Targeted Ribozyme Against Telomerase RNA Component

Thesis Submitted to Jawaharlal Nehru University
for the Award of the Degree of

DOCTOR OF PHILOSOPHY
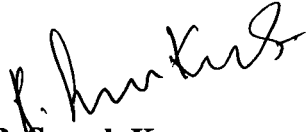
Submitted by

R. SURESH KUMAR

Applied Molecular Biology Laboratory
School of Life Sciences
Jawaharlal Nehru University
New Delhi 110067, INDIA

2008

School of Life Sciences

Jawaharlal Nehru University. New Delhi

Declaration

The research work embodied in this thesis entitled **"Targeted Ribozyme Against Telomerase RNA Component"** has been carried out at Applied Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full for the award of any other degree or diploma of any other university.

R. Suresh Kumar
(Student)

Prof. Pramod K. Yadava
(Dean)

July 23, 2008

Prof. Pramod K. Yadava
(Supervisor)

Dedicated to

My

Parents

CONTENTS

III. Results and Discussions

III. 1. Designing of Ribozyme against hTR

III. 2.   Cloning of Ribozymes and hTR

III. 3. Expression of Ribozyme and its Effect on Cells

III. 4.   Transcription Profiling of Transfectants

III. 5. Proteomic Alterations in Transfectants

III.6. Future Prospects   168

Acknowledgement

I thank all mighty God for given me enough courage and energy to face tough conditions and stay in race with perseverance.

The whole work was from the supports of friends without whom I would have perished from science. I thank all of them whomever helped and supported (if missed my apologies) to finish this endeavour.

(R. Suresh Kumar)

Date & Place : 24 | 07/08 · N.Delhi ·

Common Abbreviations:

ATP	Adenosine Tri Phosphate
bp	base pairs
BSA	Bovine Serum Albumin
Ci	Curie
cpm	counts per minute
ddNTPs	Dideoxynucleotides
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleoside Triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic Acid
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kb	Kilobase
LB	Luria Broth
M	Molar
MCS	Multiple Cloning Site
ml	milli liter
mM	milli Molar
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	messenger RNA
N	Normal
OD	Optical density
PAGE	Poly Acrylamide Gel Electrophorosis
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pI	Iso electric point
PMSF	Phenyl Methyl Sulfonyl Fluoride
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
TBE	Tris, Borate, EDTA buffer

Tris	Tris (hydroxymethyl)-aminomethane
UTR	Untranslated Region
uv	ultra violet
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
μg	micro gram
μl	micro liter
μM	micromolar

Abbreviation for Genes/ proteins

14-3-3alpha	14-3-3 refers to the characteristic migration pattern of these proteins on electrophoretic gels.
Ap-1	Activator protein -1
APC	Adenomatous polyposis coli gene
ARF	Alternate reading frame (ARF) product of the CDKN2A locus
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telangiectasia and Rad3-related
ATRIP	ATR interacting Protein
BAX	Bcl2-associated X protein
BCL2	B-cell chronic lymphatic leukemia/lymphoma 2 oncogene
BRCA1	Breast cancer 1 gene
BRCA1, 2	Breast cancer gene
Cdc	Cell division cycle
CDK	cyclin-dependent kinaseor Cell cycle dependant Kinases
CHK1	Checkpoint homolog of *S.pombe*
EBP	Emopamil Binding Protein
EGFR	Epidermal growth factor receptor gen
hCG	Human Chroionic Gonadotrophin
HEI-C	Human Enhancer of Invasion Cluster

HNPCC	Hereditary non-polyposis colorectal cancer
HPV	Human Papilloma virus
hTERT	Human telomerase reverse transcriptase
hTR	Human Telomerase RNA
IL-12R	Interleukin 12 Receptor
JNK	C-Jun N-terminal kinases
K10	Keratin 10
MDM2	Murine double minute (mdm2) oncogene
MMR	Mismatch repair
MRE	Meiotic recombination -
NBS-1	Nijmegen breakage syndrome
p21 (WAF1, CDKN1A)	cyclin-dependent kinase inhibitor 1A
p53	Tumor suppressor protein 53
PCR	Polymerase chain reaction
PiNX	Pin2 interacting protein
Pot1	Protection of Telomeres
PTEN	Phosphatase and Tensin Homolog
Rb	Retinoblastoma gene
RBP2	Retinoblastoma Binding Protein 2
RPL23,35	Ribosomal rRNA binding proteins
RT-PCR	Reverse transcriptase polymerase chain reaction
SCCHN	Squamous cell carcinoma of head and neck
TIN2	TRF interacting protein 2
TRAP	Telomere Repeat Amplification Protocol
TRF-1, 2	Telomere repeat binding factor 1, 2
β –TrCP	Beta Transducin Repeat Containing Isoform

Introduction & Review of Literature

Replication of DNA involves copying of existing sequences in a template and primer dependant action of DNA polymerase. This leaves a part of the 3' terminus of lagging strands unreplicated. Thus all eukaryotic chromosomes need the assistance of a special machinery to add terminal sequences to DNA to prevent attrition through successive replication cycles.

The chromosome ends have specialized repeat arrays of hexa- or octa nucleotides making the telomeres. Telomere is important element in chromosomes apart from centromeres and origins of replication. It prevents end to end fusion, aberrant recombination and in general it provides stability to genome. The telomere sequences are maintained by a special reverse transcriptase called telomerase which is RNA dependent DNA polymerase (TERT), having integral RNA sequence as template (TR or hTR in Humans).

In general, normal cells lack expression of telomerase (hTERT) even when there is detectable RNA component. As a result, there is progressive loss of telomeres at every round of replication eventually leading to cellular senescence. This is commonly referred to as end replication problem, which renders the ends of chromosome to get shortened at every cell division. So in normal cells telomere length reduces at every cell division, whereas in cancer cells telomerase is over expressed and telomere length is always maintained. Telomere length defines the life span of cells as the cells with longer telomeres enjoy extended population doublings. So telomeres indirectly provide proliferative potential to cells.

Cancer is a disease of uncontrollable cell divisions and has deregulated signaling and gene networks. As a disease, cancer falls next to cardiovascular diseases in causing human mortality the world over. According to estimates published in "Global Cancer Report 2007" released by American Cancer Society, more than 12 million new cases and 7.6 million deaths from the disease world wide were predicted to occur during 2007. Majority of cancer incidence, and deaths would have occurred in developing nations while 5.4 million cases and 2.9 million deaths would have ocured in economically developed countries. The three common male cancers in developed nations are prostrate, lung and colorectal (Bowel), while in women three most common cancers are breast, colorectal and lung cancer. In developing countries in male, most common are lung, stomach, liver cancer and in women, it is breast, cerivcal and stomach cancer (Global Cancer Facts and Figures, 2007).

Early diagnosis and effective targeted therapy are the main priorities in cancer treatment. This is obliterated by acquired multiple mutations underlying deregulatory events and indistinguishable morphologic characters. One of the therapeutic strategies is to restrict cancer cell doublings and induce apoptosis. As telomeres provide proliferative advantage to the cells, restricting the telomere synthesis by various means could limit the replicative potential of cells and induce senescence or cell death programmes. One of the targets selected is telomerase RNA (hTR) which acts as template in telomere synthesis, while hTERT protein can also be used as target. Gene knockdown of hTR will make telomerase as defective enzyme that would fail to maintain telomere length. Shortened telomeres could result in induction of apoptotic programme as critically reduced lengths of telomeres could be recognized as DNA damage in cellular systems.

Many molecules to date have been emerged as probable therapeutic molecule and one of among them is targeted ribozyme (catalytic RNA molecule) as a tool to knockdown the undesirable gene transcripts in cancer. Ribozymes are naturally occurring catalytic molecules that can be engineered to work against chosen targets. Among those, hammerhead ribozymes have been well characterized and used for many therapeutic interventions. Hammerhead ribozyme has conserved catalytic sequences flanked by arm of complementary to the target RNA. Ribozymes have also proven to work *in vivo* in many cellular and animal studies and offer as good therapeutic molecule for study. The present study involves a) developing ribozyme against telomerase RNA in HeLa cells, b) to observe the effect of ribozyme c) to evaluate hTR as suitable target and d) therapeutic efficacy of expressed ribozymes in HeLa cells.

I.1. Biology of Telomeres and Telomerase

I.1.1. Historical Background

The term telomere was coined by Herman J. Muller, who used to work with flies and observed that the ends of chromosomes were different from other part of genome when irradiated with X rays. Later on Barabara McClintok, while working with Zea Maize, described the rupture of chromosome resulting in fusion of their ends, with consequent formation of dicentric chromosomes and concluded that the telomeres play a crucial role in integrity of chromosome and prevent rupture fusion bridge

cycles which are catastrophic to cellular survival. The telomere research was reinitiated again 30 years later when JD Watson raised the question of replication of 3' end of the template. At the same time Russian geneticist Alexsei Olovnikov, found link between the problem of end replication and cellular senescence (Hayflick and Moorhead 1961, Hayflick 1965, Watson 1972).

Leonard Hayflick in 1958 started culturing cells *in vitro* and found the population of normal human fibroblast in culture, doubled a finite number of times after which the cells stopped dividing and entered Phase III phenomenon as per Hayflick's term. According to Hayflick, the primary culture is phase I and the phase at which cells actively dividing and grows for 10 months or so, termed as Phase II, subsequently cells enter non dividing Phase III (Shay and Wright 2000). Later on, Hayflick also proved the existence of some counting device referred it as "replicometer" to designate putative molecular event counter and such replicometer is located in nucleus, and subsequently proved telomeres as counting device (Wright and Hayflick 1975). It was the time when the convergence of end replication problem and senescence programme got impetus in scientific field (Shay and Wright 2000).

For Olovnikov, the problem in terminal replications was the cause of the progressive shortening of the telomere that acts as internal clock to determine the number of division that cell could experience throughout its life and could control the process of aging (Olovnikov 1973).

Joseph Gall and Elizabeth Blackburn studying the extra-chromosomal elements of ciliated protozoan *Tetrahymena thermophilia*. It has micronucleus with normal chromosomes and macronucleus with fragmented chromosome and found the ends of which have repetitive sequence CCCCAA. Blackburn along with her graduate student identified there is special enzyme for maintaining such repetitive ends and referred the enzyme as telomere specific "Terminal transferase", later on called as telomerase which could add telomere sequence in artificially introduced telomere like sequence in the extracts of *Tetrahymena* (Greider and Blackburn 1987).

I.1.2. Telomeres

The chromosome ends have specialized repeat arrays of hexa- or octa nucleotides making the telomeres. The telomeres are composed with a set of proteins which protect the telomeres from end to end fusion and recombination events. The telomere

3

sequence is maintained by a special reverse transcriptase called telomerase which is RNA dependant DNA polymerase (TERT), having integral RNA sequence as template (TR or hTR in Humans). The Telomerase complex extends short stretch of TTAGGG sequence. Human chromosome ends bear 3-18kb of double stranded DNA of TTAGGG repeats which are necessary for telomere functions in somatic cells (Farr et al. 1991, Hanish et al. 1994). The termini of human telomeres carry 100 to 280 base protrusions of single stranded TTAGGG repeats (Markov et al. 1997, McElligott and Wellinger 1997) which are effective substrate for telomerase in vitro.

Absence of telomerase complex leads to progressive loss of telomeres at every round of replication eventually leading to cellular senescence. This is commonly referred to as end replication problem, a replication related mechanism which renders the ends of chromosome to get shortened at every cell division. Telomere length predicts the replicative capacity of human cell as proved by Harley group in early 1990's who first time provided a model converging Hayflick limit hypothesis and later on several groups modified the model based on the status of stress the cells encountered during carcinogenesis process (Allsopp et al. 1992). Their postulation was that the loss of telomeres due to incomplete DNA replication and absence of telomerase provides mitotic clock, which thrives on signals for cell cycle exit, and limiting replicative capacity of cells. In cells with active telomerase the loss at each replication and cell division is counterbalanced with addition of telomeres.

I.1.3. End Replication Problem

As the replication fork reaches the 3' end of the (Lagging strand) template strand, a stage is reached when primer cannot be synthesized anymore. This leaves a part of the terminus unreplicated. In linear replicons, there is no DNA beyond the end of chromosome to serve as a template for an RNA priming event, the gap between the final lagging strand (Okazaki) and the end of chromosome cannot be filled in. So the 5' end of lagging strand will lose some nucleotides every time cell replicates its DNA. In the early 1970s it was first suggested that the lagging strand synthesis of linear strand DNA templates would be incomplete for two reasons. First there is no known mechanism that ensures priming of the most distal Okazaki fragment synthesis from the very end of the template molecule.

Origin of DNA replication
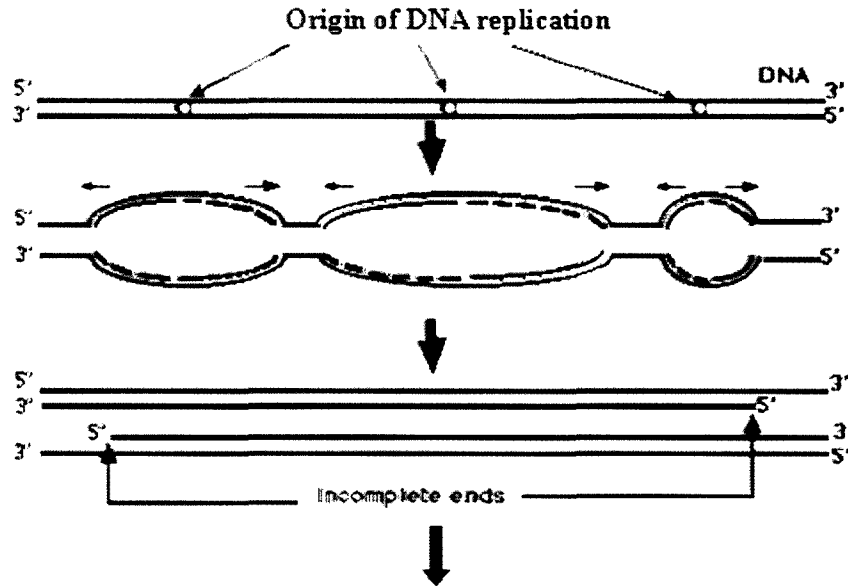
The Newly synthesized Daughter strands (in red), loss its terminal end in subsequent replication cycle, due to primer removal at 5' end which results in reducing telomere length at every division of cell cycle.

Fig I. 1: The model depicts the "End replication Problem" at chromosome ends (From Dr. Jerry Shay's Web page).

Accordingly the template sequence between the end and the most distal okazaki fragment would not be replicated. Second there is no known mechanism for the most distal RNA primer to be replaced by DNA. Consequently the sequence of the most distal RNA primer would be lost in the daughter strand. In contrast to lagging strand synthesis, leading strand synthesis is thought to continue to the very end of the template molecule (Fig I. 1) (Ohki et al. 2001).

Thus the daughter strands are always found recessed and have C rich 5' ends. The reason behind existence of about 150-200 base single stranded overhang of G rich strand could be due to special nuclease which are C strand specific may be activated or may be due to removal of RNA primer and inability of polymerase to complete the very end of chromosome (Kenneth et al. 2000, Ohki. et al. 2001).

I.1.4. Processivity of Telomerase.

Carol greider in early 90s proved that telomerase elongation rate is 74nt/min *in vitro*. The *E.coli* polymerase I and reverse transcriptase have turnover rates of about 10nt/second, and *E.coli* Pol III has turnover of 100nt/second, considering telomerase

5

is very slow in elongation. The enzyme's processivity adds many d (TTGGGG) repeats rather than disassociating after each nucleotide or each hexanucleotide. The processive character of the enzyme was proved with *Tetrahymena* which synthesized (TTGGGG)n repeats. Telomerase enzyme recognizes the characteristic telomeric repeat (TTGGGG) and elongates the 3' end adding TTG. After this step the enzyme complex translocates and positions the telomerase RNA template i.e. 3'-AACCCCAAC-5' to the newly synthesized TTG and base pairing is through 3'-AAC-5' of template to the extended telomeric repeat TTG. Now the translocated telomerase enzyme complex elongates further to complete the full tract of 5'-TTGGGGTTG-3' of the telomere. The processivity of telomerase in human telomerase also worked out and same is referred in Fig (I. 18).

I.1.5. Telomerase Accessory Factors.

Telomerase holoenzyme apart from hTERT and hTR has several accessory factors associated with it, which includes Est1A/B and Dyskerin (Fig I. 2). The functional constituents of telomerase are conserved across species from yeast to human. Human genome contains atleast three EST1 orthologs (Ever Shorter Telomeres in yeast is an accessory factor), having role in telomerase regulation.
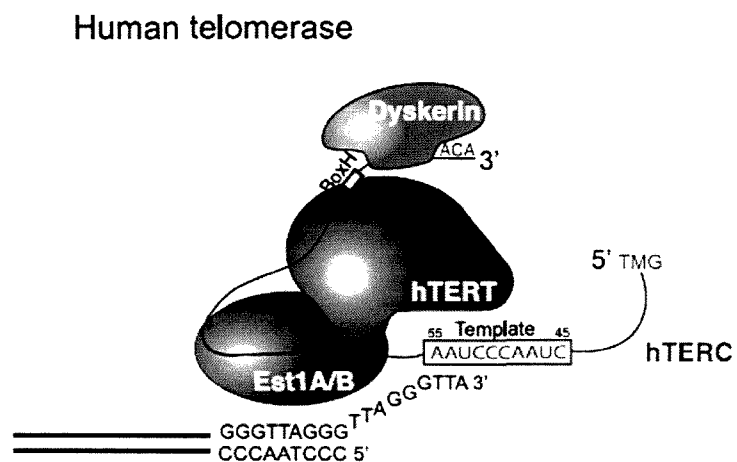
Human telomerase

Fig I. 2: A model on telomerase provides information on the interaction of telomerase enzyme with RNA component and telomere and other proteins associated with the complex (Adapted from smogorzewska and deLange 2004, with permission).

Human telomerase protein interacts with another RNA binding protein i.e. dyskerin, a putative psedouridine synthase functioning in ribosomal processing as it binds with small nucleolar RNAs (snoRNA). Very recently (Venteicher et al. 2008) it was proposed that in addition to above-mentioned complex, telomerase also associated with ATPase pontin and reptin as components for its assembly. But Cohen et al. (2007) with mass spectrometric analysis found only two proteins i.e. hTERT and Dyskerin at catalytically active telomerase complex.

I.1.6. Telomere maintenance proteins

The mammalian telomere with TTAGGG repeats are bound by multi protein complex known as telosome or shelterin. Three shelterin subunits TRF1, TRF2 (TTAGGG repeat factor 1, 2) and Pot1 (Protection of Telomeres 1) directly recognize TTAGGG repeats. They are interconnected by three additional shelterin protein TIN2, TPP1 and Rap1 forming a complex that allows cells to distinguish telomeres from sites of damage.
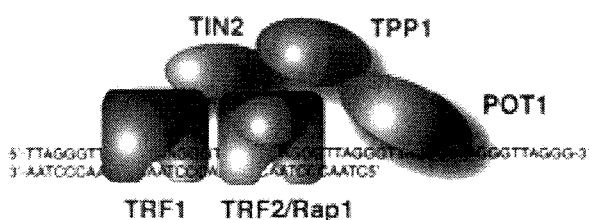
Fig I. 3: Complex formation, TRF1 along with its partners (reproduced from smogorzewska and deLange 2004 with permission)

Without protective activity of shelterin, telomeres are no longer hidden from DNA damage surveillance and chromosome ends are inappropriately processed by DNA repair pathways.

The TIN2 that tethers TPP1 and POT1 to TRF1 and TRF2, also connects TRF1 to TRF2 and this link contributes to the stabilization of TRF2 on telomeres. This shelterin complex is abundant at chromosome ends and not elsewhere, and it is present in telomeres throughout the cell cycle. The shelterin protects telomeres, by making it assume a special structure of telomeric DNA and is also implicated in the generation of T loops and in controlling the telomere synthesis by protecting telomere from accessibility for telomerase (Fig I. 3) (Smogorzewska and deLange 2004).

I. 1. 6. 1. Telomere Length control by shelterin (TRF1 and POT1)

TRF1 binds with duplex telomeric repeat (TTAGGGTTAG) and the number of TRF1 present in telomeric tract is correlated with length of repeat array (Vansteensel et al. 1997, Smogorzewska et al. 2000). TRF1 negatively regulates telomere length by occupying duplex telomeric tract and increasing the accessibility and recruiting of POT1 in single stranded region to block telomerase from accessing telomeric ends (Fig I. 4). Overexpression of TRF1 shortens telomere length and dominant negative mutant of TRF1 resulted in extended telomeric ends proving the role of shelterin complex in telomere length (Bauman and Cech 2001, Lei et al. 2002, Loayza and De lange 2003).
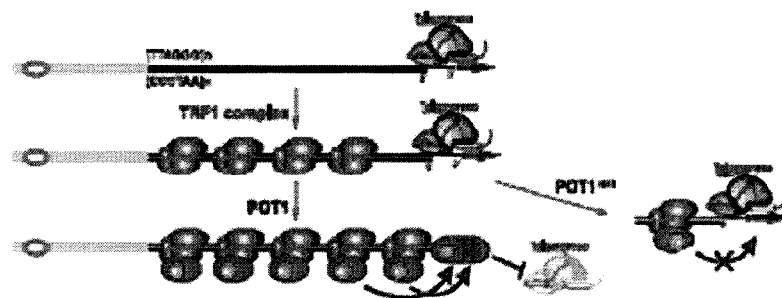
Fig I. 4: Model of POT1 regulation of Telomere Length (reproduced from smogorzewska and deLange 2004 with permission).

I.1.6.2. Telomere length homeostasis

In cells that express telomerase, the length of duplex telomeric repeat array is kept within a species and cell type specific narrow range. In humans, the average length varies between 5 and 15 kb (Smogorzewska and De Lange 2004). The primary cause of telomere shortening is incomplete DNA replication. (Huffman et al. 2000; Makarov et al. 1997; McEligott and Wellinger 1997, 1996; Wright et al. 1997). The telomerase lengthening and shortening provides a control towards activity of telomerase.

One of the model envisages telomere length dependent changes in chromatin structure influencing the productive association of telomerase with telomeres 3' ends. A long telomere would have lower probability to be in telomerase extendible state than short

telomere. In this model a telomere could shorten over several rounds of DNA replication without being elongated by telomerase, before its chromatin structure would switch and become competent for telomerase-mediated elongation. Thus association rather than activity of telomerase would be regulated element. Recently it has been argued that the homeostasis mechanism of telomeres is achieved by a switch between telomerase extendible and non-extendible states (Gael Cristofari and Joachim Lingner 2006).

I.2. Telomeres, Telomerase and Cancer.

I. 2.1. Cancer and Global Incidence.

Cancer is a disease accompanying deregulation of cell division. According to a WHO bulletin "Cancer is a generic term for a group of over 100 chronic diseases, which can affect any part of the body. A defining feature of cancer is the rapid creation of abnormal cells, which grow beyond their usual boundary and can invade adjoining parts of the body. The cells may also spread to other organs, a process referred to as metastasis". As cancer cells are morphologically and beahvioraly indistinguishable at early stages, it is very difficult to predict the transformed status of cell in the niche of normal cells.

I.2.2. Biological Aspects of Cancer.

Cancer is a disease with unresolved 'puzzles' of complex circuitry of interrelated molecular events which enable the cell survive and proliferate in an uncontrollable way as a result of accumulated mutation and overall, generation of genomic instability. Cancer cells arise as a result of continuous genetic evolution and natural selection of mutant cells. Genetically deviant cells are generated as a result of environmental insult or normal errors in replication. One of historical hypotheses on tumor evolution is that, tumor initiation occurs in single normal cells by induced change which makes it neoplastic and provides it with selective growth advantage over adjacent normal cells. From time to time as a result of genetic instability in the expanding tumor population, mutant cells are continuously produced (Nowell 1976). Through multiple rounds of proliferation, mutation and selection, a neoplastic variant

evolves to cancer. Ultimately the fully developed malignancy as it appears clinically has a new genetic constitution e.g., unique aneuploid karyotype associated with aberrant metabolic behavior and specific antigenic properties.

Tumorigenesis thus is a multistep process, which involves genetic alteration at different steps of development, resulting in permissive transformation of normal in to highly malignant derivatives (Hanahan and Weinberg 2000).

Knudson's two hit hypothesis gave impetus to identification of genes responsible for and genetic changes that lead to cancer. Discovery of tumor suppressor genes and oncogenes has helped to visualize the nature of changes underlying tumorigenesis. Loss of gene function (tumor suppressor gene) and gain of gene function (oncogene) through mutation, deletion and other genetic events are associated with cancer. There are hundreds of cancer types and subtypes of tumors within specific organs, each having its own distinct abnormal regulatory circuits. It is primary question as to what are the regulatory circuits within each type of target cell to be altered to become cancerous cell. Each subset seems to have its own distinct pattern of genetic changes, reflecting on complexities of molecular events to become cancerous (Knudson 1971).

The vast range of alterations resulting in cancer cells have been catalogued by Hanahan and Robert Weinberg (Hanahan and Weinberg 2000) into six essential alterations in molecular physiology that collectively dictate malignant growth. And those are as follows

1) Self sufficiency in growth signals

2) Insensitivity to growth inhibitory signal

3) Evasion of programmed cell death (apoptosis)

4) Limitless replicative potential

5) Sustained angiogenesis

6) Tissue invasion and metastasis

These six capabilities are shared by most of the known type of cancer. Among those, the limitless replicative potential is of our interest for study and therapeutic intervention. Understanding the basic cellular physiology with such an aberrational event would be helpful to devise possible therapeutic intervention. Still failure at the front of cancer treatment is attributed to lack of early detection and indistinguishable morphological features of cancers from normal cells. The six most possible genetic

events catalogued by Weinberg provide a window at each step for therapeutic intervention. One such effort is to intervene into the potential of cells with their acquired capability of limitless replicative potential using catalytic RNAs. Before exploring the possible interventional modalities, it would be appropriate to understand the developing concepts and molecular players that provide the **"limitless replicative potential"** to the cells.

I.2.3. Cellular Controls Over its Life span

At cellular level each cell tries to protect its genome integrity by different means and the changed one is evicted by its own planned programmes (Apoptosis). Cells in higher organisms, have defined life time (population doubling time) and DNA maintenance machanisms i.e. DNA repair machineries, gate keepers and molecular policing (P53 and pRB and other tumor suppressor genes) and apoptotic molecules (Bcl2 family and Caspases etc), to ensure the proper cellular functioning and the integrity of genome. The variant cells at certain times may be favored for propagation. Realizing the possible changes and certainty of evolution in favoring such changes, cells must have wired in such a way to limit their divisions, and genome size, life time etc to protect themselves from the possible stress induced changes.

Tumorigenic process involves acquiring multiple mutations during its course of propagation, and those ensuring their proliferation are selected. Each mutation probably requires 20-30 divisions. It is expected that such mutated cells must expand to, about 1 million cells before a second mutation to occur. As mutations are commonly recessive in nature they must be propagated either by recombination events or by clonal expansion in such a way as to eliminate the wild type alleles. Limiting the number of cell divisions would prevent the possibility of emergence of such mutated cells or preventing mutated cells from progression. So one obvious strategy to prevent cancers is to have limited cell divisions (Shay and Wright 2005).

Most cells in primary culture divide over a limited number of cycles. After a period of rapid cell proliferation, their division rate slows down, and cells ultimately cease to divide. Such cells become unresponsive to mitogenic stimuli but remain viable for extended period of time. Hayflick and Moorhead (1961) found that cells have definite life span and human cell limit is around 52, and fetal cells go around 50 ±10. This phenomenon is called as **Hayflick limit or phase III phenomenon** (Hayflick and

11

Moorhead 1961, Shay and Wright 2000). Senescence is defined as a state of permanent growth arrest in which cells enter the G0/G1 phase of the cell cycle, assume an enlarged flattened morphology and exhibit altered pattern of gene expression and metabolic activity (Artandi and Depinho 2000, Campisi 2000, Sitte et al. 2000, Narita et al. 2003). This form of senescence, which follows extended period of propagation in vitro, is termed replicative senescence (Ittai and Weinberg 2004).

Subsequent to Hayflick's discovery various studies proved different form of senescence that normal cells can enter senescence rapidly in response to various physiological stresses, often referred to as "Stress-induced Senescence". So senescence programme can be activated by the cells when such physiological stress is encountered. Mutation in p53, pRB, p16/INK 4A molecular activators of senescence make the mouse more tumor prone, suggesting senescence to be a tumor suppressing mechanism (Artandi and Depinho et al. 2000).

The finite replicative potential of cells is linked not to chronological age of culture but to the number of cell divisions and to telomeres. The telomere shortens at every cell division due to end replication problem and the shortening is wired in tumor suppressor net work. Telomere length can be extended or maintained by telomerase and doing so it can extend life time of the cells.

Cultured human cells express two distinct barriers to control the indefinite proliferation. In epithelial cells the first barrier appears to be mediated by stress induced cyclin kinase inhibitors which protect RB from inactivation through phosphorylation. Cells at this first barrier are under stasis, (a viable G1 arrest with normal karyotypes) and cannot be rescued by introduction of telomerase. This **stasis** is what is referred to as **mortality stage I** (M1). Senescence barrier or premature senescence represents this Rb mediated barrier (Fig I. 5). The second proliferative barrier is associated with shortened telomeres. This barrier is stringent in long lived animals and with strict repression and may have evolved as a mechanism of tumor suppression. Continuous telomere erosion in cells which overcome the first barrier leads to unprotected telomeric ends that may fuse with other chromosomes and cause wide spread genomic instability.

Cells lacking functional p53 exhibit crisis: A high L1 (DNA synthesis index) and massive cell death. Cells retaining functional p53 exhibit largely viable growth arrest

in all phases of cycle and show moderate L1. In both situations prior introduction of hTERT will immortalize the population. Human carcinoma development requires overcoming these senescence barriers and reactivating telomerase activity. Many of derangements observed in human cancers reflect alterations in molecules that enforce stasis and telomerase repression.

TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG TTAGGG
AATCCC AATCCCAATCC CAATCCC AATCCC

M1 -Stasis
(Stress activated,
Rbmediated,
Viable G1 arrest)

TTAGGGTTAGGGTTAGGG TTAGGG TTAGGG
AATCC CAATCCC AATCCC AATCCC

Extended
Life Span

TTAGGGTTAGGGTTAGGGT
AATCC CAATCCCA

TTAGGGTTA
AAT

M2- Crisis (p53-) Cell death
Agonscence(p53+)-Stoped division

Telomerase
Activation

TTAGGG TTAGGG
AATCCC

Extended Life span

Fig I. 5: In the absence of telomerase, telomeres shorten with each cell division. Cellular senescence (Mortality stage -M1) begins when there are on average length of telomeric repeats remaining. In fibroblast cultures, M1 requires p53 and pRb activity. If p53 or pRB mutated cells continue to divide, and telomeres get shortened to an extent to prevent further replication (Mortality stage-M2). Expression of telomerase can bypass this stage and induction of limitless replicative potential could be achieved (Wright and shay 1995, Redrawn with permission from Shay)

I.2.4. Telomere Dependent Proliferative Barrier: Agonescence or Crisis.

The human cells in culture that overcome the Rb mediated barrier still express a finite life span, and proliferation in absence of telomerase leads to erosion of telomeres and causes genomic instability and telomere dependent proliferation barrier. Where p53 function is absent or abrogated this barrier presents a **crisis** and cell death. (Rheinwald et al. 2002, Shay et al. 1993). When wild type p53 is present, this barrier termed as "**Agonescence**", produces most viable growth arrested cells (Fig I. 6) (Stampfer and Yaswen 2003).

In most of human cellular immortalization, overcoming the RB mediated barrier has been achieved utilizing viral oncogenes that obligately inactivate p53 in addition to pRB. Overcoming the second barrier relied on conditions that produce inactive p53. Human epithelial cells transduced with SV 40 large T antigen, or post stasis cells lacking functional p53 typically cease growth during a period of crisis with rare immortalization (Shay et al. 1995, Gollahon et al. 1996, Gao et al. 1996, Van Der Haegen and Shay 1993, Bartek et al. 1991). Telomerase activity was not found in these populations, while wide- spread genomic instability is present. Cells in crisis maintain proliferation and ectopic expression of hTERT can efficiently immortalize post stasis epithelial cells by escaping the second barrier (Dickson et al. 2000, Rheinwald et al. 2002, Stampfer et al. 2001, Kiyono et al. 1998). These results indicate that loss of p53 by itself or in combination with RB inactivation, is not sufficient to produce immortal transformation, whereas introduction of telomerase activity is sufficient in such (p53-/-, pRb-/-) genetic background.
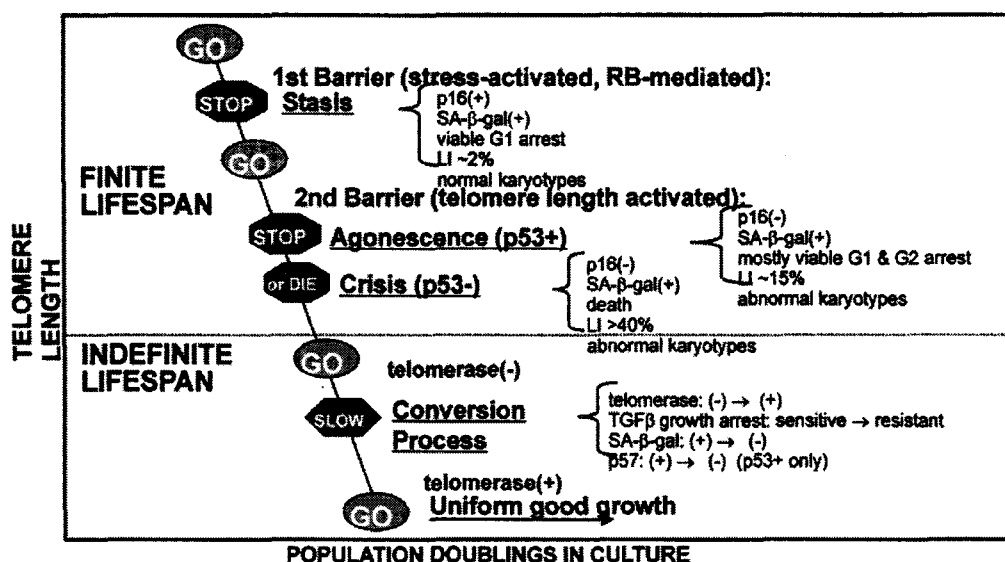
Fig I. 6: Model for Immortalization of human mammary epithelial cells cultured *In vitro* (Adapted from Stampfer and Yaswen 2003, reproduced with permission from Yaswan)

I.2.5. Overcoming the Telomere-Dependent Barrier and Turning on Telomerase

The cells cultured from human tissues do not spontaneously overcome telomere dependent barrier, and emergence of immortal cells is very rare even in p53-/-, pRB-/-

status. Telomerase introduction can overcome this barrier. The mechanism enforcing telomerase repression has not been studied. Some of the clues that human dermal tissues and cultured keratinocytes express a low level of telomerase activity correlate with the presence of stem cells within the population. The telomere dependent proliferative barrier might be possible in such telomerase expressing cells by preventing differentiation of the telomerase expressing stem cells (Bringold and Serrano, 2000).

I.2.6. Role of Telomeres in Suppressing and Facilitating Carcinogenesis.

There are different views on the role of telomeres in carcinogenesis, which can vary with different genetic backgrounds and species. Progressive telomere shortening during primary human fibroblast division in vitro leads to activation of senescence programme at a time point coinciding with Hayflick limit i.e. up to 60-80 population doublings. The telomere shortening is sensed as DNA damage and the shortened telomere signal is transduced to tumor suppressor pathways controlled by p53 and pRb. Inactivation of p53 and pRb by viral proteins leads to extended replicative life span, and prevents senescence.
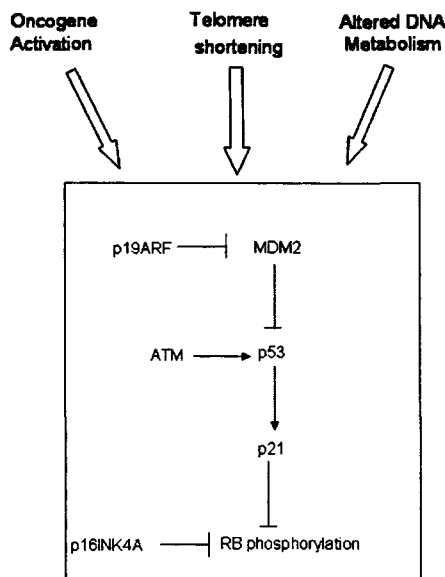
Fig I. 7: The effector programme for senescence is illustrated in centre box The p53 is activated by ATM or by p19 ARF leads to transcriptional activation of p21, inhibition of CDK4/6 and decreased Rb phosphorylation. Activation of p16 INK4A inhibits CDK4/6 and blocks Rb phosphorylation (redrawn from Steven artandi and Ronald depinho 2000).

15

The INK 4A locus encodes two tumor suppressor genes, p16INK4A and p19 ARF, that activates Rb and p53 respectively, and both are involved in senescence. Human foreskin keratinocytes and human mammary epithelial cells senesce earlier than fibroblasts after 20-30 population doublings. These epithelial cells arrest prematurely as a result of accumulation of p16INK4A. Epithelial cells escape this check point either spontaneously via repression of p16 IN4A promoter methylation or experimentally through inactivation of pRB by viral protein HPV E7 protein.

Bypassing such checkpoint allows the epithelial cells to directly encounter true telomere based replicative senescence at which point they are likely to be immortalized by hTERT. Expression of activated oncogenes H-RAS in human cells, activates the p19ARF which binds to MDM2, a regulator of p53. MDM2 (or HDM2) acts as an ubiquitin ligase for p53, targeting ubiquitin mediated degradation of p53 (Kubbutat et al. 1997, Haupt et al. 1997). Stimulation of p19ARF therefore blocks MDM2 mediated p53 ubiquitinylation, resulting in p53 activation (Pomerantz et al. 1998, Zhang et al. 1998, Kamijo et al. 1998). The p19ARF-p53 axis served to integrate a variety of growth and oncogene signals that enforce a number of responses including G1 cell cycle arrest and senescence depending on the specific signal and cellular context (Fig I. 7).

I.2.7. Telomere Shortening may Impede Tumorigenesis.

Telomere hypothesis was formulated to explain its role in senescence. Telomerase is reactivated in 90 % of cancers, and telomere lengths in tumor cell lines are often shorter than primary normal cells. The end replication problem poses threat to the cell's life as a result of attrition of telomere length. A drastic reduction in telomere length would be sensed as DNA damage, and DNA damage activates p53 which could cause cell cycle arrest.

It was proved that there is clear genetic link between the p53 and telomere, as shortened telomere activates the p53 and removing TRF2 from telomere causes chromosomal fusions and cell death in an ATM and p53 dependent manner (Fig I. 8). The functional links were provided with mouse studies with *mTR/INK4A* compound mutant mice and mTR/p53 compound mutant.

The *INK4A /ARF* deficiency inactivates only p19 ARF arm of p53 pathway, leaving the ATM-p53 /DNA damage arm that is activated by telomere dysfunction. The tumor

16

size reduced significantly in *mTR-/- INK4A-/-* mice with severe telomere dysfunction compared to *INK4A-/-* mice with intact telomere function.
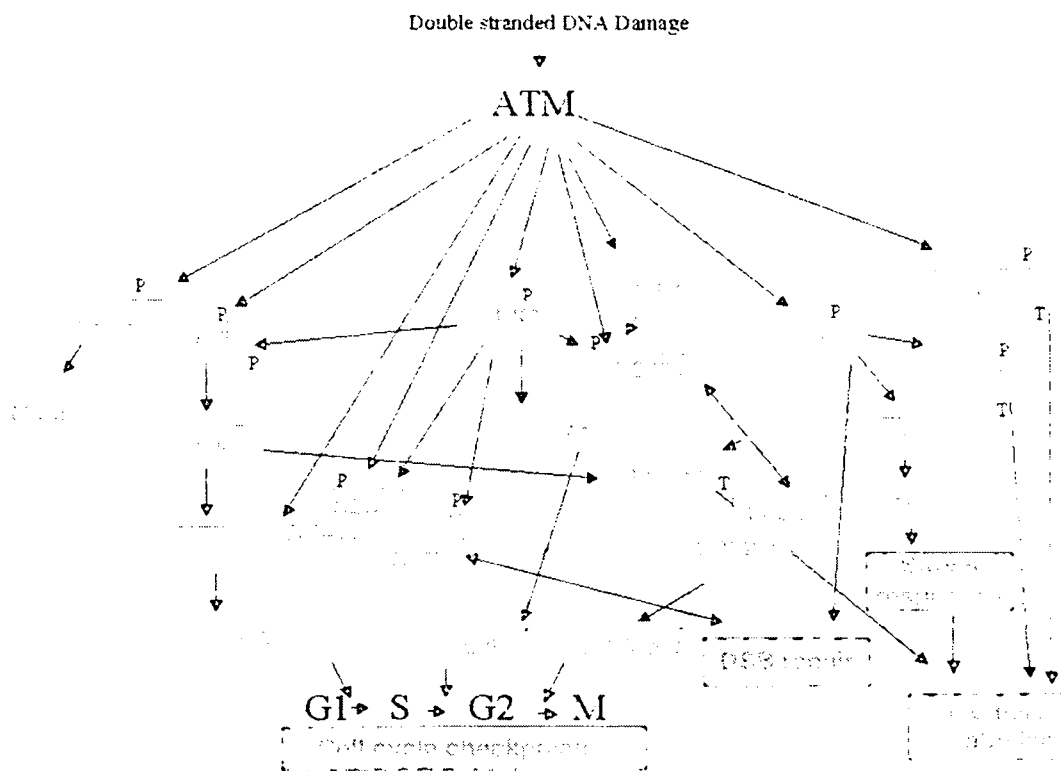
Fig I. 8: The ATM telomere pathway (redrawn with permission from Tej K Pandita from Tej K.Pandita 2002).

I. 2. 8. Telomere Crisis Promotes Tumorigenesis

Studies with mouse embryonic fibroblasts from late generation mTR -/-, p53-/- mice show enhanced transformation by telomere dysfunction in p53-/- background in contrast to the reduced transformation as a result of telomere dysfunction in INK4A-/- background (Greenberg et al. 1999).

This suggests that telomere crisis is not a tumor suppressor mechanism; instead it is a period of extreme chromosomal and genetic instability that results in cell death or cellular transformation depending on stochastic genetic changes and activation of adaptive mechanisms. In human cells the final cell division that precedes replicative senescence can be thought of as "early crisis". Senescence is dynamic programme in which the rate of cell growth slows gradually and gene expression profiles are altered. Despite telomere shortening the genome remains stable until few cell divisions at

which point the frequency of dicentric chromosomes and chromosomal fragments increases dramatically. These observations indicate that in human cells early crisis actually begins at the very end of senescence as telomere dysfunction deteriorates and chromosomal integrity is compromised. At this critical point cell growth is checked by p53 and pRb activation. So the telomere hypothesis and its role in promoting or preventing tumorigenesis is based on status of p53 and pRb pathway proteins. The modified model of telomere hypothesis and cancer, aging is represented in Fig I. 9.
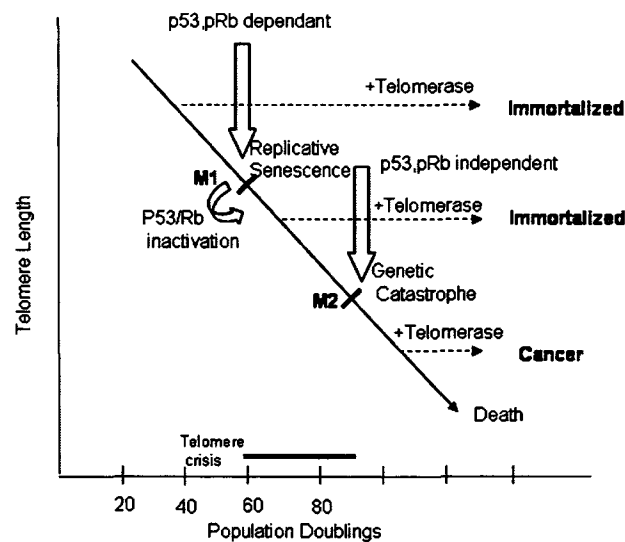
Fig I. 9: Modified view of Telomere Hypothesis. Telomere shortening in primay Human cells leads to replicative senescence and cell death (M1) dependant on status of p53 and pRb. Mutation in p53/pRb allows cells continued cell division and enter telomere-mediated crisis (M2). Expression of telomerase can bypass replicative senescence or telomere crisis lead to immortalization (dashed lines). Similarly reactivation of telomerase with such genetic catastrophe makes cells tumorigenic (Redrawn from Artandi and Depinho 2000).

I.3. Biology of Human Telomerase RNA (hTR)

I.3.1. hTR Locus

Human Telomerase RNA (hTR) is encoded by a single copy gene localized to chromosome 3, and locus 3q26.3. This locus is found to be frequently amplified in certain types of solid tumors, cancer of cervix, lung and squamous cell carcinoma of the head and neck (SCC-HN). Increased copy number of hTR was found in comparison to normal tissues of cervix and SCCHN (Soder et al. 1997). The catalytic protein

component hTERT maps to chromosome 5, at 5q15.33, region which is frequently amplified in cancer with increased copy number of transcripts too.

A 3 to 10 fold increase in steady state levels of hTR has been observed in variety of immortal cells (Yi et al. 1999, Avilion et al. 1996) indicating that some regulation of hTR is associated with acquisition of telomerase activity. *In situ* hybridization experiments show that the relative levels of hTR in human cells are significantly different from those in adjacent tissues to be clinically useful in diagnosis of cancer (Morales et al. 1998, Morales et al. 1998, Yashima et al. 1997, Yashima et al. 1997).

In normal human diploid cells, there is lack of mRNA for the catalytic subunit hTERT and presence of integral RNA component of telomerase hTR, whereas in cancers both the components are found to be present or elevated (Meyerson et al. 1997, Miller & Rosman 1989, Morales et al. 1999, Morgenstern and Land 1990, Nagano and Kelly 1994, Nakamura et al. 1997, Kilian et al. 1997, Soder et al. 1997).

I.3.2. Telomerase RNA Structure

Human telomerase RNA (hTR) is about 451 bases long, is rich in GC, and has conserved secondary structures and is transcribed by RNA polymerase II (Feng et al. 1995). The prediction of telomerase RNA structure was determined by aligning the 35 vertebrate telomerase RNA sequence using conserved sequence as anchor points. The conserved region (CR) was numbered from 5' to 3' and designated the CR1 through CR 8. The first conserved region (CR1) represents the template region and longest conserved region found to be CR2 and CR5 (Fig I. 10). The 5' end of human RNA is 45 nucleotides upstream of template (CR1) sequence, the 3' end of human telomerase RNA has been mapped to 3 nucleotides down stream of CR8 (ACA) motif. There are 10 conserved helical regions seem to be universally present in vertebrate telomerase RNA structure. These helices constitute 4 distinct structural domains. Paired region is referred as 'P' and single stranded or junction regions as 'J' in the following section (Fig I. 10) (Chen et al. 2000).

1) Pseudo knot domain.
2) CR4- CR5 domain
3) The box H/ACA domain
4) CR7 domain.

Junctions are regions between the two successive helices. The region between helices p2b and p3 is named as j2b/3. The unstructured template region will be discussed prior to structured domains.

a) Template Region

The first conserved region (CR1) consensus sequence 5'-CUAACCCU-3' represents the template region of telomerase RNA that specifies the synthesis of the 5' TTAGGG-3' telomere repeats in vertebrate. The sequence upstream of the template region pairs with the sequence located at nucleotide 187-208 to form helix P1.

In human telomerase RNA (hTR) helix P1 is the only conserved structural element located upstream of template and was good candidate for defining template boundary.

The helix P1 is divided into two base paired region helix P1a and P1b which are separated by an internal loop.

b) Pseudoknot Domain:

The pseudoknot domain contains CR2 and CR3 sequences and is stabilized by helices P2A, P2b and P3. In addition to base pairs that co-vary, putative base pairing was included that extends the helix P3 and allows a coaxial stacking between P2b and P3. Such coaxial stacking between two helical regions would significantly stabilize this type of pseudoknot structure. The pseudoknot region after the base 165 contains an unpaired stretch of 'A's and target site GUC and partially paired region which was taken for ribozyme designing and used as target site in this study. The j2b/3 region is conserved not only in sequence but also in length. This length conservation is also important for maintaining a stable conformation of this pseudoknot element (Fig I. 11).

c) CR4-CR5 Domain:

The CR4-CR5 domain lies downstream of pseudoknot domain and consists of stem loop structure established by helices p5 and p6. The intervening loop contains the conserved CR4 and CR5 sequence elements. The pseudoknot and CR4-CR5 domain can independently assemble with TERT protein and are essential for telomerase activity in vitro. This CR4-CR5 domain is designated as activation domain of telomerase. Recently the CR4-CR5 domain was modified to have internal loop, which is evident from enzyme activating structures of telomerase RNA studies (Tesmer et al. 1999, Mitchell and Collins 2000).

Human Telomerase RNA

G,A,U,C : 100% conserved

▨ : supported by universal covariation

▢ : supported by group-specific covariation

— : Watson-Crick base pair in >90% of sequences

● : G/U base pair

○ : non-canonical base pair

★ : non-universal base pair

Pseudoknot Domain (CR2/CR3)

Template (CR1)

Hypervariable Paired Region

CR4-CR5 Domain

BoxH/ACA Domain (CR6/CR8)

CR7 Domain
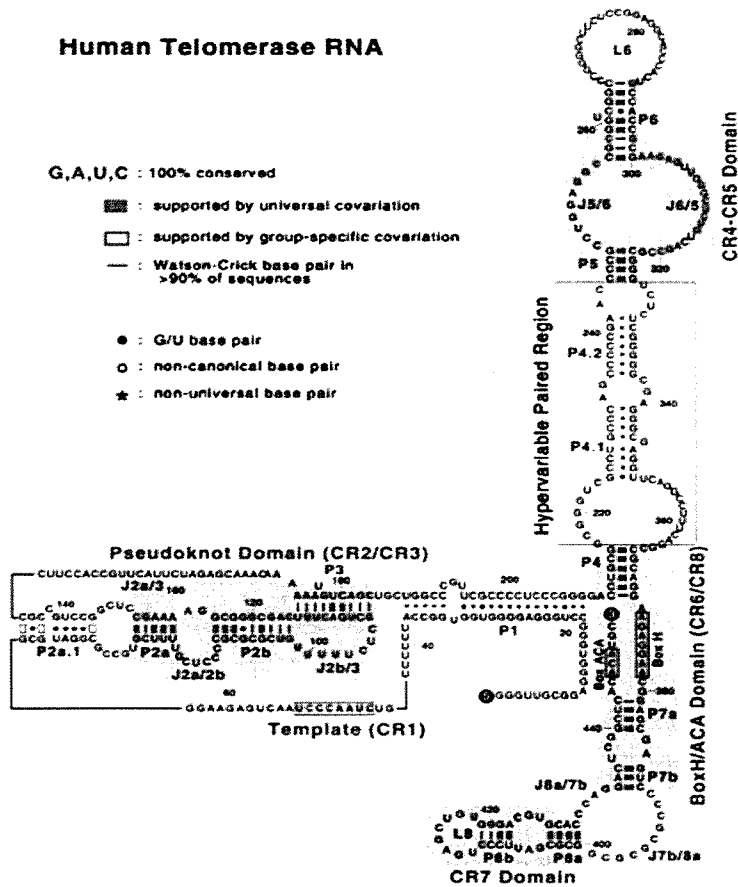
Fig I. 10: Structure proposed by Chen et al. (2000) by aligning different phylogenies of hTR (Adapted from Chen et al. (2000) with permission from Carol Greider).
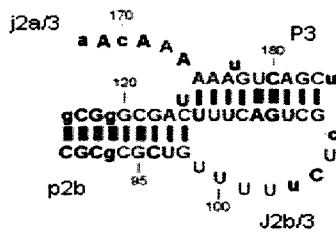
Fig I. 11: Pseudoknot region which includes the target region selected (180) for ribozyme targeting in the present study.

d) The Box H/ACA Domain:

The box H/ACA domain includes p4, Box H, P7a, P7b and box ACA, which form a conserved structure similar to the structure found in the box H/ ACA family of Sno RNAs (Gannot et al. 1997, Mitchell et al. 1999a). The conserved sequence of box H

21

(CR6) and Box ACA (CR8) motifs are both single stranded as seen in small nucleolar RNAs. The box H/ACA Sno RNAs also terminates 3 nucleotide down stream of a consensus ACA motif.

e) The CR7 Domain

CR7 domain defined by a highly conserved sequence of helices P8a, P8b and the loop L8. The sequence and length conservation of CR7 region suggests that the structure is specific to vertebrate telomerase RNA that functions in localization and processing events of hTR.

I.3.3. Comprehensive Structure of hTR

Though the four domains seem to contribute to telomerase function in vivo, telomerase catalytic activity in vitro only requires the core (pseudoknot) and CR4-CR5 domains each of which binds independently to the TERT proteins (Mitchell and Collins 2000).

The deduced structure for the core domain encompasses five short helically paired regions designated P1, P2a.1, P2a, P2b, P3 and some single stranded regions (J). Three of the paired sequences (P2a.1, P2a, P2b) together form stem of hairpin, a portion of which can base pair with sequences down stream to form the P3 helix, creating potential pseudo knot region. The P3 base pairing in pseudo knot domain is important, as the deletion in the region disrupting the P3 base pairing reduces or abolishes telomerase activity. Apart from core and CR4-CR5 region, an extensive mutagenesis study in other regions provides the comprehensive hTR structure (Fig I. 12). Residues 18-37 are postulated to form base pairs in P1. Deleting residue 33 or beyond drastically reduced telomerase activity, but the residues 1-32 are dispensable for telomerase activity which indicates that at least some base pairing in the P1 region is required for optimal function. The previously predicted stem of base paired regions P2a.1 has extended stem structure while the primary sequence of P2b and P3 stems are relevant in functional reconstitution of the human telomerase complex. The accessibility mapping analysis of hTR has failed to confirm the stem formation in hTR, suggesting that either the P3 base pairing doesn't occur or it may be dynamic. Other studies are supportive of this evidence that P3 may have dynamic structure; adopting different conformations during various stages of telomere repeat synthesis or in different physiologic states of telomere repeat synthesis.
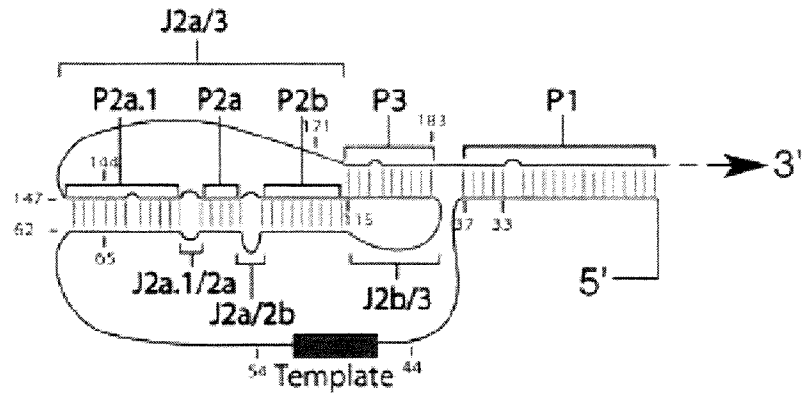
Fig I. 12: The schematic view of core sequence consisting of 210 base of the 5' of hTR. The template region has been indicated in numbers 44-54, and base pairing nucleotides in the region of nt 62-147 and 33-205. These regions were shown to contribute to optimal telomerase function. The P2a.1 helix is extended proximally by 3 bp (Adapted from Hinh ly et al 2003 with permission).

The P3 facilitates the homodimerization of hTR both in vivo and *in vitro*, and such P3 dependent dimerization is essential for ribonuclear protein complex (Mitchell and Collins. 2000). To assess the importance of helix P1 structure in telomerase function, variety of RNA truncation mutation with this 5'end at positions of 1, 15, 26, 32, 44 and end position 212, as base pairing nucleotides falls in 5' of template and 3' of pseudoknot regions. These mutants which lack 3'half of RNA can reconstitute telomerase activity provided CR4-CR5 RNA fragment are present. RNA mutants hTR 1-212, 15-212, 26-212 and 32-122 reconstituted telomerase activity and showed typical 6 nucleotide repeat patterns of elongation products (Fig I.13 and I. 14).
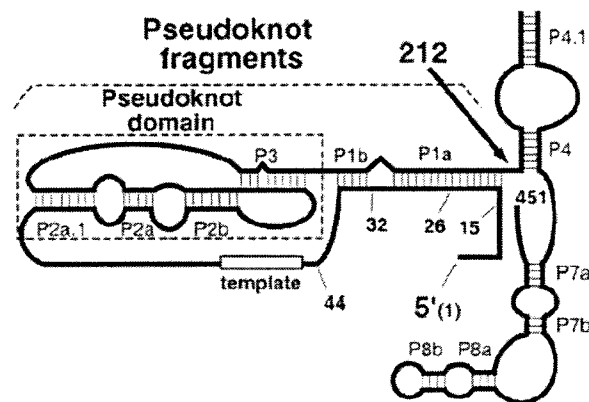
Fig I. 13: The P1 helix is shown as separate P1a and P1b helices, formed of template and 3' of pseudoknot region (Adapted from Chen and Greider 2003 with permission).

