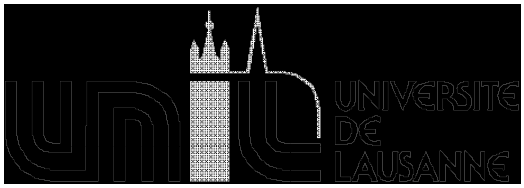

FACULTE DES SCIENCES

Regulation of the human telomerase reverse transcriptase gene

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ABSTRACT

Telomeres are protective DNA-protein structures located at the ends of chromosomes. Telomeric DNA is maintained by a reverse transcriptase called telomerase that consists of an RNA moiety, a catalytic protein subunit (TERT) and auxiliary proteins. In adult human, 85% of tumor-derived cells have detectable telomerase activity, whereas in most of somatic cells telomerase activity is not detected. Consequently, somatic cells exhibit progressive telomere shortening and proliferative failure. Evidence that telomere shortening limits proliferative potential was demonstrated by ectopic expression of hTERT. Cells that stably expressed hTERT exhibited telomerase activity and indefinite proliferation. There is a striking correlation between telomerase activity and hTERT RNA levels in the cells examined so far, therefore indicating that regulation of hTERT expression is the limiting step for inducing telomerase activity.

To understand the differential expression of hTERT mRNA between tumor and somatic cells, we determined by quantitative RT-PCR the level of hTERT mRNA in telomerase positive and negative cells. Telomerase-positive cell lines contained between 0.2 and 6 molecules of spliced hTERT RNA/cell, whereas no transcripts could be detected in telomerase negative cells (<0.004 molecules/cell). Furthermore, intron-containing, immature hTERT RNA was detected only in nuclei of telomerase positive cells. These data are consistent with a regulation of hTERT RNA at the transcription level.

To analyze hTERT 5'flanking region, we developed a new GFP-reporter system that is not limited by a low efficiency of transfection. The hTERT-GFP reporter constructs consist of fragments of the hTERT 5'flanking region fused to GFP. We found that the hTERT-GFP reporters were not expressed in telomerase negative primary cells but in telomerase positive cells but also in the telomerase negative cell line, 21NT-chromosome 3. Thus, in the latter cells, in which transfer of chromosome 3 extinguished hTERT RNA, the hTERT-GFP reporters containing 5'flanking region up to 7.4 kb upstream of the translation start site did not faithfully mimic endogenous hTERT.

We investigated the possible function of c-Myc, a known regulator of hTERT, upon transfer of chromosome 3 in 21NT-chromosome 3 cells. We found that the expression levels of c-Myc and of c-Myc target genes were not affected, indicating that the putative hTERT repressor on chromosome 3 is unlikely to affect hTERT expression via alteration of c-Myc or one of its co-regulators. We also tested whether Notch is a regulator of hTERT, since Notch and hTERT expression correlates in embryonic cells and in some cancers. Over-expression of Notch1IC modulated hTERT RNA levels in telomerase positive but not in telomerase negative cells, suggesting that Notch may be a regulator of hTERT. Though we identified

putative binding sites for an effector of Notch, CBF1, in hTERT gene, we have not yet evidence for a direct interaction of Notch1 with the hTERT gene.

RESUME

Les télomères sont formés par un complexe d'ADN et de protéines qui protègent les extrémités des chromosomes des cellules eukaryotes. L'ADN télomérique est synthétisé spécifiquement par la télomérase, une ribonucléoprotéine constituée d'un ARN et de plusieurs sous-unités protéiques dont une transciptase inverse, appelée hTERT chez l'humain. Chez l'humain, l'activité de la télomérase est détectée dans les cellules germinales, dans les lymphocytes activés et dans 85 % des lignées cellulaires dérivées de tumeurs, ces cellules sont appelées télomérase positives. Au contraire, la majorité des cellules somatiques sont télomérase négatives. Par conséquent, les télomères de la majorité des cellules différenciées raccourcissent à chaque division cellulaire et ce phénomène est probablement une cause de la sénescence cellulaire. La surexpression artificielle de hTERT dans les cellules télomérase négatives est suffisante pour induire l'activité de la télomérase, rallonger leurs télomères et leur permettre une prolifération infinie. Par ailleurs, le profil d'expression de l'ARNm de hTERT est fortement correllé à l'activité de la télomérase. Ces résultats indiquent que l'expression de l'ARNm de hTERT est le facteur limitant de l'activité de la télomérase.

Afin de comprendre comment l'expression de hTERT est régulée, nous avons déterminé par RT-PCR la quantité d'ARNm de hTERT dans des cellules télomérase positives et négatives. Dans des cellules telomérase positives, nous avons mesuré 0.2 à 6 molécules par cellule d'ARNm matures de hTERT et détecté de l'ARN immature de hTERT dans leurs noyaux. Par contre, dans des cellules télomérase négatives, le niveau de l'ARN n'est pas détectable (<0.004 molécules/cellule). Ces résultats suggèrent une régulation de hTERT au niveau de sa transcription.

Dans le but de caractériser les éléments régulateurs de hTERT, nous avons développé un système de gène rapporteur pour lequel une faible efficacité de transfection n'est pas limitante. Dans ces rapporteurs, l'expression de la GFP a été placée sous le controle de fragments de hTERT situés en 5' du site d'initiation de la traduction. Nous avons testé ces rapporteurs dans des cellules télomérase positives et négatives. De ces experiences, il en resulte qu'une région de 7.4 kb en 5' de hTERT ne suffit pas à mimer l'expression endogène de hTERT dans certaines cellules.

Comme c-Myc est un régulateur connu de hTERT, nous avons étudié son rôle lors de la répression de hTERT par un répresseur putatif codé par le chromosome 3. Par RT-PCR, nous avons montré que l'expression de c-Myc et de ses gènes cibles ne sont pas modifiés par le transfert du chromosome 3 dans ces cellules, indiquant que le répresseur putatif codé par le chromosome 3 ne diminue pas le taux d'ARNm de hTERT via c-Myc. Nous avons aussi testé

si Notch pourrait contrôler l'espression de hTERT. La cascade Notch affecte la différentiation et, de plus, Notch est surexprimé dans certains cancers. Nous avons montré que la surexpression de la partie intracellulaire de Notch (Notch1IC) module l'expression de hTERT dans des cellules télomérase positives. Aucun effet sur la transcription de hTERT n'a été détecté dans des cellules télomérase négatives. Bien que nous ayons identifié au niveau du locus hTERT des sites de liaison probables pour CBF1, un effecteur de Notch, nous n'avons pas pour l'instant de preuve d'une interaction directe de Notch avec hTERT.

1 INTRODUCTION

1.1 TELOMERE STRUCTURE AND FUNCTIONS

Telomeres are DNA-protein complexes at the ends of linear eukaryotic chromosomes (reviewed in (McEachern *et al.*, 2000) (Fig. 1). Mammalian telomeric DNA consists of tandem arrays of double-stranded TTAGGG repeats, which end with a single-stranded G-rich 3'overhang. The length of the double-stranded repeat ranges from a few to more than 10 kb (Collins, 2000), whereas the length of the 3'overhang corresponds to 150 to 200 nucleotides (Makarov *et al.*, 1997; McElligott and Wellinger, 1997; Wright *et al.*, 1997). The conventional DNA replication machinery cannot replicate the 3'overhang because the parental CA-rich strand is recessed and cannot function as template. Telomerase, a ribonucleoprotein, can solve the end replication problem by balancing telomere loss with addition of telomeric repeats to the ends of chromosomes (Lingner and Cech, 1998; Nugent and Lundblad, 1998).

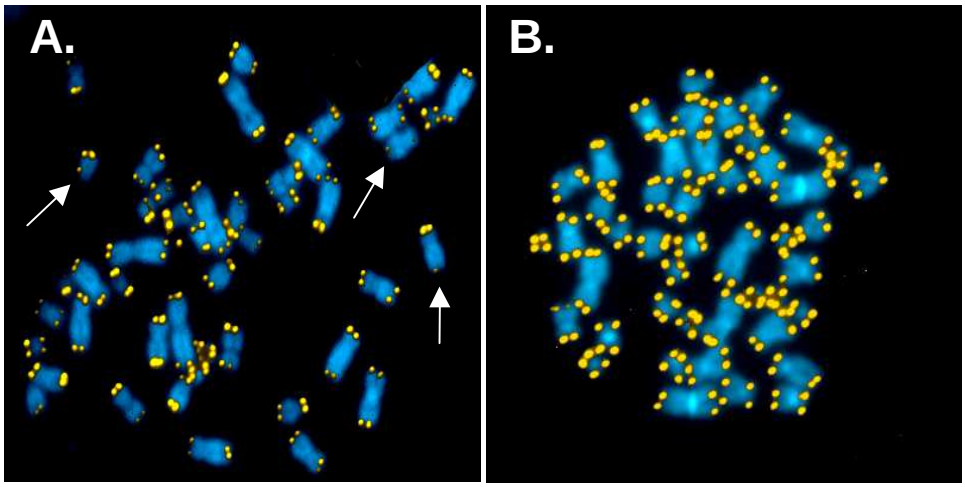

Figure 1: Telomere lengths of metaphase spread of human T cells transduced with a control (A) or a hTERT containing vector (B). Telomeres were probed with a specific PNA-probe (yellow), DNA was stained by DAPI (blue). Arrows indicate telomere loss. The pictures were kindly provided by Nathalie Rufer.

