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# **TELOMERES AND TELOMERASE IN CANCER OF ESOPHAGUS**

by

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## DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is original, independent and has not in its entirety or part been submitted to any university for a degree.

All the sources I have made use of or quoted have been acknowledged by complete references.

  
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WMJ VAN DEN HEEVER  
Datum: 30 November 2001

*Dedicated to*

*the Lord for giving me the perseverance and courage to complete this  
study, to Him all the Honour.*

# CONTENTS

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	PAGES
<b>ABBREVIATIONS</b>	i
<b>LIST OF FIGURES</b>	iv
<b>LIST OF TABLES</b>	vi
<b>1 INTRODUCTION</b>	<b>1</b>
1.1 INTRODUCTION	1
1.2 AIMS AND SCOPE OF THE PROJECT	3
1.3 REFERENCES	4
<b>2 CANCER OF THE ESOPHAGUS AND TELOMERASE</b> <b>(What we know at the end of the millennium and what we</b> <b>would like to know.)</b>	
<b>LITERATURE REVIEW</b>	<b>7</b>
2.1 INTRODUCTION	7
2.2 PREVALENCE AND INCIDENCE	7
2.3 PATHOGENESIS	8
2.3.1 Risk factors	8
<i>a) Alcohol and tobacco</i>	9
<i>b) Dietary factors</i>	9
<i>c) Specific foods</i>	10
<i>d) Vitamins and Minerals</i>	10
2.3.2 Genetic factors	12
2.3.3 Infectious agents	12
2.3.4 Predisposing conditions	13

2.3.5	Other diseases	13
2.3.6	Genetic alterations	13
2.4	TELOMERES AND TELOMERASE	15
2.4.1	Proteins at the telomere	20
2.4.2	Higher order telomere structure	21
2.4.3	Telomere capping	23
2.4.4	The telomere RNP	25
2.4.5	Telomerase – independent telomere maintenance	27
2.5	TELOMERASE ACTIVITY	29
2.6	TELOMERASE ACTIVITY ASSAY	35
2.6.1	Problems with the telomerase activity assay	37
2.7	WILL INHIBITION OF TELOMERASE ACTIVITY RESULTS IN CANCER REMISSION?	38
2.8	TELOMERASE AS A DIAGNOSTIC TOOL FOR CANCER DETECTION	39
2.9	<i>In vivo</i> AND <i>in vitro</i> MODELS TO STUDY DRUGS THAT TARGET TELOMERASE AND TELOMERES	40
2.10	TELOMERASE INHIBITORS	41
2.11	TELOMERASE TARGETING AGENTS	41
2.11.1	The DNA component: G-quartet interactive compound	41
2.11.2	The nucleoprotein complex	43
2.12	MOLECULAR TARGETING OF CANCER: TELOMERES AS TARGET	45
2.13	FUTURE PROSPECTS	45
2.14	REFERENCES	46
<b>3</b>	<b>TELOMERASE ACTIVITY IN ESOPHAGEAL CANCER</b>	<b>63</b>
3.1	INTRODUCTION	63
3.2	ARTICLE	65



3.3	RESULTS EXCLUDED FROM ARTICLE	79
3.4	DISCUSSION	84
3.5	REFERENCES	88
<b>4</b>	<b>DEVELOPMENT OF A FLOW CYTOMETRIC METHOD TO DETERMINE TELOMERE LENGTH IN TUMOR CELLS</b>	91
4.1	INTRODUCTION	91
4.2	MATERIALS AND METHODS	
4.2.1	Materials	94
	<i>a) Peripheral blood mononuclear cells</i>	94
	<i>b) SNO cell line</i>	94
	<i>c) Baboon liver cells and esophageal biopsies</i>	94
4.2.2	Fluorescence <i>in situ</i> hybridization	95
4.2.3	Flow Cytometric measurement	96
4.2.4	Q-FISH technique	96
4.3	RESULTS	97
4.4	DISCUSSION	112
4.5	REFERENCES	116
<b>5</b>	<b>DEVELOPMENT OF PEPTIDE INHIBITORS OF TELOMERASE ACTIVITY BY USING PHAGE DISPLAY TECHNOLOGY</b>	123
5.1	INTRODUCTION	123
5.2	PHAGE DISPLAY TECHNOLOGY	124
5.2.1	Introduction	124
5.2.2	The filamentous phage	125
5.2.3	Construction of a random phage library	128
5.2.4	Selection and Replication	129
5.2.5	Proposed strategy to select peptides binding to telomerase RNA	131

5.3	MATERIALS AND METHODS	131
5.3.1	Synthesis of 2 oligonucleotides	131
5.3.2	Cleavage of the pGEMEX-1 vector	133
5.3.3	Recovery of DNA from the agarose gel	133
5.3.4	Ligation of the oligonucleotides to the plasmid DNA	135
5.3.5	Transformation	135
5.3.6	Preparation of competent cells	135
5.3.7	Plasmid isolation	136
5.3.8	Digestion with Sac I	136
5.3.9	DNA purification	137
5.3.10	RNA synthesis	137
5.3.11	Isolation of RNA-binding phages	138
5.3.12	ELISA of individual rounds	139
5.3.13	Phage ELISA of single colonies	140
5.3.14	Cultured used for panning	140
5.4	RESULTS	140
5.5	DISCUSSION	146
5.6	REFERENCES	148
6	CONCLUDING REMARKS	153
7	SUMMARY/OPSOMMING	156
8	ACKNOWLEDGEMENTS	160

## ABBREVIATIONS

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7-AAD	7-aminoactinomycin D
ADP	adenosine-5'-diphosphate
ALT	Alternative Lengthening of Telomeres / alternative telomere maintenance mechanism
AML	acute myelogenous leukemia
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CaCl <sub>2</sub>	calcium chloride
CANSA	Cancer Association of South Africa
cDNA	complementary DNA
Cdk	cyclin-dependent kinase
CML	chronic myelogenous leukemia
CMV	cytomegalovirus
DAPI	4', 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP's	deoxynucleoside-triphosphate
DTT	1,4-dithiothreitol
EBV	Epstein-Barr virus
<i>E coli</i>	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immuno-sorbent assay
flow-FISH/	
Flow FISH	flow cytometry method for quantitative FISH
FITC	fluorescein isothiocyanate
h	hours
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIV	human immunodeficiency virus
HPV	human papilloma virus
HRP	horseradish peroxidase
HSV	herpes simplex virus

hTERT	human telomerase reverse transcriptase
hTR/hTER	human telomerase RNA
ISH	<i>in situ</i> hybridization
IPTG	isopropyl $\beta$ -D-thiogalactoside
KCl	potassium chloride
kD	kilodalton
LB	Luria broth
MESF	molecules of equivalent soluble fluorochrome
MgCl <sub>2</sub>	magnesium chloride
min	minutes
MRC	Medical Research Council
mRNA	mitochondrial RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCR-SSCP	PCR-single-stranded conformational polymorphism
PEG	Polyethyleneglycol
PEG-NaCl	polyethyleneglycol sodium chloride
PNA	peptide nucleic acid
Pot1	protection of telomeres 1
Q-FISH	quantitative fluorescence <i>in situ</i> hybridization
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein
RPMI	Roosevelt Park Memorial Institute medium
rNTPs	ribosomal nucleoside triphosphates
rRNA	ribosomal RNA
RT	hTERT truncation
rpm	revolutions per minute
SCC	squamous cell carcinoma
SCCE	squamous cell carcinoma of the esophagus
SDS	sodium dodecyl sulfate
Se	selenium
SnoRNA	small nuclear RNA
S-Se	selenenylsulphide

S-Se-S	selenodisulphide
SV40	simian virus 40
TRAP	telomeric repeat amplification protocol
TBE-buffer	Tris-borate/EDTA buffer
TE-buffer	Tris/EDTA buffer
TERT	telomerase reverse transcriptase
TEP1	telomerase associated protein 1
TIN 2	TRF 1-interacting nuclear protein 2
TR/TER	telomerase RNA
TRF	telomere restriction fragment
TRF1	telomeric repeat binding factor 1
TRFL	telomere restriction fragment length
Tris	Tris(hydroxymethyl)aminomethane
U	enzyme unit
UDT	upper digestive tract
UTP	uridine triphosphate
v/v	volume/volume
ZnCl <sub>2</sub>	zinc chloride
Xgal	5-Bromo-4-chloro-3-indonyl-β-D-galactoside

## LIST OF FIGURES

---

		PAGES
Fig 2.1	The end-replication problem	16
Fig 2.2	How telomerase solves the problem	18
Fig 2.3	A model of Crisis and Tumorigenesis	19
Fig 2.4	A model for mammalian telomere structure	22
Fig 2.5	A common theme in telomere capping	24
Fig 2.6	hTERT and hTER. Schematic of the human telomerase RT and telomerase RNA	26
Fig 2.7	A model for mammalian RNP assembly	28
Fig 2.8	Proposed model for cell-cycle regulation of telomerase Activity	33
Fig 2.9	The TRAP Assay for telomerase activity	36
Fig 2.10	Structure of G-quartets	44
Fig 1	Distribution of the optical density values of telomerase activity assays	71
Fig 2	Survival curves as a function of telomerase activity	72
Fig 4.1A	Dot plot A illustrates the cell gating region 1(R1), forward scatter versus side scatter and B forward versus FL3. Histogram C shows the fluorescence intensity of the FITC-labeled fluorescent calibration beads	98
Fig 4.1B	The fluorescence of the FITC-labeled fluorescent calibration beads measured in MESF units compared to the mean channel value obtained from the cytometer	98
Fig 4.2	Flow FISH analysis of lymphocytes to obtain the optimum FITC-labeled PNA probe concentration	99
Fig 4.3	Flow FISH analysis of lymphocytes	101
Fig 4.4	Flow FISH analysis of the SNO cell line	102

Fig 4.5A	Baboon liver cell nuclei after hybridization under the fluorescence microscope with a DAPI filter	104
Fig 4.5B	The same field as in A, this time using a FITC filter	104
Fig 4.6	Flow FISH analysis of baboon liver nuclei	105
Fig 4.7	Flow FISH analysis of the esophageal tumor nuclei	106
Fig 4.8	Dot plots indicating the presence of the FITC probe (B & D) in esophageal tumor cells, compared to unlabeled tumor cells (A & C)	107
Fig 4.9	Flow FISH cytometric analysis to illustrate differences in fluorescence intensity between normal esophageal tissue (A,C & E) compared to tumor tissue of the same patient (B, D & F)	108
Fig 5.1	Schematic diagram of the filamentous phage M13	127
Fig 5.2	Diagram of the construction of a phage library	127
Fig 5.3	A shematic presentation of the selection and amplification of peptides on the phage surface	130
Fig 5.4	Summary of the proposed development of peptide inhibitors of telomerase activity	132
Fig 5.5	PGEMEX-1 vector map	134
Fig 5.6A	One clone was chosen as a source of RNA and it was linearized with Sac I	
Fig 5.6B	The RNA synthesis was checked by electrophoresis on an 0.8% agarose gel	142
Fig 5.7	Blue plaques after the third round of panning	142

## LIST OF TABLES

---

	PAGES	
Table 2.1	Telomerase activity in human tumor tissue	30
Table 2.2	Telomeres and telomerase as target for new drug discovery	42
Table 1	Characteristics of patient tissue used for prognostic studies	73
Table 3.1	Characteristics of patient tissue excluded from this article	81
Table 3.2	Summary of the demographic detail of the patients from Universitas Hospital, Bloemfontein	82
Table 3.3	Summary of telomerase activity reported in this study	82
Table 3.4	A summary of the tumor classification (used by the World Health Organization) of patients from Universitas Hospital, Bloemfontein	83
Table 4.1	Results of flow-FISH analysis of paired tumor and normal biopsies	109
Table 5.1	Phage concentrations (phage/ml) after rounds 1 to 3 of the cyclic 7 mer library	143
Table 5.2	ELISA plate readings of the cyclic 7 mer library	143
Table 5.3	Phage concentrations (phage/ml) after rounds 1 to 3 of the linear 12 mer library	145
Table 5.4	ELISA plate readings of the linear 12 mer library	145
Table 5.5	ELISA plate readings of the 48 plaques tested for RNA Binding	145



# CHAPTER 1

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## INTRODUCTION

	PAGES
1.1 INTRODUCTION	1
1.2 AIMS AND SCOPE OF THE PROJECT	3
1.3 REFERENCES	4

# CHAPTER 1

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## GENERAL INTRODUCTION

### 1.1 INTRODUCTION

Squamous cell carcinoma of the esophagus is very common in South African Blacks, especially in males. As with most cancers, there is probably a long interval between exposure to the causative factors and development of the disease. Esophageal cancer, while virtually unknown in western and northern Africa, is common in circumscribed regions of southern and eastern Africa. In parts of the Transkei, Zimbabwe, Zambia, Malawi and Kenya, esophageal cancer represents one fifth to almost half of all registered cancers (Burrell, 1962; Rose, 1973). In the Transkei, the highest incidence (246 per 100 000) was recorded in the Butterworth district (Silber, 1985). The search for aetiological determinants must explain a mosaic incidence pattern that is not restricted to natural geographic boundaries but rather appears to reflect a complex of environmental factors that are intimately correlated with sociocultural and ethnic characteristics. Dramatically different patterns of occurrence are observed for esophageal cancer in different parts of the world. Its epidemiologic behaviour is marked by sharp variations in incidence within discrete geographic areas, among different ethnic groups, and between males and females. Esophageal cancer is a disease of the poor in most areas of the world. Among some populations, major risk factors have already been identified, and the potential exists for primary prevention.

Cancer of the esophagus is characterized by its cell type and anatomic location along the length of the esophagus. Squamous cell carcinomas of varying degrees of differentiation comprise the vast majority (95%) of cancers in the esophagus. They arise from the squamous cell epithelium lining the lumen of the esophagus. In most countries the incidence of squamous cell carcinoma of the esophagus is predominant in males, the male:female ratio

being 3:1. The incidence and the male: female ratio increases with increasing age (Burbank, 1971). The majority of esophagus cancers are located in the middle and lower thirds of the esophagus. It is a very difficult condition to treat and the prognosis is generally poor with a low 5-year survival period.

Primary adenocarcinoma of the esophagus is a relatively uncommon tumor, accounting for 1 to 50 percent of all malignant esophageal tumors, depending upon geographic location (Katzka, 1989). Almost all cases of primary esophageal adenocarcinoma arise from Barrett's esophagus (Katzka, 1989). In Barrett's esophagus the normal stratified squamous epithelium lining the esophagus is replaced by columnar epithelium for variable lengths from the lower esophageal sphincter region. The most common predisposing factor for Barrett's esophagus is chronic gastroesophageal reflux (Agha and Keren, 1987; Burgess *et al.*, 1971). The sequence of events leading to Barrett's esophagus has not been clearly defined. It is probable that ulceration of the squamous epithelium occurs in response to gastroesophageal reflux of acid, bile and duodenal contents and that re-epithelialization occurs via multipotential stem cells which in turn differentiate into the variety of epithelial cells found in Barrett's esophagus. There may, however, be other mechanisms.

Barrett's esophagus is most commonly diagnosed between the ages of 40 and 60 years (Duhaylongsod and Wolfe, 1991) but can also occur in children (Hassell *et al.*, 1985). There is a clear-cut predominance in white males over females, in the order of 4:1 (Polepalle and Callum, 1990). This disorder is uncommon in blacks; less than 2% of blacks with gastroesophageal reflux have Barrett's esophagus (Polepalle and Callum, 1990).

This project is part of a nation-wide effort aimed at understanding the disease and is jointly funded by the Cancer Association (CANSAs) and the Medical Research Council (MRC). The collaborative program involves researchers from the Universities of Cape Town, Stellenbosch, Western Cape, Free State, Transkei and Natal as well as the Experimental Carcinogenesis Program of the MRC. The covering title of this whole effort is "Esophageal Cancer: From Demography to DNA." Each partaking laboratory has a specific task and samples are shared when possible, so that the greatest amount of information can be extracted from them.

## 1.2 AIMS AND SCOPE OF THE PROJECT

The main purpose of this project is to look at the expression of an enzyme called telomerase in esophageal biopsies. Telomeres, at the ends of chromosomes, shorten with each cell division, ultimately resulting in cellular senescence. Telomerase, a ribonucleoprotein enzyme, replaces short stretches of repeat nucleotides (TTAGGG) that are lost from telomeric ends of chromosomes with each round of replication (Greider and Blackburn, 1985). Cells in the process of maturation usually have high levels of telomerase activity, but the moment they become mature, the activity switches off. It thus forms a component of the co-called "mitotic clock" determining the life span of normal cells. Tumor cells have to maintain their telomeres otherwise they will die. If they do not have high telomerase activity, there must be an alternative mechanism still to be elucidated. The activity of telomerase is repressed in many human somatic cells, whereas the enzyme is activated during tumor progression in most human tumors (Blackburn 1991; de Lange, 1992; Greider and Blackburn, 1985; Kim *et al.*, 1994). In many cancers, telomerase has previously been found to correlate with disease stage, as well as with prognosis, where high telomerase levels indicate poor prognosis (Kim *et al.*, 1994).

The telomerase status of squamous cell carcinoma of the esophagus was unknown at the outset of this study and its objective was to fill that gap in our knowledge. The aim of this work was to correlate telomerase activity and telomere length with disease stage, histological classification and outcome.

Since activation of telomerase occurs in most cancers and is viewed as a critical event in the multistep process of human carcinogenesis, telomerase could be a new target for cancer diagnosis and anticancer therapy. It provides the basis for speculation that telomerase inhibitors might constitute a new class of anticancer agent.

In recent years, a new generation of cancer therapies has emerged, based on a growing understanding of the molecular events that contribute to malignant transformation and which therefore represent targets for selectively killing or inhibiting growth of malignant cells. In this context, there has been considerable interest in elucidating mechanisms that regulate the capacity for cell division in normal as well as transformed cells, and substantial attention has

focused specifically on the role of telomeres as the "mitotic clock" that mediates replicative capacity (Hodes, 2001).

An alternative strategy for cancer treatment has been directed, not at inhibition of telomerase enzymatic activity, but rather at targeting immune recognition and destruction of cells that express telomerase (Minev *et al.*, 2000). In principle, this approach would result in rapid immune elimination of telomerase-expressing tumor cells without the lag involved in strategies that inhibit telomerase function and depend on gradual telomere shortening to inhibit tumor growth. Kim *et al.* (2001) worked on another approach to target telomeres in cancer cells. They concluded that expression of mutant telomerase template RNA inhibits tumor cell proliferation by a mechanism that does not involve either telomere shortening or inhibition of telomerase activity.

In addition we would like to look at the chromosomal abnormalities associated with the disease with the aim of identifying prognostic markers. The long-term aim of the national project is to eventually find a cure for this major disease in our country.

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## CHAPTER 2

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### CANCER OF THE ESOPHAGUS AND TELOMERASE

(What we know at the end of the millennium and what we would like to know.)

### LITERATURE REVIEW

	PAGES
2.1 INTRODUCTION	7
2.2 PREVALENCE AND INCIDENCE	7
2.3 PATHOGENESIS	8
2.3.1 Risk factors	8
a) <i>Alcohol and tobacco</i>	9
b) <i>Dietary factors</i>	9
c) <i>Specific foods</i>	10
d) <i>Vitamins and Minerals</i>	10
2.3.2 Genetic factors	12
2.3.3 Infectious agents	12
2.3.4 Predisposing conditions	13
2.3.5 Other diseases	13
2.3.6 Genetic alterations	13
2.4 TELOMERES AND TELOMERASE	15
2.4.1 Proteins at the telomere	20
2.4.2 Higher order telomere structure	21



2.4.3	Telomere capping	23
2.4.4	The telomere RNP	25
2.4.5	Telomerase – independent telomere maintenance	27
2.5	<b>TELOMERASE ACTIVITY</b>	29
2.6	<b>TELOMERASE ACTIVITY ASSAY</b>	35
2.6.1	Problems with the telomerase activity assay	37
2.7	<b>WILL INHIBITION OF TELOMERASE ACTIVITY RESULTS IN CANCER REMISSION?</b>	38
2.8	<b>TELOMERASE AS A DIAGNOSTIC TOOL FOR CANCER DETECTION</b>	39
2.9	<b><i>In vivo</i> AND <i>in vitro</i> MODELS TO STUDY DRUGS THAT TARGET TELOMERASE AND TELOMERES</b>	40
2.10	<b>TELOMERASE INHIBITORS</b>	41
2.11	<b>TELOMERASE TARGETING AGENTS</b>	41
2.11.1	The DNA component: G-quartet interactive compound	41
2.11.2	The nucleoprotein complex	43
2.12	<b>MOLECULAR TARGETING OF CANCER: TELOMERES AS TARGET</b>	45
2.13	<b>FUTURE PROSPECTS</b>	45
2.14	<b>REFERENCES</b>	46

## CHAPTER 2

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### CANCER OF THE ESOPHAGUS AND TELOMERASE

(What we know at the end of the millennium and what we would like to know in future.)

#### LITERATURE REVIEW

##### 2.1 INTRODUCTION

Squamous cell carcinoma of the esophagus (SCCE) is one of the most aggressive cancers and is associated with poor survival. The most common presenting symptom is dysphagia, beginning with solid foods and progressing to liquids. The duration of symptoms prior to diagnosis is often a few months. The cancer is usually invasive by the time the diagnosis is made. Cellular progression to malignancy is thought to involve multistep events, and is expressed histologically as a progression from normal epithelium to cancerous tissue (Silber, 1985).

##### 2.2 PREVALENCE AND INCIDENCE

Cancer of the esophagus has been reported as the ninth most common malignancy in the world and it is most prevalent in developing nations (Day and Varghese, 1994). There are over 300 000 new cases reported annually and about 80% of these occur in developing countries. In these countries it is mostly associated with micronutrient deficiencies and the consumption of hot beverages and soups, whereas in more developed nations, esophageal cancer is usually attributed to tobacco and alcohol use. As a result, there is marked variation in its incidence from one geographic region to another and between one ethnic group and another within a given region, reflecting differences in behaviour and diet (Day and Varghese, 1994). In the United States, there are marked differences in the incidence of esophageal

higher than it is in white men (Blot, 1994). In all parts of the world it is predominantly a disease of men, with men outnumbering women by about 3 to 1.

Geographic variations in the incidence of SCCE are greater than for any other malignancy. There are regions of high frequency in the People's Republic of China, Singapore, the Caspian Sea region of Iran, Kazakhstan, Puerto Rico, temperate South America, including Chile, Uruguay, northern Argentina and Brazil, parts of western Europe such as Switzerland and France and the Transkei region of South Africa. An "esophageal cancer belt" extends across Asia from the southern shore of the Caspian Sea in Iran, through Soviet Central Asia and Mongolia, to northern China. The incidence of cancer of the esophagus in the area within the "esophageal cancer belt" is higher than in any other area in the world (Blot and McLaughlin, 1999).

The incidence in developing countries is four times higher than in developed countries. In north-east Iran around the Caspian Sea, the incidence of esophageal SCC is second only to that in China. In South Africa, the incidence in the black population increased dramatically between 1940 and 1950 in the Transkei region, to a point where it is the most common cancer among men and second most common among women (Mannell and Murray, 1989). Environmental factors seem to be responsible for these phenomena. Certain alcoholic drinks (Kachosu, home-brewed from sugar and maize husks rather than the sorghum beer), homegrown tobacco smoked in homemade pipes, and nutritional deficiencies, may be responsible for the increased incidence.

Perhaps the areas of the world with the highest incidence and most obscure etiologic relationships are in Iran and the old Soviet Union around the Caspian Sea. There is no significant alcohol or tobacco consumption among the Muslim population in this area. Dietary factors are most suspect.

## **2.3 PATHOGENESIS**

### *2.3.1 RISK FACTORS*

Since SCCE has such a striking variation in incidence in different parts of the world and even among different population groups within the same country, it is expected that there are different risk factors in different areas.

a) *Alcohol and tobacco*

Alcohol and tobacco are two of the best known agents that have been implicated in the aetiology of cancer of the upper digestive tract (UDT) (i.e., oral cavity, pharynx and esophagus). One of the strongest associations in Western developed countries is that between SSCE and alcohol consumption. The risk of developing this tumor is 25 times greater for chronic liquor drinkers than for non-drinkers; for chronic beer drinkers, the risk is 10 times that of non-drinkers (Sons, 1987). However, this interaction may be somewhat different for cancers arising in different parts of the esophagus. As an example, in a Swedish study, 59% of the patients with cervical esophagus carcinoma were alcohol abusers, while this dropped to 43% for carcinoma in the upper thorax, 37% in the mid-thorax, and 25% in the lower thorax (Kuylenstierna and Munck-Wikland, 1985).

In the West, tobacco has been implicated as a risk factor, although not with the intensity of alcohol. However, it may also have a role in carcinogenesis in some underdeveloped countries. As an example, in the Transkei region of South Africa, the population at risk has a long tradition of smoking locally grown tobacco, beginning in adolescence (Sagar, 1989). In this population, risk factors for esophageal cancer are smoking, consumption of beer and the use of traditional medicine. In addition, nitrosamine-rich tobacco juice that accumulates in the pipe stem is swallowed. In the high-risk areas of Iran, opium use is common among the population at risk, and not only is the opium smoked, but the tar in the pipes is often eaten (Chang *et al.*, 1993). In Kerala, India, tobacco smoking or chewing is one of the most significant risk factors, along with alcohol, but only in men, not in women (Sankaranarayanan *et al.*, 1991). However, there are areas outside Europe and North America with a very high incidence of UDT cancers, particularly esophageal cancer, in which alcohol and tobacco play negligible roles. Therefore, interest has focused on the manner in which poor diets may increase the risk of these cancers (Morton, 1968; Cook, 1971; van Rensburg, 1981).

b) *Dietary factors*

In the high-risk areas, there are dietary characteristics that combine deficiencies of vitamins and minerals in the dominant foodstuffs to produce situations that are potentially conducive to cancer development. Many of these combinations lead to an increased nitrosamine content in the daily diet of the inhabitants of the various localities. Some studies have considered various

macronutrient and micronutrient deficiencies or imbalances in the aetiology of esophageal cancer (Craddock, 1987).

c) *Specific Foods*

Certain foods, many of which are dietary staples, have been implicated in the aetiology of esophageal cancer. A study conducted in the north-eastern part of Italy (Rossi *et al.*, 1982), has suggested that the geographical and temporal distributions, at least of esophageal cancer, could reflect the use of maize as a major staple food as well as the use of maize for brewing beer. Li *et al.* (1989) and Wahrendorf *et al.* (1989) also detected a fairly strong trend of increasing risk of esophageal cancer with increasing consumption of maize and wheat.

Carcinoma of the esophagus is the number one cancer of Black males in the country. The Transkei and Ciskei regions of the Eastern Cape have the highest incidence of the disease, although it occurs all over South Africa. The causes are unknown at this stage although maize, contaminated with *Fusarium moniliforme*, seems to be implicated at least partly. Life style, especially the use of tobacco and alcohol, could be important as well. Van Rensburg *et al.* (1985) found a more than fivefold elevated risk of developing esophageal cancer among Zulu men who daily *purchased* maize meal. Segal *et al.* (1988) observed a 25-fold enhanced risk of developing esophageal cancer among heavy drinkers of traditional beer made from maize in South Africa. By far the strongest evidence for the role of maize comes from surveys in Africa. Maize is an introduced crop in Africa, and its spread as a staple food and ingredient in traditional beer seems to coincide (after one allows for a latent period) with a rise in the frequency of esophageal cancer (Cook, 1971; Van Rensburg, 1981; Bradshaw *et al.*, 1982). Maize is easier to grow and more resistant to fungi and attacks by birds than other traditional grains. However, if it is refined, maize is less nutritious than other grains and can cause deficiencies of several micronutrients (chiefly riboflavin and niacin) (Darby *et al.*, 1977). Riboflavin deficiency has been suggested as one of the deficiencies that cause Plummer-Vinson syndrome, a long-recognized precancerous lesion of the UDT (Wynder *et al.*, 1957)

d) *Vitamins and Minerals*

Studies from high-risk areas have shown that vitamin deficiencies are most common among high-risk populations: Riboflavin deficiency is common in China and Iran, while the intake of

vitamin A and C is low in northern Iran (Munoz *et al.*, 1985). In China, the country with the highest mortality rate due to this disease, incidence rates have been decreasing since the 1970s, possibly reflecting increased consumption of foods rich in protein, carotene, vitamin C and E, and riboflavin (Day and Varghese, 1994).

In the Transkei, the soil has low concentrations of zinc and molybdenum, and this may be important in relation to the very high rate of esophageal cancer in this region (Sagar, 1989). A study of esophageal cancer patients who were heavy smokers and drinkers in Washington D.C., indicated that as a group they had significantly lower levels of plasma zinc and vitamin A than the group of nonalcoholic controls without SCC (Mellow *et al.*, 1983). These results suggested that zinc and vitamin A deficiencies might be carcinogenic co-factors for human esophageal SCC in this American population.

Numerous studies of animal models and more recent studies of humans have demonstrated the cancer chemopreventive effects of selenium (Se) (Ip, 1998). There is extensive evidence that monomethylated forms of Se are critical metabolites for chemopreventive effects of Se. Induction of apoptosis in transformed cells is an important chemopreventive mechanism (Thompson *et al.*, 1992). Apoptosis can be triggered by micromolar levels of monomethylated forms of Se independent of DNA damage and in cells having a null p53 phenotype (Kaeck *et al.*, 1997; Lu *et al.*, 1994). Cell cycle protein kinase cd2 and protein kinase C are strongly inhibited by various forms of Se (Sinha and Medina, 1997). Inhibitory mechanisms involving modification of cysteine residues in proteins by Se have been proposed that involve formation of Se adducts of the selenodisulfide (S-Se-S) or selenenylsulfide (S-Se) type or catalysis of disulfide formation. Se may facilitate reactions of protein cysteine residues by the transient formation of more reactive S-Se intermediates (Medina, 1986). A novel chemopreventive mechanism is proposed involving Se catalysis of reversible cysteine/disulfide transformations that occur in a number of redox-regulated proteins, including transcription factors. A time-limited activation mechanism for such proteins, with deactivation facilitated by Se, would allow normalization of critical cellular processes in the early stage of transformation. There is uncertainty at the present time regarding the role of selenium proteins in chemoprevention model systems where supranutritional levels of Se are employed (Grander, 1999).

### 2.3.2 GENETIC FACTORS

In general, esophageal SCC does not appear to be a familial cancer, although heredity may have some effect. In a Chinese study, there was an increased cancer risk if there was a family history in one high-risk geographic area, but family history was not important in a second high-risk area (Wang *et al.*, 1992). About a third of family members with the rare, hereditary, dominantly transmitted condition of *tylosis palmaris et plantaris*, have esophageal carcinoma, in addition to the abnormal cutaneous keratinization involving the palms, soles and ventral surfaces of the fingers and toes (Marger and Marger, 1993; Sons, 1987). In a study of an American family with the syndrome, the esophageal cancer risk among affected members was at least 90% (Marger and Marger, 1993).

### 2.3.3 INFECTIOUS AGENTS

Viruses including herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and Human Papilloma Virus (HPV) have been implicated in human tumorigenesis. Of these, HPV is important in the evolution of several squamous cell or squamous-like carcinomas, especially those arising in the uterine cervix, vulva, vagina and the anal transitional zone. The most commonly implicated viral types are 16 and 18. It is logical to look for viral material in other SCCs, and esophageal tumors are obvious targets. When 72 SCC samples from different parts of the world were analyzed in a single laboratory by PCR (polymerase chain reaction), HPV DNA was detected in only 10 (14%). There was no difference in the prevalence of HPV DNA in cancers in various countries including Italy, France, Japan, Iran, South Africa, and the United States, but the number of cases tested from each country was small (Togawa *et al.*, 1994). Finally, in a study from the United States, no HPV DNA was detected in 15 squamous cell carcinomas or in the adjacent mucosa by both ISH (*in situ* hybridization) and PCR (Wilson *et al.*, 1990). Thus, HPV is probably an important factor in the evolution of some, but not all, parts of the world. In South Africa, HPV may be a significant factor, with less importance in northern China and some parts of France and minimal importance elsewhere, especially in parts of the world with low overall risk for squamous cancer (Wilson *et al.*, 1990). However, the mode of transmission is unknown. Standardized HPV DNA detection systems in future studies may produce more meaningful data about population differences.

### **2.3.4 PREDISPOSING CONDITIONS**

In many of the epidemiologic studies of esophageal carcinoma, there is repeated reference to an inflammation of the esophagus that is prevalent in the high-risk populations. This inflammation is referred to simply as "esophagitis" or "chronic esophagitis" with no qualification. It is asymptomatic and not related to gastroesophageal reflux (Kuylentierna and Munc-Wikland, 1985). The non-reflux type of esophagitis in high-risk populations has been described as the most frequent finding in esophageal biopsies in these regions (Wahrendorf *et al.*, 1989). It also tends to occur in relatively young people in the high-risk areas.

### **2.3.5 OTHER DISEASES**

Esophageal SCC has been reported as a complication of acid and lye burns with resultant strictures (Sons, 1987). The carcinomas occur after a long latent period, usually 30 years or more, but the latent period may decrease as the age, in which lye ingested increases. However, since almost all cases of lye ingestion are during childhood, the age of the patient when the carcinoma develops is still lower than that of usual or common carcinoma development. Since lye strictures are most common in the upper thoracic portion of the esophagus behind the tracheal bifurcation, the complicating carcinomas occur there, rather than in the usual site of SCC, which is the middle or distal esophagus (Appelqvist and Salmo, 1980).

### **2.3.6 GENETIC ALTERATIONS**

Recent studies have demonstrated genetic alterations in esophageal SCC, especially mutational activation or overexpression of oncogenes and loss of the normal function of tumor suppressor genes (Ozawa *et al.*, 1987; Kitagawa *et al.*, 1991). In addition to such genetic alterations, cellular immortalization is required for malignancy.

The literature mostly covers the p53 tumor suppressor gene and *ras* oncogene, both of which have been implicated in the evolution of a variety of cancers. The *ras*-family of oncogenes,



commonly mutated in many cancers, has also been analyzed in esophageal SCC by PCR techniques (Bos, 1989). In contrast to frequent p53 mutations, no *ras* mutations of any type were found in all 16 carcinomas from Uruguay (Hollstein *et al.*, 1991), 25 carcinomas from a high-risk area of China (Jiang *et al.*, 1989), and 41 carcinomas from France (Hollstein *et al.*, 1988). In the study from France, amplification of the epidermal growth factor receptor gene was detected in 2 of 25 carcinomas using hybridization techniques and in 3 of 12 carcinomas there were higher levels of the epidermal growth factor mRNA than in the surrounding squamous mucosa (Hollstein *et al.*, 1988). A remarkable feature of esophageal cancer is the absence of mutations in members of the *ras* oncogenes (Montesano *et al.*, 1996). Various genetic alterations in other loci have been described in esophageal cancer, and among the most important are those that occur in components controlling the progression of the cells through the cell cycle. The orderly progression of cells through the cell cycle is governed by genes encoding proteins transmitting positive (e.g. activated cyclin and cyclin-dependent kinases [Cdks]) and negative (e.g. inhibitors of Cdk) signals (Sherr, 1993). Dysregulation of these genes can lead to premature entry into the next phase of the cell cycle prior to completion of critical macromolecular events, including the repair of DNA damage, and generate genomic instability and neoplastic transformation (Gamielien *et al.*, 1998). Negative regulation of the cell cycle occurs at G<sub>1</sub> and G<sub>2</sub> checkpoints (Hartwell, 1992). The p53 and p16/CDKN2 genes are negative regulators in cell-cycle control. Several genetic alterations in these genes have been detected among esophageal cancer and other cancers, and it has been suggested that the genetic alterations may represent molecular fingerprints of critical risk factors involved in the development of various cancers (Montesano *et al.*, 1996).