23
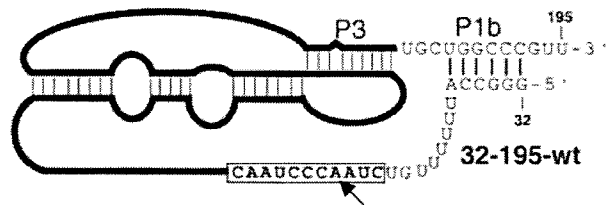
Fig I. 14: The sequence of P1b helix and changes in the sequences of linker region shown as arrow (Adapted from Chen and Greider 2003 with permission).

Removal of first 31 residues in RNA resulted in increased overall telomerase activity. But mutants lacking P1a and P1b in the 44-212 region resulted in additional bands at position +6, +12, +18 and +24, because the 5' end of hTR 44-212 RNA located only 2 residues upstream of template. This suggests that mutant truncated RNAs can fold differently and alter the function of telomerase. Changing the P1b helix 38-uuuuuu-43 to 38-AAAAAA-43 resulted in base paired structure and overall reduction in activity and abnormal pattern of telomere products (Fig I. 14) (Chen and Greider 2003).

I.3.4. RNA-RNA Interaction of hTR

The hTR itself participates in intramolecular interactions and the CR4/CR5 region, which contained the P6.1 loop involved in an RNA-RNA interaction with template region intramolecularly. Mitchell and Collins (2000) showed that deletion of the residues of 303-315 (p6.1) results in improper interaction between hTR and hTERT and abrogate telomerase activity (Fig I. 15).
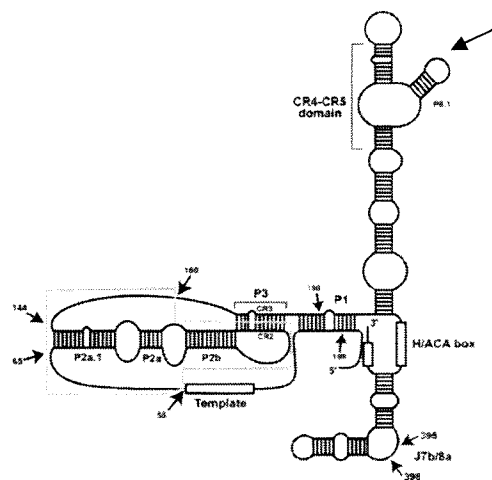
Fig I. 15: The CR4-CR5 domain structure is modified from evidences. In CR4-CR5 domain, the P6.1 region is formed through internal loops indicated with arrow (Adapted from Chen et al 2002).

24
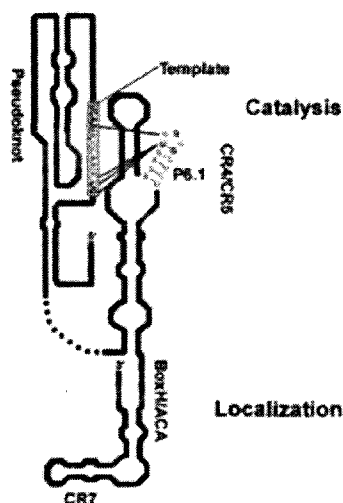
Fig I. 16: A new model for the secondary structure of hTR with interacting residues shown by gray connecting lines. The catalytic domains (pseudoknot, CR4/CR5, and template) and localization domains (Adapted from Ueda et al 2004 with permission)

The loop bases of P6.1 (U307, G308, and G309) are inaccessible *in vivo*, but accessible *in vitro* (Antal et al. 2002) suggestive of hTERT mediated protection, due to interaction of hTERT with these nucleotides. The P6.1 hairpin and template region has highly conserved bases, and such interaction would have some biological significance. Altering the nucleotides in the regions altered the telomerase activity.

The new model of RNA secondary structure argues the possibility of close proximity of template region with that of P6.1 of CR4/CR5. The hairpin might be having interaction at multiple sites in the template domain. If this interaction is dynamic then the hairpin may be involved in mechanism of translocation of the template during reverse transcription (Fig I. 16) (Lai et al 2003).

The conserved hairpin in the CR4/CR5 domain may play similar role in processivity of human telomerase. P6.1 may prepare the template domain by either stabilizing the template or positioning it for proper reverse transcription by the protein.

I.3.5. hTR Dimer Model

The purified human telomerase is found to have dimer of RNA template and most probably of two protein components. The sequences in the hTR pseudoknot P3 helix mediate TR dimerization. The P1b helix of the hTR pseudoknot template domain

25

contributes to 5' template boundary definition. Based on the observations the interaction of hTERT and hTR contributes to both DNA synthesis and processivity function of telomerase. The hTR-hTR interactions at the P3 helix interface could stabilize the hTR dimer structure, permitting one to allosterically influence the function or conformation of the other telomerase RNA molecule (Fig I. 17).

The N terminus of hTERT contains stable integrated and structurally complex template RNA. The interactions of hTERT domains RID1and RID 2 with hTR sequences are essential for processivity and DNA synthesis respectively, which facilitates dynamic hTR arrangement i.e. alternative use of the two potential active sites of telomerase that are likely involved in telomere synthesis. An alternative model for the function of telomerase dimerization is that it facilitates the simultaneous extension of two DNA substrates (Moriatry et al. 2004).
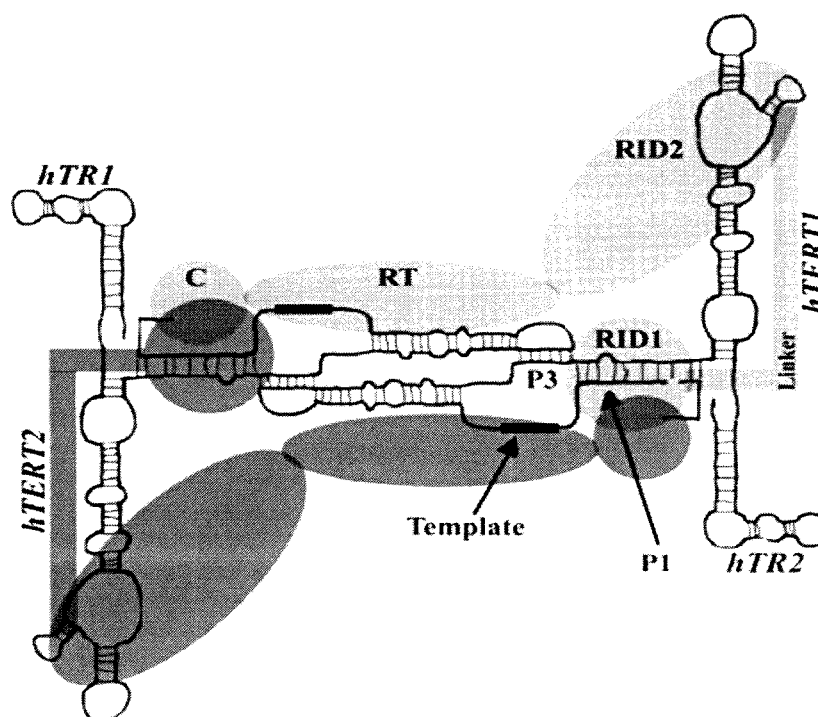
Fig I. 17: Schematic diagram showing RNA-RNA and RNA-Protein interaction viz.,. hTR-hTR and hTR-hTERT, hTERT -hTERT. RID 1 is connected to core hTERT domain RID2, RT, C terminus. The RID2-P6.1 interaction site is likely essential for DNA synthesis. RID 1 pseudoknot template and RID 1 C terminus interaction may regulate repeat addition processivity. RID1 and C terminus hTERT may interact with different hTR molecule. The P3 in hTR molecule contribute in dimerization and processivity and regulate allosteric regulation of other hTR monomer   (Adapted from Moriatry et al. 2004 with permission)

26

I.3.6. Template region of hTR processivity- Model

The template region of human telomerase consist of 11nt sequence viz., 3'-CAAUCCCAAUC-5' complementary to nearly two telomeric repeats d (GGTTAG). The template sequences of other vertebrates vary in length ranging from 8-11nt suggesting 8-nucleotide may be sufficient for telomerase function. Out of the 8 nt, six form universally conserved elongation domain (3'CCAAUC-5') which codes for single telomeric repeat. The alignment domain contiguous to the 3' end of the elongation chain consists of additional nucleotides complementary to the telomeric sequence (Fig I. 18).
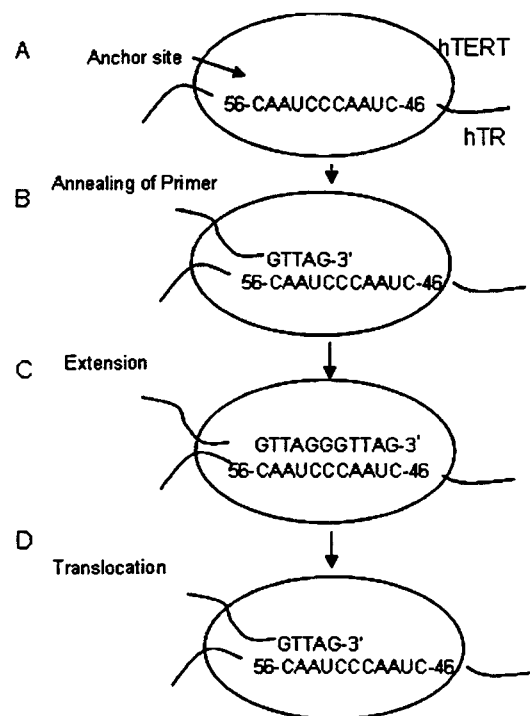
Fig I. 18: A)Human telomerase RNA (hTR) template with hTERT in a complex ,and B)annealing of primer base pairs with template region of hTR C) Primer extension of telomerase until the 5' end is reached. D) Translocation and realignment of primer terminus to the 3' end of the template for next round of extension (Redrawn from gavory G et al. 2003 with permission from Balsubramanium)

The alignment domain functions to reposition the primer substrate by base pairing during processive synthesis. Unlike elongation domain, the alignment domain of telomerase is not completely conserved throughout vertebrates and varies in length from 2nt (3'UC-5') for rodents to maximum of 5 nt (3'-CAAUC-5') for humans. This strongly suggests that not all nucleotides in the alignment domain of human telomerase are required for function.

27

Altering the sequence of the alignment domain of human telomerase at the first two positions from the 3' end (56C and 56CA55 respectively) influence some aspects of telomerase activity. Deletion of the first 3' residue (56C) of the alignment domain of the templatae sequence marginally reduced overall activity to 84 ± 8% whilst deletion of the sequence 56CA55 had more pronounced effect. So the most 3' terminal residues 56C and 55A of the alignment domain of human telomerase do contribute to optimal function of telomerase but are not essential for catalytic activity *in vitro*. At least 3 nt of the DNA substrate with RNA template is required for efficient realignment and processive synthesis.

I.3.7. hTERT Interaction with hTR

In most telomerase studied, the 5' template boundary definition is regulated by template adjacent TR stem structures and intervening template linker sequences, which have been proposed to constrain movement of the template in the active site. In hTR, this structure is the P1b helix (Fig I. 19) (Autexier and Greider 1995, Prescott and Blackburn 1997, Chen and Greider 2003, Seto et al. 2003). hTERT contains reverse transcriptase motifs that are relatively well conserved with other RTs and flanked by specific N and C terminal sequences. The C terminus may constitute the polymerase thumb of TERT and is important for nucleotide addition (Peng et al. 2001, Huard et al. 2004). The hTERT C and N termini may associate with DNA substrates and also regulate telomerase specific property of repeat additions. Two regions in hTERT- N terminus are involved in hTR interaction viz., RNA interaction domain 1 and 2 (RID1 and RID 2) that are separated by non conserved catalytically inessential linker (Xia et al. 2000).

P1b Helix

Template Linker

56 Template seq   46 ———————— UGGCC

3'-CAAUCCCAAUC UGUUUUUUACCGG-5'
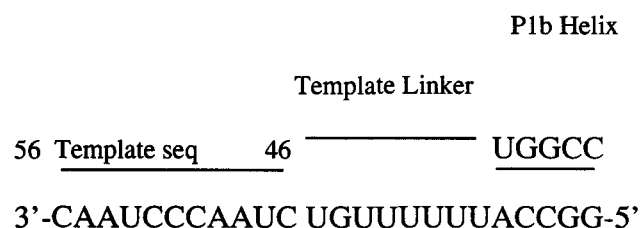
Fig I. 19: The sequence of hTR that are involved in interaction with hTERT (redrawn from Moriarty et al 2005).

28

RID 2 likely regulate telomerase assembly via interactions with the hTR pseudoknot/template domain, possibly transiently or with low affinity. This is essential for repeat addition processivity, and contributes anchor site-specific catalytic functions. A sub region of RID1 referred to as N- DAT (dissociative activities of telomerase) is not required for specificity and affinity of telomerase DNA interactions and telomere maintenance in vivo (Armbruster et al. 2001, Lee et al. 2003). A short C-DAT region has also been identified at the extreme C terminus of hTERT though C-DAT sequences contribute to human telomerase catalytic function (Banik et al. 2002, Huard et al. 2004, Moriarty et al. 2005).

I.3.8. Transcriptional and Post-transcriptional Regulation of hTR

Elevated hTR levels may be due to increased transcription rate and an increased half life as a result of the association of hTR with catalytic protein subunit or other regulatory modifications or combinations of the factors. It seems as if there is coordinated program for the derepression of telomerase during tumor formation (involving regulation of both hTR and hTERT) rather than just focal activation of hTERT.

There is an increased steady state level of hTR, due to up-regulation of hTR transcription and it is not due to presence of hTERT protein. Since increased hTR transcription is not present in cells expressing exogenous hTERT but occurs only in cells in which endogenous gene has been activated by process of in vitro Immortalization or by viral oncogenes on in vivo tumor formation. The increased transcription rate of hTR only in immortal cell types expressing their endogenous hTERT suggests that changes or mutations in immortalization process affect a coordinated telomerase reactivation program that may up regulate hTR expression. Thus increased transcription partly accounts for the elevated steady state hTR levels in cells expressing their endogenous hTERT. Association of telomerase RNAs with hTERT may also contribute its increased half-life.

Expression of telomerase catalytic subunit alone may cause moderate increase in the steady state hTR levels by increasing half-life without affecting its transcription rate. The half-life of hTR ranged from 4.4 to 32 days from different cell types. The half life of hTR in H1299 cells is the longest half-life reported for any eukaryotic RNA.

Dramatically elevated hTR levels in a wide variety of human tumors (Morales C.P 1998, Yashima et al. 1997) are attributed to hTERT protection by binding to hTR.

I.3.9. Transcriptional Regulation of hTR

A number of transcription factor play a role in transcription regulation. The hTR promoter contains the consensus TATA box and CCAAT box in close proximity to the transcriptional start site, and is transcribed by RNA polymerase II. Some of the putative sites correspond to transcription factors AP1 and ETs and response elements for gluco corticoid, progesterone and androgen steroid hormone. The minimum promoter activity was found with 272 bp upstream of the transcriptional start site, and contains DNase I protected site, which encompass the 4 consensus binding site for the Sp1 family of transcription factors in addition to the CCAAT box (Zhao et al. 2003).

Sp1.2   CCAAT box   SP1.1   TATA box   Sp1.3   Sp1.4
                                                +8   +33
-106   - 97 -62   -52   -42   -23

2923wt-GCCCGCCCGA — TTGGCCAATCCG —— GGCCGCTCCCTTTATAA — GAGGGTGGGCCTGGGAG GGTGGTC
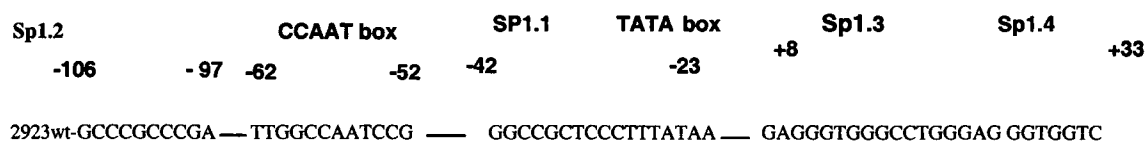
Fig I. 20: The sequence of the wild-type hTR core promoter (−107/+69) is shown above. The number on either side of the sequence is related to the transcriptional start site. The hTR template boundary region is indicated in bold. The respective transcription factor binding sites are shown above the sequence.

Binding of both Sp1 and Sp3 has a regulatory role in hTR expression. The Sp1 induces the HTR expression whereas Sp3 suppresses the hTR expression (Fig I. 20 and I. 21).

So the upstream of CCAAT box conferred a positive regulation on the promoter and 3' sites down stream of the CCAAT Box appeared to be repressive, and the site immediately down stream of CCAAT mediates the strong repressive effect, due to its positioning in relation to NF-Y binding (Zhao et al. 2003). Mutation in Sp1 site upstream of CCAAAT box from patients with aplastic anemia and lack of sp1 sites downstream of the transcriptional start site has positive effect on promoter activity, which implies a negative regulatory role for the upstream site (Keith et al. 2004).

30

Core Promoter Region
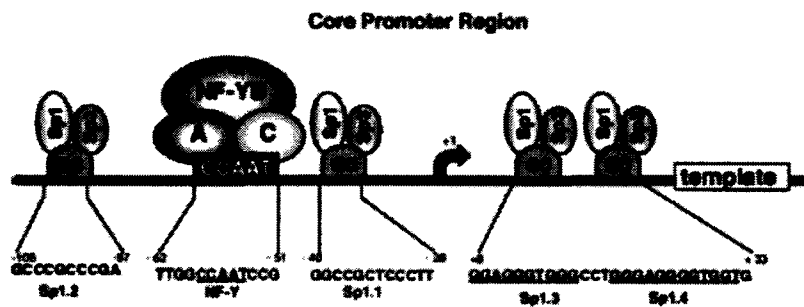
Fig I. 21: Schematic illustration of the hTR gene promoter. Regulatory sequences (GC box and CCAAT box sites) and their cognate binding factors are shown. The Sp1 and NF-Y binding nucleotide sequences are indicated (Copied from Zhao et al 2003 reproduced with permission from Nicole keith)

The Sp1 binding site has ability to bind Sp1 and Sp3, so it is possibly, cooperation among other transcription factor binding the promoter that determines whether these Sp1 sites act in a positive or negative manner. The tumor suppressor and cell cycle regulator pRB induces hTR promoter activity and pRb doesn't bind directly to DNA and instead mediates its effects through recruitment of additional transcriptional regulators, and increasing the affinity of Sp1 and NF-Y binding to hTR.

The MDM2 a master regulator of p53 activity also binds with Sp1 and represses the functional activity of transcription. The retinoblastoma protein has ability to relieve the repression by displacing the Sp1 from MDM2 and activate transcription through Sp1 (Johnson-Pais et al. 2001). Studies have shown association of MDM2 with Sp1 and over-expression of MDM2 represses hTR transcription (Zhao et al. 2005).

I.3.10. Regulation of hTR by Cell Signaling pathways.

The mitogen activated protein Kinase (MAPK) signaling cascades modulate transcription of hTERT through numerous effector binding sites within core promoter, including the c-Myc, Ap1, Ets transcription factors (Cong et al. 2002, Kyo and Inoue 2002). MAPK also has a role in HTR regulation. Co-expression of constitutive active domain of MEKK1, the major MAP3K for the JNK pathway, causes repression of hTR (Billsland et al. 2006).

Hypoxia response element (HRE) sites with in the core promoters of HTERT and hTR offers binding site for hypoxia inducible factor-1 (HIF-1), indicating that hTR may be regulated by HIF-1. Expression of hTR and hTERT is repressed by co

repressor C terminal binding protein (CtBP) that binds with various histone modifying enzymes (Glaspool et al. 2005). This suggests a role for chromatin remodeling in regulation of telomerase.

I.3.11. Epigenetic Regulation of hTR

Lack of hTR expression was associated with acetylated histones H3 and H4 and acetylated lysines 9 of H3, a modification generally associated with active gene expression, and hypermethylation of lysine 9 of H3 facilitates formation of heterochromatin and is generally associated with gene repression.

The 1765 bp encompassing the HTR gene and both 5' and 3' flank is located within a CpG island. This suggests a positive role of DNA methylation in hTR regulation. However, hTR expression levels and DNA methylation patterns didn't correlate in cell lines and tumor tissue though there is strong correlation in DNA hypermethylation with hTR levels in ALT cell lines. Some of the studies have proven the role of chromatin remodeling in hTR transcription. Such studies were undertaken with ALT cell lines SUSM-1, KMST6 and W138-SV40 (Atkinson et al. 2005) and human mesenchymal stem cell (Serakinci S.F et al. 2006). In such cell lines the lack of hTR was associated with reduced levels of acetylated histones H3 and H4 and acetylated lysine 9 of H3. Conversely, the hypermethylation of Lysine 9 of H3 facilitates formation of heterochromatin generally associated with gene repression consistent with hTR repression in those cell lines. In contrast, the telomerase positive cell lines 5637, A2780, C33a and Alt cells SKLU, GM847, which express high levels of hTR, had elevated association of acetylated histones H3 and H4, with hypomethylation at Lysine 9 of H3 (Atkinson et al 2005).

I.3.12. Telomerase RNA   Localizations

The human telomerase RNA travels through different cellular compartment, and by FISH studies it was evident that hTR accumulates within intra nuclear foci called Cajal bodies (CB). It was found in many tumor cells, but not in primary and ALT cells (where hTERT is minimal). Cajal Bodies are evolutionarily conserved domains present in nuclei in animal and plant cells (Gall 2003). They are enriched with RNA processing enzymes, RNP assembly chaperones, and other protein with structural

roles. Some of RNAs also found in Cajal bodies called small Cajal body RNAs (scaRNAs) which can guide sequence specific modifications of small nuclear RNAs (snRNAs) during their assembly into splicesomal snRNPs (Jady et al. 2003).

The human telomerase RNA contains H/ACA motif shared by many scaRNAs and large family of small nucleolar RNAs (snoRNAs) (Mitchell et al. 1999a, Henras et al. 2004). The H/ACA motif recruits proteins, including pseudouridine synthase cbf5p/Dyskerin, forming an RNP that can recognize and modify specific sites of target RNA molecule (Henras et al. 2004).

I.3.13. hTR Biogenesis

The human hTR primary transcript is synthesized by RNA polymerase II, capped with tri methyl guanosine at its 5' end, internally modified, and its 3' end is processed to make a mature hTR transcript. Unlike RNA POL II based transcripts, hTR ends in Poly A independent fashion. The proper processing is dependent on H/ACA motif in hTR, which is at 3' end of the molecule. This H/ACA motif is also present in non coding RNAs which guides in post transcriptional modifications. Such non coding RNAs undergo co transcriptional processing by ribonucleoprotein assembly (RNP). This H/ACA motif RNP assembly have a co transcriptional binding of proteins including Dyskerin (Cbf5in yeast) NHP2, NOP10 and GAR1. Human hTR also has these four proteins. The H/ACA motif alone may not be sufficient for TER accumulation. The loops of H/ACA motif 3' hairpin is proposed to interact with unknown RNP biogenesis factor in case of human telomerase RNA (Fig I.23) (Dragon et al. 2000, Dragony and Collins 2003).

It was expected that the endogenous transcription continues beyond the mature 3' end, and hTR transcripts extending beyond 500 base were also detected in RT PCR, but not by northern blotting (Wong et al. 2002), as they exist at very low steady state level. Transcription for more than 500 nt downstream of the hTR 3' end could provide a kinetic delay in 3' end processing that enhances RNP assembly. The 3' loop has two separable roles viz., its involvement in a) RNP biogenesis and b) in RNP enrichment in Cajal bodies.

The CR 7 region which contains this H/ACA, a box that is common in snoRNAs, mostly functions as guide RNA in the site specific pseudo-uridinylation of rRNAs in the nucleolus. There are small Cajal bodies specific RNAs (Sca RNAs) in CB which

carries conserved sequence elements as localization signal for cajal bodies. Conserved sequence motifs are called Cajal Body Box (CAB) and studies have shown that hTR also contains such elements. This CAB boxes are cis acting localization signals with conserved nucleotides of 411-UGAG 414, which directs for CB specific accumulation. The Cab box in hTR shown in Box (Fig I. 22) (Beata Jady et al. 2004).
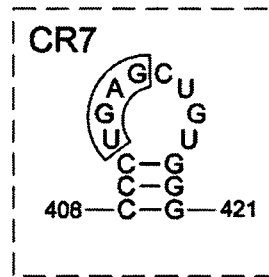
Fig I. 22: The sequences comprising Cajal Body Box (CAB).

The modification of hTR i.e. its trimethyl cap is expected to happened in CBs, as some methylase activity also associated with CBs. Moreover the hTR is not shuttling to cytoplasm for its modification, so most probably the modification takes place in CBs.
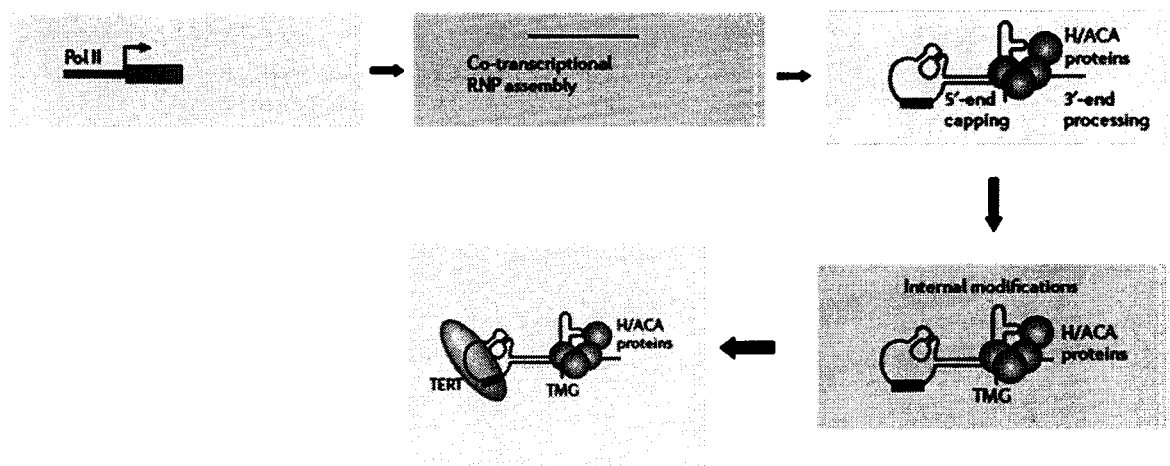
Fig I. 23: self explanatory model of hTR RNP assembly and Processing steps. The Proteins (green) are processing proteins of RNP and in orange is TERT (Reproduced with modification from Collins 2006).

I.3.14. Cell Cycle Regulated Trafficking of Telomerase RNA and TERT.

Telomere is synthesized during S phase in human cells (Ten Hagen et al., 1990). Redistribution of components of telomerase has been observed during S phase. Wong et al. (2002) showed by linking hTERT with GFP, that the fusion protein signaled predominantly nucleolar to nucleaplasmic, as cell progressed to the S phase. The recruitment of telomerase to telomeres is restricted to S phase, i.e. during the timing of telomere elongation. Telomerase accumulates at only a subset of telomeres in given cell at any given time. Telomerase may not act on every telomere during every cell cycle as demonstrated in yeast cells, where only small fraction of telomeres are extended within given cycle (Teixeira et al. 2004). Studies in yeast and human cells show the telomeres extends preferentially short telomeres in a population (Teixeira et al. 2004) Chromosomes replicate at different rates during S phase (Woodfine et al. 2004); their timing of telomere replication may vary with replication induced changes in chromatin structures and telomere accessibility at individual chromosomes.

During G1 phase, hTR and hTERT are observed in separate intra nuclear structures; hTR is present in Cajal bodies (Jady et al. 2004) and hTERT accumulates in distinct nucleoplasmic foci. Specifically during S phase, hTR and hTERT exhibit dynamic redistribution and become targeted to common intranuclear sites.
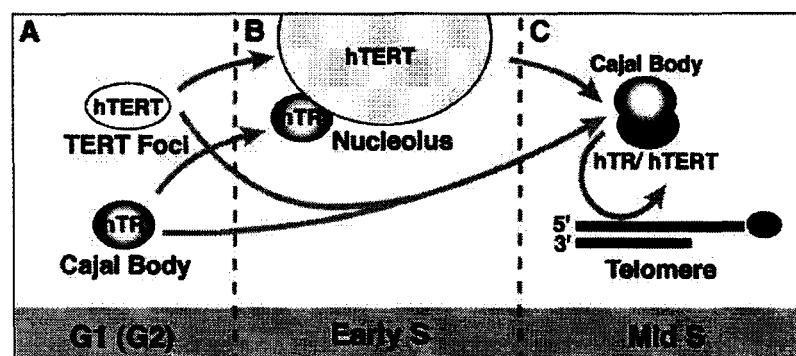
Fig I. 24: The model depicts the phase specific localization of hTR and hTERT during A) G1 phase B) early S phase C) Mid S phase and arrows are possible trafficking constructed based on observation by Terns group (reproduced with permission from Dr. Terns)

In early S phase both hTR and hTERT can be found associated with nucleoli, but not in shared compartment. hTR is present in Cajal bodies that seem to reside around the periphery of the nucleolus, whereas hTERT seems to be distributed throughout the interior of the nucleolus (Fig I. 24).

Movement of Cajal bodies to and from nucleoli has been documented previously (Paltani et al. 2000) and may account for the appearance of hTR at nucleoli in S phase (Tomlinson et al. 2006). The hTERT and hTR localization to Cajal bodies associated foci preceded localization to telomeres and suggested that Cajal bodies with compartmentalized cargo of hTR and hTERT deliver telomerase to individual telomeres throughout the cell (Tomlinson et al. 2006).

I.3.15. Functions of hTR

The telomerase RNA is known for its contribution in telomere synthesis. But still steady state meager presence of hTR and its over expression prompted to look for extra telomeric functions of hTR. Still at infancy, hTR is found to have some roles in regulation of certain functional genes in normal metabolism. The hTERC (hTR) presence increases wound healing, and tumorigenic property of overexpressed telomerase (hTERT) component. In mouse models, hTERT exerts inhibitory effect on wound healing and tumorigenesis process in absence of hTR (Cayuela et al. 2005). Inhibition of hTR expression triggers a rapid, telomerase independent growth arrest associated with p53 and CHK1 activation and hTR effects are mediated through ATR, and it is sufficient to impair the ATR mediated DNA damage repair pathways (Kedde M et al. 2007). Still more informations are needed to assign extra telomeric functions to hTR.

I.3.16. Disorders of Telomerase hTERC Variants

Inherited mutations in hTERC underlie one form of rare human disorder, dyskeratosis congenita (DC) that involves haematopoietic failure characterized by abnormal pigmentations in skin, dystrophy in nail, oral lueokoplakia, and immunodeficiency and bone marrow failure. Autosomal dominant form of DC results from germ line inheritance of mutations in hTERC and most of them are heterozygous carrying one

normal allele too. Lymphocytes from DC patients show the abnormal expressions of hTERC, reduced levels of telomerase activity and reduced telomere length.

Mutation in C116T, A117C, C 204G, G304A of hTERC have shorter telomere length of about 3.7 to 4.6 kb when compared to age matched health individuals who possess 8-12 kb and patients bearing these variants showed no telomerase activity in the primary cells from the patients in reconstituted *in vivo* telomerase assays (Fig I. 25) (Hinh Ly et al. 2005).
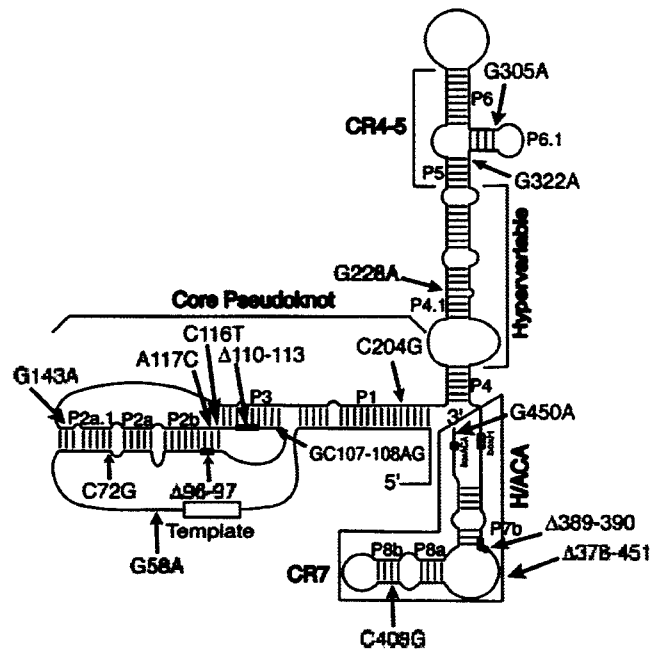
Fig I. 25: Mutations in hTR region found in different diseased phenotypes, Mutations of DC given in red color and with aplastic anemia and MDS or PNH are in green. Thick lines indicate nucleotide deletions, whereas boxed regions show a large deletion that completely removes the sequences of the box H/ACA and CR7 domains (reproduced from Hinh ly et al. 2005).

Patients heterozygous for these alleles showed lower level of telomerase activity. Variants G228A and G45A show same telomere length as the normal cells and without any effect on telomerase activity (Hinh Ly et al. 2005).

The table I.1 below shows the variants of hTR and associated clinical abnormalities.

Table I.1. hTERC variants and associated manifestations in terms of telomerase activity, telomere length and clinical symptoms.

hTERC sequence Variant	Clinical diagnosis	Telomere length	Telomerase activity
Core Domain			
C116T	Aplastic Anemia (severe pancytopenia)	+	−
C204G	Aplastic Anemia (Moderate Pancytopenia)	+	−
A117C	Aplastic Anemia (SeverePancytopenia)	+	−
G143A	Dyskeratosis Congenita	+	−
Δ96-97	Dyskeratosis Congenita	+	−
CR4-CR5			
G305A	Aplastic Anemia (moderate)	+ +	+
G322A	Myelo Dysplastic Syndrome	Not determined	+
H/ACA domain			
Δ389-390	Essential Thrombocytopenia	Not determined	−
C408G	Dyskeratosis Congenita	+	−
G450A	Aplastic Anemia (Severe)	+ + +	+++
CR7 domain			
Δ378-451	Dyskeratosis Congenita	+ +	−
Hyper variable Region			
G228A	Aplastic Anemia (moderate) or healthy	+ + +	+++

+++ With in reference range,   ++ 2-3kb less than reference range

+   3-6kb less than reference range.

I.4. Ribozyme Biology

The RNA molecules having intrinsic ability to break and form covalent bonds are called ribozymes (Cech et al. 1981, Kruger et al. 1982, Gurrier takada and Syndney altman 1983). Ribozymes were discovered in the group I intervening sequences (IVS) in the pre rRNA of *Tetrahymena thermophlia*, by Thomas Cech and his colleagues and from RNA component of *Escherichia coli* bacterial RNAse P by Sydney Altman and his colleagues (Cech et al. 1981, Gurrier Takada et al. 1983). Tom Cech and Altman together shared Nobel prize for the discovery of catalytic RNA.

These RNA molecules are typically small and can catalyze a chemical cleavage reaction or formation of phosphor-ester bond in the absence of protein. Ribozymes are found in nature to work in cis and through studies these cis-acting sequences have been engineered to give a targeted ribozymes in trans. This modification prompted their application to broader areas in gene regulation especially in gene therapy field.

Six types of catalytic motifs are known viz. Group I introns, RNase P, hammerhead ribozyme, hairpin ribozyme, and axe head ribozyme of HDV and RNA transcripts of the mitochondrial DNA plasmid of *Neurospora* (Symons 1987, Foster and Symons 1987, Kijima et al. 1995). The catalytic activity of ribozyme has been demonstrated to occur through the rearrangement of phosphodiester bonds (Von Tol et.al 1990, Kumar and Ellington 1995).

The ribozymes are classified according to their size as large and small ribozymes. The large ribozyme consist of Group I and Group II ribozyme, and RNA component of RNAse P. The class of small ribozyme includes hammerhead ribozyme, hairpin ribozyme, hepatitis delta ribozymes and varkud satellite (VS) RNA and other artificially selected ribonucleic acids (Schubert and Kurreck J 2004).

All ribozymes were believed to be metalloenzymes requiring Mg^{2+} or other divalent cations for both folding and catalysis. The two metal ion mechanisms were proposed in which hydrated Mg^{2+} ions played a role in general acid base catalysis. This prediction appears to be correct in Group I introns, but general acid base catalysis appears to be catalytic strategy and in many cases RNA itself rather than passive scaffold for metal ion binding is an active participant in acid base catalysis, in the sense that nucleotide functional groups rather than metal complexes often mimic the roles of amino acids that play crucial role in the active sites of protein enzymes . For

the present study, we used hammerhead ribozyme targeted against telomerase RNA component.

I.4.1. The Hammerhead Ribozyme

The hammerhead ribozyme was discovered in the plant viroid RNAs as satellite RNAs. These satellite RNA of tobacco ring spot virus RNA replicate only in tissues infected with tobacco ring spot virus (TRSV). It becomes encapsidated in TRSV and reduces the accumulation of TRSV and decreased the severity of symptoms as compared to infection by TRSV alone. Thus sTRSV RNA is a molecular parasite of TRSV and potentially may act as antiviral agent (Haseloff et al. 1988). The self cleavage of both (+) and (-) sTRSV was discovered by Bruening and his colleagues who showed that the cleavage products contain 5' hydroxyl and 2', 3' cyclic phosphate termini (Symons et al. 1987). The ribozyme consists of 3 base paired helices I-III connected by 2 single stranded regions and a bulged nucleotide which gives wish bone structure (Fig I. 26).

The core catalytic domains of hammerhead ribozyme has been reduced to 11 nucleotides high lighted in fig 26 (a). Stem I does not have any conserved nucleotides (Basically stem I and III are complementary arms of target) and stem II has two conserved nucleotides (10.1 and 11.1), which usually form base pair adjacent to catalytic core.

a)   b)
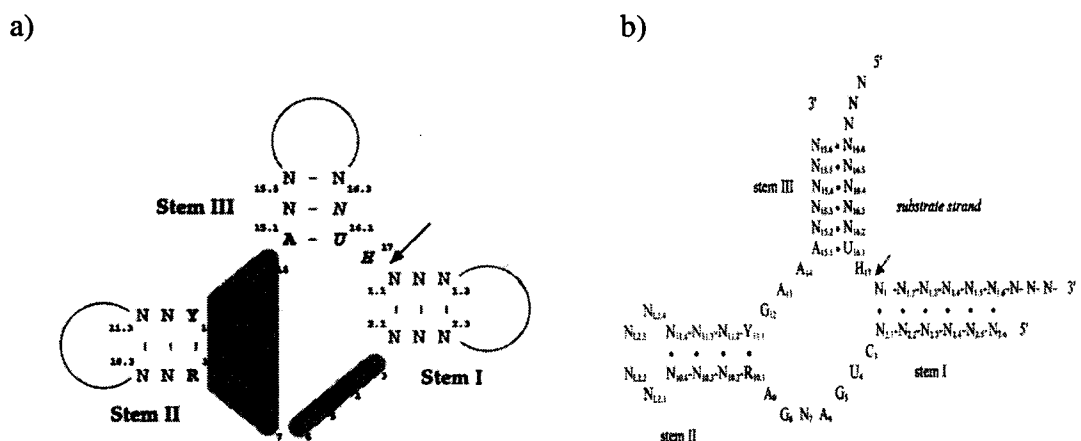
Fig I. 26: The numbering and stems of catalytic hammerhead ribozyme shown with its cleavage site. The fig a) is in cis Rz, found in nature and b) right is in Trans, engineered to modulate gene expression. The nucleotides highlighted (between stem I and III) represent core-catalytic domain of the ribozyme.

40

Ribozyme bearing shorter length of stem II (less than two nucleotide) become less active when compared with ribozymes bearing stem II length of more than two nucleotide.

The most commonly found cleavage triplet in nature is GUC (GUA, AUA have also been observed). The cleavage site in general is said to be NUX and by experiments the preferred cleavage site with in terms of Km are GUC, AUC, GUA, AUA, CUC, UUC, GUU, UUA, AUU, CUA, UUU, CUU while in terms of Kcat they are GUC, CUC, UUC, GUU, AUA, AUC, GUA, UUU, UUA, CUA, AUU, CUU.

Self cleaving reactions have been observed in satellite RNA of TRSV (Buzayan et al. 1986) the avocado sunblotch viroid (Hutchins et al. 1986) and virusoid Lucerne transient streak virus (Foster and Symons 1987). The human hepatitis delta virus (HDV) (Branch and Robertson 1991) and the ribosomal RNA (Symons 1992) have been reported to possess catalytic activities as ribozymes.

I.4.2. Sequence Requirements of Hammerhead Ribozyme

By mutational analysis, minimum nucleotide requirement, which is actually required for catalysis was determined. With exception of position 7 the substitution of any other nucleotide in the conserved single stranded region of central core destroys catalytic activity. At position 7 there is still dependence of particular nucleotide, highest cleavage rates observed with U, followed by G, A, C which have 60%, 50%, 20% activity respectively (Ruffner et al.1990). In Stem I & II no conserved nucleotides have been found but global structure of ribozyme is affected by nucleotide sequence directly adjacent to the core (Amri and Hegermann 1996).

I.4.3. Catalytic Mechanism

The presence of divalent metal ions is essential for hammerhead catalysis. The metal ion promotes proper folding of the RNA to form catalytic core, and acts as catalytic cofactor (Dahm and Ulhenbeck 1991). Once the ribozyme cleaves its substrate it targets multiple targets as like enzymatic property of turnover.
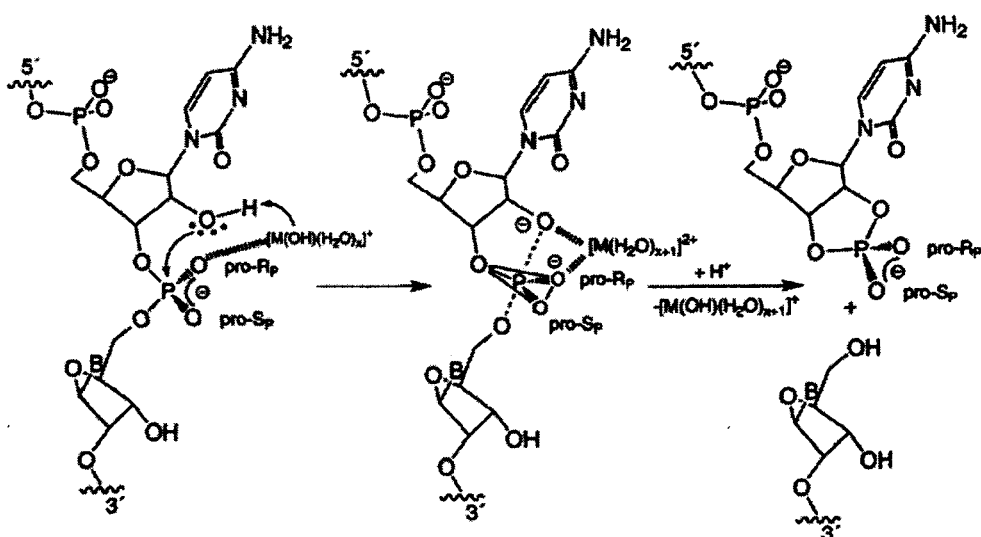
Fig I. 27: Proposed mechanism of Hammerhead ribozyme mediated phospo-diester bond cleavage.

The mechanism of cleavage proposed was the deprotonation of the 2' hydroxyl group by magnesium aqua hydroxy complex bound by the pro-R oxygen at the phosphate cleavage site followed by nucleophilic attack of the resultant 2'alkoide on the scissile phosphate, forming penta co-ordinate phosphate intermediate. The 5' leaving group departs yielding the 2' 3' cyclic phosphate with inverted configuration (fig. I. 27).

I.4.4. Ribozyme at Clinical Trials

Three ribozymes have been completed with their Phase II trials angiozyme, a ribozyme targeting VEGFR-1(Flt-1) is the receptor for vascular endothelial growth factor (flt-1), expression of which gets induced in hypoxic condition and it helps in matrix degradation, proliferation, migration and tube formation of endothelial cells. The receptor (Flt1) also gets induced by hypoxic conditions, and decrease in this receptor nullifies the effect of VEGF and prevents the formation of new blood vessels (Angiogenesis) and hypoxia induced injury of tumor cells leads to cell death.

Targeted ribozyme against Flt-1 could ensure the induction of cell death of tumor tissues (Cunningham.C 2002, Weng et al. 2005). A phase II trial for another ribozyme OZ1, against HIV-1 is underway. In another study, involves the stem cells transduced with ribozyme containing virus and then injected to the patients with non- Hodgkin's lymphoma in HIV patients (Michinzie et al. 2003, Ngok et al. 2004). Other clinical

42

trials are Herzyme against Her/neu oncogene, and ribozyme against Hepatitis C virus (HCV) that is Heptazyme LY 466700 has also passed through clinical trials. Novel tools like siRNA have taken over ribozyme therapeutics as they offer more efficacy and mechanism of action is independent of target structure.

I.5. Telomerase as a Therapeutic Target

Telomerase expression is regained in cancerous foci compared to adjacent or normal tissue, and it provides the proliferative advantage by maintaining telomere length. Hence telomerase seems to be one of the most commonly associated and the promising drug target.

I.5.1. How Telomerase Therapy Affect Cell Proliferation

Terminal DNA gets shortened by about 50-150 bp at every cell division in the absence of telomerase. After certain generations the telomere length becomes minimal and attains the critical length and it may further extend to the sub telomeric region, which will activate the DNA damage-signaling programmes. In presence of p53, telomere mediated induction of apoptotic programs are activated, in absence, p53 independent pathways may result in induction of apoptosis or differentiation programmes in cancers.

I.5.2. Strategies to Target Telomerase

Telomerase is a unique multicomponent RNA protein complex with a reverse transcriptase catalytic subunit (hTERT) along with an RNA molecule (hTR) as core components while other regulatory proteins hsp90, p23, TEP1 and dyskerin etc, are associated with this core. In view of the regulation of telomerase at transcriptional, post transcriptional and posttranslational level, different strategies have been proposed to devise appropriate treatment modalities to attenuate or suppress telomerase activity or reduce telomere length (Table I. 2)

Table I.2: Telomerase based Therapeutics

Target molecule	Inhibitors	Comments	Ref
I) Pre transcriptional **Promoter regulation** Sp1(+ regulator of hTR promoter) Sp3 ('-' regulator of hTR promoter) pRb (+ regulator of hTR promoter) C Myc(+ regulator of hTERT promoter) P53, E2F1('-' regulator of hTR promoter)	Retinoids, Tamoxifen NY Mutants " Introduction of functional p53	Signal Transduction Inhibitors, Transcription factor inhibition	Poole et al. 2001 Xu et al.2000, Takakura et al.1999, Kanaya et al.2000
II)Post Transcriptional **And Translational** Telomerase Transcripts hTERT and hTR Protein Kinase C Alpha	Antisense, Ribozymes, siRNA, Dominant Negative mutants. bis indole maleimide	Sequence specific Knock-down Phosphorylation cannot be achieved and results in reduction or abolition of telomerase activity	Feng et al, 1995, Yokoyama et al 1998, Kondo et al 2000, Folini et al 2000, Hahn et al. 1999,Yokoyama et al. 2000, Ludwig et al 2001, Murakami et al. 1999, Melana et al 1998. Li et al 1997, Yu et al 2001, Ku et al. 1997
III) Enzyme Assembly and **Telomere Interaction**	FJ 5022, AZT (Azido Thymidine, BIBR1532, BIBR, Porphyrins, BSU-1051	Small molecule inhibitors, G quadret interacting molecule, Reverse transcriptase Inhibitors Non specific to Cancer	Tanious et al. 1992, Izbicka et al. 1999, Grand et al. 2002
IV) Gene Therapy Using Telomerase Promoter (hTR and hTERT promoters)	Expression of nitro reductase and activating prodrug CB(5-(Aziridin-1-yl)-2,4-dinitro benzamide) Expression of apoptotic mediators (Bax, Caspases) Toxin genes, siRNA, ribozyme	Adenoviral suicide gene therapy(Ad-hTR-NTR) Telomerase specific Oncolytic virus –	Plumb et al 2001, Bilsland et al. 2003, Shay and Keith 2008

		prmoter region of hTERT regulating replication of adenovirus for selective expression in cancer cells not in normal cells. Specific to Cancer	
V)Telomerase Immuno Therapy	Short peptides of TERT as immuno peptides –GV 1001	Dendritic cell presentation approach. Induce CD 4^+, $CD8^+$ T cell immunity. Specific to telomerase over expressing tissues	Nair et al 2000,

I.5.3. Limitations.

The anti-telomerase therapeutics poses a problem that inhibition of telomerase will pronounce their effect after multiple generations as erosion of telomeres is gradual. Telomeres in human tumor cells are generally shorter than those in normal cells and ranges from 100 to few thousand bases long. And to observe the effect of anti telomerase, one needs longer period and in such time span lag, cells or human system may develop a drug resistance, or exposure of harmful effect of drug toxicity due to prolonged exposure (stopping treatment would result in re-growing of telomeres) or bystander effect (chemotherapy) and in such period it is also anticipated that additional mutational events may take care of telomerase independent telomere length regulation (Alternative Lengthening of Telomere). But such a time gap may be useful to the terminally ill patients if the drug is non-toxic and effective.

As most cancer cells possess a high proliferative index compared with normal stem or germ line cells anti telomerase therapy is expected to have minimal side effects such as bone marrow toxicity and this would be much safer for patients than conventional chemotherapy (Blasco et al. 1997). However, telomerase negative tumors have been identified and alternative mechanism of maintenance of telomere (ALT) exists in some tumors. So anti-telomerase therapy can't be regarded as a fool-proof way of treating all human cancers. Though it will deprive tumor cells of a growth advantage over surrounding normal cells which could result in a significant reduction in malignant progression of tumor (Shay and Bacchetti 1997).

Telomerase targeting is not limited only to the telomerase core complex. In addition the disruption of telomerase maintenance could be achieved through targeting components other than telomerase that are involved in telomere maintenance mechanism e.g., targeting TRF1 and TRF2 and Pot I proteins found in telomere maintenance. The simultaneous downregulation of telomerase and disruption of telomere maintenance mechanism by various means might be expected to produce even greater detrimental effects on cancer cell viability.

The development of anti telomerase therapy, drug developments and clinical studies have been undertaken in rapid succession and have been completed to different extents. It is likely that telomerase inhibitors will have most impact in minimal disease states such as maintenance therapy after tumor debulking by chemotherapy or in combination with cytotoxic chemotherapy. Possibilities may exist to use telomerase inhibitors as adjuvant therapy in early disease. Present scenarios have improved the field of telomerase-based therapeutics, as the assumption that telomerase has role only in maintaining telomeres is not quite valid. As extra-curricular functions of telomere including its role in carcinogenesis and other cellular events open up new avenues and promises around telomerase as therapeutic target. The strategies and tools used for targeting telomerase and telomeres are given in Table. I. 2

The usage of existing technologies and recent advancements in assays and innovative trial design would facilitate to understand the basic biology of human telomerase to strive for betterment of human health.

I.6. Objectives of the Present Study.

Telomerase RNA molecule is selected in our study to evaluate its suitability as a good target in cancers. As the copy number and transcript levels of hTR is elevated in most of the cancer, by reducing the hTR level, it was expected to alter telomerase activity, restrict telomere synthesis and induce apoptosis in cancer cells. The present study involves a) to develop and to evaluate the designed ribozyme *in vitro* and *in vivo* b) to study the alterations in telomerase associated gene expression in ribozyme expressing cells. The following objectives were charted in the present study.

❖ Designing of hammerhead ribozyme against telomerase RNA.

❖ Cloning of human telomerase RNA.

❖ Cloning of ribozyme (active and mutant Rz) in pStu I vector.

❖ *In vitro* evaluation of cleavage reaction.

❖ Cloning of ribozyme (active and mutant Rz) in mammalian expression.

vector pCi-NEO.

❖ Expression of constructs in HeLa cells.

- Analysis of ribozyme expression.

- Effect of ribozyme against hTR.

- Effect on telomere and telomerase activity.

- Effect on cell cycle.

- Effect on morphology of cells.

❖ Analysis of Global gene transcript profiling by Differential display.

❖ Proteomic profiling to identify the modulation of gene expression at translational level.

❖ *In silico* Analysis of differentially expressed genes.

Materials & Methods

II.1. Materials Used for Routine Work.

In general, molecular biology grade of chemicals obtained from SIGMA were used. Enzymes and some fine chemicals were obtained from sources as mentioned in respective sections.

Acrylamide (40%) solution

38 g of Acrylamide and 2g of bis-acrylamide to the final volume of 100ml in water, mixed properly till it dissolved and sterilized by either whatmann paper or by 0.45μ filters and stored in dark bottles in 4 °C

Agarose Gels

0.8% -2.5% of gels were used. Appropriate amount of agarose powder was taken in 1X TAE buffer and melted, after cooling ethidium bromide was added (0.5ug/ml) and mixed properly and poured in gel tray.

Agar Plates

1.5 g of agar powder was taken in 100ml of water mixed properly, autoclaved and added with antibiotics (100ug/ml) before pouring in to plate.

Ammonium Acetate (10M)

77g of ammonium acetate dissolved in 80 ml of water and volume adjusted to 100ml and sterilized by filter sterilization (Not to be autoclaved).

Antibiotic stocks

Ampicilin sodium salt (Gibco BRL) dissolved at a concentration of 100mg/ml in water.

Tetracycline hydrochloride in 50% ethanol.

Geneticin (G418), 100mg/ml stock in water.

48

Denhardt's Reagent	1% (w/v) ficoll 400, 1%(w/v)Polyvinylpyrolidone, 1% (w/v) Bovine serum albumin (fraction V) in water.
DMEM culture media	Dulbecco's modified eagle medium powder (Hi Media) of single vial was added in 1lit of autoclaved water with 3.7 g of sodium bicarbonate and sterilized by filtration.
DTT (1M)	Dithiothreitol, 1.045 g of DTT dissolved in 10 ML of 10mM sodium acetate pH (5.2) and sterilized by filtration and stored as aliquots in – 20 °C.
EDTA (0.5M)	18.6 gm of EDTA was added in 80 ml of water and dissolved by addition of NaOH and pH checked adjusted to 8.0 and volume adjusted to 100ml.
EGTA (0.5M, pH 8.0)	Appropriate amount of EGTA dissolved in water and adjusted pH to 8.0 with NaoH and final volume adjusted to 1ml with water.
Ethidium Bromide (10mg/ml)	10mg of ethidium bromide in 1 ml of water and stored by wrapping in aluminium foil at room temperature.
Formamide dye	95%Formamide, 30mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol
IPTG	Isopropylthio β-D Galactoside, 2g of IPTG by dissolving in 8 ml of distilled water and sterilized by filtration and stored at –20°C

Loading Dye (DNA)	0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 30% Glycerol and final volume adjusted with autoclaved Milli-Q water.
Lysozyme	10mg/ml in 50mM Tris–Cl PH 8.0, stored in -20°C
Luria Broth	2.5 g of Luria bertani medium (Hi Media) in 100ml of water, mixed and sterilized by autoclaving.
PBS (1X)	8g Nacl (137mM), 0.2g Kcl (2.7mM), 1.44g of Na_2HPO_4 (10mM), 0.24g KH2PO4 (2mM) in 1 lit of water and sterilized by autoclaving.
Phenol	Crystaline phenol was distilled, equilibrated with 0.5M Tris (pH 8.0) by stirring with magnetic stirrer and added with hydroxyl quinoline (0.1%). The upper phase was removed by pipetting and equal volume of 0.1M Tris was added and stirred. This step repeated thrice until pH reaches to 7.8 and stored in light protected bottle at 4 °C.
RNAse A	10mg/ml in 0.01M sodium acetate (pH5.2), heated for 10 mins at 90°C and allowed it to cool and adjusted pH by adding 0.1 vol of 1M Tris pH 7.4, stored at –20°C
Sephadex G50 beads	Appropriate amount of sephadex G-50 (SIGMA) powder in autoclaved double distilled water for swelling for 1-4 hours and changed in Tris buffer and sterilized by autoclaving.

