TELOMERE POSITION EFFECT IN HUMAN CELLS

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DEDICATION

Dedicated to my parents, Daniel and Catherine Baur, and to Amelia for their constant support and encouragement.

TELOMERE POSITION EFFECT IN HUMAN CELLS

by

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DISSERTATION

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ABSTRACT

Telomeres are tracts of repetitive DNA that cap the ends of linear chromosomes. Each time the chromosome is duplicated, a small amount of telomeric DNA is lost from the end due to factors inherent in the mechanism of DNA replication. The result is a net shortening of telomeres with each cell division, unless new repeats are synthesized through the action of the enzyme telomerase. Most human somatic cells lack telomerase activity and so continued cell division leads to telomere shortening. After a limited number of divisions (the "Hayflick limit"), it is believed that a few critically shortened telomeres trigger a state of growth arrest termed replicative senescence.

Genes near telomeres in yeast and other lower organisms have been shown to be reversibly repressed, resulting in a variegated (mosaic) phenotype. This silencing has been termed telomere position effect, or TPE. Because human telomeres shorten during cell division, a similar effect in human cells could potentially be regulated by the age of the cell.

In the present work, telomere position effect was demonstrated in human cells by comparing the expression of a luciferase reporter integrated either next to a telomere or at an internal site. Despite the expected high variability within each group, a ten-fold decrease in average luciferase activity was shown for the telomeric clones. Silencing was relieved by treatment with a histone deacetylase inhibitor or BrdU, indicting that reduced expression was not due to alterations in the gene itself. Elongation of telomeres by telomerase resulted in a two to ten-fold increase in silencing specifically in telomeric clones. When a fluorescent reporter was used, TPE in human cells produced a variegated phenotype, and spontaneous reactivation of the transgene could be detected in non-expressing subclones. A screen of candidate proteins identified hRap1 as a potential mediator of this effect. No effect of telomere length was detected on the expression of several endogenous subtelomeric genes. However, few candidates are currently available since knowledge concerning the detailed structure of most chromosome ends is limited at present. A more detailed analysis of subtelomeric gene expression will be an important future step since relief of silencing in these regions has the potential to play an important role in human aging.

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LIST OF ABBREVIATIONS

5-FOA – 5-Fluoro-Orotic Acid

ALT – Alternative Lengthening of Telomeres

bp – Base Pair(s)

BrdU – 5-Bromodeoxyuridine

cDNA – Complementary DNA

CMV – Cytomegalovirus

DAPI – 4',6-Diamidino-2-Phenylindole

DNA – Deoxyribonucleic Acid

DNA-PK – DNA-Dependent Protein Kinase

Dox – Doxycycline (Tetracycline Analog)

DsRed2 – *Discosoma sp*. Red Fluorescent Protein

dsRNA – Double-Stranded RNA

EGFP – Enhanced Green Fluorescent Protein

HDAC – Histone Deacetylase

HM – Mating Type Loci

hnRNP – Heterogeneous Nuclear Ribonucleoprotein

HSV-tk – Herpes Simplex Virus Thymidine Kinase

hTERT – Human Telomerase Reverse Transcriptase (Protein Component)

hTR – Human Telomerase RNA (Component)

kb – Kilobase Pair(s)

Mb – Megabase Pair(s)

mRNA – Messenger RNA

NAD – Nicotinamide Adenine Dinucleotide

OR – Olfactory Receptor

PARP – Poly ADP-Ribose Polymerase

PCR – Polymerase Chain Reaction

PEV – Position-Effect Variegation

rDNA – Ribosomal DNA

RNA – Ribonucleic Acid

RNAi – RNA Interference

RNP – Ribonucleoprotein

RTPCR – Reverse Transcription PCR

SIR – Silent Information Regulator

siRNA – Short Interfering RNA

snoRNA – Small Nucleolar RNA

snoRNP – Small Nucleolar Ribonucleoprotein

SV40 – Simian Virus 40

tet - Tetracycline

TPE – Telomere Position Effect

TSA – Trichostatin A

VSG – Variable Surface Glycoprotein

YAC – Yeast Artificial Chromosome

CHAPTER ONE
Introduction and Literature Review

TELOMERES AND TELOMERASE

The telomere is the structure that protects, or "caps", the end of a chromosome, derived from the Greek telos (end) and meres (part). Hermann J Muller in 1938 (Muller 1938) and Barbara McClintock in 1941 (McClintock 1941) first showed that these naturally occurring DNA ends are very different from those produced by breaks in the chromosome. While free ends resulting from DNA damage frequently fuse together, resulting in genomic rearrangements and further damage to the DNA, chromosome ends with intact telomeres remain protected (Figure 1.1).

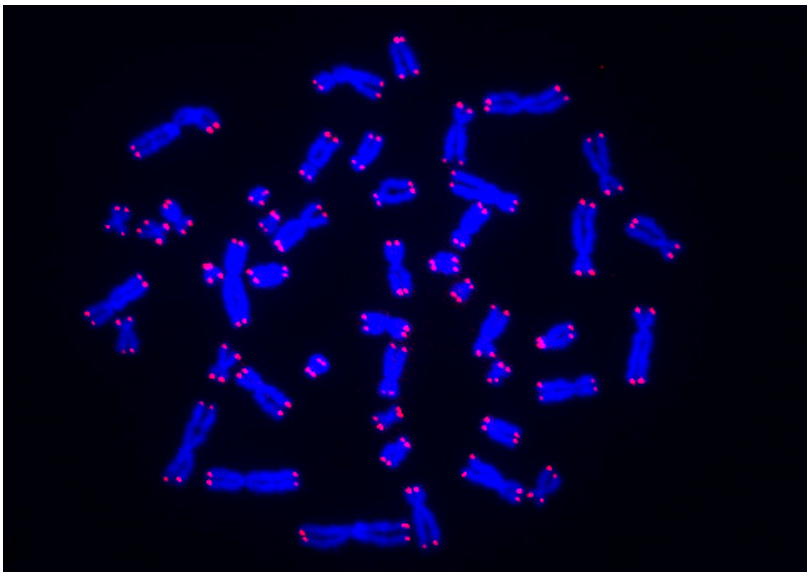

Figure 1.1 – Human chromosomes arrested in the metaphase stage of mitosis. Chromosomes were visualized using the general DNA stain DAPI (blue) and a fluorescently labeled $(CCCTAA)_3$ probe (Cy5, shown in pink), which was hybridized to telomeres. Four spots are visible because the chromosomes have been replicated but not yet separated into daughter cells. (Courtesy of Ying Zou)

Telomeres received little attention during the following three decades, but during that time a series of independent events began to unfold that set the stage for the return of the telomere to the forefront of modern science. On April 2, 1953, James Watson and Francis Crick published the structure of deoxyribonucleic acid (DNA) (Watson and Crick 1953). In 1961, Leonard Hayflick and Paul Moorhead made the discovery that human cells grown in tissue culture can divide only a limited number of times (the "Hayflick limit"), after which they enter a non-dividing state termed replicative senescence (Hayflick and Moorhead 1961). As work on the mechanisms of DNA replication continued, it became apparent that a linear molecule (such as a human chromosome) cannot be completely replicated, and instead loses a small amount of DNA from each end every time it is copied. This is often referred to as the

"end-replication problem" and is discussed in more detail below. In 1971, Alexey Olovnikov, a Russian theoretical scientist, proposed that incomplete replication of human chromosomes, due to the end-replication problem, could form the molecular basis for the Hayflick limit (Olovnikov 1971). During each round of DNA replication (i.e. each cell division), he suggested, a small amount of DNA would be lost from the telomere at the end of each chromosome. When the telomeres became too short to function properly, the cell would stop dividing. This became known as the "telomere hypothesis" of replicative aging (or by extension, of aging in general). Olovnikov's original publication was in Russian and so it is often eclipsed by Watson's independent 1972 proposal predicting the end-replication problem in T7 phage (Watson 1972) and by his own 1973 paper, published in English (Olovnikov 1973). Interest in this theory languished at first because the biological knowledge and tools needed to test it did not exist. By the early 1980's, that was beginning to change.

Working in the single-celled protozoan *Tetrahymena thermophila*, Elizabeth Blackburn, then a postdoctoral fellow working in the lab of Joseph Gall, determined that the telomeres consisted of many repeats of the DNA sequence TTGGGG (Blackburn and Gall 1978). Because *Tetrahymena* can divide indefinitely, Blackburn reasoned, they must have a way to prevent the loss of DNA from telomeres that would occur because of the end replication problem. In 1985, Blackburn and her student Carol Greider successfully demonstrated the existence of a protein (or more correctly an RNA/protein complex called a ribonucleoprotein) from *Tetrahymena* that was capable of adding new repeats onto the ends of telomeres (Greider and Blackburn 1987), which they initially called telomere terminal

transferase and later telomerase (Greider and Blackburn 1987). Loss of telomeric DNA during replication, they discovered, was counteracted by the addition of new DNA by telomerase, thereby preventing any shortening of *Tetrahymena* telomeres over multiple rounds of cell division.

In the late 1980s, work on human telomeres began with Cooke and Smith's finding that different human tissues have telomeres of different lengths (Cooke and Smith 1986) and Moyzis's publication in 1988 that human (and other mammalian) telomeres are composed of hundreds of repeats of the sequence TTAGGG (Moyzis *et al.* 1988). Unlike normal human cells, cancer cells are immortal (can divide indefinitely) and so must, like *Tetrahymena*, possess a means to counteract the end-replication problem. Following this reasoning, Gregg Morin in 1989 identified human telomerase activity in extracts from a tumor cell line (HeLa) (Morin 1989). The next year, Harley, Futcher, and Greider demonstrated that telomerase is absent in normal human cells and that telomeres in these cells shorten with each division (Harley *et al.* 1990). These findings proved that the telomere shortening predicted by the end-replication problem was real and lent substantial support to the telomere hypothesis as an explanation for replicative aging. However, proof was still lacking that shortened telomeres truly represented the cause of replicative senescence as opposed to merely correlating with it.

The importance of telomerase in cancer was underscored by the finding of Kim et al in 1994 that telomerase could be detected in over 90% of human tumors (Kim *et al.* 1994). Interest in the field grew rapidly as the simple and sensitive PCR-based assay described in this paper helped telomere biology to spread from its roots in DNA replication and aging into the enormous field of cancer research. The RNA component of human telomerase was

cloned by Feng *et al* (Feng *et al.* 1995) in 1995 and the catalytic protein subunit was cloned by both Nakamura *et al* (Nakamura *et al.* 1997) and Harrington *et al* in 1997 (Harrington *et al.* 1997b). Nakamura *et al* also showed that the telomerase integral RNA is expressed in all cells while expression of the telomerase protein component tightly correlated with telomerase activity. This led to the hypothesis, tested by Weinrich *et al* in the same year, that normal telomerase negative human cells could be made telomerase positive simply by expressing the catalytic subunit (called hTERT for human telomerase reverse transcriptase) (Weinrich *et al.* 1997).

At the same time, Woodring Wright and Jerry Shay were working to firmly establish telomere shortening as the cause of replicative senescence. In a 1996 report, they showed that when different cell types were fused together, the replicative lifespan of the hybrid was limited by the shorter telomeres (Wright *et al.* 1996a). While these experiments strengthened the link between telomere length and senescence, the highly unnatural setting of a fused hybrid cell line limited the conclusions that could be drawn. Their discovery the following year that telomerase activity could be restored in normal human cells simply by expressing hTERT (Weinrich *et al.* 1997) provided all the tools necessary for a final, definitive test of the telomere hypothesis.

Shay and Wright "telomerized" normal human cells by expressing hTERT. After careful examination to insure that telomerase activity could be detected in the cells and that telomeres were in fact getting longer, they grew two populations differing only by the expression of the hTERT protein out to the Hayflick limit. In a landmark 1998 paper, they showed that while the control cells underwent normal replicative senescence at the Hayflick

limit, the telomerized cells continued to grow indefinitely, exceeding their original lifespan by many times (Bodnar *et al.* 1998). With the completion of this experiment, Olovnikov's 25-year-old telomere hypothesis became reality, at least at the cellular level.

It has been shown that almost all normal human cells, like the ones studied by Shay and Wright, lack telomerase activity [with the exception of germ line cells (Wright *et al.* 1996b), stem cells (Engelhardt *et al.* 1997), and some cells of the immune system (Broccoli *et al.* 1995)] and will undergo replicative senescence when the Hayflick limit has been reached [reviewed in (Shay and Bacchetti 1997)]. Most cancer cells, in contrast, escape replicative senescence through expression of hTERT. These observations have lent support to the prevalent theory in the field as to why normal human tissues do not express telomerase. The best evidence available suggests that evolution may have favored a situation in which most of the cells in a human body lack telomerase as a first line of defense against cancer (Wright and Shay 2001). A cell that is in the process of accumulating all of the mutations necessary to form a life-threatening tumor is thought to reach the Hayflick limit before that occurs unless it is additionally able to activate expression of the hTERT gene.

Issues like those presented here are never as simple as they first appear. It should be pointed out that some human tumors have been shown to escape replicative senescence without detectable expression of telomerase (Murnane *et al.* 1994; Bryan *et al.* 1997). The process of maintaining telomeres in these cells is termed alternative lengthening of telomeres, or ALT (Bryan and Reddel 1997), and seems to involve recombination [reviewed in (Henson *et al.* 2002)]. Potential therapies involving the inhibition or activation of telomerase have spawned an ongoing debate over the exact relationship between telomeres,

telomerase, and cancer. While cells expressing telomerase have one less roadblock to tumor formation (Hahn *et al.* 1999), it has also been argued that most of the mutations that lead to cancer occur during genomic rearrangements that can occur when telomeres are short, which would make telomerase expression potentially protective (Harley 2002). There have also been recent claims that telomerase can be detected sporadically in some normal human cells (Hahn 2002). And of course, the most controversial issue of all remains; does replicative aging at the cellular level play a role in organismal aging?

THE END-REPLICATION PROBLEM

In order to understand the end-replication problem, it is first necessary to understand a few basic principles of DNA structure and replication. A linear piece of DNA, such as a human chromosome, contains two strands in opposite orientation. Because DNA can only be synthesized in a 5' to 3' direction, the replication of one strand must be discontinuous (Figure 1.2).

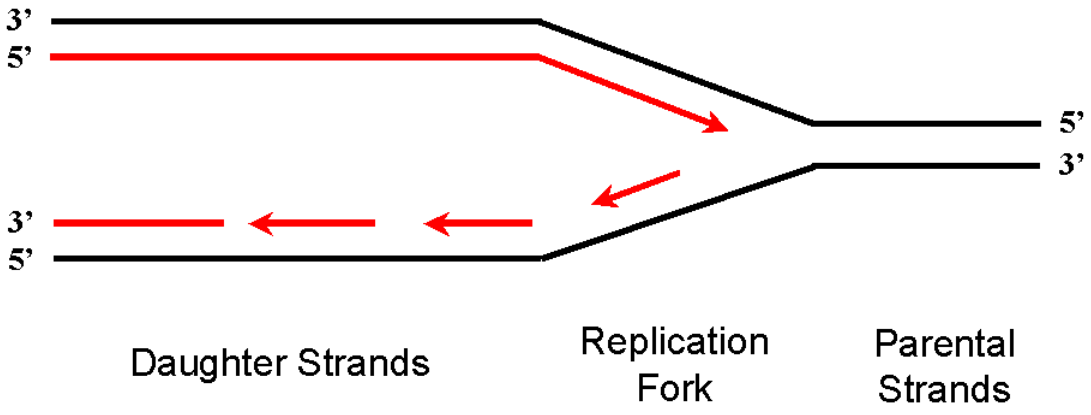

Figure 1.2 – DNA Replication. As the parental DNA strands are unwound at the replication fork, leading (upper) strand synthesis is continuous while lagging (lower) strand synthesis takes place in a series of short sections called Okazaki fragments.

These short discontinuous stretches of DNA used to make up the "lagging" strand are called Okazaki fragments. Each is begun with an RNA primer (which is subsequently degraded) and becomes ligated to the growing strand when it has extended back to the site of the previous fragment. This system works well to generate two complete copies from one parental DNA molecule, except at the end of the lagging strand. The "leading" strand poses no problem since DNA is synthesized towards the end. However, there is no way to fill in the space transiently occupied by the final RNA primer on the lagging strand, or the remaining distance (if any) to the end of the DNA molecule. The result is a net shortening of the DNA molecule over multiple rounds of replication (as well as the accumulation of single-stranded "overhangs" on the ends generated from lagging strands). This is known as the end-replication problem (Figure 1.3).

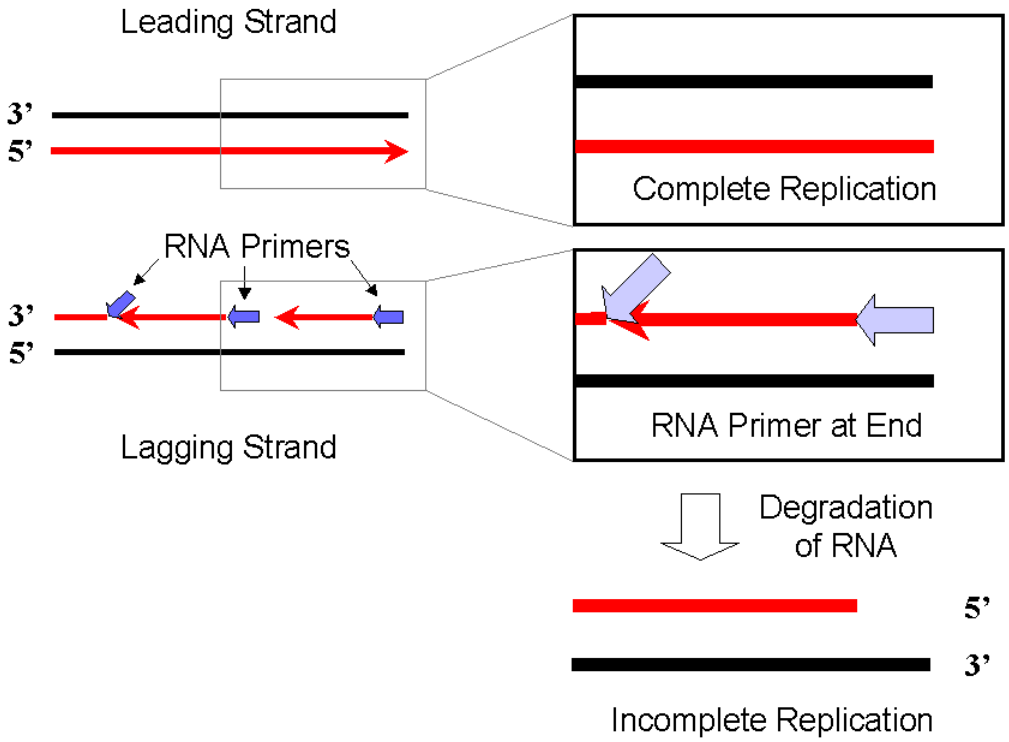

Figure 1.3 – The End-Replication Problem. During lagging strand synthesis, each Okazaki fragment starts at the site of an RNA primer. Normally, the RNA primer is degraded and extension of the next Okazaki fragment fills in the gap with DNA. At the end of the strand however, there is no next Okazaki fragment and so degradation of the RNA primer leaves an unreplicated region, resulting in a net loss of DNA from the end of a linear molecule during each round of replication. Note that the situation pictured here is the "best case scenario" where the final RNA primer is situated exactly at the end of the molecule. Single-stranded overhangs in human cells are larger than the RNA primers, indicating that placement may be random, or there may be an active process that further degrades the DNA.

A CURRENT VIEW OF THE HUMAN TELOMERE

The telomeres at the end of human chromosomes are composed of 2-15 kb of the repeated sequence TTAGGG, depending on the replicative age of the cell, the donor, and the tissue type (de Lange *et al.* 1990; Martens *et al.* 1998). Recently, a detailed analysis has shown that the length can drop to nearly zero for individual telomeres within a population of senescent cells (Baird *et al.* 2003). The end of the chromosome is not blunt (i.e. both strands do not end at the same point). Instead, the G-rich strand of the telomere extends ~125-275 bases past the complimentary C-rich strand to create a 3' single-stranded overhang (Makarov *et al.* 1997; Wright *et al.* 1997). Overhangs occur at both ends of the chromosome (Makarov *et al.* 1997), suggesting an active degradation of the C-rich strand in at least some cases, since the end-replication problem alone would leave half of the ends blunt (Figure 1.3). The average length of the overhang in different cell types correlates directly with the rate of telomere shortening (Huffman *et al.* 2000), and individual ends may have overhangs from 24 to over 400 nucleotides long (Cimino-Reale *et al.* 2001). By electron microscopy, human chromosomes have been observed to end in a loop structure, termed a t-loop (for telomere loop), suggesting that the single-stranded overhang may invade the double-stranded region of the telomere (Greider 1999; Griffith *et al.* 1999). Loop size is highly variable, ranging from several kb to including nearly the entire telomere.

The duplex region of human telomeres is bound directly by the related proteins TRF1 (TTAGGG Repeat binding Factor) (Chong *et al.* 1995) and TRF2 (Broccoli *et al.* 1997), while the single-stranded overhang is bound by several hnRNPs (heterogeneous nuclear

Ribonucleoproteins) (McKay and Cooke 1992; Ishikawa *et al.* 1993) and the more recently discovered hPot1 (Protection of telomeres) (Baumann and Cech 2001). TRF1 also exists in a second form, known as Pin2, which is derived by alternate splicing (Shen *et al.* 1997). TRF1 is bound both by the novel protein TIN2 (Kim *et al.* 1999) and by the two PARP (Poly ADP-Ribose Polymerase) enzymes tankyrase (Smith *et al.* 1998b) and tankyrase 2 (Kaminker *et al.* 2001; Kuimov *et al.* 2001; Lyons *et al.* 2001; Monz *et al.* 2001), while TRF2 is bound by hRap1 (Li *et al.* 2000). Most of these proteins seem to have direct roles in protection of the chromosome end from recognition as damaged DNA [reviewed in (de Lange 2002)] and/or telomere length regulation in telomerase positive cells (LaBranche *et al.* 1998; Kim *et al.* 1999; Li *et al.* 2000; Smith and de Lange 2000; Smogorzewska *et al.* 2000). A wide variety of other proteins, especially DNA repair factors, have also been localized to telomeres. These include the DNA-dependent protein kinase (DNA-PK), thought to be recruited by interaction of the TRF proteins with its Ku70 subunit (Hsu *et al.* 1999; Hsu *et al.* 2000; Song *et al.* 2000), and the RAD50/MRE11/NBS1 complex, which has been shown to colocalize with telomeres and TRF1 (Lombard and Guarente 2000; Wu *et al.* 2000) and to associate specifically with TRF2 (Zhu *et al.* 2000).

THE TELOMERASE RNP COMPLEX

Human telomerase contains two core components, the hTERT protein (catalytic component) and an RNA component termed hTR (human Telomerase RNA), which together are sufficient for reconstitution of the active enzyme in rabbit reticulocyte lysates (Weinrich *et al.* 1997). Since hTR is expressed in most telomerase-negative human cells, hTERT is normally the only component necessary to render cells telomerase-positive (Weinrich *et al.* 1997). The assembled telomerase ribonucleoprotein (RNP), however, is more complex than this suggests and contains many other structural and regulatory proteins supplied by the reticulocyte lysates or already present in normal cells [reviewed in (Cong *et al.* 2002)].

The hTERT protein contains a phylogenetically conserved reverse transcriptase motif, a telomerase-specific region (T motif), and a large N-terminal region containing functionally important domains (Nakamura *et al.* 1997; Xia *et al.* 2000; Armbruster *et al.* 2001; Moriarty *et al.* 2002) [reviewed in (Kelleher *et al.* 2002)]. The RNA component (hTR) provides the template for synthesis of telomeric repeats by reverse transcription (Feng *et al.* 1995). Secondary structure is highly conserved although primary sequence is not (Chen *et al.* 2000). At least two regions within hTR bind the catalytic subunit in an independent manner (Mitchell and Collins 2000), and several lines of evidence suggest that the functional unit of telomerase is a dimer (of both components) (Beattie *et al.* 2001; Wenz *et al.* 2001; Moriarty *et al.* 2002).

The chaperones p23 and hsp90 were shown to be associated with hTERT *in vitro* and *in vivo* and subsequently to be required for assembly of functional telomerase (Holt *et al.*

1999). These proteins remain stably associated with active telomerase and have been hypothesized to play a role in translocation of the complex after synthesis of each telomere repeat (Forsythe *et al.* 2001). The hTERT protein has also been shown to interact with 14-3-3 proteins, which are thought to help recruit telomerase to the nucleus (Seimiya *et al.* 2000). Disruption of this interaction results in the accumulation of hTERT within the cytoplasm. The first protein shown to interact with hTERT was TEP1 (Harrington *et al.* 1997a; Nakayama *et al.* 1997), a component of large cytoplasmic ribonucleoprotein complexes termed vaults (Kickhoefer *et al.* 1999), but the functional significance of this interaction remains unknown.

The telomerase RNA in humans (Mitchell *et al.* 1999a) and other mammals (Chen *et al.* 2000), but not in yeast (Seto *et al.* 1999) or ciliates (Collins 1999) contains a 3' region that structurally resembles a class of snoRNAs (small nucleolar RNAs) termed H/ACA (based on sequence elements). There are four common proteins that associate with snoRNAs in snoRNPs (small nucleolar Ribonucleoprotein particles) and all (hGAR1, dyskerin/NAP57, hNOP10 and hNHP2) associate with hTR (Mitchell *et al.* 1999b; Dragon *et al.* 2000; Pogacic *et al.* 2000; Dez *et al.* 2001). The hnRNPs (heterogeneous nuclear Ribonucleoproteins) A1, C1, and C2 also bind to hTR and seem to play a role in recruiting telomerase to telomeres (LaBranche *et al.* 1998; Ford *et al.* 2000; Fiset and Chabot 2001). The La antigen specifically associates with hTR, and its overexpression causes telomere shortening (Ford *et al.* 2001). L22 and hStau have also been identified as hTR-binding proteins and may play some role in processing or in the assembly or localization of telomerase (Le *et al.* 2000). A

thorough review of the current knowledge concerning telomerase regulation was recently published by Cong *et al* (Cong *et al.* 2002).

POSITION-EFFECT VARIEGATION

The discovery that the position of a gene within the genome could affect its transcription was made in 1925 by Alfred Sturtevant who showed that Drosophila with an equal number of copies of the Bar locus distributed differently among chromosomes show distinguishable phenotypes (Sturtevant 1925). In 1926, Milislav Demerec isolated two *Drosophila* strains with unstable mosaic phenotypes (Demerec 1926b; Demerec 1926a). Hermann J Muller showed in 1930 that a similar phenomenon could be induced in *Drosophila* by irradiation, that the occurrence of a "variegated" (mosaic) phenotype correlated with the occurrence of chromosome rearrangements, and that multiple genes on the same chromosome were often affected (Muller 1930). This suggested that repositioning of the affected genes, as opposed to mutation, was responsible for creating the variegated phenotypes. Historically, position effects such as the one described by Sturtevant have been referred to as S-type (stable) and have been reported in relatively rare cases while those observed by Demerec and Muller have been referred to as V-type (variegated), and have made up the majority of position effects subsequently discovered (Lewis 1950). Jack Shultz later showed that variegated expression typically occurs after a genomic rearrangement that brings the affected gene in close proximity to the pericentric heterochromatin (Schultz 1936). Position-effect variegation

(PEV) was therefore defined as the variable, but heritable inhibition of euchromatic gene activity when artificially juxtaposed with heterochromatin by chromosome rearrangement [reviewed in (Spofford 1976; Henikoff 1992; Karpen 1994)]. Particularly striking were cases in which the genes for eye pigments were affected. PEV in these genes could result in eyes that were wild type, mutant, or intermediate with speckles or blotches of a second color throughout. It was additionally observed that genes closer to the junction between the translocated DNA and heterochromatin had a higher frequency of silencing. In cases where a more distal gene was silenced, genes proximal to the breakpoint were never found to be expressed (Demerec and Slizynska 1937; Schultz 1939). This led to the proposal of a model wherein inactivating factors "spread" from the heterochromatin into the translocated chromosome fragment.

In addition to *Drosophila* PEV and to the telomeric position effects (TPE) discussed in the following sections, there have been reports of position effects in plants (Catcheside 1947), mice (Russell and Bangham 1959; Cattanach 1974; Butner and Lo 1986; al-Shawi *et al.* 1990), mosquitoes (Benedict *et al.* 2000), trypanosomes (Kohler 1999), bacteria (Clugston and Jessop 1991), at the centromeres of fission yeast (Allshire *et al.* 1994), at the ribosomal DNA (rDNA) (Smith and Boeke 1997) and mating type loci of budding yeast (Brand *et al.* 1985; Schnell and Rine 1986), and in human cell culture (Kalos and Fournier 1995; Walters *et al.* 1995; Walters *et al.* 1996) and genetic disease (Barbour *et al.* 2000; Gabellini *et al.* 2002) [reviewed in (Kleinjan and van Heyningen 1998)].

