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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, cervical and ovarian 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 and metastasis. 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.

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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 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). 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 template domain of the RNA 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 the Anti-Telomerase Ribozyme

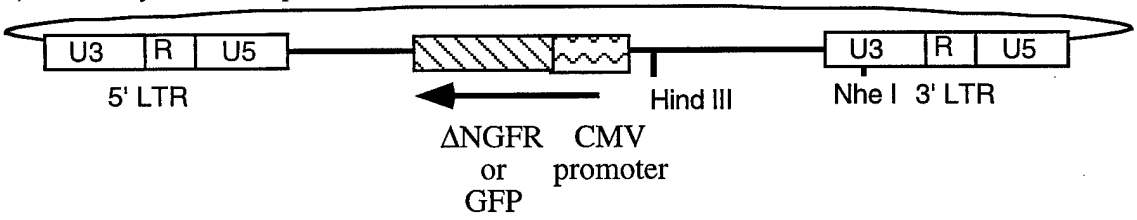
Overview: Moloney based retroviral vectors were constructed and used to deliver the therapeutic ribozyme gene to the target cancer cells. Three candidate ribozymes that target the mRNA component of telomerase are summarized with their corresponding target sequence in Table-1.

Table-1: Three possible target sequences in the RNA template of the telomerase complex and their corresponding ribozyme sequence.

RNA COMPONENT OF TELOMERASE (ACCESSION S79400)	RELATIVE POSITION	RIBOZYME SEQUENCE
5' AGCGAGTCCCGCCGCG 3'	378-393	5' TCGCaagaGGCGGCGC 3'
5' GACCAGTCCCTCAACG 3'	749-764	5' CTGGaagaGGAGTTGC 3'
5' CGCGGGTCTCTCGGGG 3'	318-333	5' GCGCaagaAGAGCCCC 3'

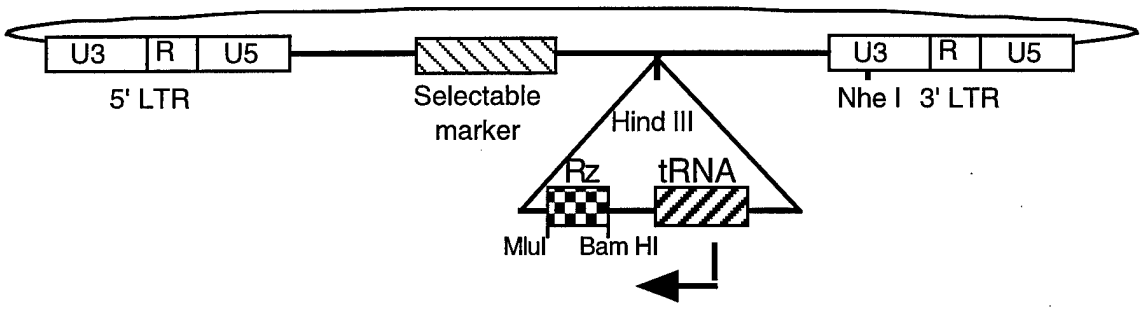
For the first set of experiments, LNL-6 based murine retroviral vectors were constructed to express a single copy of the anti-telomerase ribozyme candidates driven by pol III t-RNA^{val} promoter as well as the selectable marker (Figures 1 and 2). A truncated form of the human low affinity Nerve Growth Factor Receptor (24) (ΔNGFR) not normally expressed on the surface of the target cancer cells was chosen as the transient selectable marker for this investigation. The use of a non-functional receptor as a transient selectable marker is very rapid following retroviral transduction or transfection compared to selection of cells with cytotoxic drugs such as neomycin or puromycin (4 to 6 weeks). Drug selection requires a much longer time to isolate a drug resistant cell population which often results in a population of cells that may no longer expresses the gene of interest or has become spontaneously resistant to the selectable drug without retroviral transduction. The truncated form of the NGFR receptor (831 base pairs) includes the extracellular protein binding domain and the transmembrane portion for anchorage and orientation on the cell membrane. The intracellular signal domain has been removed to prevent possible effects on cellular function subsequent to antibody or nerve growth factor binding in vivo. The Cytomegalovirus (CMV) promoter was used to drive the expression of the selectable marker. The advantage achieved by using this promoter is the high levels of transient expression of the marker in target cancer cells may be achieved. This transient expression permits rapid selection of breast cancer cells transduced with the retroviral vector containing the anti-telomerase ribozyme gene using flow cytometry. A murine hybridoma producing a monoclonal antibody against the extracellular domain of the NGFR has been developed, allowing for the visualization, selection and sorting of cells expressing NGFR using immunofluorescent microscopy and flow cytometry. This anti-NGFR producing hybridoma (HB8737 - American Type Culture Collection (ATCC, Rockville, MD) in X-47 media and 10% serum) was cultured in our laboratory. Alternatively, retroviral vectors have been constructed that use green fluorescent protein (GFP) substituted for ΔNGFR as the selectable marker, as this protein permits rapid selection of transduced target cells using flow cytometry without the use of additional antibody staining.

Figure-1: Illustration of the LCΔNGFR/GFP plasmid containing the selectable marker (ΔNGFR or GFP) driven by the CMV promoter.



Construction of the retroviral vectors containing the candidate anti-telomerase ribozymes was confirmed by PCR, restriction digests and DNA sequencing. Following confirmation, large DNA preparations of the plasmids were prepared for retroviral packaging. GP+Am_{12} cells were used to package the retroviral-based plasmids containing a single copy of the candidate anti-telomerase ribozymes.

Figure-2: Illustration of the LCΔNGFR/GFP plasmid containing the selectable marker (ΔNGFR or GFP) driven by the CMV promoter and a single copy of the anti-telomerase ribozyme candidate.



