TELOMERE ELONGATION THROUGH THE EXPRESSION OF UP1, A DERIVATIVE OF hnRNP A1

A Senior Honors Thesis

By

NATALIE JEAN TANNER

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2002

Group: Life Sciences

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Approved as to style and content by:

Jorge Piedrahita
(Fellows Advisor)

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ABSTRACT

Telomere Elongation Through

The Expression of UP1,

A Derivative of hnRNP A1. (April 2002)

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As little as a decade ago, the cloning of an eukaryotic organism was generally thought of as an element in a science fiction novel, however, today the procedure is a reality. Genetic modification of cloned animals is also becoming a feasible idea, one that has the potential to create extensive benefits in the areas of medicine and agriculture. Transgenic animals would make it possible to develop livestock that produce high amounts of lean meat or high volumes of milk without the use of hormones. Transgenic pigs have the potential to save hundreds of thousands of peoples' lives by providing organs for xenotransplantation; the transplant of an organ across species. There are many barriers leading to the production of transgenic animals, one being that the genetic modifications must be done *in vitro*. Often, before the necessary modifications are complete, the cultured somatic cells will have reached senescence, a genetically determined event defined as cell aging and eventually death.

The specific cause of senescence has not been determined, but several contributing factors have been identified. The telomere, located at the end of the chromosome, has been proven to play a role in senescence, although it is not clear as to whether its structure or length is the larger determinant. It has been concluded that each time the cell divides, the telomere length shortens, eventually leading to the instability of the chromosome and senescence of the cell. Because telomere length continually decreases, somatic cells can undergo only a limited number of divisions before reaching senescence. My research is examining telomere lengthening in order to extend the cellular life span through the expression of a dominant-negative domain of the naturally occurring heterogeneous nuclear ribonucleoprotein A1 (hnRNP-A1), a gene that has been linked to the telomere mechanism. A dominant-negative is a protein that has been modified to express some characteristics of that protein while repressing others. In this case, the dominant-negative domain UP1 has the unique property of encasing the telomere while its mRNA splicing characteristics have been turned off. Theoretically this protective coat will encase the telomere and protect it from degradation during cell division, therefore delaying senescence of the cell.

Once the dominant-negative UP1 gene was isolated and introduced into porcine fetal fibroblast cells, colonies producing high UP1 expression were selected and expanded. These colonies were then subject to several tests in order to determine the effects of the modified protein. The technique of Population Doubling Factor (PDF) was used to determine if the somatic cells have increased in their number of divisions compared to a control set of cells. Upon completion of PDF analysis, tests for

telomerase activity, an enzyme proven to lengthen telomere length in stem cells, were conducted to rule out this confounding variable.

Our results from the PDF trials did indicate cellular life extension in the somatic cells expressing UP1. Further tests examining telomere length and cellular karyotype must be explored before any definitive conclusions can be drawn, however, the PDF experiment yielded encouraging results as to the prospect of somatic cellular life extension *in vitro*.

ACKNOWLEDGEMENTS

I would like to thank Dr. Piedrahita's lab for all of their help and support. I would especially like to recognize Dr. Jorge Piedrahita, Dr. Bashir Mir, and Scott Dindot for helping when I needed it and putting up with all of my questions. I would also like to thank Kevin Shea and Matt Dolezal for everything that they have done.

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INTRODUCTION

Over the past one hundred years, the leading cause of death around the world has shifted from infectious diseases to chronic diseases. This shift is referred to as the epidemiological transition. Modern medicine has alleviated the major threats imposed by viruses and bacteria, only to find a dead end with cancers, neurological disorders such as Alzheimer's disease, and other types of chronic ailments. The causes and cures of these diseases have been under intensive study over the past twenty years, and recently the medical world has teamed up with the areas of molecular biology and genetics in attempt to approach these disorders from a different angle.

Genetic modification and therapeutic cloning are being explored aggressively in hopes of finding new treatments for chronic diseases. These procedures are examples of research that is based in the field of biotechnology. Biotechnology has in a short time made numerous significant advances not only in medicine, but also in agriculture and environment. New cloning techniques have recently been developed in different species of animals, porcine being most pertinent to telomere elongation research. Cloning allows for the manipulation of specific genes and provides a means by which to make animal models of human diseases. These genetic manipulations also make it possible to produce elite animals that generate higher weights, leaner meat, and increased milk production without the use of supplemental hormones. Because of the versatility of the possible applications, genetic modification and cloning have the potential to benefit people around the world.

This thesis follows the style and format of BIOLOGY OF REPRODUCTION.

Extensive research has been conducted over the past twenty years in order to discover methods to genetically modify organisms. Natural mechanisms such as homologous recombination have been exploited in the past to achieve precise genetic modifications. To develop this technology, mouse embryonic stem cells (ES), which are highly efficient in homologous recombination, have a long cell life due to telomerase activity, and are efficient in nuclear transfer, were used. These properties make gene targeting in mouse ES cells somewhat straightforward. However, this has not proven to be the case in other species or in other cell types. Researchers began to investigate the use of somatic cell lines for nuclear transfer and cloning.

Unfortunately, there are two primary problems with using somatic cells in cloning. First, their cell divisions are limited, ending in senescence in a shorter time as compared to stem cells. Genetic modifications require about 100 population doublings on average, and the modification of a single allele takes about thirty population doublings. Therefore, the simple modification of two alleles would be over the typical cellular senescence plateau, which is reached anywhere from thirty to fifty population doublings, dependent upon the cells used. Secondly, somatic cells have a very low rate of homologous recombination resulting in a highly inefficient genetic modification system. In order to effectively use these cells for cloning and gene targeting, both of these problems must be alleviated.

A contributing factor to these obstacles is the fact that the mechanism of senescence is not completely understood. Several factors have been acknowledged as affecting cell death, one of the most major being the telomere. Scientists have found that the telomere does in fact affect the length of cell life, but it remains uncertain as to the

mechanism of the process. The telomere is a terminal end of a chromosome that decreases in length with each cell division. Once telomeres become too short, the chromosome becomes unstable and the cell dies. Because telomere length of somatic cells continuously decreases, these cells are able to undergo only a limited number of divisions before reaching senescence.