TELOMERE POSITION EFFECT IN *DROSOPHILA*

In 1984, two variegated *Drosophila* lines were described in which the affected gene appeared to be located near the end of a chromosome (Gehring *et al.* 1984; Hazelrigg *et al.* 1984). Although position effects had not previously been described near telomeres, this formed an attractive hypothesis because of the heterochromatic morphology that had been described near telomeres (Beermann 1962) and the observation from the previous year that repetitive sequences near telomeres and centromeres in *Drosophila* share some sequence homology (Young *et al.* 1983). In 1985, Levis *et al* successfully translocated the gene to a new location, restoring the wild type phenotype (Levis *et al.* 1985). Because this proved the variegated phenotype was not the result of a mutation in the gene, this was the experiment that formally demonstrated that telomeres in *Drosophila* could exert a position effect on adjacent genes.

It was not immediately clear whether phenomena related to *Drosophila* telomeres should be extrapolated to other organisms, largely due to the unusual, possibly unique method used by *Drosophila melanogaster* to circumvent the end-replication problem. Instead of ending in repetitive sequences generated by a telomerase enzyme, their chromosomes end in a series of retrotransposons (Biessmann *et al.* 1992; Levis *et al.* 1993; Walter *et al.* 1995) [reviewed in (Mason and Biessmann 1995)]. New copies of the retrotransposons are generated by reverse transcription from RNA intermediates and added to the ends of telomeres. With the exception of a similar system recently discovered in *Drosophila yakuba* (Casacuberta and Pardue 2002), it is not yet clear whether this method of

telomere maintenance is conserved even within other *Drosophila* and related species [reviewed in (Biessmann and Mason 1997)]. In these organisms, it seems that subtelomeric repetitive sequences rather than the terminal retrotransposons may regulate the observed telomere position effects (Cryderman *et al.* 1999). The subtelomeric repeats on chromosomes II and III are related to each other and different than those on chromosome IV. While chromosome IV telomeres require HP1 (heterochromatin protein 1) for silencing and associate with pericentric heterochromatin, chromosome II and III telomeres do not require HP1 and generally localize to the nuclear periphery. It is somewhat ironic that both the discovery of telomere position effect and Muller's original discovery of the telomere itself (Muller 1938) have taken place in the one known organism whose telomere structure, maintenance, and regulation have proven so dramatically different from all others.

TELOMERE POSITION EFFECT IN *SACCHAROMYCES CEREVISIAE*

Silencing near telomeres of *Saccharomyces cerevisiae* (budding yeast) was first reported in 1990 by Daniel Gottschling, then a postdoctoral fellow in the lab of Virginia Zakian (Gottschling *et al.* 1990). He showed that four different reporter genes that could be expressed normally from internal loci became reversibly silenced when placed near yeast telomeres. Perhaps the most dramatic example was the *ADE2* gene, which results in the formation of normal white colonies when expressed, but causes the colonies to turn red when repressed (Roman 1956). Yeast bearing a telomeric copy of the *ADE2* gene formed colonies

of both colors and moreover, sectors of opposite color could plainly be observed within a single colony (Figure 1.4). A second marker he employed for this experiment was *URA3*. This gene is particularly useful because it can be selected for (on uracil deficient media) or against [on 5-fluoro-orotic acid (5-FOA) media] (Boeke *et al.* 1987). Gottschling's strains bearing a telomeric *URA3* were able to grow on either media, due to their variegated phenotype. He also used this finding to explain the "leakiness" another group had observed while trying to inhibit the growth of a *URA3* expressing strain using 5-FOA (Hegemann *et al.* 1988). In this strain, the gene had been placed 6-8 kb from a telomere. Subsequent work by the Gottschling lab and others has made the position effect at *Saccharomyces* telomeres probably the best-characterized example of PEV.

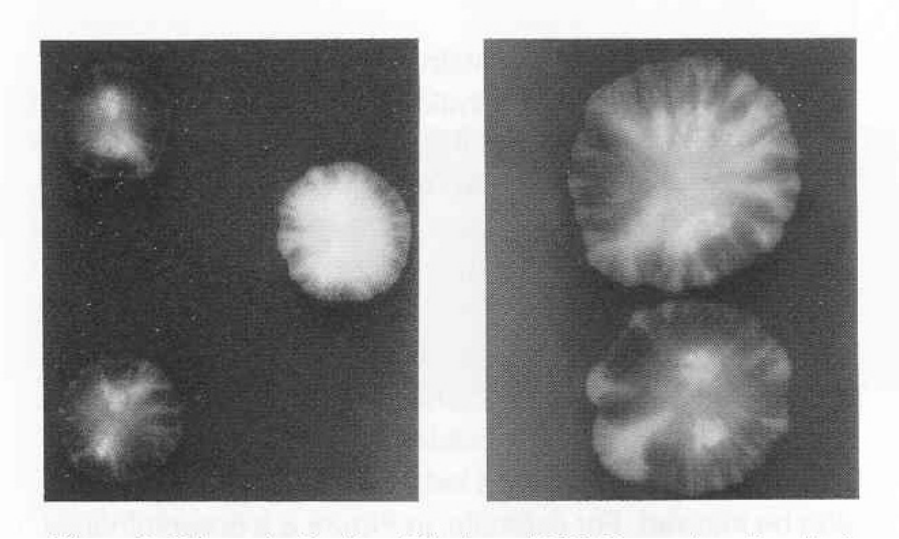

Figure 1.4 – The original image of a yeast strain bearing a telomeric copy of *ADE2*. In this image, red yeast appear as dark areas. The variegated phenotype of these yeast can easily be seen from the sectoring within individual colonies. [Reprinted from "Position Effect at S. cerevisiae Telomeres: Reversible Repression of Pol II Transcription" Cell **37**:869-78 (Gottschling *et al.* 1990) with permission from Elsevier.]

Yeast telomeres consist of approximately 350 base pairs (bp) of the repeated sequence TG_{1-3}. There are also two important classes of subtelomeric elements to consider, reviewed in (Louis 1995). The first, called Y', is found in up to four copies at about two thirds of the telomeres. The second, X, is found at almost every telomere. The effects of these subtelomeric elements on TPE are not always consistent (Fourel *et al.* 1999; Pryde and Louis 1999). Seemingly very similar elements at different telomeres can have variable effects on silencing. In some cases these elements seem to act as relays, propagating the silencing effect over large distances that can include actively transcribed regions. It has been suggested that reducing these elements to two families may be an over-simplification and that small differences between the elements on different telomeres may be functionally very significant (Tham and Zakian 2002). It is important to note that in most cases, yeast TPE has been studied in the absence of these subtelomeric elements due to the method used to place the reporter gene near a telomere. In these experiments, a linearized plasmid containing the gene of interest and a short stretch of telomeric DNA has been transfected into the cells. After selection, strains are chosen in which integration of the plasmid has caused the truncation of an endogenous chromosome. Subsequent elongation of the telomeric "seed" DNA contained in the plasmid by telomerase results in the formation of a new telomere immediately adjacent to the reporter gene (Gottschling *et al.* 1990).

The most striking feature of TPE in yeast is probably its semi-stable pattern of inheritance. Although the state of a telomeric gene (silenced or expressed) is normally conserved during cell division (budding), switching does occur about once every 15 to 20 doublings (Gottschling *et al.* 1990). This produces the characteristic sectored colonies

observed in strains with *ADE2* at a telomere. For this particular reporter gene, the probability of a given cell being in the "on" or the "off" state is about 50/50. However, the fraction of cells expressing a telomeric gene is very much dependent on both promoter strength and the distance (from the promoter) to the telomere (Renauld *et al.* 1993). Silencing is typically observed up to about 6 kilobases (kb) from the site of a chromosome truncation, or up to about 12 kb if that region contains a Y' element. By overexpressing proteins that help mediate TPE in combination with the presence of a Y' element, silencing has been observed up to 22 kb from the telomere. Telomere position effect can be overcome with a strong promoter (Aparicio and Gottschling 1994), and transcription blocks the spreading of silencing factors from the telomere (i.e. the effect is not propagated to the centromeric side of the transcribed region) in the absence of endogenous subtelomeric repeat elements (Renauld *et al.* 1993). Although yeast telomere length remains constant in a wild type context [reviewed in (Marcand *et al.* 1997b)], it has been possible through studies in mutant strains to show that the strength of silencing is proportional to telomere length (Kyrion *et al.* 1993; Buck and Shore 1995).

Quite a large number of yeast proteins have been shown to affect TPE. Most prominent among these are the Sir proteins (Silent information regulator) (Aparicio *et al.* 1991). While Sir1p does not appear to be involved in TPE, loss of Sir2p, Sir3p, or Sir4p almost completely eliminates telomere-based silencing. These three proteins form a complex that interacts with Rap1p, which itself binds to telomeric sequences directly. A mutant form of Rap1p lacking the portion that interacts with Sir proteins causes a loss of telomeric silencing (Kyrion *et al.* 1992; Kyrion *et al.* 1993). Sir2p is an NAD-dependent histone

deacetylase (Imai *et al.* 2000), and so may be directly responsible for silencing telomeric genes through deacetylation of histones H3 and H4 in the subtelomeres (Hecht *et al.* 1995). The Sir proteins are also involved in silencing at the two other major sites of repression in yeast, the silent mating type (*HM*) loci and ribosomal DNA (rDNA). In addition to increasing silencing at these loci, overexpression of Sir2p also increases yeast lifespan. A variety of processes linked to aging in yeast have now been shown to be mediated through Sir2p, and this has led to the suggestion that it might constitute a "master regulator" that controls the rate of aging in this organism (Guarente 2000).

Another essential set of factors for TPE in yeast are the *HDF*-encoded Ku proteins (Boulton and Jackson 1998; Laroche *et al.* 1998). Loss of these proteins abolishes silencing at the telomeres but not at the *HM* locus. Loss of Rif1p and Rif2p leads to an increase in telomeric silencing (Kyrion *et al.* 1993), apparently due to the ability of these proteins to compete with Sir3p for binding sites on Rap1p (Mishra and Shore 1999). A surprisingly large number of proteins have intermediate effects on TPE, as exemplified by Rrm3p, a helicase that causes about a ten-fold drop in telomeric silencing when deleted (Ivessa *et al.* 2002). Many of the proteins that have less dramatic phenotypes with respect to TPE probably act indirectly. For example, it has been shown that a silent telomeric transgene can be reactivated in G2/metaphase arrested cells but not in cells arrested at other stages in the cell cycle (Aparicio and Gottschling 1994). Proteins that affect cell cycle could therefore be expected to produce secondary effects on TPE in the absence of any direct association with telomeres (Laman *et al.* 1995).

The current model for the essential mechanism of TPE in yeast involves recruitment of a Sir protein-containing silencing complex to telomeres by Rap1p, which is bound directly to telomeric DNA. The Sir complex then spreads inward along the chromosome by virtue of the abilities of Sir3p and Sir4p to bind to the amino terminal tails of histones H3 and H4. This brings the histone deacetylase Sir2p in close proximity to its substrates in the subtelomere and provides a mechanism to establish the observed gradient of silencing that extends away from the telomere (Tham and Zakian 2002).

The analysis of gene expression at naturally occurring subtelomeric loci has been complicated by the fact that many of the endogenous genes are members of large multigene families such as *SUC*, *MAL*, and *MEL* [reviewed in (Zakian 1996)], making it hard to specifically detect transcripts from any one locus. Naturally occurring TPE was first detected in 1997 for the retrotransposon Ty5-1, which resides 1.8 kb from a telomere (Vega-Palas *et al.* 1997). Limiting the expression of a retrotransposon was hypothesized to limit the genetic damage caused by its movement to new sites, but this finding did not provide a satisfying answer to the question of whether or not true endogenous genes were regulated in this manner. It remained possible that real genes would be insulated from this silencing effect whose primary target might be unstable genetic elements such as retrotransposons. In 2000, a report was published showing that YFR057w, an endogenous yeast gene of unknown function located 1 kb from a telomere, was subject to TPE (Vega-Palas *et al.* 2000). Although no transcripts from this gene could be detected in wild type yeast strains, deletion of SIR2, 3, or 4 led to a dramatic upregulation of YFR057w while the expression of genes

adjacent to two other telomeres was not affected. This led to the second important finding that not all genes in endogenous subtelomeric regions are affected by TPE.

At least two groups have attempted to characterize the expression of telomeric genes in yeast through genome-wide analysis of transcription. By this method, the 267 genes located within 20 kb of a telomere are expressed on average at a level of 0.5 mRNA molecules per cell while more internally located genes are expressed on average at 2.4 mRNA molecules per cell (Wyrick *et al.* 1999). However, only 20 of these genes are upregulated in response to deletion of the *SIR* genes, indicating that this difference in average transcription is not entirely explained by telomere position effect. A second study examined the effects of telomerase deletion (and subsequent telomere shortening) on global transcription in yeast (Nautiyal *et al.* 2002). A wide variety of changes were observed, including changes (at least two-fold) in 77 genes located within 20 kb of a telomere. Most of these were upregulated. Because shortened telomeres may be recognized as damaged DNA, the profile of these cells was compared to others that had undergone various types of DNA damage and the changes unique to the telomerase deleted cells were compiled to create a "telomerase deletion signature". This group included 12 genes, none of which was apparently located in a subtelomeric region. This is not necessarily surprising, however, since it has long been known that DNA damage causes the delocalization of many telomeric proteins, including Sir proteins, Rap1p, and Ku (Martin *et al.* 1999; McAinsh *et al.* 1999; Mills *et al.* 1999). DNA damage would therefore be predicted to trigger a loss of telomeric silencing, which would cause subtelomeric genes to be excluded from the telomerase deletion signature. In fact, it has been suggested that one of the functions of the telomere

may be to serve as a reservoir of repair proteins that can be quickly mobilized by DNA damage (Tham and Zakian 2002). Consistent with this, the Ku complex has a well-documented role in DNA repair [reviewed in (Haber 1999)] and loss of Sir proteins has been linked to hypersensitivity to DNA damage in some strains (Martin *et al.* 1999; Mills *et al.* 1999). Interestingly, Sir proteins redistribute from the telomeres to the ribosomal DNA (rDNA) as yeast cells age (Kennedy *et al.* 1997). By repressing transcription of the rDNA, Sir proteins may slow the rate of recombination and excision of rDNA, a process that appears to be causal in yeast aging (Sinclair and Guarente 1997). The NAD dependence of Sir2p's histone deacetylase activity may link this process, and consequently the rate of yeast aging, to the metabolic state of the cell (Tissenbaum and Guarente 2002).

TELOMERE POSITION EFFECT IN OTHER LOWER ORGANISMS

Schizosaccharomyces pombe (fission yeast), although evolutionarily distant from *S. cerevisiae* (Russell and Nurse 1986), also shows TPE (Nimmo *et al.* 1994). While the behavior of telomeric genes seems very similar in some cases, it is important to note that in *S. pombe*, strains have been recovered in which the expression of *ADE6* from the telomere results in the formation of pink colonies rather than the sectored pattern of red and white observed in *S. cerevisiae*. This suggests that in some cases, either transcription is generally reduced in the absence of a switching phenotype or very rapid transition between the on and off states occurs in these cells. Loss of the telomere binding protein Taz1p in this yeast

abolishes TPE in a manner analogous to loss of Rap1p function in *S. cerevisiae* (Cooper *et al.* 1997; Nimmo *et al.* 1998).

Trypanosome brucei, the parasite responsible for sleeping sickness, has developed an interesting use for telomeric silencing. This organism escapes the immune system by repeatedly switching the identity of a single VSG (variant-specific surface glycoprotein) used to make up its outer coat [reviewed in (Rudenko *et al.* 1998)]. This is a complex process involving genomic rearrangements that place any of about 1000 VSG genes into about 20 telomeric sites where they can potentially be expressed. Expression can occur from any of these sites but only one is ever expressed at a time. Placing a reporter gene next to one of the silent telomeres results in stable repression (Horn and Cross 1995). Repetitive DNA, including the telomeres and the subtelomeric DNA at the inactive sites, but not the active transcription site in these organisms contains the novel base β-glucosyl-hydroxy-methyluracil (base J), the purpose of which is not yet well understood (Gommers-Ampt *et al.* 1993; van Leeuwen *et al.* 1998). A direct role in transcriptional repression has not been demonstrated for base J and it is thought to be more likely involved in tightening the shutdown of inactive sites rather than the initiation of silencing (Borst and Ulbert 2001).

A similar situation exists in the parasite *Plasmodium falciparum*, which causes malaria. Subtelomeric regions in this organism also contain gene families that encode virulence factors such as the var genes. After infecting a red blood cell, a *Plasmodium* cell will express only one var gene at a time, suggesting that the others may be subject to TPE (Scherf *et al.* 1998).

TPE IN MAMMALS

Recent evidence for TPE in the mouse has come from the comparison of a cell line bearing a telomeric copy of the neomycin phosphotransferase (*neo*) gene to two of its subclones (Murnane 2001). In these subclones, fusion at the marked telomere led to the recovery of cell lines in which the locus of the *neo* gene had become an internal site. In both cases, the reporter was expressed at a higher level in the subclones than in the parental line, indicating that the presence of the telomere was sufficient to decrease transcription. Mouse lines have since been identified in which integration of a transgene near a telomere (based on colocalization by *in situ* hybridization) has apparently led to the production of a variegated phenotype (Ramirez *et al.* 2001). Taken together, these initial results suggest a striking similarity between telomere position effect in yeast and mammals.

Another relevant study was published in 2001. In this case, an 800 bp tract of telomere repeats was inserted into an intron of the adenosine phosphoribosyltransferase (*APRT*) gene of Chinese hamster ovary cells (Kilburn *et al.* 2001). The major finding of the paper was that this process created chromosomal instability but a second important finding was that transcription of the *APRT* gene was reduced two-fold specifically when the telomere tract was oriented such that the promoter was on what would have been the subtelomeric side (had the telomere repeats been at a chromosome end). This is consistent with the finding in yeast that internal tracts of telomeric DNA can function as silencers, albeit less effectively than true telomeres (Stavenhagen and Zakian 1994). This finding also suggests that a reporter gene in an internal control line generated from a construct that contains telomere

repeats may be partially silenced despite being integrated far from the telomere. It is therefore more appropriate to compare expression of a telomeric gene to that of an internal control generated in the absence of telomeric DNA.

The first attempt to detect human TPE was made in 1994 using a series of Chinese hamster somatic cell hybrid lines each containing a derivative of the human X chromosome (Bayne *et al.* 1994). The main interest of the authors in this report was to study the effects of alpha satellite DNA from centromeric regions on mitotic stability and the expression of an adjacent reporter gene. For this reason, they used telomere-associated chromosome fragmentation to generate a library of truncated X chromosomes with their construct containing the hygromycin resistance (*hygro*) gene at the breakpoint. The technique is similar to that described earlier for yeast in that a linear piece of DNA containing telomere repeats is transfected into cells and integrates into the genome, often resulting in formation of a new telomere from the plasmid-based repeats and loss of the distal portion of the chromosome. Initial comparisons between X chromosomes in which the alpha satellite DNA was either completely intact or partially deleted showed no effect on mitotic stability. Because PEV within mouse pericentric heterochromatin had been previously reported, they next compared transcription levels for the *hygro* gene sandwiched within 12 kb of intact alpha satellite DNA and 1.4 kb of a newly formed telomere to those in another cell line with an integration site that was distal from the centromere, but still within 1.4 kb of a newly formed telomere (using human X chromosomes in Chinese hamster cells). Because transcription levels were not significantly different, it was concluded that pericentric sequences do not exert a position effect in this system. In order to test for position effect due

to the newly formed telomere, expression in these lines was compared to that in a third line in which integration of the *hygro* gene-containing construct had not led to chromosome truncation and so the reporter was distal to both centromeric and telomeric sequences. It was not however clear whether or not the plasmid-based telomeric sequences remained after integration in this cell line. Once again, no difference was detected and so it was concluded that telomeric sequences do not exert a position effect in this system. In is important to keep in mind when interpreting these experiments that only a single non-telomeric clone was analyzed and that the cellular environment was that of a Chinese hamster line, aside from the presence of the human X chromosome.

In 1995, data consistent with a very mild mammalian TPE were described (Cooke 1995). The cells used in this case contained the HPRT gene with a weak promoter. This gene is particularly useful because it is possible to select for cell that express it, or by changing the growth conditions select specifically for cells that do not express it. Cell lines were isolated that were capable of growing in both sets of conditions, apparently switching states and in these cases, the HPRT gene was inserted into either a telomeric or a centromeric region. By mRNA analysis, however, these cells show a low, minimally changing level of HPRT expression. This made it difficult to determine whether a threshold expression level was simply permissive to growth in either set of conditions or if a slight variegation was occurring that was sufficient to produce a switching phenotype.

The strongest evidence against the existence of TPE in human cells came from a 1996 report using an SV40-transformed human fibroblast cell line containing the neomycin phosphotransferase (*neo*) gene adjacent to a telomere (Sprung *et al.* 1996). The authors took

advantage of the highly heterogeneous telomeres in these cells to obtain subclones with telomere lengths varying between 0.5 and 25 kb. No significant difference in *neo* expression was detected between these subclones either by Northern analysis or by a colony-forming assay. Interestingly, the level of *neo* expression in a control cell line was shown to be approximately four times higher than in the subclones bearing a telomeric copy by Northern and this difference was not reflected in the colony-forming assay. An important consideration is that the cells used in this experiment have since been shown to use the ALT (alternative lengthening of telomeres) pathway (Murnane *et al.* 1994), a phenotype that involves altered telomere biology and a substantial increase in total telomeric DNA (Bryan and Reddel 1997). It is therefore possible that TPE in these cells might be disrupted through titration of essential telomere binding factors, as has been observed in yeast (Wiley and Zakian 1995), or through some other mechanism associated with the ALT pathway. The authors also suggest that specific promoters might be differentially affected by TPE, while pointing out that the HSV-tk promoter used in these studies is clearly subject to epigenetic effects at other loci (Butner and Lo 1986).

In 1999, it was reported that a human telomere could produce a position effect on replication timing (Ofir *et al.* 1999). The cells used in this study were derived from a patient with mental retardation and a microdeletion of 130 kb from the end of one copy of chromosome arm 22q, which had been repaired by the additional of telomere repeats. By comparison to the same locus on the normal copy of 22q, the authors were able to show that replication of the region adjacent to the newly formed telomere was significantly delayed. The nearest gene to the breakpoint was located approximately 54 kb away. By taking

advantage of a polymorphic site within this locus, it was possible to demonstrate that transcription continued to take place from both the normal and the truncated chromosome. However, there did appear to be a small difference in the expression levels of the two alleles. Failure to detect a convincing telomere position effect on transcription in this study may have been due to the short length of the healed telomere and/or to the relatively large (as compared to yeast studies) distance to the nearest gene that could be measured.

The experiments presented here were conducted in the Shay/Wright laboratory at the University of Texas Southwestern Medical Center from 1999 to 2003 with the goal of detecting and characterizing a position effect at human telomeres. They were first published (in part) on June 15, 2001.

CHAPTER TWO
The Discovery of Telomere Position Effect in Human Cells

INTRODUCTION

Despite the previous lack of encouraging results by others, sufficient reason existed to justify developing a sensitive test for telomere position effect in human cells. Unlike the telomeres of the other organisms in which TPE had been studied, the telomeres of human (and other large mammalian) somatic cells shorten with each cell division (Harley *et al.* 1990). If telomeric silencing was found to be proportional to telomere length, a hypothesis supported by studies in mutant yeast (Kyrion *et al.* 1993), then TPE in human cells had the potential to be regulated by cellular aging. In an extreme scenario, loss of telomeric silencing due to telomere shortening has the potential to mechanistically explain the Hayflick limit (i.e. be the trigger for replicative senescence). However due to the obvious similarities between senescence and the state of growth arrest induced by DNA damage, a model was favored in which loss of silencing due to telomere shortening would primarily be associated with some of the age-dependent phenotypic changes observed in pre-senescent cells (Hayflick 1980; Sottile *et al.* 1989; Doggett *et al.* 1992; Burke *et al.* 1994).

A luciferase reporter gene was chosen for the initial studies because its expression can be precisely measured over many orders of magnitude (Himes and Shannon 2000). Since it had been shown previously in yeast that a strong promoter could abolish telomere position effect (Aparicio and Gottschling 1994), a tetracycline-inducible system (*tet*-off)

(Gossen and Bujard 1992; Baron *et al.* 1995) was employed. The first goal was to generate a series of clones bearing the reporter either at a telomere or, as a control, at an internal locus. To accomplish this, the method of Hanish *et al* (Hanish *et al.* 1994) was employed, in which a linearized piece of plasmid DNA containing the reporter gene and a stretch of human telomere repeats was transfected into HeLa cells (Figure 2.1). This process frequently results in the truncation of an endogenous chromosome at the site of plasmid integration with subsequent extension of the plasmid-based telomere repeats to form a new telomere. (For a more detailed description and additional references, see the Materials and Methods section in this chapter.)

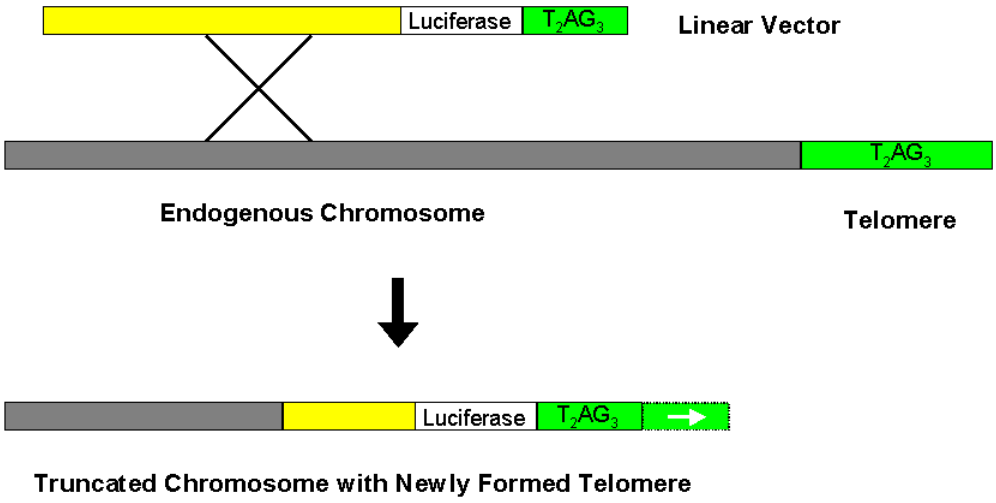

Figure 2.1 – Formation of a new telomere at the site of chromosome truncation. After transfection into the cell, a linearized plasmid containing the luciferase reporter and telomere repeats (top) recombines with an endogenous chromosome. In many cases, this results in truncation of the chromosome and formation of a new telomere by extension of the plasmid-based repeats (shown).

An important consideration is that in this system, as in most of the studies in yeast, endogenous subtelomeric elements are not present since the telomere adjacent to the reporter gene has been generated *de novo* at a previously internal site. Control insertions at (random) internal sites are generated by transfection of a similar linearized plasmid that lacks telomere repeats.

After isolating HeLa clones bearing the luciferase gene either at a newly formed telomere or an internal site, several key experiments were performed. From measurements of luciferase activity it was possible to show that while expression within each group varied over at least three orders of magnitude, there was a clear ten-fold reduction in the average for telomeric clones as compared to internal controls. Relief of silencing by the histone deacetylase inhibitor trichostatin A (TSA) demonstrated that this was in fact a position effect and not due to modification of the gene itself. Finally, by elongating telomeres through hTERT (telomerase) overexpression, it was possible to show that the strength of telomeric silencing in these cells was dependent on telomere length.

RESULTS

HeLa clones bearing the luciferase reporter at a telomere can be recovered

Telomeric clones were generated by "seeding" the formation of a new telomere at the site of transgene integration. The definitive test for successful formation of a new telomere from plasmid-based sequences is a Southern blot in which the restriction digest is such that the

probe region is left attached to the telomere repeats. If the repeats have been extended to form a new telomere, then this fragment will be heterogeneous in size (due to the variability in telomere lengths between cells) and form a characteristic smear. If the plasmid sequences have been integrated at an internal site, however, the restriction enzyme will find a second site in the genomic DNA and so the probe will reveal a single, tight band. In Figure 2.2, the additional step was taken to purify the telomeric DNA. This provides an independent confirmation of the result since smears appear in the telomere (Tel) lanes for the first two (telomeric) clones and a tight band appears only in the supernatant (Sup) fraction for the third (internal) clone. Mean length of the healed telomeres (after subtracting 3 kb of attached plasmid sequences) was estimated to be between 1.5 and 2 kb.

