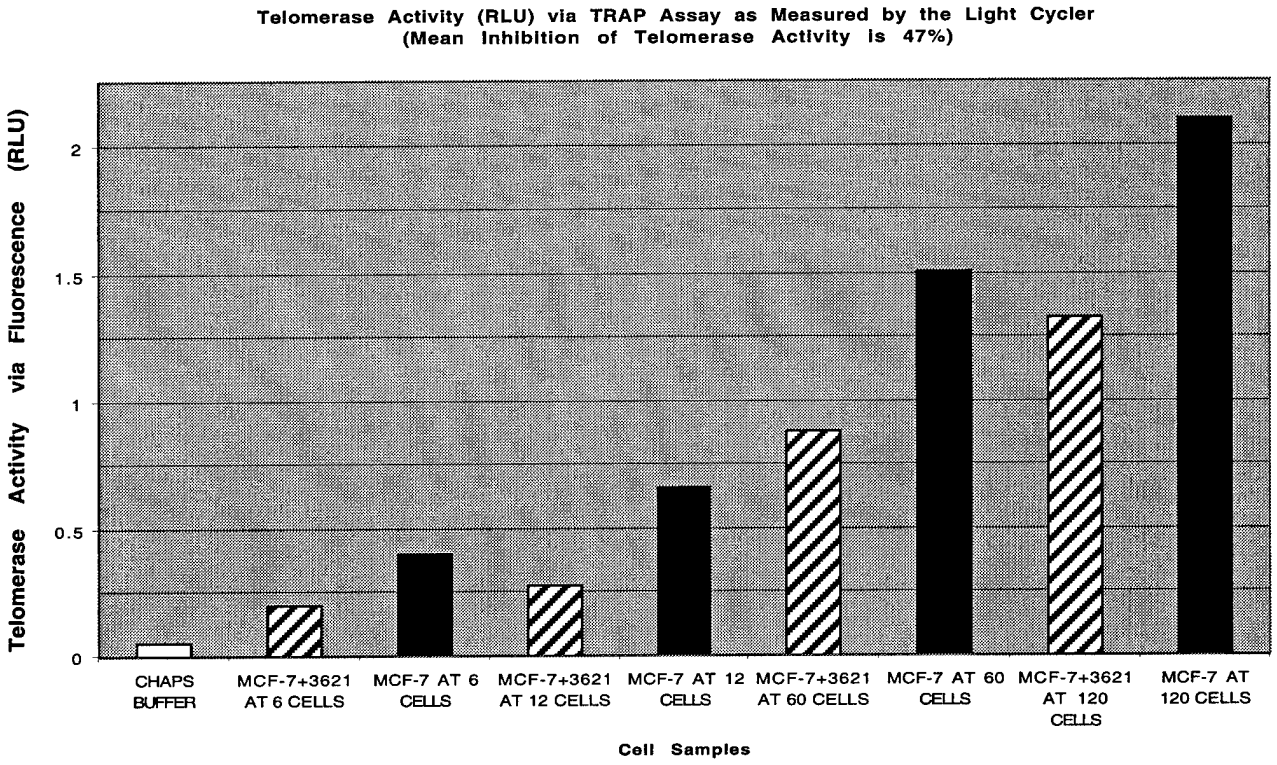
Figure-7: Conventional TRAP Assay visualized on a 12% non-denaturing acrylamide gel stained with Sybr Green I. Lanes are described in the table.

LANE	MCF-7 CELLS OR CONTROLS
Marker	20 BP Ladder
1	Chaps Buffer Only
2	MCF-7 Heat Inactivated
3	MCF-7 (500 cells)
4	MCF-7 (50 cells)
5	MCF-7 (5 cells)
6	MCF-7 + 3621 (500 cells)
7	MCF-7 + 3621(50 cells)
8	MCF-7 + 3621 (5 cells)



