THE ROLE OF TELOMERE LENGTH AND TELOMERASE ACTIVITY IN CELL IMMORTALIZATION AND TUMOURIGENESIS

By

CHRISTOPHER MORRIS COUNTER, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (1995)
(Medical Sciences)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: The role of telomere length and telomerase activity in cell immortalization and tumourigenesis.

AUTHOR: Christopher Morris Counter, B.Sc. (McMaster University)

SUPERVISOR: Dr. S. Bacchetti, Professor, Dept. of Pathology

CO-SUPERVISOR: Dr. C.B. Harley, Associate Professor, Dept. of Biochemistry

NUMBER OF PAGES: xiii, 119

THE ROLE OF TELOMERE LENGTH AND TELOMERASE ACTIVITY IN CELL IMMORTALIZATION AND TUMOURIGENESIS

Abstract

Human somatic cells have a finite lifespan. In contrast, since most cancers evolve through expansions of mutant clones with increasingly more transformed phenotypes, tumour cells may exhaust the proliferative potential of normal cells and possibly acquire an unlimited replicative capacity (immortality). Thus, one essential step in tumourigenesis may be the acquisition of an immortal phenotype. The results presented in this thesis suggest that telomeres, the terminal structures that prevent illegitimate recombination and ensure the proper segregation of chromosomes, and telomerase, the enzyme that elongates telomeres *de novo*, play critical roles in the process of immortalization of transformed cells both in tissue culture and *in vivo*.

In a tissue culture model of transformation we have shown that telomeres shorten as normal cells divided, as previously reported (Harley *et al.*, 1990), and that, consistent with this observation, the cells lacked detectable levels of telomerase activity. Cells that were driven to divide beyond their normal lifespan by transformation with viral oncogenes did not directly acquire telomerase activity; consequently telomeres continued to shorten until a proliferative crisis, characterized by cell death, was reached. At crisis, chromosome ends contained very little telomeric DNA and appeared to be unstable since the frequency of dicentric chromosomes, aberrations that can be formed by the fusion of chromosome ends, increased. These data suggest that the critically short telomeres detected at crisis may no longer be functional, resulting in genomic instability and potentially cell death. Immortal clones which survived crisis maintained short, but stable telomeres and had telomerase activity. Similarly, malignant cells from the advanced stages of different cancers also had short telomeres and were telomerase positive. Moreover, in one cancer analyzed, the telomeres of malignant cells were found to be stably maintained *in vitro* and *in vivo*. These data, although correlative in nature, strongly suggest that, in culture as well as *in vivo*, telomerase must be activated to counter the lethal loss of telomeric DNA if cells are to become immortal.

Acknowledgements

I would like to thank my supervisor and friend Dr. Silvia Bacchetti. I am eternally grateful for her help and guidance and will always cherish the laughs and long chats we had over the years. This is perhaps the only page of this thesis that she will not have to correct. I would also like to thank my co-supervisor Dr. Calvin Harley for his support and thoughtful insight throughout my project. Additionally, I would not be typing a thesis now if it were not for the efforts and support of Dr. A.D. Dingle.

I have had a memorable time working with the members of the Cancer Research Group and would like to specifically thank Dr. Jim Smiley, Dr. Dr. Ian York, Dr. Craig Smibert, Dr. Coco Chadeneau and Peter Chung for their interest and helpful comments in my project. The work of this thesis is really the product of the thoughts, ideas, and efforts of a number of people whose names can be found in the authorship of the papers within this thesis. In particular, I would like to acknowledge Drs. Hal Hirte and Brian Leber who showed me that cancer more than cells in a flask. I would also like to thank Pete Betti and the magic boys, Lee Lichen Thackeray, Kevin Dingwell and Craig Brunetti for all the memories I will take with me. I consider myself very fortunate to have made such good friends. In particular I would like to apologize to Craig for all the times I made him listen to my talks, bummed a ride home from him, and crashed his house for dinner or for a movie.

I would not have made it this far without the support of my wife, companion, and most dear friend Deanne who, many times, put her own dreams and wishes second to mine. Between the lines of this thesis is a little of Deanne. Having married into the Maw family I got the mother, Linda, and father, Dan, that I never had and in the process also acquired a new brother, sister, and a slew of loving in-laws. I can never thank them all enough for their support. I would also like to thank my sister Anita who has always been, and always will be, my friend. I know all these people share in my accomplishments.

I dedicate this thesis to my wife Deanne and daughter Carling.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A	adenine residue
Ad	adenovirus
AML	acute myeloid leukemia
B2 to 5	clonal populations of EBV transformed human B lymphocytes
BM	bone marrow
bp	base pairs
BSA	bovine serum albumin
C	cytidine residue
°C	degrees Celsius
CHAPSO	3[(3-chloramidopropyl)dimethylammino]-2-hydroxy-1-propanesulfonate
CLL	chronic lymphocytic leukemia
dATP	deoxyadenosine nucleotide triphosphate
dCTP	deoxycytidine nucleotide triphosphate
dGTP	deoxyguanidine nucleotide triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine nucleotide triphosphate
E1	early region 1 of Ad virus
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-amino ethyl ether) N,N,N',N'-tetraacetic acid
G	guanine residue
g	gram
g	gravity units
G_0	growth arrest occurring in the G_1 phase of the cell cycle
G_1	gap 1 phase of the cell cycle
G-quartet	tetraplex structure formed from the non-Watson-Crick base pairing of G rich DNA
h	hour
HA1 to 10	clonal populations of HEK cells transformed with SV40
HEK	human embryonic kidney
HeLa	human cervical carcinoma cell line
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HEY	human ovarian carcinoma cell line
HPV	human papilloma virus

HTLV	human T-cell leukemia virus
hypo buffer	hypotonic buffer
kbp	kilobase pairs
KH_2PO_4	potassium dihydrogen orthophosphate
KCl	potassium chloride
L	litre
M	Molar
MDS	myelodysplastic syndrome
MEM	minimal essential medium
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
MPD	mean population doubling
Na_2HPO_4	di-sodium hydrogen orthophosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
OD	optical density
p	probability value
p21	cyclin dependent kinase inhibitory protein of molecular weight 21 kDa
p53	tumour suppressor protein of molecular weight 53 kDa
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pd	population doubling
pRb	tumour suppressor protein encoded by the retinoblastoma susceptibility gene
PMSF	phenylmethyl sulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
S	synthesis phase of the cell cycle
S100	100 *g* supernatant fraction
SD	standard deviation
SDS	sodium dodecyl sulphate
SKOV-3	human ovarian carcinoma cell line
SSC	sodium chloride/sodium citrate buffer
SV40	simian virus 40
T	thymidine residue
t-Ag	SV40 small tumour antigen

T-Ag	SV40 large tumour antigen
TBE	Tris-borate electrophoresis buffer
TE	Tris-EDTA buffer
telomerase	telomere terminal transferase
Tris-HCl	tris(hydroxymethyl)aminomethane buffered with HCl
TRF	terminal restriction fragment
tRNA	transfer ribonucleic acid
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
U	units
V	volt
293	Ad5 E1 transformed HEK cells
293 N3S	subline of 293 adapted to grow in suspension
293 CSH	subline of 293 adapted to grow in suspension

Preface

In the work presented in chapters 2,3,4 and 5, I wrote all the initial drafts, designed the figures and tables and performed all the experiments with the exception of the following:

In Chapter 2, the cytogenetic data described in Table 2.0 and exemplified in Figure 2.3 were collected by Nancy Stewart and Catherine LeFeuvre. Telomerase activity shown in Figure 2.5 and listed in Table 2.3 was assayed by Ariel Avilion from extracts I prepared.

In Chapter 3, Fernando Botelho remeasured the TRF length of samples I had previously analyzed and the combined data is shown in Table 3.0. Fernando also produced Figure 3.0. Ping Wang collected all the cytogenetic data presented in Table 3.0.

In Chapter 4 Dr. Hal W. Hirte obtained all malignant ascites fluid samples.

In Chapter 5 Dr. Brian Leber obtained all blood and bone marrow samples with the exception of one since he was the donor. Initially Dr. Leber also isolated the low density mononuclear cells. Dr. Jyothi Gupta re-assayed telomerase activity in samples previously tested by myself as well as preparing and assaying new samples. The combined data from our experiments are listed in Table 5.0.

Chapter 1: Introduction

1.0) Overview.

Senescence is a genetically controlled cellular program that limits the proliferative lifespan of normal cells (reviewed in Goldstein, 1990). Human cells rarely, if ever, bypass this stage spontaneously indicating that the senescent phenotype is tightly regulated (reviewed in McCormick and Maher, 1988). However, most cancers arise via the successive expansions of single, progressively more transformed cells (reviewed in Cairns, 1975; Nowell, 1976; Weinberg, 1989; Fearon & Vogelstein, 1990) a process that could conceivably exhaust the cell proliferative potential and force acquisition of unlimited proliferative capacity (immortality). Senescence may therefore impede the growth of a tumour and hence the acquisition of an immortal phenotype (Sager, 1991; Stamps *et al.*,1992). The growth arrest associated with senescence may not be the only replicative block tumour cells must surmount in order to become immortal. Cells transformed by experimental means bypass senescence but eventually undergo a proliferative crisis that is physiologically and apparently also genetically distinct from senescence (reviewed in Chang, 1986; Shay *et al.*, 1991b). It is not until transformed cells overcome crisis that they become immortal.

Telomere length has been implicated in the regulation of the replicative lifespan of human cells (Harley *et al.*, 1990; Hastie *et al.*, 1990) and telomerase, the enzyme that elongates telomeric DNA (Greider & Blackburn, 1985; Morin, 1989), has been shown to be essential for the immortal phenotype of unicellular organisms (Yu *et al.*, 1990; Singer & Gottschling, 1994; McEachern & Blackburn, 1995). Based on

these observations we investigated the possible roles of telomere length and telomerase activity in the immortalization process of human cells transformed in culture and of human tumour cells *in vivo*. The following introduction will review the structure and function of telomeres and of telomerase and the process of immortalization of human cells. Finally, the rationale and experimental systems used to investigate this particular aspect of cell immortalization are discussed in relation to the state of knowledge at the time this project was initiated.

1.1.i) Telomeres.

Telomeres are DNA-protein complexes that cap and protect the ends of eukaryotic chromosome from illegitimate recombination and degradation, and are essential for the mitotic stability of chromosomes. The two components of telomeres, protein and DNA, will be described, followed by a review of telomere function.

1.1.ii) Telomeric DNA.

Telomeric DNA consists of a tandemly repeated, short (generally 5-8 nucleotides) G-rich sequence oriented 5' to 3' towards the end of the chromosome (reviewed in Blackburn & Szostak, 1984; Zakian, 1989). Most eukaryotes contain only one type of repeat although there are exceptions with organisms having a mixture of two slightly different repeats or a more complex set of repeats that conform to a simpler consensus sequence (Emery & Weiner, 1981; Shampay *et al.*, 1984; Ponzi *et al.*, 1985; Baroin *et al.*, 1987; Forney & Blackburn, 1988; Richards & Ausubel, 1988; Ganal *et al.*, 1991;

McEachern & Blackburn, 1994). Among eukaryotes, only *Drosophila* has chromosome termini that lack G-rich repeats (Richards & Ausubel, 1988; Meyne *et al.*, 1989; Levis *et al.*, 1993; Okazaki *et al.*, 1993) and are instead composed of tandem arrays of retrotransposon-like elements (Young *et al.*, 1983; Levis *et al.*, 1993; Biessmann *et al.*, 1994; Danilevskaya *et al.*, 1994). The evolutionary conservation of telomeric DNA suggests its sequence is critical for telomere function. Indeed, experimental alterations of this sequence in lower eukaryotes can be lethal (Yu *et al.*, 1990; McEachern & Blackburn, 1995) and generation of new telomeres in human cells has very stringent sequence requirements (Hanish *et al.*,1994).

The actual number of telomeric repeats is variable, ranging from as few as 4.5 in the ciliate *Oxytricha* (Klobutcher *et al.*, 1981; Pluta *et al.*, 1982) to many thousand in mice (Kipling & Cooke, 1990; Starling *et al.*, 1990). In humans, telomeres are thought to be composed of 7-20 kbp of pure TTAGGG repeats (Moyzis *et al.*, 1988; Allshire *et al.*, 1989; Brown, 1989; Cross *et al.*, 1989; de Lange *et al.*, 1990; Hastie *et al.*, 1990), although their exact length is unknown (see below). Therefore, in contrast to its sequence, the actual length of telomeric DNA is evolutionarily flexible, perhaps reflecting specialization or divergence of telomere functions.

Telomere ends are not necessarily blunt, rather the 3' end of the G-strand protrudes past the complementary C-strand in yeast (Wellinger *et al.*, 1993), slime mould (Henderson & Blackburn, 1989) and in a number of ciliates (Klobutcher *et al.*, 1981; Pluta *et al.*, 1982). This overhang can adopt a 'G-quartet' configuration *in vitro*

and possibly *in vivo*, whereby the G residues from one or more G-strands form a four cornered cage by pairing in a non-Watson-Crick fashion (Sundquist & Klug, 1989; Williamson *et al.*, 1989; Wellinger *et al.*, 1993; reviewed in Williamson, 1994). Although a G-strand overhang has not been detected in humans, the oligonucleotide $(TTAGGG)_4$ can fold into a G-quartet (Balagurumoorthy & Brahmachari, 1994), suggesting human telomeres may have a terminal structure similar to those of lower eukaryotes. Indirect evidence suggests that G-quartets are essential structures of the telomere. In yeast the *KEM1* gene product is a nuclease that binds to G-quartets and cleaves the 5' adjacent single stranded region (Liu *et al.*, 1993). *kem1* deletion mutants undergo telomere shortening during the mitotic cycle suggesting that intrastrand G-quartets may regulate telomere length by restricting access of telomerase to the telomere ends (Liu *et al.*, 1995). In agreement with this possibility, G-quartets do not serve as primers for telomerase *in vitro* (Zahler *et al.*, 1991). Also, during meiosis *kem1* deletion mutants fail to complete the pachytene stage, supporting the possibility that interstrand G-quartets may be important in homologous pairing of chromosomes (Liu *et al.*, 1995).

Proximal to the telomeric repeats is a region defined as the subtelomere. In humans, subtelomeric DNA is composed of telomeric TTAGGG repeats interspersed with short tandem arrays of degenerate repeats (Allshire *et al.*, 1989; Brown *et al.*, 1990), followed centromerically by tandem arrays of minisatellite-like repeats (Brown *et al.*, 1990; Cheng *et al.*, 1990; Cross *et al.*, 1990; de Lange *et al.*, 1990; Weber *et*

al.,1991). However, since no single subtelomere sequence is found on every chromosome (Brown *et al.*, 1990; de Lange *et al.*, 1990) and functional telomeres can be generated that completely lack the subtelomere (Wilkie *et al.*, 1990; Farr *et al.*, 1991,92; Itzhaki *et al.*, 1992; Barnett *et al.*, 1993; Lamb *et al.*, 1993; Flint *et al.*, 1994; Hanish *et al.*, 1994) there is no obvious role for subtelomeric DNA.

At present, the length of telomeres of most organisms, including humans, cannot be measured directly. There are no means to separate the entire tract of telomeric repeats from the flanking subtelomeric DNA since the telomeric repeat TTAGGG, and presumably much of the subtelomeric DNA, does not contain recognition sites for any known restriction enzyme. Instead, digestion of genomic DNA with restriction enzymes liberates terminal restriction fragments (TRFs) which are composed of telomeric DNA and variable amounts of subtelomeric DNA (Fig. 1.0). Even the length of TRFs cannot be determined accurately since these fragments are highly heterogeneous in size due to variability in the lengths of both the telomere and subtelomere (Allshire *et al.*, 1989; Brown *et al.*, 1990; de Lange *et al.*, 1990; Harley *et al.*, 1990; reviewed in Levy *et al.*, 1992). Therefore, telomere length is usually reported as a mean TRF length from all chromosomes determined by Southern hybridization with a telomeric probe.

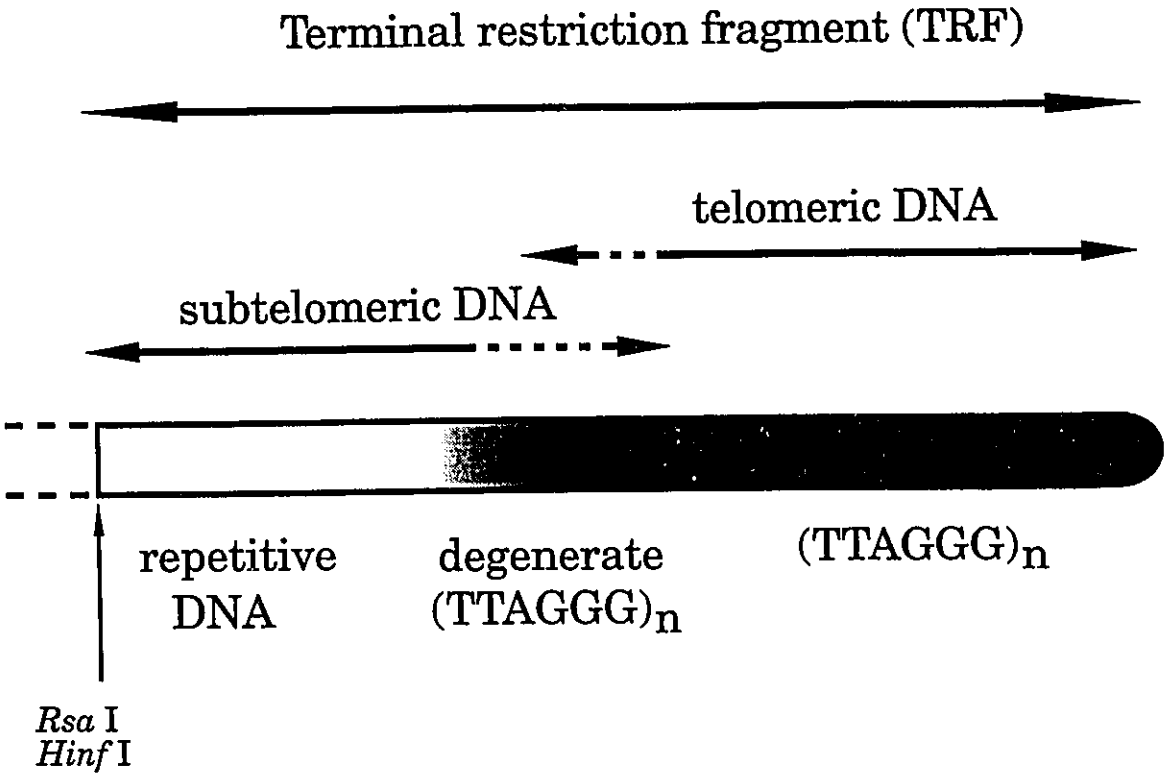

Figure 1.0

The structure of the terminal restriction fragment (TRF).
The most terminal region of the TRF is composed of telomeric TTAGGG repeats. Proximal to this DNA is the subtelomeric DNA which is composed of TTAGGG repeats interspersed with short tandem arrays of TTAGGG-like repeats, followed by arrays of minisatellite repeats.
(see text for details and references, diagram by F.L. Graham)

1.1.iii) Telomeric proteins.

Telomeric chromatin is quite different from bulk chromatin. In lower eukaryotes telomeric DNA interacts with specific telomere binding proteins to form a complex from which histones are excluded (Blackburn & Chiou, 1981; Edwards & Firtel, 1984; Gottschling & Cech, 1984; Lucchini *et al.*, 1987; Wright *et al.*, 1992). Similarly, the most terminal region of telomeres in humans and many other vertebrates has a diffuse chromatin structure which arguably could be due to a non-nucleosomal DNA-protein complex (Markarov *et al.*, 1993; Muyldermans *et al.*, 1994; Tommerup *et al.*, 1994; Lejnine *et al.*,1995). In simple organisms two principal classes of telomere specific proteins which are involved in the unique packaging of telomeric DNA have been identified (see below). Vertebrate proteins with similar binding properties have also been discovered arguing that specific nucleoprotein structures may be evolutionarily conserved features of telomeres (Fang & Cech, 1991; Gualberto *et al.*, 1992; Zhong *et al.*, 1992; Cardenas *et al.*, 1993).

The first class of proteins binds the single stranded G-rich tails of telomeres (Price, 1990; Fang & Cech, 1991; Gualberto *et al.*, 1992; Wang *et al.*, 1992; Cardenas *et al.*, 1993; Schierer & Henderson, 1994; Frantz & Gilbert, 1995), as typified by the *Oxytricha* heterodimer α/β (Gottschling & Zakian, 1986; Gray *et al.*, 1991; Fang & Cech, 1993a). Telomeric DNA bound to this protein acquires many of the physical features of telomeres that it lacks as naked DNA, such as resistance to nucleases and protection from chemical modification or end labelling (Gottschling & Zakian, 1986;

Price & Cech, 1987). Furthermore, the β subunit of the heterodimer appears to mediate the folding of telomeric DNA into a G-Quartet *in vitro* under physiological conditions (Fang & Cech, 1993b).

The other class of proteins binds to double stranded telomeric DNA (Coren *et al.*, 1991; Liu & Tye, 1991; Coren & Vogt, 1992; Zhong *et al.*, 1992; Brigati *et al.*, 1993; Zentgraf, 1995). The best characterized member of this class is the yeast protein RAP1 (Conrad *et al.*, 1990) which recruits other proteins to the telomere (Aparicio *et al.*, 1991; Hardy *et al.*, 1992; Suzuki & Nishizawa, 1994) to form a complex (Hardy *et al.*,1992; Palladino *et al.*, 1993; Moretti *et al.*, 1994). Like the *Oxytricha* α/β heterodimer, RAP1 and the complex it forms are important for many aspects of telomere function and structure, such as telomere silencing (the unstable repression of promoters adjacent to telomeres [Gottschling *et al.*,1990; Renauld *et al.*, 1993; Aparicio & Gottschling, 1994]), regulation of telomere length (Conrad *et al.*, 1990; Lustig *et al.*, 1990; Sussel & Shore, 1991; Hardy *et al.*, 1992; Kyrion *et al.*, 1992; Suzuki & Nishizawa, 1994), telomere-nuclear envelope interactions (Palladino *et al.*, 1993; Buck & Shore, 1995; Hecht *et al.*, 1995) chromosome stability (Conrad *et al.*, 1990; Kyrion *et al.*, 1992) and even G-quartet formation (Gilson *et al.*, 1994; Giraldo & Rhodes, 1994; Giraldo *et al.*, 1994).

There are also a variety of proteins whose relationship with telomeric DNA is less clear. One class of these proteins binds *in vitro* to single stranded G-rich telomeric DNA and in most cases its RNA counterpart. However, it is questionable

whether these proteins play any role in either the structure or function of telomeres *in vivo* (McKay & Cooke, 1992a,b; Ishikawa *et al.*, 1993; Lin & Zakian, 1994; Petracek *et al.*,1994). The *KEM1* gene product is another interesting protein which, as discussed above, may regulate telomere length by removing telomeric DNA that is folded into a G-quartet (Liu *et al.*, 1993, 1995). There are also proteins which do not bind telomeres directly but still manage to affect telomere length either by interacting with telomere binding proteins (Aparicio *et al.*, 1991; Hardy *et al.*, 1992; Suzuki & Nishizawa, 1994) or through as yet unknown mechanisms (Carson & Hartwell, 1985; Lustig & Petes, 1986; Lundblad & Szostak, 1989; Schulz & Zakian, 1994).

1.1.iv) Telomere functions.

As expected for a complex structure, telomeres perform a number of functions, of which a primary one is to ensure that chromosomes are retained and properly inherited with each cell division. The requirement for telomeres for the mitotic stability of chromosomes is particularly well exemplified in yeast: linearized plasmids are retained only if they are capped with telomeric DNA (Szostak & Blackburn, 1982) whereas specific removal of a telomere (Sandell & Zakian, 1993) or gradual shortening of all telomeres (Lundblad & Szostak, 1989) results in a dramatic increase in mitotic instability of the chromosome(s). Most likely the role telomeres play in this phenomenon reflects many aspects of their structures. For example, as described earlier the telomere complex protects chromosome ends from degradation (Blackburn & Chiou, 1981; Gottschling & Cech, 1984; Edwards & Firtel, 1984; Lucchini *et al.*,

1987; Wright *et al.*, 1992). Additionally, since the termini of linearized plasmids or chromosome breaks are highly recombinogenic, and in the latter case are recognized as DNA damage (Sandell & Zakian, 1993; reviewed in Blackburn & Szostak, 1984; Zakian, 1989) unless capped with telomeric repeats (Szostak & Blackburn, 1982; Bourgain & Katinka, 1991; reviewed in Biessmann & Mason, 1992), telomeres also protect chromosome ends from illegitimate recombination. These two features of telomeres ensure the structural stability, and therefore indirectly the mitotic stability, of chromosomes. However, telomeric DNA also improves the fidelity of chromosome segregation. Circular autonomously replicating plasmids in yeast segregate better if they contain telomeric DNA (Longtine *et al.*, 1992) whereas, the loss of telomeric repeats from chromosomes in *Tetrahymena* leads to improper chromosome segregation (Yu *et al.*, 1990). Although speculative, several observations suggest that telomeres may mediate chromosome segregation by correctly organizing the chromosomes in the nucleus prior to mitosis through interactions with the nuclear matrix. For example, prior to mitosis telomeres are usually found in discrete territories in most organisms and, in particular, in yeast, *Drosophila*, and plants they localize to the nuclear envelope (Avivi & Feldman, 1980; Mathog *et al.*, 1984; Chung *et al.*, 1990; Heslop-Harrison & Bennett, 1990; Rawlings & Shaw, 1990; Funabiki *et al.*, 1993; Vourc'h *et al.*, 1993; Palladino *et al.*, 1993; Broccoli & Cooke, 1994). Telomeric DNA purifies with the nuclear matrix (de Lange, 1992) and binds envelope proteins *in vitro* (Shoeman *et al.*, 1988; Shoeman & Traub, 1990). Also, the yeast telomere binding protein RAP1

associates with the nuclear envelope (Klein *et al.*, 1992). Lastly, and most convincingly, loss or mutation of the yeast genes SIR3, SIR4 or RAP1, which all encode proteins of the telomere complex, result in both a loss of pericentric nuclear localization and congregation of telomeres at interphase and a decrease in the mitotic stability of natural occurring chromosome V (but not of an artificial minichromosome; Palladino *et al.*, 1993).

In addition to the role telomeres play in the mitotic stability of chromosomes as discussed above, mutation of the *KEM1* gene in yeast suggests that telomeric DNA, or more specifically G-quartets, are essential for the homologous pairing of chromosomes during meiosis (Liu *et al.*, 1995). In agreement with this possibility, the telomeres of chromosomes of many insects and plants gather at the nuclear envelope during the pachytene stage of meiosis (reviewed in Gillies, 1975; Dancis & Holmquist, 1979; Biessmann & Mason, 1992) and in yeast, they actually appear to direct the migration of chromosomes as they move into correct positions for meiosis (Chikashige *et al.*, 1994).

Aside from the role telomeres play in chromosome integrity, segregation and pairing, telomeres also regulate gene expression at least in some organisms. Genes placed next to telomeres in yeast and *Drosophila* (reviewed in Karpen, 1994), but apparently not in humans (Bayne *et al.*, 1994), can be repressed due to the heterochromatic nature of telomeres. Lastly, and most relevant to the work discussed in this thesis, telomeres provide a buffer against, and a means to compensate for the

loss of terminal DNA due to the "end replication problem", that is the incomplete replication of the 5' ends of the daughter strands of linear DNA templates such as those of eukaryote chromosomes (Olovnikov, 1971,1973; Watson, 1972; Cavalier-Smith, 1974). Since DNA polymerase replicates DNA only in the 5' to 3' direction and requires a 5' primer (usually RNA) to initiate replication, removal of the most terminal RNA primer during the replication of a linear template leaves a gap that cannot be filled in by the enzyme owing to the absence of a 5' phosphate to prime synthesis. Therefore, each round of replication is accompanied by loss of terminal sequences resulting in the shortening of chromosome ends or telomeres (Fig. 1.1). Eukaryotes have developed a variety of mechanisms to counter the end replication problem. In *Drosophila* retrotransposon elements transpose to the ends of chromosome breaks at a rate sufficient to compensate for the loss of terminal DNA (Traverse & Pardue, 1988; Biessmann *et al.*, 1990, 92) and in yeast telomere length can be maintained through what appears to be nonreciprocal recombination (Dunn *et al.*, 1984; Wang & Zakian, 1990; Lundblad & Blackburn, 1993). However, the most conserved mechanism for telomere preservation in eukaryotes is the *de novo* addition of telomeric DNA by the enzyme telomere terminal transferase, or telomerase.

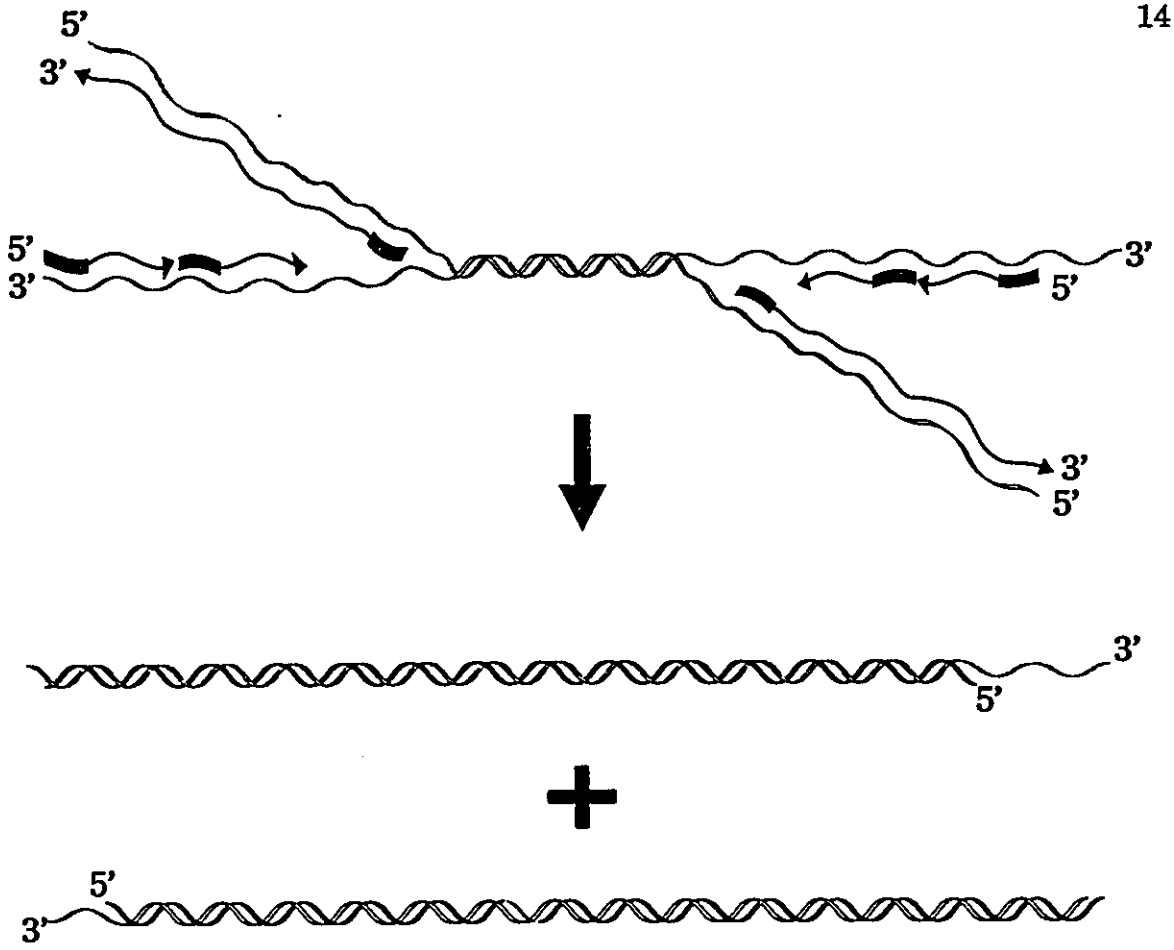

Figure 1.1

The end replication problem.
A linear double stranded DNA molecule is shown during and after replication. For simplicity, only the terminal half of two replication forks originating near the end of the DNA shown. During replication of the lagging strand, the most terminal 5' RNA primer (■) can anneal at different distances from the end of the template. Thus, once these primers are removed, the 5' ends of the newly synthesized DNA (wavy black lines) can be shorter than the template by at least the length of the RNA primer (see text for references, diagram by F.L. Graham).

1.2) Telomerase.

Telomerase was first identified in *Tetrahymena* (Greider & Blackburn, 1985) and to date the enzyme from this organism remains the best characterized and the only one that has been cloned. *Tetrahymena* telomerase is composed of a 80 and a 95 kDa protein subunit (Collins *et al.*, 1995) and a structural RNA transcribed by polymerase III (Greider & Blackburn, 1989). *In vitro* the enzyme elongates oligonucleotides that correspond to the G-strand of telomeric DNA by the *de novo* addition of the telomeric repeat GTTGGG, one nucleotide at a time, to the 3' end. The RNA component contains the nine nucleotide sequence 3'AACCCCAAC which is complementary to 1.5 telomeric repeats of the G-strand, suggesting that this region acts as a template (Greider & Blackburn, 1989). Indeed, these nine nucleotides are essential for activity *in vitro* (Greider & Blackburn, 1989) and mutations of the 5' nucleotides of this template *in vivo* result in corresponding changes of the telomere sequence during regular maintenance or *de novo* synthesis (Yu *et al.*, 1990; Yu & Blackburn, 1991).

Based on the enzymatic properties and the RNA template sequence of telomerase, the following series of events has been postulated to occur (Fig 1.2; Blackburn & Greider, 1989). First, telomerase recognizes and binds to DNA, potentially through the 95 kDa subunit (Collins *et al.*, 1995). The exact primer sequence that telomerase can bind to is variable, likely reflecting the ability of the enzyme to elongate telomeres as well as add repeats to chromosome breaks (Harrington & Greider, 1991; Collins & Greider, 1993; Lee & Blackburn, 1993). If

the enzyme is not positioned at the 3' end of the primer it may slide or loop out intervening DNA to reach the end (Harrington & Greider, 1991). Next, the first three nucleotides of the telomerase RNA sequence 3' AACCCCAAC hybridize to the 3' end of the telomere G-strand, although the initial alignment of a primer with the telomerase RNA may encompass more than the first three 3' nucleotides of the template, depending on the primer sequence. Using the remaining nucleotides 3'CCCAAC as a template, one full telomeric repeat is added (Autexier & Greider, 1994) of which the last nucleotide can be excised and replaced with a G residue in a fashion analogous to editing (Collins & Greider, 1993). Once the correct residue is added, telomerase translocates and again realigns the 3' template sequence with the last three nucleotides of the newly elongated G strand in preparation for another cycle of repeat addition. The time required for translocation and possibly 'editing' is believed to account for the pause the enzyme makes *in vitro* after addition of the last G of every GGGTTG repeat. Although telomerase can perform many cycles of elongation *in vitro* (Greider, 1991) it is not processive *in vivo* (Yu *et al.*, 1990). How processivity is repressed *in vivo* is unknown, but telomere structure could be involved since primers which adopt a G-quartet configuration are not elongated (Zahler *et al.*, 1991) unless packaged into chromatin (Shippen *et al.*, 1994).

Telomerase activity has since been detected in other ciliates (Zahler & Prescott, 1988; Shippen-Lentz & Blackburn, 1989; Lingner *et al.*, 1994; Melek *et al.*, 1994) and found to use an RNA template as in *Tetrahymena* (Shippen-Lentz & Blackburn, 1990;

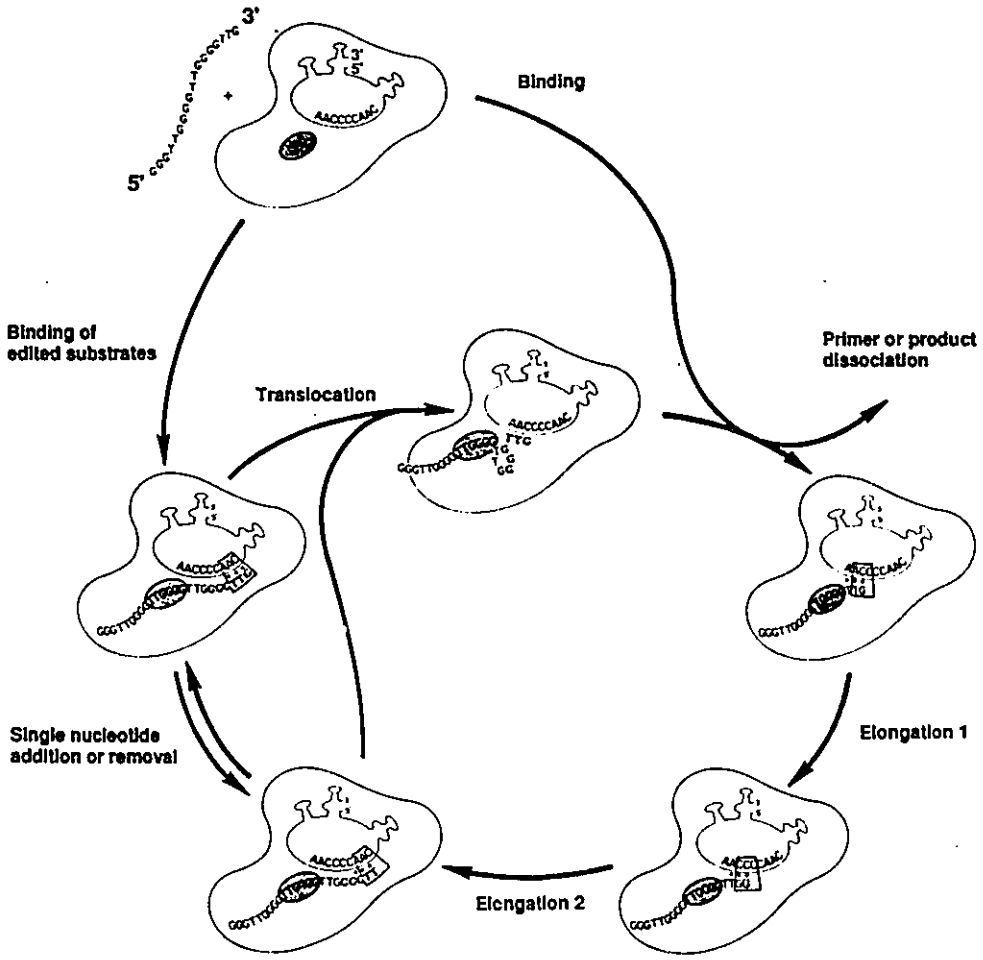

Figure 1.2

Model of the *de novo* elongation of a telomeric primer by *Tetrahymena* telomerase.

As described in the text, telomerase is thought to anchor to a primer possibly through the 95 kDa protein (shaded oval) and align the first three 3' nucleotides of the template with the DNA termini. Nucleotides complementary to the remaining template sequence are added onto the 3' end of the DNA and before translocation or dissociation of the enzyme, the last nucleotide may be cleaved off and replaced with a G residue. (see text for further details, taken from Collins & Greider, 1993)

Melek *et al.*, 1994; Lingner *et al.*, 1994). Comparison of the structural RNA sequence between subspecies of *Tetrahymena* (Romero & Blackburn, 1991) or different ciliates (Bhattacharyya & Blackburn, 1994; Lingner *et al.*, 1994) has revealed little homology outside of the template region. Instead, the length and secondary structure of the RNA is preserved between ciliates suggesting it is conformation and not sequence that is important for its function. However, neither transcript length or secondary structure are conserved between ciliates and yeast (Singer & Gottschling, 1994).