Telomeres serve different functions. First telomeres protect the end of linear chromosomes from degradation and unwanted fusion events (McClintock, 1941; van Steensel *et al.*, 1998). Damaged telomeres will be subject to DNA repair, and undergo end-to-end fusion, which causes dicentric chromosomes that impair the next cell divisions. Second they specifically position chromosomes at the nuclear periphery in yeast (Gotta *et al.*, 1996), while in mammalian cells the telomeres form nuclear matrix-associated complexes at dispersed sites troughout the nucleus (Ludérus *et al.*, 1996). Third, in budding yeast and in mammals, telomeres seem to control transcription of genes located close to them (Baur *et al.*, 2001;

Gottschling *et al.*, 1990). This phenomenon is referred as telomere position effect or telomere silencing. Finally telomere shortening may limit the replicative potential of normal human cells providing a powerful tumor-suppressive mechanism (Wright and Shay, 2001).

1.2 MAMMALIAN TELOMERIC PROTEINS

Double-stranded telomeric repeats are bound directly by at least two proteins, TRF1 (Chong *et al.*, 1995; Smith and de Lange, 1997) and TRF2 (Bilaud *et al.*, 1997; Broccoli *et al.*, 1997) (TTAGGG repeat binding factor 1 and 2). TRF2 proteins stabilize telomeres by creating the so-called T loop (Griffith *et al.*, 1999) (Fig. 2A). In this structure the 3'overhang folds back and is thought to invade the duplex of double-stranded telomeric repeats. In this way the telomeric end is sequestered and may be protected from inappropriate repair activities and end-to-end fusion and from telomerase elongation (Fig. 2B) (Broccoli *et al.*, 1997). Over-expression of wild-type TRF1 reduces telomere length and over-expression of dominant-negative TRF1 increases telomere length (van Steensel and de Lange, 1997), suggesting a role for TRF1 in controlling telomere length homeostasis. TRF1 is also implicated in maintenance of the mitotic checkpoint in response to DNA damage and it is involved in the mitotic spindle checkpoint. TRF1, also called Pin2 (Shen *et al.*, 1997), was also identified as an ATM kinase substrate (Kishi and Lu, 2001; Nakamura *et al.*, 2001). A number of interacting partners of TRF1 have been discovered: PinX1 (Zhou and Lu, 2001), TIN2 (Kim *et al.*, 1999) and Tankyrase 1 and 2 (Kaminker *et al.*, 2001; Smith *et al.*, 1998). Among them TIN2 and PinX1 appear to function as positive regulators of TRF1-dependent pairing of telomeric repeats (Kim *et al.*, 1999; Zhou and Lu, 2001). Tankyrase 1 ADP-ribosylates TRF1 in vitro thus reducing its binding affinity for telomeric DNA (Smith *et al.*, 1998). Over-expression of tankyrase 1 results in telomere elongation in telomerase positive cells (Cook *et al.*, 2002). TRF2 may not only be a negative regulator of telomere length as TRF1 but also function to protect telomeres. Over-expression of dominant negative TRF2 induces loss of the single-stranded telomeric 3' overhang leading to end-to-end fusion and ATM/p53 dependent apoptosis or cellular senescence (Karlseder *et al.*, 1999; van Steensel *et al.*, 1998). A human ortholog of the yeast telomeric protein Rap1p is recruited to telomeres by TRF2 (Li *et al.*, 2000). Its function remains unknown.

During the last couple of years proteins involved in DNA repair were also detected at telomeres. Some interact with TRF1 (Dunham *et al.*, 2000; Hsu *et al.*, 1999) or TRF2 (Zhu *et al.*, 2000). For example a small fraction of RAD50, MRE11 and the Nijmegen breakage syndrome protein (NBS1), which are components of the double strand repair machinery (DSB), are associated with TRF2 in HeLa cells.

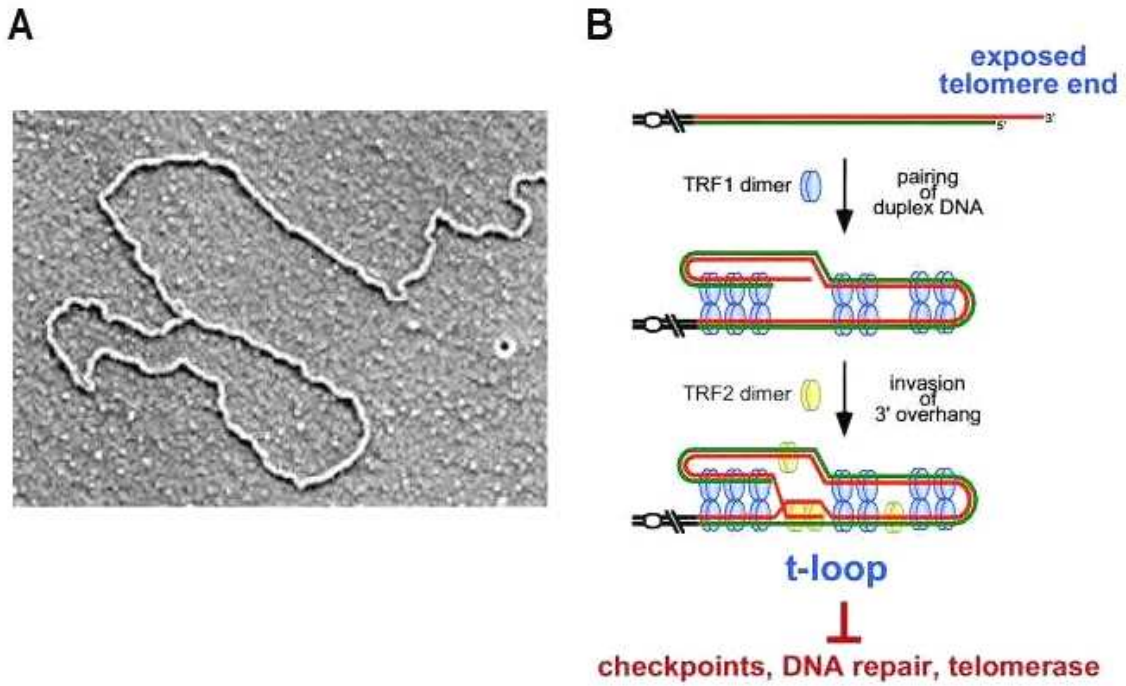

Figure 2: T-loop structure in mammalian cells. **A** telomeric DNA from mouse liver was isolated by size fractionation following psoralen/UV treatment of nuclei, deproteinization, and restriction cleavage. The DNA was spread on air-buffer interface with cytochrome c protein followed by rotary shadowcasting with platinum-paladium. The T-Loop contains around 20 kb of DNA. **B** Proposed formation and functions of T-loop. (from (Griffith *et al.*, 1999).

Interestingly, NBS1 interacts with TRF2 at telomeres specifically in S phase of the cell cycle, suggesting a role for NBS1 in telomere replication (Zhu *et al.*, 2000). Loss of Ku, which is also involved in non homologous end-joining (NHEJ), or its associated DNA protein kinase (DNA-PKcs), leads to end-to-end telomeric fusions and enhanced chromosomal instability in mammalian and yeast cells (Bailey *et al.*, 1999; Difilippantonio *et al.*, 2000; Gravel *et al.*, 1998; Nugent *et al.*, 1998; Polotnianka *et al.*, 1998). These results indicate a possible role of Ku in telomere capping (Bertuch and Lundblad, 1998). Recently, it was shown that only leading-to-leading-strand end fusions occur in TRF2 dominant-negative mutants and DNA-PKcs deficient mouse cells (Bailey *et al.*, 2001), suggesting different requirements for TRF2 and DNAPKcs in capping of lagging and leading strand after replication. Conventional DNA replication is predicted to give rise to leading strand telomeres, which are blunt ended, and to lagging strand telomeres that have a 3'G-rich single-stranded overhang. Both types of ends may be processed further perhaps by degradation of the C-rich strand or by the 3'exonuclease activity of the MRE11/RAD50/NBS1 complex to allow the action of telomerase (Makarov *et al.*, 1997; Wellinger *et al.*, 1996; Zhu *et al.*, 2000).

1.3 THE TELOMERASE RIBONUCLEOPROTEIN

Telomerase is the enzyme required for the addition of telomeric repeats to the ends of linear chromosomes. It consists of a reverse transcriptase, TERT that carries its own template in the form of an RNA moiety, TER (Feng *et al.*, 1995). TERT has homology to viral reverse transcriptases (RT) (Lingner *et al.*, 1997) (Fig. 3) and contains the conserved RT motifs and a telomerase specific motif.