There are three factors that have been linked to the rate that a cell reaches senescence, including the presence of free radicals, the initial length of the telomere, and the rate at which the telomere is degraded. Research has been conducted in the area of telomere length, but it has mainly concentrated on the effects of the protein human telomerase reverse transcriptase (hTERT).

This study examines telomere lengthening and extension of cellular life span through the expression of a shortened derivative of heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), a gene that has been linked to the telomere maintenance mechanism. UP1, the dominant-negative derivative of hnRNPA1, directly binds to the telomere and in theory protects it from shortening during cell division. In mice, it has been shown that the lack of expression of hnRNPA1 results in abnormal telomere shortening (La Branche et al.,1998). However, the replacement of the A1 expression in those A1-deficient cells increased the telomere length. HnRNPA1's mutant gene UP1 had the same effect on telomere lengthening, but failed to retain its RNA splicing ability (Dallaire et al.,2000). In this research UP1 was introduced into porcine fetal fibroblasts followed by a subsequent analysis of the cells and the effects of their dominant-negative expression.

LITERATURE REVIEW

Cell division is required for the survival of multicellular organisms that contain renewable tissues. With the exception of stem cells, somatic cells have a limited capacity to proliferate either *in vivo* or *in vitro*. Therefore, in order for precise genetic modifications of somatic cells to be achieved, replicative senescence must be overcome through the extension of cellular life. Several different elements play active roles in the cell life cycle and cellular senescence and these constituents must be understood in order to develop a logical supposition as to the contributing factors and effects of cellular senescence.

The Telomere

In 1938 Hermann J. Muller gathered the first evidence for telomeres from his light microscopic studies in *Drosophila*. After further investigation, it was discovered that the end region of a linear chromosome has a unique structure that causes it to behave in a way that is distinct from a free end or a double-stranded break. Prior to the establishment of any evidence in confirmation of his thoughts, Muller stated "...The terminal gene must have a special function, that of sealing the end of the chromosome, so to speak, and that for some reason a chromosome cannot persist indefinitely without having its ends thus 'sealed.'" Following Muller's initial discovery, much attention has been devoted to the research of the telomere and its affect on cell cycles and senescence.

Telomeres are nucleoprotein structures on the ends of linear chromosomes distinguished in vertebrates by their hexanucleotide sequence $(TTAGGG)_n$. Telomeric DNA consists of several kilobase-pairs of tandemly repeated sequences with a single-stranded overhang of the glycine-rich strand that makes up the terminal 3'-end of the chromosome. Numerous studies have shown that although the length of telomeric DNA varies between species, specific orientation is conserved in most telomeres and is, therefore, likely of functional importance (Henderson, 1987).

The word telomere literally means end-part; coming from the two Greek roots telos (end) and meros (part). These segmented end structures play an important role in chromosome stability, nuclear structure, and certain chromosome movements during mitosis. Telomere ends are different from broken ends: the telomere's specialized structure causes the natural ends of linear chromosomes to behave differently from simple double-stranded breaks. This is of major importance because the detection of a double-stranded break immediately activates cell cycle checkpoints and the arrest of cell division (Fig. 1). This defense mechanism would be detrimental if activated by the telomere end, given that maintaining the integrity of the telomeric repeats is essential for cell survival (Bodnar et al., 1998).

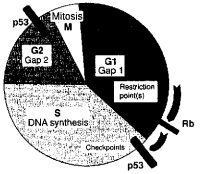

FIG. 1. Cell Cycle Checkpoints (Freshney, 2000)

A double-stranded break may result in dicentric chromosomes, translocations, or deletions. In maize breaks resulting from the action of dicentric chromosomes, cause repeated rounds of breakage-fusion-bridge cycles, which lead to gross changes in cell karyotype (McClintock, 1941, 1942). Natural chromosome ends are not reactive in this way, although both the telomere and the double-stranded break are ends of linear DNA molecules. Telomeric chromosome ends do not cause delays in cell cycle progression, nor are they subject to DNA repair as are broken chromosome ends (Kurenova and Mason; 1997). These facts indicate that the telomere plays a role in maintaining chromosome stability and there is at least one defining factor associated with the telomere that enables the cell to recognize it as a separate entity from a double stranded break.

Two related proteins, the TTAGGG repeat-binding factors TRF1 and TRF2, bind the lengths of mammalian telomeres (Chong et al., 1995; Bilaud et al., 1997; Broccoli et al., 1997). Electron microscopic examination of telomeres has presented evidence

suggesting that TRF2 induces a conformational change in the architecture of the telomeric ends, creating a t loop structure (Griffith et al., 1999). Because a large TRF2 protein complex was present at the loop-tail junction in all cases in Griffith's experiment, his data suggests that t loops are a product of the interaction between TRF2 and the telomeric substrate. This t loop consists of a large duplex loop-back structure most likely formed through the invasion of the single-stranded telomeric 3' overhang into a section of the telomeric repeats located closer to the centromere. The presence of this lasso-like structure would solve the double stranded break problem presented by linear DNA. Because the linear end is buried within itself, there is no free end for the cell cycle checkpoints to detect. The inhibition of TRF2 immediately activates a DNA damage checkpoint pathway leading to cell cycle arrest (Karlseder et al., 1999), further evidence that suggests that TRF2 and t loops are elements of the "sealing" process of the telomere.

The mechanism through which telomeres are replicated was one of the first issues addressed by inquisitive researchers and is a problem that poses the most serious obstacle for telomere elongation research. Conventional DNA replication includes the priming of DNA by short RNA molecules, the extension of the new strand in the 5' to 3' direction, and the ligation of discontinuous pieces. This presents no problem at one end of each template strand, however, at the other end, there is an unreplicated segment when the primer is removed (Watson, 1972). Because of the involvement of this RNA primer, DNA polymerase α cannot replicate the very end of a linear chromosome (Fig. 2). In somatic cells, the absence of a telomere maintenance mechanism to overcome this

"end-replication problem" results in the cells' failure to pass their complete genetic complement from generation to generation, or the degradation of the telomere each round of cell division. Approximately 50-200 base pairs (bp) of telomeric DNA are not replicated during each S phase. This constant telomeric dilapidation brings the cell closer to chromosomal instability each round of cell division, eventually leading to cell-cycle arrest and senescence, or cell death.