At the start of this project telomerase activity had been detected in the human cell line HeLa (Morin, 1989). More recently, enzyme activity has also been found in *Xenopus* (Mantell & Greider, 1994), mouse (Prowse *et al.*, 1993; Chadeneau *et al.*, 1995b; Prowse & Greider, 1995) and yeast (Singer & Gottschling, 1994; Cohn & Blackburn, 1995; Lin & Zakian, 1995) and the telomerase structural RNA has been cloned from yeast (Singer & Gottschling, 1994; McEarchern & Blackburn, 1995), mouse (Blasco *et al.*, 1995) and humans (Feng *et al.*, 1995). The detection of telomerase in such diverse organisms suggests an evolutionarily conserved role for this enzyme in countering the loss of telomeric DNA. This is not however, the only function of telomerase as the enzyme can also "heal" chromosome ends. For example, in both murine and human cells, artificially shortened telomeres are elongated to the length of the other telomeres in what appears to be a telomerase specific manner (Barnett *et al.*, 1993; Hanish *et al.*, 1994). Telomerase can also heal chromosome ends

that lack telomeric repeats. In *Tetrahymena*, and likely other ciliates, telomeres are added to chromosome breaks yielding new stable linear subchromosomes during a specific phase of development (Yu & Blackburn, 1991). Similarly in yeast (Murray *et al.*, 1988; Kramer & Haber, 1993; Singer & Gottschling, 1994) and apparently also in humans (Wilkie *et al.*, 1990; Morin, 1991; Lamb *et al.*, 1993; Flint *et al.*, 1994) artificially induced or naturally occurring chromosome breaks can be capped and thereby stabilized by the addition of telomeric repeats by telomerase. Thus, telomerase can both prevent and repair certain types of DNA damage (reviewed in Greider, 1994).

1.3) Cell senescence and immortalization.

Human somatic cells from a variety of tissues divide *in vitro* a finite number of times before they exit the cell cycle and arrest in G_0 in a viable state called "senescence" (Hayflick & Moorhead, 1961; reviewed Goldstein, 1990). The timing of growth arrest is dependent upon the number of cell divisions and not the length of time in culture (Dell'Orca *et al.*, 1973; Hayflick, 1976; Harley & Goldstein, 1978). Although cells at senescence remain metabolically active they cannot undergo DNA synthesis or division, even in the presence of physiological or mitogenic stimulation (Matsumura *et al.*, 1979a,b). This replication block is quite stable in human cells (reviewed in McCormick and Maher, 1988) suggesting that senescence may have evolved to prevent the continuous proliferation of cells in which normal growth

control mechanisms have been subverted.

Two lines of evidence indicate that there may be a genetic basis for senescence. First, cell division of normal young human fibroblasts can be halted by fusion with, or injection of mRNA from senescent cells (Norwood *et al.*, 1974; Yanishevsky & Stein, 1980; Lumpkin *et al.*, 1986). Second, senescence is dominant over unlimited proliferation since fusion of mortal with immortal cells generally results in hybrid cells with a finite lifespan (Bunn & Tarrant, 1980; Muggleton-Harris & DeSimone, 1980; Pereira-Smith & Smith, 1981, 1983). In agreement with senescence being under genetic control, a number of genes have been shown to contribute to the senescent phenotype. Among these a central and essential role is played by the tumour suppressors p53 and pRb which negatively regulate the G_1 to S transition. Through a variety of experimental means it has been demonstrated that cells can escape senescence if the functions of these tumour suppressors are impeded (Bischoff *et al.*, 1990; Hara *et al.*, 1991; Shay *et al.*, 1991b, 1995; Rogan *et al.*, 1995), and conversely that a senescent-like growth arrest is reinstated if p53 and pRb functions are restored (Brugge & Butel, 1975; Radna *et al.*, 1989; Wright *et al.*, 1989; Levine *et al.*, 1991; Hinds *et al.*, 1992; Quinlan, 1993; Rinehart *et al.*, 1993; Yamato *et al.*, 1995). Genes which regulate p53 or pRb have also been implicated in the senescence phenotype (reviewed in Barrett *et al.*, 1994; Dimri & Campisi, 1994). Indeed, p53 and pRb may operate in concert since p53 is known to increase the levels of p21, a protein which regulates pRb function (Noda *et al.*, 1994). Cell fusion experiments suggest

that the establishment of senescence may involve additional pathways. Fusion of different immortal cell lines does not always yield hybrids with unlimited growth capacity, indicating that several recessive changes complement the immortal phenotype. In fact, from a systematic series of experiments, four complementation groups for unlimited proliferation have been identified (Pereira-Smith & Smith, 1983, 1988; Whitaker *et al.*, 1992; Duncan *et al.*, 1993; Goletz *et al.*, 1994b). Introduction of single chromosomes from a normal human cell background into different immortal cell lines has lead to the identification of chromosomes containing senescent genes of the different complementation groups (Ning *et al.*, 1991, Ogata *et al.*, 1993, 1995; Hensler *et al.*, 1994; Rimessi *et al.*, 1994; Sandu *et al.*, 1994; Yoshida *et al.*, 1994). However, the identity of such genes remains to be elucidated.

The replicative blockade of senescence is not impenetrable. Transformation of cultured human cells with DNA tumour viruses (reviewed in Shay *et al.*, 1991b), with retroviruses (Miyoshi *et al.*, 1981), or with carcinogens or radiation (Harris, 1987) allows cells to escape from senescence. In the case of cells transformed by viral oncogenes, the most commonly used transforming agents, this process involves the functional inactivation of p53 and pRb by the oncoproteins (reviewed in Dimri & Campisi, 1994). However, even transformed cells do not divide indefinitely but eventually enter a proliferative crisis. Although the severity of this crisis can vary, it is generally characterized by an increase in cell death (Shein *et al.*, 1964; Girardi *et al.*, 1965; Stein, 1985; reviewed in Sack, 1981) and in some virus transformed cells, an

increase in chromosomal aberrations (primarily dicentrics, Moorhead & Saksela, 1963, 65; Wolman *et al.*, 1964; Stewart & Bacchetti, 1991). Proliferative crisis is thus phenotypically different from senescence suggesting that the two stages reflect different processes.

Generally transformed cells survive crisis and acquire an unlimited lifespan only in rare cases. The frequency of immortalization varies with cell type and transforming agent and is also influenced by clonal differences within a cell population (*ie:* Girardi *et al.*, 1965; Graham *et al.*, 1977; Byrd *et al.*, 1982; Huschtscha & Holliday, 1983; Whittaker *et al.*, 1984; Chang, 1986; Harris, 1987; Pirisi *et al.*, 1987; Shay & Wright, 1989; Band *et al.*, 1990; Karran *et al.*, 1990; Middleton *et al.*, 1990; Hering *et al.*, 1991; Wazer *et al.*, 1995). Nevertheless, even in virus transformed cells, the most efficient system for generating immortal populations, it has been estimated that only one in 10^5 to 10^7 cells will become immortal (Huschtscha & Holliday, 1983; Shay & Wright, 1989; reviewed in Shay *et al.*, 1991b). The rarity of this event suggests that oncogenes are either inefficient at (Strauss & Griffin, 1990) or incapable of (Chang, 1986; Wright *et al.*, 1989) immortalizing cells. Both these possibilities are consistent with the requirement for oncogene expression, and hence p53 and pRb suppression, for immortalization (Brugge & Butel, 1975; Radna *et al.*, 1989; Wright *et al.*, 1989; Shay *et al.*, 1991; Quinlan, 1993; Rinehart *et al.*, 1993). However, fusion of immortal virus transformed cells with mortal cells gives rise to mortal hybrids despite high levels of oncoprotein expression. This observation suggests that the expression of

these proteins is insufficient for immortalization and that other event(s) are required for this process (Pereira-Smith & Smith, 1987). Given the similarity between immortalization frequency and mutation rates (reviewed in Shay *et al.*, 1991b) it seems likely that these event(s) are mutations of cellular gene(s). Indeed, cells can become immortal if treated solely with mutagens (Harris, 1987), and the genetic instability of virus transformed cells (Moorhead & Saksela, 1963, 65; Stich *et al.*, 1964; Wolman *et al.*, 1964; McDougall, 1971; Caporossi & Bacchetti, 1990; Schramayr *et al.*, 1990; reviewed in Sack, 1981; Mansur & Andophy, 1993) can conceivably be considered mutagenic. Based on these observations it has been proposed that immortalization is a two step process. Initially viral oncogenes suppress the senescence signal thereby endowing cells with an extended lifespan. In the second step the infrequent acquisition of a randomly generated mutation perturbs a second unrelated cellular process permitting transformed cells to escape from crisis and thereby become immortal (Wright *et al.*, 1989).

As *in vitro*, somatic cells appear to have a finite lifespan *in vivo* unless transformed. A number of observations suggest that replicative senescence may in fact occur *in vivo* and potentially be a component of organismal ageing. For example, the replicative capacity of cells in culture decreases with donor age, possibly as a result of prior proliferation *in vivo* (Martin et al., 1970; Dell'Orco *et al.*, 1973; Goldstein *et al.*, 1978; Mets *et al.*, 1983; Schneider & Mitsui, 1986). Fibroblasts obtained from patients with premature ageing syndromes divide fewer times in culture than age

matched controls (Martin *et al.*, 1965; Goldstein, 1969, 1979; Norwood *et al.*, 1979; Mills & Weiss, 1990). Similarly, cultured cells from long-lived animals reach senescence after more cell divisions than those from short-lived animals (LeGuilly *et al.*, 1973; Goldstein, 1974; Rohme, 1981). Finally, murine tissues can only be serially transplanted a finite number of times (Daniel, 1972). Given the strong evidence that cells have a finite proliferative capacity *in vivo*, senescence could therefore represent a replicative barrier that malignant cells, like cells transformed *in vitro*, must overcome to acquire an immortal phenotype. Several lines of evidence suggest that tumour cells do in fact overcome senescence and become immortal. For examples, the tumour suppressors p53 and pRb which must be inactivated for cellular immortality in culture (see above) are often inactivated in tumours (*ie.* Skuse & Ludlow, 1994). Also, most cancers arise through multiple expansions of single, progressively more transformed cells (reviewed in Cairns, 1975; Nowell, 1976; Fearon & Vogelstein, 1990). Although a single clonal expansion could generate a large tumour within the normal lifespan of cells (Tannock 1987; Stamps *et al.*, 1992) repeated expansions could easily exhaust the proliferative capacity of malignant cells. Minimally, clonal expansions associated with the process of metastasis or tumour recurrence should have such an outcome. Lastly, although it is difficult to establish tumour cell in culture, permanent cell lines have been generated from a number of different tumours. Thus, the immortalization of cultured cells may reflect a process that occurs during tumour evolution *in vivo*.

1.4) The telomere hypothesis for cell senescence and immortalization.

Molecular dissection of the various stages that cultured cells must traverse to become immortal has failed to reveal the underlying mechanism which limits the lifespan of normal and transformed cells. In 1971 Olovnikov proposed that the loss of telomeric DNA due to the end replication problem (Fig. 1.1) would eventually lead to the inactivation of a gene essential for growth thus resulting in senescence unless, as in the case of immortal cells, the loss of telomeric DNA was circumvented.

A large body of evidence supports many aspects of this model. Telomeres do in fact shorten as human somatic cells divide. The first evidence for this was the observation that TRFs of sperm were much larger than those of embryonic and adult somatic tissues, suggesting that there was a loss of telomeric DNA during development (Cooke & Smith, 1986; Cross *et al.*, 1989, 1990; Hastie *et al.*, 1990; de Lange *et al.*, 1990; Allsopp *et al.*, 1992). TRFs of human somatic cells have subsequently been shown to decrease both with cell division *in vitro* (Harley *et al.*, 1990; Lindsey *et al.*, 1992; Allsopp *et al.*, 1992) and with increasing donor age *in vivo* (Hastie *et al.*, 1990; Harley *et al.*, 1990; Allsopp *et al.*, 1992; Vaziri *et al.*, 1993). However, in contrast with Olovnikov's predictions TRFs are still quite large in senescent cells (Harley *et al.*, 1990; Lindsey *et al.*, 1992; Allsopp *et al.*, 1992; Allsopp & Harley, 1995).

A number of models have been proposed to explain how partial loss of telomeric DNA could induce senescence. One model is based on the observation that in yeast and *Drosophila*, genes placed next to telomeres can be silenced because the

heterochromatic nature of telomeres (Karpan, 1994). It was therefore postulated that telomere shortening alters the extent with which telomere heterochromatin invades the subtelomeric DNA. Alteration of the amount of terminal DNA packaged into heterochromatin could, in turn, affect the expression of nearby genes essential for growth control (Wright & Shay, 1992). However, recently it was demonstrated that human telomeric DNA does not affect the expression of adjacent genes (Bayne *et al.*, 1994). A second model proposes that a single chromosome loses all telomeric DNA by senescence (Harley *et al.*, 1990; reviewed in Harley, 1991). This chromosome end becomes recombinogenic and fuses to form dicentrics which, when resolved, generate chromosome breaks. Alternatively the naked end could fail to correctly interact with the nuclear matrix, resulting in aberrant chromosome segregation. Indeed, dicentrics, chromosome breaks as well as polyploidy and aneuploidy have been reported in senescent cells (Saksela & Moorhead, 1963; Wolman *et al.*, 1964, Benn, 1976; Sherwood *et al.*, 1988) and all these types of damage may, or are known to (Di Leonardo *et al.*, 1994) induce a senescent-like p53 dependent growth arrest. In the absence of telomerase activity chromosome ends would not be repaired and thus the proliferative block would become permanent. A third model proposes that it is short telomeres themselves, and not the absence of telomeres, that are detected by the p53 pathway as damage, resulting in the induction of senescence (Harley *et al.*, 1990; reviewed in Harley, 1991). The latter two models are consistent with many of the characteristics of the senescent phenotype.

Irrespective of the role telomeres play at senescence it is clear that immortal cells must overcome the ultimately lethal process of telomere shortening. Indeed, uncontrolled loss of telomeric DNA in lower eukaryotes has been shown to eventually lead to the death of most cells in the population (Lundblad & Szostak, 1989; Yu *et al.*, 1990; Singer & Gottschling, 1994; McEachern & Blackburn, 1995; Liu *et al.*, 1995) whereas telomere length is stable in immortal cells (*ie.* Bernards *et al.*, 1983; Larson *et al.*, 1987; Lundblad & Szostak, 1989; Allsopp *et al.*, 1992). Telomerase was shown to be the primary means by which telomere length is maintained in yeast and *Tetrahymena* (Singer & Gottschling, 1994; McEachern & Blackburn 1995; Yu *et al.*, 1990) and detection of the enzyme in an immortal human cell line suggested a similar role for telomerase in humans (Morin, 1989). Based on these observations we postulated that when the proliferative block of senescence is overcome by transformation in culture or *in vivo*, the telomere shortening associated with division of normal somatic cells would eventually lead to cell death unless countered by the activation of telomerase. Therefore, stabilization of telomere length by the enzyme would be a necessary event in the acquisition of the immortal phenotype.

1.5.i) Statement of purpose.

At low frequency it is possible to generate immortal cells in culture. Similarly, several lines of evidence argue that malignant cells can acquire an immortal phenotype. Given that telomeres shorten as normal human somatic cells divide and that this process will inevitably be lethal, human immortal cells must, at some point, overcome telomere shortening. As described, one means of achieving stabilization of telomere length may be through the activation of telomerase. The purpose of the experiments presented in this thesis was to determine if the loss of telomeric DNA observed in normal cells must be arrested by the activation of telomerase for cells to acquire an unlimited lifespan. To address this question we initially studied telomere length and telomerase activity in *in vitro* transformed cells because this system has been well characterized in terms of immortalization and is considered a model system for tumourigenesis. Following these studies we addressed the clinical significance of these observations by investigating the role of telomere length and telomerase in human tumours.

1.5.ii) A tissue culture model of cell immortalization.

If, as predicted, telomere shortening must be arrested by the activation of telomerase in order for cells to become immortal, then irrespective of cell type or transforming agent *any* immortal cell population should have stable telomeres and be telomerase positive. Conversely, mortal cells, whether transformed or not, should be telomerase negative. To investigate these possibilities telomere length and telomerase

activity were measured throughout the lifespan of mortal and immortal clones derived from human embryonic kidney (HEK) cells transformed by Ad5 E1 or SV40 large (T) and small (t) t-antigen (Ag) and from EBV transformed human B cells. As a control, untransformed HEK cells were similarly characterized. In addition, previous work had shown that the frequency of dicentric chromosomes increases strikingly in SV40 transformed cells during crisis (Moorhead & Saksela, 1963, 65; Wolman *et al.*, 1964; Stewart & Bacchetti, 1991). Since dicentrics can be generated by the loss of telomeric repeats (reviewed in Blackburn & Szostak, 1984) we investigated the relationship between telomere length and terminal recombination by measuring the frequency of chromosomal aberrations during the lifespan of different clonal populations of transformed cells. The results of these studies are described in chapters 2 and 3.

1.5.iii) An *in vivo* model of cell immortalization

Since most tumours are derived from somatic tissues and telomeres of somatic cells decrease with cell division, it is likely that telomeres shorten as tumour cells divide. Indeed, the telomeres of Wilms' tumours (de Lange *et al.*, 1990) and colorectal carcinomas (Hastie *et al.*, 1990) were found to be generally shorter than those of the control tissues. Moreover, since tumour cells found in advanced malignancies may acquire unlimited proliferative capacity, we postulated that such cells would express telomerase to counter the inevitable loss of telomeric DNA observed during tumour growth. To investigate this possibility, telomere length was measured and telomerase activity was assayed in two different malignancies: ovarian carcinoma and leukemia.

Ovarian carcinoma was chosen as the first experimental system for a number of reasons. The tumours are generally detected late, often after they have metastasized throughout the peritoneal cavity (Clarke-Pearson & Creasman, 1986). The chances of obtaining malignant cells that are immortal is therefore maximized. Second, sufficiently large quantities of relatively pure tumour cells (required to assay telomerase activity) can be readily obtained from the ascites fluid several times over the course of the disease, permitting telomere length and telomerase to be studied temporally. Moreover, the malignant cells can be cultured and therefore studied in the same fashion as virus transformed cells. Finally, non-malignant mesothelial and fibroblastic cells can be separated from the tumour cells and expanded in culture to serve as isogenic controls. Using this system, the telomere length and status of telomerase of tumour cells from a number of patients was assessed temporally both *in vivo* and in culture and compared to control non-malignant isogenic cells and ovarian epithelial cells from non-diseased donors. The results from this study are presented in chapter 4.

One disadvantage of the ovarian system was that cells were only available from tumours that were already at a clinically advanced stage. Therefore, to follow telomere length and telomerase activity throughout disease progression *in vivo*, and also to extend our observations to another human cancer, we studied leukemic cells. To compare patterns of telomerase expression in leukemias with different growth properties, samples were collected from patients with chronic lymphoid leukemia

(CLL) acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS).

CLL is characterized by a slow but progressive clonal accumulation of functionally incompetent partially mature B cells (Rai, 1991) which, for the most part are arrested in G_0 (Vincent, 1990). Given the slow progression of CLL it has been possible to identify several distinct steps in the evolution of this leukemia making CLL an excellent *in vivo* counterpart to the transformed cell model in which telomere length and telomerase activity were followed during the process of immortalization.

In sharp contrast to CLL, AML is a rapidly growing malignancy characterized by the continual division and hence rapid accumulation of immature myeloid cells arrested at different stages of early maturation (reviewed in Löwenberg & Delwel, 1991). Due to this ever expanding pool of self-renewing cells, by the time AML is detected the disease has progressed to the end stage, very much like ovarian cancer. However, MDS, a neoplastic bone marrow disorder, can be considered analogous to early stage AML since MDS resembles less severe forms of AML and can, if a patient survives long enough with the disease, progress to AML (reviewed in Tricot, 1991).

In these two very different leukemias telomere length and telomerase activity were monitored at 'early' (early CLL, MDS) and 'late' (late CLL, AML) stages of disease progression. As in the ovarian carcinoma studies, normal control tissues (bone marrow and peripheral blood leukocytes) were similarly assayed. The results of these studies are described in chapter 5.

Chapter 2: Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity.

2.0) Foreword.

The process of telomere shortening observed during the finite lifespan of human cells (Harley *et al.*, 1990) cannot continue indefinitely in human cells which acquire an unlimited lifespan. One mechanism by which immortal human cells may overcome telomere shortening is through the activation of telomerase, the enzyme which elongates telomeres *de novo* (Greider & Blackburn, 1985). Consistent with this possibility, the human cell line HeLa was shown to be telomerase positive (Morin, 1989). Therefore, to determine if, when, and at what telomere length the enzyme is activated during the immortalization process *in vitro*, telomere length and telomerase activity were monitored throughout the lifespan of clonal populations of SV40 transformed HEK cells and in the immediate postcrisis phase of Ad5 transformed HEK cells. In addition, the frequency of dicentric chromosomes, which can arise by the fusion of chromosome ends lacking telomeres, was also determined throughout the lifespan of the SV40 transformed cells as an indirect method to potentially assess telomere function.

The EMBO Journal vol.11 no.5 pp.1921 – 1929, 1992

Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity

Christopher M.Counter[1,2], Ariel A.Avilion[3], Catherine E.LeFeuvre[1], Nancy G.Stewart[1,4], Carol W.Greider[3], Calvin B.Harley[2] and Silvia Bacchetti[1,5]

[1]Department of Pathology and [2]Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada and [3]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
[4]Present address: Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba R3E OV9, Canada
[5]Corresponding author

Communicated by J.A.Steitz

Loss of telomeric DNA during cell proliferation may play a role in ageing and cancer. Since telomeres permit complete replication of eukaryotic chromosomes and protect their ends from recombination, we have measured telomere length, telomerase activity and chromosome rearrangements in human cells before and after transformation with SV40 or Ad5. In all mortal populations, telomeres shortened by ≈65 bp/generation during the lifespan of the cultures. When transformed cells reached crisis, the length of the telomeric TTAGGG repeats was only ≈1.5 kbp and many dicentric chromosomes were observed. In immortal cells, telomere length and frequency of dicentric chromosomes stabilized after crisis. Telomerase activity was not detectable in control or extended lifespan populations but was present in immortal populations. These results suggest that chromosomes with short $(TTAGGG)_n$ tracts are recombinogenic, critically shortened telomeres may be incompatible with cell proliferation and stabilization of telomere length by telomerase may be required for immortalization.

Key words: chromosome rearrangements/immortality/telomerase/telomeres

Introduction

Human cells can undergo only a limited number of divisions *in vitro*. This phenomenon, termed replicative senescence, has often been used as a model for cellular ageing (Hayflick and Moorhead, 1961; reviewed in Goldstein, 1990). Cell senescence can be at least partially overcome by carcinogen- or virus-induced transformation, a complex process that results in the acquisition of altered morphology and growth properties. Most transformed human cells acquire an extended lifespan compared with their untransformed counterparts, but ultimately cease to divide and die (crisis). At a very low frequency, some cells overcome both replicative senescence and crisis and become immortal (reviewed in Sack, 1981; DiPaolo, 1983; Chang, 1986).

As somatic cells age *in vivo* or *in vitro*, their telomeres become progressively shorter (Harley *et al.*, 1990; Hastie *et al.*, 1990). Telomeres are specialized structures at the ends of eukaryotic chromosomes, consisting of proteins and simple repeated DNA sequences which are highly conserved throughout evolution (Blackburn and Szostak, 1984; Moyzis *et al.*, 1988; Meyne *et al.*, 1989; Zakian, 1989). In humans, 5–15 kbp of TTAGGG repeats are found at the ends of all chromosomes (Allshire *et al.*, 1988; Moyzis *et al.*, 1988; Brown, 1989; Cross *et al.*, 1989; de Lange *et al.*, 1990). Telomeres are elongated by the ribonucleoprotein enzyme telomerase which adds telomeric sequences *de novo* (Greider and Blackburn 1985, 1987). Their structure and mode of synthesis thus allow for the complete replication of chromosome ends. In addition, telomeres protect chromosome ends against illegitimate recombination and may direct chromosome attachment to the nuclear membrane (reviewed in Blackburn and Szostak, 1984; Blackburn, 1991; Zakian, 1989).

Loss of chromosome terminal sequences with each round of replication had previously been predicted based on the inability of DNA polymerases to completely replicate linear DNA molecules (Olovnikov 1971, 1973; Watson, 1972). Olovnikov (1973) further proposed that telomere shortening would ultimately lead to cell death and thus might play a role in cell senescence by limiting proliferation of somatic cells. Unicellular organisms and germline cells, however, should have acquired a mechanism to overcome incomplete replication of chromosome ends, thus allowing for their unlimited proliferative capacity (Olovnikov, 1973; reviewed in Harley, 1991). In agreement with these predictions, telomerase activity has been detected in unicellular eukaryotes where telomere length is stable (Greider and Blackburn, 1985, 1989; Zahler and Prescott, 1988; Shippen-Lentz and Blackburn, 1989) and cell senescence results from mutations in the *Est1* (ever shorter telomeres) gene of yeast (Lundblad and Szostak, 1989) or in the RNA component of *Tetrahymena* telomerase (Yu *et al.*, 1990). Moreover, in human germline cells, telomeres are significantly longer than those from somatic tissues (Allshire *et al*, 1989; Cooke *et al.*, 1989; Cross *et al.*, 1989; de Lange *et al.*, 1990; Hastie *et al.*, 1990) and are stable regardless of donor age (Allsopp,R.C., Vaziri,H., Patterson,C., Goldstein,S., Younglai,E.V., Futcher,A.B., Greider,C.W. and Harley,C.B., submitted). Presumably, telomerase is active in the germline, but inactive in somatic cells where telomere shortening occurs.

A role for telomere shortening in cellular senescence would further imply that immortalized cells should have telomerase activity and stable telomeres (reviewed in Harley, 1991). Enzyme activity has indeed been detected in HeLa cells, which are tumour-derived and immortal (Morin, 1989). However, loss of telomeric sequences can occur in tumour cells since often their telomeres are shorter than those in the tissue of origin (Cooke *et al.*, 1985; de Lange *et al.*, 1990; Hastie *et al.*, 1990). Tumour and transformed cells, like senescent cells, also have dicentric chromosomes which may arise from telomere association and fusion as a direct

result of sequence loss (Wolman *et al.*, 1964; Benn, 1976; Blackburn and Szostak, 1984; Pathak *et al.*, 1988; Zakian, 1989).

The existence of shortened telomeres in many tumour cells suggests that telomere stabilization and expression of telomerase activity occur relatively late during oncogenesis. This might also be the case for cell transformation *in vitro*. We hypothesized that loss of telomeric sequences would not be arrested in transformed cells which have acquired only an extended lifespan and might in fact play a role in the proliferative crisis of these cells. However, activation of telomerase, and thus telomere stabilization, would necessarily occur during the development of the immortal phenotype.

To test this hypothesis we measured the length and amount of telomeric DNA, incidence of dicentric chromosomes and telomerase activity throughout the lifespan *in vitro* of human embryonic kidney cells, untransformed or transformed by SV40 or Ad5 oncogenes. Our results indicate that telomeric sequences were initially lost at a similar rate in all cell populations. This process continued unchecked in transformed populations which had acquired only an extended lifespan and died at crisis. In such populations no telomerase activity was detected and loss of telomeric DNA was associated with an increase in dicentric chromosomes. In contrast, populations which had become immortal expressed telomerase and in these cells we observed stabilization of telomeric DNA length and of the frequency of dicentric chromosomes.

Results

Decrease in length and amount of telomeric DNA with cell age in vitro

To define the relationships between telomeres and cell proliferation, the length and amount of telomeric DNA were measured throughout the lifespan of transformed cells generated by transfection of human embryonic kidney (HEK) cells with the plasmid pSV3neo (Stewart and Bacchetti, 1991; Bacchetti,S. and LeFeuvre,C.E., unpublished). Under G418 selection a total of 11 colonies expressing SV40 tumour antigens were isolated and expanded. All of the populations (Table I) were found to have an extended lifespan compared with the control untransfected HEK cells, which could be subcultured for only 16 mean population doublings (MPD). However, ten of the populations (HA2-EL to HA11-EL) entered a crisis between 75–120 MPD, from which viable cell lines could not be rescued. One population, HA1-IM, underwent a brief period of slow growth from which, at ≈90 MPD, evolved a faster growing subclonal population characterized by the presence of a marker chromosome. HA1-IM cells have since continued to proliferate for >250 MPD and are therefore considered immortal. A second immortal population, the 293 cell line generated by transformation of HEK cells with adenovirus 5 DNA (Graham *et al.*, 1977), was also cultured for analysis.

Genomic DNA was isolated every two or four MPD throughout the lifespan of control cells and of a subset of the SV40 transformed cells. For the 293 cells, DNA was obtained at one time point just prior to crisis from the pre-immortal population and periodically between 12 and 135 MPD from the post-crisis immortal population. The DNA was cleaved with *Hin*fI and *Rsa*I to liberate the terminal restriction fragments (TRFs, Figure 1A) which comprise both subtelomeric repetitive DNA, X, and telomeric TTAGGG repeats, T. TRFs were resolved on agarose gels and detected by hybridization to the telomeric probe $(CCCTAA)_3$ (Figure 1B). Heterogeneity in TRF size and its multimodal distribution are due to variations in both the number of TTAGGG repeats and the length of the subtelomeric region X, but the latter accounts for most of these effects (Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted). Thus, upon autoradiography the TRF specific signal appears as a smear often divided in subpopulations.

TRF length decreased with increasing MPD in all cell populations analysed (Figure 1B). This trend was most evident for the high molecular weight band (≈17 kbp), particularly in the HA1-IM DNA, but was also easily detected for all subpopulations of TRFs sizes in both HA1-IM and HA5-EL DNA. In control HEK cells, analysis of the limited number of samples which could be obtained indicated an overall reduction in TRF size, but the relatively high molecular weight of the TRFs precluded detection of subpopulations. A reduction in TRF length with increasing MPD also occurred in the 293 cells, since TRFs in the population just prior to crisis were shorter than in HEK cells but slightly longer than in the immortal population which emerged from crisis (not shown).

A decrease in the total hybridization signal, i.e. in the amount of DNA complementary to the $(CCCTAA)_3$ probe, was also apparent in all samples analysed. This suggested that shortening of TRFs is due to specific loss of the TTAGGG repeats in the T region, rather than to a decrease in the subtelomeric region X (Figure 1A). Low molecular weight bands of ≈2, 1.6 and 0.9 kbp did not decrease in size or intensity with age of the cells (Figure 1B) and were insensitive to digestion of genomic DNA with exonuclease Bal31 (not shown), which progressively removes telomeric sequences (reviewed in Blackburn and Szostak, 1984). These

Table I. Growth and cytogenetic properties of cells

Cells	Transforming viral oncogenes	Lifespan	Increase in dicentrics near crisis
HEK		mortal, cell death at MPD 16	–
HA2,3,4,5-EL	SV40 T and t	extended, crisis at MPD 75	+
HA6,7,8,9,10,11-EL	SV40 T and t	extended, crisis between MPD 100 and 120	+[a]
HA1-IM	SV40 T and t	immortal (passaged >250 MPD), crisis MPD 65–90	+
293	Ad5 E1	immortal	n.d.

[a]HA6,8,9 and 11-EL were not tested
n.d. not determined

bands represent nontelomeric DNA fragments which hybridize at high stringency to the $(CCCTAA)_3$ probe and they were used as an internal control for the amount of DNA loaded.

The average TRF length and hybridization signal were quantified by densitometry (Figure 2 and Table II). Telomeric sequences were lost at an average rate of ≈65 bp/MPD in the extended lifespan and pre-immortal populations (ΔTRF/MPD in Table II). The data for HEK cells indicated a loss of ≈150 bp/MPD. However, this higher loss rate may not be significant since HEK TRFs could only be measured in a limited number of samples and their large size made accurate measurements difficult. The rate at which telomeric sequences were lost in pre-crisis 293 cells was not determined.

In all SV40 transformed cell populations, TRFs decreased until crisis when their average length was ≈4 kbp. In the immortal HA1-IM population, past 90 MPD, TRF length stabilized indicating no further loss of telomeric sequences for at least 120 additional MPD (Figure 2A). Loss of telomeric DNA also ceased in the second immortal line analysed, the post-crisis 293 cells, where stable TRFs of ≈3 kbp average length were maintained for at least 135 MPD (Figure 2B; Table II). The 293 cells used in these experiments were from monolayer cultures. TRFs of similar length were present in the nonadherent 293-N3S cells, whereas in the nonadherent 293(CSH) subline the TRFs had an average size of ≈7.5 kbp. Since both nonadherent lines were derived from immortal adherent cells well beyond crisis, this suggests that telomere elongation might have occurred in the 293(CSH) cells, although clonal variation cannot be ruled out (see Discussion).

The amount of telomeric DNA, as quantified from the total TTAGGG hybridization signal, also decreased at a similar rate in both extended lifespan and pre-immortal populations transformed by SV40 (Figure 2B; Table II: ΔS/MPD). The results for control HEK cells indicated a higher rate of decrease in signal intensity than for the transfected cells but, as noted above, quantification of these samples was less accurate. In the HA1-IM cells no further loss of TTAGGG signal was detected past MPD 90. Similarly, the amount of telomeric DNA was also stable in the immortal 293 cell line past crisis (Figure 2D).

The mean telomeric TTAGGG DNA length at crisis is ≈ 1.5 kbp

It has been hypothesized that loss of telomeric sequences may play a role in cell mortality (Olovnikov, 1973; Harley *et al.*, 1990; Hastie *et al.*, 1990). Since the TRF contains both subtelomeric and TTAGGG sequences, we determined

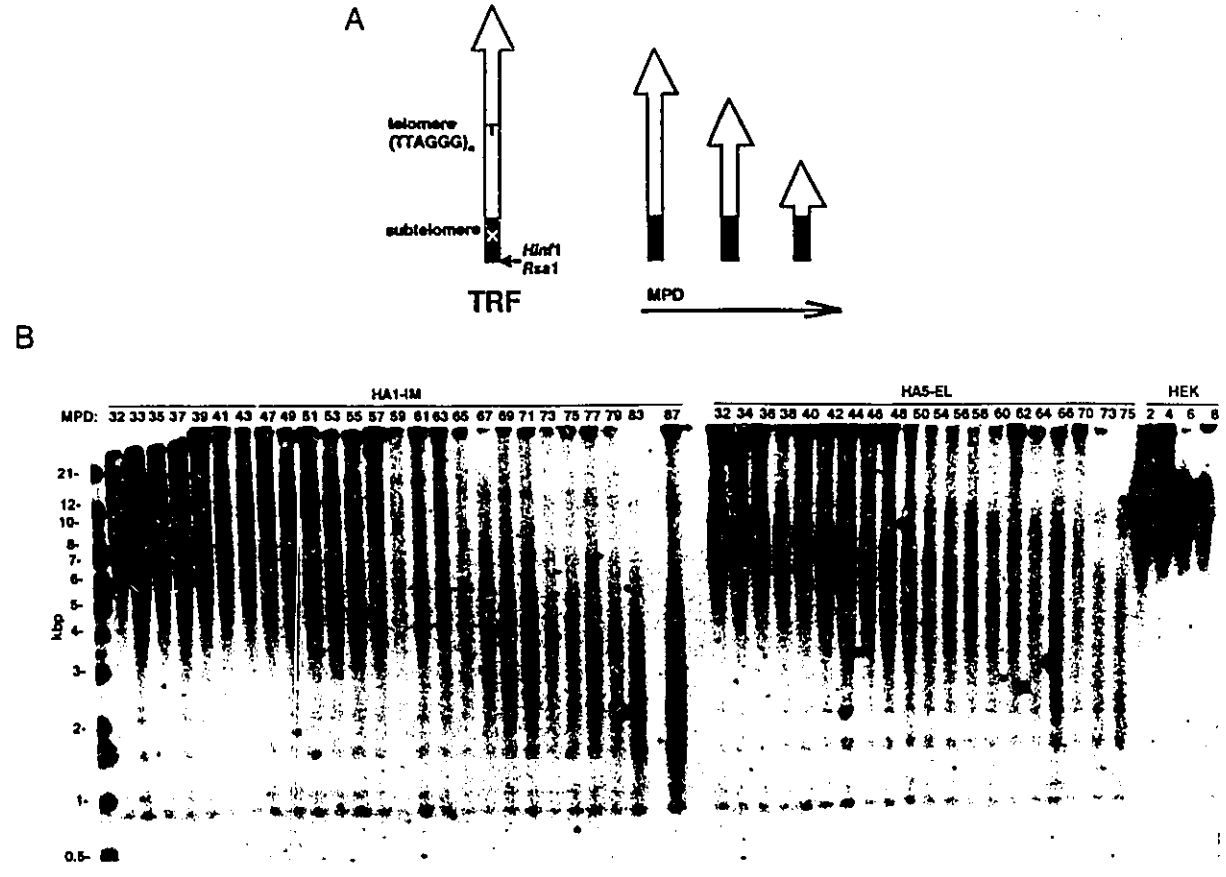

Fig. 1. (A) Structure of terminal restriction fragments (TRFs) and model for TRF shortening. TRFs are comprised of telomeric DNA, T, consisting of TTAGGG repeats and of subtelomeric DNA, X, containing degenerate arrays of TTAGGG and other sequences. As cells proliferate, TRFs become progressively shorter through loss of T. (B) Length of TRFs versus age (MPD) in immortal (HA1-IM), extended lifespan (HA5-EL) and control HEK cells. DNA was extracted from each cell population at the indicated MPD, digested with restriction enzymes *Hin*fI and *Rsa*I, separated by electrophoresis on a 0.5% agarose gel and hybridized to the human telomeric probe $[^{32}P](CCCTAA)_3$. In all cases 1.0 μg of DNA was loaded, except for the HA1-IM sample at 87 MPD which was overloaded. The auroradiograph represents a typical result and is one of several experiments with these cell populations. Similar patterns were observed for all other cell populations analysed.

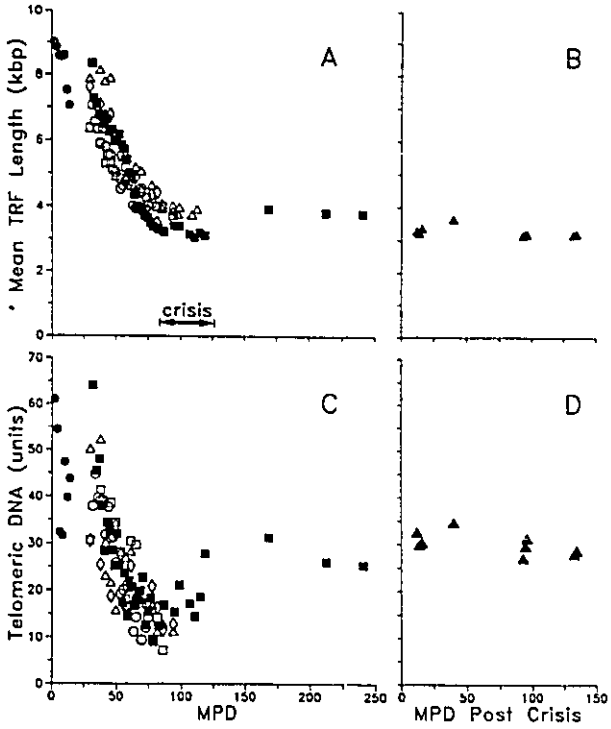

Fig. 2. Mean TRF length and amount of telomeric DNA as a function of age of control and transfected cells. For each data point a minimum of three autoradiographs (such as that of Figure 1B) were scanned with a densitometer over the size range 2–21 kbp and the densitometric values were used to determine the mean TRF length in kbp (panel A and panel B) and the amount of telomeric DNA (panel C and panel D). The latter is expressed in arbitrary units and was derived from the intensity of the total hybridization signal as previously described (Harley *et al.*, 1990). The average standard deviation of the data points for mean TRF length was 0.5 kbp with the largest deviation being 1.1 kbp. For the amounts of telomeric DNA the average and the largest standard deviations were 9.1 units and 28.6 units, respectively. Values generated from: ●, control HEK; ○, HA5-EL; □,HA6-EL; △, HA7-EL; ◇, HA10-EL; and ■, HA1-IM populations are plotted in panels A and C; the span of MPD at which individual populations enter crisis is indicated by the bar. Values generated from 293 cells (▲) are shown in panels B and D.