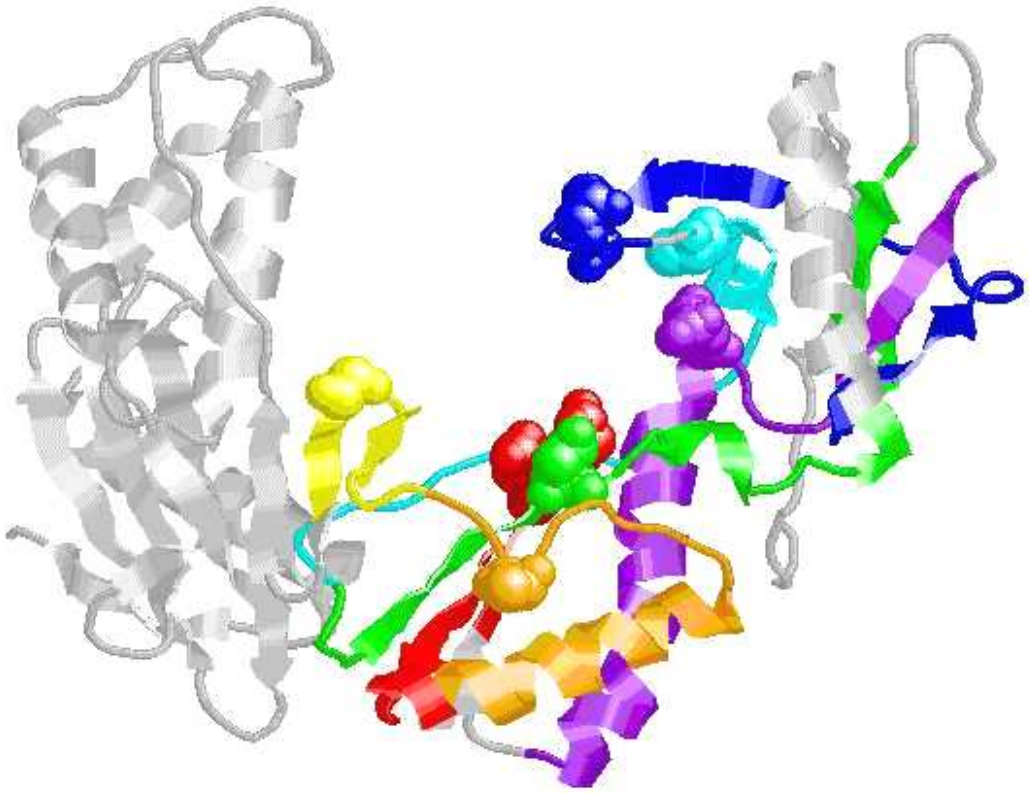

Figure 3: Reverse transcriptase (RT) domains of HIV as a model for TERT RT domains. As in most polymerases, the active site is present in a cleft, the structure of which is compared to a half-open right hand with fingers, palm and thumb.The red and green motifs contain the critical asparagine residues for activity. (Reprinted with permission from (Nakamura *et al.*, 1997).

The RNA moiety includes the template sequence for synthesis of telomeres. These two components are both necessary and sufficient to mediate telomerase activity *in vitro*, although a variety of additional molecules regulate its *in vivo* activity (see below). TERC has a conserved secondary structure found in ciliates and vertebrates (Chen *et al.*, 2000; Lingner *et al.*, 1994), including a pseudoknot, which is essential for activity and stable assembly with TERT (Gilley and Blackburn, 1999) and a H/ACA box (Mitchell *et al.*, 1999). The H/ACA motif of telomerase RNA is essential for the accumulation of TERC and for telomerase activity in vivo (Mitchell and Collins, 2000). The members of the H/ACA snoRNA family function in ribosomal RNA maturation, specifying sites of pseudouridine modification or processing (Ganot *et al.*, 1997). The human dyskerin, that was previously cloned as the mutated gene responsible for X-linked dyskeratosis (DKC) is a component of H/ACA

snoRNPs and stabilizes TERC (Mitchell *et al.*, 1999; Vulliamy *et al.*, 2001). Other proteins were found to be associated with TERC such as Staufen (Le *et al.*, 2000), L22 (Le *et al.*, 2000) and hnRNP A1 (Fiset and Chabot, 2001; Labranche *et al.*, 1998). The roles of these proteins remain unclear. It is also unclear whether the assembly of the telomerase holoenzyme occurs in the cytoplasm or in the nucleus. Two proteins, p23 and hsp90, are involved in the assembly of active telomerase (Holt *et al.*, 1999). They remain associated with the telomerase complex (Forsythe *et al.*, 2001).

Recent experiments indicate that telomerase functions as a dimer (Beattie *et al.*, 2001; Wenz *et al.*, 2001). In one study telomerase activity was reconstituted in cells expressing two different inactive fragments of hTERT suggesting a role of hTERT dimerization in the generation of active telomerase (Beattie *et al.*, 2001). The second study established that the active reconstituted holoenzyme has a molecular weight that is consistent with a dimer of both hTERT and hTERC and that the isolated complexes contain two hTERC molecules. They also showed that a reconstituted enzyme that consisted of a heterodimer of wild-type and mutant hTERC had a dramatic reduction in telomerase activity (Wenz *et al.*, 2001), indicating that the two molecules of hTERC cooperate for extension of telomeres 3'ends. Several potential roles for hTERT/hTERC multimerization have been proposed. They include enhancement of telomerase processivity and formation of a binding interface that recognizes telomeric DNA.

1.4 RECRUITMENT OF TELOMERASE

Molecular mechanisms underlying the recruitment of telomerase to the chromosome ends, and the coordination of DNA replication with telomerase action are currently being investigated. In budding yeast, at least five genes are necessary for the *in vivo* activity of telomerase: *EST1-3*, *TLC1* and *CDC13* (Lundblad and Szostak, 1989). Cdc13p is a single-stranded G rich DNA binding protein that is required to protect telomere ends from degradation and to recruit the telomerase complex to chromosome ends (Evans and Lundblad, 2000). Recently, in human, a single-stranded G-rich binding protein, Pot1 (for protection of telomeres) that may serve the same function was identified (Baumann and Cech, 2001). Yeast Est1p interacts specifically with the single-stranded telomeric DNA overhang (Nugent *et al.*, 1998). Est1p and Est3p were shown to be associated with the telomerase holoenzyme (Hughes *et al.*, 2000) and Est1p recruits in cooperation with cdc13p telomerase to the end of the chromosome (Evans and Lundblad, 1999).

Recent work in several systems support the hypothesis that telomeres may switch between at least two states: capped and uncapped (Blackburn, 2000). The capped state would preserve telomeres and thereby chromosome integrity, whereas the uncapped state would

allow the access of enzymes to telomeres for restoring its cap. The cap function may be fulfilled by T-loops in mammalian cells (Fig. 2B) or Pot1p, binding to free G-rich 3'overhangs. The uncapped state may permit the elongation by telomerase while preventing end-joining reactions at telomeres.

1.5 TELOMERE MAINTENANCE IN HUMAN

In human embryonic cells telomerase activity is detected in germ-line, blastocysts and up to 16 to 20 weeks old fetal tissues (Ulaner and Giudice, 1997; Wright *et al.*, 1996). In adult humans the enzyme is present in cells of the germline that give rise to mature gametes, as well as in at least some stem cell populations and in activated lymphocytes but not in differentiated cells (Chiu *et al.*, 1996; Wright *et al.*, 1996). Thus most somatic human cells lack the telomerase enzyme (Kim *et al.*, 1994) and their telomeres shrink with each replication cycle by approximately 30 to 100 bp (Counter *et al.*, 1992; Harley *et al.*, 1990; Huffman *et al.*, 2000). Since short telomeres induce cellular senescence in tissue culture (Bodnar *et al.*, 1998), it has been proposed that telomere shortening may limit the replicative potential of normal cells providing a powerful tumor-suppressive mechanism (Wright and Shay, 2001). In contrast to somatic cells 85% of human tumor-derived immortal cells have detectable telomerase activity (Kim *et al.*, 1994). In a minority of tumor cells an alternative non-telomerase dependent mechanism (ALT) is responsible for telomere stabilization (Bryan *et al.*, 1997). In several somatic cell types ectopic expression of human TERT (hTERT) is sufficient to induce in vitro and in vivo telomerase activity, to elongate their telomeres and to extend the life span of these cells (Bodnar *et al.*, 1998; Morales *et al.*, 1999; Vaziri and Benchimol, 1998; Yang *et al.*, 1999). Moreover among the number of telomerase-positive and negative cells so far examined, the expression of hTERT mRNA correlates with the presence of telomerase activity (Ducrest *et al.*, 2001; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). This indicates that expression of hTERT is the limiting step for the induction of telomerase activity in most cells.

1.6 GOAL OF THE THESIS

In my thesis, I have addressed the question of how hTERT expression is regulated. In chapter 2, we present the possible models for controlling hTERT expression and review the different hTERT regulators that have been identified so far. To elucidate the mechanism controlling hTERT expression we used three different approaches. First, we attempted to characterize the regulatory elements of hTERT gene using a reporter assay. As described in Chapter 3, we set up a GFP reporter system that can be analyzed at the single-cell level by

flow cytometry. With this system, we can reliably measure the activity of weak promoters even in cells transfected with low efficiency. Based on this technique, we analyzed hTERT-GFP reporter constructs containing 5'flanking region of the hTERT gene in telomerase positive and negative cells. In chapter 4, we showed that a 7.4 kb fragment upstream of the translation start site of hTERT, placed in a reporter construct, is not sufficient to mimic endogenous hTERT gene in some cells. Second, we measured levels of different hTERT RNAs by quantitative RT-PCR (Chapter 4). This study led us to the conclusion that hTERT RNA is controlled at the level of gene transcription, but, however, failed to exclude that regulation involves changes in the efficiency of nuclear processing of primary transcripts. Third, we tested a candidate regulator of hTERT for its effect on hTERT RNA expression (Chapter 5). We reasoned that in most carcinomas hTERT expression may not be due to the reactivation of the hTERT gene but reflects the advantage, during tumor progression of cells in which differentiation is partially blocked and hence hTERT expression maintained (Chapter 2). Notch may be a good candidate to modulate hTERT expression, since Notch1 controls cell differentiation in embryonic cells and its abnormal expression was detected in some cancers. We found that in some telomerase positive cells Notch1IC modulated hTERT expression, but in telomerase negative cells no effect could be detected.