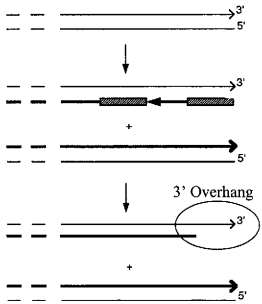

FIG. 2. The End Replication Problem (Blackburn and Greider, 1995)
The removal of the primer after DNA replication results in a 3' overhang.

Senescence

In 1881 the German biologist August Weismann speculated that somatic cells of higher animals would be found to have a limited doubling potential because "a worn-out tissue cannot forever renew itself, and because a capacity for increase by means of cell

division is not everlasting but finite" (Weismann, 1891). Weismann's conjecture proved to be plausible when in 1961 the finite replicative capacity of normal human fibroblasts was described (Hayflick and Moorhead, 1961). It was shown that the death of cultured normal human cells was not due to some trivial cause involving medium components or culture conditions, but was an inherent property of the cells themselves (Hayflick, 1997).

Senescence is cell death after a limited number of cell divisions. Cells that have undergone senescence are irreversibly arrested in the G_1 phase of the cell cycle and are no longer sensitive to growth factor stimulation (Seshadri and Campisi, 1990). This genetically determined event is associated with several factors, one being the inability of the terminal sequences of telomeric DNA to replicate at each cell division. Although the exact mechanism of replicative senescence is unknown, several factors have been identified that affect this phenomenon, including free radicals present in the cell or its medium, telomere length, and the rate at which the telomere is degraded.

In the past, researchers have supposed that the main cause behind the initiation of senescence was severely shortened telomere length. Although this appears to affect the rate at which the senescent phenotype appears, recent data supports that the telomere structure plays a significant role in this area as well. The sequence specific telomere binding protein TRF2 has shown to delay senescence by protecting critically short telomeres from fusion, which suggests that replicative senescence is induced by a change in the protected status of shortened telomeres rather than the complete loss of telomeric DNA (Karlseder et al., 2002). Other research involving telomerase, a telomere specific polymerase, indicates that its introduction into the cell does not immediately result in net

telomere lengthening, but rather that the telomeres continue to shorten for a period of time before a stable, short length is reached and cell life is extended (Zhu et al., 1999; Yang et al., 1999). This presents further evidence supporting that telomere maintenance, not exclusively telomere length, is critical for cell survival. A method to overcome the continual shortening of the telomere with every DNA replication may be necessary to achieve immortality in cells, however, cellular life extension could possibly be achieved through protection of the telomeric structure.

The first evidence that human somatic cells might show terminal fragment loss, and therefore exhibit telomere degradation, was derived from observations that human telomeres are longer in sperm cells than in hemocytes (Cooke and Smith, 1986; Allshire et al., 1998, 1999; Cross et al., 1989; de Lange et al., 1990; Hastie et al., 1990). Human telomeres from somatic cells that have eroded from their maximum size of 10-15 kb (in the germ line) to an average size of 4-6 kb have shown to irreversibly arrest growth, producing a characteristic (senescent) phenotype (Campisi, 1997; Chiu and Harley, 1997). Thus, the progressive shortening of the telomeres continues until the cell is unable to divide further (Bodnar et al., 1998).

Oxidative damage is another cellular stress that can cause premature cellular aging and growth arrest. Fibroblast cells cultured in conditions of mild hyperoxia showed a shortened replicative lifespan and exhibited an increased rate of telomere degradation (von Zglinicki et al., 1995). Eukaryotic cells use strong oxidants housed in cytoplasmic organelles to destroy cellular waste products and subsequently remove them from the cell via exocytosis. Cells also use oxidizing agents to undergo cell-mediated

apoptosis by the programmed release of these oxidants. In healthy cells, there will be some degree of oxidation or reactive oxygen species (ROS) present at any given time, however at a low concentration. Specific culture conditions can significantly affect these oxidant levels, therefore having a direct affect on the rate in which cultured cells undergo replicative senescence.

Several theories have identified senescence as functioning in part to eliminate or decrease the development of cancer (oncogenesis). The process of cell division innately puts the cells at risk for developing cancer as genomes are continually damaged by oxidative metabolism, the environment, and, in actual dividing cells, errors in DNA replication and mitosis. Maintenance and repair mechanisms are present in the cell, but this machinery is not always one hundred percent efficient. Dividing cells have the highest risk for genomic mutations, which are generated by failures or mistakes in repair. To protect against accumulation of these replicative errors, eukaryotic organisms undergo cellular senescence, an all-encompassing protection mechanism, to suppress the growth of cells at risk for oncogenic transformation. Cellular senescence is a major barrier that cells must overcome in order to progress to full-blown malignancy (Smith and Pereira-Smith, 1996; Campisi, 2000). This anti-oncogenic function is not without its adverse affects on the cell *in vivo*. Because senescent cells can, in theory, disrupt local tissue integrity, they might also contribute to age-related pathology (Campisi, 2001). The emerging idea is that when senescent cells accumulate, as they do later in life, they might actually facilitate tumorigenesis (Krtolica et al., 2001).

Genetic Modifications

Different methods have been explored in attempts to delay the onset of senescence *in vitro*. This delay is of the utmost importance, as genetic modifications of eukaryotic cells *in vitro* require long periods of time in culture and multiple population doublings. The replicative potential of a cell is measured using units of population doublings (pds), or the number of times that a cell colony divides over a predetermined amount of time. Untreated somatic cells will become senescent after thirty to fifty pds, dependent on the cell type and the cell age upon initiation of the culture. On average, genetic modifications require about one hundred pds to complete. The discrepancy between the average pds of normal cells *in vitro* and the number of pds required for genetic modifications is one of the issues addressed by telomeric elongation research (Fig. 3).