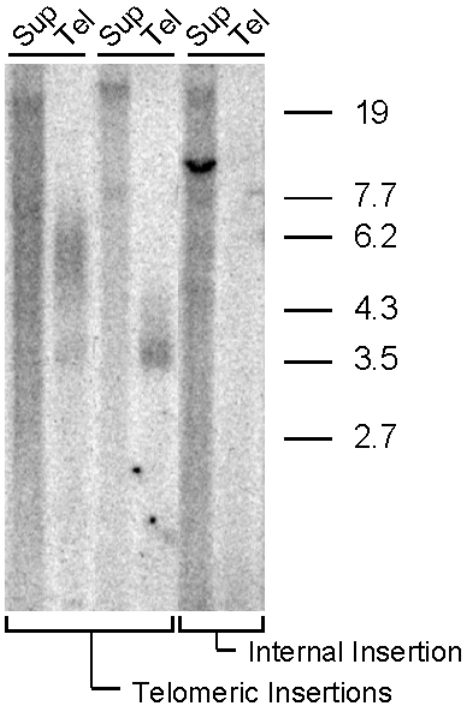

Figure 2.2 – Identification of telomeric clones by Southern blotting of purified telomeres. Genomic DNA was digested with *Stu* I, leaving the luciferase gene attached to the plasmid telomere sequences. Telomeres were then separated from bulk genomic DNA and subjected to Southern blotting as described in Materials and Methods. Telomeric luciferase genes appear as a smear in the telomere fraction due to the heterogeneous lengths of the attached telomeres while internally integrated genes appear as a discrete band in the supernatant fraction. Multiple integrations were noted in several of the internal control clones, however the average was less than two. Markers shown are λ *Sty* (kb).

The recovery of clones bearing the luciferase gene at a telomere can also be verified visually by florescence *in situ* hybridization. The overlap (yellow) between the signals for telomeric DNA (green) and the luciferase gene (red) in the three telomeric clones but not in the internal clone (Figure 2.3) shows that the reporter has been successfully placed next to a telomere.

By these criteria, it was possible to verify the generation of HeLa clones bearing the luciferase reporter immediately adjacent to a newly formed telomere.

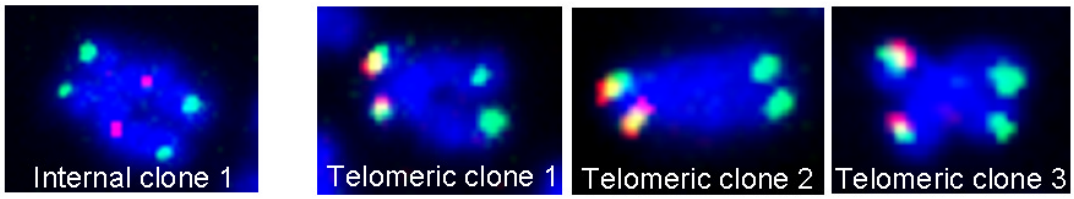

Figure 2.3 – Verification of telomeric clones by *in situ* hybridization. Cells were fixed and probed simultaneously with the luciferase plasmid labeled with Spectrum Orange (Vysis, Downers Grove, IL), shown in red, and a FITC-tagged peptide nucleic acid (PNA) complementary to telomere sequences [$(CCCTAA)_3$], shown in green as described in the Materials and Methods section. DAPI staining is shown in blue. The left panel shows a clone with an internal integration site while remaining panels demonstrate the colocalization of the telomere and luciferase signals in three independent telomeric clones. This hybridization was performed by Ying Zou.

Average luciferase expression is reduced ten-fold in telomeric clones

After growing cells throughout the process of isolation and identification in the fully induced state (absence of tetracycline), the telomeric and internal clones were assayed for luciferase activity. As expected with stable transfectants (Kalos and Fournier 1995; Walters *et al.* 1995; Walters *et al.* 1996; Wright 2003) [reviewed in (Martin and Whitelaw 1996; Dorer 1997)], the level of transgene expression varied widely (~1000-fold) between members within each of the two groups. This variability most likely represents the "permissiveness" of the loci into which the transgene randomly integrated in each clone. However as can be seen in Figure 2.4, there was a clear and significant ($P < 0.0001$) ten-fold reduction in the

average luciferase activity in the telomeric group as compared to internal controls. Thus, telomeres appeared to exert a position effect on even a strong (fully induced) promoter.

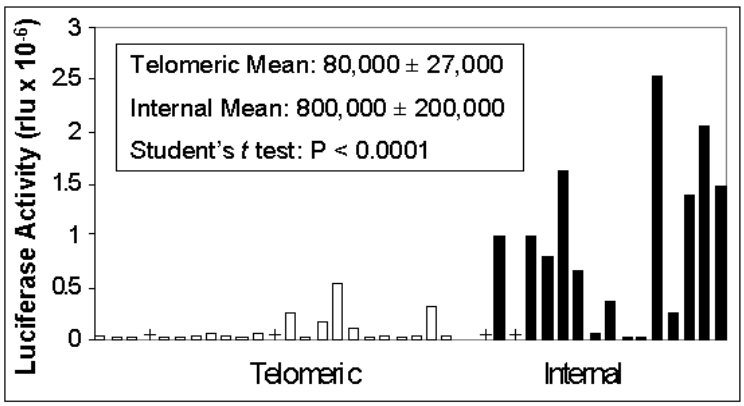

Figure 2.4 – Telomeric clones show a 10-fold lower level of luciferase activity. Puromycin resistant clones were screened using a Luciferase Assay System (Promega, Madison, WI) on an Optocomp I luminometer (MGM Instruments, Hamden, CT). The results for 23 telomeric and 15 internal integrations are shown. The "+" symbols indicate clones with a level of expression too low to be visible on this scale.

One possible alternative interpretation is that duplication of the luciferase gene could have occurred specifically at internal loci, resulting in a higher level of expression in these clones. This is not likely the case, as the Southern blotting technique should (and did in some cases) reveal the presence of multiple copies of the luciferase gene. Because one of the restriction sites used to generate a luciferase fragment comes from genomic DNA flanking the insertion site, integrations at discreet loci should each produce a band of unique size on a Southern blot. Amplification at the site of a single integration should produce a dark band resulting from the repeated copies and a smear (telomeric) or single band of unique size (internal) resulting from the final copy of the luciferase gene. Some examples of these types of

patterns are shown in Figure 2.5. Because the average number of integrations was less than two per clone, and not all of those were shown to be functional, copy number does not explain the observed differences in luciferase activity.

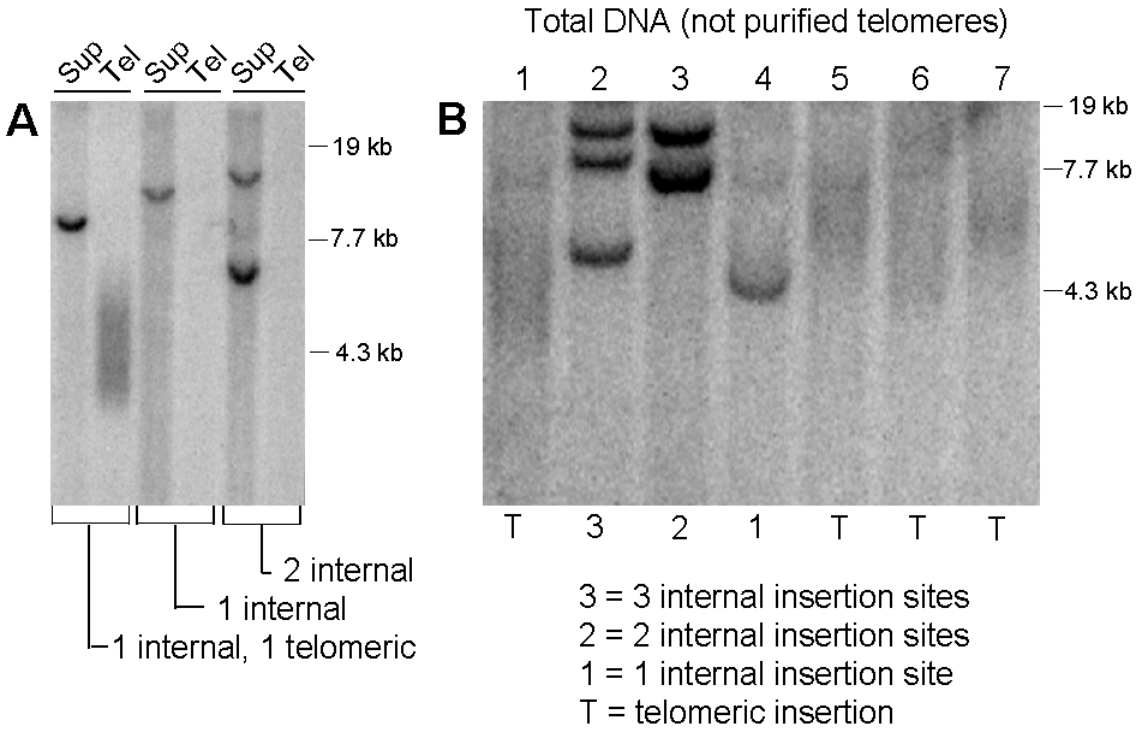

Figure 2.5 – Multiple integrations of the luciferase sequences in some clones. (A) Additional clones were analyzed as described in Figure 2.2. From left to right they are a clone with both a telomeric and an internal integration, a clone with a single internal integration, and a clone with at least two internal integrations (lower band is darker, possibly indicating more than one copy at that site). Genomic DNA was digested with *Stu* I, leaving the luciferase gene attached to the plasmid telomere sequences. Telomeres were then separated from bulk genomic DNA as described in materials and methods. Both the telomere and supernatant (bulk genomic DNA) fractions were analyzed by Southern blotting. The fact that the signal seen in the telomere fraction for the first clone appears as a smear provides additional confirmation that the clone is telomeric since the attached telomere sequences are heterogeneous in length. (B) Multiple bands appear in some clones. Genomic DNA was digested using *Stu* I and analyzed by Southern blotting without prior separation of telomeres. Four telomeric clones with no internal sites (lanes 1, 5, 6, and 7), a single internal integration site (lane 4), two internal integration sites (lane 3) and three internal integration sites (lane 2) are shown.

Reducing promoter strength does not significantly enhance TPE

To test whether weakening the luciferase promoter would enhance telomere position effect, doxycycline (a tetracycline analog) was added to the media. In the *tet*-off system used for these experiments (see Materials and Methods), adding doxycycline to the media reduces promoter strength by shifting the transactivator protein (tetracycline transactivator protein, or tTA) to an inactive conformation. As shown in Figure 2.6, telomeric and internal clones were affected to approximately the same degree.

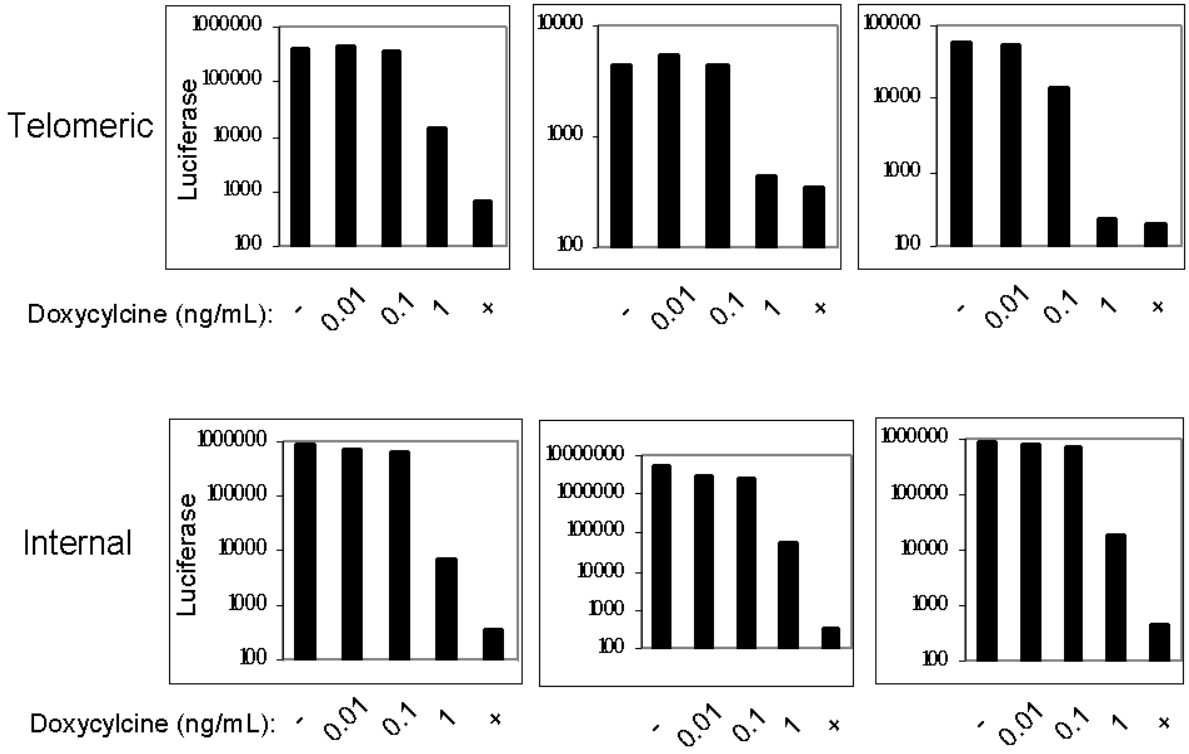

Figure 2.6 – Telomeric clones and internal controls are silenced to an equivalent degree in response to doxycycline. Three telomeric (upper) and three internal (lower) clones were grown in the presence of 0 (-), 0.01, 0.1, 1, or 1000 (+) ng/mL doxycycline for >4 days. Luciferase activity was measured and expressed on a logarithmic axis. As can be seen, telomeric clones were not generally affected to a greater degree.

In a second experiment, the rate of silencing after the addition of doxycycline to the media was tested. Again, there was not a dramatic difference between the telomeric clones and the internal controls (Figure 2.7).

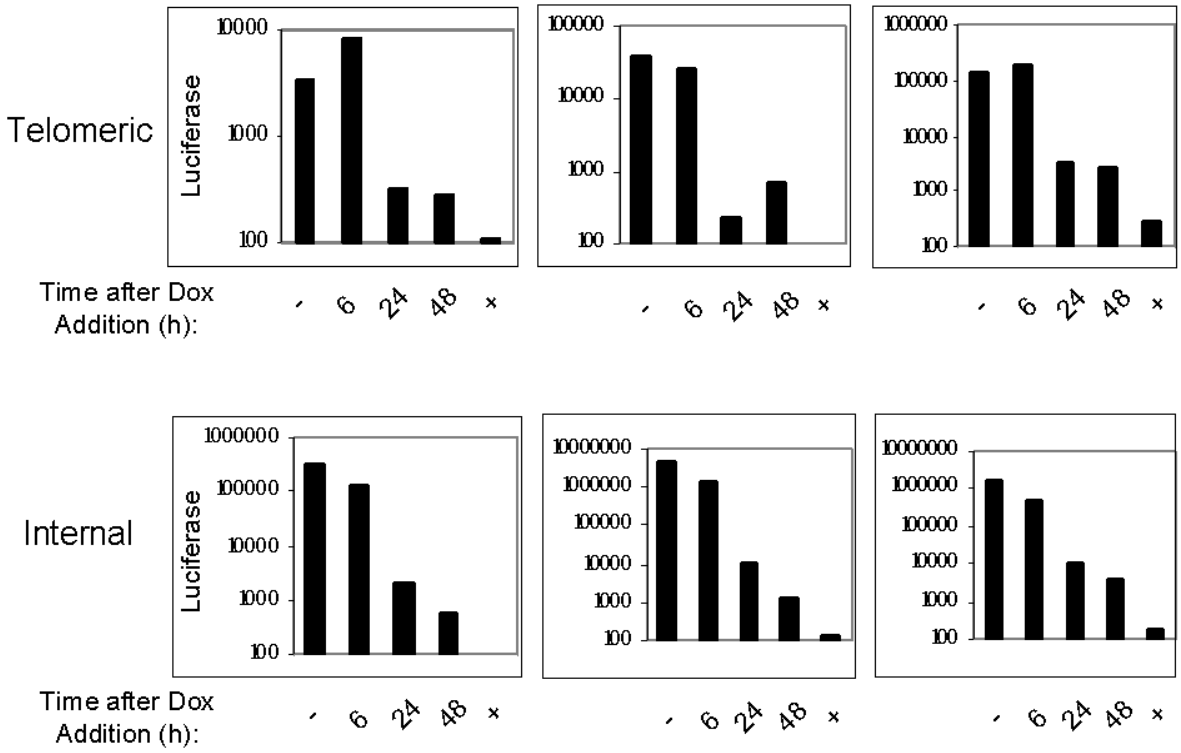

Figure 2.7 – Telomeric clones and internal controls are silenced at equivalent rates in response to doxycycline. Three telomeric (upper) and three internal (lower) clones were grown in the presence of 1 μg/mL doxycycline for 0 (-), 6, 24, 48, or >96 (+) hours. Luciferase activity was measured and expressed on a logarithmic axis. As can be seen, telomeric clones were not generally affected to a greater degree.

It can also be seen in these figures that subtle differences in doxycycline concentration have the potential to produce significant changes in luciferase activity and thereby complicate the analysis of these clones. Even after growth in the repressed state for > 1 month, telomeric

and internal clones could be re-induced to express luciferase at approximately their original levels, indicating that no additional silencing near telomeres was established in the absence of transcription. For these reasons, it was determined to proceed with all clones kept in the fully induced state for the remainder of these experiments.

Silencing can be relieved using the histone deacetylase inhibitor trichostatin A or 5-bromodeoxyuridine

The hallmark of a position effect is its dependence on a gene's location, and not factors within the gene itself. The histone deacetylase inhibitor trichostatin A (TSA) was used to relieve silencing, demonstrating that the luciferase gene was intact and capable of high-level expression in both telomeric and internal clones (Figure 2.8). TSA enhanced expression of the internal and telomeric clones by 2.6 ± 0.4 and 51 ± 37-fold respectively, indicating that the initial difference observed was histone deacetylase-dependent.

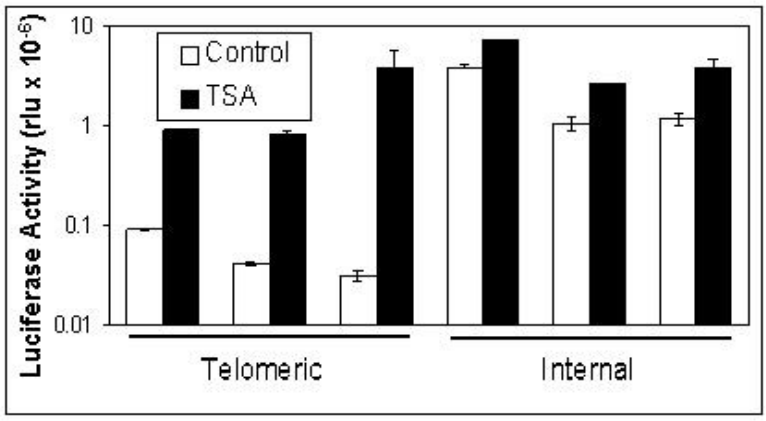

Figure 2.8 – Silencing is relieved by the histone deacetylase inhibitor trichostatin A. Three telomeric and three internal clones were treated with 200 ng/mL TSA (Sigma, St. Louis, MO) for 24 hours. The media was replaced and the cells were incubated for an additional 24 hours before collection for luciferase assays. Note the logarithmic scale.

Promoter methylation has been reported to result in transcriptional silencing that can be relieved by treatment with the demethylating agent 5-azacytidine (5-aza), reviewed in (Karpf and Jones 2002). However, treatment of these clones with 5-aza produced relatively minor and inconsistent increases in activity and was not pursued (data not shown). Treatment with 5-bromodeoxyuridine (BrdU) however, dramatically relieved silencing in these cells, mirroring the effects of TSA (Figure 2.9). The mechanism by which BrdU relieves silencing is not understood, although it has been speculated that its incorporation alters the binding sites for many DNA-interacting proteins (Suzuki *et al.* 2001). Although less clear mechanistically, this method has the advantage that toxicity is greatly reduced compared to that observed with TSA. The actions of both TSA and BrdU are likely non-specific, with greater relief of silencing in telomeric clones being the result of a greater initial degree of silencing. These experiments should be taken primarily as evidence that the luciferase gene is intact and capable of being expressed in all clones.

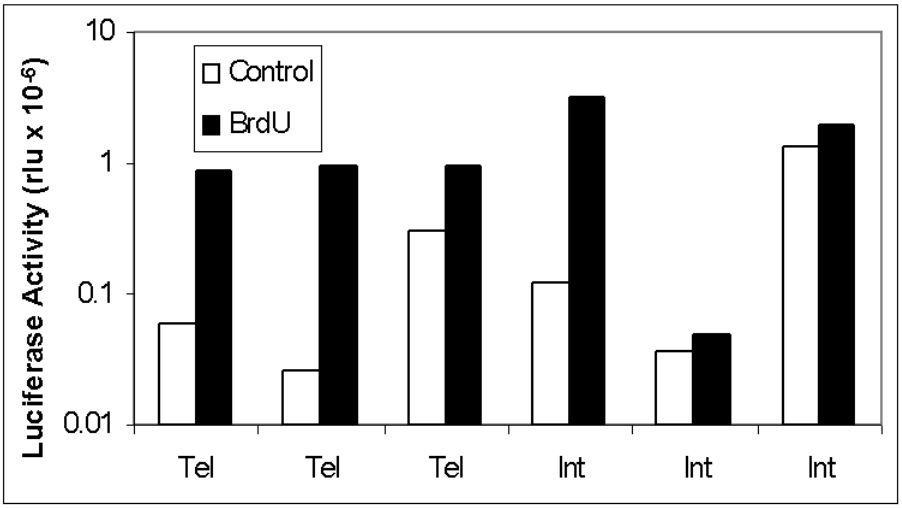

Figure 2.9 – Luciferase activity is restored by 5-bromodeoxyuridine (BrdU). Silencing is relieved by the nucleotide analog BrdU through an unknown mechanism. Three telomeric and three internal clones were treated with 50 μM BrdU for 3 days. Note the logarithmic scale.

Telomere elongation by telomerase overexpression enhances silencing specifically in telomeric clones.

HeLa cells are immortal and express telomerase (Morin 1989). [In fact, HeLa was the first cell line ever grown in culture, isolated from a cervical tumor over 50 years ago, reviewed in (Masters 2002).] Even though HeLa cells possess endogenous telomerase, the addition of exogenous telomerase, using a retroviral vector, is sufficient to greatly increase the lengths of their telomeres (Figure 2.10A). Telomere lengths increased from roughly 4 kb to about 14 kb by three weeks after infection in these cells.

After telomere elongation, silencing was enhanced specifically in the telomeric clones (Figure 2.10B). This correlation between telomere length and the strength of silencing

agrees well with previous studies in yeast. It also provides additional proof of the existence of a position effect, since a 2 to 10-fold increase in silencing is produced in the absence of any mutation in the gene or its promoter. Unlike the relief of silencing by TSA and BrdU, this effect is highly specific to telomeric clones.

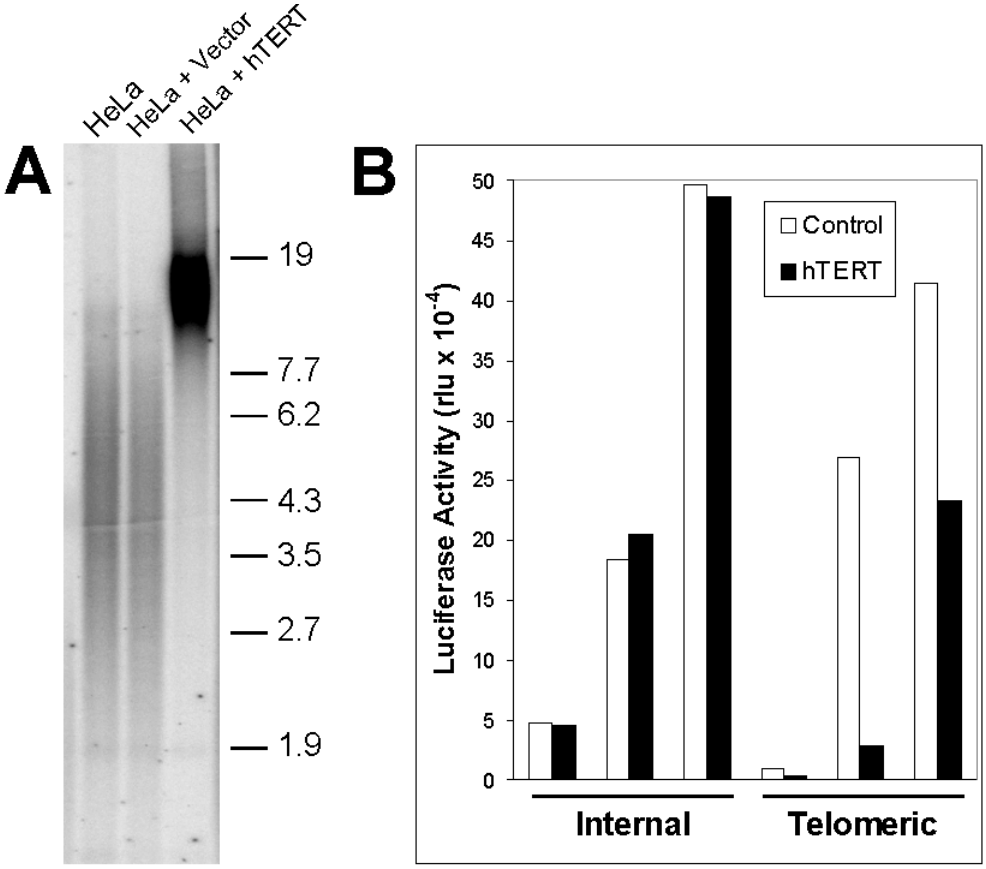

Figure 2.10 – Silencing in telomeric clones is enhanced by an increase in telomere length. (A) Infection of HeLa cells with an hTERT-encoding retrovirus causes telomere elongation as demonstrated by terminal restriction fragment (TRF) analysis. Mean telomere length increased from approximately 4 kb to almost 14 kb. Genomic DNA was digested with six restriction enzymes to degrade non-repetitive sequences. Samples were then separated on a 0.7% agarose gel and probed with an oligonucleotide complementary to telomere repeats. Markers shown are λ *Sty* (kb). (B) Telomeric clones infected with hTERT express 2 to 10-fold lower levels of luciferase activity as compared to control, vector-only infections. Internal clones having comparable initial values retain full expression of the luciferase reporter after infection with hTERT.

DISCUSSION

In this chapter, it has been shown that a telomere position effect can be detected in human cells. Placement of a luciferase reporter next to a telomere resulted in an approximately 10-fold reduction in average expression level as compared to random insertion. This silencing could be relieved through inhibition of histone deacetylase or treatment with BrdU, indicating that the effect was not due to a change within the gene itself or the promoter, but rather by the genomic environment. Furthermore, increasing telomere length through overexpression of telomerase resulted in a 2 to 10-fold enhancement of silencing, demonstrating that the terminal array of TTAGGG repeats mediates this effect. These findings support the hypothesis that a telomere length-dependent position effect could play a role in the replicative aging of human cells and by extension, in human aging.

While these findings show that telomere position effect in principle could occur in human cells, the important question remains of whether or not endogenous genes are affected in a similar manner. The construct used here resulted in the placement of the luciferase gene only about 500 bp from the base of the telomere. Since the gene was oriented to be transcribed toward the telomere, the promoter was about 2 kb from the base of the telomere repeats. Although many pseudogenes are closer and many regions have yet to be accurately mapped, the promoter of the nearest known bona-fide single-copy gene to a human telomere lies approximately 35 kb from the start of the TTAGGG repeats [based on available information from the human genome project, (Ciccodicola *et al.* 2000), (Daniels *et al.* 2001), and H. Riethman, unpublished data]. By analogy to the well-characterized *S. cerevisiae*

system, this would be too great a distance over which to expect TPE to act, at least at a truncated telomere. However there are several problems with this analogy. Human telomeres are on the order of 20 to 30-fold longer than yeast telomeres. Given that the yeast TPE typically extends about 6 kb in the absence of subtelomeric sequences and overexpressed proteins (Renauld *et al.* 1993), human TPE could reasonably be predicted to act over a distance of 120 to 180 kb from the site of a truncated telomere. A second problem is that this approximation, in both yeast and human, ignores the influence of endogenous subtelomeric sequences. In yeast, these sequences have been proposed to act in some cases as "relays", propagating TPE well beyond the limits observed for truncated telomeres (Fourel *et al.* 1999; Pryde and Louis 1999). In humans, a similar process might allow telomeres to influence gene expression over a distance of a megabase or more. Alternatively, the distance over which TPE can act in both yeast and in human cells might be limited intrinsically by the mechanism the silencing factors use to spread. It could also be that these two processes, while apparently conserved in terms of function, utilize almost entirely different mechanisms, causing the analogy to fail outright.