In a study conducted by Gamielien *et al.* (1998) the genetic alterations of p53 and p16/CDKN2 genes were examined in a series of 76 primary human esophageal SCCs, as well as 9 adjacent tissue samples and 50 blood samples from the same patients. The patients originated from the Transkei region of the Eastern Cape Province of South Africa, a high-risk area for esophageal cancer (Marasas *et al.*, 1988). Exons 5-8 of the p53 gene and exons 1-2 of the p16/CDKN2 gene were examined for mutations using PCR-SSCP procedures and DNA sequence analysis. The results showed that 17% of the tumors contained small deletions, insertions and point mutations, resulting in frame-shifts or amino acid changes in the p53 gene. Among the mutations in the structural p16/CDKN2 gene, 9 were point mutations, 4 were deletions and 3 were insertions. The relatively high frequency of the small deletions or

insertions in the p53 gene has not been previously noted in esophageal cancer, and the origin of these defects is unknown. However, it would tend to support the slippage/misalignment model (Jego *et al.*, 1993). Slippage or misalignment can occur spontaneously or can be initiated by a spontaneous or adduct-induced base misincorporation that creates a context of slippage. Environmental agents or dietary deficiencies can induce mutations.

In two Japanese studies, mutations of the p53 gene were found in 4 out of 5 cases of invasive SCC using PCR analysis; p53 gene overexpression suggesting mutations was detected in 17 out of 20 carcinomas by immunohistochemistry (Imazeki *et al.*, 1992; Sasano *et al.*, 1992). However, not all studies have found equally high frequencies, so there may be geographic differences. For instance, in a study of 34 cases from two high incidence areas, viz. Uruguay and Normandy in France, using PCR analysis only 15 cases had p53 point mutations (Hollstein *et al.*, 1991). This is about half the rate of the two Japanese studies. In a study from a high incidence area in China, the p53 mutation rate was about 50%, also much lower than the rate in the Japanese studies (85%) (Bennett *et al.*, 1991).

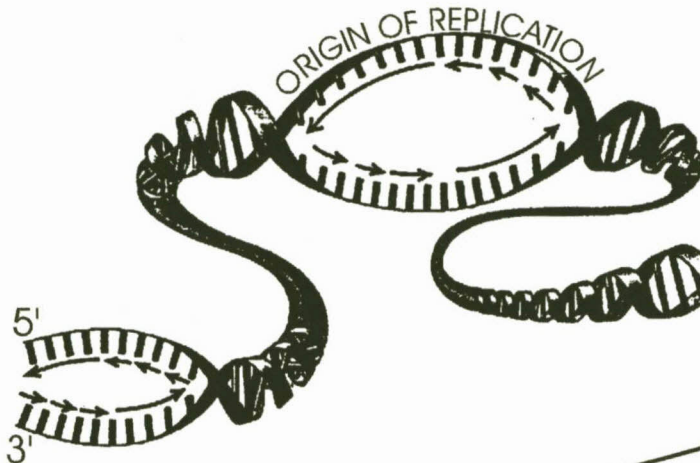
#### 2.4 TELOMERES AND TELOMERASE

Telomeres are the end regions of linear chromosomes, and in normal somatic cells the length of the telomeres shortens with successive cell divisions. Telomerase, a ribonucleoprotein enzyme, maintains the length of the telomeres in immortal and germline cells. Although present in human foetal tissue, shortly after birth telomerase activity becomes undetectable except in germline cells, hematopoietic cells, and most human primary tumors (Kim *et al.*, 1994).

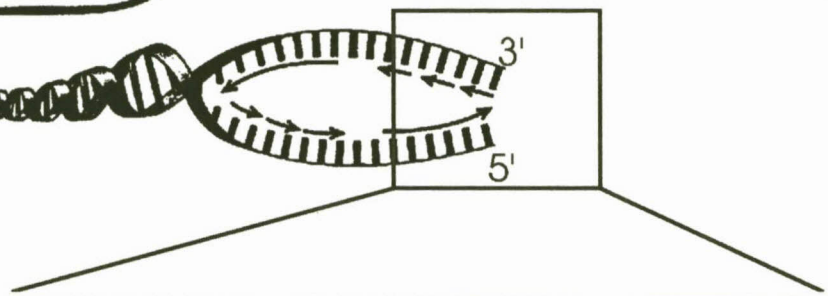
Telomeres are DNA-protein complexes of eukaryotic chromosomes. These chromosomal end regions are made up of several thousand copies of a repeating nucleotide sequence (TTAGGG) and are important in stabilizing the chromosome during replication (Greider, 1994). They cap and protect the ends of chromosomes from degradation and illegitimate recombination. The termini of a linear template cannot, however, be completely replicated in normal human cells as DNA polymerase is unable to replicate the very ends of the linear

The so-called end replication problem arises from the inability of standard mechanisms for replicating chromosomes to do a complete job. When enzymes known as DNA polymerases copy the two original, or "parent", DNA strands in a chromosome, they leave each new "daughter" strand shortened at one tip (at the end traditionally labelled 5'). If cells did not compensate for this flaw in the replication mechanism, chromosomes would inexorably shrink. The existence of telomerase, the enzyme that elongates telomeres, was proposed in the early 1980s as a solution to the end-replication problem.

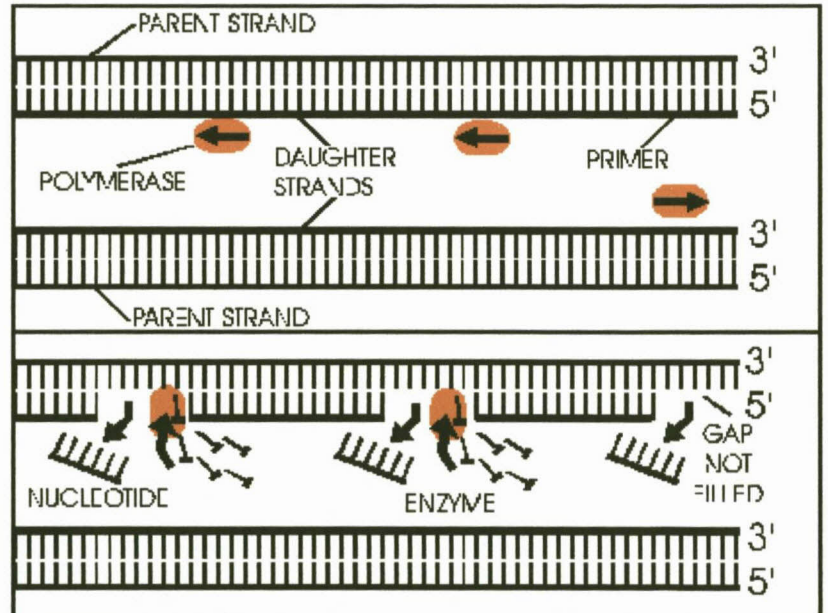
1. Copying of chromosomal DNA, shown highly schematically, proceeds from regions known as origins of replication, at which the parent strands separate. Synthesis of each daughter strand occurs continuously on the side of the region and discontinuously on the opposite side (black arrows).



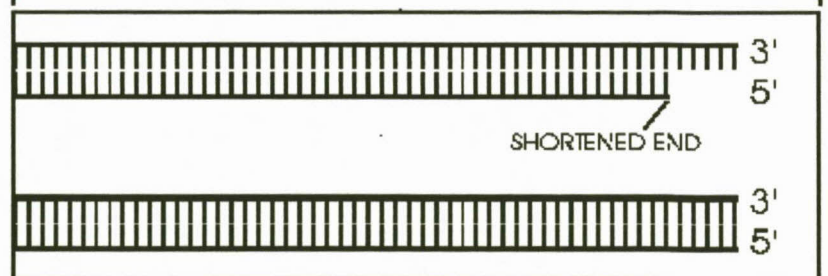
2. Separated parent strands serve as templates on which polymerases synthesize daughter strands. The parts constructed as fragments extend from bits of RNA called primers.



3. Other enzymes remove primers and fill the gaps between adjacent fragments.



4. But the enzymes cannot fill the gap remaining at one end (the 5' tip) of each daughter strand.



Greider and Blackburn (1996)

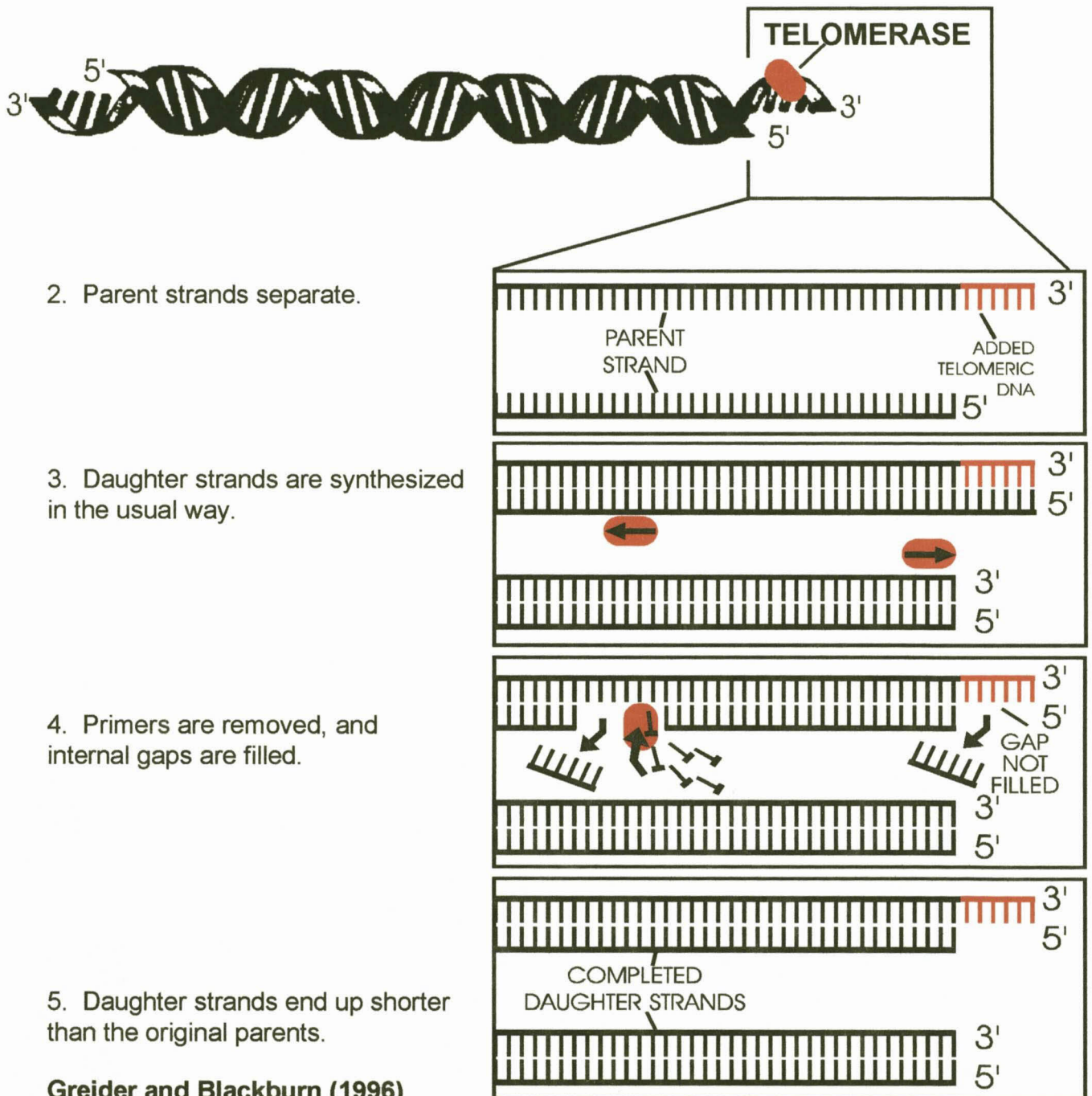
**Figure 2.1 The end-replication problem**

DNA (Watson, 1972; Olovnikov, 1973). The inability to completely replicate telomeric sequences results in the loss of 50 – 200 nucleotides from the telomere with each cell division (Allsopp *et al.*, 1995) (Fig 2.1). Telomere shortening may have been adopted to limit the life span of somatic cells. Human somatic cells have a finite proliferative capacity and eventually enter a viable growth arrested state, called replicative senescence. Life span appears to be governed by cell division, not time. The regular loss of telomeric DNA could therefore serve as a mitotic clock in the senescence program, counting cell divisions (Harley, 1991; Harley *et al.* 1992). Consequently, a decline in telomere length correlates with the increasing age of a cell and it is likely to play a role in events related to cellular senescence (Shay, 1995). Immortal cells, such as the human germ line or tumor cell lines, yeast and ciliates, all maintain a stable telomere length through the action of telomerase. It has been suggested that activating or re-expressing telomerase may impart the chromosomal stability necessary to produce an immortal phenotype (Shay *et al.*, 1994). By maintaining a constant telomere length, telomerase ensures retention of chromosomal stability attributed to functional telomeres and alters the cell's biological clock (Fig 2.2).

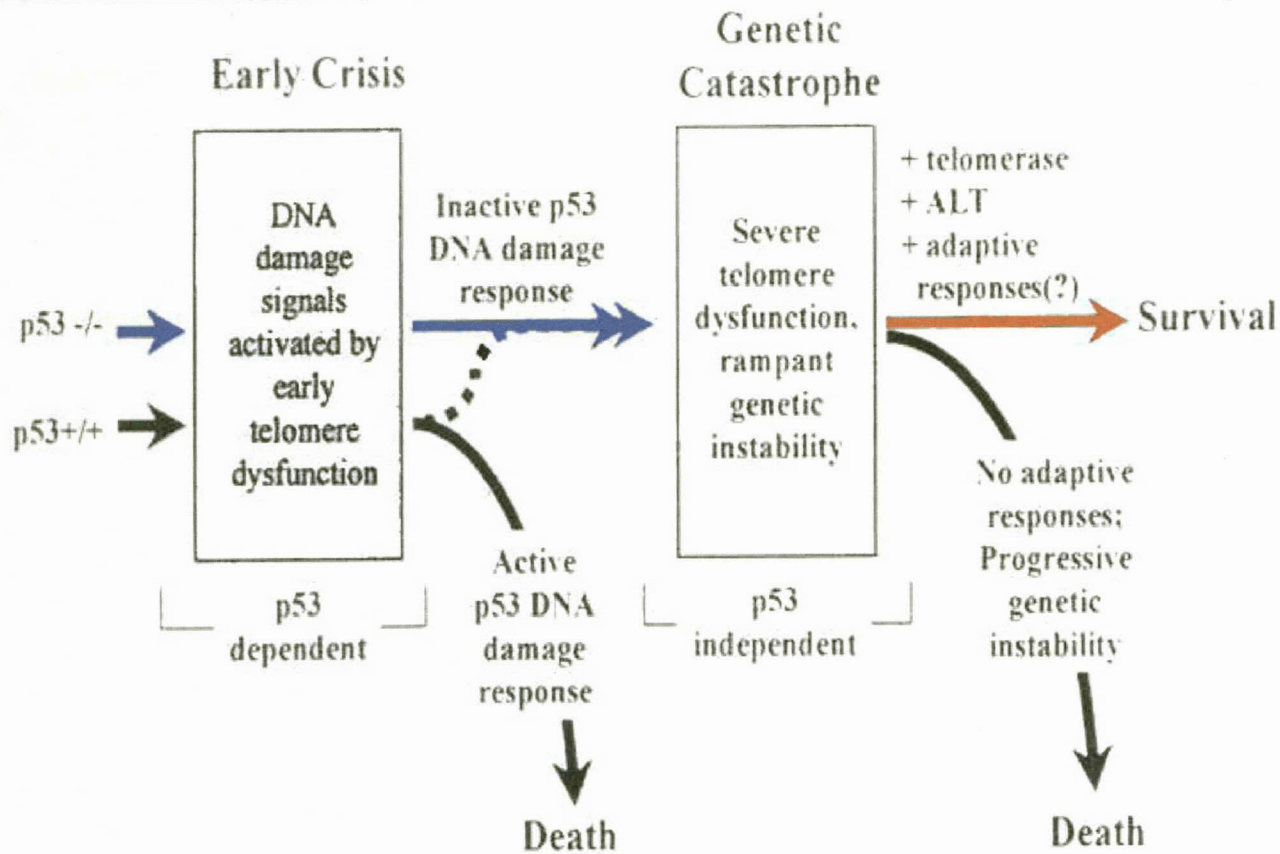
Cell division in the absence of telomerase results in telomere shortening. Oncogene activation or tumor suppressor deletion can facilitate growth beyond senescence, leading to further telomere shortening. In early crisis, p53 is activated, leading to cell death or permanent growth arrest. Inactivation of the p53 DNA damage pathway allows rare cells to continue to divide and bypass the p53 checkpoint. Alternatively, cells that deactivate p53 DNA damage responses early, proceed unimpeded through early crisis. Both pathways lead to genetic catastrophe – massive genetic instability in the setting of the telomere dysfunction and p53 loss. This results in cell death by p53-independent mechanisms unless cells reestablish telomere maintenance either via telomerase or ALT or develop adaptive responses to tolerate genomic instability (Chin *et al.*, 1999) (Fig 2.3).

One scheme for how telomerase solves the end-replication problem proposes that the enzyme adds DNA to chromosomes before replication begins. The added DNA consists of one or more telomeric sub-units: the short sequences of nucleotides that are repeated over and over in telomeres. The addition ensures that a daughter strand will be at least as long as its parent.

1. Before replication begins, telomerase adds some number of telomeric repeats to one end of each parent strand.



**Figure 2.2 How telomerase solves the problem.**



Cell division in the absence of telomerase results in telomere shortening. Oncogene activation or tumor suppressor deletion can facilitate growth beyond senescence, leading to further telomere shortening. In early crisis, p53 is activated, leading to cell death or permanent growth arrest. Inactivation of the p53 DNA damage pathway (dotted black arrow) allows rare cells to continue to divide and bypass the p53 checkpoint. Alternatively, cells that deactivate p53 DNA damage responses early proceed unimpeded through early crisis (blue arrow). Both pathways lead to genetic catastrophe—massive genetic instability in the setting of telomere dysfunction and p53 loss. This results in cell death by p53-independent mechanisms unless cells reestablish telomere maintenance either via telomerase or ALT or develop adaptive responses (red arrow) to tolerate genomic instability.

**Figure 2.3 A Model for Crisis and Tumorigenesis**  
**(Chin et al., 1999)**

When primary cells (taken directly from an animal) are grown in culture, their telomeres gradually shorten to a critical length, at which point the cells undergo a crisis and for the most part die (Harley *et al.*, 1995). However, a small fraction of these cells survive and, once immortalized, are able to replicate indefinitely in culture. Although the molecular basis of this immortalization is not well understood, it appears to be associated with the activation of telomerase, which reinstates telomere repeat units and thus stabilizes the telomere length (Counter *et al.*, 1992; Kim *et al.*, 1994). It is possible that telomere stabilization is a consequence rather than a cause of immortalization; the association is further strengthened by the demonstration that inhibition of telomerase activity in cultured cells reverses the immortalization process. Telomere length is stable in several immortal human cell lines, suggesting that a regulatory mechanism exists for limiting telomere elongation by telomerase (Counter *et al.*, 1992).

#### **2.4.1 PROTEINS AT THE TELOMERE**

Results from recent studies of mammalian telomeres and telomerase demand new structural models. In mammalian cells, double-stranded telomeric repeats are bound directly by two proteins, TRF1 (TTAGGG repeat binding factor 1) and TRF2. Overexpression of wild-type TRF1 reduces telomere length, whereas overexpression of dominant-negative TRF1 increases telomere length (Van Steensel and de Lange, 1997). Van Steensel and de Lange (1997) showed that the human telomeric-repeat binding factor TRF1 is involved in this regulation. The results identified TRF1 as a suppressor of telomere elongation and indicate that TRF1 is involved in the negative feedback mechanism that stabilizes telomere length. As TRF1 does not detectably affect the expression of telomerase, van Steensel and de Lange (1997) propose that the binding of TRF1 controls telomere length *in cis* by inhibiting the action of telomerase at the ends of individual telomeres. Although TRF1 may function less directly, the authors favour the possibility that TRF1 controls telomere maintenance by binding to telomeres. Overexpression of wild-type TRF2 also reduces telomere length (Griffith *et al.*, 1999; Smorgorzewska *et al.*, 2000). In addition, overexpression of dominant negative TRF2 or a different truncated form of TRF2 can induce proliferative senescence (Griffith *et al.*, 1999). Collectively, these studies could be interpreted to suggest that TRF1 functions to regulate telomere length, whereas TRF2 functions independently to protect telomeres from non-homologous end joining and other DNA repair or DNA damage response pathways.

However, the roles of TRF1 and TRF2 in creating the higher order telomere structures below suggest that end maintenance and end protection are interdependent processes.

Two TRF1-interacting proteins have been discovered: tankyrase (Smith *et al.*, 1998) and TRF1-interacting nuclear protein 2 (TIN2) (Kim *et al.*, 1999). Both proteins were identified by the two-hybrid interaction assay in yeast. Tankyrase interacts with the acidic amino-terminal region of TRF1. TIN2 interacts with the homodimerization region of TRF1. Similarly to the phenotypes observed with overexpression of TRF1 described above, overexpression of wild-type TIN2 reduces telomere length; overexpression of truncated, presumably dominant-negative, TIN2 increases telomere length. *In vitro*, the interaction of TIN2 with TRF1 does not block binding of TRF1 to telomeric repeats (Collins, 2000).

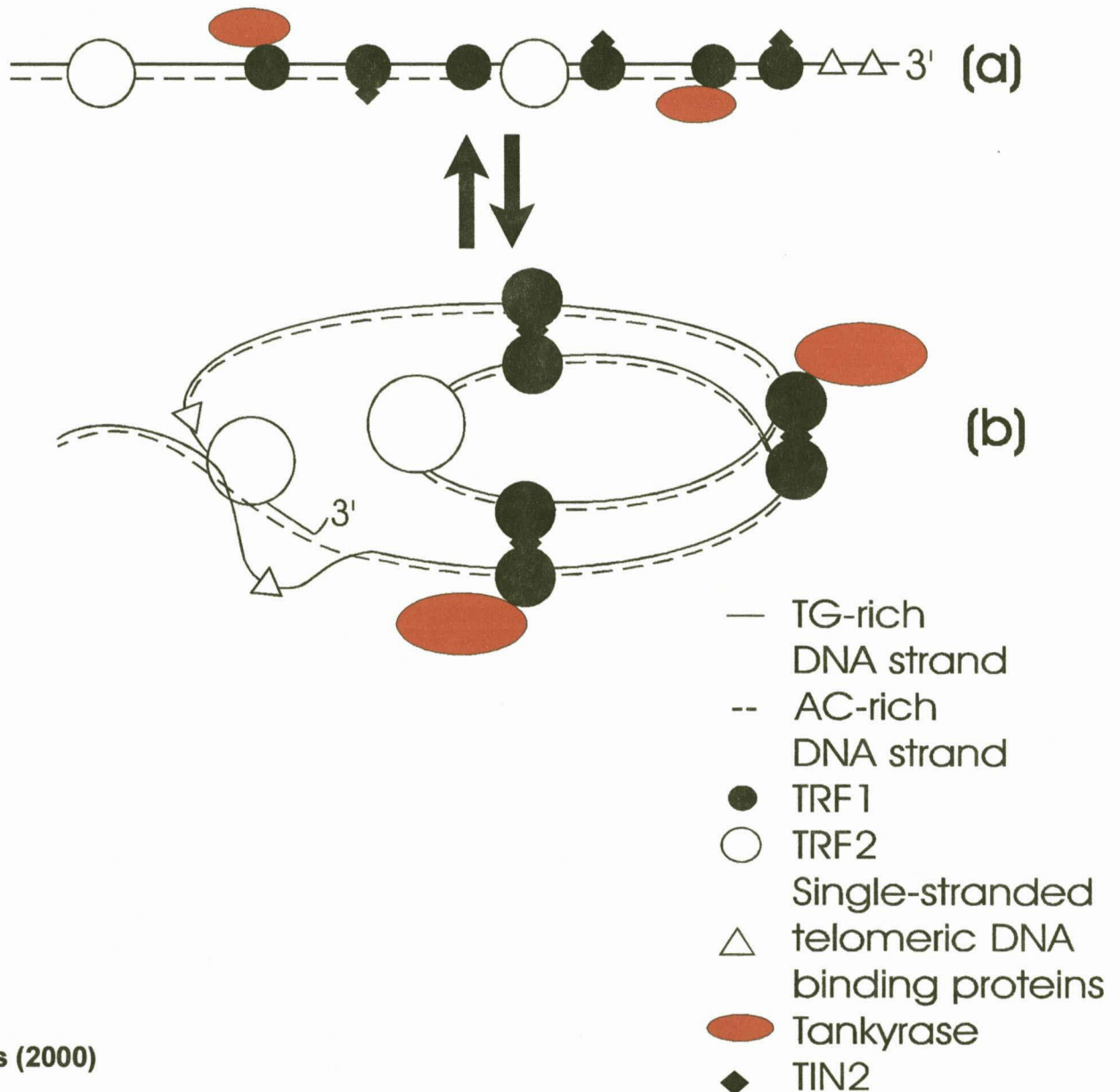
#### **2.4.2 HIGHER ORDER TELOMERE STRUCTURE**

Representations of entire linear chromosomes include loops of internal sequence. The chromosome end joins the ranks of topologically constrained chromosome domains, with the demonstration that at least half of the mammalian telomeres form loops of duplex DNA, termed t-loops (Griffith *et al.*, 1999). Loops form by the 3' single-stranded overhang invading a region of double-stranded repeats, probably establishing base pairs between the formerly single-stranded overhang and the complementary strand of the invaded duplex. *In vitro*, t-loop formation is dependent on three features of telomere structure: double-stranded repeats, single-stranded repeats in a 3' overhang and the telomere-binding protein TRF2. TRF2 binds to the site of loop formation on t-loops formed *in vitro* (Griffith *et al.*, 1999).

TRF1 may promote three-dimensional t-loop architecture. TRF1 can repair bound telomeric repeats (Griffith *et al.*, 1998). Because mammalian chromosome ends do not appear to interact with each other constitutively, a TRF1-dependent pairing of double-strand repeats would be most likely to organize repeats on the same chromosome end into a higher order structure rather than organizing a telomere cluster (Fig 2.4). If internally situated repeats are more likely to be constrained by TRF1-dependent pairing, TRF2-dependent t-loop formation would be more likely to occur with repeats located towards the telomeric/non-telomeric sequence junction, as observed in purified cellular DNA. A role for TIN2 in stimulating or stabilizing TRF1-mediated pairing would be consistent with its biochemical activities *in vitro*



- (a) The linear model. Telomeric DNA is composed of double-stranded TG/AC-rich repeats with a 3' overhang of the TG-rich strand. TRF1, TRF2 and putative single-stranded telomeric repeat binding proteins interact directly with DNA. Tankyrase and TIN2 interact with TRF1.
- (b) Three-dimensional model. Once TRF1 has bound, it can pair telomeric repeats, perhaps stimulated or regulated by TIN2. TRF2 joins double-stranded telomeric repeats and the 3' single-stranded overhang, displacing the formerly double-stranded TG-rich strand to base-pair the AC-rich strand with the overhang. Single-stranded-DNA binding proteins could interact with the displaced strand or unpaired regions of the 3' overhang. No specific prediction of topology is intended.



Collins (2000)

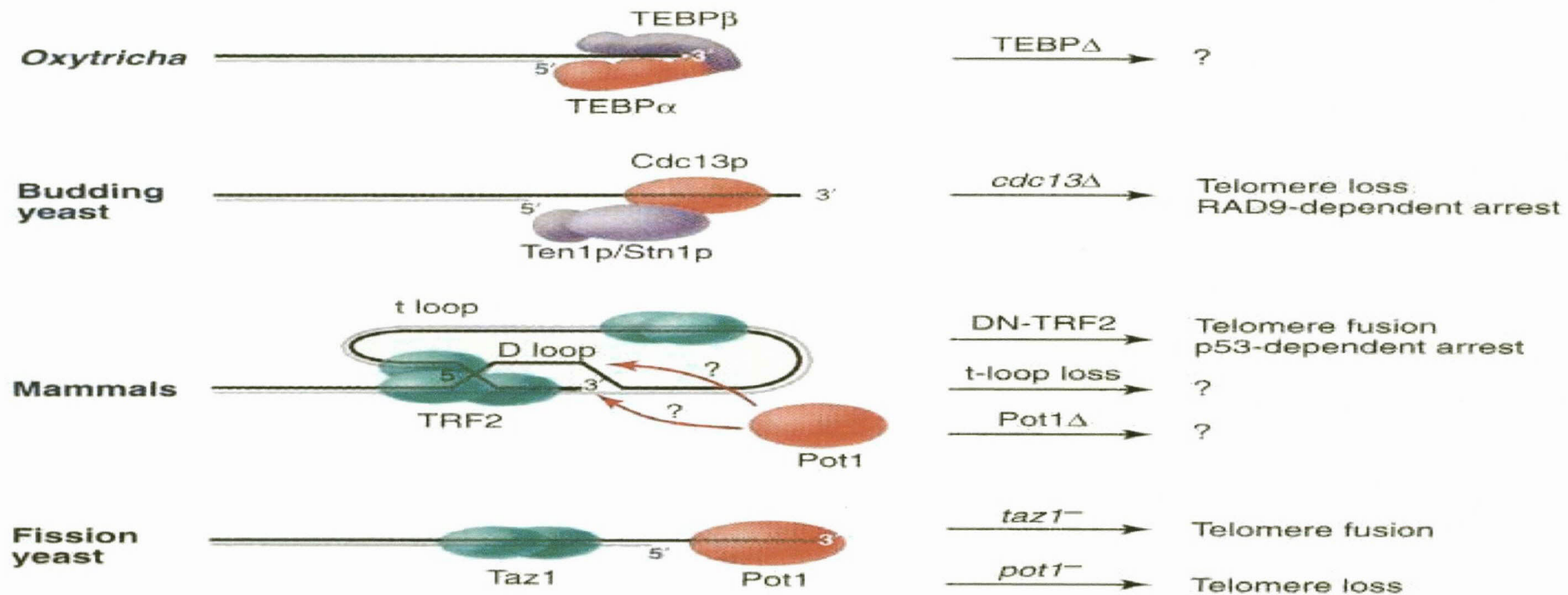
**Figure 2.4 A model for mammalian telomere structure.**

and its impact on telomere length *in vivo*. A telomere structure such as in Figure 2.4 could also promote the release of double-stranded telomeric repeats by recombination, which would account for the extra-chromosomal telomeric repeats observed in human cells with abnormally long telomeres (Yeager *et al.*, 1999).

The telomere shortening and subsequent loss of function observed in cells that lack telomerase may be due to disruption of the t-loop structure. In several organisms in which telomere shortening is seen, telomeres lose the ability to protect against chromosome fusion well before all telomere sequence is lost (Greider, 1999). Perhaps a minimal length is needed to form a t-loop and once telomeres are sufficiently short, they can no longer protect the end. The minimal length for t-loop formation may also be species specific, as are both double-stranded telomere tracts and G strand overhang length (Greider, 1999). Much remains to be learned about the formation and regulation of t-loops.

### 2.4.3 TELOMERE CAPPING

Chromosome termini must be “capped”; otherwise they will be identified as damaged and subjected to DNA repair, an outcome that would cause loss of chromosome integrity and cell viability. In essence, the capping problem has been solved by the presence of telomeres, complexes of DNA and proteins that protect the ends of the chromosomes. However, the way in which various organisms protect their telomeres appears to be remarkably idiosyncratic (de Lange, 2001). Ciliates, budding yeast and mammals each seem to have arrived at a different system (Fig 2.5), providing a perplexing diversity of mechanisms to solve such a fundamental problem (de Lange, 2001). Baumann and Cech (2001) now provide evidence for a common theme in telomere protection among ciliates, yeast and mammalian cells. Their findings indicate that all eukaryotes use a single-stranded DNA binding protein to cap the telomere. Apart from telomere synthesis by telomerase, this may be the first truly conserved aspect of eukaryotic telomeres. *In vitro*, Pot1 (protection of telomeres) protein binds specifically to the single-stranded G-rich telomeric overhang of fission yeast; biochemical analysis suggests that this capping factor might bind along the length of the single-stranded DNA tail of the telomere, as well as at its end. Using human sequence databases, Baumann and Cech (2001) identified a human ortholog of Pot1 and found that, like its yeast counterpart, this protein binds specifically to the G-rich DNA overhang of human



**A common theme in telomere capping.** Diverse telomere-capping strategies have in common a single-stranded telomeric DNA binding protein. Telomeres of the ciliate *Oxytricha nova* contain a 16-nucleotide 3' overhang bound by the single-stranded DNA binding protein TEBP $\alpha$  $\beta$ . The function of this telomeric complex has not yet been tested. Budding yeast telomeres are protected by the single-stranded telomeric DNA binding protein Cdc13p, which recruits the capping proteins Stn1p and Ten1p. Loss of any component of this complex results in degradation of 5' chromosome ends and cell cycle arrest. Mammalian telomeres are protected by TRF2, perhaps through its ability to form t loops. Loss of TRF2 results in cell cycle arrest and end-to-end ligation of telomeres. Fission yeast telomeres are protected by Taz1, an ortholog of TRF2. The newly discovered capping factor Pot1 binds to single-stranded telomeric DNA and protects fission yeast telomeres from degradation. A human ortholog of fission yeast Pot1 has been identified. Because this protein binds to single-stranded G-rich telomeric DNA *in vitro*, its *in vivo* binding sites could be the 3' overhang (if it is unpaired) or the D loop (see arrows) of human telomeres. In all telomeres, the dark line indicates the G-rich telomeric repeat strand that extends as a 3' overhang beyond the double-stranded region of the telomere.

**Figure 2.5** A common theme in telomere capping (de Lange, 2001).

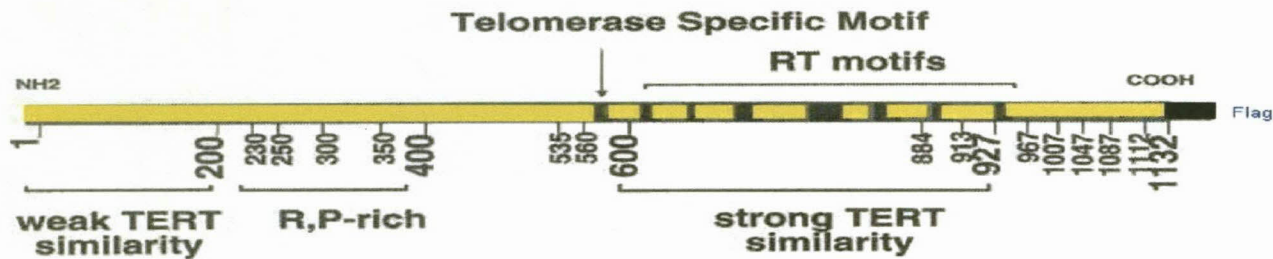
telomeres *in vitro*. Mammalian telomeres end in a very long 3' overhang (up to 300 nucleotides), so that multiple copies of Pot1 may have to bind along the single-stranded tail. In addition, Pot1 could bind to the G-rich telomeric DNA in the D-loop and stabilize the t-loop configuration (Fig 2.5).

It now appears that at least in mammalian cells, telomeres may exist in at least three interconvertible states: as t-loops, Pot1 protein-bound and engaged with telomerase. Although these different states could correlate with particular stages of the cell cycle, they need not be mutually exclusive. As indicated by Buamann and Cech (2001) the Pot1 protein may be involved in actively recruiting telomerase. Alternatively or in addition, the 3' end of telomeric DNA could be capped by Pot1 protein within the structure of a t-loop, which would prevent the chromosome end from being used as a primer for conventional DNA synthesis (Baumann and Cech, 2001).

