

**TELOMERASE ACTIVITY AND TELOMERE LENGTHS IN HUMAN  
FIBROBLAST CELLS TREATED WITH EPENDYMIN  
PEPTIDE MIMETICS**

A THESIS

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## ABSTRACT

Telomerase is an enzyme that helps maintain the telomeric ends of chromosomes during DNA replication. Telomere lengths represent a balance between telomerase activity attempting to elongate their ends, and cell division that causes telomere shortening. As cells age, diminished telomerase activity allows a shortening of telomere lengths until they reach a target length that stimulates apoptosis. Identifying a drug capable of upregulating telomerase activity may help increase cell (and even organismal) lifespan. The purpose of this thesis was to determine whether treatment of human primary foreskin fibroblast cultures with a 14 amino acid (aa) ependymin peptide mimetic upregulates (or at least maintains) telomerase activity and telomere lengths during cellular ageing. The 14aa peptide was previously shown to significantly increase the murine lifespan by 25%, so its activity was a logical candidate to test in this thesis. In a preliminary set of experiments, the human primary fibroblast cells were shown to respond to the 14aa drug by upregulating the antioxidative enzyme superoxide dismutase (SOD), thus human fibroblast cells likely contain the appropriate receptor for binding this drug. This same dose proved optimal for upregulating telomerase activity in the fibroblast cells an average of 57% relative to untreated cells ( $p$  value = 0.003). The upregulation appears to be specific for the sequence of aa in the 14aa drug since a “scrambled” peptide containing the same aa but in a different order showed no upregulation, even at doses 10-fold higher. Treatment of mice once per day or twice per day with the 14aa peptide was also found to upregulate telomerase activity *in vivo* in brain and heart. The activity was optimal at a 3.3 mg/kg dose for each aged organ, and was generally high in young organs. The activity observed in heart was a total surprise since

heart cells are generally thought to be quiescent, and telomerase is usually associated with cell division, so perhaps telomerase has a function other than in cell division.

The second part of the hypothesis tested whether treatment of fibroblast cells with the 14aa drug elongated (or prevented from shortening) telomere lengths in aged cells. A telomere length assay (TLA) based on a Southern hybridization approach using a telomere probe appeared to work well, since marker DNAs showed appropriate differences in their “telomere smears”, and aged fibroblast cells showed shorter smears than young cells. However, no difference was observed between drug-treated versus vehicle-treated cells, even at the 10 ng/ml dose previously shown to strongly upregulate telomerase activity. So perhaps the upregulation of telomerase activity was not sufficient to provide a measurable increase in telomere lengths. Telomerase has been shown to extend the lifespan of virus-transformed human cells without showing any visible telomere lengthening (Blackburn et al, 1999), so perhaps telomerase can increase cell lifespan without increasing telomere lengths.

To our knowledge, this is the only drug demonstrated to upregulate telomerase activity. Transforming cells with the viral T-antigen can upregulate telomerase, but T-antigen is not a therapeutic drug since it also causes cancer. Telomerase upregulation is known to occur during oncogenesis, but telomerase itself is not an oncogene since oncogenesis also requires the upregulation of oncogenes. Our lab previously showed this peptide does not upregulate the potent oncogene *myc*. If this proves to be the case for other oncogenes, using this 14aa drug to upregulate telomerase activity without activating oncogenes could prove extremely useful for helping prove telomerase is not an oncogene, and for extending cell lifespan.

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## BACKGROUND

### Telomeres

Telomeres are “caps” on the ends of chromosomes that were first observed in *Drosophila* in the early 1930's (Muller, 1938) in a genetic analysis of chromosome terminal deletions and inversions. However, their function was not uncovered until the 1980's, when Elizabeth Blackburn showed that telomeres protect the genetic material during replication (Blackburn and Gall, 1978). Telomeres are composed of a G-rich strand of repetitive DNA sequence that also contains telomere-binding proteins (Haussman et al., 2003). In the human genome, surprisingly about one part in 3000 by weight is telomeric DNA (Blackburn, 1990). Telomere sequences in eukaryotic chromosomes are most often tandem repeats of a short sequence TTAGGG in a hexamer repeat unit (Moyzis et al., 1988; Morin, 1989), and are associated with non-histone structural proteins in the nucleus (Blackburn, 1990). The complexes of proteins associate into large polymers *in vitro*, and include nuclear lamins and vimentin. Telomeres are considered the most conserved cis-acting chromosomal DNA elements. When attached to linearized plasmids, telomeric DNA can stabilize plasmid half-life in the cytoplasm, so telomeres can stabilize DNA even apart from the chromosome end-replication problem (discussed below). In fact, telomeric DNA isolated from one species can stabilize the chromosomes in cells of a different species even when the two species have different telomeric sequences (Blackburn, 1990). Telomere lengths are not static (discussed below) and vary considerably depending on the number of TTAGGG repeats present (de Lange et al., 1990). They can even vary for different chromosomes within the same cell (Blackburn, 2000).

## **Telomeres and the End Replication Problem**

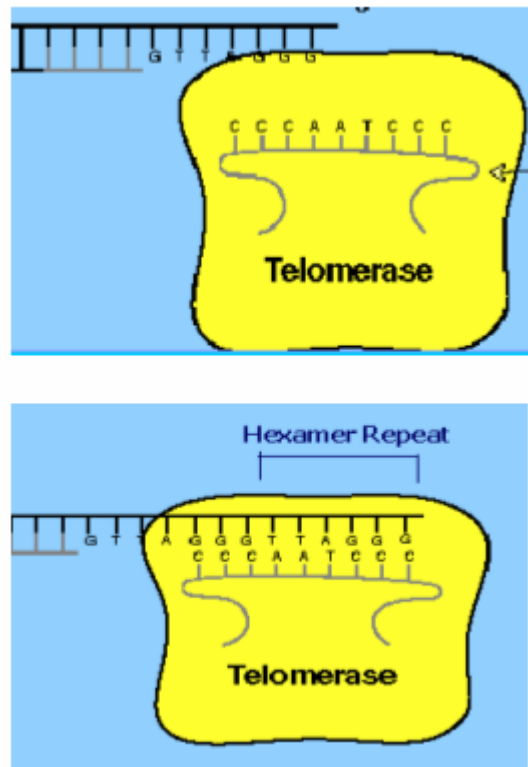
Telomeres solve the DNA end replication problem, the inability of DNA polymerase to replicate the ends of linear chromosomes (Kowald, 1997). Normal cellular DNA polymerases can only replicate DNA in the 5' → 3' direction, and are unable to start DNA synthesis *de novo*, thus DNA-dependent RNA polymerase is used to synthesize an 8 to 12 bp stretch of RNA that is able to prime DNA synthesis by way of its free 3' hydroxyl end. That piece of primer RNA is annealed to the 3' single-stranded DNA, where it primes synthesis of the daughter strand in the 5' → 3' direction. The RNA primer is later removed and filled by the DNA polymerase that synthesizes short DNA Okazaki fragments upstream. However, linear chromosomes are unable to fill in their extreme 3' ends because the placement of the RNA template leaves a 8-12 bp gap from the end of the strand. Thus, the ends of linear chromosomes shorten continuously during each cell division (Saldanha et al., 2003). If a telomere did not have a mechanism for filling the 3' gap by capping the end of the chromosome then a small piece of the sequence would be removed after each round of replication (Saldanha et al., 2003). Without telomeres, the ends of chromosomes could fuse with other chromosomes or lead to a progressive loss of DNA sequence (Blackburn, 1990). With each cell cycle about 50-150 base pairs of terminal DNA is lost, leading to shortened 5' termini in the daughter strand (Sozou and Kirkwood, 2001).

## **Telomerase**

Telomerase is an enzyme that maintains the telomeric repeats at the end of chromosomes. It was first discovered in *Tetrahymena* (Greider and Blackburn, 1985) as a ribonucleoprotein (Greider and Blackburn, 1989) that elongates telomeres by adding a motif to the daughter strand termini complementary to the telomeric DNA sequence (Blackburn, 1991;



Allsopp et al., 1995) (see Figure 1). Telomerase acts as a reverse transcriptase that synthesizes the G-rich strand of telomeric DNA (Blackburn, 1990) to compensate for the shortening observed with the DNA end replication problem. Thus the lengths of telomeres appear to represent a balance between the number of cell cycles a cell has undergone versus the activity of telomerase in those cells. If the telomerase activity present in a cell is not sufficient to prevent shortening to a critical point, the cell undergoes senescence. In normal somatic cell division, the absence of telomerase leads to the reduction of telomere length and the wearing down of telomeric repeats (Saldanha et al., 2003). Both telomerase RNA (TER) and telomerase reverse transcriptase protein (TERT) are needed for enzymatic activity in vitro. Telomerase activity in humans and mice corresponds directly to the expression of TERT (Blackburn et al., 1999), so perhaps the protein component is rate-limiting within cells. Although telomerase activity is high in cancer cells, its activity does not directly lead to cancer; oncogenes must also be upregulated to cause oncogenicity. Thus telomerase is not an oncogene (Holt et al., 1996; Belair et al., 1997; Kiyono et al., 1998; Jiang et al., 1999).



**Figure 1: Diagram of the Function of Telomerase.** The upper panel depicts the movement of telomerase (yellow) containing its RNA template (grey) to the single-stranded 3' terminus of the chromosome during replication. The lower panel shows the hybridization of the CCC portion of the telomerase RNA template to the GGG portion of the chromosome, and the extension of the TTAGGG using telomerase RNA as template (Slish, 2005).

## Telomeres and Aging

Telomere lengths shorten with age (Blackburn, 1987). Telomere shortening can be as small as a few base pairs per population doubling in *Drosophila*, to almost 200 base pairs per population doubling in human endothelial cells. Normal CD8<sup>+</sup> T-cells enter senescence after about 25 cell divisions, at which point their telomeres have shortened from 10-11 kb to 5-7 kb (Harley et al., 1990). The differences in telomere rate shortening between species can be accounted for by species differences in RNA primer location and length during lagging strand

synthesis, alterations of overhang length, and levels of telomerase activity (Allsopp et al., 1995). Cells with long lifespans (cancer cells, reproductive cells, and immortal cell lines) have stable telomeres that do not shorten with cell division (de Lange et al., 1990; Hastie and Dunlop, 1990; Allsopp et al., 1992; Greider, 1994).

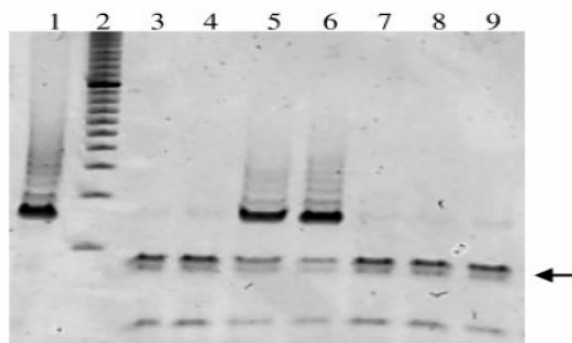
In the 1960's, Leonard Hayflick made the discovery that non-immortalized cultured cells undergo a limited number of cell divisions. This population doubling limit is known as the Hayflick Limit (Hayflick, 1992). Since each cell cycle leads to a loss of telomeres at the 5' end of the new daughter molecule, and a minimal telomere length is needed to maintain chromosomal integrity, then short telomere lengths could limit the replicative lifespan of cells. Telomere shortening is believed to be a “mitotic clock” that counts the number of cell divisions and determines when the cell should undergo senescence (Harley, 1991; Shay et al., 1996).