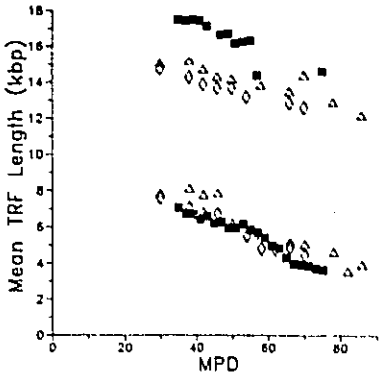

Fig. 3. Comparison between the length of the high molecular weight TRF subpopulation and the mean TRF length. The high molecular weight bands (see Figure 1B) in DNA isolated at the indicated MPD from HA1-IM (■), HA7-EL (△) and HA10-EL (◇) were scanned with a densitometer and their mean TRF lengths determined. These values ranging from 18 to 13 kbp are plotted together with those of the average of all TRF (ranging from 8 to 3 kbp) for the same cell populations and MPD.

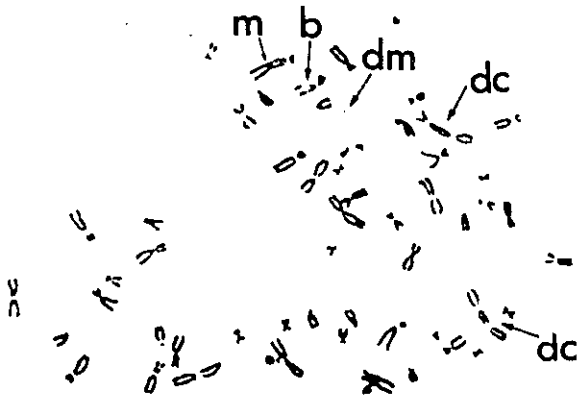

Fig. 4. Cytogenetic analysis of SV40 transformed cells. Chromosome spreads were prepared and scored as described in Materials and methods. A metaphase with different types of aberrations is shown: dc, dicentric chromosome; dm, double minute; b, break; m, marker chromosome.

the length of the latter component in cells undergoing crisis. Assuming that the decrease in TRF length and signal intensity results solely from loss of terminal TTAGGG repeats, the length of the subtelomeric component of the TRF, X, can be calculated by comparing the rate of signal loss with the rate of decrease in TRF length (Levy *et al.*, 1992). The average subtelomere length, X, for all DNA samples we have analysed by *Hin*fI and *Rsa*I digestion was ≈2.5 kbp (Table II). In other human cell strains, the length of X ranged from ≈3 to 4 kbp (Levy *et al.*, 1992; Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted). Since the mean TRF length at crisis was ≈4 kbp in all populations, we conclude that the TTAGGG component, T, was on average ≈1.5 kbp (Table II).

Terminal restriction fragments longer than 3–4 kbp at crisis are clearly seen in Figure 1B. We have analysed three subpopulations of long TRFs: the ≈17 kbp band from HA1-IM cells (Figure 1B) and the ≈15 kbp bands from HA7-EL and HA10-EL cells (not shown). We found that these TRFs decreased in length at the same rate as the mean of the total TRF population (Figure 3). Calculations such as those described above indicated that the average length of TTAGGG DNA present in the high molecular weight TRFs at crisis was 1.5 kbp, as was that of the total TRF population (Table II). Thus long TRFs do not necessarily represent long TTAGGG tracts. In agreement with data obtained from analysis of human fibroblasts (Levy *et al.*, 1992; Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted) it appears that the heterogeneity in TRF length derives primarily from a variable size of the X region.

Chromosome fusion products increase near crisis

Dicentric chromosomes increase in frequency during ageing of cultured fibroblasts (Saksela and Moorhead, 1963; Benn, 1976; Sherwood *et al.*, 1988; reviewed in Harley, 1991) and are abundant in transformed and tumour cells (Moorhead and Saksela 1963, 1965; Wolman *et al.*, 1964; Pathak *et al.*, 1988). Since chromosomes which have lost their telomeric DNA are highly recombinogenic (Blackburn and Szostak, 1984; Pathak *et al.*, 1988; Zakian, 1989), we investigated whether the existence of very short telomeric DNA in cells

Table II. Characteristics of telomeres in control and transfected cells

Cells	ΔTRF/MPD[a] (bp/MPD)	ΔS/MPD[a] (units/MPD)	TRF length at crisis (kbp)	T length at crisis (kbp)	X length (kbp)
HEK	−150	−1.2	n.d.	n.d.	2.6
HA5-EL, HA6-EL, HA7-EL, HA10-EL	−62 ± 17	−0.5 ± 0.2	3.9 ± 0.1	1.5 ± 1.0	2.3 ± 0.9
HA1-IM	−82[b]	−0.7[b]	3.5[c]	1.0[c]	2.5
293 (adherent)	n.d.	n.d.	3.2[c]	n.d.	n.d.

[a]ΔTRF/MPD and ΔS/MPD are the decrease in TRF length and signal intensity respectively, as a function of MPD. They are derived from the slopes of the linear regressions of the data points of each cell population from Figure 3 [upper (A) and lower (C) panels respectively].
[b]Before MPD 90
[c]Average values from post-crisis cells
n.d. not determined. HEK cells do not undergo crisis, thus no values for the lengths of TRF and T at crisis are presented. Since adherent 293 cells were assayed at one time point prior to crisis and the TRF length and the amount of telomeric DNA were stable after crisis (Figure 3), no values were calculated for ΔTRF/MPD, ΔS/MPD, T and X lengths. Note also that the average values of X, T and TRF at crisis for all HA clones tested are 2.5 kbp, 1.5 kbp and 4 kbp, respectively.

at crisis was accompanied by an increase in the incidence of chromosome rearrangements which could potentially derive from telomere association and fusion. At early times after isolation all transformed populations contained on average less than one dicentric per 50 metaphases (Figure 4). Just prior to crisis, when the least amount of telomeric DNA was present, we observed a dramatic increase in the frequency of these rearrangements (Figure 5 and Table I). Ring chromosomes were also detected but at a very low frequency. Beyond crisis, in the HA1-IM cells past 90 MPD, the number of dicentrics stabilized or possibly even decreased (Figure 5). Dicentric chromosomes can arise from mechanisms other than telomere fusion and in fact other types of aberrations, unrelated to telomeres, increased prior to crisis and stabilized thereafter (not shown; Stewart and Bacchetti, 1991). Nevertheless, the striking correlation between the kinetics of telomeric DNA loss and of appearance of dicentric chromosomes, suggests the possibility that at least a fraction of these rearrangements might have arisen from telomere fusion. No chromosome aberrations were detected in 300 metaphases of control HEK cells at 10 MPD, beyond which time the low mitotic index of the cultures precluded significant cytogenetic analysis (not shown).

Telomerase is active in immortal cells

Telomeric DNA decreased in length in all populations analysed until crisis, when the cells either died or became immortal and maintained constant telomere length. To determine if telomerase activity was correlated with telomere stability, we assayed extracts from the SV40 transformed cells and the Ad5 transformed 293 cells. Telomerase activity *in vitro* is detected as a characteristic 6 nt repeat ladder on sequencing gels (Greider and Blackburn, 1985; Morin, 1989). This pattern was visible using extracts from the immortal 293 cells (Figure 6A) and HA1-IM cells at 269 MPD (Figure 6A) or 123 and 165 MPD (Table III). In both cell lines, pre-incubation of the extracts with RNase, which inactivates telomerase, abolished formation of the 6 nt repeat ladder. The RNase-insensitive 10 nt repeat ladder seen in lanes 9 and 10 between the 75 and 142 size markers, as well as other RNase insensitive bands, were observed sporadically in reactions with extracts from monolayer cultures harvested by scraping (Materials and methods). These patterns were independent of the presence of the telomeric oligonucleotide primer and arise from *in vitro* labelling of contaminating DNA. No telomerase activity was detected in extracts from the extended lifespan populations, HA5-EL and HA10-EL (Figure 6A), the HA1-IM population at 45 or 75 MPD, or the control HEK cells (Table III). All these cells were assayed before crisis or senescence when telomere shortening was occurring. The lack of enzyme activity in these extracts was not due to limited sensitivity of the assay since activity was detected in extracts of 293 cells diluted by as much as 80-fold (not shown), nor was it due to a diffusible inhibitor since mixing of HA5-EL and 293 cell extracts gave similar levels of activity as the 293 extract alone (Figure 6B). Lastly, telomerase negative extracts were as active as positive extracts in DNA polymerase assays (not shown) attesting that no generalized protein inactivation had occurred. Although the lack of detectable telomerase activity is not definitive proof for the absence of the enzyme, the detection of activity in two independently immortalized cell lines was quite striking. These results suggest that activation of the telomerase occurred during the establishment of the immortal populations.

Discussion

We have documented three novel aspects of the process of cell immortalization. Firstly, we have shown that critically short telomeres are associated with an increased frequency of dicentric chromosomes and with crisis of transformed cell populations. Secondly, in immortal cells, shortening of telomeres is arrested and the frequency of dicentric chromosomes stabilizes. Lastly, we have found that telomerase activity is not detectable in populations undergoing telomere loss, but is present in immortal cells where a constant telomere length is maintained.

Telomere shortening occurs in transformed cells which are not immortal

All transfected HEK cell populations expressing SV40 tumour antigens were found to lose telomeric TTAGGG repeats progressively till the onset of crisis. The average rate of loss of ≈65 bp/MPD, although possibly slower than that in untransfected HEK cells, was similar to that previously reported for human diploid skin fibroblasts (Harley *et al.*, 1990). Thus, acquisition of transformed properties such as altered growth rate and extended lifespan did not prevent the shortening of telomeres which is characteristic of senescing cells (Harley *et al.*, 1990) and which is probably

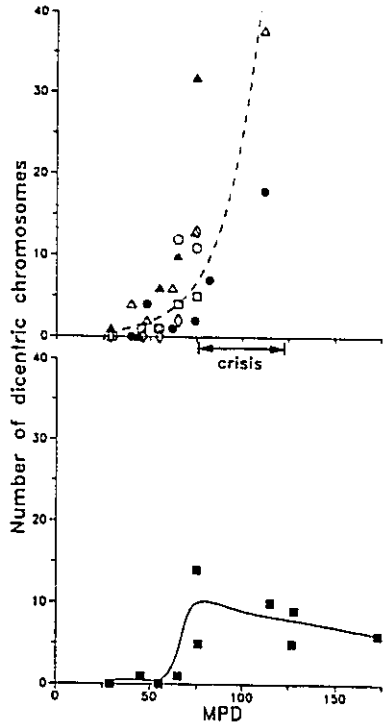

Fig. 5. Frequency of dicentric chromosomes as a function of age of transformed cell populations. At the indicated MPD chromosome spreads were prepared from: ●, HA2-EL; □, HA3-EL; ▲, HA4-EL; ○, HA5-EL; △, HA7-EL; ◇, HA10-EL **(upper panel)** and ■, HA1-IM **(lower panel)**. For each sample, 50 metaphases were analysed for the presence of chromosome aberrations. The number of dicentric chromosomes per 50 metaphases is plotted versus the age of the populations in MPD. Similar curves were obtained when plotting the frequency of cells with dicentrics, since in the majority of cases one dicentric was present per cell. As indicated, crisis refers to the range of MPD at which individual populations enter this period.

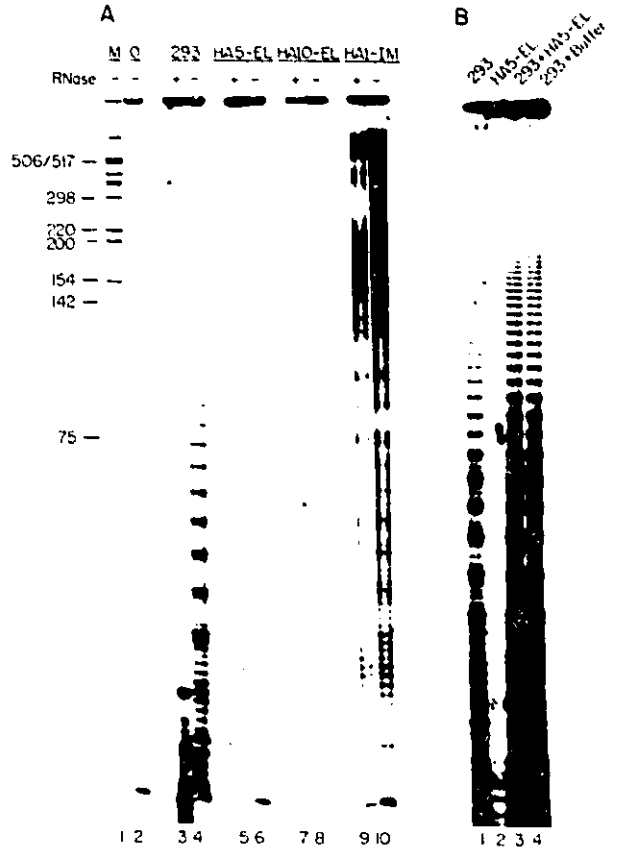

Fig. 6. Telomerase activity in extended lifespan and immortalized populations. **(A)** Each of the indicated cell populations was tested for telomerase activity using 80 μl reactions in the presence (lanes 4, 6, 8 and 10) or absence (lanes 3, 5, 7 and 9) of RNase. A ^{32}P-labelled 1 kbp ladder (lane 1, M) and the [^{32}P](TTAGGG)$_4$ oligonucleotide (lane 2, O) were run as markers. 293 cells (lanes 3 and 4) and HA1-IM cells (lanes 9 and 10) are immortal. In this experiment 293(CSH) cells and HA1-IM cells at 269 MPD were assayed. Telomerase activity was also detected in the adherent 293 cells and in the 293-N3S subline (Table III). HA5-EL (lanes 5 and 6) and HA10-EL (lanes 7 and 8) are populations with extended but finite lifespan; these cells were assayed at 47 and 57 MPD, respectively. **(B)** Extracts from HA5-EL and 293 cells were mixed and assayed for telomerase activity to determine if an inhibitor was present in the HA5-EL extract. Both extracts were assayed individually (lanes 1 and 2) using 20 μl of extract in a 40 μl reaction volume. In the mixing experiment, 20 μl of HA5-EL extract was combined with 20 μl of 293 extract (lane 3) or 20 μl of buffer (lane 4) in a final volume of 80 μl.

a consequence of incomplete replication of chromosome ends (Watson, 1972; Olovnikov, 1973; Levy *et al.*, 1992).

We calculated that the average length of telomeric TTAGGG DNA was ≈1.5 kbp at crisis. Even long restriction fragments were found to contain short TTAGGG tracts, in agreement with the suggestion that heterogeneity in TRF length derives primarily from the variable size of the subtelomeric region (Levy *et al.*, 1992; Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted). Since the value of ≈1.5 kbp is an average for the entire TRF population, it seems likely that at least some cells contain chromosomes with little or no protective telomeric DNA. Indeed, given the average rate of base pair loss per MPD and assuming that the TTAGGG region of the telomeres is roughly the same length in all chromosomes, we estimated that TRFs should lose all telomeric repeats by ≈100 MPD. Support for the generation of unprotected chromosome ends comes from the large increase in the number of dicentric chromosomes detected in cells at crisis. Association and fusion of chromosome ends occur in the absence of telomeric DNA (Blackburn, 1991; Zakian, 1989) and have been frequently observed in senescent and tumour cells and in cells transformed by SV40 (Benn, 1976; Fitzgerald and Morris, 1984; Walen, 1987; Meisner *et al.*, 1988; Pathak *et al.*, 1988 and references therein). It seems likely that this type of illegitimate recombination might have contributed to the formation of dicentrics in the transformed populations we have analysed. The fact that stabilization of telomeres in the immortal HA1-IM population correlates with a decrease in the frequency of these rearrangements is in agreement with this hypothesis. Thus, crisis in SV40 transformed cells could in part result from the existence of one or more chromosomes with 'critically' short telomeres.

Untransformed HEK cells, like other cells of epithelial origin, have an unusually short lifespan in culture compared with human embryonic fibroblasts. In part, this might reflect a lack of specific growth factors in the medium (Stampfer *et al.*, 1980; Chang, 1986), as also suggested by the absence of chromosome aberrations associated with true senescence in late cultures of these cells (this study; Stewart and Bacchetti, 1991). Nevertheless, loss of telomeric sequences occurred in HEK cells and such loss (from ≈9 to 7 kbp) was comparable to that observed during the lifespan of

Table III. Correlation between telomerase activity and immortalization

	Cells	MPD	Telomerase activity[a]
	HEK	≈10	–
pre-crisis	HA5-EL	47	–
	HA10-EL	57	–
	HA1-IM	45	–
		75	–
post-crisis	HA1-IM	123	+
		165	+
		269	+
	293 (adherent)	22	+
	293 (CSH and N3S)	n.d.[b]	+

[a]Telomerase activity in S100 extracts from cells at the indicated MPD was detected by extension of a telomeric oligonucleotide as described in Figure 6. Extracts were considered negative if the characteristic 6 nt repeat pattern was not detected on sequencing gels within a two week exposure of the film.
[b]Cumulative MPD of nonadherent 293 cells was not determined.

human embryonic fibroblasts (from ≈8 to 6 kbp over 60 MPD; Harley *et al.*, 1990). Transformation with viral oncogenes confers an extended lifespan to both HEK cells and fibroblasts (Girardi *et al.*, 1965; Stein, 1985; Radna *et al.*, 1989; Shay and Wright, 1989; Wright *et al.*, 1989). Thus, senescence in primary human cells may represent a checkpoint rather than an absolute limit to cell proliferation (Wright *et al.*, 1989; reviewed in Goldstein, 1990). Telomere shortening could be one of the signals that triggers this checkpoint and causes cell cycle exit and cell response or threshold levels may differ in various tissues. Viral transformation may override this signal and cause cells to resume division but, in the absence of telomerase, loss of telomeric DNA would continue until the cells reach crisis.

Telomerase may be required for cell immortality

The most interesting observation from our analysis of telomere dynamics in transformed cells was that loss of telomeric DNA ceased in two distinct populations of immortalized cells, HA1-IM and 293. In HA1-IM cells, where telomere shortening could be measured throughout the pre-crisis growth, stabilization of telomere length occurred quite abruptly at ≈90 MPD. This trend most likely reflects the outgrowth of a subclonal population which had acquired the immortal phenotype including the ability to halt telomere loss. The HA1-IM population which emerged from an indistinct crisis associated with slow growth prior to 80–90 MPD, was characterized by a higher growth rate and the presence of a marker chromosome. The progenitor of the immortal subclone must, however, have been present in the HA1-IM population earlier since two different stocks of early passage HA1-IM cells were successfully grown past crisis and yielded immortal lines with the same marker chromosome. It is tempting to speculate on the potential role of this aberrant chromosome. It was first detected in one of 50 cells at 75 MPD but might conceivably have existed in the population even earlier (Stewart and Bacchetti, 1991). However, its occurrence at similar low frequencies in transformed cell populations which could not be rescued from crisis (e.g. HA4-EL and HA5-EL; Stewart and Bacchetti, 1991), suggests that it may represent a genetic change which is necessary but not sufficient for the acquisition of the immortal phenotype. Other events must presumably occur to allow unlimited proliferation of cells.

We propose that expression of telomerase is one of the events required for a cell to acquire immortality. Telomerase activity was not detected in untransfected HEK cells nor in cells which expressed the SV40 tumour antigens and some of the transformed phenotypes but still retained a limited lifespan. In contrast, the enzyme was present in extracts of SV40 or Ad5 transformed cells which had become immortal and its presence was correlated with that of stable telomeres. This pattern of expression indicates that the enzyme is not induced by the viral proteins and suggests the involvement of mutational events. It is possible that one or more of the chromosomal rearrangements associated with shortened telomeres resulted in telomerase reactivation. In addition to the two types of virus transformed HEK cells reported here, telomerase activity has been detected in two human tumour cell lines, HeLa (Morin, 1989) and HL-60 (Allsopp,R.C., Vaziri,H., Patterson,C., Goldstein,S., Younglai,E.V., Futcher,A.B., Greider,C.W. and Harley,C.B., unpublished). We have previously argued that shortening of telomeres might contribute to replicative senescence both *in vivo* and *in vitro* (Harley *et al.*, 1990), a hypothesis which was at variance with the existence of immortal cells with short telomeres (de Lange *et al.*, 1990; Hastie *et al.*, 1990). Our findings that in immortalized cells telomeres are stable and telomerase activity is present resolve this apparent contradiction.

Our data allow us to define an approximate time in the growth of the immortal HA1-IM population when the enzyme is first expressed. Although extracts from cells before crisis (45 and 75 MPD) were negative for telomerase, enzyme activity could have been present but undetected in a small fraction of the population. However, the telomere length of HA1-IM cells past 90 MPD indicated that the progenitor of the immortal population acquired telomerase activity when its telomeres were critically shortened. Similar conclusions, as to the time of telomerase expression, can be drawn for the immortal 293 cell line, which also has stable but very short telomeres. Formally, however, enzyme activation could occur at any time during the development of the immortal phenotype. An imbalance between telomerase activity and incomplete replication could also favour telomere elongation. Indeed, the TRFs of the 293(CSH) subline are twice as long as those of the parental line; long TRFs are also present in one of three sublines of HeLa cells (deLange *et al.*, 1990) and in a subset of colon mucosal tumours (Hastie *et al.*, 1990).

Significance and implications

Our *in vitro* model of cellular transformation can be summarized as follows. Growth control of normal somatic cells is overridden by oncogene expression and the replicative limit of these cells is extended. In the absence of telomerase, telomeres shorten until cells become nonviable (crisis), presumably due to chromosome instability. If an additional mutation activates telomerase, an immortal clone may survive the crisis period with stabilized telomeres.

This model may have direct relevance to tumourigenesis *in vivo*. For example, the finite lifespan of partially transformed (pre-immortal) cells which lack telomerase might explain the frequent regression of tumours after limited growth *in vivo*. In bypassing the checkpoint representing normal replicative senescence, transformation may confer

an additional 20–40 population doublings during which an additional ≈2 kbp of telomeric DNA is lost. Since 20–40 doublings (10^6–10^{12} cells in a clonal population) potentially represents a wide range of tumour sizes, it is possible that many benign tumours may lack telomerase and naturally regress when telomeres become critically shortened. We predict that more aggressive, perhaps metastatic tumours would contain immortal cells which express telomerase. To test this hypothesis, we are currently attempting to detect telomerase in a variety of tumour tissues and to correlate activity with proliferative potential. Anti-telomerase drugs or mechanisms to repress telomerase expression could be effective agents against tumours which depend upon the enzyme for maintenance of telomeres and continued cell growth.

Materials and methods

Cells and transfection

Human embryonic kidney (HEK) cells obtained by trypsinization of fetal organs were seeded at high density, harvested when confluent, frozen and stored in liquid nitrogen. Upon thawing, cells were seeded at approximately half confluence and were grown to confluence in α-MEM with 10% fetal calf serum. This stage of growth was arbitrarily denoted 0 MPD. For transfections, cells were seeded at a density of 2.5×10^5 cells/100 mm plate and grown for 24 h. Transfection with pSV3neo [encoding the SV40 early region and the bacterial neomycin gene (Southern and Berg, 1982)] was performed by the calcium phosphate technique (Graham and van der Eb, 1973). The cells were incubated for 8 h in the presence of the DNA–calcium phosphate precipitate, refed with fresh medium and 48 h after transfection were reseeded at low density in G418-containing medium. Eleven independent colonies (HA1-IM and HA2-, HA3-, HA4- and HA5-EL: Stewart and Bacchetti, 1991; HA6-, HA7-, HA8-, HA9-, HA10- and HA11-EL: LeFeuvre,C.E. and Bacchetti,S., unpublished) surviving G418 selection were isolated and expanded. All of the cells had altered morphology and expressed SV40 tumour antigens. Cells were subcultured when confluent and reseeded at a split ratio of 1:4 or 1:8 in α-MEM with 10% fetal calf serum. Thus each successive passage represents two or three MPD. In all populations cell viability remained high up to the onset of crisis, as indicated by their replating efficiency and stable growth rate. Five of the eleven transformed populations generated from kidney cells from two different donors were chosen for in depth analysis. Aliquots of the 293 cell population frozen prior but close to crisis and of the immortal (post-crisis) 293 cell line (Graham *et al.*, 1977) were obtained from F.L.Graham (McMaster University). These cells were grown in monolayers in MEM-F11 plus 10% newborn calf serum. The 293 cell line adapted for growth in suspension, 293(CSH), was obtained from B.Stillman (Cold Spring Harbor Laboratory). These cells and the 293-N3S subline derived from passage in mice (Graham, 1987) were grown in spinner flasks in Joklik's modified MEM with 10% horse serum. All three lines were used for DNA analysis and assayed for telomerase activity.

DNA extraction

Cells were lysed and proteins were digested in 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K at 48°C overnight. Following two extractions with phenol and one with chloroform, DNA was precipitated with ethanol and dissolved in 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA (TE).

Determination of TRF length and amount of telomeric DNA

Genomic DNA was digested with *Hin*fI and *Rsa*I, extracted and precipitated as above and redissolved in TE. The DNA concentration was measured by fluorometry (Morgan *et al.*, 1979). DNA samples (1 μg each) were loaded onto a 0.5% agarose gel and electrophoresed for 13 h at 90 V. The gel was dried at 60°C for 30 min, denatured in 1.5 M NaCl and 0.5 M NaOH for 15 min, neutralized in 1.5 M NaCl, 0.5 M Tris–HCl (pH 8.0) for 10 min and hybridized to a 5′-[^{32}P](CCCTAA)$_3$ telomeric probe in 5× SSC (750 mM NaCl and 75 mM sodium citrate), 5× Denhardt's solution (Maniatis *et al.*, 1982) and 0.1× P wash (0.5 mM pyrophosphate, 10 mM Na_2HPO_4) at 37°C for 12 h. Following three high stringency washes in 0.1× SSC at 20–22°C (7 min each), the gel was autoradiographed on pre-flashed (OD = 0.15) Kodak XAR-5 X-ray films for 24–48 h with enhancing screens. Each lane was scanned with a densitometer and the data were used to determine the amount of telomeric DNA and the mean TRF length as previously described (Harley *et al.*, 1990).

Cytogenetic analysis

Cells were seeded at a 1:8 split ratio onto plates containing coverslips and grown to half confluence. Following addition of colcemid (0.1 μg/ml) for 5 h to arrest chromosomes at metaphase, the cells were incubated in hypotonic KCl (0.075 M) at 37°C and fixed in cold methanol:acetic acid (3:1 v/v). Chromosome were stained with 5% Giemsa and metaphases were scored regardless of ploidy, unless chromosome overlaps precluded analysis. Aberrations were identified according to Buckton and Evans (1973).

Preparations of S-100 cell extracts

Approximately 6×10^8 cells were used for each extracts. Cells growing in suspension were collected by centrifugation for 10 min at 1800 r.p.m. (500 *g*) at 4°C with no brake in a Beckman JA-10 fixed angle rotor. Cells growing in monolayer were harvested by scraping with a rubber policeman and centrifuged as above. The pellets were rinsed twice in cold PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl and 8 mM Na_2HPO_4) followed by centrifugation for 3 min at 2000 r.p.m. (570 *g*) at 4°C in a swing-out rotor. The final pellet was rinsed in cold 2.3× Hypo buffer (1× Hypo buffer: 10 mM HEPES pH 8.0, 3 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 10 U/ml of RNasin, 1 μM leupeptin and 10 μM pepstatin A; in some experiments the latter two reagents were omitted), centrifuged for 5 min and resuspended in 0.75 vol of 2.3× Hypo buffer. After incubation on ice for 10 min the sample was transferred to an ice cold 7 ml Dounce homogenizer and homogenized on ice using a B pestle (25–55 μm clearance). After a further 30 min on ice the sample was centrifuged for 10 min at 10 000 r.p.m. (16 000 *g*) at 4°C in a Beckman J3–13.1 swing-out rotor. One-fiftieth volume of 5 M NaCl was added and the sample was centrifuged for 1 h at 38 000 r.p.m. (100 000 *g*) at 4°C in a Beckman Ti50 rotor. Glycerol was added to a final concentration of 20% and the extract aliquoted and stored at −70°C. Protein concentration in a typical extract was ≈4 mg/ml.

Telomerase and DNA polymerase assays

Telomerase activity was assayed by a modification of the method of Morin (1989). Aliquots (20 μl) of S-100 cell extract were diluted to a final volume of 40 μl containing 2 mM dATP, 2 mM dTTP, 1 mM $MgCl_2$, 1 μM $(TTAGGG)_3$ primer, 3.13 μM (50 μCi) [α-^{32}P]dGTP (400 Ci/mmol), 1 mM spermidine, 5 mM β-mercaptoethanol, 50 mM potassium acetate and 50 mM Tris–acetate (pH 8.5). In some experiments reaction volumes were doubled. The reactions were incubated for 60 min at 30°C and stopped by addition of 50 μl of 20 mM EDTA and 10 mM Tris–HCl (pH 7.5) containing 0.1 mg/ml RNase A, followed by incubation for 15 min at 37°C. To eliminate proteins, 50 μl of 0.3 mg/ml proteinase K in 10 mM Tris–HCl (pH 7.5), 0.5% SDS was added for 10 min at 37°C. Following extraction with phenol and addition of 40 μl of 2.5 M ammonium acetate and 4 μg of carrier tRNA, the DNA was precipitated with 500 μl of ethanol at −20°C. DNA pellets were resuspended in 3 μl of formamide loading dye, boiled for 1 min, chilled on ice and loaded onto an 8% polyacrylamide–7 M urea sequencing gel and run at 1500 V for 2.5 h using 0.6× TBE buffer. Dried gels were exposed to Kodak XAR-5 pre-flashed film at −70°C with enhancing screen. Typical autoradiograph exposures were between 2 and 7 days. DNA polymerase was assayed according to Bauer *et al.* (1988) in 25 μl reactions containing 12.5 μl of S-100 extract and 20 mM Tris–HCl pH 7.8, 8 mM $MgCl_2$, 0.5 mg/ml BSA, 5 mM DTT, 2 mM spermidine, 80 μM dGTP, TTP and dCTP, 20 μM dATP, 4% glycerol, 0.5 μCi [α-^{32}P]dATP (3000 Ci/mmole) and 4 μg of high molecular weight gapped calf thymus DNA (Spanos *et al.*, 1981). Following incubation at 37°C for 30 min and addition of 10 μl of 0.5 M EDTA, samples were spotted in duplicate on DE81 paper. Percentage incorporation was calculated from the ratio of radioactive counts on paper washed extensively in 0.5 M Na_2HPO_4, water and ethanol to input counts on unwashed paper.

Acknowledgements

We are grateful to Frank Graham, Jim Smiley and Hara Ghosh for critical reading of the manuscript. This work was supported by grants from the Sloat Foundation, the Medical Research Council of Canada and the Cancer Research Society of Quebec (to C.B.H. and S.B.) and from an American Cancer Society Institutional Grant #IN-153 (to C.W.G.). C.M.C. is the recipient of an Ontario Graduate Scholarship. C.W.G. is a Pew Scholar of the Biomedical Sciences. S.B. is a Terry Fox Research Scientist of the National Cancer Institute of Canada.

References

Allshire,R.C., Gisden,J.R., Cross,S.H., Cranston,G., Rout,D., Sugawara,N., Szostak,J.W. and Hastie,N.D. (1988) *Nature*, **332**, 656–659.

Allshire,R.C., Dempster,M. and Hastie,N.D. (1989) *Nucleic Acids Res.*, **17**, 4611–4627.

Bauer,G.A., Heller,H.M. and Burgers,P.M.J. (1988) *J. Biol. Chem.*, **263**, 917–924.

Benn,P.A. (1976) *Am. J. Human Genet.*, **28**, 465–473.

Blackburn,E.H. (1991) *Nature*, **350**, 569–573.

Blackburn,E.H. and Szostak,J.W. (1984) *Annu. Rev. Biochem.*, **53**, 163–194.

Brown,W.R.A. (1989) *Nature*, **338**, 774–776.

Buckton,K.E. and Evans,H.J. (1973) *Methods for the Analysis of Human Chromosome Aberrations*. World Health Organization, Geneva.

Chang,S.E. (1986) *Biochim. Biophys. Acta*, **823**, 161–194.

Cooke,H.J., Brown,W.R.A. and Rappold,G.A. (1985) *Nature*, **317**, 687–692.

Cross,S.H., Allshire,R.C., McKay,S.J., McGill,N.I. and Cooke,H.J. (1989) *Nature*, **338**, 771–774.

de Lange,T., Shiue,L., Myers,R.M., Cox,D.R., Naylor,S.L., Killery,A.M. and Varmus,H.E. (1990) *Mol. Cell. Biol.*, **10**, 518–527.

DiPaolo,J.A. (1983) *J. Natl. Cancer Inst.*, **70**, 3–8.

Fitzgerald,P.H. and Morris,C.M. (1984) *Hum. Genet.*, **67**, 385–390.

Girardi,A.J., Jensen,F.C. and Koprowski,H. (1965) *J. Cell. Comp. Physiol.*, **65**, 69–84.

Goldstein,S. (1990) *Science*, **249**, 1129–1133.

Graham,F.L. (1989) *J. Gen. Virol.*, **68**, 937–940.

Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **64**, 456–467.

Graham,F.L., Smiley,J.R., Russell,W.C. and Nairn,R. (1977) *J. Gen. Virol.*, **36**, 59–72.

Greider,C.W. and Blackburn,E.H. (1985) *Cell*, **43**, 405–413.

Greider,C.W. and Blackburn,E.H. (1987) *Cell*, **51**, 887–898.

Greider,C.W. and Blackburn,E.H. (1989) *Nature*, **337**, 331–337.

Harley,C.B. (1991) *Mutat. Res.*, **256**, 271–282.

Harley,C.B., Futcher,A.B. and Greider,C.W. (1990) *Nature*, **345**, 458–460.

Hastie,N.D., Dempster,M., Dunlop,M.G., Thompson,A.M., Green,D.K. and Allshire,R.C. (1990) *Nature*, **346**, 866–868.

Hayflick,L. and Moorhead,P.S. (1961) *Exp. Cell Res.*, **25**, 585–621.

Levy,M.Z., Allsopp,R.C., Futcher,A.B., Greider,C.W. and Harley,C.B. (1992) *J. Mol. Biol.*, in press.

Lundblad,V. and Szostak,J.W. (1989) *Cell*, **57**, 633–643.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Meisner,L.F., Wu,S.-Q., Christian,B.J. and Reznikoff,C.A. (1988) *Cancer Res.*, **48**, 3215–3220.

Meyne,J., Ratliff,R.L. and Moyzis,R.K. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7049–7053.

Moorhead,P.S. and Saksela,E. (1963) *J. Cell. Comp. Physiol.*, **62**, 57–83.

Moorhead,P.S. and Saksela,E. (1965) *Hereditas*, **52**, 271–284.

Morgan,A.R., Lee,J.S., Pulleyblank,D.E., Murray,N.C. and Evans,D.H. (1979) *Nucleic Acids Res.*, **7**, 547–569.

Morin,G.B. (1989) *Cell*, **59**, 521–529.

Moyzis,R.K., Buckingham,J.M., Cram,L.S., Dani,M., Deaven,L.L., Jones,M.D., Meyne,J., Ratliff,R.L. and Wu,J-R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6622–6626.

Olovnikov,A.M. (1971) *Doklady Biochem.*, **201**, 394–397.

Olovnikov,A.M. (1973) *J. Theor. Biol.*, **41**, 181–190.

Pathak,S., Wang,Z., Dhaiway,M.K. and Sachs,P.C. (1988) *Cytogenet. Cell Genet.*, **47**, 227–229.

Radna,R.L., Caton,Y., Jha,K.K., Kaplan,P., Li,G., Tragnos,F. and Ozer,H.L. (1989) *Mol. Cell. Biol.*, **9**, 3093–3096.

Sack,G.H. (1981) *In Vitro*, **17**, 1–19.

Saksela,E. and Moorhead,P.S. (1963) *Proc. Natl. Acad. Sci. USA*, **50**, 390–395.

Shay,J.W. and Wright,W.E. (1989) *Exp. Cell Res.*, **184**, 109–118.

Sherwood,S.W., Rush,D., Ellsworth,J.L. and Schimke,R.T. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9086–9090.

Shippen-Lentz,D. and Blackburn,E.H. (1989) *Mol. Cell. Biol.*, **9**, 2761–2764.

Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327–341.

Spanos,A., Sedgwich,S.G., Yarranton,G.T., Hubscher,U and Banks,G.R. (1981) *Nucleic Acids Res.*, **9**, 1825–1839.

Stampfer,M., Hallowes,R.C. and Hackett,A.J. (1980) *In Vitro*, **16**, 415–425.

Stein,G.H. (1985) *J. Cell. Physiol.*, **125**, 36–44.

Stewart,N.G. and Bacchetti,S. (1991) *Virology*, **180**, 49–57.

Walen,K.H. (1987) *Cancer Genet. Cytogenet.*, **25**, 149–159.

Watson,J.D. (1972) *Nature*, **239**, 197–201.

Wolman,S.R., Hirschhorn,K. and Todaro,G.J. (1964) *Cytogenetics*, **3**, 45–61.

Wright,W.E., Pereira-Smith,O.M. and Shay,J.W. (1989) *Mol. Cell. Biol.*, **9**, 3088–3092.

Yu,G.L., Bradley,J.D., Attardi,L.D. and Blackburn,E.H. (1990) *Nature*, **344**, 126–132.

Zahler,A.M. and Prescott,D.M. (1988) *Nucleic Acids Res.*, **16**, 6953–6972.

Zakian,V.A. (1989) *Annu. Rev. Genet.*, **23**, 579–604.

Received on November 1, 1991; revised on January 13, 1992

Chapter 3: Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B Lymphocytes

3.0) Foreword.

As described in the previous chapter, it appeared that telomerase positive immortal cells were expanded under the selective pressure of very short and potentially nonfunctional telomeres, suggesting that activation of telomerase arrests telomere shortening thereby permitting cells to divide indefinitely.

EBV transformed human B cells immortalize more readily, and with a less severe crisis, than either SV40 or Ad transformed cells (Middleton *et al.*, 1991). One possible exception for these differences is that telomerase is activated in EBV transformed cells before telomeres become very short. To address this possibility and to assess the validity of our findings in a completely different cell type transformed by a virus belonging to another family, telomere length, telomerase activity and the number of dicentric chromosomes were monitored throughout the lifespan of EBV transformed human B cells.