These problems may be rectified by aborting the PCR amplification step of the TRAP assay at 33 cycles or using the Light Cycler to run the TRAP Assay. The Light Cycler performs real time quantitative RT-PCR and permits the visualization of "primer dimer" formation and artifact amplification in control samples. Thus, the Light Cycler allows the user to arrest the TRAP assay just as artifacts are beginning to be amplified. We completed several TRAP assays using the Light Cycler and MCF-7 breast cancer cells. The raw data reveals that telomerase generated templates from non-transduced MCF-7 cells at each concentration amplify earlier in the PCR protocol than templates from MCF-7 cells transduced with the 3621 anti-telomerase ribozyme (Appendix B). Earlier amplification suggests that there is a higher concentration of telomerase generated template in non-transduced MCF-7 cells following the initial RT or telomerase-mediated step of the TRAP assay. This is directly proportional to the level of telomerase activity in these breast cancer cells; the lower the telomerase activity, the less template generated and the longer time required for template amplification in the PCR protocol. Using the Light Cycler and the quantitative analysis mode, the TRAP assay was performed using various concentrations of cell lysates from MCF-7 cells transduced with the 3621 anti-telomerase ribozyme candidate and non-transduced controls (Figure-8). In general, the Light Cycler method reveals that MCF-7 cells transduced with the 3621 anti-telomerase ribozyme have approximately 47% less telomerase activity than non-transduced MCF-7 cells. The Light Cycler was able to determine differences in the telomerase activity using lysates with the equivalent of 6 cells, which was not easily evaluated using the 12% non-denaturing acrylamide gel. Correlation of these results with the RT-PCR data that will be obtained in subsequent experiments will provide further insight into the activity of the anti-telomerase ribozymes and verify this novel approach to quantitatively analyzing the TRAP assay results using real-time RT-PCR.

Figure-8: Chart plotting telomerase activity for MCF-7 breast cancer cells transduced with the 3621 anti-telomerase ribozyme candidate and controls as measured via the Light Cycler. The overall mean inhibition of telomerase activity in transduced breast cancer cells was 47%.