SSC (20X)	175.3 g of NaCl and 88.2 g sodium citrate in 800ml of water and pH was adjusted with 14N HCl and water to the final volume of 1 liter.
Sodium Acetate (3M, pH 5.2)	40.8 g of sodium acetate in 80 ml of water and pH adjusted to 5.2 with glacial acetic acid and final volume adjusted with water to 100ml, autoclaved and stored at RT
Sodium Chloride (5M)	29.2 g of NaCl in 80 ml of water and volume adjusted with water and sterilized by autoclaving.
SDS (10%)	10g of Sodium Dodecyl sulfate dissolved in 90 ml of water completely and final volume adjusted to 100ml. (Not to be autoclaved)
TAE (50X)	24.2 g of Tris Base, 5.7ml of glacial acetic acid, 10 ml of EDTA (pH 8.0) and final volume adjusted with water and autoclaved and stored in room temperature.
TBE (10X)	10.8 g of Tris base, 5.5 g of boric acid, 4ml of EDTA and final volume adjusted with 100ml and autoclaved and stored at RT.
TCM solution	0.3M $CaCl_2$, 0.3M $MgCl_2$, 0.1M Tris-Cl (pH 7.5) and final volume adjusted with water.
Tris Cl (pH 8, 7.5, 8.3)	12.112 g of Tris base in 80ml of water and pH adjusted with HCl and water to the final volume of 100ml.

TE	Tris-Cl (pH 8.0) 10ul (10mM) and EDTA 1μl (1mM) in 989μl of water to make 1 ml.
X gal	(5 bromo-4 chloro-3 indolyl β-D galactoside) 20mg of X gal dissolved in 1 ml of dimethyl formamide, wrapped with aluminum foil and stored in –20°C.
X gal, IPTG Plates	Pre-made agar plates are added with 20μl of X-gal (20mg/ml) and 100 μl of IPTG mixed together and spreaded in plate till it fully absorbed in plate and incubated at 37 °C for 30 mins for complete drying and used immediately for transformation or stored at 4 °C covered with aluminium foil.
Restriction enzymes	Most of the enzymes are from New England Biolabs(NEB)
Other common enzymes	NEB, Promega, Bangalore Genei
Invitro Transcription Kit	Promega's T7-T3 riboprobe transcription kit.
Differential display PCR Kit	Gene Hunter's Differential display PCR kit no 2.
TRI- Reagent	Sigma.
Transfast transfection kit	Promega.

Bacterial Strains

Escherichia coli (XL-1 Blue)   [F', *proAB*, *lac* I^q Z M15, Tn10 (tet^R)], λ-, recA, *end* A1, *gyr* A96, *thi*-1, *hds*17 (r$_K$⁻, mk$_K$⁻), *sup* E44, *rel* A1, Δ (lac).

Escherichia Coli (DH5 α)   φ80 dlac ZΔ M15, recA1, *end*A1, *gyr* A96, *thi*-1, *hsd*17 (r$_K$⁻, mk$_K$⁻), *sup* E44, *rel*A1, deoR, (*lac*ZY A-*arg* F) U169.

Plasmids

pStuI   Modified Bluescript SK vector introduced with Stu I site immediate downstream to T7 promoter.

pCI Neo   Mammalian expression vector with cytomegalovirus (CMV) immediate early enhancer promoter sequences with intronic sequences at upstream of multiple cloning site with neomycin phosphotransferase resistance gene under SV 40 promoter.

pGEM-T   Modified Bluescript vector containing T7-SP6 promoters flanking predigested multiple cloning sequences, having "T" overhang at digested blunt ends.

II.2. Methods

II.2.1. Preparation of Competent Bacterial Cells

E. *coli* strains XL1 blue or DH5α were used for cloning the constructs. XL1 blue cells were invariably pre selected for the tetracycline resistance. The single pre selected colony was grown by shaking overnight in 2-10ml cultures. This culture was inoculated (1:100, v/v) in fresh LB (Tet) medium and shaken at 37° C to an OD at 550nm of 0.6-0.7. This OD corresponds to approximately 6×10^7 cells /ml. The cells were pelleted at 3500 rpm for 5 mins at 4° C, resuspended in half the original culture volume of pre cooled 50mM $CaCl_2$, and incubated for 10-20 minutes. The cells were centrifuged again as above and suspended in one tenth of the original volume of pre cooled 50mm $CaCl_2$ with 20% glycerol. The cells were aliquoted in 200-600 µl volumes and stored at -80° C until use.

II.2.2. Transformation of Plasmid DNA in *E.coli* Cells

For transformation typically 20ng of plasmid DNA was pre treated with 2µl of ice cold TCM solution (100mM Tris and 300mM of $CaCl_2$, 300 mM of $MgCl_2$) in ice for 5 minutes. The frozen competent cells were kept in ice for thawing, and 150µl of cell suspension was added to the TCM pre-treated plasmid DNA, and incubated for 40 min in ice. After 40 min, the transformation mixture was given heat shock at 42°C for 90 sec and immediately kept in ice for 5 mins. To this 850µl of LB was added and incubated under moderate agitation at 37°C for 60mins, to allow the expression of antibiotic resistance gene. An aliquot of transformed cells (100-125µl) was spreaded on agar plates containing selective antibiotic and incubated overnight for selection of the transformants.

For blue- white selection of transformants, the transformed cells were spreaded on LB agar plates containing Xgal and IPTG (plates were initially spread with 50µl of IPTG (100mM in water), 20µl of X gal (50mg/ml in dimethyl formamide) before plating the cells) and incubated at 37°C overnight.

II.2.3. Isolation of Plasmid DNA (Mini Prep)

Single colonies were inoculated into 5ml of Luria broth and grown by shaking the culture overnight at 37°C at 200 rpm. Next day 1.5ml of overnight culture was spun in

micro centrifuge at 5000 rpm for 3-5mins. The cells were resuspended in 100µl of TELT (Tris –EDTA-LiCl-Triton X) and lysed by adding 10µl of freshly prepared lysozyme (10mg/ml), mixed by pipetting and left in ice for 5'minutes and kept in boiling water bath exactly for 1min, and subsequent snap chilling in ice, and left for 5'min. After the lysate cooled down, it was centrifuged for 10 min at 12000 rpm, and debris formed was removed using sterile tooth picks and to the resulting lysate, 2.5 volume of ice cold 100% ethanol was added and kept at -20°C for 60 mins or overnight for precipitation. This was followed by centrifugation at 12000 rpm for 15mins and subsequent wash with 70% ethanol. The pellet was dried and resuspended with 30µl of TE and the plasmid quality was checked in 1.5% agarose gel.

II.2.4. Plasmid Isolation (Midi Preparation)

The selected bacterial colony was inoculated in 25 ml LB medium with antibiotic and kept in shaking water bath overnight at 37 °C. Next day the cells were taken in Oakridge tube and centrifuged at 5000 rpm for 5 minutes at 4°C. The cell pellet was washed once with solution I (50mM Glucose, 25mM Tris-Cl, 10mM EDTA) and re pelleted and again resuspended with 1.25 ml of solution I and kept in ice for 5 minutes. To this 2.5 ml of solution II (0.2N NaOH, 1% SDS) was added and gently swirled around and kept in ice for 5 minutes. After incubation, 1.875 ml of solution III (3M sodium acetate pH 5.2) was added and mixed gently and kept in ice for 20 minutes.

After incubation, samples were centrifuged at higher speed at 4°C to precipitate the chromosomal debris and the supernatant was taken to fresh tubes. To this supernatant 6 µl of RNase A (10mg/ml) was added and incubated at 37 °C for 1 hour. To this 0.6 volume of iso-proponal was added and mixed by inverting gently and kept at room temperature for 30 minutes. The tubes were centrifuged at 13000 rpm for 15 minutes and supernatant was decanted. The dried pellet was resuspended with 300µl of TE or water and kept at room temperature for 20 minutes. The dissolved nucleic acids were taken into fresh eppendorf tubes and extracted once with phenol chloroform, followed by chloroform iso-amyl alcohol. To the supernatant 1/10 volume of 1 M NaCl was added and DNA was precipitated with 2.5 volume of ethanol at -20 °C for 2 hours or over night. The tube was centrifuged at 13000 rpm and washed with 70 % ethanol and

dried. The pellet was reconstituted with double distilled water and checked for quantity and quality spectrophotometrically and by agarose gel electrophoresis.

II.2.5. Restriction Digestion of Plasmid DNA

The plasmid DNA was cleaved with restriction enzyme using the following composition of reaction mixture.

Plasmid DNA	1-5µg
10 X Buffer	2µl
Restriction Enzyme (1U/µg)	0.5 -1µl
DD H$_2$O to the final volume	20 µl

The reaction mix was properly mixed by vortexing and incubated at 37°C over night.

II.2.6. De-phosphorylation of the Vectors

The plasmid DNA (5-10µg) was digested with desired restriction enzyme and directly purified by phenol chloroform extraction method if the vector was digested with single site cutting enzyme. The desired band of DNA was extracted from agarose gels by freeze thaw method. The purified vector was treated as follows

Vector DNA	5µg
10X Buffer	2µl
Calf intestine Phosphatase enzyme	0.5µl
Double Distilled water to the final	
volume of	20µl

The reaction was incubated for 30 -60 minutes and deactivated by incubating at 65-75°C for 15 minutes, and phenol chloroform purified and precipitated with ethanol.

II.2.7. Ligation of Inserts in Vectors.

Ligation was performed with purified vector with desired sticky ends or with blunt ends with appropriate insert ratios with that of vector. The reaction mix consists of the following.

Vector DNA	50 ng (~25 f mol)
Insert to be cloned	10 ng (~25 f mol)
Buffer (10X)	2 µl(1X)
T4 DNA Ligase Enzyme	1 µl (5 Weiss Units)
PEG 30% (optional)	1µl
Double distilled water to the final volume of	20 µl

The reaction was mixed properly and incubated at 16°C overnight and stored at 4° C untill transformation.

II.2.8. Polymerase Chain Reaction

To amplify specific gene from genomic or plasmid DNA, PCR reaction were set as follows. The reaction mixture for PCR consisted of

Genomic DNA	25ng
Forward Primer (10pmol)	1µl (10pmol)
Reverse Primer (10pmol)	1µl (10pmol)
10 X Buffer	2.5 µl (1X)
dNTPs (2.5 mM)	1µl (100µM)
Taq polymerase(3U/µl)	0.2µl (0. 2units)
Double distilled water to final volume of	25 µl

The PCR reaction was typically programmed for the following thermal regimens

Initial Denaturation   94°C for 4 minutes

Denaturation   94 °C for 30 seconds ⎤

Annealing   55 °C for 30 seconds ⎬ 35 cycles

Extension   72 °C for 30 seconds ⎦

Final extension at 72 °C for 7 minutes and stored at 4 °C or -20 °C.

II.2.9. Reverse Transcription -PCR

Total RNA was isolated from HeLa cells using TRI Reagent (SIGMA) and checked for its quality by gel electrophoresis and quantitated spectrophotometrically. The DNA free RNA (0.2-1µg) was taken for RTPCR. The reverse transcription reaction was performed with target specific primers for 45 minutes at 45°C and the same was carried with PCR using target specific sense and antisense primers.

Primers for hTR

5'- cct ggg agg ggt ggt ggc cat-3' forward primer

5'-aac tgc agc atg tgt gag ccg ag-3' reverse primer

RT PCR Reaction mix Concentrations

RNA sample or control	Xµl (0.2-1µg)
AMV/Tfl (5X Reaction Buffer)	10µl (1X)
dNTP Mix (10mM each dNTP)	1µl (200µM)
Downstream primer	50 pmol (1µM)
Upstream primer	50 pmol (1µM)
MgSO4 (25mM)	2µl (1mM)
AMV Reverse Transcriptase (5U/µl)	1µl (0.1U/µl)
Tfl DNA Polymerase (5U/µl)	1µl (0.1U/µl)
Nuclease-Free Water to the final volume of	50µl

Reaction conditions

For reverse transcription

      45 °C for 45 minutes

      94 °C for 2 minutes

Second strand synthesis and PCR

      94 °C for 30 seconds ⎫

      52 °C for 1 minute   ⎬   40 cycles

      68°C for 2 minute   ⎭

      68° C for 7 minutes for finishing the synthesis before storing at 4°C

II.2.10. Sanger's di-Deoxy Sequencing of Cloned DNA Template

The *in vivo*, *in vitro* expression constructs were sequenced to confirm the desired inserts, using promega's f Mol cycle sequencing kit. The template plasmid DNA was isolated using midi prep method of DNA isolation.

Kinasing of the sequencing Primers

Primer (15p mol/μl)	1μl
10x T4 polynucleotide kinase buffer	1μl
γ ^{32} P-ATP (3000mci/mmol, 10uCi/ul)	2μl
Autoclaved double distilled water	5μl
T4 polynucleotide kinase (10U/μl)	1μl
Total volume	10μl

The reaction mixture was incubated at 37°C for 30min and kinase was inactivated at 90°c for 2mins.

59

Sequencing Reaction

The Master mix:

Template DNA	9µl (200-800ng)
5X DNA sequencing buffer (5X)	5µl
Labeled Primer (1.5 pmol/µl)	1.5µl
Sterile water to the final volume of	16µl
Taq DNA polymerase (5U/µl)	1µl
Final volume of reaction mix	17µl

4ul of the above was added to 2µl with ddG, ddC, ddA, ddT in 0.5 ml eppendorf tubes. A drop of mineral oil was overlaid and the tubes briefly spun and put up for PCR for following regimen.

Initial denaturation	95°C for 2 minutes followed by 30 cycles of
Denaturation	95°C for 30 seconds
Annealing	42°C for 30 seconds
Extension	70°C for 1 minute

After 30 cycles, the tubes were stored at 4°C.

3.0µl of stop dye was added to each tube and stored in -20°C till further use. The samples were heated for 3min at 80°C and loaded directly from the denatured state to the sequencing gel (6% acrylamide, 8M Urea).

Glass plates were treated with SIGMA COTE on both the plates, and 0.4mm /poly acrylamide gel (6%) was used. A pre run was performed using sequencing dye before the actual sample running at 2000 volts, which could maintain the plates at 45-50°C during electrophoresis.

After the run was over for 2-4 hours or the run of dye front to 5-7cms from the end of the plate, then the glass plates were separated and gel was fixed with methanol/acetic acid mixture and excess urea removed. The gel was transferred to Whatmann filter paper (3mm), dried for one hour at 80°C in the drier and kept for imaging for 20hrs.

II.2.11. Automated DNA Sequencing

The PCR products or the plasmid DNA containing inserts were confirmed by sequencing using automated DNA sequencer. The purified plasmid of 100ng or 200ng of purified PCR products were taken for reaction along with 1.5 pmole of forward primer and ready reaction mix. The PCR conditions were as follows

Initial denaturation	94 °C	30 seconds followed by 25 cycles of
Denaturation	94°C	10 seconds
Annealing	52°C	10 seconds
Extensions	60°C	4 minutes

The PCR products were purified using ethanol EDTA method as suggested by manufacturer's protocol. The air-dried PCR products were resuspended with template suppressor reagent and denatured at 95°C for 3 minutes, loaded in 310-sequencer machine. The data was collected and analyzed using Chromas software.

II.2.12. Designing of Ribozyme Against Telomerase RNAs

The telomerase RNA sequence was retrieved from NCBI's nucleotide database and analyzed for possible folds using Michael Zuker's RNA fold. We focused on GUC sequences as target cleavage site and looked for possible secondary structures with conserved features in folding pattern and allowed possible foldings with changing ▯G (free energy patterns).

The GUCs in the non-base paired regions were selected by comparing the structures that are conserved in most of the energy levels. The flanking sequences of GUC were considered for ribozyme base pairing in the target region. The ribozyme catalytic core sequence along with chosen flanking sequence (complementary to the target) was appended with target sequence and analyzed for its specificity upon folding. The designed ribozyme was qualified only if it annealed with the proposed target.

II.2.13. *In vitro* Run Off Transcription

In vitro run off transcription was performed using plasmid constructs linearized by restriction enzyme cleavage at a site closely following the insert. pStuI digested with

61

Stu I enzyme and pGEM-T vector linearized with NcoI enzyme, were used for generating ribozyme and target transcripts respectively. The ribozyme constructs were under T7 promoter system and hTR of pGEM-T system under SP6 promoter and respective polymerases were used for *in vitro* transcription reaction, typically consisting of:

Transcription Buffer (5X)	-	4µl
DTT (100mM)	-	2µl
Recombinant RNaseIn(RNase Inhibitor)	-	20U
rATP, rGTP, rCTP (2.5mM)	-	4µl
rUTP(Cold) (100µM)	-	2.4µl
Linearized Template (0.2-1mg/ml)	-	3µl
α^{32} p UTP (10µci /µl, 3500Ci/mmol)	-	2µl (20µci)
Sp6,T7 RNA polymearse	-	15-20 U
Final Volume	-	20µl

All the mixture was kept at Room Temperature during addition of each successive component as DNA precipitates in presence of Spermidine if kept at 4° C. The reaction was incubated for 1hour at 37° C. After the reaction was over, RNase free DNase 1µl was added to reaction tube and incubated at 37° C for 30 min and subsequently inactivated by incubating at 65° C for 15 mins. After transcription the reaction tube can be kept at -20° C or taken for further purifications.

II.2.14. Column Purification and Percentage Incorporation Radioactive label

The reaction volume of 20 µl was increased by addition of 80µl of DEPC treated water to make the final volume to 100µl. From this 100µl volume, 1µl aliquot was taken for quantifying the transcript from the radioactive incorporation. The 1µl of reaction volume was taken in scintillation vial for 32P reading, which represents the total radioactive count of 100µl. The 100µl reaction volume was taken for column purification and added to the pre-equilibrated Sephadex 50 in-house made columns

and centrifuged at the same speed at which the column was equilibrated (speed of 1000 rpm for 1min). The eluate should be 100µl and the same was used for determining the radioactive incorporation. An aliquot (1µl) was taken out from 100µl and counted in scintillation vial, and the counts were noted. The RNAs can be used directly or it can be further purified with phenol chloroform and precipitated with sodium acetate pH 5.2, and 2 volume of ethanol.

II.2.15. Freeze Thaw method of Purification of DNA from Agarose gels

The band of interest from digest or PCR generated fragment was electrophoresed in low percent agarose gel, and band of interest was cut with sterile blade and the gel was crushed properly using 1ml pipette tips. To this 100-200µl of 10mM Tris-EDTA buffer or water was added, followed by addition of equal volume of phenol, vortexed, and kept at -70°C for 15 minutes. This was thawed at room temperature. This process was repeated for 3 times and centrifuged and the supernatant was extracted with chloroform. To this 0.1 volume of 3M sodium acetate and 2.5 volume of ethanol were added for precipitation for 30 minutes to 2 hours at -20°C and pelleted at high-speed centrifugation. The pellet was washed with 70% ethanol and solubilized with TE.

II.2.16. Genomic DNA Isolation

The scraped cells were washed twice in Cold PBS and to that 300µl of SE solution (75 mM NaCl, 20 mM EDTA) was added and mixed thoroughly with pipetting to disrupt clumps. 10µl/ml of proteinase K (10mg/ml) was added and mixed properly by pipetting and kept for 5-10 minutes. 60 µl of 10 % SDS was added and mixed properly. The eppendorf tubes were kept at 37°C over night (or at 50°C for two hours). From this procedure onwards cut tips were used for pipetting to avoid shearing of DNA. Next day the lysed cell extracts were added with equal volume of phenol chloroform isoamyl alcohol (25:24:1) and kept for gentle shaking for 5-10 minutes. The procedure repeated twice and the supernatant extracted once with chloroform isoamyl alcohol and precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol and mixed gently to visualize the thread kind of formations

and kept at RT for 30 minutes. The sample tubes were centrifuged for 15 minutes at 18°C and washed twice with 70 % ethanol and dried. The pellets were reconstituted with TE or with autoclaved double distilled water and kept at RT for 15-20 minutes and taken for further utilization.

II.2.17. Total RNA Isolation

The trypsinized cells were washed twice with cold PBS 1ml of Tri- reagent (for 5-10* 10^6cells) (SIGMA) was added. Cells were disrupted by continuous pipetting and kept in ice for 5 minutes. Chloroform (200µl) was added and vortexed or shaken vigorously for 15-20 seconds and kept at 4°C for 15 minutes. The samples were centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant transferred to fresh tubes (without disrupting the interphase white layer which is of DNA) and 0.5 ml of isopropanol was added and mixed gently and kept at room temperature. Again it was centrifuged at higher speed for 15 minutes at 4°C. The supernatant was removed and the pellet was washed with 75 % ethanol and dried at room temperature. The pellet was reconstituted with DEPC treated water and kept at 4°C for 15 minutes and taken for qualitative and quantitative analysis.

II.2.18. Trypsinizing and Sub Culturing Cells from Monolayer

Pre-cultured plates and flasks of monolayer cells were washed with PBS twice with gentle rinsing. To this washed plates, trypsin EDTA solution to a concentration of 1X (Stock 100X Gibco BRL) was added and kept in CO_2 incubator at 37° C for 1-2 minutes. This facilitates the dislodging of the cells from culture flask. Immediately 2ml of complete medium was added to neutralize the effect of trypsin EDTA. The flask was flushed with medium and transferred to fresh eppendorf tube and centrifuged at 4°C at 3000 rpm. To the washed cells 500µl of complete medium was added and part of the cells was taken for counting. For seeding, cells were added to the pre filled flask with 4ml of complete medium and labeled with date and the name of cell strain and if it was transfected then labeled with the plasmid construct details. The seeded plates were kept in CO_2 incubator programmed to maintain 5% CO_2. After a day cells can be taken for analysis.

II.2.19. Freezing Human Cells Grown in Monolayer Culture

The flasks were viewed with an inverted microscope to assess the health and confluence of the cells. The flasks with 50-60% of confluence were trypsinized. Cells were suspended in 2ml complete medium, and centrifuged at 1500-3000 rpm under aseptic conditions. The cells were washed with PBS once, and centrifuged to remove the PBS. To the sterilized cryo-vials, the cells were added with complete medium along with 10% DMSO as freezing medium (10% or 20 % (v/v) FCS and 10% DMSO). Generally cells were stored as 1ml aliquot and kept at -20°C for an hour, then at -80°C for overnight and shifted to Liquid nitrogen container for storage.

II.2.20. Thawing and Recovering Human Cells.

The cryo vials were removed from liquid nitrogen container and immediately placed on to 37° C preset water bath. The thawed cell suspension were transferred to new sterile centrifuge tube and centrifuged to remove the medium and washed twice with complete medium to remove the residual DMSO, gently resuspended in small amount of complete medium with 10 or 20% FCS and seeded in to fresh culture plates for growth in CO_2 incubator. After a day the medium was changed and antibiotics (Penicillin/Streptomycin 100X GIBCO BRL) were added in order to avoid contamination. After the cells were grown and complete confluence was observed, the cells were split in to new flasks or taken for further analysis.

II.2.21. Splitting Cells in to Fresh Flasks

The cells of already grown and fully confluent flask were taken from CO_2 incubator and caps were closed tightly and wiped with 70% alcohol and kept at laminar flow hood. The culture flask was opened in front of flame, and the medium decanted or taken out by pipetting. Cells were rinsed with two changes of sterile PBS with or without antibiotics.

1ml of PBS or medium was added either by flushing against the wall of tissue culture flask or added with trypsin EDTA in the PBS. In case of trypsin EDTA solution along with PBS, the flask was kept in CO_2 incubator for 5-7 minutes and gently taped the base of the cell platform. Cells will be coming off the surface and taken to fresh eppendorf tube in ice. Cells were centrifuged and added with fresh PBS

to remove the traces of trypsin followed by addition of 1 ml medium .Aliquots were taken for counting in the microscope and transfer to new flask.

II.2.22. Determining the Viability of Cells

To 0.1ml cell suspension, 0.4% Trypan blue and 0.3ml of PBS was added in small tube. Cells were mixed thoroughly and let stand for 5 minutes before loading on Haemocytometer (non viable cells will take up the dye whereas the live cells will be impermeable to it). The cells were counted for total number of viable cells and the viable cells were calculated as follows.

$$V = \frac{U}{T} * 100$$

Percentage of Viable cells(V) = $\frac{\text{Number of Unstained Cells(U)}}{\text{Total number of cells (T)}} * 100$

The cover slip and Hemocytometer were decontaminated by rinsing with ethanol and washing with sterile deionized water.

II.2.23. Transfection of Cloned Vectors in Mammalian Cells

HeLa cells were grown to 70-80% confluence, in the 6 well plates and used for transfection purposes. TRANSFAST reagent (promega) was used for transfection .The cells were washed once and fresh DMEM with serum was added and kept in CO_2 incubator for an hour. The TRANSFAST reagent was used for transfection as per manufacturer's recommendations. The ribozyme coding DNA in mammalian expression vector pCI-Neo (around 1-1.25 µg of DNA in 10µl solution) mixed with 3.0µl or 6.0µl of TRANSFAST reagent by vortexing and incubated at room temperature for 15 minutes. In the meanwhile the medium with serum was removed and plates kept ready. Just before transfection, the mixture was briefly vortexed and added to the plates containing cells in 1 ml of DMEM medium. Plates were incubated for 1 hour in CO_2 incubator. After one hour the cells were added with fresh medium with serum and the plates were kept in CO_2 incubator.

II. 2.24. Selection of Stable Transformants

After transfections, the cells were maintained for 48-72 hours and either to the same flask or sub cultured flasks, 400 µg /ml G418 was added and maintained for 21 days. The cells which were resistant to the drug were further sub cultured in antibiotic medium periodically. The selected cells were considered as transfected cells and used for further experiments.

II.2.25. Northern Blotting

For Northern blotting, the RNA samples were quantitated spectro- photometrically and checked for quality by agarose gel electrophoresis. About 15-30 µg of RNA along with sample buffer was taken from each sample and denatured in PCR machine at 65 C for 10-15 minutes and snap chilled in ice.

RNA samples were prepared for electrophoresis as follows (1X MOPS, 2.2M formaldehyde, 50% formamide)

RNA	9 µl (15-30 µg)
10X MOPS	2.5 µl
Formaldehyde	3.6 µl (12.2M formaldehyde)
Formamide	10 µl (100% formamide)
Total	25.0 µl

Agarose gel (1%) prepared as follows

Agarose	0.4g
DEPC treated water	36ml
Boiled in microwave oven at 85°C	
Cooled to 55°C and the following was added	
Formaldehyde	9.0 ml
10X MOPS	5.0 ml
Total	50ml

The preheated samples of RNA were loaded with marker and electrophoresed at 40-60V for 6-8hrs; electrophoresis was carried out in 1x MOPS (0.02M MOPS, 8mM Sodium Acetate, 1mM EDTA) buffer.

Running Buffer

10 X MOPS   62.5 ml

DEPC water   563 ml

Gel tanks were pretreated with H_2O_2 and all solutions were prepared in DEPC water except Tris buffers.

After electrophoresis the gel was washed in DEPC water 3 times at intervals of 5 minutes to remove formaldehyde. The gel was equilibrated in 10X SSC for 10-15 minutes. The membrane and Whatmann papers were pre-soaked in 10X SSC and gel apparatus was utilized to assemble the membrane and blotting pads. .

The gel and membrane were marked with pencil and the membrane removed from sandwich. The membrane now can be checked in UV trans-illuminator for confirming the transfer. The membrane was kept in UV for 2-3 minutes and directly taken for hybridization or stored in aluminium foil at 4°C.

Pre-hybridization was done with sodium phosphate buffer containing sheared salmon Sperm DNA with or without formamide. The membrane was packed in the plastic sheet along with pre-hybridization buffer, sealed and kept at 60°C for 4 hours. With same composition, purified probe of denatured hTR was added and kept for hybridization at 60°C and left for overnight.

Next day the membrane was washed sequentially as follows.

2X SSC with 0.1 % SDS and

1 X SSC with 0.1%SDS, and

0.5 X SSC with 0.1 % SDS, and

0.2 X SSC with 0.1 % SDS and all washes done at 60°C for 10 minutes .

Membrane was air dried and covered with Saran wrap and kept for exposure for autoradiography.

Pre-hybridization and Hybridization Buffer (Sodium Phosphate Buffer)

Sodium Phosphate	0.5 M
SDS	7 %
EDTA pH 8.0	1mM

100μg of sheared, denatured salmon sperm DNA

Pre hybridization and Hybridization Buffer (Formamide buffers)

Formamide	5 ml	(50%)
SSC or SSPE 20X	3 ml	(6X)
SDS 10%	0.5 ml	(0.5 %)
Denhard't (50X)	1 ml	(5X)
H₂O to the final volume of	10ml	

100 μg of sheared salmon Sperm DNA

II.2.26. Terminal Restriction Fragment Length Assay (TRF) (Telomere Length Analysis)

The Genomic DNA (10μg) from transfected cells was digested with Hinf I and RsaI overnight and loaded in 0.8% to 1% agarose gel along with marker, electrophoresed at 80 V for 5-6 hours and the gel was checked in UV transilluminator. The gel was depurinated in 0.25 M HCl for 5 minutes and washed in distilled water twice. The DNA in gel was denatured in 1.5M NaCl and 0.5M NaOH for 15 minutes with gentle shaking. The H⁺ nylon membrane and Whatmann sheets were cut to the size of gel for stacking. The gel apparatus was used as transfer apparatus, and on both sides the transfer buffer of 10XSSC was used. Three sheets of whatmann paper soaked in 10XSSC were placed on top of wick with its ends immersed in buffer. On top of that denatured agarose gel was placed and membrane placed above the gel. Three sheets of whatmann paper also stacked carefully without air bubble on the membrane. Finally dry sheets of paper towels were stacked for 7 cm and a glass plate with weight was placed above all the stacks. The set up was left undisturbed overnight and next day the membrane was taken and processed further.

The membrane was washed in 2X SSC for 5 minutes, dried and cross-linked in UV transilluminator or UV crosslinker. The membrane was directly taken for hybridization.

Prehybridization solution

50 X Denhardt	5X	4ml
20X SSC	6X	10ml
10 % SDS	0. 1%	0.4 ml
Sheared salmon sperm DNA	100μg	100μl
Formamide	50%	20ml
Water to final volume of		40 ml

The membrane was packed in plastic sheet and pre hybridization carried out for 2-4 hours. After pre-hybridization end labeled purified telomeric probe was added in hybridization solution, heat sealed and hybridization performed at 42°C overnight. After hybridization, the membrane was washed with solution I (2X SSC, 0.1% SDS) for 10 mins at room temperature and with solution II (1 X SSC, 0.1% SDS) for 10 minutes and solution III (0.1 X SSC with 0.1% SDS) for 30 minutes at 65°C with gentle agitation, and finally washed with 0. 1xSSC. The membrane was dried in air and kept for autoradiography.

II.2.27.Colony Blotting

The transformed cells with clone of interest were screened by colony blotting. After spreading the transformed cells, the plates were incubated at 37°C and next day the colonies were transferred to membrane by gently overlaying the membrane on agar plate. A mark was put in membrane and respective mark in plate to identify clones. The membrane was allowed to sit for 3-5 minutes and gently removed without disturbing the plate. Now membrane was air dried few minutes and treated with 0.2N NaOH and 1 M NaCl for 5 minutes. Now the membrane was transferred to the glass plate poured with 0.4M Tris-Cl, pH 7.6 and 2X SSC for 5 minutes. The membrane was transferred to Whatmann paper and dried for 90-120 minutes at 80°C. Next day the colonies could be seen completely dried and the membrane was taken for pre hybridization and hybridization and further processed as described under southern blotting.

II.2.28. Slot Blotting

The Slot blotting apparatus was washed properly and dried completely. The apparatus were packed with whatmann sheet soaked in 2X SSC and soaked nylon membrane, and packed in apparatus. The DNA samples were diluted to 100µl and boiled for 5-10 minutes, and equal volume of 0.4 M NaOH was added to DNA sample to maintain the denatured state. Similarly 0.4M NaOH was added in the slots and vaccum manifold was applied to have suction force. The DNA samples were loaded in slots and vaccum was applied for suction. The membrane was further processed as described under colony blotting procedures.

II.2.29. Fluorescence Activated Cell Sorter (FACS) Analysis

To analyze the cellular phases, the cells were either scraped or trypsinized and washed with PBS and fixed in ethanol by adding drop by drop in Falcon tubes held on vortex mixer containing 4ml of 70% ethanol, and stored at $-20°$ C overnight to 1 month. The ethanol was carefully removed and the cells were re-suspended in propidium Iodide master mix at a final density of 0.5 $\times 10^6$ cells. PI master mix consisted of

Propidium iodide (1mg/ml stock)	40µl
RNase (10mg/ml)	10µl
PBS	950µl

The propidium Iodide mix was added to cells and incubated at 37°C for 1 hour and taken for FACS analysis and the diploid and hypo diploid, tetra ploid fractions were estimated.

II.2.30. Telomere Repeat Amplification Protocol (TRAP)

i) Cell Lysis and protein extraction for TRAP

All the steps were performed in ice strictly. The cells were harvested either by scrapping or by trypsin EDTA treatment and the cells were washed twice with PBS and centrifuged to remove the residual PBS. After this step the cells can be stored at -80°C for future use or can be used immediately.

To the cell pellet 200μl of cell lysis buffer was added and mixed with pipette to ensure proper lysis. As an alternative repeated freeze thaw cycles were also used (thawing should not be performed in hot water). The lysed cell extracts were centrifuged at 4°C at higher speed for 30 minutes to remove the cell debris. After centrifuging, the cell supernatant was removed to DEPC treated RNase free tubes as multiple aliquots and stored at -80°C for future use. Part of an aliquot was taken for total protein quantitation spectrophotometrically either by Bradford estimation or by direct 280nm readings (TS Template strand or Telomerase substrate, Acx-Anchored Reverse primer, TSNT control, NT is reverse primer of TSNT internal control).

Cell Lysis Buffer for TRAP

Buffer components	Needed Concentration	final volume of 5ml
1M Tris Hcl pH 7.5	10mM	50μl
1M Mgcl₂	1mM	5μl
0.5M EGTA	1mM	10μl
10% CHAPS	0.5%	250μl
100% Glycerol	10%	0.5ml
DEPC treated water	to the final volume of	5ml

ii) TRAP Reaction oligo components and concentrations

ii a) The following primers were used

TS-5'- AATCCGTCGAGCAGAGTT-3'

ACX -5'- GCGCGGCTTACCCTTACCCTTACCCTAACC-3'

TSNT-5'- AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3'

NT-5'- ATCGCTTCTCGGCCTTTT-3'

Final concentrations of the primers and the telomerase substrate in TRAP reaction

ACX	10 pmol / reaction	
NT	1.6 pmol / reaction	
TSNT	0.01 Atto mol / reaction	(Internal control)
TS	16 pmol /reaction	

iii) Composition of 10X TRAP PCR Buffer

Buffer Components	Needed concentration	Volume to be taken
Tris HCl pH 8.3(1M)	(200mM)	200µl
MgCl₂ (1M)	(15mM)	15µl
KCl (1M)	(630mM)	630µl
Tween 20	(0.5%)	5µl
EGTA (0.5M)	(10mM)	20µl
BSA 100X	(0.1%)	1µl
DD water of	to final volume	1000µl

iv) TS primer labeling

TS primer	1.6µl (100pmol/µl)
T4 poly nucleotide kinase buffer	2.0µl
γ^{32} p ATP	2.5 µl
Enzyme	1.0µl
Water	4.5µl
Final volume	20.0µl

Reaction tube kept at 37°C for 1hour and deactivated at 85°C for 10mins.

v) TRAP PCR

Cell extract	2ul (0.1µg to 1µg of protein)
10X TRAP PCR Buffer	5µl(1X)
TS labelled Oligo	2µl
Primer Mix	1µl
dNTPs (2.5mM)	1µl (200uM)
RNAse free water	32µl
BSA (100X)	0.2µl (1X)
Taq Polymerase (3U/µl)	0.5µl
Total volume	50µl

The reaction tubes were kept in ice while setting the reaction and reaction was mixed properly and kept for initial extension by telomerase at 25°C for 30 minutes. After 30 minutes of incubation at 25°C the tubes were set for PCR, with the following thermal regimen.

Denaturation	94°C	30sec
Annealing	60°C	30sec
Extension	72°C	30sec

Kept for 27 cycles and finally soaked at 4°C and stored at -20°C.

The post PCR TRAP products are run in 10%-12% non-denaturing PAGE gel at constant voltage and 40-50 watts and the run was kept until the bromo phenol blue reaches the bottom of the gel. The ladder generally comes above the xylene cyanol and predicted TSNT co-migrate in xylene cyanol.

10% Non denaturing PAGE for TRAP

Acrylamide 40%	20ml
10X TBE	8ml
TEMED	20µl
APS(10%)	1ml
DD Water to the final volume of	80ml

II.2.31. Differential Display PCR

Differential display PCR was done with the RNA samples derived from cells transfected with vector, wild type ribozyme, and mutant ribozyme. The RNA was quantitated spectrophotometrically and checked for quality following agarose gel electrophoresis to observe the integrity of RNA samples and for DNA contaminations. The stable transformants of HeLa cells representing vector alone, wild type ribozyme and mutant ribozyme were taken for RNA isolation. Equal amount of RNA from these three different experimental sets were taken for global cDNA synthesis and subsequently PCR was done with different (forward) primers according to the instructions given in the Gene Hunter differential display kit manual.

Each RNA sample was amplified with three different oligo dT (having A or C or G at the 3' end of primer to differentiate the transcripts) for making cDNA. From these three different cDNA pools aliquots were taken for PCR reaction with eight different forward primers. So each sample comprises 24 PCR. The Following oligo components were taken for RT reaction and each sample was labeled with HT-11A, HT-11G, HT-11C (oligo- dT primers ending with A, G, C respectively, in general referred as HT11M primer).

Reverse Transcription Reaction

5X RT Buffer	4.0
dNTP(250µm)	1.6
total RNA (DNA free)	2.0 (0.1µl/µl)
HT-11M (2µM)	2.0 µl
DD H₂O	9.4
Total volume	19.0 µl

The components were properly mixed and set at the PCR thermal cycler. The conditions were as follows

RNA denaturation	65°C for 5 minutes
cDNA extension	37°C for 60 minutes
Final Inactivation of RT enzyme	75 °C for 5 minutes

When the sample reaches 37°C, after 10 minutes 1µl of MMLV reverse transcriptase was added and kept back in the thermal cycler for incubation at 37° C for cDNA synthesis. At the end of transcription PCR tubes were spun down for any condensation. The cDNA samples can be stored at -20°C. From the cDNA made from the samples the PCR was performed with eight different forward (HAP) primers.

PCR Reaction

DD H₂O	10.0
10X PCR buffer	2.0
dNTP (25µM)	1.6µl

75

HAP primer (2μM)	2.0μl
HT11- M (2μM)	2.0μl
RT Mix from the cDNA	2.0μl
α ^{32} P dATP	0.1μl
Taq DNA polymearase	0.2μl
Double distilled water to the final	
Volume	20μl

The conditions were as follows

Initial denaturation	94°C	2mins
Denaturation	94°C	30sec
Annealing	40°C	30sec
Extension	72°C	2 minutes

The PCR reactions were performed for 40 cycles. The PCR products were electrophoresed in 6% denaturing PAGE gel in 0.5X TBE at 40-55 watts and at 1000 volts. The 3.5μl of PCR product mixed with 2μl of formamide dye heated for 2 minutes and loaded immediately, and electrophoresed for 8hrs or overnight at 600 volts. Electrophoresis was continued till the Xylene cyanol dye reaches the bottom of the gel, and the gel was transferred to 3MM Whatmann paper and dried for 2hours. The dried gel was kept for autoradiography for two days at -20°C.

From the developed X-ray film, differentially expressed genes were identified and marked. Then the gel was aligned with film and gel slices corresponding to differentially expressed transcripts were extracted with water by boiling for 10 minutes in a hot water bath and kept at 4°C over night for gel elution. The samples were centrifuged for 2 minutes at high speed and watery part excluding gel pieces were taken to fresh tube and precipitated with 5μl of (20mg/ml) of glycogen 1/10 volume of 3M sodium acetate and 2.5 volume of ethanol and kept for overnight at -20°C. The extracted sample was taken as template for re-PCR with the corresponding

primer pairs to re-amplify the same. The PCR conditions for the isolated fragments of differentially displayed products are as follows.

Re PCR from Isolated PCR fragments.

10X PCR Buffer	4.0µl
dNTP(250µM)	3.2µl
H-AP primer(2µM)	4.0µl
H-T11M primer (2µM)	4.0µl
cDNA template from the purified sample	8.0µl
Taq DNA polymerase	0.2µl
DDH$_2$O	16.4µl
Total volume	40.0µl

Five to seven micro liter of PCR sample were loaded for eletrophoresis (5% PAGE or agarose gel, 1.5% or 2%) to analyze the amplification. If the amplification failed, the samples were diluted 1:100, and 4µl or 8 µl of the same diluted PCR sample was taken for re-PCR with corresponding primers.

The reamplified products were purified and cloned in to pGEM T vector, and sequencing was done. The clones are also probed with labeled cDNA pool for reverse northern to avoid false positives arising among differential display products. The sequenced clones were analyzed *in silico* using NCBI's BLAST to find corresponding genes.

II.2.32. Reverse Northern Blotting

Total RNA from HeLa cell was reverse transcribed by internally labeling the cDNAs using oligo-dT. The clones of differentially expressed genes were denatured and blotted in membrane as for slot blotting procedures. The α^{32}P labeled cDNAs were used as probe in hybridization reaction and further processed as described under Southern blotting. The autoradiograph was aligned with membrane and the positive clones identified for further characterization.

II.2.33. 2-D Gel Electrophoresis and Mass analysis of proteins

The stable transformants of vector, ribozyme and mutant ribozyme were frozen untill use. The cell pellets were solubilized with UREA lysis buffer (8M urea, 4% CHAPS

and 2% Ampholytes (3-10) (Amersham)), and immediately quantitated using Nanodrop, with BSA as standards. A urea lysis buffer was used as a blank. About 60μg of protein from each transfectant were taken and resolubulized with buffer (8M urea, 2% CHAPS, 0.002% bromophenol blue) containing ampholytes of pH range 3-10. The precast isoelectric focusing gels (pH 3-10) were used for first dimension electrophoresis. The precast gels were passively re-hydrated with protein samples in sample tray for 12-16 hrs. The re-hydrated gels were electrophoresed in IPGphor IEF electrophoresis unit (Amershem/GE Healthcare) at 500 V for 2 hrs, 1000V for 30 mins, 2000 V for 30', 2500V for 30' and 5000V for 2 hrs. After electrophoresis the gels were gently rinsed with water and stored in falcon tubes at -80° C. until use.

The 1D gels after focusing was equilibrated first with a buffer (50mM Tris Cl pH 8.8, urea 6M, glycerol 30%, SDS 2%, bromo phenol blue 0.002%) containing DTT (10mg/ ml) for 30 mins followed by equilibration with a buffer of same composition but having iodo acetamide (25mg/ml) instead of DTT for another 30 min to reduce the cystein sulphydryls and further corbomidomethylated the proteins. Gels were directly placed in PAGE gel containing stacking (5%) and resolving (12%) phases and electrophoresed for 2 hrs at 20 mA and for 5 hrs at 40 mA. The gels were fixed in methanol acetic acid for 30 minutes; silver stained and compared for differentially appeared proteins.

The differentially expressed proteins were identified and isolated from the gel and trypsin digested for mass analysis (MALDI –TOF). The resulting peptides were desalted and concentrated by zip tip and crystallized with acetonitrile at room temperature. The spectrometer was normalized calibrated with known standards before starting the firing. The peptides were crystallized with matrix, acetonitrile and fired by laser for time of flight. The MALDI spectra were acquired at multiplicates and cumulative of peak intensities were represented. The peaks at mass to charge (m/z) ratios above 800 were considered for analysis. The peaks observed were searched against available peak standard to identify the peptide fragments using MASCOT software. The peptides showing significant score were considered and those which gave insignificant score, were processed for MS/MS analysis and further confirmed for peptide. The deduced proteins were further analyzed for interactions by STRINGS software.

Results and Discussion

III. 1. Designing of Ribozyme against hTR

III.1.1. Designing Ribozyme and Visualizing Secondary Structure of Human Telomerase RNA (hTR)

To find out the possible target sites for ribozyme designing, the full length human telomerase RNA was analyzed for its possible secondary structures employing an M Fold program of Michael Zuker. RNA secondary structure is formed through hydrogen bonds between complementary RNA molecules {G-C, A-U, G-U}. These interactions can bring distant complementary sections of one RNA molecule in to close proximity (Zuker 2003).

The "M Fold" was developed in late 1980s and the 'M' simply refers to multiple; the software views possible secondary structure based on minimum free energy (ΔG). The prediction of RNA secondary structures by energy minimization using nearest neighbor energy parameters began with Tinoco and colleagues (1971) and later on by others (Borer et al. 1974, Tinoco et al. 1971, Tinoco et al. 1973, Ulhenbeck et al. 1973 and Delisi and Crothers 1971). The efficient RNA secondary structure algorithms using dynamic programming methods were borrowed from sequence alignment programs developed by a number of different groups (Waterman and Smith 1978, Waterman 1978, Nussinov et al. 1978, Nussinov and Jacobson 1980, Zuker and Stiegler 1981, Sankoff et al. 1983).

The early programs computed single minimum free energy foldings of RNA sequence. The modified version of this was incorporated in University of Wisconsin GCG (UWGCG) package and later on as separate GCG package. The M FOLD server not only predicts secondary structure, it also predicts the hybridization, and melting temperatures which served as separate applications.

III.1.2. Folding parameters

RNA and DNA sequences may be linear or circular; the software by default treats a given sequence as linear. Still circular nucleic acids also can be used for the same. The folding temperature is fixed at 37°C for RNA folding, but any integral temperature may be selected from 0°C to 100 °C.

Ionic conditions may be altered only for DNA, and for RNA, the ionic conditions are fixed at $[Na^{+}]$ = 1M and $[Mg^{++}]$ = 0 M for folding and these are equivalent to

physiological conditions. The choice of salt could be other than Na⁺. So Na⁺ may be considered equivalent to Li $^+$, K⁺ and NH₄ $^+$ while Mg⁺⁺ is equivalent to Ca⁺⁺.

Bases in plotted structure may be annotated by pNum values which represents the number of ways that a base may pair in all foldings with in δδG from the minimum energy. Low values indicate well defined bases, and value 0 or 1 indicates that a base is single stranded or always paired to a unique partner. The number of times that a base is single stranded in a computed folding is called its SS count number (Zuker 2003).

III.1.3. Single Stranded Regions in Muliple Foldings of hTR

To assess the target accessibility by ribozyme, target RNA was analyzed for its optimal and suboptimal secondary structures using M FOLD software package. For targeting we selected GUC sites in hTR regions, and keeping in mind that GUC should fall in single stranded region .This M Fold program generated structures differed minimally in energies for suboptimal foldings. The full length sequence of hTR is represented below.

```
  1 gggttgcgga gggtgggcct gggaggggtg gtggccattt  tttgtctaac cctaactgag

 61 aagggcgtag gcgccgtgct tttgctcccc  gcgcgctgtt tttctcgctg  actttcagcg

121 ggcggaaaag cctcggcctg ccgccttcca  ccgttcattc  tagagcaaac aaaaaatgtc

181 agctgctggc  ccgttcgccc ctcccgggga cctgcggcgg gtcgcctgcc cagcccccga

241 accccgcctg gaggccgcgg tcggcccggg gcttctccgg aggcacccac tgccaccgcg

301 aagagttggg ctctgtcagc  cgcgggtctc tcggggggcga gggcgaggtt caggcctttc

361 aggccgcagg aagaggaacg gagcgagtcc ccgcgcgcgg cgcgattccc tgagctgtgg

421 gacgtgcacc  caggactcgg  ctcacacatg c
```

The table 3.1 below shows the GUC sequences of hTR transcript that were used for folding. We have selected a few GUC of hTR and analyzed for conserved structures of single stranded regions. The foldings generates multiple secondary structure of target which displays varying degrees of differences in folded substrates. However,

some of the sequences still show conservation of their structural components at different energy levels. High conservation of these structures among suboptimal secondary structures increases the possibility of existence of true single strandedness of target. First we analyzed the SS count which determines the frequency of certain bases being single stranded at multiple foldings.

1	37'-5'- CCATTT TTT GTC TAAC CCTAA-3' 55
2	171-5'-CAAAAAAT GTC AGCTGCT -3' -187
3	215-5'-CGGCGG GTC GCCTGCC-3'-230
4	307-5'-TTGGGCTCT GTC AGCCGC-3'-323
5	318-5'- GCCGCGG GTC TC TCGGG-3' 335
6	381-5' GAGCGA GTC CCCGCGC -3'

Table 3.1: The Presence of GUC in full length sequence of hTR shown in table for secondary structure analysis for its possible sub optimal structures.

The present analysis performed 21 folding at different energy levels and represented the frequency of single stranded sequence at multiple foldings. The SS count results of hTR shows the bases after 32 till 62nd base and 360-400 base to have wider window of single strandedness. We chose the GUC sequence at 44th and 180th nucleotide with in a single stranded region for designing ribozyme.

The structures were analyzed with truncated form of sequence in which 225 base sequence were analyzed for single stranded regions (Fig 1A). The result shows the GUC bases at 44 and 180th position falls 16 times as single stranded, and same is repeated with full length hTR sequence of 451 bases (Fig 1B). In such a long substrate RNA, the position of 44 and 180 shows same frequency of single strandedness. Other structures found to be in single stranded regions were at the bases of $360-400^{th}$ base regions. One of GUC falls in such region may also fold in to unpaired regions, and this window has sequence stretches with highest probability of assuming single stranded forms. Further the structures created out of multiple foldings were viewed as plots for conserved secondary structures.
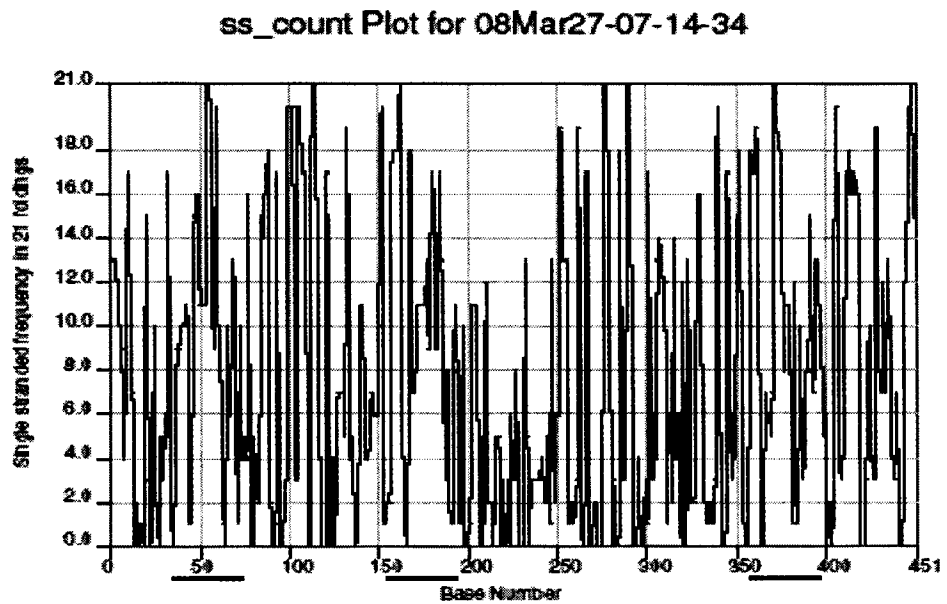
A)
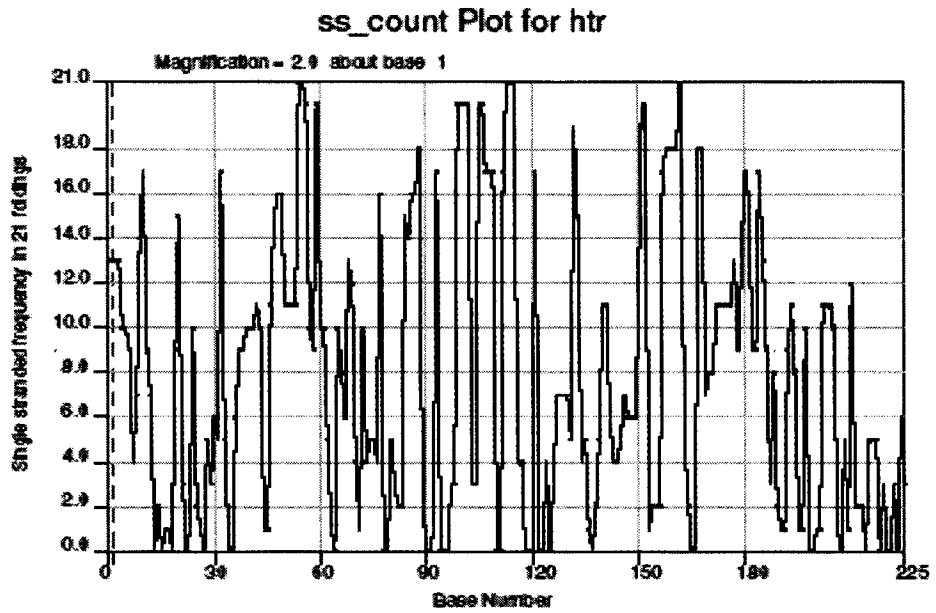
B)

Fig 3.01: Analysis of single strandedness of bases and its frequency in multiple folding of A) hTR 225 bases and B) 451 full length of hTR. The GUC at 180th position falls 16 times as single stranded structure. Bars below the base numbers are the stretch of window having single stranded sequence (40-60,150-200,350-400).

III.1.4. RNA Fold Finds Conserved Secondary Structures of hTR

Some of the selected GUC's seem to be conserved at structural level and the one finally chosen was 180th position of GUC. Out of 21 structures more than 7 structures were found to have (Fig 3.02-13) conserved structures at different energy levels.

The foldings from Fig 3.02-3.13 show alternative folding in which the selected GUC at 180th position was found to be in non base paired region except at 4 incidences out of 21 foldings and also found to have similar structures at different energy levels. The foldings of subset sequences show the sequence with conserved secondary structures (Fig 3.14: A, B, E, F) and dynamic nature of 180th sequence being single stranded (fig3.14: G, I, J) for its full access of ribozyme bindings.

The selected GUC at 180th nucleotide was further folded after appending the designed ribozyme, and as expected the ribozyme finds its target by base pairing with flanking arms of GUCs of 180th position (Fig 3.16 and 3.17). Such designed ribozymes further has to be tested for invitro cleavage activity.

III.1.5. Folding of Ribozyme Appended with hTR Sequence

Once the target sequence has been folded and analyzed for conserved secondary structures, it is necessary to fold along with ribozyme sequence designed to check the ribozyme binding with its designated target sequence. The appended ribozyme has to find its target and bind the flanking arms of target GUC sites sequence. The result shown in the folding pattern reveals that the ribozyme at a few energy levels assumes its structure and binds the target site sequence specifically. The target sites in Fig 3.15 shows the sequences of hTR at 180 nt and Fig 3.16 and 3.17 show the ribozyme binding with target site sequence specifically.