JOURNAL OF VIROLOGY, May 1994, p. 3410–3414
0022-538X/94/$04.00+0

Vol. 68, No. 5

Stabilization of Short Telomeres and Telomerase Activity Accompany Immortalization of Epstein-Barr Virus-Transformed Human B Lymphocytes

CHRISTOPHER M. COUNTER,[1] FERNANDO M. BOTELHO,[1] PING WANG,[1] CALVIN B. HARLEY,[2]† AND SILVIA BACCHETTI[1]*

Molecular Virology and Immunology Programme, Department of Pathology,[1] and Department of Biochemistry,[2] McMaster University, Hamilton, Ontario L8N 3Z5, Canada

Received 11 October 1993/Accepted 9 February 1994

We have measured telomere length and telomerase activity throughout the life span of clones of human B lymphocytes transformed by Epstein-Barr virus. Shortening of telomeres occurred at similar rates in all populations and persisted until chromosomes had little telomeric DNA remaining. At this stage, some of the clones entered a proliferative crisis and died. Only clones in which telomeres were stabilized, apparently by activation of telomerase, continued to proliferate indefinitely, i.e., became immortal. Since loss of telomeres impairs chromosome function, and may thus affect cell survival, we propose that telomerase activity is required for immortality. We have now detected this enzyme in a variety of immortal human cells transformed by different viruses, indicating that telomerase activation may be a common step in immortalization.

Transformation by DNA tumor viruses usually confers on cultured human cells an extended life span (reviewed in references 3, 30, 33, and 34). In the case of simian virus 40 (SV40), adenovirus, and human papillomavirus, most transformed populations eventually reach a proliferative crisis and perish (13, 14, 32), only rarely yielding immortal clones. The low efficiency of immortalization by these viruses (14, 20, 28, 32, 34) has suggested that viral proteins are insufficient for this process and that additional events, such as mutations of host genes, are required (29, 34, 35, 37). In contrast, Epstein-Barr virus (EBV) (23, 34) and polyomavirus (34, 35) reportedly immortalize human cells at high frequency with no detectable crisis, suggesting that these viruses may encode most, or all, of the functions required for immortalization (34).

Cell life span and telomere length appear to be related. Telomeres are the most distal structures of chromosomes, composed of highly repetitive DNA, $(TTAGGG)_n$ in humans (6, 11), and protein, which together maintain the stability of chromosome ends (reviewed in references 4, 5, and 44). In cells transformed by SV40 and adenovirus type 5 (9) or by human papillomavirus type 16 (21a, 32), telomeres shorten until crisis, at which point little or no telomeric DNA remains. Concomitantly, dicentric chromosomes increase in frequency, compatible with loss of functional telomeres and fusion of chromosomal ends (9). After crisis, telomere shortening is arrested, apparently by activation of telomerase (9), the enzyme which elongates telomeres de novo (15, 16, 26, 43), and frequency of dicentric chromosomes stabilizes (9). On the basis of these observations, we proposed that virus transformation overcomes the checkpoint regulating the life span of normal cells (senescence) but does not prevent loss of telomeric DNA. Telomeres eventually reach a critically short length, leading to chromosome rearrangements and finally to cell death. Only by stabilizing telomere length by addition of telomeric repeats through activation of telomerase can cells continue to proliferate (9, 18).

Given the ability of EBV and polyomavirus to immortalize cells at high efficiency and apparently in the absence of proliferative crisis, we speculated that activation of telomerase in cells transformed by these viruses may occur early, circumventing the possible consequences of telomere loss. To investigate this hypothesis, we measured telomere length, chromosome aberrations, and telomerase activity throughout the lifespan of clonal populations of EBV-transformed human B lymphocytes. As with other viruses (9, 32), we find, however, that reactivation of telomerase is a late event in the generation of immortal cells, and that the presence of critically short telomeres correlates with proliferative crisis of nonimmortal cells. Shortening of telomeres during growth of EBV-transformed lymphocytes has recently been reported by others (17).

Growth properties of EBV-transformed clones. Human B lymphocytes from two different donors were infected with EBV (strain B95-8), and independent clonal populations were isolated and subcloned (38). The latter populations were provided to us at early passage by B. Sugden (McArdle Laboratories, University of Wisconsin) and were grown in RPMI with 10% fetal calf serum and antibiotics and, for ~5 population doublings (pd), were maintained on human fibroblast feeder layers (38). Two of the clones, B2 and B5, ceased proliferating at pd 45 and 95, respectively (Table 1), at what appeared to mimic the proliferative crisis of adenovirus type 5- and SV40-transformed cells (13, 14). This event was reproducible, as shown by culturing of B2 a second time from frozen early-passage cells. Loss of cell viability was likely not due to activation of the lytic cycle since EBV-transformed populations release very little virus and maintain a constant copy number of viral genomes throughout their life span (36, 39). Our data suggest that proliferative crisis does occur in EBV-transformed cells and is detectable when clonal populations are analyzed. This crisis is likely masked in nonclonal populations because of the high rate of immortalization by EBV (23) (Table 1).

Clones B3 and B4 reproducibly yielded immortal populations (Table 1). The doubling time of these cultures did not

* Corresponding author. Phone: (905) 525-9140, ext. 22296. Fax: (905) 525-9940. Electronic mail address: Bacchett@FHS.CSU.McMaster.CA.

† Present address: Geron Corporation, Menlo Park, CA 94025.

TABLE 1. Properties of EBV-transformed B lymphocytes

Clone	Lifespan (pd)[a]	ΔTRF (bp/pd)	TRF length at crisis (kbp)	Telomere length at crisis (kbp)[b]	Dicentrics[c]			Telomerase activity[d]	
					Precrisis	Crisis	Posterisis	Precrisis	Posterisis
B2	Mortal, crisis at pd 45	−120	4.5	ND[e]	−	ND	ND	−	ND
B3	Immortal, crisis at pd 60–100[f]	−100	3.5	1.5	−	++	−	−	+
B4	Immortal, crisis at pd 70–100	−90	3.5	2.0	−	+	−	−	+
B5	Mortal, crisis at pd 95	−80	5.0	ND	−	++	ND	−	ND
Mean (± SD)		−100 ± 20	4 ± 1	1.8 ± 0.1					

[a] Life span is recorded as pd after isolation of subclones. Crisis was defined as the time when cell death exceeded cell proliferation (B2 and B5) or when cell viability decreased, as determined by trypan blue exclusion (B4). Immortal clones were cultured for more than 150 pd.

[b] Amount of telomeric $(TTAGGG)_n$ DNA at crisis was calculated, as previously described (9, 22), by comparing the rate of loss of hybridization signal (Fig. 1C) with the rate of decrease in TRF length (Fig. 1B), and by assuming that decrease in both parameters is due exclusively to loss of $(TTAGGG)_n$ repeats.

[c] A minimum of 50 metaphases was examined at different time points before, during, and after crisis. The average number of dicentrics per 50 metaphases in each period was as follows: 0 to 1 (−), 2 to 4 (+), and more than 4 (++).

[d] Extracts were considered positive (+) if the 6-nucleotide pattern was detected in a 4-day exposure and negative (−) if no pattern was detected in a 10-day exposure.

[e] ND, not determined.

[f] Cell viability was not measured for clone B3, and crisis was defined as the period in which TRFs were critically short and the frequency of dicentrics dramatically increased.

vary appreciably with age; however, in the case of B4 cells, for which viability was measured, there was a detectable increase in the number of nonviable cells (5 to 12%) between pd 70 and 100 (Table 1). Whether this represents crisis is unclear. Even in SV40 and adenovirus transformants, the severity of crisis can vary, and immortalization with little or no overt crisis has occasionally been detected (7, 9, 24, 31).

Telomerase activity is detected in immortal populations concomitantly with stabilization of telomeres. To measure telomere length as a function of cell age, genomic DNA was isolated at regular intervals throughout the life span of each of the clones. The DNA was digested with restriction enzymes *Hin*fI and *Rsa*I which liberate terminal restriction fragments (TRFs), composed of telomeric TTAGGG repeats and of subtelomeric DNA containing short stretches of TTAGGG DNA, degenerate TTAGGG, and unique repeats (1, 12). Digested DNA was resolved in agarose gels and hybridized with the telomeric probe $^{32}P(CCCTAA)_3$. Gels were stringently washed and exposed to PhosphorImager screens (Molecular Dynamics) to visualize the TRFs, as described elsewhere (2, 9). In all populations, TRFs had heterogeneous distributions (Fig. 1A) since their length varies between different chromosomes and even the same chromosomes in different cells (9, 12, 22). Distinct DNA fragments of 2 kbp and smaller (Fig. 1A) were present in all clones. These species were resistant to exonuclease *Bal* 31 (not shown) and thus likely correspond to nontelomeric interstitial TTAGGG repeats (2, 9, 40, 44). In addition, DNA from B3 and B4 cells contained a unique fragment of 4 kbp which disappeared beyond pd 44 to 47 (Fig. 1A). This suggests that cells containing this polymorphic sequence were lost from the populations. Since B3 and B4 were derived from the same donor and cloned twice, this polymorphism may have been present in the parental cells. Its loss is consistent with the occurrence of genetic heterogeneity in transformed populations, as indicated by the existence of polyploid cells (see below).

As shown in Fig. 1A for B3, both TRF length and intensity of telomeric signal decreased with cell age, indicating an actual loss of TTAGGG repeats (9, 19). Similar results were obtained for all the clones and are plotted as TRF length and telomeric signal versus pd in Fig. 1B and C. Specifically, the rate of decrease in TRF length was, on average, 100 bp/pd (Table 1), similar to previous reports (2, 9, 40). In the terminal passages of B2 and B5 (pd 45 and 95, respectively), TRF length was ~4.7 kbp, on average (Table 1; Fig. 1B). On the basis of the data for B3 and B4 (Table 1), a TRF length of 4.7 kbp corresponds to a calculated average length of <2 kbp of residual true telomeric DNA (TTAGGG). TRF length is quite heterogeneous, and thus, it is likely that some chromosomes in these populations actually lack telomeric DNA (9). Indeed, since the residual hybridization signal may be due to subtelomeric TTAG GG repeats, many chromosome ends may be composed of nontelomeric DNA. The actual sequence at the termini of chromosomes appears to be critical since single-nucleotide permutations of the telomeric repeat cause cell senescence and death in *Tetrahymena thermophila* (43). This suggests that shortening of telomeres may not be detrimental per se until a chromosome lacks TTAGGG repeats at its end, in agreement with the observation that large terminal deletions are tolerated only if broken ends acquire telomeric DNA (5, 21, 42–44). In clones B3 and B4, TRFs reached their shortest length of 3.5 kbp by pd 100 and thereafter were stable. It is intriguing that the limited decrease in cell viability occurred in B4 cells between pd 70 and 100. It is also interesting to note that the difference in initial telomere length in B5 compared with B2 (≈13 versus ≈10 kbp [Fig. 1B]) may account for the longer replicative capacity (45 pd longer) of this clone, as suggested for cultured human fibroblasts (2).

To assay telomerase activity, extracts were prepared by collecting 10^7 to 10^8 cells in mid-log phase from each of the clones before and, if applicable, after crisis. S100 extracts were prepared either as previously described (9, 10) or by first washing 10^7 cells in 1 ml of hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol), before permeabilization in 200 μl of lysis buffer (10 mM Tris-HCl [pH 8], 1 mM $MgCl_2$, 10% glycerol, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.4% Nonidet P-40) as described by Wang et al. (41). Telomerase was assayed by incubating at 30°C for 1 to 2 h 20 μl of extract with buffer (10, 11) or 10 μl of extract with an equal volume of 2× buffer {100 mM Tris-HCl [pH 8], 6 mM $MgCl_2$, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 2 mM spermidine, 0.2 mM spermine, 2 mM β-mercaptoethanol, 2 μM $(TTAGGG)_3$, 5 mM dATP, 5 mM TTP, and 1.25 μM [α-^{32}P]dGTP [10 mCi/ml] [41]}. Reactions were terminated by digestion with proteinase K, followed by phenol and chloroform extractions and removal of unincorporated nucleotide by Sephadex GS-50 spin columns (9, 10). DNA was precipitated, washed with ethanol, resuspended in loading buffer, and resolved on sequencing gels which were then exposed to PhosphorImager screens (9, 10). Since the enzyme contains an RNA template (16, 26, 43), RNase was added to duplicate reaction mixtures as a control prior to the addition of [α-^{32}P]dGTP. This treatment abolishes the 6-nucleotide ladder

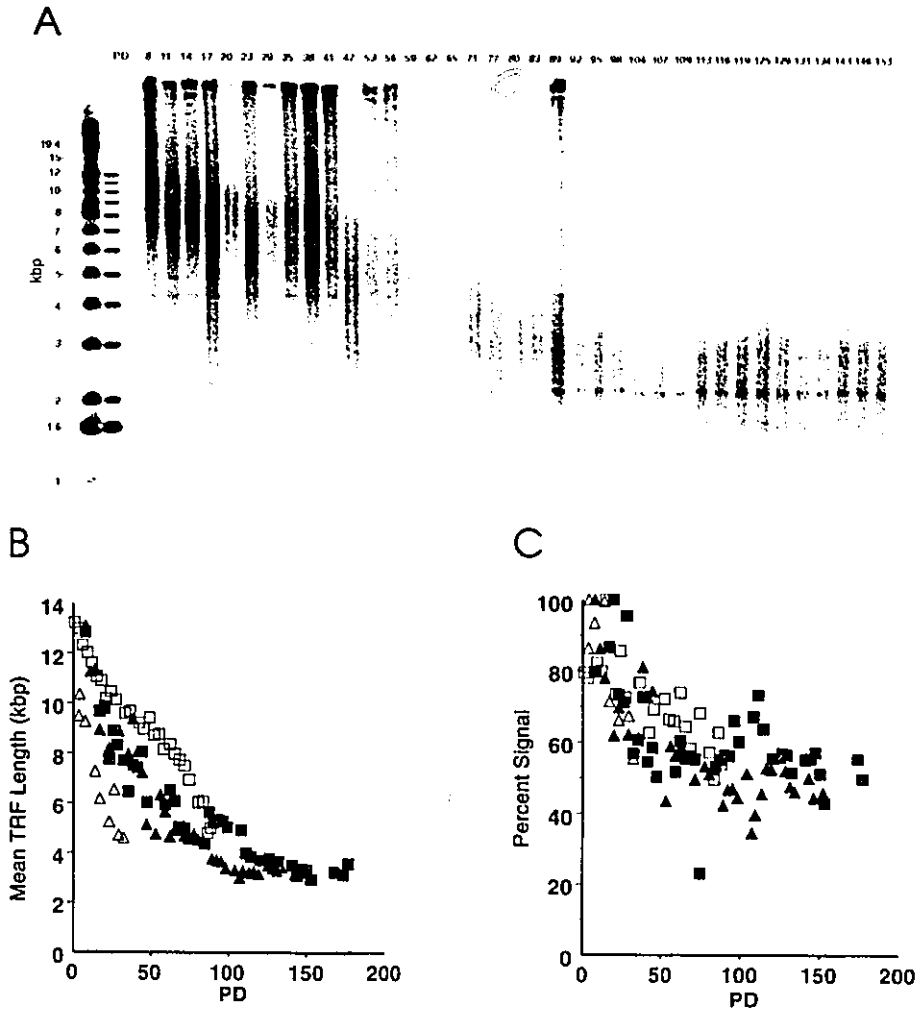

FIG. 1. TRF length and amount of telomeric DNA (intensity of hybridization signal) in clonal populations of EBV-transformed B lymphocytes. (A) Southern hybridization of the telomere probe $(CCCTAA)_3$ to *Hin*fI- and *Rsa*I-digested DNA from clone B3 at the indicated pd. (B and C) The TRF distribution was quantified from 21 to 2 kbp (2, 9) for a minimum of three separate experiments for the mortal clones B2 (△) and B5 (□) and the immortal clones B3 (▲) and B4 (■). The values were used to determine mean TRF length (B) and signal intensity, expressed as percentage of the maximum signal (C).

characteristic of the pausing of telomerase during elongation of a primer (9, 16, 26, 43).

An RNase-sensitive 6-nucleotide ladder comigrating with an identical pattern from telomerase-positive 293 cell extracts (9) was detected in extracts from the immortal populations derived from clones B3 and B4 (Fig. 2; Table 1). No activity was detected, even in longer exposures, in either of these clones or in the mortal clones B2 and B5 before crisis (Fig. 2; Table 1) and in peripheral blood leukocytes from normal donors (10). All extracts were prepared from the same number of cells and had similar protein concentrations and DNA polymerase activity (not shown). Lack of detectable telomerase activity in precrisis and control cells was confirmed by using both methods for preparation and testing of extracts (9, 10, 41). We occasionally detected an RNase-sensitive 5-nucleotide ladder (not shown) or an RNase-insensitive single-nucleotide ladder (Fig. 2) but found that these patterns were not template-dependent when permutated oligonucleotides were used, indicating that they were not due to telomerase (8, 26, 27). Thus, as in the case of cells transformed by other viruses (9), telomerase activation or up-regulation is not an early event in EBV transformants but occurs when telomere length is stabilized near crisis. The existence of stable but short telomeres in immortal populations, moreover, argues against selection of preexisting telomerase-positive cells, since the presence of these cells at early passages should yield immortal clones with long stable telomeres.

Cytogenetic properties of transformed B lymphocytes. SV40-transformed cells exhibit a dramatic increase in dicentric chromosomes at crisis (9, 25, 30, 37), possibly because of the loss of functional telomeres (9, 18). To determine whether dicentrics also form at crisis in EBV-transformed cells, metaphase spreads were prepared at regular intervals throughout the life spans of the populations and scored for chromosomal aberrations and ploidy (37). All populations contained a fraction of polyploid cells, except for B3 which was almost exclusively polyploid. The incidence of aberrations was very low throughout the life span of all clones (<2/50 metaphases, on average, excluding dicentrics) compared with SV40-transformed cells (9, 25, 30, 37), although in some cases aberrations

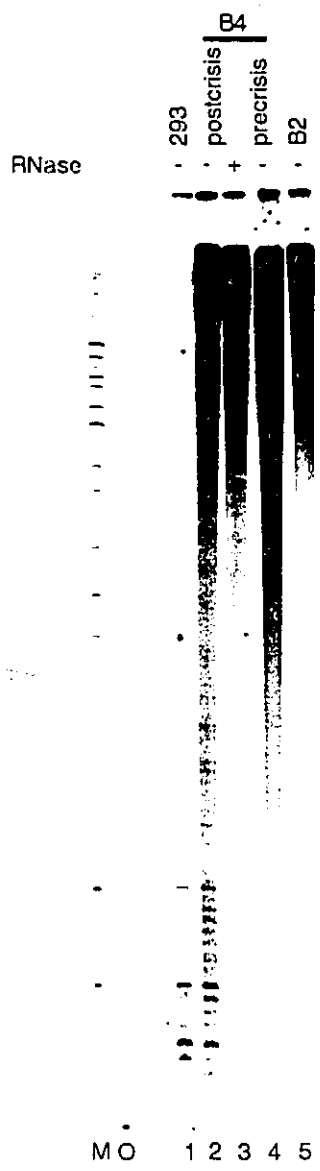

FIG. 2. Telomerase activity in EBV-transformed B lymphocytes. S100 extracts from the telomerase-positive cell line 293 (lane 1), the immortal clone B4 after and before crisis (lanes 2 to 4), and the mortal clone B2 before crisis (lane 5) were incubated with buffer, TTP, dATP, the telomeric oligonucleotide $(TTAGGG)_3$, and [α-^{32}P]dGTP. As a control, RNase was added to the B4 postcrisis extract prior to addition of [α-^{32}P]dGTP (lane 3). Reaction products were resolved on a sequencing gel and detected after exposure to a PhosphorImager screen (Molecular Dynamics) for 6 days (9, 39). M, molecular weight marker; O, ^{32}P$(TTAGGG)_3$ oligonucleotide.

increased near crisis. Few or no dicentrics were detected in transformed populations at early times when telomeres were long (Fig. 3; Table 1). Interestingly, however, when telomeres became critically short, the frequency of dicentrics increased dramatically, at least in B5 cells at terminal passage and in B3 cells between pd 40 and 80 (Fig. 3; Table 1). Thus, as in the case of SV40 transformants (9), dicentric formation may correlate with critically short telomeres, reduced cell viability, and absence of telomerase activity. These observations agree with data from a variety of systems supporting the notion that

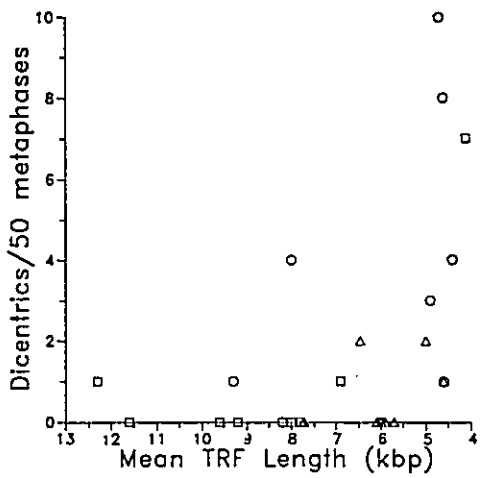

FIG. 3. Dicentrics per 50 metaphases versus mean TRF length in EBV-transformed B lymphocytes: B5 (□), B4 (△), and B3 (○). The number of dicentrics detected at crisis, when TRFs are 4 to 5 kbp in length, was found to be significantly different from the values corresponding to TRFs 1 kbp longer (5 to 6 kbp; $P = 0.004$) or to all larger TRFs (up to 13 kbp; $P < 0.0001$).

lack of telomeric DNA leads to highly recombinogenic chromosomes (4, 5, 9, 21, 44). Since EBV transformants contained few aberrations which could serve as precursors for dicentrics (e.g., breaks), it is tempting to speculate that the latter structures may result exclusively from fusion of chromosomes lacking telomeres. After crisis, only one dicentric was detected in B3, and none were detected in B4 cells, out of 150 and 300 metaphases, respectively. Telomerase presumably balances telomere shortening by addition of TTAGGG repeats to chromosome ends, thus potentially restoring telomere function (chromosome stability). Lack of dicentrics in immortal populations may be a consequence of this process.

Activation of telomerase may be a common step in the acquisition of immortality. Clonal populations of EBV-transformed B lymphocytes undergo a proliferative crisis, indicating that the virus does not encode all the necessary functions required for immortalization. Presumably, the remaining events involve mutations of host genes. It is clear that the high rate of immortalization by EBV (23) relative to SV40 and adenovirus is not due to early activation of telomerase, since neither stable telomeres nor telomerase activity were detected until after pd 80. Perhaps EBV can directly induce more of the events required for immortalization than other viruses, or immortalization efficiency is dependent upon the cell type. As in the case of SV40- and adenovirus type 5-transformed cells, in EBV transformants telomere shortening, and potentially crisis, are overcome concomitantly with detection of telomerase activity, suggesting that this enzyme may be universally required for immortality. We have recently detected telomerase in late-stage ovarian carcinomas (10). Our present and previous (9) results render virus-transformed cells a suitable system for the study of the enzyme in tumor development and the testing of telomerase-inhibitory drugs.

We are in debt to B. Sugden for the EBV-transformed human B-lymphocyte clones and for helpful discussions and critical review of the manuscript. We thank J. J. McCormick for communicating his protocol for preparing and assaying human cell extracts for telomerase, prior to publication.

This work was supported by grants from the Cancer Research Society of Quebec (S.B. and C.B.H.) and the National Cancer Institute of Canada (S.B.). C.M.C. is a recipient of the Longstaffe Scholarship. S.B. is Terry Fox Research Scientist of the National Cancer Institute of Canada.

REFERENCES

1. **Allshire, R. C., M. Dempster, and N. D. Hastie.** 1989. Human telomeres contain at least three types of G-rich repeat distributed non-randomly. Nucleic Acids Res. **17**:4611–4627.
2. **Allsopp, R. A., H. Vaziri, C. Patterson, S. Goldstein, E. V. Younglai, A. B. Futcher, C. W. Greider, and C. B. Harley.** 1992. Telomere length predicts replicative capacity of human fibroblasts. Proc. Natl. Acad. Sci. USA **89**:10114–10118.
3. **Benjamin, T., and P. K. Vogt.** 1990. Cell transformation by viruses, p. 317–368. *In* B. N. Fields et al. (ed.), Virology, 2nd ed. Raven Press, Ltd., New York.
4. **Blackburn, E. H.** 1991. Structure and function of telomeres. Nature (London) **350**:569–573.
5. **Blackburn, E. H., and J. W. Szostak.** 1984. The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. **53**:163–194.
6. **Brown, W. R. A.** 1989. Molecular cloning of human telomeres in yeast. Nature (London) **338**:774–776.
7. **Byrd, P., K. W. Brown, and P. H. Gallimore.** 1982. Malignant transformation of human embryo retinoblasts by cloned adenovirus 12 DNA. Nature (London) **298**:69–71.
8. **Collins, K., and C. W. Greider.** 1993. *Tetrahymena* telomerase catalyzes nucleolytic cleavage and nonprocessive elongation. Genes Dev. **7**:1364–1376.
9. **Counter, C. M., A. A. Avilion, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, and S. Bacchetti.** 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J. **11**:1921–1929.
10. **Counter, C. M., H. W. Hirte, S. Bacchetti, and C. B. Harley.** Telomerase activity in human ovarian carcinoma. Proc. Natl. Acad. Sci. USA, in press.
11. **Cross, S. H., R. C. Allshire, S. J. McKay, N. I. McGill, and H. J. Cooke.** 1989. Cloning of human telomeres by complementation in yeast. Nature (London) **338**:771–774.
12. **DeLange, T., L. Shiue, R. M. Myers, D. R. Cox, S. L. Naylor, A. M. Killery, and H. E. Varmus.** 1990. Structure and variability of human chromosome ends. Mol. Cell. Biol. **10**:518–527.
13. **Girardi, A. J., F. C. Jensen, and H. Koprowski.** 1965. SV40 induced transformation of human diploid cells: crisis and recovery. J. Cell. Comp. Physiol. **65**:69–84.
14. **Graham, F. L., J. R. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus 5. J. Gen. Virol. **36**:59–72.
15. **Greider, C. W., and E. H. Blackburn.** 1985. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell **43**:405–413.
16. **Greider, C. W., and E. H. Blackburn.** 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. Nature (London) **337**:331–337.
17. **Guerrini, A. M., B. Camponeschi, F. Ascenzioni, E. Piccolella, and P. Donini.** 1993. Subtelomeric as well as telomeric sequences are lost from chromosomes in proliferating B lymphocytes. Hum. Mol. Genet. **2**:455–460.
18. **Harley, C. B.** 1991. Telomere loss: mitotic clock or genetic time bomb? Mutat. Res. **256**:271–282.
19. **Harley, C. B., A. B. Futcher, and C. W. Greider.** 1990. Telomeres shorten during ageing of human fibroblasts. Nature (London) **345**:458–460.
20. **Huschtscha, L. I., and R. Holliday.** 1983. Limited and unlimited growth of SV40-transformed cells from human diploid MRC-5 fibroblasts. J. Cell Sci. **63**:77–99.
21. **Jager, D., and P. Philippsen.** 1989. Stabilization of dicentric chromosomes in *Saccharomyces cerevisiae* by telomere addition to broken ends or centromere deletion. EMBO J. **8**:247–254.

21a. **Klingelhutz, A. J., S. A. Barber, P. P. Smith, K. Dyer, and J. K. McDougall.** 1994. Restoration of telomeres in human papillomavirus-immortalized human anogenital epithelial cells. Mol. Cell. Biol. **14**:961–969.

22. **Levy, M. Z., R. C. Allsopp, A. B. Futcher, C. W. Greider, and C. B. Harley.** 1992. Telomere end-replication problem and cell aging. J. Mol. Biol. **225**:951–960.
23. **Miller, G.** 1990. Epstein-Barr virus: biology, pathology and medical aspects, p. 1921–1958. *In* B. N. Fields et al. (ed.), Virology, 2nd ed. Raven Press, Ltd., New York.
24. **Miranda, A. F., G. J. Duigou, E. Hernandez, and P. B. Fisher.** 1988. Characterization of mutant human fibroblast cultures transformed with simian virus 40. J. Cell Sci. **89**:481–493.
25. **Moorhead, P. S., and E. Saksela.** 1965. The sequence of chromosome aberrations during SV 40 transformation of human diploid cell strain. Hereditas **52**:271–284.
26. **Morin, G. B.** 1989. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell **59**:521–529.
27. **Prowse, K. R., A. A. Avilion, and C. W. Greider.** 1993. Identification of a nonprocessive telomerase activity from mouse cells. Proc. Natl. Acad. Sci. USA **90**:1493–1497.
28. **Radna, R. L., Y. Caton, K. K. Jha, P. Kaplan, G. Li, F. Traganos, and H. L. Ozer.** 1989. Growth of immortal simian virus 40 tsA-transformed human fibroblasts is temperature dependent. Mol. Cell. Biol. **9**:3093–3096
29. **Ray, F. A., D. S. Peabody, J. L. Cooper, L. S. Cram, and P. M. Kraemer.** 1990. SV40 T antigen *alone* drives karyotype instability that precedes neoplastic transformation of human diploid fibroblasts. J. Cell. Biochem. **42**:13–31.
30. **Sack, G. H.** 1981. Human cell transformation by simian virus 40—a review. In Vitro **17**:1–19.
31. **Shay, J. W., and W. E. Wright.** 1989. Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. Exp. Cell Res. **184**:109–118.
32. **Shay, J. W., W. E. Wright, D. Brasiskyte, and B. A. Van Der Haegen.** 1993. E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. Oncogene **8**:1407–1413.
33. **Shay, J. W., W. E. Wright, and H. Werbin.** 1991. Defining the molecular mechanisms of human cell immortalization. Biochim. Biophys. Acta **1072**:1–7.
34. **Strauss, M., and B. E. Griffin.** 1990. Cellular immortalization—an essential step or merely a risk factor in DNA virus-induced transformation. Cancer Cells **2**:360–365.
35. **Strauss, M., S. Hering, L. Lubbe, and B. E. Griffin.** 1990. Immortalization and transformation of human fibroblasts by regulated expression of polyoma virus T antigens. Oncogene **5**:1223–1229.
36. **Sternas, L., T. Middleton, and B. Sugden.** 1990. The average number of molecules of Epstein-Barr nuclear antigen 1 per cell does not correlate with the average number of Epstein-Barr virus (EBV) DNA molecules per cell among different clones of EBV-immortalized cells. J. Virol. **64**:2407–2410.
37. **Stewart, N., and S. Bacchetti.** 1991. Expression of SV40 large T antigen, but not small t antigen, is required for the induction of chromosomal aberrations in transformed human cells. Virology **180**:49–57.
38. **Sugden, B., and W. Mark.** 1977. Clonal transformation of adult human leukocytes by Epstein-Barr virus. J. Virol. **23**:503–508.
39. **Sugden, B., M. Phelps, and J. Domoradzi.** 1979. Epstein-Barr virus DNA is amplified in transformed lymphocytes. J. Virol. **31**:590–595.
40. **Vaziri, H., F. Schachter, I. Uchida, L. Wei, X. Zhu, R. Effros, D. Cohen, and C. B. Harley.** 1993. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am. J. Hum. Genet. **52**:661–667.
41. **Wang, Q., V. M. Maher, and J. J. McCormick.** Personal communication.
42. **Wilkie, A. O. M., J. Lamb, P. C. Harris, R. D. Finney, and D. R. Higgs.** 1990. A truncated human chromosome 16 associated with α thalassaemia is stabilized by the addition of telomeric repeat $(TTAGGG)_n$. Nature (London) **346**:868–871.
43. **Yu, G. L., J. D. Bradley, L. D. Attardi, and E. H. Blackburn.** 1990. *In vivo* alteration of telomere sequences and senescence caused by mutated *tetrahymena* telomerase RNAs. Nature (London) **344**:126–132.
44. **Zakian, V. A.** 1989. Structure and function of telomeres. Annu. Rev. Genet. **23**:579–604.

Chapter 4: Telomerase activity in human ovarian carcinoma

4.0) Foreword.

The research described in the previous chapters indicates that irrespective of cell type or transforming virus, all transformed cells which became immortal had short stable telomeres and telomerase activity, suggesting that the activation of telomerase is a general requirement for the acquisition of an immortal phenotype. Therefore it is reasonable to propose that *in vivo* transformed cells which may have an unlimited replicative capacity should also be telomerase positive and maintain a stable telomere length. Although it is debatable whether all tumours acquire an unlimited lifespan we speculated that tumours that arise from many clonal expansions may contain cells that have divided sufficient number of times to be considered immortal. Therefore, to ascertain if cell transformed *in vivo* behave as those transformed *in vitro* with respect to enzyme activation, telomere length and telomerase activity were monitored temporally *in vitro* and *in vivo* in tumour cells from late stage ovarian cancers patients. As a control, normal tissues were similarly assayed.

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 2900–2904, April 1994
Cell Biology

Telomerase activity in human ovarian carcinoma

(telomere/ovarian cancer/cell immortalization/aging)

CHRISTOPHER M. COUNTER*, HAL W. HIRTE†, SILVIA BACCHETTI*‡, AND CALVIN B. HARLEY‡§¶

*Molecular Virology and Immunology Programme, Department of Pathology; and Departments of †Medicine and §Biochemistry, McMaster University, Hamilton, ON L8N 3Z5, Canada

Communicated by James D. Watson, January 3, 1994

ABSTRACT **Telomeres fulfill the dual function of protecting eukaryotic chromosomes from illegitimate recombination and degradation and may aid in chromosome attachment to the nuclear membrane. We have previously shown that telomerase, the enzyme which synthesizes telomeric DNA, is not detected in normal somatic cells and that telomeres shorten with replicative age. In cells immortalized *in vitro*, activation of telomerase apparently stabilizes telomere length, preventing a critical destabilization of chromosomes, and cell proliferation continues even when telomeres are short. *In vivo*, telomeres of most tumors are shorter than telomeres of control tissues, suggesting an analogous role for the enzyme. To assess the relevance of telomerase and telomere stability in the development and progression of tumors, we have measured enzyme activity and telomere length in metastatic cells of epithelial ovarian carcinoma. We report that extremely short telomeres are maintained in these cells and that tumor cells, but not isogenic nonmalignant cells, express telomerase. Our findings suggest that progression of malignancy is ultimately dependent upon activation of telomerase and that telomerase inhibitors may be effective antitumor drugs.**

Telomeres contain both DNA and protein that together appear to stabilize the ends of eukaryotic DNA (reviewed in refs. 1–3). The DNA component of telomeres is generally characterized by a G-rich strand composed of a simple tandemly repeated sequence (TTAGGG in humans) (1–3). The correct sequence of this repeat is required for telomere function, since addition of telomeric DNA harboring a mutated telomeric sequence to the ends of the endogenous *Tetrahymena* telomeres lead to telomere length instability and death (4). In addition, it now appears that the length of these repeats may play a role in the lifespan of yeast (5) and human cells (6–12). A number of studies have shown conclusively that telomere length decreases with both *in vitro* and *in vivo* division of human cells (6–12). Since cultured fibroblasts still contain telomeric repeats at senescence, it was proposed that shortening of telomeres to a critical size may act as a mitotic clock, signaling a cell cycle exit (reviewed in ref. 13).

In contrast to normal cells, transformation *in vitro* subverts normal growth control, yielding populations with extended but still finite lifespan, although immortal clones may emerge at low frequency (14–17). We have previously shown that telomere shortening continues during the extended lifespan of transformed cells until crisis, where some chromosomes may have actually lacked TTAGGG repeats (9). Interestingly, during this period the frequency of dicentric chromosomes dramatically increased. Clones overcoming crisis exhibited a stabilization of telomere length and frequency of dicentrics, apparently due to the activation of telomerase (9), the enzyme which elongates telomeres *de novo* (4, 18–20). Thus, telomerase may have restored telomere function by the addition of TTAGGG repeats and thereby permitted continued cell division in immortal clones (9). If telomerase is required for immortalization *in vitro*, it may also be necessary during tumorigenesis. We reasoned that tumor metastases or recurrent tumors, arising from repeated clonal expansion, should contain immortal cells with stable telomeres and telomerase activity and that if telomerase activation were a late event, telomeres would be short. Indeed, in some cancers it appears as though telomere length decreases with increasing tumor grade (21, 22), and tumors with very short telomeres have been detected in a variety of cancers (7, 21–26, 35). We report that late-stage ovarian carcinoma tumor cells maintain short stable telomeres both *in vitro* and *in vivo* and that telomerase is specifically activated in tumor cells, but not in normal somatic cells.

MATERIALS AND METHODS

Cell Culture. 293 CSH cells, a subline of 293 (27), were obtained from B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and cultured in Joklik medium supplemented with 5% fetal bovine serum. Ovarian carcinoma cell lines HEY and SKOV-3 (American Type Culture Collection) and normal ovarian epithelium (see below) were cultured in E3 medium [a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 supplemented with epidermal growth factor (5 ng/ml), insulin (5 μg/ml), transferrin (10 μg/ml), phosphoethanolamine (50 μM), ethanolamine (50 μM), and 3% fetal bovine serum]. The nonmalignant fraction of ascites cells were maintained in α minimal essential medium (αMEM) supplemented with 10% fetal bovine serum, whereas the tumor fraction and lines derived from them were cultured in E3 medium. Some cell lines established were tested for the ability to grow in serum-free medium (E3 medium with 0.3% bovine serum albumin replacing serum).

Isolation of Cells. Ascitic fluid was withdrawn from ovarian carcinoma patients at the time of diagnostic laparotomy or by subsequent paracentesis (28) and centrifuged to obtain a cell pellet as described (29). Leukocytes were obtained from the pooled buffy coats of three normal males, and normal ovarian epithelium was obtained from the surface of the ovary (30).

Fractionation of Ascites Cells. Fibroblasts and mesothelial cells (the nonmalignant fraction) were separated from the tumor cells based on their ability to adhere to plastic more readily than tumor cells or most leukocytes (31, 32). Specifically, cells were resuspended in αMEM supplemented with 10% fetal bovine serum and left undisturbed for 12–18 hr in culture plates. Nonadhering tumor cells were removed and expanded in culture in E3 medium, whereas adherent nonmalignant cells were expanded in αMEM with 10% fetal

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: TRF, terminal restriction fragment.

‡Reprint requests should be addressed to either corresponding author.
¶Present address: Geron Corp., 200 Constitution Dr., Menlo Park, CA 94025; email charley@geron.com.