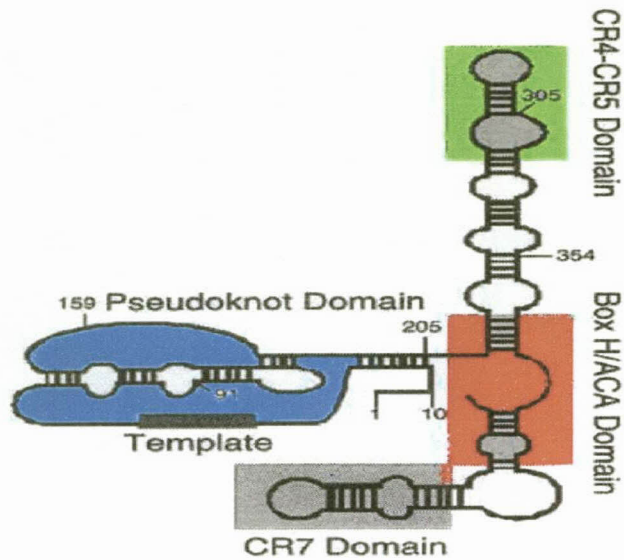
#### **2.4.4 THE TELOMERASE RNP (ribonucleoprotein)**

The minimal, active core of human telomerase is postulated to contain two components, the telomerase RNA (hTR) and the telomerase reverse transcriptase (hTERT) (Fig 2.6).

In previous years, mammalian telomerase RNA, telomerase-associated protein 1 (TEP1) and telomerase reverse transcriptase (TERT) were described (Nugent and Lundblad, 1998). Although most of the cells produce telomerase RNA and TEP1, it was initially thought that only cells with active telomerase produce TERT. Telomerase regulation was envisioned as a simple on/off switch mediated by transcriptional on/off regulation of TERT mRNA expression. However, regulation of telomerase activity now appears more complex. In addition to the first TERT isoform characterized, additional isoforms have been discovered, which are encoded by alternatively spliced TERT mRNA (Kilian *et al.*, 1997). Because some of these isoforms are catalytically inactive, cells containing TERT can lack telomerase activity (Ulaner *et al.*, 1998). Expression of an inactive TERT that assembles into telomerase RNP would titrate telomerase RNA away from interaction with other TERT isoforms, reducing the maximum attainable telomerase activity. In addition, some evidence suggests that telomerase activity is regulated by post-translational modification of TERT (Liu *et al.*, 1999; Li *et al.*, 1998; Kang *et al.*, 1999). Beattie *et al.* (2000) found that TEP1 could interact with hTERT truncations that contain the RT domain. Because TEP1 was able to interact



B



(A) The first 200 amino acids contain very weak sequence similarity to the TERT proteins from other species. The region between amino acids 200 and 400 contains high arginine and proline content, which is thought to be important in protein-protein interactions. The region between amino acids 536 and 600 has a weak homology to cyclin E. Amino acid 560 begins the region of highest similarity to other TERT family members, and includes the telomerase specific motif. The region of highest similarity to other RTs begins at amino 601. C-terminal truncations of hTERT included a truncation at amino acid 884, which deletes RT motif 6 (GVPEYGCVVNLRKTVV) of hTERT, a truncation at amino acid 913, and a truncation at amino acid 927, which removes motif 7 (hLGxxh), and ends at the highly conserved RT motif. Other C-terminal truncations were made at increments of ~50 amino acids from 927 to the C terminus of the protein. Each of these specific regions was deleted to ascertain its role in the telomerase complex with regard to activity, hTER binding, and interaction with TEP1. (B) Secondary structure of the human telomerase RNA adapted from Chen *et al.* (2000). The template region of hTER falls between nucleotides 44 and 52. Highly conserved motifs such as the pseudoknot domain; conserved regions (CR) 4, 5, and 7; and the H/ACA box are indicated in gray. Specific 5' and 3' truncations were made at the positions indicated to ascertain the roles of specific RNA sequences and structures in the reconstitution of human telomerase activity.

**Figure 2.6 hTERT and hTER. Schematic of the human telomerase RT and telomerase RNA (Beattie et al., 2000).**

with an hTERT truncation (RT domain) that could not bind to the telomerase RNA, Beattie *et al.* (2000) suggest that the interaction may occur independently of the telomerase RNA.

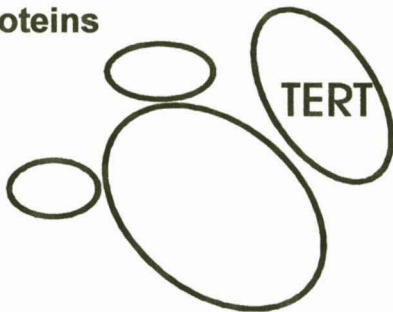
New findings have provided a surprising explanation for the mysterious evolutionary expansion of telomerase RNA length and telomerase RNP mass from ciliates to mammals. The increase in size of the mammalian telomerase RNAs derives from an addition of an independently stable domain at the telomerase RNA 3' end, containing a box H/ACA small nuclear RNA (snoRNA) motif (Mitchell *et al.*, 1999a). All other members of the box H/ACA snoRNA family function in ribosomal RNA (rRNA) maturation, specifying sites of pseudouridine modification or processing (Mitchell *et al.*, 1999a). The H/ACA motif of the telomerase RNA is essential for RNA accumulation and 3' end processing *in vivo*, roles that can be provided by a heterologous H/ACA snoRNA, when substituted for the telomerase H/ACA domain (Kang *et al.*, 1999). However, the telomerase RNA H/ACA domain is also essential for telomerase activity *in vivo*, a role not provided by the heterologous H/ACA snoRNA sequence. This extra function of the telomerase RNA H/ACA domain may be mediated by the extra sequence not present in other H/ACA RNAs. Recent biochemical studies have demonstrated that dyskerin is a component of both human H/ACA snoRNPs and the human telomerase RNP (Mitchell *et al.*, 1999b). These results suggest that mammalian telomerase incorporates an entire H/ACA snoRNP, not just an H/ACA RNA motif. Initial assembly of an H/ACA sub-domain RNP may facilitate its subsequent interaction with TERT, consistent with a step-wise telomerase RNP assembly process and the dependence of *in vivo* telomerase RNA stability on interaction with H/ACA proteins, but not on interaction with an active or inactive TERT (Fig 2.7).

#### **2.4.5 TELOMERASE –INDEPENDENT TELOMERE MAINTENANCE**

Short telomeres in cultured human primary cells induce proliferative senescence (de Lange, 1998; Price, 1999). If telomerase is inactivated in an immortal tumor cell will proliferation similarly cease? Three separate studies in 1999 reported that telomerase-positive cells driven to short telomere lengths by telomerase inhibition undergo apoptosis (Zang *et al.*, 1999; Hahn *et al.*, 1999a; Herbert *et al.*, 1999).

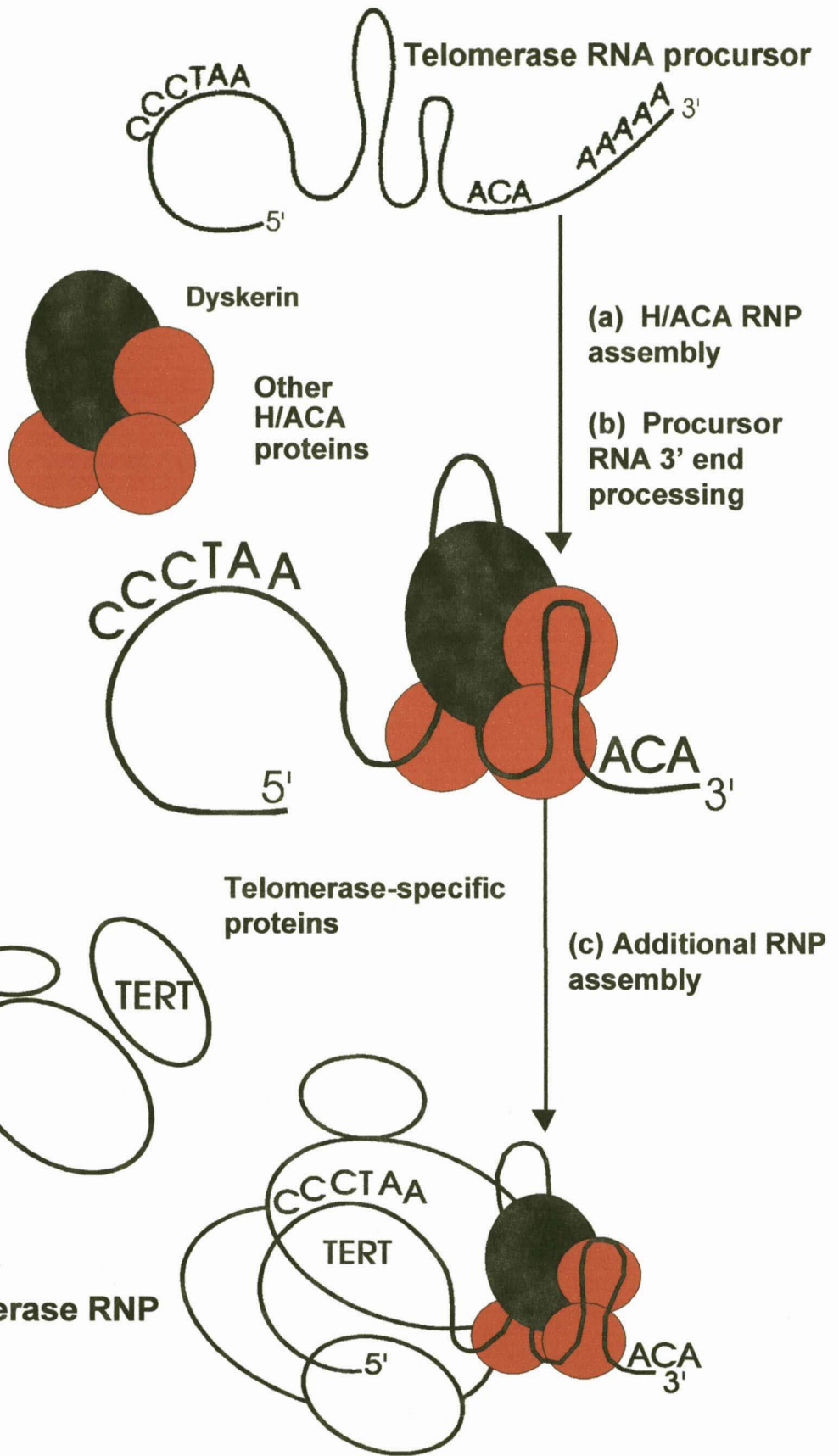
A step-wise RNP assembly pathway first associates H/ACA proteins with the telomerase RNA H/ACA motif. Processing of the telomerase RNA precursor to form a mature RNA 3' end could occur before or after this initial RNP assembly reaction. Subsequent assembly of telomerase-specific proteins such as TERT and additional shared proteins including molecular chaperones would complete the telomerase RNP assembly process.

**Additional shared telomerase proteins**



**Assembled telomerase RNP**

Collins (2000)



**Figure 2.7 A model for mammalian RNP assembly.**

Some cells will adopt a telomerase-independent alternative telomere maintenance mechanism (ALT) (Colgin and Reddel, 1999). The induction of ALT in human cells appears to be rare, consistent with a stimulatory role for telomerase activation in increasing the tumorigenicity of oncogenically transformed cells (Hahn *et al.*, 1999b). ALT has been identified in about one third of cell lines immortalized *in vitro* using simian virus 40 (SV 40), human papillomavirus (HPV) and chemical carcinogens or spontaneously immortalized cells. ALT has recently been detected in four tumor-derived cell lines and a small number of tumor samples, indicating that ALT is a phenomenon involved in *in vitro* tumor development (Bryan *et al.*, 1997). The mechanism of telomere lengthening in ALT is currently unknown. It does not appear to be due to a burst of telomerase activity that has subsequently been switched off, since a gradual increase in telomere length in two ALT cell lines occurred in the absence of telomerase activity (Bryan *et al.*, 1995). Bryan *et al.* (1997) conclude that the ALT mechanism is not dependent on the expression of hTR, and does not involve mutations in the hTR sequence.

Recent findings provide the first evidence that telomerase activity is required for health and viability within a single human lifespan (Mitchell *et al.*, 1999b). Single amino-acid substitutions in dyskerin are responsible for DKC, a disease characterized by anaemia, immune deficiency, skin and nail lesions, chromosomal instability and cancer (Dorak, 1996).

## 2.5 TELOMERASE ACTIVITY

Most normal tissues lack telomerase activity, while at least 90% of primary human tumors exhibit significant telomerase activity (Table 2.1). Since activation of telomerase occurs in most cancers and is viewed as a critical event in the multistep process of human carcinogenesis, telomerase could be a target for cancer diagnosis and anticancer therapy. It provides the basis for speculation that telomerase inhibitors might constitute a new class of antitumor agent. Since nearly all tumors express telomerase activity, such inhibitors might serve as universal anticancer agents and since most normal cells lack telomerase activity, these agents might act specifically on tumor cells. Finally, telomeres of tumors are close to the critical minimum length, so even a partial inhibition of telomerase activity might profoundly affect tumor cell viability.



Table 2.1 Telomerase activity in human tumor tissue

Tumor type	Tested positive	Tested Total	%	References
Acute myeloid leukemia	8	12	67	Raymond <i>et al.</i> , 1996
Acute lymphoid leukemia	18	25	72	Raymond <i>et al.</i> , 1996
Chronic myeloid leukemia	12	12	100	Raymond <i>et al.</i> , 1996
Chronic lymphoid leukemia	5	9	55	Raymond <i>et al.</i> , 1996
Myeloma	1	1	100	Raymond <i>et al.</i> , 1996
Low-grade lymphoma	2	3	67	Raymond <i>et al.</i> , 1996
High-grade lymphoma	9	9	100	Raymond <i>et al.</i> , 1996
Breast	130	140	95	Hiyama <i>et al.</i> , 1996
Prostate	21	25	84	Sommerfeld <i>et al.</i> , 1996
Lung	88	115	76	Raymond <i>et al.</i> , 1996
Colon	22	23	96	Hastie <i>et al.</i> , 1990; Vogelstein <i>et al.</i> , 1988
Ovarian	13	14	93	Counter <i>et al.</i> , 1994a
Head and neck	14	16	87	Kim <i>et al.</i> , 1994; Voravud <i>et al.</i> , 1993
Kidney	41	55	74	Kim <i>et al.</i> , 1994
Melanoma	7	7	100	Kim <i>et al.</i> , 1994
Neuroblastoma	76	79	96	Brodeur, 1995
Glioblastoma	38	51	75	Langford <i>et al.</i> , 1995
Oligodendroglioma	19	19	100	Langford <i>et al.</i> , 1995
Bladder	72	75	96	Muller <i>et al.</i> , 1996
Basal cell carcinoma	73	77	95	Taylor <i>et al.</i> , 1996
Nonpolyposis colorectal	10	13	77	Li <i>et al.</i> , 1996
Hepatocellular carcinoma	28	33	85	Kim <i>et al.</i> , 1994
Gastric	56	66	85	Kim <i>et al.</i> , 1994
<b>TOTAL</b>	<b>763</b>	<b>879</b>	<b>87</b>	

Blasco *et al.* (1996) have studied telomerase activity and telomere regulation in two mouse models that permit the precise analysis of the multi-stage development of cancer. In one model, expression of a transgenic viral oncogene sets into motion a well-defined series of events that culminate in pancreatic islet cell carcinoma whereas in the second, the end-stage cancer is squamous cell carcinoma of the skin (Blasco *et al.* 1996). They followed the expression of the RNA component of telomerase as well as the activity of the telomerase. Both the expression of the RNA component and the telomerase activity increase in preneoplastic and early stage tumor, and increase further in the late stage tumors. By contrast, telomerase activity is evident only in end stage tumors in humans (Axelford, 1996). The discrepancy between RNA expression and telomerase activity during tumorigenesis could be quite complex and confirms and extends an earlier finding that telomerase RNA is expressed in several normal human cells and tissues that lack telomerase activity (Feng *et al.*, 1995). However, it should be noted that possible differences in the structure and length of human and mouse telomeres raise some doubts, about the value of mouse models for the study of human cancer and it remains to be seen whether the findings concerning mice are applicable to humans (de Lange, 1995).

The association of the end replication problem with DNA replication has led to the assumption that telomerase synthesizes telomeres during the S phase of the cell cycle. In *Saccharomyces cerevisiae*, single-stranded G-rich overhangs appear late in the S-phase of the cell cycle, suggesting that late S phase is the time when telomerase is actually active (Wellinger *et al.*, 1993). Currently, little is known about the regulation of telomerase activity in vertebrates. If telomerase synthesizes telomeric repeats only during the S phase, a variety of mechanisms may be involved in the regulation of telomerase during the other phases of the cell cycle. For instance, it is possible that telomerase is expressed and active only during the S phase when telomeres are replicated, or is present during all phases of the cell cycle, but is able to add repeats only during S phase, or is continuously synthesizing telomeric DNA throughout the cell cycle.

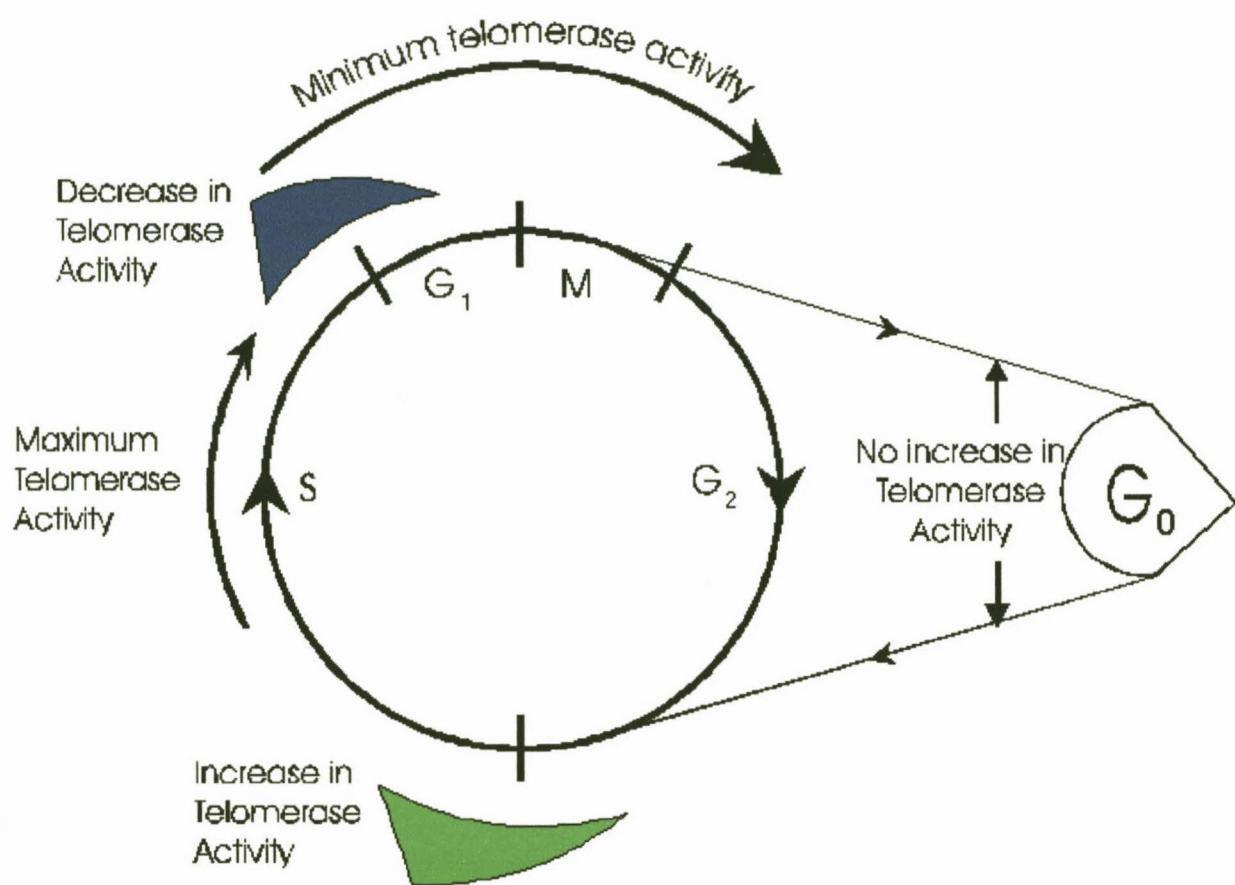
Zhu *et al.* (1996) demonstrated that in tumor cell lines, telomerase activity changes as cells progress through the cell cycle. Withdrawal from the cell cycle by serum deprivation did not cause a reduction of telomerase activity. Quiescent cells do retain telomerase activity, which finding is consistent with the recent detection of telomerase activity in noncycling

hematopoietic cells, including leukocytes from peripheral blood, cord blood and bone marrow (Counter *et al.*, 1995) and resting T and B lymphocytes (Broccoli *et al.*, 1995).

Telomerase activity has been observed during both the S and M phases of the cell cycle in *Xenopus* oocytes (Mantell and Greider, 1994), suggesting that telomerase activity is not restricted exclusively to the S phase.

If telomerase is required for the maintenance of telomeres, it must be active during the DNA-replication stage (S phase) but not necessarily in the nonreplicative phase of the cell cycle, for example, the G<sub>2</sub>/M phase. As cells progress through the G<sub>1</sub>/S phase of the cell cycle, telomerase activity gradually increases (Fig 2.8). The highest levels of telomerase activity are present at the replicative S phase, with a virtual loss of activity at the G<sub>2</sub>/M phase. However, withdrawal from the cell cycle did not cause a reduction of telomerase activity, and reentry of quiescent cells into the cell cycle mediated by cytokines and growth factors did not cause any further increase in telomerase activity. The lack of telomerase activity in tumor cells synchronized at the G<sub>2</sub>/M phase is striking. Two different G<sub>2</sub>/M blockers (nocodazole and doxorubicin) caused a complete abolition of telomerase activity (Zhu *et al.*, 1996). Although mixing experiments with G<sub>2</sub>/M extracts did not reveal the presence of an inhibitory molecule(s), it is tempting to speculate that one or several proteins that are expressed and/or activated during the G<sub>2</sub>/M phase may inhibit telomerase activity. Telomerase activity was inhibited by TGF- $\beta$ 1, a G<sub>1</sub>/S phase blocker (Zhu *et al.*, 1996), in comparison to the thymidine/aphidicolin-mediated G<sub>1</sub>/S block, which showed no effect. Similarly, methotrexate- and 5-fluorouracil-mediated S phase block (Zhu *et al.*, 1996) caused inhibition of telomerase activity, whereas a hydroxyurea-mediated S phase block did not. These results suggest that inhibition of telomerase activity by these agents is complex and may be indirect. The results of cell cycle-dependent regulation of telomerase activity are consistent with the hypothesis that telomeric DNA synthesis, telomere structure and telomerase function are subject to cell cycle regulation.

In the model proposed by Shay *et al.* (1991) a cell must bypass two checkpoints, viz. Mortality stage 1 (M1) and Mortality stage 2 (M2), before immortalization. M2 corresponds with the stage of proliferative crisis and during M2 activation of telomerase might occur, leading to synthesis of TTAGGG repeats and a balanced compensation of the end replication



Zhu et al. (1996)

**Figure 2.8 Proposed model for cell-cycle regulation of telomerase activity.**

problem. This might be an essential and rate-limiting step in the immortalization process of a tumor cell.

To gain insight into the regulation of telomerase in cell lines and tumors, Avillion *et al.* (1996) compared the levels of telomerase activity to the levels of human telomerase RNA (hTR). Whereas many cell lines and tumors had both increased hTR and telomerase activity, they found that hTR was present in cell lines and tissues that lacked telomerase activity. This indicates that the RNA is not limiting for telomerase activity and that the RNA component is not a good predictor of the presence of enzyme activity. The presence of telomerase RNA in cell cultures that lack telomerase activity suggests that telomerase is regulated at several different levels. The lack of activity in cells that contain high levels of hTR may be due to the absence of a protein component of telomerase or to specific down-regulators of telomerase present in primary cells and tissues (Ohmura *et al.*, 1995). Cell fusion experiments between telomerase-positive immortal cells and telomerase-negative immortal or normal cells have shown that telomerase activity is down-regulated in at least some cases, suggesting that a trans-repression activity may be present in telomerase-negative cells (Ohmura *et al.*, 1995; Bryan *et al.*, 1995; Wright *et al.*, 1996). This trans-acting repression could act directly on the availability of one or more telomerase polypeptides or it may represent a direct repressor of the telomerase enzyme activity.

Tatematsu *et al.* (1996) proposed that the different patterns of telomere dynamics observed in their study on leukemias are related to the competency of cancer as to clonal evolution through chromosomal dynamics. Short telomeres with low telomerase activity, found in chronic phase CML (chronic myelogenous leukemia) and early stage AML (acute myelogenous leukemia), lead to chromosomal instability. Thus, in these cases, the chance for the disease to progress through chromosomal changes, such as deletions inactivating anti-oncogenes, is low. In contrast, when telomere shortening is accompanied by sufficient telomerase activity, aberrant chromosomes can be rescued from broken chromosomes and can survive. In the latter situation, typically found in CML crisis and advanced stage AML, the cancer cells can undergo efficient clonal evolution towards more malignant phenotypes.

Many *in vitro*-immortalized human cell lines, however, lack telomerase activity and all have very long and heterogeneous telomeres, up to about 20-50 kb (Kim *et al.*, 1994; Murnane *et*

*al.*, 1994; Bryan *et al.*, 1995), distinct from those of telomerase-positive lines. This suggests that their telomeres have been lengthened by a novel mechanism, which has been termed ALT (Alternative Lengthening of Telomeres) (Bryan and Reddel, 1997).

## 2.6 TELOMERASE ACTIVITY ASSAY

*In vivo* human telomerase activity was first detected in ovarian carcinoma cells growing in suspension and in human hematopoietic B-cell neoplasias (Counter *et al.*, 1994b; Nilsson *et al.*, 1994) using the conventional primer-extension telomerase assay (Greider and Blackburn, 1985). A conventional assay for telomerase activity requires only a single-stranded (TTAGGG)<sub>n</sub> DNA primer mimicking the chromosome end, nucleoside triphosphates and the telomerase enzyme (Shay *et al.*, 1994). The low sensitivity of the conventional telomerase assay and the requirement for large amounts of tissue have made it difficult to detect telomerase activity in solid tumors.

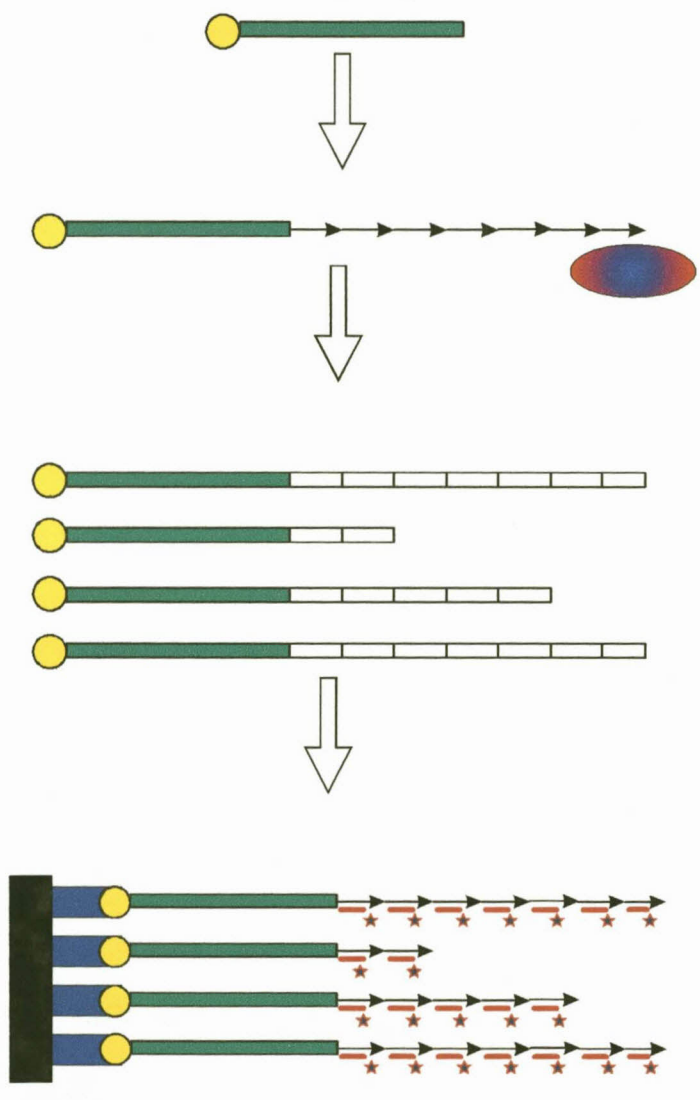
Kim *et al.* (1994) developed a procedure consisting of a detergent-based extraction and a PCR-based assay designated TRAP (Telomeric Repeat Amplification Protocol). The simplicity and 10<sup>4</sup> – fold increased sensitivity of this assay has resulted in a dramatic growth in investigations of telomerase expression. Using the TRAP assay, telomerase activity has been detected in high frequency in various tumor types.

Shay and Wright (1996) have developed a highly sensitive, PCR-based assay for TRAP to measure telomerase activity, in collaboration with scientists at the Geron Corporation (Menlo Park, CA), that includes an improved detergent lysis method to allow a more uniform extraction of telomerase from small number of cells.

The conventional TRAP assay achieves full sensitivity only when performed with a hazardous radioactive label and visualization of results requires time-consuming gel electrophoresis and autoradiography. The new Telomerase PCR ELISA combines a one-step/one tube TRAP assay with non-radioactive detection in a highly sensitive photometric ELISA (see Fig 2.9). The Telomerase PCR ELISA, developed by Boehringer Mannheim, is an extension of the original method described by Kim *et al.* (1994). It allows highly specific amplification of

**T**elomeric  
**R**epet  
**A**mplification  
**P**rotocol

**P1**



- ◆ Biotinylated substrate
- ◆ Extended by telomerase in extract
- ◆ PCR using P1 and a repeat reverse primer
- ◆ Denature, capture biotinylated strands on streptavidin-coated microtitre plates.
- ◆ Hybridize with DIG labeled repeat probes.
- ◆ Develop colour reaction, read.

**Figure 2.9 The TRAP Assay for telomerase activity**

telomerase-mediated elongation product combined with non-radioactive detection following an ELISA protocol. The assay can be separated into the following steps:

#### *Elongation/amplification:*

In the first step, telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin-labeled synthetic P1-TS-primer. In the second step, these elongation products are amplified by PCR using the primers P1-TS and P2, generating PCR products with the telomerase-specific 6 nucleotide increments. As opposed to other TRAP assay formats, the Telomerase PCR ELISA contains all compounds required for the telomerase reaction and PCR in a ready-to-use reaction buffer for combining both reactions in one-step/one-tube reaction. An additional advantage over the conventional assay is the use of optimized primer sequences eliminating the need for hot-start PCR or separation of the primers by wax barrier and this avoids amplification artifacts, such as primer dimers.

#### *Detection by ELISA:*

An aliquot of the PCR product is denatured and hybridized to a digoxigenin-(DIG)-labelled, telomeric repeat-specific detection probe. The resulting product is immobilized via the biotin-labelled primer to a streptavidin-coated microtiter plate. The detection probe and the hybridization conditions have been optimized for obtaining the highest specificity and sensitivity. The immobilized PCR product is then detected with an antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxidase. Finally, the probe is visualized by virtue of peroxidase metabolizing TMB to form a coloured reaction product.

### **2.6.1 PROBLEMS WITH THE TELOMERASE ACTIVITY ASSAY**

Although the development of the PCR-based telomerase assay is capable of analyzing small tissue biopsies and has permitted a large number of tumor samples to be analyzed, several problems remain: 1) the assay is non-linear, making statements about relative levels of activity difficult; 2) some samples may contain inhibitors of Taq polymerase and thus give a false negative result using the standard 6 µg of protein extract per assay but a positive result when diluted 10- to 100-fold (Piatyszek *et al.*, 1995; Hiyama *et al.*, 1995); 3) The necessity of preparing a cell or tissue extract limits the application of the technique to small, but not microscopic samples. These difficulties have largely been overcome with recent protocol



modifications (Wright *et al.*, 1995). The inclusion of a 150-bp internal standard in the assay, which is sufficiently long so that it appears as a single band on the gel and does not interfere with the visualization of the amplified telomeric fragments, results in more precise quantification of telomerase activity. The intensity of the telomerase ladder compared with that of the internal standard permits the assay to become highly linear, allowing for accurate comparisons between samples. The inclusion of the internal standard also immediately identifies false-negative results caused by Taq-polymerase inhibitors.

## 2.7 WILL INHIBITION OF TELOMERASE ACTIVITY RESULT IN CANCER REMISSION?

Because telomerase activity appears necessary for the sustained proliferation of cancer cells, anticancer agents based on telomerase inhibition may potentially provide an effective therapy, possibly with limited side-effects. In addition, because telomerase appears to be required for telomere maintenance, the enzyme may be a novel target in cancer cells (Harley *et al.*, 1995). However, there are potential risks inherent in the use of such therapy that must be considered, for example, the effects of inhibitors on telomerase expressing stem cells (Rhyu, 1995).

It is likely that this approach will be less toxic than conventional chemotherapy, which affects all proliferating cells, including stem cells. Thus, some of the side effects of standard chemotherapy such as nausea and hair loss may be reduced by the use of telomerase inhibitors. Another concern regarding this proposed treatment regimen is the prolonged time potentially required for the telomerase inhibitor to be effective (Harley *et al.*, 1995). Because the mode of action of telomerase inhibitors may require telomeric shortening before inhibition of cell proliferation, there may be a significant delay in effect. Thus, methods may have to be devised to increase the rate of telomere shortening when telomerase inhibitors are used therapeutically. Telomerase inhibitors will most likely be used together with or following conventional therapies to prevent the regrowth of resistant cancer cells. They may also be used in early stage cancer to prevent overgrowth of metastatic cells.

## 2.8 TELOMERASE AS A DIAGNOSTIC TOOL FOR CANCER DETECTION

Besides the potential therapeutic benefits of telomerase inhibitors, telomerase activity may also be useful in cancer diagnosis and serve as a prognostic indicator of clinical outcome. An assay for telomerase activity may also be very useful as a screen for the detection of early cancer or early metastasis, assuming that metastatic tumor cells can be distinguished from blood-derived stem cells. For cancer patients, early detection of tumors having telomerase activity could lead to more aggressive adjuvant chemotherapy (e.g., the use of telomerase inhibitors when the patient has a low tumor burden). Low doses of telomerase inhibitors for a long-term treatment of cancer patients to prevent metastases and routine screening of peripheral blood for the presence of telomerase as an early means of tumor detection, are optimistic expectations.

Tumor cells with active telomerase can maintain their telomere lengths, whereas tumor cells lacking telomerase activity should lose telomeric sequences with each round of cell division due to the end replication problem. If telomerase-negative tumor cells have no other means of compensating for this loss, then their growth should be self-limiting and patients with such tumors may not require the most aggressive forms of therapy. This hypothesis is supported by the work of Hiyama *et al.* (1995) in which it was observed that spontaneous regressions in neuroblastoma cases correlated with telomerase-negative status.

Koyanagi *et al.* (1999) suggested that measurements of telomerase activity in normal epithelium of patients with SCCE are a highly sensitive method of detecting the microinvasion of cancer cells. Telomerase activity could be used before operation to define appropriate treatment for a patient with esophageal SCC. If telomerase activity was detected in biopsy samples of normal epithelial regions obtained from a patient with SCCE by preoperative endoscopic examination, the patient might be at high risk of metastases and could benefit from a combination of radical lymph node dissection and adjuvant systemic chemotherapy (Koyanagi *et al.*, 1999).

## 2.9 *In vitro* AND *in vivo* MODELS TO STUDY DRUGS THAT TARGET TELOMERASE AND TELOMERES

Several observations suggest that specific models should be considered to study the anticancer effects of telomerase inhibitors. Cytotoxicity and telomere length have initially been selected to evaluate the antiproliferative effects of telomerase- and telomere-targeting agents, because telomere length is maintained in tumor cells by telomerase. It has been calculated that more than 20 cell-population doublings would be necessary before chromosome instability causes cancer cell death (Kipling, 1995). Traditional methods of evaluating the growth inhibitory activity of anticancer drugs may not be applicable to telomerase inhibitors, which require a very long-term culture before any biological effects are observed. Unspecific cytotoxicity is likely to occur before telomere shortening. The cytotoxicity assay remains a very interesting way to determine candidate compounds that target DNA by mechanisms other than telomerase inhibition or telomere interaction (Kipling, 1995).