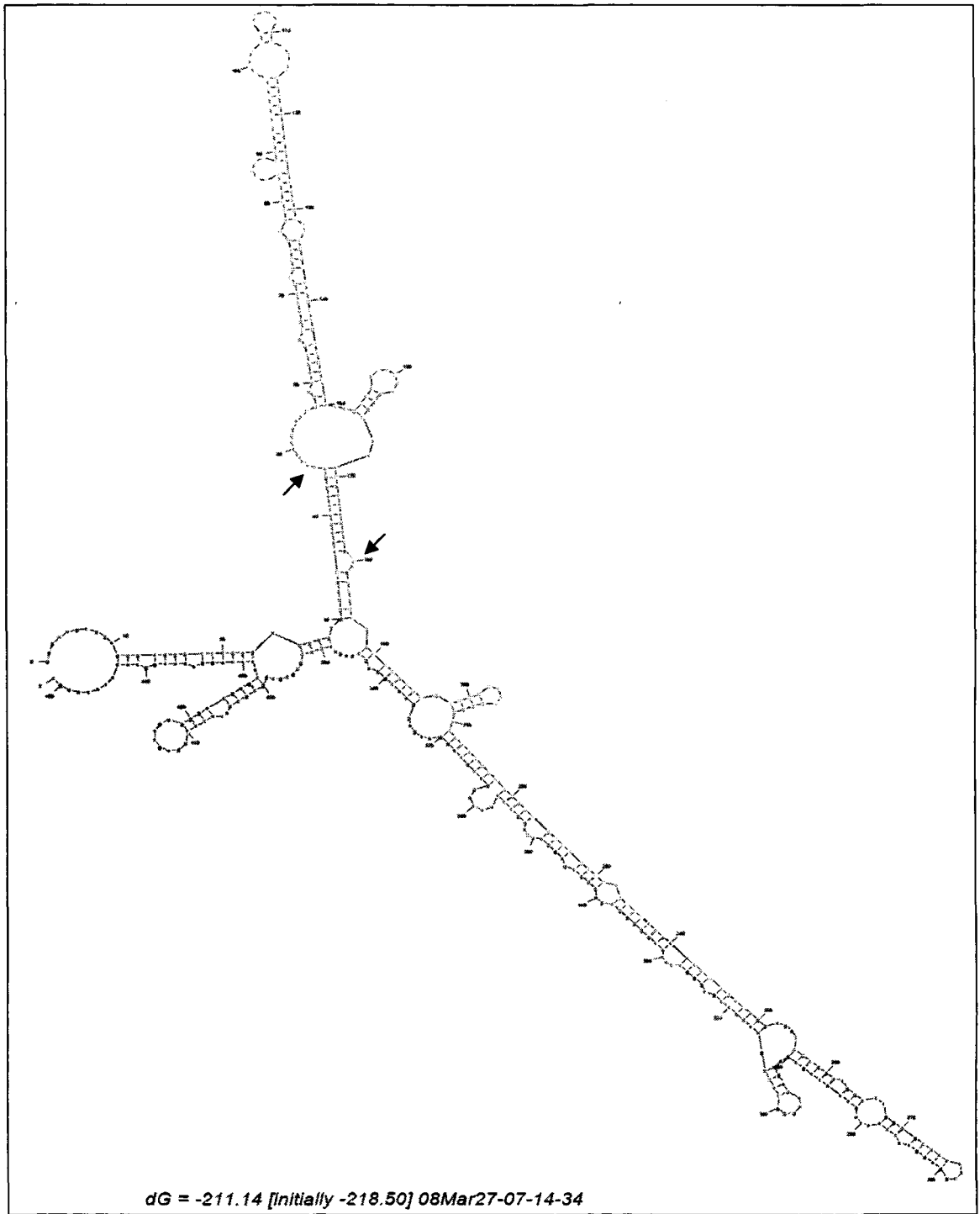*dG = -211.14 [Initially -218.50] 08Mar27-07-14-34*

Fig 3.02: Folding of full length hTR showing the different structures including bulge, loops, internal loops. The presence of GUC in loops are shown by arrow which may serve as target.

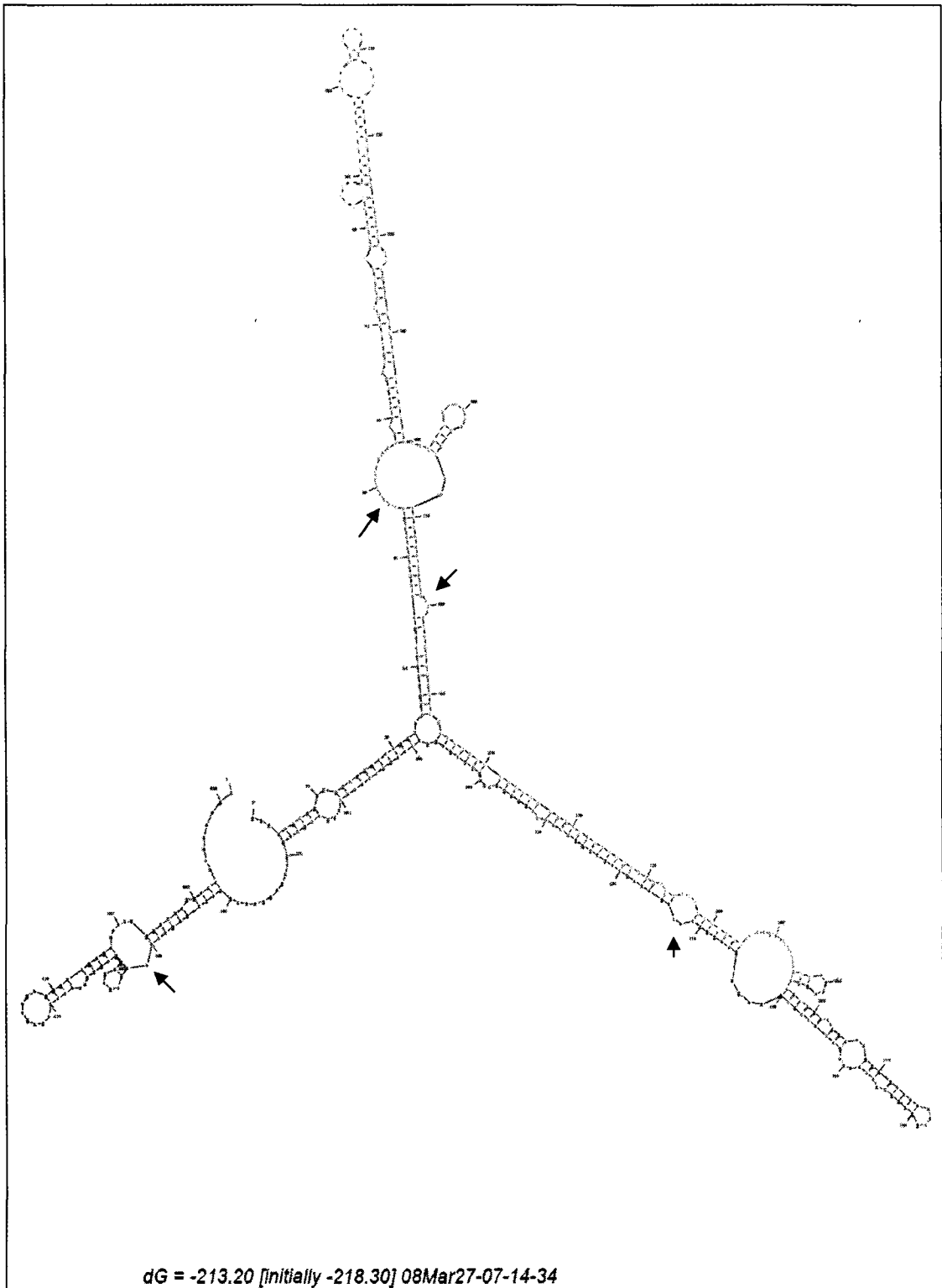dG = -213.20 [initially -218.30] 08Mar27-07-14-34

Fig 3.03: Folding of hTR showing bulges at reducing folding energy levels. Multiple folds are to compare the conserved structures of possible target sequences. Targets are shown by arrows.
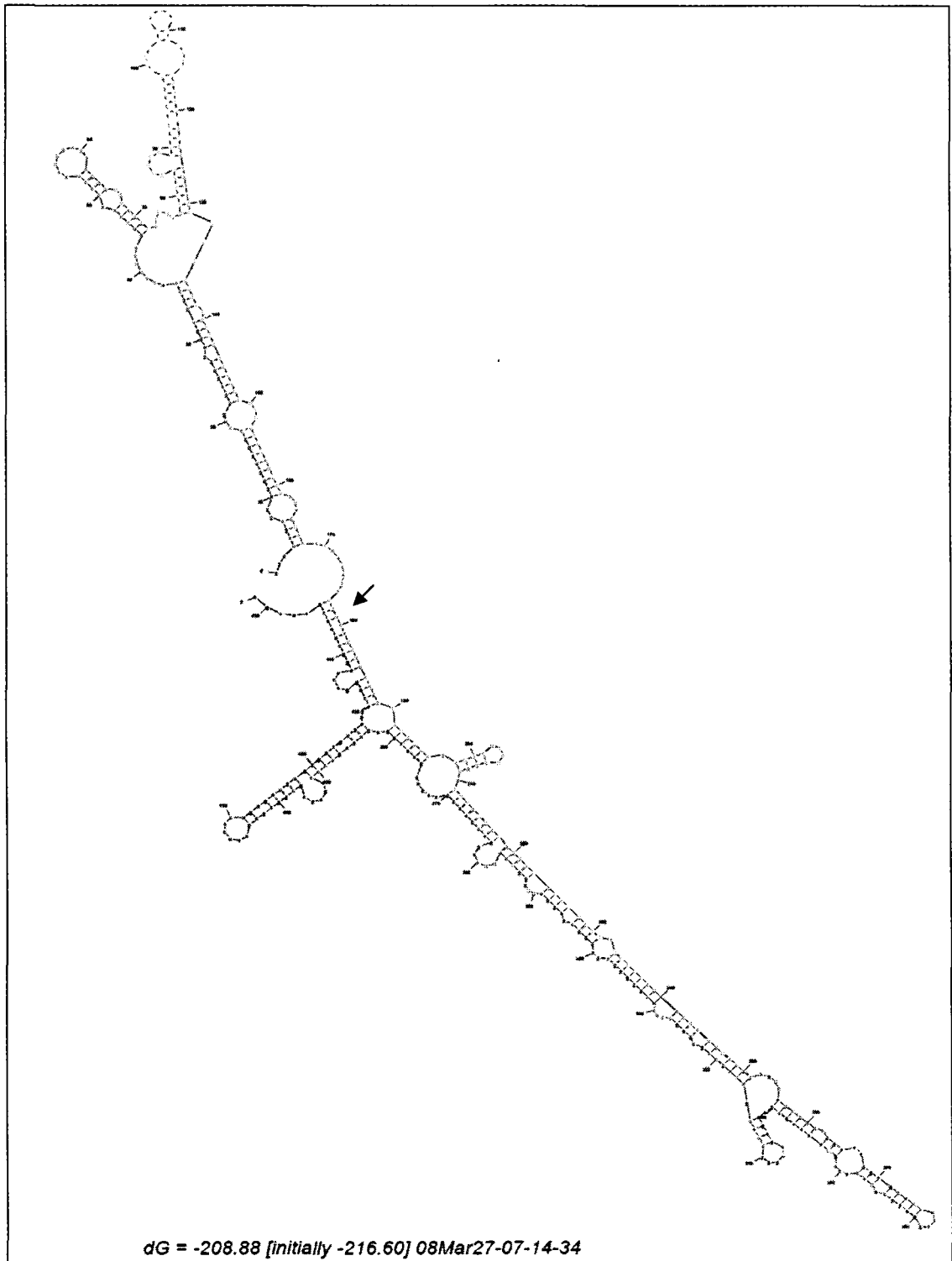
dG = -208.88 [initially -216.60] 08Mar27-07-14-34

Fig 3.04: folding of hTR at ΔG of -208.88 kcal/mole for comparing the conserved secondary structures.

dG = -209.91 [initially -216.50] 08Mar27-07-14-34
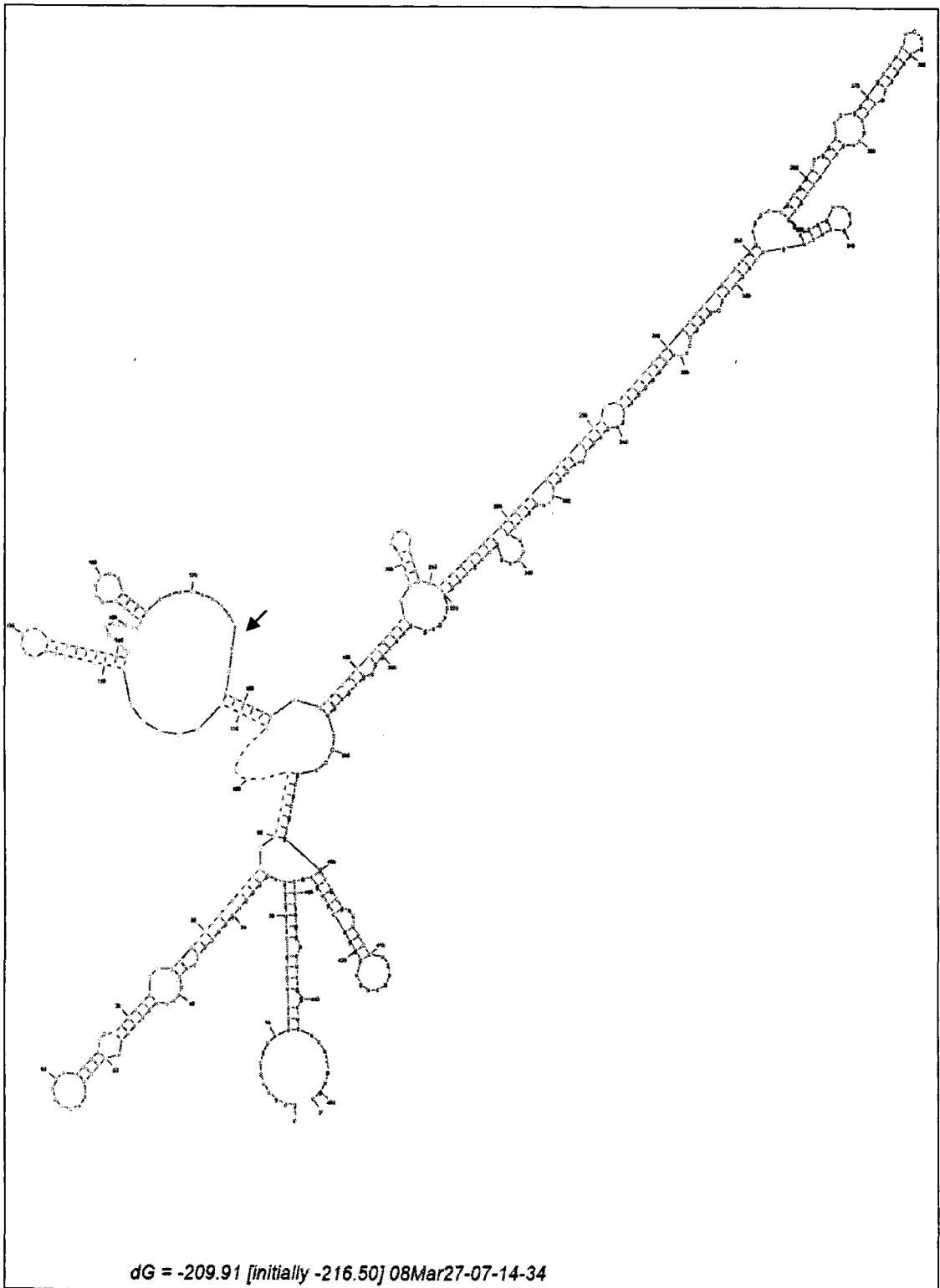
Fig 3.05: Folding of hTR at ΔG = -209.91 k cal mol^{-1} the predicted target site forms base pairing at this energy levels. But the flanking sequences are found to be single stranded.

dG = -208.44 [initially -216.30] 08Mar27-07-14-34
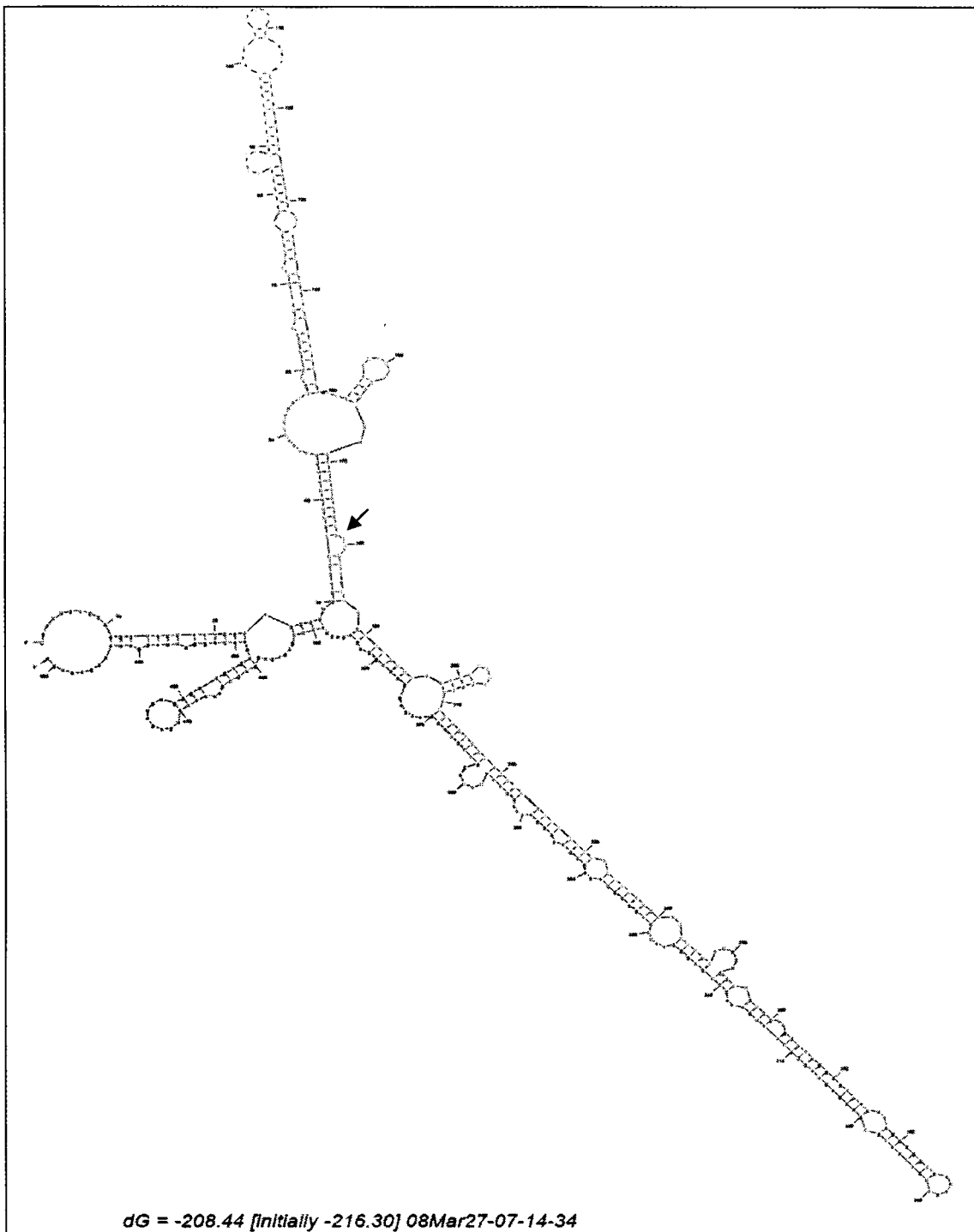
Fig 3.06: Folding of hTR at ΔG = -208.44 kcal mol⁻¹ shows the conserved structures of non base paired region of GUC at 180 the position shown with arrows.

dG = -210.94 [initially -216.30] 08Mar27-07-14-34
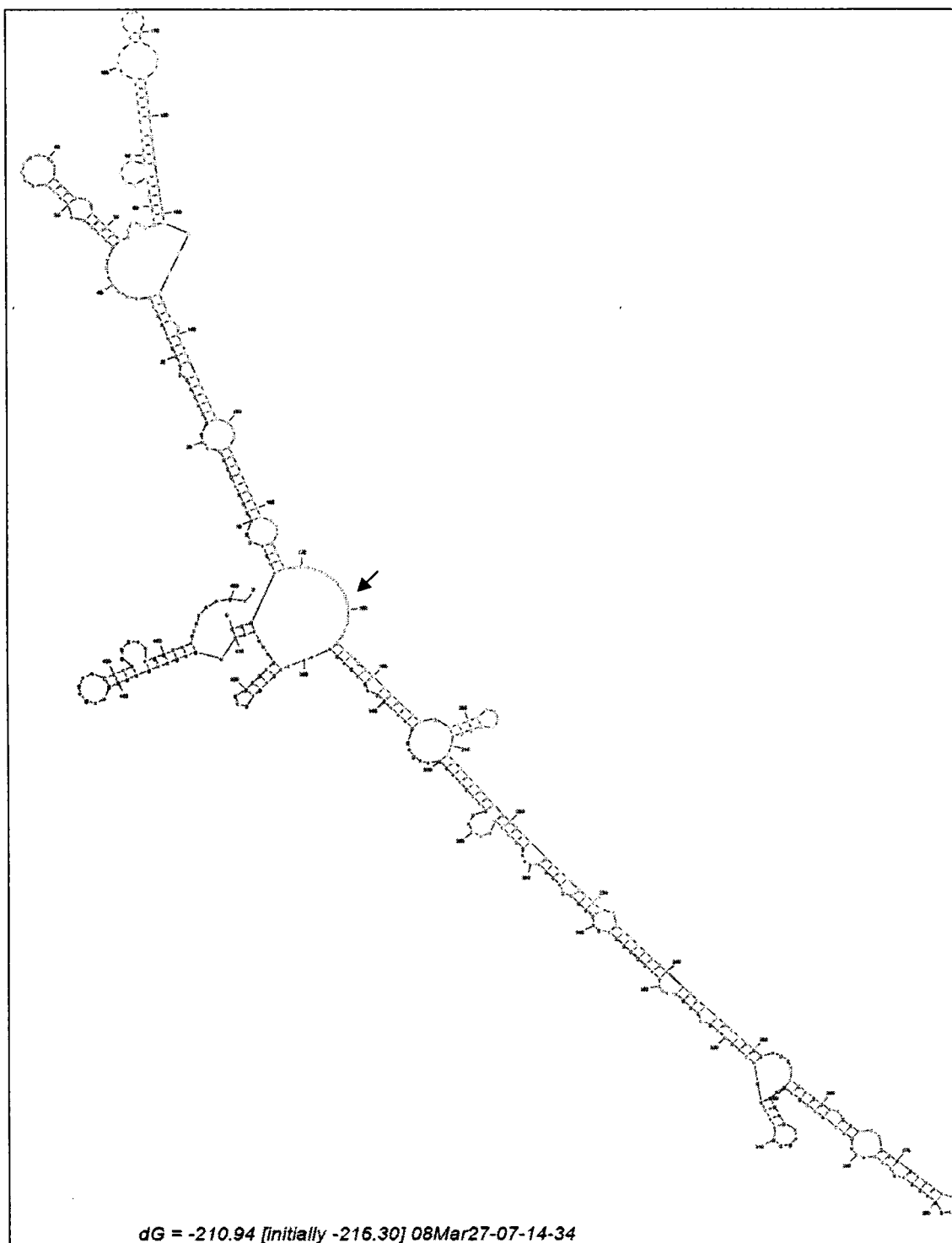
Fig 3.07: Folding of hTR at ΔG = -210.94 kcal mol⁻¹ which shows the accessibility of GUC at 180th position shown with arrows.

dG = -207.58 [initially -216.20] 08Mar27-07-14-34
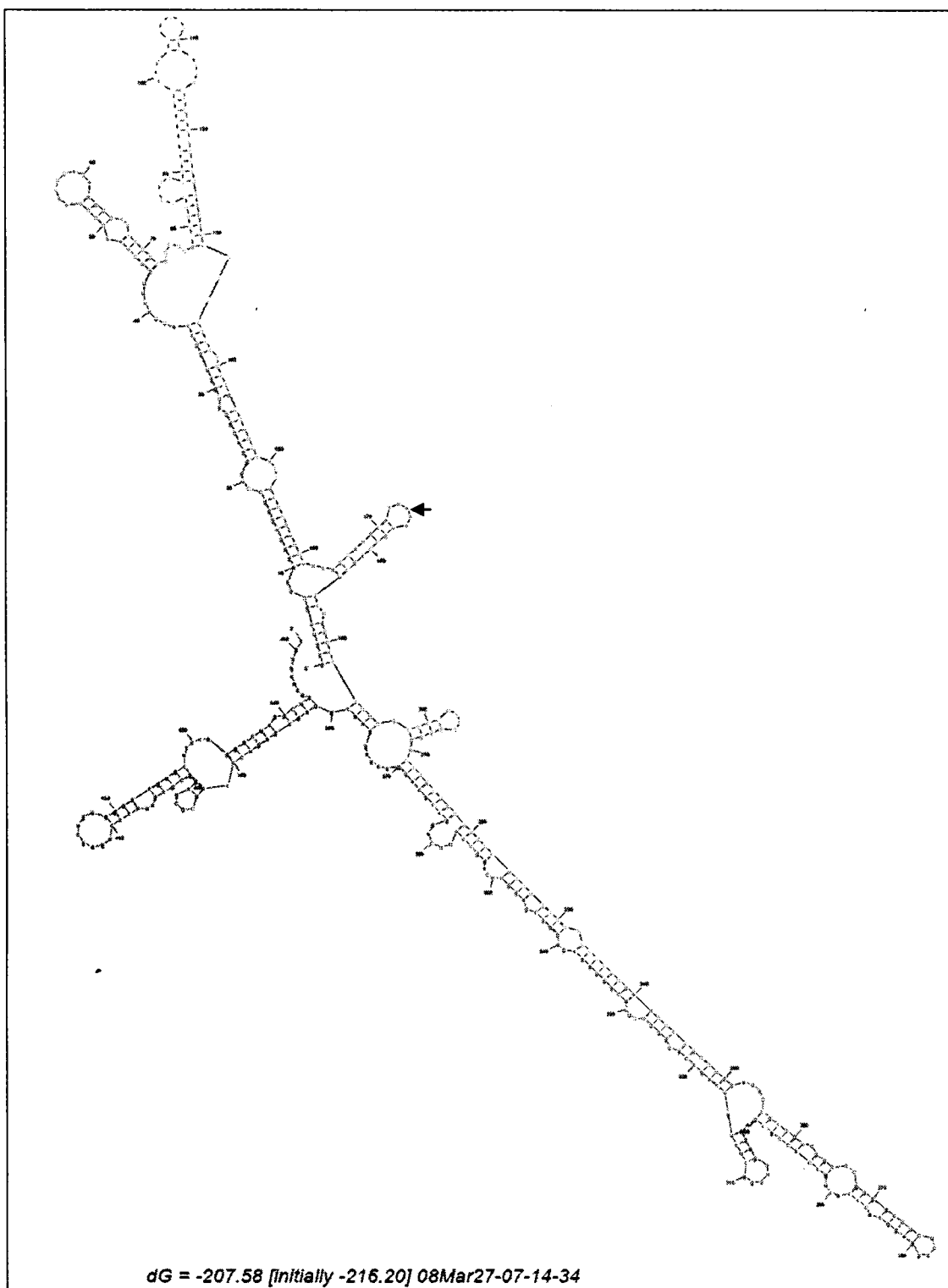
Fig 3.08: Folding of hTR at ΔG = -207.58 kcal mol^{-1}, showing the presence of selected GUC at stem but in non base paired bulge region

dG = -210.80 [initially -215.80] 08Mar27-07-14-34
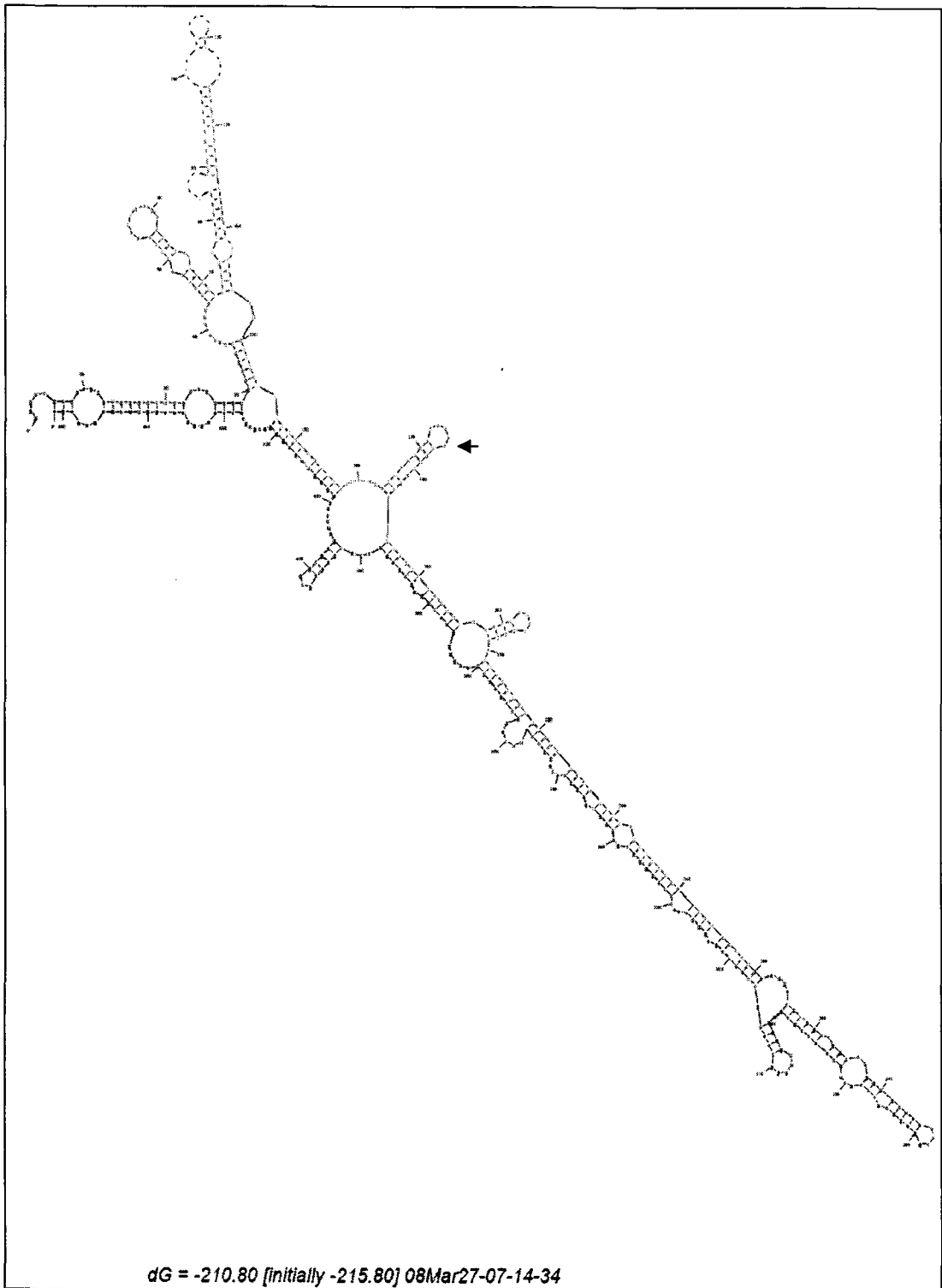
Fig 3.09: Folding of hTR at ΔG = -210.80 kcal mol⁻¹ shows the conserved single stranded ness of GUC though present in stem of the loop structures

dG = -210.77 [initially -215.70] 08Mar27-07-14-34
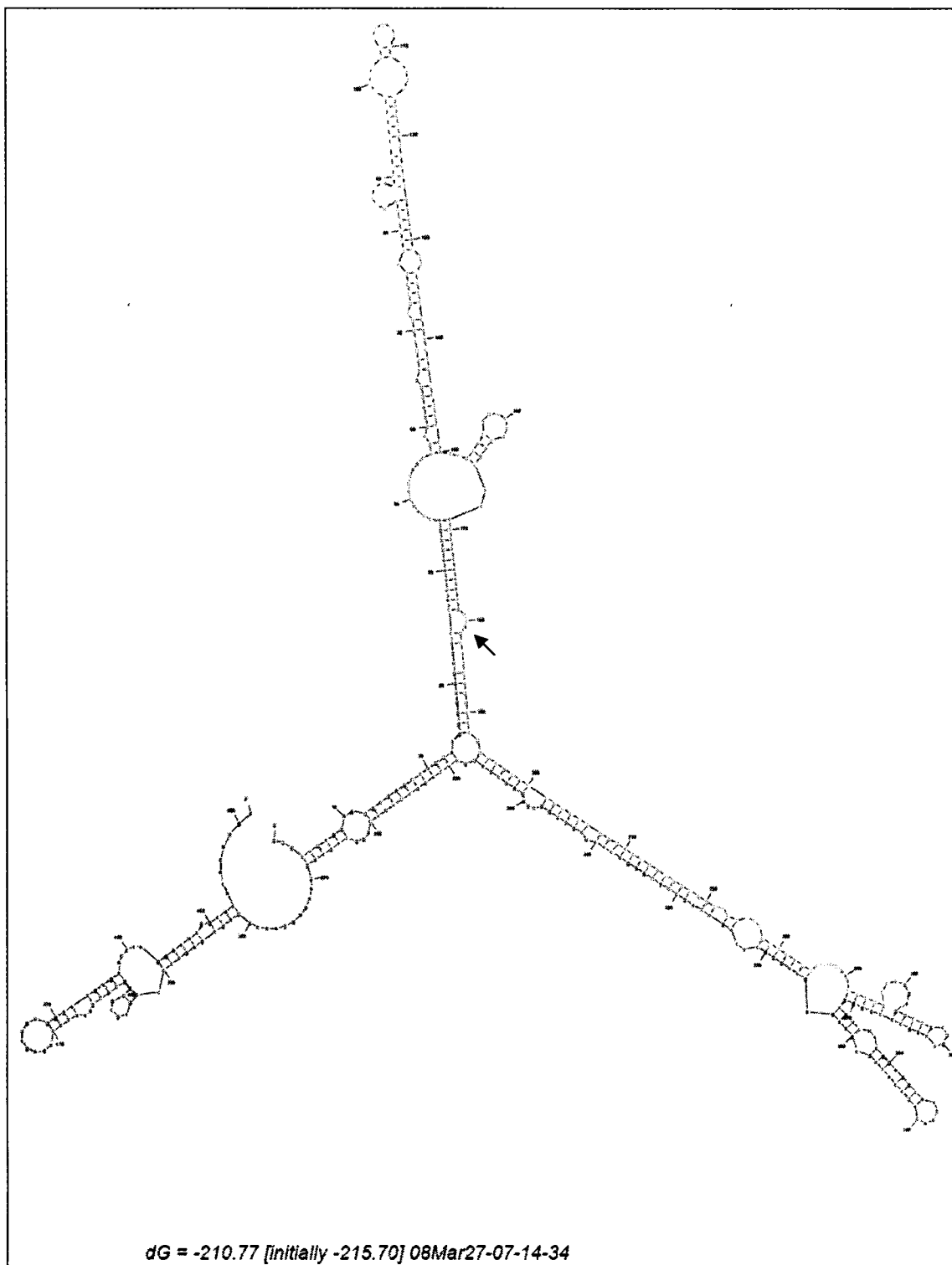
Fig 3.10: Folding of hTR at ΔG = -210.77 k cal mol^{-1} showing conserved 180th position of GUC

dG = -205.01 [initially -215.50] 08Mar27-07-14-34
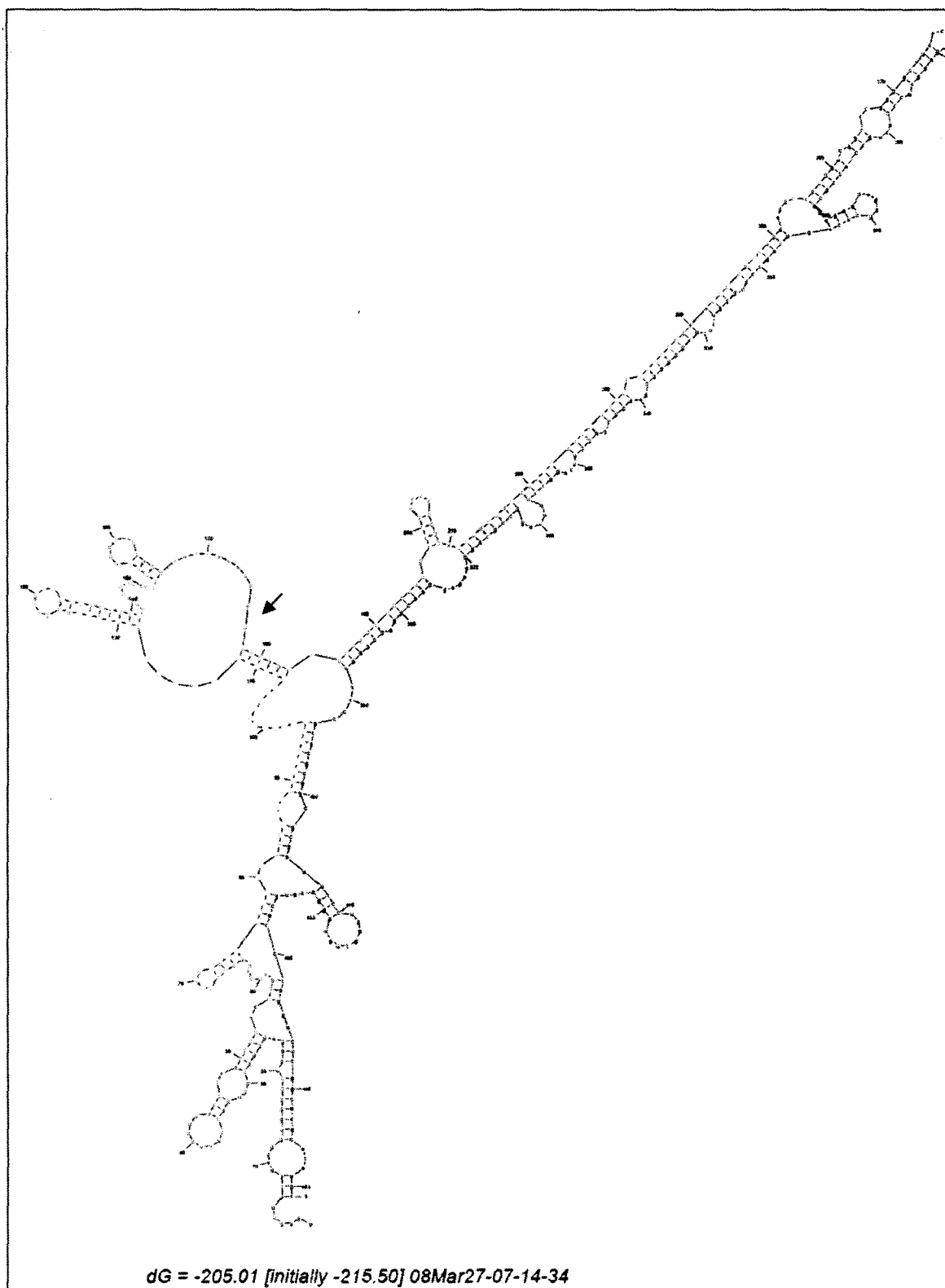
Fig 3.11: Folding of hTR at ΔG= -205 kcal mol⁻¹ showing selected GUC site at single stranded regions shown by arrow.

dG = -202.98 [initially -213.10] 08Mar27-07-14-34
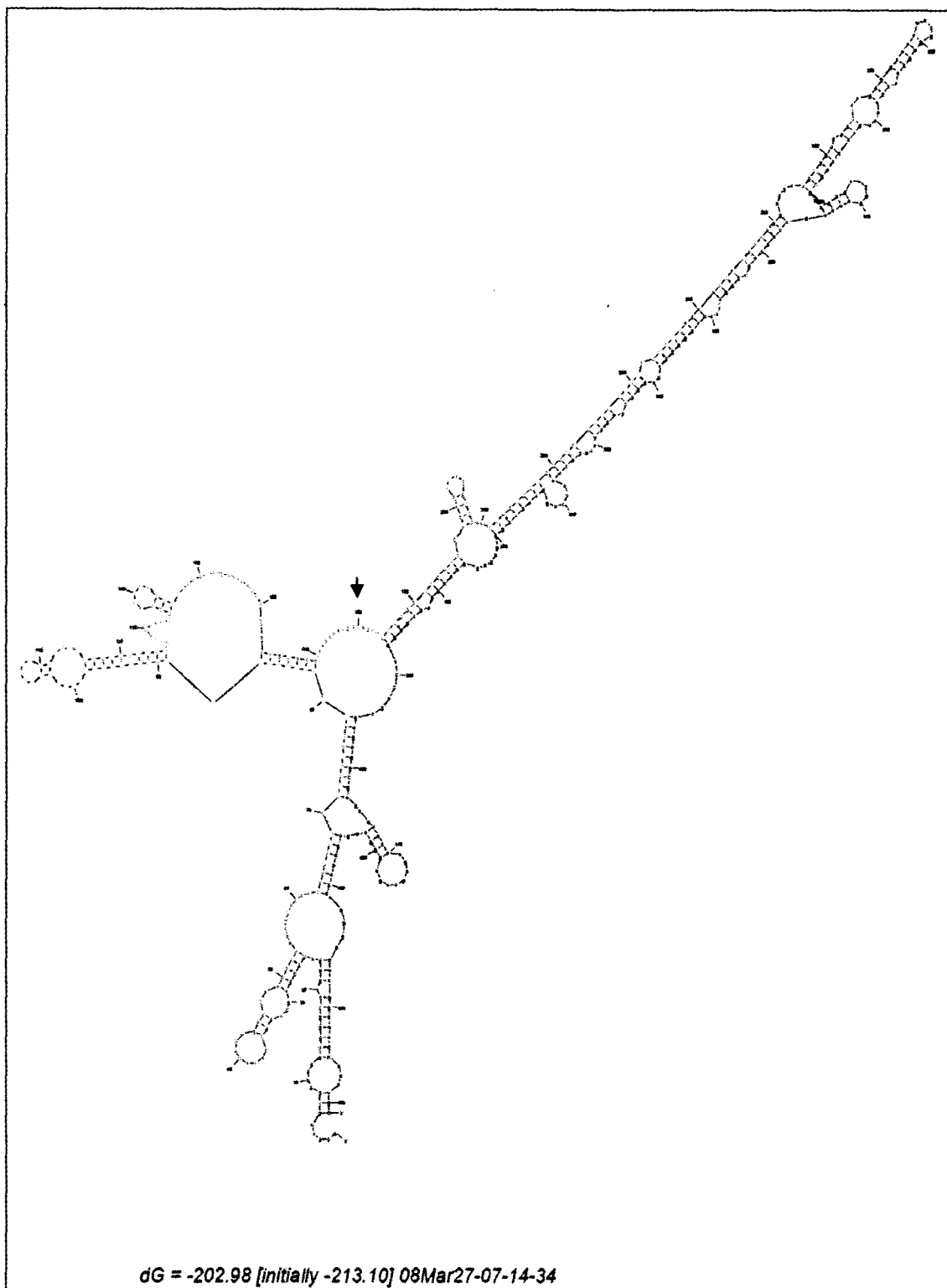
Fig 3.12: Folding of hTR at ΔG = -202 kcal mol^{-1} showing the conserved single stranded ness of GUC at 180th position shown by arrow.
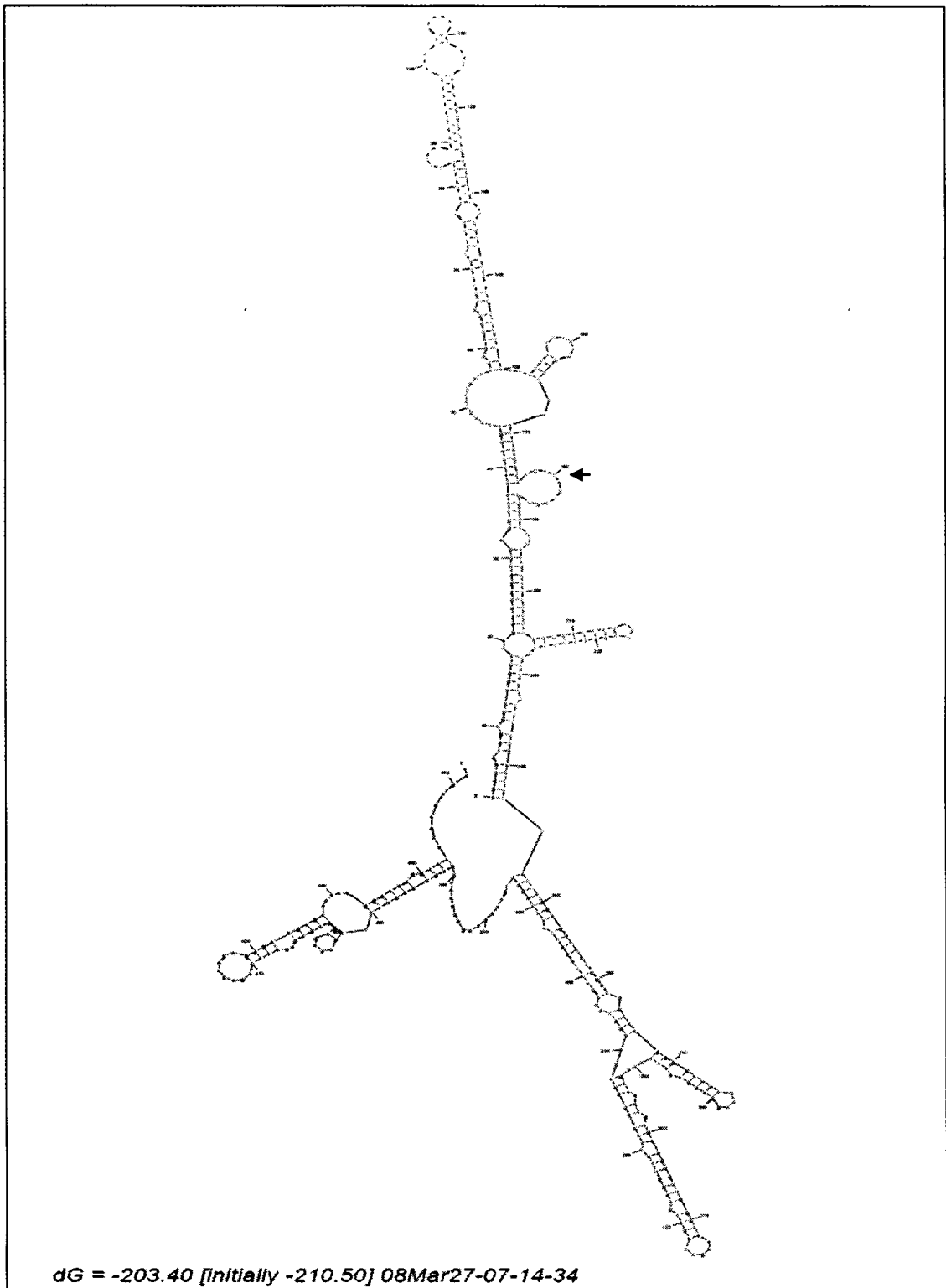
dG = -203.40 [initially -210.50] 08Mar27-07-14-34

Fig 3.13: hTR folding at ΔG = -203 kcal mol^{-1} showing the single stranded ness of 180th nucleotide shown by arrow.
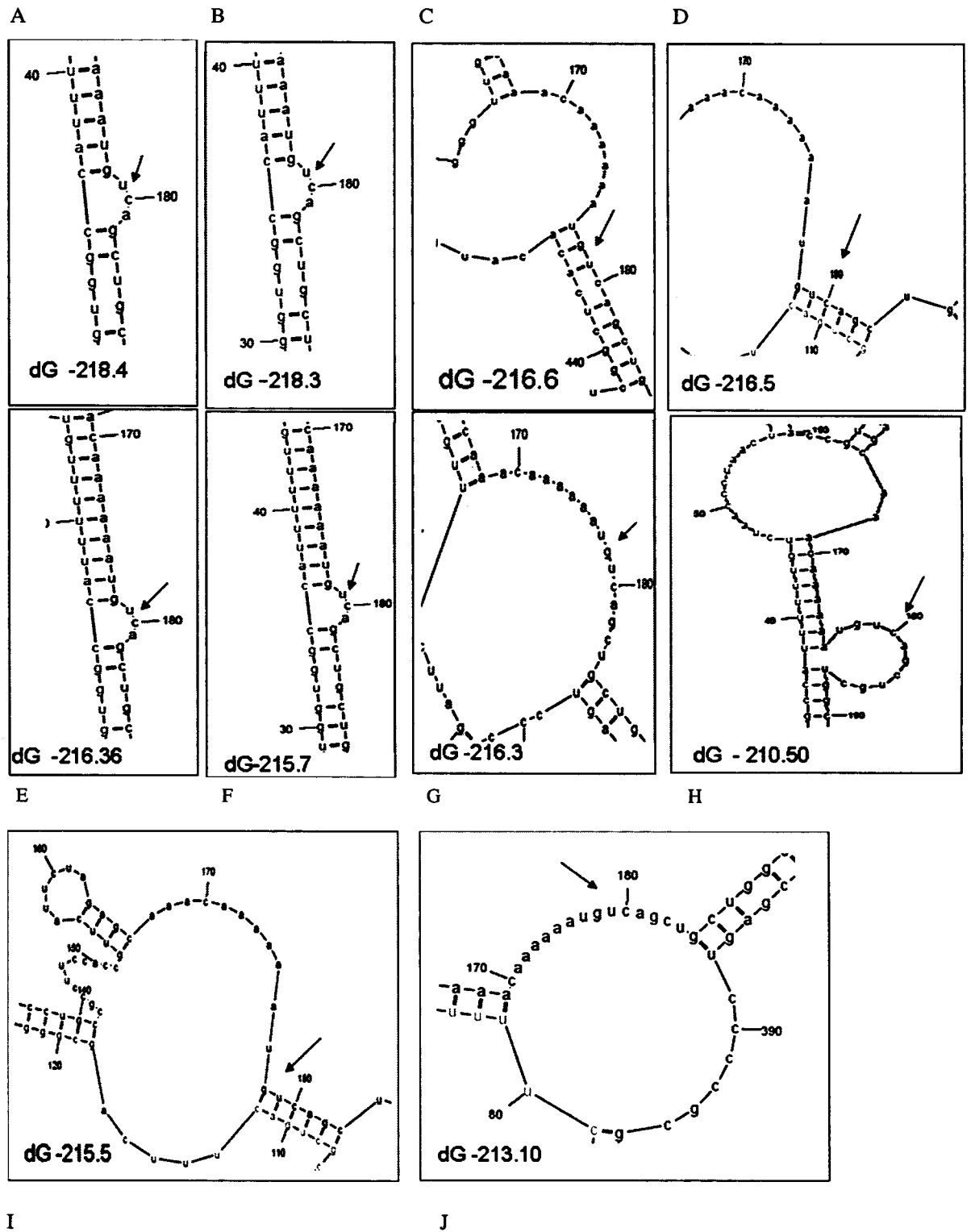
Fig 3.14: Conserved structure of hTR with respect to GUC at 180th position and its single stranded form in most of the foldings (A-J).
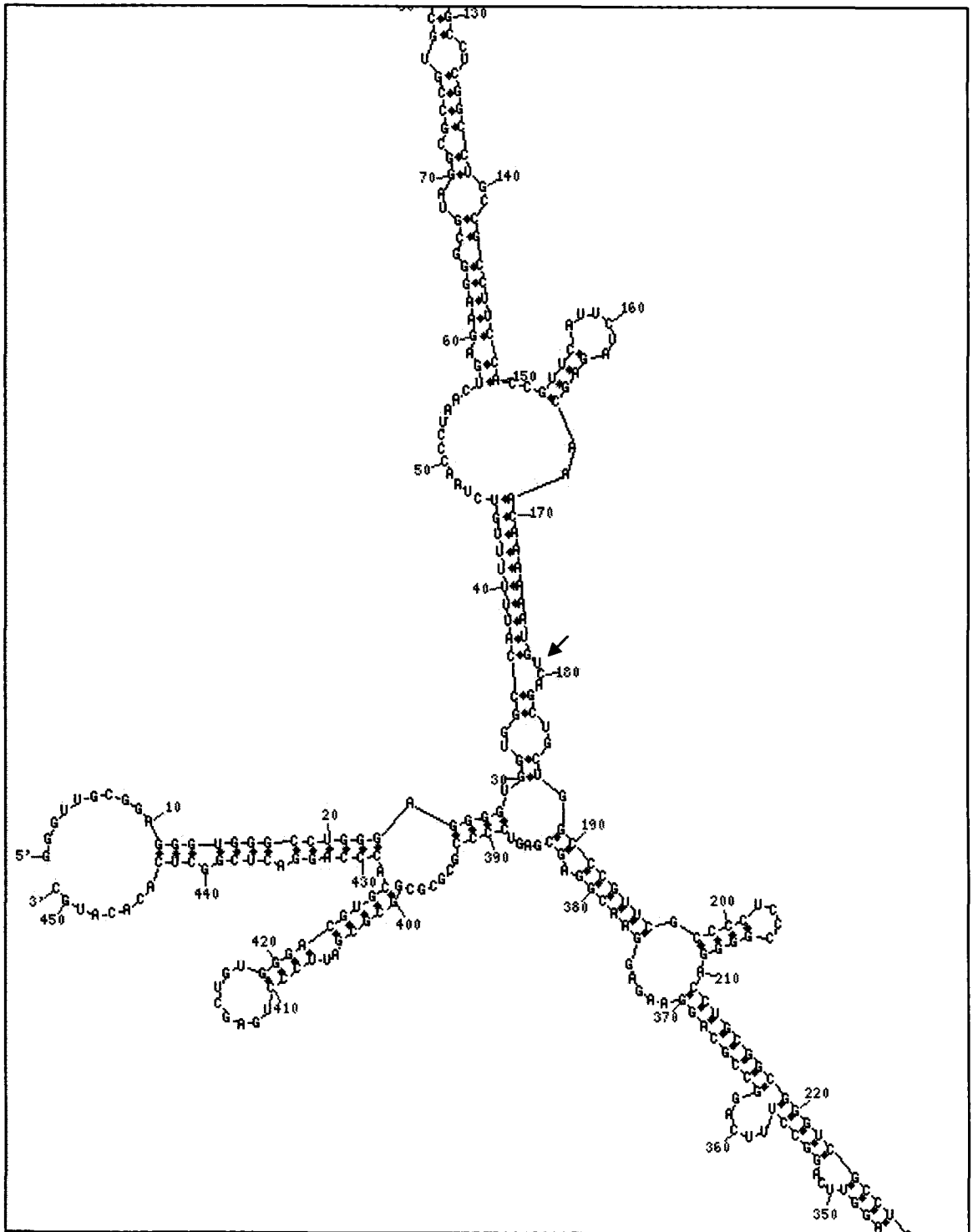
Fig 3.15: Folding of hTR and 180th position shown clearly as conserved single stranded internal bulge sequence.
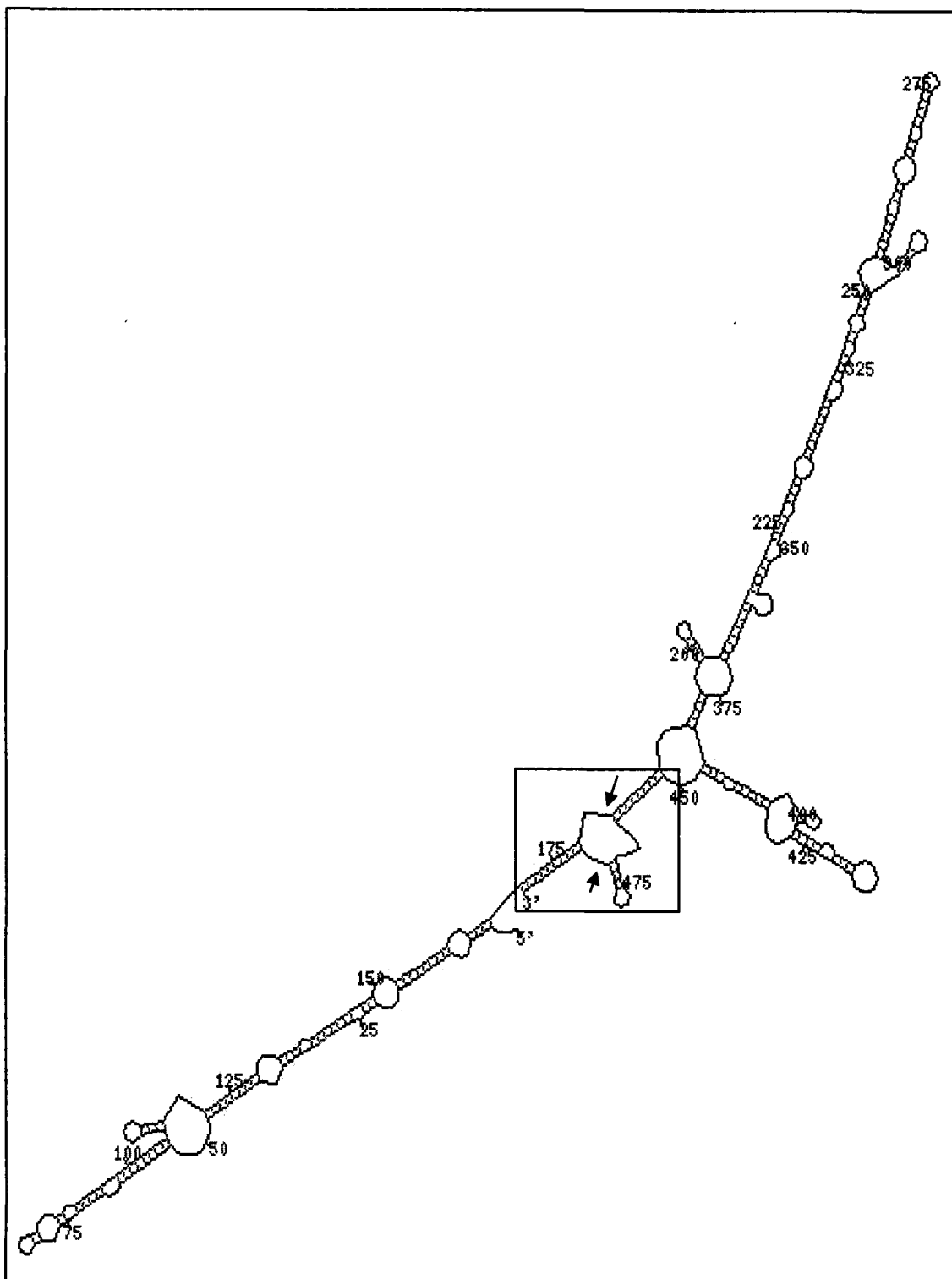
Fig 3.16: Folding of hTR appended with designed ribozyme finding its target at 180th nucleotide sequence specifically, highlighted in box and shown with arrows.
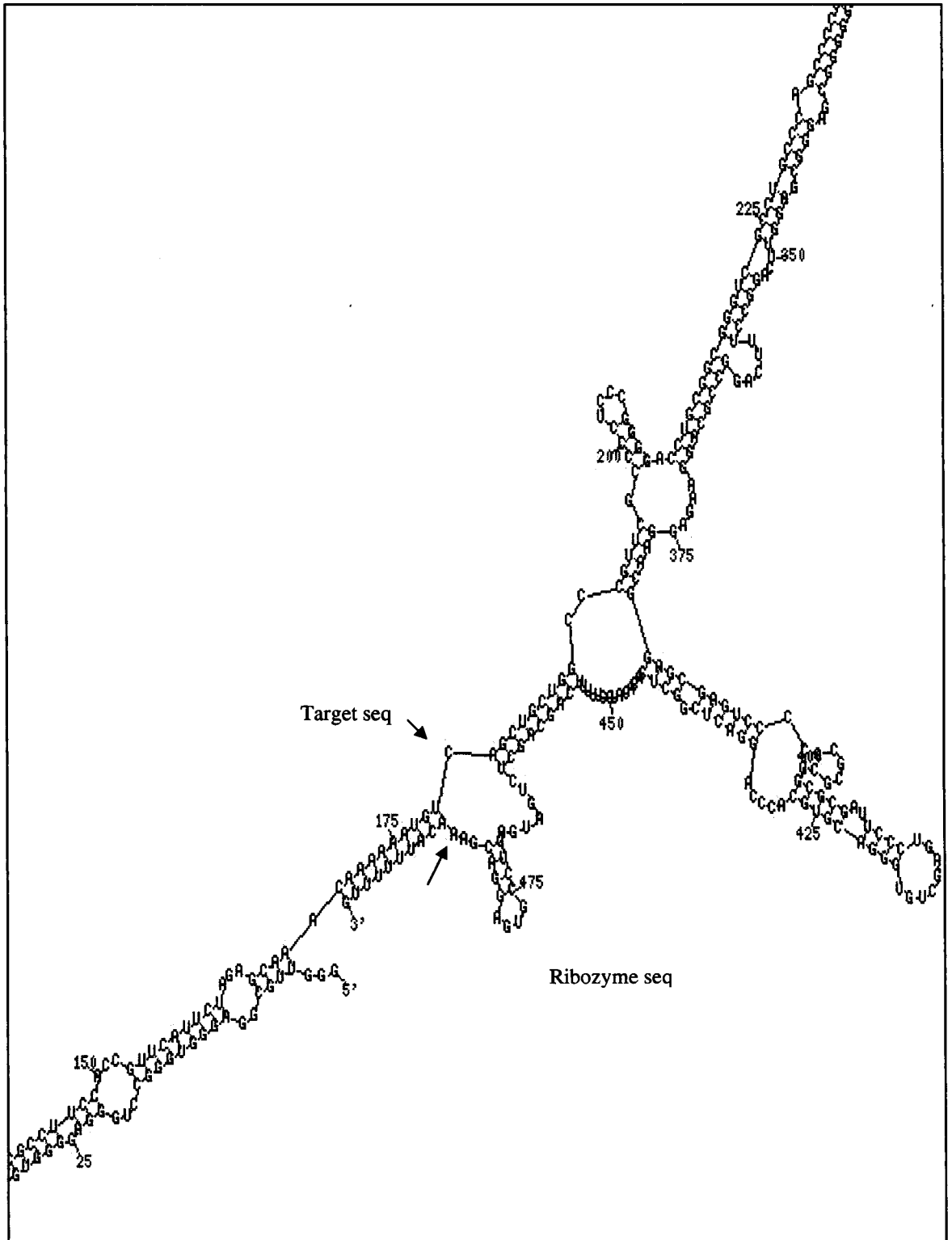
Fig 3.17: Folding of hTR appended with designed ribozyme targeting 180th nucleotide showing its sequence specific target binding.