In addition to finding endogenous genes affected by TPE, there are several other ways in which the current findings can be extended to learn more about telomere position effect in human cells. These ideas make up the basis for the following chapters. By using the luciferase reporter gene, it was possible to gain increased sensitivity over other systems but it was not possible to examine expression at the single-cell level. By analyzing single cells, it was possible to distinguish whether silencing at telomeres represents a graded, uniform decrease in expression in all cells, or a decrease in the fraction of cells expressing the

reporter (at a relatively constant level), or possibly a combination of the two. A second approach was to test known telomere binding proteins and the homologs of proteins known to affect TPE in yeast for the ability to influence TPE in human cells. Besides increasing general knowledge of the process, this would allow a better comparison of human TPE to yeast and possibly provide tools that could be used to identify affected human genes. For example, the first endogenous yeast gene known to be subject to TPE was discovered through a comparison of wild type and *SIR*-deleted (loss of TPE) strains (Vega-Palas *et al.* 2000). The results presented here clearly demonstrate that TPE in human cells is possible, but leave open the questions of whether it is present for endogenous genes and what the exact nature of the silencing effect might be.

MATERIALS AND METHODS

Vector Construction

Design of the initial construct for these studies was complicated by the finding that telomere repeats in certain positions and orientations destabilized the plasmid to an unacceptable degree. The starting material was the pBI-2 variant of the plasmids developed by Baron *et al* (Baron *et al.* 1995) that contained a bi-directional *tet*-inducible promoter (7 *tet* operator sequences fused to minimal promoters sequences derived from the cytomegalovirus immediate early promoter) simultaneously driving expression of luciferase and the puromycin resistance gene. It was designed this way to permit drug selection in the presence

of a strong (induced) promoter, followed by growth and establishment of potential TPE under non-induced (weak promoter) conditions. A Bam HI/Bgl II fragment containing 1.6 kb of T_2AG_3 repeats was excised from the vector pSXneo (a gift from T. de Lange) and inserted into the Bgl II site of PBI-2, destroying the site proximal to the promoter. Next, the plasmid was cut with Nhe I/Hpa I to generate a fragment and a separate aliquot was cut with Spe I/Eco RV to generate a backbone. Ligation effectively flipped the fragment so that the luciferase gene was placed downstream of the telomere repeats and both the luciferase and puromycin resistance genes were terminated by SV40 poly adenylation signals. A "repeatless" variant was also generated by digestion with Bst XI/Not I and blunt ligation.

The Tetracycline-Inducible System

The tetracycline-inducible system was created by fusing the *tet* repressor with the activating domain of virion protein 16 from herpes simplex virus (Gossen and Bujard 1992). When expressed in cells, this fusion protein binds and activates transcription from *tet* operator sequences. In the presence of tetracycline or an analog (1 μg/mL doxycycline unless otherwise stated in these studies), the binding is disrupted (hence *tet*-off). The HeLa cells in these studies are the same ones described by Gossen et al and express this fusion protein, termed the tetracycline transactivator (tTA). More recently, a variant form of this protein has been produced that requires tetracycline in order to activate transcription (*tet*-on) (Gossen *et al.* 1995).

Generation of Clones Containing Telomeric Luciferase Reporters.

The previously described plasmid containing a 1.6 kb tract of telomere repeats adjacent to a luciferase reporter was linearized using *Not* I and transfected into HeLa cells using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Clones were recovered in which integration of the plasmid had caused the truncation of a chromosome and subsequent extension of the plasmid sequences to form a new telomere (Figure 2.11).

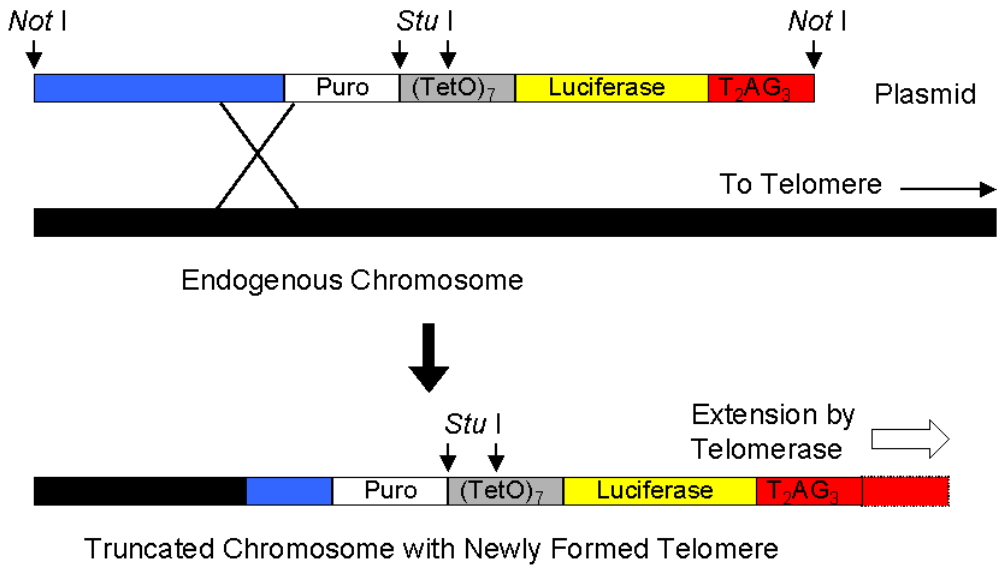

Figure 2.11 – Generation of telomeric clones. Transfection of the linearized plasmid containing 1.6 kb of telomere repeats frequently results in chromosome breakage followed by healing of the plasmid-based repeats into a new telomere. $(TetO)_7$ represents seven tet operator sites fused to create a bi-directional, tetracycline-responsive promoter. The indicated *Stu* I sites were used to analyze chromosomal insertion sites.

Internal controls were generated using the "repeatless" version of this plasmid cut with Pvu I (replacing the telomere repeats with junk DNA). This method was adapted from Hanish *et al*

(Hanish *et al.* 1994) whose experiments were based on several previous studies in mammalian cells (Farr *et al.* 1991; Farr *et al.* 1992; Barnett *et al.* 1993).

Cell Culture

HeLa tTA cells (Clontech, Palo Alto, CA) were grown in X media (4:1 DMEM:Medium 199) supplemented with 10% tetracycline-free serum (Donor Calf from Gibco, Gaithersburg, MD) and 50 μg/mL gentamycin. Cells were passaged by trypsinization and subsequent inactivation with serum-containing media, followed by cell counting when necessary and replating.

Telomere Purification

4.5 pmol of a biotinylated $(CCCTAA)_6$ oligonucleotide (complementary to the single-stranded overhang of a human telomere) were added to a restriction digest containing 15 μg genomic DNA. Sufficient 20X SSC and Triton X-100 were added to bring the (final) concentrations to 1X and 0.15% respectively after diluting to 250 μL with water. Annealing was achieved by a series of incubations in a PTC-100 programmable thermal cycler (MJ Research) consisting of 15 minutes at 80°C, 20 minutes at 65°C, 20 minutes at 55°C, 15 minutes at 45°C, and 15 minutes at 35°C. 20 μL of a 10 mg/mL stock of streptavidin-coated magnetic beads (Dynabeads produced by Dynal, Oslo, Norway) that had previously been washed in 1X SSC, coated in 5X Denhardt's solution for 30 minutes at room temperature, and resuspended in 1X SSC were then added to each tube. Tubes were rotated (~ 2 rpm) overnight at 4°C. On the second day, a magnet, 1X SSC with 1% Triton X-100, 0.2X SSC

with 1% Triton X-100, and TE (pH 8) were pre-chilled and the entire procedure was performed on ice. Beads were pulled to the bottom of the tube with the magnet and supernatant was saved for later analysis. Beads were then gently resuspended in 150 µL 1X SSC (with 1% Triton X-100), pulled down and the supernatant discarded. The second wash step was identical except that the slurry after resuspension was transferred to a new tube in order to exclude DNA bound non-specifically to the walls of the original tube. The third wash was in 0.2X SSC. Finally, holding the tube against the magnet, the beads were rinsed with 50 µL TE by pipeting in the solution and quickly removing it with no resuspension of the beads. Beads were then resuspended in 20 µL TE, heated to 65°C for 10 minutes to melt the interaction between the telomeres and the biotinylated oligonucleotide, and pulled down with the (pre-warmed) magnet. Supernatant containing purified telomeres was then recovered.

Southern Blotting

Samples were separated on a 0.7% agarose gel and transferred to a Zeta-Probe blotting membrane (Bio-Rad, Hercules, CA) by capillary transfer in 10X SSC. The membrane was then crosslinked twice on the "Autocrosslink" setting using a Stratagene Stratalinker 2400 UV crosslinker. The membrane was pre-hybridized with 7% SDS in 0.25 M sodium phosphate (pH 7.2) at 65°C for 45 minutes and probed for 8-16 hours in the same buffer. Probes were generated from plasmid sequences by randomly primed incorporation of α-^{32}P-labeled dCTP using the Radprime kit (Invitrogen, Carlsbad, CA). The membrane was then washed twice with 5% SDS in 0.2 M sodium phosphate buffer and twice with 1% SDS in 0.2

M sodium phosphate for 30 minutes each at 65°C. The membrane was then exposed to a Phosphor screen and scanned using a Storm 860 PhosphorImager system (Molecular Dynamics/Amersham, Piscataway, NJ).

In Situ Hybridization

The telomere probe was a fluorescein isothiocyanate (FITC)-conjugated oligonucleotide N3'-N5' phosphoramidate [$(CCCTAA)_3$] that was kindly provided by the Geron corporation (Menlo Park, CA). The luciferase probe was labeled with Spectrum Orange (Vysis, Downers Grove, Illinois) by nick translation according to the manufacturer's instructions and both were stored at a concentration of 500 ng/µL at –20°C. Cells were dropped onto wet slides, which were then allowed to sit at room temperature for several days. Slides were rehydrated in 1x PBS (pH=7.0-7.5) for 15 min, fixed in 4% formaldehyde in PBS (pH= 7.0-7.5) for 2 min, and washed in 1 x PBS for 3 X 5 min. Slides were then treated with working pepsin solution (1mg/ml in 37°C) for 10 min [pepsin should be prepared fresh in acidified water (pH=2)], and rinsed with 1 x PBS for 5 min x 2. Slides were fixed in 4% formaldehyde in PBS (pH= 7.0-7.5) for 2 min, washed 3 in PBS for 5 min, dipped through an ethanol series (70%, 90% and 100% ethanol) for 2-5 min, and allowed to air dry. (Optional: Add 80 µl of RNase to each side, cover with coverslip and let sit for 10 min at 37 °C. Rinse with 1 x PBS for 5 min x2, go through the ethanol series, and air-dry.) 20 µL hybridization solution was added (70% formamide, 12.5 mM $MgCl_2$, 2.5% MEN blocking solution (Invitrogen, Carlsbad, CA), 17 ng telomere probe, 300 ng luciferase probe, and water up to 20 µL). Slides were heated to 78 °C for 10 minutes and covered or placed in a dark humidified

chamber at 37°C for 16 hours. Probe was rinsed off with 70% formamide buffer, 10 mM Tris, 0.1% BSA, pH=7.0-7.5, for 2 x 15 min, 0.1M Tris, 0.15 M NaCl, 0.08% Tween-20, pH = 7.0-7.5 for 3 x 5 min, then slides were drained and dipped through the ethanol series and air dried in dark. 2 x 10 µl drops of Vectashield (Vector Laboratories, Burlingame, CA) containing 200 ng DAPI were placed on a coverslip, covered with the slide (upside down), and the slide was turned up for microscopy. Slides were stored in a light-protected storage box at –20°C.

Luciferase Assays

Cell pellets were stored at –80°C prior to assay. Pellets were resuspended at 1000 cells/µL in 1X reporter lysis buffer (Promega, Madison, WI) and incubated on ice for 20 minutes, followed by a 2-minute centrifugation at 13 000 rpm (Eppendorf 5415 C centrifuge) to remove insoluble debris. 20 µL of each sample was assayed on an Optocomp I luminometer (MGM Instruments, Hamden, CT) using a Luciferase Assay System (Promega, Madison, WI). The instrument was set such that 100 µL of the substrate reagent was injected, followed by a 1-second delay and a 10-second count.

Trichostatin A Treatment

Cells were treated with 200 ng/mL trichostatin A (Sigma, St. Louis, MO) in regular media for 24 hours. Media was then replaced and the cells were incubated an additional 24 hours before collection for reporter assays. Significant toxicity (> 50% cell death) was observed in HeLa cells for this concentration of the drug. Stock was 1 mg/mL in DMSO.

5-Bromodeoxyuridine Treatment

Cells were treated with 50 μM BrdU for 2-5 days in regular media. A decrease in growth rate was noted, however, toxicity was dramatically reduced as compared to TSA treatment.

Retroviral Infections

The amphotrophic retroviral packaging cell line PA317 (Miller 1990) was infected using supernatants from PE501 cells that had been transiently transfected with retroviral plasmid DNA. Following selection, supernatants were harvested from PA317 cells, purified by passage through a 0.45 μm sterile filter, and stored at –80°C for later use. Infection of target cells was carried out by 8-16 hour exposure to supernatant diluted 1:2 in regular media with 4 μg/mL (final) polybrene (Sigma, St. Louis, MO). Cells were then allowed to recover for 12-24 hours before selection.

Terminal Restriction Fragment (TRF) Analysis

Cells were suspended in 100 mM NaCl, 100 mM EDTA, and 10 mM Tris (pH 8) at 20 000 cells/μL. Genomic DNA was extracted by bringing the final concentrations of Triton X-100 and proteinase K up to 1% and 2 mg/mL respectively and incubating for 12 hours at 55°C, followed by inactivation of proteinase K at 70°C for 30 minutes. Samples were then dialyzed overnight against TE (pH 8). After dialysis, 1 μg DNA was digested with a mixture of six restriction enzymes (Alu I, Cfo I, Hae I, Hinf I, Msp I, and Rsa I) with 4 bp target sites and run on a 0.7% agarose gel overnight at 70 V. The gel was denatured for 20 minutes in 0.5 M NaOH and 1.5 M NaCl, rinsed 10 minutes in water, dried 1 hour at 55°C, neutralized

for 15 minutes in 1.5 M NaCl and 0.5 M Tris (pH 8), and probed with ^{32}P-labeled $(T_2AG_3)_4$. After washing in 2X SSC for 15 minutes and 0.1X SSC with 0.1% SDS twice for 10 minutes, the gel was exposed to a Phosphor screen and analyzed using a Storm 860 PhosphorImager (Molecular Dynamics/Amersham, Piscataway, NJ).

CHAPTER THREE
Analysis of Telomere Position Effect at the Single-Cell Level

INTRODUCTION

One of the hallmarks of a position effect is the production of a variegated phenotype. Because the assay for luciferase activity involves the extraction of protein from ~ 100 000 cells, the experiments described in Chapter 2 did not allow differentiation between uniform, graded changes in expression and "on" or "off" type switching (in which average expression level reflects the fraction of positive cells, rather than the expression level within each cell). This type of switching is implied by the phenotype in classic position-effect variegation in *Drosophila* (Muller 1930) and directly observed for telomeric genes in yeast (Gottschling *et al.* 1990) and transgenes in mouse cells (Ronai *et al.* 1999). In order to examine human TPE at the single-cell level, another series of clones was generated bearing a reporter either at the telomere or at an internal locus. In this case, however, instead of luciferase the reporter was the fluorescent protein DsRed2 (Clontech, Palo Alto, CA). This approach facilitated single cell analysis by both fluorescence microscopy and fluorescence-activated cell sorting (FACS).

After some initial difficulties that may have been related to the structure of the plasmid used to generate these clones, several lines with telomeric integrations were isolated and characterized. All showed clear variegated expression, as did many of the internal control lines. This result is not surprising since it has long been known that expression can

occur sporadically in stably transfected cells (Kalos and Fournier 1995; Walters *et al.* 1995; Walters *et al.* 1996; Wright 2003) [reviewed in (Martin and Whitelaw 1996; Dorer 1997)]. It is worth noting, however, that while the level of expression in internal controls varied widely, with some lines showing strong uniform expression, all telomeric lines showed expression in only a few percent of cells, placing them among the lowest expressing and most variegated clones. Similar results were published by another group during the course of this work (Koering *et al.* 2002). It was found that silencing could be relieved in these cells by BrdU treatment (used instead of TSA because of its reduced toxicity) and, somewhat surprisingly, by serum starvation. Importantly, it was shown through subcloning that expression in these cells spontaneously switches on and off, in contrast with the findings of Koering *et al* in C33-A cells (Koering *et al.* 2002). Thus, the observed variegation is generated by a stochastic process of switching between expression states, as opposed to progressive stable repression, or by fixing of expression states at some point during clonal expansion (possibly as chromatin domains were re-established in response to the chromosome truncation event). Evidence is presented in this chapter to suggest that the decrease in expression of the telomeric reporter following telomere elongation is primarily the result of a decrease in the fraction of positive cells as opposed to a reduction in the average level of expression within positive cells.

RESULTS

The frequency of telomeric clones was reduced with this construct

Initial attempts to isolate clones bearing the fluorescent reporter by chromosome truncation were disappointing since only internal insertions were identified. By screening an increased number of clones, three telomeric insertions were eventually identified. This represented a frequency of telomeric insertion of about 9%, whereas for both the luciferase clones discussed in the previous chapter and in the experiments of Hanish *et al* from which the method had been derived, the frequency of telomeric insertions was approximately 70% (Hanish *et al.* 1994). This may have been due to the plasmid construct used in these experiments, which had several important differences as compared to the luciferase construct used in the previous chapter. Transcription in this construct was oriented away from the telomere with almost no intervening sequences between the cytomegalovirus (CMV) promoter and the start of telomere repeats (Figure 3.1).

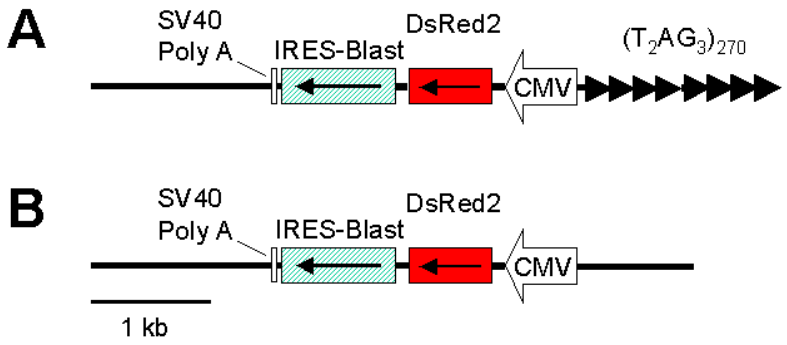

Figure 3.1 – Structure of the DsRed2 chromosome truncation vector. The CMV promoter in the truncation vector (A) sits immediately adjacent to the telomere repeats, driving transcription of the DsRed2 reporter toward the centromere (after the plasmid has been integrated). The control vector for generating internal integrations (B) has approximately 1 kb of plasmid sequence in place of the telomere repeats. The DsRed2 transcript also includes an internal ribosome entry site (IRES) in front of the coding sequence for the blasticidin resistance gene. All blasticidin-resistant clones would therefore be expected to express DsRed2 at some level during selection.

A major factor in the design of these constructs is the presence of the telomere repeats, which are notoriously difficult to work with in plasmids, frequently triggering deletions or loss of the entire plasmid when placed in certain positions or orientations. Transcriptional orientation is not expected to affect TPE [based on yeast experiments (Renauld *et al.* 1993)] and the CMV promoter was chosen because it removes the possibility of errors arising through tetracycline contamination (common in regular serum). In addition, the CMV promoter produces a sufficient level of expression to allow visualization of fluorescent proteins and TPE was shown in the previous chapter to be established even in the presence of a strong promoter.

One possible reason for the reduced frequency of telomeric clones recovered with this construct is the close proximity of the CMV reporter to the telomere repeats. To test this hypothesis, a 750 bp spacer was inserted into the original construct to provide some physical separation. Although the absolute number of stable transfectants recovered with this construct was significantly increased (~ 3-fold), only 2 of 13 clones analyzed in the first experiment contained a telomeric insertion. One of those additionally contained three internal insertions, rendering it unusable for most purposes. In the same experiment, 1 of 12 clones generated using the original construct contained a telomeric insertion. This suggests that a larger spacer is needed, that the transcriptional orientation is playing a larger role than previously suspected, or that the identity of the selectable marker used may be important (blasticidin in this case as compared to puromycin for the luciferase construct and neomycin in the original Hanish *et al* version of this experiment).

Expression in these cells is variegated

Expression in all telomeric clones was clearly variegated while expression in internal controls ranged from variegated to high uniform levels. These results (for the internal controls) are consistent with the majority of experiments concerning the behavior of stably integrated transgenes in human cells, although many of these experiments remain unpublished or buried deep within other reports (Kalos and Fournier 1995; Walters *et al.* 1995; Walters *et al.* 1996; Wright 2003) [reviewed in (Martin and Whitelaw 1996; Dorer 1997)]. As can be seen in Figure 3.2, the pattern of expression in these cells appears to involve more than a simple on/off switch. Although the majority of cells are not expressing

the transgene, differences in the brightness of cells that are expressing can clearly be seen in panel C and were observed at times in all clones.

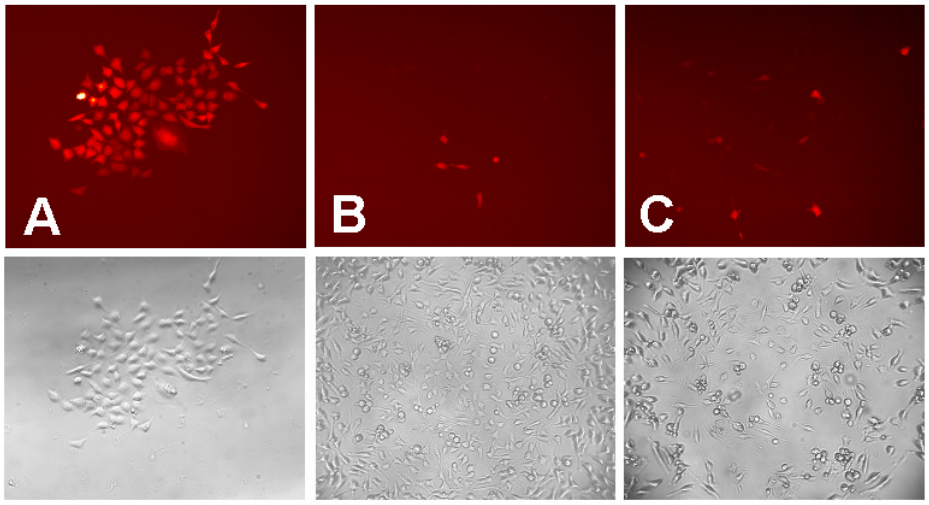

Figure 3.2 – HeLa clones expressing DsRed2. A bright field image for each clone is shown below it. Clones A and B are respectively high and low expressing internal clones. Clone C is a telomeric clone representative of the pattern observed in all telomeric clones that were obtained in this study.

This suggests a combination of the switching and graded response models. Alternatively, the appearance of these cells could be entirely explained by the switching model if the cells appearing to express at a lower level actually represent populations that have either just begun transcription of the reporter or have recently switched it off but have not yet degraded all of the DsRed2 protein. Consistent with the switching model, elongation of telomeres by overexpression of hTERT appears primarily to cause a reduction in the number of cells that are fluorescing rather than affect the average brightness of positive cells. Note that the

maximal brightness does not appear to change and that only the brightest cells in each panels A, B, C and F are microscopically visible (Figure 3.3).

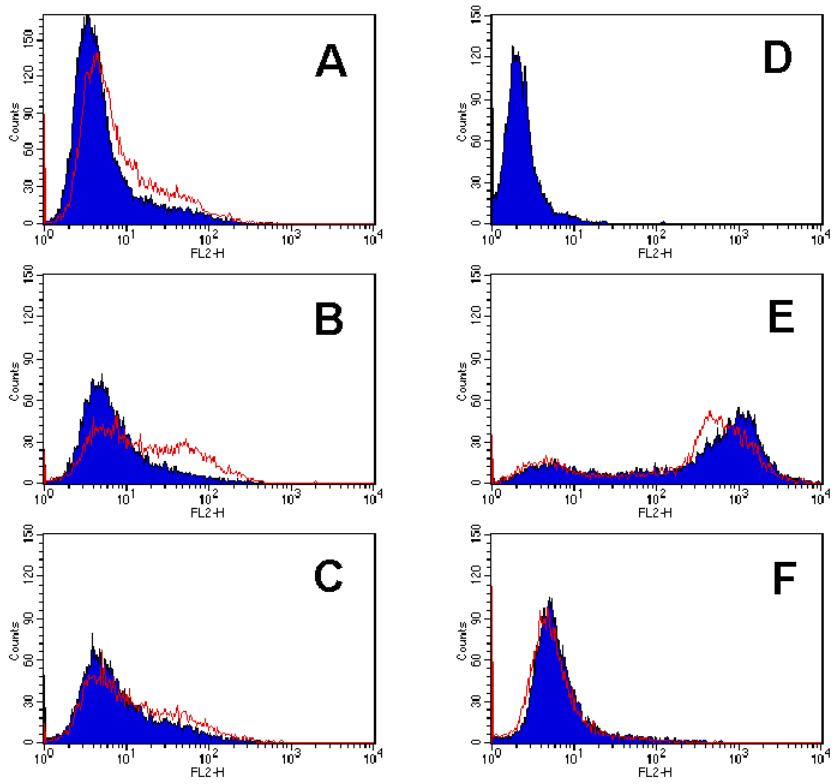

Figure 3.3 – Effect of telomere elongation on fluorescence in telomeric and internal clones. Cells infected with hTERT (telomerase) are shown as blue histograms. A red outline shows the histogram for the corresponding vector-only infected cells. The clones in panels A-C are telomeric, D is the parent cell line (negative control), and E and F are internal clones with high and low expression respectively. The unexpected tendency for internal clones to express slightly more DsRed2 after hTERT infection may be due to a reduction in oxidative stress or other growth advantage conferred by telomerase (Sharma *et al.* 2003). In clones A-C it can be seen that while the number of positive cells goes down after telomere elongation, the maximal level of expression is not significantly changed.

Silencing is relieved by BrdU or growth in serum-free conditions

Figure 3.4 shows that treatment of these cells with BrdU nearly abolishes silencing as was observed in the previous chapter using the luciferase reporter. The degree to which BrdU affected the cells was somewhat variable between clones and experiments.

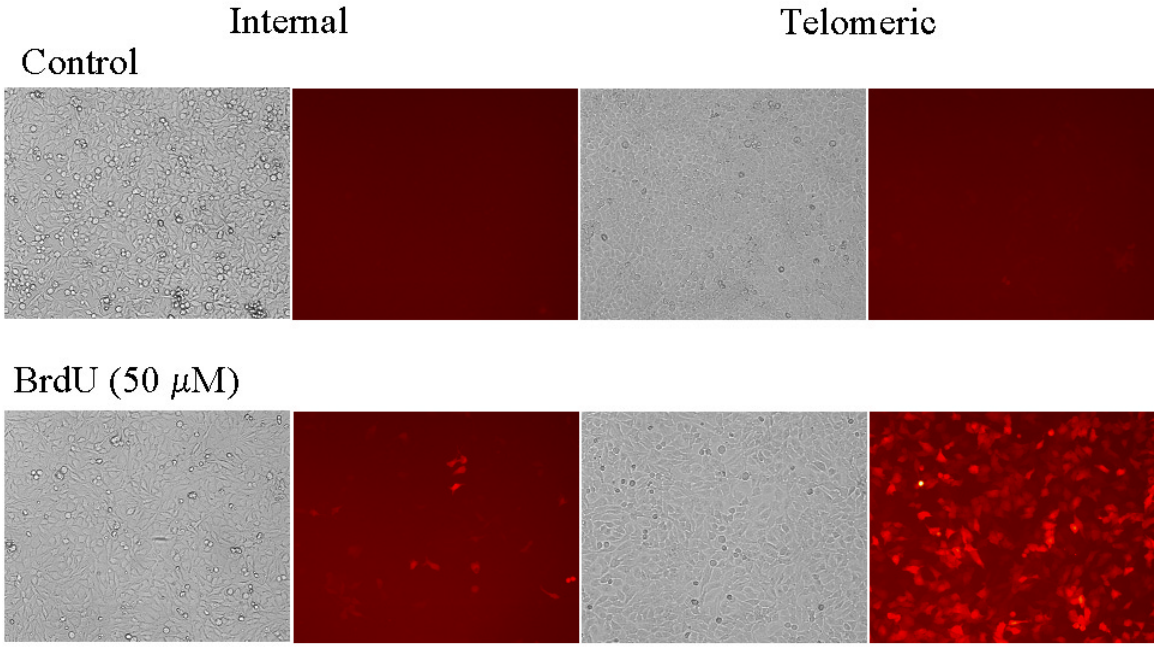

Figure 3.4 – BrdU relieves silencing of DsRed2. Bright field and fluorescent images are shown for an internal and a telomeric clone before and after a 72-hour treatment with 50 μM BrdU (5-bromodeoxyuridine). The result that telomeric clones were affected to a greater degree was not consistent for all clones and experiments.