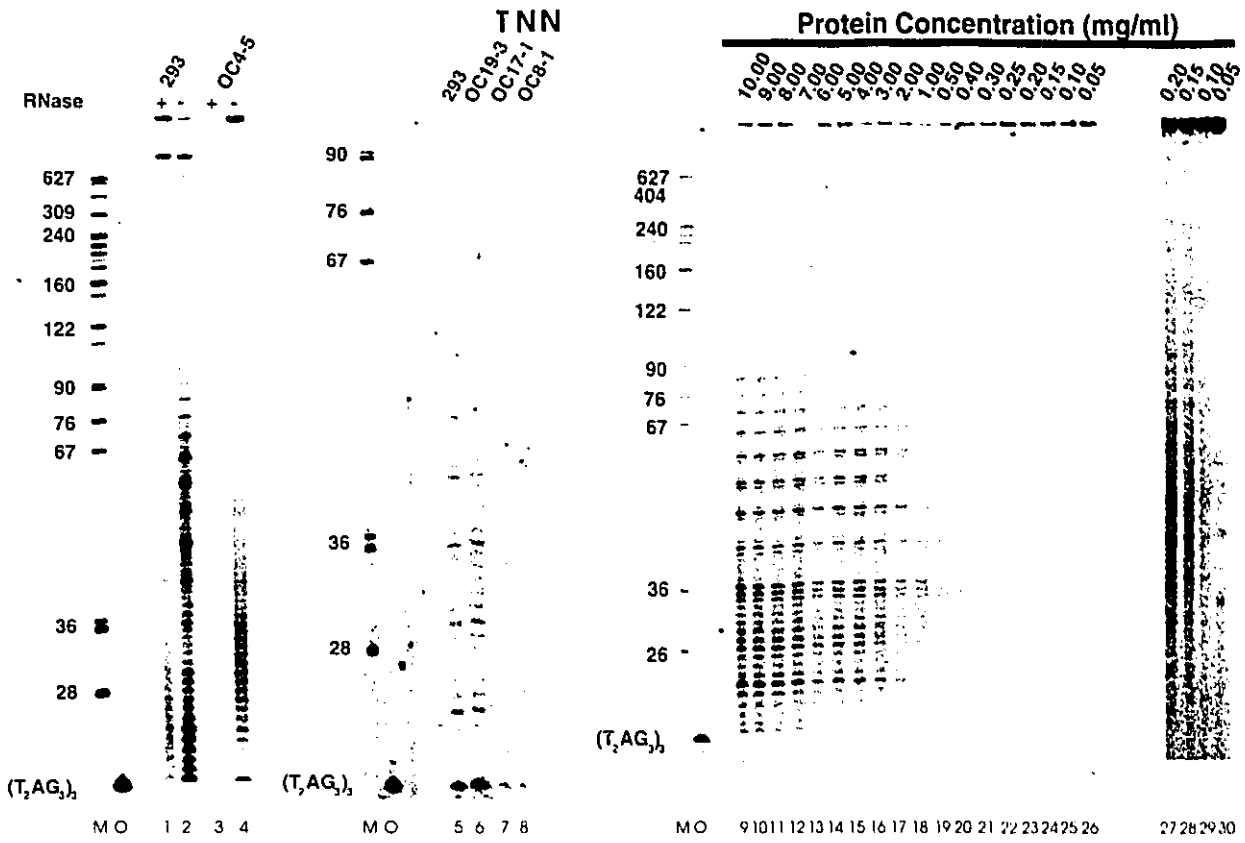

FIG. 1. Telomerase activity in cells from ascitic fluid. Telomerase activity in extracts prepared and assayed at a protein concentration of ≈11 mg/ml is shown for 293 CSH cells (lane 2) and unfractionated ascites cells from patient OC4 (lane 4; patients were assigned individual codes followed by the paracentesis number). For lanes 1 and 3, RNase was added to the extracts prior to the addition of [α-^{32}P]dGTP. Activity was detected in extracts from 293 CSH cells (lane 5), and the tumor (T, lane 6) but not the nonmalignant (N, lanes 7 and 8) cell fractions from ascites. Protein concentration in the tumor cell extract was 3.3 mg/ml, and in the normal cell extracts 2.1 and 1.9 mg/ml, respectively. Consequently, the 293 cell extract was diluted and assayed at 1.5 mg/ml for proper comparison with the negative extracts. Lanes 9–26 show a serial dilution of 293 CSH cell extract. Lanes 27–30 show the concentration range 0.2–0.05 mg/ml at greater sensitivity. Exposure to PhosphorImager screens was 1 week in all cases. Lane M, size markers (lengths in nucleotides at left); lane O; ^{32}P-labeled $(TTAGGG)_3$ oligonucleotide.

bovine serum. Cells fractionated in this manner were used to prepare S100 extracts for assaying telomerase activity (see below). Highly pure tumor cells for DNA analysis were obtained by fractionating ascites cells through two consecutive cycles of differential attachment. Additionally, attempts were made to establish some of these pure tumor cells in culture. Sometimes it was possible to obtain enough cells for analysis by filtering ascites cells through a 30-μm nylon mesh (Spectrum), which retains tumor clumps but not the smaller leukocytes, fibroblasts, or mesothelial cells. Filters were backwashed to yield essentially only tumor cell clumps (29, 31). Results with this technique were similar to those obtained by fractionating cells by differential attachment.

Preparation of S100 Extracts. Extracts were prepared from $>10^8$ cells from fresh material (leukocytes and unfractionated ascites cells) and cultured cells (nonmalignant and tumor cell fractions, normal ovarian epithelium, and cell lines HEY, SKOV-3, and 293 CSH) as described (9). Protein concentrations were determined by Bradford assay (Bio-Rad) and enzymatic activity was determined by the level of DNA polymerase activity (9).

Telomerase Assay. The assay was performed as described (9) with minor modifications. In brief, reaction mixtures containing 0.5 volume of S100 extract, 3.5 mM $MgCl_2$, 1 mM spermidine, 5 mM 2-mercaptoethanol, 50 mM potassium acetate, 2.5 mM EGTA, 50 mM Tris acetate (pH 8.5), 1 μM telomere primer $(TTAGGG)_3$, 3.1 μM [α-^{32}P]dGTP (800 Ci/mmol; 1 Ci = 37 GBq), 2 mM dTTP and 2 mM dATP were incubated at 30°C for 1 hr. As a control, RNase A was added to parallel reactions before the addition of [α-^{32}P]dGTP. Reactions were terminated by treatment with RNase A followed by proteinase K (9). Unincorporated [α-^{32}P]dGTP was removed with Nick Spin columns (Pharmacia). Products were resolved by electrophoresis in a sequencing gel and exposed to a PhosphorImager screen (Molecular Dynamics). Extracts were considered positive (++) if the characteristic 6-nucleotide pattern was detected in a 7-day exposure, and weakly positive (+) or negative (−) if detected or not detected, respectively, after 2 weeks. Protein concentration in all extracts was >1 mg/ml—i.e., 10 times the lowest concentration at which activity was detected in control 293 CSH extracts. Dilution of 293 CSH extract was performed with 1× hypobuffer (9) with the addition of NaCl to a final concentration of 0.1 M.

Analysis of DNA. DNA was isolated, digested with restriction enzymes *Hin*fI and *Rsa* I, and quantitated by fluorometry from $>10^7$ cells of unfractionated ascites or nonmalignant or tumor ascites fractions or cultured cells as described (6, 9–11). Restriction enzyme digestion liberates terminal restriction fragments (TRFs), which are composed of telomeric DNA (TTAGGG) at the most distal end, followed by subtelomeric DNA consisting of degenerate TTAGGG and other unrelated repetitive DNA (26, 33). TRFs were resolved in agarose gels, hybridized with the telomere-specific ^{32}P-labeled $(CCCTAA)_3$ probe, and visualized on film as described (9–11). The mean TRF length was determined from the values obtained from densitometric scanning of at least two autoradiographs over the size range 2–21 kbp (9–11). For simplicity, this value is

Table 1. Telomerase activity of tumor and nonmalignant cells

Tumor		Nonmalignant	
Sample	Activity	Sample	Activity
Unfractionated ascites		Normal tissues	
OC1-1	++	OE1*	–
OC2-1	+	OE2*	–
OC4-1	++	LEU†	–
OC4-5	++		
OC23-1	++		
Fractionated ascites		Fractionated ascites	
OC18-2	++	OC8-1	–
OC19-3	++	OC16-7	–
Cell lines		OC17-1	–
HEY	++	OC24-1	–
SKOV-3	+	OC25-1	–

Ascites cells were isolated and selected samples were fractionated into tumor and nonmalignant fractions and assayed for telomerase activity as described in *Materials and Methods*.
*Ovarian epithelium.
†Leukocytes.

recorded as the TRF length. When the average of several mean TRF lengths is calculated this value is called the average TRF length.

RESULTS AND DISCUSSION

To test the hypothesis that telomerase was present in tumor cells which had undergone many cell divisions, ascitic fluid from patients with end-stage (metastatic) epithelial ovarian carcinoma was obtained by diagnostic laparotomy or therapeutic paracentesis. Assays for telomerase were performed with extracts from ascites cells, ovarian carcinoma cell lines, and control cells. A telomerase-positive extract elongates single-stranded telomere primers by repetitive addition of nucleotides, yielding products which resolve in a sequencing gel as a 6-nucleotide repeat ladder. The characteristic pausing at each cycle is thought to reflect the translocation step (4, 18–20). Since telomerase uses its integral RNA as template, pretreatment of extracts with RNase abolishes this pattern (4, 18–20). Telomerase activity was present in ascites cells from patients and in established carcinoma lines (Fig. 1, lane 4; Table 1). Extracts from normal epithelial cells from the ovarian surface, however, had no detectable activity (Table 1), suggesting that telomerase may be present exclusively in tumor cells. Extracts from a given patient (OC4, Table 1), at presentation and 8 months thereafter, indicated persistence of telomerase activity *in vivo*. These data provide direct evidence of specific telomerase activity in human tissue.

We estimated that cell samples from ascites were composed of ≈95% tumor cells with the remaining fraction consisting of mesothelial cells, fibroblasts, and leukocytes. To identify the source of telomerase activity, tumor and nonmalignant cells from ascites were separated and assayed. Purified tumor cells were found to be telomerase-positive, whereas the nonmalignant cell fraction and peripheral blood leukocytes from normal donors lacked detectable activity (Fig. 1; Table 1). Lack of activity did not appear to be due to diffusible inhibitor(s), since the activity of 293 CSH extracts was not altered upon mixing with negative extracts (data not

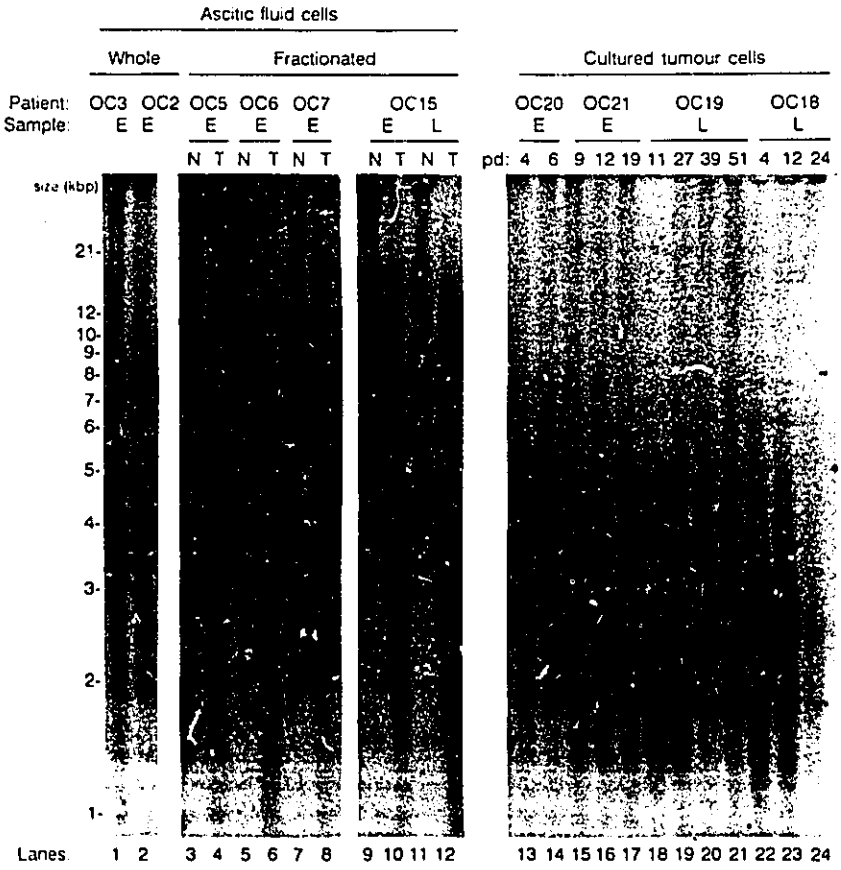

FIG. 2. TRF lengths in cells from ascitic fluid. Southern hybridization with the telomere-specific probe $(CCCTAA)_3$, which detects TRFs, is shown for genomic DNA isolated from unfractionated ascites cells (lanes 1 and 2) and nonmalignant cell (N, lanes 3, 5, and 7) and highly pure tumor cell (T, lanes 4, 6, and 8) fractions. DNA from nonmalignant and tumor cells from the same patient was analyzed at presentation [early (E), lanes 9 and 10] and just prior to death [late (L), lanes 11 and 12]. Tumor cells from both early and late paracentesis were cultured and analyzed at the indicated population doublings (pd) (lanes 13–24).

Table 2. TRF lengths of tumor and nonmalignant cells

Unfractionated ascites*		Fractionated ascites			Cultured tumor cells†	
			TRF length, kbp			
Sample	TRF length, kbp	Sample	Non-malignant	Tumor	Sample	TRF length, kbp
OC1-1	3.8	OC5-1	7.0	5.0	OC18-2	3.4
OC2-1	5.5	OC6-1	9.2	5.4	OC19-3	3.4
OC3-1	5.4	OC7-1	8.0	5.4	OC20-1	4.2
OC3-2	4.4	OC8-1	7.7	4.3	OC21-1	3.3
OC4-1	4.5	OC9-1		5.2	OC5-1	4.3
		OC10-1		3.9	OC22-13	6.9
		OC11-2		3.7		
		OC12-1		3.8		
		OC13-1		5.1		
		Serial				
		OC14-1 (E)	7.3	4.1		
		OC14-5 (L)		4.7		
		OC15-1 (E)	9.4	5.0		
		OC15-5 (L)	9.3	5.2		
		OC16-1 (E)		3.9		
		OC16-2 (E)		3.4		
		OC16-7 (L)		3.9		
		OC17-1 (E)	7.7	4.3		
		OC17-15 (L)		4.7		
Average‡	4.7 ± 0.7	Average‡	8.2 ± 0.9	4.5 ± 0.6	Average‡	4.2 ± 1.4 (3.7 ± 0.5)§

TRF lengths were determined from densimetric scans of multiple autoradiographs (similar to that in Fig. 2). Average standard deviation was 0.5 kbp, with the largest deviation being 2 kbp. Samples defined as E (early) were obtained near the time of presentation, and samples L (late) near death. Paracenteses were performed 4–15 times over the course of 4–22 months.

*Since TRF length in unfractionated ascites cells is almost identical to that of the tumor fraction (4.7 versus 4.5 kbp) and since we assume that both the nonmalignant (TRF of 8.2 kbp) and tumor fractions contribute to the composite TRF distribution in proportion to their relative abundance, the fraction of tumor cells (x) can be determined in the equation $8.2(1 - x) + 4.5x = 4.7$. This yields $x = 0.95$, indicating that 95% of the ascites cells are of tumor origin, a value similar to those reported by others (32).

†TRF length was determined for each sample over at least 30 population doublings. Values were averaged since TRFs were stable in all populations.

‡Average ± SD of the mean TRF lengths of all samples.

§Average value excluding OC22-13.

shown). In 293 CSH cells, telomerase activity was approximately proportional to total protein concentration (Fig. 1, lanes 9–26) and was detectable at 0.1 mg/ml (lane 29). Thus, lack of activity in extracts from nonmalignant cells, which were assayed at 1–3 mg/ml, was most likely unrelated to low protein concentration. In support of this, extracts from the ovarian tumor cell line SKOV-3, with half the protein concentration of nonmalignant cell extracts, were positive (Table 1). The extent of primer elongation and frequency of initiations, as judged by the intensity of bands, was less in reactions with extracts from unfractionated ascites or purified tumor cells than that with 293 CSH cells (Fig. 1, compare lanes 2 and 4 for extracts assayed at the same protein concentration). This was also the case when the cell extracts were assayed for DNA polymerase (data not shown). Whether these results reflect an inherent property of tumor cells, heterogeneity of the tumor cell population, or some feature of the isolation procedures is not known.

We have previously shown that telomerase activity is associated with maintenance of telomere length in cultured human cell lines (9). To determine whether this was the case for fresh tumor cells, lengths of TRFs were measured by Southern analysis of DNA from unfractionated ascites cells, using a $(CCCTAA)_3$ telomeric probe. Consistent with results from studies of other tumors (7, 21–26), the average TRF in these cells was short (4.7 ± 0.7 kbp; Table 2). The TRF size distribution, however, was quite heterogeneous, ranging from 2 to >12 kbp (Fig. 2, lanes 1 and 2). To assess whether differences in telomere length existed among cell types, ascites cells were separated into nonmalignant (fibroblast and mesothelial cells) and tumor cell fractions. Southern analysis of DNA demonstrated that the TRF length of nonmalignant cells was much greater than that of tumor cells from the same ascites (Fig. 2; compare samples N and T; Table 2; 8.2 ± 0.9 kbp versus 4.5 ± 0.6 kbp, $P = 0.001$). Thus, TRF heterogeneity in unfractionated ascites resulted from the presence of at least two cell populations with quite different TRF lengths.

Telomeres were shorter in tumor cells than in normal cells from the tissue of origin. In ovarian epithelial cells obtained from three nondiseased donors, TRFs were ≈12 kbp long (data not shown), somewhat longer than those in nonmalignant ascites cells or in other aged-matched somatic cells (6–8, 10, 11). If rate of telomere loss in tumor cells *in vivo* is similar to that in transformed cells *in vitro* (65 bp per population doubling) (9), the loss of ≈7.5 kbp in tumor cells relative to normal cells represents in excess of 100 doublings. This is most likely an underestimate, since tumor cell populations maintain neither 100% viability nor exponential growth (34).

Telomere length in tumor cells was stable *in vivo*. Over the course of the disease, a dozen or more ascites samples, each containing 10^9–10^{10} tumor cells, may be withdrawn from a given patient. Since TRF lengths in tumor cells from a patient at presentation and just prior to death remained essentially constant, telomere length was maintained *in vivo* over a large number of cell doublings (Fig. 2, lanes 10 and 12; Table 2; 4.3 ± 0.5 kbp for early samples versus 4.6 ± 0.5 kbp for late samples; $P = 0.5$). Although TRF size did not change appreciably during the disease in nonmalignant cells from the

ascitic fluid (Fig. 2, lanes 9 and 11; Table 2), these cells are predicted to undergo relatively few cell divisions, as judged from the rate of telomere loss of normal somatic cells *in vivo* (7, 10, 11). Similarly, when put in culture, the limited lifespan of nonmalignant cells (≈10 doublings) (31) precluded detection of telomere shortening normally associated with somatic cell proliferation (6, 8–13).

When end-stage tumor cells from 151 patients were cultured, ≈25% (41) of the samples readily established and continued proliferating for months with little or no discernible crisis, suggesting that the cells were already immortal *in vivo*. Moreover, the cells had a transformed morphology and they formed foci, and many of the clones tested grew in an anchorage-independent manner in serum-free medium. DNA was collected from six cultures every two to four population doublings, and TRF lengths were determined. Samples OC19-3, OC18-2, OC20-1, OC21-1, and OC5-1 (Fig. 2, lanes 13–24; Table 2) had a short but stable average TRF length of 3.7 ± 0.5 kbp *in vitro*, confirming our *in vivo* observations. Notably, one sample, OC22-13, contained stable telomeres of much greater length (6.9 kbp; Table 2), as occasionally reported for other tumors (7, 21–23, 26).

It is interesting that in almost all of the tumor samples analyzed, telomerase was detected after telomeres had become very short. In our studies with cells transformed *in vitro* we proposed that activation of the enzyme was most likely due to mutational event(s) which could conceivably occur at any time and telomere length (9). Although one tumor (OC22-13) had long, stable telomeres, compatible with early activation of the enzyme, elongation may have occurred *in vivo* after initial shortening, as observed in some immortalized cell lines (9, 26, 36). Whether selective pressure operates on telomerase-positive cells only when telomeres are short or whether telomerase activation occurs late in the transformation process is unknown. It is possible that short telomeres and the concomitant decrease in chromosome stability (9) trigger activation of the enzyme in rare cells. Alternatively, activation may depend upon multiple mutational events which are only achieved after many cell divisions.

We propose that tumor cells, like cells transformed *in vitro* (9, 12), lose telomeric DNA until a critically short telomere length is reached. At this stage, only cells which can maintain functional chromosome ends—for example, through the expression of telomerase—are capable of continued proliferation. Thus, drugs which specifically inhibit telomerase activity could critically limit the lifespan of late-stage tumor cells. Side effects from this therapeutic approach should be limited, since telomerase is believed to be absent or minimally expressed in somatic tissues (9, 13).

We are grateful to D. W. Andrews, C. W. Greider, and J. R. Smiley for critical review of the manuscript and to B. Eigl and F. M. Botelho for technical assistance. C.M.C. thanks C. Brunetti, I. York, P. Betti, and C. Smibert for helpful discussions. This work was supported by a grant from the Cancer Research Society of Quebec (Canada) and the National Institute on Aging (CBH, AG09-383). C.M.C. is the recipient of a Longstaffe Scholarship for Graduate Studies, and S.B. is a Terry Fox Research Scientist of the National Cancer Institute of Canada.

1. Blackburn, E. H. & Szostak, J. W. (1984) *Annu. Rev. Biochem.* **53**, 163–194.
2. Zakian, V. A. (1989) *Annu. Rev. Genet.* **23**, 579–604.
3. Blackburn, E. H. (1991) *Nature (London)* **350**, 569–573.
4. Yu, G. L., Bradley, J. D., Attardi, L. D. & Blackburn, E. H. (1990) *Nature (London)* **344**, 126–132.
5. Lundblad, V. & Szostak, J. W. (1989) *Cell* **57**, 633–643.
6. Harley, C. B., Futcher, A. B. & Greider, C. W. (1990) *Nature (London)* **345**, 458–460.
7. Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K. & Allshire, R. C. (1990) *Nature (London)* **346**, 866–868.
8. Lindsey, J., McGill, N. I., Lindsey, L. A., Green, D. K. & Cooke, H. J. (1991) *Mutat. Res.* **256**, 45–48.
9. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B. & Bacchetti, S. (1992) *EMBO J.* **11**, 1921–1929.
10. Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W. & Harley, C. B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 10114–10118.
11. Vaziri, H., Schachter, F., Uchida, I., Wei, L., Zhu, X., Effros, R., Cohen, D. & Harley, C. B. (1993) *Am. J. Hum. Genet.* **52**, 661–667.
12. Shay, J. W., Wright, W. E., Brašiškyte, D. & Van Der Haegen, D. (1993) *Oncogene* **8**, 1407–1413.
13. Harley, C. B. (1991) *Mutat. Res.* **256**, 271–282.
14. Girardi, A. J., Jensen, F. C. & Koprowski, H. (1965) *J. Cell. Comp. Physiol.* **65**, 69–84.
15. Sack, G. H., Jr. (1981) *In Vitro* **17**, 1–19.
16. Huschtscha, L. I. & Holliday, R. (1983) *J. Cell. Sci.* **63**, 77–99.
17. Shay, J. W., Wright, W. E. & Werbin, H. (1991) *Biochim. Biophys. Acta* **1072**, 1–7.
18. Greider, C. W. & Blackburn, E. H. (1985) *Cell* **43**, 405–413.
19. Greider, C. W. & Blackburn, E. H. (1989) *Nature (London)* **337**, 331–337.
20. Morin, G. H. (1989) *Cell* **59**, 521–529.
21. Hiyama, E., Hiyama, K., Yokoyama, T., Ichikawa, T. & Matsuura, Y. (1992) *Jpn. J. Cancer Res.* **83**, 159–164.
22. Smith, J. K. & Yeh, G. (1992) *Am. J. Obstet. Gynecol.* **167**, 1883–1887.
23. Nürnberg, P., Thiel, G., Weber, F. & Epplen, S. T. (1993) *Hum. Genet.* **91**, 190–192.
24. Holzmann, K., Blin, N., Welter, C., Zang, K. D., Seitz, G. & Henn, W. (1993) *Genes Chromosomes Cancer* **6**, 178–181.
25. Adamson, D. J. A., King, D. J. & Haites, N. E. (1992) *Cancer Genet. Cytogenet.* **61**, 204–206.
26. de Lange, T., Shiue, L., Myers, R. M., Cox, D. R., Naylor, S. L., Killery, A. M. & Varmus, H. E. (1990) *Mol. Cell. Biol.* **10**, 518–527.
27. Graham, F. L., Smiley, J. R., Russell, W. C. & Nairn, R. (1977) *J. Gen. Virol.* **36**, 59–72.
28. Clarke-Pearson, D. T. & Creasman, W. T. (1986) in *Comprehensive Textbook of Oncology*, eds. Moossa, R. A., Robson, M. C. & Schimpff, S. C. (Williams & Wilkins, Baltimore), pp. 845–855.
29. Hirte, H., Clark, D. A., Mazurka, J., O'Connell, G. & Rusthoven, J. (1992) *Gyn. Oncol.* **44**, 223–226.
30. Kruk, P. A., Maines-Bandiera, S. L. & Auersperg, N. (1990) *Lab. Invest.* **63**, 132–136.
31. Allen, H. J., Porter, C., Gamarra, H., Piver, M. S. & Johnson, E. A. Z. (1987) *Exp. Cell Biol.* **55**, 194–208.
32. Crickard, K., Marinello, M. J., Crickard, U., Satchidanand, S. K., Yoonessi, M. & Caglar, H. (1986) *Cancer Genet. Cytogenet.* **23**, 135–143.
33. Allshire, R. C., Dempster, M. & Hastie, N. D. (1989) *Nucleic Acids Res.* **17**, 4611–4627.
34. Tannock, I. F. (1987) in *The Basic Science of Oncology*, eds. Tannock, I. F. & Hill, R. P. (Pergamon, Toronto), pp. 140–159.
35. Mehle, C., Ljungberg, B. & Roos, G. (1994) *Cancer Res.* **54**, 236–241.
36. Klingelhutz, A. J., Barber, S. A., Smith, P. P., Dyer, K. & McDougall, J. K. (1994) *Mol. Cell. Biol.* **14**, 961–969.

Chapter 5: Telomerase activity in normal leukocytes and in hematologic malignancies

5.0) Foreword.

As described in the last chapter, malignant cells of ovarian cancer were found to have short stable telomeres *in vitro* and *in vivo* and to be telomerase positive, suggesting that telomerase is activated during tumourigenesis. Since ovarian cancer is detected when cells are already telomerase positive, the point in tumour progression when the enzyme is activated could not be determined. To address this issue, telomere length and the levels of telomerase activity were measured in early and late stages of CLL, the end stage acute leukemia AML and the pre-AML condition known as MDS.

RAPID COMMUNICATION

Telomerase Activity in Normal Leukocytes and in Hematologic Malignancies

By Christopher M. Counter, Jyothi Gupta, Calvin B. Harley, Brian Leber, and Silvia Bacchetti

Telomeres are essential for function and stability of eukaryotic chromosomes. In the absence of telomerase, the enzyme that synthesizes telomeric DNA, telomeres shorten with cell division, a process thought to contribute to cell senescence and the proliferative crisis of transformed cells. We reported telomere stabilization concomitant with detection of telomerase activity in cells immortalized in vitro and in ovarian carcinoma cells, and suggested that telomerase is essential for unlimited cell proliferation. We have now examined the temporal pattern of telomerase expression in selected hematologic malignancies. We found that, unlike other somatic tissues, peripheral, cord blood, and bone marrow leukocytes from normal donors expressed low levels of telomerase activity. In leukocytes from chronic lymphocytic leukemia (CLL) patients, activity was lower than in controls in early disease, and comparable with controls in late disease. Relative to bone marrow, telomerase activity was enhanced in myelodysplastic syndrome (MDS) and more significantly so in acute myeloid leukemia (AML). Regardless of telomerase levels, telomeres shortened with progression of the diseases. Our results suggest that early CLL and MDS cells lack an efficient mechanism of telomere maintenance and that telomerase is activated late in the progression of these cancers, presumably when critical telomere loss generates selective pressure for cell immortality.

NORMAL HUMAN somatic cells have a limited proliferative capacity, both in culture and in vivo, and senesce.[1] Transformation in vitro confers to cells an extended lifespan, but transformed cells eventually undergo a proliferative crisis accompanied by cell death, from which rare immortal clones emerge.[2] Circumstantial evidence suggests that acquisition of extended proliferative capacity, and even of immortality, can also occur in vivo during the development of tumors.[3]

Recent studies have implicated telomeres and telomerase in the regulation of cell lifespan.[4] Telomeres, composed of protein and of tandemly repeated simple DNA sequences, cap the ends of eukaryotic chromosomes and protect them from illegitimate recombination and degradation.[5] Telomeric DNA is lost every time somatic cells divide,[4] presumably because of the inability of DNA polymerase to fully replicate the ends of a linear DNA template[6,7] and because of the lack of telomerase activity,[8-10] the enzyme that elongates telomeric DNA de novo.[11] Such shortening may act as a mitotic clock regulating the number of divisions a normal cell can undergo.[4] We and others have shown that this control is overridden by transformation in vitro because transformed cells continue to lose telomeric DNA.[8,12-15] At crisis, telomeres are shortened to the point that they may no longer stabilize chromosome ends, and the ensuing genomic instability may contribute to the observed cell death.[8,12] Immortal cells emerging from crisis still have short telomeres[8,12,13,15]; however, they express telomerase activity[8,12] and hence their telomeres are stable[8,12,13] or even elongated.[15] These observations support the hypothesis that unlimited cell proliferation requires telomerase activity to arrest the potentially lethal loss of telomeric DNA and to maintain functional telomeres.[8,10] Telomerase may similarly be required in tumorigenesis because tumor cells often have short, or critically short, telomeres.[10] In support of this hypothesis we have shown that in metastatic ovarian carcinoma telomerase activity is present and short telomeres are stabilized in vivo.[16] Enzymatic activity has since been detected in other advanced malignancies, including those of the hematopoietic lineage.[9,17]

The presence of short telomeres despite detectable telomerase activity and the fact that complete loss of telomeric DNA entails a substantial number of cell divisions, suggest that selection for telomerase activation is likely to be a relatively late event in carcinogenesis.[10,16] To address this question, we investigated the temporal pattern of telomerase expression in two hematologic malignancies with different progression: chronic lymphoid leukemia (CLL) and acute myeloid leukemia (AML). CLL is initially characterized by a very slow accumulation of terminally differentiated B lymphocytes, whose number may double in as long as 5 years. The disease becomes gradually more aggressive but rarely undergoes blast transformation.[18,19] On the other hand, AML is a highly aggressive disease resulting from aberrant proliferation and maturation of progenitor stem cells.[18,20] In addition, we assayed samples of myelodysplastic syndrome (MDS), a neoplasia of the bone marrow (BM) characterized by variable, but often high, proliferation rates. MDS may progress through a variety of stages and convert to AML,[21,22] and can therefore be considered a pre-AML condition.

MATERIALS AND METHODS

Isolation and culturing of cells. Normal human skin fibroblasts were cultured in α-minimal essential medium (α-MEM), supplemented with 10% fetal calf serum (FCS). 293 CSH cells[23] were cultured in Joklik medium supplemented with 5% FCS. B4 cells, an immortal line derived from Epstein-Barr virus (EBV)-infected human B lymphocytes,[12] were grown in RPMI with 10% FCS serum.

From the Departments of Pathology and Medicine, McMaster University, Hamilton, Ontario, Canada; and Geron Corp, Menlo Park, CA.

Submitted January 20, 1995; accepted February 17, 1995.

Supported by the Medical Research Council of Canada and Geron Corp (USA). C.M.C. holds a Studentship from the National Cancer Institute of Canada (NCIC) and S.B. is a Terry Fox Cancer Research Scientist of the NCIC.

Address reprint requests to Silvia Bacchetti, ScD, Department of Pathology, McMaster University, 1200 Main St W, Hamilton, Ontario, L8N 3Z5 Canada.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/95/8509-0012$3.00/0

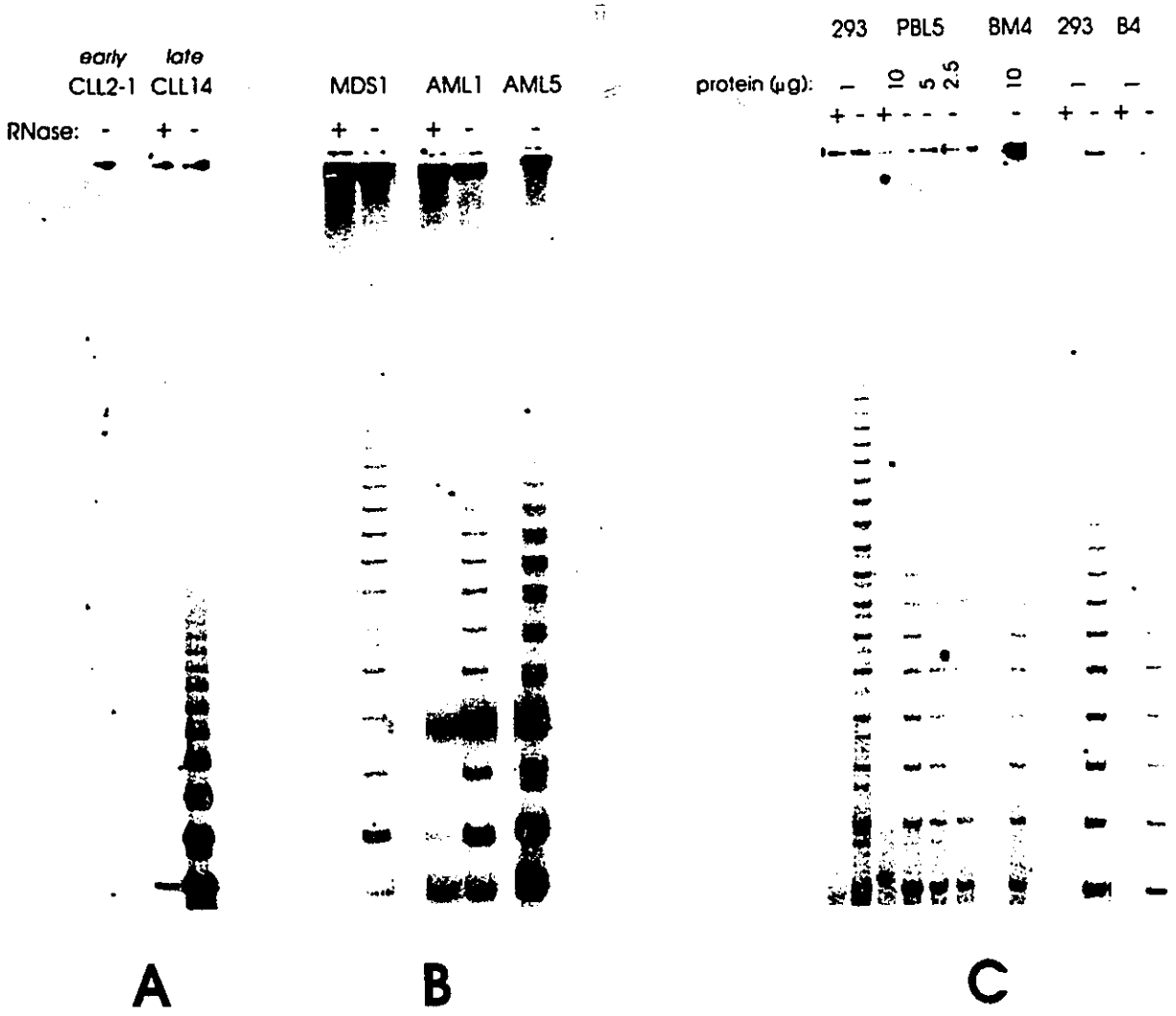

Fig 1. Telomerase activity in normal and leukemic cells. Cell extracts were incubated with the TS oligonucleotide in the presence of dNTPs and α^{32}PdCTP and, after addition of the CX primer, elongated TS oligonucleotides were amplified by PCR. As a control, RNase, which abolishes telomerase activity, was added to duplicate reaction before addition of α^{32}PdCTP. Reaction products were resolved on acrylamide gels and visualized after exposure to PhosphorImager screens. (A) Telomerase activity in representative samples of leukocytes from patients with early (CLL2-1) or late (CLL14) CLL (10 μg protein per assay). (B) Activity in extracts from patients with MDS or AML (5 μg protein per assay). (C) Activity in peripheral blood (PBL5) and BM (BM4) leukocytes from normal individuals, and in control 293 and B4 cell lines, assayed at the indicated amounts of protein.

Samples were obtained with informed consent from normal donors, or from cancer patients at diagnosis or during follow-up. The age of normal adult donors ranged from 25 to 55 years, and that of patients from 40 to 80 years, without significant differences in the average age between groups. CLL was staged according to Rai[10] and samples were obtained from blood when white blood cell (WBC) counts exceeded 15×10^9/L. MDS samples were isolated from BM whereas normal and AML samples came from both sources. All samples were processed immediately after collection without expansion in culture. Following two washes in phosphate-buffered saline (PBS), low-density mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation and assayed.

Analysis of DNA. Genomic DNA was isolated and digested to liberate the terminal restriction fragments (TRFs), as previously described.[8] Digested DNA was resolved in 0.5% agarose gels, which were dried, hybridized with the telomeric probe $^{32}P(C_3TA_2)_3$, stringently washed, and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA).[8] TRFs are comprised of telomeric and subtelomeric DNA, and variability in the lengths of both components gives rise to their heterogeneous size.[5] A mean TRF length was calculated using the total counts between 21 and 2 kbp, determined using ImageQuant software (Molecular Dynamics),[8,9] and recorded for simplicity as TRF length.

Telomerase assay. Most S100 extracts were prepared from 10^7 cells using hypotonic-detergent lysis as previously described,[12] except that the lysis buffer contained 0.5% CHAPSO (Sigma, St Louis, MO) instead of 0.5% Nonidet P-40 (Sigma).[9] A few extracts were prepared from 10^8 cells using a hypotonic-Dounce homogenization method.[8] Extracts were treated with RNase to a final concentration

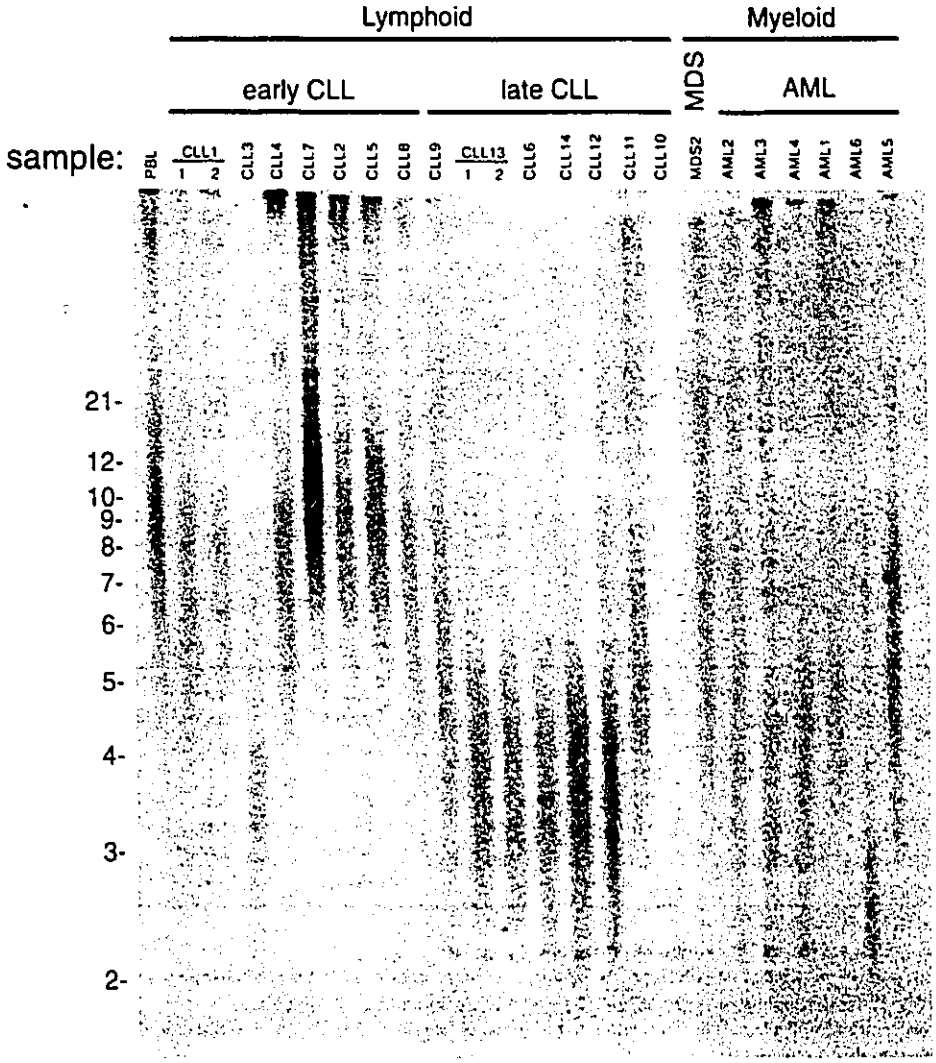

Fig 2. TRF lengths of normal and leukemic cells. Southern hybridization with the telomere specific probe $(CCCTAA)_3$ to *HinfI*/*RsaI*-digested genomic DNA isolated from leukocytes from a normal individual (PBL), or from patients with early or late stage CLL, MDS, and AML. Samples obtained from the same patient at different times are denoted by numbers after the sample's code. Position of molecular weight markers, in kilobase pairs, is shown on the left.