III. 1. 6. Discussion

Folding the hTR sequence at varied energy levels shows a few conserved single stranded regions. The sequence around 40-52nd nt nucleotides showed highest possible single stranded region in the sequence. At 52nd nt it shows the highest probable single stranded ness, but absence of GUC at these region prevents its use as target. The structure of bases at or around 180th GUC nucleotides found to be conserved throughout multiple foldings.

In the present study we concentrated on targeting the catalytically relevant regions of hTR in order to inactivate telomerase. However, it will be interesting to target other regions of hTR (nt 44, 380) functioning as template, signal sequence respectively.

Though the target sequence at 44th nt also seems to be good target, still it lacks conservation of structures at different energy levels, which prompted to take 180th rather than 44th nt as target sequence. The sequence at 3' end of the hTR is omitted since these sequences participate as signal sequence for localization of hTR rather than catalytically important. Studies have proved that the truncated version of hTR (32-224) can very well act as template and is essential and sufficient for catalytically active telomerase complex *in vitro*. The pseudoknot sequence also has a role in dimer formation of hTR and the region 180 is found to be highly dynamic in nature and could serve as a better target for ribozyme targeting. The sequence of ribozyme appended hTR showed sequence specific targeting and could be manipulated for further analysis *in vitro* and *in vivo*.

Further folding of hTR appended with ribozyme shows the specificity at different energy levels though it also fails to identify its target sequences at few energy levels. The folding at Fig 3.16 and 3.17 confirms the sequence specificity of designed ribozyme against the 180th nt of hTR target sequence. The sequence of hTR at 180th 5'-GUC-3', the ribozyme pairing and cleavage sites are shown with arrow.

III. 2. Cloning of Ribozyme and hTR

III.2.1. Cloning of Ribozyme under T7 Promoter (pStuI vector)

To assess designed ribozyme, it has to be verified *in vitro* for its cleavage efficiency. To make ribozyme transcripts *in vitro*, the ribozyme- coding oligos were cloned under T7 or T3 promoter. To minimize the vector sequences co transcribed along with ribozyme, a modified bluescript vector with StuI restriction site overlapping with the transcription intiation nucleotide with reference to T 7 promoter was used (Yadava and Yadava 2000). The StuI digestion creates blunt ends for cloning of ribozyme coding sequences for creation of precise ribozyme transcripts *in- vitro* which would be free from vector sequences. As an alternative, ribozyme coding oligos with T7 promoter were used for the same purpose.

III.2.2.Ribozyme Cloning and Sequencing

For cloning, the ribozyme coding oligos were end labeled, annealed and purified from non denaturing polyacrylamide gel following electrophoresis. The oligos contain degenerate base at conserved catalytic center of ribozyme (A/C) to clone wild type and mutant ribozyme (Fig 3.18, 19). The clones were further screened by digesting with Pvu II enzyme which creates 400 bp fragment in original constructs without insert and with insert it creates 440 bp (Fig 3.20A, B). In Fig 3.20A, lane 1 and 3 shows the presence of insert i.e., 440bp and lane 2 shows the absence of insert. In Fig 3.20B, lane 3 shows the presence of insert and other lanes represents clones without insert. The digested clones that show the differences in mobility when running in gel were considered for further confirmation with sequencing. The sequencing of positive clones shows the conserved sequences of ribozyme and mutant ribozyme (Fig 3.21).
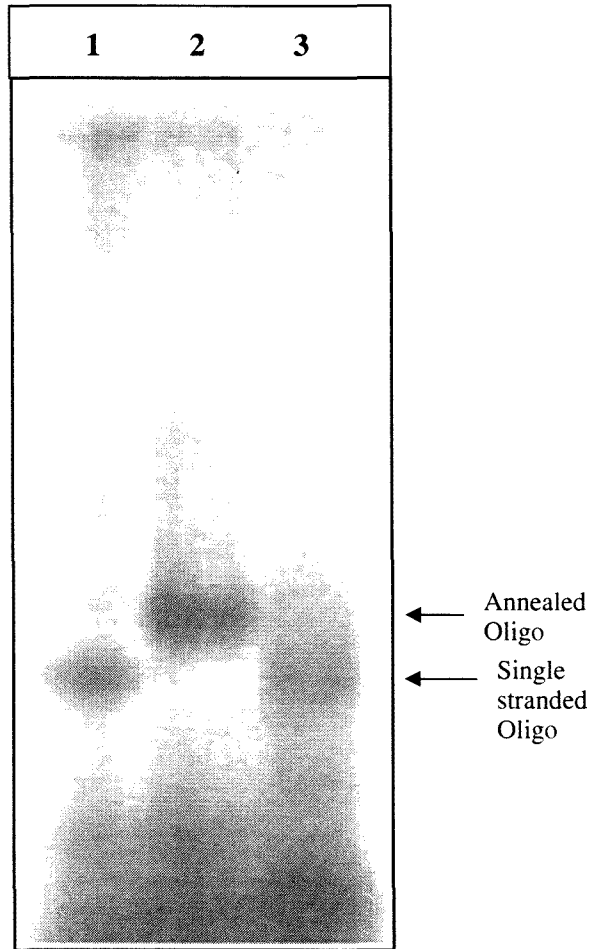
Fig 3.18: Ribozyme cloning in pStuI vector shown schematically. The StuI site is used for cloning the duplexed oligos corresponding to sense and antisense sequence of the ribozyme with conserved catalytic sequence shown in red and blue colors respectively). M* is an 'A' in wild type and a 'C' in mutant ribozyme .
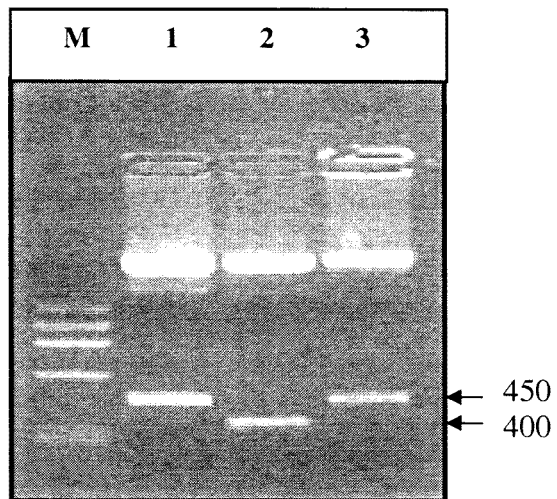
Fig 3.19: Electrophoresis of + and - strand oligonucleotide and their duplex through non-denaturing PAGE gel. Lanes 1 and 3 are sense and anti sense respectively and Lane 2 is annealed oligo showing shift compared to unannealed single strand oligos. The part of annealed oligo was eluted from gel for cloning purposes (15% non denaturing acrylamide gel).
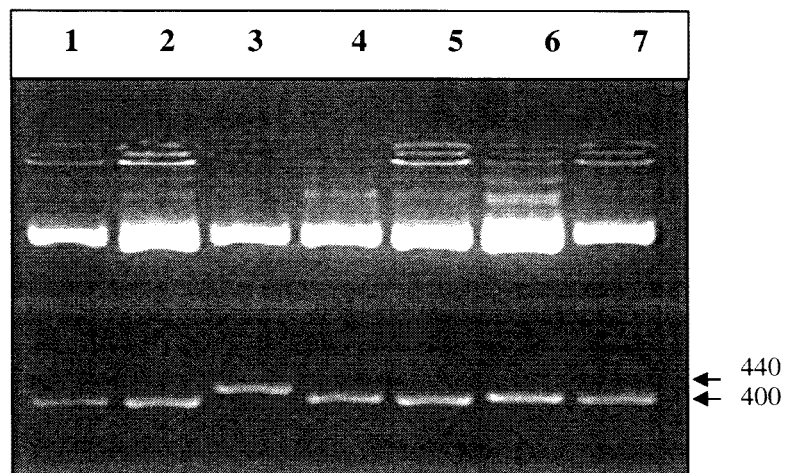
A)

B)

Fig 3.20:   A) Representative picture of Screening patterns upon digestion with PvuII for presence of insert. Presence of insert has shown in lanes 1 and 3. B) Lane 3 shows larger Pvu II fragment than the vector and was verified by sequencing.
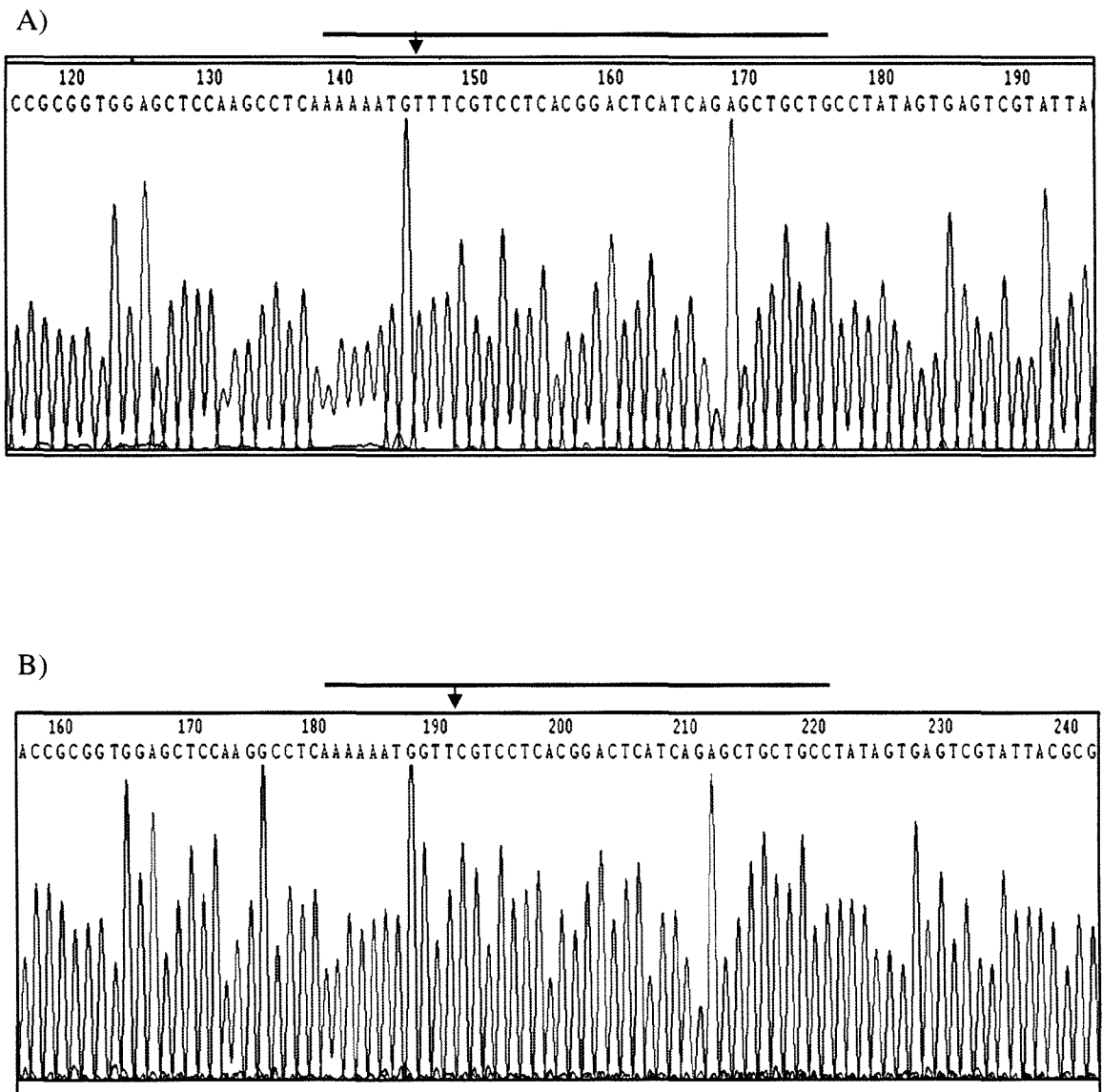
A)

B)

Fig 3.21: Sequencing complementary strands of ribozyme clones shown positive from restriction digestions. A) Sequencing of active ribozyme from T3 promoter site, showing the 5' GTTTC 3' (corresponding to 5'GAAAC 3' in the wild type ribozyme) and B) Sequencing of clone shows 5' GGTTC3' (corresponding to 5'- GAACC-3' in the mutant ribozyme).

III.2.3.Cloning of Human Telomerase RNA Component

The full length cDNA for human telomerase RNA component was prepared by using RNA from HeLa cells, amplified with telomerase RNA specific primers. The cDNA samples were further reamplified with modified temperature regime to avoid the co-migrants, which were found in cDNA samples (Fig 3.22A, B). The PCR products were purified and cloned in pGEM- T vector which contains T7 and SP6 promoter flanking the cloning site (Fig 3.23).

The hTR clones were screened by sequencing and confirmed with NCBI's BLAST to confirm the sequences (Fig 3.24). The hTR was found to be cloned in reverse orientation with respect to T7 promoter, and sense strand with SP6 promoter. Sense strand transcripts of hTR for ribozyme cleavage assays, could be generated by SP6 RNA polymerase.

III. 2. 4. In vitro Cleavage by the Designed Ribozyme

To assess the designed ribozyme's catalytic efficiency, *in vitro* cleavage assay were performed. The Constructs bearing ribozyme inserts were linearized and used for generating transcripts labeled with $\alpha^{32}P$ UTPs. For monitoring cleavage products, generally the target sequences were labeled and ribozymes unlabeled. The transcripts were purified by G-50 column and quantitated in terms of specific radioactivity.

The equimolar amounts of transcript or 2 fold to 10 fold ribozymes (100 f mole target and 1000 fmol of Rz) were used to test the cleavage. The transcript of ribozyme and target sequences were mixed and denatured shortly and made to anneal slowly at 37°C. Addition of 10 mM $MgCl_2$ provides catalytic efficiency to ribozyme and cleavage reaction is started at this point. The cleavage reaction was set for 3 hours. Fig 3.28 shows the truncated version of target RNA created with T7 appended primer and transcribed and cleavage reaction set with active Rz and mutant Rz. The mutant ribozyme serves as control. The cleavage of truncated hTR of 85 base produces 69 and 16 base RNA fragments confirming the cleavage activity of the ribozyme (Fig: 3.25). The present ribozyme 180 (Rz180) was taken for study because ribozyme against the template region of telomerase RNA has been found to be effective (Yokayama et al. 1998) in cell culture studies and already proven.
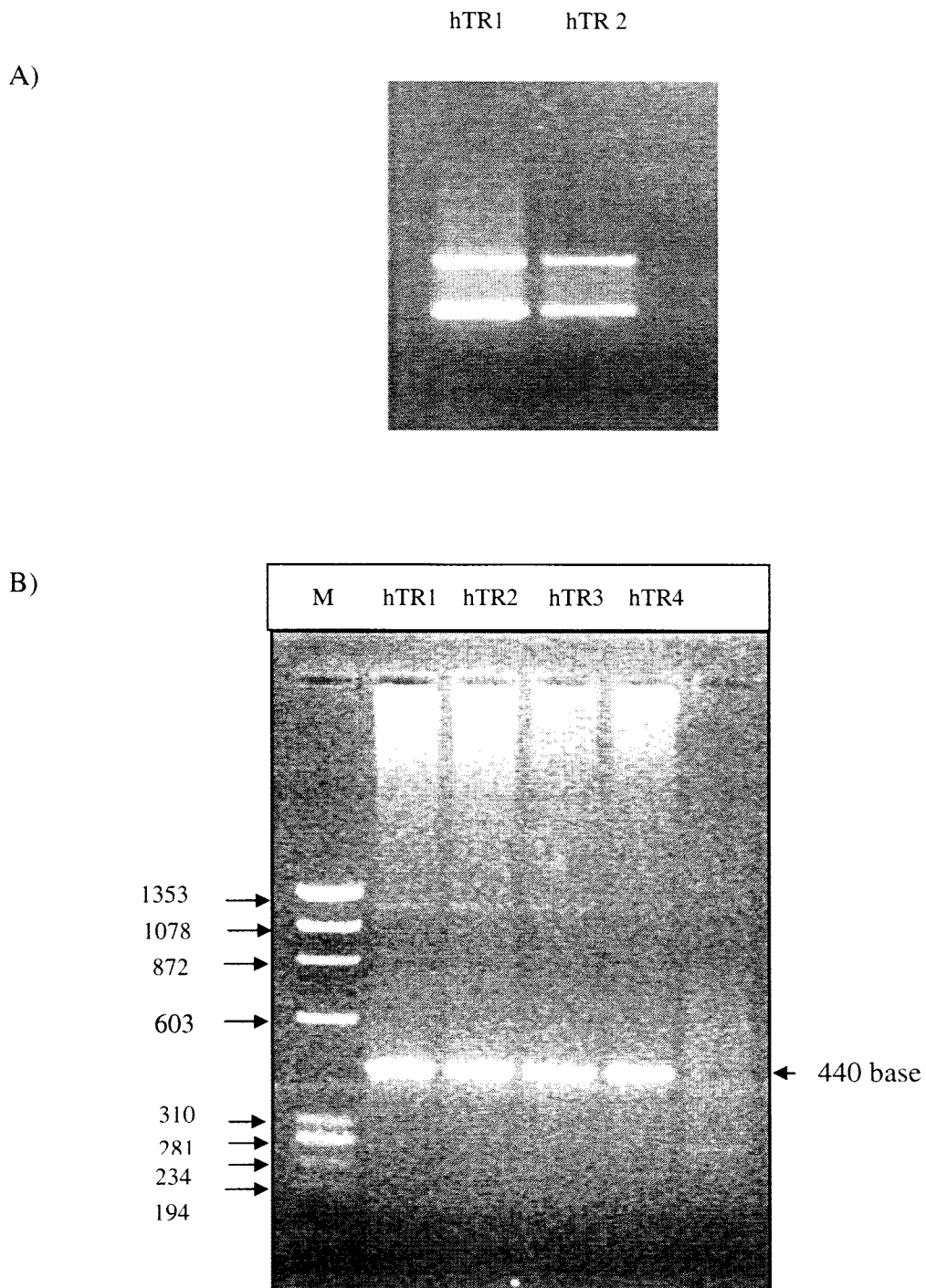
Fig 3.22: A) Typical pattern of RT-PCR amplicons of hTR showing co amplification of unprocessed transcript. B) Re amplification of cDNA with modified annealing temperatures. The reamplified products were used for cloning in pGEM-T vector.
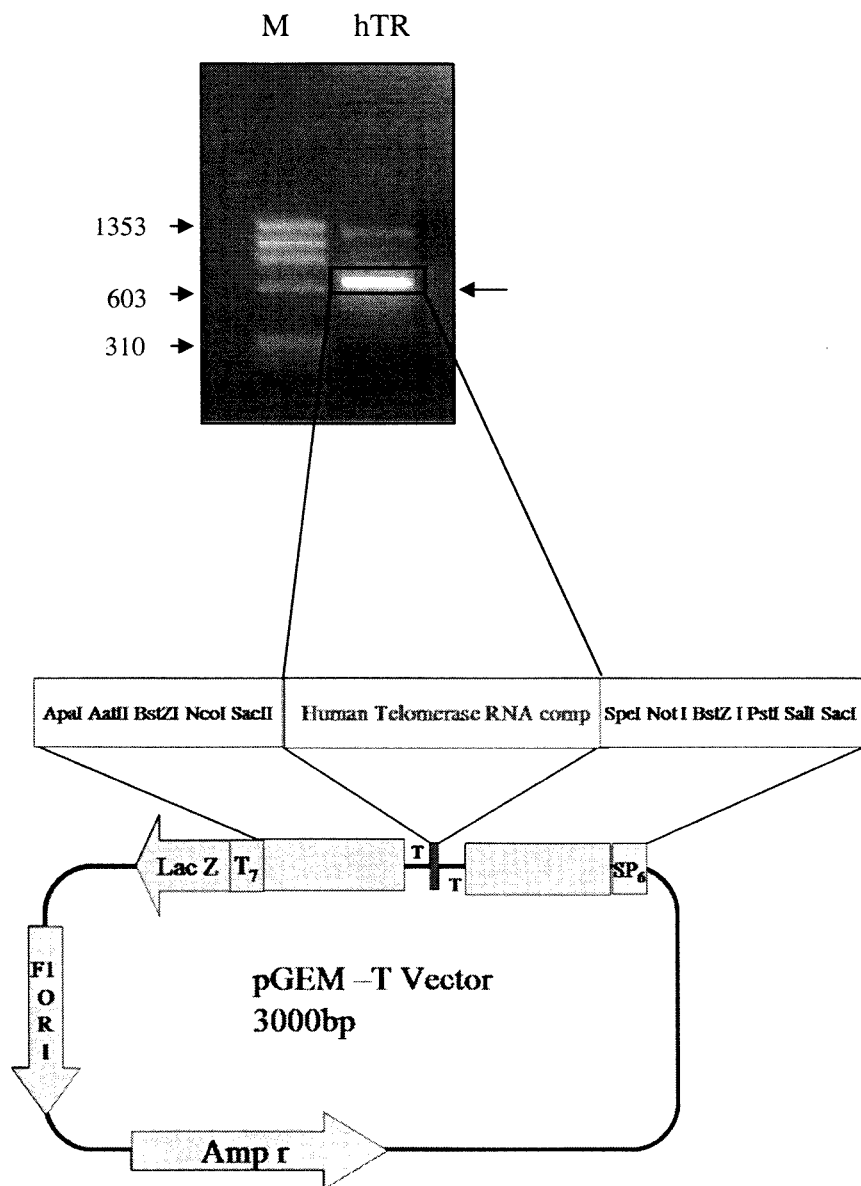
Fig 3.23: Cloning of Full length Human Telomerase RNA component (hTR). The gel picture shows the RT PCR product used for cloning.

TGGCGCGCGATGTCCGNCCCGTGGCCGCGGGATACTCCAGCCTGTGTGAGCCGAGCCTGGGTGACGTCCCACAGCTCAGGGAATCGCGCCGCGCGCGGGGACTCGCTCCGTTCCTCTTCCTGCGGCCTGAAAG
10   20   30   40   50   60   70   80   90   100   110   120   130   1·

GGCCTGAACCT CGCCCTCGCC CCCGAGAGAC CCGCGGCTGA CAGAGCCCAA CTCTTCGCGG TGGCAGTGGG TGCCTCCGGA GAAGCCCCGG GCCGACCG:G GCCTCCAGGC G
40   150   160   170   180   190   200   210   220   230   240   250

GGGTTCGGG GGCTGGGCAG GCGACCCGCC GCAGGTCCCC GGG AGGGGCG AACGGGCCAG CAGCTGACAT TTTTTGTTTG CTCTAGAATG AACGGTGG AAGGCGGCAGGC CGA
260   270   280   290   300   310   320   330   340   350   360

GGCTTTT CCGCCCGCTG AAAGTCAGCG AGAAAACAGC GCGCGGGGAG CAAAGCACGG CGCCTACGCCCTTCTCAGTT AGGGTTAGACAAAAATGGCC AATCACTAGTGCGGCCGCCTGCAGG
370   380   390   400   410   420   430   440   450   460   470   480
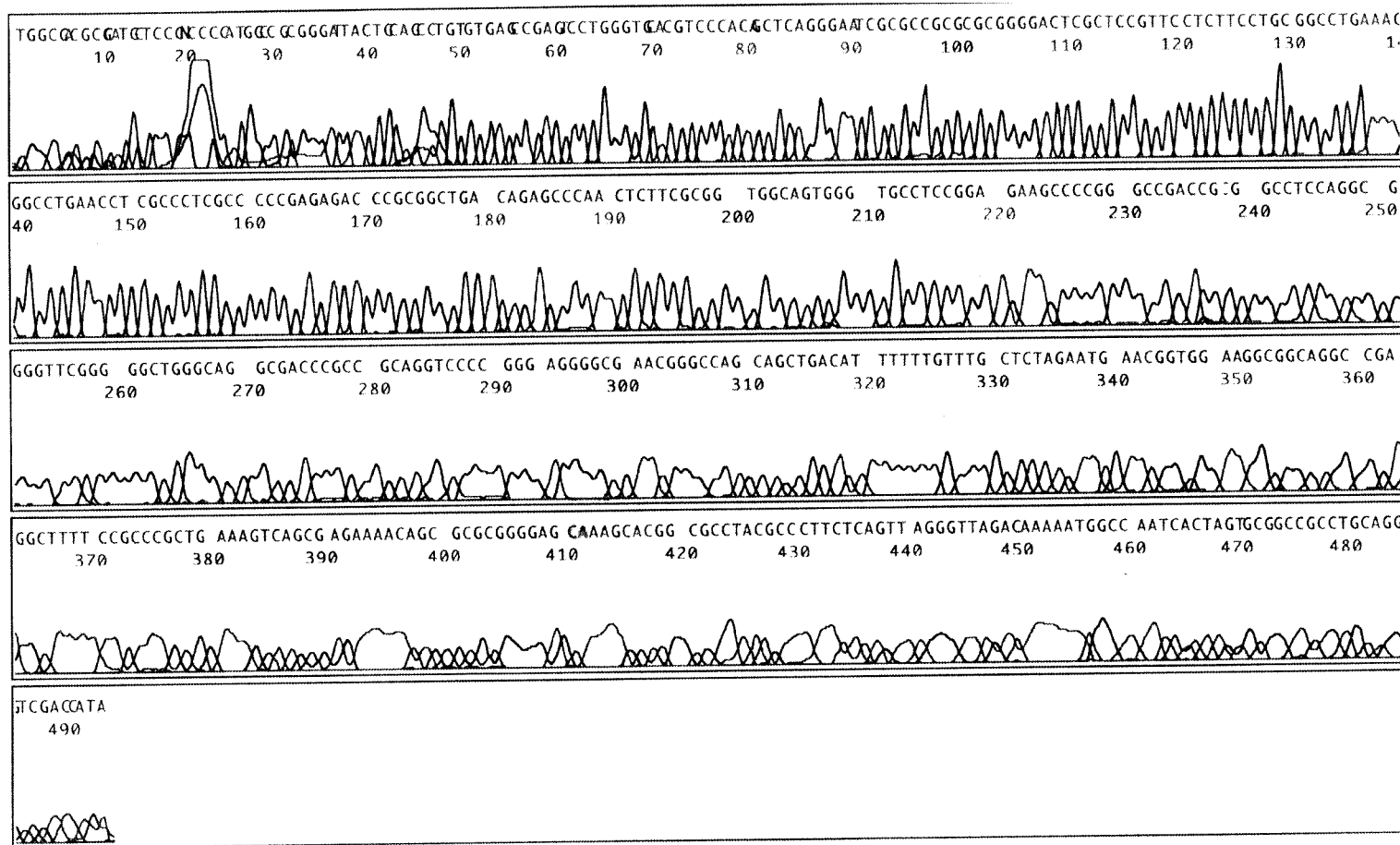
GTCGACCATA
490

Fig 3.24: Sequencing of Cloned full length human telomerase RNA, using T7 primer. In this particular clone, hTR cDNA is cloned in reverse orientation with respect to T7 promoter.
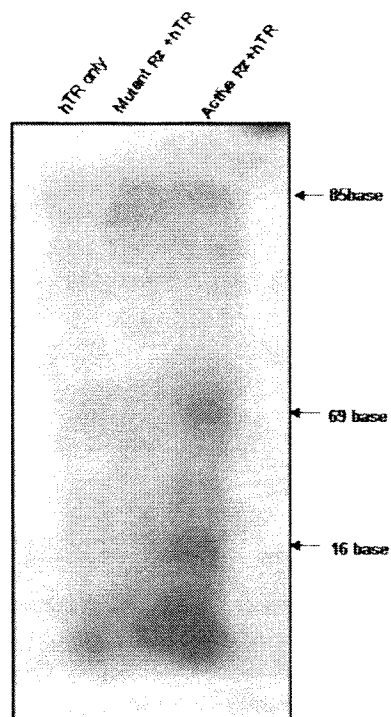
Fig 3.25: Ribozyme mediated cleavage carried out with PCR generated truncated transcript of 85 base having target site, tested with active and mutant ribozyme. The undried denaturing PAGE gel showing the cleavage products of 69 and 16 bases as expected.

III.2.5. Cloning of Ribozyme in Mammalian Expression Vector

The pCI Neo contains mainly CMV promoter followed by intronic sequence to increase the stability of RNAs expressed from its cassettes. Down stream of introns have multiple cloning sites flanked by T7 and T3 promoters. The transcripts that are expressed form this construct will bear T7 sense and T3 sequence. The presence of T7 and T3 offers possibility of in vitro transcription and PCR amplification. The pCI-Neo has another transcriptional unit bearing SV-40 promoter containing neomycin phospho-transferase gene which confers the resistance against drugs for selection purposes. The continual exposure of cells to neomycin or G 418 selects the clones that got the vector stably integrated in to genomic DNA. The pCI Neo integrates in to genomic DNA of Cells during replication process and favors stable expression of cloned genes.

III.2.6. Cloning and Screening of Ribozyme

For cloning of Ribozyme pCi Neo was digested with SmaI and annealed oligos were cloned by blunt end ligation at SmaI site. The schematic diagram as shown in Fig 3.26 depicts the method used to clone ribozyme oligos in mammalian expression vector. The ribozyme bearing clones were screened by colony blotting. After cloning the vectors were transformed and cells were plated on agar. The membrane was cut to the size of plate and placed in plate to take the imprint of colonies and processed and probed with end labeled ribozyme-coding oligos (Fig 3.27).

The autoradiograph was superimposed on plates to identify positive clones for further screening. The clones, which were positive for inserts, were randomly taken for sequencing to confirm their orientation with respect to CMV promoter and screening of active and mutant ribozyme. The sequencing result confirms the integrity of ribozyme bearing clone as well as orientation with respect to CMV promoter system (Fig 3.28).
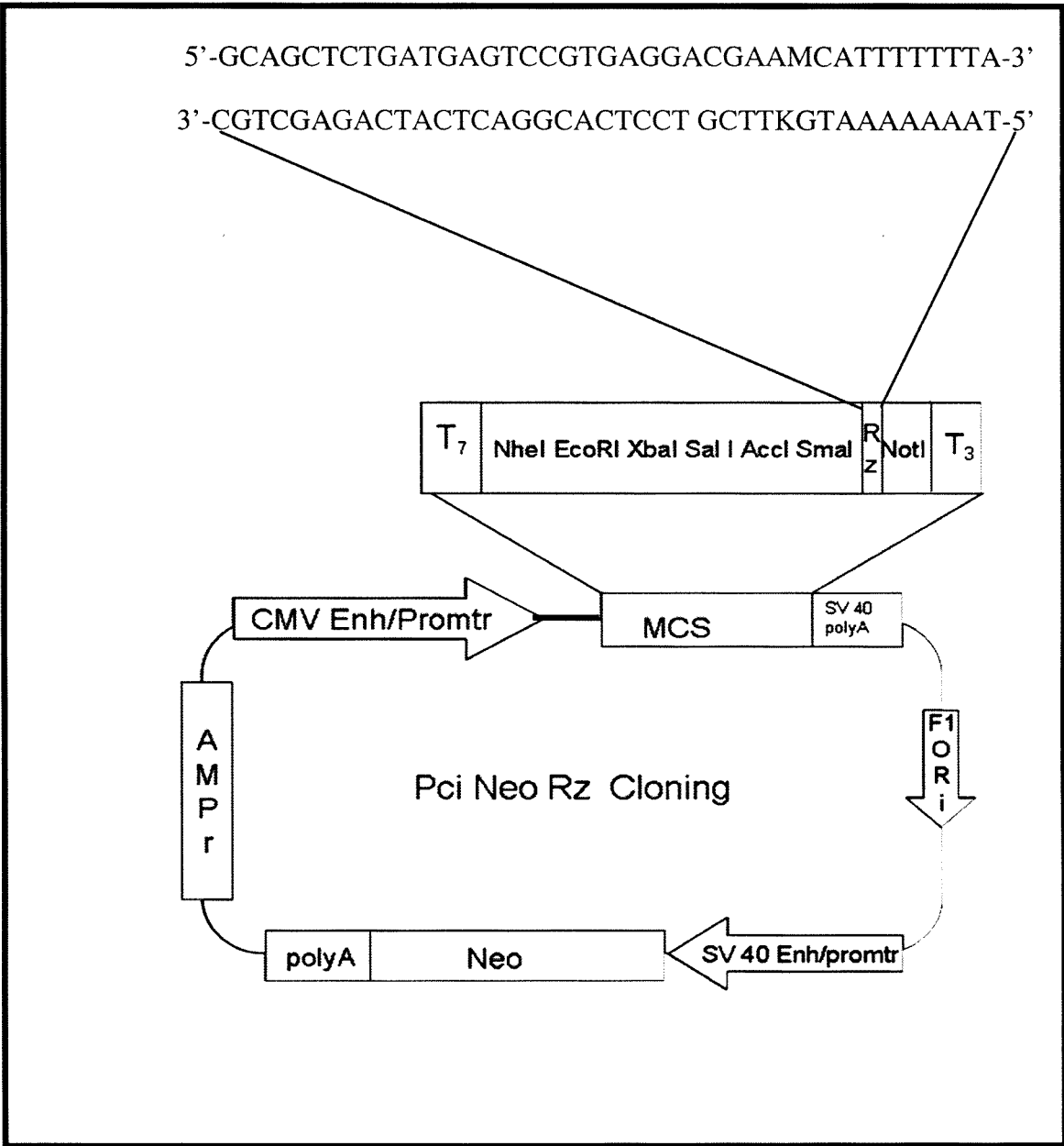
Fig 3.26: Cloning of Ribozyme Coding Oligos in pCI Neo mammalian expression vector. Immediately after CMV promoter there is intronic sequence which is followed by MCS as shown above (M in sequence denotes A or C nucleotide and K denotes T or G).
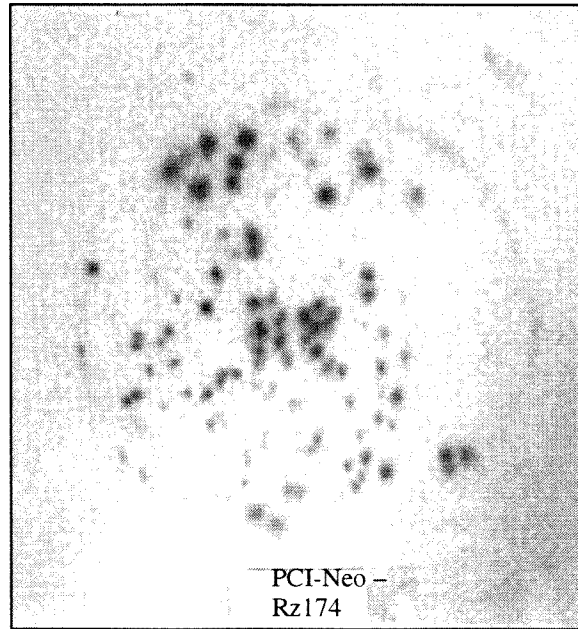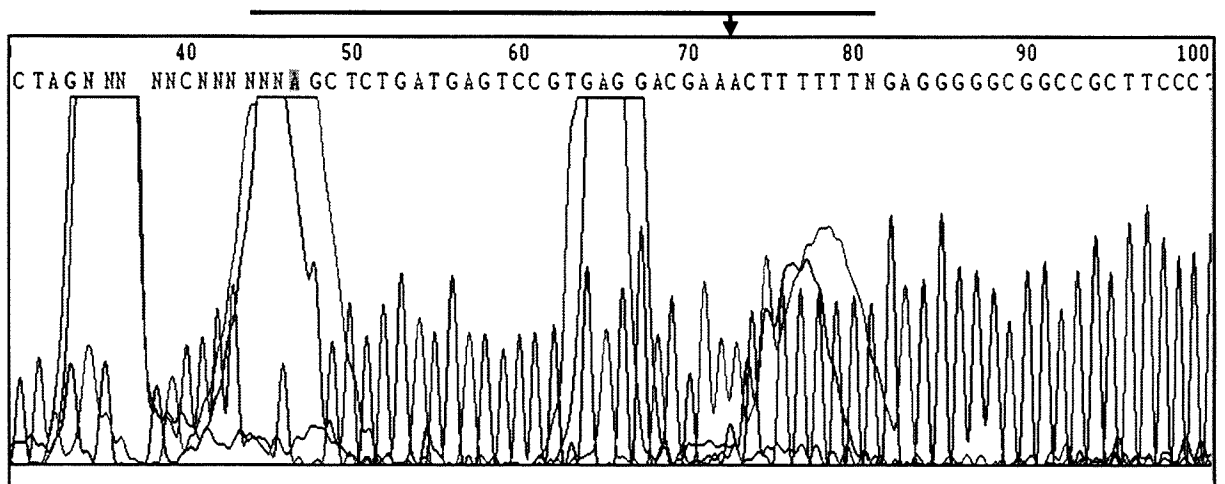
Fig 3.27: Colony blotting showing the positive colonies having Rz inserts, used for further confirmation by sequencing.
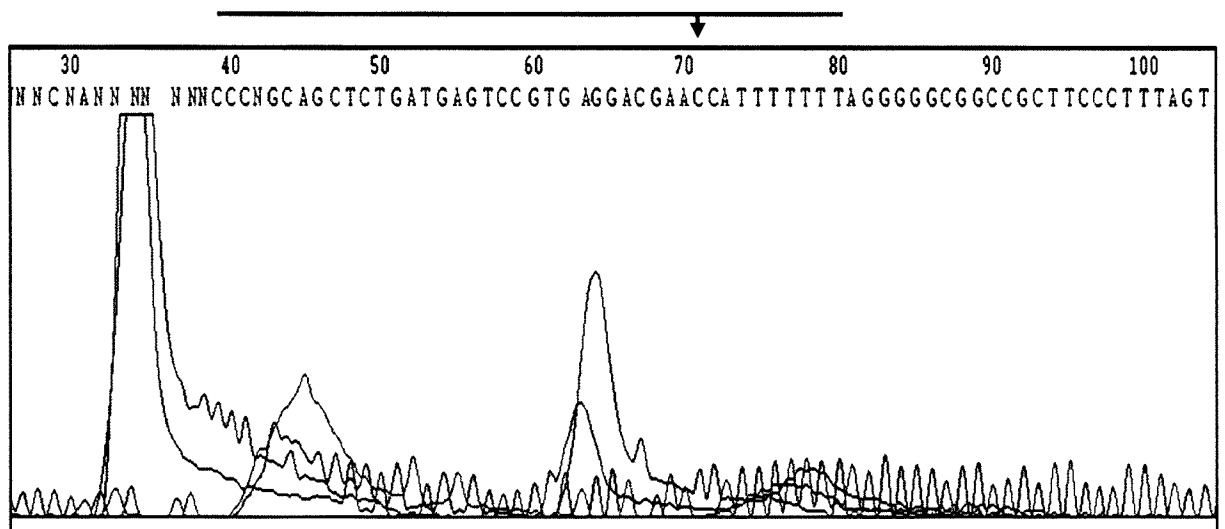
A) Active Rz

B) Mutant Rz

Fig 3.28: Sequencing of Rz positive clones identified from colony blotting A) Sequence of Active ribozyme B) Sequence of Mutant Ribozyme. Arrows shows the base 5'-GAAAC-3' for active Rz and as 5'-GAACC-3' for mutant Ribozyme, cloned in pCI -Neo mammalian expression vector.

III.2.7. Discussion

The cloning of ribozyme involves purification of annealed oligos to minimize or avoid single stranded, un-annealed oligo from interfering with cloning procedures. Once the annealed products were purified from gel with mild treatments it could be used directly for cloning purpose. The usage of pStu-I vector serves for transcribing only the catalytic RNA, as conventional vector system gives additional vector sequences in transcript, which may interfere with ribozyme's kinetic efficiency. To avoid the vector derived sequences, pStu-I or T7 appended ribozyme sequences were used for *in vitro* transcription purposes. This vector also offers a good system for screening for inserts using restriction enzyme digestion patterns. The relatively low frequency of positive clones may be due to the secondary structure assumed by the ribozyme-coding oligonucleotides and the blunt end ligation used for cloning them.

The cloning of full-length hTR was carried out following RT-PCR with hTR specific primers. The designed primer falls upstream of template sequence of hTR and the amplicons consist of 440 bp out of 451 bp of full length hTR cDNA. We used stringent temperature regimens for PCR in order to avoid any non specific amplicons or any unprocessed transcripts. The hTR RNA can be transcribed in vitro using SP6 RNA polymerase from pGEM-T-hTR.

In vitro ribozyme cleavage assay was assessed by mixing the *in vitro* transcribed ribozyme and target hTR with appropriate cations ($MgCl_2$) and followed by (12% denaturing PAGE) gel electrophoresis. The cleavage of 85 base truncated version of target RNA (having the desired GUC sequence) theoretically should produce 69 and 16 base and same is confirmed from gel electrophoresis. After electrophoresis the denaturing gel was directly exposed for autoradiography as higher percentage of gels tend to be brittle upon drying. The ribozyme employed in our study though less efficient compared to the ribozyme against template region of hTR, still it is catalytically active *in vitro*.

Cloning of ribozyme under CMV promoter ensures high level of expression in the introduced cellular environment. The CMV promoter offers greater stability in terms of half life of the transcribed RNA under its promoter system. It may be mentioned that CMV based vector system is not specially designed for RNA expression, as it was mainly developed for protein expressions. Still CMV proved to be effective in

ribozyme studies. The neomycin transferase gene serves as a good selection marker in mammalian system.

Using colony-blotting approach allows numerous parallel clones to be screened. The pCI- Neo contains T7-T3 promoter containing multiple cloning site, which is 35 base pair sequence. Introduction of Rz oligo further increase to 75 base sequences and selecting the restriction site farthest of these sites doesn't resolve the fragment unambiguously on agarose gels. As an alternative, colony blotting approach was carried out to screen the presence of inserts and to save the clones that are positive for inserts. Sequencing results further confirms the orientation of inserts with respect to promoter.

III. 3. Expression of Ribozyme and its Effect on Cells

III.3.1. Transfection, Selection and Ribozyme Expression in HeLa Cells

To assess the ribozyme's efficiency *in vivo*, the constructs representing vector alone, active ribozyme and mutant ribozyme were transfected in HeLa cells and selected with antibiotic G418 (400µg/ml), for 21 days. Cells surviving prolonged selection were propagated and analyzed for expression studies. The schematic of method carried out is shown in Fig 3.29.

Expression of transfected ribozyme was analyzed by RT-PCR coupled slot blot representing cells at varied time points post transfection. RNA from the transfected cells was reverse transcribed with T3 primer and amplified with T7-T3 primers, which is a component of expressed transcripts. The amplification was carried out at different PCR cycles to determine the level of expression as well as to avoid PCR cycle limited threshold parameters (Fig 3.30A). Slot blotting was carried out with control oligos and the PCR products confirmed that sequences are ribozyme specific (Fig 3.30B). The same PCR product was sequenced to confirm further the integrity of expressed ribozyme sequences (Fig 3.31). The sequencing reaction confirmed the integrity of expressed ribozyme sequences, importantly the conserved catalytic core of ribozyme shown with arrows and red bars above the sequence (Fig 3.31).

III.3.2. Analysis of cellular phenotype and cell cycle

Morphology and cell cycle related events of cells at different time points after transfection were recorded. The cells after transfection seemed to be under stress from 4th day onwards as the cells were exposed to high concentration of drug after 48 hrs of transfection. The cells had high granularity and abnormal shapes (Fig 3.32A, B). Most of the cells return to normal morphology after 15-17 days and still few cells were under stress. The selection continued for 21 days, by the time the surviving resistant cells proliferated as colonies. These polyclonal populations of selected cells were maintained for 120 days as stable transformants. Abnormal shapes such as rounded outline, granularity in the nuclei and vacuoles characterized the cells during selection. The selected cells become normal and properly flattened with normal cellular morphology.

Interestingly subset of the ribozyme transfected cells were showing elongated cellular phenotype from 60th day post selection, but very few such cells were observed in mutant ribozyme expressed cells (Fig 3.32.C). The elongated cells resemble the dendritic cells in morphology. Such transitions may reflect on induced terminal differentiation programmes in the cells.

In parallel the cells were examined with cell cycle event and presence of any apoptotic events by FACS. The cells were stained with propidium iodide and analyzed for DNA content and granularity. The forward and side scattering of general module was employed in FACS. The cells were initially gated from forward and side scattering to exclude the large cells or doublets of aggregated cells or cell debris. Fig 3.33A, B shows the gating of cells of same size and increasing DNA content. Those gated cells were further analyzed for DNA content and presence of apoptotic signatures. Generally alcohol extracts the apoptotic DNA, a small hypo diploid peak appears on as sub G1 peak in flow cytometry, indicating apoptosis.

In terms of DNA content and cell cycle events, there was no variation immediately following transfection. Cell cycle events were similar in cells transfected with wild type or mutant ribozyme transfected cells (Fig 3.34). The numerical representation also shows the uniformity of cellular phases and events which is indicative of undisturbed cell cycle events in ribozyme transfected cells.
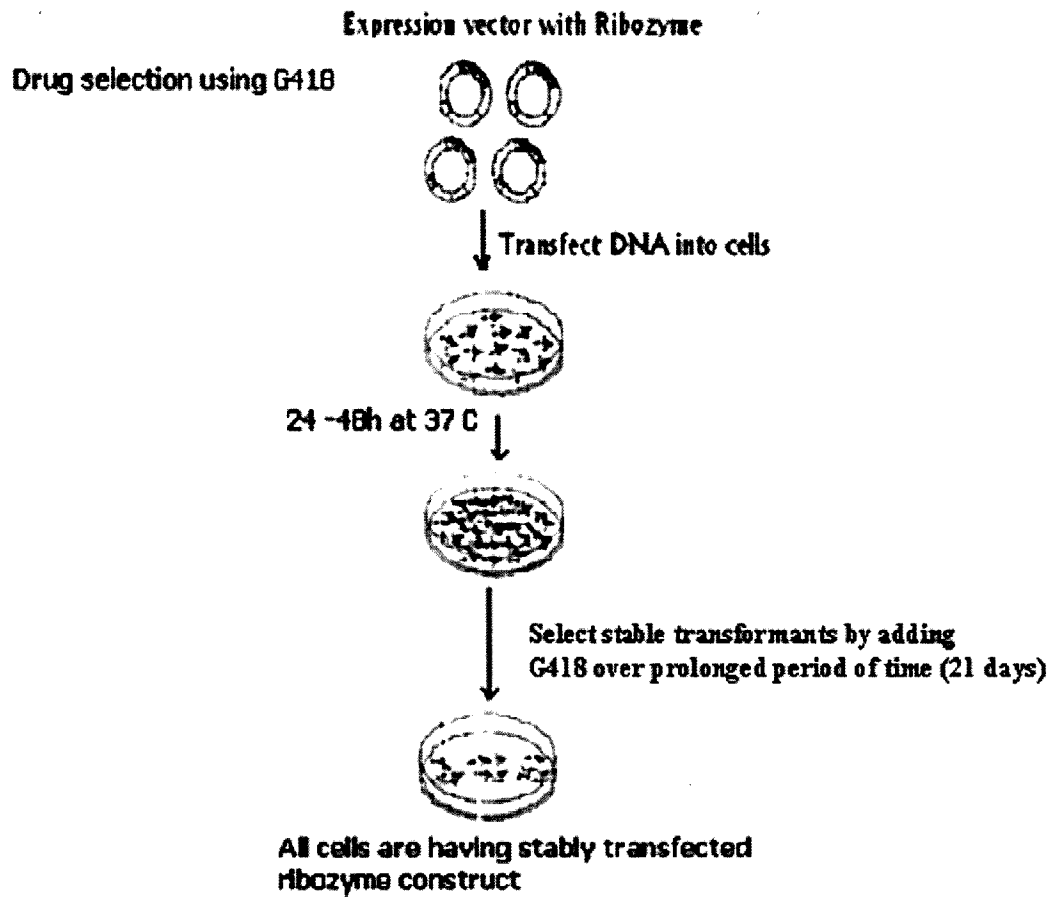
Expression vector with Ribozyme

Drug selection using G418

Transfect DNA into cells

24 ~48h at 37 C

Select stable transformants by adding
G418 over prolonged period of time (21 days)

All cells are having stably transfected
ribozyme construct

Fig 3.29: Schematic representation of methods carried out for assaying ribozyme expression in tissue
culture systems.

A)

B)

30   35   40   Cycles

A Mut Rz
(0.5µg
RNA)

B Active Rz
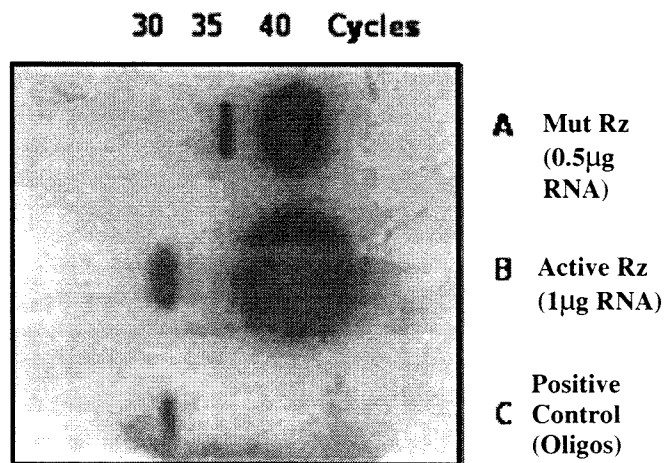**(1µg RNA)**

Positive
C Control
(Oligos)
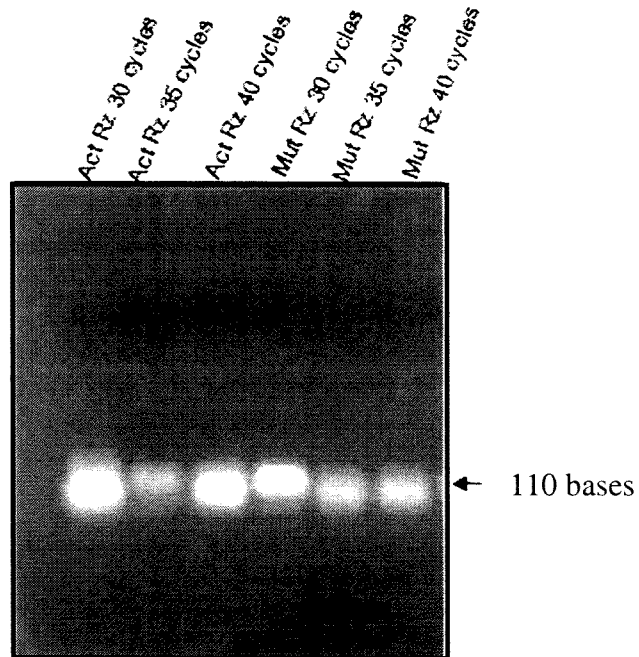
Fig 3.30: A) Individual RT-PCR reactions using T7-T3 primers of RNAs expressed from cells transfected with Active ribozyme and mutant ribozyme constructs at different PCR Cycles. B) The PCR Products were further Slot blotted and probed with ribozyme coding oligos, showing the expression of ribozyme slot at 30th cycle of row C is only loaded with Rz Oligo and other slots at 35th, 40th cycle of C were left unloaded.
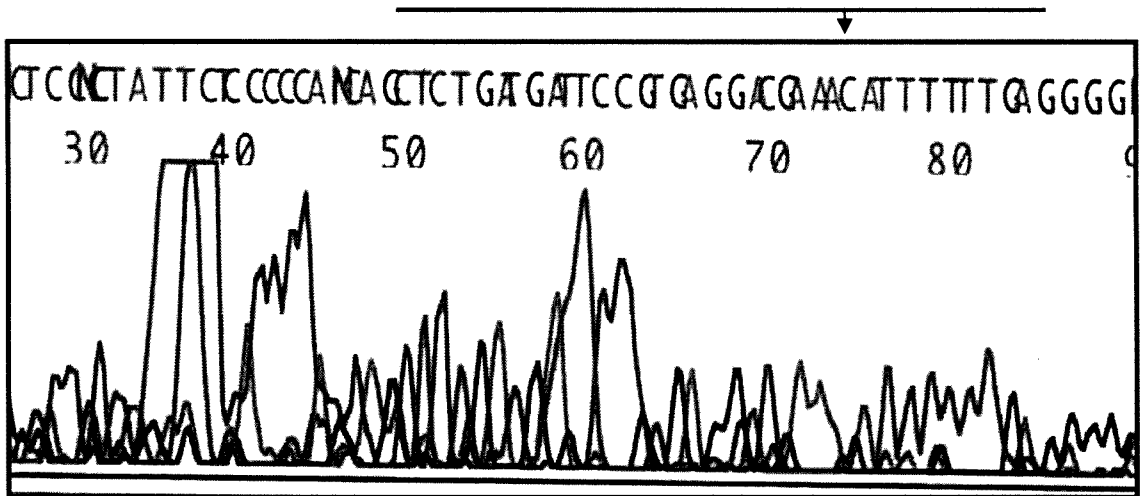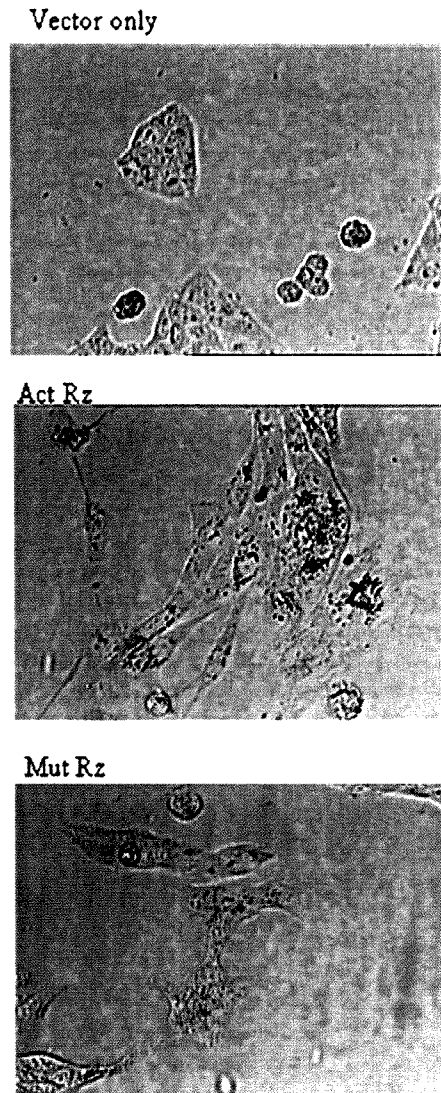
Fig 3.31: Sequencing of RTPCR products showing the conserved sequences of active ribozyme expressed from transfected cells. The sequence shown with red bar is ribozyme and arrow to show the conserved 5'-GAAAC-3' which is characteristic of active ribozyme sequences.