### **Increase of Cell Life-Span by Telomerase Activation**

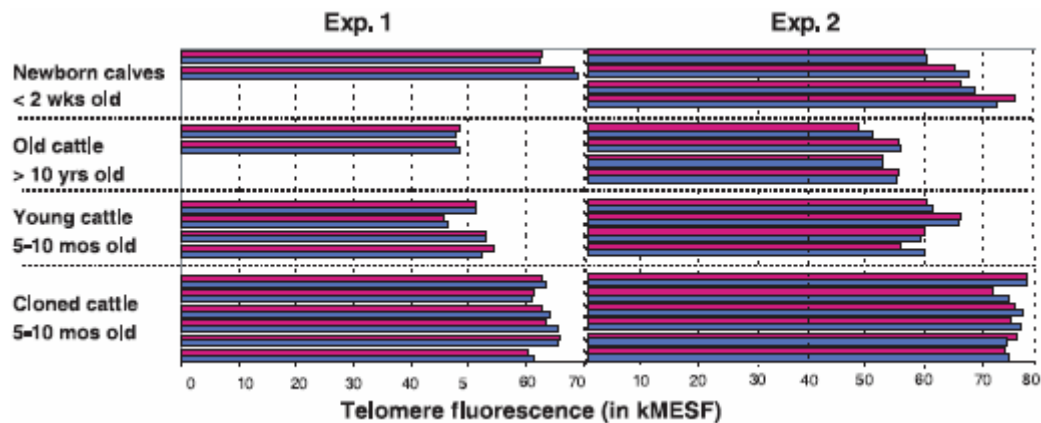
The activation of telomerase in cells has been associated with an increase in cell life-span. Bodnar et al (1998) transfected telomerase-negative normal human cell types, including foreskin fibroblasts, with vectors containing the human telomerase catalytic subunit. They found that the telomerase expressing clones had elongated telomeres and divided vigorously. The cell types far exceeded their normal 20 population doublings. In another study (Vaziri and Benchimol, 1998), telomerase activity was increased in normal diploid fibroblast cells (naturally low in telomerase activity) by transfection with an hTERT gene, causing an increase in the length of telomeric DNA and an extension of cellular lifespan. Extending cellular lifespan by increasing telomeres and telomerase activity does not appear to affect cellular differentiation or functional phenotype (Yang et al., 1999). The introduction of hTERT into epithelial cells

allowed the cells to resist apoptosis under different conditions, while parental non-transfected cells underwent senescence (Yang et al., 1999). Thus cellular lifespan can be increased by the introduction of telomerase activity, without a change to functional phenotype, in cells that normally undergo a finite number of replications.

Lanza *et al.* (2000) showed an increase in cell population doubling potential, telomerase activity, and telomere lengths in cells cloned from aged bovine fibroblast nuclei, indicating that the cloning process may have the ability to reset the telomere clock. Figure 2 shows high telomerase activity in the cloned bovine embryos, and low activity in the aged fibroblast cells providing donor nuclei for the cloning. Figure 3 shows a telomere length analysis performed by hybridizing a fluorescently labeled telomere probe to the ends of the DNA. Cloned cattle had almost the same length telomeres as newborn calves, while the aged cattle showed short telomeres (Lanza et al., 2000).

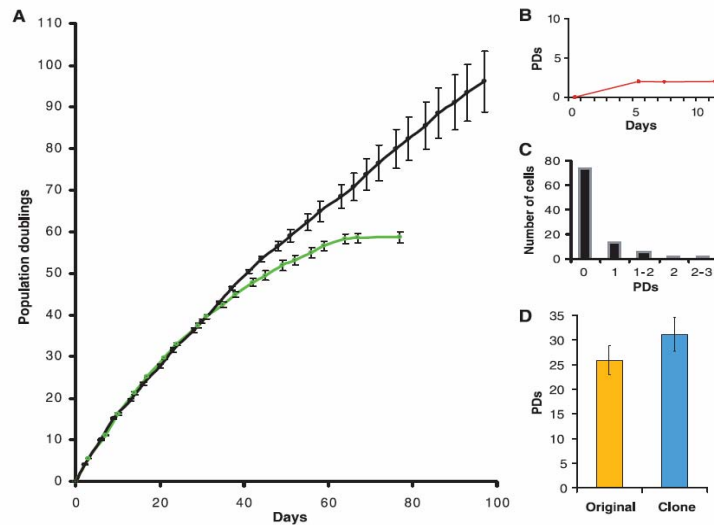


**Figure 2. Telomerase Activity Increases in Embryos Cloned from Bovine Fibroblast Nuclei.** Telomere repeat amplification protocol (TRAP) analysis of reconstructed embryos and donor bovine fibroblasts (Lanza et al., 2000). Strong telomerase activity (hexamer ladder) is seen in lanes 5 and 6 representing cloned cattle embryos, but not in the aged embryos providing donor nuclei (lanes 7-9). The arrow denotes the position of a PCR control.



**Figure 3. Analysis of Telomere Lengths in Peripheral Blood Samples from Cloned and Control Cattle.** The X-axis values represent telomere lengths as measured by hybridization of a fluorescently labeled telomere probe to chromosomes. The greater the fluorescent signal, the longer the telomere population in those cells. Samples analyzed are listed to the left of the figure. Two independent experiments were performed (left and right columns) on different cattle. Each blood sample was analyzed twice (red versus blue histobars) (Lanza et al., 2000).

Figure 4 shows the population doublings obtained for cloned and non-cloned cells. *In vitro*, human diploid fibroblasts possess only a limited capacity for cell division (Sozou and Kirkwood, 2001), fibroblasts from older donors have a shorter lifespan than fibroblasts from younger donors (Park et al., 2001). The data of Figure 4 show that the population doublings increased in the cloned fibroblast cells (Panel A, upper black line) compared to the female bovine fetus (BFF) cell line they were cloned from (Panel A, lower line). The Lanza *et al.* article demonstrated that cloned fibroblast nuclei from cattle are capable of resetting their telomere clocks.



**Figure 4. Cloned Bovine Cells Show Greater Population Doublings.** Figure shows the growth curve of aged bovine female fetus cells (lower curve, green, in Panel A) used to provide donor nuclei, versus bovine cells cloned from those aged donor nuclei (upper curve, Panel A) (Lanza et al., 2000).

A key point with the Lanza study was that the donor nuclei were obtained from fibroblast cells, not mammary cells as with Dolly the world's first cloned mammal. Dolly apparently died of premature ageing, so the mammary nuclei she was cloned from apparently lack the ability to reset their telomere clock. The apparent ability of fibroblast cells (or nuclei) to reset their telomere clock was, in part, a key reason for analyzing fibroblast cells in this thesis.

## Ependymin

Ependymin is a secretory protein found in abundance in the extracellular fluid (ECF) and cerebrospinal fluid (CSF) of goldfish brains. It was first discovered in the zona ependyma of goldfish brains due to its high turnover rates following various learning events (Shashoua, 1976). Ependymin has a role in long term memory formation, and optic nerve elongation (for a review see Shashoua, 1991). Our lab has cloned and characterized ependymin genes from 4 teleosts,

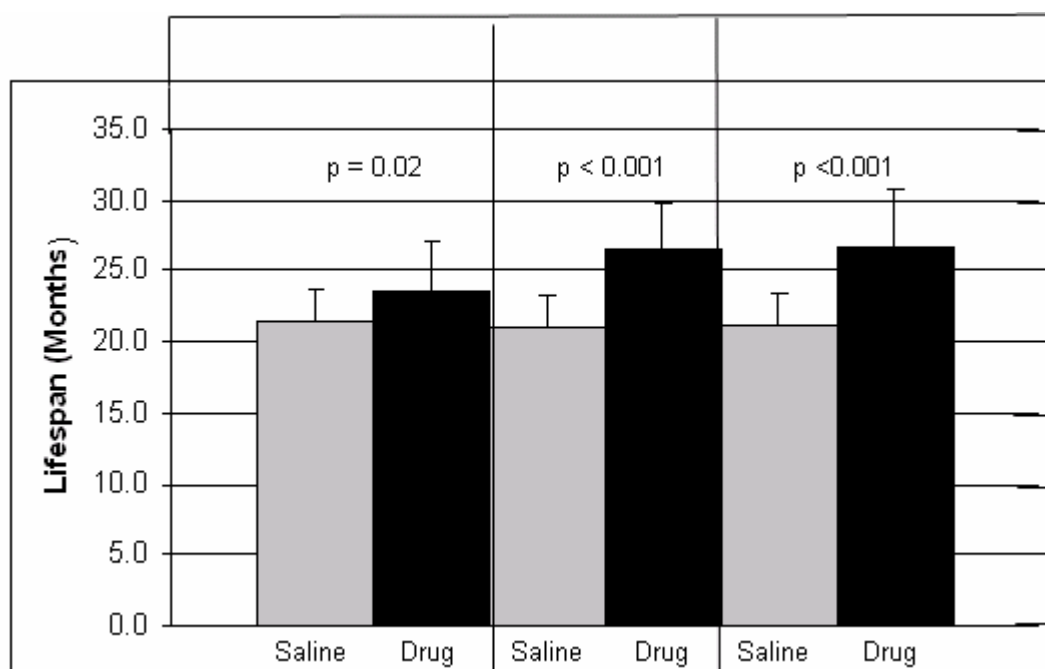
and has elucidated several key signal transduction events activated by treatment of neuronal cells with endymnin or its peptide mimetics (Adams et al., 2003).

Ceremedia Inc. (Maynard, MA) who funds our lab on endymnin projects, focuses on designing endymnin peptide mimetics that are short enough to cross the blood brain barrier when delivered intravenously (i.v.) or intraperitoneal (i.p.), yet retain biological activity. The sequence of the 14aa length endymnin mimetic used in this thesis is ESCKKETLQFRKHL, representing a central domain of goldfish endymnin that we hypothesize binds the putative endymnin receptor. That peptide (or its KKETLQFR core, designated CMX-8933) has previously been shown to retain biological activity (Adams et al., 2003; Shashoua et al., 2004).

### **Fort Worth Ageing Study**

Because the treatment of cultured neuronal cells with endymnin or its mimetics was found to upregulate the synthesis of several antioxidative enzymes (Shashoua et al., 2004) which has been directly correlated with an increase in *Drosophila* lifespan (Orr and Sohal, 1994; Parkes et al., 1998), Ceremedia, Inc. funded a study termed the “Fort Worth Ageing Study” that analyzed the effects of an i.p. delivery of the 14aa endymnin peptide on the lifespan of mice (Figure 5). The mice were treated with 1 mg/kg peptide once a week (first two histobars), once a day (middle two histobars), or twice a day (right two histobars). Twenty mice were used in each group. While there is a slight increase in lifespan with the group of mice that were treated once a week, a significant 6 months increase was seen in the animals treated either once per day or twice per day with the peptide. This study indicated that endymnin mimetics are capable of increasing the mouse lifespan. Perhaps telomerase, a known factor in increased lifespan, was

being upregulated in the drug treated animals (in addition to the antioxidative enzymes whose observation initiated the study).