Telomere-length measurements are subject to important variations due to the heterogeneity of cancer cell populations and will require a long-term culture to observe significant shortening. Several cytogenetic anomalies have been observed in tumor cells in relation to telomeric degradation (Counter *et al.*, 1992; Park *et al.*, 1992).

The antitumor effects of telomerase- and telomere-targeting drugs represent other challenges. Chadeneau *et al.* (1995) have determined the telomerase activity in malignant tissues in transgenic mice. Telomerase in mice tumor cells seems to possess nonprocessive characteristics distinct from human telomerase. This could be a limiting factor in the study of telomerase inhibitors in transgenic tumor models (Maine *et al.*, 1995; Bednarek *et al.*, 1995; Bacchetti *et al.*, 1995; Burger *et al.*, 1996). Human xenografts in athymic mice would be closer to clinical situations and would allow the selection of tumors that demonstrate high telomerase activity *in vitro*.

## 2.10 TELOMERASE INHIBITORS

One strategy for identifying telomerase inhibitors is based on the design of inhibitors exploiting the properties of telomerase or on a concomitant screening of a library of compounds interacting with DNA, RNA and proteins. There are several rational approaches including the strategies presented in Table 2.2.

There is evidence suggesting that some advanced tumors are not composed of immortalized cells (Eddington *et al.*, 1995). Therefore, primary resistance to telomerase inhibitors is initially likely to occur in these non-immortalized telomerase-negative tumor cells (Kim *et al.*, 1994). The TRAP assay is able to detect less than one positive cell in one thousand. With this assay, telomerase-negative tumors account for about 15% of human tumors (Counter *et al.*, 1992). In tumors showing telomerase activity, variations of the signal using a semi-quantitative TRAP assay suggest that the number of telomerase-positive cells varies markedly from one tumor to another (Raymond *et al.*, 1996). Considering that some tumors are composed of both telomerase-positive and negative cells, initial debulking by conventional therapy would be required in the design of clinical trails with telomerase inhibitors. Other concerns are the secondary mechanisms of resistance, including the multidrug-resistance mechanisms that could develop in tumor cells exposed to telomerase inhibitors. Another major problem could be the ability of tumor cells to solve the problem of telomere shortening by mechanisms that do not involve telomerase, for example, by DNA recombination (Ezzell, 1995). This was initially proposed in *Saccharomyces* (Wang and Zakian, 1990; Lundblad and Blackburn, 1993) and *Drosophila* (Levis *et al.*, 1993; Biessmann *et al.*, 1990), and it has been recently suggested in immortalized human tumor cells that maintain their telomere length without telomerase activity (Bryan *et al.*, 1995).

## 2.11 TELOMERASE TARGETING AGENTS

### 2.11.1 *The DNA component: G-quartet interactive compound*

Because of its unique mode of replication and its special G-rich structure, telomeric DNA has been described as the Achilles heel of chromosomes. A large variety of chemicals may interact with varying degrees of specificity with this region of the chromosome. Direct

Table 2.2 Telomeres and telomerase as targets for new drug discovery.

Agents	Targets under investigation	Strategy
Telomerase-interacting compounds	<p>RNA component</p> <p>Indirect inhibition</p> <p>Protein components?</p> <p>Telomerase-gene modulation?</p>	<ul style="list-style-type: none"> <li>◦ Antisense strategy</li> <li>◦ Targeting RNA components with antibiotics</li> <li>◦ Oligonucleotides that mimic telomere sequences</li> <li>◦ Modification of telomere structures</li> <li>◦ Reverse transcriptase inhibitors</li> <li>◦ ?</li> </ul>
Telomere-interacting compounds	<p>Alkylation of telomeric DNA repeats</p> <p>G-quartet</p> <p>The nucleoprotein complex?</p>	<ul style="list-style-type: none"> <li>◦ ?</li> <li>◦ Nucleoside analogs 7-deaza-dGTP</li> <li>◦ Intercalation of chemical into the telomere</li> <li>◦ G-quartet structure</li> <li>◦ ?</li> </ul>

telomere-targeting would lead to rapid destabilization of chromosomes and presumably interact with telomerase and various telomerase-independent mechanisms that maintain telomere length in the tumor cells.

The (TTAGGG)<sub>n</sub> repeats that characterize telomeric DNA can form a secondary structure in the form of a tetraplex held together by G-quartets (Fig 2.10). G-quartets may contribute to the regulation of telomere length *in vivo* through the direct inhibition of telomerase binding to long telomeric sequences or through the regulation of the dissociation of telomerase from telomere DNA. Thompson *et al.* (1996) and Salazar *et al.* (1996) developed several G-quartet-interacting agents. They showed high affinity for the structure and formed stable complexes that may inhibit the proper recognition of telomeric DNA by telomerase. Some promising compounds have been selected for their ability to inhibit telomerase activity *in vitro* and are currently under investigation *in vivo* (Thompson *et al.*, 1996; Salazar *et al.*, 1996). Fletcher and Chen (1996) used telomerase to incorporate 7-deaza-dGTP, a nucleoside analog, into telomeric DNA. This strategy was associated with telomerase inhibition and cell-growth inhibition. In this model, telomerase inhibition was thought to result in the inhibition of the translocation step because 7-deaza-dGTP lacks the 7 nitrogen, which is essential for the formation of G-quartets.

### **2.11.2 The nucleoprotein complex**

Although telomeres could be considered distinct targets for developing specific inhibitors, the complex of telomerase and telomere structural proteins could be considered as a nucleoprotein complex that could be studied in order to understand the biological effects of telomerase- and telomere-targeting compounds (Blackburn, 1991). For a long time, an interaction between telomeres and the nuclear matrix has been suggested by the position held by chromosome ends in the interphase nuclei. The direct evidence that telomerase is anchored to the nuclear matrix via the (TTAGGG)<sub>n</sub> repeats was provided by de Lange (1992). This study provides further evidence that the TTAGGG sequence by itself maintains the nuclear matrix association, but requires anchorage proteins. Those proteins are likely to provide additional protection against exonucleic degradation of chromosome ends and could be essential for proper chromosome segregation and, therefore, nuclear division (Chong *et al.*, 1995). Further characterization of that process would provide interesting new targets for drug development.

The (TTAGGG) repeats that characterize telomeric DNA can form secondary structures in the form of a tetraplex held together by G-quartets. G-quartet-interacting agents stabilize this structure and inhibit the proper recognition of telomeric DNA by telomerase.

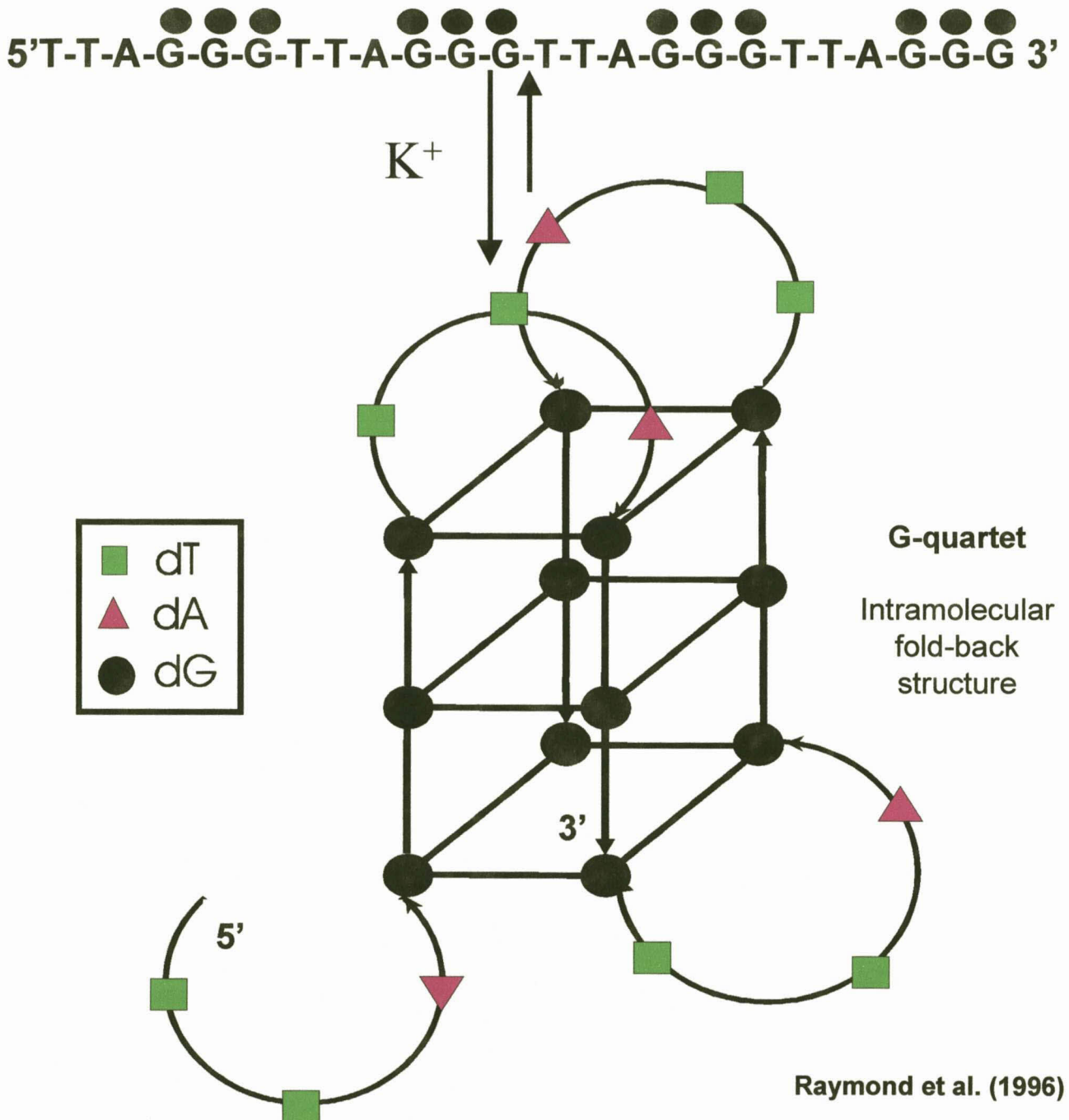


Figure 2.10 Structure of G-quartets.

## 2.12 MOLECULAR TARGETING OF CANCER: TELOMERES AS TARGET

In recent years, a new generation of cancer therapies has emerged, based on a growing understanding of the molecular events that contribute to malignant transformation and which therefore represent targets for selectively killing or inhibiting growth of malignant cells. In this context, there has been considerable interest in elucidating mechanisms that regulate the capacity for cell division in normal, as well as transformed cells, and substantial attention has focused specifically on the role of telomeres as the "mitotic clock" that mediates replicative capacity (Hodes, 2001).

An alternative strategy for cancer treatment has been directed (Fig 2.10) not at inhibition of telomerase enzymatic activity, but rather at targeting the immune recognition and destruction of cells that express telomerase. It has been suggested that telomerase activity *in vivo* be determined primarily by regulated expression of TERT and that telomerase-expressing tumor cells, but not telomerase-negative cells, express TERT protein. Immune responses, specifically cytotoxic T cell responses, have been generated against peptide sequences of the TERT protein. It has been demonstrated that these cytotoxic T cells are capable of selectively lysing target cells that express TERT protein and therefore present recognizable TERT target peptide in the context of cell surface class I MHC molecules (Minev *et al.*, 2000). In principle, this approach would result in rapid immune elimination of telomerase-expressing tumor cells without the lag involved in strategies that inhibit telomerase function and depend on gradual telomere shortening to inhibit tumor growth (Fig 2.10). Kim *et al.* (2001) worked on the third approach to target telomeres in cancer cells. They have concluded that expression of mutant telomerase template RNA inhibits tumor cell proliferation by a mechanism that does not involve either telomere shortening or inhibition of telomerase activity.

## 2.13 FUTURE PROSPECTS

Our understanding of the biology, epidemiology and clinical management of cancer of the esophagus continues to evolve. The changing epidemiology of this disease should continue to provide new areas of investigation into the biology and etiology of esophageal cancer. The clinical application of molecular understanding of esophageal cancer is still in its infancy.



Nonetheless, there is significant promise for translating these developments into screening and diagnosis and areas of treatment and prevention.

Identification of telomerase inhibitors and their use in clinical trials are also imminent. The pathways connecting telomere shortening, cellular senescence, telomerase expression and human cancer are presently under intense investigation and many surprises are likely to emerge. The manipulation of telomere length may change the rate of aberrant gene expression associated with aging or other degenerative disease processes. Simply slowing the rate of telomere shortening in normal cells and tissues may also profoundly affect the age of onset and severity of many age-associated illnesses. In conclusion, telomerase fulfills many of the criteria of an ideal target for cancer therapy, and although there are still many unanswered questions, the optimism surrounding the telomere hypothesis appears justified (Morin, 1995).

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## CHAPTER 3

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### TELOMERASE ACTIVITY IN ESOPHAGEAL CANCER

	PAGES
3.1 INTRODUCTION	63
3.2 ARTICLE	65
3.3 RESULTS EXCLUDED FROM ARTICLE	79
3.4 DISCUSSION	84
3.5 REFERENCES	88

## CHAPTER 3

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### TELOMERASE ACTIVITY IN ESOPHAGEAL CANCER

#### 3.1 INTRODUCTION

During the last number of years a variety of molecular lesions have been noted in SCCE tumors, among others p53, p16, cyclin D1, c-myc and RB1, and their prognostic values are being investigated (Gamielden *et al.*, 1998; Maesawa *et al.*, 1994; Nishihira *et al.*, 1993). Because of the high incidence of this disease and the generally dismal outcome, additional prognostic markers are in great demand.

The maintenance of telomeric repeats throughout many cycles of cell division requires telomerase, a telomere-specific ribonucleoprotein reverse transcriptase (Nugent and Lundblad, 1998). Telomerase copies a template sequence carried within its integral RNA and adds single-stranded telomeric repeats to the chromosome 3' ends. Single-strand synthesis by telomerase and accompanying complementary-strand synthesis can balance the telomere erosion inherent in incomplete end-replication, allowing telomere length homeostasis. Telomerase is active in foetal cells, but is switched off shortly after birth, except in germline cells, haemopoietic cells, inflamed or regenerating tissue (Hiyama *et al.*, 1995; Taylor *et al.*, 1996; Yasumoto *et al.*, 1996) and a variety of tumors, e.g. head and neck (Mutirangura *et al.*, 1996), prostate (Sommerfeld *et al.*, 1996), breast (Hiyama *et al.*, 1996; Umbricht *et al.*, 1999), bladder (Muller *et al.*, 1996) neuroblast (Hiyama *et al.*, 1995) and esophagus (Takubo *et al.*, 1997; Morales *et al.*, 1998). Some immortalized human cell lines survive by keeping their telomeres above the critical length by an alternative, non-telomerase dependent process, the mechanism of which is not yet fully understood (Bryan *et al.*, 1997). Inhibition of telomerase may be an alternative target for limiting tumor growth and cell line experiments have

produced promising results using a variety of telomerase inhibitors (Hebert *et al.*, 1999; Murakami *et al.*, 1999).

The main focus of this chapter is to examine at the expression of telomerase in esophageal cancer. A number of reports have been published on the role of telomerase in cancer progression and it seems to be finding use as a diagnostic tool (Counter *et al.*, 1994a and b; Hiyama *et al.*, 1995a; Kim *et al.*, 1994). In many cancers, telomerase has previously been found to correlate with disease stage, as well as with prognosis, where high telomerase levels indicate poor prognosis. The telomerase status of squamous cell carcinoma of the esophagus was unknown at the start of this project and we would like to fill that gap in our knowledge. The aim of this chapter is to correlate telomerase activity with disease stage, histological classification and outcome. The previously reported studies of SCCE tumors were on Japanese patients (Takubo *et al.*, 1997). This study reports an investigation of telomerase activity of SCCE tumors of South African origin and its possible prognostic significance in a third-world setting.

This chapter will be presented as an article with an appendix. The appendix contains data excluded from the article, and an additional discussion. The excluded data from the article concerns the patients without histological information or follow-up information for survival analysis. We also received esophageal biopsies from Tygerberg hospital. These biopsies were also excluded from the article because we have no histological information or follow-up information on these patients.

## 3.2 ARTICLE

# Telomerase activity and survival of South African esophageal carcinoma patients

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**Short title:** Telomerase and survival

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## Abstract

Squamous cell carcinoma of the esophagus is a cancer with a high incidence in South Africa. We have investigated the prognostic value of telomerase activity in tumors as well as in nearby normal tissue. Biopsies from 98 patients (71 males and 27 females) were analyzed using an adaptation of the TRAP assay. We found all tumor biopsies to have moderate to high telomerase activity, while one third of biopsies from normal mucosa were negative. The telomerase activity level of the tumors had no prognostic value ( $P=0.95$ ) as determined by the log rank test. A  $P$ -value of 0.02 was found when the telomerase-negative and moderately positive normal biopsies were grouped together and compared to those with high activity. Our results show that telomerase activity of normal mucosa in the vicinity of the tumor can identify a population of patients with significantly worse prognoses, even in late stage patients.

Key words: epithelium, gastroscopy, field cancerization

## Introduction

Squamous cell carcinoma of the esophagus (SCCE) is one of the most prevalent cancers in South Africa [1,2]. The epicentre of the disease is along the eastern seaboard, that is the Eastern Cape/KwaZulu-Natal Provinces, although it occurs all over the country at a lower level [2]. Various factors have been suggested to be responsible for the high incidence, e.g. maize contaminated with mycotoxins [3], nutritional deficiencies [4] and soil fertility factors [5], but at this stage a clear answer is not yet emerging. During the last number of years, a variety of molecular lesions have been noted in SCCE tumors, among others in p53, p16, cyclinD1, c-myc and RB1, and their prognostic values are being investigated [6,7,8]. A national effort is underway to study this disease in the hope of eradicating it eventually. Due to the high incidence of the disease and the generally dismal outcome, additional prognostic markers are in great demand.

Telomeres are specialized structures found at the ends of linear chromosomes, functioning to prevent interchromosomal recombination and enabling maintenance of the extreme ends [9] of the chromosomes. This is accomplished by telomerase, a reverse transcriptase containing an integral RNA molecule used as template for extending the telomeric repeat structure. Telomerase is active in foetal cells, but is switched off shortly after birth, except in germline cells, haemopoietic cells, inflamed or regenerating tissue [10,11,12] and a variety of tumors, e.g. head and neck [13], prostate [14], breast [15,16], bladder [17] neuroblast [18] and esophagus [19,20]. Some immortalized human cell lines survive by keeping their telomeres above the critical length by an alternative, non-telomerase dependent process, the mechanism of which is not yet fully understood [21]. Inhibition of telomerase may be an attractive target for limiting tumor growth, and cell line experiments using a variety of telomerase inhibitors have produced promising results [22,23].

Recent data [6,7] may indicate that the molecular basis of the disease in South Africa could be different from that found elsewhere, e.g. Japan, as the mutation profiles of the p53 gene were found to be significantly different in these two populations. The previously reported telomerase studies of SCCE tumors were on Japanese patients [19]. Here we report on our investigation of the telomerase activity of SCCE tumors of South African origin and its possible prognostic significance in a third-world setting.

## Materials and Methods

### *Patients, tissue and cell lines*

Newly diagnosed, untreated patients referred to the weekly gastroscopy clinic at Universitas Hospital, Bloemfontein, for esophageal dilatations, were asked to participate in the study. The time period covered is from the beginning of April 1998 to the end of September 2000. All patients electing to participate signed a consent form and provided demographic detail. The province of origin was taken to be the one where the patient had spent most of his/her life. Clearance for this project was given by the Ethics Committee of the Faculty of Health Sciences of the University of the Free State (ETOVS 45/98). Endoscopic biopsies were taken from the tumor mass (3 biopsies) and at least 2 cm above any sign of abnormal tissue (2 biopsies) before the dilatation procedure. The esophagus epithelial cell lines WHCO1, 3 and 6 were kindly supplied by Prof R Veale of the University of the Witwatersrand, Johannesburg and the SNO cell line was supplied by Highveld Biological Association, affiliated to CANSA, and were grown in RPMI 1640 medium containing 10% foetal calf serum.

### *Sample processing*

Biopsies were individually quick-frozen in liquid nitrogen in 1.5 ml microcentrifuge tubes and stored at  $-70^{\circ}\text{C}$  until analysed. Cultured cells were harvested after trypsin detachment, washed with RPMI 1640 medium and the cell pellets frozen and stored as described for the biopsies.

### *Telomerase activity determination*

Batches of frozen biopsies were thawed on ice and homogenized in telomerase assay buffer [24]. Particulates were sedimented by centrifugation for 5 minutes in a refrigerated microcentrifuge and the protein concentration of the supernatant determined using the BCA reagent (Pierce). One microgram of protein was then analyzed using the Telomerase ELISA kit (Roche Molecular Biochemicals), which is an adaptation of the original TRAP (Telomeric Repeat Amplification Protocol) approach [24], enabling better quantification of telomerase activity than the original method. Briefly, it consisted of incubating the protein lysate with an artificial telomerase template in order for the telomerase to extend it. The elongated products



were then amplified by PCR using biotin-labeled telomere-specific primers. Aliquots of the PCR product were denatured, hybridized to a DIG-labeled telomere repeat-specific probe and bound to streptavidin-coated microtiter plates. The immobilized extension products were detected with peroxidase-conjugated anti-DIG antibodies and TMB as substrate. Optical density was read at 650 nm against a boiled extract as blank and values were grouped as follows: 0-0.2, negative (-); 0.21-0.6, moderately positive (+); higher than 0.61, strongly positive (++)

### ***Statistical analysis***

Kaplan-Meier survival curves were constructed with the aid of the Prism 3.0 (Graphpad) software package. Statistical significance of difference was evaluated by the log rank test with  $P < 0.05$  indicating significance.

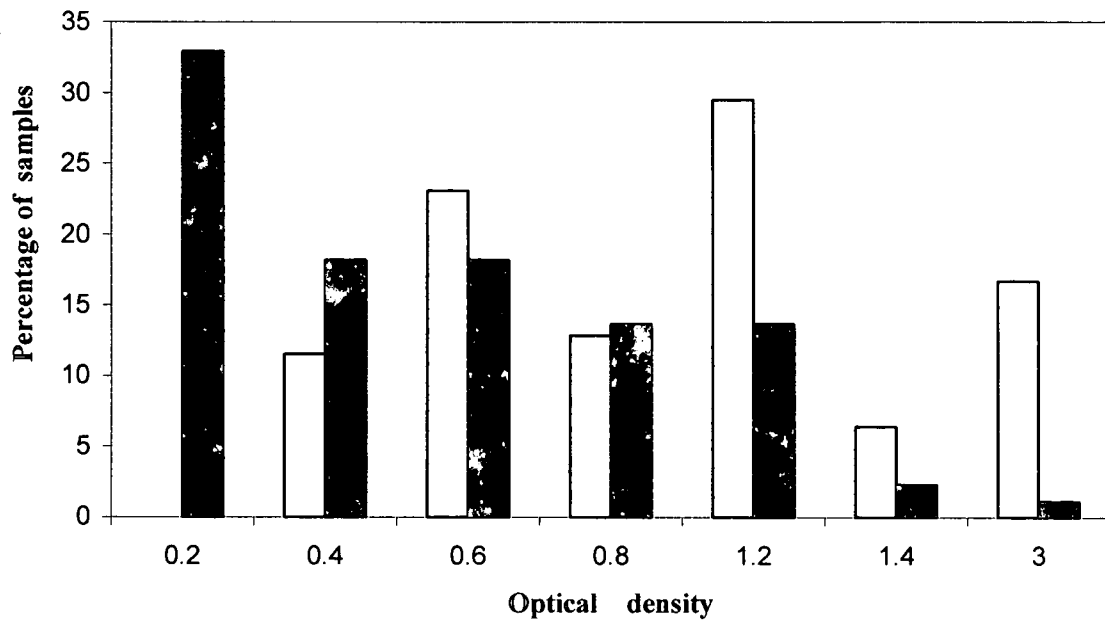
## Results

We have analyzed material from 98 patients in this study. Males were in the majority with 71 (average age 59 years) as opposed to 27 females (56 years), reflecting the gender imbalance of this disease [2]. Most of the patients treated locally came from the Free State (68) or Northern Cape Province (14) with the remainder (16) coming from the rest of the country. All patients had squamous cell carcinoma and the average tumor depth was  $29 \pm 2$  cm, locating it in the central region of the esophagus. Histological analysis of the tumor samples revealed mostly moderately differentiated tissue (48%), followed by poorly differentiated (20%) and well differentiated (12%), while both infiltrating and carcinoma *in situ* stood at 3%. For the remaining 14% of patients no histopathological results were available.

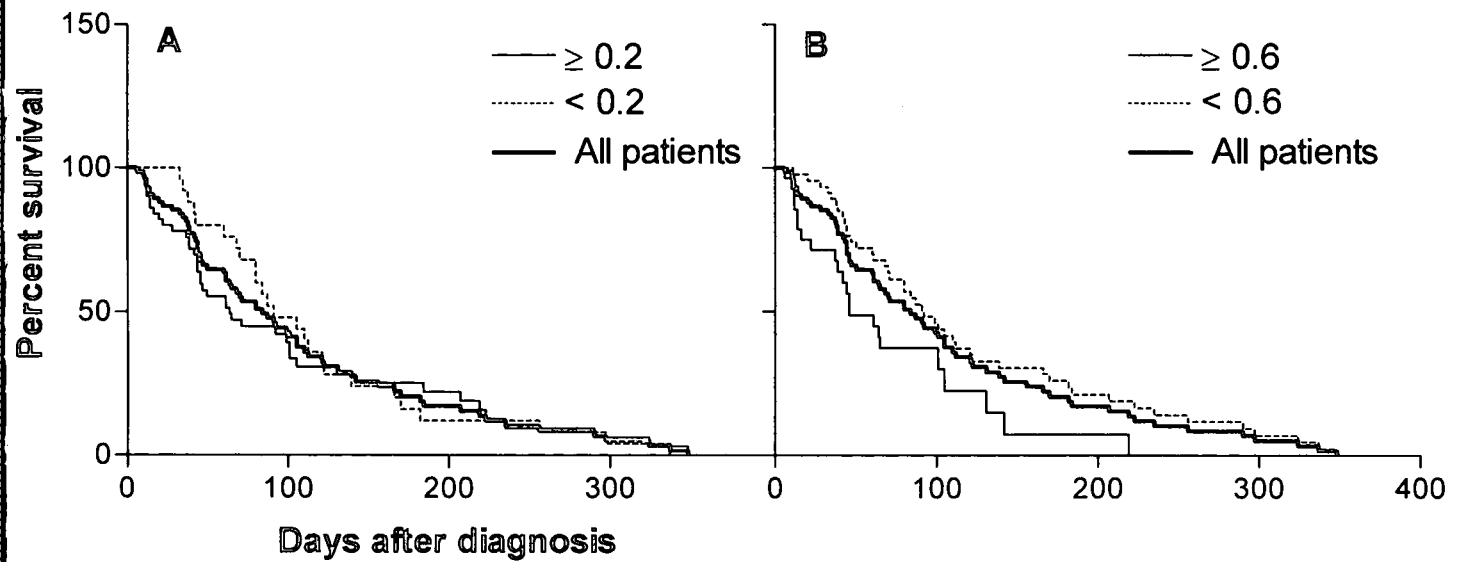
The cell lines had extremely high telomerase activities, probably as a result of selection for being able to sustain proliferation under tissue culture growth conditions (Results not shown). Using serially diluted high activity extracts from the cell lines, we found good linearity of dose response, provided the number of PCR cycles did not exceed 25 (Results not shown). We then proceeded to analyze our biopsies. As in the case of Japanese patients [19], all tumor samples in our study had moderate to high telomerase activity (Figure 1), while the normal tissue samples showed a more even spread of activity levels.

We had enough follow-up information on 75 patients (Table 1) to use their data for survival analysis. The telomerase activity levels of tumor tissue had no discriminating power, as the survival curves were virtually identical (Results not shown;  $P=0.7$ ). We then used the telomerase activity of the normal tissue samples and the resulting survival curves are shown in Figure 2. When the discriminator was set at an optical density of 0.2A, the lines in Figure 2A were obtained. This approach discriminated between telomerase positive and negative samples and was followed by Koyanagi *et al* [25]. In their case, a clear difference was seen between the survival times of patients having detectable vs non-detectable telomerase activity in normal esophageal tissue. However, when we set the discriminator at an optical density of 0.6A to distinguish between high activity and low/moderate activity, the lines began to diverge to a significant extent ( $P=0.02$ ).

**Figure 1.** Distribution of the optical density values of telomerase activity assays. Open bars indicate tumor biopsies while the black bars represent the biopsies taken in normal mucosa above the tumors.



**Figure 2.** Survival curves as a function of telomerase activity. In A the curves are shown for positive ( $OD \geq 0.2$ ) versus negative biopsies taken from normal mucosa, while in B the discriminator has been set at an optical density of 0.6.



**Table 1. Characteristics of patient tissue used for prognostic studies.**

Patient Number	Sex	Origin <sup>1</sup>	Age	Histology <sup>2</sup>	Telomerase Activity <sup>3</sup>		Survival (Days)	Patient Number	Sex	Origin	Age	Histology	Telomerase Activity		Survival (Days)
					Tumor	Normal							Tumor	Normal	
1	M	EC	61	PD	++	++	142	39	F	FS	66	WD	+	++	65
2	M	NC	60	WD	+	-	43	40	M	NC	59	MD	+	-	84
3	F	FS	64	MD	++	+	28	41	M	FS	57	MD	++	-	68
4	M	FS	66	PD	++	-	122	42	M	FS	57	PD	++	++	12
5	F	FS	45	PD	++	++	42	43	M	NW	41	MD	+	-	110
6	F	WC	48	MD	++	+	62+	44	F	FS	49	CIS	+	-	91
7	F	NC	59	WD	++	-	112	45	M	FS	51	PD	++	+	39
8	M	FS	42	NA	++	+	42	46	M	FS	56	MD	+	-	70
9	M	FS	74	NA	++	++	86+	47	M	FS	58	MD	++	+	47
10	F	FS	65	MD	++	+	44	48	M	FS	60	MD	++	+	71
11	F	NC	41	MD	++	-	297	49	M	FS	62	MD	++	++	46
12	M	FS	55	WD	+	-	42	50	M	FS	74	MD	++	++	46
13	M	FS	70	MD	++	+	223	51	M	FS	42	PD	++	++	6
14	M	FS	48	MD	++	+	207	52	M	NP	71	MD	++	++	44
15	M	FS	72	MD	++	+	184	53	M	FS	40	PD	++	+	235
16	M	LE	74	MD	++	++	64	54	M	FS	60	MD	++	++	39
17	M	FS	41	MD	+	-	121	55	M	NC	27	MD	++	+	61
18	M	FS	60	MD	++	++	14	56	M	FS	54	MD	++	++	61
19	M	FS	64	MD	+	-	182	57	F	FS	57	CIS	++	++	35+
20	M	FS	76	MD	++	++	37	58	M	FS	37	PD	++	++	73+
21	M	FS	61	MD	++	+	50	59	M	EC	75	PD	++	++	86+
22	F	FS	51	MD	++	-	337	60	M	FS	63	PD	++	++	12
23	M	FS	74	MD	++	++	91+	61	F	FS	65	WD	++	-	105
24	M	FS	48	MD	++	++	86+	62	F	FS	72	WD	++	+	110
25	M	FS	74	WD	++	++	131	63	F	FS	47	MD	++	+	324
26	M	FS	78	PD	++	+	11	64	F	FS	57	PD	+	-	139
27	M	FS	74	MD	++	++	105	65	M	FS	44	PD	+	-	87
28	F	FS	57	MD	++	+	14	66	M	FS	57	MD	+	+	290
29	F	FS	54	MD	++	++	219	67	M	FS	85	WD	++	+	16
30	F	NC	56	WD	+	-	60	68	M	FS	40	MD	+	-	170
31	F	FS	61	MD	+	+	33	69	M	FS	51	MD	+	-	99
32	M	FS	95	MD	++	++	10	70	M	FS	51	MD	+	+	44
33	F	FS	50	NA	++	-	80	71	M	FS	47	MD	++	+	348
34	M	FS	57	WD	++	-	256	72	M	FS	48	NA	+	-	88
35	M	FS	57	MD	++	++	101	73	M	FS	73	PD	++	++	22
36	M	FS	45	WD	++	+	156+	74	F	FS	58	MD	+	-	80
37	M	FS	64	MD	++	+	92	75	M	FS	46	PD	+	-	166
38	M	FS	65	PD	+	-	35								

1. FS: Free State, NC: Northern Cape, EC: Eastern Cape, WC: Western Cape, LE: Lesotho, NW: Northwestern Province, NP: Northern Province

2. CIS: Carcinoma in situ, MD: Moderately differentiated, PD: Poorly differentiated, WD: Well differentiated, NA: Not available.

3. -: Negative, +: Moderately positive, ++: Strongly positive

## Discussion

Cancer of the esophagus is one of the major causes of death in South Africa, especially among African males. In this study we have evaluated telomerase activity not only as a prognostic marker, but also to learn more about the characteristics of telomerase expression in squamous epithelial tumors of the esophagus. At the outset, no such information was available in the literature, but during the course of our study, a number of papers [25-27] were published on this subject. Koyanagi *et al* [25] has found a strong prognostic indicator in the absence of telomerase activity in iodine-reactive tissue adjacent to primary tumors. Telomerase activity in the normal tissue was interpreted as an indication of early stage field cancerization. Our study differs from theirs in two respects: firstly, we have not used iodine staining to identify regions of normal mucosa in the vicinity of the tumors, instead taking samples without any sign of abnormal growth at least 2 centimeters above the tumors. It is possible that this way of sampling may have led to some "normal" biopsies being more advanced than others, which in turn could explain why such a high percentage of these biopsies had high telomerase activity. Secondly, our patients were all at a much later stage of disease than those in the Japanese study [25]. Most patients have been referred to the tertiary hospital from rural areas, which takes time, and this is compounded by a tendency to present at a very late stage of disease. We also do not have any form of early detection screening program in place, even in high-risk areas.

In the light of the above-mentioned factors, we were surprised to discover that the telomerase activity of normal mucosa still could identify a group of patients with significantly decreased survival rate (Figure 2B), although even those with the better prognosis had a survival probability of months only. It is our hope that with future improvement in health service delivery in rural areas, cancer of the esophagus would be detected earlier and that telomerase activity tests could have a role to play in identifying patients needing more aggressive treatment at an early stage.

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### 3.3 RESULTS EXCLUDED FROM ARTICLE

A total of 108 patients were analyzed in this study (91 from hospitals in Bloemfontein, Table 3.1 and 17 from Tygerberg, Hospital Cape Town, kindly supplied by Dr W Gelderblom). Males were in the majority with 82 (average age 59 years) as opposed to 26 females (56 years). Most of the patients treated locally came from the Free State (76) or Northern Cape (8) with the rest (7) coming from the rest of the country (see Table 3.2), while the Tygerberg patients came from either the Western Cape (14) or the Eastern Cape (3).

All patients had squamous cell carcinoma and the average tumor depth was  $28 \pm 2$  cm. As in the case of Japanese patients (Tabuko *et al.*, 1997), all tumor samples in this study showed moderate to high telomerase activity (Table 3.3), while the normal tissue showed a more even spread of activity levels. In the case of the Tygerberg tumor samples, about one third showed moderate activity, while for the locally procured samples, the equivalent fraction is less than one fifth. This may reflect differences in handling of the samples prior to storage. The cell lines had extremely high activities, probably due to selection for maximal proliferation. Most biopsies of the normal tissue near the tumors (2 cm away) had a more even spread of telomerase activity. Sixty-eight percent of the normal biopsies were also positive; indicating that telomerase activation could be an early event in the disease progression.

There was no direct correlation between telomerase activity and tumor differentiation assayed histologically (results not shown). Less differentiated and more penetrating growth generally denotes more advanced cancer. One complicating factor was that the histology and molecular biology were done on separate biopsies, which may not be identical in tumor content. Li *et al.* (1996) also found no apparent correlation between stage and telomerase activity in colorectal cancer.