Key Research Elements

A) Construction of NGFR Deletion, Evaluation and GFP Substitution

A truncated form of NGFR was constructed using PCR. A 849 base pair fragment of NGFR was amplified from a plasmid containing the full length NGFR (contributed by Dr. Asad Bashey, UCSD, San Diego, CA) using the following primers: 5' cccgctcgagATGGGGGCAGGTGCCACCGGC and 3' GAGGTGGAACAGCTGCAAGCAGtagtcgacgc. This PCR amplified fragment of NGFR has a 444 nucleotide deletion of the 3' end and introduced two unique restriction enzyme sites, 5' Xho I site and 3' Sal I, for subsequent cloning of the ΔNGFR. The deleted region of NGFR encoded for the intracellular signaling domain of the protein. The PCR fragment was subcloned into an expression vector pcDNA3.1 to determine that the expressed portion of the ΔNGFR protein is displayed in a useful manner in the transfected eukaryotic cells.

Cos cells, an adherent, primate cell line that does not express endogenous NGFR, were transfected with the pcDNA3.1/ΔNGFR to evaluate expression of the ΔNGFR selectable marker in vitro using immunofluorescence. Transfected cells were washed and fixed at 24 hrs, 48 hrs, and 72 hrs post-transfection and incubated with anti-NGFR monoclonal antibody. Following stringent washes, transfected cells were incubated with a FITC-labeled secondary antibody and assessed for ΔNGFR expression using a fluorescent microscope. Control Cos cells transfected with the LNL-6 plasmid without the ΔNGFR marker presented no fluorescent signal above background. Approximately 10 to 15% of Cos cells were observed to express the ΔNGFR marker at 24 hours, with levels raising to 25 to 30% by 48 and 72 hours (Appendix A-Pictures A to H). These observations are consistent with conventional percentages of cells transfected using the calcium-phosphate method (usually transfect 7 to 15% of cells). Thus, we confirmed the isolated ΔNGFR cloned into the pcDNA3.1 expression plasmid could be detected using the anti-NGFR monoclonal antibody as early as 24 hours post-transfection/transduction.

For the proposed study, transduced cells expressing the candidate ribozyme and the ΔNGFR were to be isolated using flow cytometry. The transduced cells could not be fixed and must be viable for further investigation. Thus, unfixed or viable Cos cells transfected with the pcDNA3.1/ΔNGFR were incubated with anti-NGFR monoclonal antibody and stained with FITC-labeled secondary antibody at 24 and 48 hours post-transfection (Appendix A - Picture I). Transfected cells expressing the ΔNGFR were sorted using flow cytometry. However, multiple attempts at using the ΔNGFR system to isolate transfected cells with flow cytometry were not successful. This poor result may be attributed to the low levels of ΔNGFR expressed on the surface of viable, transfected cells. The initial experiments used Cos cells fixed in 1:1 methanol:acetone which permits the detection of proteins both on the surface of cells and in the intracellular or cytoplasmic regions. For flow cytometry, Cos cells were not fixed and sorting was

dependent upon sufficient levels of ΔNGFR expression on the surface of transfected cells. The levels of ΔNGFR expression on the surface of viable cells may not be sufficient, even under the control of the powerful CMV promoter, for efficient sorting of transfected/transduced cells. Expression of the ΔNGFR on the cell surface may be hindered by the deletions we made in the full length protein. Future directions with the ΔNGFR as a selectable marker may include using smaller deletions that prevent biological signaling but permit higher levels of protein expression on the surface of the transfected/transduced cells for effective cell selection with flow cytometry. The truncated form of NGFR (ΔNGFR) stills remains a viable option as a selectable marker for future investigations.

Due to the expression problem encountered with the ΔNGFR marker, an alternative selectable marker was chosen for further investigation. Green fluorescent protein (GFP) is a conventional and well-characterized selectable marker that may be utilized in the selection and isolation of viable cells using flow cytometry. Although GFP is also expressed in the intracellular region of transfected/transduced cells, its fluorescence is sufficient for sorting viable cells without fixation or the addition of antibodies. To avoid further delay, GFP was substituted for ΔNGFR as the selectable marker for this investigation.

B) Construction of LNL-6 Based GFP Vectors with the Anti-Telomerase Ribozyme

The gene encoding for the GFP was inserted by blunt-end ligation into an existing LNL-6 based vector containing a SV40 promoter and a poly A signal (LNPT). In detail, the GFP was cloned into the LNPT vector, which was completely cut with Age I and partially digested with Xba I and treated with T4 DNA polymerase to produce compatible blunt ends. The two DNA fragments were ligated to create the vector called LC-GFP (Figure-1). There are three possible target sequences located in the RNA template domain of the telomerase complex for ribozyme targeting and cleavage (Table-1). The first two anti-telomerase ribozyme candidates listed in Table-1 were investigated initially due to the location of their target sequences at opposing ends of the RNA template. The candidate ribozymes were cloned into unique restriction enzyme sites Bam HI and Mlu I of an already existing t-RNA^{val} promoter/terminator cassette containing LNL-6 based plasmid. The resulting plasmid contained internally a single copy of the ribozyme candidate (Figure-2). The t-RNA^{val} ribozyme cassette can be released by digesting the DNA with Hind III. Following the construction of the retroviral plasmids containing a single copy of the candidate ribozymes, the plasmids underwent PCR, restriction digests and DNA sequencing to confirm the proper anti-telomerase ribozyme sequence and positioning was free of mutation. Once construction of the single copy plasmids was confirmed, large DNA preparations of the plasmids were prepared for retroviral packaging.