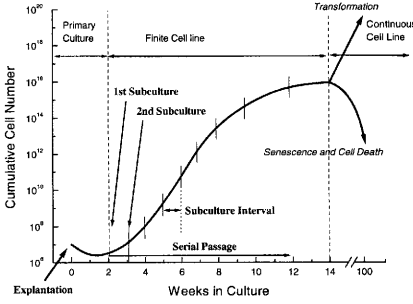

FIG. 3. Standard Cell Growth Curve *in vitro* (Freshney, 2000)

Telomerase

The process of telomere degeneration can be overcome by the expression of hTERT, the catalytic subunit of the ribonucleotide complex of telomerase (Bodnar et al., 1998). Telomerase is a specialized DNA polymerase that has been shown to synthesize the terminal repeat sequences of chromosomes (TTAAGGG) and add them *de novo* to the G-rich 3' ends of the telomeres. In this way it counters the constant telomere degradation typical of the DNA polymerase α mechanism. Thus, the telomeres of immortal cells do not continue to shorten with successive passage *in vitro* (Harley et al., 1990).

This enzyme was first discovered in the ciliated protozoan *Tetrahymena thermophila* (Greider and Blackburn, 1985). Telomerase expression has been identified in four additional species, the human and the mouse being the only eukaryotes. In these eukaryotic organisms only specific cells express it, as it is active in germ cells and has moderate activity in stem cells (no normal expression is seen in somatic cells). Some somatic cells *in vitro* develop the expression of telomerase after an extended number of population doublings, but this expression is often the first stage of oncogenesis. Furthermore, telomerase activity has been detected in cancerous tumor cells *in vivo*. The level of telomerase activity found in normal cell populations, such as germ and stem cells, is significantly less per cell than that found in cancer cell populations (Wright et al., 1996). It is possible that the high level of repression of telomerase in most somatic cells has been selected in long-lived species, such as humans, as a mechanism for reducing the frequency of cancer (Harley et al., 1994).

Normal levels of telomerase activity seem to have no adverse consequences to the cell. Research is currently being pursued concerning the initiation of telomerase activity in somatic cells in order to achieve fibroblast immortality *in vitro*. This involves the difficult process of inserting the hTERT subunit into a cell. Cells expressing hTERT have a normal karyotype and behave as normal cells do in response to cell-cycle inhibitors, serum deprivation, high cell density, and irradiation (Yang et al., 1999; Jiang et al., 1999; Morales et al., 1999; Vaziri et al., 1999). It is also indicated that only a very small average telomere elongation by hTERT resulted in a 50% increase in life span of human fibroblasts (Steinert et al., 2000). These results suggest the potential safe use of hTERT in tissue engineering.

Heterogeneous Nuclear Ribonucleoprotein A1 and UP1

In recent years several proteins have been identified as having involvement with mammalian telomeres. These proteins all have different binding specificity; some associating exclusively with the telomeres, others with the protein complexes that are built at the chromosome end, and still others have a further affinity for additional cellular sites. While every one of the identified proteins' exact roles has not yet been identified, many of them appear to have some function in maintaining the length and/or structure of the telomere.

The telomere's 3' overhang of the G-rich strand is subject to the most oxidative and replicative degradation. During DNA replication it is thought that the G-rich strand serves as the substrate for the synthesis of the complementary strand (Greider et al.,

1989; Nugent and Lundblad, 1998). This is the process previously defined with its involvement of the RNA primer causing the incomplete replication of the lagging strand. Because the protein heterogeneous nuclear ribonucleoprotein (hnRNP) A1 has been shown to associate with this area of the chromosome, it has received much attention. Moreover, hnRNP A1 is capable of interacting with telomerase, which further suggests it performs an important role in telomere biology (LaBranche et al., 1998).

HnRNP A1 is one of the most abundant core proteins of hnRNP complexes in eukaryotic nuclei. Along with its DNA annealing activity, the protein also shuttles continuously between the nucleus and the cytoplasm, a process managed by an export signal within its C-terminal glycine-rich domain (Piñol-Roma and Dreyfuss, 1992; Michael et al., 1995). This shuttling suggests hnRNP A1 may be involved in transporting mature mRNA to the cytosplasm (Xu et al., 1997). It has been identified as having not only an association with the telomere, but also participating in telomere biogenesis (LaBranche, 1998) (Fig. 4). Increased expression of hnRNP A1 and its shortened derivative UP1 promote telomere elongation in mammalian cells through direct interactions with the telomere (LaBranch, 1998). It is not clear whether hnRNP A1 does this by protecting the telomere from shortening or by conserving the telomere's structure.

FIG. 4. Proposed Manner in which UP1 Binds Telomeric DNA (Ding et al., 1999)
UP1 is represented by the bi-colored ovals, the light half representing RRM1 and the dark half representing RRM2.

The N-terminal region of hnRNP A1, designated unwinding protein 1 (UP1), preferentially binds to the G-rich telomeric overhangs at the 3' terminus (Dallaire et al., 2000). UP1 runs the first 196 base pairs and functions exactly as hnRNP A1 sans splicing ability (Mayeda et al., 1994). This UP1 fragment, or domain of hnRNP A1, is composed of two stable, independently folded subdomains – subdomain I and subdomain II. Within subdomain I and subdomain II reside two highly conserved RNA-recognition motifs (RRMs), RRM1 and RRM2, respectively (Xu et al., 1997) (Fig. 5). The co-crystal structure of UP1 suggests that both RNA binding domains can interact with telomeric DNA (Ding et al., 1999), however it has been proposed that the two RRMs have distinct specificities: RRM1 binds to telomeric DNA sequences while RRM2 simultaneously interacts with the RNA component of telomerase (Fiset and Chabot, 2001). These results are significant as the simultaneous interaction of A1/UP1 with telomerase and telomeric DNA may help position telomerase for the extension of 3' overhangs (Fiset and Chabot, 2001).

FIG. 5. Structure of UP1 Bound to DNA (Ding et al., 1999)
The protein is represented by the ribbon structures and the bound DNA is represented by the overlaid helices.

HnRNP A1 and its proteolytic fragment UP1 have shown to play a direct role in telomere biogenesis (LaBranche, 1998). Both UP1 and hnRNP A1 have proven to bind directly to telomeric DNA and it has subsequently been shown that the A1/UP1 binding to a long single-stranded oligonuceotide protects DNA from endonuclease and exonuclease attack (Dalliare et al., 2000). The complete mechanism through which the

telomere is protected is not clear, but there is evidence that the over-expression of A1/UP1 has some affect on cell life in culture (Dallaire et al., 2000; LaBranche et al., 1998). Furthermore, recent data has additionally indicated that the binding of A1/UP1 to telomeric DNA may help shield the ends of chromosomes from nucleolytic attack and from surveillance mechanisms that detect double-stranded DNA breaks (Fiset and Chabot, 2001).