**Figure 5. Fort Worth Mouse Ageing Study.** The effects intraperitoneal (i.p.) injection of 1 mg/kg 14 aa ependymin mimetic on mouse lifespan. Three different treatment modes were tested: once a week (first two histobars), once a day (middle two histobars), or twice a day (right two histobars). Each histobar represents the mean of 20 mice. Significant increases were seen for the latter two treatment modes (Ceremedix Inc).



## THESIS PURPOSE

The Fort Worth Mouse Ageing Study funded by Ceremedix, Inc. showed that i.p. treatment of mice with a 1 mg/kg dose of a 14aa ependymin peptide mimetic significantly increased lifespan when administered once or twice per day. Increases in cellular and organismal lifespan have previously been correlated with increases in telomerase activity (and in some cases telomere lengths), so perhaps the mimetic has the capacity to upregulate telomerase activity in mammalian cells. The purpose of this thesis was to test this hypothesis *in vitro* using cultured primary human fibroblasts as a model system, and *in vivo* using mice. Previous experiments with cattle (Lanza et al., 2000) demonstrated that fibroblasts have the capacity to upregulate telomerase activity, telomere lengths, and cell population doublings, and fibroblast cells have long served as a model for ageing studies.

## **MATERIALS AND METHODS**

### **Fibroblast Cultures**

Human foreskin primary fibroblast cultures were maintained by Ceremedix, Inc. (Maynard, MA). Various doses of 14aa ependymin mimetic peptide (usually 10 ng/ml) were added to the cultures on a continual basis for telomerase studies, or for 3 hours for SOD westerns.

### **Preparation of Whole Cell Lysates**

Whole cell extracts were prepared from human foreskin primary fibroblast cultures to prepare protein samples for immunoblots. The cells were scraped into the medium, then collected by centrifugation at 500 x g for 5 minutes. The supernatant was discarded, and the cell pellet washed once with ice cold phosphate buffered saline (PBS). The washed pellet was partially dried by inverting the tube onto toweling, then resuspended in 200 µl of Complete Lysis Buffer (20 mM HEPES pH 7.9, 10 mM KCl, 300 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 20% Glycerol, and freshly added 0.5 mM DTT and 0.5 mM PMSF). The pellet was re-suspended in the buffer, then transferred to a 1.5 ml eppendorf tube on ice. The suspension was incubated on ice for at least 10 minutes (with occasional vortexing) to thoroughly lyse the cells. The lysate was then microcentrifuged at 4°C for 5 minutes to pellet cell debris, and the supernatant was aliquotted into several 0.5 ml eppendorf tubes (about 40 µl per tube). A 1 µl aliquot of the supernatant used for protein determination using Coomassie Reagent (Pierce). Samples were stored at -80°C.

## **SOD Western**

### *Gel Polymerization*

One BRL V-16 unit was used with two 0.8 mm spacers. The large glass plate was placed on the bench and the two side spacers were added. The small plate was then placed on top of the large plate. The bottom spacer and clamps were added. The plates were then placed vertically, and then 20 ml of lower resolving gel (7.6 ml distilled water, 6.7 ml of 30% Acrylamide, 5.1 ml of 1.5 M Resolving Gel Buffer, 200  $\mu$ l of 10% SDS, 400  $\mu$ l of 5% Ammonium Persulfate, and 10  $\mu$ l of 100% TEMED )was made. The TEMED was added last because it was the polymerization catalyst. The tube was then capped and inverted slowly to avoid bubble formation. The solution was then immediately poured between the glass plates. The gel solution was overlaid with dH<sub>2</sub>O to prevent bubble formation at the surface, and the gel was left for 40 minutes at room temperature to polymerize. The excess water was poured off and 10 ml of upper stacking gel (5.52 ml of water, 1.67 ml of 30% Acrylamide, 2.5 ml of 0.5 M Stacking Gel Buffer, 100  $\mu$ l of 10% SDS, 200  $\mu$ l of 5% Ammonium Persulfate, and 10  $\mu$ l of 100% TEMED) was added between the glass plates. A 20 stall comb was then inserted at an angle (to avoid making air bubbles) into the upper stacking gel, and it was allowed to polymerize for 45 minutes to overnight. If left overnight, the gel was wrapped in plastic wrap to prevent dehydration.

### *Gel Electrophoresis*

Once the gel had polymerized, the clamps, lower spacer, and comb were removed. The outer surface of the small plate was then cleaned with ethanol and mounted into the V-16 unit with two clamps on each side. Protein electrode buffer was then poured up to an inch above the

stalls. Before protein electrode buffer was added to the lower reservoir, the unit was checked for leaks. A syringe with a bent needle was used to remove any air bubbles at the base of the gel, and well stalls were flushed with buffer to remove air bubbles or gel debris. The gel was then pre-run for one hour at approximately 150 V.

A biotinylated marker was prepared by mixing 1  $\mu$ l of Biotinylated SDS-PAGE Standard (Biorad #161-0319) with 10  $\mu$ l of 1 X Protein Sample Buffer. The fibroblast lysate samples were loaded at 5  $\mu$ g of protein per lane, and were mixed with two volumes of 1 X protein sample buffer per volume of cell extract. All protein samples and markers were boiled for 2 minutes before loading to unwind protein secondary structure. The tubes were then briefly microfuged. Following pre-electrophoresis, the gel was turned off and the well stalls were cleaned with buffer, and air bubbles were removed from underneath the gel. The samples were loaded with a V-16 thin pipette tip. The gel was then electrophoresed at 150 V until the BPB dye was 2/3 of the way down the gel (approximately 3 hours).

### *Gel Transfer*

The glass plates were removed from the V-16 unit, and the plates were separated with a pizza cutter. A small piece of gel was removed from the lower right hand corner for orientation purposes. A piece of nitrocellulose membrane and two pieces of 3MM filter paper were cut to the same size as the gel. The nitrocellulose membrane, with a piece of the lower right corner cut out, was dipped into 4°C pre-chilled transblot buffer (58 g Trizma Base, 29 g Glycine, 2 L Methanol, distilled water to make 10 L, and 3.7 g SDS Powder, the SDS was added last to prevent bubble formation) and placed on top of the gel. All air bubbles were removed with a gloved finger. The 3 MM paper was then soaked in transblot buffer and one piece was put over

the membrane. The air bubbles were removed from the paper, and a transblot sponge was put over the 3 MM paper. The transfer unit plastic grid marked positive was then put over the 3 MM paper and sponge, and the gel was flipped over. A pizza cutter was used to remove the remaining glass plate from the gel and a second piece of pre-soaked 3 MM paper was placed over the gel. Air bubbles were removed and a transfer unit sponge was put over the 3 MM paper. The transfer unit plastic grid marked negative was then put over the sponge. The grids were locked into place and put in the transfer unit. The plastic grids were tapped to allow trapped air bubbles out. The transblot unit was then placed in the refrigerator on an electromagnetic stirrer. The lid was placed on the transblot unit with the negative cathode facing the negative marking on the grid. The electronic timers were set up to control power to the transblot unit and the electromagnetic stirrer. The transblot unit was set at 50 V and the timers set for 2 hours. After the two hour transblotting was complete, the unit was removed from the refrigerator and the plastic grids holding the membrane were removed. The plastic grids were then unlocked and tweezers were used to separate the membrane from the gel. The side of the membrane facing the gel was marked with a felt pen (to denote the side containing protein). The membrane was then placed in a Tupperware® container protein side up, and covered with 25- 50 ml (depending on the size of the membrane) of fresh blocker solution (50 ml of 20 X PBS pH 7.5, 10 gm Casein, 2 ml 100% Tween-20, and dH<sub>2</sub>O to make 1 liter, stored overnight to remove fines). The Tupperware® container was then placed on the red rocker shaker at medium speed for at least 1 hour at 25°C.

### *Antibody Incubations*

Using tweezers, the membrane was held above the Tupperware® and the solution was discarded. 25 ml of fresh blocker was then added to the Tupperware® container plus 25 µl of 10 mg/ml rabbit SOD antibody (Rockland) (but not directly on the membrane). The membrane was then gently rocked on the Red Rocker Shaker for at least 2 hours at 25°C. The primary antibody solution was discarded, and the membrane was washed on a gyrotory shaker vigorously twice with enough PBS-Tween (1 X PBS, 0.05% Tween) to keep the membrane submerged. After the washes, 25 ml of fresh blocker was added to the Tupperware® plus 25 µl of 0.4 µg/ml goat anti-rabbit-HRP (secondary antibody) (Pierce) (not directly on the membrane). The membrane was then incubated on the Red Rocker shaker at a medium speed for 2 hours at 25°C. After the membrane was incubated with the secondary antibody, 3 vigorous washes with PBS-Tween were performed for two minutes each. Then, the membrane was rinsed briefly with 1 X PBS. The membrane was placed onto a sheet of foil with the protein side up.

### *Chemiluminescent Detection*

The water in the darkroom was turned on to 25°C. In a 15 ml tube, 5 ml of Luminol/Enhancer Solution was mixed with 5 ml of Stable Peroxide Solution (Pierce #34080). The detection solution was then poured over the membrane. A glass pipette was used to roll the solution over the membrane to ensure that it was evenly covered. The membrane was incubated for 5 minutes at 25°C. The membrane was removed from the foil and a small corner of it was touched to a paper towel to remove excess luminol solution. The membrane, with the protein side up, was placed between two plastic sheets and air bubbles removed. The membrane was put

into a film cassette with no intensifying screen, and the membrane was exposed to Kodak XAR-5 X-ray film for 1-5 seconds at 25°C.

### **TRAP (Telomerase Repeat Amplification Protocol) Assay**

This protocol (Kim et al., 1994; Wright et al., 1995) was performed as described in the manual (Intergen) with a few alterations.

#### *Cell Extract/ Lysate Preparation*

Fibroblast whole cell extracts were prepared using 1 X CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-Mercaptoethanol, 0.5% CHAPS, 10% Glycerol) supplied with the TRAPeze telomerase detection kit (Intergen). Cell pellets representing one T-25 flask were resuspended in 20 µl of CHAPS lysis buffer. The suspension was then incubated on ice for 30 minutes to facilitate lysis, then spun in a microcentrifuge at 10,000 x g for 20 minutes at 4°C to pellet cell debris. The supernatant was aliquoted and stored at -80°C.

#### *Determination of Protein Concentration*

Lysate protein concentrations were determined using a Coomassie Protein Reagent (Pierce) and a BSA standard curve. Standard BSA dilutions were prepared at the following concentrations 1.25, 2.5, 5, 10, 20, and 40 µg/ml. The “blank” tube contained 500 µl of distilled water. Sample tubes contained 5 µl of cell extract diluted with 495 µl of distilled water. The tubes were incubated at 37°C for 1 minute to equalize their temperatures. Then, 0.5 ml of

Coomassie Reagent (Pierce) was added to each tube. The samples were mixed, and the OD was read at 595 nm relative to the blank.

#### *TS Primer Kination*

End labeling of the TS primer was performed according to Intergen's TRAPeze Telomerase detection protocol. The TS primer (5'-AATCCGTCGAGCAGAGTT-3') was 5' end labeled with [ $\gamma$ - $^{32}$ P]-ATP (ICN) using T4 polynucleotide kinase. All the reagents were thawed and kept on ice. The following reagents were combined in a 0.5 ml eppendorf tube to make a 20  $\mu$ l reaction: 10  $\mu$ l of TS primer, 2.5  $\mu$ l of [ $\gamma$ - $^{32}$ P]-ATP, 2  $\mu$ l of 10 X kinase buffer, 0.5  $\mu$ l T4 polynucleotide kinase, and 5  $\mu$ l of PCR grade water. These reagents were then mixed and spun briefly in a microcentrifuge. The reagent mix was incubated for 20 minutes at 37°C to label the primer, then for 5 minutes at 85°C to inactivate the kinase. The kinased samples were then stored at -20°C in a lead pig. 2  $\mu$ l of kinase-labelled TS primer were used per TRAP reaction.