C) Packaging of the Retroviral Vectors Containing the Anti-Telomerase Ribozyme

Vector producing cell lines were generated by transfecting (calcium-phosphate precipitation method) these vectors into a retroviral packaging cell line, GP+Am$_{12}$ (ATCC, Rockville, MD). This packaging cell line was preferred because it is the least likely to generate Replication Competent Retrovirus (RCR) due to further splitting of the viral structural genes. PA317 cells are an alternative packaging cell line, but are more susceptible to recombination and the generation of RCR. Transfected cells were selected using the GFP marker and flow cytometry. Clones of vector producing cells were screened for vector titer using Cos-transduction assays. Briefly, subconfluent Cos cells were incubated with virus-laden supernatants of the packaging cell clones. At 24 to 36 hours, the cos cells were counted and sorted using flow cytometry. The packaging cell clone that produced the highest number of transduced cos cells (gfp expressing cells) was identified as a high titer producer clone. Of these, the highest titer clones were tested for RCR contamination using a cocultivation assay, generally accepted as the most sensitive assay for RCR. This assay amplifies RCR by co-cultivating vector producing cells with a replication permissive cell line, Mus dunni, followed by a plaque assay on the indicator S+L cell line, PG4. Only clones tested negative for RCR were used for this study. For each candidate ribozyme, a high titer packaging cell line was identified and found to be free of replication competent retrovirus, thereby permitting further use of these producer cell lines in this investigation.

II. Selection and Evaluation of Cancer Cells Expressing the Anti-Telomerase Ribozyme

Overview: Human breast cancer cell lines, MCF-7, MDA-453, SK-BR-3, and BT-474 (ATCC, Rockville, MD) are known to have various levels of telomerase activity and will be used to assess the efficacy of anti-telomerase ribozyme for this investigation. However, for the initial period of this research project, the human cervical carcinoma cell line, Hela, was used to evaluate changes in telomerase activity following delivery of the anti-telomerase ribozymes. Hela cells were chosen because they have been well-characterized for both telomerase activity and growth characteristics following retroviral transduction. Hela cells have a moderate to high level of telomerase activity and have been used extensively by other research efforts characterizing telomerase dynamics(12, 16). Thus, the preliminary experiments with Hela cells would permit the standardization of telomerase detection assays, while permitting preliminary evaluation of the anti-telomerase ribozyme candidates.

For the first set of experiments, hela cells were transfected (calcium-phosphate method) with the retroviral-based plasmid containing the anti-telomerase ribozyme candidate and the GFP selectable marker. For comparison, control cultures were transfected with the control LC-GFP vector that does not contain the anti-telomerase therapeutic gene. 24 to 36 hours following transfection the cancer cells were prepared for selection using flow cytometry. From preliminary experiments with the GFP driven by the CMV promoter, we observed that transduced cancer cells will begin to express the GFP selectable marker as early as 20 to 24 hours post-transduction. This rapid expression and the use of flow cytometry provides a fast and efficient means of selecting therapeutically altered cells that cannot be achieved with selection by drug resistance. Selected and sorted cancer cell samples were assayed for viability using trypan blue stain and a hemocytometer. The isolated cancer cell populations were placed in culture and prepared for comparative evaluation of telomerase activity using the commercial Telomerase Activity Detection Kit or TRAP assay (Boehringer-Mannheim). The telomeric repeat amplification protocol or TRAP assay was used to evaluate telomerase activity in these transfected Hela cells. The TRAP is a PCR based assay designed to detect telomerase activity in cellular samples. In the TRAP assay, telomerase synthesizes extension products, which then serve as templates for amplification by PCR. The TRAP assay can be completed in a single tube as conditions were devised that were suitable to both telomerase and Taq polymerase. A positive signal in a TRAP assay requires a ribonucleoprotein in an immortal cell extract capable of extending the TS oligonucleotide with three or more TTAGGG repeats, thereby validating the assay for specific detection of telomerase activity. The commercial kit states that a minimum of 10^2 cells may be used to detect telomerase activity in human cell lines. We set our cell samples sizes at 3×10^3 Hela cells for adequate detection of telomerase activity in these samples. Hela cells transfected with the control LC-GFP vector were used as a positive control and negative controls were prepared from untreated Hela cells as described by the commercial protocol. The data from the TRAP assays are reported in the following section.

2.1. Preliminary Observations from Experiments with the Anti-telomerase Ribozymes Against the RNA Component of Telomerase

A) Transfection and Selection of Cancer Cells using the GFP marker and Flow Cytometry

Subconfluent cultures of Hela cells were transfected with 5μg of the retroviral-based plasmid DNA containing the GFP marker and a single copy of an anti-telomerase ribozyme candidate. Control cultures of Hela cells were transfected with a similar plasmid without the therapeutic ribozymes. At 24 hours post-transfection, hela cells were washed and prepared for flow cytometry where transfected cells would be sorted via expression of the GFP marker. The GFP cloned in the retroviral-based plasmid DNA is human codon optimized and contains a chromophore mutation which produces fluorescence 35 times more intense than wild-type GFP at 488nm due to an increase in its extinction coefficient. This makes the GFP selectable marker ideal for fluorescence microscopy and fluorescence-activated cell sorting (FACS). Transfected Hela cells were sorted by FACS and placed in 6-well culture plates at a concentration of 10^5 cells per well.

B) Observations and Telomerase Activity in Transfected Hela Cells

Transfected cells were harvested at 1 day, 7 days, 14 days, 30 days and 45 days post-selection and prepared for the TRAP assay. No changes in cell proliferation or characteristics were observed over this time period compared to Hela controls. Results from the TRAP assay suggested expression of the anti-

telomerase ribozymes had no effect on telomerase activity at any time point post-selection. When the telomerase activity level for Hela cell controls was set at 100% activity, the activity levels for Hela cells expressing the anti-telomerase ribozyme candidates were not significantly different. These results were confirmed by repetitive experiments done in triplicate and may be attributed to the following:
1) The target RNA sequence is either not present in Hela cells or is in accessible by the ribozymes due to tertiary RNA structure.
2) The anti-telomerase ribozymes were not expressed in the target cells.
3) The TRAP assay is too sensitive to detect small changes in telomerase activity in transfected Hela cells.
4) The anti-telomerase ribozymes cleaved the target RNA domain of telomerase but were not sufficient to change overall telomerase activity and alter cell division.