Table 3.4 is a summary of tumor classification (used by the World Health Organization) of the patients in Bloemfontein. Forty-nine percent of the patients were classified with

moderately differentiated tissue, followed by poorly differentiated (23.1%) and well differentiated (14.3%), while carcinoma *in situ* stood at 3.3%.

We had enough follow-up information on 75 patients (Table 3.1) to use their data for survival analysis. The telomerase activity levels of tumor tissue had no discriminating power, as the lines of moderate and high activities were virtually identical (results not shown;  $P=0.7$ ). I then plotted the telomerase activity of the normal tissue samples and the resulting survival curves are shown as figure 2 (in the article). When the discriminator was set at an optical density of 0.2A, the lines in figure 2A (in the article) were obtained. This approach discriminated between telomerase positive and negative samples and was followed by Koyanagi *et al.* (1999). In their case, a clear difference was seen between the survival times of patients having detectable vs. non-detectable telomerase activity in normal esophageal tissue. However, when the discriminator was set at an optical density of 0.6A to discriminate between high activity and low/moderate activity, the lines began to diverge to a significant extent ( $p=0.02$ ) (Fig 2B in the article).

Table 3.1 Characteristics of patient tissue excluded from the article.

Patient Number	Sex	Origin <sup>1</sup>	Age	Histology <sup>2</sup>	Tel Act <sup>3</sup> Tumor	Tel Act <sup>3</sup> Normal	Survival Time (days)
1	M	FS	64	NA	++	++	-
2	F	NC	52	PD	++	-	-
3	M	FS	48	WD	++	++	-
4	M	LE	72	PD	++	++	-
5	M	FS	62	MD	++	++	-
6	M	FS	54	NA	++	-	-
7	F	FS	45	NA	++	++	-
8	F	FS	60	NA	++	++	-
9	M	FS	53	CIS	++	+	-
10	F	FS	60	PD	++	++	-
11	F	FS	57	PD	++	++	-
12	F	FS	78	MD	++	++	-
13	M	NC	56	WD	++	++	-
14	M	FS	75	PD	++	++	-
5	M	FS	48	NA	++	-	-
16	F	FS	62	MD	++	++	-

1 FS: Free State, NC: Northern Cape, LE: Lesotho.

2 CIS: Carcinoma in situ, WD: Well Differentiated, MD: Moderately Differentiated, PD: Poorly Differentiated, NA: Not available.

3 -: Negative, +: Moderately Positive, ++: Strongly Positive.

Table 3.2 Summary of the demographic detail of the patients from Universitas Hospital, Bloemfontein.

Province	Females	Males	TOTAL
Free State	21 (23.1%)	55 (60.4%)	76 (83.5%)
Northern Cape	4 (4.4%)	4 (4.4 %)	8 (8.7%)
Western Cape	1 (1.1%)	0	1 (1.1%)
Eastern Cape	0	2 (2.2%)	2 (2.2%)
North-West	0	1 (1.1%)	1 (1.1%)
Northern	0	1 (1.1%)	1 (1.1%)
Lesotho	0	2 (2.2%)	2 (2.2%)
<b>Total:</b>	<b>26 (28.6%)</b>	<b>65 (71.4%)</b>	<b>91 (100%)</b>

Table 3.3 Summary of telomerase activity reported in this study.

Sample origin	n	Telomerase activity		
		< 0.2	0.21-0.6	> 0.61
Bloemfontein (tumor)	91	0	22 (24.2%)	69 (75.8%)
Bloemfontein (normal)	91	28 (30.8%)	25 (27.5%)	38 (41.8%)
Tygerberg (tumor)	17	0	6 (35.3%)	11 (64.7%)
Cell lines	4	0	0	4 (100 %)

Table 3.4 A summary of the tumor classification (used by the World Health Organization) of the patients from Universitas Hospital, Bloemfontein.

<b>Tumor classification</b>	<b>Number</b>	<b>Percentage%</b>
Well differentiated	13	14.3
Moderately differentiated	45	49.5
Poorly differentiated	21	23.1
CIS (carcinoma <i>in situ</i> )	3	3.3
Not available	9	9.9
<b>TOTAL</b>	<b>91</b>	<b>100</b>

### 3.4 DISCUSSION

Dramatically different patterns of occurrence are observed for esophageal cancer in different parts of the world. Its epidemiologic behaviour is marked by sharp variations in incidence within discrete geographic areas, among different ethnic groups and between males and females (Day and Varghese, 1994). In South Africa, the prevalence of esophageal cancer in rural people has been found to be high, especially in parts of the Eastern Cape and kwaZulu-Natal (Rose, 1973; Silber, 1985). In the Transkei the highest incidence (246 per 100 000) was recorded in the Butterworth district (Silber, 1985). Although it is suspected that traditional food choices and preparation methods could contribute to the condition, the precise aetiology of this high prevalence remains to be determined. It is clear, however, that the causes are multifactorial and may differ in different parts of the world.

Previous studies concerning diet and cancer of the esophagus in South Africa concentrated on the traditional areas of high incidence. The population in the Free State and Northern Cape has never been studied before and make out part of this study. From the demographic data entered on the consent form, we found 93% of the patients to be born and bred in this part of the country and not migrants from the Eastern Cape as expected (see Table 3.2). The department of Oncotherapy, University of the Free State, reports an average of 140 new cases per year and if most are from within the Free State or Northern Cape, this could indicate an area of moderate incidence. It would have been interesting to compare the ethnic differences between the Universitas and Tygerberg population, but unfortunately, as stated in the introduction, we have no histological or demographic detail on the Tygerberg patients.

The typical patient with esophageal cancer will usually be a man between 55 and 65 years of age with a long-standing history of cigarette smoking and heavy alcohol intake (Lewin and Appelman, 1996). Dysphagia and weight loss are the initial symptoms of carcinoma of the esophagus in 90% of patients. Difficulty swallowing does not occur until the circumference of the esophagus is narrowed to a third or half of normal size. The



average age of the patients in this study was 59 years for the males and 56 years for the females. Li (1981) reported that the incidence or mortality rate for males in the Transkei areas was the highest at the age of 60 years. The incidence of squamous cell carcinomas of the esophagus in most countries is predominant in males, the male: female ratio is 3:1. The incidence and the male: female ratio increase with increasing age (Burbank, 1971). The age and gender distribution of patients in this study closely mirrors that found elsewhere. In this study the males were in the majority with 82 as opposed to 26 females, and the male:female ratio was 3:1.

Cancer of the esophagus may be characterized by its cell type and anatomic location along the length of the esophagus. Anatomically, the esophagus is divided into thirds. The cervical esophagus and the upper thoracic esophagus are the upper third (above the aortic arch); the middle thoracic esophagus forms the middle third (between the aortic arch and the inferior pulmonary vein); while the lower thoracic esophagus (below the inferior pulmonary vein) forms the lower third (Vincent *et al.*, 1989). The anatomical classification is the most practical, because it is the simplest. The majority of squamous cell carcinomas of the esophagus are located in the middle and lower thirds of the esophagus (Schottenfeld, 1984). The most frequent site of squamous cell carcinoma of the esophagus in Black patients is the middle third of esophagus (Mannell, 1980). The location of the squamous cell carcinoma in this study correlates with the above data. The average depth of the esophageal tumor samples was  $28 \pm 2$ cm, also in the middle third of the esophagus.

Primary adenocarcinoma of the esophagus is a relatively uncommon tumor, accounting for 1 to 50 percent of all malignant esophageal tumors, depending upon geographic location (Katzka, 1989). Almost all cases of primary esophageal adenocarcinoma arise from Barrett's esophagus (Katzka, 1989). In Barrett's esophagus the normal stratified squamous epithelium lining the esophagus is replaced by columnar epithelium for variable lengths from the lower esophageal sphincter region. The most common predisposing factor for Barrett's esophagus is chronic gastroesophageal reflux (Agha and

Keren, 1987; Burgess *et al.*, 1971). The sequence of events leading to Barrett's esophagus has not been clearly defined. It is probable that ulceration of the squamous epithelium occurs in response to gastroesophageal reflux of acid, bile and duodenal contents and that re-epithelialization occurs via multipotential stem cells which in turn differentiate into the variety of epithelial cells found in Barrett's esophagus. There may, however, be other mechanisms.

Barrett's esophagus is most commonly diagnosed between the ages of 40 and 60 years (Duhaylongsod and Wolfe, 1991) but can also occur in children (Hassell *et al.*, 1985). There is a clear-cut predominance in white males over females, in the order of 4:1 (Polepalle and Callum, 1990). This disorder is uncommon in blacks; less than 2% of blacks with gastroesophageal reflux have Barrett's esophagus (Polepalle and Callum, 1990).

More than 90% of malignant esophageal tumors are squamous cell carcinomas, arising from the squamous cell epithelium lining of the lumen of the esophagus (Vicent *et al.*, 1989). Well-differentiated cancers have the characteristic features of keratin formation (epithelial pearls), intercellular bridges and minimal pleomorphism. Poorly differentiated tumors do not contain keratin nor demonstrate intercellular pleomorphism. The moderately differentiated tumors are intermediate between these two. Table 3.4 is a summary of tumor classification (used by the World Health Organization) of the patients in Bloemfontein. Forty-nine percent of the patients were classified with moderately differentiated tissue, 23.1% with poorly differentiated tissue and 14.3% with well-differentiated tissue.

In tumor-derived cell lines telomeres are maintained by the ribonucleoprotein enzyme telomerase. Telomerase activity is repressed in almost all the normal human somatic cells. Due to the end replication problem, progressive telomere shortening occurs in normal somatic cells, leading to a limited replicative capacity and eventually resulting in cellular senescence. In the presence of viral oncogenes or some somatic mutations that block cellular senescence, cells continue to divide and telomere erosion continues. This

continuing telomere erosion ultimately leads to the activation of telomerase, a necessary event for the sustained growth of most human tumors.

Telomerase activity in foetal cells is switched off shortly after birth, except in germline cells, haematopoietic cells, inflamed or regenerating tissue (Taylor *et al.*, 1996; Yasumoto *et al.*, 1996) and a variety of tumors, e.g. head and neck (Mutirangura *et al.*, 1996), prostate (Sommer-field *et al.*, 1996), breast (Hiyama *et al.*, 1996; Umbricht *et al.*, 1999), bladder (Muller *et al.*, 1996) neuroblast (Hiyama *et al.*, 1995) and esophagus (Takuba *et al.*, 1997; Morales *et al.*, 1998). As in the case of Japanese patients (Tabuko *et al.*, 1997), all tumors in this study showed moderate to high telomerase activity (Table 3.3), while the normal tissue samples showed a more even spread of activity levels. In the case of the Tygerberg tumor samples, about one third showed moderate activity, while for the locally procured samples, the equivalent fraction is less than one fifth. This may reflect differences in handling of samples prior to storage. The cell lines had extremely high activities, probably due to selection for maximal proliferation. The most biopsies of the nearby normal tissue near the tumor (2 cm away) had low or moderate telomerase activity. Sixty-eight percent of the normal biopsies were also positive; indicating that telomerase activation could be an early event in disease progression. In certain tumor types, such as neuroblastoma (Hiyama *et al.*, 1995), telomerase activity is generally expressed at low levels in early stage cancers and at very high levels in late-stage cancers.

There was no direct correlation between telomerase activity and tumor differentiation assayed histologically (results not shown). Less differentiated and more penetrating growth denotes more advanced cancer. One complicating factor was that the histology and molecular biology were done on separate biopsies, which may not be identical in tumor content. Li *et al.* (1996) also found no apparent correlation between stage and telomerase activity in colorectal cancer.

Koyanagi *et al.* (1999) detected telomerase activity in normal epithelial tissue of the esophageal patients. In four of the five telomerase positive normal tissues cancer cells

infiltrating blood or lymphatic vessels of the mucosal or submucosal layer in the normal epithelial region were detected on detailed histopathological examination. The evidence strongly suggested that microinvasion of cancer cells was responsible for the positive activity of telomerase in the normal tissue. Telomerase activity in normal epithelial regions may thus be a useful prognostic factor in patients with SSCE. Telomerase activity could be used before operation to define appropriate treatment for a patient with SSCE. If telomerase activity was detected in biopsy samples of normal epithelial regions obtained from a patient with SSCE by preoperative endoscopic examination, the patient might be at high risk of metastases and could benefit from a combination of radical lymph node dissection and adjuvant systemic chemotherapy. In addition, telomerase activity in normal epithelial regions may assist in selecting the appropriate treatment for patients with SSCE.

Because telomerase activity appears necessary for the sustained proliferation of most cancer cells, anticancer agents based on telomerase inhibition may potentially provide a highly effective therapy with possible limited side effects. In addition, knowledge of telomerase activity may be useful in cancer diagnostics and may serve as a prognostic indicator of clinical outcome.

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## CHAPTER 4

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### DEVELOPMENT OF A FLOW CYTOMETRIC METHOD TO DETERMINE TELOMERE LENGTH IN TUMOR CELLS

	PAGES
4.1 INTRODUCTION	91
4.2 MATERIALS AND METHODS	94
4.2.1 Materials	94
a) <i>Peripheral blood mononuclear cells</i>	94
b) <i>SNO cell line</i>	94
c) <i>Baboon liver cells and esophageal biopsies</i>	94
4.2.2 Fluorescence <i>in situ</i> hybridization	95
4.2.3 Flow Cytometric measurement	96
4.2.4 Q-FISH technique	96
4.3 RESULTS	97
4.4 DISCUSSION	112
4.5 REFERENCES	116

## CHAPTER 4

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### DEVELOPMENT OF A FLOW CYTOMETRIC METHOD TO DETERMINE TELOMERE LENGTH IN TUMOR CELLS

#### 4.1 INTRODUCTION

In recent years, a new generation of cancer therapies has emerged, based on a growing understanding of the molecular events that contribute to malignant transformation and which therefore represent targets for selectively killing or inhibiting the growth of malignant cells. In this context, there has been considerable interest in elucidating mechanisms that regulate the capacity for cell division in normal as well as transformed cells and substantial attention has focused specifically on the role of telomeres as the “mitotic clock” that mediates replicative capacity (Hodes, 2001).

Telomeres are specialized structures at the ends of eukaryotic chromosomes that are important for chromosome stability (Blackburn and Greider, 1985). In human somatic cells the length of telomere repeats decrease with cell divisions *in vitro* (Harley *et al.*, 1990; Allsopp *et al.*, 1992) and *in vivo* (Hastie *et al.*, 1990; Vaziri *et al.*, 1993). Telomerase is a unique reverse transcriptase that has the ability to extend the 3' end of telomeres (Greider and Blackburn, 1985; Lingner *et al.*, 1997). Telomerase is required to maintain telomere length in germline cells (Blasco *et al.*, 1997) and is typically upregulated in cancer cells (Autexier and Greider, 1996; Shay and Wright, 1996). A linkage between telomere shortening and cellular senescence has been established by showing that transfection of the telomerase reverse transcriptase gene into various human



mortal cells may result in telomere length elongation and extension of the *in vitro* replicative lifespan (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998).

Telomeres play an essential role in maintaining chromosomal stability and integrity. They protect chromosomes from illegitimate recombination and degradation by nuclease attack (Blackburn, 1991). Telomeres also appear to play a role in the correct pairing and movement of chromosomes at meiosis (Liu *et al.*, 1995; Chikashige *et al.*, 1994; Dernburg *et al.*, 1995). Furthermore, telomeric attachment to the nuclear matrix may play a role in nuclear organization (de Lange, 1992). Although telomeres consist of transcriptionally non-coding DNA, telomere position effects suggest a gene regulatory role (de Lange, 1992).

Telomere biology has had perhaps an even greater impact on the fundamental understanding of cell immortalization and tumor formation. It has been estimated that approximately 90% of primary tumors and perhaps up to 98% of immortal cell lines possess short telomeres and express increased amounts of telomerase activity as opposed to the negligible expression in normal somatic cells and benign tissue (Kim *et al.*, 1994). However, it has been reported that telomerase activity is an indicator of cell proliferation rather than of transformation (Belair *et al.*, 1997) and that expression of telomerase in somatic cells induces an extended lifespan (Bodnar *et al.*, 1998) but not phenotypic changes consistent with tumorigenesis (Jiang *et al.*, 1999; Morales *et al.*, 1999). Furthermore, those cell lines that do not express detectable levels of telomerase are often found to possess alternative methods for maintaining telomere length (Ogino *et al.*, 1998). Therefore, telomere maintenance seems to be a key component in the multistep process of tumorigenesis. It is conceivable that monitoring telomere length may be predictive, in conjunction with other factors, in tracking malignant tumor formation.

The gold standard for measurement of telomere length is Southern blotting (Allsopp *et al.*, 1992; Allshire *et al.* 1989; Harley *et al.*, 1990; de Lange *et al.*, 1990) which, although accurate and reproducible, is relatively cumbersome; thereby precluding its use for large-scale analysis (Norwood and Dimitrov, 1998; Rufer *et al.*, 1998; Hultdin *et al.*, 1998).

This method requires a relatively large number of cells (at least  $5 \times 10^5$  to produce  $\geq 2 \mu\text{g}$  of DNA) that are lysed to release the DNA. Several improvements to the Southern blot technique have been proposed. Pulse-field electrophoresis has been used to increase the resolution of the TRFL differences on the autoradiographs (Feng *et al.*, 1999). Slot blots have been employed to measure telomere-specific DNA content (Bryant *et al.*, 1997). However, each of these refinements to the Southern blot method increases rather than decreases the complexity of the assay.

An alternative method of telomere measurement is quantitative fluorescence *in situ* hybridization (Q-FISH) performed on metaphase spreads (Zijlmans *et al.*, 1997; Lansdorp *et al.*, 1996; Blasco *et al.*, 1997). It is analysed by an image analysis system coupled to a fluorescence microscope. Q-FISH is a labour-intensive, time-consuming procedure that requires a high degree of technical expertise, thus limiting its general applicability.

An alternative method for examining telomere length is required to provide information at the cellular level, which may distinguish critical changes in telomere length in cellular subpopulations. There have been recent reports from Rufer *et al.* (1998) and Hultdin *et al.* (1998) describing flow cytometric methods for the measurement of telomere length in whole cells. Both protocols provide quantitative, highly reproducible results that correlate well with the Southern blot. The original description of flow-FISH by Rufer *et al.* (1998) has recently been revised (Rufer *et al.*, 1999) and clearly shows the utility of the methodology in the examination of a large number of samples. So far, flow-FISH has only been applied to leukocytes which are an easily isolated, relatively homogenous cell population.

Our initial goal was to develop a technique to determine telomere length on the flow cytometer, followed by determination of the telomere length of esophageal biopsies, so as to establish whether telomere length could be used as a prognostic marker and to compare it with telomerase activity.

## 4.2 MATERIALS AND METHODS

### 4.2.1 *Materials*

#### a) *Peripheral blood mononuclear cells*

Peripheral blood mononuclear cells (PBMCs) were obtained following Ficoll-Hypaque density centrifugation. The PBMCs isolated from healthy donors were washed with PBS (phosphate-buffered saline) containing 0.1% BSA (bovine serum albumin), and the pellets were resuspended in 2 ml FACS™ permeabilizing solution (Becton Dickinson) and incubated at room temperature in the dark for 10 minutes. Thereafter the cells were washed by adding 5 ml PBS/ 0.1% BSA and centrifuged at 3000xg for 5 min. For fixation of the cells, the pellet was resuspended in 2 ml 2% paraformaldehyde and incubated for 10 min at room temperature. The cells were centrifuged at 300xg for 5 min. The pellets were washed with PBS containing 0.1% BSA (4.2.2).

#### b) *SNO cell line*

The SNO esophageal cancer cell line was supplied by Highveld Biological Association affiliated to CANSA and was grown in RPMI medium containing 10% fetal calf serum. The cultured cells were harvested by centrifugation after trypsin digestion, washed with RPMI medium, and the pellet was resuspended in 2 ml FACS™ permeabilizing solution and incubated at room temperature in the dark for 10 minutes. Thereafter the cells were washed by adding 5 ml PBS/ 0.1% BSA and centrifuged at 3000xg for 5 min. For fixation of the cells, the pellet was resuspended in 2 ml 2% paraformaldehyde and incubated for 10 min at room temperature. The cells were centrifuged at 300xg for 5 min. The pellet was washed with PBS/ 0.1% BSA.

c) *Baboon liver cells and esophageal biopsies*

Baboon liver was obtained from the UFS Experimental Animal Facility and was removed from a female that died of hemorrhage after surgery. The baboon liver cells and the esophageal biopsies were handled identically. The tissue was cut into very small pieces with the aid of 2 scalpel blades. Then 500 µl of lysis buffer (320 mM sucrose, 5mM MgCl<sub>2</sub>, 10 mM HEPES at pH 7.4 and 1% Triton X-100) and 1% collagenase (Sigma, Type III fraction A) were added to the diced tissue. The mixture was incubated for 15 min at 37°C, cooled down for 5 min on ice and pelleted by centrifugation at 3 000xg for 5 min. Thereafter the pellet was resuspended in 1 ml PBS/ 0.1% BSA.

**4.2.2 Fluorescence *in situ* hybridization**

The fluorescence *in situ* hybridization method of Rufer *et al.* (1998) was modified. The cells were washed in PBS containing 0.1% BSA and placed in 1.5 ml microcentrifuge tubes. Cells were spun down at 3000xg for 5 minutes and resuspended in 1 ml PBS/ 0.1% BSA. Each sample was then divided into two equal parts between two eppendorf tubes followed by centrifugation for 15 seconds at 13 000xg in an Eppendorf Microfuge. The supernatants were removed and the pellets were resuspended in 500 µl hybridization buffer (70% deionized formamide, 20 mM Tris-HCl, pH 7.0, 0.1% BSA and 0.3µg/ml telomere PNA probe). The probe was a telomere specific FITC conjugated PNA oligomer (C<sub>3</sub>TA<sub>2</sub>)<sub>2</sub> supplied by Dr Bruce Armitage, School of Biological Sciences, Carnegie-Mellon University, Pittsburgh, USA. The volume of the hybridization buffer was adjusted to between 200 µl and 500 µl. The samples were subjected to heat denaturation for 10 minutes at 80 °C in a Thermomixer followed by hybridization for 2 hours at room temperature in the dark. The cells were spun down at 3000xg for 5 minutes. The supernatant was removed and the cells were washed twice with 1 ml wash buffer (70% deionized formamide; 10 mM Tris-HCl, pH 7.0; 0.1% BSA and 0.1% Tween 20) and once with PBS/ 0.1% BSA and 0.1% Tween 20. Centrifugation of all the washing steps was done at 3000xg for 5 minutes except the last wash (5 minutes at 2000xg). After the last washing step the cells were resuspended according to the cell

number (100  $\mu$ l per  $10^5$  cells) in PBS/ 0.1% BSA containing RNase A (10  $\mu$ g/ml) and 7 AAD (0.06  $\mu$ g/ml, Molecular Probes). The mixture was incubated for 15 minutes at room temperature and immediately analyzed on a flow cytometer (4.2.3) or stored at 4°C for up to 2 days prior to analysis.

#### 4.2.3 Flow Cytometric measurement

The average length of the telomere repeats at the chromosome ends in individual cells (normal and tumor biopsies of each patient) were measured by flow-FISH (Rufer *et al.*, 1998), using the following modifications. To correct for the daily shifts in the linearity of the flow cytometer (Becton Dickinson FACS Calibur), fluctuations in the laser intensity, alignment, and to allow expression of results in standard fluorescence units, FITC-labeled fluorescent calibration beads (Quantum<sup>TM</sup>-24 Premixed; Flow Cytometric Standards Corp.) were used. At the beginning and end of each experiment the fluorescence signals from calibration beads suspended in PBS/0.1% BSA were acquired. The bead solution contains four populations ranging from 5 500 - 83 000 MESF (Molecules of Equivalent Soluble Fluorochrome) units (Henderson *et al.*, 1998), as well as non-fluorescent beads.

Voltage and amplification of the FL1 parameter were set in such a way that blank, 5.585, 13 643, 26 783 and 82 957 MESF units of the average microbeads ( $n = 6$ ) gave 2.42, 27.71, 59.38, 105.63 and 288.80 FL1 channels on a log-scale, respectively (Fig 4.1A). The resulting calibration curve (Fig 4.1B) ( $y=0.0036x$ ) was then used to convert telomere fluorescence data to MESF ( $\times 10^{-3}$ ), allowing comparison. For the instrument settings, chicken red blood cells (Becton Dickinson) were used and 10 000 events per gate were counted.

#### 4.2.4 Q-FISH technique

The baboon liver cells were resuspended in 4 ml freshly prepared fixative (1:3 acetic acid: methanol, ice cold). The first ml was added drop by drop while mixing. After centrifugation at 2 500 rpm for 5 min in a Heraeus CHRISTcentrifuge, the supernatant

was removed. Ten  $\mu\text{l}$  of cell suspension was dropped on a microscope slide, the cells were spread out and dried. Ten  $\mu\text{l}$  hybridization buffer (70% deionized formamide, 20 mM Tris-HCl, pH 7.0, 0.1% BSA and 0.3  $\mu\text{g}/\text{ml}$  telomere PNA probe) was added to the spread out cells and the slide was covered with a coverslip and sealed with glue. The slide was subjected to heat denaturation for 10 minutes at 80 °C on the *in situ* hybridization block of a Hybaid PCR machine followed by hybridization for 2 hours at room temperature in the dark. The coverslip was removed and the cells were washed twice with 10 ml washing buffer (70% deionized formamide; 10 mM Tris-HCl, pH 7.0; 0.1% BSA and 0.1% Tween 20) and once with PBS/ 0.1% BSA and 0.1% Tween 20. The slide was air-dried in the dark and then counterstained with 10  $\mu\text{l}$  DAPI in antifade solution (Vectashield, Vector Laboratory). The cells were viewed using a triple band filter and a Nikon E400 fluorescence microscope.

### 4.3 RESULTS

For the development of this technique, lymphocytes were used, then SNO cells, followed by baboon liver cells and eventually esophageal biopsies. Flow cytometry methods for quantitative FISH (flow-FISH) on whole cells pose a unique problem related to providing the probe access to the target DNA while maintaining cell structure. The first obstacle is getting the probe inside the cell. This is generally a delicate balance between fixing the cell sufficiently to maintain cellular structure without cross-linking the proteins to such an extent that the probe can no longer penetrate the cell or irreversibly binding the DNA-associated proteins to the chromosome. The lymphocytes were first permeabilized to make it easier for the probe to enter and then fixed with paraformaldehyde to keep the cell structure intact.

At the beginning and the end of each experiment the fluorescence signals from calibration beads were acquired (Fig 4.1A). This enabled comparison of experiments done on different days.

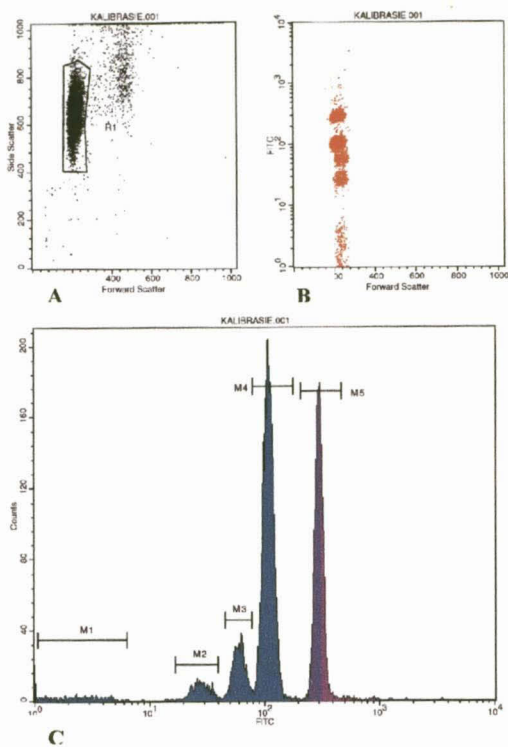


Figure 4.1A Dot plot A illustrates the cell gating region 1(R1), forward scatter versus side scatter and B forward scatter versus FL3. Histogram C shows the fluorescence intensity of the FITC-labeled fluorescent calibration beads.

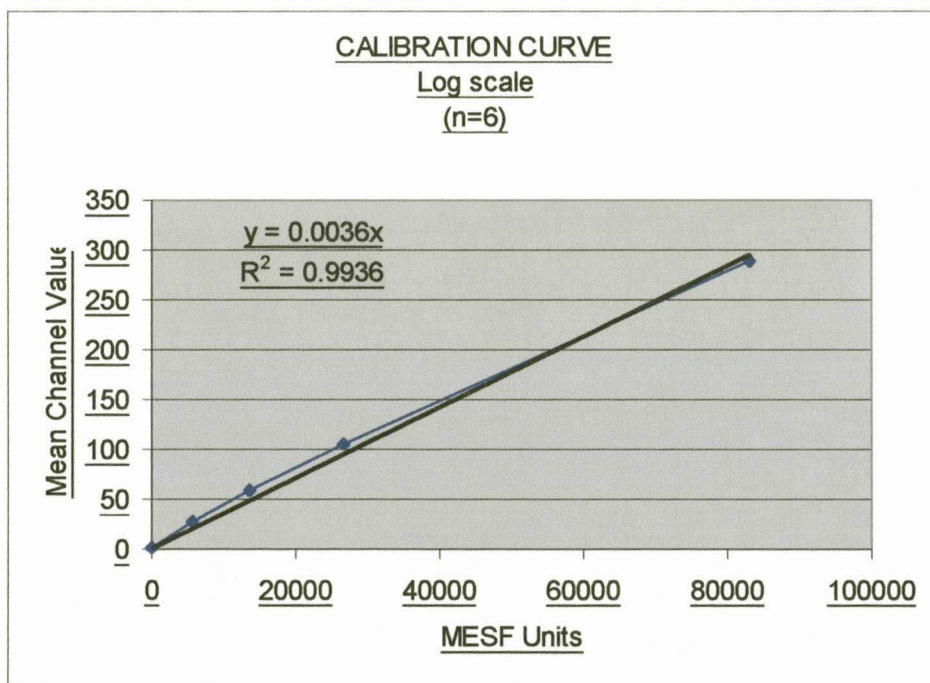
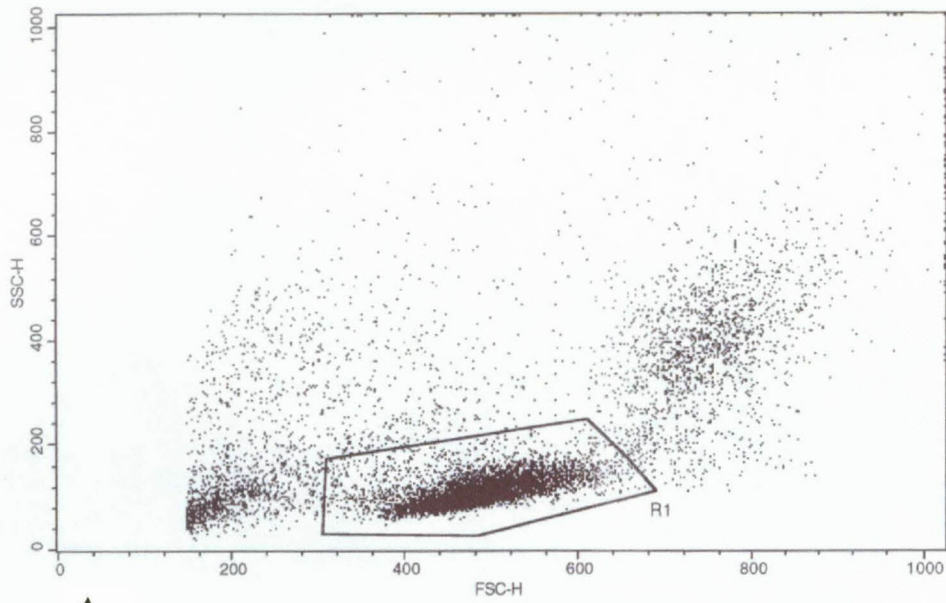
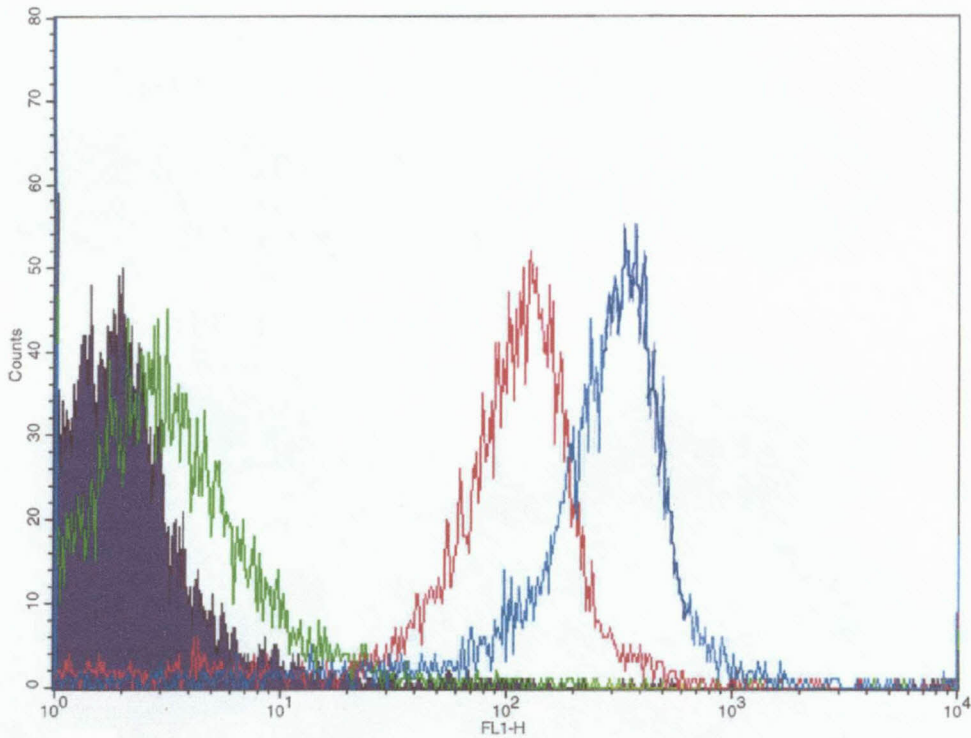


Figure 4.1 B The fluorescence of the FITC-labeled fluorescent calibration beads measured in MESF units compared to the mean channel value obtained from the cytometer.



**A**



**B**

**Figure 4.2** Flow FISH analysis of lymphocytes to obtain the optimum FITC-labeled PNA probe concentration. The cells were gated on region 1 (R1), forward scatter versus side scatter. Histogram B illustrates the different probe concentrations: Purple (0.1  $\mu\text{g/ml}$ ); green (0.2  $\mu\text{g/ml}$ ); pink (0.3  $\mu\text{g/ml}$ ) and blue (0.4  $\mu\text{g/ml}$ ).

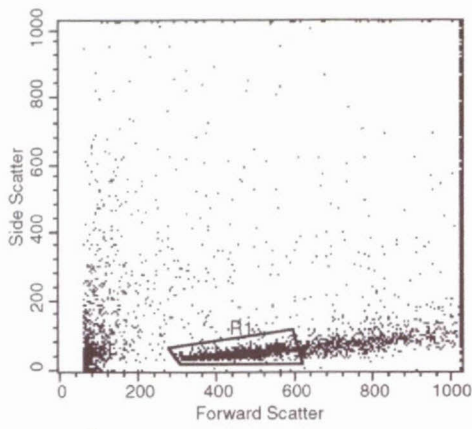


The second obstacle was to find the optimum concentrations of the FITC-labeled probe and the 7 AAD counterstain. With a 7 AAD concentration higher than 0.06  $\mu\text{g/ml}$ , too much background was detected (results not shown). Fig 4.2 shows the optimum concentration of the PNA probe. The 0.3  $\mu\text{g/ml}$  concentration was the best to use, because it showed almost no overlapping in FL3. The 0.1  $\mu\text{g/ml}$  and 0.2  $\mu\text{g/ml}$  concentrations peak in the  $10^1$  region. The  $10^1$  region was used for adjustment of the instrument settings to get rid of the background.