MATERIALS AND METHODS

Production of the Dominant-negative UP1

Production of mRNA

DNA was extracted from porcine fetal fibroblast cells grown in monolayer. The cells were lysed in the culture dish by trypsinization followed by the addition of 1 ml TRI REAGENT®. RNA was then extracted by way of a kit from TRI REAGENT® (Invitrogen, #15596026).

The homogenate was stored at room temperature for five min followed by addition of 0.2 ml chloroform. After the sample was shaken vigorously for 15 sec, the resulting mixture was stored at room temperature for ten min and subsequently centrifuged at 12,000 g for 15 min at 4°C. Following centrifugation, the colorless upper aqueous phase containing the RNA (0.6 ml) was removed and transferred to a fresh tube. The interphase and organic phase left in the original tube was discarded.

The RNA was precipitated from the aqueous phase by the addition of 0.5 ml isopropanol to the solution. The sample was then stored at room temperature for 5-10 min followed by centrifugation at 12,000 g for 8 min at 4°C. The supernatant was removed and the resulting pellet was washed by the addition of 1.5 ml of 75% ethanol, vortexing, and centrifugation at 7,500 g for 5 min at 4°C. The RNA pellet was then air-dried for ten min. At this point the RNA was solubilized in 15 μl of nuclease-free water.

Reverse Transcription

Reverse transcription was performed using the ProSTAR RT-PCR Kit (Stratagene, #200420). The control reaction was prepared by adding 2 μl of control mRNA to 36 μl of diethylpyrocarbonate (DEPC)-treated water. A separate experimental reaction of 10 μg of fibroblast RNA and 28 μl of DEPC-treated water was also prepared. 3 μl of random primers (100 ng/μl) were added to both the control and experimental reactions and mixed gently. The samples were then incubated at 65°C for 5 min, followed by a cooling period at room temperature for ten min. The following components were then added to both reactions in the order listed:

5 μl of 10x first-strand buffer
1 μl of Rnase Block Ribonuclease Inhibitor (40 U/μl)
2 μl of 100 mM dNTPs
1 μl of StrataScript™ reverse transcriptase (50 U/μl)

The control and the experimental reactions were mixed gently and subsequently incubated at 42°C for 1 hour.

Primer Design

Two primers were designed for the isolation of the UP1 sequence. These primers, coined "pUP1" and "pUP2," were designed to anneal to the DNA strand before and after the UP1 sequence (amino acids 1-196).

A porcine UP1 sequence is currently not available. Therefore, DNA conservation between species was relied on to design the primers. A sequence comparison was made between human and mouse DNA (Fig. 6), and the sequences for the UP1 primers were taken from a region conserved between the two species.

Additionally, the region seleceted incorporated three enzyme cut sites. The custom primers were then produced by Sigma-Genosys (Houston, TX).

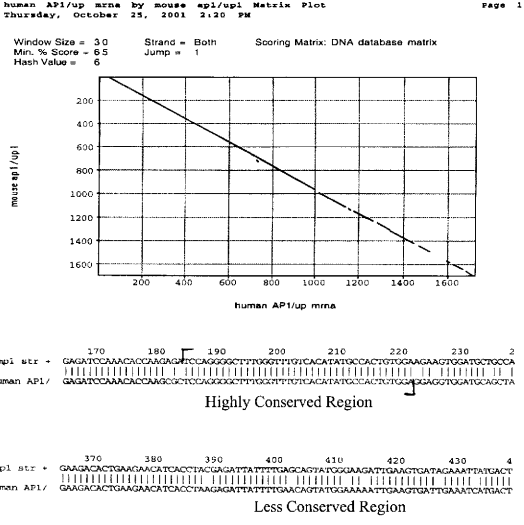

FIG. 6. MacVector Human and Mouse Conservation Graph

Polymerase Chain Reaction

The UP1 RT-PCR products were pooled and 50 μl of water was added to make a total volume of 300 μl. The product was washed by adding an equal volume of phenol chloroform (300 μl) and centrifuging. The upper aqueous layer was extracted and transferred to a fresh tube. An equal amount of isopropanol was added to the sample and mixed gently, followed by a 5 min centrifugation. The supernatant was removed and the pellet was washed with 300 μl of 70% ethanol. The UP1 fragment was then dried for 3 min in a spin-dry vacuum and immediately resuspended in 30 μl of water. The DNA was sequenced through reverse transcriptase was sequenced with the custom Sigma-Genosys primers through a PCR reaction (94°C for 10 sec, 60°C for 30 sec, 68°C for 1 min, 30 cycles).

pCR® 2.1-Topo Vector

Following the TOPO TA Cloning® protocol, 4 μls of the purified sequence product/water solution was mixed with 1 μl of salt solution and 1 μl Topo 2.1 vector (Invitrogen, #K4500-40). The resulting solution was then incubated at room temperature for 5 min and 3 μl were added to one vial of One Shot® Top 10 E. coli cells. The mixture was then ready for transformation as described by the Promega protocol. The mix was incubated for 30 min on ice and afterward heat shocked for 30 sec at 42°C. Immediately following the heat shock, the cells were placed on ice for 2 min. The transformed cells were transferred into 200 μl of LB broth and placed at 37°C for one

hour with shaking at about 100 rpm. All 200 μl were spread onto agar selection plates, which contained 60 μl/ml X-Gal and 100 μl/ml carbenicillin (Carb). The plates were incubated overnight at 37°C. The white colonies were picked and screened by restriction digestion with Fse I for the presence of the UP1 insert (Fse I site located on pUP1).