This result shows that the transgene was intact and capable of being expressed, indicating a possible role for position effect in the silenced cells. A surprising result was that growth in serum-free media also led to desilencing (Figure 3.5).

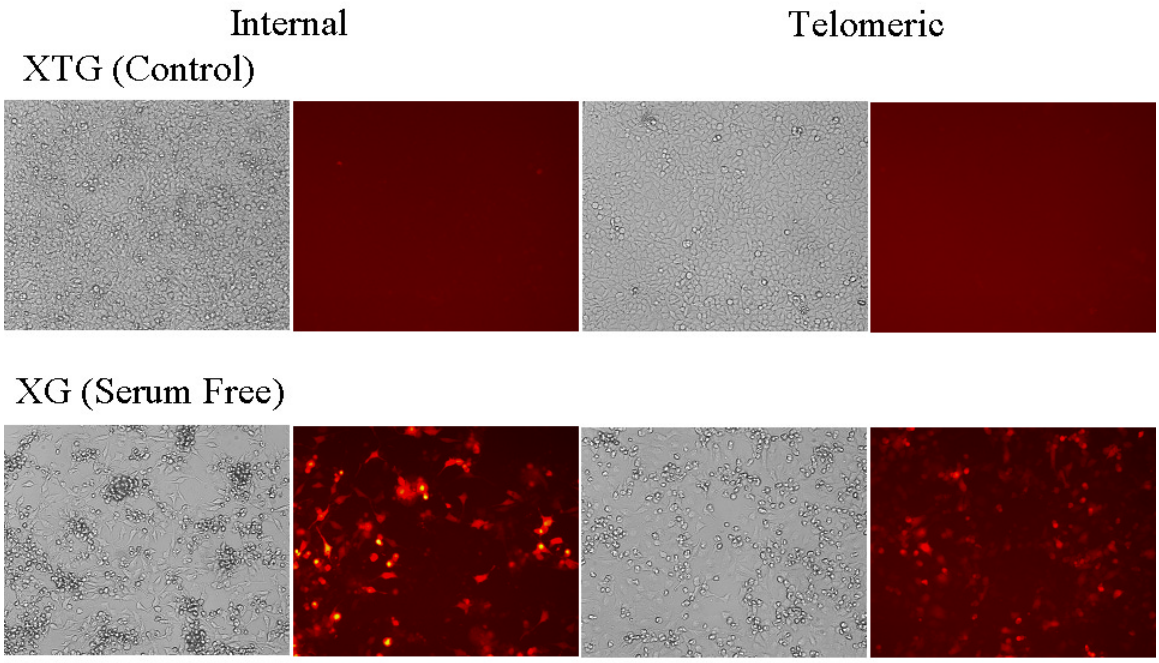

Figure 3.5 – Growth in serum-free media for 72 hours relieves silencing of DsRed2. Bright field and fluorescent images are shown for an internal and a telomeric clone in regular and serum free media. Like the BrdU treatment, serum free media produced somewhat variable increases in DsRed2 expression from experiment to experiment.

This result also held true in the luciferase containing clones in which (due to the much shorter half-life of the luciferase protein) it could be shown that growth in serum-free media triggers a distinct spike in reporter transcription after about 48 hours. Replacing the serum-free media at 24-hour intervals completely abrogated this effect, indicating that some factor was either being depleted from, or accumulating in, the media. Like the effects observed with TSA and BrdU, the serum starvation-induced loss of silencing was not specific to telomeric clones but did appear to affect them to a greater degree (Figure 3.6).

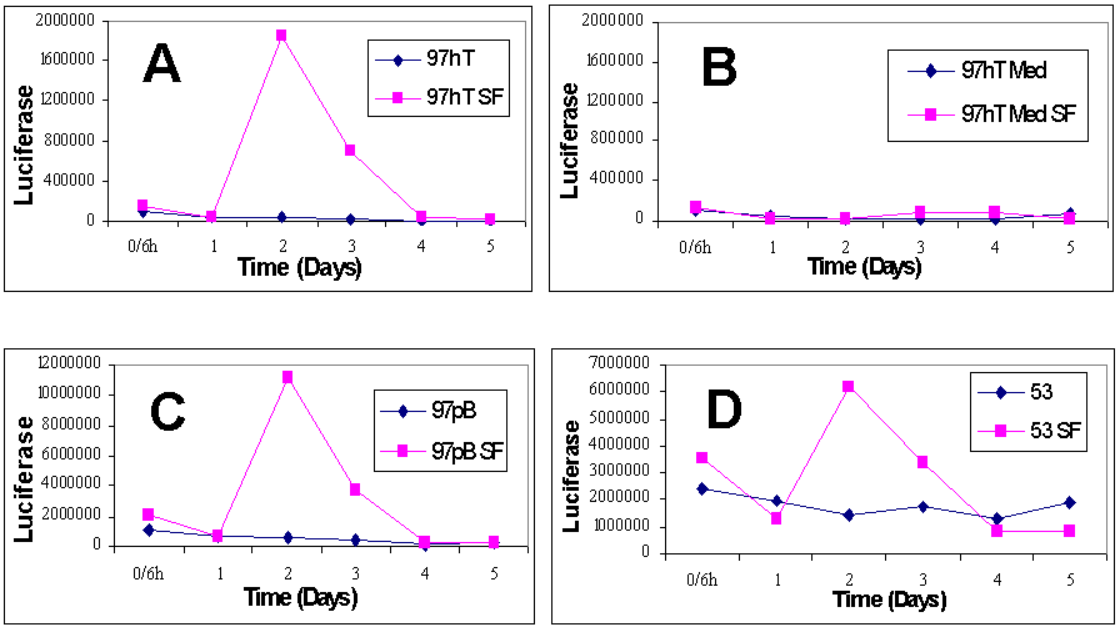

Figure 3.6 – Growth in serum-free media triggers a spike in luciferase expression at about 48 hours. Luciferase activity is shown for a telomeric clone with long telomeres (97hT) in regular (blue line) or serum-free media (pink line) in panel A. The x-axis is time in days. Panel B shows the same experiment with media changes at 24-hour intervals. In panel C, the same clone is shown with short telomeres (97pB) and in panel D an internal clone is shown (53). Note that the y-axis (luciferase activity) is scaled for each clone. The telomeric clone in panel C has a lower baseline activity than the telomeric clone in panel D but is induced to a higher level of expression by growth in serum-free media. Day 0 time points were harvested 6 hours after plating, when the cells were switched to serum-free media.

Spontaneous reactivation of the silent telomeric transgene can be directly detected in subclones

In order to detect spontaneous reactivation of the transgene, the progeny of a single cell were subcloned and followed by fluorescence microscopy. Each subclone was first observed at the 1 to 4-cell stage and was grown approximately 20 population doublings in most cases. In 18 of 19 subclones that were initially negative, DsRed2-expressing cells were detected within

1 to 2 weeks (Figure 3.7A-C), with 11 of the subclones becoming similar in appearance to the parent clone by week 3. In subclones that were initially weakly positive, both strongly positive and completely negative cells were detected within 1 to 2 weeks (Figure 3.7D).

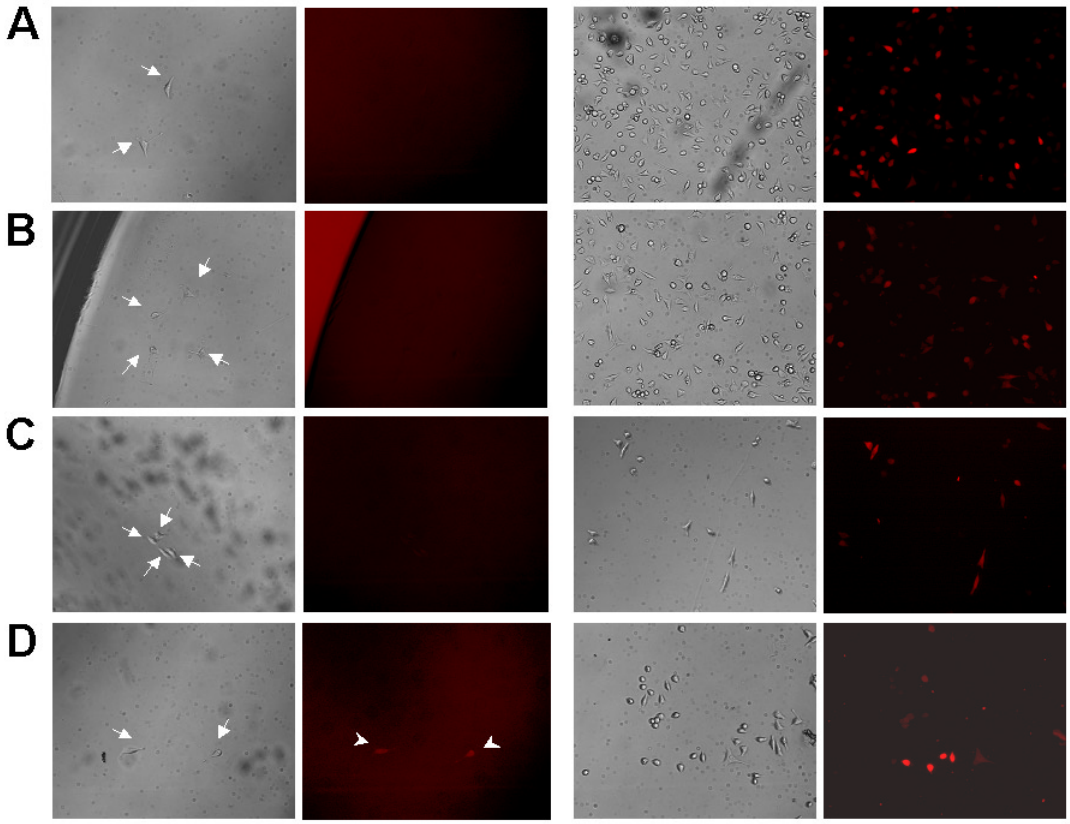

Figure 3.7 – Spontaneous reversal of silencing in cells bearing a telomeric reporter gene. Each row represents a different subclone derived from a parental clone in which the gene for DsRed2 fluorescent protein has been placed next to a newly formed telomere. Subclones were initially negative (A-C) or very weak (D) for DsRed2 expression but by 3 weeks after subcloning most had developed sporadic expression in a small fraction of cells, resembling the pattern of expression in the parental cell line. Arrows indicate the positions of cells in the original bright field images. Arrowheads indicate weakly positive cells in the fluorescent image of subclone D.

This data suggest that telomere position effect in these cells resembles the variegation observed in other systems such as at yeast telomeres (Gottschling *et al.* 1990) and some loci within the mouse genome (Ronai *et al.* 1999). This is the first demonstration of switching in the "off" to "on" direction near human telomeres and, apparently, the first direct observation that variegation in human cells can be generated through fluctuations in transcription, as opposed to progressive silencing.

DISCUSSION

Here it has been shown that the phenotype of cells bearing a telomeric transgene is variegated. The loss of silencing observed after BrdU treatment shows that the telomeric transgenes are intact and not modified any permanent way. Surprisingly, growth in serum-free conditions also led to desilencing. Spontaneous reversal of silencing was observed in subclones derived from initially negative cells. Taken as a whole, these data suggest that the position effect at human telomeres produces a phenotype fundamentally similar to what has been observed for yeast bearing telomeric reporter genes (Gottschling *et al.* 1990) and for other mammalian position effects (Butner and Lo 1986; Dorer 1997; Ronai *et al.* 1999).

The variegated phenotype is also seen for some clones with internal integration sites, consistent with previous observations by many groups (Kalos and Fournier 1995; Walters *et al.* 1995; Walters *et al.* 1996; Wright 2003) [reviewed in (Martin and Whitelaw 1996)]. Based on experiments comparing transgene expression in the presence or absence of an

enhancer it has been suggested that the primary function of enhancer elements in the human genome is to act as binary switches that turn the expression of adjacent genes from "off" to "on" without having an appreciable effect on the level of transcription for genes in the "on" state (Walters *et al.* 1995; Walters *et al.* 1996). By the classical definition of a position effect (an effect on gene expression not related to the gene or its promoter) it could be argued that the vast majority of the human genome exerts positions effects based on local enhancer strength, repetitive sequences and proximity to heterochromatin, as shown by the high frequency of variegated phenotypes within stable transfectants. Following this line of reasoning, it is important to note that the critical feature of telomere position effect is its dependence on the constantly changing length of the adjacent telomere. This and its unusual strength as a silencer, demonstrated by the 10-fold reduced average level of luciferase expression at telomeres as compared to internal sites, make the telomere region potentially unique in terms of gene regulation.

The finding that serum-starvation relieved silencing was surprising. Since changing the media at 24-hour intervals prevented the loss of silencing, it seems that either some essential factor in the media was exhausted over time or something secreted by the cells built up to a critical threshold level. It was not a response to stress caused by the lack of serum growth factors because fresh, serum-free media was sufficient to prevent the effect. Also the induction of luciferase activity by serum-free media was not an indication of tetracycline contamination in the serum for the same reason and because the effect was also seen in the CMV promoter-driven DsRed2 construct, which is not responsive to tetracycline.

The finding that spontaneous switching between "on" and "off" states can occur for a transgene next to a human telomere reinforces the idea that human and yeast telomere position effect may be very similar phenomena. Whereas in human cells, it is easy to envision a role for TPE simply based on the fact that shorter telomeres exert less of an effect, in yeast cells having a regulated telomere length (Marcand *et al.* 1997a) it seems likely that the generation of a heterogeneous population through switching is the key feature of TPE. Because this process has apparently been evolutionarily conserved, at least in some sense, from the level of a single-celled organism in which telomere length is relatively constant, it seems reasonable to hypothesize that hTPE might play a role in human cells that is at least partially dependent on this ability to switch states.

MATERIALS AND METHODS

Construction of the Truncation Vectors

The Afl III/Bfr I fragment (containing the DsRed1 protein) from pDsRed1-N1 (Clontech, Palo Alto, CA) was blunt ligated into the Sma I/Hpa I backbone from pSXneo1.6T_2AG_3 (kindly provided by T. de Lange) such that the CMV promoter was placed at the base of the telomere repeats. Next, a blunted fragment containing an IRES (internal ribosome entry site) and the blasticidin resistance gene (from pWZL Blast Sal I/ Cla I) was ligated into the Hpa I site in the same orientation as the DsRed1 protein. When DsRed2 became available, the new coding region was inserted by exchanging the DsRed fragment defined by Sal I/Not I (this

required a partial digest with Not I) and the resulting vector was designated pSXD2. The control vector lacking repeats was generated by blunt ligation after excision of the Cla I/ Sac II fragment. Due to the lack of available restriction sites, it was necessary to take an additional step before inserting "filler" DNA between the T_2AG_3 repeats and the CMV promoter. The Cla I/Bam HI fragment from pSXD2 was replaced with the Cla I/ Bam HI fragment from pSXneo1.6T_2AG_3, effectively removing the CMV promoter and part of the multi-cloning site. The Dra II/Afl III fragment from pZeoSV (Invitrogen, Carlsbad, CA) was then blunt ligated into the Sma I site, restoring the CMV promoter and adding a spacer of approximately 750 bp (consisting mainly of the F1 origin). Clones were generated as described in Chapter two except that vectors were linearized with Cla I/Pvu I (with repeats) or Pvu I alone (without repeats).

Cell Cloning

Cells were cloned by either standard ring-cloning methods or by sorting single cells into each well of a 96 well plate using a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO) and a FACStar Plus (Becton Dickinson, San Jose, CA). In the ring-cloning method, cells were plated at clonal density (~200-300 cells on a 10 cm dish for HeLa) and allowed to grow until clones reached a size that was easily visible (~1.5-2 weeks or several hundred cells). Individual clones were then isolated by placing a plastic ring over them (sealed with vacuum grease) and transferred to separate dishes by standard trypsinization methods. Sorting into 96-well plates was an automated feature of the cell sorters. 100 µL media was placed in each

well prior to sorting and cells were selected based on size criteria only (to avoid fragments and doublets).

Fluorescence Microscopy

Analysis was performed primarily on a Zeiss Axiovert 100M inverted microscope attached to a MacIntosh G4 computer using Openlab imaging software.

Fluorescence Activate Cell Sorting (FACS)

Cell were scanned on a FACScan (Becton Dickinson, San Jose, CA). For sorting during cloning and subcloning, see above.

Relief of Silencing by Serum-Free Growth

Cells were grown in regular medium (4:1 DMEM:Medium 199) supplemented with 50 μg/mL gentamicin. It is important to note that the cells require serum for attachment to the dish. It was therefore necessary to plate cells in regular medium overnight, wash at least twice in serum-free medium, and add a final volume of serum-free medium before returning them to the incubator.

Subcloning

Subcloning was carried out by sorting single cells into 96-well plates as described above. Wells containing cells were identified by microscopy the following day (2-4 cell stage) and observed regularly for 2-3 weeks before being discarded or transferred to larger dishes.

CHAPTER FOUR
In Search of the Protein Mediators of Human TPE

INTRODUCTION

In yeast, identification of the protein factors involved in telomeric silencing has proven invaluable both in terms of elucidating the mechanism of the position effect and in terms of identifying affected endogenous genes, reviewed in (Tham and Zakian 2002). A candidate approach to this problem was chosen in human cells because extensive study of yeast TPE and normal regulation of human telomeres had already implicated a long list of proteins as having possible roles in human TPE. The approach relied primarily on two techniques, overexpression of proteins and the relatively new technology of RNA interference (RNAi, described below). By the application of one or both of these techniques to a wide range of candidate proteins, it was possible to identify several factors whose expression level seems to correlate with the strength of TPE, as measured by the expression of a telomeric luciferase reporter.

RNA Interference (RNAi)

RNA interference is a term used to describe the ability of double-stranded RNA (dsRNA) to trigger the degradation, and consequent loss of expression, of single-stranded messenger RNA (mRNA) containing the same sequence. The phenomenon was discovered and named by Fire *et al* in 1998 based on the result that attempts to silence expression using antisense

RNA (single-stranded RNA complementary to a target mRNA that would theoretically bind and block translation into protein) were less successful if the antisense RNA was purified (Fire *et al.* 1998). They showed that impurities in the RNA included fragments that could anneal to create double-stranded regions (a by-product of the method used to produce the RNA) and that it was these double-stranded RNAs rather than the single-stranded antisense RNA that mediated the bulk of the observed silencing. By deliberately creating double-stranded RNA, they were able to demonstrate "potent and specific" silencing of target genes with only a few molecules of dsRNA per cell.

RNAi has since been studied extensively, almost immediately taking its place as a standard tool in the manipulation of invertebrate organisms (Bosher and Labouesse 2000). Its translation into mammalian systems however was initially thought to be impossible due to the presence of a variety of other, more drastic responses to dsRNA in these cells (Hope 2001). Primarily, this involves the two-pronged interferon response pathway, leading initially to a halt in protein production and non-specific degradation of mRNA mediated by PKR and RNase L respectively, and if prolonged, to cell death by apoptosis (Kumar and Carmichael 1998). More recently, Elbashir *et al* made the important observation that the small fragments of dsRNA that exist as an intermediate step in normal RNAi remain active yet are too small to elicit the interferon response (Elbashir *et al.* 2001). By exposing cells to these 21-23 nucleotide-long dsRNAs (referred to as small interfering RNAs or siRNAs), it is possible to induce RNAi and reduce the expression of a specific target mRNA in mammalian cells.

RESULTS

Testing of the RNAi system and initial screening of candidates.

In order to validate the RNAi system, control genes were used as the initial targets. The extremely sensitive and relatively simple assay for luciferase made it an ideal choice. Dr. Michael White's lab had previously demonstrated that this technique could be used effectively to silence expression of caveolin (unpublished observations), and so this control was also included. As can be seen in Figure 4.1, residual expression could be detected (as is typical for mammalian but not invertebrate systems) but an impressive degree of silencing was achieved for both luciferase (panel A) and caveolin (panel B).

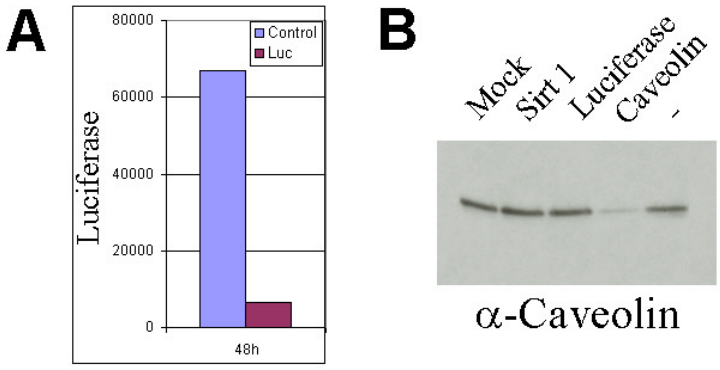

Figure 4.1 – RNAi can be used effectively to silence expression of two control genes in HeLa clones. Panel A shows luciferase activity after cells were mock-transfected (blue) or treated with a specific siRNA against luciferase (burgundy). The specific siRNA reduced luciferase activity to approximately 10%. Panel B shows a Western blot using an anti-caveolin antibody after cells were mock-transfected, treated with siRNAs against several targets, or untreated (-). Only the caveolin specific siRNA caused a reduction in the level of protein expression.

This importantly showed that the transfection efficiency for these experiments was high, indicating that a failure to observe silencing for a given target in this system is most likely related to the specific siRNA or target mRNA chosen.

In Figure 4.2, the effects of targeting various candidate proteins are shown as measured by the effect on expression of a telomeric luciferase report. Note that the lanes with the largest reduction in luciferase are controls in which luciferase itself is the target.

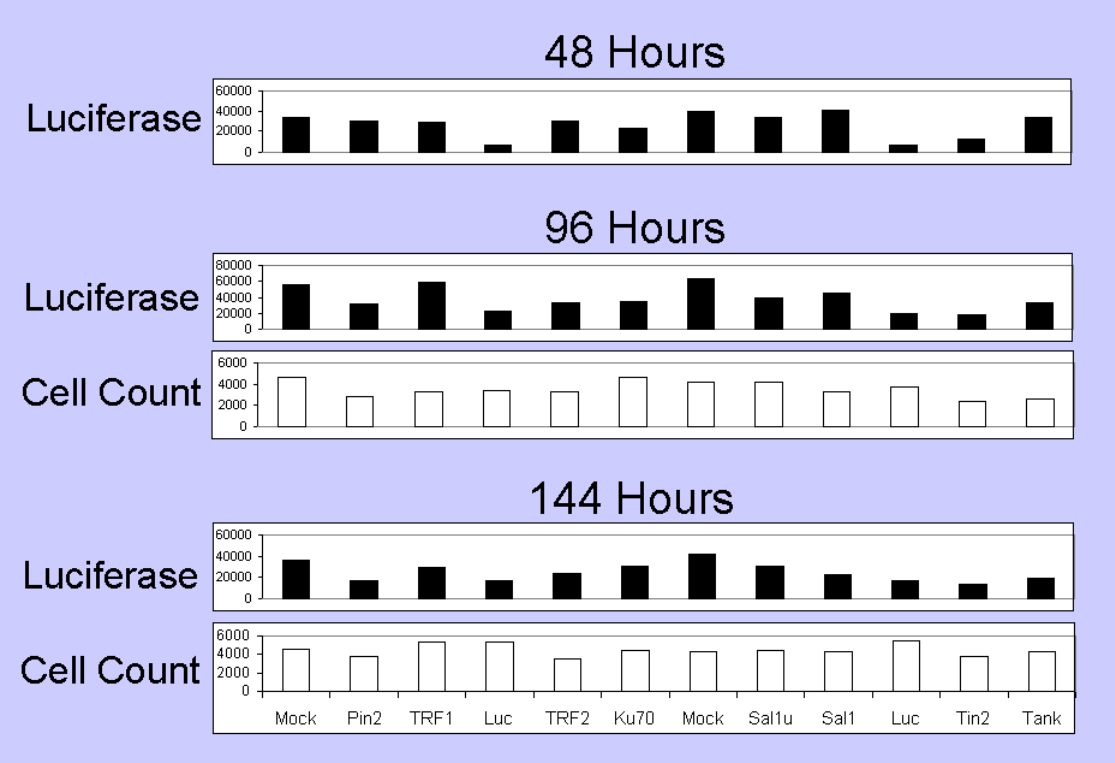

Figure 4.2 – A sample RNAi experiment in which candidate genes predicted to have a possible effect on TPE have been targeted. The target in each column is listed across the bottom. Each column shows the activity of a telomeric luciferase reporter at 48, 96, and 144 hours as well as cell count at 96 and 144 hours (open bars). Note that in initial experiments such as this one, successful silencing of the target gene was not confirmed by Western blot (i.e. a target having no effect may truly not affect TPE or may not have been silenced). Two of the lanes are controls in which luciferase has been targeted. Inhibition of none of the candidates shown here was found to relieve silencing although several, including TIN2, appeared to mildly enhance it (up to ~ 3-fold with about 50% cell death at 96 hours). Apparent increases in silencing may also have been due to non-specific toxicity.

As can be seen in this figure, there is a general trend toward a mild reduction in both luciferase activity and cell viability after treatment with siRNAs. This effect is likely to be completely nonspecific as far as TPE is concerned since most of these proteins affect telomere function, which is necessary for viability, and general toxicity seems to reduce luciferase activity in these cells. An extremely important consideration in this preliminary experiment is that antibodies were not available for most targets and consequently a negative result indicates either that the protein is not involved in TPE or that the RNAi in that case was not effective. Notable among these candidates is TIN2, a protein that interacts with human telomeres through TRF1. When targeted, this protein triggers the greatest reduction in luciferase activity, with a loss of viability that is also high but does not appear to be in proportion. However, this candidate was not pursued since the loss of a protein directly involved in TPE would be expected to cause in increase in luciferase expression. Effects of TIN2 on TPE are therefore likely to be secondary and complicated by toxicity.

hRap1

The first protein that did result in a relief of telomeric silencing when targeted with siRNAs was hRap1. Targeting of hRap1 consistently produced about a 1.5-fold increase in the activity of a telomeric reporter (Figure 4.3).

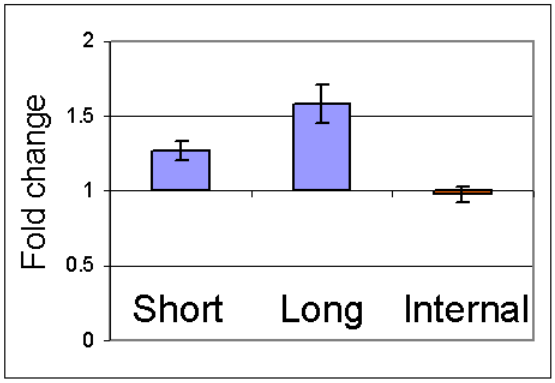

Figure 4.3 – Fold change in the expression of a telomeric luciferase reporter after RNAi against hRap1. Cells with a luciferase reporter next to a short telomere, a long telomere or at an internal site were treated with siRNA targeting hRap1. As can be seen, this resulted in a small relief of silencing near the short telomere, an increase of more than 1.5-fold near a long telomere, and no effect at the internal site. While the relief of silencing is somewhat modest, it is interesting since most non-specific effects result in a decrease in luciferase activity.

This was an especially exciting result since the yeast (*S. cerevisiae)* homolog of this protein, Rap1, is essential for yeast telomere position effect. An important difference in the human form of this protein, and one that delayed its discovery, is that it does not bind telomeres directly. Instead, it is linked though the telomere-binding protein TRF2 (Li *et al.* 2000). In this case, *S. cerevisiae* seems to be the exception since in fission yeast (*S. pombe*) the Rap1 homolog is also linked to telomeres through a second protein (Taz1p) (Park *et al.* 2002).

At the time points when relief of silencing was observed (~3-6 days), little if any reduction in the expression of hRap1 protein was detected. When lysates were taken at 24 hours, however, there was a modest but clear reduction in protein level. A second hRap1-targeting construct targeted to a different site within the hRap1 mRNA (5/6, kindly provided

by T. de Lange along with the hRap1 antibody) was more effective in reducing the hRap1 protein level (Figure 4.4).