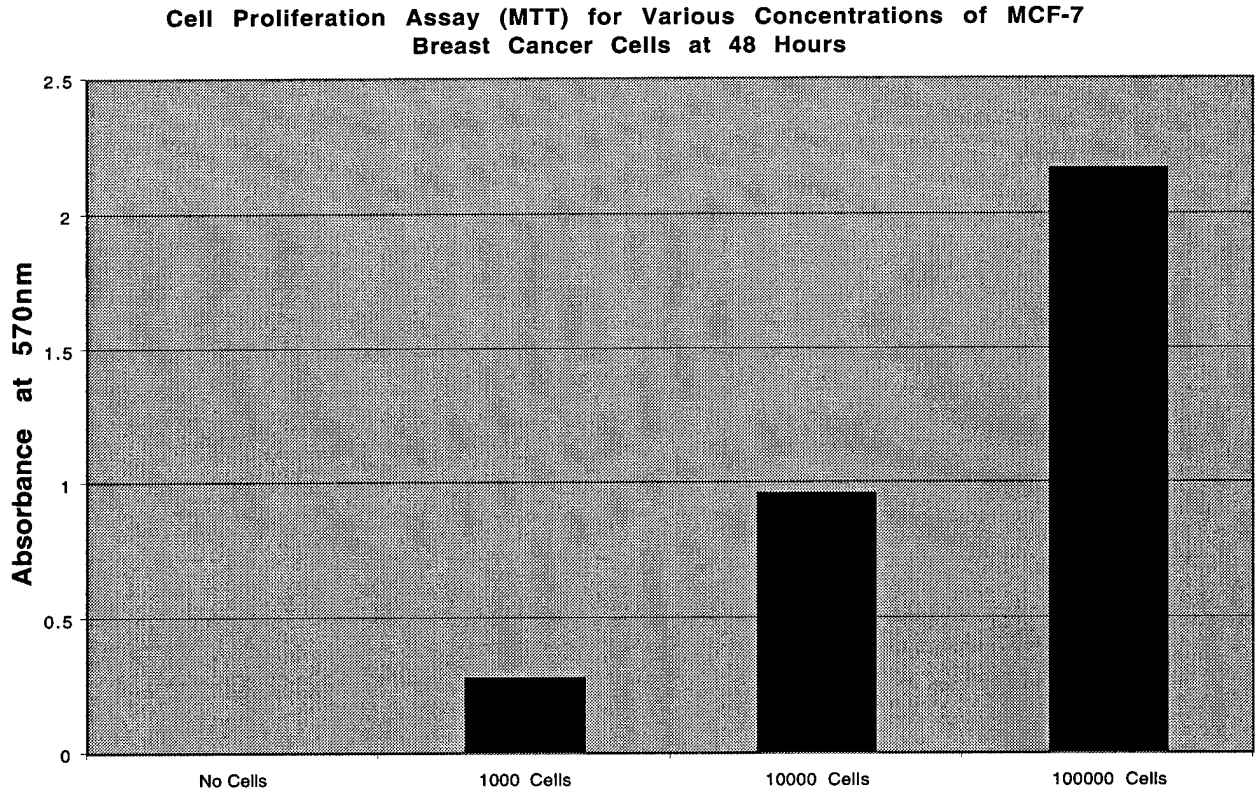


3.5. Assessment of Cancer Cell Line Growth and Proliferation Following Retroviral Transduction with the Anti-Telomerase Ribozyme

As a safe and non-radioactive alternative, colorimetric assays have become available for analyzing the number of viable, proliferating cells by the cleavage of tetrazolium salts added to the culture medium. This technique requires neither washing nor harvesting of cells, and the complete assay from the onset of microculture to data analysis by an ELISA reader is performed in the same microtiter plate. MTT was the first tetrazolium salt described. It is cleaved to formazan by the "succinate-tetrazolium reductase" system, which belongs to the respiratory chain of mitochondria and is active only in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. For this research project, cell proliferation of each of the transduced breast cancer cell lines and controls at 7, 14, 30, 45, 60 and 120 days post-transduction and selection will be determined by the level of formazan dye formation via the Cell Proliferation Kit I (MTT) (Roche Biochemicals, Indianapolis, IN). This form of cell proliferation assay eliminates the need to use radioisotopes and improves the overall safety of the project. Briefly, transduced cancer cell lines and control cell lines are split into quadruplicate wells of a U-bottomed 96 well plate at 10^4 cells per 100µl/well. 100µl of culture media supplemented with 10% FBS containing MTT is then added to each well at the designated time point. Following 4 hours incubation, the microtiter plate is analyzed at 570 nm for formazan dye formation using an ELISA reader. Levels of formazan dye production will be compared between each of the transduced cancer cell lines and their respective controls to assess changes in cancer cell proliferation. Breast cancer cells expressing the an effective anti-telomerase ribozyme will have less dye production over time compared to control cells, indicating the activity of the ribozyme retards or prevents further cancer cell division. To date, the number of MCF-7 cells

transduced with 3621 anti-telomerase ribozyme candidate obtained following selection have not been sufficient to run this assay. As reported in Section 3.1, selected MCF-7 cells transduced with the 3621 anti-telomerase ribozyme candidate have survived but have not expanded past small colonies of 50 to 100 cells through 45 days. As these experiments are repeated, this assay will be used to evaluate changes in cell proliferation in breast cancer cells expressing anti-telomerase ribozyme candidates and inactivated controls. We would expect cell proliferation to be retarded in breast cancer cells expressing an effective anti-telomerase ribozyme and could be correlated with morphological observations as reported in Section 3.1. We have verified the use of this assay with the MCF-7 and other breast cancer cell lines by measuring their proliferation over a set time period (Figure-9).

Figure-9: MTT-based cell proliferation assay in MCF-7 breast cancer cells over a period of 48 hours beginning with three different concentrations of cells. Formazan dye formation is measured at 570 nm.