A)   B)

Vector only   Vector only

Act Rz   Act Rz
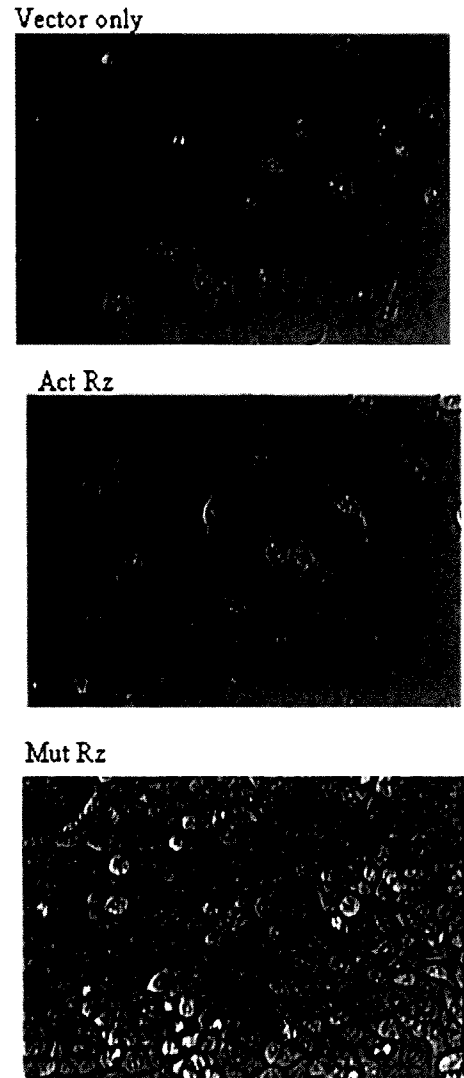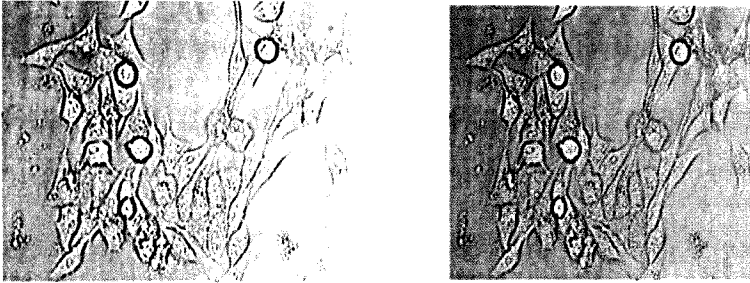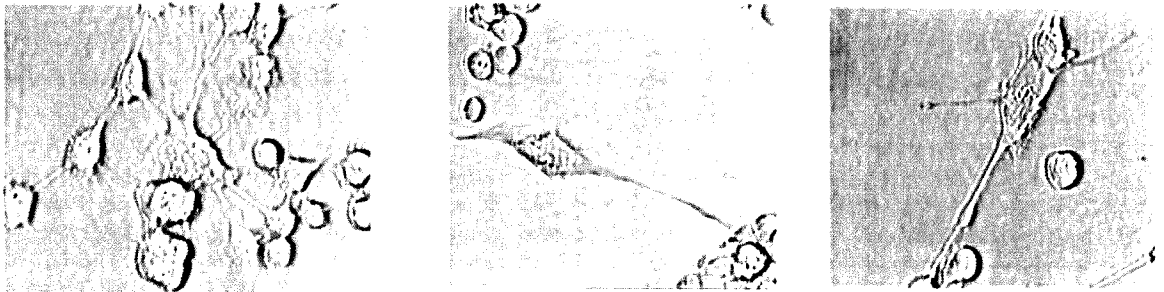
Mut Rz   Mut Rz

Fig 3.32 A: Phase Contrast Images showing the phenotype of cells transfected with constructs carrying vector alone, active ribozyme and mutant ribozyme. Cells were at stress for first 5 days. The images were obtained at 4th day after transfection. Fig 3.32. B) Images were taken at 8th day after transfection (Images at 400x).

Vector Only

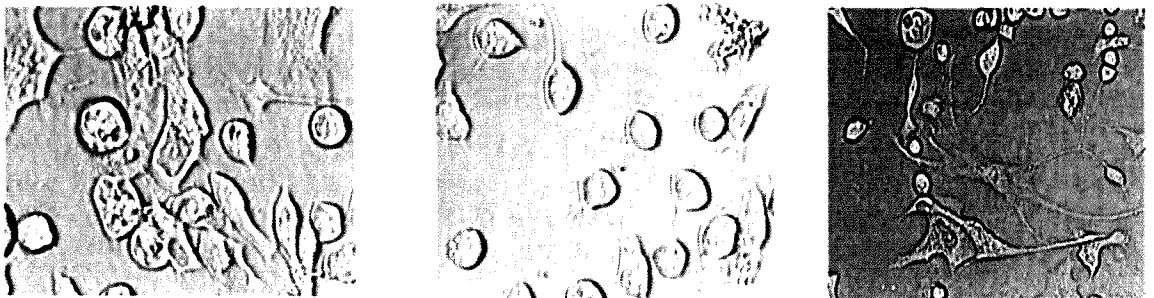**Active Rz**

Mutant Rz

Fig 3.32 C: Comparison of Cellular phenotype transfected with vector, active ribozyme and mutant ribozyme carrying constructs. Active Rz expressing cells appeared as elongated cells and very few cell also observed as elongated in mutant Rz expressing cells. Vector alone transfected cells appear as normal in phenotype. Images were taken at 69th day after transfection.
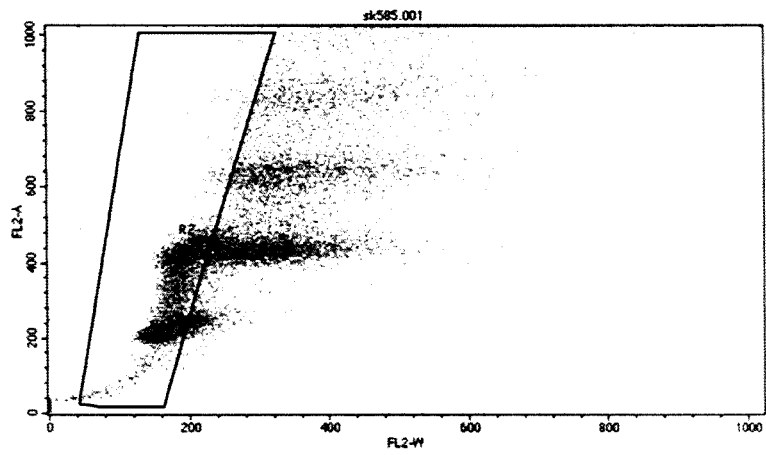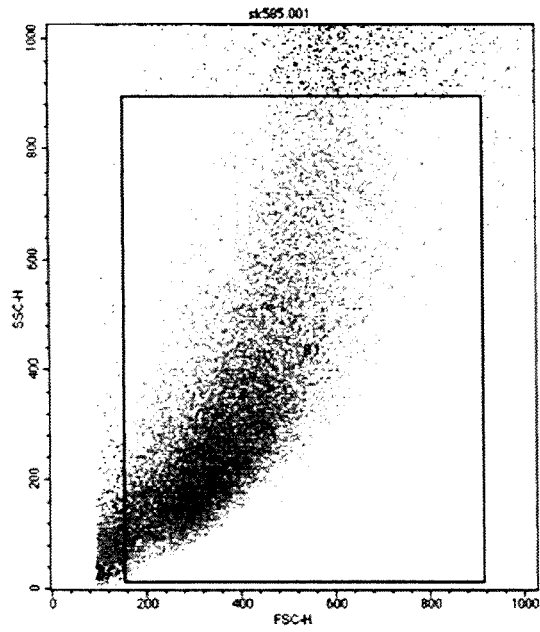
Fig 3.33A: Gating of cells by forward and side scattering of active Rz transfected cells. The lower panel shows gating of 2N and 4N cells and excluding the doublets and debris for analysis.
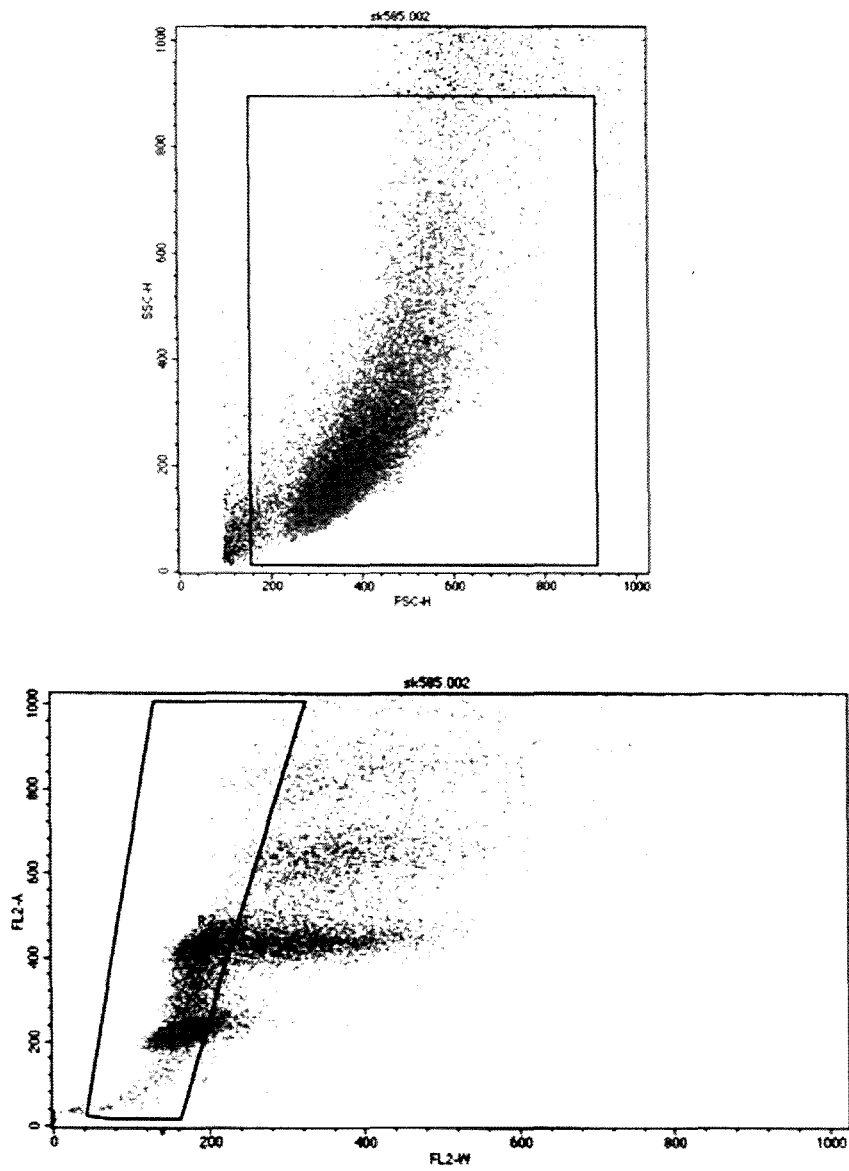
Fig 3.33B: Forward and side scattering of cells transfected with mutant ribozyme. The lower panel shows gating of cells (green color) and excluding enlarged cells.

125
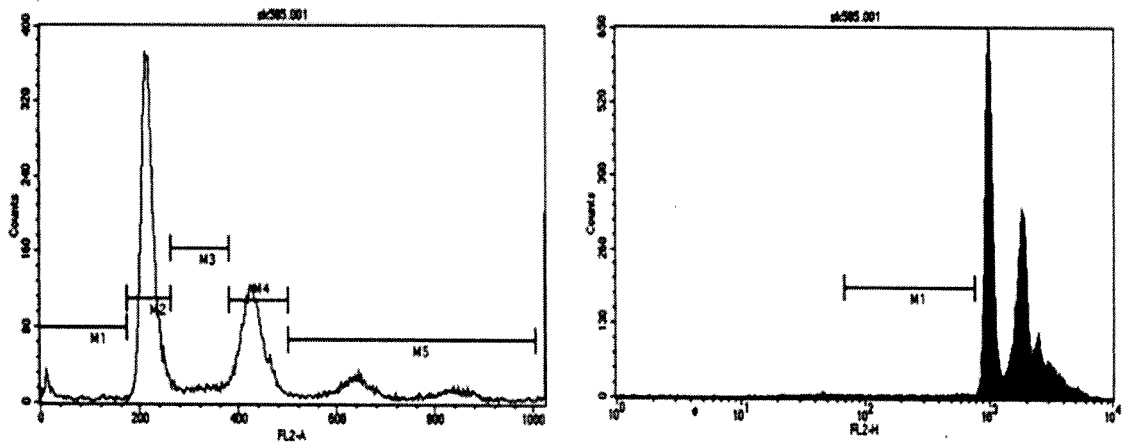
A) Active Rz
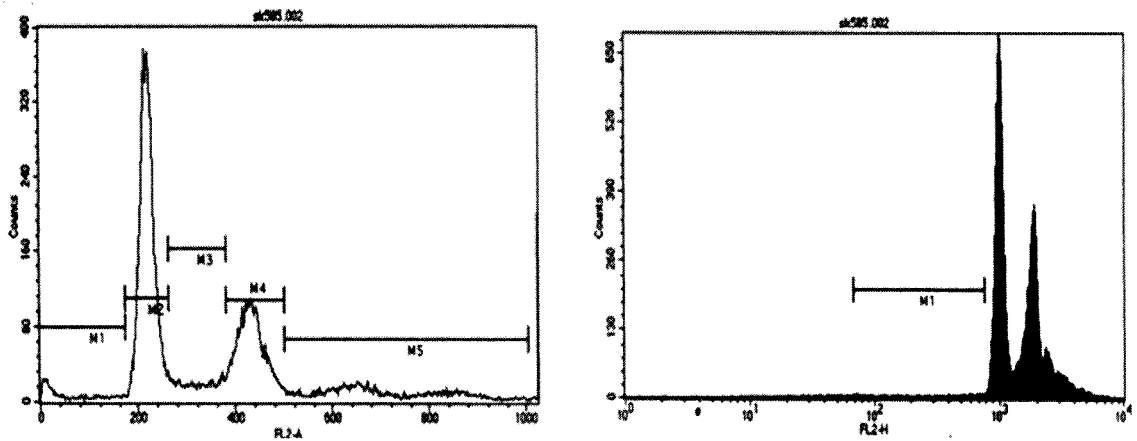
B) Mutant Rz

Transfection	Sub G1(M1)	G1/G0 (M2)	S phase(M3)	G2/M (M4)
Active Rz	2.87	34.95	6.33	21.47
Mutant Rz	2.64	38.93	5.04	20.91

Fig 3.34: Histogram showing population of cells transfected with A) Active ribozyme B) Mutant ribozyme. Table in the bottom represents fraction of cells in different phases of cell cycle.

III.3.3. Reduction of Telomerase RNA level in Ribozyme Expressing Cells.

To assess the effect of ribozyme on hTR levels, we carried out northern blotting and semi-quantitative RT-PCR in transfected cells. In general hTR is 451 bases long and associated with telomerase catalytic component. Free hTR is also found in cells as a varied length product which is expected as unprocessed transcripts. Collins et al (2006) reported that there must be telomerase RNA longer than previously defined length as unprocessed transcripts.

Northern blots of RNA from HeLa cells show reduction of hTR signal in ribozyme transfected cells (Fig 3.35). The observation of additional signals over the expected length may be attributed to unprocessed transcripts which are not confirmed in our study.

Semi quantitative RT-PCR was carried out to assess the level of hTR in ribozyme transfected cells. The RT-PCR carried out as two steps, once cDNA is made, an aliquot of cDNA was used for amplification purpose. The PCR products were internally labeled with α-^{32}P label to monitor the signal intensity which is proportionate to amount of amplified transcripts. The results show that amplified products could be seen from 20th cycle onward in vector and mutant transfected cells (Fig 3.36A), and absent in ribozyme transfected cells. The products start appearing from 25th cycle onward in ribozyme transfected cells, and those levels are not comparable to the products in 20th cycle of vector and mutant ribozyme transfected cells, confirming the reduction of telomerase RNA in ribozyme transfected cells. The same results represented as semi logarithmic plot (Fig. 3.36B) and showing reduction of hTR by an order of magnitude.
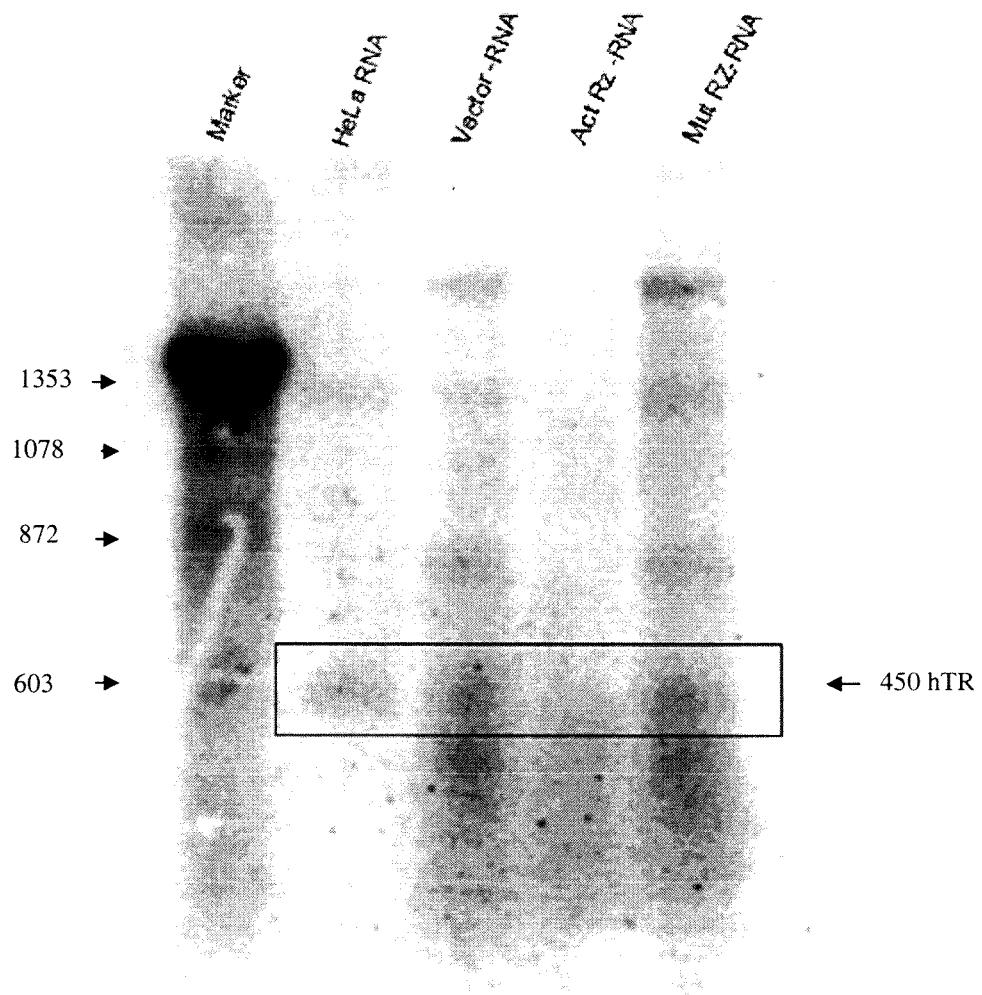
Fig 3.35: Northern Blotting of hTR, probed with antisense truncated hTR sequence. Boxed area shows the actual expected hTR, but appearance of higher molecular weight bands may be co migrants or unprocessed hTR.

A)

Vector only transfected

Active Rz transfected

Mutant Rz transfected
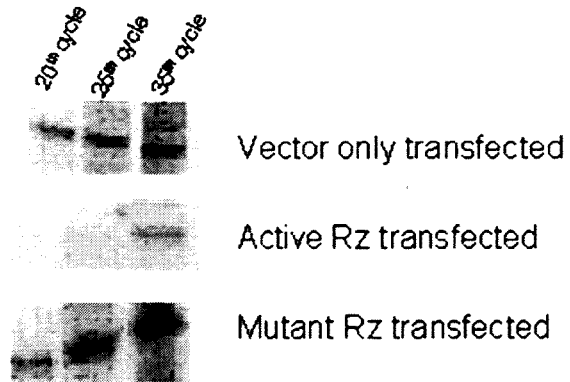
B)
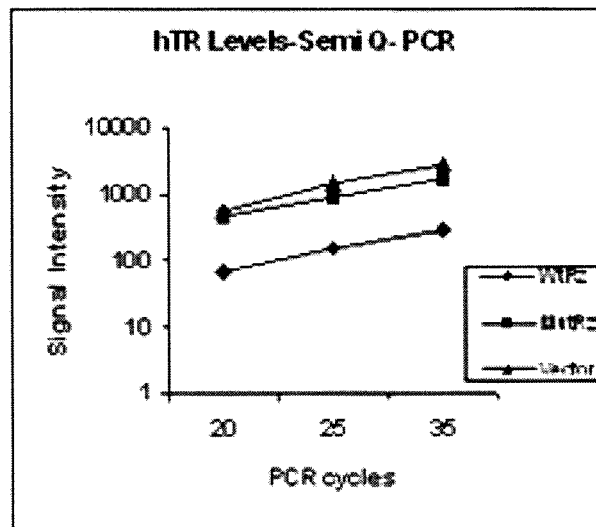
Fig 3.36: A) Semi quantitative radiolabled RT-PCR at different cycle from RNAs derived from vector alone and active ribozyme transfected and mutant vector transfected cells. B) Semi logarithmic representation of hTR levels from the densitometric analysis (Fuji Phosphor imager) showing reduction of hTR levels over an order of magnitude in cells transfected with active ribozyme(—◆—) that those transfected with vector alone (—■—) and mutant ribozyme (—▲—).

III.3.4. Reduction of Telomerase Activity in Ribozyme Expressing Cells

The reduction in hTR should reflect in telomerase activity. To assess ribozyme efficiency in telomerase inhibition, extracts from transfected constructs were analyzed with TRAP assay. The samples from different time points post transfection were analyzed for telomerase activity. Telomerase extended products were found inspite of very feeble reduction of signal intensity in ladders compared to vector alone transfected cellular extracts (Fig. 3.37A). TRAP assay with 100ng extract showed visible reduction of telomerase activity in cells transfected with active ribozyme when compared to controls (Fig 3.37B). Next, serially diluted extract (100- 300 ng) from all three cell lines were compared. The results show reduction or absence of products in cells transfected with active ribozyme (Fig 3.38, lane 4,7) as compared to those transfected with vector and mutant ribozyme at 100ng concentration (Fig 3.38, lane 3,6 and 5,8 respectively). But telomerase extended products started appearing in ribozyme transfected (lane 10) cells compared to vector and mutant transfected cells (Fig 3.38, lane11) at higher concentration of protein extracts. This suggests partial lowering of telomerase activity.

III.3.5. Reduction of Telomere Length in Ribozyme Expressing Cells

Telomere length in transfected cells was visualized by southern blotting. The DNA from transfected cells was digested with Hinf I and RsaI and probed with end labeled telomere specific probes. There is reduction of telomere length in ribozyme transfected cells (Fig 3.39, lane 3) compared to vector (lane 2) and mutant ribozyme (lane 4). The reduction in telomere length is not different in cells transfected with vector or mutant ribozyme.
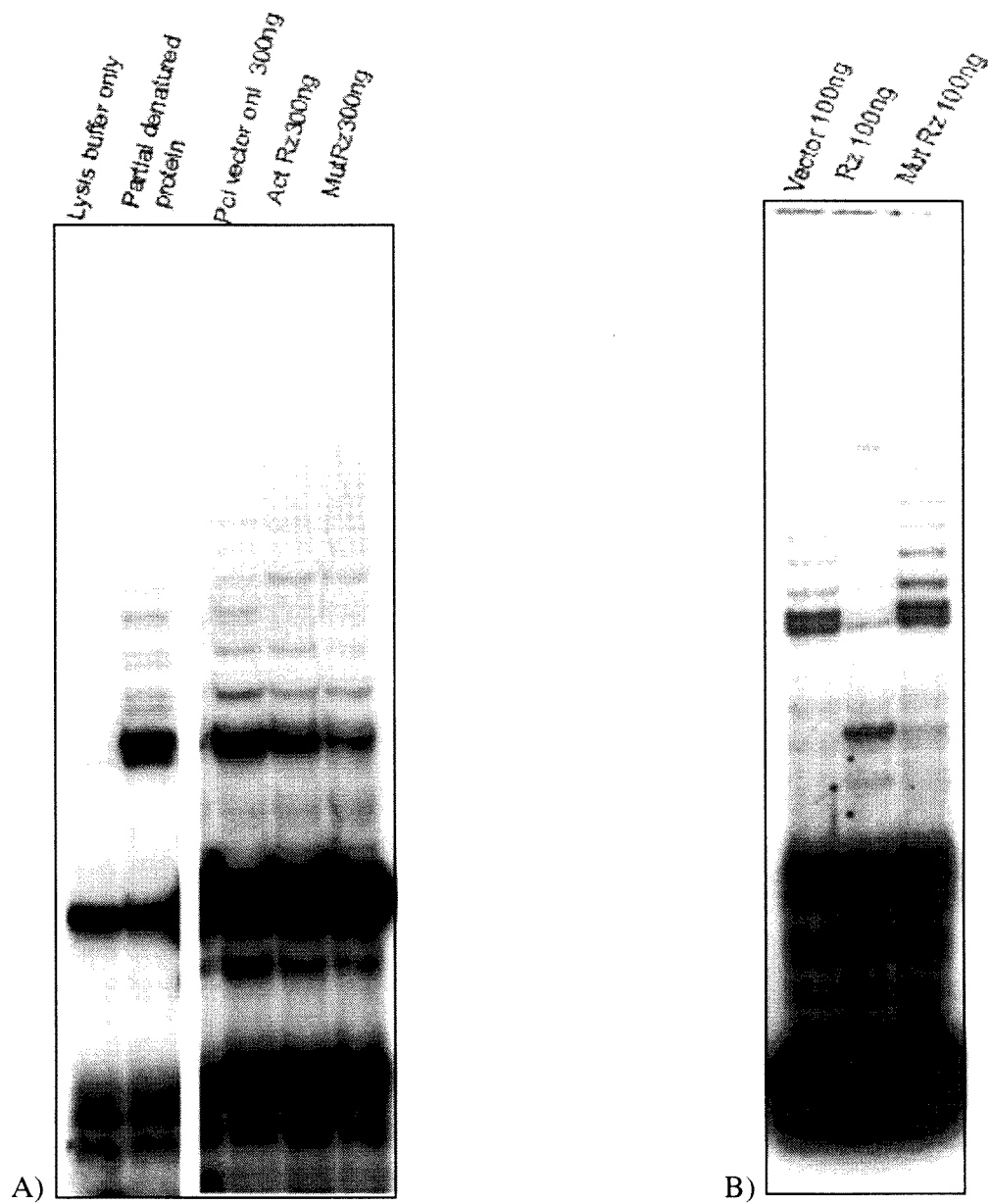
Fig 3.37: TRAP assay showing telomerase activity in cells transfected with vector, active ribozyme and mutant ribozyme employing 100ng or 300ng of protein equivalent in cell extract. A) TRAP assay for 60 days old culture, discernible reduction in telomerase activity in ribozyme transfected when compared with vector only at 300ng concentration. B) TRAP assay at 100ng extract showing reduction in ribozyme (Rz) transfected cells compared to proteins from vector and mutant ribozyme transfected cells.

Controls   + + - - - - - - - - -
Vector alone - - + - - + - - + - -
Mut Rz   - - - - + - - + - - +
Wt RZ   - - - + - - + - - + -

1 2 3 4 5 6 7 8 9 10 11
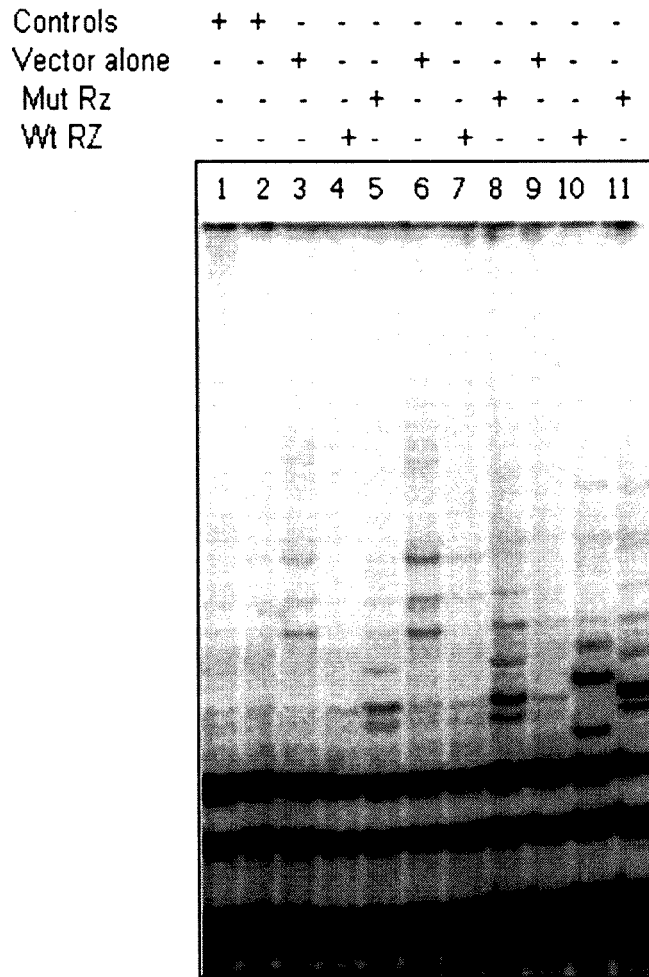
Fig 3.38: Serial dilution of proteins from vector, ribozyme and mutant ribozyme transfected cells. At lower concentration of protein (100ng at lanes 3-5 and 200ng at lanes 6-8)), reduction in telomerase activity observed in Rz transfected (lane 4 and 7) compared with vector (Lane 3, 6) and mutant ribozyme (lane 5, 8). TRAP assay with increasing concentration (300ng lanes 9,10, 11) of protein reveal, appearance of telomerase extended product in ribozyme expressing cells also (Lane 10) compared with vector and mutant Rz (Lane 9, 11).

132

Fig 3.39: Terminal restriction fragment analysis (TRF) in vector (lane 2), active ribozyme (lane 3), and mutant ribozyme (lane 4) transfected cells. Lane 1 is DNA from un-transfected cells as a control. Attrition of telomere length observed in ribozyme transfected cells (the maximum signal intensity shown with dents).

III. 3. 6. Discussion

For *in vivo* expression HeLa cells were used as it was earlier shown to have higher telomerase activity as well as it harbors human papilloma virus type 18 which is associated with inhibition of p53 and pRB (Horner et al 2004). Th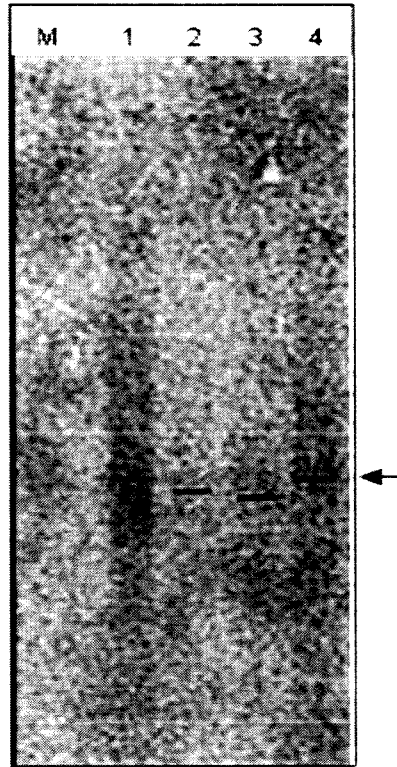is cell line also offers indirectly the p53-/- status and induced status of telomerase expression due to HPV E6, and E7 genes. These are considered as main oncogenes which deregulate most of the cell cycle control events directly or indirectly. It also facilitates the oncogenic status of cells and continued expression of telomerase. Introduction of p53 in HPV infected cells could inhibit the telomerase expression (Xu et al 2000, Kanaya et al 2000).

The method adapted to analyze ribozyme expression was by using T7-T3 promoter primers. These promoter sequences are phage specific and absent in mammalian cells. The transcripts from CMV promoter have T7 and T3 phage promoter sequence flanking the ribozyme. The CMV promoter of mammalian expression vector (pCI-Neo) is followed by intronic sequence, which confers the stability of transcripts along with multiple cloning sites having termination sequence (for transcripts) that ends at the 3' into the T3 promoter sequences. The vector also contains neomycin phosphor transferase (neo) genes which can metabolize the G 418 drug used for selection purposes. The RNA used for RT-PCR were predigested with DNase and purified with phenol choloroform. The amplification was separately carried out as the expression level may be low or high and if it is very low it may need increased amplification cycle for detectable PCR products. From our studies we could visualize RTPCR products at 30 cycles onwards and carried out till 40th cycle. The PCR product is about 105 bases in length and same is confirmed by hybridizing the slot blots with ribozyme specific probe.

The sequencing of RT-PCR products further confirms the expressed sequence is active ribozyme and the sequences are conserved *in vivo*. The cells were maintained with periodic drug selection and expressions were analyzed at different time points after transfections.

The elongated cellular phenotype is characteristic of differentiation event in certain cells, and it is expected that the cells could be induced for terminal differentiation which may be telomerase dependent or independent. The cancer cells have uncontrollable proliferative capacity due to maintenance of telomeres. One way to

control its proliferation is to induce the differentiation programmes. The notion of treating tumor malignancies by forcing them to complete terminal differentiation was first suggested by Pierce (1961). The cancer cells are mostly differentiated cells but not terminally differentiated cells. They are metabolically active quiescent cells and many of therapeutic strategies opt for inducing terminal differentiation in different cancers including breast and colon cancer (Mueller et al. 1998, Nojiri et al. 1999). From our study the phenotype observed was similar to dendritic cells and studies with antisense and ribozyme against telomerase proved that the cells attain elongated phenotype and undergo differentiation programme (Kondo et al. 1999, Bagheri et al. 2007).

Northern blotting shows lowering of telomerase RNA in ribozyme transfected cells. Northern blot also reveals appearance of signal above the expected size limits, which may be due to unprocessed transcripts and to less extent due to non specificity, as hybridizations and washings were carried out under stringent conditions. The semi quantitative RT PCR product reveal the appearance of hTR from 16th cycle onward from vector and mutant ribozyme transfected cells while in ribozyme transfected cells those amplicons could be seen after a prolonged PCR.

Telomerase activity is assessed by standard enzymatic assay in which extracts were incubated with telomere-specific oligos. The efficiency of telomerase enzyme is monitored by the amount of product it generated *in vitro*. Telomerase extends the telomeric primer and further amplified with forward and reverse primer by PCR. The extended products are measured by its signal intensity with that of control 36 base ladder signal intensity. Arbitrarily, the reduction of telomerase activity can be assessed visually by absence or reduction of telomerase extended bands. Telomerase activity was diminished in ribozyme transfected cells at minimal concentration of protein extracts (3.37B). But when higher concentration of proteins was used, telomerase extended products start appearing (Fig 3.37A, 3.38 lanes 9, 10, 11). This indicates the partial inhibition of telomerase RNA and the residual hTR may be enough to carry out the telomerase function. At higher protein concentrations (300ng), the products seem to be reduced in vector transfected cell (Fig 3.38, lane 9). This is attributed to inhibitory activities of cellular debris or other inhibitors present in extracts.

Southern blotting reveals discernible reduction in telomere length in ribozyme transfected cells. The cells were maintained as stable transformants for 4 months and

observable reduction in telomere length needs prolonged maintenance of cultures. The present blot from our study was carried out at 45th day; it may need DNA from cells which are maintained for prolonged period of time *in vitro*. In TRF analysis, the signal intensity is maximal at a point which is used as reference for length analysis. Appearance of a streak of bands is a measure of telomere length. The southern blotting has a limitation that it cannot differentiate the length difference if not more than 1000 bases. The reduction of telomere length was not to the expected level which is also evident from partial inhibition of telomerase in our study. Yet some telomerase molecules are present which maintain telomere length, though there is reduction in telomerase and telomere length in ribozyme transfected cells. At this stage we do not rule out activation of alternative mechanisms of telomere maintenance in the ribozyme transfected cells.

III.4. Transcription Profiling of Transfectants

Ribozyme mediated effects on telomerase RNA, telomeric DNA and on telomerase activity were observed in transfected cells. Observation of changes in cellular morphology prompted us to compare the global gene expression profiling of transfected cells. Global gene expression analysis could be analyzed by subtractive cDNA hybridization, differential display PCR (Liang and Pardee 1992), serial analysis of gene expression (Velculescu et al. 1995) and by micro array. Though each technique has its own advantages and disadvantages, we opted for differential display technique considering the possibility of observance of novel genes and requirement of minimal amount of starting RNA material and ease of handling. Differential display technique is basically based on usage of "defined arbitrary primers" with the cDNA created with oligo dT producing arbitrary length products. We performed differential display PCR (DD-PCR) to analyze the differential expression pattern among the stable transformants and the molecular alterations upon ribozyme expressions. The differential display PCR was done in two batches corresponding to 30th day and 45th day post transfections. The schematic of the method carried out is presented in Fig 3.40.

III. 4. 1. Analysis for Differentially Expressed Genes

We examined the role of telomerase in modulating gene expression patterns which may in turn result in phenotypic changes observed. We explored the possibility of change in global gene expression pattern.

Aliquots of total RNA from cells transfected with vector, active ribozyme and mutant ribozyme were predigested with DNase, further purified and quantified. Equal amount (0.2µg) of RNA was taken from transfected cells and reverse transcribed with oligo dT primer terminating into G or C or A at the 3' end. Each sample has 3 RT-PCR products and was amplified with 8 different defined forward primers. The products of all three sample amplified with primers were compared by electrophoresis and the products showing differential intensity were considered for further analysis.

The amplicons in Figs 3.41A (and 3.41 B), in lanes G-9, G-12 and 13 show differences in expression pattern (bands within boxes in lanes 9, 12, 13). Specifically transfectants with active ribozyme show signs of down regulation of some of the transcripts. Amplicons of oligo dT primers ending in A and C from 30 day old stable transfectants are not shown in the figure. The second batch DD-PCR was performed

with 45 day old stable cell transfectants of vector, active and mutant ribozyme constructs (Figs 3.42, 43). The amplicons appearing in DDPCR of 45 days old transfectants differed from the amplicons of 30 day old transfectants. The boxed bands represent the differentially displayed products. A10, 12, 16 primer pairs show the differentially displayed products in Fig 3.42, and C-9, 11, 13, 15 of Fig 3.43. The differentially displayed bands were eluted and reamplified with same primers and either cloned or directly used for sequencing. Re-amplification of DNA from these bands yielded 150-220 bp amplicons (Fig 3.44).

III. 4.2. Reverse Northern Blotting, Sequencing and *In Silico* Analysis of Differentially Expressed Genes.

The cloned sequences were further confirmed to eliminate false positives arising out of arbitrary PCR primers. To eliminate false positive, reverse northern blotting was performed with PCR products blotted and probed with total cDNA as probe. Reverse northern blots of these amplicons probed with total cDNA probes confirmed the consistency of the amplicons as being part of expressed genes. The reverse northern blotting in Fig 3.45 shows the rows of 6 different differentially displayed products as a clone or PCR product. Only slots in the rows of c, d, and e showed positive transcripts and others proven to be false positive. The true positive DDPCR products were sequenced for further analysis (Fig .3.46-51)

The sequences of true positive clones were analyzed further for the gene it denotes. The *in silico* analysis of the sequences using BLAST (www.ncbi.nlm.nih.gov/) reveals identity of some of the genes with those down-regulated specifically in ribozyme transfected cells. It will be of interest to define functions of respective gene products and their interplay with telomerase. The gene products so deduced were further analyzed with 'STRINGS' online software (a protein interaction network, www.strings.embl.de), for possible cross talks with cellular proteins (Fig 3.52). The genes corresponding to the differentially displayed PCR products include an enhancer invasion cluster (HEI-C) functioning in spindle fiber assembly, the ribosomal L23 and

Differential Display PCR
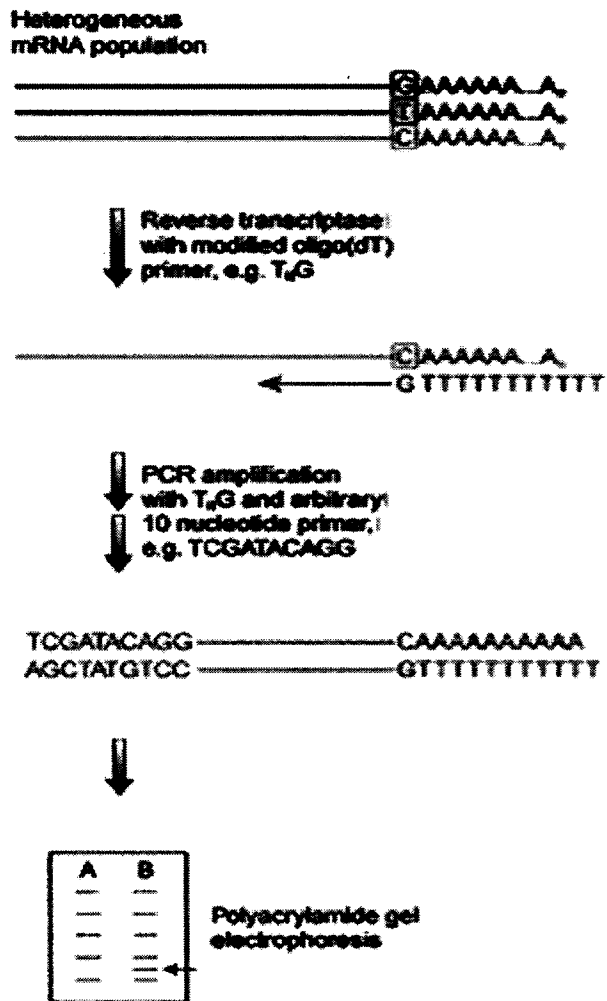
Fig 3.40: A schematic representation of differential display PCR technique and methods to identify the differential displayed products.

139
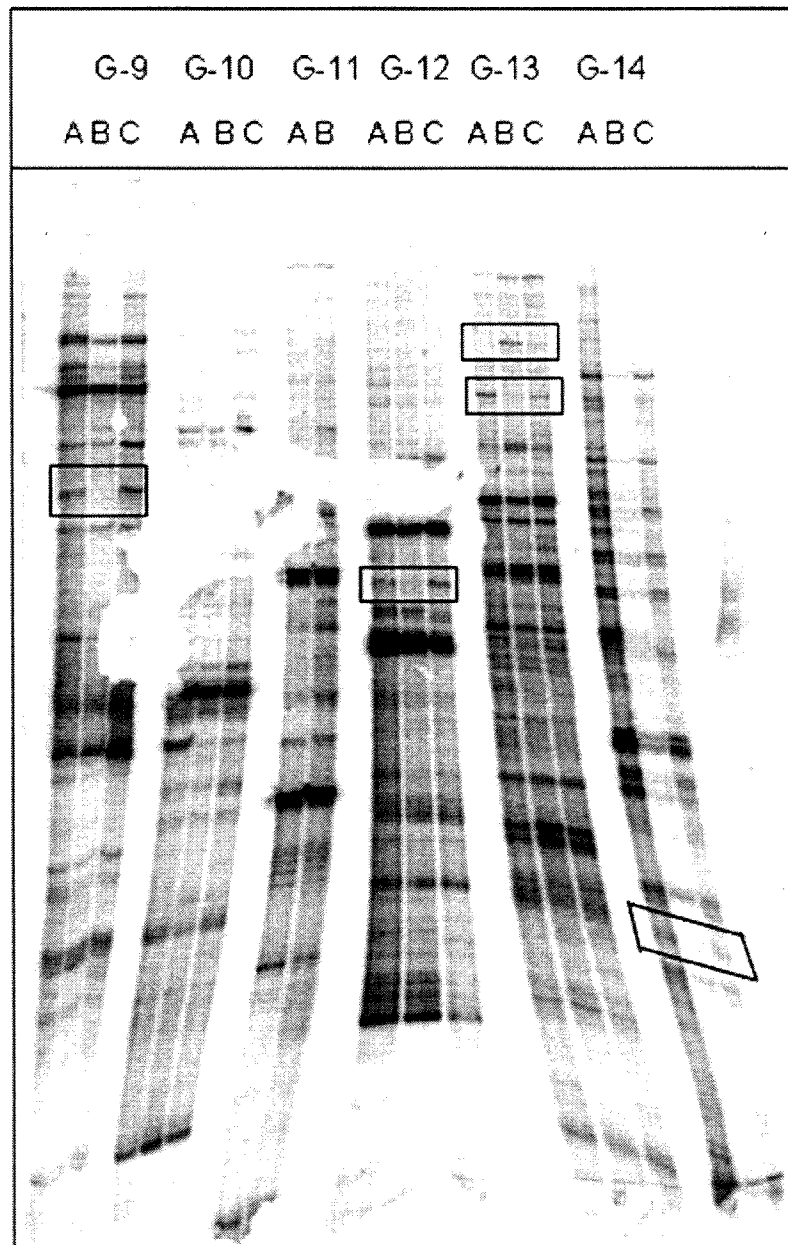
Fig 3.41 A: The differentially displayed product with transcripts amplified with oligo dT-G and with eight different primers (only six are shown). The boxed bands identified as differentially displayed sequences. First batch i.e., 30 days old post transfected cells. A-Vector alone, B –Active ribozyme and C- Mutant ribozyme transfected cellular transcripts.

140
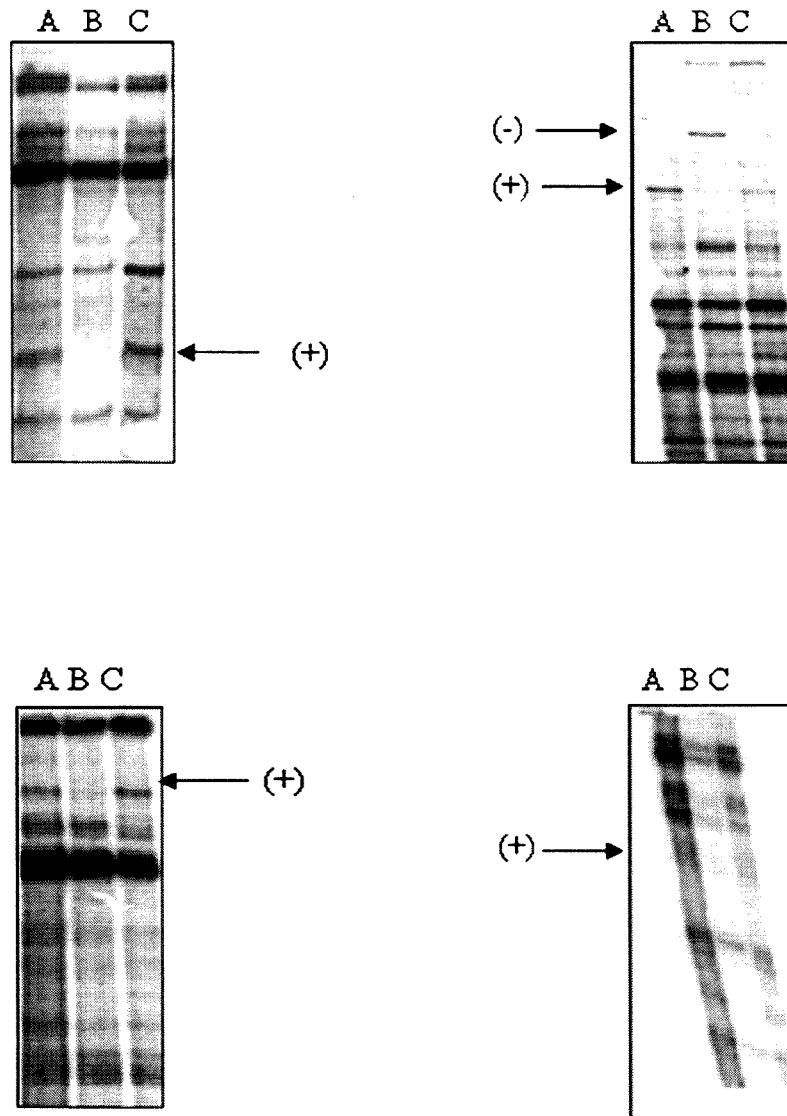
Fig 3.41 B: Subsets of differential displayed products predicted to have positive or negative association with hTR and telomerase activity. A-Vector alone, B –Active ribozyme and C- Mutant ribozyme transfected cellular transcripts.
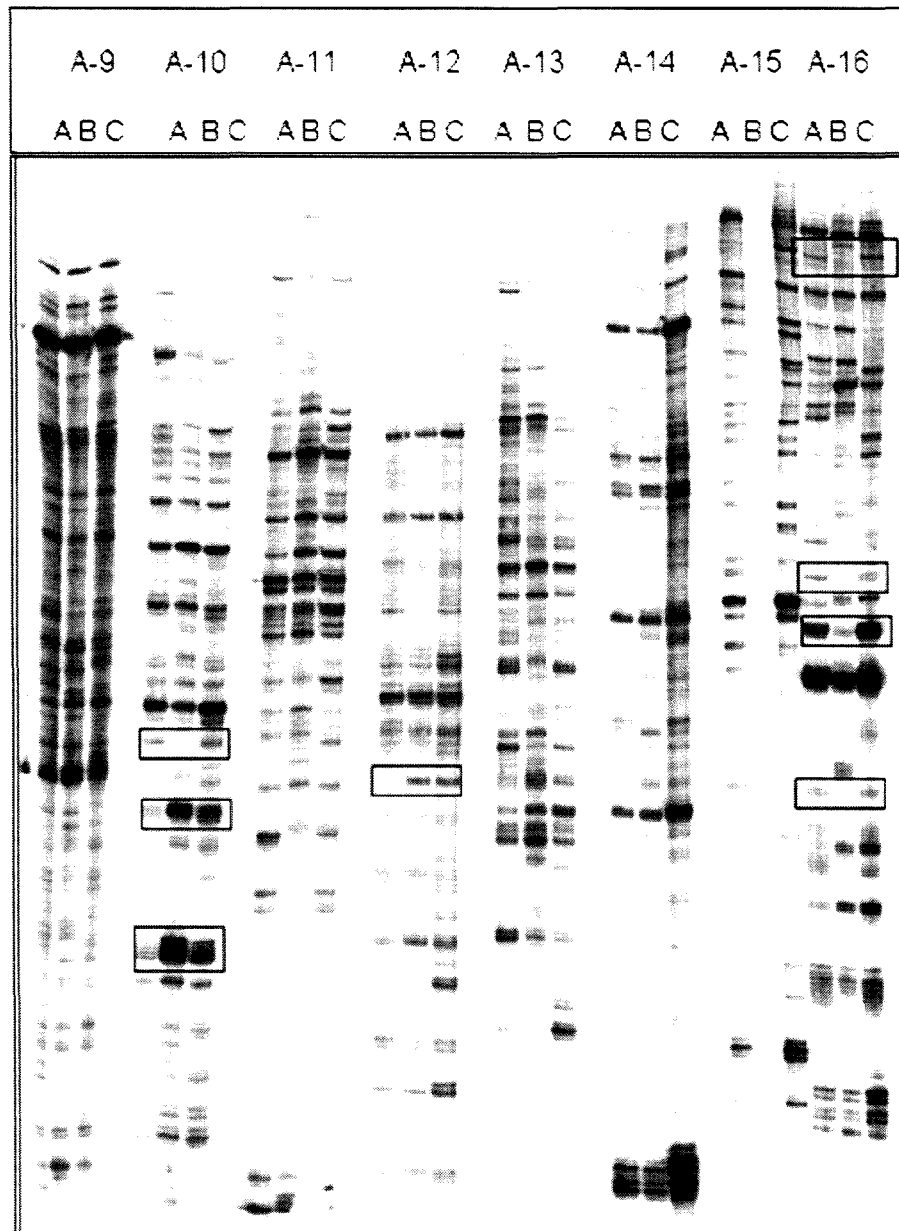
Fig 3.42: DD PCR of second batch 45 day post transfections, reveals the differential displayed products with oligo dT-A with eight different arbitrary primers. Boxed bands are differentially displayed products and varied with 30 day old post transfected gene expression profiles. A-Vector alone, B – Active Ribozyme, C- Mutant Ribozyme transfected cellular transcripts.