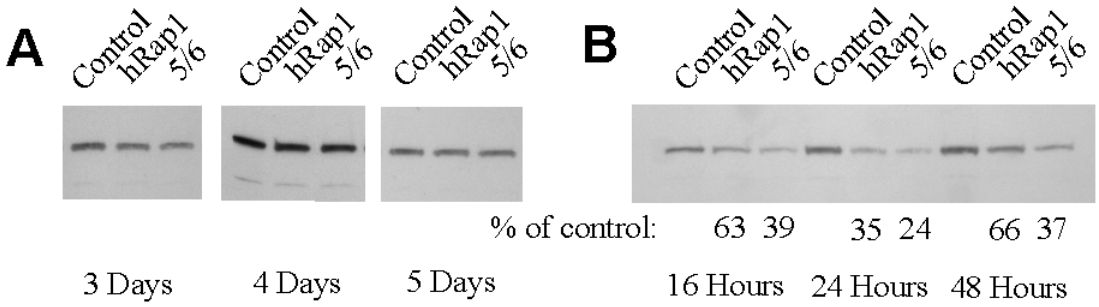

Figure 4.4 – hRap1 protein level is reduced at 24 hours after RNAi and returns to normal levels by about day 4. Control cells were treated with siRNA targeting either luciferase or hTERT, neither of which is expected to affect hRap1 expression. The lanes labeled "hRap1" were treated with the original hRap1-targeting siRNA and lanes labeled "5/6" were treated with a second siRNA targeting a different sequence within the hRap1 mRNA. Panel A shows no change in hRap1 expression at the times when luciferase expression is affected. Panel B shows that there is a reduction in protein expression immediately after transfection, peaking at about 24 hours.

These data are consistent with an essential role for hRap1 in human TPE given that the observed relief of silencing occurs with only a modest (~3-fold in the best case for the original hRap1 siRNA) and transient reduction in protein level.

An approximately 3-fold relief of silencing was achieved by transfecting cells with the original siRNA hRap1-targeting for three days in a row. A baffling result in this experiment was that the second siRNA (5/6), which was more effective at reducing protein level, was less effective at relieving telomeric silencing (Figure 4.5).

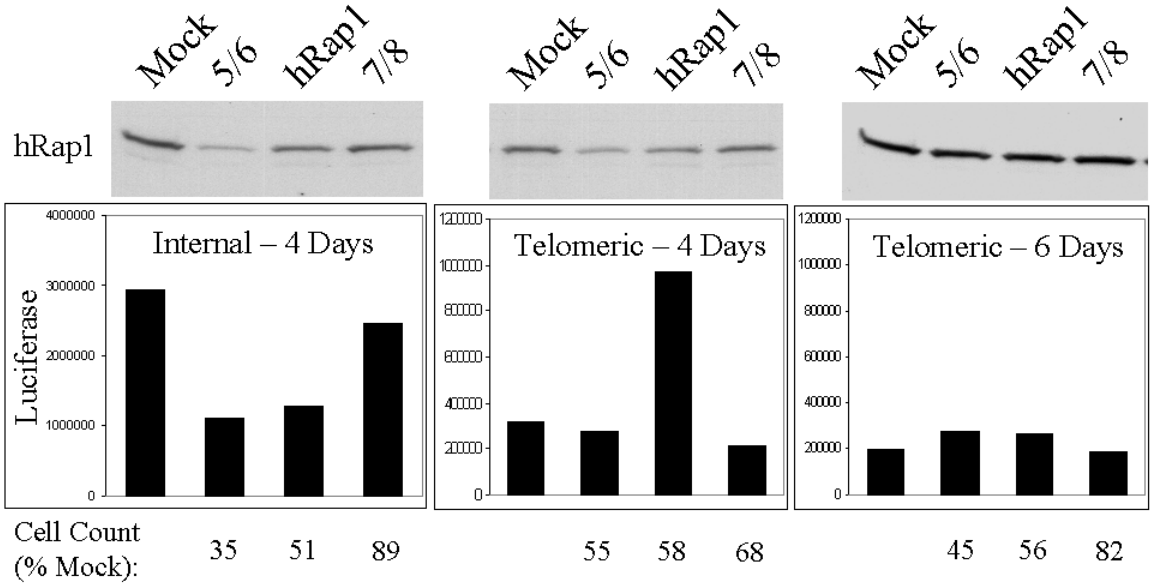

Figure 4.5 – Expression of a telomeric luciferase reporter is increased 3-fold after three sequential transfections. Cells were transfected with siRNAs or mock-transfected on days 0, 1, and 2, then harvested on days 4 and 6 for luciferase assays and Western blots. hRap1 is the original siRNA, 5/6 targets a second site in the hRap1 mRNA and, 7/8 is an siRNA known not to affect hRap1 levels. While 5/6 is more effective at reducing protein level, it has a slight negative effect on expression of the luciferase reporter on day 4 when hRap1 relieves silencing 3-fold. By day 6, both cause a modest relief of silencing. Part of the reason for this discrepancy may be the higher toxicity observed for the 5/6 siRNA but a complete explanation is lacking at present.

It is not likely that altered protein produced from truncated mRNAs played a role in generating this result since the antibody used for Western blots was polyclonal and should have detected any fragment of the protein. Toxicity may contribute to the explanation since after multiple transfections, more cell death was observed in the clones treated with the second siRNA construct (5/6). By optimizing the transfection technique, including switching to serum-free conditions, it was possible to get similar results with a single transfection, eliminating much of the toxicity (Figure 4.6).

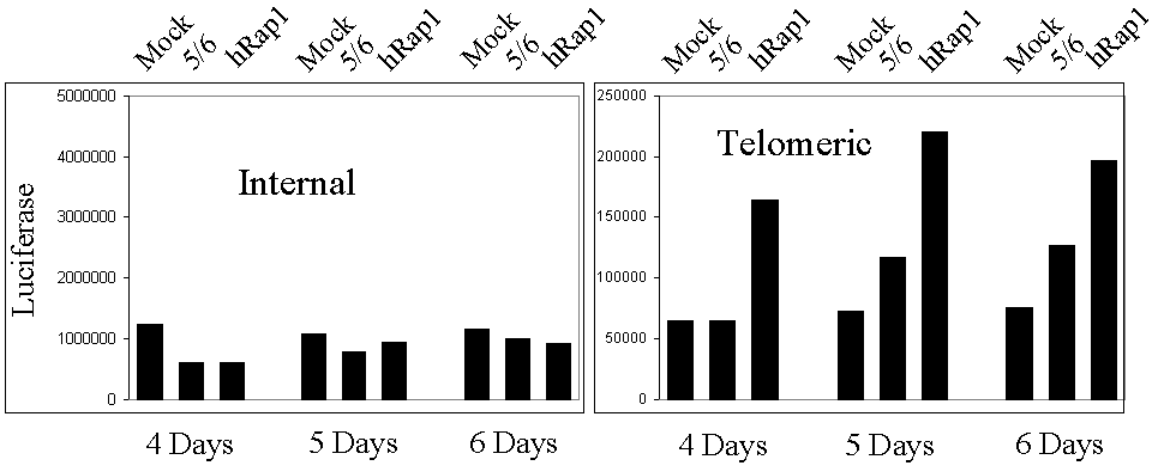

Figure 4.6 – Relief of silencing of a telomeric luciferase reporter after a single transfection of siRNAs under serum-free conditions. Cells were mock-transfected or treated with the original hRap1-targeting siRNA (hRap1) or a second siRNA (5/6). Cells bearing either a telomeric or an internal luciferase reporter were harvested after 4, 5, or 6 days and assayed for luciferase activity. Over 80% of the cells were viable (compared to mock-transfected) at all time points.

Even under conditions where most cells survive, the hRap1 siRNA is more potent at relieving telomeric silencing than is the 5/6 siRNA. Further reduction in the expression of this protein may not be possible since it is likely to be essential.

In a second series of experiments, hRap1 and three different deletion mutants were overexpressed in these cells. Surprisingly, this also led to relief of silencing although in this case a weaker effect was also apparent in internal clones (Figure 4.7).

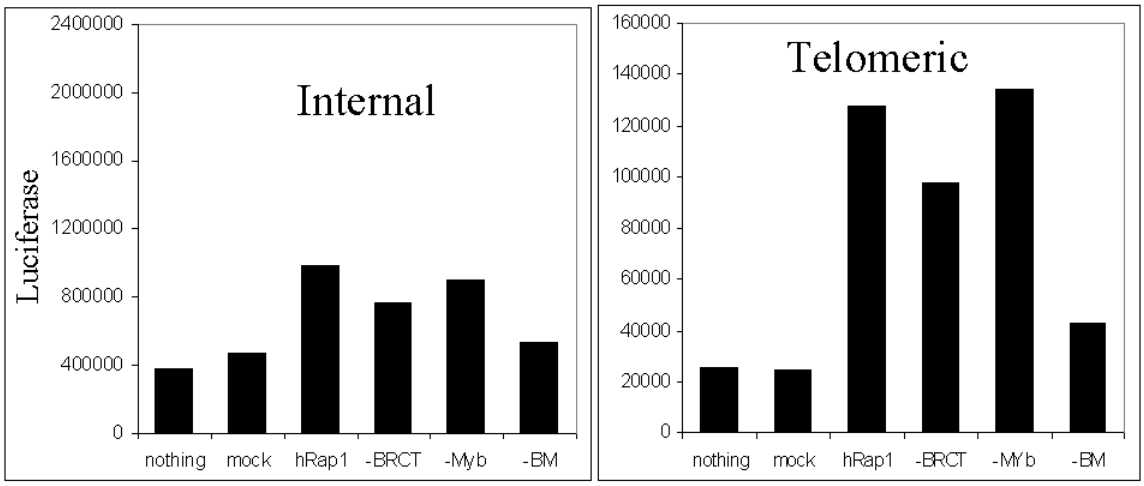

Figure 4.7 – Transient overexpression of hRap1 or deletion mutants relieves silencing of a telomeric luciferase reporter. Cells were either untreated (nothing), mock-treated, or transfected with an overexpression construct containing hRap1 or a deletion mutant lacking the BRCT domain, the Myb domain, or both (-BM). Cells were collected 48h after transfection. Although the effect is not completely specific, there is a preferential relief of silencing in the telomeric clone. The double deletion (-BM) initially appeared to be incapable of inducing this loss of silencing but was later found to be only marginally expressed (Figure 4.8).

Neither the Myb nor the BRCT domain appeared to be necessary for this relief of silencing since single deletion mutants were still effective. The double-deletion mutant did not appear to have a dramatic effect on silencing but was subsequently shown to be expressed at a lower level (Figure 4.8). These results were consistent with those obtained by stable overexpression of hRap1 and the deletion mutants.

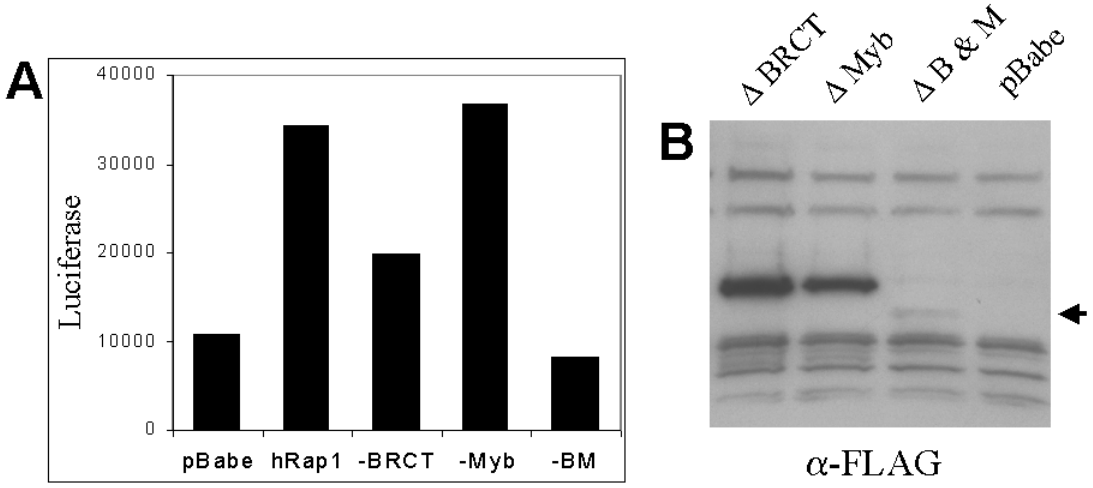

Figure 4.8 – Stable overexpression of hRap1 or deletion mutants relieves silencing of a telomeric luciferase reporter. Cells were transfected with either an empty vector (pBabe), hRap1, or a deletion mutant lacking the BRCT domain, the Myb domain or both and selected to isolate a population in which the vector had stably integrated. Cells were then collected for luciferase assay and Western blotting. Panel A shows that the BRCT-lacking mutant was less effective at relieving silencing than wild type hRap1, while the Myb-lacking mutant was approximately equally effective, and the double deletion mutant had almost no effect. Panel B shows that consistent with this, the double deletion mutant was only marginally expressed (band indicated by an arrow) while the single deletions were highly expressed. Deletion mutants were FLAG-tagged and an anti-FLAG antibody was used. The BRCT domain may have a role in the relief of silencing since the BRCT-lacking mutant is expressed more highly than the Myb-lacking mutant despite being less effective at relieving silencing.

Relief of silencing by either a reduction or overexpression of hRap1 is puzzling but may be explained in terms of a model where important silencing factors are titrated away from the telomere by the overexpressed protein. Both the recruitment of silencing factors by hRap1 and the inhibition of telomeric silencing by competition for these factors are consistent with the available data in yeast (Wiley and Zakian 1995; Cockell *et al.* 1998; Smith *et al.* 1998a; Perrod *et al.* 2001). Internal controls were affected to a lesser degree by hRap1 overexpression but were not affected at all by RNAi.

hnRNP K

A second protein that caused an upregulation of luciferase when targeted by RNAi was hnRNP K (Figure 4.9).

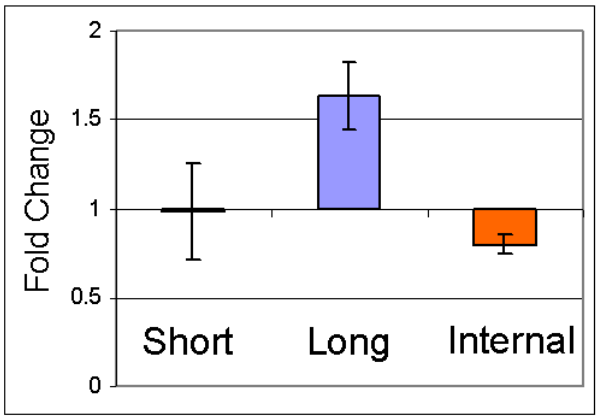

Figure 4.9 – Relief of silencing in a clone bearing a telomeric luciferase reporter after treatment with siRNA targeting hnRNP K. Cells with the reporter next to a short telomere, a long telomere or at an internal site were collected 48 hours after transfection and assayed for luciferase activity. The fold change relative to controls is shown. This treatment was highly toxic, possibly explaining the decrease in expression observed at the internal site.

This protein is not known to play a role at human telomeres but was tested based on its homology to yeast proteins known to affect TPE (Denisenko and Bomsztyk 2002). Loss of this protein was found to be extremely toxic, which was reflected by the reduction of luciferase expression in the internal control clones. Most likely, this protein had no effect on internal loci but the toxicity led to a general loss of luciferase activity. Human hnRNP K has the additional complications that it is known act as a transcription factor in some cases (Michelotti *et al.* 1996) and to be involved in the regulation of translation for certain mRNAs that contain a DICE (DIfferentiation Control Element) (Ostareck-Lederer *et al.* 2002).

Although there is no obvious occurrence of such an element in the luciferase mRNA, it is possible that some key components, or an element with similar function are present. The yeast homologs of this protein were proposed to act through elongation of the telomere adjacent to the transgene over multiple rounds of division (Denisenko and Bomsztyk 2002). This mechanism was not compatible with the effects observed here less than 48 hours after transfection of human cells with siRNAs. For these reasons and because reagents such as overexpression vectors and antibodies for this protein were not readily available, this result has not been further pursued at present.

TRF2

Because of the promising results using hRap1, overexpression of TRF2 was also tested. TRF2 is the protein that links hRap1 to the telomere. Its overexpression resulted in a loss of silencing consistent with the overexpression data for hRap1 (Figure 4.10).

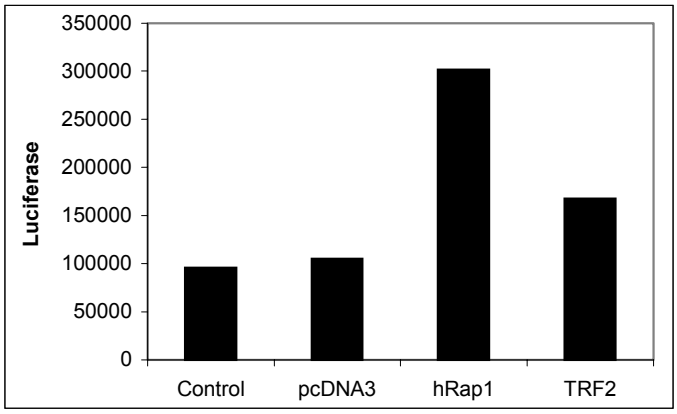

Figure 4.10 – Overexpression of TRF2 relieves silencing of a telomeric luciferase reporter. Cells were harvested 72 hours after transfection with an overexpression construct containing nothing (pcDNA3), hRap1 (known to relieve silencing) or TRF2. Control cells were not transfected. As can be seen, overexpression of TRF2 relieves silencing, albeit less efficiently than hRap1.

This is in agreement with the model that important hRap1-bound silencing factors can be titrated away from the telomere since overexpressed TRF2 would be expected to compete with endogenous, telomere-bound TRF2 for hRap1. Like the hRap1 phenotype, this relief of silencing was detectable, to a lesser degree, for internal clones.

Pot1

Pot1 is a protein that binds to the single-stranded overhang at human telomeres. When targeted by RNAi, this protein results in a decrease in the expression of a telomeric reporter (enhanced silencing) as shown in Figure 4.11.

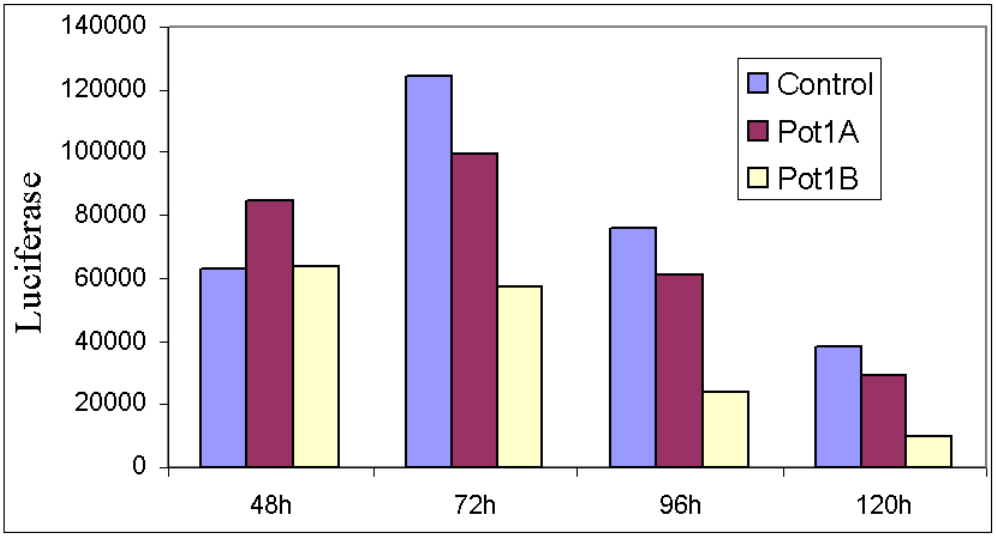

Figure 4.11 – Inhibition of Pot1 expression by RNAi enhances silencing of a luciferase reporter. Cells were either mock-transfected (control) or treated with one of two different siRNAs targeting Pot1. Unlike other targets that apparently enhance silencing, loss of Pot1 is completely non-toxic. The cells shown here contained a telomeric luciferase reporter but this effect could also be observed at internal loci.

In contrast with previous candidates producing this phenotype, loss of Pot1 (at the levels achieved by RNAi) is not detectably toxic, eliminating the most trivial explanation. Targeting Pot1 however, does not seem to produce a telomere-specific effect since internal controls are also affected. This indicates that Pot1 may be a general inhibitor of silencing.

HDAC4

Histone deacetylases (HDACs) are normally enzymes that mediate silencing. HDAC4 was tested because of some preliminary evidence in our lab indicating it might be present at telomeres. Surprisingly, eliminating expression of this protein by RNAi increased silencing in both telomeric and internal clones, as was the case with Pot1. A precedent for this puzzling result has been set by the histone deacetylase Rpd3 in yeast. Mutation of this protein enhances yeast TPE and can even restore silencing in certain defective strains (Vannier *et al.* 1996). Elucidation of the role played by HDAC4 in mammalian silencing will be an interesting challenge, however, the protein does not appear to have telomere-specific effects.

DISCUSSION

In this chapter it was shown that altering the expression of a variety of human proteins affects the degree of silencing observed at telomeres. A disappointing number of these effects were not specific to telomeric genes, having either reduced or equal effects on internal controls. A

notable exception was that reduction of hRap1 by RNAi specifically relieved silencing of telomeric and not internal luciferase genes.

An important consideration in interpreting these results is the fundamentally different organization of heterochromatin within the yeast and mammalian genomes. Silent domains in yeast are rare, primarily consisting of the telomeres, rDNA genes, and silent mating type loci, reviewed in (Huang 2002). Yeast chromosomes are too small to allow simple microscopic examination for heterochromatin (evident as darkly staining regions that may be condensed even in interphase) (Grunstein 1998) but it has been demonstrated that well over half, and possibly all, of the foci containing Sir (Silent information regulator) proteins colocalize with subtelomeric sequences (Gotta and Gasser 1996). The mammalian genome, in contrast, is riddled with heterochromatic regions (Wreggett *et al.* 1994; Cheutin *et al.* 2003). This is probably a reflection of the necessity for each human cell type to express a specific group of genes while suppressing genes appropriate to other cell types. It is easy to see after isolating transgene-containing human clones that the fraction of cells expressing and the intensity of expression within individual cells vary drastically, indicating a wide variety of genomic environments (Kalos and Fournier 1995; Walters *et al.* 1995; Walters *et al.* 1996; Wright 2003) [reviewed in (Martin and Whitelaw 1996; Dorer 1997)]. For these reasons, changes that either generally relieve or generally enhance silencing may have a tendency to appear specific in yeast but not in human cells. For example, deletion of *SIR2* in yeast relieves silencing at telomeres and has no effect on the level of expression at typical internal loci. However these yeast are also defective in silencing at rDNA and mating type loci, making them essentially devoid of heterochromatin. The failure to observe desilencing at

other internal loci may simply be an indication that these regions are fully expressed under normal conditions. In human cells, where heterochromatin is widespread and likely influencing expression of the majority of transgenes, a similar phenotype (loss of all silencing) would not appear specific to telomeres. Instead, due to the stronger than average repression observed at telomeres (10-fold lower expression for telomeric insertions as compared to random insertion), it might be predicted that desilencing of telomeric genes would occur to a greater degree but in a similar fashion to that of internal controls, as has been observed after targeting several proteins with siRNAs in this chapter.

Reduction of hRap1 expression did appear to specifically affect telomeric genes in human cells. This is not completely surprising since hRap1 is known to localize specifically to telomeres (Li *et al.* 2000) and it is consistent with the idea that hRap1 could form a specific link between the telomere and silencing machinery. In yeast mutant for the homologous protein (Rap1p), silencing is also specifically lost at the telomeres and to a lesser degree at the mating type loci (Kyrion *et al.* 1993), but not at the rDNA. Again this is expected since Rap1p binds directly to yeast telomeres and sequences near the mating type loci where it can function in recruiting the Sir-containing silencing complex. To avoid confusion, it should be pointed out that Rap1p plays multiple roles in controlling transcription within the cell, including functions within the rDNA (Miyoshi *et al.* 2001). However it is a different protein, Net1p, that recruits a silencing complex including Sir2p and Cdc14p to the rDNA (Shou *et al.* 1999; Straight *et al.* 1999).

The current data suggest that most of the silencing machinery is relatively nonspecific. In contrast to a model where a distinct set of proteins mediate all aspects of

telomeric silencing, it seems that only a few key factors such as hRap1 may be involved specifically in directing silencing to the telomere regions. These findings are consistent with the model proposed for silencing in yeast, where telomeres are thought to cluster at the nuclear periphery in a physical domain of the nucleus that contains high concentrations of silencing factors (Palladino *et al.* 1993; Gotta *et al.* 1996; Maillet *et al.* 1996). Tethering of a weak silencer to the nuclear periphery enhances its action (Andrulis *et al.* 1998) and *HML* silencers have recently been shown to physically interact with the telomeres (Lebrun *et al.* 2003), supporting the idea that localization of DNA in these repressive domains facilitates silencing. Centromeric heterochromatin seems to exist in similar repressive domains in *Drosophila* (Csink and Henikoff 1996; Dernburg *et al.* 1996), murine (Brown *et al.* 1997; Brown *et al.* 1999), and human cells, in which enhancers have been shown to function at least partially by preventing the localization of genes to these regions (Francastel *et al.* 1999). A relatively small number of proteins might therefore be predicted to specifically be involved in localization of telomeres to these domains and subsequent recruitment of a larger set of more general silencing factors.

MATERIALS AND METHODS

RNA interference

Short interfering RNAs (siRNAs) were generated by an on-campus facility and annealed to generate 21 base pair duplexes with 2 base 3' overhangs at a concentration of 20 μM as

described in Elbashir *et al* (Elbashir *et al.* 2001). Cells were transfected on 6-well dishes (on which 200 000 cells had been plated the previous day) using Oligofectamine reagent (Invitrogen, Carlsbad, CA). The transfection was carried out according to the manufacturer's instructions except that 6 μL annealed oligos and 4 μL Oligofectamine reagent were used per well and the incubation was carried out overnight (~12-16 hours) before growth medium was replaced. Initial experiments were done in the presence of serum while later experiments were serum-free (during transfection). Target sequences are listed in Appendix A.

Cell Counting

Cells were counted using a Z1 Dual Coulter Counter.

Antibodies

Caveolin – Polyclonal rabbit anti-caveolin 1 (13630 from Transduction Labs, Lexington, KY) was used at 1:5000 for Western. hRap1 – Polyclonal rabbit anti-hRap1 (a gift from T. de Lange) was used at 1:2500 for Western. FLAG – Monoclonal mouse anti-FLAG (F4042 from Sigma, St. Louis, MO) was used at 1:1000 for Western.

Western Blotting

Cell pellets were lysed in 1X Western extraction buffer (0.05M Tris, pH 7, 2% SDS, 5% sucrose) at a concentration of 10,000 cells/μL. Lysates were sonicated using a probe sonicator for 9 seconds at 50 J/Ws and stored at -20°C. Samples were diluted 1:2 in 2X Laemmli buffer (0.125M Tris, pH 6.8, 10% β-mercaptoethanol, 0.002% bromophenol blue,

4% SDS, and 20% glycerol for 2X) heated to 95º C for 2 minutes and run on an appropriate concentration SDS-PAGE gel (~10% for hRap1). Gels were transferred to PVDF membrane (Millipore, Billerica, MA) for 75 minutes at 100 mA and blocked in PBS plus 0.05% Tween-20 containing 5% milk. Primary antibody was diluted as stated above in PBS plus 0.05% Tween-20 containing 0.5% milk and incubated for 1 hour at room temperature, followed by a 1-hour incubation in a 1:8000 dilution of goat anti-rabbit antibody (31463 from Pierce, USA) or sheep anti-mouse antibody (NA 931 from Amersham) as appropriate, coupled to horseradish peroxidase. Blots were washed extensively and signal was detected using the ECL detection system (RPN2109 from Amersham, Piscataway, NJ) and X-ray film.

Transfections

Cells were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. For transient transfections, cells were assayed after 48-72 hours. For stable transfections, cells were allowed to recover overnight, then selected depending on the resistance gene being used (~1 μg/mL blasticidin or 600 ng/mL puromycin 5-7 days for these cells).

CHAPTER FIVE
The Hunt for Endogenous Genes

INTRODUCTION

Now that the ability of human cells to suppress the expression of a transgene placed artificially close to a newly formed telomere has been established, a key question to ask is does this happen to endogenous genes in human subtelomeres? It is not an easy question to answer, since human subtelomeres contain a great deal of repetitive DNA, pseudogenes, and many duplicated regions [reviewed in (Mefford and Trask 2002)]. On top of this, human subtelomeres are polymorphic within the population, with some individuals possessing hundreds of kilobases of subtelomeric DNA that others lack (Wilkie *et al.* 1991; Riethman 2003). These sequences are among the last to be sequenced by the human genome project and have frequently been mismapped. Today, detailed information is known for only a few chromosome ends (Ciccodicola *et al.* 2000; Daniels *et al.* 2001; Heilig *et al.* 2003).