(0-II) and late (III-IV) stage CLL, with MDS, and with AML were assayed for enzyme activity. As controls, leukocytes from cord, peripheral blood and BM of normal donors were used. A telomerase-positive extract elongates a single-stranded primer by addition of T_2AG_3 repeats and the elongated products are amplified in the PCR step.[9] Pretreatment of the extract with RNase will abolish telomerase activity by degrading the templating RNA of the enzyme.[11]

Assaying of samples from normal individuals showed that telomerase was present in leukocytes from cord (3/3), peripheral blood (6/6), and BM (3/3) (Fig 1 and Table 1). Activity was low in all three tissues (on average ≈0.8% of that in the 293 cell line, and ≈2% of that of the B4 lymphocyte cell line), suggesting that enzyme expression may be limited to a small subset of normal leukocytes or may be insufficient for telomere maintenance. In agreement with these possibilities, telomeres were significantly shorter in adult versus newborn leukocytes (Table 1), as reported by others.[24,25] Leukocytes from early stage CLL patients (n = 14) expressed on average lower telomerase activity than control samples (Fig 1 and Table 1). In the vast majority (12/14 or 85%) activity was reduced on average by 70% (P = .03), and only two cases exceeded control values. Of interest, the latter samples (CLL19 and 20) were from patients with significant increase in WBC count and lymph nodes in the month before sampling. Conversely, in late-stage CLL (n = 7), four of the seven samples assayed (or 57%) had elevated enzyme levels compared to early stage samples (P = .016), although cases with no or negligible activity still persisted (Fig 1 and Table 1). The average value for late CLL patients was comparable with that of normal blood. In the myeloid diseases (Fig 1 and Table 1), MDS (n = 6) and AML (n = 7) samples on average had higher levels of telomerase than normal BM (≈twofold for MDS and ≈fourfold for AML). The significance of these differences was borderline except for the comparison between AML and controls (P = .029). However, there was substantial variability in enzyme levels among samples and a subgroup of MDS patients (4/6 or 67%) with significantly higher activity than controls

Table 1. TRF Length and Telomerase Activity in Normal and Leukemic Cells

Sample	TRF* (kbp)	Telomerase Activity† (% of B4 cells)
Normal Blood		
PBL1	13.3	3.3
PBL2	12.7	1.5
PBL3	11.6	1.3
PBL4	13.0	4.5
PBL5	11.6	1.5
PBL6	12.0	2.1
Mean:	12.4	
CBL1	16.7	1.2
CBL2	16.2	0.4
CBL3	15.2	0.7
Mean:	16.0	1.8§
Normal BM		
BM1	ND	0.6
BM2	10.3	2.2
BM3	10.3	2.4
BM4	ND	3.0
Mean:	10.3	2.1
Early Stage CLL		
CLL1-1	8.1	0.5
-2	8.0	0.7
CLL2-1	ND	0
-2	10.1	1.3
CLL3-1	4.5	0
-2	3.7	0.9
CLL4-1	8.4	ND
-2	11.4	ND
CLL5	10.4	0.2
CLL6-1	3.6	NA
CLL7	12.9	0.7
CLL8-1	8.4	0.2
-2	ND	1.4
-3	ND	0.2
CLL11	5.5	0.9
CLL19	ND	2.3
CLL20	ND	2.7
Mean:	7.9	0.9
MDS		
MDS1	ND	4.0
MDS2	7.5	4.6
MDS3	ND	1.1
MDS4-1	20.1	1.5
-2	ND	7.6
MDS5	5.6	8.8
Mean:	11.1	4.6
Late-Stage CLL		
CLL6-2	3.8	0.5
CLL9	6.0	1.3
CLL10	3.8	0
CLL11	4.1	2.3
CLL13-1	4.3	NA
-2	4.2	2.5
CLL14	3.8	3.1
CLL15	3.9	NA
CLL16	6.3	NA
CLL17	4.2	NA
CLL18	ND	4.3
Mean:	4.4	2.0
AML		
AML1	4.4	5.9
AML2	4.8	12.0
AML3	5.1	2.9
AML4	4.2	6.9
AML5	6.5	14.3
AML6	4.3	1.9
AML7	3.6	18.1
Mean:	4.7	8.9

Abbreviations: ND, not determined; NA, not available.

* The SD of TRF measurements ranged from 0 to 1.4 kbp, with an average of 0.3 kbp.

† Activity of normal leukocytes from peripheral (PBL), cord (CBL), or BM is expressed as percent of B4 cells which have ~40% the activity of 293 cells. The SD of telomerase activity of the normal tissues ranged from 0.4% to 4.2%, with an average of 1.5%.

§ Mean telomerase activity of PBL and CBL samples.

of 0.1 μg/μL (negative control) or left untreated for 10 minutes at 21°C.

Telomerase was initially assayed by incubating cell extracts for 10 minutes at 21°C with 0.1 μg of TS oligonucleotide (5′-AATCCG-TCGAGCAGAGTT-3′) in 20 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L $MgCl_2$, 63 mmol/L KCl, 0.005% Tween 20 (Sigma), 1 mmol/L EGTA, 50 μmol/L each dNTP, 0.5 mmol/L T4-gene 32 protein, 2 μCi α^{32}PdCTP (3,000 Ci/mmol), and 2 U Taq polymerase in a total volume of 50 μL in a tube containing 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) separated from the reaction by a wax barrier.[9] After elongation of the TS oligonucleotide by telomerase, products were polymerase chain reaction (PCR)-amplified by 27 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds. The first denaturation step inactivates telomerase and melts the wax barrier, releasing the CX primer for first-strand synthesis. This protocol results in a 10^4 increase in sensitivity[9] compared with previous methods.[8] In later experiments, the CX primer and Taq polymerase were added to reactions prewarmed to 92°C to reduce background. Lastly, kinase-labeling of the TS oligo, substitution of the second primer with one (ACT) unable to dimerize with TS, and the use of a standard for measuring PCR amplification were used to increase sensitivity and to provide better quantitation and comparison between assays (Kim N.W., Prowse K.R., Chiu C.-P., Harley C.B., in preparation). Results from all three assays were qualitatively consistent. Serial dilutions of S100 extracts were assayed in triplicate to establish the linear range of enzyme activity for the purpose of quantitation. Reaction products were resolved in 15% nondenaturing polyacrylamide gels and exposed to PhosphorImager screens. Extracts were considered negative if no products were detected on a 7-day exposure. Enzyme activity was expressed in arbitrary units as total counts in the RNase-sensitive reaction products, determined using ImageQuant software, after normalization to total protein content. A minimum of two separate assays was used to determine the mean percentage activity of each sample, except for CLL5, CLL9, MDS2, AML6, and AML7, for which a single assay was performed.

RESULTS AND DISCUSSION

To establish the temporal pattern of telomerase expression during leukemogenesis, leukocytes from patients with early

(≈threefold on average) could be identified. Similarly, the majority (6/7 or 86%) of AML samples expressed on average ≈fivefold more telomerase than normal BM.

As previous studies in different tumors[10] reported decreasing TRF length with increasing tumor grade or disease stage, we measured TRFs during leukemogenesis. In early stage CLL, TRF length varied considerably among samples but on average was 7.9 kbp, (Fig 2 and Table 1), a value consistent with those of leukocytes from age-matched normal donors.[24,26] Furthermore, there was no detectable loss of telomeric DNA in samples taken 2 years apart from patients CLL1 and CLL4, as expected if telomere shortening occurred at the same rate as in normal leukocytes (≈40 bp/yr).[26] These observations are in agreement with a normal turnover of CLL leucocytes.[18,19] TRF lengths in late CLL were more homogeneous and much reduced, with an average size of 4.4 kbp, comparable with that detected for late-stage ovarian carcinoma.[16] A similar trend was observed for the myeloid diseases (Fig 2 and Table 1), with TRFs being longer and extremely variable in size in MDS patients (average = 11.1 kbp), and consistently and significantly shorter in AML samples (average = 4.7 kbp), in agreement with previous studies.[10]

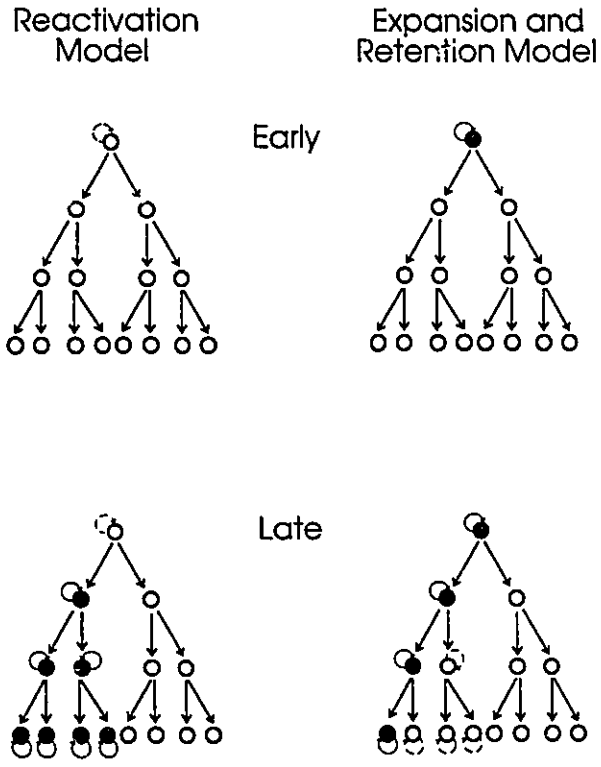

Fig 3. Models for telomerase activation during leukemogenesis. The "reactivation model" proposes that the progenitor leukemic cell lacks telomerase activity (○) and will ultimately senesce (---→). As the disease progresses and telomeres shorten, selective pressure will lead to reactivation of telomerase (●) and the predominance of a telomerase-positive immortal subclone (→). The "expansion and retention" model, on the other hand, proposes that the leukemic stem cell is telomerase positive (●) and self-renewing (→) but also gives rise to mortal telomerase negative progeny (○, ---→), thus accounting for telomere shortening in the population.

The present study shows that telomerase activity is present in leukocytes from BM and peripheral blood from normal donors. Activity in blood has also been detected by others (Kim N.W., Chiu C.-P., Vaziri H., Weinrich S.L., Harley C.B., in preparation). To date, these are the only adult somatic tissues in which telomerase has been detected. The level of activity and the age-related shortening of telomeres in both tissues[24,26] are compatible with enzyme expression being restricted to a subset of cells. The biologic significance of these findings and the identity of the positive cells is unclear at present. However, loss of telomeric DNA during in vitro culturing has been reported for the most primitive BM stem cell ($CD34^+$ $CD38^{lo}$), suggesting that telomerase-positive cells may belong to a more differentiated compartment.[27] Despite the constitutive activity of the normal tissues, we detected distinct patterns of telomerase expression in samples from lymphoid and myeloid leukemias. In both diseases leukocytes from early stage patients generally have less telomerase activity and longer TRFs than those from late-stage patients. However, we also found samples not fitting either pattern, with short TRFs but low or no telomerase activity (CLL 3, 6, 10, and possibly AML 6). These may represent populations in transition, although inability to detect enzyme activity for technical reasons cannot be excluded. Kim et al[9] have also detected telomerase activity in 2/2 late-stage CLL and in 14/16 AML samples. By contrast, Nilsson et al[17] reported no activity in four AML samples, possibly because of the lower sensitivity of their assay that did not include an amplification step. Indeed, we were unable to detect activity in peripheral blood lymphocytes without amplification of products.[12]

In the majority of early CLL, telomerase activity was undetectable or substantially reduced compared with control leukocytes. The most likely explanation is that activity present in a subset of normal cells is diluted by the more numerous telomerase-negative CLL cells, resulting in reduced levels in the whole population. Although normal cells may also contribute to telomerase levels in MDS samples, in 4/6 of these cases enzyme levels were significantly elevated over control. Our data suggest an inverse correlation between telomere length and telomerase activity that may reflect the proliferative history of the leukemic clone. Although our sample size is small, this pattern is consistent with recent observations on TRF length by Ohyashiki et al,[28] who identified three classes of MDS patients with: (1) short TRFs at diagnosis and no change during disease evolution; (2) large TRFs decreasing in length as the disease evolved; or (3) large and stable TRFs. These data and ours suggest that telomerase is variably activated in MDS. Similar to our observations on late CLL, AML was associated with elevated telomerase activity relative to MDS. However, in the late stage of both leukemias activity was substantially lower (at most ≈16%) than that in a clonal population of immortalized B cells (Table 1), suggesting a preponderance of negative cells in the leukemic samples. Lastly, CLL and AML differed with respect to levels of telomerase, with CLL samples being generally less active. We speculate that this difference

may reflect the lower number of proliferating cells and/or the higher degree of cell differentiation characteristic of CLL.

Overall, our data are compatible with both CLL and AML arising in a progenitor cell that lacks, or has insufficient, telomerase activity to maintain telomere length during clonal expansion and differentiation. The presence of elevated telomerase levels in advanced leukemias suggests two alternative models for disease evolution (Fig 3). A "reactivation" model postulates that despite the capacity for self renewal, the leukemic stem cells arises from a precursor that is telomerase negative and will ultimately senesce. Thus, cellular expansion results in shortening of telomeres. During disease progression, telomerase becomes illegitimately activated, as proposed for other malignancies[9,10,16,17] and a telomerase-positive subclone will ultimately predominate. Reactivation presumably occurs late, in terms of cell proliferation, because telomeres are short. An "expansion and retention" model, on the other hand, invokes the existence of a telomerase positive leukemic stem cell ab initio. This cell is capable of cell renewal but gives rise to telomerase-negative, possibly more differentiated, progeny, thus accounting for the observed shortening of telomeres. A prediction of this model is that telomere length in the leukemic population will ultimately increase as a result of replacing telomerase-negative cells having short telomeres and finite lifespan with positive cells that have not undergone telomere erosion.

ACKNOWLEDGMENT

We are very grateful to N.W. Kim and S. Weinrich for providing us with the TRAP assay before publication, and to N.W. Kim for alerting us to the presence of telomerase activity in blood samples. C. Chadeneau, N.W. Kim, and C-P Chiu are acknowledged for critical review of the manuscript.

REFERENCES

1. Hayflick L: The limited in vitro lifespan of human diploid cell strains. Exp Cell Res 37:614, 1965

2. Girardi AJ, Jensen FC, Koprowski H: SV40-induced transformation of human diploid cells: crisis and recovery. Int J Natl Cancer Inst 42:867, 1969

3. Stamps AC, Gusterson BA, O'Hare MJ: Are tumours immortal? Eur J Cancer 28A:1495, 1992

4. Harley CB, Vaziri H, Counter CM, Allsopp RC: The telomere hypoyhesis of cellular ageing. Exp Gerontol 27:375, 1992

5. Blackburn EH: Structure and function of telomeres. Nature 350:569, 1991

6. Olovnikov AM: Principles of marginotomy in template synthesis of polynucleotides. Doklady Biochem 201:394, 1971

7. Watson JD: Origins of concatemeric T7 DNA. Nat New Biol 239:197, 1972

8. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S: Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11:1921, 1992

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

10. Bacchetti S, Counter CM: Telomerase: A key to cell immortality? The role of the enzyme in cell transformation and cancer. Cancer Invest 1995 (in press)

11. Blackburn EH: Telomerases. Annu Rev Biochem 61:113, 1992

12. Counter CM, Botelho F, Harley CB, Bacchetti S: Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. J Virol 68:3410, 1994

13. Shay JW, Wright WE, Brašiškytė D, Van der Haegen BA: E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. Oncogene 8:1407, 1993

14. Guerrini AM, Camponeschi B, Ascenzioni F, Piccolella E, Donini P: Subtelomeric as well as telomeric sequences are lost from chromosomes in proliferating B lymphocytes. Hum Mol Genet 2:455, 1993

15. Klingelhutz AJ, Barber SA, Smith PP, Dyer K, McDougal JK: Restoration of telomeres in human papillomavirus-immortalized anogenital epithelial cells. Mol Cell Biol 14:961, 1994

16. Counter CM, Hirte HW, Bacchetti S, Harley CB: Telomerase activity in human ovarian carcinoma. Proc Natl Acad Sci USA 91:2900, 1994

17. Nilsson P, Mehle C, Remes K, Roos G: Telomerase activity in vivo in human malignant hematopoietic cells. Oncogene 9:3043, 1994

18. Vincent PC: Kinetic of leukemia and control of cell division and replication, in Henderson ES, Lister TA (eds): William Dameshek and Fredrick Gunz's Leukemia (ed 5). Toronto, Canada, Saunders, 1990, p 55

19. Rai KR: Chronic lymphocytic leukemia, in Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ (eds): Hematology—Basic Principles and Practice. New York, NY, Churchill Livingstone, 1991, p 990

20. Löwenberg B, Delwel FR: The pathology of human acute myeloid leukemia, in Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ (eds): Hematology—Basic Principles and Practice. New York, NY, Churchill Livingstone, 1991, p 708

21. Tricot GJK: Myelodysplasias, in Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ (eds): Hematology—Basic Principles and Practice. New York, NY, Churchill Livingstone, 1991, p 805

22. Mayer RJ, Canellos GP: Primary myelodysplastic syndrome and secondary preleukemic disorders, in Henderson ES, Lister TA (eds): William Dameshek and Fredrick Gunz's Leukemia (ed 5). Toronto, Canada, Saunders, 1990, p 613

23. Stillman BW, Gluzman Y: Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. Mol Cell Biol 5:2051, 1985

24. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC: Telomere reduction in human colorectal carcinoma and with ageing. Nature 346:866, 1990

25. Lindsey J, McGill NI, Lindsey LA, Green DK, Cooke HJ: In vivo loss of telomeric repeats with age in humans. Mutat Res 256:45, 1991

26. Vaziri H, Schachter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB: Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet 52:661, 1993

27. Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp PM: Evidence for a mitotic clock in human hematopoietic stem cells: Loss of telomeric DNA with age. Proc Natl Acad Sci USA 91:9857, 1993

28. Ohyashiki JH, Ohyashiki K, Fujimura T, Kawakubo K, Shimamoto T, Iwabuchi A, Toyama K: Telomere shortening associated with disease evolution patterns in myelodysplastic syndromes. Cancer Res 54:3557, 1994

Chapter 6: Discussion

6.0) Overview.

The studies presented in this thesis have provided a body of evidence that, although correlative in nature, strongly supports the involvement of telomere maintenance by telomerase in the processes of cell immortalization *in vitro* and tumourigenesis. The data addressing this possibility are presented and discussed in detail in chapters 2 ,3, 4 and 5. The role of telomeres and telomerase in cell immortalization within the context of the most recent published observations, and the practical implications of telomerase activation are re-examined below.

6.1.i) A model for the role of telomeres and telomerase during the process of cell immortalization *in vitro*.

In the work described in the previous chapters it was found that, with the exception of leukocytes from blood and bone marrow, all human somatic cells assayed were negative for telomerase activity and that, consistent with this phenotype, TRF length in the one cell strain analyzed (HEK cells) decreased with cell division (chapters 2-5). Lack of telomerase activity in most human somatic tissues and cultured cells has since been reported by others (Kim *et al.*,1994; reviewed in Bacchetti & Counter, 1995) and erosion of somatic telomeres is well documented in the literature (Hastie *et al.*, 1990; Harley *et al.*, 1990; Lindsey *et al.*, 1992; Allsopp *et al.*, 1992; Vaziri *et al.*, 1993). All these observations are compatible with loss of telomeric DNA acting as a "mitotic clock" that signals senescence in normal somatic

cells (Harley, 1991). We further observed that cells which were experimentally driven to divide past senescence by transformation with viral oncogenes did not directly acquire telomerase activity nor other mechanisms for telomere maintenance. Consequently, TRF length in these cells continued to decrease with cell division and became extremely short (and significantly shorter than those of senescent cells) by the time the transformed cell populations reached crisis. Concomitant with this pronounced telomere erosion, we observed a dramatic increase in the number of dicentric chromosomes. Based on these results we have proposed that at crisis some or all chromosome ends completely lack telomeric DNA thereby becoming recombinogenic, and that the dicentric structures generated by terminal fusions contribute to the cell death occurring at this stage. Lastly, we observed a temporal correlation between stabilization of telomeres, detectable telomerase activity and acquisition of immortality by the transformed cells (chapters 2,3). These data lead us to postulate that these events are causally related, and in particular that expression of the enzyme is necessary for unlimited cell proliferation. Similar findings have since been reported by others (Shay *et al.*, 1993; Kim *et al.*,1994; Klingelhutz *et al.*, 1994; reviewed in Bacchetti & Counter, 1995). Moreover, our model, which was initially supported by the mitotic instability and loss of viability induced in ciliates by telomerase inhibition (Yu *et al.*, 1990), has since been validated in yeast (Singer & Gottschling, 1994; McEachern & Blackburn, 1995) and most recently in human cells (Feng *et al.*, 1995).

6.1.ii) Limitations of the study.

There are a number of technical limitations which caution our interpretations of some of the data presented. For example, there is no direct evidence to indicate that telomere shortening results from the end replication problem. Nevertheless, the inability of DNA polymerase to completely replicate a linear template remains the most likely explanation to account for the loss of telomeric DNA with cell division since telomere shortening, in the absence of telomerase, is evolutionarily conserved (*ie.* Harley *et al.*, 1990; Hastie *et al.*, 1990; Yu *et al.*, 1990; Singer & Gottschling 1994; McEachern & Blackburn, 1995) and actually occurs at the rate predicted by this hypothesis (Biessmann *et al.*, 1992; Levy *et al.*, 1992). Another limitation in this study was the inability to measure the actual lengths of individual telomeres. Instead the average telomere length was estimated by calculating the average subtelomere length and subtracting this value from the mean TRF length. The difficulty with this approach is that the calculation of subtelomere length is strongly dependent upon the intensity of the hybridization signal for TRF in Southern blots, which is in itself extremely variable. Not surprisingly, using this method to calculate the length of the subtelomere different studies have reported that this region ranges from 1.5 to 4 kbp (chapters 2,3: Levy *et al.*, 1992, Allsopp *et al.*, 1992). Thus, there is no direct proof for, nor the means to determine if some chromosomes lack telomeres at crisis. Nevertheless, irrespective of the subtelomeric value used to calculate telomere length, the average size of telomeres in cells at crisis is always less than 2 kbp. Given the

heterogeneity of telomere length, our data are therefore still consistent with the assumption that some chromosome ends may lack TTAGGG repetitive DNA by crisis. There is also no direct evidence that short telomeres are the cause of the dicentric chromosomes and cell death detected at crisis. However, it is well established that chromosomes which lack telomeres are recombinogenic and can form dicentrics and that resolution of these structures through the breakage-fusion-bridge cycle can lead to further genomic instability which can be lethal. Lastly, since a functional assay was used to measure telomerase activity, we could never exclude the possibilities that a telomerase negative population contained a small number of telomerase positive cells or that all cells had undetectable levels of the enzyme. Yet, the reproducible detection of telomerase activity only in immortal cells in which loss of telomeric DNA is arrested, strongly suggests that telomerase is activated, or at the very least upregulated to levels sufficient to counter telomere shortening only in cells which acquire an unlimited lifespan.

6.1.iii) Telomeres and cell senescence.

Since our studies on telomere dynamics during immortalization involved the analysis of cells driven to divide beyond their normal lifespan, our observations of telomere shortening in transformed cells are relevant to the process of senescence. Most models of senescence that invoke telomeres in a causal role assume that erosion of telomeres to a critical length activates the p53 checkpoint, the key pathway mediating the senescence growth arrest signal (Harley, 1991). One way telomeres

could function in this manner is if some chromosomes lost all telomeric repeats by senescence, thereby becoming recombinogenic and yielding chromosome aberrations that could cause a p53-dependent proliferation arrest. In agreement with this possibility, it has been calculated that the average amount of TTAGGG repeats remaining on chromosome ends at senescence is between 2 to 3.5 kbp, depending on the estimated length of the subtelomere, and thus, given the heterogeneous size of telomeres, some chromosome may theoretically lack TTAGGG repeats at senescence (chapters 2,3; Levy *et al.*, 1992; Allsopp & Harley, 1995). Moreover, the number of dicentric chromosomes increases when fibroblasts cease proliferation at the end of their normal lifespan (Saksela & Moorhead 1963; Wolman *et al.*, 1964; Benn 1976; Sherwood *et al.*, 1988). These types of aberrations can be formed from chromosomes lacking telomeric repeats and, when resolved at mitosis, do lead to types of damage known to induce a senescent-like p53-dependent growth arrest (Di Leonardo *et al.*, 1994). Inconsistent with the existence of "naked" chromosome ends at senescence however, is our finding that TRF length continued to decrease during the extended lifespan of transformed cells without repercussions on growth rate, cell viability or chromosome stability until crisis (chapters 2,3). This observation suggests that telomeres that are significantly shorter than those of senescent cells are still functional, at least in a transformed cell background. One explanation as to how chromosome ends lacking telomeric DNA at senescence could shorten during the extended lifespan following transformation but still retain functionality is if the distal region of the

subtelomere contained tracts of TTAGGG and TTAGGG-like repeats which could substitute for telomeric DNA in transformed cells (Fig. 6.0A).

Another way telomeres could be involved in the p53 checkpoint is if this pathway recognizes short telomeres themselves as damage, an assumption compatible with the observations that telomeres shorten during the extended lifespan of transformed cells (Fig. 6.0B). Although somewhat speculative, the fact that plasmids containing short tracts of telomeric DNA can cap a chromosome break and act as a functional telomere only in cells that lack p53 (Barnett *et al.*, 1993) supports the possibility that the p53 checkpoint is sensitive to short telomeres. It would not be until crisis that some chromosomes would lack telomeric repeats and become recombinogenic. This model therefore predicts that the dicentric chromosomes detected in senescent fibroblasts must be generated in a telomere-independent manner. In agreement with this prediction, we and others have failed to detect dicentrics (or detected these aberrations at low frequency) in transformed cells with TRFs far shorter that those of fibroblasts at senescence (chapter 3; Saltman *et al.*, 1993; Pandita *et al.*, 1995). However, it still remains formally possible that the absence of dicentric chromosomes in transformed cells having telomeres shorter than those of senescent fibroblasts may reflect a cell type difference or some aspect of transformation.

At present, it is equally plausible that a p53-dependent growth arrest at senescence is signalled by either the exposure of nontelomeric DNA (Fig. 6.0A) or the shortening of telomeric DNA (Fig. 6.0B). Complete sequencing of individual

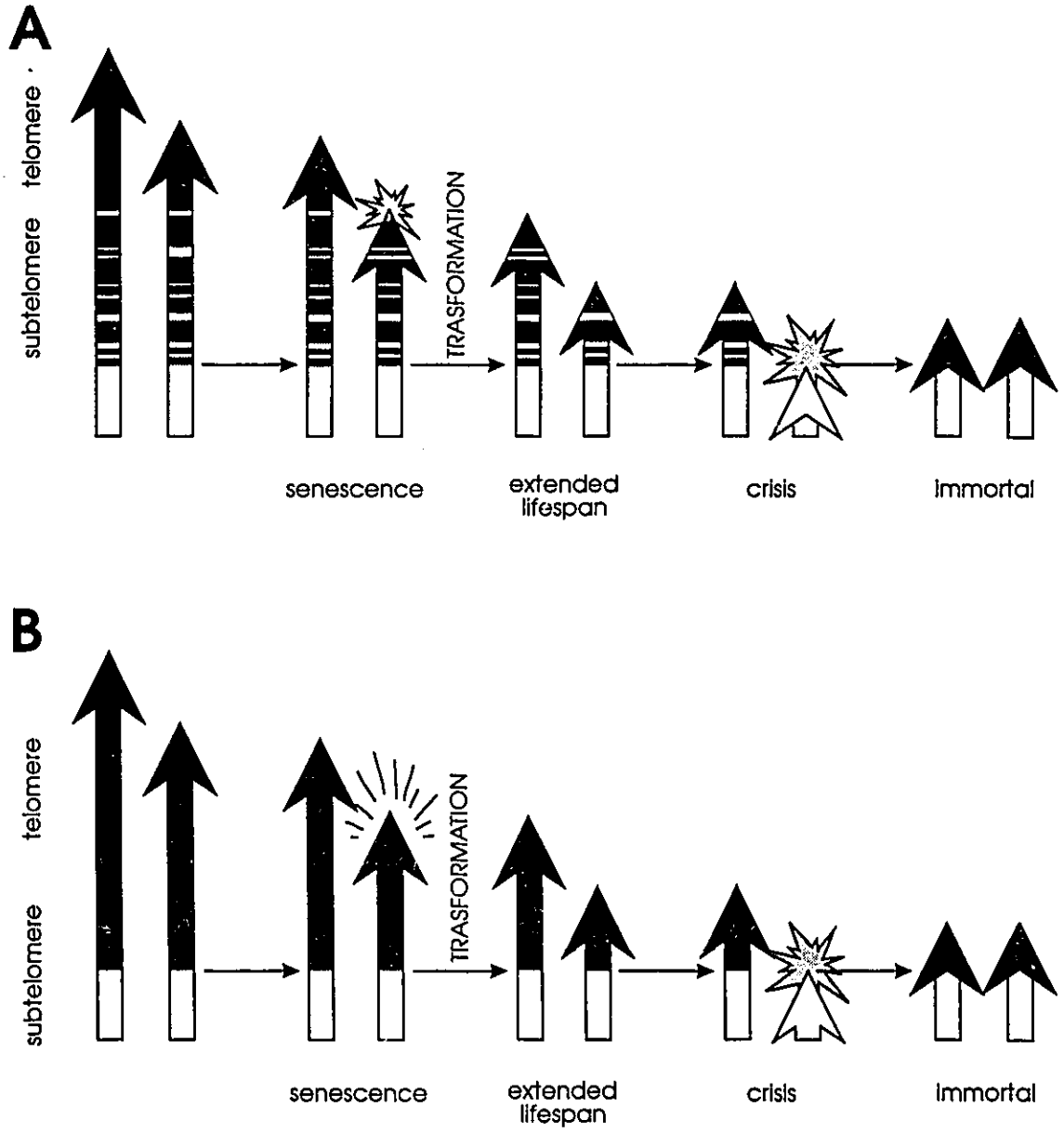
A
telomere
subtelomere
TRASFORMATION
senescence
extended lifespan
crisis
immortal
B
telomere
subtelomere
TRASFORMATION
senescence
extended lifespan
crisis
immortal

Figure 6.0

Figure 6.0

Models for the role of telomere length in senescence and crisis.

A) At senescence all terminal TTAGGG repeats (■) are lost on one or more chromosomes exposing non-telomeric DNA (▭) which is recombinogenic (✸). This may lead to terminal fusions which when resolved could yield chromosome breaks that are detected by the p53 pathway, resulting in the growth arrest of senescence. In transformed cells this growth arrest is bypassed and cells can continue to divide since the TTAGGG and TTAGGG-like (■) repeats of the subtelomere can mediate at least partial telomere function. At crisis a number of chromosome ends have insufficient subtelomeric repeats to maintain telomere function and global genomic instability and cell death ensue (✸). All telomeres in immortal cells, however short, are capped with telomeric repeats and are therefore non-recombinogenic.

B) At senescence short telomeres are recognized as damage by the p53 pathway (✋), resulting in growth arrest. Transformed cells overcome senescence and telomeres continue to shorten until one or more chromosome ends lack telomeric DNA (crisis), thereby becoming recombinogenic leading to global genomic instability and cell death. As above, telomeres are stabilized by the addition of telomeric DNA by telomerase in immortal cells.

telomeres would uncover exactly what type of DNA caps chromosomes at senescence and hence which of the above two models may best explain the role of telomeres in growth arrest. However, it is still formally possible that telomere shortening and senescence may simply be two unrelated processes that occur concomitantly or two independent mechanisms that are activated by the same event. At least in some organisms telomere shortening does not appear to play a role in senescence. For example, in yeast, *Paramecium* and possibly some strains of mice, a senescence-like phenomenon occurs even though the cells are known or thought to be telomerase positive (Singer & Gottschling, 1994; Lin & Zakian, 1995; Cohn & Blackburn, 1995; Prowse *et al.*, 1993; Chadeneau *et al.*, 1995b; Prowse & Greider, 1995; Blasco *et al.*, submitted) and the telomeres are, or appear to be, maintained (Kipling & Cooke, 1989; Starling *et al.*,1989; D'Mello & Jazwinski, 1991; Gilley & Blackburn, 1994; Kennedy *et al.*, 1995). Nevertheless, the observation that human fibroblasts always have TRFs of similar size at senescence weighs heavily in favour of a direct role for telomeres in limiting the lifespan of human cells (Harley *et al.*, 1990; Allsopp *et al.*, 1992; Lindsey *et al.*, 1991; Levy *et al.*, 1992; Allsopp & Harley, 1995). Once it is understood how telomerase is regulated it may be possible to induce the expression of the enzyme in normal cells to directly test the prediction that telomere shortening is causally involved in the process of senescence.

6.1.iv) Telomere stability.

Based on our observations we have proposed that at crisis the loss of telomeric DNA from one or more chromosomes results in terminal recombination, leading to an increase in dicentric chromosomes and cell death. In apparent contradiction with this possibility, we found that although the frequency of dicentrics decreased when cells became immortal, the length of TRFs at crisis and postcrisis was virtually identical (chapters 2,3). We believe that these data reflect a change in one or more features upon immortalization, most likely the status of telomerase (Fig. 6.0). A number of studies in many organisms, including man, indicate that telomerase is essential for cell viability, presumably because the enzyme maintains chromosome stability by capping the ends with telomeric DNA (Yu *et al.*, 1990; Wilkie *et al.*, 1990; Yu & Blackburn, 1991; Morin, 1991; Itzhaki *et al.*, 1992; Barnett *et al.*, 1993; Kramer & Haber, 1993; Greider, 1994; Hanish *et al.*, 1994; Singer & Gottschling, 1994; Feng *et al.*, 1995). Therefore, it is possible that in immortal cells all chromosome termini are capped with telomeric repeats, however short, and are thus protected from illegitimate recombination, whereas in telomerase negative cells at crisis chromosomes which have lost most or all telomeric DNA may become recombinogenic.

Of course it is unknown whether all of the short telomeres of immortal cells are fully functional since dicentrics are still detected in some immortal cell lines and at low frequency in a wide range of tumours (chapter 2,3; Saltman *et al.*, 1993; Pandita *et al.*, 1995; de Lange, 1995). This raises the possibility that stable but short

telomeres may provide a genetic motor that drives chromosome instability and hence tumour progression. However, the frequency of dicentric chromosomes does not always correlate with TRF length indicating that these terminal recombinations are the result of more than one type of damage or are dependent upon multiple events (Saltman *et al.*, 1993). Also, it is unknown for how many cell divisions short possibly "pseudo-stable" telomeres are maintained in telomerase positive cells. For example, telomeres of some HPV-16 transformed cells (Klingelhutz *et al.*, 1994) or chromosome breaks in HeLa or mouse cells which are capped with a plasmid containing short stretches of telomeric DNA, are actually elongated by the addition of telomeric repeats (Barnett *et al.*, 1993). Since telomerase has been detected in all three cell types (Morin, 1989; Prowse *et al.*, 1993; Chadeneau *et al.*, 1995b; Prowse & Greider, 1995; Shay *et al.*, 1995) it is plausible that telomere elongation may result from telomerase adding more repeats than necessary to counter telomere shortening. Nevertheless, chromosome ends with very little telomeric DNA may still be sufficiently recombinogenic, even if for only a limited period, to generate genomic instability which could, in turn, promote tumour evolution.

6.1.v) Activation of telomerase during cell immortalization.

It has been hypothesized that cell immortalization requires the mutation of a cellular gene(s) because viral oncogene expression alone does not immortalize human cells and the frequency of immortalization is similar to the mutation rate (reviewed in Shay *et al.*, 1991b). As described above, we found that telomerase was detectable

only after the cells had acquired an unlimited lifespan. Taken together these data suggest that the enzyme is probably activated by a mutational event. At present we can only speculate on how a mutation may result in an increase in the level of telomerase activity in immortal cells (Harley, 1991). One possibility is that a positive regulator of telomerase expression is upregulated in immortal cells, although such an event is difficult to envisage in view of the recessive nature of immortality. More likely, and consistent with the observation that some factor(s) from senescent cells limit the lifespan of immortal cells (Lumpkin *et al.*, 1986), a mutation could instead inactivate a repressor of telomerase activity or of the synthesis of one or more of the enzyme's components. Given that *in vitro* mixing of telomerase positive and negative extracts gives no indication of a diffusible inhibitor (chapter 2), inhibition of telomerase in normal cells may be at the transcription/translation level. Whatever the mechanism, *a priori* a mutation activating telomerase could occur at any time in the proliferative history of a cell, and hence at any telomere length. Yet, with one possible exception (Klingelhutz *et al.*, 1994) telomerase is activated only at crisis when telomeres are generally critically short (chapters 2,3; Shay *et al.*, 1993, 1995). Based on these data it is formally possible that telomerase activation may be dependent upon short telomeres. For example, the genetic instability generated by short telomeres could translocate a telomerase gene(s) beside a strong promoter causing upregulation of the enzyme. Alternatively, since repeated cycles of dicentric formation and resolution (breakage-fusion-bridge cycle) have been shown to result in gene

amplification (Smith *et al.*, 1992), it is possible that the short telomeres at crisis may lead to the amplification of telomerase genes. Both these possibilities predict that the same region of a specific chromosome will be altered in some fashion in all cells immortalized either *in vitro* or *in vivo* yielding, for example, immortalization specific chromosome markers, which have not however been identified. Perhaps more likely are the following two models. First, activation of telomerase may require prior mutations or simply multiple mutations, and should therefore occur after many cell divisions, that is once telomeres have become very short. Second, a mutation(s) which activates telomerase may indeed occur at any time but because a large proportion of the cells (75-90%) are discarded with each subculturing, the chance of a telomerase positive cell being retained is very low. Therefore, at crisis under the selective pressure of short, perhaps non-functional telomeres, only cells which have recently acquired telomerase activity (and hence have short telomeres) are expected to be present and are expanded.