Expression of the anti-telomerase ribozymes was confirmed by PCR. The sensitivity of the TRAP ELISA was controlled for using various dilutions of cell lysate containing the ribonucleoprotein from the Hela cells. Furthermore, retroviral transduction of hela cells with vectors containing the anti-telomerase ribozyme candidates yielded similar results, with no measureable effect on telomerase activity. Finally, further evaluation of the RNA component of human telomerase using Gene Bank suggested that the target RNA had been changed or corrected. These corrections to the original sequence would have rendered the anti-telomerase ribozymes ineffective as their target sequences were no longer present. Furthermore, sequences submitted for the RNA domain of telomerase suggest variability in the RNA template. Sequence variability is a major detriment to the design and function of catalytic ribozymes, rendering them ineffective or impotent. Thus, the candidate anti-telomerase ribozymes against the RNA domain of telomerase would be ineffective in preventing telomerase activity in cancer cells that are prone to mutations. However, during the sequence analysis of the RNA domain of telomerase, several submissions had been made to the Gene Bank over the past year indicating that the protein component of telomerase had been sequenced and cloned. Further analysis of these sequences suggested that DNA sequence encoding for the protein component of telomerase was highly conserved across species and cell types, suggesting the telomerase protein component may be a more suitable target for catalytic RNAs. Thus, rather than trying to design anti-telomerase ribozymes against the variable RNA component of telomerase, we shifted our focus to ribozymes that target the mRNA encoding for the conserved protein domain of the target ribonucleoprotein.

III. Design, Construction and Evaluation of the Anti-Telomerase Ribozymes Against the mRNA Encoding for the Protein Component of Telomerase

Overview: Several target sites for ribozyme therapy have been identified in the sequence of the mRNA encoding for the protein component of the telomerase complex (Table-2). Each of these ribozyme candidates will be cloned into retroviral vectors and evaluated for anti-telomerase activity in Hela and breast cancer cell lines. As described previously, Moloney based retroviral vectors will be constructed and used to deliver the therapeutic ribozyme gene to the target cancer cells. This amphotrophic 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-2: Target sequences in the mRNA of the protein component (hRT) of the telomerase complex and their corresponding ribozyme sequence. T815 and T275 anti-telomerase ribozymes are in bold print.

mRNA TARGET SEQUENCE OF TELOMERASE PROTEIN (hRT)	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′	3641-3656	5′ GCTCaagaGTCGGTTC 3′
5′ TGAGTGTCCAGCACAC 3′	3660-3675	5′ ACTCaagaGTCGTGTG 3′
5′ TGGGGGTCCCTGTGGG 3′	3933-3948	5′ ACCCaagaGGACACCC 3′

The LNL-6 based murine retroviral vector will contain one to three copies of the gene encoding for the anti-telomerase ribozyme candidate driven by pol III t-RNA^{val} promoter as well as the selectable marker (Figures 1-4). Initially, the single copy vector will be transfected into Hela cells to evaluate anti-telomerase activity in a well-characterized cell line with moderate to high levels of telomerase activity. The 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). GFP will be used as the selectable marker in these constructs. Following construction of the retroviral vectors containing one to three copies of the therapeutic ribozyme candidate or an inactivated anti-telomerase ribozyme control and preliminary evaluation in hela cells, these vectors will be packaged for in vitro evaluation of anti-telomerase activity in breast cancer cells.

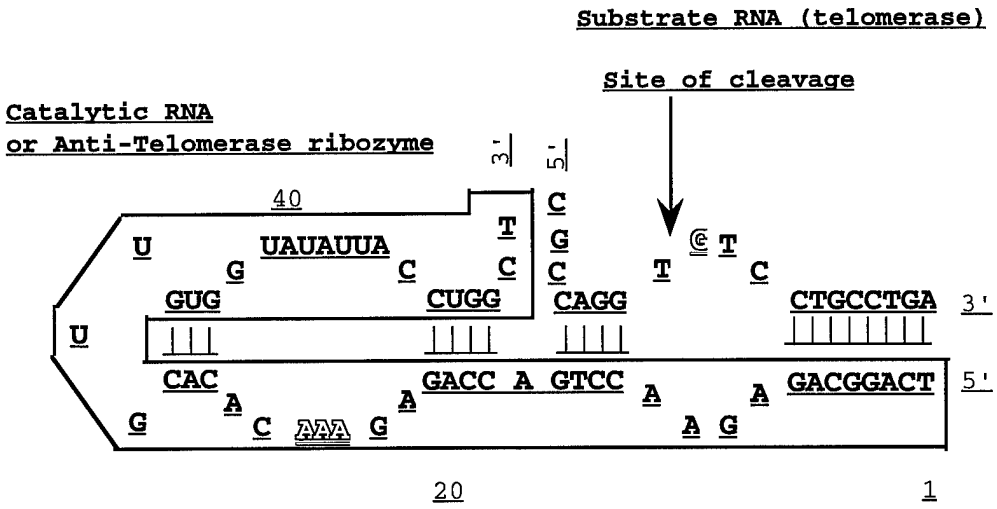
3.1 Construction of LNL-6 Based GFP Vectors with the Anti-Telomerase Ribozyme

Moloney based retroviral vectors will be constructed and used to deliver the therapeutic ribozyme gene or inactivated ribozyme control to the target cancer cells. The LNL-6 based murine retroviral vectors will contain one to three 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 GFP selectable marker driven by the CMV promoter (Figure-2). The basic LNL-6 retroviral plasmid containing the GFP 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 (Table-2).

The two anti-telomerase ribozymes in bold (T275 and T815) listed in Table-1 will be investigated for the first set of experiments due to the location of their target sequences at opposite ends of the target mRNA. For each therapeutic ribozyme candidate, inactivated anti-telomerase ribozymes will be synthesized which include the specific hybridization regions to control for anti-sense effect but do not

possess the cleavage sequence, rendering them inactive (Figure-3). Initially, retroviral vectors expressing a single copy of the anti-telomerase ribozyme candidates will be evaluated in Hela cells for anti-telomerase activity prior to retroviral packaging.