Even in yeast, where the genome has been complete since 1996 and genetic manipulation is relatively simple, it took until the year 2000 (10 years from the first report of TPE in yeast), to demonstrate silencing of a true endogenous gene (Vega-Palas *et al.* 2000). In this case expression of the gene was not detectable in wild type yeast but was strongly upregulated in a SIR deleted strain, and the second important result was presented that many genes at endogenous yeast telomeres are not subject to TPE. The equivalent experiment is currently not possible in human cells due to the lack of a non-essential protein known to be

required for TPE, although it may be feasible to conduct similar experiments using RNAi to partially eliminate hRap1.

Initially, the identification of endogenous genes in human cells that are subject to TPE was approached by selecting candidate genes from known telomeric regions to test by RTPCR and Northern blotting. In the absence of a known mutation that could abolish TPE, expression was compared in cells with short and long telomeres. If the primary hypothesis that telomere position effect in human cells is regulated by telomere length is correct then a detectable difference in the expression of subtelomeric genes should be present in these two populations. The possibility existed at the time that a large proportion of subtelomeric genes would be strongly influenced by telomere length. This does not appear to be the case, however, and so this problem may need to be tackled in the future through the use of cDNA arrays to test as many genes in as many tissues as possible.

RESULTS

The hTERT (telomerase) gene is not affected by TPE in HeLa and is not located as close to the telomere as previously suspected

Probably the most attractive potential role for TPE at the time of its discovery was in the regulation of the human telomerase protein component (hTERT). At the time, this gene was thought to be the most distal on chromosome 5p (Bryce *et al.* 2000), suggesting the attractive hypothesis that a position effect due to long telomeres could suppress expression of the gene

for telomerase, effectively regulating telomere length. While the converse, that short telomere length could trigger activation of the telomerase gene made sense for telomerase-positive cells, it had disturbing implications for telomerase-negative cells since this mechanism would tend to aid in escape from senescence.

As can be seen in Figure 5.1, telomere length had little effect on telomerase expression in HeLa cells.

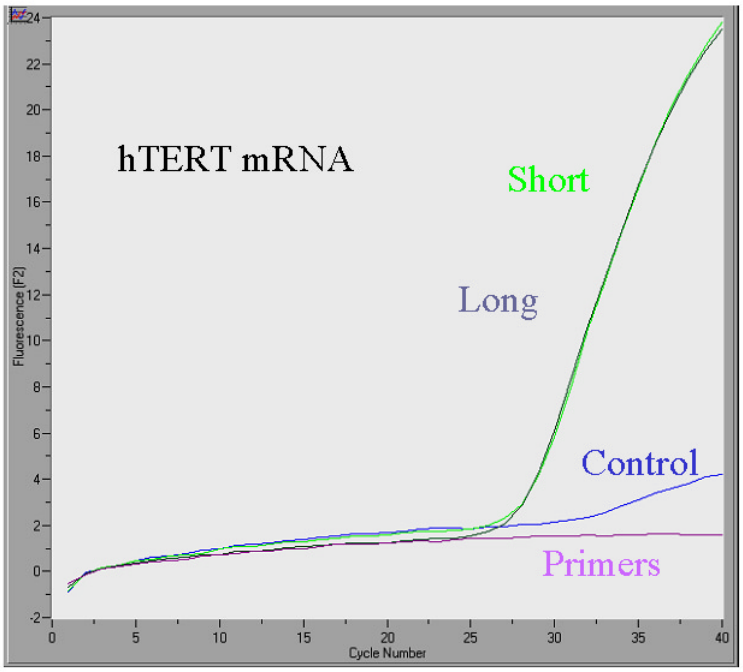

Figure 5.1 – The level of hTERT mRNA in HeLa cells is not affected by telomere length. Real-time RTPCR was performed on total RNA from HeLa cells with short or long telomeres, telomerase negative BJ fibroblasts (control), or water (primers). The target region was within the 3' UTR of the endogenous hTERT mRNA, which is not present it the constructs used to elongate telomeres through exogenous telomerase expression. Telomere length was not found to significantly affect expression of telomerase in these cells.

This experiment was not entirely conclusive however since HeLa cells (and any other spontaneously telomerase-positive cell line) may have lost normal regulation of telomerase. It is even possible that the hTERT gene escapes a position effect in some cells by translocation or because of a mutation in one of the mediators of the position effect. Translocation is not likely the cause of telomerase activation in a subset of immortal cells that includes HeLa since the introduction of a single wild type copy of chromosome 3 (Ohmura *et al.* 1995), 4 (Backsch *et al.* 2001), 5 (Kugoh *et al.* 2003), 6 (Steenbergen *et al.* 2001), 10 (Nishimoto *et al.* 2001), or 17 (Yang *et al.* 1999), depending on the cell line, has the potential to restore silencing. In normal cell lines, any activity of the hTERT promoter is below the threshold of detection (Harley *et al.* 1990) and so any telomere-length dependent changes in its expression cannot be measured. Interest in the idea that hTERT might be regulated by telomere position effect waned after several remappings of the hTERT gene by Celera (Rockville, MD) and the Human Genome Project placed it more than a megabase from the chromosome end. Although action of TPE over such a large distance cannot be ruled out by the current data, it does not seem likely and given the difficulty in measuring telomerase expression in any situation where it is not known to be disregulated [working with stem cells is problematic and telomerase expression in hematopoetic cells is regulated by stimulation (Weng 2002)], other genes located closer to telomeres should be more informative at present.

Promising candidates from existing literature

Other candidates were selected based on published work placing them within a short distance (~ 100 kb in most cases) of the telomere. These included POLR3K (Daniels *et al.* 2001), RABL2B (and A as a control) (Wong *et al.* 1999), PGPL (Gianfrancesco *et al.* 1998) and HMRT1L1 (Katsanis *et al.* 1997). All were analyzed by RTPCR and if expression level permitted, by Northern. Normalizing expression when comparing cells with different telomere lengths is not trivial. Older cells (short telomeres) divide slower and are much larger than younger cells (long telomeres) and so a variety of transcripts are altered in a way that is probably not directly related to TPE. β-2-microglobulin was initially selected as a control gene based on a published report showing it was superior to other control genes (including β-actin and GAPDH) for comparing normal to serum-starved cells (Schmittgen and Zakrajsek 2000). Using this method, the data supporting a position effect looked promising (Figure 5.2A). After a more extensive analysis however, it was determined that the expression β-2-microglobulin itself was changing in these cells since β-actin, GAPDH, and all telomeric genes maintained their relative proportions at various telomere lengths (Figure 5.2B). Thus, no position effect was detected.

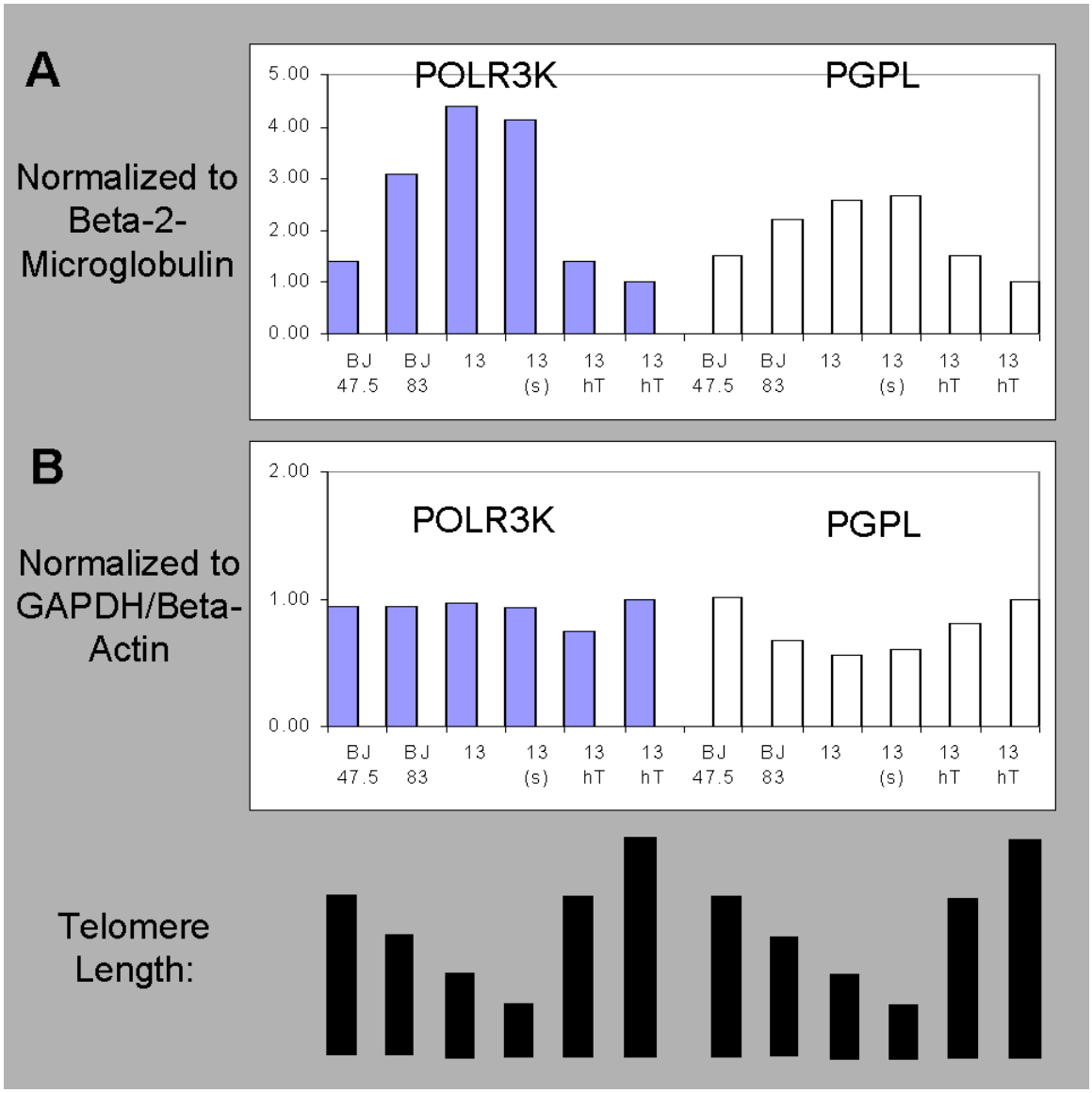

Figure 5.2 – Telomere length does not affect expression of two telomeric genes, POLR3K and PGPL, in BJ fibroblasts. Data presented are Northern blot quantitations normalized to control genes. Expression of POLR3K is shown in blue and expression of PGPL is shown in burgundy. Samples are BJ cells at population doubling (PD) 47.5 and PD 83, a clone with short telomeres (13), the same clone at senescence (s), and the clone after infection with hTERT (telomerase). Telomere length in each sample is represented schematically at the bottom of the figure. Panel A shows an apparent position effect dependent on telomere length when expression is normalized to β-2-microglobulin. Panel B, however, shows that this effect disappears when other control genes (average of β-actin and GAPDH shown) are used. The most likely interpretation is therefore that β-2-microglobulin is a poor choice for a control gene in theses studies and no position effect is present.

Genes supplied by Dr. Harold Riethman

During the course of this work, a collaboration was established with Dr. Harold Riethman (The Wistar Institute, University of Pennsylvania). His lab is involved in completing the telomeric regions of the human genome project through sequencing of half-YAC (Yeast Artificial Chromosome) clones (Riethman *et al.* 2001). In this process, segments of human DNA are captured with a linear vector that contains only one functional telomere. The other end of the vector must therefore be protected by a human telomere and so the captured DNA is normally the terminal fragment of a human chromosome (Riethman *et al.* 1989). From this data, Dr. Riethman has generated a list of telomeric genes, which he kindly shared. Using this information, a second series of RTPCR primers was generated in order to test a new set of telomeric genes. As can be seen in Figure 5.3, no effect of telomeric length on the expression of these genes was detected.

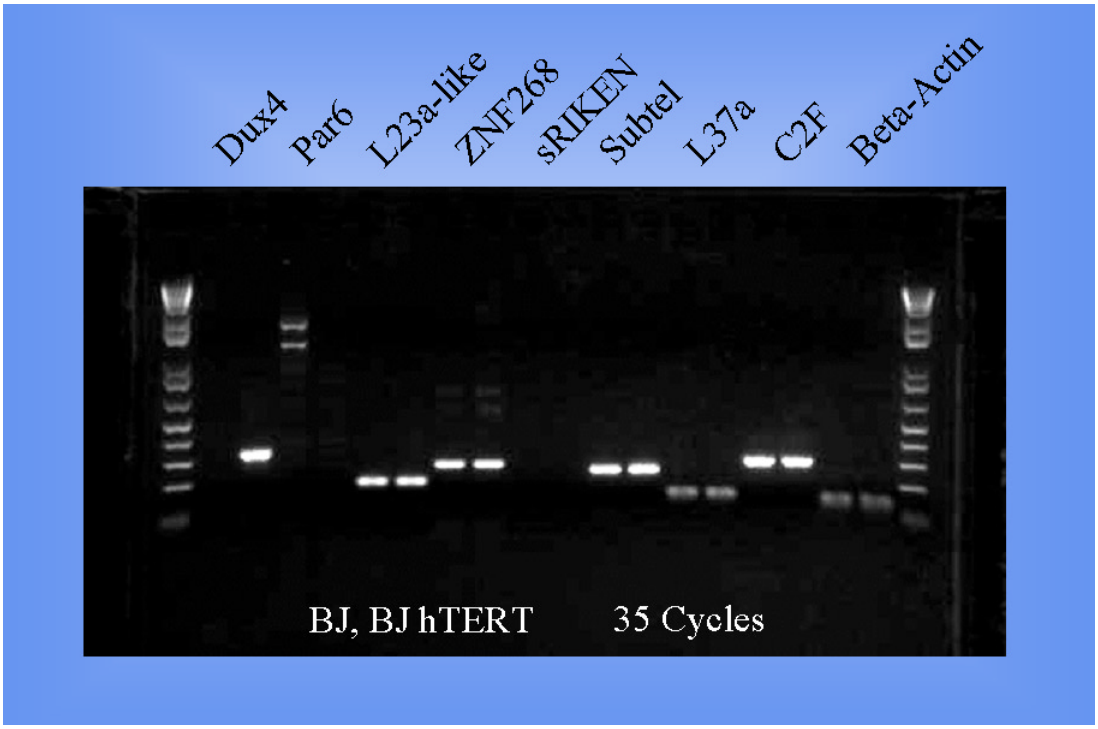

Figure 5.3 – No effect of telomere length on the expression of seven subtelomeric sequences in BJ fibroblasts. RTPCR was performed on total RNA from BJ fibroblasts or BJ hTERT with elongated telomeres. Results were verified at various cycle numbers for each mRNA (the C2F signal in this image is saturated but also shows no difference within the linear range of amplification). No signal was detected for Dux4, Par6, or sRIKEN. The visible Par6 bands are non-specific and the Dux4 signal in the BJ hTERT lane is the result of low-level DNA contamination. Dux4 can be present in up to several hundred copies within the genome (Hewitt *et al.* 1994) and so can produce a signal from a tiny amount of contaminating DNA that does not affect the signal from other genes (verified by DNase treatment).

DISCUSSION

It is difficult to know how to interpret the failure to detect TPE in these candidate genes. As discussed in the introduction to this chapter, it is difficult even to be certain that a gene is truly telomeric in a given cell line. For example POLR3K, which was examined in this

chapter, is located on chromosome arm 16p, which is known to be polymorphic within the population (Wilkie *et al.* 1991). In the BJ cells used for most of these studies, it is not known whether the POLR3K was actually at the telomere as assumed or whether it was located hundreds of kilobases away. Based on yeast data, it is expected that not all endogenous telomeric genes (if any) will be affected by TPE (Vega-Palas *et al.* 2000). It is also possible that this process will be tissue specific as is clearly seen for position-effect variegation in whole organisms [e.g. *Drosophila* eye color (Muller 1930) or mouse coat color (Rakyan *et al.* 2002)]. For these reasons, it is almost impossible to thoroughly demonstrate a lack of TPE.

The next step will likely be to use cDNA arrays to test a much larger sample of subtelomeric genes simultaneously. Others have recently taken this approach in a variety of unpublished experiments, but their work has been subject to various limitations such as a very limited sampling of telomeric genes [either due to the lack of available information or their under-representation in commercially available products (Mefford and Trask 2002)] and the use of senescent cells as the "short telomere" population since senescence involves a massive shift in gene expression.

MATERIALS AND METHODS

RNA Isolation

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) or the RNAqueous kit (Ambion, Austin, TX) according to the manufacturer's instructions.

Real-Time RTPCR

Real-time RTPCR for the 3' UTR of endogenous hTERT message was performed on a LightCycler (Roche, Basel, Switzerland) using the hybridization probes method. Primers and probes were obtained from IT Biochem (Salt Lake City, UT). Primers were sense: CGCACCGCTGGGAGTCTG, and antisense: GGTGAACAATGGCGAATCTGG. Probes were AGCCAAGGGCTGAGTGTCCAGCA-FITC and LCRed640-CCTGCCGTCTTCACTTCCCCACAG-phosphate. The standard hybridization probes kit (2 015 145 from Roche) was used as recommended. Final Mg^{2+} concentration was 6.0 μM. Primers were used in a 1:2 ratio (sense:antisense) to give final concentrations of 0.67 μM and 0.33 μM respectively. Probes were used in a ratio of 1:2 (FITC:LCRed640) to give final concentrations of 0.2 μM and 0.4 μM respectively. Samples were denatured for 30 seconds at 95°C, then subjected to 40 cycles of 95°C for 5 seconds, 60°C for 10 seconds [followed by fluorescence resonance energy transfer (FRET) measurement], and 72°C for 15 seconds.

Northern Blotting

1/5 volume of RNA denaturing loading dye (80 µL 10X MOPS, 140 µL formaldehyde, 400 µL deionized formamide, 20 µL of 1 mg/mL ethidium bromide, and 90 µL 10X loading stock (50% glycerol, 1 mM EDTA, 4 mg/mL bromophenol blue, and 4 mg/mL xylene cyanol)) was added to ~10 µg total RNA, which was then heated to 68°C for 5 minutes. Samples were run on a 1% denaturing (8% formaldehyde) gel in 1X MOPS buffer and capillary transferred to a nylon (Hybond-N+) membrane overnight in 20X SSC. The membrane was crosslinked twice on the "Autocrosslink" setting using a Stratagene (La Jolla, CA) Stratalinker 2400 UV crosslinker, pre-hybed with 7% SDS in 0.25 M sodium phosphate (pH 7.2) at 65°C for 45 minutes and probed for 8-16 hours in the same buffer. Probes were generated from plasmid-based sequences (β-Actin and GAPDH) or RTPCR products (see below) by randomly primed incorporation of α-^{32}P-labeled dCTP using the Radprime kit (Invitrogen, Carlsbad, CA). The membrane was then washed twice with 5% SDS in 0.2 M sodium phosphate buffer and twice with 1% SDS in 0.2 M sodium phosphate for 30 minutes each at 65°C. Finally, the membrane was exposed to a Phosphor screen and scanned using a Storm 860 PhosphorImager system (Molecular Dynamics/Amersham, Piscataway, NJ).

RTPCR

RTPCR was carried out using total RNA and the One-Step RTPCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In this kit, reverse transcription (primed by the PCR primers) is carried out in the same tube as the PCR amplification. PCR reactions were denatured for 30 seconds at 94°C, annealed for 30 seconds at 55-60°C, and extended for

1 minute per kilobase at 72°C (30 seconds minimum). Samples were then run on a 1% agarose gel in 0.5X TBE. Primer sequences are listed in Appendix B.

CHAPTER SIX
Discussion

While the phenomenon of telomere position effect in human cells is of some interest in its own right, its true importance will ultimately depend on whether or not it provides insight into the behavior of endogenous subtelomeric genes in a natural setting. The following discussion will highlight several areas that merit further consideration and future investigation.

RANGE OF TPE

An important and obvious question to address is over what distance can TPE be predicted to act? The luciferase construct used in the current experiments placed a tetracycline-inducible promoter approximately 2 kb from the start of telomere repeats such that the gene was transcribed toward the telomere. The DsRed2 construct placed the CMV promoter within a few base pairs of telomere repeats such that the gene was transcribed away from the telomere. During the course of this work, another report appeared in which TPE was detected using an EGFP reporter that was transcribed toward the telomere from a CMV promoter placed just over 1 kb from the start of telomere repeats (Koering *et al.* 2002). In all of these cases, the reporter lies well within the approximately 6 kb limit observed for the spreading of yeast TPE from a truncated telomere (Renauld *et al.* 1993). But is that limit

likely to apply to human telomeres? As discussed briefly in Chapter 5, yeast telomeres are about 350 (± 75) bp in length (Tham and Zakian 2002) while telomere lengths in young human cells can easily reach 20 to 30 times that number (de Lange *et al.* 1990). Since TPE in both of these systems is dependent on telomere length, the human equivalent limit for spreading of TPE could be predicted to be 120-180 kb. Besides transcriptional status, position effects can influence other properties of chromatin such as replication timing. It has been shown that in a human cell line, the presence of a telomere (due to a naturally-occurring chromosome truncation) delays the replication of a region at least 40 kb away (Ofir *et al.* 1999). Although transcription of a gene located 54 kb from the telomere (nearest available) does occur from both alleles in these cells, it is not entirely clear whether or not the expression level is affected by the presence of a telomere.

It could be [and in fact has been (Wright and Shay 1992)] suggested that it is the chromosome end itself, and not the double-stranded region that is important for TPE. In this model, the ends of human and yeast telomeres would be considered equivalent and moreover, the tips of human telomeres are separated from the genes they might influence by a long region of double-stranded telomere repeats. This model has proven flawed, however, as data has accumulated. In both human and yeast TPE, when telomeres shorten and the chromosome end gets closer to subtelomeric genes, silencing is relieved, not enhanced (Kyrion *et al.* 1993; Buck and Shore 1995). In yeast, the double-stranded telomeric DNA binding protein Rap1p is responsible for recruiting the Sir-containing silencing complex [reviewed in (Tham and Zakian 2002)]. The current data suggests that the human homolog

of this protein, hRap1, may play a similar role after being recruited by the double-stranded telomeric DNA binding protein TRF2 (Li *et al.* 2000).

Given that the extent of silencing is almost certainly determined by the double-stranded region, and having limited information with which to predict the distance over which telomeric heterochromatin could spread, one might turn to other systems such as classic position-effect variegation (PEV) in *Drosophila*. In this system, variegation has been reported to extend as much as 100 kb (Weiler and Wakimoto 1995), or 50 (Demerec 1941), 22-80 (Demerec 1940), or 65 (Hartmann-Goldstein 1967) polytene chromosome bands from a breakpoint near centromeric heterochromatin. Since an average polytene band represents about 20 kb (Hartl *et al.* 1994) (ranging from 5-50 kb), the latter three numbers represent distances in the area of one megabase (Mb). In *Drosophila virilis*, variegation was observed for a gene located approximately one third of the entire chromosome (chromosome 5) from a breakpoint (Baker 1954). In the mouse, position-effect variegation at endogenous loci has been shown to result from translocations involving the inactive X chromosome (Russell and Bangham 1959; Cattanach 1974). An effect has been reported for a gene 25 crossover units (centiMorgans, cM) from the junction with inactive X chromosome sequences (Russell and Montgomery 1965). Since an average crossover unit is about 2 Mb and the average mouse chromosome is about 75 cM long, this distance represents about 50 Mb, or one third of a chromosome (Silver 1995). Similar X chromosome effects have been reported in humans (Engel *et al.* 1971; Thelen *et al.* 1971), and data consistent with variegated gene expression has been correlated with a delay in replication timing (Couturier *et al.* 1979). In that report, variegation in the expression of Cu-SOD at 21q22.1 was observed in a translocation between

Xq27 and 21q11, indicating the effect was propagated approximately 20 Mb. X inactivation is a process that is designed to target (most of) an entire chromosome so it is perhaps unlikely that position-effects attributable to other causes would display such long-range effects. Position effects on transgene expression in mice have been shown to result from insertion near centromeric heterochromatin (Butner and Lo 1986; Festenstein *et al.* 1996; Graubert *et al.* 1998) or telomeres (Murnane 2001; Ramirez *et al.* 2001), but in the majority of cases unknown factors at seemingly random genetic loci mediate these variegating effects (Graubert *et al.* 1998) [reviewed in (Martin and Whitelaw 1996)]. Because PEV in mouse cells can be the result of unknown mechanisms, no firm conclusions can be drawn about the distance over which these effects might act. Variegation at many random sites could be due to long-range action of centromeric or telomeric heterochromatin, and variegation near centromeres or telomeres might be coincidental, and more correctly attributed to some other factor. A notable exception is the telomere position effect observed by Murnane *et al* (Murnane 2001), which was reversed by elimination of the telomere (by chromosome fusion).

With so little detailed knowledge to build on, all things remain possible at this stage. A final consideration in the potential distance over which human telomere position effect could act is the influence of endogenous subtelomeric sequences. In *Drosophila*, subtelomeric repeats, rather than the terminal retrotransposons are thought to mediate telomeric silencing (Cryderman *et al.* 1999) and in fact the retrotransposons have recently been shown to have an activating influence (Golubovsky *et al.* 2001). In *S. cerevisiae*, at least the two major subtelomeric repeated elements, X and Y', have been shown to

dramatically modify TPE (Fourel *et al.* 1999; Pryde and Louis 1999). The current hypothesized result is that the position effect at endogenous yeast telomeres might be propagated in some cases well beyond the limits observed at truncated telomeres, and that sub-domains permissive for transcription may exist within this region (Fourel *et al.* 1999; Pryde and Louis 1999). Supportive of this model, some heterochromatic sequences in Drosophila have been shown to induce variegation only in the presence of a threshold amount of repetitive DNA or some addition enhancer of variegation (Tartof *et al.* 1984; Pokholkova *et al.* 1993). Interestingly, the silencing elements found at the yeast mating type loci (E and I) function only when in the vicinity of a telomere (Maillet *et al.* 1996). Creation of a new telomere 13 kb from an integrated cassette containing these elements and a reporter gene restores silencing. Failure to silence at internal loci (Marcand *et al.* 1996) seems to be related (at least partially) to subnuclear compartmentalization of the DNA since the same silencers on a freely diffusing plasmid are able to associate with telomeres and function normally (Lebrun *et al.* 2003). Lastly, while TPE is observed at all truncated telomeres, the degree of silencing is highly variable between different endogenous telomeres (Pryde and Louis 1999). One out of three endogenous opening reading frames adjacent to different yeast telomere was found to be subject to TPE (Vega-Palas *et al.* 2000). The potential therefore exists for silencing near human telomeres to extend over vast portions of the genome, or to be neutralized by subtelomeric sequences before reaching even the first pseudogene.

SUBTELOMERIC GENES

Another issue to consider is the arrangement and identity of subtelomeric genes. Within a human diploid nucleus there are 92 chromosome ends. Since there are two copies of each chromosome, this represents 46 different ends [48 in males due to the pairing of X and Y chromosomes, but the ends of these two chromosomes are made up of pseudoautosomal regions that are in fact homologous, reviewed in (Graves *et al.* 1998)]. Five chromosomes (13, 14, 15, 21, and 22) are acrocentric, meaning they possess little DNA on the p arms beyond the centromeric sequences. To date, the human genome project has not mapped a gene within the terminal 7 Mb of 21p, or the terminal 10 Mb of the other four. There are therefore 41 regions in the human genome potentially affected by TPE (ignoring allelic variation between homologous chromosome ends).

Mapping the precise structure of these ends, however, has proven more difficult than originally suspected. Human subtelomeres contain a great deal of repetitive DNA, including stretches of degenerate short telomeric repeats and long segmental duplications that appear at many different chromosome ends as well as other internal loci in some cases [reviewed in (Mefford and Trask 2002)]. This has led to frequent confusion and mismappings. For example, the finished sequence of chromosome 22q does not reach the T_2AG_3 repeats. A clone exists in GenBank containing a 66 kb region > 98% identical to the end of the published 22q sequence along with additional DNA that was thought to be the more telomeric segment of the chromosome. Instead, it has been shown that this entire clone including the homologous region is actually derived from internal sequences on chromosome

2q13 at the site of an ancestral telomere fusion (Mefford and Trask 2002). Additionally, subtelomeres are underrepresented in the clone libraries used by sequencing centers and in the draft sequence of the human genome (Bailey *et al.* 2001). This is most likely due to the absence of restriction sites in telomeric and short degenerate repeats and the lack of a convenient pattern of sites within segmental duplications (Mefford and Trask 2002). Finally, there is significant allelic variation within the population at about half the chromosome ends for which information is available (Riethman 2003). The first and most impressive published case is for chromosome 16p, where short and long alleles differ by 260 kb (Wilkie *et al.* 1991). Although the sequence for the short allele has been published (Flint *et al.* 1997; Daniels *et al.* 2001), the sequence of the long allele (accounting for ~30% of alleles) is unknown (Mefford and Trask 2002). The result of this combination of factors is that sequences from the "complete" human genome normally terminate at an undefined point, short of the T_2AG_3 repeats. Even in the four "finished" chromosomes [22 (Dunham *et al.* 1999), 21 (Hattori *et al.* 2000), 20 (Deloukas *et al.* 2001), and 14 (Heilig *et al.* 2003)], the known sequence does not extend into the telomeric DNA, although the sequence of (acrocentric) chromosome 14 is estimated to be nearly complete on the q arm, reaching within 5-8 kb of telomere repeats (Heilig *et al.* 2003).