The pShooter Expression Vector

Isolation and Purification of the UP1 Sequence

The UP1 fragment was cut out of the pCR 2.1 Topo vector with the enzymes Not I and Nco I (located on pUP1 and pUP2). The fragment was then isolated and purified using QIAquick Gel Extraction Kit (Qiagen, #28704). The DNA was run on a 1% agarose gel and excised. 390 μl of Buffer QG was added to the excised gel. The sample was incubated at 50°C for 10 min, with vortexing every 2 min during the incubation. The sample was then applied to a QIAquick spin column placed in a 2-ml collection tube, centrifuged for 1 min, and the flow-through discarded.

To wash, 0.75 ml of Buffer PE was added to the QIAquick column and centrifuged for 1 min. The flow-through was discarded and the column centrifuged for an additional min at 13,000 rpm. The QIAquick column was then placed into a clean 1.5-ml microfuge tube and the DNA eluted by adding 30 μl of water to the center of the QIAquick membrane, followed by centrifugation of the column for 1 min at maximum speed.

Cloning of UP1 into pShooter Vector and Transformation

The pShooter vector was cut by Nco I and Not I enzyme sites and purified by the same protocol as described for the UP1 fragment. Upon pShooter purification, the purified UP1 fragment was cloned into the vector. 100 ngs of the pShooter/UP1 sample were added to one vial of One Shot® E. coli competent cells and the mixture was incubated on ice for 30 min. Transformation was performed by the same protocol as described previously for the pCR 2.1 Topo vector. Positive colonies were selected and DNA was harvested.

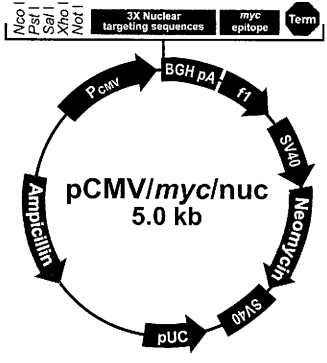

FIG. 7. The pShooter Vector
The Nco I and the Not I sites correspond to the sites on the UP1 fragment.

Electroporation

Digestion and DNA Preparation for Electroporation

70 μls of pShooter/UP1 DNA, 270 μl water, 40 μl buffer, and 20 μl Sca I enzyme (Roche Diagnostics, #775258) were combined and incubated at 37°C overnight. The resulting DNA was separated on a 1% agarose gel to confirm the complete digestion.

400 μl phenol-chloroform isoamyl was added and the sample was shaken vigorously to achieve a homogenous mixture. The mixture was centrifuged at full speed for 5 min and the aqueous phase was extracted. An equal volume of isopropanol was added to the aqueous layer, the mixture shaken by hand, and left at room temperature for 5 min. This was followed by centrifugation, again at full speed, for 5 min. The supernatant was removed and 500 μl of 70% ethanol added followed by 5 more min of centrifugation. The supernatant was poured out and the sample was centrifuged for 1 min. The remaining liquid was aspirated with a pipette tip without disturbing the pellet. The pellet was vacuum dried for 5 min and resuspended in 50 μl endotoxin free TE buffer. The solution was set at 37°C for 30 min and centrifuged for 1 min at full speed. The concentration of the resulting DNA was checked using the spectrometer.

Electroporation

Porcine fetal fibroblast cells were grown from frozen stock to a sub-confluency of 70-80%. The cells were then trypsinized and counted. Ten million cells were pelleted down and resuspended in 800 μl of F10. 20 μg of the pShooter/UP1 linearized DNA were added to the mix and the solution was left at room temperature for 10 min.

During the incubation, 30 ml of media (DMEM/F12 based, 10% fetal bovine serum) were prepared in a 50 ml tube. The DNA-cell mixture was added to a 4mm cuvette and electroporated (450V, 4 pulses, 1 ms pulse length). The contents were then transferred to the prepared medium and mixed properly. The electroporated cells were added to fifteen plates at 2 ml/plate. 6 ml of additional media was added to each plate to make a volume of 8 ml of media per 100mm plate. 24 hours following the electroporation, the media was changed and neomycin, or G418, (Gibco, #1811) was added to the media at a predetermined concentration (ranging from 500 μl/ml to 1,100 μl/ml dependent on plate surface area). In addition to the electroporated cells, a control set of 1.5 million cells (from the same frozen stock) was seeded. These cells did not undergo electroporation and were not exposed to antibiotic. The remaining cells from the frozen stock were refrozen at -80°C for later assays.

Population Doublings

Colony Selection and Expansion

Protocols for the isolation of cloned colonies followed those in *Culture of Animal Cells; A Manual of Basic Technique* protocol. Ten cloned colonies expressing G418 resistance were marked, media removed, and the plate washed gently with PBS. With sterile forceps, a cloning ring was dipped in silicone grease and placed around each colony. 0.4 ml of trypsin were added to each cloning ring, left on for 20 sec, and removed. The plates were then closed and incubated at 37°C for 15 min. 0.4 ml of

medium was then added to each ring. Taking each colony in turn, the medium was pipetted up and down to disperse the cells, and transferred to one well of a 96-well plate. The ring was washed out with another 0.4 ml of medium, and the media transferred to the same well with the cells. Cells were gradually expanded from 24 wells, to 6-well plates, and eventually to 100mm plates.

Population Doubling Factor

Population doubling factor (PDF) was calculated as log (D/D_o), where D and D_o are the density of cells at the time of harvesting and seeding, respectively. Sub-confluent (70-80%) plates were harvested by trypsinization once per week, cells counted, and PDF values calculated and plotted as a function of time.

The cells were passed according to *Culture of Animal Cells; A Manual of Basic Technique* protocol. First the media was removed from each plate, followed by 5 ml of PBS wash with Magnesium. The PBS wash was removed and 2.5 ml of trypsin added per 100 mm plate of cells, followed by immediate incubation for 4-6 min at 37°C. All cells were removed from the plate, counted, and centrifuged at 3,000 rpm for 10 min. $6x10^5$ cells were resuspended in fresh media containing the determined amount of G418 and plated at a density of $2x10^5$ cells per 100mm plate. The cells unused for further population doublings were frozen in 0.5-1.0 of freezing media (40% fetal bovine serum, 10% DMSO, DMEM-F12 base), labeled according to their group, and kept at -80°C for later analysis. The population doublings were recorded for all sets of cells beginning day 1 as the day of electroporation and ending with day 98.