2 REGULATION OF THE HUMAN TELOMERASE REVERSE TRANSCRIPTASE GENE[1]

2.1 ABSTRACT

Most somatic human cells lack telomerase activity because they do not express the telomerase reverse transcriptase (hTERT) gene. Conversely, most cancer cells express hTERT and are telomerase positive. For most tumors it is not clear whether hTERT expression is due to their origin from telomerase positive stem cells or to reactivation of the gene during tumorigenesis. Telomerase negative cells lack detectable cytoplasmic and nuclear hTERT transcripts; in telomerase positive cells 0.2 to 6 mRNA molecules/cell can be detected. This suggests that expression is regulated by changes in the rate of hTERT gene transcription. In tumor cell lines hTERT expression behaves like a recessive trait, indicating that lack of expression in normal cells is due to one or several repressors. Studies with monochromosomal hybrids indicate that several chromosomes may code for such repressors. A number of transcription factors, tumor suppressors, cell cycle inhibitors, cell fate determining molecules, hormone receptors and viral proteins have been implicated in the control of hTERT expression; but these studies have not yet provided a clear explanation for the tumor specific expression of the hTERT gene, and the cis-acting elements which are the targets of repression in normal cells still have to be identified.

2.2 INTRODUCTION

Telomerase is the enzyme required for the addition of telomeric repeats to the ends of linear chromosomes. It consists of a reverse transcriptase, TERT that carries its own template in the form of an RNA moiety, TER. In vitro this complex can add telomeric repeats to artificial substrates. Its activity in vivo depends on other components some of which probably control the access of the enzyme to chromatid ends (Evans and Lundblad, 2000). In the absence of telomerase the telomeres of normal cells shorten by about 50 nt per cell population doubling (Counter *et al.*, 1992; Harley *et al.*, 1990; Huffman *et al.*, 2000). In adult humans the enzyme is present in the germ line stem cells that give rise to mature gametes as well as in at least certain stem cell populations and in activated lymphocytes, but not in differentiated cells (Chiu *et al.*, 1996; Wright *et al.*, 1996). In the absence of telomerase activity human somatic epithelial cells and fibroblasts can undergo approximately 50 to 60 population doublings

[1] This review was published in Oncogene 2002, 21, 541-552 with the following authors: Anne-Lyse Ducrest, Henrietta Szutorisz, Joachim Lingner and Markus Nabholz. The main contribution of Henrietta Szutorisz is the

before telomere shortening leads to replicative senescence (see e.g.(Bodnar *et al.*, 1998). In rodents TERT expression is maintained during differentiation, and cellular senescence is not due to absence of telomerase (Russo *et al.*, 1998). Observations on TER-deficient mice indicate that the enzyme is not required for the development and normal life span of laboratory mice in early generations (Blasco *et al.*, 1997). However, propagation of mTERC -/- mice for three or more generations leads to extensive telomere shortening and affected development and function of multiple tissues (Lee *et al.*, 1998). In several human cell types ectopic expression of human TERT (hTERT) is sufficient to induce in vitro and in vivo telomerase activity and to «immortalize» the cells, indicating that none of the other components is limiting (Bodnar *et al.*, 1998; Morales *et al.*, 1999; Vaziri and Benchimol, 1998; Yang *et al.*, 1999).

There is a striking correlation between the presence of hTERT mRNA and telomerase activity (see e.g.(Ducrest *et al.*, 2001), and this has been taken to suggest that hTERT expression is regulated through changes in the rate of transcription, but direct evidence for this is scarce (see below). Post-transcriptional regulation of hTERT expression through alternative splicing has been observed during human development (Ulaner *et al.*, 2001), and there have been claims that posttranslational modifications can affect TERT activity (Kang *et al.*, 1999; Kharbanda *et al.*, 2000; Liu *et al.*, 2001; Yu *et al.*, 2001), but the role of such mechanisms in tumor specific telomerase expression is, as yet, quite unclear. The finding that most tumors express hTERT and telomerase activity (Kim *et al.*, 1994), and that in vitro transformation of telomerase negative human cells requires activation of hTERT expression (Hahn *et al.*, 1999) indicates that maintenance of telomeres is required for the unlimited proliferative potential of tumor cells. This conclusion is supported by the finding that telomerase negative in vitro transformed cells maintain telomeres through an alternative (ALT) pathway that is based on somatic recombination (Bryan *et al.*, 1995; Dunham *et al.*, 2000).

For oncology the importance of understanding the mechanisms that control hTERT expression in tumors is two-fold; on the one hand, it may lead to the discovery of targets for new cancer therapies, and on the other hand it might provide cis-acting regulatory elements that could contribute to tumor targeting of tumoricidal genes or viruses. Thus, it is not surprising that there have been a large number of groups that have tried to dissect the mechanisms that control hTERT expression. In this review we discuss this work, limiting ourselves to efforts to elucidate the mechanisms regulating hTERT mRNA levels, and try to

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preparation of Table 1 and she tested the role of TCF in hTERT regulation. She is currently investigating the cromatin structure of the hTERT gene by nuclease hypersensitivity assay and by ChIP.

explain why so far it has provided few if any conclusive answers that would be helpful to oncologists.

2.3 MAINTENANCE OF EXPRESSION OR ACTIVATION OF THE hTERT GENE?

Human skin or lung fibroblasts do not express hTERT and senesce after 50 to 60 population doublings. Ectopic expression of hTERT renders these as well as endothelial cells «immortal» without inducing any changes in their karyotype or other signs of transformation (Bodnar *et al.*, 1998; Jiang *et al.*, 1999; Morales *et al.*, 1999; Vaziri *et al.*, 1999; Yang *et al.*, 1999). There is no report of spontaneous immortalization of normal fibroblasts, but SV40 infection, by blocking the p53 and p16 dependent pathways that arrest cells when they reach senescence, extends their life span (see (Duncan and Reddel, 1997) for review). These cells eventually hit a «crisis» during which almost all cells die with the exception of a few transformed survivors that either maintain their telomeres by the ALT pathway (see (Reddel, 1997) for review) or express hTERT. In this case there is no doubt that hTERT expression has been reactivated. Whether this occurs in tumors is much less clear (for a discussion of this issue see (Greaves, 1996; Shay and Wright, 1996). There is evidence that some, perhaps most, tumors are derived from cells that have already gained their first alterations towards malignant transformation before undergoing differentiation, close to a stem-cell like stage when hTERT may still have been expressed. The clearest case can probably be made for colorectal carcinoma. Colorectal adenomas are derived from crypt cells some of which can express hTERT, as detected by in situ hybridisation (Kolquist *et al.*, 1998). Many adenomas themselves contain hTERT expressing cells but a proportion of them lack detectable telomerase activity (Yan *et al.*, 2001). This may reflect the fact that most adenoma cells undergo differentiation and eventually die, while a small variable number of undifferentiated cells ensure the survival of the tumor. These may be the hTERT positive cells detected in situ.

Thus, in most carcinomas hTERT expression may not be due to reactivation of the hTERT gene but to the fact that the cells which maintain the tumor are prevented from differentiating and maintained in a stage at which their normal counterparts still express hTERT. The finding that the frequency of telomerase negative sarcomas is higher than that of carcinomas (Yan *et al.*, 1999) suggests that sarcomas might be more frequently derived from hTERT negative cells for which there is no preferential choice of the mechanism through which they stabilize chromosome ends (Carroll *et al.*, 1999).

2.4 WHY DO TUMOR CELLS NEED TELOMERASE?

Telomeres are structures that prevent the ends of a linear chromosome to be mistaken for a double strand break (Godhino Ferreira and Promisel Cooper, 2001; McClintock, 1941; van Steensel *et al.*, 1998). If these structures are disrupted, the cell attempts to repair the break and, in doing so, generates fusions between the telomeres of different chromatids. Fusions occur when the number of telomeric repeats drops below a critical level, in cells which lack telomerase (Blasco *et al.*, 1997; Hackett *et al.*, 2001) and do not express an ALT pathway. Thus, most tumor cells need telomerase to maintain telomeres sufficiently long to keep the incidence of chromosome fusions low. However, telomere attrition to a level at which telomeres cease to protect chromosome ends requires 50 to 60 cell doublings, and it is not clear whether the cells in a tumor have indeed undergone that many divisions, even taking into account cell loss due to differentiation and death. It seems important to consider alternative reasons for the hTERT expression by most tumors. One explanation may lie in the chromosomal instability that characterizes most cancer cells (Parshad and Sanford, 2001) (see (Sen, 2000) for review). At least some of this instability arises from the breakage and fusion of chromosomes. Indeed, breakage is involved in the amplification of oncogenes or genes conferring drug resistance, through breakage-fusion-bridge cycles (Coquelle *et al.*, 1997; Coquelle *et al.*, 1998)[2]. Although chromosome breaks can provide the cellular substrate for the selection of more aggressive tumor cells, they will also give rise to non-viable cells. One way to keep these processes in check is through de novo addition, by telomerase, of telomeres to the ends of broken chromosomes (Friebe *et al.*, 2001; Hande *et al.*, 1998; Varley *et al.*, 2000). This would mean that premalignant cells, which express hTERT, have an advantage over the others not only when cells have undergone more than 50 to 60 divisions, but at a much earlier stage when chromosome breakage becomes frequent.