Nevertheless, a picture of the general organization of subtelomeres, both in humans and in other organisms, is beginning to emerge. T_2AG_3 repeats in humans are separated from chromosome-specific single-copy DNA by zones containing 10-300 kb of duplicated segments [reviewed in (Mefford and Trask 2002)]. Individual segments can contain genes and may be very small or greater than 50 kb in size. Some are found at many ends while

others are present at only a few. The subtelomere of chromosome 3q is related to at least 35 other ends (Mefford and Trask 2002) while 7q has very little homology to other subtelomeres (Riethman *et al.* 1993). Based on the limited information available, a 2-domain model has been proposed in which a distal subtelomeric domain containing shorter, more frequently duplicated elements is separated from a more proximal (centromeric) subtelomeric domain by a tract of degenerate telomere repeats. This general organization of subtelomeric duplicated regions separating telomeres from unique sequence DNA is conserved in yeast, *Plasmodium falciparum*, and even in *Drosophila* where the structure and maintenance of the telomere itself is not conserved. In fact, of the eukaryotic organisms for which information is available, only in *Caenorhabditis elegans* do the subtelomeres lack homology to one another (Wicky *et al.* 1996).

Although ESTs (expressed sequence tags) and probable pseudogenes have been detected within a few kb of human telomeres, the closest true genes known at present are the interleukin 9 receptor (IL9R) on Xq/Yq, with the promoter about 35 kb from the telomere (Ciccodicola *et al.* 2000), and the RNA polymerase III CII subunit (POLR3K) on 16p, with the promoter located about 44 kb from the telomere (Daniels *et al.* 2001). Illustrative of the problems encountered in dealing with subtelomeric genes, ILR9 has at least four pseudogenes (Kermouni *et al.* 1995), including one on 16p and that chromosome arm, containing POLR3K, has an allelic variant in which an additional 150-260 kb of unknown sequence are present. Human subtelomeres are gene-rich regions and code for proteins involved in a wide variety of processes (Riethman *et al.* 2001). Perhaps the most interesting pattern noted to date is the abundance of olfactory receptor (OR) genes in these regions

(Trask *et al.* 1998). At least 8 subtelomeric OR genes have been identified, 4 of which are polymorphic in number and location (Mefford and Trask 2002) and transcription of one of these genes has been demonstrated to occur from multiple chromosomal locations (Linardopoulou *et al.* 2001). Individuals have been found to carry up to 56 copies of subtelomeric OR genes and one segment containing three of these genes has been found at 14 different chromosome ends (Mefford *et al.* 2001). The same segment is single-copy in non-human primates.

SUBTELOMERES AND EVOLUTION

The distribution of OR genes and other observations of plasticity within human (Brown *et al.* 1990) and non-human (Corcoran *et al.* 1988) subtelomeres have prompted the suggestion that these regions might function to increase genetic diversity, allowing for rapid adaptive evolution (Trask *et al.* 1998). One supporting example is the subtelomeric var genes in *P. falciparum*, which encode antigens. Diversification of these genes is beneficial to the parasite and this process is enhanced by increased recombination within the subtelomeres (Freitas-Junior *et al.* 2000). Another is the subtelomeric genes that function in carbon-source metabolism in *S. cerevisiae*, which have spread and amplified differentially in strains subjected to different environments. The RTM genes, conferring resistance to the toxicity of molasses, are found at the subtelomeres of all brewing strains of yeast, for which resistance to molasses toxicity is crucial, but are not found in most strains used for wine production, for

which it is not (Denayrolles *et al.* 1997). Strains that carry many MEL genes, encoding α-galactosidase, have no subtelomeric SUC genes, encoding β-fructofuranosidase (invertase), and vice versa (Naumov *et al.* 1990). Subtelomeric MAL genes, encoding enzymes involved in maltose metabolism, have been shown to diversify by ectopic recombination and gene conversion (Wang and Needleman 1996; Gibson *et al.* 1997). Finally, the further characterization of subtelomeric OR genes in humans has provided additional evidence of recent evolution. 180 copies of the *OR-A* gene (derived from 12 different chromosomes in 22 different individuals) were sequenced and found to encode 14 different proteins differing by 1-5 amino acids (Linardopoulou *et al.* 2001). In the chimpanzee and gorilla, *OR-A* is a pseudogene but it is intact in the orangutan. In humans, *OR-A* has expanded to 7-11 copies (from 2 in chimpanzee). This variation in copy number and protein sequence has been speculated to affect olfactory sensitivity, the variety of odorants that can be detected, or both (Mefford and Trask 2002). A detailed analysis of the finished chromosome 22 sequence has recently provided support for the role of segmental duplications in evolution (Bailey *et al.* 2002). More than 10% of the total chromosome sequence was found to be involved in duplications (counting regions over 1 kb with at least 90% identity) in contrast to previous findings by chromosome painting that suggested this chromosome was highly conserved (Muller *et al.* 1999). Interchromosomal duplications on chromosome 22 were found to cluster within the pericentromeric and subtelomeric regions (this enrichment was previously estimated to be about 10-fold on average for the entire genome (Bailey *et al.* 2001)) and intrachromosomal duplications were found to be clustered within the proximal third of the chromosome arm. 11 new transcripts resulting from duplication events were identified and

since chromosome 22 represents ~1% of the genome it was extrapolated that ~1100 transcripts may have arisen from this process over the past 35 million years. The authors conclude that segmental duplication is an ongoing process that has been active throughout recent primate evolution, with a particular preference for pericentromeric regions. PEV in mammals is also more prevalent near centromeres (Butner and Lo 1986; Ramirez *et al.* 2001) and these regions share with subtelomeres the presence of repetitive DNA and several features of heterochromatin (Hennig 1999). The shortening of human telomeres with replicative age, however, makes subtelomeres potentially unique in terms of their role in evolution.

THE ROLE OF SILENCING

It has been suggested that one function of subtelomeric regions might be to buffer single-copy portions of the genome from telomere position effect (Mefford and Trask 2002). This may be a part of but not the whole explanation for the structure and behavior of human subtelomeres since very small insulating elements in yeast (Fourel *et al.* 1999; Pryde and Louis 1999), *Drosophila* (Kellum and Schedl 1991) and humans (Burgess-Beusse *et al.* 2002) are capable of preventing the spread of silencing effects. The hypothesis can be extended by supposing that variegation due to telomeric (or centromeric) silencing might allow the sampling of potentially harmful genetic intermediates during protein evolution. Since these regions support high frequencies of duplications and rearrangements, the

potential to silence expression of certain transcripts would seem to be extremely beneficial. A similar role in allowing the progression through harmful genetic intermediates has been suggested for prion proteins (True and Lindquist 2000).

The hypothesis as presented to this point does not put any special emphasis on the role of subtelomeres, as opposed to other regions capable of duplication and production of variegated phenotypes, most notably centromeres (Butner and Lo 1986). Indeed in most organisms such as yeast, where telomere length is maintained, and mice, where telomeres are very long and not expected to shorten dramatically within one generation (Wright and Shay 2000), it might be predicted that any variegating locus is roughly equivalent in terms of its influence on nearby genes, despite a few hints of unique features near telomeres (see below). In humans however, relief of silencing as telomeres shorten could be predicted to have a major effect specifically on subtelomeric genes. Remarkably, although yeast telomeres do not shorten with replicative age, relief of silencing is observed near telomeres as yeast cells approach the end of their replicative lifespan (Kim *et al.* 1996) and is associated within redistribution of Sir proteins from telomeres to the nucleolus (Kennedy *et al.* 1997). Although it has yet to be tested experimentally, the possibility exists that in mice, telomere shortening due to oxidative damage could provide the rapid reduction in telomere length that would be predicted to be necessary to trigger relief of telomeric silencing. Note that the observation that the telomerase knockout mouse is viable for six generations (Lee *et al.* 1998) is an indication of telomere status in germ-line cells only. It is therefore possible, given the current state of knowledge, that significant relief of silencing of endogenous subtelomeric genes during aging could occur in humans (and possibly other long-lived

mammalian species, with the yeast findings being anecdotal), or more generally in many species.

The effects of loss of telomeric silencing during aging, if any, are likely to be indirect since most would be observed within the post-reproductive lifespan of an organism (see below for possible exceptions). In terms of evolution, it is hard to imagine how traits could be selected for that would manifest so late in life. Instead, loss of telomeric silencing might contribute to aging due to the misregulation of subtelomeric genes or the increased expression of many non-functional or even harmful genes having arisen from evolutionary subtelomeric duplications and rearrangements that were previously repressed. This would represent a textbook case of antagonistic pleiotrophy where the subtelomeric plasticity that facilitates evolution is positively selected for early in life but is harmful late in life (post-reproduction, and hence post-evolutionary influence). The harmful effect, relief of silencing, would be triggered by the same process, telomere shortening, that mediates an important survival-promoting trait (limited replicative lifespan as a cancer defense). In fact, at least two reviews have focused on the loss of heterochromatin as a cause of cellular (Imai and Kitano 1998) and organismal (Villeponteau 1997) aging. Both of these reviews discussed the possibility that relief of telomeric silencing could serve as the trigger for replicative senescence in human cells. This has since proven unlikely since the existing data is adequately explained by a DNA damage type response to a critically short telomere (Steinert *et al.* 2000; Itahana *et al.* 2001) and a similar phenotype is produced by the exposure of cells to oligonucleotides mimicking the single-stranded G-rich overhang (Li *et al.* 2003). However, loss of TPE is useful in explaining changes observed prior to senescence in human

cells. No other mechanism has yet been proposed that can explain the increase in collagenase expression (Sottile *et al.* 1989; Burke *et al.* 1994), the decrease in (inducible) pigment epithelium-derived factor (Doggett *et al.* 1992), or a variety of other gradual changes observed in aging human cells (Hayflick 1980). Note that these genes are not telomeric and TPE is suggested to provide only a root cause for modification their expression that is mediated through more complex mechanisms.

Functioning as the trigger for senescence (and therefore as a cancer defense) was a role that made sense evolutionarily for TPE since tumors occur well within the reproductive lifespan of humans. Despite the fact that TPE is most likely not involved in this process, the example serves to illustrate the possibility that specific roles for telomeric silencing (as opposed to a non-specific role in aging) could exist. Shorter telomere lengths within a specific tissue, particularly a glandular tissue with high turnover, could easily lead to organismal changes within the reproductive lifespan of a human. Through such a mechanism, TPE has the potential to explain changes at the organismal level that consistently occur in different individuals after decades, such as redistribution of fat from the shoulders to the stomach in males.

One argument against more specific, beneficial roles for TPE in mammalian species comes from the example of the muntjac (Liming *et al.* 1980). The Chinese muntjac has 23 pairs of chromosomes while, due a series of recent fusions, the Indian muntjac has only 3, the fewest of any mammal. A superficial search of the internet (yielding information primarily from the websites of zoos and enthusiasts) reveals that estimates for the lifespan of the Chinese muntjac range from about 10-19 years while estimates for the lifespan of the Indian

muntjac range from 15-25 years. This admittedly soft data is very much compatible with the idea that non-specific relief of silencing in subtelomeres contributes to aging since the animal with fewer subtelomeres seems to live longer but if relief of silencing of specific subtelomeric genes were playing beneficial roles in the aging process then chromosome fusions would be expected to have the opposite effect. While the possibility exists that specific genes on the remaining chromosome ends in Indian muntjacs play important, beneficial roles in aging, or that some material from joined ends was translocated to the remaining subtelomeres during each fusion, it seems more likely that any contributions by subtelomeric regions to aging in this animal are non-specific and accelerate the process.

Several counterarguments can be made. The first is that roles for TPE in aging may have evolved specifically in very long-lived animals such as humans. This argument has been made for the process of replicative senescence, thought to function in humans as a cancer defense but not to occur within a normal mouse lifespan (Wright and Shay 2000). Another is that since so much subtelomeric DNA is duplicated between chromosomes (Mefford and Trask 2002), there may be redundancy at many ends for certain beneficial or harmful effects from specific genes. Silencing at human telomeres, in addition to its regulation by telomere length, may also be found to have important properties that further separate telomere position effect from the phenomena observed at centromeres and other variegating loci, as is suggested by studies in fission yeast (Nimmo *et al.* 1994) and *Drosophila* (Wallrath and Elgin 1995). For instance, telomeres have been implicated in both preferentially absorbing DNA damage (Bar-Or *et al.* 2001; Oikawa *et al.* 2001; von Zglinicki 2002) and in functioning as a reservoir of repair factors (Nakamura *et al.* 2002; Tham and

Zakian 2002; Wei *et al.* 2002). Subtelomeric genes could exist that need to be switched on and off effectively during DNA repair, or it could be that expression of subtelomeric genes is simply disruptive in assembling these repair complexes. For either reason, shortening of telomeres could lead to a decrease in the efficiency of DNA repair.

Although TPE could be envisioned to play an active role in a wide variety of human biological processes, the most likely manifestation of this silencing effect seems to be in the establishment of genomic regions that facilitate the evolution of new genes through low-level, variegated expression. The collapse of these regions (loss of silencing) as telomeres shorten could potentially play a major role in creating the aging phenotype at the cellular and organismal levels in humans. Completion of the sequencing of human subtelomeres combined with more detailed expression data from multiple human tissues should allow these hypotheses to best tested in the near future.

POSTION EFFECTS AND HUMAN DISEASE

Position effects have been implicated in a growing number of human diseases including aniridia (*PAX6*), campomelic dysplasia (*SOX9*), X-linked deafness (*POU3F4*), Rieger syndrome (*PITX2*), holoprosencecephaly (*SHH*), Greig cephalopolysyndactyly (*GLI3*), Saethre-Chotzen syndrome (*TWIST*), γβ-thalassemia (HBB complex), sex reversal (*SRY*), split hand/foot malformation (*SHFM1*), and facioscapulohumural dystrophy (*FSHD*) [reviewed in (Kleinjan and van Heyningen 1998)]. Typically, these diseases manifest in a

severe form in cases where the relevant locus is deleted, or in a milder form in cases where a translocation (with preservation of the intact locus) has occurred. FSHD occurs in patients with fewer than 11 copies of a 3.3 kb repeat termed D4Z4 in the chromosome 4q subtelomere. It has recently been shown that sequences within this repeat bind a protein complex that is involved in transcriptional repression and that, in FSHD patients, expression of at least three genes on the centromeric side of these repeats is dramatically elevated (Gabellini *et al.* 2002). These repeats might represent the first mammalian equivalent of the subtelomeric repeated elements that propagate TPE in yeast. Notably, expression of the three genes characterized in this study correlates to their distance from both the D4Z4 repeats and the telomere in the normal controls. α-Thalassemia, resulting from disruptions in the α-globin genes near the chromosome 16p telomere, has also been shown in at least one case to be produced by a chromosomal position effect (Barbour *et al.* 2000). In this case, an 18 kb deletion juxtaposes the intact α2 gene with region rich in *Alu* and MER repeats that is proposed to be heterochromatic in character. Despite the fact that no positive regulatory sequences were deleted and no specific silencing sequences were detected in the flanking DNA, this deletion resulted in the complete silencing of α-globin genes. This could represent a case where deletion of an insulating element has allowed telomere position effect to influence gene expression. A telomere position effect has recently been proposed to explain some of the clinical features of chromosome 14q terminal deletion syndrome in patients having a ring chromosome (van Karnebeek *et al.* 2002). A silencing effect is thought to spread from the fused p arm telomere into q arm genes near the breakpoint. As more information becomes available, TPE may be found to have a major effect in cases

where the telomere has become separated from its natural subtelomeric environment. For example, telomere abnormalities have been detected in about 4.6% of all cases of mental retardation using available techniques, and that number is likely to rise since new techniques are constantly being developed and a cause is currently known for only about 50% of cases (Xu and Chen 2003). Wolf-Hirschhorn syndrome has recently been identified in a patient with a truncated chromosome but retaining the previously accepted critical region (Zollino *et al.* 2003). The authors propose an additional critical region distal to the original but an alternative interpretation could be made that a position effect from the new telomere mediates the syndrome in this patient. A more complete understanding of the effects of TPE in abnormal situations will have to wait until more detailed data can be accumulated.

CONCLUSIONS

It has been demonstrated that human telomeres exert a position effect on an adjacent reporter gene and that this effect is proportional to the length of the telomere. The reporter is expressed in a variegated fashion, similar to the behavior of genes near yeast telomeres and in other repressive environments in several species. Like its yeast homolog, hRap1 seems to play an important role in mediating telomere position effect. Expression of several endogenous subtelomeric genes has been shown to be unaffected by the length of the adjacent telomere tract, however effects on the transcription of most of these genes are still unknown and form the basis for important future experiments. Because telomeres shorten

with each cell division, it is possible that upregulation of subtelomeric genes over time could play an important role in human aging.

APPENDIX A
siRNA Sequences

Target	Strand	Sequence	Evidence
Luciferase (Tuschl)	sense	CUAUGAAGAAGUGUUCGUCTT	Luciferase Assay (Better)
	antisense	GACGAACACUUCUUCAUAGTT	
Luciferase 2	sense	CAUCACGUACGCGGAAUACTT	Luciferase Assay
	antisense	GUAUUCCGCGUACGUGAUGTT	
Luciferase 3	sense	GUUGCGCGGAGGAGUUGUGTT	Luciferase Assay (Better)
	antisense	CACAACUCCUCCGCGCAACTT	
hRap1	sense	GAUCUAAAUCAGACAGGAGTT	Western (24h), TPE, RTPCR
	antisense	CUCCUGUCUGAUUUAGAUCTT	
5/6 (hRap1) (de Lange)	sense	CGCCUUGUGGAAAGCGAUGUU	Western (Best, 24h), TPE
	antisense	CAUCGCUUUCCACAAGGCGUU	
7/8 (hRap1) (de Lange)	sense	GCGUCGGCUGUCGACGCUCUU	Western (No effect)
	antisense	GAGCGUCGACAGCCGACGCUU	
TRF1	sense	CCUAUAAGCAUGACAUACUTT	
	antisense	AGUAUGUCAUGCUUAUAGGTT	
TRF2	sense	CUGGACCAGAAGGAUCUGGTT	
	antisense	CCAGAUCCUUCUGGUCCAGTT	
Tin2	sense	AGCAGGAUCUGAGGAAGAUTT	
	antisense	AUCUUCCUCAGAUCCUGCUTT	
Tankyrase	sense	CCGUGUAGAAGUCUGCUCUTT	
	antisense	AGAGCAGACUUCUACACGGTT	
SIRT1	sense	GUUGACCUCCUCAUUGUUATT	Western (Best, 72h)
	antisense	UAACAAUGAGGAGGUCAACTT	
SIRT1.2	sense	GUGAUGAGGAGGAUAGAGCTT	Western (Better, 72h)
	antisense	GCUCUAUCCUCCUCAUCACTT	
SIRT1.3	sense	GUACAAACUUCUAGGAAUGTT	Western (Slight, 72h)
	antisense	CAUUCCUAGAAGUUUGUACTT	
CAF1	sense	GAUGAGGACGAUGGUUUCUTT	
	antisense	AGAAACCAUCGUCCUCAUCTT	
Ku70	sense	CCCCGUGCUGCAGCAGCACTT	
	antisense	GUGCUGCUGCAGCACGGGGTT	
Caveolin	sense	AGACGAGCUGAGCGAGAAGCA	Western (6 Days)
	antisense	CUUCUCGCUCAGCUCGUCUGC	
SALL1	sense	GCCACCAAAUGUCACUGCCTT	
	antisense	GGCAGUGACAUUUGGUGGCTT	

SALL1-UTR	sense	CAUUUAUGGCAAUUGCAAGTT	
	antisense	CUUGCAAUUGCCAUAAAUGTT	
PIN2	sense	CAACCGGACAAGUGUCAUGTT	
	antisense	CAUGACACUUGUCCGGUUGTT	
hnRNP K-1	sense	CACUGAUGAGAUGGUUGAATT	TPE, RTPCR, Toxic
	antisense	UUCAACCAUCUCAUCAGUGTT	
hnRNP K-2	sense	UCCGUCAUGAGUCGGGAGCTT	
	antisense	GCUCCCGACUCAUGACGGATT	
p16 (+ p14)	sense	GCGCACAUUCAUGUGGGCATT	
	antisense	UGCCCACAUGAAUGUGCGCTT	
p16-B	sense	GGUCCCUCAGACAUCCCCGTT	Western (Slight, 72h)
	antisense	CGGGGAUGUCUGAGGGACCTT	
hTERT	sense	GAACGUGCUGGCCUUCGGCTT	TRAP (No effect)
	antisense	GCCGAAGGCCAGCACGUUCTT	
hTERT-2	sense	UGAGGCCAGCAGUGGCCUCTT	TRAP (No effect)
	antisense	GAGGCCACUGCUGGCCUCATT	
hTERT-3	sense	GUUUGGAAGAACCCCACAUTT	TRAP
	antisense	AUGUGGGGUUCUUCCAAACTT	
hTR-template	sense	CCCUAACUGAGAAGGGCGUTT	TRAP (No effect)
	antisense	ACGCCCUUCUCAGUUAGGGTT	
hTR-2	sense	AAUGUCAGCUGCUGGCCCGTT	TRAP (No effect), Toxic
	antisense	CGGGCCAGCAGCUGACAUUTT	
Pot1A	sense	AUGGUAGAAGCCUUACGUGTT	Silencing
	antisense	CACGUAAGGCUUCUACCAUTT	
Pot1B	sense	UCAGAACCUGACGACAGCUTT	Silencing (Better), RTPCR
	antisense	AGCUGUCGUCAGGUUCUGATT	
HDAC4A	sense	GCCGAGGUUCACGACAGGCTT	Silencing
	antisense	GCCUGUCGUGAACCUCGGCTT	
HDAC4B	sense	GCCAUCGGAAGAUGCGAGUTT	Silencing
	antisense	ACUCGCAUCUUCCGAUGGCTT	

All RNA oligos were synthesized at the on-campus core facility except for initial samples of 5/6 and 7/8 (kindly provided by T. de Lange). Oligos were annealed according to the method of Elbashir *et al* (Elbashir *et al.* 2001) to generate a 19 bp duplex region with 2 bp 3' deoxythymidine overhangs in most cases. In cases where TPE is cited as evidence of efficacy, an effect on target protein level was inferred from an effect on telomeric silencing. If silencing of internal controls was also affected, this is noted as simply "silencing". Except where noted as "No effect", evidence is included if any effect on protein or mRNA level was detected. Many targets were not analyzed at the protein level because specific bands could not be detected with commercially available antibodies or no antibody was available.

APPENDIX B
PCR and RTPCR Primers

Target	D	Name	Sequence	A	Pro
Luciferase	S	Luciferase s	AAACCGTGATGGAATGGAAC	51	Yes
	A	Luciferase a	AGATGCACATATCGAGGTGAAC		
Luciferase Poly-A Region	S	Poly A s	CTTGTTTATTGCAGCTTATAATGG	52	Yes
	A	Poly A a	GATCGTGGATTACGTCGCC		
β(+ γ1?)-Actin	S	B-Actin s	GCGCAAGTACTCCGTGTGGATCG	63	Yes
	A	B-Actin a	CCTAGAAGCATTTGCGGTGGACGATG		
POLR3K	S	POLR3K F	AGGGACAACGCTGCCACCGC	65	Yes
	A	POLR3K R	CGAGGGACAAGGCAAGCACACAC		
RABL2A/B	S	RABLF1	CCACAGCAGCTGTCCACGTA	59	Yes
	A	E0.91F	TCCTCTGATGGGGTCTCGAT		
β2-Microglobulin	S	hBeta2micro	TGAGTATGCCTGCCGTGTGAACC	63	Yes
	A	hBeta2micro	CACCTCTAAGTTGCCAGCCCTCC		
PGPL	S	PGPL917F	GTCTCGCTCCCAGCCATTTGCTGGGATGAC	65	Yes
	A	PGPL917R	GGAAACATTCCGAGGGAAAGCAGTTCACAG		
HRMT1L1	S	HRMT1L1-S	CTGGCTGAAGGAGGACGGGGT	63	Yes
	A	HRMT1L1-A	GTGCCTTCTCCACACTGGGTTTCTC		
hnRNP K	S	hnRNP K-S	GAAACTGAACAGCCAGAAGAAACC		Yes
	A	hnRNP K-A	ATGGGAGACTCAGATATAAGATCAAGG		
hRap1	S	hRap1-S	GGTCCCGACAAGATGACATAGATT		Yes
	A	hRap1-A	GATTACTTATGCTGCCTGAAATGG		
Par6	S	PAR6-F	ATGATCGCCAACAGCCACAA	59	
	A	PAR6-R	TCGTTGTCCTCATCGCTCTCC		
ZNF268	S	ZNF268-F	GGGACCTTTGTCATTCATGGATG	59	
	A	ZNF268-R	ACCAAACCTGCCAGCACCTT		
sRIKEN	S	sRIKEN-F	AGAGAGTTACAGGTCCTTCTCCAAG	59	
	A	sRIKEN-R	TGGGAACAACCTCTGCAGTTAG		
X92108.1	S	SUBTEL-F	TACGCTGGGCCAGAAACCTCT	60	
	A	SUBTEL-R	GAACAGACCCACTCTTGGCTGA		
L23a-like	S	L23AL-F	AGCACCCCCAGGAGAAACAA	60	
	A	L23AL-R	TGGTGACCTTGGCCACATCA		
L37a	S	L37a-F	ATCAGCCAGCACGCCAAG	57	
	A	L37a-R	ACAGCGGAAGTGGTATTGTAC		
C2F	S	C2F-F	GTGAACGAAGCGGTGGGG	57	
	A	C2F-R	ATGGATATAAACCTGTAGCAAGCC		

DUX4	S	DUX4-F	CAAGACTCCCACGGAGGTTCA	59	No
	A	DUX4-R	CCCCTTCATGAATGGCGGGT		
hG3PDH	S	hG3PDH-F	TGAAGGTCGGAGTCAACGGATTTGGT	64	Yes
	A	hG3PDH-R	CATGTGGGCCATGAGGTCCACCAC		
hPot1(6-12)	S	Pot1(6-12)F	ACACCCCTGAATCAACTTAAGGGTGGT	63	
	A	Pot1(6,9-12)R	ATTGACAGATAACATCTGAATGCTGATTGGCTGTC		
hPot1(9-12)	S	Pot1(9-12)F	GGGCAAAGCAGAAGTGGACGGAGCATC	63	
	A	Pot1(6,9-12)R	ATTGACAGATAACATCTGAATGCTGATTGGCTGTC		
hPot1(12-16)	S	Pot1(12-16)F	TTCAGATGTTATCTGTCAATCAGAACCTG	58	
	A	Pot1(12-16)R	ATGTATTGTTCCTTGTATAAGAAATGGTGC		
hPot1(16-20)	S	Pot1(16-20)F	CAGCACCATTTCTTATACAAGGAACAATAC	59	
	A	Pot1(16-20)R	GATTACATCTTCTGCAACTGTGGTGTCA		
hTERT	S	hTERT sense	TCCTGCGTTTGGTGGATGATTTCTTG	62	Yes (209)
	A	hTERT antisense	GCCGCACCAGGGGAATAGGC		
hTERT 3' UTR	S	hTERT-3UTR s2	CGCACCGCTGGGAGTCTGAGG	65	Yes (344)
	A	hTERT-3UTR a	ACAATGGCGAATCTGGGGATGGAC		
hTERT 3' UTR (Real-Time)	S	3UTRsensepure	CGCACCGCTGGGAGTCTG		
	A	3UTRantipure	GGTGAACAATGGCGAATCTGG		
PEDF/EPC-1	S	PEDF/EPC-1 S	GTCTCCACCTTCGGCTATGA	57	
	A	PEDF/EPC-1 A	ATGTCTGGGCTGCTGATCAA		
B Actin	S	B-Actin Sense	ATCATGTTTGAGACCTTCAA	52	
	A	B-Actin Anti	CATCTCTTGCTCGAAGTCCA		

"D" indicates Direction (Sense or Antisense), "A" is Annealing temperature (used or estimated from calculated T_ms and expressed in °C), and "Pro" is Product (Yes or No for whether or not a band was observed and the expected or observed size is indicated in bp).

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