UP1 Expression

DNA samples of each of the five UP1 expressing colonies were extracted and mRNA was prepared through the protocol previously described. The mRNA was manipulated through reverse transcription and the resulting cDNA was analyzed through PCR reactions.

Telomere Repeat Amplification Protocol

Telomerase activity was measured using telomere repeat amplification protocol (TRAP) using a kit from Intergen (#S7700). DNA was isolated from each of the samples and manipulated according to the TRAPeze kit instructions. 10 ng of protein extract were used per reaction and positive controls were included with the gel. The tubes were first incubated for 30 min at 30°C and then a 2-step PCR (94°C for 30 sec, 59°C for 30 sec at 30 cycles) was performed following the PCR reaction. The fragments were separated on a 6% denaturing polyacrylamide gel electorphorisis (600V, 45 min). The gel was scanned using an Alpha Innotech image acquisition and an analysis system then assessed the telomerase activity. Activity was calculated as a ratio of the intensity of the telomerase ladder to the intensity of the 36bp standard.

RESULTS AND DISCUSSION

Production of the Dominant-negative UP1

Production of mRNA and cDNA

The mRNA was successfully extracted and purified from porcine fetal fibroblast cells and cDNA was produced without difficulty. An adequate amount of cDNA was obtained through the original RT-PCR reaction so that further amplification was not necessary.

Primer Design

An important step in the research design was the creation of the UP1 primers. Because it was only necessary to amplify a segment of the DNA in order to achieve the over-expression of UP1, two primers were designed and introduced into the PCR reaction. Three specific enzyme cut sites were incorporated into the primers (Fig. 8). By incorporating these sites, potential problems were eliminated in succeeding steps of the methodology. The Fse I enzyme (Promega) site created a system through which UP1 expression could be verified in the transformed bacterial colonies. Likewise, the incorporation of Nco I and Not I (Promega) cut sites on pUP1 and pUP2, respectively, ensured that the UP1 fragment would orient correctly into the pShooter expression vector. The primer design created through the comparison of human and mouse DNA was adequate and the UP1 fragment was effectively isolated and amplified from the cDNA template.

pUP1: **GGCCGG/CCATGG**TGACGCCGCCGAAGAAGCATCG
- Fse I and Nco I sites (respectively)

pUP2: CCGGCC**GCGGCCGC**CCACTGCGACCTCTTTGGCTGGATGA
- Not I site

FIG. 8. Sequences of UP1 Primers and the Incorporated Enzyme Sites.

Polymerase Chain Reaction

The PCR reaction combined the designed primers and the porcine cDNA. Production of the UP1 fragment was confirmed through electrophoresis. The reaction was run on a 1% gel and a band between 500 and 600 bps was identified.

pCR® 2.1-Topo Vector

The plasmid vector Topo 2.1 was chosen for several reasons. Its replication is unidirectional, it has a smaller size that allows it to transform at higher frequencies than a larger plasmid, and it is encoded with selective antibiotic resistance. To confirm the successful ligation of the UP1 segment into the 2.1-Topo vector, the DNA extracted from the selected positive colonies was cut with Fse I enzyme (Promega). The samples were run as uncut and cut, side by side on the same gel in order to facilitate a simple comparison (Fig. 9). From these results, three colonies were selected for transformation.

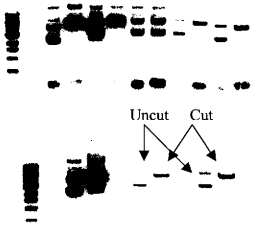

FIG. 9. Topo 2.1/DNA Samples Run On a Gel.
The two samples indicated with arrows are positive for the vector and UP1 insert.

The transformation of the DNA into E. coli cells resulted in numerous white colonies (white colonies are indicative of a positive transformation due to the X-gal supplemented agar plates). There was no visible contamination and the transformed colonies grew quickly.

The pShooter Expression Vector

Isolation and Purification of the UP1 Sequence

Pshooter (pCMV/myc/nuc) is a nuclear localization vector that signals the transformed cells to shuttle the protein being expressed (UP1) to the cells' nuclei. In addition, the pShooter vector encodes for neomycin (G418) and ampicillin antibiotic resistance. The UP1 fragment was successfully excised from the 2.1-Topo vector and purified with the Qiagen Gel Extraction Kit (Qiagen, #28704).

Cloning of UP1 into pShooter Vector

The UP1 primer design ensured the correct orientation of the UP1 fragment into the pShooter vector. Because the two separate enzyme sites (located on opposite ends of the UP1 sequence) only match in one direction with the pShooter sites, the possibility of incorrect orientation was eliminated. UP1 was cloned effectively into the pShooter expression vector and transformed into competent E. coli cells following the Promega protocol for transformation. The transformation resulted in multiple positive bacterial colonies.

Electroporation

Digestion and DNA Preparation for Electroporation

The pShooter/UP1 DNA was harvested from the positive colonies and purified. Portions of the samples were then run through the spectrometer and their concentration was recorded (3.3 μg/ml, 1.79 $^{260}/_{280}$). The results indicated the amount of DNA needed for the electroporation.

The DNA was then linearized by the Sca I enzyme. There is one Sca I cut site located on the pShooter vector at base 4535. The digested DNA was run on a 1% agarose gel. A band at 5.5 kb was located that confirmed the sample consisted of the pShooter vector and the UP1 fragment and was in fact linear (data not shown). The linearization of DNA prior to electroporation is important as it increases the efficiency of the transformation.

Electroporation and Colony Selection

Upon linearization of the DNA, the cells were electroporated into 10 million porcine fetal fibroblast cells and plated at about $6.7x10^5$ cells per plate. Prior to electroporation, wild-type cells were grown in culture and exposed to different concentrations of G418 in order to achieve the ideal antibiotic concentration for selection of the electroporated cells (kill curve). The cells were plated at a density of $1.0x10^5$ cells per well of a 6-well plate and observed over fourteen days. Additional cells were plated in 100mm plates at the concentrations of 1,000 μg/ml and 1,100 μg/ml and observed for the same amount of time. The concentrations 700 μg/ml was identified as the ideal for a 6-well plate, and 1,000 μg/ml was identified for a 100mm plate. The electroporated cells were then subjected to these concentrations.