2.5 IS hTERT EXPRESSION REGULATED BY CHANGES IN THE LEVEL OF GENE TRANSCRIPTION?

As pointed out above there is a very strong correlation between telomerase expression and the presence of detectable hTERT mRNA (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). We have compared the numbers of hTERT molecules per cell, determined by quantitative RT-PCR, in a number of cell lines from different tissue origins (Ducrest *et al.*, 2001). In all

[2] Telomere loss itself can lead to chromosomal instability, and experiments with telomerase-deficient mice show that this correlates with a very strong increase in the incidence of carcinomas. What the contribution of telomere loss to genome instability in the evolution of human tumors is remains to be analysed. The answer to this question depends, in part, on what fraction of tumors is derived from telomerase positive stem cells.

telomerase positive cells hTERT transcripts are detectable but rare (0.2 to 6/cell) whereas no transcripts (<0.004/cell) could be detected in telomerase negative cells. This correlation has been widely assumed to reflect regulation of hTERT expression via control of the rate of transcription. But it is equally compatible with regulation of transcript processing or changes in the mRNA half-life. Although a considerable number of transcription factors have been implicated in the control of hTERT expression (see below), direct evidence that hTERT gene transcription is regulated is scarce. Specifically it is unclear whether the tumor specific expression of hTERT is controlled at the level of transcription. The finding that activation of a c-Myc-estrogen receptor ligand binding domain fusion can increase hTERT mRNA levels in the absence of protein synthesis shows that ectopic c-Myc can indeed directly stimulate transcription of the gene (Greenberg *et al.*, 1999; Oh *et al.*, 2000; Wu *et al.*, 1999). We will discuss the biological role of c-Myc in hTERT regulation below.

The classical assay detecting changes in the rate of gene transcription, run-on nuclear experiments which measure the average loading of RNA-polymerase molecules on the gene, has been reported for one leukemia cell line (U937) (Gunes *et al.*, 2000). This study indicated that in these blood cells hTERT is regulated at the level of transcription rather than RNA stability. We have made attempts to obtain similar evidence for a tumor cell line derived from fibrosarcoma (HT1080) that contains relatively high numbers of hTERT transcripts among the cell lines screened by us, and have been unable to detect run-on transcription signal above background. The probable reason for this failure is that the rate of transcription is too low in HT1080 cells to be detectable by this approach. Comparing the levels of spliced cytoplasmic mRNA with that of intron-containing nuclear transcripts in different telomerase positive and negative cell lines, we observed that telomerase negative cells did not contain detectable levels (< 0.004 molecules/cell) of either cytoplasmic mRNA or nuclear transcripts, whereas telomerase positive cells contained both transcript forms. These results clearly suggest that hTERT mRNA levels are indeed controlled at the level of gene transcription, but they do not exclude that regulation involves changes in the efficiency of nuclear processing of primary transcripts.

2.6 POSSIBLE MODELS OF hTERT REGULATION

In a sense hTERT behaves like a protooncogene; abnormal maintenance or reactivation of expression contributes to tumorigenesis. Thus, one would expect that genomic changes that can lead to improper expression of protooncogenes, such as translocations that include the regulatory regions, would also be found in the hTERT genes of tumors.

Indeed, there is one report (Horikawa and Barrett, 2001) suggesting that the integration of the hepatitis B viral genome into the 5'flanking region of the hTERT gene might induce its expression in a hepatocellular carcinoma. But so far, there is no other evidence indicating a role of cis-acting gene rearrangements in the activation or maintenance of hTERT expression in tumors. We have found no evidence for rearrangements in the 5'flanking region and the 5'half of the gene (-10 to +25 kb) screening a number of cell lines of divers origin. The second intron of the hTERT gene contains a meiotically unstable minisatellite with several putative binding sites for c-Myc (Szutorisz *et al.*, 2001; Wu *et al.*, 1999). Size rearrangements of that minisatellite are not required for telomerase expression in colon carcinomas (Szutorisz *et al.*, 2001). In 31 of 33 colon carcinomas that were heterozygous for the polymorphic minisatellite the 1:1 ratio of hTERT alleles was maintained, indicating that there had been no gene amplification in these tumors. In the two remaining tumors there was a change compared to normal tissue from the same patient, compatible with amplification of one hTERT allele. Amplification of the hTERT gene was also detected in another study, in 20% of primary tumors and 40% human cancer derived cell lines (Zhang *et al.*, 2000). Amplification may be the result of selection for higher expression of an active hTERT gene. It might also lead to the expression of an inactive gene as a consequence of the genomic rearrangements that give rise to amplification, or through titration of a gene specific repressor.

Another modification that might affect hTERT expression is DNA methylation. Turning off the expression of tumor suppressor genes or genes involved in DNA repair, through methylation of their promoter, can contribute to carcinogenesis. Comparison of the methylation status of the hTERT promoter in telomerase positive and negative cells has not provided any compelling clues that this type of modification controls tumor specific hTERT expression (Dessain *et al.*, 2000; Devereux *et al.*, 1999).

hTERT expression due to cis-acting gene rearrangements should behave like a dominant trait. Dominant expression would also be likely if demethylation of the hTERT promoter were the mechanism through which hTERT expression is activated in tumors. However, so far no cross in which hTERT expression is dominant has been reported. On the other hand there is a number of tumor lines in which hTERT expression behaves like a recessive trait; expression is extinguished in hybrids with telomerase negative cells or by transfer of single chromosome from a normal cell (Table 1) (Bryan *et al.*, 1995; Cuthbert *et al.*, 1999; Horikawa *et al.*, 1998; Nishimoto *et al.*, 2001). This suggests that hTERT expression in normal cells is repressed by a mechanism, which is no longer functional in tumors. The simplest model that accounts for these observations is that hTERT transcription is under the control of a repressor, absent in cancer cells, that acts via a cis-acting element in the hTERT gene. Note, that the repressor may not itself be a sequence specific DNA-binding

protein, but could be a co-repressor interacting with a transcription factor. The data are equally compatible with a model according to which the repressor controls a gene coding for an obligatory activator of hTERT transcription, and so on. The finding that single normal chromosomes can repress hTERT expression in tumors has led to attempts to clone the genes coding for such repressors, by positional cloning (see next section).

2.7 HOW MANY hTERT REPRESSORS ARE THERE?

Table 1 lists the chromosome transfer experiments that have addressed the question of hTERT regulation. The data summarized are not homogeneous, and different studies testing the same chromosome have not always used the same chromosome donor cells. It should also be kept in mind that a normal chromosome may undergo changes in the donor cells. This might explain that chromosome 6 represses hTERT in the cervical carcinoma line SiHa in one study but fails to do so in another. Alternatively, the different result may reflect changes in the cell line. Given these limitations the studies listed in Table 1 strongly suggest that there is no single chromosome that represses hTERT expression in all cells. Chromosome 3, e. g. represses hTERT expression in several but not all of the recipient lines tested. This is not unexpected; even if there were a single molecular complex that is responsible for the repression of the hTERT gene in normal cells, mutations in both copies of any gene coding for a component of the complex should lead to inactivation of the repressor and expression of hTERT. It would certainly be interesting to determine whether a large-scale chromosome screen would reveal patterns, e.g. consistent repression of hTERT expression by chromosome 6 in HPV16-transformed tumors. The available data do not permit to detect such patterns. Transfer of chromosomes from irradiated donor cells can be used for attempts to positionally clone a putative hTERT repressor gene. The chromosome for which this approach is most advanced is chromosome 3. Upon introduction of a normal chromosome 3, two renal, one breast, and one cervical carcinoma line ceased to express hTERT. Two groups using either a renal carcinoma (Tanaka *et al.*, 1998) or a breast cancer derived line (Cuthbert *et al.*, 1999) as recipients have narrowed the region that confers repression to 3p14.2-21.1. This region overlaps with a segment of chromosome 3 that undergoes frequent LOH in breast cancer (Maitra *et al.*, 2001). LOH and deletions of smaller parts of 3p have been identified in breast, cervix, colon, lung, and renal carcinomas (Kok *et al.*, 1997).

In a single study both chromosome 3 and 4 have been found to shut off hTERT expression in HeLa cells. This suggests multiple independent pathways of repression. Since mutations affecting a repressive pathway are recessive, activation of hTERT expression

through such mutations would be expected to be an extremely rare event. It might explain why spontaneous immortalization of normal fibroblasts has never been observed.

TABLE 1 Effect of normal human chromosomes on hTERT expression in telomerase positive cell lines

Transferred chromosome	*Recipient cell line*		*Phenotype of hybrids*		*Other chromosomes tested*[b]	*Source of chromosomes*[c]	*Reference*
	Name	*Cell type*[a]	*Telomerase activity*	*hTERT mRNA*			
# 1	TE85	osteosarcoma	+	nd		(Koi *et al.*, 1989)	(Hensler *et al.*, 1994)
	B16-F10	*mouse* melanoma	+	nd		(Koi *et al.*, 1989)	(Oshimura and Barrett, 1997)
# 2	SiHa	cervical carcinoma	+	nd	**3, 6, 7**, 9, 11, 12	(Koi *et al.*, 1989)	(Tanaka *et al.*, 1999)
	B16-F10	mouse melanoma	+	nd		(Koi *et al.*, 1989)	(Oshimura and Barrett, 1997)
# 3	RCC23	renal cell carcinoma	-	-	**7**, 11	(Koi *et al.*, 1989)	(Horikawa *et al.*, 1998; Tanaka *et al.*, 1999)
	KC12	renal cell carcinoma in VHL	-	nd	11	(Koi *et al.*, 1989)	(Tanaka *et al.*, 1998)
	21NT	breast carcinoma	-	-	8, 12, 20	chr 3, 8, 20: (Cuthbert *et al.*, 1995); chr 12: (Ning *et al.*, 1992)	(Cuthbert *et al.*, 1999)
	HeLa	cervical carcinoma	-	nd	**6**, 11	chr 3: MCH 922.5; chr 6: MCH 226; chr 11: MCH 556	(Backsch *et al.*, 2001)
	TS1	lung adenocarcinoma	+	nd		(Koi *et al.*, 1989)	(Ohmura *et al.*, 1995)
# 4	HeLa	cervical carcinoma	-	nd	**6**, 11	chr 4: HA(4)A9; chr 6: MCH 226; chr 11: MCH 556	(Backsch *et al.*, 2001)
# 6	FK16A	HPV16-immortalised keratinocyte	-	-	11	chr 6: (Cuthbert *et al.*, 1995); chr 11: (Koi *et al.*, 1989)	(Steenbergen *et al.*, 2001)
	SiHa	cervical carcinoma	-	-	11	chr 6: (Cuthbert *et al.*, 1995); chr 11: (Koi *et al.*, 1989)	(Steenbergen *et al.*, 2001)
# 7	CC1	choriocarcinoma	+	nd	1, 2, **6**, 9, 11	(Koi *et al.*, 1989)	(Tanaka *et al.*, 1999)
	MeT5A	SV40-transformed mesothelial cell	-	-		(Koi *et al.*, 1989)	(Nakabayashi *et al.*, 1999)
# 10	Li7HM	hepatocellular carcinoma	-	-	2, **4**, 5, 16	chr 2, 4, 5, 10: (Kugoh *et al.*, 1999); chr 16: (Koi *et al.*, 1989)	(Nishimoto *et al.*, 2001)
# 11	JTC-32	bladder carcinoma	+	nd	**7**	(Koi *et al.*, 1989)	(Tanaka *et al.*, 1999)
# 17	BP1-E	immortalized breast epithelial cell	-	nd	11	(Koi *et al.*, 1989)	(Yang *et al.*, 1999)