We initially proposed that telomerase activation was necessary for cells to acquire an unlimited lifespan. In contradiction with this hypothesis, numerous unrelated immortal human cell lines have recently been described that lack telomerase activity (Kim *et al.*, 1994; Murnane *et al.*, 1994; Bryan *et al.*,1995; Rogan *et al.*, 1995; Shay *et al.*, 1995) but still maintain very large TRFs. Interestingly, in one such cell line individual telomeres were found to be highly dynamic, undergoing cycles of gradual shortening followed by rapid elongation. Based on this pattern of variable

telomere length it was proposed that in these populations telomere shortening is countered by the selection of cells in which chromosomes have acquired long tracts of telomeric repeats (Murnane *et al.*, 1994). These telomerase negative cell lines probably acquired this type of telomere maintenance through mutations since telomere elongation is not observed in normal, or for that matter most immortal cells. Although speculative, it is possible that in telomerase negative cell lines a mutation which inactivates a telomere binding protein leads to the amplification of telomeric DNA by, for example, permitting telomeres to recombine inappropriately. In support of this possibility, an as yet undefined mutation in yeast can lead to telomere maintenance solely by what appears to be nonreciprocal terminal recombination (Lundblad & Blackburn, 1993). Alternatively, loss of a telomere binding protein may permit telomeric DNA to form secondary structures during replication such as G-quartets which could imped DNA polymerase. As proposed to account for the expansion of small G-rich repeats in such diseases as fragile X syndrome (Fu *et al.* 1991; reviewed in Kukl & Caskey, 1993), impeding DNA polymerase may result in multiple terminations and reinitiations which, when resolved, could lead to large increase in the length of repetitive (telomeric) DNA. There is however insufficient data to completely rule out the possibility that telomeres in telomerase negative immortal cells are still elongated by some form of telomerase. For example, the cells may encode a mutated form of telomerase that is highly processive but expressed at such low levels or infrequently that telomeric DNA is only rarely elongated (but to a great extent) *in*

vivo, but never in the *in vitro* telomerase assay. Nevertheless, irrespective of how telomere length is maintained in these cells, the very fact that a mechanism exists to counter telomere shortening argues that loss of telomeric DNA, by whatever means, must be arrested for cells to divide indefinitely, as proposed.

6.1.vi) Telomerase positive somatic cells and cell immortalization.

In contrast with earlier predictions (Chapter 2; Harley, 1991), there are in fact somatic tissues that are telomerase positive. Weak levels of activity have been detected in leukocytes from adult peripheral and fetal cord blood and bone marrow as well as in the fetal placenta and adrenal gland, and the liver (Chapter 5; Broccoli *et al.*, 1995; Hiyama *et al.*, 1995a,b). Since the one common feature shared by these tissues is that they do or may contain self-renewing (stem) cells, it is possible that these cells are responsible for the activity detected. However, the level of activity in partially purified stem cells does not appear sufficient to account for the activity detected in peripheral blood leukocytes (Hiyama *et al.*, 1995b) and activity does not appear to fractionate with any particular blood cell type (Broccoli *et al.*, 1995; Hiyama *et al.*, 1995b). Nevertheless, the activity detected in some somatic tissues may still be related to stem cells if stem cells give rise to weakly telomerase positive cells. Perhaps once reagents such as antibodies against the protein components of telomerase become available this issue can be addressed by assaying for the presence of the enzyme components *in situ*, rather than depending on a functional assay. Another puzzling aspect of telomerase positive tissues is that the level of enzyme activity

appears to be too low to counter telomere shortening given that TRF length clearly decreases in PBLs with donor age (Hastie *et al.*, 1990; Vaziri *et al.*, 1993) and possibly even in stem cells (Vaziri *et al.*, 1994) although in the latter case only a slight difference in TRF length was detected between a single young and old donor. The significance of telomerase activity in some somatic tissues therefore remains unknown.

If, as predicted, leukocytes are unique among the somatic cells because they express weak levels of telomerase, it is reasonable to speculate that full activation of the enzyme may occur more readily in these cells. Since arrest of telomere shortening by the activation of telomerase is proposed to be an essential step in the immortalization process, it stands to reason that leukocytes should immortalize at a higher frequency than telomerase negative cells. Circumstantial evidence supports this possibility. EBV transformed B lymphocytes are one of the most readily immortalized type of transformed cell (Middleton *et al.*, 1991). The ease with which these transformed cells become immortal appears to be cell type specific since human mammary epithelial and fetal kidney cells transformed with a subgenomic DNA fragment of EBV immortalize much less frequently, and with a pronounced crisis, compared to B cells transformed by EBV (Karran *et al.*, 1990). Similarly, human T-cell leukemic virus type 1 (HTLV-1) transformed T lymphocytes are easily immortalized and although the virus can infect other cell types, it is apparently involved only in neoplasia of lymphoid cells (Miyoshi *et al.*, 1981; Clapham *et al.*, 1983). Lastly, leukocytes share some common property related to cell immortalization

(weak levels of telomerase?) since these cells belong to the same complementation group of indefinite division (Goletz *et al.*, 1994a).

6.2.i) The role of telomeres and telomerase during the process of cell immortalization *in vivo*.

The most important aspect of the work presented in this thesis is the observation that telomerase is activated during tumourigenesis. Our studies (chapters 4,5) and those that followed (Kim *et al.*, 1994; Nilsson *et al.*, 1994; Broccoli *et al.*, 1995; Chadeneau *et al.*, 1995a; Hiyama *et al.*, 1995a,b,d; Schwartz *et al.*, 1995) have consistently found telomerase activity in most tumour cells but generally not in normal somatic tissues or cultured cells (reviewed in Bacchetti & Counter, 1995). However, unlike in tissue culture, there is no correlation between TRF length and telomerase activity in tumour cells. Although most tumour are telomerase positive, TRF length varies between tumours indicating that tumour cells with almost any telomere length may express the enzyme (reviewed in Bacchetti & Counter, 1995). Indeed, this is the case in most cancers in which telomere length and the status of telomerase were determined in the same samples (chapter 5; Nilsson *et al.*, 1994; Broccoli *et al.*, 1995; Hiyama *et al.*, 1995a,b,d).

At present it is not known why telomerase positive cells in culture have short telomeres whereas *in vivo* they have variable telomere lengths. As in culture, cells transformed *in vivo* appear to lose telomeric DNA during tumourigenesis. For

example, in every cancer studied to date there are tumours with TRFs significantly shorter than in the control tissues (reviewed in Bacchetti & Counter, 1995), and at least some metastatic lesions have shorter TRFs than primary tumours implying that telomeres shorten during the process of clonal expansion (Hiyama *et al.*, 1995d). Moreover, TRF length decreases with cell division *in vitro* in a variety of cultured tumour cells (Rogalla *et al.*, 1994; Ohyashiki *et al.*, 1994b) and in spite of its variability among tumour samples, there is a trend of decreasing TRF length with increasing disease severity in neuroblastoma (Hiyama *et al.*, 1992), endometrial adenocarcinoma (Smith & Yeh, 1992), breast cancer (Odagiri *et al.*, 1994) and MDS (Ohyashiki *et al.*, 1994a). The difference in telomere length of telomerase positive cells transformed *in vitro* and *in vivo* can also not be explained by a difference in the mechanism of telomerase activation, since *a priori* there is no reason to believe that the enzyme is not activated by a mutational event *in vivo*, as proposed to be the case *in vitro*. Therefore, if both the processes of telomere shortening and telomerase activation in transformed cells are identical *in vitro* and *in vivo*, the expansion of telomerase positive cells with variable lengths during tumourigenesis may reflect some aspect of tumour evolution or tumour cell proliferation. For example, cell death and/or clonal expansion could affect the probability that a tumour cell which acquires telomerase activity early will be retained. Thus, depending on when telomerase is activated, malignant telomerase positive cells with different size telomeres could be expanded. On the other hand, age of onset (and hence the initial telomere length of the

transformed cell) or mutation frequency could affect the timing of telomerase activation and thus the length of telomeres maintained in positive cells. However, the telomere length detected in telomerase positive tumours may not neccessarily reflect the length of the initial telomerase positive cell that was expanded since long TRFs could be generated by telomerase activation when telomeres were short, followed by telomere elongation, as observed occasionally in transformed cells in culture (Klingelhutz *et al.*, 1994; Shay *et al.*, 1995). Also, the actual telomerase positive cells which give rise to the activity detected in a tumour specimen may in fact have short telomeres which are masked by the long telomeres of either contaminating normal tissue (Hastie *et al.*, 1990; Adamson *et al.*, 1992; Hiyama *et al.*, 1992, 1995d; Smith & Yeh, 1992; Kim *et al.*, 1994; Mehle *et al.*, 1994) or other tumour cell with shorter proliferative histories. Given the number of variables affecting the expansion of a telomerase positive cell during tumourigenesis, longitudinal studies in which telomere length and telomerase activity are carefully monitored *in vivo* will be required to address this issue.

6.2.ii) Clinical applications.

Aside from contributing to our understanding of the process of cell immortalization, the possible requirement for telomerase activation during tumourigenesis suggests that the enzyme may have diagnostic, prognostic and even therapeutic applications. Diagnostically the strict correlation of enzyme activity in tumour but not most somatic cells indicates that telomerase is a prevalent tumour

marker. In terms of prognostic value, we have proposed that the selection of a telomerase positive cell may occur after many cell doublings and thus a telomerase negative tumour is predicted to be less advanced in malignancy than a telomerase positive tumour. In support of this possibility, benign (Kim *et al.*, 1994; Chadeneau *et al.*, 1995a; Hiyama *et al.*, 1995a) or early stage (chapter 5; Hiyama *et al.*, 1995b) tumours from many, but not all cancers are telomerase negative. Moreover, in some cases the types of benign or early stage tumours which are telomerase positive show a tendency to progress to either full malignancy or more advanced stages (chapter 5; Kim *et al.*, 1994; Schwartz *et al.*, 1995). Based on our tissue culture observations, we would also predict that a tumour in which telomerase was not activated should reach a "proliferative crisis" and perish. Thus, the absence of telomerase activity could potentially forecast tumour remission. Indeed, stage IVs neuroblastoma tumours which lack telomerase activity regress (Hiyama *et al.*,1995a). However, as stated above, by the time most tumours are detected, the malignant cells are already telomerase positive indicating that assaying for telomerase activity for prognostic purposes may be of value only in selected types of cancers.

Perhaps most importantly, telomerase may serve as a target for anti-cancer therapy. Since most tumours express telomerase, loss of enzyme activity in tumour cells from potentially any type of cancer is predicted to reinitiate telomere shortening, leading to chromosome instability and cell death. Somatic tissues would be spared from the lethal effects of telomerase inhibition because, in general, they lack enzyme

activity. In support of the therapeutic value of inhibiting proper telomerase action, cell lines with short telomeres which express antisense RNA transcripts of the structural RNA component of telomerase underwent telomere shortening and entered a "crisis" (Feng *et al.*, 1995). Although these data clearly demonstrated the requirement of telomerase activity for the immortal phenotype the experimental system was optimized to be sensitive to a loss of telomerase activity since the cells analyzed had short telomeres and stably expressed an antisense RNA. Even using this approach high enough expression of an antisense RNA could not be achieved to effectively eradicate the population. Perhaps a better system to experimentally assess the lethality of disrupting telomerase function, which does not require total inhibition of the enzyme, would be to overexpress in malignant cells a structural RNA of telomerase containing mutations in the template region. Using this approach non-telomeric sequences could be added to telomere termini of telomerase positive tumour cells thereby compromising telomere function, regardless of telomere lengths within the cell or population and, at least in theory, in as little as a single cell division. In lower eukaryotes altering telomere sequences in this fashion has been shown to be lethal (Yu *et al.*, 1990; McEachern & Blackburn, 1995). However, it will be some time before this approach could be tested *in vivo* since at present there are no techniques to effectively deliver DNA vectors (and hence the mutated RNA) to all tumour cells. Moreover, a vector would also have to be specifically targeted to tumour cells for selective treatment since telomerase positive normal tissues would also acquire non-

telomeric repeats with the same efficiency as tumour cells.

The most promising approach to alter telomerase function in tumour cells is to inhibit enzyme activity by a chemical agent. Such an agent could be administered systemically with presumably little effect on most normal somatic tissues. Telomerase positive cells, for example those of the germline or blood, would however undergo telomere shortening and even though their long telomeres should protect chromosome ends from becoming recombinogenic, there is a possibility that loss of telomeric DNA may result in senescence of some of the cells. Of greater concern however, is the possibility that telomerase inhibitors may by insufficient or even incapable of eradicating certain types of tumours. For example, telomerase positive tumours with long telomeres would require long periods of exposure in order for telomeres to become critically short, thus such tumours may reach a size or stage no longer compatible with life within the period of treatment. More importantly, telomerase negative tumour cells with long telomeres or tumour cells which maintain telomere length in a telomerase-independent manner would be completely resistant to a telomerase inhibitor. If these cells were present in a telomerase positive tumour they could act as a reservoir, regenerating a malignant lesion after treatment with an anti-telomerase agent (reviewed in Bacchetti & Counter, 1995; Harley *et al.*, 1994). Nevertheless, the presumed specificity of an inhibitor for telomerase positive cells is a great advantage over current chemotherapeutic agents which target all dividing cells.

6.3) Future Directions

As discussed above, activation of telomerase may be a required step during tumourigenesis. I foresee two areas of research which may investigate this possibility and expand our understanding of the normal role this enzyme plays *in vivo*.

First, since the murine structural RNA of telomerase has recently been cloned (Blasco *et al.*, 1995), it will be only a matter of time before the gene is knocked out in transgenic mice. Such knockout mouse would therefore lack a functional telomerase holoenzyme and since mice appear to have long telomeres, loss of telomerase activity should not disrupt development, at least in the first few generations, unless the enzyme is required for more than countering the loss of telomeric DNA. Knockout mice should therefore prove invaluable in unravelling the process of immortalization in murine cells and in furthering our understanding of this process in human cells. For example, if telomerase is required for the immortalization of mammalian cells, cells from knockout mice which are transformed with viral oncogenes or which spontaneously overcame senescence should have a finite lifespan whereas the counterparts from normal sibling mice should be immortal. The actual lifespan of cells from knockout mice should be dependent upon their telomere length prior to being cultured. On the other hand, if knockout mice cells were immortal in culture then mice may have alternative mechanisms of maintaining telomeres, a finding which could have important implications for the use of telomerase inhibitors in the treatment of human cancers. The measurement of telomere lengths in tissues from the knockout

mice may also have developmental implications. Since telomeres shorten at a constant rate, at least in somatic cells in humans (Allsopp *et al.*, 1992; Allsopp & Harley, 1995), the difference between the telomere length of the parental germline cells and the tissues of their offsprings could be used to estimate the number of cell divisions required to generate a tissue. Similarly, by determining which organs fail in the adult knockout mouse, it may be possible to identify those tissues that may require telomerase positive cells for their upkeep, and hence which tissues may be most severely affected by a telomerase inhibitor.

Knockout cells may also provide a wonderful opportunity to manipulate telomere sequences by introducing into them telomerase RNAs with different template sequences. Presumably an RNA harbouring a template mutation would be incorporated into a functional holoenzyme which should then cap each chromosome with altered telomeric sequences. This approach could prove invaluable as an experimental system to study the relationship of telomere sequence and function in mammals.

The second important step in understanding the role of telomerase, both in normal and malignant cells, will be the cloning of, and generation of antibodies against the protein subunits of the human enzyme. Such antibodies could be used to identify telomerase positive cells in the blood or bone marrow (or in any tissue) *in situ*, which may further our understanding of the hematopoietic system. Antibodies could also be employed to address the question of how telomerase is normally

regulated. For example, it has been postulated that telomerase may be activated through the increase in the expression of a protein subunit that is normally repressed, since the RNA component is expressed in all human cells and only slightly increased in telomerase positive cells (Feng *et al.*, 1995; Avilion *et al.*, personal communication). The latter data would however also be consistent with the holoenzyme being expressed in all cells, but functionally repressed in normal somatic cells. Western blot analysis could therefore be used to determine whether all or only some protein subunits of telomerase are expressed in telomerase negative tissues. Moreover, if all the enzyme components are detected in telomerase negative tissues, antibodies could be used to purify potential protein(s) that bind to and repress telomerase. The pattern of telomerase activity in the cell cycle could also be addressed, particularly in yeast since not only have many of the genes that regulate the cell cycle been identified and disrupted in this organism, but telomerase activity and the cloning of the enzyme's structural RNA have recently been reported in this eukaryote (Singer & Gottschling, 1994; Cohn & Blackburn, 1995; Lin & Zakian, 1995; McEachern & Blackburn, 1995). In addition, the relationship between telomere length regulation and telomerase could also be investigated in yeast since there are a number of mutations that result in an alteration of telomere length in this organism (*ie.*Carson & Hartwell, 1985; Lustig & Petes, 1986; Aparicio *et al.*, 1991; Hardy *et al.*, 1992; Schulz & Zakian, 1994; Suzuki & Nishizawa, 1994; Liu *et al.*, 1995). Extracts from these mutant strains could be assayed for telomerase activity *in vitro* to determine if the defect in telomere length

regulation is related to telomerase activity. Such an approach has already been attempted with a strain of yeast which, due to the lack the *EST1* gene, undergoes inappropriate telomere shortening. The results from these experiments suggest that the *EST1* gene product may possibly regulate telomerase function in a subtle fashion (Cohn & Blackburn, 1995; Lin & Zakian, 1995).

With the cloning of the telomerase protein components of *Tetrahymena* (Collins *et al.*, 1995) the enzyme may now be reconstituted from purified or recombinant proteins *in vitro* allowing the holoenzyme to be dissected biochemically and possibly even crystallized. The *in vitro* reconstitution of the human enzyme will also aid in the screening of telomerase inhibitors. With the current interest in this field from a broad spectrum of laboratories it is inevitable that telomerase will soon be cloned in its entirety from many different organisms, opening up a new chapter in the study of telomerase and its role in cellular immortality.

References

Adamson, D.J.A., King, D.J., and Haites, N.E. (1992) Significant telomere shortening in childhood leukemia. *Cancer Genet. Cytogenet.* **61**:204-206.

Allshire, R.C., Dempster, M., and Hastie, N.D. (1989) Human telomeres contain at least three types of G-rich repeat distributed non-randomly *Nucl. Acids Res.* **17**:4611-4627.

Allsopp, R.C., and Harley, C.B. (1995) Evidence for a critical telomere length in senescent human fibroblasts. *Exp. Cell Res.* **219**:130-136.

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

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. (1991) Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell*, **66**:1279-1287.

Aparicio, O.M. and Gottschling, D.E. (1994) Overcoming telomeric silencing: A *trans*-activator competes to establish gene expression in a cell cycle-dependent way. *Genes Dev.* **8**:1133-1146.

Autexier, C., and Greider, C.W. (1994) Functional reconstitution of wild-type and mutant *Tetrahymena* telomerase. *Genes Dev.* **8**:563-575.

Avivi, L., and Feldman, M (1980) Arrangement of chromosomes in the interpahse nucleus of plants. *Hum. Genet.* **55**:281-295.

Bacchetti, S., and Counter, C.M. (1995) Telomeres and telomerase in human cancer (Review). *Int. J. Oncol.* **7**: 423-432.

Balagurumoorthy, P., and Brahmachari, S.K. (1994) Structure and stability of human telomeric sequence. *J. Biol. Chem.* **269**:21858-21869.

Band, V., Zajchowski, D., Kulesa, V., and Sager, R. (1990) Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc. Natl. Acad. Sci. USA*, **87**:463-467.

Baroin, A., Prat, A., and Caron, F. (1987) Telomeric site position heterogeneity in macronuclear DNA of *Paramecium primaurelia*. *Nucl. Acids Res.* **15**:1717-1728.

Barnett, M.A., Buckle, V.J., Evans, E.P., Porter, A.C.G., Rout, D., Smith, A.G., and Brown, W.R.A. (1993) Telomere directed fragmentation of mammalian chromosomes. *Nucl. Acids Res.* **21**:27-36.

Barrett, J.C., Annab, L.A., Alcorta, D., Preston, G., Vojta, P., and Yin, Y. (1994) Cellular senescence and cancer. *Cold Spring Harbor Symp. Quant. Biol.* **59**:411-418.

Bayne, R.A.L., Broccoli, D., Taggart, M.H., Thomson, E.J., Farr, C.J. and Cooke, H.J. (1994) Sandwiching of a gene within 12 kbp of a functional telomere and alpha satellite does not result in silencing. *Hum. Mol. Genet.* **3**:539-546.

Benn, P.A. (1976) Specific chromosome aberrations in senescent fibroblast cell lines derived from human embryos. *Am. J. Hum. Genet.* **28**:465-473.

Bernards, A., Michels, P.A.M., Lincke, R.C., and Borst, P. (1983) Growth of chromosome ends in multiplying *trypanosomes*. *Nature,* **303**:592-597.

Bhattacharyya, A., and Blackburn, E.H. (1994) Architecture of telomerase RNA. *EMBO J.* **13**:5721-5731.

Biessmann, H., and Mason, J.M. (1992) Genetics and molecular biology of telomeres. *Adv. Genet.* **30**:185-249.

Biessmann, H., Champion, L.E., O'Hair, M., Ikenaga, K., Kasravi, B., and Mason, J.M. (1992) Frequent transposition of *Drosophila melanogaster* HeT-A transposable element to receding chromosome ends. *EMBO J.* **11**:4459-4469.

Biessmann, H., Kasravi, B., Bui. T., Fujiwara, G., Champion, L.E., and Mason, J.M. (1994) Comparison of two active HeT-A retroposons of *Drosophila melanogaster*. *Chromosoma,* **103**:90-98.

Biessmann, H., Kasravi, B., Jakes, K., Bui, T., Ikenaga, K., and Mason, J.M. (1993) The genomic organization of HeT-A retroposon in *Drosophila melanogaster*. *Chromosoma,* **102**:297-305.

Biessmann, H., Mason, J.M., Ferry, K., d'Hulst, M., Valgeirsdottir, K., Traverse, K.L., and Pardue, M.L. (1990) Addition of telomere-associated HeT DNA sequences "heals" broken chromosome ends in *Drosophila*. *Cell,* **61**:663-673.

Bischoff, F.Z., Yim, S.O., Pathak, S., Grant, G., Siciliano, M.J., Giovanella, B.C., Strong, L.C., and Tainsky, M.A. (1990) Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome: Aneuploidy and immortalization. *Cancer Res.* **50**:7979-7984.

Blackburn, E.H., and Chiou, S.-S. (1981) Non-nucleosomal packaging of a tandemly repeated DNA sequence at termini of extrachromosomal DNA coding for rRNA in *Tetrahymena, Proc. Natl. Acad. Sci. USA*, **78**:2263-2267.

Blackburn, E.H., and Szostak, J.W. (1984) The molecular structure of centromeres and telomeres. *Ann. Rev. Biochem.* **53**:163-194.

Blasco, M.A., Funk, W., Villeponteau, B., and Greider, C.W. (1995) Functional characterization and developmental regulation of mouse telomerase RNA. *Science,* **269**:1267-1270.

Bourgain, F.M., and Katinka, M.D. (1991) Telomeres inhibit end to end fusion and enhance maintenance of linear DNA molecules injected into the *Paramecium primaurelia* macronucleus. *Nucl. Acids Res.* **19**:1541-1547.

Brigati, C., Kurtz, S., Balderes, D., Vidali, G., and Shore, D. (1993) An essential yeast gene encoding a TTAGGG repeat-binding protein. *Mol. Cell. Biol.* **13**:1306-1314.

Broccoli, D., and Cooke, H.J. (1994) Effect of telomeres on the interphase location of adjacent regions of the human X chromosome. *Exp. Cell Res.* **212**:308-313.

Broccoli, D., Young, J.W., and de Lange, T. (1995) Telomerase activity in normal and malignant hematopoietic cells. *Proc. Natl. Acad. Sci. USA*,**92**:9082-9086.

Brown, W.R.A. (1989) Molecular cloning of human telomeres in yeast. *Nature,* **338**:774-776.

Brown, W.R.A., MacKinnon, P.J., Villasanté, A., Spurr, N., Buckle, V.J., and Dobson, M.J. (1990) Structure and polymorphism of human telomere-associated DNA. *Cell,* **63**:119-132.

Brugge, J.S., and Butel, J.S. (1975) Role of simian virus 40 gene A function in maintenance of transformation. *J. Virol.* **15**:619-635.

Bryan, T.M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R.R. (1995) Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.* **14**:4240-4248.

Buck, S.W., and Shore, D. (1995) Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between HMR and telomeres in yeast. *Genes Dev.* **9**:370-384.

Bunn, C.L., and Tarrant, G.M. (1980) Limited lifespan in somatic cell hybrids and cybrids. *Exp. Cell Res.* **37**:385-396.

Byrd, P., Brown, K.W., and Gallimore, P.H. (1982) Malignant transformation of human embryo retinoblasts by cloned adenovirus 12 DNA. *Nature,* **298**:69-71.

Byrd, P.J., Grand, R.J.A., and Gallimore, P.H. (1988) Differential transformation of primary human embryo retinal cells by adenovirus E1 regions and combinations of E1A + *ras. Oncogene,* **2**:477-484.

Caporossi, D., and Bacchetti, S. (1990) Definition of adenovirus type 5 functions involved in the induction of chromosomal aberrations in human cells. *J. Gen. Virol.* **71**:801-808.

Cardenas, M.E., Bianchi, A., and de Lange, T. (1993) A *Xenopus* egg factor with DNA-binding properties characteristic of terminus-specific telomeric proteins. *Genes Dev.* **7**:883-894.

Carson, M.J., and Hartwell, L. (1985) CDC 17: An essential gene that prevents telomere elongation in yeast. *Cell,* **42**:249-257.

Cairns, J. (1975) The cancer problem. *Sci. Am.* **233**:64-78.

Cavalier-Smith (1974) Palindromic base sequences and replication of eukaryote chromosome ends. *Nature,* **250**:467-470.

Chadeneau, C., Hay, K., Hirte, H.W., Gallinger, S., and Bacchetti, S. (1995a) Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res.* **55**:2533-2536.

Chadeneau, C., Siegel, P., Harley, C.B., Muller, W.J., and Bacchetti, S. (1995b) Telomerase activity in normal and malignant murine tissues. *Oncogene,* **11**:893-898.

Chang, S.E. (1986) In vitro transformation of human epithelial cells. *Biochim. Biophys. Acta,* **823**:161-194.

Clapham, P., Nagy, K., Cheingsong-Popov, R., Exley, M., and Weiss, R.A. (1983) Productive infection and cell-free transmission of human T-cell leukemia virus in a nonlymphoid cell line. *Science,* **222**:1125-1127.

Cheng, J.-F., Smith, C.L., and Cantor, C.R. (1990) Structure and transcriptional analysis of a human subtelomeric repeat. *Nucl. Acids Res.* **19**:149-154.

Chikashige, Y., Ding, D.-Q., Funabiki, H., Haraguchi, T., Mashiko, S., Yanagida, M., and Hiraoka, Y. (1994) Telomere-led premeiotic chromosome movement in fission yeast. *Science,* **264**:270-273.

Chung, H.-M.M., Shea, C., Fields, S., Taub, R.N., and Van der Ploeg, H.T. (1990) Architectural organization in the interphase nucleus of the protozoan *Trypanosoma brucei:* location of telomeres and mini-chromosomes. *EMBO J.* **9**:2611-2619.

Clarke-Pearson, D.L., and Creasman, W.T. (1986) Ovarian cancer. *In:* Moossa, A.R., Robson, M.C., and Schimpff, S.C. (*eds*) Comprehensive textbook of oncology. Williams and Wilkins, Los Angeles, USA, pp 845-854.

Cohn, M., and Blackburn, E.H. (1995) Telomerase in yeast. *Science,* **269**:396-400.

Collins, K., and Greider, C.W. (1993) *Tetrahymena* telomerase catalyzes nucleolytic cleavage and nonprocessive elongation. *Genes Dev.* **7**:1364-1376.

Collins, K., Kobayashi, R., and Greider, C.W. (1995) Purification of *Tetrahymena* telomerase and cloning of genes encoding the two protein components of the enzyme. *Cell,* **81**:677-686.

Conrad, M.N., Wright, J.H., Wolf, A.J., and Zakian, V.A. (1990) RAP1 protein interacts with yeast telomeres in vivo: Overproduction alters telomere structure and decreases chromosome stability. *Cell,* **63**:739-750.

Cooke, H.J., and Smith, B.A. (1986) Variability at the telomeres of the human X/Y pseudoautosomal region. *Cold Spring Harbor Symp. Quant. Biol.* **51**:213-219.

Coren, J.S., Epstein, E.M., and Vogt, V.M. (1991) Characterization of a telomere-binding protein from *Physarum polycephalum*. *Mol. Cell. Biol.* **11**:2282-2290.

Coren, J.S., and Vogt, V.M. (1992) Purification of a telomere-binding protein from *Physarum polycephalum*. *Biochim. Biophys. Acta,* **1171**:162-166.

Cross, S.H., Alishire, R.C., McKay, S.J., McGill, N.I., and Cooke, H.J. (1989) Cloning of human telomeres by complementation in yeast. *Nature,* **338**:771-774.

Cross, S., Lindsey, J., Fantes, J., McKay, S., McGill, N., and Cooke, H. (1990) The structure of a subterminal repeated sequence present on many human chromosomes. *Nucl. Acids Res.* **18**:6649-6657.

Danilevskaya, O., Slot, F., Pavlova, M., and Pardue, M.L. (1994) Structure of the *Drosophila* HeT-A transposon: a retrotransposon-like element forming telomeres. *Chromosoma,* **103**:215-224.

Dancis, B.M., and Holmquist, G.P. (1979) Telomere replication and fusion in eukaryotes. *J. Theor. Biol.* **78**:211-224.

Daniel, C. (1972) Aging of cells during serial propagation *in vivo*. *Adv. Gerontol. Res.* **4**:167-199.

de Lange, T. (1992) Human telomeres are attached to the nuclear matrix. *EMBO J.* **11**:717-724.

de Lange, T., (1995) Telomere dynamics and genome instability in human cancer. *In:* Blackburn, E.H., and Greider, C.W. (*eds*) Telomeres. *Cold Spring Harbor Press,* in press.

de Lange, T., Shiue, L., Myers, R.M., Cox, D.R., Naylor, S.L., Killery, A.M., and Varmus, H.E. (1990) Structure and variability of human chromosome ends. *Mol. Cell. Biol.* **10**:518-527.

Dell'Orco, R., Merteans, J., and Kruse, P. (1973) Doubling potential, calender time and senescence of human diploid cells in culture. *Exp. Cell. Res.* **77**:356-360.

Di Leonardo, A., Linke, S.P., Clarkin, K., and Wahl, G.M. (1994) DNA damage triggers a prolonged p53-dependent G_1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Gene Dev.* **8**:2540-2551.

Dimri, G.P., and Campisi, J. (1994) Molecular and cell biology of replicative senescence. *Cold Spring Harbor Symp. Quant. Biol.* **59**:67-73.

D'Mello, N.P., and Jazwinski, M. (1991) Telomere length constancy during aging of *Saccharomyces cerevisiae. J. Bacteriol.* **173**:6709-6713.

Duncan, E.L., Whitaker, N.J., Moy, E.L., and Reddel, R.R. (1993) Assignment of SV40-immortalized cells to more than one complementation group for immortalization. *Exp. Cell Res.* **205**:337-344.

Dunn, B., Szauter, P., Pardue, M.L., and Szostak, J.W. (1984) Transfer of yeast telomeres to linear plasmids by recombination. *Cell,* **39**:191-201.

Edwards, C.A., and Firtel, R.A. (1984) Site-specific phasing in the chromatin of the rDNA in *Dictyostelium discoideum. J. Mol. Biol.* **180**:73-90.

Emery, H.S., and Weiner, A.M. (1981) An irregular satellite sequence is found at the termini of linear extrachromosomal rDNA in *Dictyostelium discoideum. Cell,* **26**:411-419.

Fang, G., and Cech, T.R. (1991) Molecular cloning of telomere-binding protein genes from *Stylonychia mytilis. Nucl. Acids Res.* **19**:5515-5518.

Fang, G., and Cech, T.R. (1993a) *Oxytricha* telomere-binding proteins: DNA dependent dimerization of the α and β subunits. *Proc. Natl. Acad. Sci. USA*, **90**:6056-6060.

Fang, G., and Cech, T.R. (1993b) The β subunit of *Oxytricha* telomere-binding protein promotes G-quartet formation by telomeric DNA. *Cell,* **74**:875-885.

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

Farr, C.J., Stevanovic, M., Thomson, E.J., Goodfellow, P.N., and Cooke, H.J. (1992) Telomere-associated chromosome fragmentation: Applications in genome manipulation and analysis. *Nature Genet.* **2**:275-282.

Fearon, E.R., and Vogelstein, B. (1990) A genetic model for colorectal tumourigenesis. *Cell,* **61**:759-767.

Feng, J., Funk, W.D., Wang, S-.S., Weinrich, S.L., Avilion, A.A., Chiu, C-.P., Adams, R.R., Chang, E., Allsopp, R.C., Yu, J., S., Le, West, M.D., Harley, C.B., Andrews, W.H., Greider, C.W., and Villeponteau, B. (1995) The RNA component of human telomerase. *Science,* **269**:1236-1241.

Flint, J., Craddock, C.F., Villegas, A., Bentley, D.P., Williams, H.J., Galanello, R., Cao, A., Wood, W.G., Ayyub, H., and Higgs, D.R. (1994) Healing of broken chromosomes by the addition of telomeric repeats. *Am. J. Hum. Genet.* **55**:505-512.

Forney, J.D., and Blackburn, E.H. (1988) Developmentally controlled telomere addition in wild-type and mutant *Paramecia*. *Mol. Cell. Biol.* **8**:251-258.

Frantz, J.D., and Gilbert, W. (1995) A yeast gene product, G4p2, with a specific affinity for quadruplex nucleic acids. *J. Biol. Chem.* **270**:9413-9419.

Fu, Y.-H., Kuhl, D.P.A., Pizzuti, A., Pieretti, M., Sutcliffe, J.S., Richards, S., Verkerk, A.J.M.H., Holden, J.J.A., Fenwick Jr., R.G.F., Warren, S.T., Oostra, B.A., Nelson, D.L., and Caskey, C.T. (1991) Variation of the CGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox. *Cell,* **67**:1047-1058.

Funabiki, H., Hagan, I., Uzawa, S., Yanagida, M. (1993) Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. *J. Cell Biol.* **121**:961-976.

Ganal, M.W., Lapitan, N.L.V., and Tanksley, S.D. (1991) Macrostructure of the tomato telomeres. *Plant Cell,* **3**:87-94.

Gilley, D., and Blackburn, E.H. (1994) Lack of telomere shortening during senescence in *Paramecium*. *Proc. Natl. Acad. Sci. USA*, **91**:1955-1958.

Gillies, C.B. (1975) Synaptonemal complex and chromosome structure. *Ann. Rev. Genet.* **9**:91-108.

Gilson, E., Müller, T., Sogo, J., Laroche, T., and Gasser, S. (1994) RAP1 stimulates single- to double-strand association of yeast telomeric DNA: Implications for telomere-telomere interactions. *Nucl. Acids Res.* **22**:5310-5320.

Giraldo, R., Suzuki, M., Chapman, L., and Rhodes, D. (1994) Promotion of parallel DNA quadruplexes by a yeast telomere binding protein: A circular dichroism study. *Proc. Natl. Acad. Sci. USA*, **91**:7658-7662.

Giraldo, R., and Rhodes, D. (1994) The yeast telomere-binding protein RAP1 binding to and promotes the formation of DNA quadruplexes in telomeric DNA. *EMBO J.* **13**:2411-2420.

Girardi, A.J., Jensen, F.C., and Koprowski, H. (1965) SV_{40}-induced transformation of human diploid cells: Crisis and recovery. *J. Cell. Comp. Physiol.* **65**:69-84.

Goldstein, S. (1969) Lifespan of cultured cells in progeria. *Lancet,* **I**:424.

Goldstein, S. (1974) Ageing *in vitro*: Growth of cultured cells from the Galapagos tortoise. *Exp. Cell Res.* **83**:297-302.

Goldstein, S. (1979) Studies on age-related diseases in cultured skin fibroblasts. *J. Invest. Dermatol.* **73**:19-23.

Goldstein, S. (1990) Replicative senescence: The human fibroblast comes of age. *Science,* **249**:1129-1133.

Goldstein, S., Moerman, E.L., Soeldner, J.S., Gleason, R.E., and Barnett, D.M. (1978) Chronological and physiologic age affect replicative life span of fibroblasts from diabetic, pre-diabetic, and normal donors. *Science,* **199**:781-782.

Goletz, T.J., Robetorye, S., and Pereira-Smith, O.M. (1994a) Genetic analysis of indefinite division in human cells; Evidence for a common immortalization mechanism in T and B lymphoid cell lines. *Exp. Cell Res.* **215**:82-89.

Goletz, T.J., Smith, J.R., and Pereira-Smith, O.M. (1994b) Molecular genetic approaches to the study of cellular senescence. *Cold Spring Harbor Symp. Quant. Biol.* **59**:59-66.

Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. (1990) Position effect at *S. cerevisiae* telomeres: Reversible repression of pol II transcription. *Cell,* **63**:751-762.

Gottschling, D.E., and Cech, T.R. (1984) Chromatin structure of the molecular ends of *Oxytricha* macronuclear DNA: Phased nucleosomes and a telomeric complex. *Cell,* **38**:501-510.

Gottschling, D.E., and Zakian, V.A. (1986) Telomere proteins: specific recognition and protection of the natural termini of *Oxytricha* macronuclear DNA. *Cell,* **47**:195-205.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human Adenovirus type 5. *J. Gen. Virol.* **36**:59-72.

Gray, J.T., Celander, D.W., Price, C.M., and Cech, T.R. (1991) Cloning and expression of genes for the *Oxytricha* telomere-binding protein: Specific subunit interactions in the telomeric complex. *Cell,* **67**:807-814.

Greider, C.W. (1991) Telomerase is processive. *Mol. Cell. Biol.* **11**:4572-4580.

Greider, C.W. (1994) Mammalian telomere dynamics: Healing, fragmentation, shortening and stabilization. *Curr. Opin. Genet. Dev.* **4**:203-211.

Greider, C.W., and Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell,* **43**:405-413.

Greider, C.W., and Blackburn, E.H. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature,* **337**:331-337.

Gualberto, A., Patrick, R.M., and Walsh, K. (1992) Nucleic acid specificity of a vertebrate telomere-binding protein: evidence for G-G base pair recognition at the core-binding site. *Genes Dev.* **6**:815-824.

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

Hara, E., Tsuri, H., Shinozaki, S., and Oda, K. (1991) Cooperative effect of antisense-Rb and antisense-p53 oligomers on the extension of lifespan in human diploid fibroblasts, TIG-1. *Biochem. Biophys. Res. Commun.* **179**:528-534.

Hardy, C.F.J., Sussel, L., and Shore, D. (1992) A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. *Genes Dev.* **6**:801-814.

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

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

Harley, C.B., and Goldstein, S. (1978) Cultured human fibroblasts. Distribution of cell generations and a critical limit. *J. Cell. Physiol.* **97**:509-516.

Harley, C.B., Kim, N.W., Prowse, K.R., Weinrich, S.L., Hirsch, K.S., West, M.D., Bacchetti, S., Hirte, H.W., Counter, C.M., Greider, C.W., Piatyszek, M.A., Wright, W.E., and Shay, J.W. (1994) Telomerase, cell immortality and cancer. *Cold Spring Harbor Symp. Quant. Biol.* **59**:307-316..

Harrington, L.A., and Greider, C.W. (1991) Telomerase primer specificity and chromosome healing. *Nature,* **353**:451-454.