142
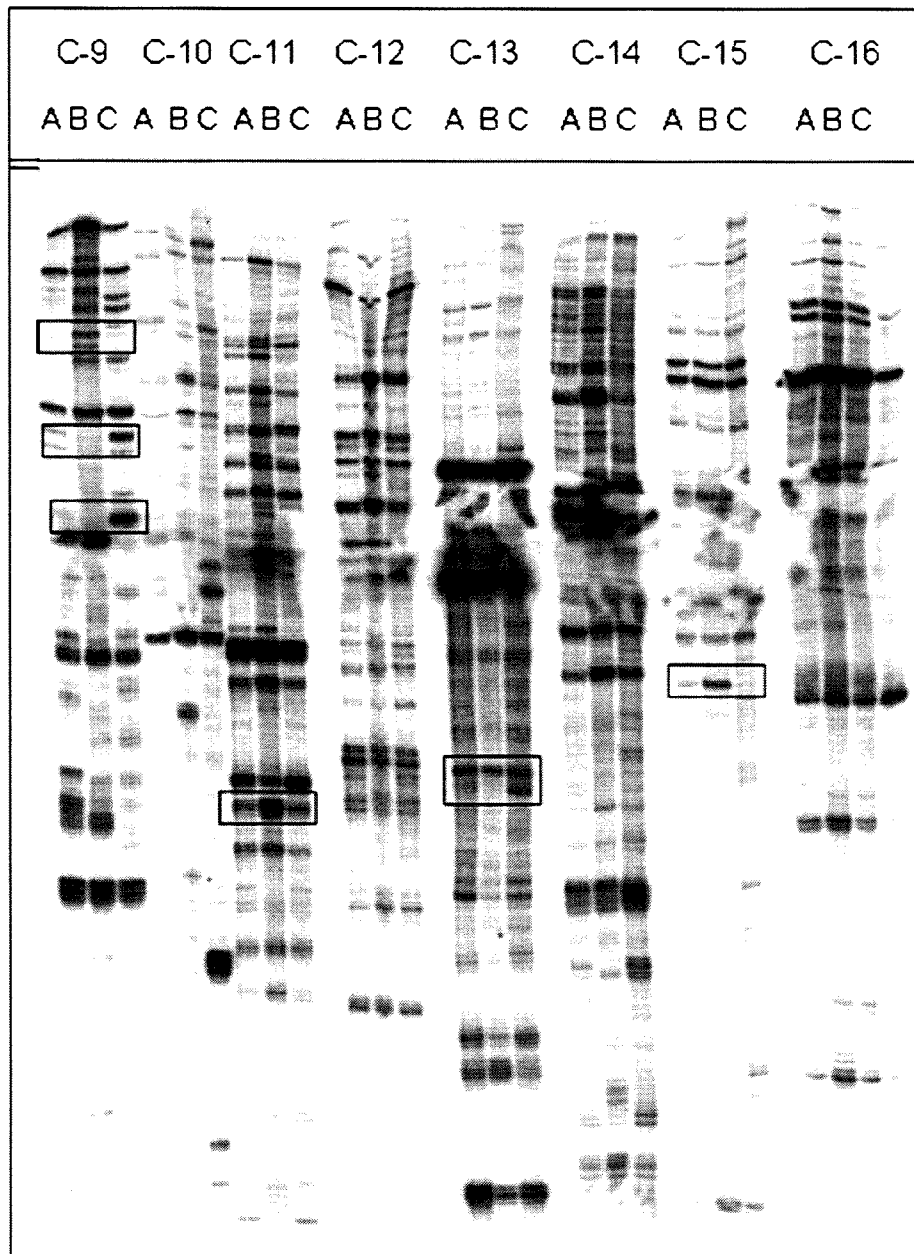
Fig 3.43: DD PCR products of cells 45 days after transfections, products were of oligo dT-C with eight different arbitrary primers. Boxed bands are differentially displayed products. A-Vector alone, B – Active Ribozyme, C- Mutant Ribozyme transfected cellular transcripts.
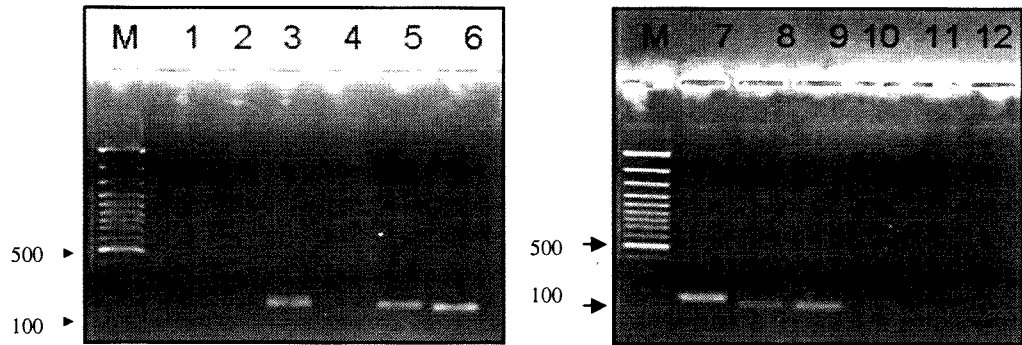
Fig 3.44: Reamplified products from differentially displayed amplicon, reveals variation in size and the products were of 150-180 base pairs. M -100base pair markers, Numbers are different PCR product of differential displayed products. Many primary amplicons could not be amplified from eluate of the corresponding gel slices.
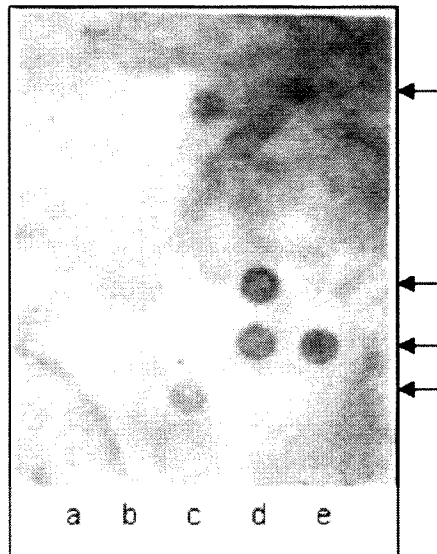
Fig 3.45: Reverse northern blotting of differentially displayed products, using total c DNA as probe. The a, b, c, d, e are column containing many spots of differentially displayed products and only positive transcripts are confirmed with hybridizations and elimination of false positive PCR products. Arrows show the positive products. Absence of positive signals in a and b is false positive transcripts from DDPCR.

ref|NM 000978.3| Homo sapiens ribosomal protein L23 (RPL23), mRNA
Length=594 Strand=Plus/Plus


```
Query  52   TCATTCCGGAGAAAAGATGGTGTGTTTCTCTATTTTGAGGATAATGCGGGGGTCATAGTA  111
            ||||  |||  |||||||||||  ||||||||  ||||||||  ||||||||  |  |||||||||
Sbjct  349  TCATACCGTAGAAAAGATGGCGTGTTTCTTTATTTTGAAGATAATGCAGGAGTCATAGTG  408

Query  112  AATAATAAAGGTGAAATGAAAGGTTCTGCTATTACAGGACCAGTCGCAAAGGAGTGTGCA  171
            ||  ||||||||  ||  |||||||||||||||  ||||||||||||||  |||||||||||||||
Sbjct  409  AACAATAAAGGCGAGATGAAAGGTTCTGCCATTACAGGACCAGTAGCAAAGGAGTGTGCA  468


Query  172  GACTTATGGCCCAGGATTGCTTCCAATGCTGGCAGCATTGCATGATTCTTTGGTGTATTT  231
            ||||| |||||| |||||||| ||||||||||||||||||||||||||||||||||    || ||||||
Sbjct  469  GACTTGTGGCCCCGGATTGCATCCAATGCTGGCAGCATTGCATGATTCTCCAGTATATTT  528
```


Fig 3.46: Sequencing of Differentially displayed products and *in silico* analysis shows the sequence of ribosomal protein L23 gene.

145

757367 bp at 5' side: methionine adenosyltransferase II, beta
isoform 1

```
Query  300       CGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAAATTCCACAC  359
                 ||||||||||||||||||||||||||||||||||||||| || ||||| |||||| ||||||||
Sbjct  37364847  CGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATGGT-ATCCGGTCACAA-TTCCACAC
37364904

Query  360       AACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAA  404
                 ||||||||||||| ||||| ||||||||||||||| ||||||||||||
Sbjct  37364905  AACATACGAGCCCGAAGC-TAAAGTGTAAAGCC-GGGGTGCCTAA  37364947
```

Fig 3.47: Sequencing of Differentially displayed products and *insilico* analysis shows the sequence of methionine adenosyl transferase II gene.

146
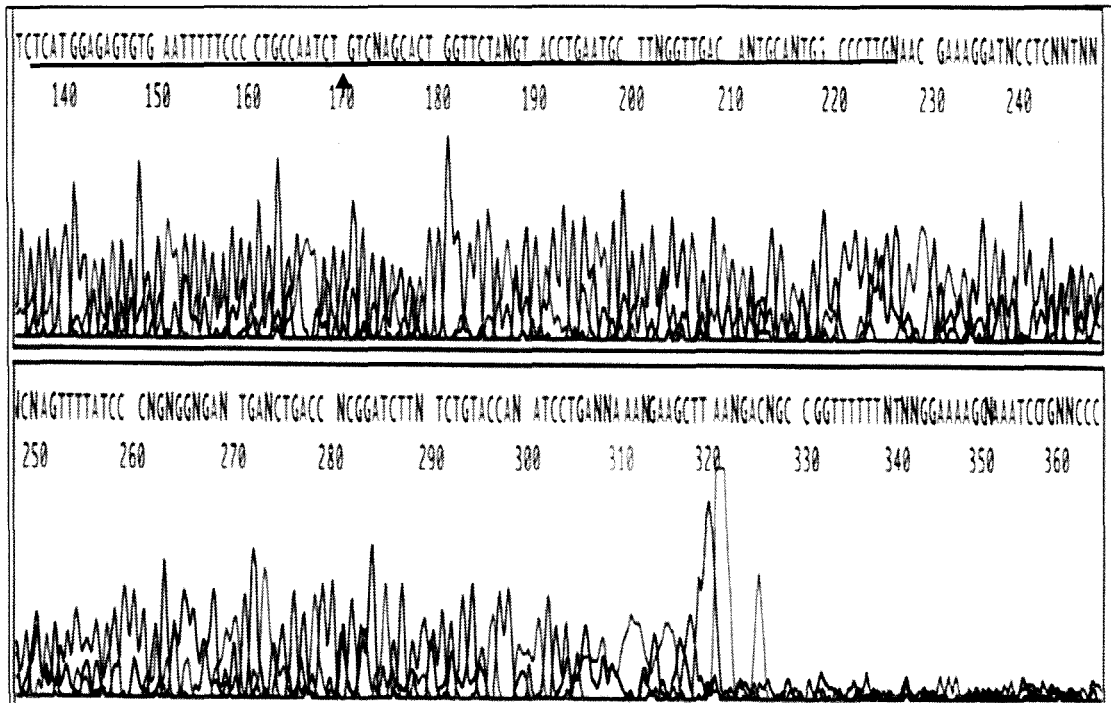
>gi|113427929|ref|XM_001129608.1| PREDICTED: Homo sapiens coiled-coil
domain containing 5 (spindle associated) (CCDC5), mRNA

Length=1002

```
Query  1    TCTCATGGAGAGTGTGAATTTTTCCCCTGCCAATCTGTCTAGCACTGGTTCTAGGT 56
            |||||||||||||||||||||||||||||| |||||||| |||||||||||||| ||||
Sbjct181    TCTCATGGAGAGTGTGAATTTTTCCCCCGCCAATCTCTCTAGCACTGGTTCCAGGT236
```

Fig 3.48: Sequencing and BLAST search identifies the DDPCR product as Coiled coil domain domain
containing 5 (CCDC5) or enhancer of invasion cluster (HEI-C).

147
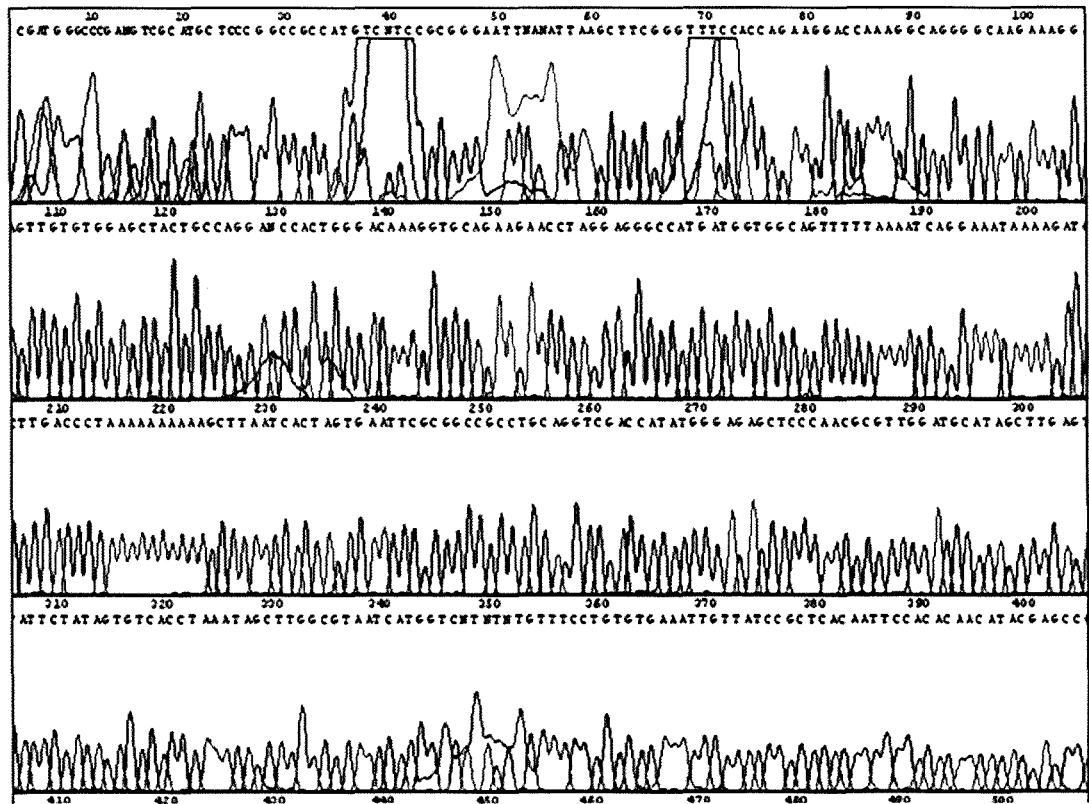
ref|NM_006579.1| Homo sapiens emopamil binding protein (sterol isomerase) (EBP), mRNA Length=1073 GENE ID: 10682 EBP

```
 Score =  120 bits (132),  Expect = 1e-24
 Identities = 105/131 (80%), Gaps = 0/131 (0%)
 Strand=Plus/Plus

Query  92    GGGCAAGAAAGGAGTTGTGTGGAGCTACTGCCAGGANCCACTGGGACAAAGGTGCAGAAG151
             ||| || |||||| | |||||| ||  ||||| ||| ||| | | ||| || | || ||
Sbjct  941   GGGGAATAAAGGGGCTGTGTGAAGGCACTGCTGGGAGCCATTAGAACACAGATACAAGAG
1000

Query  152   AACCTAGGAGGGCCATGATGGTGGCAGTTTTTAAAATCAGGAAATAAAAGATCTTGACCC211
             || | ||||||| | ||||||||| |  ||||||||||||||||||||||||||||||| |
Sbjct  1001  AAGCCAGGAGGTCTATGATGGTGACGATTTTTAAAATCAGGAAATAAAAGATCTTGACTC
1060
Query  212   TAAAAAAAAA  222
             ||||||||||
Sbjct 1061   TAAAAAAAAAA 1071
```

Fig 3.49: Sequencing and BLAST search identifies DDPCR product as emopamil binding protein (sterol isomerase)

148

```
ref|NM_007209.3|Homo sapiens ribosomal protein L35 (RPL35), mRNA
Length=475 GENE ID: 11224 RPL35 | ribosomal protein L35 [Homo
sapiens]

Query  76    GTACAAGCCCCTGGATCTGCGGCCCAAGAAGACACGTGCCATGCGCCGCCGGCTNANCAA 135
             ||||||||||||||||| ||||||||| |||||||||||||||||||||||||||| | |||
Sbjct  280   GTACAAGCCCCTGGACCTGCGGCCTAAGAAGACACGTGCCATGCGCCGCCGGCTCAACAA
339

Query  136   GCATGAGGAAAATCTGAAGACCAAGAAGCAACAGCGGAAGGAGCGGCTGTACCCTCTGCG 195
             ||| |||||| || |||||||||||||||| |||||||||||||||||||||||| |||||
Sbjct  340   GCACGAGGAGAACCTGAAGACCAAGAAGCAGCAGCGGAAGGAGCGGCTGTACCCGCTGCG 399

Query  196   GAAGTATGCGGTCAAGGCCTGAG   218
             ||||||  ||||||||||||||||
Sbjct  400   GAAGTACGCGGTCAAGGCCTGAG   422
```

Fig 3.50: Sequencing and BLAST search identifies DDPCR product as ribosomal protein L 35 coding sequence.

ref|NT 026437.11|Hs14 26604   Homo sapiens chromosome 14 genomic
contig, reference assembly Length=88290585

Features in this part of subject sequence:

   retinoblastoma-binding protein 11 isoform II

Query 1   GTCCATCCACTTCTCTCCAGGTGAACCTTTGTTCTAAAAGATCCCTTAAATATGAATGCT 60
              |||||||| |||||||||||||||||||| | |||||||| | ||||||||| ||||||
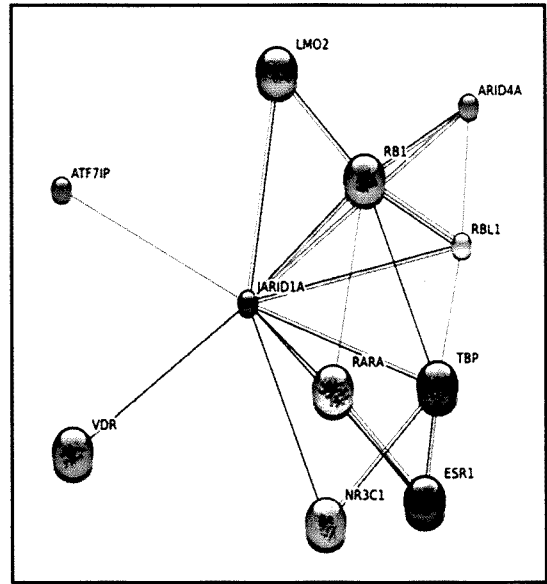Sbjct3'2013 GTCCATCCCTATCTCTCCAGGTGAACCTTTAT-CTAAAAGGTACCTTAAATACAAATGCT
39772071

Query 61   GAATCCAATATTCAGTGTTTCTGTAAAAAAAAA 93
                 ||||||||||||||| | ||||||| ||| ||||
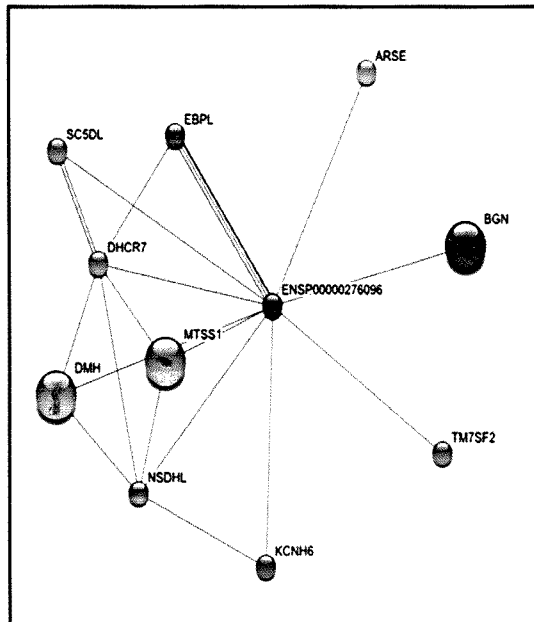Sbjct 39772072 GAATCCAATATTCACTATTTCTGTGAAAGAAAA 39772104

Fig 3.51: Sequencing and BLAST search of positive clone identifies as retinoblastoma binding protein 2 isoform II
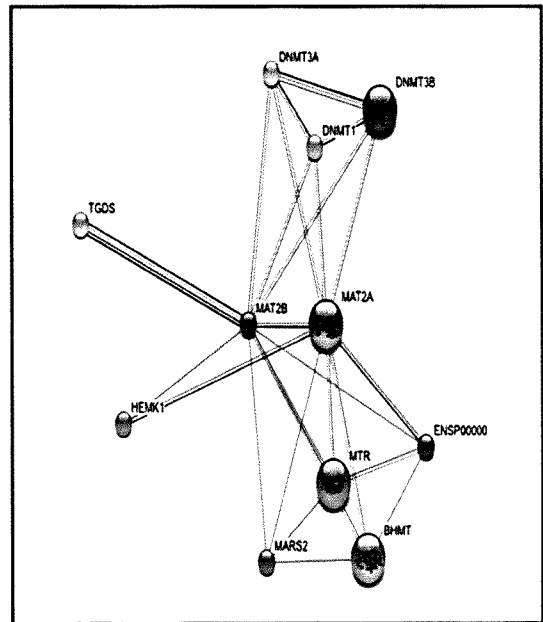
150
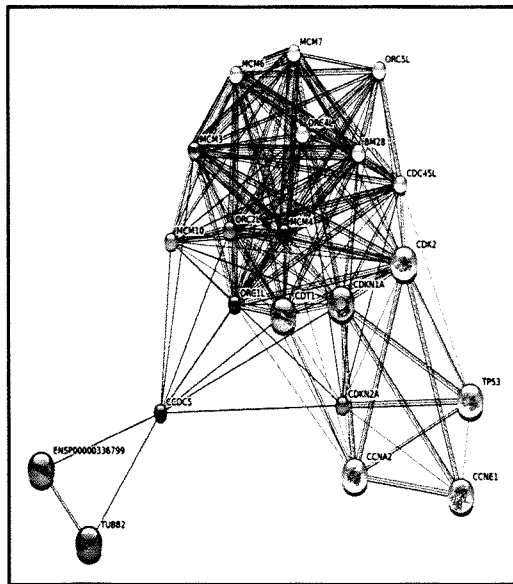
CCD 5

RBP2

EBP

MAT2A

Fig 3.52: STRINGS search of protein networks (CCD5-Coiled Coil domain containing protein 5, JARDIA-Retioblastoma binding protein 2, MAT2B-Methionine adenosyl transferase, of differentially expressed genes identified from DDPCR. The red color line shows the experimentally proven interaction with the reported proteins.

151

III. 4. 3. Discussion

Differential display PCR is a convenient technique to identify differentially expressed genes. DDPCR from cells 30 day and 45 day post transfection reveals alteration of transcripts in ribozyme transfected cells. Most of the transcripts were absent or down regulated in ribozyme transfected cells, and in two instances a new transcript appeared in ribozyme, the same was absent in both vector and mutant ribozyme transfected cells. Each group had 24 samples for comparison with 24 samples of other groups. Second batch DD-PCR results representing cells of 45 days post transfection came out with many differentially displayed products.

The main focus in cancer research and therapeutics is on identifying the underlying principles that govern the balance of tumor suppressor and activation of proto-oncogene and its involvement on cell proliferation and differentiation activities either in isolation or in concert. The behavior of molecules at different genetic status alters the patterns that are followed by strictly regulated molecular circuit. Carcinogenesis involves induction of proliferation and inhibition of cell differentiation and abrogation of cell cycle arrest programmes along one or more convergent pathways.

The main players of cell cycle p53 and pRb, in most types of cancer are either mutated or deleted. The tumor suppressor function of p53 involves activating its down stream effectors whose protein products may control cell cycle, apoptotic mechanism and senescence in response to various cellular stresses, preventing transformed and potentially tumorigenic cells from proliferation. The pRb products can control cell proliferation and promote differentiation (e.g., osteogenesis, adipogenesis and myogenesis) by enforcing several differentiation inducing transcription factors such as Myo D, CCAAT/enhancer binding protein ß, Runx2, and glucocorticoid receptor. The p53 gets inactivated by either endogenous proteolysis or exogenous viral factors. Several viral oncoproteins, including simian virus 40 large T antigen and adenovirus E1A, have been found to bind strongly to hypophosphorylated Rb (DeCaprio et al. 1988; Whyte et al. 1988). By binding to strategic domains they functionally inactivate Rb, probably by displacing cellular proteins important for normal functions in negative cell cycle regulation (Jones et al. 1997)

In our studies, telomerase inhibition did not seem to induce any apoptotic signal although it is evidenced from other studies (Yatabe et al. 2002, Hahn et al. 1999). Alternatively, the non-induction of apoptosis in our studies, could be due to the

absence of functional p53 or pRB protein in the HeLa cells, as HeLa harbors human papilloma virus, (HPV18) and proteins coded by the viral early genes E6 and E7 are known to target p53 and pRb respectively causing their inactivation/degradation and these cellular proteins are main players in cell cycle regulation and apoptotic signaling events (Scheffner et al.1990, Boyer et al. 1996).

Appearance of elongated cellular phenotypes may also result from the interaction of HPV E6 and E7 gene products with their respective targets p53 and pRb. In such status cells might have lost the signals for G0/G1 arrest and instead, the ribozyme-transfected cells are promoted along the differentiation pathway. Some of the genes which are down regulated upon telomerase targeting may presumably in cross talk with hTR or be influenced by telomerase activity or under the regulatory net work of telomerase.

Most of the genes noticed for differential display of cDNAs corresponding to their transcripts, showed reduction of transcripts in cells expressing wt ribozyme; however we did find a few genes that were over expressed. Reverse northern blotting indicated the truly positive clones for further characterization. The list we present here of differentially displayed genes is by no means a comprehensive one, as the primer combination in this differential display PCR experiment could cover only 45% of the represented transcripts. Studies on genome-wide expression patterns associated with telomerase therefore gain in value (Liang et al. 1992, 1994).

Genes down regulated by hTR-targeting ribozyme and thus showing a positive association with hTR expression/availability are likely to be positively regulated by hTR or telomerase activity and function of those down regulated genes may open up novel therapeutic options in addition to telomerase itself.

One of the deduced genes codes fore L23a protein, a component of ribosome along with other proteins, which has also a role in rRNA maturation and in early stages of ribosome biogenesis. It also has a role in protein interaction with chaperone trigger factor in later stages of translation and in preventing the MDM2, an oncogene which triggers p53 degradation. The independent role of L23a have been studied in p53 -/- background as the reduction of L23a would disturb the ribosome biogenesis and rRNA maturation events, and the translation process will be at halt or disturbed, a molecular phenotype, which may be a prerequisite for apoptosis. It has been demonstrated that p53 and pRb deficient cells would not opt for a sudden apoptosis or

show any signals of apoptosis rather they proliferate and progressive reduction of ribosome contents and impaired biogenesis results in to cell death (Mantanoro et al.2007). Another downregulated gene, the L35 protein is a basic protein from large subunit (50S) of ribosome in *E.coli* and 39S of mouse. L23a and L35 are close to the nascent polypeptide chain exit point in ribosome, having a role as a bridge with translocon in the translation process (Pool et al., 2002). The reduction of L35 again affects the translation process.

The methionine s-adenosyl transferase catalyzes the formation of s-adenosyl methionine (SAM), a donor of methyl group. In cancer SAM levels are decreased due to increased utilization of methyl groups as compared to abundant levels of SAM in normal cells. When the methionine adenosyl transferase level is reduced or inhibited, the methyl group abundance would be unavailable, preventing cancer cells from developing and maintaining their altered methylation patterns. In such instances, we expect methylation status changed in cancer cells and some of the genes may be relieved of methylation and may trigger other regulatory network which is necessary for cell cycle control or normal maintenance. SAM is also necessary for normal cell maintenance; the disturbance and un availability of methyl group donor may result in many abnormal expressions which may select the cells for apoptosis (Chiang et al. 1996). Role of SAM as methyl donor is not limited to DNA methylation events but also extends to protein methylation and t-RNA maturation process etc (Amalric et al. 1977). From our studies we found reduction in methionine s-adenosyl transferase in cells with wt ribozyme targeting hTR, suggesting that it could be positively associated with telomerase activity or hTR.

Another gene which seems to be positively regulated by telomerase is the enhancer of invasion cluster since it is down regulated in ribozyme expressing cells. This is a conserved coiled coil domain containing protein 5 (HEI-C) which functions in maintaining spindle formation during mitosis and its reduction or knock down by siRNA causes perturbed metaphase to anaphase transition and disorganized mitotic spindles, and subsequent disintegration of cells (Einarson et al. 2004). Disintegration happened in such cells due to intact cell cycle check point machineries. We didn't observe cellular disintegration perhaps due to their p53 and pRb negative status.

The role of pRB binding protein 2 (RBP2) is likely to be of significance since pRB is best known tumor suppressor; in addition, it also blocks proliferation and induces differentiation (Thomas et al. 2003). The free RBP2 blocks differentiation and pRb

inhibits the effect by binding with RBP2, and induces differentiation. In absence of pRB, down regulation of RBP2 can phenocopy the reintroduction of pRB in pRB-/- cells (Benevolenskaya et al 2005, 2007). RBP2 is also found to be modulating the chromatin structures. Recently it was shown to have a demethylase activity on trimethylated histones, and removes the methylated states of histone (H3K4 me3). In view of the association of trimethylated histones with euchromatic state of genome (Christensen 2007), the above observation would suggest that telomerase modulates chromatin through RBP2 protein in absence of functional pRBs (Ahmed et al.2004, Benevolenskaya et al. 2005). Hence reduction in RBP 2 level might cause heterochromatic region to switch in to active euchromatic region of chromosome and induce differentiation.

Emopamil binding protein (Sterol isomerase) belongs to sigma family of receptor, mutations that disrupts EBP's 3β –hydroxysteroid sterol Δ^8 Δ^7 isomerase activity impair cholesterol biosynthesis and cause X chromosomal dominant chondroplasia punctata, a rare disorder that is lethal in most males and causes patches of skin and bone in females due to random X inactivation process (Moebius et al 2003). Uptake of low density lipoprotein is through formation of coated pits and processed through endocytosis and hydrolyzed by lysosomal enzymes in to basic elements cholesterol, fatty acid and amino acids. The cholesterol liberated by lysosomal degradation is used for cell membrane synthesis. Cancer cells need large quantities of cholesterol for cell membrane synthesis and the control mechanism is some how deregulated. The reduction or absence of emopamil binding protein in ribozyme expressing cells may be defective in cholesterol biosynthesis. Impaired membrane synthesis may restrict cell propagation. Association of EBP in telomerase associated diseases like chondroplasia punctata also confirms direct or indirect association of EBP with telomerase.

In conclusion, the present study proves the association of some cellular genes with hTR, and also validated telomerase as a therapeutic target in p53-/- and pRb-/- status, and such treatment modalities will be effective in targeting telomerase. The study using ribozyme prompts to propose a hypothetical model of possible cross-talk or direct or indirect effect of hTR with necessary cellular events depicted as in Fig 3.59. The pathways mediating those gene and telomerase cross talk have to be established with experimental proofs.

III.5. Proteomic Alterations in Transfectants

III.5.1. Proteomic Profiling of Transfected Cells

The changes in whole genome transcript profiling prompted us to analyze the possible alterations in protein expression patterns. To analyze the proteome, 2D gel electrophoresis was carried out, which includes isoelectric focusing as 1st dimension and normal SDS PAGE as second dimension gel electrophoresis. Two-dimensional electrophoresis (2DE) of proteins is one of the highest-resolution analytical techniques available for the study of global protein expression patterns. The proteomic profiling by 2D gel electrophoresis methods allows one to identify a number of proteins that had been altered in their expression pattern.

III.5.2. Expression of Ribozyme Alters Proteomic Profile of Stable Transfectants

Cellular pellets of stable transfectants of vector, ribozyme and mutant ribozyme were lysed with urea buffer and isoelectric focusing was carried out in precast gels. After electrofocusing, conventional SDS PAGE gel was used for 2nd dimension PAGE gels as described under materials and methods. The spots were compared to locate the differentially expressed proteins. There were 9 proteins differentially expressed in ribozyme expressing cells and 4 from vector transfected cells (Fig 3.53A, B). Most of the protein species were slightly basic in nature and were between 17 to 82 Kd molecular mass. At this stage we could extract significant mass characteristics for four spots from Ribozyme transfected and one spot from the vector transfected cells.

To identify the proteins, mass spectrometer was used i.e, MALDI-TOF (Matrix Assisted Laser Desorbtion and Ionization). The proteins of differentially expressed spots were lysed by trpsin digestion, resulting peptides were ionized by laser and fired and analyzed by MALDI-TOF (Bruker Daltonics). The represented peptide's peaks m/z values were compared manually. A consistent presence of certain peaks was observed at 2370 and 2770 and 3250 (Fig 3.54-58). Those peaks considered as noise or contaminations were not taken for the analysis. The peptide peaks obtained for each sample were searched for their homology with the database of NCBI, EXPASY and SWISS PROT.

Spot 1 (Figs 3.53A, 54) is found to be exclusively expressed in vector transfected and absent from ribozyme transfected cells. The protein mass falls between 82-126 Kd

and MALDI created around 60 peaks and matched with interleukin 12 receptor beta 2 precursor, and 35 peaks created in MALDI TOF were matched with MASCOT's database. The main peaks were 1024, 1306, 1474, 2241, 2382 m/z values. The molecular mass and gel pictures were compared and identity assigned.

The 6th spot (Figs 3.53B, 55) appeared between 82 -126 Kda marker. MALDI yielded 73 peaks spanning between 800-2800 of m/z ratios. The peaks were 904, 1045, 1064, 1233, 1261, 1319, 1492, 1583, 1604 and 1838 daltons and are identified to be ATR interacting protein. The highest peak observed had a m/z value of 1319 representing peptide 'LQSLQSELQFK'.

The spot 7 of protein (Figs 3.53B, 56) from ribozyme transfected cells fell in the mass range of approximately to 82 Kda. The MALDI analysis created about 75 peaks and those are mainly 1035, 1178, 1191, 1233, 1299, 1319, 1739, 1790 and 1946. The peaks 1790 and 1946 converged, as single amino acid difference of cleavable amino peptide repeated (RR) at those sites (SIAVWDMASPTDITLRR). The matched peaks found homology to nearby significant value to the Beta transducin repeat containing isoform CRA-d.

The 8th spot (Fig 3.53B, 57) which has mass of between 38 to 82 kda from 2 D gel and the MALDI analysis created 100 peaks spanning from 800 to 3300 m/Z and those peaks with good intensities and matched with the available data base were identified as keratin 10. The matched peaks correspond to m/z of 806, 992, 994, 1002, 1089, 1117, 1164, 1200,1261,1299, 1356, 1364, 1389, 1433, 1492 and 1706, matched with Keratin 10 from the entire data base with significant score.

The 11th spot (Figs 3.53B, 58) eluted from 2D gel were between 17 to 32 Kd, and from MALDI analysis, we obtained 64 peaks including the noise and salt created peaks. The 64 peaks were searched for their homology and found hCG with significant score. The peptides1046, 1196, 1706, 3222 matched with hCG and the peptide peak of 3222 m/z confirms the hCG as represented protein in MALDI.
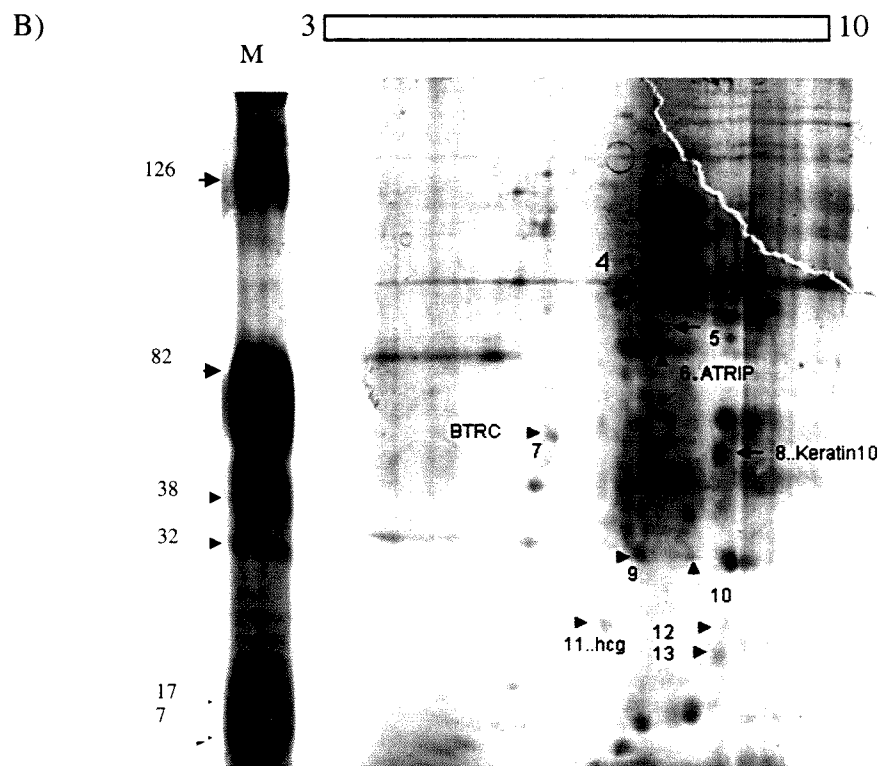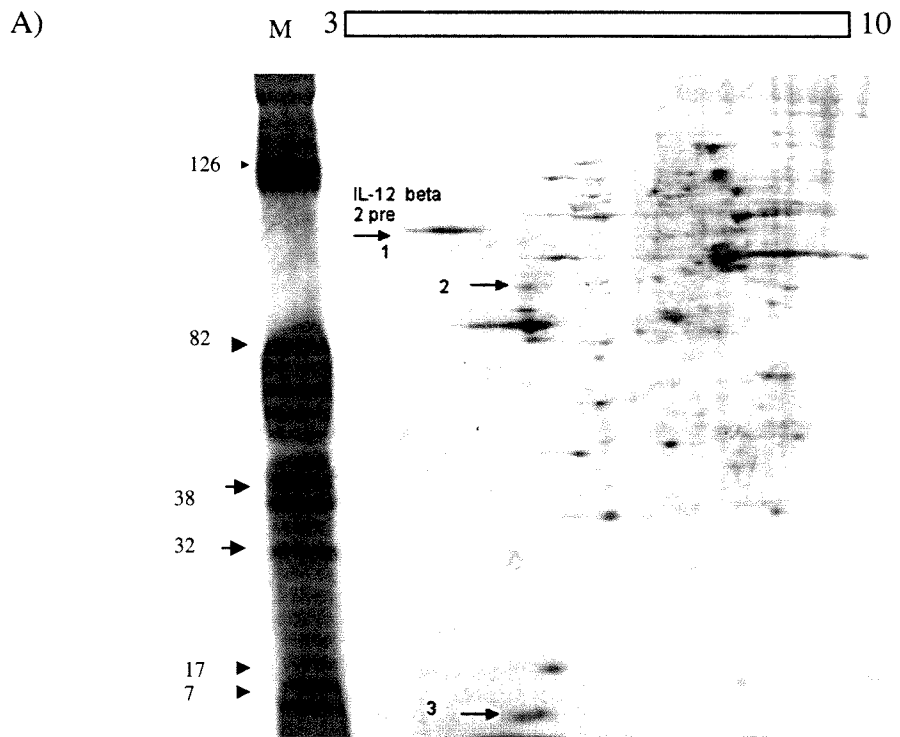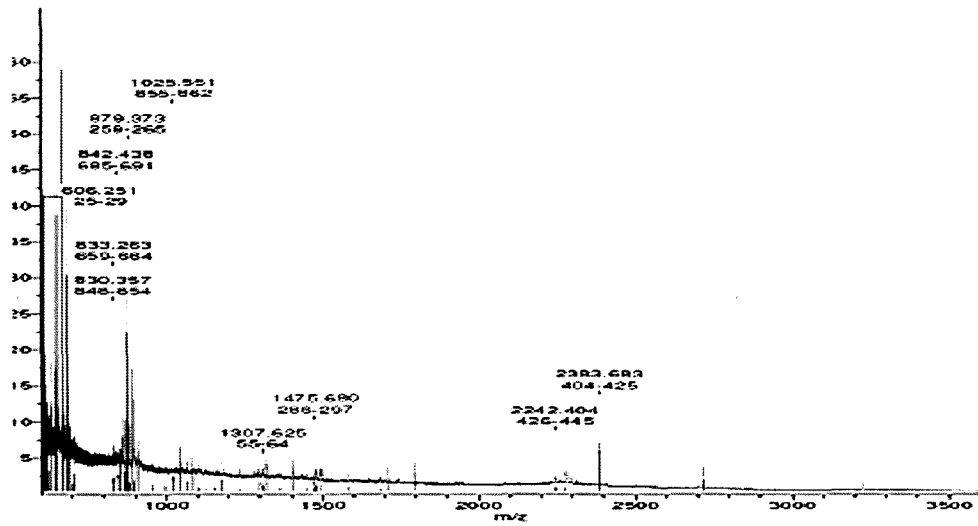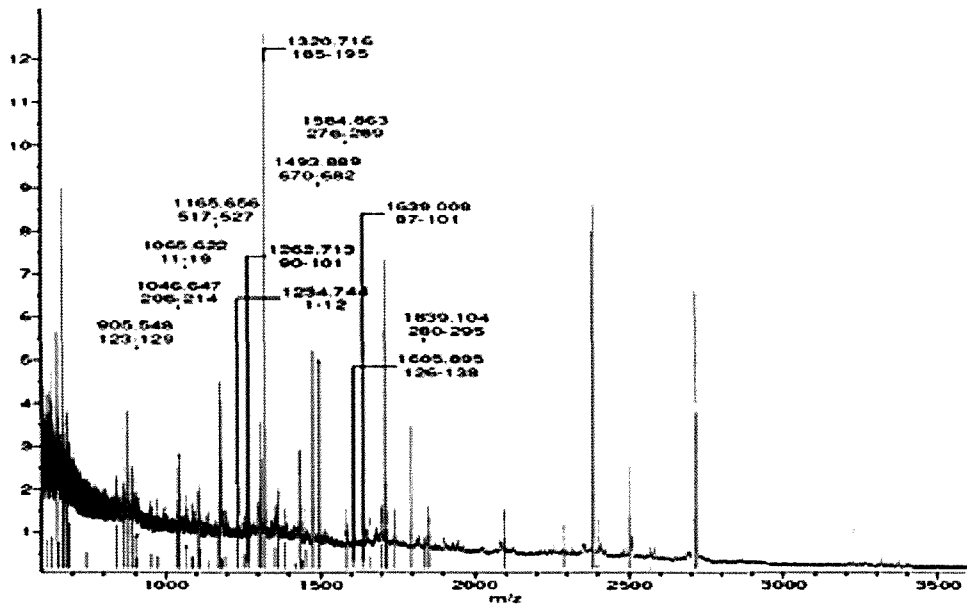
Fig 3.53: 2D gel electrophoresis of protein isolated from stable transfectants A) Proteins from vector transfected and B) from ribozyme transfected cells in 12% PAGE gel. M represents marker lane (3-10 represents pI values).

Protein spot 1: Interleukin 12 receptor, beta 2 precursor (Homo sapiens)

Fig 3.54: MALDI Analysis of peaks generated with digested peptides, and deduced as   Interleukin 12 receptor, beta 2 precursor and its sequence. The sequences highlighted in red are matching peaks generated from peptides.

Protein spot 6: ATR interacting protein (Homo sapiens)

Fig 3.55: MALDI Analysis of peaks generated with digested peptides, and deduced as ATR interacting protein (ATRIP) and its sequence. The sequences highlighted in red are matching peaks generated from peptides.

Protein spot 7: Beta –transducin repeat containing, isoform CRA-d (Homo sapiens)

Fig 3.56: MALDI Analysis of peaks generated trypsin fragments, and deduced as beta –transducin repeat containing, isoform CRA-d and its sequence. The sequences highlighted in red are matching peaks.

Protein spot 8:   Keratin 10 (Homo sapiens)

Fig 3.57: MALDI analysis of peaks generated with digested peptides, and deduced as protein keratin10 and its sequence. The sequences highlighted in red are matching peaks generated from peptides.
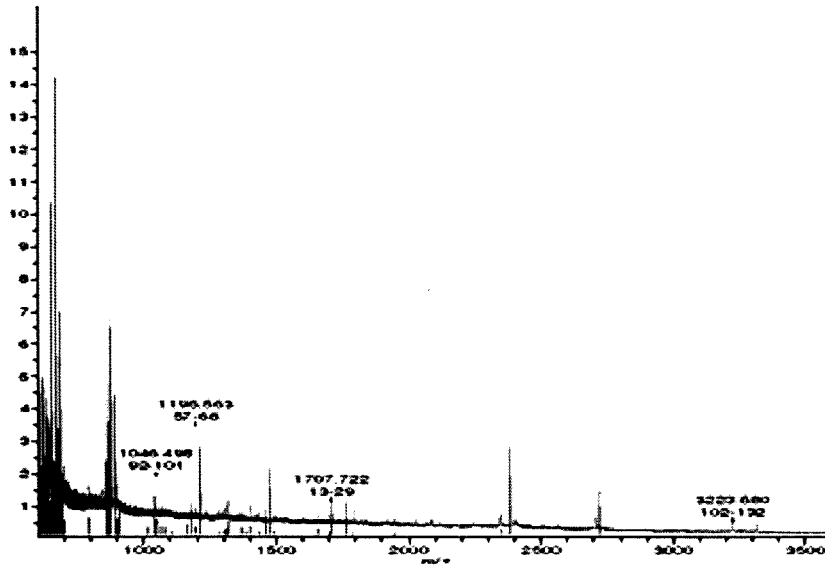
	10	20	30	40	50	60	70
	KLGLVRCEVA	HRPACVAGTG	QPSPPSPERS	ACGCGLHLSR	RKSAPRGGPS	VLPGTKLNSF	CAASGSSRLG

	80	90	100	110	120	130	140
	DQRQEVRAAA	GGVPGEAGAR	RARSSAQLAS	RTAPAAPPCA	PGLLASRCPP	EDSAGPFLPL	SREHVRGERD

	150	160	170	180	190	200	210
	VSCQRCCEGK	FGRRYPDCLP	RQRRFAAUGH	SRRFGILPSS	PFERSQFRLL	SEKRAEVTAP	ELRSGVGV

Protein spot 11: hCG 1993711

Fig 3.58: MALDI Analysis of peaks generated with digested peptides, and identified it as hCG and its sequence. The sequences highlighted in red are matching peaks generated from peptides.

III. 5.3. Discussion

Two dimensional PAGE gel electrophoresis resolves a large number of proteins from whole cell proteome. The proteomic profiling by 2D gel electrophoresis and MALDI analysis can identify not only differentially expressed proteins, but also the phosphorylation and other post translational modifications. The advent of new techniques like MALDI –TOF simplifies the identification of peptide finger print in mass scale analysis as the whole genome sequencing, proteomic efforts have already defined the possible peptide reference of the proteins from particular cellular and tissue types (Walker et al 2002). Comparative proteomic profiling of normal cell and pathologic cell allows one to identify the proteins that may play role in pathology of diseases.

The proteomic profiling of vector and ribozyme transfected cells shows that there is marked difference in expression patterns as about 13-15 identifiable protein species were differentially expressed . The 2D gel electrophoresis pattern reveals that most of the proteins have mild basic pI (iso electric point) and fall in low molecular weight ranges (17-82 kda). Only very few were identified as high molecular weight proteins.

In view of some of the differentially expressed proteins like keratin 10, and IL12R, ATRIP, we try to propose the pathway that may be involved in induction of differentiation in the ribozyme expressing cells.

One of the proteins that is specifically down regulated in ribozyme transfected and over expressed in vector transfected cells is Interleukin 12 receptor (IL-12R), a trans-membrane protein. Binding of IL12 with IL-12R leads to activation of JAK2, Tyk2, STAT3 and STAT4 transcription factors. TGF- β blocks the IL-12 induced activation of JAK/ STAT pathway. TGF-β also blocks the telomerase hTERT promoter. From our studies it can be suggested that down regulation of IL-12R may inhibit the JAK / STAT oncogenic pathway, and thereby inhibit cell proliferation.

Keratin 10 (K10) is major structural protein of the epidermis and recently its role was also proved in cell proliferation and differentiation. K10 expression is restricted to post mitotic cells of supra basal layer, and it is suppressed in wound and tumor cells. K10 inhibits proliferation by reducing cyclin D1 expression and pRb phosphorylation.

K10 binds with Akt,   PKC ζ is a key signaling protein in phosphatidyl ionositol 3 kinase pathways (PI-3-K). Akt promotes cell survival by phosphorylating BAD

164

(Proapoptotic), and activate NFkB via regulating IkB Kinase (IKK), resulting in transcription of pro-survival genes. K10 binds with Akt and PKC ζ and prevents the translocation of these kinases to the membrane. Through this mechanism keratin 10 affects proliferation, differentiation and apoptosis (Paramio et al. 2001).

ATR (ATM and Rad 3 related) is homologous to Ataxia Telangietasia–mutated (ATM) and is related to PI-3-K. These kinases respond to DNA damage events and initiate signals for cell cycle arrest. Loss of ATR destabilizes genome and increases the risk of transformation in to cancer. ATR exists as stable complex with associated protein ATRIP (ATR interacting protein). Loss of ATRIP by siRNA causes the same phenotype as ATR loss. Depletion of ATRIP from cell causes a decrease in the intracellular levels of ATR and vice versa. ATR is proved to be negatively regulated by hTR (Kedde et al. 2006), when the level of hTR is increased the ATR level is decreased. ATR and ATRIP are positively associated with each other. Ribozyme mediated knocking down of hTR in the present study seems to cause ATRIP over expression and induction of DNA damage signaling events. Since HeLa cells have abrogated p53, cell cycle arrest does not follow.

β-transducin repeat containing protein isoform (β-TrCP), role of which is still not proved, is a components of β-catenin and Iκ Bα for proteasomal degradation. It negatively regulates Wnt/ β-catenin signaling and positively regulates NFκB signaling. Over expression of β-TrCP down regulates the catenin, and the isoform is predicted to have same function (Hart et al. 1999). In our study over expression of β-TrCP may inhibit β-catenin, and also raise the question about the plasticity of partly differentiated cells having stem cell like properties.

Specifically, the human telomerase RNA (hTR) is expressed in normal cells, though telomerase activity is nil. This raises the question about its function in absence of its catalytic counterpart. Recent evidences suggests that hTR increases wound healing potency of mouse skin (Gonzalez-Suarez et al. 001), and induce transformation capability together with RAS oncogene (Stewart et al. 2002). Its role in ATR regulation further proves that telomerase RNA have extra telomeric function.

In conclusion, from our studies including DDPCR and 2D –MALDI- TOF analyses, we could infer that in absence of the master regulators of cell cycle p53 and pRB proteins (as in the HPV-transformed cells like HeLa), cells fail to induce apoptotic signaling and adopting anti-apoptotic pathway generally mediated by PI-3-K, JAK-

STAT and Wnt pathways. This pathway may be converged together to maintain the proliferation status along with telomerase and hTERT expression to get the proliferative advantage. Upon ribozyme expression against hTR, cellular genes for retinoblastoma binding protein 2 (RBP2), human enhancer of invasion cluster (HEi-C or CCD5), ribosomal protein 23, 35 (RPL 23, RPL35), emopamil binding protein (EBP), methionine- s-adenosyl transferase (MAT 2A) and interleukin 12 receptor are predicted to have positive relation with hTR function whereas keratin 10, β - transducin repeat containing isoform (β -TrCP-CRA-d) and ATR interacting protein (ATRIP) have negative regulation or association with hTR. Haplo-insufficiency levels of hTR, and complete knockdown studies were not performed; we could create analogous situation with the help of the ribozyme. Increased level of hTR has an effect on cellular survival and to a certain degree regulates other cellular genes.

Our results for the first time, seem to suggest that knocking down hTR results in induction of differentiation and during the process, the cells inhibit PI-3-K pathway and may adopt TGF β pathway. It should be mentioned that HeLA cells are p53-/-. From our own experimental proofs, STRING search of MALDI and DD-PCR results and other published sources we propose a hypothetical model on telomerase and its association with certain genes which provide proliferative, anti-apoptotic and differentiation functions to the cells.

166
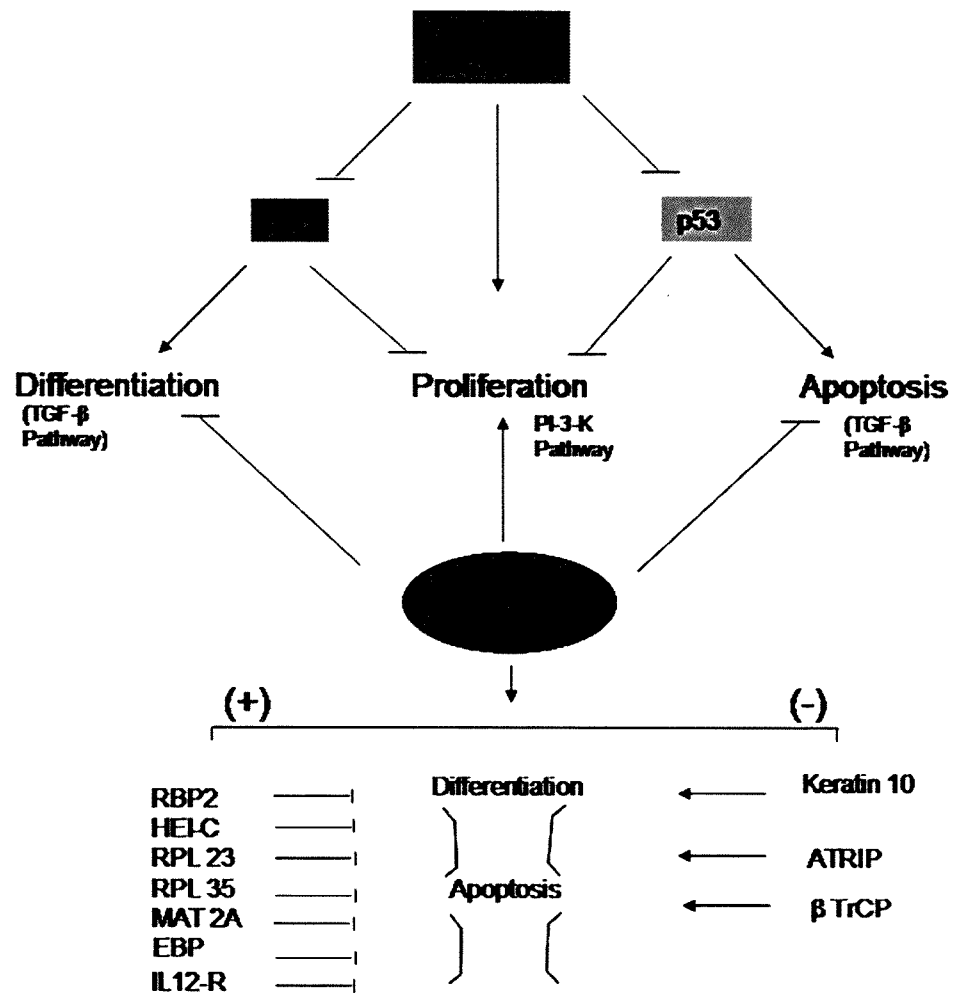
Fig 3.59: Model depicting the pathway and genes that are positively and negative association with telomerase RNA possibly by inhibiting PI-3-K, Wnt, JAK/STAT pathway and integrate with TGF- β pathway. The positively associated genes RBP2 suppresses differentiation while HEI-C, RPL23, RPL35, MAT2A, EBP and IL12-R promote proliferation and inhibit apoptosis. Similarly, the negatively associated gene Keratin10 promotes differentiation while ATRIP and βTrCP promote apoptosis.

III.6. Future Prospects.

Since hTR is found to have role in regulation of certain cellular genes, it would be interesting to make a comprehensive study of the pathways involved. Complete knock out of hTR (instead of knockdown) in presence and in absence of hTERT and its changes in molecular signatures in cancer cells and in normal cells with functional p53 and pRb status need to be studied in greater details. In knock down studies, either ribozyme or siRNA can be expressed by strong promoter-expression system like lenti-viral systems and co-localizing the therapeutic molecules to its target in nuclear or cytoplasmic compartment using certain signal sequences can add to the efficacy of the process. Usage of athymic or null mice for assessing the proliferative efficiency of hTR knocked out cells would give additional information on its role in proliferation.

Summary and Conclusions

Telomerase is overexpressed in most of the cancers and it provides proliferative advantage to the cells by maintaining telomere length as well as its contributions in tumorigenesis by unknown mechanism. Telomerase reverse transcriptase (hTERT) and telomerase RNA component both play role in maintenance of telomere length and associated cellular signaling events. As cancer is a disease caused by accumulated mutations and deregulated signaling and cell cycle events. Restricting its population doublings would be one of the effective strategies for combinatorial therapies. Targeting telomerase and its RNA component could prove to be an effective approach in suppressing cancer specific events and it would be appropriate to evaluate the global gene expression effects on targeting telomerase in cancer cells.

One of the approaches is using ribozyme to knockdown gene expression and targeting telomerase RNA by ribozyme would be one of the strategies in cancer therapeutics. Ribozymes are catalytic RNA molecules that can be directed to base pair with the specified target sequence and can cleave the transcript in to functionally inactive fragments that are further degraded by endogenous RNAse digestion.

Hammerhead ribozyme was designed using Zuker's M Fold programme by energy minimization and the conserved secondary structures of hTR were compared at multiple foldings. We selected 180th GUC of pseudo knot region of hTR for targeting.

Ribozyme coding oligos were annealed and cloned in pStu I vector and confirmed with sequencing. The positive clones were taken for cleavage assays. For *in vivo* expression ribozymes were cloned in pCI Neo mammalian expression vector and screened by colony blotting and further sequencing for confirmation of intactness of conserved sequence.

Human telomerase RNA was reverse transcribed and cloned in pGEM-T vector and sequenced to confirm the presence of target sequence. The ribozyme and hTR was *in vitro* transcribed and *in vitro* cleavage reaction was analyzed for the designed ribozyme. The ribozyme cleaves the target with relatively low turn over. Therefore it is necessary to use more than stochiometric concentration of ribozymes while a catalyst is characterized by non stochiometric relation with the substrate.

The evaluated ribozymes were cloned in mammalian expression vector (pCI-NEO) and transfected to HeLa cells and maintained as stable transfectants for 120 days. The ribozyme expressions were monitored and confirmed with sequencing for intactness

of its sequences. Telomerase RNA levels were found to be reduced in ribozyme expressed cells compared to vector only and mutant ribozyme transfected cells.