nd, not determined. [a] Cells are of human origin, unless mentioned otherwise. [b] Chromosomes that did not affect cell immortality (when the chromosome indicated in the first column had no effect on telomerase) or telomerase activity. In bold chromosomes which repress telomerase activity or hTERT expression in another cell line.
[c] Reference to the panel of chromosome donor cells used.

2.8 OTHER APPROACHES TO STUDY REGULATION OF hTERT EXPRESSION

2.8.1 Screening candidate molecules

The mapping and cloning of genes on normal chromosomes that shut off hTERT expression in tumor cells is one approach towards the elucidation of the regulation of hTERT expression. Other, complementary approaches consist in (1) the testing of candidate molecules for their effect on the expression of the endogenous hTERT gene, or (2) attempts to identify the cis-acting elements in the hTERT gene that control its expression. The former approach is based on guesses as to what molecules might be involved in hTERT regulation which can be tested either through the ectopic expression of such putative positive regulators in hTERT negative cells, or through the expression of dominant negative version of such molecules in hTERT expressing cells. The latter is a better approach that can provide informative data even if the results are negative. Candidates include molecules whose abnormal expression in tumor cells prevents their differentiation, such as c-Myc, TCF or Notch. The second approach aims at the identification of cis-acting regulatory sequences in or near the hTERT gene through experiments using reporter gene constructs and including in vitro assays for DNA binding proteins, nuclease hypersensitivity, in vivo footprinting assays and ChromatinIP.

Numerous molecules, including transcription factors, regulators of differentiation and the cell cycle and proteins of viruses implicated in tumorigenesis, have been proposed to regulate hTERT expression. We have attempted to summarize the most relevant findings in Table 2 and Fig. 1, without being exhaustive in our literature citations. Many studies were based on the ectopic expression of positive regulators. The interpretation of such experiments is often difficult. An example is provided by the studies on the effect of c-Myc on hTERT expression. The published data show that overexpression of c-Myc can increase the level of hTERT mRNA in B-cell lines or induces its appearance in fibroblasts. This effect does not depend on protein synthesis and is therefore likely to be due to a direct action of c-Myc protein on the hTERT gene (Greenberg *et al.*, 1999; Oh *et al.*, 2000; Wu *et al.*, 1999). Mad, the antagonist of c-Myc was shown to be a potential repressor of hTERT. Mad was a candidate repressor identified in a gene screen for hTERT regulators (Oh *et al.*, 2000), and a rise in endogenous Mad RNA and protein levels was inversely correlated with hTERT RNA levels (Gunes *et al.*, 2000; Oh *et al.*, 2000; Xu *et al.*, 2001). Finally, while c-Myc protein was found associated with the hTERT gene in vivo in telomerase positive promyelocytic leukemia HL60 cells as determined in chromatin immunoprecipitation assays (Xu *et al.*, 2001),

differentiation of these cells by DMSO led to downregulation of hTERT, loss of association with c-Myc and binding of the c-Myc antagonist Mad1. These results show that the c-Myc/Mad regulatory network can regulate hTERT expression, but the role of this network in tumor specific hTERT expression is not yet clear. Deregulation of the c-Myc/Mad balance is unlikely to be sufficient for the activation of the hTERT gene in cancers, for several reasons: (1) In most cases overexpression of c-Myc is expected to behave like a dominant trait in somatic cell crosses, unlike of what has been observed for hTERT expression. (2) In exponentially growing fibroblasts c-Myc is expressed at lower levels than in tumor derived cell lines (Gewin and Galloway, 2001; Kyo *et al.*, 2000; Oh *et al.*, 2000), and declines even further when fibroblasts are serum deprived. Restimulation with serum induces a transient, high level of c-Myc and downregulation of Mad (Grandori *et al.*, 2000; Obaya *et al.*, 1999), but there is no evidence that this change is sufficient to induce hTERT expression. (3) Overexpression of HPV16 E7, which is important for immortalization of keratinocytes, induces high level of c-Myc protein but is unable to activate telomerase expression (Gewin and Galloway, 2001; Veldman *et al.*, 2001). (4) In the breast cancer derived cell line 21NT chromosome 3 transfer leads to immediate repression of the hTERT gene but expression of c-Myc, Mad1 and c-Myc target genes remained unchanged (Ducrest *et al.*, 2001). Therefore, the putative repressor on chromosome 3 does not regulate hTERT through c-Myc or one of its coregulators. In conclusion, it seems likely that normal changes in the c-Myc/Mad ratio control hTERT transcription in cells in which the gene is not “closed” by one or several repressors, but that the levels of c-Myc in most tumors are not high enough to overcome repression. One obvious possibility is that in normal cells competent to express the gene c-Myc links hTERT expression to the proliferative status of the cell. Other genes involved in the control of cell cycle progression have been suggested to repress hTERT expression such as p53, p16, p21 and E2F-1 (Table 2 and Fig.1). However the effect of these genes on hTERT expression remains ambiguous. The best case can be made for p53, which was shown to downregulate hTERT expression. This effect seems to be independent of p53 induced cell cycle arrest and apoptosis (Kanaya *et al.*, 2000; Kusumoto *et al.*, 1999; Xu *et al.*, 2000). Another case in which hTERT can be regulated independently of differentiation and/or growth inhibition is the acute promyelocytic leukemia cell line NB4-R1, in which treatment with retinoic acid dowregulates hTERT without inducing maturation (Pendino *et al.*, 2001).

As suggested above hTERT expression in most carcinomas may not be due to a reactivation of the hTERT gene but reflect the advantage, during tumor progression of cells in which differentiation is partially or completely blocked and, as a consequence, hTERT expression maintained. This view would predict that pathways which control cell differentiation and which are frequently deregulated in cancer, such as the Notch and the Wnt

pathways may be implicated in hTERT regulation. We have found that in the breast cancer cell line, 21NT, overexpression of the intracellular part of the Notch 1 protein increases the levels of hTERT transcripts as well as of HES-1, a known Notch1 target (A.D.; unpublished data). Similarly, arguing that TCF activity may be required for hTERT expression in colon carcinoma cells we have determined the levels of hTERT mRNA in four colon carcinoma cell lines carrying tetracycline inducible constructs coding for dominant negative version of TCF1 or TCF4. These lines were prepared by Marc van de Wetering in the laboratory of Hans Clevers. Tetracycline treatment of such cells leads to a significant down-regulation of a number of TCF target genes expressed in colon carcinomas, but had no effect on hTERT transcript levels which were comparable to that in control cells from the same tumors lacking the dominant negative TCF constructs. These results quite strongly argue that TCF does not play a role, direct or indirect, in controlling hTERT expression in colon carcinomas.

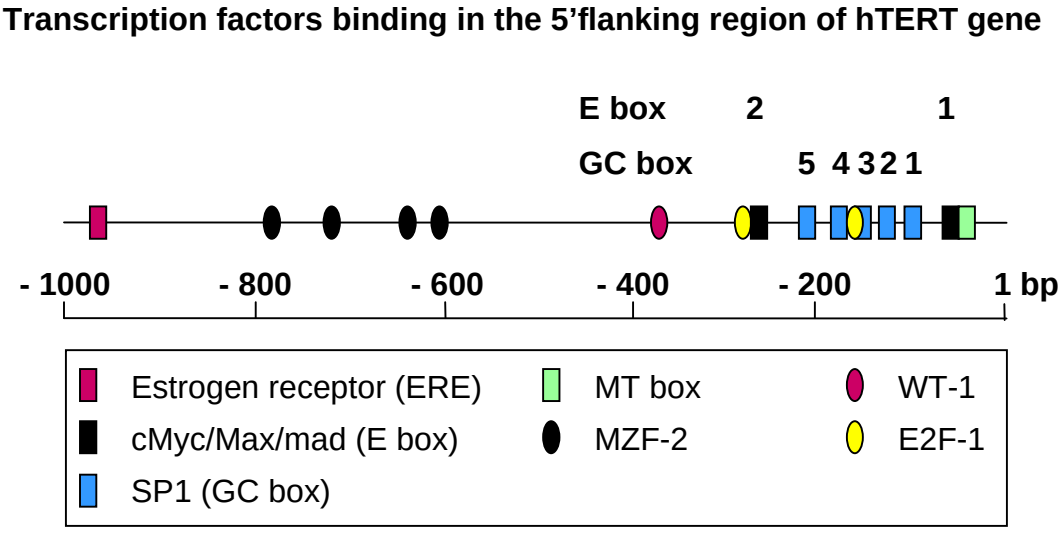


Figure 1: Schematic representation of the potential cis-acting regulatory elements in the first 1000 bp upstream of the translation start site of the hTERT gene. Rectangles represent putative activator binding sites (except for Mad), ovals represent putative repressor binding sites.