#### *Telomerase Reaction and PCR*

A "Master Mix" was prepared for the PCR amplification according to Intergen's TRAPeze Telomerase detection protocol. The master mix was prepared by combining the following reagents in a 1.5 ml eppendorf tube. All reagents were thawed and kept on ice. The amount of reagents used for each assay was as follows: 5  $\mu$ l of 10X TRAP reaction buffer (200 mM Tris-HCl, pH 8.3, 15 mM MgCl<sub>2</sub>, 630 mM KCl, 0.5% Tween 20, 10 mM EGTA), 1  $\mu$ l of 50 X dNTP mix (2.5 mM each of dATP, dTTP, dGTP, dCTP), 2  $\mu$ l 32P-labeled TS primer, 1  $\mu$ l TRAP primer mix (RP primer, K1 primer, TSK1 template), 0.4  $\mu$ l of Taq polymerase (5 units/ $\mu$ l, Amersham Pharmacia Biotech) and 38.6  $\mu$ l of PCR grade water. The tubes were vortexed and



spun briefly in a microcentrifuge. For each assay, 48  $\mu$ l of the “Master Mix” was aliquoted into a 0.5 ml eppendorf tube. One of the following sample cell extracts or controls was added to the master mix: 2  $\mu$ l of CHAPS lysis buffer (negative control-1), 2  $\mu$ l of heat-inactivated extract (negative control-2), 2  $\mu$ l of cancer cell line (positive control), or a volume of fibroblast cell extract containing 1 $\mu$ g of protein. The tubes were then mixed and spun briefly in a microcentrifuge. The tubes were placed in a thermocycler and incubated at 30°C for 30 minutes to allow hexamer ladder extensions to the TS primer. A 2-step PCR was then performed at 94°C/30 seconds, and 59°C/30 seconds for 27 cycles. Following PCR, the samples were stored at 4°C, or were immediately analyzed on a 10% non-denaturing polyacrylamide gel.

#### *TRAP Gel Electrophoresis*

The TRAP reaction products were analyzed on a 0.8 mm 10% non-denaturing polyacrylamide gel containing 0.5 X TBE. First, the BRL V-16 glass plates were set up using 0.8 mm, thick spacers and comb. A narrow toothed comb was used to analyze more samples. 30 ml of gel solution was prepared by mixing 10 ml of 30% polyacrylamide/ bisacrylamide, 1.5 ml of 10X TBE, 0.6 ml of 5% ammonium persulfate (to make 0.1%), dH<sub>2</sub>O to make 30 ml, and 15  $\mu$ l TEMED to make a 0.8 mm thick, 7 inches long, 10% gel. The gel was left to polymerize for 30 minutes, then the comb and lower spacer were removed. The gel was mounted into the electrophoresis unit, and the upper and lower reservoirs were filled with 0.5 X TBE buffer. Before loading the samples, the gel was pre-electrophoresed at 287 V for 15 minutes. 5  $\mu$ l of 10X loading dye (containing bromophenol blue and xylene cyanol (0.05% each) and 10% glycerol) was added to each PCR reaction. The tubes were then vortexed and spun in a microfuge. Five  $\mu$ l from each reaction was loaded per lane. The remaining reaction mixes were

stored at 4°C. The gel was then electrophoresed at 287 V for 1 hour and 30 min, until the xylene cyanol ran 70-75% of the gel length.

#### *Gel Drying and Autoradiography*

After electrophoresis, the radioactive electrode buffer was carefully discarded in the isotope sink, and the PAGE unit was dismounted. The gel was separated from the glass plates, and the lower right corner of the gel was cut to mark for orientation. The gel was then carefully spread on 2 layers of 3 MM filter paper and was covered with plastic wrap. The gel covered with plastic wrap was placed in the gel drier and dried for 1 hour at 80°C. The telomerase reaction products on the dry gel were then visualized by autoradiography using Kodak X-OMAT XAR-5 X-ray film.

#### *TRAP Assay Quantitation*

The telomerase products were quantified using a Dupont Benchtop Radioisotope Counter. Radioactive India ink was used to orient the gel with the X-Ray film. Then that portion of the gel corresponding to the P<sup>32</sup>-labeled telomerase reaction products (all the bands greater than 50 bp) was carefully cut out from the gel, squished into an eppendorf tube, and placed in the counter. The radioactive signal was read as counts per minute (CPM). Experiments analyzed when the P<sup>32</sup> was less than one week old produced the best results.

#### **Telomere Length Assay**

This Assay (Chang and Harley, 1995; Landsorp et al., 1996) was performed essentially as described in the specification manual (Roche) with a few differences.

### *Digestion of Genomic DNA*

Genomic DNA was isolated from fibroblast cultures using a proteinase K / phenol extraction protocol (Adams et al., 1994). The digestion of genomic DNA was performed according to Roche's TeloTAGGG Telomere Length Assay protocol (#2209136). 1 µg of extracted DNA was diluted with nuclease-free water (from the TeloTAGGG kit) to a final volume of 16 µl. The solutions and pipetting were performed over ice. To make a 20 µl reaction the following reagents were added to the 16 µl: 2 µl of 10 X digestion buffer, 1 µl of HinF1 (40 U/µl) and 1 µl of Rsa 1 (40 U/µl) (both Roche). 1 µg of high molecular weight control DNA (containing long telomeres, 100 ng/µl), and low molecular weight control DNA (containing short telomeres, 100 ng/µl) in a 16 µl volume were used as a control. The reaction mixture was incubated for 6 hours at 37°C, and then 1 µl of both enzymes was added again and left overnight at 37°C. 5 µl of 5 X loading buffer was added to each 20 µl reaction tube to make a final volume of 25 µl.

### *Genomic DNA Electrophoresis*

Digested genomic DNA was separated by agarose gel electrophoresis. A 0.8% horizontal agarose gel was prepared as follows: 0.8 g of nucleic acid grade agarose (International Biotechnologies Inc.) was added to 100 ml of 1 X TAE buffer in an Erlenmeyer flask. The solution was heated in a microwave oven for 2 to 3 minutes until the agarose was fully dissolved. The agarose solution was then poured into an 8 cm x 10 cm electrophoresis tray, and left to solidify at room temperature for 45 minutes. Once the gel solidified, the gel comb was removed and the electrophoresis unit was filled with 1 X TAE running buffer. The digoxigenin DIG

molecular weight marker mix was prepared just before loading the samples onto the gel by mixing in a 0.5 ml eppendorf tube: 4 µl of DIG molecular weight marker, 12 µl of nuclease free water, and 4 µl of 5 X loading buffer. This 20 µl marker sample was microfuged briefly and incubated at 65°C for 10 min. 25 µl of each sample were loaded per lane and 10 µl of the DIG labeled molecular weight marker were loaded on each side of the gel. The gel was electrophoresed at 20 V for 8 hours until the Bromophenol blue tracking dye had traveled approximately  $\frac{3}{4}$  the length of the gel.

### *Southern Blotting*

Southern transfer of the digested genomic DNA was performed by high salt capillary transfer to nitrocellulose membrane using a 20 X SSC (Saline Sodium Citrate, 3 M NaCl, 0.3 M Sodium Citrate, pH 7.0) transfer buffer. After electrophoresis, a small piece from the lower right corner of the gel was cut for orientation purposes. All the gel-washing steps were performed with gentle agitation on a gyrotory shaker at 25°C in a Tupperware® dish. The gel was first submerged in HCl solution (0.25 M HCl) for 5-10 min until the BPB went yellow. This step was performed to fragment the DNA, to facilitate the transfer. The gel was rinsed twice with distilled water, and then was denatured to single strands by submerging twice for 15 minutes in denaturizing solution (0.5 M NaOH, 1.5 M NaCl). This was followed by rinsing the gel two times with distilled water, and neutralization by submerging it two times for 15 minutes in Neutralization solution (0.5 M Tris-HCl pH 7.5, 3 M NaCl). All washes were decanted to waste.

Nitrocellulose membrane (BA-45, 0.45 µm pore size) and two 3 MM filter papers cut to the size of the gel were pre-soaked in 2 X SSC buffer for 30 minutes before blotting the gel to the membrane. This was done to decrease the chance of bubble formation and to help the

transfer of the DNA. The digested DNA from the gel was blotted to the nitrocellulose membrane by capillary transfer at 25°C using 20 X SSC as a transfer buffer. The southern blot transfer was performed as follows: a Tupperware® dish was used as the transfer unit, and a piece of dry 3 MM filter paper served as a wick in the transfer unit, and a piece of dry 3 MM filter paper served as a wick in the transfer unit. The Tupperware® dish was then filled with 20 X SSC buffer and the ends of the wick were submerged in the buffer. Extra buffer was poured over the wick, and all air bubbles were removed by smoothing out the wick using a gloved hand. One of the pre-moistened 3 MM filter paper squares was then placed on top of the wick. The gel was placed on the 3 MM sheet, and all the air bubbles were removed. The pre-moistened nitrocellulose membrane was then placed over the gel, and the corner corresponding to the gel was also cut, and all air bubbles were removed. Another pre-moistened 3 MM filter paper was then layered over the membrane. Next, a sheet of plastic wrap was placed over the whole unit and the center of the plastic wrap corresponding to the size of the gel was cut out. The plastic wrap was then overlayed with a piece of dry 3 MM paper, which in turn was overlayed with several layers of dry paper towels to make a stack about 10 cm thick. The paper towels were placed so that they did not directly touch the SSC buffer in the Tupperware® dish because that would short-circuit the flow of buffer through the gel. The paper towels were covered with a glass plate, and a large book was placed on top of the plates as a weight. The blot was allowed to sit overnight to allow maximum sensitivity and reproducibility of transfer. After blotting, the membrane was washed in 2 X SSC solution. The membrane was then placed between two sheets of dry 3 MM filter paper cut to the size of the membrane, and baked at 120°C in a glassware drying oven for 2 hours. If not used immediately for hybridization and chemiluminescence's detection, the membrane was wrapped in a foil and stored at 4°C.

### *DNA Hybridization*

The hybridization and chemiluminescence detection steps were performed according to Roche's TeloTAGGG telomere length assay protocol (Roche # 2209136). The hybridization and wash temperatures were precisely controlled for maximum sensitivity and reproducibility of results. The hybridization was performed as follows: the DIG hybridization solution was pre-warmed to 42°C. For pre-hybridization, the membrane was submerged in 10 ml of pre-warmed DIG hybridization solution in a hybridization bag, and incubated for 30-60 minutes at 42°C on gyrotory shaker. Hybridization solution was prepared by adding 1 µl of telomere probe (DIG labeled telomere specific hybridization probe, (Roche, # 2209136) to 5 ml pre-warmed hybridization solution, and mixed. After the pre-hyb incubation of membrane, the pre-hyb solution was discarded, and the 5 ml of hybridization solution containing the telomere probe was immediately added. The membrane was incubated in a hybridization bag for 3 hours at 42°C on a gyrotory shaker. After hybridization, the hybridization solution was discarded, and the membrane was washed twice with 100 ml stringent wash buffer-1 (2 X SSC, 0.1 SDS) at 50°C with gentle agitation. These washes were followed by rinsing the membrane in washing buffer-1X (supplied with the Roche kit # 2209136) for 1-5 minutes at 25°C on a gyrotory shaker.