From the ten colonies originally marked, seven were moved to a 24-well plate after two days by the protocol described in the methodology section. From these seven colonies, five were transferred to a 6-well plate after an additional five days. All five of these colonies were selected for the population doubling experiment and were eventually expanded to three 100mm plates each.

Population Doublings

Population Doubling Factor

Over a 300% increase in population doubling potential was seen in the porcine fetal fibroblast cells electroporated with the UP1 insert (Fig.10). The control cells reached senescence around thirty pds, a characteristic distinctive of the wild-type somatic cell life span. In contrast, the sample groups electroporated with the UP1 insert achieved a higher number of pds in the same amount of time and generally did not express a decreased rate of division. Therefore, these results indicate cellular life extension of somatic cells expressing UP1 *in vitro*.

A comparison of the growth rates amongst the cell groups reaffirmed a significant difference in life span between the control and UP1 cells. Illustrated by the graphical data (Fig. 10), the growth rates between the control cells and the UP1-expressing cells match closely days 4 – 48 (indicated by comparison of the slopes of the separate lines). Around day forty-eight the control cells begin to display signs corresponding to the senescent phenotype as they diverged from the initial growth rate (forty-eight days is equivalent to 25 pds). From day 48 on, the data plot representing the control's growth curve begins to level out as the rate of growth decreases. This is indicated by the control graph exhibiting a diminishing slope after day 48.

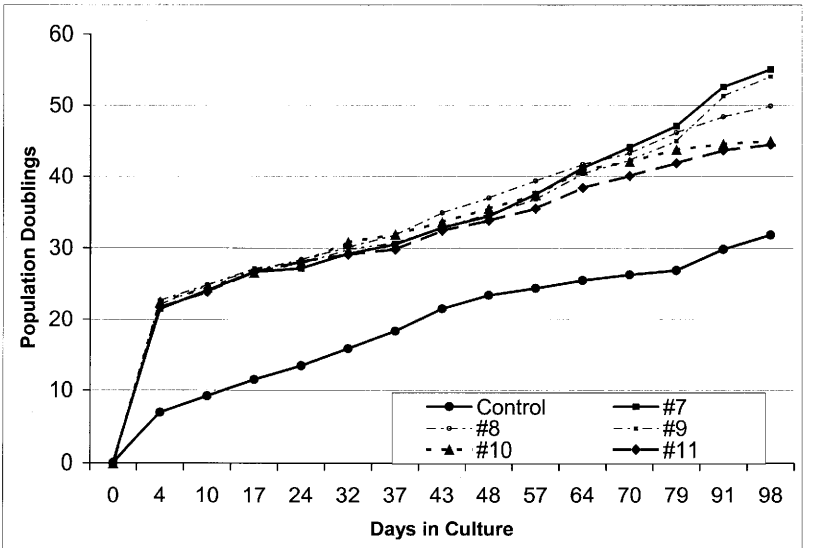

FIG. 10. PDF Data

In contrast to the control set, UP1-expressing cells exhibit a stabilized growth rate, characteristic of the initial 48 days in culture, until the PDF experiment was ceased. Between the UP1 samples themselves there was some variability present as groups #10 and #11 appear to approach senescence more rapidly than the others. While #10 and #11 did move toward a senescent phenotype, it is important to note that at the time their growth rate slows, the colonies are well above the standard number of pds typical of wild-type somatic cells *in vitro*. The variation observed between the UP1 over-expressing cells could be due to the location of the UP1 insert in the porcine cells' DNA. This positional effect could be caused by several different factors including the number and location of promoters in the DNA.

UP1 Expression

DNA samples of each of the five UP1 expressing colonies were extracted and mRNA was prepared. The mRNA was manipulated through reverse transcription and the resulting cDNA was analyzed through PCR reactions. The data produced from these reactions concluded that UP1 was being expressed to a higher level in the cloned cells (Fig. 11).

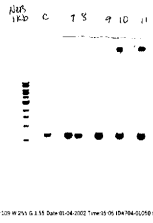

FIG. 11. UP1 Expression
Higher expression is seen in samples 7-11 as compared to the control (labeled C).

Telomere Repeat Amplification Protocol

No telomerase activity was found in the control cells or the UP1 expressing cells (Fig. 12). The implications from this data confirm that the extended cell life shown in the UP1 cells is not the result of telomerase activity, and therefore attributable to some other factor.

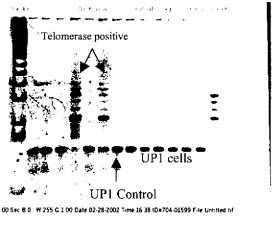

FIG. 12. Telomerase Expression

CONCLUSIONS

It has previously been shown that the heterogeneous nuclear ribonucleoprotein A1 participates in telomere biogenesis (LaBranche et al., 1998). Furthermore, it has been indicated that UP1, the dominant-negative domain of hnRNP A1, binds to telomeric DNA and provides protection against the activity of endo- and exonucleases (Dallaire et al., 2000). This research project investigates the effects of UP1, the dominant-negative derivative of hnRNP A1, on porcine fetal fibroblasts *in vitro*.

The cells expressing the UP1 derivative exhibited a definite increase in cellular lifespan. However, without further testing, it cannot be definitively concluded that UP1 over-expression is responsible for this abnormal phenotype. A karyotyping assay is necessary to examine the chromosome stability and identify if the extended growth is due to abnormalities within the chromosome as opposed to an affect of the UP1 expression. Karyotyping results indicating normal chromosomes would suggest UP1 had some affect on the extended life of the porcine cells in culture.

If normal karyotyping results did occur, telomere restriction fragment analysis could be conducted. This assay would measure the length of telomeres throughout the stages of the experiment using the cells frozen at each passage. The results from this experiment could provide information involving whether the elongation of the telomere or the maintenance of telomeric structure is more important in the extension of cellular lifespan.

The results from the PDF trials did indicate cellular life extension in somatic cells expressing UP1. Further tests examining cellular karyotype and telomere length must be explored before any definitive conclusions can be drawn; however, the PDF experimental data yielded encouraging results as to the prospect of somatic cellular life extension *in vitro*.

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