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



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 two 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-4).
Following construction of the retroviral vectors with therapeutic or inactivated forms of the anti-telomerase ribozyme candidates, the vectors will be packaged in vector producing cell lines. Viral particles produced by these cell lines will be used to transduce breast cancer cell lines in future experiments. These transduced cancer cells expressing multiple copies of the anti-telomerase catalytic RNA will be examined for ribozyme expression, telomerase activity and changes in cell proliferation.

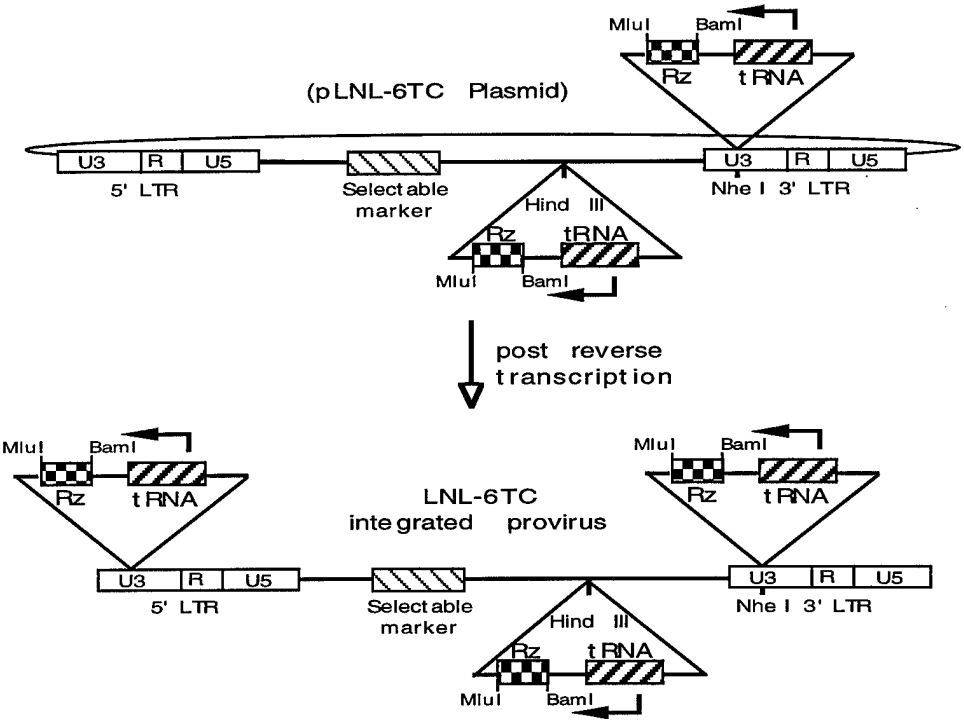
3.2. Preliminary Evaluation and Observations of the Anti-telomerase Ribozyme Candidates in Hela Cells

Preliminary experiments were completed which included the design and evaluation of anti-telomerase ribozyme candidates in a cervical cancer cell line (Hela cells). This cell line has been used for many years and has been well-characterized for telomerase activity and growth characteristics. Several ribozyme target sequences were identified in the mRNA sequence encoding for the protein component (hRT) of telomerase (Table-1). Two candidates were chosen for these preliminary experiments in Hela cells and were called Anti-telomerase ribozymes T275 and T815, thereby identifying their point of hybridization and cleavage in the target mRNA sequence. Moloney based retroviral vectors were constructed and used to deliver the therapeutic ribozyme gene to the target cancer cells. The LNL-6 based murine retroviral vectors contained one copy (pLNL-6 SC) of the gene encoding for the respective anti-telomerase ribozyme candidates driven by pol III t-RNA^{val} promoter. The ribozyme cassette, including the t-promoter and the candidate ribozyme, is located between the LTRs and is orientated in the opposite direction of the selectable marker (GFP) which is driven by the CMV promoter.

Hela cells were transfected with plasmid DNA containing a single copy of the candidate ribozyme. Transfected cells were sorted using FACS and the GFP selectable marker at 24 hours post-transfection.

Selected cells were placed in culture in triplicate prior to evaluation for telomerase activity. Morphology and cell growth was observed over a 10 day period prior to harvesting the treated cells for Telomerase Activity assays (TRAP Assay). Transfected Hela cells under both types of culture to reach confluency in the 10 day period, whereas normal Hela cells reached confluency within 2 to 3 days.

Figure-4: Illustration of the murine retroviral vector producing three copies of the anti-telomerase ribozyme (Rz) 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 GFP driven by the CMV promoter.



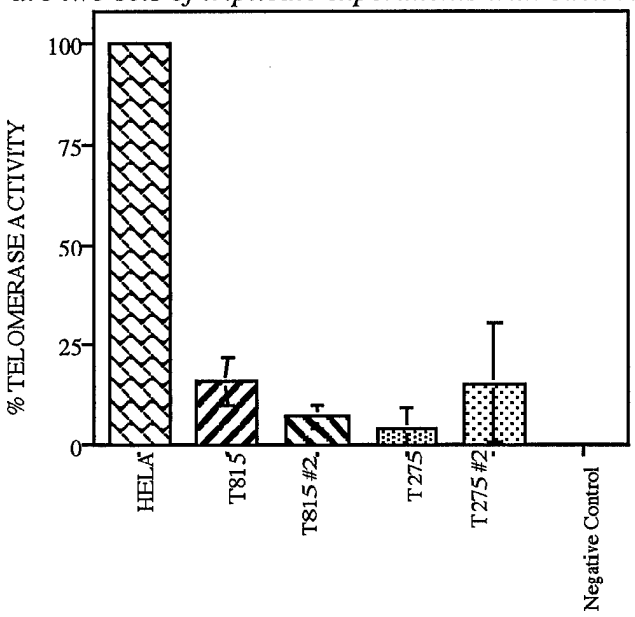
Possibly, the anti-telomerase ribozymes were interfering with telomerase protein expression and preventing efficient cell division in these cultures. By 10 days, all flasks containing Hela cells transfected with the anti-telomerase ribozymes were approximately 20% confluent.