### *DIG Antibody Binding*

The membrane was then incubated in freshly prepared blocking solution (by mixing 15 ml of 10 X Roche Blocking solution with 135 ml maleic acid buffer) for 30 minutes on a gyrotory shaker at 25°C. The antibody solution was prepared as follows: The vial containing the Anti-DIG –AP antibody (0.75 µg/µl, Fab fragments of polyclonal antibody from sheep,

conjugated to alkaline phosphatase (AP), Roche, #2209136) was microfuged at 13,000 rpm for 5 minutes to remove particulates to reduce background by aggregated antibody. An antibody aliquot was then diluted 1:10,000 with fresh blocking solution by adding 5  $\mu$ l antibody to 50 ml blocking solution. The membrane was incubated in this solution for 30 minutes at 25°C on a gyrotory shaker. This was followed by washing the membrane 2 times with 100 ml washing buffer-1X at 25°C on a gyrotory shaker.

#### *TLA Chemiluminescence Detection*

The membrane was then incubated in 100 ml detection buffer-1X for 2-5 minutes at 25°C on a gyrotory. The membrane with the DNA side up was then placed on a dry 3MM filter paper, placed on top of a clear plastic sheet, so that the membrane did not dry completely. 3 ml of substrate solution (containing CDP-Star, a highly sensitive chemiluminescence substrate) was applied immediately. A second plastic sheet was immediately used to cover the membrane so that the substrate solution spread evenly. All bubbles over the membrane were removed, and the membrane was incubated for 5 minutes at 25°C. Excess substrate solution was squeezed out from the plastic sheets, and the membrane was exposed to Kodak XAR-5 X-ray film for 1 hour at 25°C. Luminescence continued for 24 hours allowing multiple exposures when necessary. The signal intensity increased during the first few hours, so weak initial exposures were sometimes strengthened by waiting 1-2 hours.

## RESULTS

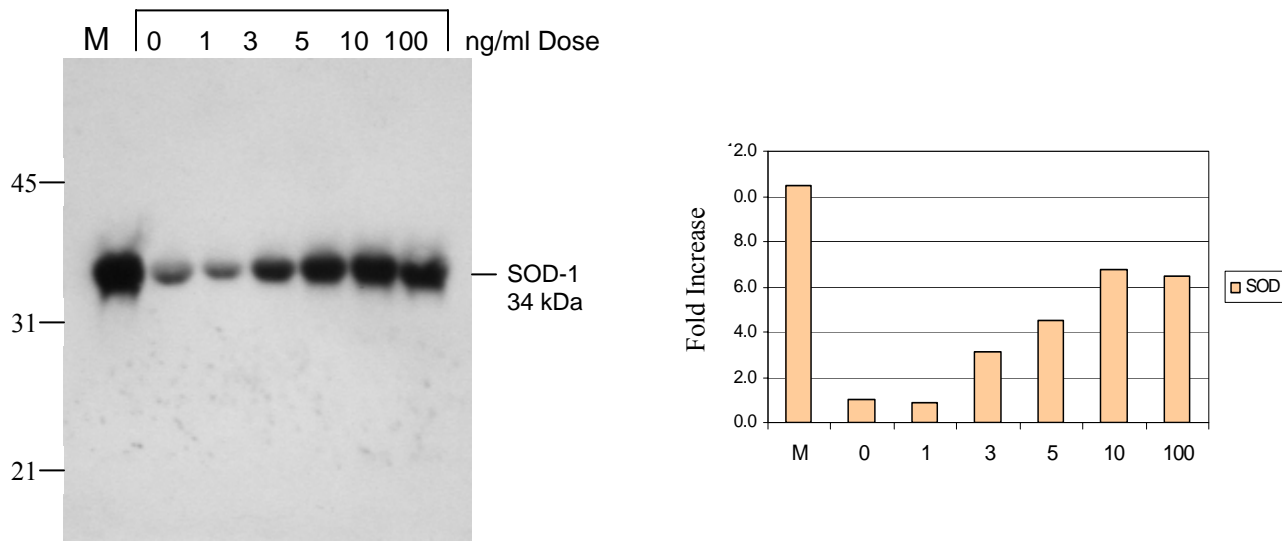
The purpose of this project was to test the hypothesis that a 14aa ependymin neurotrophic peptide that increases murine lifespan increases telomerase activity and telomere lengths. Fibroblast cells were analyzed since previous experiments (Lanza et al., 2000) showed these cells are capable of upregulating telomerase activity, increasing telomere lengths, and increasing cell lifespan, and a substantial body of work shows such cells to be an excellent model for ageing studies. The effects of the peptide were also tested on mice *in vivo*.

### **Fibroblast Cells Respond to the Peptide Treatment (SOD Western)**

Our laboratory previously showed that cultured mouse Neuro-2a cells or rat primary neuronal cultures upregulate antioxidative enzymes in response to treatment with an ependymin peptide mimetic (Shashoua et al., 2004). All known growth factors act via cell surface receptors, so presumably mouse and rat neurons are able to bind this mimetic. To determine whether human newborn foreskin fibroblast cells cultured to 30 cell divisions are also capable of responding to this mimetic, the levels of superoxide dismutase (SOD) were analyzed using an immunoblot in cell lysates prepared from cells treated for 3 hrs with various doses of peptide. Figure 6 shows a strong dose-related response for SOD upregulation. The 10 ng/ml dose produced the highest cellular levels of SOD, which is also the optimal dose for neuronal cells (Shashoua et al., 2004). A visible response was also observed as low as 3 ng/ml. The level of response decreased slightly at the 100 ng/ml dose, perhaps due to receptor desensitization.

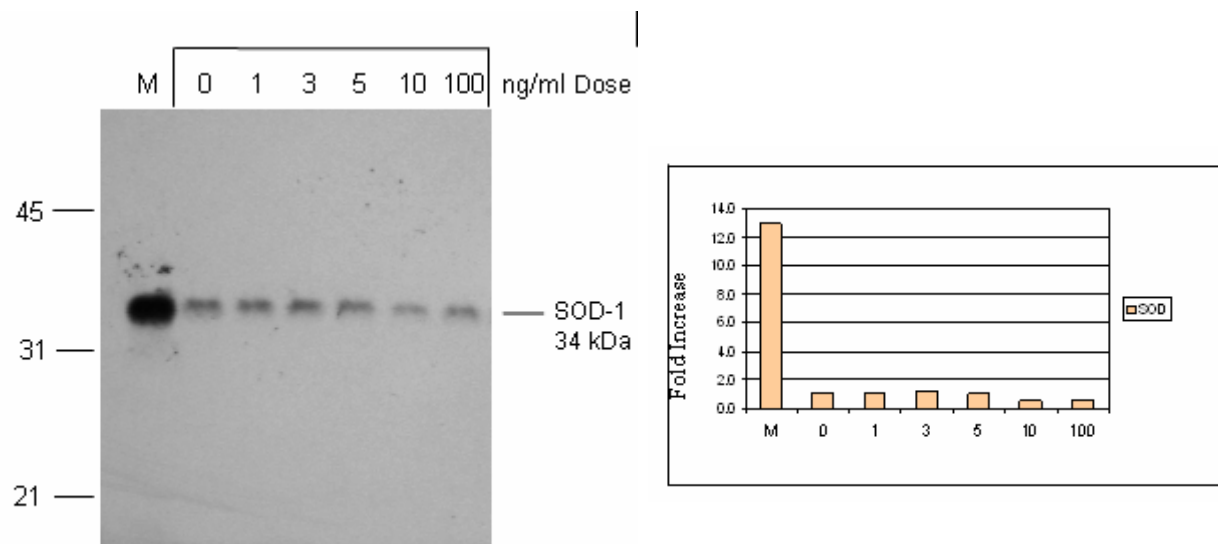


These data show that the human fibroblast cells are indeed capable of responding to the mimetic, so telomerase experiments were initiated.



**Figure 6. Assay of SOD Levels in Primary Human Fibroblast Cells Treated With 14aa Ependymin Peptide Mimetic.** Fibroblast cells at 30 cell divisions were treated with various doses of peptide (noted above each lane) for 3 hrs. Cell lysates were prepared and analyzed by western blot for SOD levels. A dose of 10 ng/ml proved optimum. Numbers to the left of the blot denote marker band sizes (in kDa). The lane labeled “M” denotes a marker consisting of mouse neuronal cells treated with 10 ng/ml peptide. The right panel represents a quantification of the western signals by Scion Image Software.

The upregulation of SOD in the fibroblast cells does not appear to be caused by a non-specific reagent present in the peptide solution since a peptide with a scrambled sequence (same amino acid composition but different sequence) failed to elicit a detectable response, even at a dose as high as 100 ng/ml (Figure 7). The data of Figures 6 and 7 show that the human fibroblast cells are indeed capable of responding to the mimetic, so telomerase experiments were initiated.



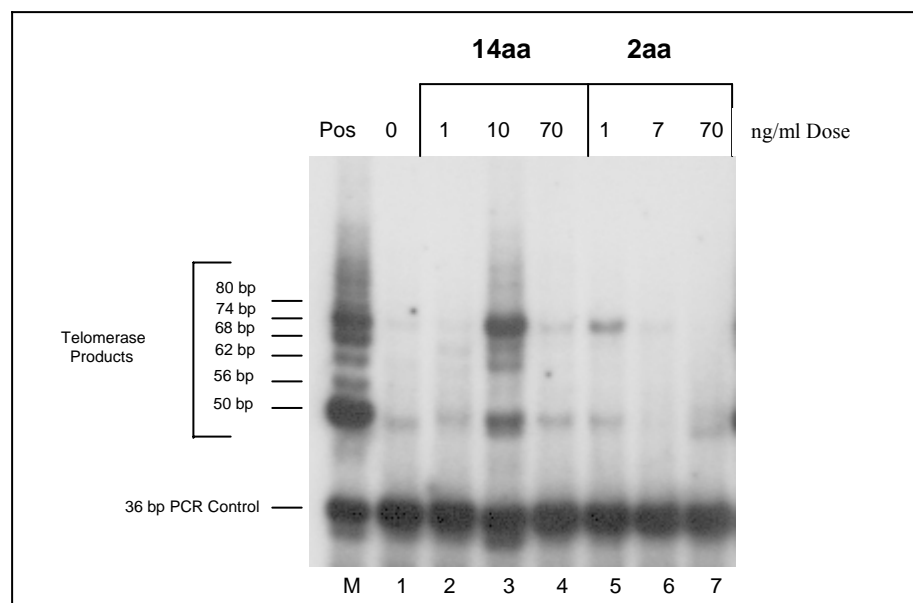
**Figure 7. Assay of SOD Levels in Primary Human Fibroblast Cells Treated With Scrambled 14aa Ependymin Peptide Mimetic.** Fibroblast cells at 30 cell divisions were treated with various doses of scrambled peptide (doses noted above each lane) for 3 hrs. Cell lysates were prepared and analyzed by western blot for SOD levels. Numbers to the left of the blot denote marker band sizes (in kDa). The lane labeled “M” denotes a marker consisting of mouse neuronal cells treated with regular 14aa peptide at 10 ng/ml. The right panel represents a quantification of the western signals by Scion Image Software.