In estrogen-targeted tissues, such as endometrium (Kyo *et al.*, 1997; Saito *et al.*, 1997; Takakura *et al.*, 1999), prostate (Meeker *et al.*, 1996) and epithelial cells with high renewal potential (Bednarek *et al.*, 1998) estrogen-responsive cells may be more prone to form tumors (Hilakivi-Clarke, 2000; Liehr, 2000) because they are telomerase positive. Estrogen was shown to activate hTERT promoter constructs through estrogen responsive elements (ERE) in the hTERT 5'flanking region. This activation was dependent on the presence of estrogen receptor-α. Genomic footprinting indicated that one ERE element, 950 bp upstream of the translation start site, is occupied in vivo in cells expressing, but not in cells lacking, the estrogen-receptor-α (Misiti *et al.*, 2000). This is in agreement with the finding that tamoxifen,

an antagonist of estrogen, reduces telomerase activity in the breast cancer cell line MCF-7 cells (Aldous *et al.*, 1999). Since in this line estrogen also increases c-Myc levels (Kyo *et al.*, 1999), c-Myc may contribute to activation of hTERT transcription.

2.8.2 On the use of hTERT-reporter constructs

There have been many attempts to identify cis-acting regulatory elements in the hTERT gene through the use of reporter constructs (see Table 2 and Fig. 1). The main a priori limitation of this approach is that it makes assumptions on the location of the regulatory elements, which can be at considerable distance, 3' or 5' of the gene, or in introns. Furthermore, certain regulatory elements might not work outside of their endogenous context. Therefore the use of the basic reporter construct should be validated in experiments testing whether it contains the cis-acting elements controlling the expression of the endogenous gene, e.g. by transfection into appropriate cell lines. Claims that hTERT reporter expression reflects that of the endogenous gene have been based on the comparison of reporter expression in normal cells with that in various cell lines. However, in vitro transformed or tumor cells differ from normal cells in many respects that can affect the rate of gene transcription in ways, which are unrelated to gene specific regulation. To solve this problem we (Ducrest *et al.*, 2001) have compared the expression of a series of reporter constructs containing the hTERT promoter and up to 7.5 kb of 5' flanking region in two SV40 transformed fibroblast lines. One of these is telomerase positive, whereas the other uses the ALT pathway and contains no detectable hTERT transcripts. All reporter constructs were more strongly expressed in either line than in normal fibroblasts, and there were no significant differences between the activity of any of the reporters in the telomerase positive and the ALT line. Knight *et al* also have reported hTERT promoter activity in an ALT cell line SUSM-1 when using another reporter containing 1.7 kb of the hTERT flanking region (Knight *et al.*, 2001). Even more strikingly, we observed no differences in the expression of the same hTERT reporters when we compared them in a breast carcinoma line and its derivatives in which transfer of a single normal chromosome 3 has reduced hTERT mRNA by at least 30 fold, to undetectable levels (Ducrest *et al.*, 2001). Thus, by these stringent criteria the validation of hTERT reporters containing the longest 5' flanking segment tested so far has completely failed, and the significance of the results obtained with similar constructs in other cells (see table 2) has to be assessed in the light of this failure.

Of course, this does not mean that regulatory sites identified in constructs expression of which does not mimic that of the endogenous gene have no role in the regulation of the latter, but without strong additional evidence such identifications provide only very weak arguments. The finding that an element identified in this way indeed binds a transcription

factor that might be implicated in the regulation in vitro adds very little weight to the argument. Strong evidence that binding of a transcription factor to a putative regulatory site plays a role in the control of gene expression requires demonstration that the factor occupies the site in vivo, most convincingly by ChromatinIP with antibodies against the putative regulator. But even such experiments cannot, by themselves, prove that the transcription factor in question controls the difference in hTERT gene expression in normal versus tumor cells. It is quite possible that certain transcription factor binding sites are indeed occupied in hTERT expressing but not in telomerase negative cells, and that occupation is required for hTERT transcription. But occupancy may reflect that fact that in cells competent to express the gene these sites are “open” i.e. accessible to the transcription factor, due to chromatin alterations that depend on other proteins which bind elsewhere and are higher up in the hierarchy of control.

2.8.3 Screening for changes in hTERT chromatin

The search for differences between the conformation of the chromatin containing the hTERT gene in hTERT expressing and non-expressing cells provides a complementary approach to the identification of cis-acting elements. The classical method used is to screen the locus of interest for sites with differential sensitivity to nucleases such as DNaseI or Micrococcal nuclease (MNase). In numerous instances the activity of a regulatory element correlates with the presence of a nuclease hypersensitive site or region at or near the element. Compared to ChromatinIP, this type of analysis has the advantage that it can be applied to very large genomic segments without previous assumption about the possible localization of regulatory elements, but it has the disadvantage that there are no strict rules describing the relationship between, say, transcription factor occupancy of a regulatory site and its nuclease sensitivity. Thus, lack of nuclease sensitive sites in a segment does not exclude that it plays regulatory role. Application of the technique to the hTERT gene has to face another uncertainty; as discussed above the rate of hTERT gene transcription is probably very low (Ducrest *et al.*, 2001), and even in a cloned hTERT positive cell line not all cells may transcribe the gene at a given moment. This may – or may not – mean that important regulatory elements in the gene are not always occupied, and that the corresponding nuclease hypersensitive sites are invisible in the background of chromatin from non-transcribed genes. Nevertheless, comparison of different telomerase positive and negative cell lines points to the existence of two nuclease sensitive sites in the second intron of telomerase expressing cells, and the significance of these sites has been validated by the stringent type of criteria outlined above for reporter construct analysis (H.S., manuscript in preparation). It remains to be seen whether these sites are the primary targets of the molecules that induce hTERT transcription

in tumors, or whether these chromatin alterations are the downstream consequence of the activity of cis-acting elements elsewhere in the gene.

2.9 OUTLOOK

One impression that emerges from this review is that in spite of considerable efforts by many groups, our understanding of the mechanisms that are responsible for the tumor specific expression of the human TERT gene is still very poor. This raises two questions. On the one hand one has to ask what new or at least modified approaches are most likely to be more successful than the attempts carried out so far, and on the other one is lead to consider the possibility that the models which determine the choice of methods are inappropriate or wrong.

At this time it seems that the approach which is most likely to provide insight into the regulation of hTERT expression is the positional cloning of genes on chromosomes that shut off hTERT expression upon microcell mediated transfer into tumor cell lines. However, it is by no means certain that such genes once they have been identified provide immediate clues as to the mechanisms through which they affect hTERT expression, and to unravel these mechanisms it would certainly be extremely useful if not essential to have a reporter system which does mimic the expression pattern of the endogenous hTERT gene according to the stringent criteria outlined above. To build such a system may require the use of much larger genomic segments as they are available, e.g., in BAC clones. BAC clones containing the hTERT gene are accessible but their sequence is not yet publicly available. Reporter constructs based on BAC clones of other genes have been successfully used for the study of regulation, but the technical investment required is not trivial, and one needs to take into account the risk that the experiments fail because of the very low level of hTERT transcription.

If a reporter system that faithfully reproduces the tumor-specific regulation of the hTERT gene were available, it might be informative to determine its expression pattern in transgenic mice. As pointed out earlier, the TERT of the mouse (mTERT) and other rodents is not shut off in differentiated somatic cells (Russo *et al.*, 1998). This difference between rodents and man may reflect changes in the cis-acting elements or in the expression of transacting factors. If hTERT gene expression in the mouse resembles that in man this would argue strongly that repression of hTERT expression during differentiation is due to differences in cis-acting elements only. What evolutionary pressure may have led to the somatic repression of hTERT expression? A simple idea is that this may be related to the species' life-span; in species that reach reproductive age late, repression of telomerase activity which provides an important barrier to malignant disease should confer a stronger selective

advantage than in species with a short life-span. Not enough species have been analyzed to allow evaluation of this hypothesis.

There are several aspects of hTERT expression that are puzzling and apparently contradictory. The finding that immortalization of normal fibroblasts by spontaneous activation of hTERT expression has never been observed, and that it is a rare event even after viral transformation, is hard to reconcile with the finding that it is quite easy to turn on hTERT expression in normal cells, through overexpression of c-Myc or treatment with an inhibitor of histone deacetylases (Cong and Bacchetti, 2000; Takakura *et al.*, 2001; Xu *et al.*, 2001). The indication, from monochromosomal tumor cell hybrids, that there are different genetic loci which can shut down hTERT expression, suggests that perhaps repression of hTERT is due to diffuse mechanisms that affect the chromatin structure in and around the hTERT gene, rather than to a few well defined target sites of sequence specific repressors or activators. In this context it may be relevant that the hTERT gene is close to the telomere of the short arm of chromosome 5. This raises the possibility that the gene is subject to telomeric repression which has recently been shown to exist in human cells (Baur *et al.*, 2001). The precise position of the hTERT gene has not yet been determined (Bryce *et al.*, 2000). It will be interesting to test whether expression of other genes close to the telomere of chromosome 5p correlates with that of hTERT.