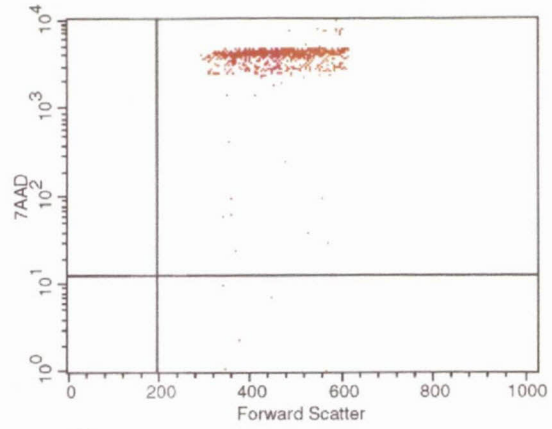
The lymphocytes were analyzed on the flow cytometer after the settings and calibration was done (Fig 4.3). Only cells located within in the gate shown in Fig 4.3A were analyzed. The lymphocytes were stained with 7AAD (Fig 4.3B) and labeled with the PNA probe (Fig 4.3C).

Ater the initial technique had been established on lymphocytes, it was tested on the SNO cell line (Fig 4.4) before we switched to solid tissue.

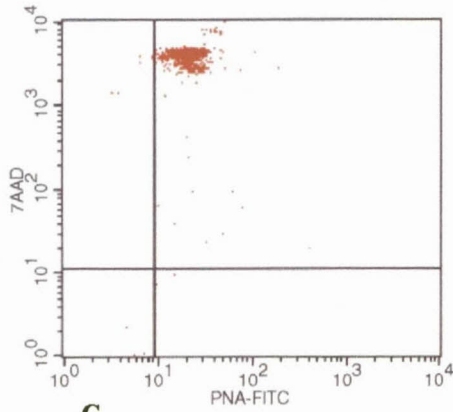
It became clear that cells from solid tissue have to be treated even more carefully than lymphocytes. The tumor biopsies were very small and it was difficult counting enough cells for a proper gate. The resulting profiles were very sensitive to the way in which cells were prepared and handled. The cells were fixed to keep the membrane intact and permeabilized to make it easier for the probe to enter; the same method as used with the lymphocytes and the SNO cell line. Still, too many cells were lost during all the washing processes and the prefixing resulted in reduced accessibility of target nucleic acid sequences and variable fluorescence signals. It was clear that we could not use the same method for the esophageal biopsies, so we used a substitute tissue for technique development. The choice fell on baboon liver cells, since we had a handy supply in the freezer. We assumed that the baboon's telomere repeats would be similar enough to those of humans for the probe to hybridize, a valid assumption, as all vertebrate repeats studied so far turned out to be identical (König and Rhodes, 1997).



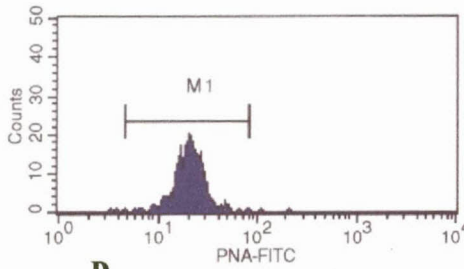
**A**



**B**

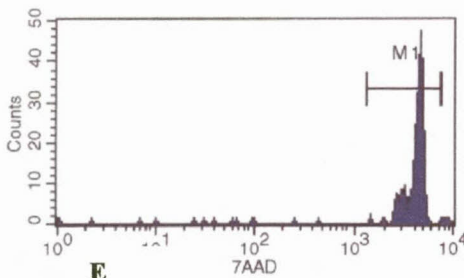


**C**



**D**

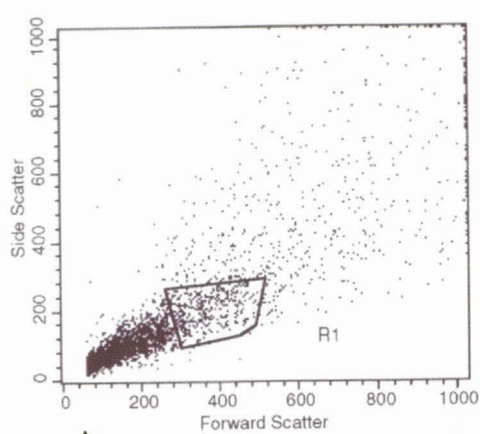
Marker	% Gated	Mean
All	100.00	21.53
M1	99.59	21.30



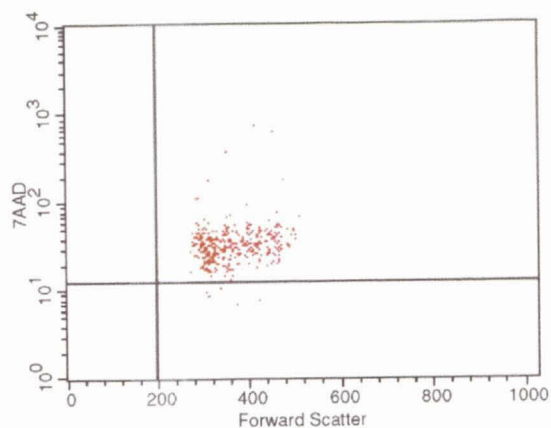
**E**

Marker	% Gated	Mean
All	100.00	3958.45
M1	97.73	3980.46

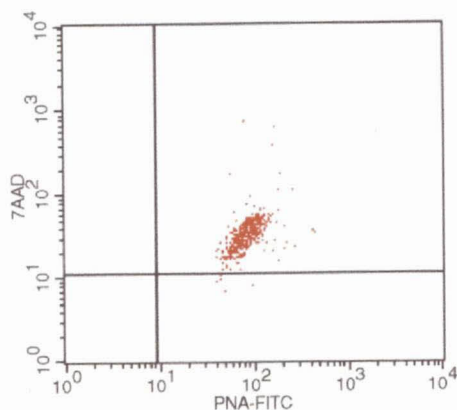
**Figure 4.3** Flow FISH analysis of lymphocytes. The dot plots illustrate the gating of the cells in the different regions: forward scatter versus side scatter (A), forward scatter versus FL3 (B) and FL1 versus FL3 (C). Histogram D illustrates the fluorescence intensity of the FITC probe and E the fluorescence intensity of the 7AAD counterstain.



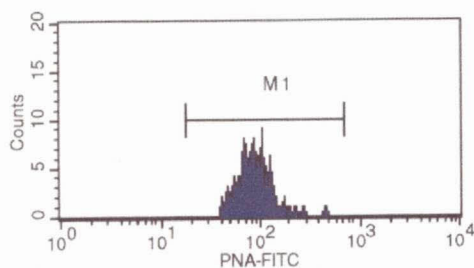
**A**



**B**

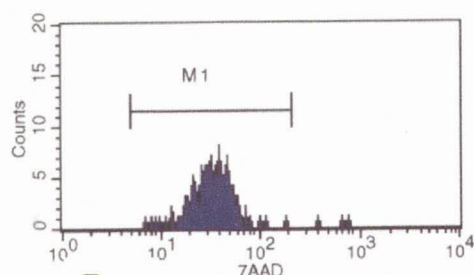


**C**



**D**

Marker	% Gated	Mean
All	100.00	90.05
M1	100.00	90.05



**E**

Marker	% Gated	Mean
All	100.00	37.84
M1	99.20	33.57

**Figure 4.4** Flow FISH analysis of the SNO cell line. The dot plots illustrate the gating of the cells in the different regions: forward scatter versus side scatter (A), forward scatter versus FL3 (B) and FL1 versus FL3 (C). Histogram D illustrates the fluorescence intensity of the FITC probe and E the fluorescence intensity of the 7AAD counterstain.

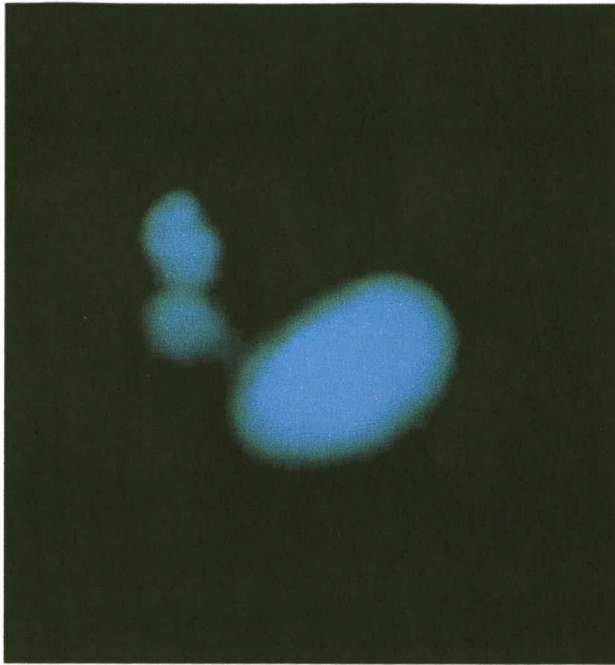
A number of laboratories have described nuclei isolation protocols for DNA content analysis (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993), eliminating the need for DNA dyes to cross a permeabilized plasma membrane. Therefore we decided to develop a method for small-scale isolation of nuclei from baboon liver cells. A modified technique was developed by using mechanical disruption with scalpel blades followed by incubation in a sucrose lysis buffer supplemented with collagenase to separate the cells. After the method was established, the optimum probe concentration was established and it was found to be the same as for the lymphocytes and the SNO cells (results not shown). Without permeabilization and fixation of the cells we rely entirely on the denaturation conditions to provide access for the probe into the cell. This strategy avoids the potential probe-accessibility problem associated with excessive fixation. It also means less handling of the cells, reducing loss.

The success of hybridization with the newly developed technique was tested with Q-FISH on the baboon liver cells. In Fig 4.5A two nuclei are clearly visible using a DAPI filter (we had to use DAPI because the mercury light source of the microscope cannot excite 7 AAD). Successful hybridization resulted in easily visible fluorescent signals (spots) within the nucleus (Fig 4.5B) when viewed through a FITC filter.

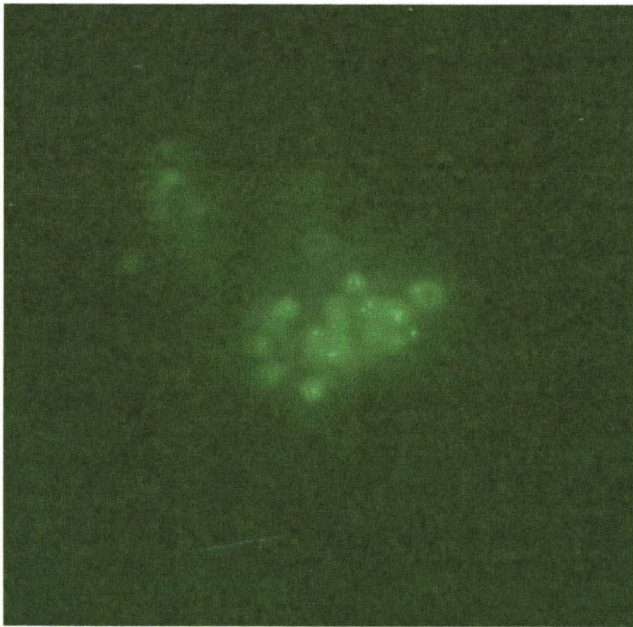
The baboon liver cells were then analyzed on the flow cytometer to see if there were enough cells to get a proper gate and to evaluate the technique before switching to tumor tissue. The results are shown in Fig 4.6 and this gave us confidence to carry on.

Esophageal biopsies (Fig 4.7) were then used. After homogenization of the tumor biopsies the mixture contains intact nuclei, debris and doublets, so that much more background was detected than for the lymphocytes (Fig 4.3) and the SNO cell line (Fig 4.4). To overcome this problem, gate 2 was used (R2) to back-gate on the 7 AAD to eliminate the background (Fig 4.7).

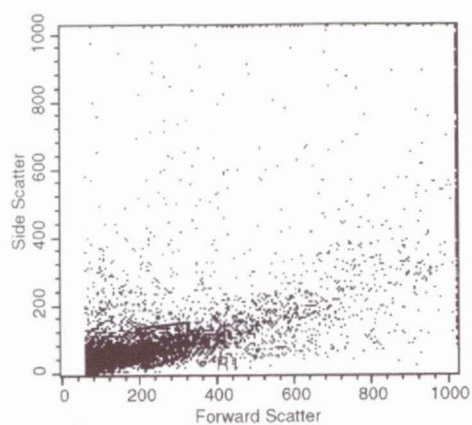
For each esophageal tumor cell population, the telomere specific fluorescence was calculated by subtracting the mean fluorescence of the background control (unlabeled



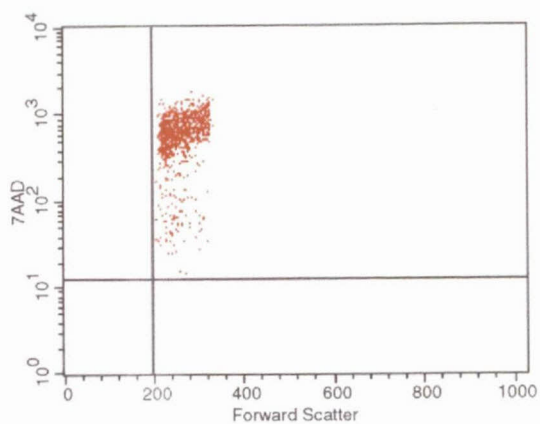
**Figure 4.5A** Baboon liver cell nuclei after hybridization under the fluorescence microscope with a DAPI filter.



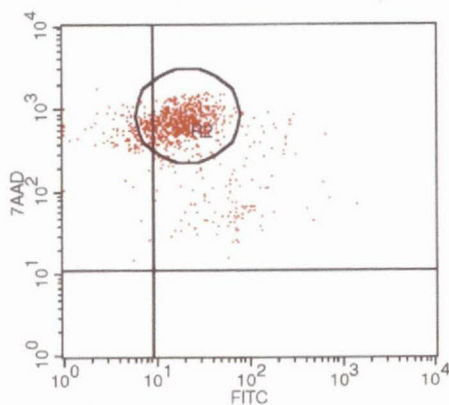
**Figure 4.5B** The same field as in A, this time using a FITC filter.



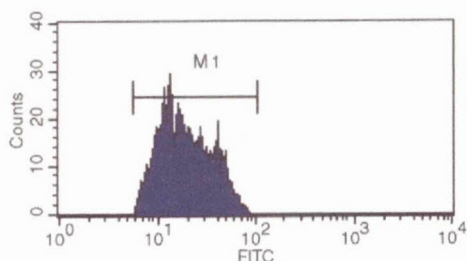
**A**



**B**

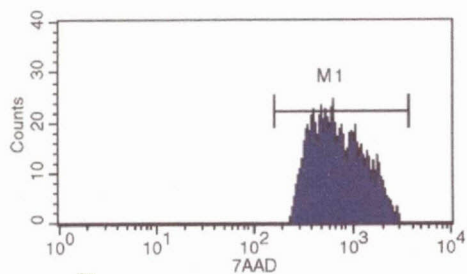


**C**



**D**

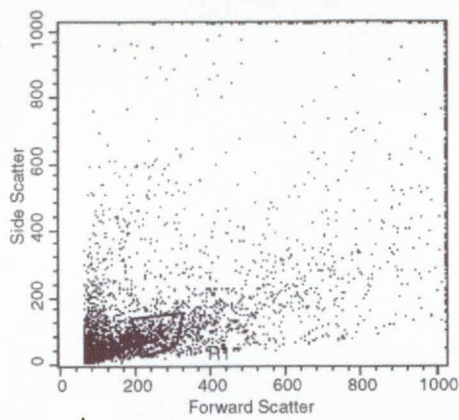
Marker	% Gated	Mean
All	100.00	21.32
M1	100.00	21.32



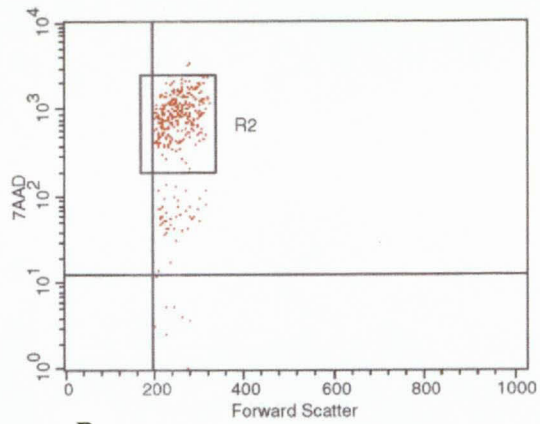
**E**

Marker	% Gated	Mean
All	100.00	760.51
M1	100.00	760.51

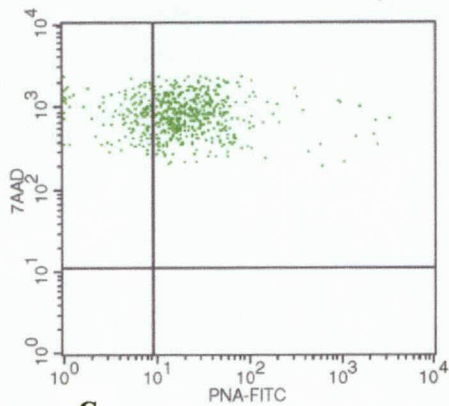
**Figure 4.6** Flow FISH analysis of baboon liver nuclei. The dot plots illustrate the gating of the nuclei in the different regions: Forward scatter versus side scatter (A), forward scatter versus FL3 (B) and FL1 versus FL3 (C). Histogram D illustrates the fluorescence intensity of the FITC probe and E the fluorescence intensity of the 7AAD counterstain.



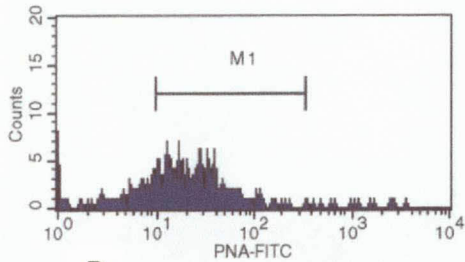
**A**



**B**

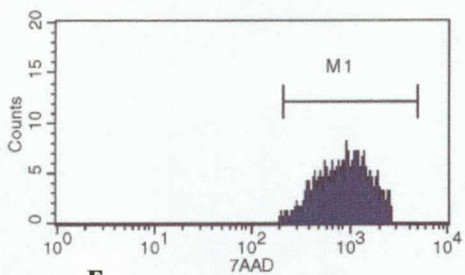


**C**



**D**

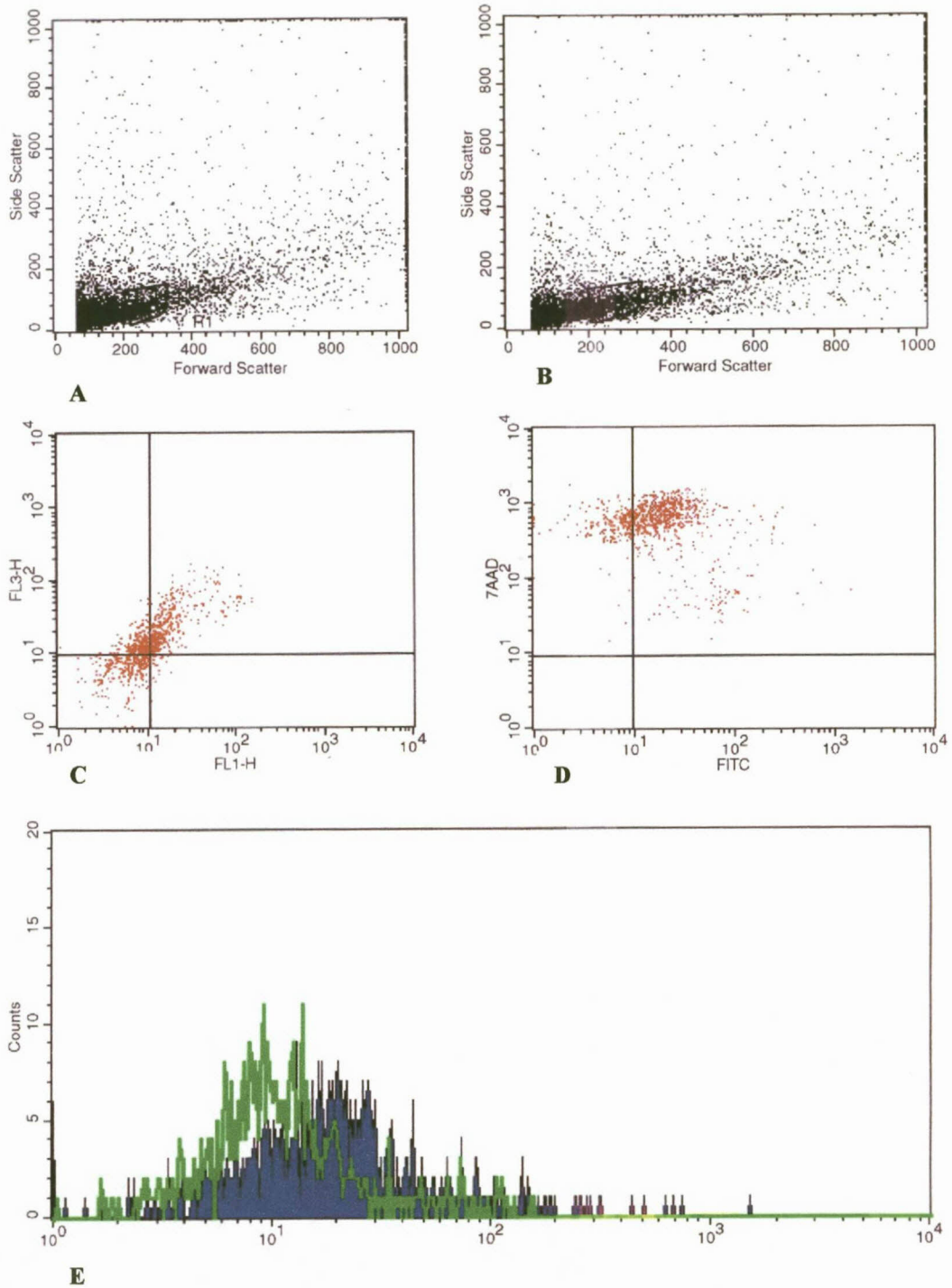
Marker	% Gated	Mean
All	100.00	57.02
M1	75.34	32.63



**E**

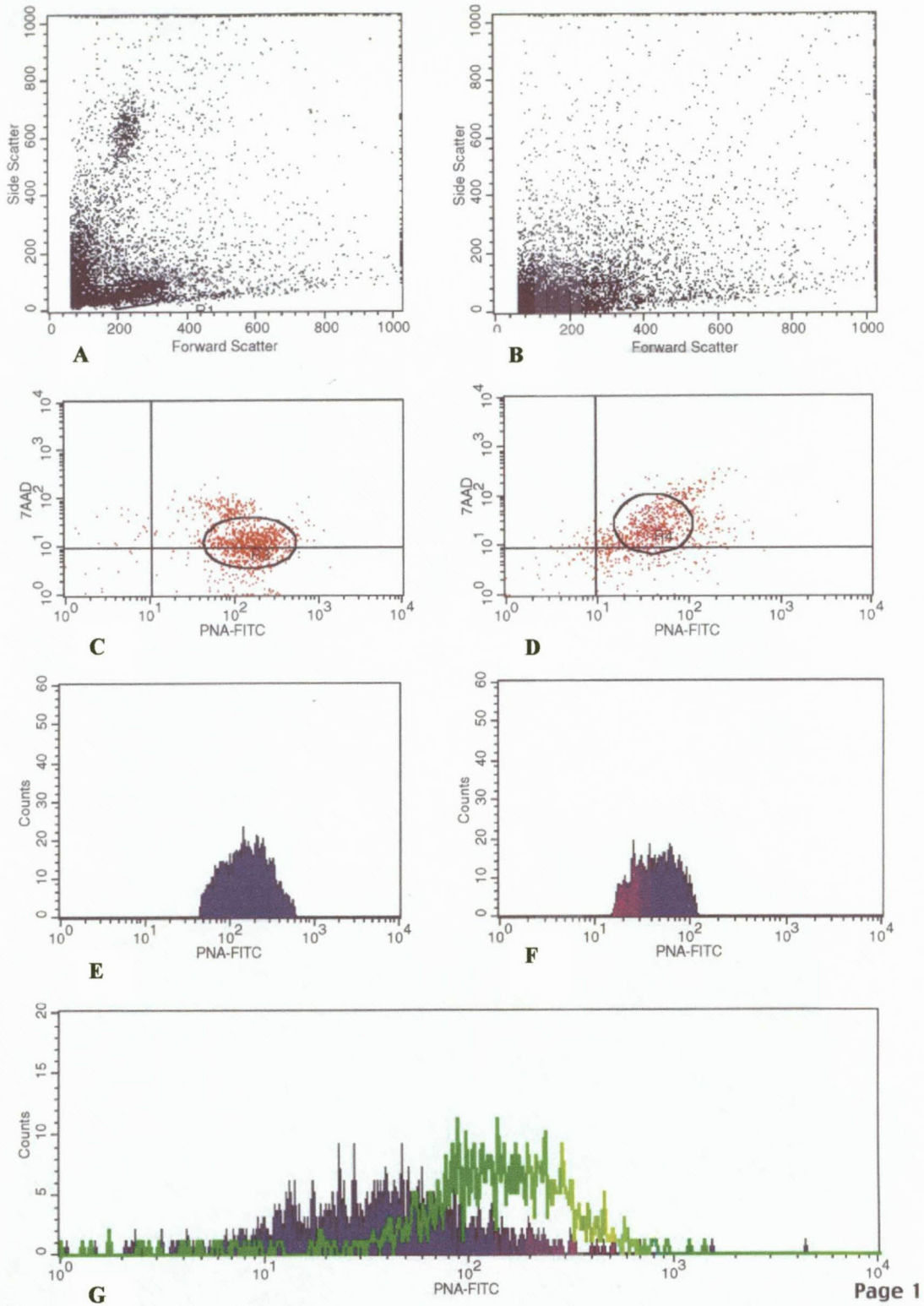
Marker	% Gated	Mean
All	100.00	908.24
M1	99.66	910.64

**Figure 4.7** Flow FISH analysis of the esophageal tumor nuclei. The dot plots illustrate the gating of the nuclei in the different regions: forward scatter versus side scatter (A), forward scatter versus FL3 (B) and FL1 versus FL3 (C). An additional region 2 (R2) was selected from the forward scatter versus the FL3 to back-gate on the 7AAD. Histogram D illustrates the fluorescence intensity of the FITC probe and E the fluorescence intensity of the 7AAD counterstain.



**Figure 4.8** Dot plots indicating the presence of the FITC probe (B & D) in esophageal tumor cells, compared to unlabeled tumor cells (A & C). The histogram (E) illustrates the difference in fluorescence intensity of positive cells (blue) overlaid with the negative control (green).





**Figure 4.9** Flow cytometric analysis to illustrate differences in fluorescence intensity between normal esophageal tissue (A,C & E) compared to tumor tissue of the same patient (B,D & F). The histogram (G) confirms the shift in fluorescence intensity between normal esophageal tissue (green) and tumor tissue (blue).

**Table 4.1 Results of flow-FISH of paired tumor and normal biopsies.**

Patient	Normal Tissue						Tumour Tissue				Ratio	
	Telomerase Activity <sup>1</sup>	Corrected MESF <sup>2</sup>	Sorted According to Telomerase Activity		Survival (days)	Sorted According to Telomerase Activity		Telomerase activity <sup>1</sup>	Corrected MESF <sup>1</sup>	Sorted According to Telomerase Activity		Tumour/Normal MESF
			Moderate	High		Moderate	High			Moderate	High	
1	++	125110		125110	142		142	++	21553		21553	0.17
2	-	6077	6077		43	43		+	3360	3360		0.55
3	+	5525	5525		28	28		++	5549		5549	1.00
4	-	2690	2690		122	122		++	12969		12969	4.82
5	++	5419		5419	42		42	++	100		100	0.02
6	+	9015	9015		62	62		++	4433		4433	0.49
7	-	6072	6072		112	112		++	3568		3568	0.59
8	+	24648	24648		42	42		+	2305	2305		0.09
9	++	5159		5159	86		86	++	1997		1997	0.39
10	+	3646	3646		44	44		++	5065		5065	1.39
11	-	4557	4557		297	297		++	4667		4667	1.02
12	-	2818	2818		42	42		+	1941	1941		0.69
13	+	5582	5582		223	223		++	2656		2656	0.48
14	+	1348	1348		207	207		++	6121		6121	4.54
15	+	23695	23695		184	184		++	7533		7533	0.32
16	++	2989		2989	64		64	++	53175		53175	17.79
18	++	95108		95108	14		14	++	13500		13500	0.14
19	-	20454	20454		182	182		+	544	544		0.03
21	+	16327	16327		50	50		++	2393		2393	0.15
22	-	20886	20886		337	337		++	2980		2980	0.14
23	++	392		392	91		91	++	8652		8652	22.07
24	++	8258		8258	86		86	++	23407		23407	2.83
25	++	5923		5923	131		131	++	14103		14103	2.38
26	+	1894	1894		11	11		++	12220		12220	6.45
27	++	2276		2276	105		105	++	2618		2618	1.15
28	+	2667	2667		14	14		++	15649		15649	5.87
29	++	7939		7939	219		219	++	5887		5887	0.74
30	-	6969	6969		60	60		+	7072	7072		1.01
31	+	2760	2760		33	33		+	2339	2339		0.85
32	++	1441		1441	10		10	++	1195		1195	0.83
33	-	4335	4335		80	80		++	4802		4802	1.11
34	-	36245	36245		256	256		++	12388		12388	0.34
35	++	3889		3889	101		101	++	18268		18268	4.70
36	+	618	618		156	156		++	2883		2883	4.67
37	+	4579	4579		92	92		++	2788		2788	0.61
38	-	428	428		35	35		+	1127	1127		2.63
39	++	1123		1123	65		65	+	1983	1983		1.77
40	-	15741	15741		84	84		+	2622	2622		0.17

Patient	Normal Tissue						Tumour Tissue				Ratio	
	Telomerase Activity <sup>1</sup>	Corrected MESF <sup>2</sup>	Sorted According to Telomerase Activity		Survival (days)	Sorted According to Telomerase Activity		Telomerase activity <sup>1</sup>	Corrected MESF <sup>1</sup>	Sorted According to Telomerase Activity		Tumour/Normal MESF
			Moderate	High		Moderate	High			Moderate	High	
41	-	5964	5964		68	68		++	2623		2623	0.44
42	++	771		771	12		12	++	1592		1592	2.06
43	-	5021	5021		110	110		+	1871	1871		0.37
44	-	9136	9136		91	91		+	9177	9177		1.00
46	-	250	250		70	70		+	702	702		2.81
48	+	271	271		71	71		++	560		560	2.07
49	++	999		999	46		46	++	1621		1621	1.62
50	++	3670		3670	46		46	++	281		281	0.08
51	++	3227		3227	6		6	++	377		377	0.12
53	+	14136	14136		235	235		++	921		921	0.07
54	++	647		647	39		39	++	394		394	0.61
55	+	882	882		61	61		++	801		801	0.91
56	++	437		437	61		61	++	1379		1379	3.16
57	++	991		991	35		35	++	1194		1194	1.20
58	++	1089		1089	73		73	++	1742		1742	1.60
59	++	532		532	86		86	++	293		293	0.55
60	++	1551		1551	12		12	++	1602		1602	1.03
61	-	704	704		105	105		++	1280		1280	1.82
62	+	922	922		110	110		++	481		481	0.52
63	+	1685	1685		324	324		++	1303		1303	0.77
64	-	949	949		139	139		+	2724	2724		2.87
65	-	486	486		87	87		+	1246	1246		2.56
66	+	1357	1357		290	290		+	321	321		0.24
67	+	1024	1024		16	16		++	1365		1365	1.33
68	-	772	772		170	170		+	392	392		0.51
69	-	911	911		99	99		+	1625	1625		1.78
70	+	1764	1764		44	44		+	527	527		0.30
71	+	1243	1243		348	348		++	1811		1811	1.46
72	-	1141	1141		88	88		+	524	524		0.46
73	++	3417		3417	22		22	++	1518		1518	0.44
74	-	807	807		80	80		+	771	771		0.96
75	-	809	809		166	166		++	1147		1147	1.42
	<b>Average</b>	<b>8031</b>	<b>6083</b>	<b>11765</b>	<b>102.3</b>	<b>121</b>	<b>66.4</b>		<b>4865</b>	<b>2159</b>	<b>5948</b>	<b>1.90</b>
	<b>SD</b>	<b>18949</b>	<b>7971</b>	<b>30143</b>	<b>84.5</b>	<b>93</b>	<b>51.1</b>		<b>7792</b>	<b>2244</b>	<b>8899</b>	<b>3.40</b>

1. Telomerase activity as determined in Chapter 3

2. Calculated as follows:  $(F/A)*F$ , where F is the probe MESF and A that of the DNA counterstain.

tumor cells) (Fig 4.8A & C) from the mean fluorescence obtained from cells hybridized with the probe (Fig 4.8B & D). The histogram (E) confirms the shift in fluorescence intensity.

In most cases differences were detected in the mean fluorescence intensity between the normal and the tumor tissue of the same patient (Fig 4.9). The histogram in Fig 4.9(G) shows the mean fluorescence intensity of the normal biopsy and the tumor biopsy of the same patient. It is clear from the overlapping that there was a difference in the fluorescence intensity of probe between these two samples.

Table 4.1 is a summary of the results obtained on 70 of the 75 patients discussed in the previous chapter. In five cases no analyses could be done since one or both of the biopsies were insufficient. All previous flow-FISH work had been done on noncancerous cells where a normal chromosomal complement could be assumed for most of the cells in the population. Since we are working with cancer cells and presumed normal controls, we had to find a way of distinguishing between changes in telomere length and gross chromosomal abnormalities like aneuploidy or polyploidy. We thus normalized the probe fluorescence intensity to the counterstain intensity for each sample, since the 7 AAD fluorescence can be taken as a measure of the amount of DNA present per cell. This normalized value is indicated as "Corrected MESF" in the table.

The most striking result is the wide range of the MESF values in both the normal and tumor samples. In all cases the standard deviation is larger than the mean values, making it very difficult to make valid conclusions about the relevance of the results. It clearly shows that the method is not optimized yet. That said, it is still tempting to make a number of comparisons of the average values.

In the first place the average corrected MESF value of the normal tissue is 8031 while the tumor value is 4865, hinting that telomeres may have shortened on average in the tumor tissue. This would confirm findings by Kim *et al.* (1994). The individual ratios of tumor to normal values span the range from 0.02 to 22.7, both difficult to accept biologically,

but the average of 1.9 confirms the possibility of telomere shortening. Sorting the ratios into increasing vs decreasing values or separating them according to the telomerase values of either the normal or tumor tissue did not cause differences to appear (results not shown).

Secondly, if the MESF values for both tumor and normal tissue are sorted according to each tissue's telomerase activity, an interesting tendency appears: In both cases, the group with the low telomerase activity showed lower averages than the group with high telomerase activity. Against the background of an apparent decrease in telomere length in the tumor tissue in spite of higher telomerase activity levels, this result confirms the complexity of the relationship between telomerase activity and telomere length. If confirmed by Southern hybridization, it could be interpreted to signify reactivation of telomerase in response to telomere shortening.

The survival times of the 70 patients were also included in the table as a control and if it is sorted into moderate vs high telomerase activity for the normal tissue, the average survival times are significantly different at 66 versus 121 days. This result is similar to that reported in the Kaplan-Meier survival curve shown in Chapter 3. It also shows that at this stage of development, flow-FISH is inferior to telomerase activity as prognostic indicator.

The best way to validate the current method is to analyze the same samples by Southern hybridization and flow-FISH, preferably well defined tumor and normal tissue. We have been unable to obtain such tissue until very recently, when a colleague (Dr Richard Naidoo of the Nelson Mandela Medical School in Durban) informed us of his collection of tissue from esophagus resections. This is the perfect type of material for the purpose of comparing the two methods and we have established collaboration on this matter. Unfortunately, it came too late for inclusion in this thesis, but it will be done early next year.