The telomeric repeat amplification protocol or TRAP assay was used to evaluate telomerase activity in these transfected Hela cells. The TRAP is a PCR based assay designed to detect telomerase activity in cellular samples. In the TRAP assay, telomerase synthesizes extension products, which then serve as templates for amplification by PCR. The TRAP assay can be completed in a single tube as conditions were devised that were suitable to both telomerase and Taq polymerase. A positive signal in a TRAP assay requires a ribonucleoprotein in an immortal cell extract capable of extending the TS oligonucleotide with three or more TTAGGG repeats, thereby validating the assay for specific detection of telomerase activity.

The commercial kit states that a minimum of 10^2 cells may be used to detect telomerase activity in human cell lines. We set our cell samples sizes at 3 x 10^3 Hela cells for adequate detection of telomerase activity in these samples. Non-transfected Hela cells were used as a positive controls and

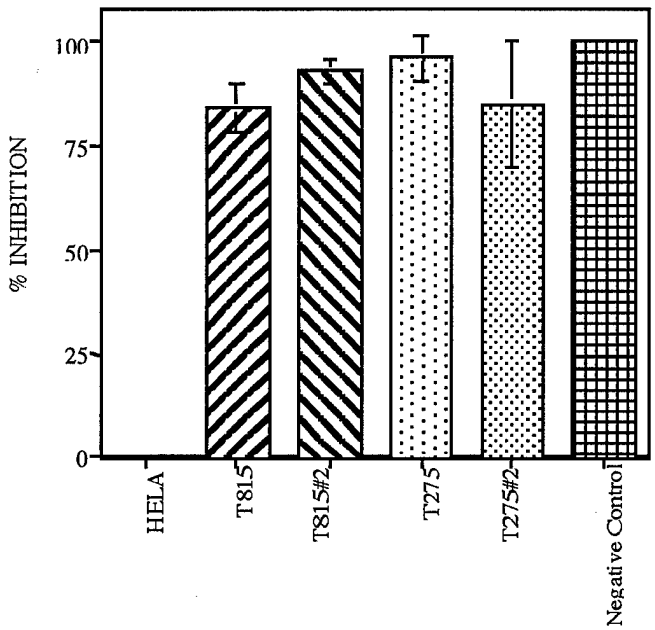
negative controls were prepared from normal Hela cells as described by the commercial protocol. The data from the TRAP assays are reported as Telomerase Activity (%) and Inhibition of Telomerase Activity (%) as summarized in Figures 7 and 8. Telomerase levels in normal or non-transfected Hela cells (positive control) were designated as 100%. Telomerase activity for each ribozyme (T815 and T275) was compared to the positive and negative controls. Telomerase activity measured for each culture was similar and reduced compared to the positive control. Telomerase activity in hela cells transfected with the anti-telomerase ribozymes ranged from 4 to 16% of normal activity (Figure-7). The values were not significantly different between the two ribozymes (T815 and T275) to determine which construct elicited the most inhibition. By inverting the measurements for telomerase activity and comparing them to the negative controls, levels of telomerase inhibition were determined for both ribozymes (Figure-8). Both anti-telomerase ribozymes were observed to significantly inhibit telomerase activity in Hela cells.

Figure-7: The percentage of telomerase activity for each anti-telomerase ribozyme compared to the positive control cells. There are two sets of triplicate experiments with each ribozyme candidate.



In summary, both anti-telomerase ribozyme candidates, T815 and T275, were observed to significantly inhibit telomerase activity in a well characterized cervical cancer cell line. Additional investigation is required to evaluate the other ribozyme candidates, ribozyme expression in target cells, target mRNA cleavage, and quantitatively evaluate changes in cell proliferation. In addition, further experimentation will reveal the therapeutic efficacy of these anti-telomerase ribozymes in other forms of cancer that have elevated levels of telomerase such as malignant breast tumors. Future investigations will focus on the use of cell lines and, more importantly, primary cancer cells samples from cancer patients to evaluate the potential therapeutic application of the anti-telomerase ribozymes.

Figure-8: The percent inhibition of Telomerase activity for each anti-telomerase ribozyme candidate compared to the negative control. There are two sets of triplicate experiments with each ribozyme candidate.



IV. Current Status and Future Experiments

Following the promising preliminary results in Hela cells with the anti-telomerase ribozymes that target the mRNA encoding for the protein domain, we have continued with the proposed work for this proposal. The two candidates chosen for these preliminary experiments in Hela cells are called Anti-telomerase ribozymes T275 and T815, thereby identifying their point of hybridization and cleavage in the target mRNA sequence. The retroviral vectors containing these anti-telomerase ribozyme have been packaged in a retroviral packaging cell line, GP+Am_{12}. Several producer cell clones were selected and isolated for each ribozyme construct. Retroviral particle production and adverse toxicity was assessed using a simple colony forming assay with Cos cells and selection for neomycin resistance. No adverse effects were observed in transduced cells, and the range of transduction efficiency was observed to be between 85 and 95%. Three producer clones with high viral particle production were chosen for each candidate ribozyme and further evaluation. Currently, human breast cancer cell lines, MCF-7, MDA-453, SK-BR-3, and BT-474 are being cultured and prepared for transduction with the retroviral particles containing a single copy of each ribozyme candidate. Each breast cancer cell line will be evaluated in triplicate for telomerase activity using the TRAP Assay to standardize the telomerase activity for each cell line. Breast cancer cell lines will be transduced with these retroviral particles and transduced cells will be selected using the GFP marker and flow cytometry. Selected cell will be evaluated for anti-telomerase ribozyme expression, changes in cell proliferation and telomerase activity following retroviral transduction.