Harris, C.C. (1987) Human tissues and cells in carcinogenesis research. *Cancer Res.* **47**:1-10.

Hastie, N.D., Dempster, M., Dunlop, M.G., Thompson, A.M., Green, R.C., and Allshire, R.C. (1990) Telomere reduction in human colorectal carcinoma and with ageing. *Nature,* **346**:866-868.

Hayflick, L. (1976) The cell biology of human aging. *N. Engl. J. Med.,* **295**:1302-1308.

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

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., and Grunstein, M. (1995) Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: A molecular model for the formation of heterochromatin in yeast. *Cell,* **80**:583-592.

Henderson, E.R. and Blackburn, E.H. (1989) An overhanging 3' terminus is a conserved feature of telomeres. *Mol. Cell. Biol.* **9**:345-348.

Hensler, P.J., Annab, L.A., Barrett, J.C., and Pereira-Smith, O.M. (1994) A gene involved in control of human cellular senescence on human chromosome 1q. *Mol. Cell. Biol.* **14**:2291-2297.

Hering, S., Griffin, B.E., and Strauss, M. (1991) Immortalization of human fetal sinusoidal liver cells by polyoma virus large T antigen. *Exp. Cell Res.* **195**:1-7.

Heslop-Harrison, J.S., and Bennett, M.D. (1990) Nuclear architecture in plants. *Trends Genet.* **6**:401-405.

Hinds, P.W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S.I., and Weinberg, R.A. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell,* **70**:993-1006.

Hiyama, E., Hiyama, K., Yokoyama, T., Ichikawa, T., and Matsuura, Y, (1992) Length of telomeric repeats in neuroblastoma: Correlation with prognosis and other biological characteristics. *Jpn. J. Cancer Res.* **83**:159-164.

Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M.A., and Shay, J.W. (1995a) Correlating telomerase activity levels with human neuroblastoma outcomes. *Nature Med.* **1**:249-255.

Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M.A., Shay, J.W., Ishioka, S., and Yamakido, M. (1995b) Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immun.* in press.

Hiyama, E., Yokoyama, T., Hiyama, K., Yamakido, M., Santo, T., Kodama, T., Ichikawa, T., and Matsuura, Y. (1995c) Alteration of telomeric repeat length in adult and childhood solid neoplasias. *Int. J. Oncol.* **6**:13-16.

Hiyama, K., Hiyama, E., Ishioka, S., Yamakido, M., Inai, K., Gazdar, A.F., Piatyszek, M.A. and Shay, J.W. (1995d) Telomerase activity in primary and metastatic lung cancer: Do all lung cancers consist of immortal cells? *JNCI*, **87**:895-902.

Hiyama, K., Ishioka, S., Shirotani, Y., Inai, K., Hiyama, E., Murakami, I., Isobe, T., Inamizu, T., and Yamakido, M. (1995d) Alterations in telomeric repeat length in lung cancer are associated with loss of heterozygosity in p53 and Rb. *Oncogene,* **10**:937-944.

Huschtscha, L.I., and Holliday, R. (1983) Limited and unlimited growth of SV40-transformed cells from human diploid MRC-5 fibroblasts. *J. Cell Sci.* **63**:77-99.

Ishikawa, F., Matunis, M.J., Dreyfuss, G., and Cech, T.R. (1993) Nuclear proteins that bind the pre-mRNA 3' splice site sequence r(UUAG/G) and the human telomeric DNA sequence $d(TTAGGG)_n$. *Mol. Cell. Biol.* **13**:4301-4310.

Itzhaki, J.E., Barnett, M.A., MacCarthy, A.B., Buckle, V.J., Brown, W.R.A., and Porter, A.C.G. (1992) Targeted breakage of a human chromosome mediated by cloned human telomeric DNA. *Nature Genet.* **2**:283-287.

Karpen, G.H. (1994) Position-effect variegation and the new biology of heterochromatin. *Curr. Opin. Genet. Dev.* **4**:281-291.

Karran, L., Teo, C.G., King, D., Hitt, M.M., Gao, Y., Wedderburn, N., and Griffin, B.E. (1990) Establishment of immortalized primate epithelium cells with sub-genomic EBV DNA. *Int. J. Cancer,* **45**:763-772.

Klein, F., Laroche, T., Cardenas, M.E., Hofmann, J.F., Schweizer, D., and Gasser, S.M. (1992) Localization of RAP1 and topoismerase II in the nuclei and meiotic chromosomes of yeast. *J. Cell Biol.* **117**:935-948.

Kennedy, B.K., Austriaco Jr., N.R., Zhang, J., and Guarente, L. (1995) Mutation in the silencing gene *SIR4* can delay aging in *S. cerevisiae. Cell,* **80**:485-496.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science,* **266**:2011-2015.

Kipling, D., and Cooke, H.J. (1990) Hypervariable ultra-long telomeres in mice. *Nature,* **347**:400-402.

Klingelhutz, A.J., Barber, S.A., Smith, P.P., Dyer, S., and McDougall, J.K. (1994) Restoration of telomeres in human papillomavirus-immortalized human angenital epithelial cells. *Mol. Cell. Biol.* **14**:961-969.

Klobutcher, L.A., Swanton, M.T., Donini, P., and Prescott, D.M. (1981) All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus. *Proc. Natl. Acad. Sci. USA*, **78**:3015-3019.

Kramer, K.M., and Haber, J.E. (1993) New telomeres in yeast are initiated with a highly select subset of $TG_{1\text{-}3}$ repeats. *Genes Dev.* **7**:2345-2356.

Kuhl, D.P.A., and Caskey, C.T. (1993) Trinucleotide repeats and genome variation. *Curr. Opin. Genet. Dev.*, **3**:404-407.

Kyrion, G., Liu, K., Liu, C., and Lustig, A. (1993) RAP1 and telomere structure regulate telomere position effects in *Saccharomyces cerevisiae. Genes Dev.* **7**:1146-1159.

Kyrion, G., Boakye, K.A., and Lustig, A.J. (1992) C-terminal truncation of RAP1 results in the deregulation of telomere size, stability, and function in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **12**:5159-5173.

Lamb, J., Harris, P.C., Wilkie, A.O., Wood, W.G., Dauwerse, J.G., and Higgs, D.R. (1993) *De novo* truncation of chromosome 16p and healing with $(TTAGGG)_n$ in the alpha-thalassemia/mental retardation syndrome (ATR-16). *Am. J. Hum. Genet.* **52**:668-676.

Larson, D.D., Spangler, E.A., and Blackburn, E.H. (1987) Dynamics of telomere length variation in *Tetrahymena thermophila*. *Cell*, **50**:477-483.

Lee, M.S., and Blackburn, E.H. (1993) Sequence-specific DNA primer effects on telomerase polymerization activity. *Mol. Cell. Biol.* **13**:6586-6599.

Le Guilly, Y., Simon, M., Lenoir, P., and Bourel, M. (1973) Long term culture of adult human liver cells: Morphological changes related to *in vitro* senescence and effect of donor age on growth potential. *Gerontologia*, **19**:303-313.

Lejnine, S., Makarov, V.L., and Langmore, J.P. (1995) Conserved nucleoprotein structure at the ends of vertebrate and invertebrate chromosomes. *Proc. Natl. Acad. Sci. USA*, **92**:2393-2397.

Levine, A.J., Momand, J., and Finlay, C.A. (1991) The p53 tumour suppressor gene. *Nature*, **351**:453-456.

Levis, R.W., Ganesan, R., Houtchens, K., Tolar, L.A., and Sheen, F.-M. (1993) Transposons in place of the telomeric repeats at the *Drosophila* telomere. *Cell*, **75**:1083-1093.

Levy, M.Z., Allsopp, R.C., Futcher, A.B., Greider, C.W., and Harley, C.B. (1992) Telomere end-replication problem and cell aging. *J. Mol. Biol.*, **225**:951-960.

Lin, J.J., and Zakian, V.A. (1994) Isolation and characterization of two *Saccharomyces cerevisiae* genes that encode proteins that bind to $(TG_{1\text{-}3})_n$ single strand telomeric DNA *in vitro*. *Nucl. Acids Res.* **22**:4906-4913.

Lin, J.J., and Zakian, V.A. (1995) An *in vitro*-assay for *Saccharomyces* telomerase requires *EST1*. *Cell*, **81**:1127-1135.

Lindsey, J., McGill, N.I., Lindsey, L.A., Green, D.K., and Cooke, H.J. (1991) *In vitro* loss of telomeric repeats with age in humans. *Mutat. Res.* **256**:45-48.

Lingner, J., Hendrick, L.L., and Cech, T.R. (1994) Telomerase RNAs of different ciliates have a common secondary structure and a permuted template. *Genes Dev.* **8**:1984-1998.

Liu, Z., Frantz, J.D., Gilbert, W., and Tye, B.-K. (1993) Identification and characterization of a nuclease activity specific for G4 tetrastranded DNA. *Proc. Natl. Acad. Sci. USA*, **90**:3157-3161.

Liu, Z., Lee, A., and Gilbert, W. (1995) Gene disruption of a G4-DNA-dependent nuclease in yeast leads to cellular senescence and telomere shortening. *Proc. Natl. Acad. Sci. USA*, **92**:6002-6006.

Liu, Z., and Tye, B.-K. (1991) A yeast protein that binds to vertebrate telomeres and conserved yeast telomeric junctions. *Genes Dev.* **5**:49-59.

Longtine, M.S., Enomoto, S., Finstad, S.L., and Berman, J. (1992) Yeast telomere repeat sequence (TRS) improves circular plasmid segregation, and TRS plasmid segregation involves the *RAP1* gene product. *Mol. Cell. Biol.* **12**:1997-2009.

Löwenberg, B., and Delwel, F.R. (1991) The pathobiology of human acute myeloid leukemia. *In:* Hoffman, R., Benz Jr., E.J., Shattil, S.J., Furie, B., and Cohen, H.J. (*eds*) Hematology: Basic principles and practice. Churchill Livingstone, NY, USA, pp 708-714.

Lucchini, R., Pauli, U., Braun, R., Koller, T., and Sogo, J.M. (1987) Structure of the extrachromosomal ribosomal RNA chromatin of *Physarum polycephalum*. *J. Mol. Biol.* **196**:829-843.

Lumpkin Jr., C.K., McClung, J.K., Pereira-Smith, O.M., and Smith, J.R. (1986) Existence of high abundance antiproliferative messenger RNA in senescent human diploid fibroblasts. *Science*, **232**:393-395.

Lundblad, V., and Blackburn, E.H. (1993) An alternative pathway for yeast telomere maintenance rescues *est1*⁻ senescence. *Cell*, **73**:347-360.

Lundblad, V., and Szostak, J.W. (1989) A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell*, **57**:633-643.

Lustig, A.J., Kurtz, S., and Shore, D. (1990) Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. *Science*, **250**:549-553.

Lustig, A.J., and Petes, T.D. (1986) Identification of yeast mutants with altered telomere structure. *Proc. Natl. Acad. Sci. USA*, **83**:1398-1402.

Makarov, V.L., Lejnine, S., Bedoyan, J., and Langmore, J.P. (1993) Nucleosome organization of telomere-specific chromatin in rat. *Cell*, **73**:775-787.

Mansur, C.P., and Androphy, E.J. (1993) Cellular transformation by papillomavirus oncoproteins. *Biochem. Biophys. Acta*, **1155**:323-345.

Mantell, L.L., and Greider, C.W. (1994) Telomerase activity in germline and embryonic cells of *Xenopus*. *EMBO J.* **13**:3211-3217.

Martin, G.M., Gartler, S.M., Epstein, C.J., and Motulsky, A.G. (1965) Diminished lifespan of cultured cells in Werner syndrome. *Fed. Proc.* **24**:678.

Martin, G.M., Sprague, C.A., and Epstein, C.J. (1970) Replicative lifespan of cultivated human cells. Effects of donor's age, tissue and genotype. *Lab. Invest.* **23**:86-92.

Mathog, D., Hochstrausser, M., Gruenbaum, Y., Saumweber, H., and Sedat, J. (1984) Characteristic folding pattern of polytene chromosomes in *Drosophila* salivary gland nuclei. *Nature*, **308**:414-421.

Matsumura, T., Pfendt, E.A., and Hayflick, L. (1979a) DNA synthesis in the human diploid strain WI-38 during *in vitro* aging: An autoradiographic study. *J. Gerontol.* **34**:323-327.

Matsumura, T., Zerrudo, Z., and Hayflick, L. (1979b) Senescent human diploid cell in culture: Survival, DNA synthesis and morphology. *J. Gerontol.* **34**:328-334.

McCormick, J.J., and Maher, V.M. (1988) Towards an understanding of the malignant transformation of diploid human fibroblasts. *Mutat. Res.* **199**:273-291.

McDougall, J.K. (1971) Adenovirus-induced chromosome aberrations in human cells. *J. Gen. Virol.* **12**:43-51.

McEachern, M.J., and Blackburn, E.H. (1994) A conserved sequence motif within the exceptionally diverse telomeric sequences of budding yeasts. *Proc. Natl. Acad. Sci. USA*, **91**:3453-3457.

McEachern, M.J., and Blackburn, E.H. (1995) Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature,* **376**:403-409.

McKay, S.J., and Cooke, H. (1992a) A protein which specifically binds to single stranded $TTAGGG_n$ repeats. *Nucl. Acids Res.* **20**:1387-1391.

McKay, S.J., and Cooke, H. (1992b) hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeats $TTAGGG_n$. *Nucl. Acids Res.* **20**:6461-6464.

Mehle, C., Ljungberg, B., and Roos, G. (1994) Telomere shortening in renal cell carcinoma. *Cancer Res.* **54**:236-241.

Melek, M., Davis, B.T., and Shippen, D.E. (1994) Oligonucleotides complementary to the *Oxytricha nova* telomerase RNA delineate the template domain and uncover a novel mode of primer utilization. *Mol. Cell. Biol.* **14**:7827-7838.

Mets, J., Bekaert, E., and Verdonk, G. (1983) Similarity between *in vitro* and *in vivo* cellular aging. *Mech. Age. Dev.* **22**:71-78.

Meyne, J., Ratliff, R.L., and Moyzis, R.K. (1989) Conservation of the human telomere sequence $(TTAGGG)_n$ among vertebrates. *Proc. Natl. Acad. Sci. USA*, **89**:7049-7053.

Middleton, T., Gahn, T.A., Martin, J.M., and Sugden, B. (1991) Immortalizing genes of Epstein-Barr virus. *Adv. Virus Res.* **40**:19-55.

Mills, R.G., and Weiss, A.S. (1990) Does progeria provide the best model of accelerated ageing in humans? *Gerontology,* **36**:84-98.

Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K., and Hinuma, Y. (1981) Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature,* **294**:770-771.

Moorhead, P.S.. and Saksela, E. (1963) Non-random chromosomal aberrations in SV_{40}-transformed human cells. *J. Cell. Comp. Physiol.* **62**:57-84.

Moorhead, P.S., and Saksela, E. (1965) The sequence of chromosome aberrations during SV 40 transformation of a human diploid cell strain. *Hereditas,* **52**:271-284.

Moretti, P., Freeman, K., Coodly, L., and Shore, D. (1994) Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. *Genes Dev.* 8:2257-2269.

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

Morin, G.B. (1991) Recognition of a chromosome truncation site associated with α-thalassaemia by human telomerase. *Nature,* 353:454-456.

Moyzis, R.K., Buckingham, J.M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, P.L., and Wu, J.-R. (1988) A highly conserved repetitive DNA sequence, $(TTAGGG)_n$ present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. USA,* 85:6622-6626.

Muggleton-Harris, A., and DeSimone, D.W. (1980) Replicative potentials of various fusion products between WI-38 and SV40 transformed WI-38 cells and their components. *Somatic Cell Genet.* 6:689-698.

Murnane, J.P., Sabatier, L., Marder, B.A., and Morgan, W.F. (1994) Telomere dynamics in an immortal human cell line. *EMBO J.* 13:4953-4962.

Murray, A.W., Clause, T.E., and Szostak, J.W. (1988) Characterization of two telomeric DNA processing reactions in *Saccharomyces cerevisiae. Mol. Bell. Biol.* 8:4642-4650.

Muyldermans, S., De Jonge, J., Wyns, L., and Travers, A.A. (1994) Differential association of linker histones H1 and H5 with telomeric nucleosomes in chicken erythrocytes. *Nucl. Acids Res.* 22:5635-5639.

Nilsson, P., Mehle, C., Remes, K., and Roos, G. (1994) Telomerase activity *in vivo* in human malignant hematopoietic cells. *Oncogene,* 9:3043-3048.

Ning, Y., Weber, J.L., Killary, A.M., Ledbetter, D.H., Smith, J.R., and Pereira-Smith, J.R. (1991b) Genetic analysis of indefinite division in human cells: Evidence for a cell senescence-related gene(s) on human chromosome 4. *Proc. Natl. Acad. Sci. USA,* 88:5635-5639.

Noda, A., Ning, Y., Venable, S.F., Periera-Smith, O.M., and Smith, J.R. (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* 211:90-98.

Norwood, T.H., Hoehn, H., Salk, D., and Martin, G.M. (1979) Cellular ageing in Werner's syndrome: A unique phenotype? *J. Invest. Dermatol.* **73**:92-96.

Norwood, T.H., Pendergrass, W.R., Sprague, C.A. and Martin, G.M. (1974) Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells. *Proc. Natl. Acad. Sci. USA*, **71**:2231-2235.

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

Nürnberg, P., Thiel, G., Weber, F., and Epplen, J.T. (1993) Changes of telomere lengths in human intracranial tumours. *Hum. Genet.* **91**:190-192.

Odagiri, E., Kanda, N., Jibiki, K., Demura, R., Aikawa, E., and Demura, H. (1994) Reduction of telomeric length and c-*erb*B-2 gene amplification in human breast cancer, fibroadenoma and gynecomastia. *Cancer*, **73**:2978-2984.

Ogata, T., Ayusawa, D., Namba, M., Takahashi, E., Oshimura, M., and Oishi, M. (1993) Chromosome 7 suppresses indefinite division of nontumourigenic immortalized human fibroblast cell lines KMST-6 and SUSM-1. *Mol. Cell. Biol.* **13**:6036-6043.

Ogata, T., Oshimura, M., Namba, M., Fuji, M., Oishi, M., and Ayusawa, D (1995) Genetic complementation of the immortal phenotype in group D cell lines by introduction of chromosome 7. *Jpn. J. Cancer Res.* **86**:35-40.

Ohyashiki, J.H., Ohyashiki, K., Fujimura, T., Kawakubo, K., Shimamoto, T., Iwabuchi, A., and Toyama, K. (1994a) Telomere shortening associated with disease evolution patterns in myelodysplastic syndromes. *Cancer Res.* **54**:3557-3560.

Ohyashiki, K., Ohyashiki, J.H., Fujimura, T., Kawakubo, K., Shimamoto, T., Saito, M., Nakazawa, S., and Toyama, K. (1994b) Telomeric shortening in leukemic cells is related to their genetic alterations but not replicative capability. *Cancer Genet. Cytogenet.* **78**:64-67.

Okazaki, S., Tsuchida, K., Maekawa, H., Ishikawa, H., and Fujiwara, H. (1993) Identification of a pentanucleotide telomeric sequence, $(TTAGG)_n$, in the silkworm *Bombyx mori* and in other insects. *Mol. Cell. Biol.* **13**:1424-1432.

Olovnikov, A.M. (1971) Principle of marginotomy in template synthesis of polynucleotides. *Doklady Biochem.* **201**:394-397.

Olovnikov, A.M. (1973) A theory of marginotomy. *J. Theor. Biol.* **41**:181-190.

Palladino, F., Laroche, T., Gilson, E., Axelrod, A., Pillus, L., and Gasser, S.M. (1993) SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. *Cell*, **75**:543-555.

Pandita, T.K., Pathak, S., and Geard, C.R. (1995) Chromosome end associations, telomeres and telomerase activity in ataxia telangiectasia cells. *Cytogenet. Cell Genet.* **71**:86-93.

Pereira-Smith, O.M., and Smith, J.R. (1981) Expression of SV40 T antigen in finite lifespan hybrids of normal-SV40 transformed fibroblasts. *Somatic Cell Genet.* **7**:411-421.

Pereira-Smith, O.M., and Smith, J.R. (1983) Evidence for the recessive nature of cellular immortality. *Science*, **221**:964-966.

Pereira-Smith, O.M., and Smith, J.R. (1987) Functional simian virus 40 T antigen is expressed in hybrid cells having finite proliferative potential. *Mol. Cell. Biol.* **7**:1541-1544.

Pereira-Smith, O.M., and Smith, J.R. (1988) Genetic analysis of indefinite division in human cells: Identification of four complementation groups. *Proc. Natl. Acad. Sci. USA*, **85**:6042-6046.

Petracek, M.E., Konkel, L.M.C., Kable, M.L., and Berman, J. (1994) A *Chlamydomonas* protein that binds single-stranded G-strand telomere DNA. *EMBO J.* **13**:3648-3658.

Pirisi, L., Yasumoto, S., Feller, M., Doniger, J., and DiPaolo, J.A. (1987) Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J. Virol.* **61**:1061-1066.

Pluta, A.F., Kaine, B.P., and Spear, B.B. (1982) The terminal organization of macronuclear DNA in *Oxytricha fallax*. *Nucl. Acids Res.* **10**:8145-8154.

Ponzi, M., Pace, T., Dore, E., and Frontali, C. (1985) Identification of a telomeric DNA sequence in *Plasmodium berghei*. *EMBO J.* **4**:2991-2995.

Price, C.M. (1990) Telomere structures in *Euplotes crassus*: Characterization of DNA-protein interactions and isolation of a telomere-binding protein. *Mol. Cell. Biol.* **10**:3421-3431.

Price, C.M., and Cech, T.R. (1987) Telomeric DNA-protein interactions of *Oxytricha* macronuclear DNA. *Genes Dev.* **1**:783-793.

Prowse, K.R., Avilion, A.A., and Greider, C.W. (1993) Identification of a nonprocessive telomerase activity from mouse cells. *Proc. Natl. Acad. Sci. USA*, **90**:1493-1497.

Prowse, K.R., and Greider, C.W. (1995) Developmental and tissue specific regulation of mouse telomerase and telomere length. *Proc. Natl. Acad. Sci. USA*, **92**:4818-4822.

Quinlan, M.P. (1993) Expression of antisense E1A in 293 cells results in altered cell morphologies and cessation of proliferation. *Oncogene*, **8**:257-265.

Radna, R.L., Caton, Y., Jha, K.K., Kaplan, P., Li, G., Traganos, F., and Ozer, H.L. (1989) Growth of immortal simian virus 40 *tsA* -transformed human fibroblasts is temperature dependent. *Mol. Cell. Biol.* **9**:3093-3096.

Rai, K.R. (1991) Chronic lymphocytic leukemia. *In:* Hoffman, R., Benz Jr., E.J., Shattil, S.J., Furie, B., and Cohen, H.J. (*eds*) Hematology: Basic principles and Practice. Churchill Livingstone, NY, USA, pp 990-1002.

Rawlings, D.J., and Shaw, P.J. (1989) Localization of ribosomal and telomeric DNA sequences in intact plant nuclei by *in-situ* hybridization and three-dimensional optical microscopy. *J. Microscopy*, **157**:83-89.

Ray, F.A., Peabody, D.S., Cooper, J.L., Cram, L.S., and Kraemer, P.M. (1990) SV40 T antigen *alone* drives karyotype instability that precedes neoplastic transformation of human diploid fibroblasts. *J. Cell. Biochem.* **42**:13-31.

Renauld, H., Aparicio, O.M., Zierath, P.D., Billington, B.L., Chhablani, S.K., and Gottschling, D.E. (1993) Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. *Genes Dev.* **7**:1133-1145.

Richards, E.J., and Ausubel, F.M. (1988) Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell*, **53**:127-136.

Rimessi, P., Gualandi, F., Morelli, C., Trabanelli, C., Wu, Q., Possati, L., Montesi, M., Barrett, J.C., and Barbanti-Brodano, G. (1994) Transfer of human chromosome 3 to an ovarian carcinoma cell line identifies three regions on 3p involved in ovarian cancer. *Oncogene,* **9**:3467-3474.

Rinehart, C.A., Laundon, C.H., Mayben, J.P., Lyn-Cook, B.D., and Kaufman, D.G. (1993) Conditional immortalization of human endometrial stromal cells with a temperature-sensitive simian virus 40. *Carcinogenesis,* **14**:993-999.

Rogalla, P., Kazmierczak, B., Rohen, C., Trams, G., Bartnitske, S., and Bullerdiek, J. (1994) Two human breast cancer lines showing decreasing telomeric repeat length during early *in vitro* passaging. *Cancer Genet. Cytogenet.* **77**:19-25.

Rogan, E.H., Bryan, T.M., Hukku, B., Maclean, K., Chang, A.C.-M., Moy, E.L., Englezou, A., Warneford, S.G., Dalla-Pozza, L., and Reddel, R.R. (1995) Alterations in p53 and $p16^{INK4}$ expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. *Mol. Cell Biol.* **15**:4745-4753.

Rohme, D. (1981) Evidence for a relationship between longevity of mammalian species and the life spans of normal fibroblasts *in vitro* and erythrocytes *in vivo*. *Proc. Natl. Acad. Sci. USA*, **78**:5009-5013.

Romero, D.P., and Blackburn, E.H. (1991) A conserved secondary structure for telomerase RNA. *Cell,* **67**:343-353.

Sack Jr., G.H. (1981) Human cell transformation by simian virus 40- A review. *In Vitro,* **17**:1-18.

Sager, R. (1991) Senescence as a mode of tumor suppression. *Envir. Health Perspectives,* **93**:59-62.

Saltman, D., Morgan, R., Leary, M.L., and de Lange, T. (1993) Telomeric structure in cells with chromosome end associations. *Chromosoma,* **102**:121-128.

Sandell, L.L., and Zakian, V.A. (1993) Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell,* **75**:729-739.

Sandu, A.K., Hubbard, K., Kaur, G., Jha, K.K., Ozer, H.L., and Athwal, R.S. (1994) Senescence of immortal human fibroblasts by the introduction of normal chromosome 6. *Proc. Natl. Acad. Sci. USA*, **91**:5498-5502.

Schierer, T., and Henderson, E. (1994) A protein from *Tetrahymena thermophila* that specifically binds parallel-stranded G4-DNA. *Biochemistry*, **33**:2240-2246.

Schneider, E., and Mitsui, Y. (1986) The relationship between *in vitro* cellular aging and *in vivo* human age. *Proc. Natl. Acad. Sci. USA*, **73**:3584-3588.

Schramayr, S., Caporossi, D., Mak, I., Jelinek, T., and Bacchetti, S. (1990) Chromosomal damage induced by human adenovirus type 12 requires expression of the E1B 55-kilodalton viral protein. *J. Virol.* **64**:2090-2095.

Schulz, V.P., and Zakian, V.A. (1994) The *Saccharomyces* PIF1 DNA helicase inhibits telomere elongation and *de novo* telomere formation. *Cell*, **76**:145-155.

Schwartz, H.S., Juliao, S.F., Sciadini, M.F., Miller, L.K., and Butler, M.G. (1995) Telomerase activity and oncogenesis in giant cell tumor of bone. *Cancer*, **75**:1094-1099.

Shampay, J., Szostak, J.W., and Blackburn, E.H. (1984) DNA sequences of telomeres maintained in yeast. *Nature*, **310**:154-157.

Shay, J.W., Pereira-Smith, O.M., and Wright, W.E. (1991a) A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.* **196**:33-39.

Shay, J.W., Tomlinson, G., Piatszek, M.A., and Gollahon, L.S. (1995) Spontaneous *in vitro* immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. *Mol. Cell. Biol.* **15**:425-432.

Shay, J.W., and Wright, W.E. (1989) Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. *Exp. Cell Res.* **184**:109-118.

Shay, J.W., Wright, W.E., Brašiškytė, D., and Van Der Haegen, B. (1993) E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. *Oncogene*, **8**:1407-1413.

Shay, J.W., Wright, W.E., and Werbin, H. (1991b) Defining the molecular mechanism of human cell immortalization. *Biochim. Biophys. Acta*, **1072**:1-7.

Shein, H.M., Enders, J.F., Palmer, L., and Grogan, E. (1964) Further studies on SV40-induced transformation in human renal cell cultures: I. Eventual failure of subcultivation despite a continuing high rate of cell division. *Proc. Soc. Exp. Biol. Med.* **115**:618-621.

Sherwood, S.W., Rush, D., Ellsworth, J.L., and Schimke, R.T. (1989) Defining cellular senescence in IMR-90 cells: Flow cytometric analysis. *Proc. Natl. Acad. Sci. USA*, **85**:9089-9090.

Shippen, D.E., Blackburn, E.H., and Price, C.M. (1994) DNA bound by the *Oxytricha* telomere protein is accessible to telomerase and other DNA polymerases. *Proc. Natl. Acad. Sci. USA*, **91**:405-409.

Shippen-Lentz, D., and Blackburn, E.H. (1989) Telomere terminal transferase activity from *Euplotes crassus* adds large number of TTTTGGGG repeats onto telomeric primers. *Mol. Cell. Biol.* **9**:2761-2764.

Shippen-Lentz, D., and Blackburn, E.H. (1990) Functional evidence for an RNA template in telomerase. *Science,* **247**:546-552.

Shirotani, Y., Hiyama, K., Ishioka, S., Inyaku, K., Awaya, Y., Yonehara, S., Yoshida, Y., Inai, K., Hiyama, E., Hasegawa, K., Inamizo, T., and Yamakido, M. (1994) Alteration in length of telomeric repeats in lung cancer. *Lung Cancer,* **11**:29-41.

Shoeman, R.L., and Traub, P. (1990) The *in vitro* DNA-binding properties of purified nuclear lamin proteins and vimentin. *J. Biol. Chem.* **265**:9055-9061.

Shoeman, R.L., Wadle, S., Scherbarth, A., and Traub, P. (1988) The binding *in vitro* of the intermediate filament protein vimentin to synthetic oligonucleotides containing telomere sequences. *J. Biol. Chem.* **263**:18744-18749.

Singer, M.S., and Gottschling, D.E. (1994) *TCL1*: Template RNA component of *Saccharomyces cerevisiae* telomerase. *Nature,* **266**:404-409.

Skuse, G.R., and Ludlow, J.W. (1994) Tumour suppressor genes in disease and therapy. *Lancet,* **345**:902-906.

Smith, J.K., and Yeh, G. (1992) Telomere reduction in endometrial adenocarcinoma. *Am. J. Obstet. Gynecol.* **167**:1883-1887.

Smith, K.A., Stark, M.B., German, P.A., Stark, O.R. (1992) Fusion near telomeres occur very early in the amplification of CAD genes in Syrian hamster cells. *Proc. Natl. Acad. Sci. USA*, **89**:5427-5431.

Stamps, A.C., Gusterson, B.A., and O'Hare, M.J. (1992) Are tumours immortal? *Eur. J. Cancer*, **28A**:1495-1500.

Starling, J.A., Maule, J., Hastie, N.D., and Allshire, R.C. (1990) Extensive telomere repeat arrays in mouse are hypervariable. *Nucl. Acids Res.* **18**:6881-6888.

Stein, G.H. (1985) SV40-transformed human fibroblasts: Evidence for cellular aging in precrisis cells. *J. Cell. Phys.* **125**:36-44.

Stewart, N., and Bacchetti, S. (1991) Expression of SV40 large T antigen, but not small t antigen, is required for the induction of chromosomal aberrations in transformed human cells. *Virology*, **180**:49-57.

Stich, H.F., van Hooseir, G.L., and Trentin, J.J. (1964) Viruses and mammalian chromosomes. Chromosome aberrations by human adenovirus type 12. *Exp. Cell Res.*, **34**:400-403.

Strauss, M., and Griffin, B.E. (1990) Cellular immortalization- An essential step or merely a risk factor in DNA virus-induced transformation? *Cancer Cell*, **2**:360-365.

Sundquist, W.I., and Klug, A. (1989) Telomeric DNA dimerizes by formation of guanine tetrads between hairpin loops. *Nature*, **342**:825-829.

Sussel, L., and Shore, D. (1991) Separation of transcriptional activation and silencing functions of the *RAP1*-encoded repressor/activator protein 1: Isolation of viable mutants affecting both silencing and telomere length. *Proc. Natl. Acad. Sci. USA*, **88**:7749-7753.

Suzuki, Y., and Nishizawa, M. (1994) The yeast GAL11 protein is involved in regulation of the structure and the position effect of telomeres. *Mol. Cell. Biol.* **14**:3791-3799.

Szostak, J.W., and Blackburn, E.H. (1982) Cloning yeast telomeres on linear plasmid vectors. *Cell*, **29**:245-255.

Tannock, I.F. (1987) Tumour growth and cell kinetics. *In:* Tannock, I.F., and Hill, R.P. (*eds.*) The basic science of oncology. Pergamon Press, Toronto, Canada, pp 140-159.

Tommerup, H., Dousmanis, A., and de Lange, T. (1994) Unusual chromatin in human telomeres. *Mol. Cell. Biol.* **14**:5777-5785.

Traverse K.L., and Pardue, M.L. (1988) A spontaneously opened ring chromosome of *Drosophila melanogaster* has acquired He-T DNA sequences at both new telomeres. *Proc. Natl. Acad. Sci. USA*, **85**:8116-8120.

Tricot, G.J.K. (1991) The myelodysplastic syndromes. *In:* Hoffman, R., Benz Jr., E.J., Shattil, S.J., Furie, B., and Cohen, H.J. (*eds)* Hematology: Basic principles and practice. Churchill Livingstone, NY, USA, pp 805-816.

Vaziri, H., Schächter, F., Uchida, I., Wei, L., Zhu, X., Effros, R., Cohen, D., and Harley, C.B. (1993) Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am. J. Hum. Genet.* **52**:661-667.

Vincent, P.C. (1990) Kinetics of leukemia and control of cell division and replication. *In:* Henderson, E.S., and Lister, T.A. (*eds*) William Dameshek and Fredrick Gunz's Leukemia. 5th Edition. W.B. Saunders Company, Toronto, Canada, pp 55-104.

Vourc'h, C., Taruscio, D., Boyle, A.L., and Ward, D.C. (1993) Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. *Exp. Cell Res.* **205**:142-151.

Wang, S.-S., and Zakian, V.A. (1990) Telomere-telomere recombination provides an express pathway for telomere acquisition. *Nature,* **345**:456-458.

Wang, W., Skopp, R., Scofield, M., and Price, C. (1992) *Euplotes crassus* has genes encoding telomere-binding proteins and telomere-binding protein homologs. *Nucl. Acids Res.* **20**:6621-6629.

Watson, J.D. (1972) Origins of concatemeric T7 DNA. *Nature New Biol.* **239**:197-201.

Wazer, D.E., Lui, X.-L., Chu, Q., Gao, Q., and Band, V. (1995) Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. *Proc. Natl. Acad. Sci. USA*, **92**:3687-3691.

Weber, B., Collins, C., Robbins, C., Magenis, R.E., Delaney, A.D., Gray, J.W., and Hayden, M.R. (1990) Characterization and organization of DNA sequences adjacent to

the human telomere associated repeat $(TTAGGG)_n$. *Nucl. Acids Res.* **18**:3353-3361.

Weinberg, R.A. (1989) Oncogenes, antioncogenes and the molecular bases of multistep carcinogenesis. *Cancer Res.* **49**:3713-3712.

Wellinger, R.J., Wolf, A.J., and Zakian, V.A. (1993) *Saccharomyces* telomeres acquire single-strand TG_{1-3} tails late in S phase. *Cell,* **72**:51-60.

Whitaker, N.J., Kidston, E.L., and Reddel, R.R. (1992) Finite lifespan of hybrids formed by fusion of different simian virus 40-immortalized cell lines. *J. Virol.* **66**:1202-1206.

Whittaker, J.L., Byrd, P.J., Grand, R.J.A., and Gallimore, P.H. (1984) Isolation and characterization of four Adenovirus type 12-transformed human embryo kidney cell lines. *Mol. Cell. Biol.* **4**:110-116.

Wilkie, A.O.M., Lamb, J., Harris, P.C., Finney, R.D., and Higgs, D.R. (1990) A truncated human chromosome 16 associated with α thalassaemia is stabilized by addition of telomeric repeat $(TTAGGG)_n$. *Nature,* **346**:868-871.

Williamson, J.R. (1994) G-quartet structures in telomeric DNA. *Annu. Rev. Biophys. Biomol. Struct.* **23**:703-730.

Williamson, J.R., Rughuraman, M.K., and Cech, T.R. (1989) Monovalent cation-induced structure of telomeric DNA: the G-quartet model. *Cell,* **59**:871-880.

Wolman, S.R., Hirschhorn, K., and Todaro, G.J. (1964) Early chromosomal changes in SV_{40}-infected human fibroblast cultures. *Cytogenetics,* **3**:45-61.

Wright, J.H., Gottschling, D.E., and Zakian, V.A. (1992) *Saccharomyces* telomeres assume a non-nucleosomal chromatin structure. *Genes Dev.* **6**:197-210.

Wright, W.E., Pereira-Smith, O.M., and Shay, J.W. (1989) Reversible cellular senescence: Implications for immortalization of normal human diploid fibroblasts. *Mol. Cell. Biol.* **9**:3088-3092.

Wright, W.E., and Shay, J.W. (1992) Telomere positional effects and the regulation of cellular senescence. *Trends Genet.* **8**:193-195.

Yamato, K., Yamamoto, M., Hirano, Y., and Tsuchida, N. (1995) A human temperature-sensitive p53 mutation $p53^{Val-138}$: Modulation of the cell cycle, viability and expression of p53-responsive genes. *Oncogene,* **11**:1-6.

Yanishevsky, R.M., and Stein, G.H. (1980) Ongoing DNA synthesis continues in young human diploid cells (HDC) fused to senescent HDC, but entry into S phase is inhibited. *Exp. Cell Res.* **126**:469-472.

Yoshida, M.A., Shimizu, M., Ikeuchi, T., Tonomura, A., Yokota, J., and Oshimura, M. (1994) *In vitro* growth suppression and morphological change in a human renal cell carcinoma cell lie by the introduction of normal chromosome 3 via microcell fusion. *Mol. Carcinog.* **9**:114-121.

Young, B.S., Pession, A., Traverse, K.L., French, C., and Pardue, M.L. (1983) Telomere regions in *Drosophila* share complex DNA sequences with pericentric heterochromatin. *Cell,* **34**:85-94.

Yu, G.-L., and Blackburn, E.H. (1991) Developmentally programmed healing of chromosomes by telomerase in *Tetrahymena. Cell,* **67**:823-832.

Yu, G.-L., Bradley, J.D., Attardi, L.D., and Blackburn, E.H. (1990) *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature,* **344**:126-132.

Zahler, A.M., and Prescott, D.M. (1988) Telomere terminal transferase activity in the hypotrichous ciliate *Oxytricha nova* and a model for replication of the ends of linear DNA molecules. *Nucl. Acids Res.* **16**:6953-6972.

Zahler, A.M., Williamson, J.R., Cech, T.R., and Prescott, D.M. (1991) Inhibition of telomerase by G-quartet DNA structures. *Nature,* **350**:718-720.

Zakian, V.A. (1989) Structure and function of telomeres. *Annu. Rev. Genet.*, **23**:579-604.

Zentgraf, U. (1995) Telomere-binding proteins of *Arabidopsis thaliana. Plant Mol. Biol.* **27**:467-475.

Zhong, Z., Shiue, L., Kaplan, S., and de Lange, T. (1992) A mammalian factor that binds telomeric TTAGGG repeats in vitro. *Mol. Cell. Biol.* **12**:4834-4843.