#### 4.4 DISCUSSION

Measurement of telomere length are most commonly performed by Southern analysis of genomic DNA digested with selected restriction enzymes. Using a  $(C_3TA_2)_n$  oligonucleotide probe, the result is a smear, which represents the heterogeneity of the telomere restriction fragment (TRF) length of all cells analyzed. However, such analysis requires a large number of cells ( $> 10^6$ ) and misses detailed information about the length of individual telomeres. In order to circumvent these limitations, Landsdorp *et al.* (1996) have established a Q-FISH technique, which allows an estimation of the telomere length on specific chromosome arms from metaphase cell preparations. Q-FISH has become possible by using directly labeled  $(C_3TA_2)_3$  peptide nucleic acid (PNA) probes. These DNA mimics have several advantageous properties compared with regular oligonucleotides probes, most important of which is that PNA probes can hybridize to complementary telomere repeats under conditions of low ionic strength, which favour annealing of the probe but disfavour renaturation of the DNA strands (Egholm *et al.*, 1993).

Despite the usefulness and amount of information obtained from Q-FISH for telomere length analysis, there are some limitations: (1) the need for metaphase preparations, which are sometimes difficult to obtain (e.g. pre-senescent cells); (2) the restriction to dividing cells represents a potential selection of cells, and (3) Q-FISH is a very time-consuming procedure. For this reason, *in situ* hybridization of telomeres on fixed suspended cells was extended to a very convenient and rapid technique using a flow cytometry (Rufer *et al.*, 1998). This method is called flow-FISH, and provides information on the mean telomere repeat content of individual cells. When combined with the flow cytometer's extensive range of discriminating capacities, the possibilities become enormous.

An ideal telomere-length assay must be rapid, permit the examination of small numbers of cells, require few steps and produce reliable, reproducible and accurate results. Furthermore, it should ideally discriminate between small changes in telomere length

(i.e., be quantitative), measure telomere length *in situ*, and allow for the simultaneous detection of cell phenotype. FISH and flow cytometry hold a great deal of promise in these respects.

A significant advance to facilitate the transition of Q-FISH protocols to *in situ* flow cytometry is the use of peptide nucleic acid (PNA)-labeled probes. PNA probes tagged with fluorescein bind to the telomeres on densely charged chromosome tips, making it possible to count the number of fluorescent molecules present. Cells with longer telomeres will have more fluorescent molecules bound to the ends of their chromosomes than will cells with shorter telomeres. The key to the success of the PNA probes is the neutral charge of the peptide-like backbone. PNA-DNA interactions are more stable than DNA-DNA or DNA-RNA interactions under hybridization conditions (Lansdorp et al., 1997). This property may yield more reproducible results and a stronger fluorescence signal than can be obtained with conventional DNA oligonucleotides.

Flow cytometry methods for quantitative FISH (flow-FISH) on whole cells pose a unique problem related to providing the probe access to the target DNA while maintaining the cell structure. Hultdin *et al.* (1998) employed the standard *in situ* hybridization method of fixation followed by permeabilization. Rufer *et al.* (1998) did without these steps entirely, relying instead on the denaturation conditions to provide access for the probe into the cell. For the analysis of the tumor biopsies we also relied on the denaturation conditions to provide access for the probe into the cell. The small size of the probe and the increased fluidity of the cell membrane at the temperatures used to denature the DNA must permit sufficient permeability to the PNA probe. This strategy avoids the potential probe-accessibility problems associated with excessive fixation (Rufer *et al.*, 1998)

Flow-FISH involves many steps through which relatively minor human or systematic error can translate into large shifts in fluorescence readout. Therefore, a great deal of effort was spent addressing controls necessary to negate or at least assess the contribution of these potential errors in the procedures. The method focused primarily on checking for variations in the cytometer and monitoring intra- and interexperimental errors,

probably because the lack of fixation and permeabilization steps limit some of the inherent tube-to-tube variations. In this procedure, fluorescein isothiocyanate (FITC)-labeled beads were run at the beginning and the end of each experiment, and the resulting calibration curve was used to correct for daily fluctuations in laser intensity, alignment and linearity.

Telomeres in cancer cells are often significantly shorter than those in normal somatic tissue (Couner *et al.*, 1994; de Lange *et al.*, 1990; Hastie *et al.*, 1990; Holzmann *et al.*, 1993; Schmitt *et al.*, 1994). It has been estimated that approximately 90% of primary tumors and perhaps up to 98% of immortal cell lines possess short telomeres and express increased amounts of telomerase activity as opposed to the negligible expression in normal somatic cells and benign tissue (Kim *et al.*, 1994). Hence it was suggested that, because of their increased number of divisions, cancer cells that may initially lack telomerase lose more telomeric repeats than do surrounding cells, and that when telomerase is reactivated in these cancer cells, telomere lengths stabilize, albeit at a shorter length (Counter *et al.*, 1992; Counter *et al.*, 1994). However, in some tumor samples, the reported telomere lengths were much greater than those of normal surrounding tissues, while other tumor samples showed no changes compared with normal donor cells (Hiyama *et al.*, 1995; Nilsson *et al.*, 1994; Schmitt *et al.*, 1994). Reports of telomere lengths in immortalized cell lines have given variable results. Rogalla *et al.* (1994) reported decreased mean telomere length in immortalized cells compared with cells from the originating tumor. On the other hand, telomeres in some immortalized HeLa cell lines can be very long (> 20 kb) (de Lange *et al.*, 1990). While many malignant tumors have detectable telomerase activity, some do not (Hiyama *et al.*, 1995; Kim *et al.*, 1994). Nilsson *et al.* (1994) reported that malignant hematopoietic (acute leukemia) cells could be either positive or negative for telomerase activity but that the telomere lengths were no different in both classes. All these results suggest that the relationship between telomerase activity and telomere length is not a simple one.



Furthermore, those cell lines that do not express detectable levels of telomerase are often found to possess alternative methods for maintaining telomere length (Ogino *et al.*, 1998). Therefore, telomere maintenance seems to be a key component in the multistep process of tumorigenesis. It is conceivable that monitoring telomere length may be predictive, in conjunction with other factors, in tracking malignant tumor formation.

A static picture of telomere length cannot be used as an indicator of telomerase activity. Even a dynamic picture of TRF length increasing or decreasing should not be relied on as reflecting the presence or absence of telomerase, since at least three events could yield a false interpretation of telomerase activity: (1) a changing population of cells within a tissue, (2) an imbalance between telomerase activity and telomere loss and (3) recombination. Caution should be exercised in the interpretation of all TRF length data, especially in tumors in which mixed populations of cells are likely to be present.

Flow cytometry provides a powerful tool for examining telomere length in different cell and tissue types. The method described in this chapter enables the routine quantitative analysis of telomere length, that surpasses other available methods for ease and rapidity, to be used. It permits the large-scale screening of samples for changes in telomere length that will be important in the research laboratory in the study of HIV and cancer. This new methodology may be clinically applicable to the screening of stem-cell and bone marrow samples intended for transplantation to ensure sufficient telomere length to provide hematopoietic reconstitution in the recipient and sufficient residual proliferative potential for a normal life span. In conclusion, studies in telomere biology appear to be on the brink of being revolutionized with the introduction of flow-FISH technology.

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## CHAPTER 5

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### DEVELOPMENT OF PEPTIDE INHIBITORS OF TELOMERASE ACTIVITY BY USING PHAGE DISPLAY TECHNOLOGY

	PAGES
5.1 INTRODUCTION	123
5.2 PHAGE DISPLAY TECHNOLOGY	124
5.2.1 Introduction	124
5.2.2 The filamentous phage	125
5.2.3 Construction of a random phage library	128
5.2.4 Selection and Replication	129
5.2.5 Proposed strategy to select peptides binding to telomerase RNA	131
5.3 MATERIALS AND METHODS	131
5.3.1 Synthesis of 2 oligonucleotides	131
5.3.2 Cleavage of the pGEMEX-1 vector	133
5.3.3 Recovery of DNA from the agarose gel	133
5.3.4 Ligation of the oligonucleotides to the plasmid DNA	135
5.3.5 Transformation	135
5.3.6 Preparation of competent cells	135
5.3.7 Plasmid isolation	136
5.3.8 Digestion with Sac I	136



5.3.9	DNA purification	137
5.3.10	RNA synthesis	137
5.3.11	Isolation of RNA-binding phages	138
5.3.12	ELISA of individual rounds	139
5.3.13	Phage ELISA of single colonies	140
5.3.14	Cultered used for panning	140
5.4	<b>RESULTS</b>	140
5.5	<b>DISCUSSION</b>	146
5.6	<b>REFERENCES</b>	148

## CHAPTER 5

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# DEVELOPMENT OF PEPTIDE INHIBITORS OF TELOMERASE ACTIVITY BY USING PHAGE DISPLAY TECHNOLOGY

### 5.1 INTRODUCTION

Telomerase has attracted intense interest as a possible target for cancer therapeutics. The apparent absence of measurable telomerase activity in many (but not all) normal cells raises the possibility that telomerase inhibitors might have a place in cancer therapy (Morin, 1989; Kim *et al.*, 1994). Besides the potential therapeutic benefits of telomerase inhibitors, telomerase activity may also be useful in cancer diagnosis and serve as a prognostic indicator of clinical outcome. Assay for telomerase activity may also be very useful as a screen for the detection of early cancer or early metastasis, assuming metastatic tumor cells can be distinguished from blood-derived stem cells. For cancer patients, early detection of tumors having telomerase activity could lead to more aggressive adjuvant chemotherapy e.g. the use of telomerase inhibitors when the patient has a low tumor burden. Low doses of telomerase inhibitors for a long-term treatment of cancer patients to prevent metastases and routine screening of peripheral blood for the presence of telomerase as an early means of tumor detection, are optimistic expectations. Despite a strong causal link between activity and malignancy, a definitive proof of principle that telomerase activity would effectively inhibit the growth of tumors is yet to be established. Furthermore, to date no regulators of telomerase activity have been described. Similar to other epithelial cancers, we have found esophageal tumors to have high telomerase activities, while the normal biopsies showed lower activities (Chapter 3). This suggested that telomerase activity could be a possible target for chemotherapy in

this cancer. Previous attempts at inhibiting telomerase activity utilized antisense oligonucleotides targeted at the template region of the RNA subunit of the enzyme (Friesen and Darby, 1997 and 1998). Although it worked very well in cell extracts, getting the oligonucleotides into intact cells are difficult.

In this study we attempted a peptide-based approach to overcome the transport problem. A phage-display selection strategy was designed to isolate peptides capable of binding to the RNA template with high affinity and in so doing preventing the enzyme from elongating existing telomeres.

## **5.2 PHAGE DISPLAY TECHNOLOGY**

### **5.2.1 Introduction**

Random peptides displayed on phage have been used in a number of applications, including epitope mapping (Scott and Smith, 1990), mapping protein-protein contacts (Hong and Boulanger, 1995), and identification of peptide mimics of non-peptide ligands (Devlin *et al.*, 1990). Bioactive peptides have been identified either by panning against immobilized purified receptor, or against intact cells (Doorbar and Winter, 1994). Protease substrates have been identified by attaching an affinity tag upstream from the randomized region, and separating cleaved from uncleaved phage with the appropriate affinity matrix (Smith *et al.*, 1995). Conversely, larger proteins [antibodies (Barbas *et al.*, 1991), hormones (Lowman *et al.*, 1991), protease inhibitors (Roberts *et al.*, 1992) and DNA binding proteins (Soumillion *et al.*, 1994)] have been displayed on phage, and variants with altered affinity or specificity have been isolated from libraries of random mutants.

The power of this technique lies in the binding of functional activity with the genetic information. Peptide displays enable fast selection and amplification of the chosen peptide encoded by the genetic information.

For a better understanding of this technique, the main features are discussed in more detail in the following sections.

### 5.2.2 The filamentous phage

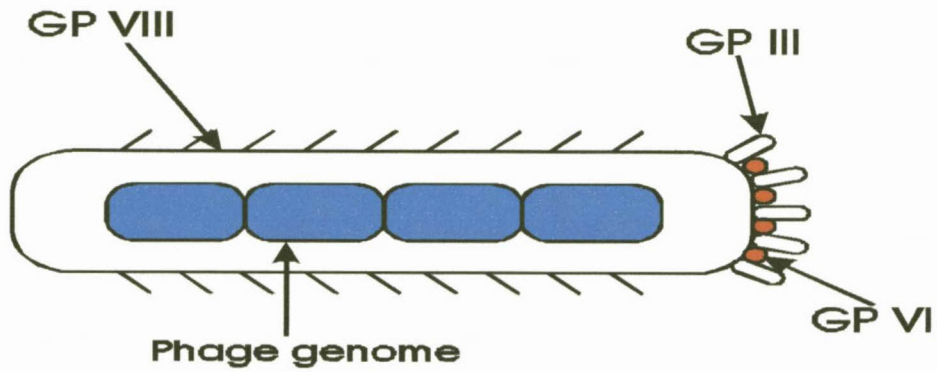
The filamentous bacteriophage M13 or fd can be used to display foreign peptides and proteins, allowing the construction of therapeutic, diagnostic and technological tools of broad utility. Foreign peptides are readily fused with a structural protein by insertion of the corresponding nucleotide sequence into the gene coding for the protein (Smith, 1991). This insertion results in a display of protein or peptide on the surface of the phage, providing that the insert does not interfere with the essential functions of the protein. If the peptide is well exposed on the surface of the phage, it will be available to act as a ligand, enzyme, immunogen, or otherwise actively participate in a biochemical process. Insertion of random oligonucleotide sequences provide a means of constructing peptide libraries that may be screened to select peptides with specific affinities or activities (Scott, 1992). To realize the full potential of this technique one must understand the structure of the viral particle, the effect that inserts have on the essential functions of the gene products and how the structural proteins interact with other proteins during assembly and infection.

*E-coli* phage M13 presents an alternative mode of cloning, namely, cloning in a single-stranded DNA molecule (Markland *et al.*, 1991). M13 is a filamentous phage that can only be adsorbed onto strains of *E-coli* containing the F sex plasmid. The phage particle contains one circular single-stranded DNA molecule, called the (+) strand. Following infection, the single-stranded DNA is converted to a double-stranded circular replicating form, by synthesis of a complementary strand termed the (-) strand. More replicating form molecules are made throughout the life cycle (Kornberg, 1980) while later in the life cycle, progeny (+) strands are made preferentially and packaged in the phage coat. Packaging of M13 differs from most other phages in that there seems to be no limit to the amount of DNA that can be packaged. Since the particle is filamentous, packaging of a larger circular DNA molecule merely results in formation of a longer filament. Another

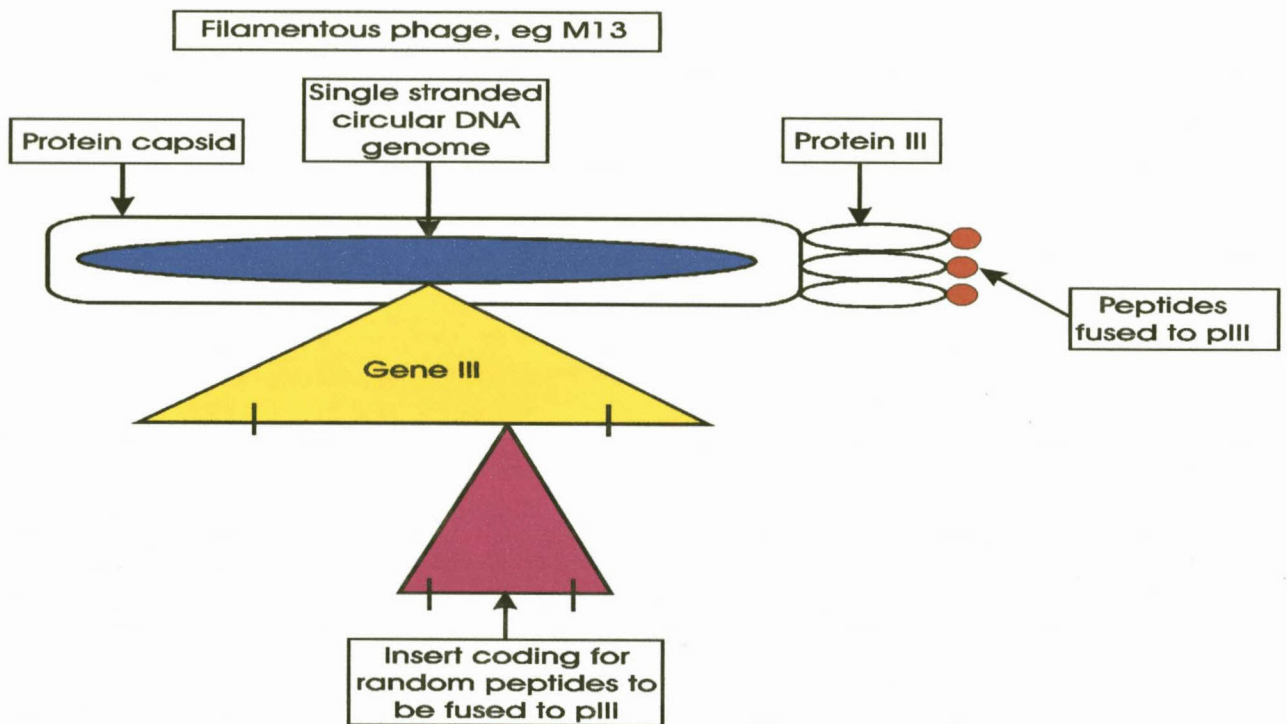
unusual feature of the life cycle is that infected cells do not lyse, instead, progeny phage particles are extruded from an infected cell. This property has a significant effect on the morphology and timing of the appearance of plaques, because plaques are normally the result of lysis of bacteria in a bacterial lawn. Infected cells grow more slowly than uninfected cells, thus an M13 plaque is a region in which cells are growing more slowly than in neighbouring cells (Kornberg, 1980), giving rise to turbid zones.

The *E-coli* filamentous bacteriophages, such as M13, are ideal as *in vitro* selection vehicles: they have small genomes in which large libraries are easily constructed. The viral particles (virions) are stable to potential elution conditions such as low pH and they accumulate to high titers ( $10^{12}$  ml<sup>-1</sup>), so that every clone in the library can be well represented (Smith, 1991).

The M13 phage is about 65 Å in diameter with a length dependent on the length of the enclosed genome (Ilychev *et al.*, 1989). A large fraction of the virion mass is made up of many copies of the 50 amino acid coat protein, gpVIII, that forms a 15-20 Å thick flexible cylinder around the single-stranded viral genome. On the distal end of the virus, which is assembled first, there are five copies each of the small, hydrophobic peptides gpVII (33 amino acids) and gpIX (32 amino acids) (Simons *et al.*, 1981; Lopez and Webster, 1983). At the proximal end of the virus are five copies each of gpIII (406 amino acids) and gpVI (113 amino acids) (Simons *et al.*, 1981) (Fig. 5.1). The gpIII protein is involved in binding to the host cell, which is the first step in infection (Henry and Pratt, 1969). GpIII is a protein required for cell infection, attaching to the F-pilus of the male *E-coli*, and is encoded by gene III. The first phage display constructs were made using sites near the amino terminus of gpIII (Smith, 1985), a region where a broad range of peptides and proteins have been successfully inserted. The amino terminus appears to be highly exposed, very near the proximal tip of the virion.



**Figure 5.1 Schematic diagram of the filamentous phage M13.**



**Figure 5.2 Diagram of the construction of a phage library**

GpIII suffers from at least two problems as a peptide vehicle. First, there are only five copies of gpIII per virion, or about one site for each 3MDa of virus. For many applications this is not an important limitation. For applications where dose is important, this may be an unacceptably low number of sites. Secondly, larger inserts at the amino terminus appear to interfere with the infection process (Smith, 1991 and 1993). This suggests that the host-binding site is located near the insertion site. Large inserts may also promote periplasmic degradation of gpIII before assembly into the phage particle (Smith and Scott, 1993). These problems appear to be remedied to some extent by the use of hybrid constructions.

### 5.2.3 Construction of a random phage library

Random phage display peptide libraries are constructed by inserting random oligonucleotides into the protein III gene of phage M13 genome (Fig. 5.2). In the very similar M13 bacteriophage gene III display system described by Devlin *et al.* (1990), synthetic oligonucleotides are cloned into a site two to three amino acid residues downstream from the cleavage site. Peptides encoded by the inserted oligonucleotides are therefore displayed near the amino terminus of the mature pIII in knob-like structures that extend from the surface of the virus particle (Crissman and Smith, 1984). The cyclic 7 mer and linear 12 mer phage display peptide libraries used in this study are based on combinatorial libraries of random 7-mer and 12-mer peptides fused to minor coat protein pIII of phage M13 (Ph D Phage Display Peptide Library Kit, New England, Biolabs, Inc).

In the cyclic 7 mer phage display library the randomized sequence is flanked by a pair of cysteine residues. Under nonreducing conditions the cysteines will spontaneously form a disulfide cross-link, resulting in display of cyclized peptides. Disulphide bond formation during phage assembly is very efficient, and proteins containing multiple correctly formed disulphide bonds can be expressed on the surface of the phage.

In the linear 12 mer phage display peptide library the displayed peptides are expressed at the N-terminus of pIII, i.e., the first residue of the mature protein is the first randomized

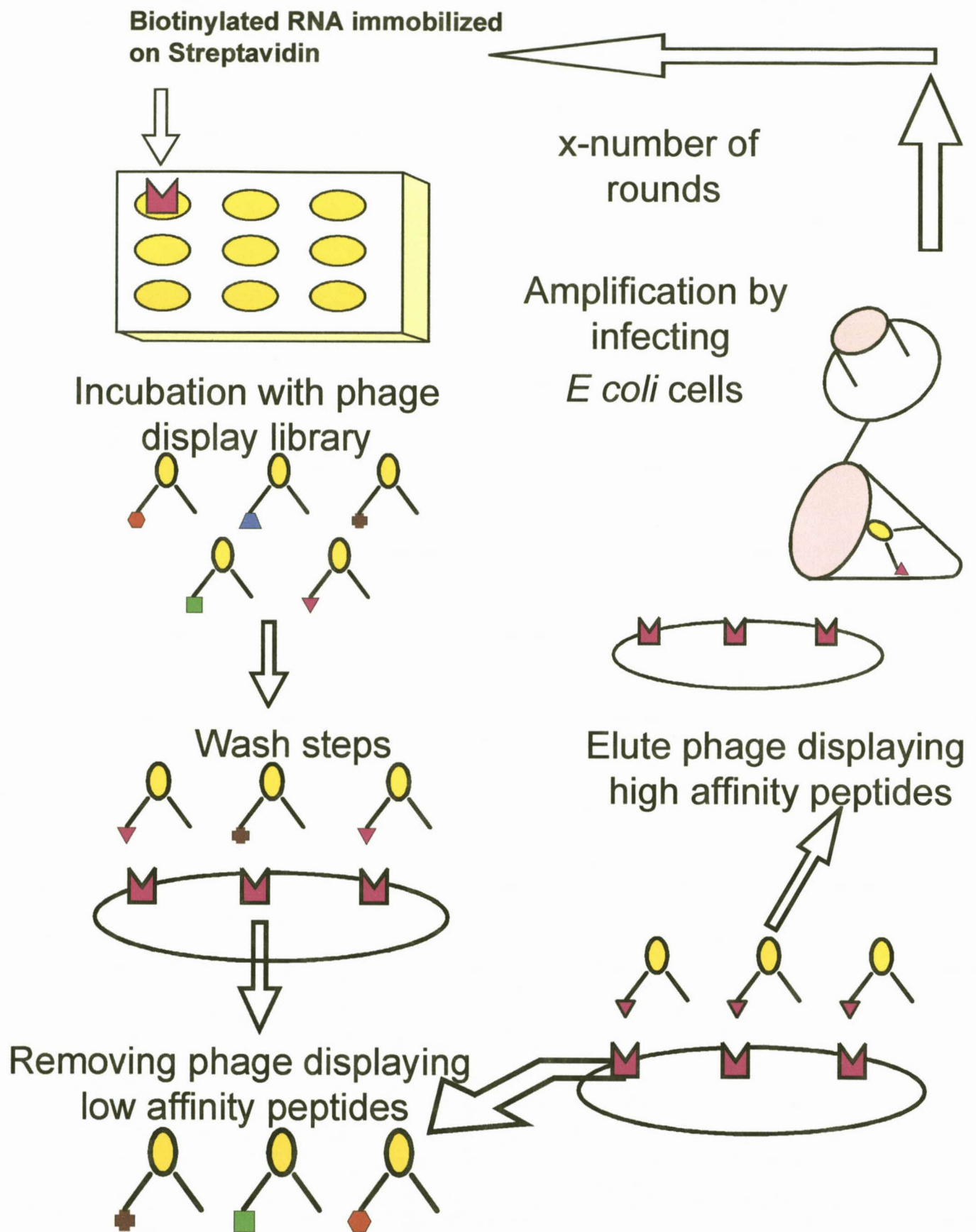
position. A short spacer (Gly-Gly-Gly-Ser) and then the wild-type pIII sequence follow the peptide.

#### 5.2.4 Selection and Replication

Selection of the phage is done by immobilizing the target protein (receptor, antibody, ligand etc.) to a plastic immuno-tube or to magnetic beads and blocked by incubation with fat free milk powder and BSA (Fig. 5.3). After blocking the non-specific binding sites the target protein is incubated with the phage library. After interaction, the unbound phage is washed away. The bound phage can be non-specifically eluted with a high acid solution or specifically eluted with an antibody or inhibitor of the target protein. After the acid elution the phage solution is neutralized.

The eluted phage is then incubated overnight with F-positive *E coli* in the exponential phase. The infected *E coli* therefore produces phages. The *E-coli* culture is then centrifuged and purified by two polyethyleneglycol sodium chloride (PEG-NaCl) precipitation and resuspension cycles. The selected phage is then used for selection and replication in two, three or four subsequent rounds. The amount of binding phage can be amplified between 1000 and 100 000 times per round. The success of this selection process is measured by collecting a small amount of the infected bacteria in the last selection round and plating it out on agar plates, to grow single colonies. Due to the fact that a phage can only infect one bacterium, each colony produces a group of identical phages. Single plaques can be picked up and cultured in micro-titer plates. This produces monoclonal phage that can be collected after the centrifuge steps. The phage plaques are then tested with an ELISA technique for binding to the target protein. To summarize this ELISA technique: the phage is transferred to a micro-titer plate coated with the target protein. After incubation, the non-binding phage is washed away and an anti-phage antibody, conjugated to horseradish peroxidase (HRP), is added to the well. After another wash step the colour substrate is added to identify the binding phage plaques. High affinity binders are then cultered, DNA is extracted and sequenced to determine the amino acid sequence of the peptides.





**Figure 5.3 A schematic presentation of the selection and amplification of peptides on the phage surface.**

### 5.2.5 Proposed strategy to select peptides binding to telomerase RNA

The human telomerase RNA is 450 bp in size and the most important part for enzyme activity is the template region, upon which new telomere repeats are synthesized. In order to select for peptides that could bind to this stretch of RNA and hopefully inhibit enzyme activity, a gene was designed coded for the core template and short flanking regions (Fig 5.4). When cloned into a plasmid containing SP6 and T3 promoters flanking the cloning site, large amounts of RNA can be synthesized *in vitro*, depending on which strand needs to be read. Digesting the plasmid DNA with Sac I in this case leads to a linear transcription template and a product of defined size when using the SP6 promoter.

In order to immobilize the target RNA during selection, a 20 mer biotinylated oligonucleotide was designed to capture the RNA by binding to the upstream part of the molecule.

## 5.3 MATERIALS AND METHODS

### 5.3.1 *Synthesis of 2 oligonucleotides*

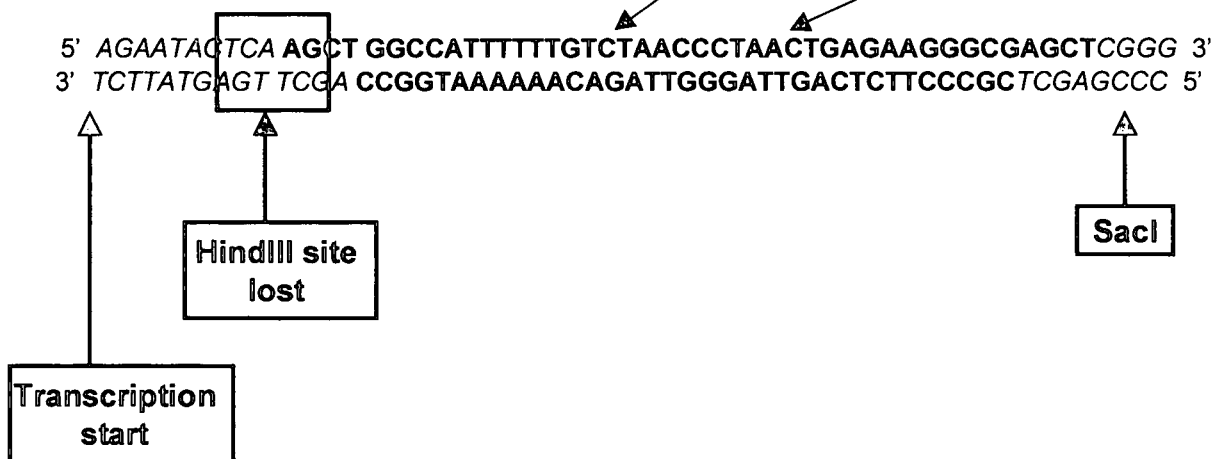
Two complementary oligonucleotides were designed to span the core template region of the telomerase RNA: Teltop (5'-AGC TGG CCA TTT TTT GTC TAA CCC TAA CTG AGA AGG GCG AGC T-3') and Telbot (5'-CGC CCT TCT CAG TTA GGG TTA GAC AAA AAA TGG CC-3') were synthesized by Integrated DNA Technologies. They were annealed to one another and ligated to linearised pGEMEX-1. For the annealing reaction 10 µl of 100 pmol/µl of Teltop and Telbot were added to a 1.5 ml microcentrifuge tube containing 80 µl of STE-buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA; 100 mM NaCl). The mixture was incubated at 94 °C for 2 minutes, cooled down slowly and stored at 4°C.

**Biotinylated capture oligonucleotide (Captel)**

3' BCTTATGAGTTCGACCGGTAA

Template

mRNA 5' GAAUACUCAAGCUGGCCAUUUUUUGU **CUAACCCUAAC** UGAGAAGGGCG 3'



1. Synthesize 2 oligonucleotides Teltop & Telbot.
2. Clone into pGEMEX-1, Hind III & Sac I sites.
3. Digest with SacI, transcribe RNA with SP6 RNA polymerase.
4. Hybridize RNA with capture oligo.
5. Immobilize on Streptavidin-Sepharose.
6. Screen phage display library to isolate RNA binders.

**Figure 5.4 Summary of the proposed development of peptide inhibitors of telomerase activity.**

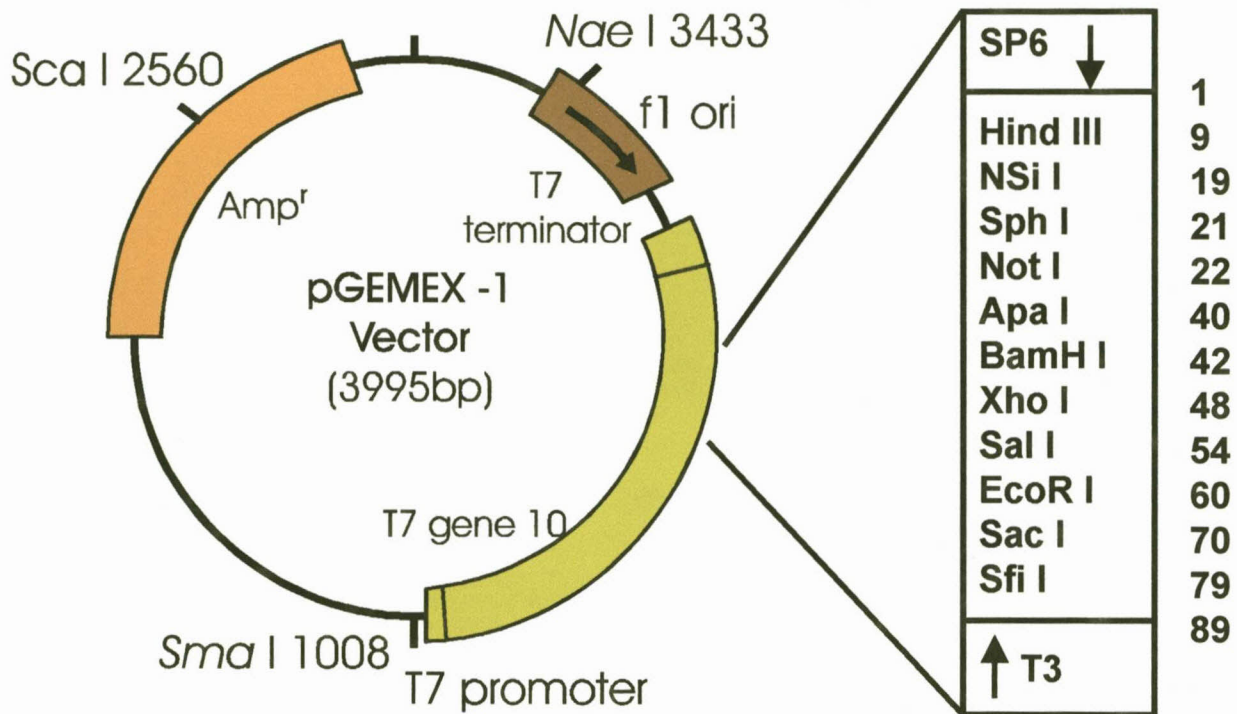
### 5.3.2 *Cleavage of the pGEMEX-1vector*

The pGEMEX-1vector was supplied by Promega (Fig. 5.5). One hundred  $\mu$ l plasmid DNA (40  $\mu$ g/ml) was added to 15  $\mu$ l restriction buffer (100 mM Tris-HCl, pH 7.5; 100 mM Mg Cl<sub>2</sub>; 500 mM NaCl; 10 mM DTT). Ten units Hind III and Sac I and 25  $\mu$ l water were added to the reaction mixture and incubated at 37 °C overnight.

The cleavage of the plasmid DNA was checked on a 0.8% agarose gel (Sambrook et al., 1989).

### 5.3.3 *Recovery of DNA from the agarose gel*

The digested plasmid DNA was recovered by using a Nucleon extraction and purification kit supplied by Amersham. A 1.5 ml microcentrifuge tube was weighed. The gel slice was cut out under long wavelength UV light, removing as much of the non-DNA-containing regions of the gel as possible. The gel fragments were placed in the pre-weighed microtube, weighed and the volume of the DNA was calculated. 4.5 Volumes of 6M sodium iodide was added to the DNA sample volume and incubated at 55 °C, with occasional shaking, until the gel slice had melted completely. Once melted, 10  $\mu$ l resuspended resin per 1-2  $\mu$ g DNA in the gel slice was added, incubated at room temperature for 1 minute and spun down for 30 seconds at 16 000xg. The supernatant was discarded and 1 ml of wash buffer was added to the pellet and spun for 30 seconds at 16 000xg. The wash buffer was discarded and the pellet was left with the lid open at 55 °C for 3 minutes to remove any remaining ethanol from the tube. Ten  $\mu$ l of sterile water was added to the pellet and incubated at room temperature for 1 minute and then spun for 30 seconds at 16 000xg, and the eluted DNA sample was removed to a clean tube. An 0.8% agarose gel was used to check if the plasmid DNA was recovered.