Retroviral vectors containing three copies of the anti-telomerase ribozyme candidates (T275 and T815) are under construction. They will be packaged and evaluated using the same parameters as described for the single copy vectors. Other anti-telomerase ribozyme candidates from Table-1 will be constructed and evaluated for anti-telomerase activity as well.

Conclusions

The following is a summary of the results with their implications towards the proposed research.

1) The truncated Nerve Growth Factor Receptor (ΔNGFR) was not a suitable selectable marker for viable cells. If transfected cells were fixed prior to staining with the anti-NGFR antibody and FITC-labeled secondary antibody, the ΔNGFR was very useful for cell visualization. However, ΔNGFR expression was not sufficient for selection of non-fixed viable cells via flow cytometry. These observations are most likely due to poor expression of the truncated receptor on the surface of transfected cells. It is possible that the ΔNGFR may be a useful selectable marker for future investigations if the intracellular domain is extended, but still inactive, for improved surface expression. For the remainder of this investigation, green fluorescent protein (GFP) will be used as a selectable marker. Cells expressing GFP can be easily selected using FACS at 24 to 48 hours post-transduction, permitting the rapid isolation of cells expressing the anti-telomerase ribozymes.

2) Anti-telomerase ribozymes designed to recognize and cleave the RNA component of the ribonucleoprotein complex did not inhibit levels of telomerase activity in well-characterized Hela cells. The TRAP assay ELISA suggested that ribozyme expression had no effect on telomerase activity and no effect on cell proliferation over time. These observations could be attributed to the target RNA sequence is either not present in Hela cells or is in accessible by the ribozymes due to tertiary RNA structure; the anti-telomerase ribozymes were not expressed in the target cells; the TRAP assay is too sensitive to detect small changes in telomerase activity in transfected Hela cells; or the anti-telomerase ribozymes cleaved the target RNA domain of telomerase but were not sufficient to change overall telomerase activity and alter cell division. Upon review of the sequences for the RNA domain submitted to the Gene Bank, we observed that there was variability in the RNA domain of telomerase between cell types. Furthermore, over the past year, several sequences were submitted for the protein component of telomerase from several cell types and species. The sequences for the protein domain of telomerase are highly conserved and have several potential targets for ribozyme therapy. Thus, we decided to pursue the design and evaluation of ribozymes which recognize and cleave the mRNA encoding for the protein component of telomerase in breast cancer cells.

3) Anti-telomerase ribozymes designed to recognize and cleave the mRNA encoding for the protein component of the ribonucleoprotein complex inhibited telomerase activity in well-characterized hela cells. Hela cells were transfected with retroviral plasmids expressing single copies of two different anti-telomerase ribozymes candidates (T275 and T815). Transfected cells were observed to grow at significantly slower rates than non-transfected controls and had significant reductions in telomerase activity at 10 days post-transfection. These promising results led to the packaging of the retroviral constructs containing a single copy of the anti-telomerase ribozymes for transduction of breast cancer cell lines. Triple copy vectors are currently under construction for both T275 and T815 anti-telomerase ribozymes. These multiple copy vectors will be packaged and used to transduce breast cancer cell lines. Breast cancer cell lines transduced with the retroviral vectors containing the anti-telomerase ribozymes will be evaluated for telomerase activity (TRAP Assay), ribozyme expression (RT-PCR), cleavage of the mRNA encoding for the protein domain of telomerase (Northern Blot), and changes in breast cancer cell proliferation (thymidine incorporation assays). Other anti-telomerase ribozymes from Table-2 will also undergo preliminary evaluation.

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Appendices

APPENDIX A: Micrographs (A to I) of immunofluorescent stained Cos cells expressing the ΔNGFR selectable marker.

Legend for Appendix A

Micrograph A: Cos Cell Control: Non-transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (4X). No immunofluorescent labeling indicating no endogenous expression of NGFR in the primate cell line.

Micrograph B: Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (4X). Note approximately 10 to 20% of the cells are labeled for ΔNGFR expression., as expected with the calcium-phosphate transfection method. Confirms expression of the ΔNGFR selectable marker.

Micrograph C: Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (4X).

Micrograph D: Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (10X).

Micrograph E: Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (40X). Note the heavy staining for ΔNGFR in the cytoplasm of fixed cells.

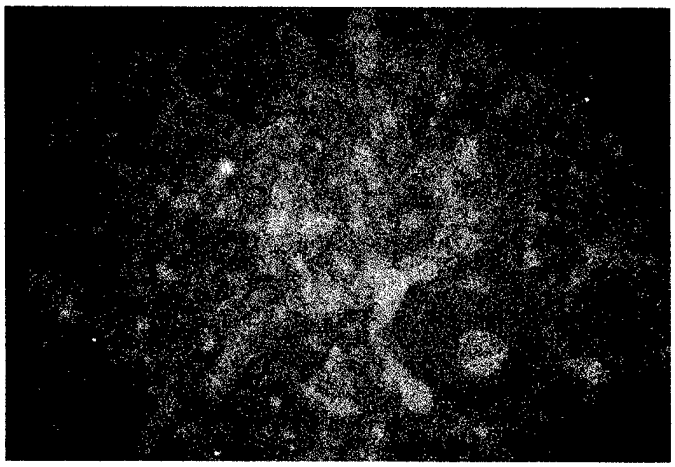
Micrograph F: Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (40X). Note the heavy staining for ΔNGFR in the cytoplasm of fixed cells and the well-defined unstained nuclei.

Micrograph G: Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (40X).