## Measurement of Telomerase Activity

Telomerase activity was measured using a TRAP (Telomerase Repeat Amplification Protocol). For this protocol, a 36 bp internal PCR control should be present in all lanes to show that the samples did not contain different amounts of a Taq Polymerase inhibitor which would affect the signal. The positive control used in these experiments was a cancer cell line extract (provided with the InterGen kit) that is naturally high in telomerase activity. All samples were quantified in relation to this marker lysate using a benchtop counter.

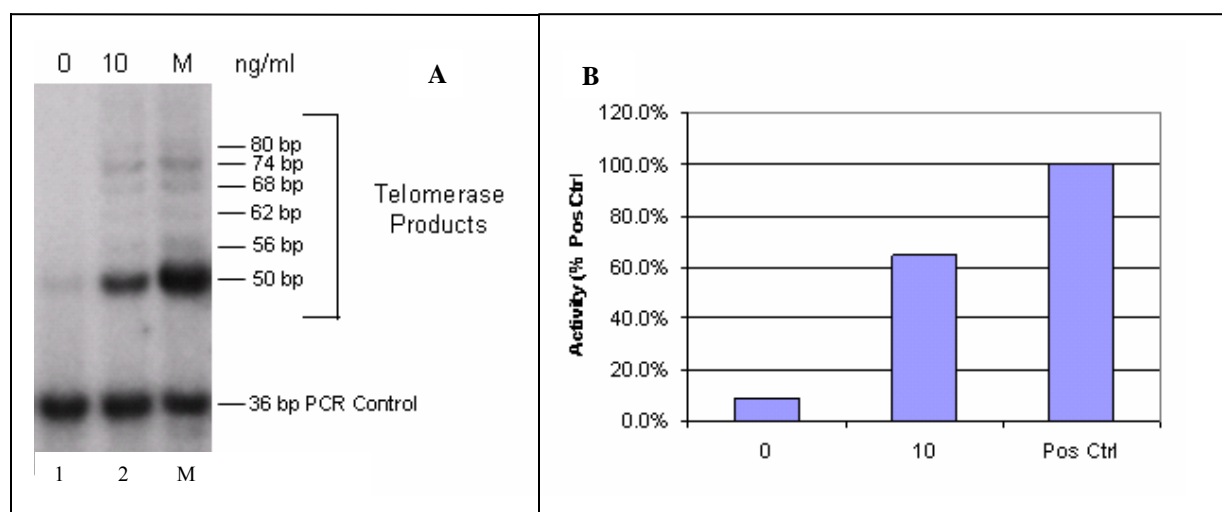
### *Comparison of Telomerase Activity Induced by the 14aa Peptide vs a 2aa Peptide*

Telomerase activity was analyzed using the TRAP procedure in primary fibroblast cells (cultured to 30 cell divisions and treated with peptide continuously for the duration of the experiment) treated with either the 14aa peptide or a 2aa peptide representing the middle two aa (Figure 8). Strong telomerase activity is evident for the positive control (left lane) shown by a strong hexamer ladder beginning at 50 bp (marked with a bracket to the left of the figure). The 36 bp internal PCR control can be seen in equal amounts in all the lanes. Lane 1 contains an untreated sample and therefore has very little telomerase activity in these aged fibroblast cells. The 14aa peptide (middle of figure) showed a large amount of telomerase activity at the 10 ng/ml dose, with little activity observed at the 1 and 70 ng/ml doses. The 2aa peptide (right side of figure) showed no increase in telomerase activity even at 70 ng/ml, so the extra aa of the 14aa drug appear to be required for activity. These data show that the 14aa drug appears to upregulate telomerase activity maximally at the same optimum dose for upregulating SOD in neuronal cells (Shashoua et al., 2004) or upregulating SOD in fibroblast cells (noted above).

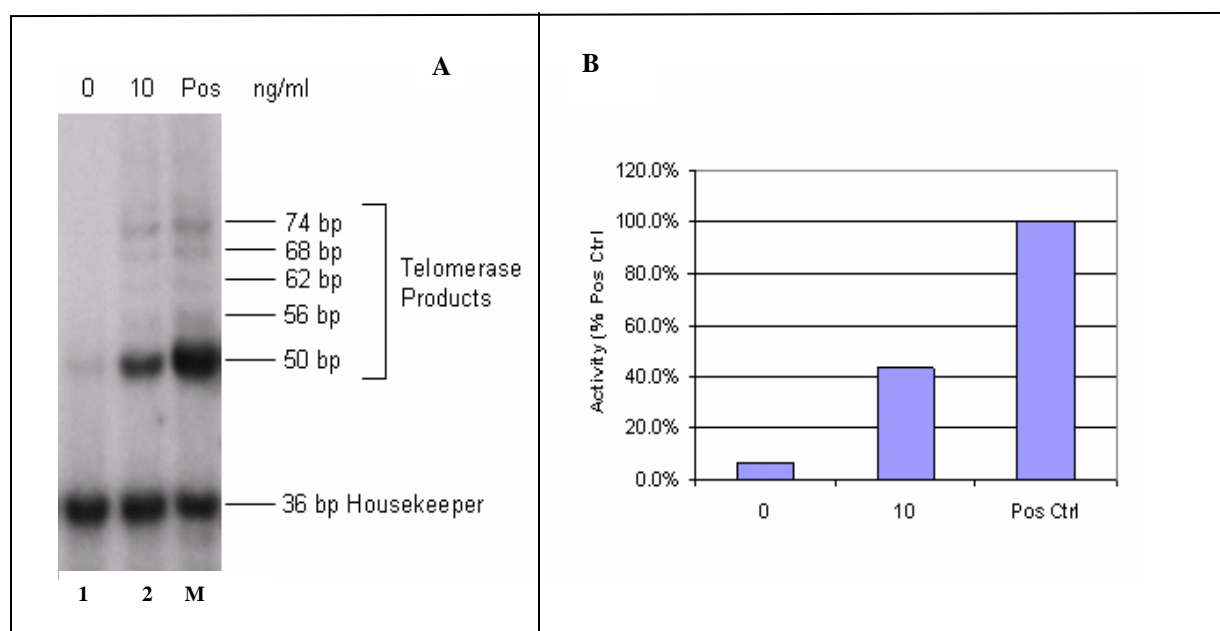


**Figure 8. The 14aa Peptide Increases Telomerase Activity in Human Fibroblast Cells.** The TRAP protocol was used to measure telomerase activity in human primary fibroblast cells cultured to 30 population doublings in the continual presence of various doses of 14aa peptide (middle lanes) or 2aa peptide (right lanes). Telomerase activity is indicated by hexamer ladder bands greater than or equal to 50 bp (bracketed on the left side).

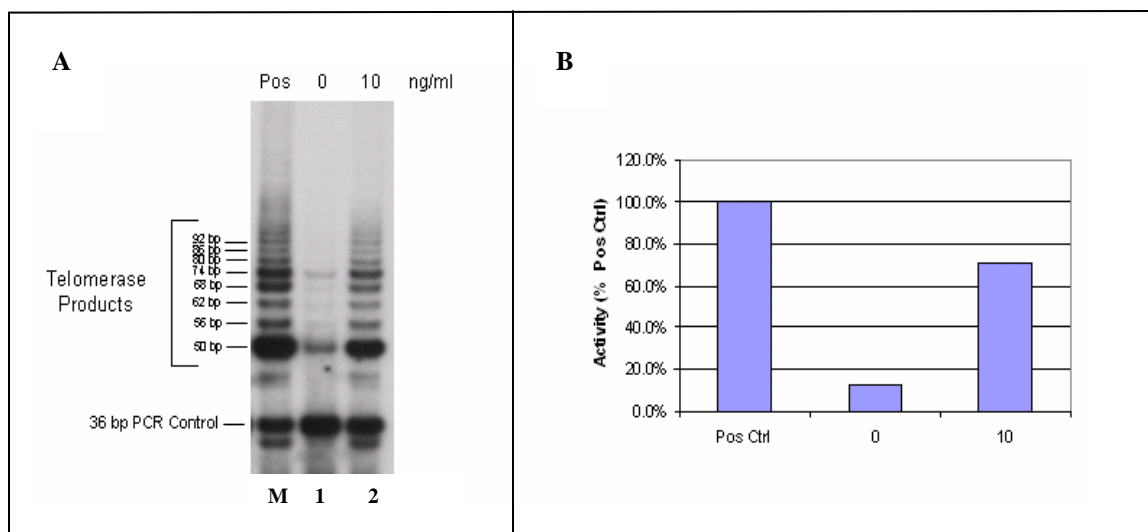
In order to determine whether the apparent upregulation of telomerase activity by the 14aa drug is significant, the experiment shown in Figure 8 was repeated 3 more times (Figures 9-11). In all experiments, the 10 ng/ml dose was optimal. The telomerase activity in each experiment was quantitated by excising all  $P^{32}$ -labeled bands greater than or equal to 50 bp (the shortest rung on the hexamer ladder). The means of the 4 independent trials are shown in Figure 12. The optimal 10 ng/ml dose (right histobar) shows an average 57% increase in telomerase activity relative to untreated control (middle histobar), with a p value of 0.003 calculated by a two-tailed T-test, thus the upregulation appears to be significant.



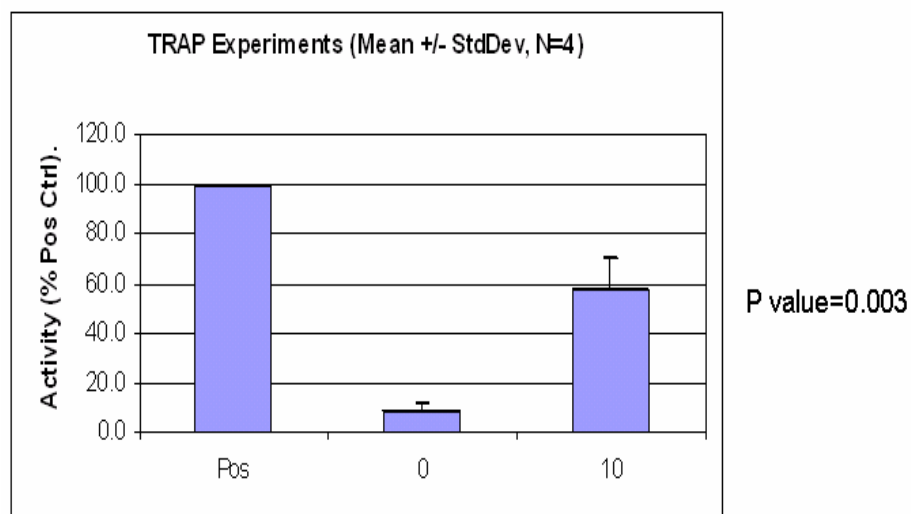
**Figure 9. Trial 2 of the TRAP Analysis .** Panel A shows an increase in telomerase activity (shown in brackets) with the 10 ng/ml 14aa treatment. Panel B represents the quantitation of the results in Panel A shown as percent activity of the positive control sample. Quantitations were performed by excising all P32 bands greater than or equal to the 50 bp lowest rung on the hexamer ladder, and quantitating in a Benchcount.