**Vector Map notes:**

1. Sequence reference points:

- |   |           |
|---|-----------|
| a. SP6 RNA Polymerase transcription initiation site | 1         |
| b. T3 RNA Polymerase transcription initiation site  | 89        |
| c. SP6 RNA Polymerase promoter                      | 3979-6    |
| d. T3 RNA Polymerase promoter                       | 84-106    |
| e. multiple cloning sites                           | 9-84      |
| f. T7 RNA Polymerase transcription initiation site  | 968       |
| g. T7 RNA Polymerase promoter                       | 969-985   |
| h. T7 gene 10 start codon                           | 905       |
| i. T7 terminator region                             | 3841-3931 |
| j. phage f1 region                                  | 3306-3761 |
| k. β-lactamase (Amp <sup>r</sup> ) coding region    | 2007-3867 |

**Figure 5.5 PGEMEX-1 Vector map.**

#### 5.3.4 *Ligation of the oligonucleotides to the plasmid DNA*

The annealed Teltop and Telbot were ligated to the cleaved plasmid DNA by using a Fast-Link DNA Ligation and Screening Kit, supplied by Epicenter Technologies. For the ligation reaction the following reagents were mixed: 5  $\mu$ l plasmid DNA, 1  $\mu$ l annealed oligonucleotides, 1  $\mu$ l T4 DNA ligase (2.5 U/ $\mu$ l), 2  $\mu$ l 10 mM ATP, 2  $\mu$ l 10x ligase buffer and 4  $\mu$ l water. Then the reaction was incubated at 14 °C overnight.

#### 5.3.5 *Transformation*

Two hundred  $\mu$ l of competent cells (See 5.3.6) was thawed and immediately dispensed into the tube containing the ligated DNA reaction mixture, and gently mixed. The DNA and the cells were placed on ice for 30 minutes. Thereafter, the cells were heat-shocked by placing the tube into a 42 °C water bath for 2 minutes. Then 800  $\mu$ l LB medium was added to the tube and incubated for 1 hour at 37 °C. The mixture was centrifuged at 5000xg for 5 minutes and the pellet was resuspended in 100  $\mu$ l TE buffer (pH 7.6). Aliquots of the transformation culture were plated on LB plates containing 50  $\mu$ g/ml ampicillin. The plates were incubated at 37 °C overnight.

Ten transformants were picked, plasmids were isolated (5.3.7) and analyzed by agarose gel electrophoresis for the presence of inserts. Three of the positives were then subjected to fluorescent dye terminator sequencing on an Applied Biosystems 377 automated sequencer. All three had the insert error-free in the correct orientation. One clone was chosen as a source of RNA.

#### 5.3.6 *Preparation of competent cells*

A single colony of *E coli* NM 522 cells was inoculated into 5 ml LB medium and grown overnight at 37 °C with moderate shaking (250 rpm). One ml of the overnight culture was inoculated into 40 ml LB medium in a sterile 2-litre flask and grown at 37 °C with moderate shaking (250 rpm) to an OD<sub>590</sub> of 0.375. The culture was transferred to 50 ml

prechilled, sterile polypropylene tubes and left on ice for 5-10 minutes. Thereafter, the cells were centrifuged at 2000xg for 7 minutes at 4 °C. The pellet was resuspended in 1 ml ice cold 100 mM MgCl<sub>2</sub> and maintained on ice for 10 minutes. Then the cells were centrifuged at 2000xg for 7 minutes at 4 °C. The pellet was resuspended in a total volume of 8 ml ice cold 100 mM CaCl<sub>2</sub> containing 15% (v/v) glycerol. The cells were stored in 200 µl aliquots at -70 °C until use.

### 5.3.7 *Plasmid isolation*

Five ml LB medium was inoculated with a single colony and grown overnight at 37 °C with moderate shaking (250 rpm). The cells were centrifuged at 2000xg for 1 minute. The pellet was resuspended in 100 µl TE buffer (pH 7.6) and maintained at room temperature for 5 minutes. Thereafter 200 µl of 0.2N NaOH containing 1% SDS was added to the mixture, mixed gently and placed on ice for 5 minutes. After the incubation time, 150 µl 3M potassium acetate was added to the mixture, vortexed and placed on ice for 5 minutes. The cells were centrifuged at 15 000xg for 1 minute. The supernatant was transferred to a fresh tube, 900 µl 100% ethanol was added to the supernatant and maintained at room temperature for 5 minutes to precipitate nucleic acids. The mixture was centrifuged at 15 000xg for 2 minutes. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and the pellet was resuspended in 20 µl TE buffer (pH 7.6).

### 5.3.8 *Digestion with SacI*

Twenty µl plasmid DNA was added to 6 µl restriction buffer (100mM Tris-HCl, pH 7.5; 100 mM Mg Cl<sub>2</sub>; 500 mM Na Cl; 10 mM DTT). Ten units Sac I and 28 µl of water were added to the reaction mixture and incubated at 37 °C overnight.

The cleavage of the plasmid DNA was checked on a 0.8% agarose gel and the remainder was purified (see 5.3.9).

### 5.3.9 DNA purification

The Sac I digested plasmid DNA was purified by using a High Pure PCR Product Purification Kit supplied by Boehringer Mannheim. Five hundred  $\mu\text{l}$  binding buffer [3 M guanidine-thiocyanate, 10 mM Tris-HCl, pH 6.6, 5% ethanol (v/v)] was added to 100  $\mu\text{l}$  DNA reaction and mixed well. A High Pure filter tube was coupled to the collection tube and the sample was pipetted to the upper reservoir. The tube was centrifuged at 13 000xg for 30 seconds at room temperature. The flow through was discarded and the filter was again coupled to the same collecting tube. Washing buffer (20 mM NaCl and Tris-HCl, pH 7.5) was added to the upper reservoir and the tube was centrifuged at 13 000xg for 30 seconds at room temperature. The washing buffer flow was discarded and the filter was coupled to the same collecting tube. This step was repeated twice. Thereafter the collecting tube was discarded and the filter inserted into a clean 1.5 ml microcentrifuge tube. One hundred  $\mu\text{l}$  elution buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.5) was added to the filter tube and the tube was centrifuged at 16 000xg for 30 seconds at room temperature.

### 5.3.10 RNA synthesis:

The RiboMax<sup>TM</sup> Large Scale RNA Production System (Promega), based on two published protocols that use HEPES buffer (Gurevich *et al.*, 1991) and yeast inorganic pyrophosphatase (Cunningham and Ofengand, 1990), was used for the synthesis of RNA. The following reaction mixture was set up in a 1.5 ml microcentrifuge tube: 40  $\mu\text{l}$  SP6 Transcription Buffer, 40  $\mu\text{l}$  of 100mM rNTPs, 50  $\mu\text{l}$  linearized plasmid DNA, 52  $\mu\text{l}$  of water and 20  $\mu\text{l}$  of SP6 Enzyme mix, to make up a total volume of 200  $\mu\text{l}$ . The reaction mixture was gently mixed and incubated for 2-4 hours at 37 °C. After performing the *in vitro* transcription reaction, 1u/ $\mu\text{g}$  of RQ RNase was added to the mixture and it was incubated for 15 minutes at 37 °C. The mixture was extracted with 1 volume of TE (pH 4.5)-saturated phenol:chloroform:isoamyl alcohol (25:24:1), vortexed for 1 minute and centrifuged at 16 000xg for 2 minutes at room temperature. The upper, aqueous phase was transferred to a clean tube and 1 volume of chloroform:isoamyl alcohol (24:1) was



added to the tube. The mixture was then vortexed for 1 minute and centrifuged at 16 000xg for 2 minutes at room temperature. The upper, aqueous phase was transferred to a clean tube and 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol (or 2.5 volumes of 95% ethanol) were added to the tube. The content of the tube were mixed and placed on ice for 5 minutes, then centrifuged at 16 000xg for 10 minutes at room temperature. The supernatant was carefully poured off and the pellet washed in 70% ethanol. The pellet was dried under vacuum for 10 minutes and the RNA sample was suspended in 100 µl TE buffer (or nuclease-free water) and stored at -70 °C. The RNA was captured by mixing 65 µl of the plasmid RNA and 10 µl of a 200 pmol/µl 20 mer Captel primer in a 1,5 ml eppendorf and the mixture was incubated at 95°C for 1 min and cooled down slowly. In the later experiments, RNA was biotinylated during the synthesis of RNA, by replacing UTP by 25 mM biotinylated UTP in the reaction mixture. In these cases, streptavidin-magnetic particles (Promega) were used for the capturing of the RNA.

#### 5.3.11 *Isolation of RNA-binding phages*

A cyclic 7- and a linear 12-mer phage display peptide library was purchased from New England Biolabs (Beverly, MA, USA). Streptavidin-magnetic particles (1.5 mg; Boehringer Mannheim, Germany) were first blocked for 2 hours at 4 °C with 2% skimmed milk (DIFCO, Detroit, MI, USA) solution in Tris-buffered Saline (TBS) at room temperature. Phage (approximately  $2 \times 10^{11}$  phages) was added to half of the blocked streptavidin magnetic particles and incubated for 1 hour at room temperature. To the other half of the magnetic particles, 30 µg of biotinylated RNA was added and incubated for 30 minutes at room temperature. The phage supernatant, i.e. phages that did not bind to the magnetic beads, was then transferred to the RNA-bound magnetic particles and incubated overnight at 4°C. After incubation, the magnetic particles were washed ten times with washing buffer (100mM KCl, 1mM MgCl<sub>2</sub>, 10µM ZnCl<sub>2</sub>, 5% glycerol, 5mM DTT, 10mM Tris-HCl, pH 7.5) to remove the non-binding phages. Phages bound to RNA were eluted with 500 µl elution buffer (4M NaCl, 5mM MgCl<sub>2</sub>, 10µM ZnCl<sub>2</sub>, 5% glycerol, 15mM HEPES, pH 7.8) for 15 minutes at room temperature.

A high salt solution buffer was used for the elution of the bound phage, because RNA can be hydrolyzed by a high acid solution. The titre of the eluted phages was estimated and half of the eluted fraction was used for amplification. Two additional rounds of selection were performed, phages were amplified by infection of *Escherichia coli* ER2537 cells and purified from the supernatant by two polyethylene glycol precipitations. A fraction (10 µl) of the eluted phages of selection round 4 was subjected to serial ten-fold dilutions with LB culture media and plated out on agar plates. To 10 µl of each of the phage dilutions, 200 µl of log-phase *E coli* cells were added and the mixture was incubated for 5 minutes at room temperature to allow the phage to infect the *E coli* cells. Each dilution was spread onto agar plates containing IPTG (isopropyl-β-D-thiogalactoside) and Xgal (5-Bromo-4-chloro-3-indonyl-β-D-galactoside) and incubated overnight at 37 °C. Since the phage is derived from the common cloning vector M13mp19, which carries the LacZα-gene, phage plaques appear blue when plated on media containing Xgal and IPTG.

#### 5.3.12 ELISA of individual rounds

A 96-well ELISA plate (Maxisorp, Nunc International) was coated with 100 µg/ml Streptavidin overnight at 4 °C in a humidified container. The next morning the wells were blocked for 2 hours at room temperature with 200 µl 4% skimmed milk solution. After incubation the wells were washed three times with washing buffer (100mM KCl, 1mM MgCl<sub>2</sub>, 10µM ZnCl<sub>2</sub>, 5% glycerol, 5mM DTT, 10mM Tris, pH 7.5). Then the wells were filled with 5pmol of biotinylated RNA and binding buffer (0.05% Nonidet p-40, 90mM KCl, 10µM ZnCl<sub>2</sub>, 5mM DTT, 8% glycerol, 1mM MgCl<sub>2</sub>, 20 mM Tris, pH 7.5), 100 µl in each well, for 1 hour. After incubation the wells were washed six times with washing buffer. One hundred µl of the *E coli* culture supernatants of rounds 1, 2 and 3 were added to each well with 100 µl of 4% skimmed milk, and incubated at room temperature for 2 hours. After incubation the wells were washed nine times with washing buffer. Bound phages were detected after a 1 hour incubation with a polyclonal anti-M13 horseradish peroxidase (HRP)-conjugated antibody (Pharmacia, USA), washed

12 times with washing buffer, and visualisation was performed with ortho-phenylenediamine (OPD, Sigma). The reaction was stopped with 100  $\mu$ l 4M H<sub>2</sub>SO<sub>4</sub> and absorbance was determined at 490-630nm.

#### 5.3.13 Phage ELISA of single colonies

Forty-eight blue phage plaques from the third panning round of the linear 12 mer library were picked from the agar plate and grown overnight at 37 °C in 1 ml LB-medium containing 1:100 diluted *E-coli* cells from a preculture. These 48 phage plaques were tested for RNA binding in an ELISA. The same technique was used as described for the previous ELISA (5.3.12).

#### 5.3.14 Cultured used for panning

A pre-culture of *E coli* was prepared by inoculating 10 ml LB medium with *E coli* cells. The pre-culture was incubated overnight at 37°C with gentle agitation. The next day the pre-culture was diluted 1:100 in 40 ml LB medium in a 250 ml Erlenmeyer flask, incubated at 37°C with vigorous shaking for 4 hours (until log-phase). Three hundred  $\mu$ l eluted phages were then added to the 40 ml log-phage *E coli* culture and incubated overnight at 37°C with gentle agitation.

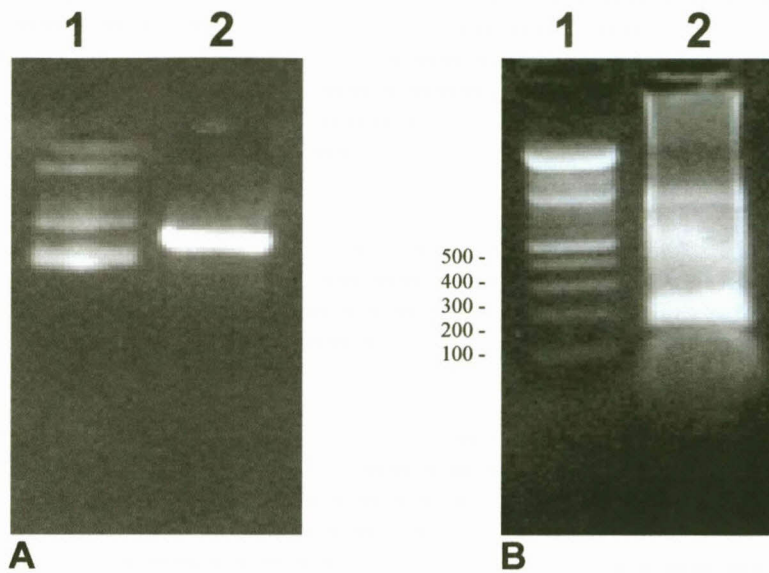
## 5.4 RESULTS

Previous attempts at inhibiting telomerase activity utilized antisense oligonucleotides targeted at the template region of the RNA subunit of the enzyme (Pitts and Corey, 1998; Norton *et al.*, 1996; Hahn *et al.*, 1999). Although it worked very well in cell extracts, getting the oligonucleotides into intact cells are difficult. We attempted a peptide-based approach to overcome the transport problem by designing a phage-display selection strategy. This was done to isolate peptides capable of binding to the RNA template with high affinity and in so doing preventing the enzyme from elongating existing telomeres.

The pGEMEX-1 plasmid DNA was cleaved by Sac I and Hind III. The cleavage of the plasmid was checked on a 0.8% agarose gel. The plasmid DNA was recovered from the agarose gel. The Teltop and Telbot oligonucleotides were first annealed to each other and then ligated to the cleaved plasmid DNA. The ligated pGEMEX-1 was used to transform *E. coli* NM 522. Ten transformants were picked, the plasmids were isolated and analyzed by agarose gel electrophoresis. Three of the clones were randomly picked and subjected to fluorescent dye terminator sequencing. All three had the insert error-free in the correct orientation. One clone was chosen as template for RNA synthesis and it was linearized with Sac I (Fig 5.6A) before inclusion in an *in vitro* transcription reaction using SP6 RNA polymerase (Fig 5.6B). It is clear that a large amount of RNA had been synthesized and the smear is probably due to secondary structure, since it was run on a non-denaturing gel.

In order to use the RNA as target for peptides displayed on phages, it needed to be immobilized. For that purpose a capture oligonucleotide was synthesized, but despite many attempts, only a small fraction of the RNA was immobilized (as determined by electrophoretic analysis of the RNA after binding). Subsequently, RNA was biotinylated during synthesis of the RNA by replacing UTP by biotinylated UTP in the reaction mixture.

The cyclic 7 mer phage display peptide library was screened first. The phage titre after each round was high (Table 5.1). The phages were transferred to a microtiter plate coated with biotinylated RNA, incubated and an ELISA was performed. There was no difference in the readings between the RNA treated wells and the untreated wells, (Table 5.2), meaning no specific binding to the RNA.



**Figure 5.6A** One clone was chosen as a source of RNA and it was linearized with *Sac* I.

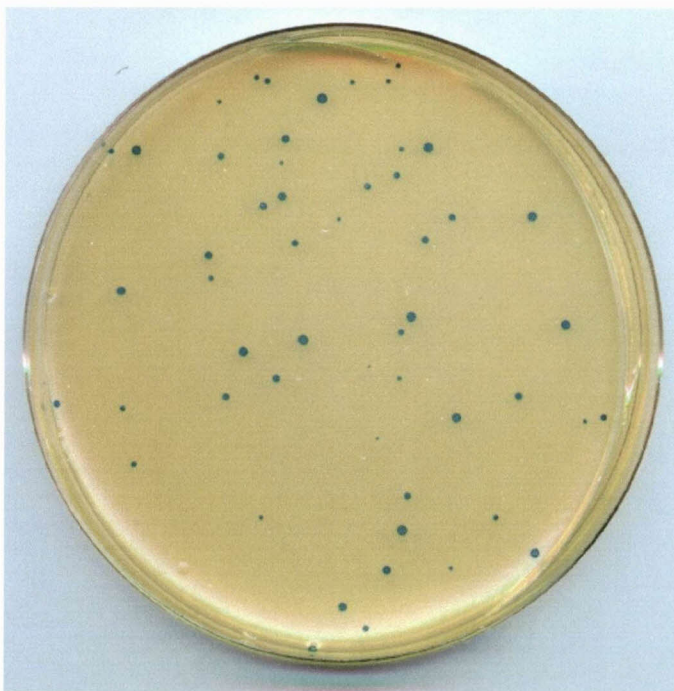
Lane 1: undigested plasmid DNA

Lane 2: digested plasmid DNA (3350 bp)

**Figure 5.6B** The RNA synthesis was checked by electrophoresis on an 0.8% agarose gel.

Lane 1: marker 100bp (DNA marker)

Lane 2: synthesized RNA



**Figure 5.7** A typical plate with blue plaques after three rounds of panning.

Table 5.1 Phage concentrations (phage/ml) after rounds 1 to 3 of the cyclic 7 mer library

Rounds of panning	1	2	3
Dilution 10x	$4.04 \times 10^{12}$	$5.75 \times 10^{12}$	$4.90 \times 10^{12}$
Dilution 20x	$4.36 \times 10^{12}$	$5.92 \times 10^{12}$	$5.31 \times 10^{12}$
Average	$4.20 \times 10^{12}$	$5.84 \times 10^{12}$	$5.11 \times 10^{12}$

Table 5.2 ELISA plate readings of the cyclic 7 mer library

With RNA					Without RNA			
Dilu- tion	Round 1	Round 2	Round 3	No phage	Round 1	Round 2	Round 3	No phage
1	1.023	0.295	0.549	0.003	0.754	0.406	0.560	0.003
1/2	0.471	0.171	0.366	0.002	0.405	0.171	0.334	0.003
1/4	0.294	0.099	0.258	0.003	0.302	0.083	0.086	0.003
1/8	0.171	0.074	0.162	0.003	0.192	0.039	0.078	0.002

We then switched to the longer linear 12 mer library. The phage titre after each selection round was high (Table 5.3). The ELISA plate readings showed differences at low dilutions between the RNA treated wells and the untreated wells (Table 5.4). With the higher dilutions the differences become progressively smaller between the RNA treated wells and the untreated wells. Although the differences between the treated and untreated RNA wells were not large, forty-eight blue plaques from the third panning round for the linear 12 mer library were then picked and tested individually for RNA binding (Fig 5.7). The phages were transferred to a microtiter plate coated with biotinylated RNA. After incubation, the non-binding phages were washed away. The wells coated with biotinylated RNA and the untreated wells had the same readings (Table 5.5). Although blue colonies, i.e. phages that display a peptide sequence on the surface (Fig 5.7), have been picked and amplified, none of the selected peptide sequences bound to the RNA.

The difference in OD between Table 5.4 and 5.5 could be explained by the fact that the cultures used for panning were done in Erlenmeyer flasks, giving good aeration and a high phage titer. The single plaques on the other hand, were inoculated into test tubes where mixing and aeration were not optimal, even with shaking. Even then, if there had been any high-affinity binders, they would have stood out above the background. This interpretation is corroborated by the behaviour of the phages in Table 5.4 upon dilution. At low dilution, a difference is evident between wells with and without RNA. Upon higher dilution however, this difference becomes progressively smaller. This is typical of low affinity interactions.

Table 5.3 Phage concentrations (phage/ml) after rounds 1 to 3 of the linear 12 mer library

Rounds of panning	1	2	3
Dilution 10x	$3.48 \times 10^{12}$	$6.28 \times 10^{12}$	$3.93 \times 10^{12}$
Dilution 20x	$3.44 \times 10^{12}$	$6.50 \times 10^{12}$	$4.15 \times 10^{12}$
Average	$3.46 \times 10^{12}$	$6.39 \times 10^{12}$	$4.04 \times 10^{12}$

Table 5.4 ELISA plate readings of the linear 12 mer library

With RNA					Without RNA			
Dilution	Round 1	Round 2	Round 3	No phage	Round 1	Round 2	Round 3	No phage
1	1.556	1.681	0.917	0.019	0.729	0.786	0.454	0.018
½	0.946	0.829	0.584	0.026	0.479	0.519	0.312	0.013
¼	0.411	0.575	0.243	0.018	0.333	0.385	0.201	0.010
1/8	0.254	0.248	0.177	0.015	0.212	0.209	0.176	0.012

Table 5.5 ELISA plating readings of the 48 plaques tested for RNA binding

With RNA							Without RNA					
	1	2	3	4	5	6	1	2	3	4	5	6
<b>A</b>	0.390	0.334	0.377	0.365	0.392	0.397	0.383	0.360	0.370	0.410	0.366	0.369
<b>B</b>	0.358	0.422	0.398	0.362	0.362	0.305	0.376	0.375	0.360	0.378	0.363	0.354
<b>C</b>	0.346	0.371	0.377	0.372	0.378	0.378	0.365	0.364	0.343	0.373	0.352	0.328
<b>D</b>	0.383	0.381	0.353	0.393	0.376	0.389	0.339	0.401	0.425	0.365	0.355	0.381
<b>E</b>	0.371	0.356	0.349	0.378	0.350	0.342	0.352	0.394	0.379	0.370	0.358	0.349
<b>F</b>	0.355	0.449	0.352	0.363	0.328	0.331	0.325	0.346	0.351	0.354	0.347	0.350
<b>G</b>	0.336	0.349	0.331	0.363	0.344	0.333	0.351	0.349	0.328	0.347	0.357	0.333
<b>H</b>	0.393	0.346	0.326	0.393	0.364	0.329	0.342	0.335	0.368	0.395	0.333	0.351



## 5.5 DISCUSSION

Phage display technology is becoming a tool for many purposes, ranging from protein engineering to gene therapy (Scott and Smith, 1990; Hong and Boulanger, 1995; Devlin *et al.*, 1990). It has had a major impact on the fields of immunology, cell biology, protein engineering, physiology and pharmacology. The screening of combinatorial peptide libraries has emerged as a promising tool in drug discovery. In the field of vaccines, phage technology has contributed to the isolation of immunogenic epitopes, dispensing with, or greatly reducing, the laborious preliminary work usually required to identify and characterize pathological antigens (Cortese *et al.*, 1995). Among the recently developed random combinatorial methods, phage display of peptide libraries has many advantages: its concepts are straightforward, it is inexpensive to set up, its methods are simple, and its range of potential applications is very wide. The display of functional proteins and antibody repertoires has become a routine method in many academic and industrial laboratories.

We have embarked on a search for short (7 and 12 amino acids) peptides capable of binding to the core template region of human telomerase RNA. As far as we could ascertain, no one has ever reported finding such a short motif having sequence specific nucleic acid affinity. It could be that others have attempted it and found nothing, or it has never been attempted because everybody has believed that nucleic acid binding domains need to be large. Since we could not distinguish between these possibilities, we took the decision to find out for ourselves. We thus employed a phage display based search for RNA binding peptide motifs.

Since the library phage is derived from the common cloning vector M13mp19, which carries the lacZ $\alpha$  gene, phage plaques appear blue when plated on media containing Xgal and IPTG. Environmental filamentous phage will typically yield white plaques when plated on the same media. After the three rounds of panning individual colonies were tested for binding to RNA. Forty-eight plaques of the linear 12-mer phage display peptide library were picked and tested for binding to RNA. None of these colonies

showed any binding to the RNA. One explanation could be that the ideal ligand sequence is not represented in the library. Alternatively, any potential ligand sequences are not able to adopt a conformation necessary for target binding. In the case of the 7-mer library, where all of the displayed peptides are structurally constrained in a 7-residue disulfide loop, a ligand sequence where the imposed constraint allows a productive binding conformation will bind more tightly than the same sequence expressed in a linear library. If the imposed constraint does not allow a productive binding conformation, then that sequence will likely not bind to the target at all.

The complexity of the peptide libraries (even if they appear as large) could be insufficient in comparison with the total sequence diversity of the RNA. Another possible reason why the displayed peptides did not bind to the RNA, could be the fact that RNA is not very stable and could have been degraded during the panning process. No RNase inhibitors were present during panning.

The most probable explanation, however, is that such short peptides are just incapable of binding sequence-specifically to nucleic acids. Most known nucleic acid binding proteins have defined domains e.g. helix-loop-helices or zinc fingers to mediate the binding. Searching the scientific literature databases using a variety of search terms and search engines produced only one example of a small RNA binding domain. This domain resides in the c-terminal part of NIPP-1 and spans 22 amino acids (Jin *et al.*, 1999). It is capable of binding to RNA and even shows endoribonuclease activity, but nothing is currently known about its sequence specificity.

A solution can be the use of an alternative phage display system, such as antibody display. In this system antibodies in which the variable parts are randomly chosen are displayed on filamentous phages. Such a system would eliminate the problem of too small RNA binding proteins. The diversity of antibody libraries is also higher than that of peptide libraries.

There is considerable interest in molecules that bind to telomeric DNA sequences and G-quadruplexes with specificity. Such molecules are valuable probes for testing hypotheses for telomere length regulation and may have therapeutic potential. In particular the 3' single stranded end of the telomere appears to be critical for regulation and the G-quadruplex nucleic acid conformation may be significant.

Another solution can be to use phage display to screen a library of zinc finger proteins (a three finger library) and to select clones that bind a deoxyligonucleotide containing the human telomeric repeat sequence (GGTTAG)<sub>5</sub> folded into G-quadruplex conformation.

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## CHAPTER 6

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### CONCLUDING REMARKS



## CHAPTER 6

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### CONCLUDING REMARKS

Squamous cell carcinoma of the esophagus (SCCE) is one of the most aggressive cancers known and is associated with poor survival rate. SCCE is one of the major causes of death in South Africa, especially among African males. Various factors have been suspected for being responsible for the high incidence, e.g. maize contaminated with mycotoxins, nutritional deficiencies and soil fertility factors, but at this stage a clear answer is not yet emerging. The cancer is usually invasive by the time diagnosis is made. Cellular progression to malignancy is thought to involve multistep events, and is expressed histologically as a progression from normal epithelium to cancerous tissue. Recent studies have demonstrated genetic alterations in SCCE, especially mutational activation or overexpression of oncogenes and loss of normal function of tumor suppressor genes. In addition to such genetic alterations, cellular immortalization is required for malignancy.

The study was part of a national effort funded by the Cancer Association of South Africa to study cancer of the esophagus with the aim of eradicating it in the long run. We approached our involvement in this enterprise on two levels:

The first objective was to study an aspect of the disease not yet covered by other laboratories. It needed to be something of value in terms of gaining a deeper understanding of the origin and/or progression of the disease. The choice fell on the enzyme telomerase and its substrate, the telomeres. At the outset of this study, nothing was known about the status of the enzyme and substrate in squamous carcinoma of the

esophagus and is important enough to warrant this study, as it addresses the important subject of cellular immortality.

Secondly, to do something which could contribute to treatment of patients with cancer of the esophagus. Once again, the choice fell on telomerase, as tissue culture research has shown that inhibition of telomerase leads to loss of immortality. We thus felt that setting up the technology and expertise to study telomeres and telomerase would be a good investment.

Firstly, collaboration was established with the staff from the Department of Surgery and the Gastroscopy Clinic at Universitas Hospital to collect endoscopic biopsies from consenting patients. These samples were taken from the tumor and surrounding normal tissue and analyzed for telomerase activity. The most important result emanating from it is that even for late stage patients, telomerase activity of normal tissue in the vicinity of the tumor has prognostic value. The survival times recorded in our study showed the seriousness of the disease and the lack of early detection. Future work on this cancer must involve a broader community of health workers to get an infrastructure in place for early detection. In this regard, the People's Republic of China is a prime example of what can be done in a resource poor setting once the epidemiology and the progression of a disease is known. They have started a huge public awareness program in high-incidence areas backed up by endoscopic screening and referral of patients with early cancer to clinics where mucosectomies are done.

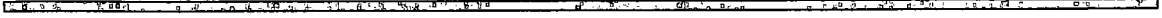
The decision to develop a flow cytometric method for telomere length measurement meant that many obstacles had to be surmounted, the most difficult of which was the dissociation of the solid tissue and the resulting debris which threatened to bury the nuclei in noise. We are not yet confident about the method and would like to thrash out remaining issues using normal and tumor tissue obtained from our colleague Dr Richard Naidoo. Despite the many unknowns, some of tendencies we saw made sense. If this method could validate, it would mean a major technical advance in our ability to study small solid tissue samples. A variety of solid tumors would then become amenable to

flow cytometric analysis, not only for cell cycle analysis (which is being done currently), but also for telomere length determination.

On the treatment side, we have embarked on a search for short peptides capable of binding to telomerase RNA using phage display technology. Although no positive results were obtained the search will continue, using alternative display libraries.

The work presented in this thesis can be regarded as a basis for extending studies on telomeres and telomerase to other systems. Our future prospective is to evaluate the utility of telomerase activity and telomere length changes as early markers in bladder and cervical tumors. The advantage of these two systems is that the analyses are done on detached cells, skipping the cell dissociation step used for solid tissue.

# CHAPTER 7



## SUMMARY / OPSOMMING

## CHAPTER 7

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### SUMMARY

Squamous cell carcinoma of the esophagus is a cancer with a high incidence in South Africa. We have investigated the prognostic value of telomerase activity in tumors as well as in nearby normal tissue. Biopsies from 98 patients were analyzed using an adaptation of the TRAP assay. We found all tumor biopsies to have moderate to high telomerase activity, while one third of biopsies from normal mucosa were negative. The telomerase activity level of the tumors had no prognostic value ( $P=0.95$ ) as determined by the log rank test. A  $P$ -value of 0.02 was found when the telomerase-negative and moderately positive normal biopsies were grouped together and compared to those with high activity. Our results show that telomerase activity of normal mucosa in the vicinity of the tumor can identify a population of patients with significantly worse prognosis, even in late stage patients.

Telomerase has attracted intense interest as a possible target for cancer therapeutics. Previous attempts at inhibiting telomerase activity utilized antisense oligonucleotides targeted at the template region of the RNA subunit of the enzyme. Although it worked well in cell extracts, getting the oligonucleotides into intact cells are difficult. We attempted a peptide-based approach to overcome the transport problem. A phage-display selection strategy was designed to isolate peptides (7 and 12 amino acids) capable of binding to the RNA template with high affinity and in so doing preventing the enzyme from elongating existing telomeres. Both libraries showed no high affinity binding to the RNA. The most probable explanation is that such short peptides are incapable of binding sequence-specifically to nucleic acids.

Many studies have indicated the importance of telomerase activity as an independent prognostic indicator in a variety of cancers, but recent results have shown that telomere length is actually a better indicator, as it shows the final result of telomerase activity. We thus decided to develop a flow cytometric method that would allow us to analyze telomere length in small tissue samples. After the initial technique had been established on lymphocytes, it was tested on the SNO cell line before we switched to solid tissue. The background of cellular debris and cell clusters was much worse than for lymphocytes, but differences in signal strength could be seen. From the results obtained it is clear that this method is not optimized yet. The results also indicate that at this stage of development, flow-FISH is inferior to telomerase activity as prognostic indicator. The best way to validate the current method is to analyze the same samples by Southern hybridization and flow-FISH, preferably well defined tumor and normal tissue.

**KEY WORDS:**

Esophageal cancer, epithelium, gastroscopy, telomeres, telomerase, phage display technology, flow-FISH

## HOOFTUK 7

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### OPSOMMING

Plaveiselepiteelkarsinoom is een van die volopste kankers in Suid-Afrika, veral in swart mans. Hierdie projek is deel van 'n nasionale navorsingsprogram om die oorsake, verloop en behandeling van die siekte te bestudeer. Daar is op telomere en telomerase gekonsentreer aangesien daar geen inligting hieroor bekend was toe die studie begin het nie.

Die moontlik prognostiese waarde van telomerase aktiwiteit in tumorbiopsies en in naasliggende normale weefsel, is ondersoek. Die telomerase aktiwiteitsvlakke van tumorweefsel het geen korrelasie met oorlewing gehad nie (75 pasiënte,  $P=0.95$ ). Indien die normale weefsel verdeel word in hoë aktiwiteit vs matig/negatief, het lg groep 'n betekenisvolle langer oorlewing as die met hoë aktiwiteit ( $P=0.02$ ). Die telomerase aktiwiteit van die normale weefsel mag 'n vroeë aanduiding wees van verspreiding.

Telomerase word ook beskou as 'n moontlike terapeutiese teiken, aangesien inhibisie van die ensiem in tumorsellyne lei tot seldood. Die meeste inhibitore is oligonukleotiede of PNAs gemik op die templaatgebed van die ensiem se RNA-subeenheid. Hoewel dit goed gewerk het *in vitro*, is transport van die molekules na die kern 'n probleem. Ons het dus inhibitore gesoek in 2 peptiedbiblioteke (7 en 12 aminosure) wat op die oppervlak van bakteriofage geanker is. Die teiken was die telomerase RNA templaats wat mbv *in vitro* transkripsie vervaardig is. Ongelukkig was daar geen peptiede wat die RNA met hoë affiniteit gebind het nie, waarskynlik omdat die peptiedgroottes kleiner is as die minimum wat vir RNA binding benodig word.

Telomeerlengte is die produk van telomerase aktiwiteit, daarom het ons besluit om te kyk of daar veranderings in lengte is tussen tumor en normale weefsel. Aangesien die biopsies uiters klein is, kon ons nie Southernhibridisasie daarop doen nie en moes 'n alternatiewe metode ontwikkel word. Vloesitometrie is gekies, aangesien dit 'n kragtige tegniek is waarmee individuele selle bestudeer kan word. Die homogenisering van soliede weefsel was egter 'n probleem wat eers oorkom moes word. Die metode soos dit nou daar uitsien maak gebruik van 'n kollagenaseverteringstap om die weefsel af te breek, dit geskied in 'n lisebuffer wat die plasmamembrane laat breek, maar die kernmembrane behou. Die kernsuspensie word dan verhit om die DNA te denatureer en gehibridiseer met 'n FITC-gemerkte PNA peiler wat uit telomeerherhalings bestaan. Die DNA in die kern word gekleur met 7 AAD en die suspensie word deur 'n vloesitometer gestuur. Die metode is stapsgewys op limfosiete, weefselkultuurselle, bobbejaanlewer en uiteindelik esofagusbiopsies ontwikkel. Daarna is gepaarde biopsies (normaal en tumor) van 70 pasiënte geanaliseer.

Die resultate van hierdie eksperimente moet as voorlopig beskou word, aangesien die metode nog nie gevalideer is deur vergelyking met Southernhibridisasie nie. Desnieteenstaande is daar aanduidings dat die gemiddelde telomeerlengte afneem tussen normale en tumorweefsel. Verdere afleidings sal moet wag totdat die metode finaal gevalideer is.



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