IV. Current Status and Future Experiments

The current status of the project closely follows the original statement of work. Although their have been a few delays due to testing of detection systems and standardizing protocols, the project will be completed by the summer of 2000 as planned. Currently, all anti-telomerase ribozyme candidates and inactivated controls have been constructed, cloned into retroviral vectors and packaged into transduction particles by producer cell lines. Some of the ribozyme candidates such as 272, 812 and 2721 are being re-evaluated (DNA sequencing, PCR and re-cloning) due to unexplained results including the lack of colony formation following G418 selection in a cell line that does express the hTERT target mRNA. The supernatants from these producer cell lines will be used to transduce the four breast cancer cell lines MCF-7, MDA-453, SK-BR-3 and BT-474. These cell lines are all currently propagated and passaged in our laboratory and are ready to be transduced with the retroviral vectors containing the anti-telomerase ribozymes and controls. Now that standard protocols for detection of ribozyme expression and levels of

target hTERT mRNA via RT-PCR have been established, the project can move forward in all breast cancer cell lines. We will be using the Light Cycler along side with conventional commercial kits to complete the TRAP assays to evaluate telomerase activity in breast cancer cells following retroviral transduction. Finally, we will evaluate changes in breast cancer cell growth and proliferation following transduction using colorimetric cell proliferation assays. These assays will be completed for all anti-telomerase ribozyme candidates and controls, followed by data analysis and final publication of results in the late summer of 2000.

Key Research Accomplishments

I. Completed the design and construction of all anti-telomerase ribozymes and inactivated ribozyme controls described in Table-1.

II. Completed the construction of retroviral vectors containing multiple copies of each anti-telomerase ribozyme candidate. Retroviral construction was confirmed by PCR, restriction enzyme digests and conventional DNA sequencing.

III. Packaged all retroviral vectors containing the anti-telomerase ribozyme candidates using PA317 cells. For all of the anti-telomerase ribozyme constructs and controls, producer cell clones were selected that generated high-titer, replication incompetent, retroviral particles with transduction efficiencies ranging from 90 to 95%. Supernatants from each of these producer cell clones corresponding to an anti-telomerase ribozyme candidate or inactivated control are being used to transduce breast cancer cell lines.

IV. Began experiments designed to evaluate anti-telomerase ribozyme activity in breast cancer cell lines. All experiments to date have utilized the MCF-7 breast cancer cell line. To date, only the 3621 anti-telomerase ribozyme candidate has been observed to have anti-cancer characteristics. Other ribozyme candidates are currently being evaluated or being re-examined for deficiencies.

V. The 3621 anti-telomerase ribozyme was observed to retard MCF-7 breast cancer cell proliferation through 45 days post-selection. Morphological data is being compared to TRAP assays and RT-PCR evaluations of telomerase activity.

VI. The polyclonal antibody, NB100-141, designed to recognize the hTERT component of telomerase is useful for Western Blot Analysis but provides mixed results. Continue to search for a monoclonal antibody to improve this form of analysis.

VII. Standardized the RT-PCR, TRAP and cell proliferation assays for MCF-7 breast cancer cells. Currently doing the same for MDA-453, SK-BR-3 and BT-474 breast cancer cell lines.

VIII. Developed RT-PCR protocols for the Roche Light Cycler to evaluate anti-telomerase ribozyme expression and assess changes in the levels of hTERT mRNA. The Light Cycler accomplishes quantitative real-time PCR and is more sensitive than conventional assays for expression such as Northern Blot Analysis.

IX. Designed protocols for use of the TRAP assay for telomerase activity on the Roche Light Cycler. Once again this system can detect and quantify very small differences in telomerase activity that are otherwise not detectable by conventional PAGE gels.