**Figure 10. Trial 3 of the TRAP Analysis.** The legend is the same as figure 9.



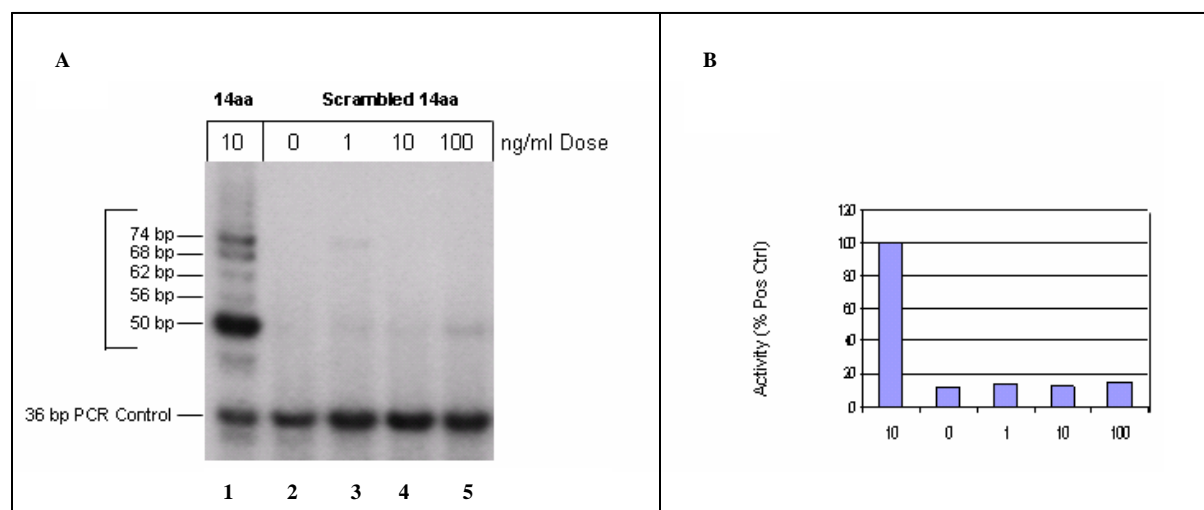
**Figure 11. Trial 4 of the TRAP Analysis.** The legend is the same as figure 9.



**Figure 12. Means of the 4 TRAP Trials.** The optimal 10 ng/ml dose (right histobar) shows an average 57% increase in telomerase activity relative to untreated control (middle histobar), with a p value of 0.003 calculated by a two-tailed T-test.

### *TRAP Analysis of the 14aa Peptide vs a Scrambled 14aa Peptide*

To ensure that the 14aa peptide sequence used in these experiments was causing the upregulation of telomerase activity instead of a contaminant of the peptide solution, its activity was compared to that of a scrambled 14aa peptide containing the same aa composition as the normal drug but with a different order of aa (Figure 13). In this experiment, the 10 ng/ml 14aa treated fibroblast sample served as positive control (lane 1). The scrambled peptide (lanes 2-5) produced no measurable telomerase upregulation, thus the activity of the regular 14aa peptide appears to reside in its sequence.



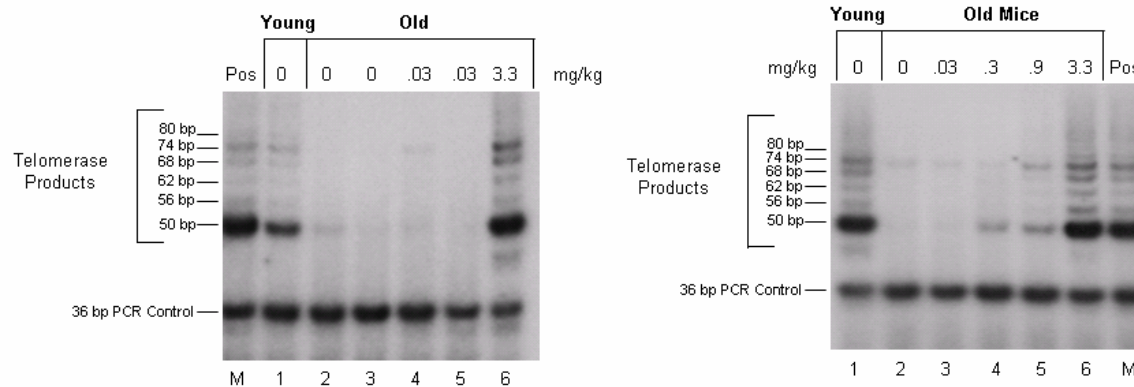
**Figure 13. TRAP Analysis of the 14aa Peptide vs a Scrambled 14aa Peptide.** The legends is the same as the previous TRAP legends, except the positive control in this experiment was fibroblast cells treated with the regular 14aa drug (lane 1). Lanes 2-5 represent fibroblast cells treated with a 14aa peptide with the same composition as the regular drug but with a different sequence. The scrambled peptide induces no measurable upregulation of telomerase activity.

### *TRAP Analysis of Telomerase Activity In Vivo*

Telomerase activity induced by the 14aa peptide was also analyzed in mouse brain and heart *in vivo* (Figure 14). Mice of various ages were injected i.p. with various doses of the 14aa

peptide once per day. The previous Ft. Worth Ageing Study (Figure 5) had shown a significant increase in mouse lifespan using a daily dose of 1 mg/kg 14aa, so our telomerase experiment used a dose range that surrounded that dose. Lane M represents the positive control cancer cell line. Young untreated animals showed strong telomerase activity (Lane 1, both panels) while untreated aged animals showed almost no detectable activity (Lane 2, both panels). This result supports that of numerous previous findings that telomerase activity declines with cellular age, but represents the first observation to our knowledge demonstrating this decline at a whole organ level. Strong telomerase activity was seen in the brain (left panel, lane 6) and heart (right panel, lane 6) of old mice treated with a high dose (3.3 mg/kg) of peptide. This data shows that the 14aa drug has the capacity to either maintain youthful levels of telomerase activity, or upregulate it to those found in young mice. A slight increase in telomerase activity was observed in the old mice at the 0.9 mg/kg dose (right panel, lane 5) but the activity was not as high as at 3.3 mg/kg, so perhaps the Ft. Worth Ageing Study could be repeated at this higher dose to see if further improvements to lifespan are observed. The presence of telomerase activity in heart (and its upregulation by drug) was a surprise since heart myocytes are generally thought to be non-replicating cells, and telomerase is usually associated with cell replication. This topic is discussed further in the Discussion.

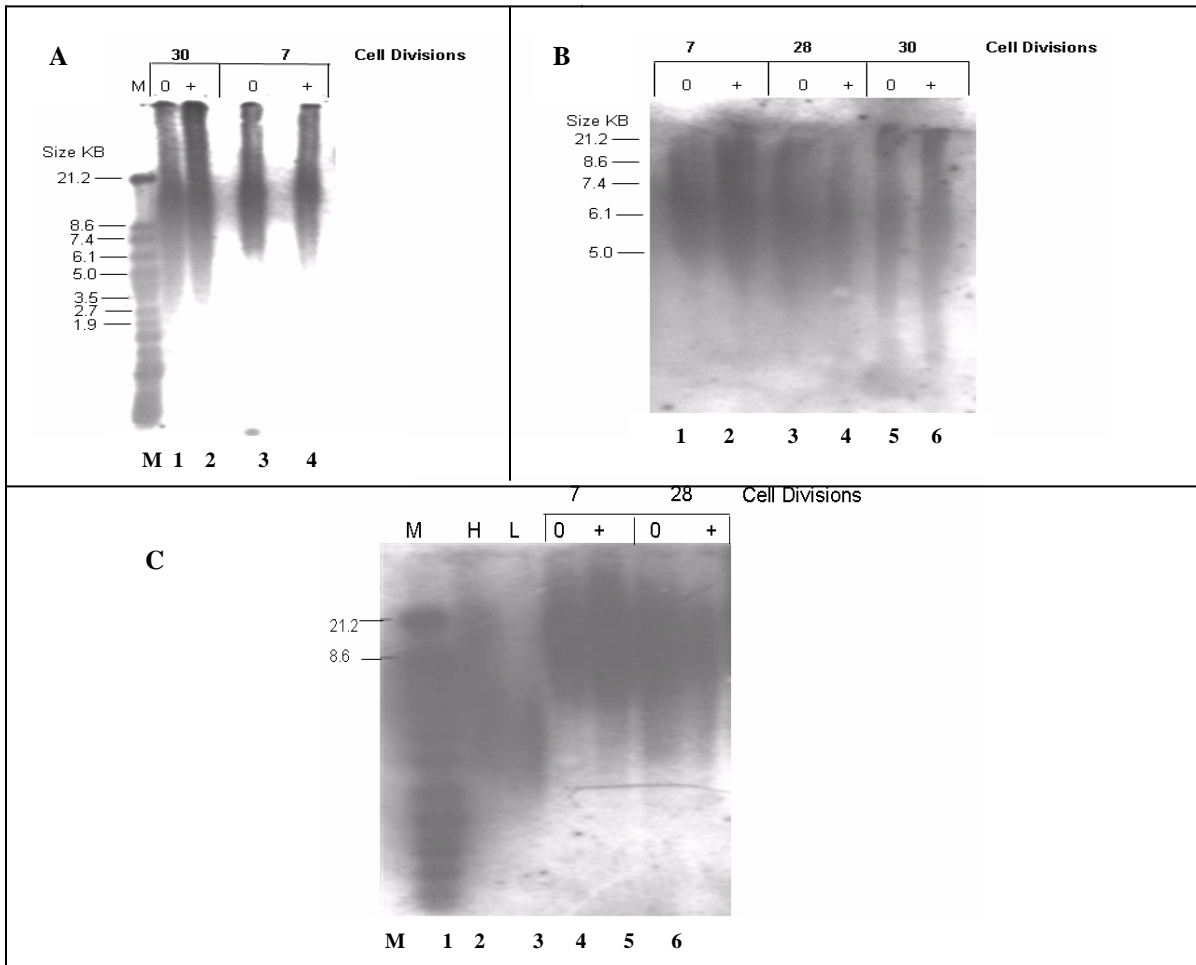




**Figure 14. TRAP analysis of telomerase activity *In Vivo* in Mouse Brain and Heart.** The TRAP procedure was used to measure telomerase activity in brain (left panel) or heart (right panel) in mice treated with various doses (listed above each lane) of 14aa peptide delivered once per day i.v.

## Telomere Length Analysis

Since the 14aa peptide was found to upregulate telomerase activity in fibroblast cells, it is possible the upregulation may help elongate the telomeres (or prevent them from shortening). To test this, human fibroblast cells were cultured to 7, 28, or 30 cell divisions in the continuous presence or absence of 10 ng/ml 14aa drug, then telomere lengths were measured by the telomere length assay (TLA) (Figure 15). In this assay, the telomere lengths appear as “smears” on the Southern blots since the assay shows the sum total of all the telomeres in the cell population which vary considerably. The cells cultured to 30 divisions appear to show lower smears than younger cells cultured to only 7 divisions in all three trials shown (panels A, B, C). The “low” and “high” DNA markers (containing short and long telomeres, respectively) also show appropriate signal differences. These two findings indicate the TLA is working, and is capable of monitoring changes in telomere lengths. However, no change was observed in drug-treated versus non-drug treated samples for any of the trials.



**Figure 15. The 14aa Peptide Does Not Appear to Affect Telomere Lengths in Fibroblast Cells.** Human primary fibroblast cultures were treated continuously with 10 ng/ml of the 14aa peptide, or with vehicle, then telomere lengths measured by the TLA. Three independent trials (Panels A, B, C) were tested. The number of cell divisions at harvest are listed above each panel. Cells receiving drug are denoted (+), and those receiving vehicle are denoted (0).