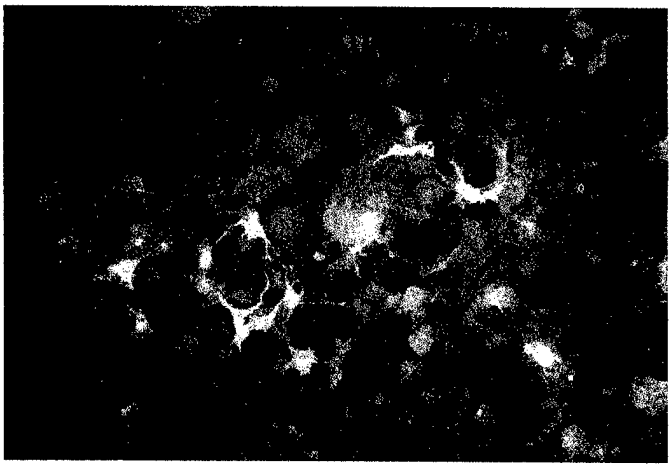
Micrograph H: Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells fixed with methanol:acetone and stained with FITC-labeled anti-NGFR monoclonal antibody (40X).

Micrograph H: Unfixed or Viable Cos Cells Transfected with pcDNA3.1/ΔNGFR: Transfected Cos cells were stained with FITC-labeled anti-NGFR monoclonal antibody and washed prior to analysis by flow cytometry (4X). Note the lack of staining for ΔNGFR on the cell surface of unfixed or viable cells. FACS analysis could not detect any signal for ΔNGFR expression and transfected cells could not be sorted from the total cell population.

A



B



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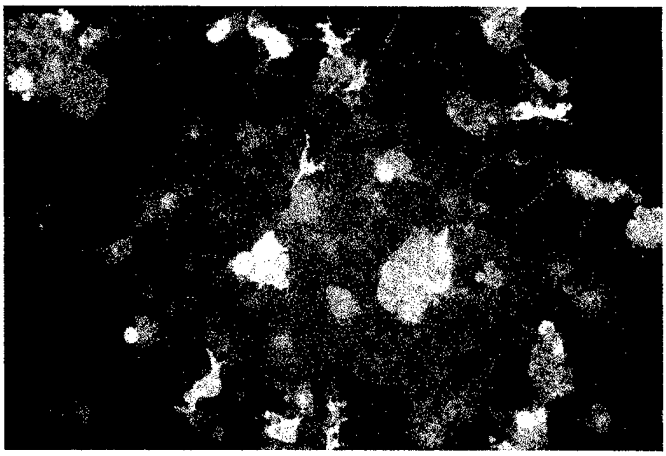

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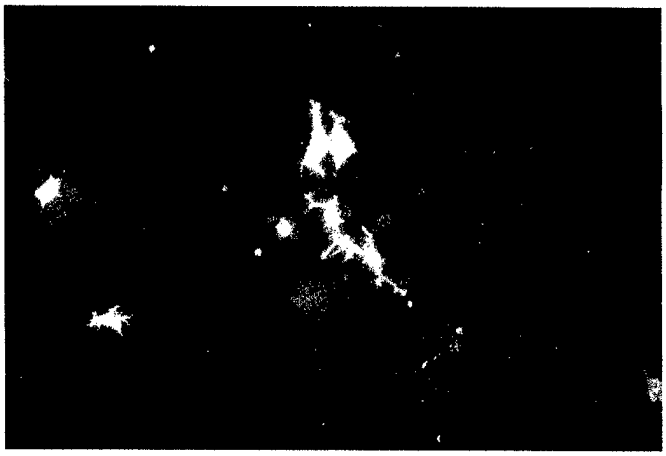

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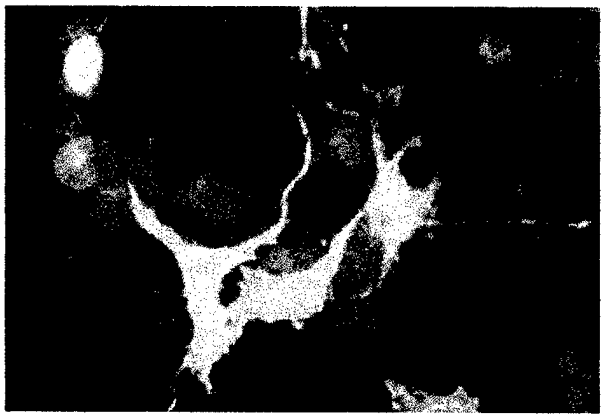

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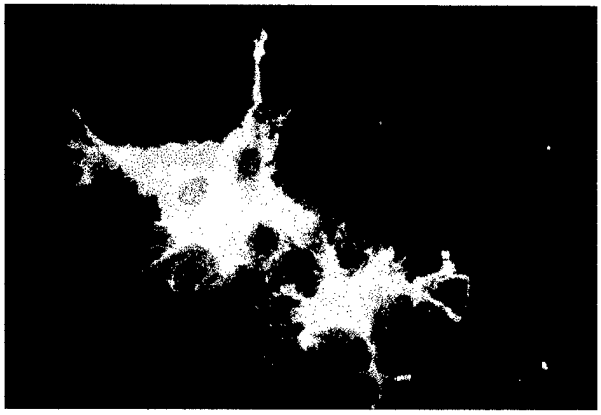
G



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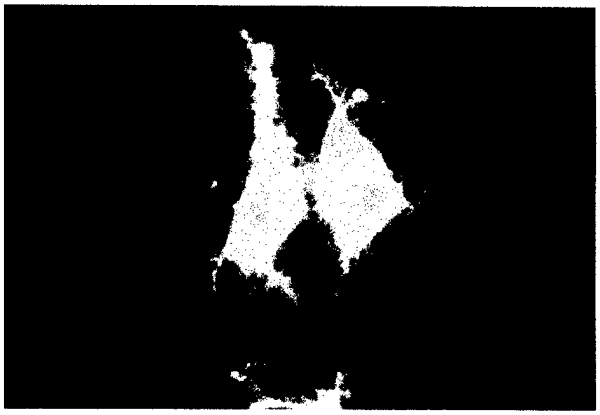

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