Conclusions

Although complete evaluations of all of the anti-telomerase ribozyme candidates in various breast cancer cell lines will not be completed until May 2000, preliminary indications suggest that at least one of the catalytic RNAs will be effective in reducing telomerase activity in breast cancer cells. Indeed, the 3621 anti-telomerase ribozyme has been observed to retard MCF-7 breast cancer cell proliferation and

reduced telomerase activity as detected by TRAP assays. However, further evaluation is required in MCF-7 cells and the other breast cancer cell lines before definitive conclusions can be made regarding the ribozyme's efficacy. We have noted that sensitivity of the various assays designed to evaluate telomerase activity in cancer cells is limited by cell number. This is extremely important if a candidate ribozyme prevents further breast cancer cell division and thereby limits the number of cells available for assessment. With these limitations in mind, we have adapted and standardized several of the assays designed to assess ribozyme expression and cleavage and telomerase activity to the Light Cycler. The sensitivity of this system permits the detection of small changes in activity and directly quantifies these changes in telomerase activity. With these more sensitive assays in place, we will be able to complete a comprehensive assessment of each anti-telomerase ribozyme candidate in each of the breast cancer cell lines.

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Appendices

APPENDIX A: Micrographs (A to D) of MCF-7 cells transduced with a retroviral vector expressing the 3621 anti-telomerase ribozyme candidate.

Legend for Appendix A

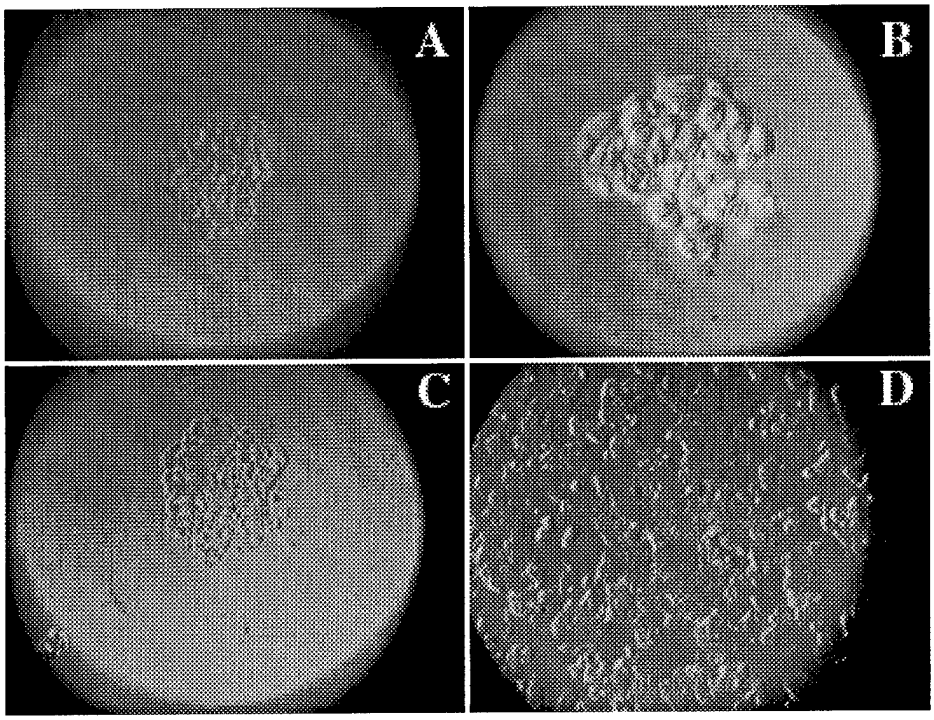
Micrograph A: MCF-7 cell colony transduced with the retroviral vector containing the 3621 anti-telomerase ribozyme candidate at 20 days post-transduction and selection (10X). These pictures are taken using a Varicel Light Distribution System (Zeiss) under phase contrast conditions.

Micrograph B: Same MCF-7 cell colony as in Micrograph A transduced with the retroviral vector containing the 3621 anti-telomerase ribozyme candidate at 45 days post-transduction and selection (40X). These pictures are taken using a Varicel Light Distribution System (Zeiss) under phase contrast conditions.

Micrograph C: MCF-7 cell colony transduced with the retroviral vector containing the 3621 anti-telomerase ribozyme candidate at 45 days post-transduction and selection (10X). These pictures are taken using a Varicel Light Distribution System (Zeiss) under phase contrast conditions.

Micrograph D: MCF-7 cells transduced with the retroviral vector containing no anti-telomerase ribozyme candidate at 20 days post-transduction and selection (10X). These cells have been passaged once at 15 days and have reached *0% confluence at 5 days post-passaging. These pictures are taken using a Varicel Light Distribution System (Zeiss) under phase contrast conditions.