Telomerase activity was also found to be reduced in ribozyme transfected cells, but at higher concentration of cellular extracts, telomerase activity was found also in ribozyme transfected cells suggesting only partial inhibition of telomerase by ribozyme. The telomere length was reduced in ribozyme expressing cells.

The cell sorting analysis didn't show any change of cellular profiling between active ribozyme and mutant ribozyme transfected cells, revealing there is no change in cell cycle and cell proliferation and division cycles are not disturbed 45 days post transfection.

Morphology of cells was modified in ribozyme transfected cells compared to vector and mutant transfected cells. The ribozyme expressing cells showed extending morphology that is typical to differentiation phenotype. Most of the cells have shown such extended morphology in ribozyme transfected cells, though some similar cells were also observed in mutant ribozyme expressing cells.

Transcription profiling of vector, ribozyme and mutant ribozyme transfected cells reveals the disappearance of certain transcripts in ribozyme expressed cells compared to vector and mutant ribozyme transfectants. Disappeared transcripts were cloned and identified by sequencing and found to be coiled coil domain containing protein 5 (HEI-C), retinoblastoma binding protein -2 (RBP2), methionine s-adenosyl transferase (MAT-2B), ribosomal binding protein 23 and 35 (RPL23 and RPL 35) and Sterol isomerase.

Proteomic profiling of vector and ribozyme transfected cells reveals change in profiling and ribozyme transfected cells found to have appearance of spots which are absent in cells transfected with vector only. The proteins identified were IL12R from vector only transfected, and ATRIP, keratin10, β transducin repeat containing isoform and hCG from ribozyme transfected cells.

The proteomic and transcriptome of ribozyme expressing cells provide hints on crosstalk of telomerase, and telomerase RNA component with other cellular genes. From the existing evidences and from our studies, it seems that during proliferation, phosphatidyl Ianositol-3 kinase pathway plays role along with MAP kinase pathway and suppression of hTR inhibits these pathways and activates the TGF β pathway.

The following objectives were accomplished

- Ribozyme was designed against human telomerase RNA by comparing conserved secondary structures.

- Designed ribozyme was cloned in pStu I and pCI neo vector.

- Human telomerase RNA's cDNA was cloned in to pGEM-T vector.

- Cleavage assay of ribozyme reveals designed ribozyme cleaves the target but less efficiently.

- Expression of ribozyme in mammalian cells shows the expression of intact ribozymes and reduction of telomerase RNA and reduction of telomerase activity in ribozyme transfected cells.

- Discernible reduction of telomere length was observed in ribozyme transfected cells compared with vector and mutant transfected cells.

- There is no change in cell cycle profiling in transfectants but change in morphology observed in ribozyme transfected cells.

- Transcript profiling reveals there is change in profiles in ribozyme transfected, mostly disappearance of transcripts in ribozyme compared to vector and mutant ribozyme transfected cellular RNAs.

- Proteomic profiling reveals the expression of certain peptides in ribozyme transfected compared to vector only transfected cells.

- The transcriptome and proteomic profiling reveals the possible cross talks of telomerase RNA with other cellular genes and opening new avenues for extracurricular activities of telomerase and proves that telomerase can be effective target in cancer therapeutics.

Bibiliography

Agata Smogorzewska and Titia DeLange (2004) Regulation of Telomerase by Telomeric proteins. Ann Rev Biochem 73:177-208.

Ahmed .S, Palermo. C, Wan.S. and Walworth. N.C (2004). A novel protein with similarities to Rb binding protein 2 compensates for loss of Chk1 function and affects histone modification in fission yeast. Mol. Cell. Biol. 24: 3660–3669.

Aldous, W.K. et al. (1999) Effects of tamoxifen on telomerase activity in breast carcinoma cell lines. Cancer 85: 1523-1529,

Allsopp, R.C., H. Vaziri, C. Patterson, S. Goldstein, E.V. Younglai, A.B. Futcher, C.W. Greider, and C.B. Harley (1992). Telomere length predicts replicative capacity in human fibroblasts. Proc. Natl. Acad. Sci. USA. 89:10114–10118.

Amalric.F, Bachellerie.J.P and Caboche.M (1977) RNA methylation and control of eukaryotic RNA biosynthesis : Processing and Utilization of undermethylated tRNAs in CHO cells. Nucleic Acid Res Vol 4 12: 4357-4370.

Amiri, K. M. A. & Hagerman, P. J. (1996) The global conformation of an active hammerhead RNA during the process of self-cleavage, J.Mol. Biol. 261:125-134.

Antal, M., Boros, E., Solymosy, F., and Kiss, T.(2002). Analysis of the structure of human telomerase RNA in vivo. Nucleic Acids Res. 30: 912–920.

Armbruster, B., Banik, S., Guo, C., Smith, A., and Counter, C. (2001) N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. Mol. Cell. Biol. 21: 7775–7786.

Artandi S.E and Depinho.R (2000) Critical role of telomeres in suppressing and facilitating carcinogenesis. Current opinion in Genetics and development 10:39-46.

Atkinson S.P., Hoare S.F, Glasspool R.M., Keith W.N. (2005) Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the hTR and hTERT gene promoters, Cancer Res. 65 7585-7590.

Autexier, C., and Greider, C.W (1995). Boundary elements of the Tetrahymena telomerase. RNA template and alignment domains. Genes & Dev. 9: 2227-2239.

Avilion AA, Piatyszek MA, Gupta J, Shay JW, Bacchetti S, Greider CW(1996) Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues.Cancer Res.56(3):645-50.

Bagheri.S, Nosrati.M, Li.S, Fong.S, Torabian.S, Javier Range.J, Moore.D.H, Federman.S, LaPosa.R.R, Baehner.F.L,Sagebiel.R.W, Cleave.J.E, Haqq.C, Debs.R.J, Blackburn.E.H, and Kashani-Sabet.M (2006) Genes and pathways downstream of telomerase in melanoma metastasis. Proc Natl Acad Sci.USA.vol. 103: 11306-11311.

Banik, S.S.R., Guo, C., Smith, A.C., Margolis, S.S., Richardson, D.A., Tirado, C.A., and Counter, C.M. (2002) C-terminal regions of the human telomerase catalytic subunit essential for in vivo enzyme activity. Mol. Cell. Biol. 22: 6234–6246.

Bartek J., Bartkova J, Kyprianou N, Lalani E.-N,.Staskova Z,. Shearer M, Chang S, Taylor-Papadimitriou J (1991) Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. Proc. Natl. Acad. Sci. USA 88: 3520–3524.

Bassi, G. S., Mollegaard, N. E., Murchie, A. I. H., von Kitzing, E. &Lilley, D. M. J. (1995) Ionic interactions and the global conformations of the hammerhead ribozyme, Nat. Struct. Biol. 2: 45-55.
Baumann, P., and Cech, T. R. (2001) Pot1, the putative telomere end-binding protein in fission yeast and humans. Science 292, 1171–1175.

Benevolenskaya, E.V., Murray, H.L., Branton, P., Young, R.A., and Kaelin, W.G., Jr. (2005). Binding of pRB to the PHD protein RBP2 promotes cellular differentiation. Mol. Cell. 18: 623–635.

Bernhardt SL, Gjertsen MK, Trachsel S, Moller M, Eriksen JA, Meo N,Buanes T, Gaudernack G (2006) Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: a dose escalating phase I/II study. Br J Cancer 95: 1474–1482.

Bert Vogestein and Kenneth W.Kinzler (2004) Cancer Genes and the pathways they Control. Nature Medicine.10:789-799.

Bilsland AE, Anderson CJ, Fletcher-Monaghan AJ, McGregor F, Evans TR, Ganly I,Knox RJ, Plumb JA, Keith WN (2003) Selective ablation of human cancer cells by telomerase-specific adenoviral suicide gene therapy vectors expressing bacterial nitroreductase. Oncogene. 22(3): 370–380.

Bilsland. A.E, Stevenson. K, Atkinson.S, Kolch.W and Nicol Keith.W (2006) Transcriptional Repression of Telomerase RNA Gene Expression by c-Jun-NH2-Kinase and Sp1/Sp3. Cancer Res. 66: 1363-1370.

Blasco M.A,.Lee H W, Hande M.P,. Samper E, Landorp PM, Depinho R.A and C.W Greider (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 91: 25-34.

Borer,P.N., Dengler,B., Tinoco,I.,Jr and Uhlenbeck,O.C. (1974) Stability of ribonucleic acid double-stranded helices. J. Mol. Biol. 86: 843–853.

Boyer, S. N., Wazer D. E, and V. Band. 1996. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res. 56:4620-4624.

Branch A.D and Robertson. H.D (1991) Efficient trans cleavage and a common structural motif for the ribozymes of the human hepatitis delta agent. Proc Natl Acad Sci. 88(22):10163-10167.

Bringold F, Serrano M (2000) Tumor suppressors and oncogenes in cellular senescence. Exp. Gerontol. 35:317–329.

Brunsvig PF, Aamadal S, Gjertsen MK, Kvalheim G, Markowski-Grimsrud CJ, Sve I, Dyrhaug M, Trachsel S, Moller M, Eriksen JA, Gaudernack G (2006) Telomerase peptide vaccination: a phase I/II study in patients with non-small cell lung cancer. Cancer Immunol Immunother 55:1553– 1564.

Bucholc, M., Park, Y., and Lustig, A.J. (2001). Intrachromatid excision of telomeric DNA as a mechanism for telomere size control in Saccharomyces cerevisiae. Mol. Cell. Biol. 21, 6559–6573.

Buzayan J.M., Gerlach W. L. and Bruening G. (1986) Non-Enzymatic Cleavage and Ligation of RNAs Complementary to a Plant Virus Satellite RNA. Nature: 323:349-353.

Buzayan J.M., Hampel A. & Bruening G. (1986). Nucleotide Sequence and Newly Formed Phosphodiester Bond of Spontaneously Ligated Satellite Tobacco Ringspot Virus RNA. Nucl Aci Res. 14: 9729-9743.

Campisi, J. (2000) Cancer, aging and cellular senescence. In Vivo.14:183–188.

Cayuela M.L, Flores J, Blasco M.A (2005)The Telomerase RNA component Terc is required for the tumor –promoting effects of Tert over expression. EMBO reports .6:268-274.

Cech.T.R, Zaug.A.J and Grabowski.P.J(1981)In vitro splicing of the ribosomal RNA precursor of tetrahymena: Involvement of a guanosine nucleotide in the excision of the intervening sequence. Cell. 27: 487-496.

Chen J.L and .Greider. C.W (2003) Template boundary definition in mammalian Telomerase. Genes and Development 17:2747-2752.

Chen J.L, Blasco.M and Greider. C.W (2000) Secondary structure of vertebrate telomerase RNA. Cell .100:503-514.

Chen J.L, Opperman. K.K and Greider. C.W (2002) A critical stem–loop structure in the CR4–CR5 domain of mammalian telomerase RNA Nucl Aci.Res.30:592-597.

Chiang PK, Gordon RK, Tal J, Zeng C, Doctor BP, Parthasarathy K and McCann P (1996) S adenosyl methionine (SAM)and methylation. FASEB J, 10: 471-480.

Christensen J.K, Agger p.A, Cloods D, Pasini .S, Rose et al. (2007) RBP2 belongs to a family of demethylases, specific for tri-and dimethylated lysine 4 on histone 3. Cell. 128: 1063–1076.

Cohen S B., Mark E. Graham, George O. Lovrecz, Nicolai Bache, Phillip J. Robinson, Roger R. Reddel (2007) Protein Composition of Catalytically Active Human Telomerase from Immortal Cells. Science .315:1850-1853.

Colgin, L, Baran, K, Baumann, P, Cech, T, Reddel, R. (2003) Human POT1 facilitates telomere elongation by telomerase. Current biology: Current Biol.13: 942-6.

Collins, K. (2006) The biogenesis and regulation of telomerase holoenzymes. Nature Reviews Molecular and Cell Biology. 6: 484-494.

Cong YS, Woodring E. Wright, and Jerry W. Shay (2002) Human Telomerase and Its Regulation. Micro biol and Molecular biol Rev. 66: 407-425.

Cunningham C. C (2002) New modalities in oncology: ribozymes. BUMC Proceedings 15:247–249. Dahm, S. C & Uhlenbeck, 0. C. (1991) Role of divalent metal ions in the hammerhead RNA cleavage reaction, Biochemistry 30: 9464 -9469.

DeCaprio.J.A,Ludlow.J.W,Figge.J,Shew.J.Y,Huang.C.H, Lee.W.H, Marsilio.E, Paucha.E, and Livingston.D.M.(1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell. 54: 275-283.

Delisi,C. and Crothers,D.M. (1971) Prediction of RNA secondary structure. Proc. Natl Acad. Sci. USA. 68: 2682–2685.

Dahm SC, Uhlenbeck OC (1991). Role of divalent metal ions in the hammerhead RNA cleavage reaction. Biochemistry.30(39):9464–9469.

Dickson M.A, Hahn W.C, Ino Y, Ronfard V, Wu J.Y, Weinberg R.A., Louis D.N, Li F.P, Rheinwald J.G (2000) Human keratinocytes that express hTERT and also bypass a p16INK4a-enforced mechanism that limits lifespan become immortal yet retain normal growth and differentiation characteristics. Mol.Cell Biol. 20:1436–1447.

Diede, S.J. and Gottschling, D.E (1999). Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta. Cell 99, 723–733.

Dragon, F., Pogacic, V., and Filipowicz, W. (2000). In vitro assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs. Mol. Cell. Biol. 20, 3037–3048.

Dragony Fu, and Collins, K. (2003) Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. Mol. Cell .11: 1361–1372.

Einarson M, Cukierman E, Compton DA,Golemis EA (2004) Human enhancer of Invasion-Cluster,a coiled coil protein required for passage through mitosis. Mol.Cell Biol. 24: 3967-3971.

Evans, S.K., and Lundblad, V (2000). Positive and negative regulatory mechanism for telomere shortening. Cell 88, 657–666.

Farr C., Fantes J., Goodfellow P. and Cooke H. (1991) Functional reintroduction of human telomeres into mammalian cells. Proc. Natl Acad. Sci. USA, 88: 7006–7010.

Fedor, M. J. & Uhlenbeck, 0. C. (1990) Substrate sequence effects on 'hammerhead' RNA catalytic efficiency. Proc. Nutl Acad. Sci. USA 87: 1668-1672.

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, and al. et (1995) The RNA component of human telomerase. Science.269:1236-1241.

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, et al.(1995) The RNA component of human telomerase. Science. 269 (5228) : 1236-41.

Folini M., Colella G, Villa R, Lualdi S, Daidone MG, Zaffaroni N (2000) Inhibition of telomerase activity by a hammerhead ribozyme targeting the RNA componant of telomerase in human melanoma calls .J Invest dermatol 114:259-267.

Ford, L. F., Suh, J. M., Wright, W. E. & Shay, J. W.(2000) Heterogeneous nuclear ribonucleoproteins C1 and C2 associate with the RNA component of human telomerase. Mol. Cell. Biol. 20: 9084–9091.

Forster A. C. and Symons R. H. (1987) Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. Cell. 49: 211–220.

Fujiwara T, Urata Y, Tanaka N (2007) Telomerase-specific oncolytic virotherapy for human cancer with the hTERT promoter. Curr Cancer Drug Targets 7: 191–201.

Gael Cristofari and Joachim Lingner (2006) Telomere length homeostasis requires that telomerase levels are limiting. EMBO.J .25:565-574.

Gall, J.G. (2003) The centennial of the Cajal body. Nat. Rev. Mol. Cell Biol. 4: 975-980.

Gannot, P., Bortolin, M.-L., & Kiss, T. (1997) Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. Cell, 89, 799-809.

Gao Q., Hauser S.H, Liu X.-L, Wazer D.E, Madoc-Jones H, Band V. (1996) Mutant p53-induced immortalization of primary human mammary epithelial cells, Cancer Res. 56: 3129–3133.

Gavory G, Farrow .M, Balasubramanian .S (2002) Minimum Length Requirement of the alignmemnt domain of human telomerase RNA to sustain catalytic activity in vitro. Nuclic Acid Res .30: 4470-4480.

Glasspool R.M., Burns S, Hoare S.F., Svensson C., Keith N.W. (2005) The hTERT and hTERC telomerase gene promoters are activated by the second exon of the adenoviral protein, E1A, identifying the transcriptional Co repressor CtBP as a potential repressor of both genes, Neoplasia 7: 614-622.

Global Cancer Facts & Figures (2007) American Cancer society .1-46.

Gollahon L.S, Shay J.W (1996) Immortalization of human mammary epithelial cells transfected with mutant p53 (273his).Oncogene 12:715–725.

Gonzalez-Suarez.E, Samper.E, Ramirez.A., Flores.J.M, Martin-Caballero.J, Jorcano .J.L, Blasco.M.A (2001). Increased epidermal tumors and increased skin wound healing in transgenic mice over expressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes. EMBO.J. 20: 2619-2630.

Grand CL, Han H, Muñoz RM, Weitman S, Von Hoff DD, Hurley LH, Bearss DJ (2002) The cationic porphyrin TMPyP4 down-regulates c-MYC and human telomerase reverse transcriptase expression and inhibits tumor growth in vivo. Mol Cancer Ther. 1(8):565-73.

Greenberg R.A, Chin.L, Femino.A, Lee. K.H, Gottlieb.G.J, Singer. R.H, Greider, C.W and DePinho. R.A (1999) Short Dysfunctional Telomeres Impair Tumorigenesis in the INK4an2/3 Cancer-Prone Mouse. Cell. 97: 515-525.

Greider CW, Blackburn EH (1987) The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell. .51(6): 887–898.

Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T (1999) Mammalian telomeres end in a large duplex loop. Cell. 97(4): 503-514.

Guerrier-Takada .C, Gardiner.K, Marsh.T, Pace.N, and Altman.S (1983)The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell. 35: 849-857.

Hahn C.W, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, Beijersbergen RL, Knoll JHM, Mayerson M, Weinberg RA (1999) Inhibition of telomerase limits the growth of human cancer cells. Nature Medicine. No5, 1164-1170.

Hanahan D and Weinberg R.A (2000) The Hall marks of Cancer. Cell .100:57-70.

Hanish J.P., Yanowitz J.L. and de Lange T. (1994) Stringent sequence requirements for the formation of human telomeres. Proc. Natl Acad. Sci. USA, 91: 8861–8865.

Harley, C.B., Futcher, A.B. and Greider, C.W (1990). Telomeres shorten during ageing of human fibroblasts. Nature 345, 458–460.

Hart M., Concordet J-P, Lassot .I, Albert .I, del los Santos. R, Durand. H., Perret. C., Rubinfeld B., Margottin F, Benarous R. and Polakis .P. (1999). The F-box protein b-TrCP associates with phosphorylated b-catenin and regulates its activity in the cell. Current Biology. 9:207–210.

Haseloff, J. and Gerlach, W.L (1988) Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature. 334: 585–591.

Haseloff, J. and Gerlach, W.L (1989) Sequences Required for Self-Catalyzed Cleavage of the Satellite RNA of Tobacco Ringspot Virus. Gene: 82: 43-52.

Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. Nature 387:296-299.

Hayflick, L (1965). The limited *in vitro* lifetime of human diploid cell strains. Exp. Cell Res. 37, 614–636.

Hayflick, L. and Moorhead, P. S (1961). The serial cultivation of human diploid cell strains. Exp. Cell Res. 25, 585–621.

Hayflick,L. and Moorhead,P.S. (1961) The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res., 25, 585--621.

Henras, A.K., Dez, C., and Henry, Y. (2004). RNA structure and function in C/D and H/ACA sno RNPs. Curr. Opin. Struct. Biol. 14: 335-343.

Herbert B-S, Gellert GC, Hochreiter A, Pongracz K, Wright WE, Zielinska D, Chin AC, Harley CB, Shay JW, Gryaznov SM (2005) Lipid modification of oligonucleotide N30-P50 – thio-phosphoramidates enhances the potency of telomerase inhibition. Oncogene 24: 5262– 5268.

Hinh Ly, Elizabeth H. Blackburn and Tristram G parslow (2003) Comprehensive structure –Function Analysis of the Core Domain of human telomerase RNAs. Molecular and cellular Biology .6849-6856.

Hinh Ly, Rodrigo T. Calado, Paulette Allard, Gabriela M. Baerlocher, Peter M. Lansdorp, Neal S. Young, and Tristram G. Parslow (2005) Functional characterization of telomerase RNAvariants found in patients with hematologic disorders. Blood. 105: 2332-2339.

Hochreiter A.E, Xiao.H, Goldblatt E.M, Gryaznov S.M, Miller K.D, Badve .S, Sledge G .Wand Brittney-Shea Herbert (2006)Telomerase Template Antagonist GRN163L disrupts Telomere Maintenance, Tumor Growth, and Metastasis of Breast Cancer . Clinical Res :12.3184-3192.

Horner S.M, DeFilippis R.A, Manuelidis.L and DiMaio.D (2004) Repression of the Human Papillomavirus E6 Gene Initiates p53-Dependent, Telomerase-Independent Senescence and Apoptosis in HeLa Cervical Carcinoma Cells. Journal of Virology. 78: 4063-73.

Huard, S. and Autexier, C.(2004) Human telomerase catalyzes nucleolytic primer cleavage. Nucleic Acids Res. 32: 2171–2180.

Huffman, K.E., Levene, S.D. Tesmer, V.M., Shay, J.W., and Wright, W.E. (2000). Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang. J. Biol. Chem. 275, 19719–19722.

Hutchins C.J,. Rathjen P. D. Forster A. C and Robert H. Symons (1986) Self Cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. Nucl Aci.Res.14: 3627-3640.

Ittai .B.P and Weinberg.R (2004) When cells get stresses: an integrative view of cellular senescence .J Clin. Invest.113:8-13.

Izbicka E, Wheelhouse RT, Raymond E, Davidson KK, Lawrence RA, Sun D, Windle BE, Hurley LH, Von Hoff DD (1999) Effects of cationic porphyrins as G-quadruplex interactive agents in human tumor cells. Cancer Res.59(3):639-44.

Jack D. Griffith, Laurey Comeau, Soraya Rosenfield, Rachel M Stansel, Alessandro Bianchi, heidi Moss and Titia de Lange (1999) Mammalian Telomeres end in a Large Duplex Loop. Cell.97:503-514.

Jády D.X , Verheggen B.E, Kiss. C., Bertrand A.M. and Kiss, T. (2002). Cajal body-specific small nuclear RNAs: A novel class of 2-O-methylation and pseudouridylation guide RNAs. EMBO J. 21: 2746-2756.

Jady, B. E., Darzacq, X., Tucker, K. E., Matera, A. G., Bertrand, E. and Kiss, T. (2003). Modification of Sm small nuclear RNAs occurs in the nucleoplasmic Cajal bodies following import from the cytoplasm. EMBO J. 22, 1878-1888.

Jády, B.E., Bertrand, E., and Kiss, T. (2004). Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. J. Cell Biol. 164: 647-652

Jerry Shay and Woodring Wright (2005) Senescence and Immortalization: role of telomeres and telomerase. Carcinogenesis 26: 867-874.

Johnson-Pais T., Degnin C., Thayer M.J (2001) pRB induces Sp1 activity by Relieving inhibition mediated by MDM2, Proc. Natl. Acad. Sci. USA 98 2211-2216.

Jones LD, Alani RM and Münger K. (1997).The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. Genes Dev. 11: 2101–2111.

Kamijo. T,. Weber J. D, Zambetti. G., Zindy.F, Roussel M.F, and Sherr. C.J (1998) Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. Proc Natl Acad. Sci.USA 1998. 95: 8292-8297.

Kanaya T, Kyo S, Hamada K, Takakura M, Kitagawa Y, Harada H, Inoue M (2000) Adenoviral expression of p53 represses telomerase activity through down-regulation of human telomerase reverse transcriptase transcription. Clin Cancer Res. 6(4):1239-47.

Karlseder J., Broccoli,D., Dai,Y., Hardy,S. and de Lange,T. (1999) p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science, 283: 1321–1325.

Karlseder J., Smogorzewska,A. and de Lange,T. (2002) Senescence induced by altered telomere state, not telomere loss. Science, 295: 2446–2449.

Kathleen Collins (2006) The Biogenesis and Regulation of telomerase Holoenzyme. Nature reviews in Molecular cell biology .7: 484-494.

Kedde.M, Sage.C.L, Duursma. A, Zlotorynski.E, Leeuwen.B.V, Nijkamp.W, Beijersbergen. R, and Agami.R (2006). Telomerase-independent Regulation of ATR by Human Telomerase RNA. J.Biol.Chem. 281: 40503-40514.

Keith W.N., Vulliamy T., Zhao J., Ar C., Erzik C.,. Bilsland A, Ulku B., Marrone A., Mason P.J, Bessler M., Serakinci N., Dokal I. (2004) A mutation In a functional Sp1 binding site of the telomerase RNA gene (hTERC) Promoter in a patient with Paroxysmal Nocturnal Haemoglobinuria. BMC Blood Disord . 4 :3.

Keith WN, Bilsland A (2008) Targeting telomerase: therapeutic options for cancer treatment. In Telomeres and Telomerase in Ageing, Disease, and Cancer, Rudolph KL (ed) Springer-Verlag: Berlin, Heidelberg, Germany. pp 247–284.

Keith WN, Bilsland A, Hardie M, Evans TR (2004) Cancer cell immortality: telomerase as a target for novel cancer gene therapies. Nat Clin Pract Oncol 1: 88–96.

Keith WN, Thomson CM, Howcroft J, Maitland NJ, Shay JW (2007) Seeding drug discovery: integrating telomerase cancer biology and cellular senescence to uncover new therapeutic opportunities in targeting cancer stem cells. Drug Discov Today 12: 611–621.

Kenneth E.Huffman,Stephen D.Levene, Valerie M Tesmers, Jerry w. Shay and Woodwring Wright (2000) Telomere shortening is proportional to the size of the G rich telomeric 3'-overhang. J.Biol ,Chem . 275; 26:19719-19722.

Kiehntopf, M., Esquivel, E. L.,Brach, M. A. & Herrmann. F (1995) Ribozymes: Biology, biochemistry, and implications for clinical medicine, J. Mol. Med. 73, 65-71.

Kijima H, Ishida H, Ohkawa T, Kashani-Sabet M, Scanlon KJ (1995) Therapeutic applications of ribozymes. Pharmacol Ther.68:247-67.

Kilian. A., Bowtell D. D, Abud H.E, Hime G. R., Venter D. J., Keese P. K, Duncan E.L, Reddel R. R and. Jefferson R. A (1997) Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum. Mol. Genet. 6:2011–2019.

Kiyono T., Foster S.A, Koop J.J, McDougall J.K,.Galloway D.A, Klingelhutz A.J. (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cell. Nature. 396: 84–88.

Klara R. Birikh, Paul A. Heaton and Fritz Eckstein(1997) Structure function and application of the hammerhead Ribozyme .European Journal of Biochemistry .245:1-16.

Klaus Damm et al. (2001) A highly selective Telomerase Inhibitor limiting Human cancer cell proliferation. EMBO .J. 20: 6958-6968.

Klose R.J, Yan.Q, Tothova. Z, Yamane.K, Erdjument-Bromage.H, Tempst. P, Gilliland. D.G, Zhang .Y, and Kaelin,W.G (2007). The Retinoblastoma Binding Protein RBP2 Is an H3K4 Demethylase. Cell.128:889-900.

Knudson, A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68, 820-823.

Koga S, Kondo Y, Komata T, Kondo S (2001) Treatment of bladder cancer cells *in vitro* and *in vivo* with 2-5 A antisense telomerase RNAs. Gene therapy 8:654-658.

Kondo .Y, Koga S, Komata T, Kondo S (2000) Treatment of prostate cancer *invitro* and invivo with 2-5 A antitelomerase RNA componant. Oncogene 19:2205-2211.

Kondo S, Kondo Y, Li G, Silverman RH, Cowell JK (1998) Targeted therapy of human malignant glioma in a mouse model by 2-5A antisense directed against telomerase RNA.Oncogene.16:3323-30.

Kondo S, Tanaka.Y, Kondo.Y, Hitomi.M, Barnett.G H, Ishizaka.Y, Liu.J, Haqqi.T, Nishiyama.A, Villeponteau.B, Cowell.J.K, and Barna B.P (1998) Antisense telomerase treatment: induction of two distinct pathways, apoptosis and differentiation . FASEB J. 12: 801-811.

Konig P., Fairall L. and Rhodes D. (1998) Sequence specific DNA recognition by the Myb-like domain of the human telomere binding protein TRF1: a model for the protein–DNA complex. Nucleic Acids Res., 26: 1731–1740.

Kruger.K, Graboeski P.J, Zaug >A.J,Sands.J, Gottschiling.D.E, Cech.T.R (1982) Self splicing RNA: Autoexcision and Autocyclization of the ribosomal RNA intervening sequence of Tetrahymena.Cell.31: 147-157.

Ku, W.C., Cheng, A.J. and Wang, T.C. (1997) Inhibition of telomerase activity by PKC inhibitors in human nasopharyngeal cancer cells in culture. Biochem Biophys Res Commun.241:730-736.

Kubbutat MH, Jones SN, Vousden KH (1997) Regulation of p53 stability by Mdm2 Nature 387:299-303.

Kumar, P. K. R. & Ellington, A. D. (1995) Artificial evolution and natural ribozymes, FASEB J. 9, 1183-1195.

Kushner D.M., Paranjape JM, Bandyopadhyay B, Cramer H, Leaman, DW. Kennedy AW,Silverman RH , Cowell JK (2000) 2.5A antisense Directed against telomerase RNA produces apoptosis in ovarian cancer cells. Gynecol Oncol 76:183-192.

Kyo S and Inoue .M (2002) Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy?. Oncogene. 21(4):688-97.

LaBranche, H. et al.. (1998) Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. Nature Genet. 19:199–202

Lai, C.K., Miller, M.C. and Collins, K. (2003) Roles for RNA in telomerase nucleotide and repeat addition processivity. Mol. Cell 11: 1673–1683.

Leclerc, F., and Karplus, M (2006). Two-metal-ion mechanism for hammerhead-ribozyme catalysis. J. Phys. Chem. B 110: 3395–3409.

Lee, S.R., Wong, M.Y., and Collins, K. (2003) Human telomerase reverse transcriptase motifs required for elongation of a telomeric substrate. J. Biol. Chem. 278: 52531–52536.

Li, B., and Lustig, A.J. (1996). A novel mechanism for telomere size control in Saccharomyces cerevisiae. Genes Dev. 10, 1310–1326.

Liang P, Pardee AB (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science. 257:967-71.

Liang P, Zhu W, Zhang X, Guo Z, O'Connell RP, Averboukh L, Wang F, Pardee AB (1994). Differential display using one-base anchored oligo-dT primers. Nucleic Acids Res. 25; 22(25):5763-4.

Lingner, J., and Cech, T. R. (1996). Purification of telomerase from Euplotes aediculatus: requirement of a primer 3' overhang. Proc Natl Acad Sci U S A 93, 10712-10717

Loayza, D., and T. De Lange (2003). POT1 as a terminal transducer of TRF1 telomere length control. Nature 423:1013-1018

Lott, W.B., Pontius, B.W., and von Hippel, P.H (1998). A two-metal ion mechanism operates in the hammerhead ribozyme-mediated cleavage of an RNA substrate. Proc. Natl. Acad. Sci. USA 95: 542–547.

Ludwig .A, Saretzki .G, Holm P.S, Tiemann F, Lorenz M, Emrich .T, Harley C.B, Von Zglinicki.T (2001) Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial calls to inhibitors of topo isomerase. Cancer Res 61: 3053-3061.

Makarov, V.L., Hirose, Y., and Langmore, J.P. (1997). Long G tails at both ends of human chromosomes suggest a C strand degradation for telomere shortening. Cell. 88:657–666.

Martin-Rivera, L. and Blasco, M.A.(2001) Identification of functional domains and dominant negative mutations in vertebrate telomerase RNA using an in vivo reconstitution system. J. Biol. Chem. 276: 5856-5865.

McElligott, R. and Wellinger, R.J. (1997). The terminal DNA structure of mammalian chromosomes. EMBO J. 16, 3705–3714.

Melana, S.M., Holland, J.F. and Pogo, B.G.(1998) Inhibition of cell growth and telomerase activity of breast cancer cells in vitro by 3'-azido-3'-deoxythymidine. Clin Cancer Res 4: 693-696.

Meyerson, M., C. M. Counter, E. N. Eaton, L. W. Ellisen, P. Steiner, S. D.Caddle, L. Ziaugra, R. L. Beijersbergen, M. J. Davidoff, Q. Liu, S. Bacchetti, D. A. Haber, and R. A. Weinberg. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell .90:785–795.

Michael Z. Levy, Richard C. Allsopp, A. Bruce Futcherc, Carol W. Greiderc and Calvin B. Harley (1992) Telomere end-replication problem and cell aging. Journal of Molecular Biology. 225; 951-960.

Michienzi, A. et al. (2003). RNA-mediated inhibition of HIV in a gene therapy setting. Ann.NY Acad. Sci. 1002: 63–71.

Miller, A. D. and G. Rosman. (1989) Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980–990.

Misiti.S. et al. (2000) Induction of hTERT expression and telomerase activity by estrogens in human ovary epithelium cells. Mol Cell Biol.20: 3764-3771.

Mitchell, J. R., and K. Collins. (2000) Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase. Mol. Cell 6:361–371.

Mitchell, J.R., Cheng, J., and Collins, K.(1999a) A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. Mol. Cell. Biol. 19: 567-57.

Moebius FF, Fitzky. B.U, Wietzorrek.G, Haidekker.A, Eder.A, Glossmann. H (2003). Cloning of an emopamil-binding protein (EBP)-like protein that lacks sterol delta 8-delta 7 isomerase activity .Biochem J. 374:229-37.

Montanaro L., Mazzini G, Barbieri M, Vici. S, Nardi-Pantoli .A., Govoni. M, Donati.G, Treré.D and. Derenzini .M (2007) Different effects of ribosome biogenesis inhibition on cell proliferation in retinoblastoma protein- and p53-deficient and proficient human osteosarcoma cell lines.Cell Prolif 40(4) 532-49.

Morales, C. P., Burdick J. S, Saboorian M. H, Wright W. E, and.Shay J. W. (1998) In situ hybridization for telomerase RNA in routine cytologic brushings for the diagnosis of pancreatic obiliary malignancies. Gastrointest. Endosc. 48:402–405.

Morales, C. P., Holt S. E., Ouellette M., Kaur K. J., Yan Y., Wilson K. S.,. White M. A, Wright W. E., and Shay J. W. (1999). Absence of cancer associated changes in human fibroblasts immortalized with telomerase. Nat. Genet. 21:115–118.

Morales, C. P., Lee E. L, and. Shay J. W. (1998). In situ hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. Cancer 83:652–659. Morgenstern, J. P., and Land H. (1990). Advanced mammalian gene transfer :high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18:3587–3596.

Moriarty T J, Delphine T, Marie-Egyptienne and Chatal Autexier (2004) Functional organization of Repeat addition processivity and DNA Synthesis determinants in the Human Telomerase Multimer . Mol Cell Biol.24:3720-3733.

Moriarty, T. J., Marie-Egyptienne and Chatal Autexier. (2005). Regulation of 5' template usage and incorporation of noncognate nucleotides by human telomerase. RNA 11: 1448-1460.

Moriarty, T. J., Ward, R. J., Taboski, M. A.S., Autexier, C. (2005). An Anchor Site-Type Defect in Human Telomerase That Disrupts Telomere Length Maintenance and Cellular Immortalization. Mol. Biol. Cell 16: 3152-3161.

Mueller E, Sarraf P, Tontonoz .P, Evans R.M, Martin.K.J, Zhang.M, Fletcher.C, Singer.S, Spiegelman B.M (1998).Terminal differentiation of human breast cancer through PPARγ. Molecular Cell, 1: 465–470.

Murakami, J. et al. (1999) Inhibition of telomerase activity and cell proliferation by a reverse transcriptase inhibitor in gynaecological cancer cell lines. Eur J Cancer 35: 1027-1034.

Murray, J. B., Adams, C. J., Arnold, J. R. P. & Stockley, P .G. (1995) The roles of the conserved pyrimidine bases in hammerhead ribozyme catalysis - evidence for a magnesium ion-binding site, Biochem. J.311: 487-494.

Nagano, M., and. Kelly P. A. (1994). Tissue distribution and regulation of rat prolactin receptor gene expression. J. Biol. Chem. 269:13337–13345.

Nair SK, Heiser A, Boczkowski D, Majumdar A, Naoe M, Lebkowski JS, Vieweg J, Gilboa E (2000) Induction of cytotoxic T lymphocyte responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. Nat Med 6:1011– 1017.

Nakamaye, K. L. & Eckstein, F. (1994) AUA-Cleaving hammerhead ribozymes: Attempted selection for improved cleavage, Biochemistry 33, 1271 - 1271.

Nakamaye, K. L. & Eckstein, F. (1994) AUA-Cleaving hammerhead ribozymes: Attempted selection for improved cleavage, Biochemistry.33,1271-77.

Nakamura, T. M., Morin G. B., Chapman K. B., Weinrich S. L, H.Andrews W., Lingner J., Harley C. B., and Cech T. R.. (1997) Telomerase catalytic subunit homologs from fission yeast and human. Science 277:955–959.

Narita M., Nunez S., Heard E., Lin A.W., Hearn S.A., Spector D.L., Hannon G.J., and Lowe, S.W. (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113: 703–716.

Ngok, F.K. et al. (2004). Clinical gene therapy research utilizing ribozymes: application to the treatment of HIV/AIDS. Methods Mol. Biol. 252: 581–598 .

Nicholas S. Y Ting, Yaping Yu, Brant Poherelic,, Susan P Lees-Miller and Tara L. Beattie.(2005) Human Ku70/80 interacts directly with hTR, the RNA component of human telomerase. Nucleic Acid Res. 33: 2090-2098.

Nojiri.H, Manya H.N, Isono.H, Yamana.H, Nojima.S (1999).Induction of terminal differentiation and apoptosis in human colonic carcinoma cells by brefeldin A, a drug affecting ganglioside biosynthesis. FEBS letters. 453:140-144.

Nowell .P.C. (1976) The Clonal evolution of tumor cell populations. Science 194 :23-28.

Nussinov,R. and Jacobson,A.B. (1980) Fast algorithm for predicting the secondary structure of single-stranded RNA. Proc. Natl Acad. Sci. USA. 77: 6309–6313.

Nussinov,R., Pieczenik,G., Griggs,J.R. and Kleitman,D.J. (1978) Algorithm for loop matchings. SIAM J. Appl. Math.. 35: 68–82.

Ohki. R , Tsurimoto. T, and Ishikawa.F (2001)In Vitro Reconstitution of the End Replication Problem. Mol.Cell.Biol.21:5753-5766.

Olovnikov, A. M. (1973) A theory of marginotomy: The incomplete copying of template margin in enzyme synthesis of polynucleotides and biological significance of the problem.J. Theor. Biol. 41, 181–190.

Paramio.J.M, Segrelles.C, Ruisz.S and Jorcano.J.L (2001). Inhibition of Protein Kinase B (PKB) and PKCζ Mediates Keratin K10-Induced Cell Cycle Arrest. Mol cell biol. 21:7449-7459.

Pendino, F. et al. (2001) Retinoids down-regulate telomerase and telomere length in a pathway distinct from leukemia cell differentiation. Proc Natl Acad Sci U S A 98: 6662-6667.

Peng, Y., Mian, I., and Lue, N. (2001) Analysis of telomerase processivity: Mechanistic similarity to HIV-1 reverse transcriptase and role in telomere maintenance. Mol. Cell 7: 1201–1211.

Pierce G.B, Andverney E.L. (1961).An in Vitro and in Vivo Study of Differentiation in Teratocarcinomas. Cancer. 14: 1017-29.

Pierce, G.B. (1961). Teratocarcinomas. Can. Cancer Conf. 4: 119–137.

Platani, M., Goldberg, I., Swedlow, J. R., and Lamond, A. I. (2000). In vivo analysis of Cajal body movement, separation, and joining in live human cells. J. Cell Biol. 151: 1561–1574.

Pley, H. W., Flaherty, K. M. & McKay, D. B. (1994) Three-dimensional structure of a hammerhead ribozyme, Nature 372, 68-74.

Plumb JA, Bilsland A, Kakani R, Zhao J, Glasspool RM, Knox RJ, Evens TRJ, Keith WN (2001) Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954. Oncogene 20: 7797–7803.

Pomerantz, J., N. Schreiber-Agus, N.J. Liegeois, A. Silverman, L. Alland, L. Chin, J. Potes, K. Chen, I. Orlow, H.W. Lee, C. Cordon-Cardo, and R.A. DePinho. (1998). The INK4a tumor suppressor gene product, p19ARF, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 92: 713-723.

Pool, M.R., Stumm. J, Fulga. T.A, Sinning. I, and B. Dobberstein (2002). Distinct modes of signal recognition particle interaction with the ribosome. Science. 297:1345–1348.

Poole, J.C., Andrews, L.G. and Tollefsbol, T.O. (2001) Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene 269: 1-12.

Prescott, J. and Blackburn, E.H. (1997) Telomerase RNA mutations in Saccharomyces cerevisiae alter telomerase action and reveal non-processivity in vivo and in vitro. Genes & Dev. 11: 528–540.

Rebecca L Tomlinson, Tania D Ziegler,Teerawit supakorndej,Rebecca Terns and Michael.P. Terns (2006) Cell cycle regulated Trafficking of human telomerase to telomeres. Mol Biology of cell 17: 955-965.

Redon. S, Reichenbach.P and Lingner.J (2007) Protein-RNA and Protein-Protein interaction mediates association of human EST1A/SMG6 with telomerase. Nucleic Acid Res . 35: 7011-7022.

Rheinwald J.G, Hahn W.C, Ramsey M.R, Wu J.Y, Guo Z, Tsao H., De Luca M., Catricala C., O'Toole K.M (2002) A two-stage p16INK4a-and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol. Cell. Biol. 22:5157–5172.

Rieko Ohki, Toshiki Tsurimoto, and Fuyuki Ishikawa (2001) In Vitro Reconstitution of the End Replication Problem. Molecular and Cellular Biology, 21: 5753-5766.

Ruffner, D. E., Stormo G. D. & Uhlenbeck, O.C. (1990) Sequence requirements of the hammerhead RNA self-cleavage reaction. Biochemistry. 29: 10695-10702.

Sachsinger, J. et al. (2001) Telomerase inhibition in RenCa, a murine tumor cell line with short telomeres, by over expression of a dominant negative mTERT mutant, reveals fundamental differences in telomerase regulation between human and murine cells. Cancer Res 61: 5580-5586.

Sankoff,D., Kruskal,J.B., Mainville,S. and Cedergren,R.J. (1983) Fast algorithms to determine RNA secondary structures containing multiple loops. chapter 3. In Sankoff,D. and Kruskal,J.B. (eds), Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison, Addison-Wesley Reading, MA, pp. 93–120.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 63: 1129–1136.

Schubert S and Kurreck J (2004) Ribozymes and Deoxyribozyme strategies for medical applications. Current drug targets. 5:667-681.

Scott, W. G., Finch, J. T. & Klug, A. (1995) The crystal structure of an all-RNA hammerhead ribozyme - a proposed mechanism for RNA catalytic cleavage, Cell 81: 991 – 1002.

Schubert..S and Kurreck. J (2004) Ribozyme and Deoxyribozyme-Strategies for Medical Applications. Curr Drug Targets 5:667-681.

Serakinci N, Hoare S.F, Kassem M., Atkinson S.P., Keith W.N (2006), Telomerase promoter reprogramming and interaction with general transcription factors in the human mesenchymal stem cell, Regen. Med 1 125-131.

Seto, A.G., Umansky, K., Tzfati, Y., Zang, A.J., Blackburn, E.H., and Cech, T.R. (2003) A template-proximal RNA paired element contributes to Saccharomyces cerevisiae telomerase activity. RNA 9: 1323–1332.

Shay J W. and Bacchetti S (1997) A survey of telomerase activity in human cancer. Eur.J.Cancer 33:787-791.

Shay JW, Keith WN.(2008)Targeting telomerase for cancer therapeutics.Br. J. Cancer.98:677-83

Shay J.W, Tomlinson G, Piatyszek M.A, Gollahon L.S (1995) Spontaneous in vitro immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. Mol. Cell. Biol. 15425–432.

Shay J.W, Van Der Haegen B.A, Ying Y., Wright W.E (1993) The frequency of immortalization of human fibroblasts and mammary epithelial cells transfected with SV40 Large T antigen. Exp. Cell Res. 209: 45–52.

Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer. 1997 Apr;33(5):787–791.

Shay.J.W and Wright.W (2000) Hayflick, his Limit and Cellular Aging. Nature Reviews Molecular cell biology. 1: 72-76.

Shay JW, Wright WE (2005): Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis. 26:867-874.

Sitte N., Merker K, Von Zglinicki T., Grune T., and Davies K.J (2000) Protein oxidation and degradation during cellular senescence of human BJ fibroblasts.Part I- Effects of proliferative senescence. FASEB J.14:2495-2502.

Smogorzewska, A. and de Lange, T (2004). Regulation of telomerase by telomeric proteins. Ann. Rev. Biochem. 73, 177–208.

Smogozewska A., van Steensel B., Bianchi A., Oelmann S., Schaefer M.R., Schnapp G. and de Lange T. (2000) Control of human telomere length by TRF1 and TRF2. Mol. Cell. Biol., 20: 1659–1668

Soder A.I.,. Hoare S.F, Muir S., Going J.J., Parkinson E.K., Keith W.N. (1997) Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer, Oncogene .14 :1013-1021.

Stahley, M.R.,and Strobel. S.A (2005). Structural evidence for a two-metal-ion mechanism of group I intron splicing. Science .309:1587–1590.

Stampfer M., Garbe J., Levine G., Lichsteiner S, Vasserot A, Yaswen P (2001) Expression of the telomerase catalytic subunit hTERT, induces resistance to transforming growth factor b growth inhibition in p16INK4 (2) human mammary epithelial cells. Proc. Natl. Acad. Sci. USA 98: 4498–4503.

Stampfer M.R and Paul Yaswen (2003) Human Epithelial cell immortalization as a step in carcinogenesis. Cancer letters . 194:199-208.

Stewart.S.A, Hahn.W.C, O'Connor.B.F, Banner.E.N, Lundberg.A.S, Modha.P, Mizuno.H, Brooks.M.W, Fleming.M, Zimonjic.D.B, Popescu. N.C. and Weinberg.R.A (2002). Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. Proc.Natl Acad Sci. 99(20): 12606-12611.

Symons R. H., Hutchins C. J., Forster A. C., Rathjen P. D.,Keese P. and Visvader J. E. (1987) Self-cleavage of RNA in the replication of viroids and virusoids. J. Cell. Sci. Suppl. 7:303–318.

Symons, R. H. (1992) Small catalytic RNAs, Annu. Rev. Biochem. 61,641-671.

Takakura, M. et al. (1999) Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res 59: 551-557.

Tanious, F.A. et al. (1992) Substituent position dictates the intercalative DNA-binding mode for anthracene-9,10-dione antitumor drugs. Biochemistry 31 11632–11640.

Teixeira, M.T., Americ, M., Sperisen, P., and Lingner, J. (2004). Telomere length homeostasis is achieved via a switch between telomerase-extendible and –non extendible states. Cell. 117, 323–335.

Tej K Pandita (2002) ATM function and telomere stability. Oncogene 21:611-618.

Ten Hagen, K. G., Gilbert, D. M., Willard, H. F. and Cohen, S. N. (1990). Replication timing of DNA sequences associated with human centromeres and telomeres. Mol. Cell. Biol. 10: 6348–6355.

Tesmer V.M, Ford L. P, Holt S.E, Frank B.C, Yi .X, Aisner D. L, Ouellette. M. Shay J.W, and. Wright W. E (1999) Two Inactive Fragments of the Integral RNA Cooperate To Assemble Active Telomerase with the Human Protein Catalytic Subunit (hTERT) In Vitro.Molecular and Cellular Biology. 19: 6207-6216.

Thomas D M, Yang H S, Alexander K, Hinds PW (2003) Role of the retinoblastoma protein in differentiation and senescence. Cancer Biol Ther.2 (2): 124-30.

Tinoco,I.,Jr and Uhlenbeck,O.C. (1971) Estimation of secondary structure in ribonucleic acids. Nature. 230: 362–367.

Tinoco,I.,Jr, Borer,P.N., Dengler,B., Levine,M.D., Uhlenbeck,O.C., Crothers,D.M. and Gralla,J. (1973) Improved estimation of secondary structure in ribonucleic acids. Nature New Biol. 246: 40–41.

Titia de Lange (2005) Shelterin: the protein complex that shapes and safeguards human telomeres .Genes and Development 19:2100-2110.

Tomlinson RL, Ziegler TD, Supakorndej T, Terns RM, Terns MP.(2006)Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell. 17(2): 955-65.

Tuschl, T. & Eckstein. F. (1993) Hammerhead ribozymes: Importance of stem-loop II for activity, Proc. Natl Acud. Sci. USA 90:6991-6994.

Tuschl, T., Gohlke, C., Jovin, T. M., Westhof, E. & Eckstein, F. (1994) A three-dimensional model for the hammerhead ribozyme based on fluorescence measurements, Science 266: 785 -789.

Tuschl, T., Thomson, J. B. & Eckstein, F.(1995) RNA cleavage by small catalytic RNAs, Curt: Opin. Struct. Biol. 5: 296-302.

Ueda C T and Richard W. Roberts (2004) Analysis of a Long Range Interaction Between conserved Domains of Human Telomerase RNA. RNA 10: 139-147.

Uhlenbeck,O.C., Borer,P.N., Dengler,B. and Tinoco,I.,Jr (1973) Stability of RNA hairpin loops: A6-Cm-U6. J. Mol. Biol. 73: 483–496.

Van Der Haegen B.A, Shay J.W (1993) Immortalization of human mammary epithelial cells by SV40 large T-antigen involves a two step mechanism, In Vitro Cell. Dev. Biol. 29A 180–182.

Van Steensel, B., and de Lange, T. (1997). Control of telomere length by the human telomeric protein TRF1. Nature 385, 740–743.

van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell 92: 401–413.

Van Tol, H., Buzayan, J. M., Feldstein, P. A,, Eckstein, F. & Bruening, G. (1990) Two autolytic processing reactions of a satellite RNA proceed with inversion of configuration, Nucleic Acids Res. 18, 1971 -1975.

Velculescu, V. E., Zhang, L., Vogelstein. B., and Kinzler, K.W. (1995). Serial Analysis of Gene Expression. Science 270, 484-487.

Venteicher.A.S , Meng. Z, Mason.P. J, Veenstra. T. D and Artandi.S .E (2008) Identification of ATPases Pontin and Reptin as Telomerase Components Essential for Holoenzyme Assembly. Cell. 132: 945-957.

Verheggen, C., Lafontaine, D.L., Samarsky, D., Mouaikel, J., Blanchard, J.M., Bordonne, R., and Bertrand, E.(2002). Mammalian and yeast U3 snoRNPs are matured in specific and related nuclear compartments.EMBO J. 21: 2736–2745.

Walker.A.K, Rymar.G, and Andrews.P.C (2002)Virtual 2-D Gel Electrophoresis by MALDI Mass Spectrometry.Proteomics Protocols Handbook. PP 417-429.

Waterman, M.S. (1978) Secondary structure of single-stranded nucleic acids. In Rota, G.-C. (ed.), Studies in Foundations and Combinatorics number 1 in Advances in Mathematics, Supplementary Studies. Academic Press, NY, pp. 167–212.

Waterman,M.S. and Smith,T.M. (1978) RNA secondary structure: a complete mathematical analysis. Math. Biosci. 42: 257–266.

Watson, J. D. (1972) Origin of concatemeric T7 DNA. Nature New Biol. 239, 197–201

Weng DE, Masci PA, Radka SF, Jackson TE, Weiss PA, Ganapathi R, Elson PJ, Capra WB, Parker VP, Lockridge JA, Cowens JW, Usman N, Borden EC.(2005) A phase I clinical trial of a ribozyme-based angiogenesis inhibitor targeting vascular endothelial growth factor receptor-1 for patients with refractory solid tumors. Mol Cancer Ther. 4(6): 948-55.

Wenz.C, Enenkel.B, Amacker. M, Kelleher. C,K, Damm.K and Lingner.J (2001) Human telomerase contains two cooperating telomerase RNA molecules. EMBO .J. 20:3526-3534.

WHO Facts on Cancer. http://www.who.int/cancer/en/

Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E.(1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature.334: 124-9.

William Scott (2007) Ribozymes. Current Opinion in Structural Biology. 17:1-7.

Wong, J. M., Kusdra, L., and Collins, K. (2002). Subnuclear shuttling of human telomerase induced by transformation and DNA damage. Nat. Cell Biol. 4:731–736.

Woodfine, K., Fiegler, H., Beare, D. M., Collins, J. E., McCann, O. T., Young,B. D., Debernardi, S., Mott, R., Dunham, I., and Carter, N. P. (2004). Replication timing of the human genome. Hum. Mol. Genet. 13: 191–202.

Wright, W. E. and Hayflick, L. (1975) Nuclear control of cellular ageing demonstrated by hybridization of anucleate and whole cultured normal human fibroblasts. Exp. Cell Res. 96:113-21.

Xia, J., Peng, Y., Mian, I., and Lue, N. (2000) Identification of functionally important domains in the N-terminal region of telomerase reverse transcriptase. Mol. Cell. Biol. 20: 5196–5207.

Xu D, Wang Q, Gruber A, Björkholm M, Chen Z, Zaid A, Selivanova G, Peterson C, Wiman KG, Pisa P (2000) Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. Oncogene. 19(45):5123-33.

Yadava R and Yadava PK (2000): Vectors for generation of precise transcripts of inserted sequence. Molecular Biology Today. 1:17-22.

Yashima, K., Litzky L. A., Kaiser L., Rogers T., Lam S, Wistuba I. I., Milchgrub S., Srivastava S., Piatyszek M. A., Shay J. W., and. Gazdar A. F.(1997). Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. Cancer Res. 57:2373-2377.

Yashima, K., Piatyszek M. A.,. Saboorian H. M, Virmani A. K., Brown D., Shay J. W., and Gazdar A. F (1997). Telomerase activity and in situ telomerase RNA expression in malignant and non-malignant lymph nodes. J. Clin. Pathol. 50:110–117.

Yatabe N., Kyo S, Kondo S, Kamaya T, Wang Z, Maida Y, Takakura M, Nakamura M, Tanaka M, Inoue M (2002) 2.5A antisense therapy directed against human telomerase RNA inhibits telomerase activity and induces apoptosis without telomere impairment in cervical cancer cells. Cancer Gene Therp 9:624-630.

Yi.X, Tesmer V.M, Savre-Train.I, Shay J.W., Wright W.E (1999) Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. Mol. Cell. Biol. 19:3989-3997.

Yokoyama Y, Takahashi .Y, Shinohara. A, Wan X., Takahashi S, Niwa K,. Tamaya T (2000) The 5' end of hTERT mRNA is a good target for hammerhead ribozyme to suppress telomerase activity. Biochem Biophys. Res Commun 273: 316- 321.

Yokoyama Y, Takahashi Y, Shinohara A, Lian Z, Wan X, Niwa K, Tamaya T (1998) Attenuation of telomerase activity by a hammerhead ribozyme targeting the template region of telomerase RNA in endometrial carcinoma cells. Cancer Res .58:5406-5410.

Yu, C.C., Lo, S.C. and Wang T.C. (2001) Telomerase is regulated by protein kinase C-zeta in human nasopharyngeal cancer cells. Biochem J .355: 459-464.

Zhang, Y., Y. Xiong, and W.G. Yarbrough (1998) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 92: 725-734.

Zhao J, Bilsland A, Hoare S.F, Keith.W.N (2003) Involvement of NF-Y and Sp1 binding sequences in basal transcription of the human telomerase RNA gene. FEBS Letters.536:111-119.

Zhao J., Bilsland A, Jackson K., Keith W.N. (2005) MDM2 negatively regulates the human telomerase RNA gene promoter, BMC Cancer 5: 6.

Zhao J.Q., Glasspool R.M.,. Hoare S.F, Bilsland A., Szatmari I., Keith W.N (2000) Activation of telomerase RNA gene promoter activity by NF-Y, Sp1, and the retinoblastoma protein and repression by Sp3.Neoplasia : 2 531-539.

Zhu, Y., Tomlinson, R.L., Lukowiak, A.A., Terns,R.M. and Terns,M.(2004)Telomerase RNA accumulates in Cajal bodies in human cancer cells. Mol. Biol. Cell 15: 81-90.

Zuker,M. and Stiegler,P. (1981) Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. 9: 133–148.

Zuker. M (2003) M fold web server for nucleic Acid folding and hybridization prediction. Nuclei Acid Res. Vol 31: 3406-3415.