In summary, the data demonstrate that telomerase activity is upregulated in fibroblast cells treated with 10 ng/ml of the 14aa ependymin peptide, and this upregulation appears to be specific to the sequence of the peptide since upregulation does not occur with a peptide containing the same aa composition but an altered sequence. However this upregulation in fibroblast cells does not appear to be sufficient to cause a measurable increase in telomere lengths. The upregulation of telomerase activity also occurs *in vivo* in both brain and heart.

## DISCUSSION

The purpose of this thesis was to determine whether treatment of human primary fibroblast cultures with a 14aa ependymin peptide mimetic upregulates (or at least maintains) telomerase activity and telomere lengths during cellular ageing. The 14aa peptide was previously shown to significantly increase the murine lifespan by 25%. Because telomerase activity has been linked to diminished cellular ageing (Bodnar et al., 1998; Vaziri and Benchimal, 1998; Yang et al., 1999; Lanza et al., 2000), its activity was a logical candidate to test in this thesis. Lanza et al. (2000) had also shown that fibroblast cells are capable of resetting their telomere clocks, so fibroblast cells were chosen for analysis. In a preliminary set of experiments, the human primary fibroblast cells were shown to respond to the 14aa drug by upregulating the antioxidative enzyme superoxide dismutase (SOD), thus human fibroblast cells likely contain the appropriate receptor for binding this drug. The optimal dose for SOD upregulation (10 ng/ml) was the same as previously shown to upregulate SOD in murine neuroblastoma cells (Saif, 2004). This same dose proved optimal for upregulating telomerase activity in the fibroblast cells an average of 57% relative to untreated cells ( $p$  value = 0.003). The upregulation appears to be specific for the sequence of aa in the 14aa drug since a “scrambled” peptide containing the same aa but in a different order showed no upregulation, even at doses 10-fold higher. Moreover, a 2aa center portion of the 14aa peptide was not sufficient for upregulating telomerase activity, so perhaps the 2aa peptide is not long enough to engage the putative receptor.

The 14aa peptide was also found to upregulate telomerase activity *in vivo* in brain and heart in mice treated once per day or twice per day. The activity was optimal at a 3.3 mg/kg dose

for each aged organ, and was generally high in young organs. The upregulation in brain was not surprising given our lab's previous finding of activity against a murine neuronal cell line (Saif, 2004), but the activity observed in heart was a total surprise since heart cells are generally thought to be quiescent, and telomerase is usually associated with cell division. A review of the literature revealed we were not alone in our finding, Leri et al. (2000) found telomerase activity in young, mature, and senescent rat cardiac myocytes. They also found that ageing decreased telomerase activity by 31%, and that telomerase activity was higher in female myocytes than male. So perhaps telomerase has a function other than in cell division.

The second part of the hypothesis tested whether treatment of fibroblast cells with the 14aa drug elongated (or prevented from shortening) telomere lengths in aged cells. The telomere length assay used was shown to work, since marker DNAs showed appropriate differences in their "telomere smears", and aged fibroblast cells showed overall shorter smears than young cells. However, no difference was observed between drug-treated versus vehicle-treated cells, even at the 10 ng/ml dose previously shown to strongly upregulate telomerase activity. So perhaps the upregulation of telomerase activity was not sufficient to provide a measurable increase in telomere lengths. Telomerase has been shown to extend the lifespan of virus-transformed human cells without showing any visible telomere lengthening (Blackburn et al., 1999), so perhaps telomerase can increase cell lifespan without increasing telomere lengths. Future experiments could include increasing the dose to see whether that produces a measurable increase in telomere lengths.

In conclusion, the 14aa ependymin mimetic peptide was found to upregulate telomerase activity in human primary fibroblast cells, but not their telomere lengths. To our knowledge, this is the only drug demonstrated to upregulate telomerase activity. Transforming cells with the

viral T-antigen can upregulate telomerase, but this is not a therapeutic drug since it also causes cancer. Telomerase upregulation is widely known to occur during oncogenesis (Kim et al., 1994), but telomerase itself is not an oncogene since oncogenesis also requires the upregulation of oncogenes (Belair et al., 1997; Holt et al., 1996; Jiang et al., 1999; Kiyono et al., 1998). Our lab previously showed this peptide does not upregulate the potent oncogene *myc*. If this proves to be the case for other oncogenes, using this drug to upregulate telomerase activity without activating oncogenes could prove extremely useful for proving telomerase is not an oncogene, or for extending cellular lifespans.

Other future studies should be conducted into uncovering the mechanism of how the cell upregulates telomerase. Is it transcriptional? Levels of telomerase RNA (TER) and protein (HTERT) could be measured by RT-PCR and immunoblots, respectively. Footprint analyses of the TER and HTERT gene promoters could allow determination of which transcription factors are important in the activation process.

## BIBLIOGRAPHY

- Adams et al. (1994) Cloning and sequencing the genes encoding goldfish and carp ependymin. *Gene* **2**:237-41.
- Adams et al. (2003) A peptide fragment of ependymin neurotrophic factor uses protein kinase C and the mitogen-activated protein kinase pathway to activate c-Jun N-terminal kinase and a functional AP-1 containing c-Jun and c-Fos proteins in mouse NB2a cells. *J Neurosci Res* **3**: 405-16.
- Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci* **21**: 10114-10118.
- Allsopp RC, Chang E, Kashefi-Aazam M, Rogaev EI, Piatyszek MA, Shay JW, Harley CB (1995) Telomere shortening is associated with cell division in vitro and in vivo. *Exp Cell Res* **220**: 194-200.
- Belair CD et al (1997) Telomerase activity: a biomarker of cell proliferation, not malignant transformation. *Proc Natl Acad Sci* **94**: 13677-13682.
- Blackburn EH and Gall JG (1978) A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. *J Mol Biol* **120**: 33-53.
- Blackburn EH et al (1987) Dynamics of Telomere length variation in Tetrahymena thermophila. *Cell* **50**: 477-483.
- Blackburn EH (1990) Telomere structure and synthesis. *J Biol Chem* **265**: 5919-5921.
- Blackburn EH (1991) Structure and function of telomeres. *Nature* **350**: 569-573.
- Blackburn EH, Zhu J, Wang H, Bishop JM (1999) Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Proceedings of the National Academy of Science* **96**: 3723-3728.
- Blackburn EH (2000) The end of the (DNA) line. *Nat Struct Biol* **7**: 847-50.
- Bodnar AG et al (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**: 349-352.
- Chang E, Harley CB (1995) Telomeres and ageing. In: *Telomeres*. Cold Spring Harbor Laboratory, New York.

de Lange T, Myers RM, Cox DR, Varmus HE (1990) Telomeres shorten with age. *Mol Cel Bio* **10**: 518-527.

Greider CW and Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* **43**: 405-413.

Greider CW and Blackburn EH (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* **337**: 331-337.

Greider CW, Mantell LL (1994) Telomerase activity in germline and embryonic cells of *Xenopus*. *Journal of Embryogenesis* **13**: 3211-3217.

Harley CB, Futcher AB and Greider CW (1990) Telomeres shorten during aging of human fibroblasts. *Nature* **345**: 458-460.

Harley CB (1991) Telomere loss: Mitotic clock or genetic time bomb? *Mutat Res* **256**: 271-282.

Hastie ND, Dunlop NG (1990) Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346**: 866-868.

Hayflick L (1992) Aging, longevity, and immortality in vitro. *Experimental Gerontology* **27**: 363-368.

Haussman MF, Winkler DW, O'Reilly KM, Huntington CE, Nisbet IC, Vleck CM (2003) Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proc Bio Sci* **1522**: 1387-1392.

Holt SE et al (1996) Refining the telomere-telomerase hypothesis of ageing and cancer. *Nat Biotech* **14**: 836-839.

Jiang XR et al (1999) Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat Genet* **21**: 111-114.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011-2015.

Kiyono T et al (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* **396**: 84-88.

Kowald A (1997) Possible mechanisms for the regulation of telomere length. *Journal of Molecular Biology* **273**: 814-825.

Landsorp PM, Verwoerd NP, Van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK, Tanke HJ (1996) Heterogeneity in telomere length of human chromosomes. *Hum Mol Genet* **5**: 685-691.

Lanza RP, Cibelli JB, Blackwell C, Cristofalo VJ, Francis MK, Baerlocher GM, Mak J, Schertzer M, Chavez EA, Sawyer N, Lansdorp PM, West MD (2000) Extension of cell lifespan and telomere length in animals cloned from senescent somatic cells. *Science* **5466**: 665-669.

Morin GB (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* **59**: 521-529.

Moyzis RK et al (1988) A highly conserved repetitive DNA sequence, (TTAGGG)<sub>n</sub>, present at the telomeres of human chromosomes. *Proc Natl Acad Sci USA* **85**: 6622-6626.

Muller HJ (1938) The Remaking of chromosomes. *Collecting Net* **8**: 182-198.

Orr WC, Sohal RS (1994) Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* **263**: 1128-1130.

Park WY, Hwang CI, Kang MJ, Seo JY, Chung JH, Kim YS, Lee JH, Kim H, Kim KA, Yoo HJ, Seo JS (2001) Gene profile of replicative senescence is different from progeria or elderly donor. *Biochemical and Biophysical Research Communications* **282**: 934-939.

Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, Boulianne GL (1998) Extension of *Drosophila* lifespan by overexpression of human SOD1 in motoneurons. *Nature Genetics* **19**: 171-174.

Saif S (2004) AP-1 is required for CMX-8933-induced SOD upregulation and is translocated in response to a human EPN mimetic. WPI Thesis.

Saldanha SN, Andrews LG, Tollefsbol TO (2003) Assessment of telomere length and factors that contribute to its stability. *European Journal of Biochem* **270**: 389-403.

Shashoua V (1976) Identification of specific changes in the pattern of brain protein synthesis after training. *Science* **4259**:1264-6.

Shashoua V (1988) The role of ependymin in the development of long lasting synaptic changes. *J Physiol (Paris)* **83**: 232-239.

Shashoua V (1991) Ependymin, a brain extracellular glycoprotein, and CNS plasticity. *Ann N Y Acad Sci* **627**: 94-114.

Shashoua V, Adams DS, Volodina NV, Li H (2004) New synthetic peptides can enhance gene expression of key antioxidant defense enzymes in vitro and in vivo. *Brain Res* **1024**: 34-43.

Shay JW, Werbin H, Wright WE (1996) Telomeres and telomerase in human leukemias. *Leukemia* **10**: 1255-1266 Review



Slis D (2005) The function of telomerase in protecting the genome. Available [online] <http://faculty.plattsburgh.edu/donald.slis/Telomerase.html>.

Sozou PD, Kirkwood TB (2001) A stochastic model of cell replicative senescence based on telomere shortening, oxidative stress, and somatic mutations in nuclear and mitochondrial DNA. *Journal of Theoretical Biology* **213**: 573-586.

Vaziri H, Benchimol S (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative lifespan. *Curr Biol* **8**: 279-282.

Wright WE, Shay JW, Piatyszek MA (1995) Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity, and sensitivity. *Nucleic Acid Res.* **23**: 3794-3795.

Yang JY et al (1999) Human endothelial cell life extension by telomerase expression. *J Biol Chem* **274**: 26141-26148.