APPENDIX B: Raw data for the TRAP assay completed using the Light Cycler. Note that for each cell concentration, MCF-7 cells transduced with the 3621 anti-telomerase ribozyme amplified telomerase-mediated template later than the comparable MCF-7 control and produced less PCR product over the same number of cycles.



Micrographs (A to D) of MCF-7 cells transduced with a retroviral vector expressing the 3621 anti-telomerase ribozyme candidate and control culture.

LightCycler Baseline Adjustment Report

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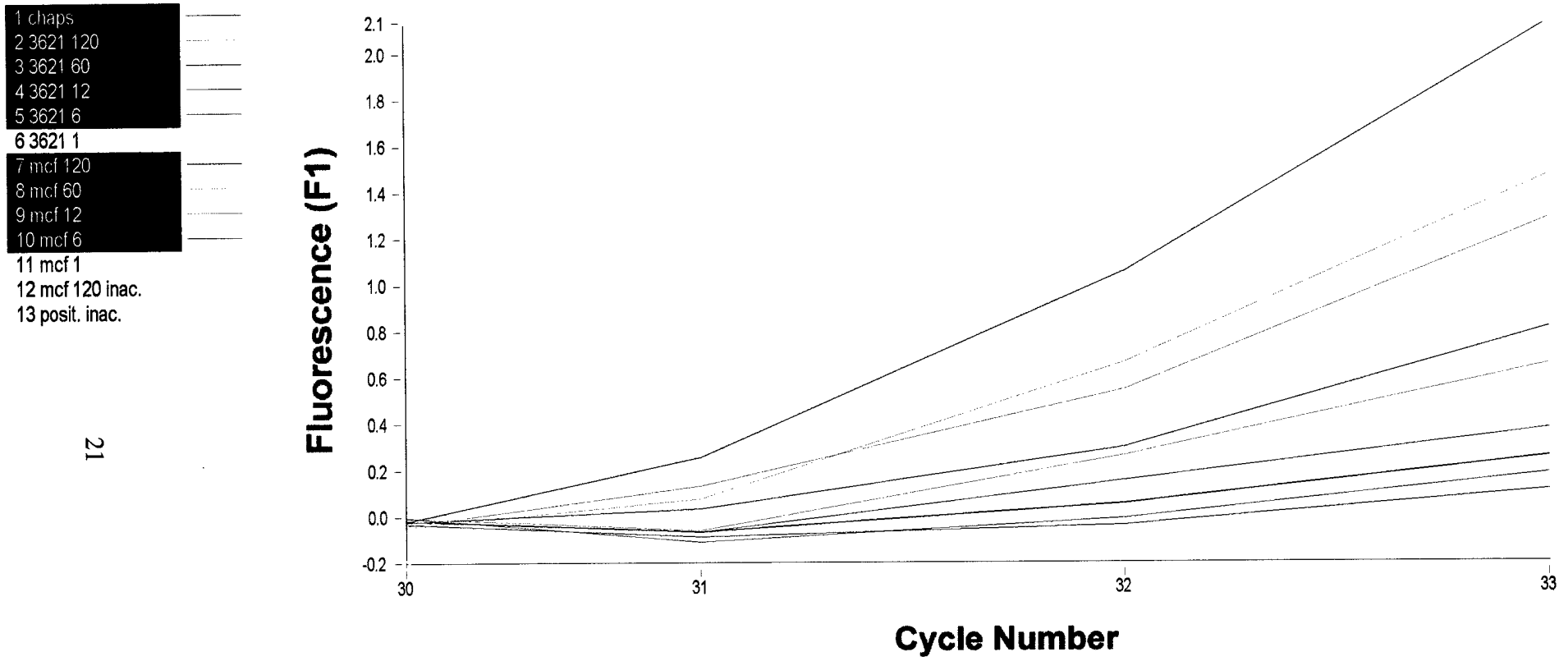

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