Table 2 Molecules implicated in the regulation of hTERT expression

Proposed regulator			*Cells*				*Endogenous gene*		*Reporter constructs*				
Name	*Type*[1]	*hTERT Activator/ Repressor*	*Name*	*Cell type*	*Telome-rase*	*Tech-niques*[2]	*mRNA*[3]	*Chromatin*[4]	*Transfected cells*	*Gene segment analysed*[5]	*Putative cis-acting elements*[6]	*In vitro DNA binding assays*[7]	*References*
E2F-1	TF	R	SCC25	tongue carcinoma	+	WT	down			-1453/-1	-251/-247 and/or -175/-171	BS: REP	Crowe et al., 2001
c-Myc	TF	A	EREB CB33[8]	EBV-immortalized B lymphocytes	+	WT	up		293T	-800/-1 intron 2	mE1-2, mE2	BS: MT	Wu *et al.*, 1999
			HL60	promyeleucytic leukemia	+	DMSO	down	ChIP-296/+20				BS: MT, S	Xu *et al.*, 2001
			HMEC	breast epithelial cells	-	WT	up						Wang *et al.*, 1998
			IMR90[8]	embryonic lung fibroblasts	-	WT	up		NIH3T3	-2500/-1	-35/-2		Greenberg *et al.*, 1999
									C33A[9] ME180 SiHa NHK	-260/-1	mE1-2, mE1, mE2	BS: REP, S	Kyo *et al.*, 2000
			WI38[8]	embryonic lung fibroblasts	-	WT	up		293	-4000/-1	mE1-2	BS: REP	Oh *et al.*, 2000
			WI38	embryonic lung fibroblasts	-	WT	up		HeLa	-300/-1		BS: MT	Oh *et al.*, 1999b
									C33A[9,10]	-260/-1	mE1-2		Kyo *et al.*, 2000
Mad	TF	R	WI38[8]	embryonic lung fibroblast	-	WT	down		WI38 293T	-260/-1	mE1-2	BS: REP	Oh *et al.*, 2000
			U937	monoblastoid leukemia	+	TPA	down			-2500/-1	mE1[11]		Gunes *et al.*, 2000
			HL60	promyeleucytic leukemia	+	DMSO	down	ChIP-296/+20					Xu *et al.*, 2001
MZF-2[12]	TF	R	C33A SiHa	cervical carcinoma	+		?[13]			-1450/-1	-763/-757, -696/-689, -620/-614, -591/-584[14]	BS: REP -763/-757	Fujimoto *et al.*, 2000
p53	TF, TS	R	SiHa	cervical carcinoma	+	WT	down			-3410/-1	-110/-1		Kanaya *et al.*, 2000
			PaCa-2	pancreatic cancer	+	WT	down						Kusumoto *et al.*, 1999
			BL41	Burkitt's lymphoma	+	MT[15]	down[16]		HeLa	-4000/-1	-330/-1	BS: MT, S (SP1)[17]	Xu *et al.*, 2000
SP1	TF	A							C33A[9] ME180 SiHa	-260/-1	mGC1: ne, single mGC2 to mGC5, mGC1-5		Kyo *et al.*, 2000
TCF	TF	no effect	DLD1 LS174 HCT116	colon carcinoma	+	DN[18]	ne						H.S., H. Clevers, M. van de Wetering, unpublished
WT1[19]	TF, TS	R	293	adenovirus type 5 transformed embryonic kidney	+	WT	down		293 HeLa	-1000/-1	-358/350[20]	BS: REP	Oh *et al.*, 1999a
?, binds to MT box	TF	A							HEY[21] SKOV-3 OVCAR-3	-5870/-1	-31/-24	BS: REP	Braunstein *et al.*, 2001

Proposed regulator			*Cells*			*Endogenous gene*		*Reporter constructs*				*References*
Name	*Type*[1]	*hTERT Activator/ Repressor*	*Name*	*Cell type*	*Telomerase*	*Tech-niques*[2]	*mRNA*[3]	*Chromatin*[4]	*Transfected cells*	*Gene segment analysed*[5]	*Putative cis-acting elements*[6]	*In vitro DNA binding assays*[7]	
HDAC[22]	ChME	R	HL60	promyeleucytic leukemia	+	DMSO, TSA	up	ChIP-296/+20					Xu *et al*., 2001
			HRCE	renal cortical epithelial	-	TSA	up			-3420/-1	mE1-2:ne mGC1-5		Takakura *et al*., 2001
			BJ MRC5 WI38	primary fibroblast	-	TSA	up		HA1[23]	-4080/-1	-260/-1 but mE1-2:ne		Cong & Bacchetti, 2000
p16INK4A	CKI, TS	R	TSU-PR1	prostate carcinoma	+	ACT	down			-1450/-1	mE1-2[24]		Kitagawa *et al*., 2000
p21WAF1	CKI	?	PaCa-2	pancreatic cancer	+	WT	ne						Kusumoto *et al*., 1999
			BL41	Burkitt's lymphoma	+	AS, mimosine[2]	ne						Xu *et al*., 2000
			T47-D	breast carcinoma	-[26]	AS	down[27]						Wang *et al*., 2000
ER[28]	HR	A	MCF-7	breast cancer	+	E2	up		MCF-7 SiHa[29] NHK[29]	-3410/-1	mE1-2[30]	BS: MT,S -2754/-2742	Kyo *et al*., 1999
			LLO/LEA	ovarian surface epithelial cells	-	E2	up						
			OVCA-433[31]	ovarian surface epithelium,	+	E2		iv-949/-935	WOO[29]	-1000/-1	-949/-935	BS: REP	Misiti *et al*., 2000
			MCF-7[31]	breast cancer	+	E2		iv-949/-935	NIH3T3[29]		-330/-1: ne		
			HeLa[32]	cervical carcinoma	+	E2		no footprint					
			MDA[32]	breast cancer	+	E2		no footprint					
PR[33]	HR	R	T-47D	breast carcinoma	-[26]	WT + E2	up and down[34]			-3400/-1			Wang *et al*., 2000
RAR[35]	HR	R	NB4	acute promyelocytic leukemia	+	ATRA	down						Pendino *et al*., 2001
Notch1	CFD	A	21NT	breast carcinoma	+	WT[36]	up						A.D., unpublished
HPV16 E6	VP	A	HFK	foreskin keratinocytes	-	WT, MT[37]	up[38]			-710/-1	mE1-2		Gewin & Galloway, 2001
					-	WT	up			-1180/-15	-185/-50		Veldman *et al*., 2001
					-	WT	up		HFK C33A	-800/-18 -260/-1	mGC1-5, mE1-2:ne single mGC1 to mGC5: ne		Oh *et al*., 2001
HPV16 E7	VP	?	HFK	foreskin keratinocytes	-	WT	ne or up						Oh *et al*., 2001
							ne						Gewin & Galloway, 2001; Veldman *et al*., 2001
KSHV-LANA[39]	VP	A	293	adenovirus type 5 transformed embryonic kidney	+	WT	up		BJAB	-1720/-50	-185/-50	BS: MT, S (SP1)	Knight *et al*., 2001

Regulation of hTERT expression

[1]Transcription factor (TF), tumor suppressor (TS), chromatin modifying enzyme (ChME), cyclin dependent kinase inhibitor (CKI), hormone receptor (HR), cell fate determining protein (CFD), viral protein (VP). [2] Ectopic expression of wildtype (WT), dominant negative (DN) or mutant (MT) protein; antisense (AS); treatment with phorbol ester (TPA), trichostatin A (TSA), DMSO or estrogen (E2), 5-aza-cytidine (ACT), all-trans-retinoic acid (ATRA). [3]No effect (ne), increase (up) or decrease (down). [4]Positions of chromatin IP (ChIP) or in vivo footprint (iv). [5]Position +1 corresponds to translation start site. The major transcription start site lies between -55 and -77 (Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). Indicated is the longest fragment tested. [6]Two putative c-Myc/Max or Mad/Max binding sites (E boxes) and 5 putative SP1 binding sites (GC boxes) were identified and mutated in several studies. E boxes: E1: -34/-29; E2: -242/-237. mE1, mE2: Mutation of one of the sites in one reporter. mE1-2: Mutation of both E boxes in the same reporter construct. GC boxes are GC1: -84/-79; GC2: -113/-105; GC3: -133/-124; GC4: -165/-159; GC5: -187/-179. mGC1-5: Mutation of all five GC boxes in the same reporter construct. mGC1, mGC2, ...: Mutation of individual site. No effect: ne. [7]Bandshift (BS) assays; mutant probes or competition with mutant oligonucleotides (MT); effect of mutations on reporter expression (REP); supershift experiments (S). [8]EREB, IMR90 and WI38 cells contain a Myc-estrogen receptor fusion protein (Myc-ER). The cells were treated with 4-hydroxy-tamoxifen (4-OH-T) and cycloheximide (CX). [9]C33A, ME180, SiHa: cervical carcinoma cell lines. NHK: primary keratinocytes. [10]Ectopically expressing Mad and Max. [11]Ectopic expression of Mad1. [12]Myeloid-specific zinc finger protein 2. [13]Telomerase activity reduced in cells ectopically expressing MZF-2. [14]All sites mutated in the same reporter construct. [15]Cell line expressing p53 ts mutant. [16]Effect not through p21. [17]Extracts from SL2 insect cells ectopically expressing SP1 and p53. [18]Stable transfectants inducibly expressing dominant negative versions of TCF-1 or TCF-4. [19]Wilms' tumor 1. [20]No effect in HeLa. [21]Ovarian carcinomas. Effect only in HEY cells. [22]Histone deacetylase. [23]SV40 T transformed embryonic kidney. [24]p16 reduces c-Myc expression. [25]In BL41-p53^{ts}, at permissive temperature. [26]Expression dependent on E2 or progesterone. [27]Decrease of progesterone dependent expression. [28]Estrogen receptor. [29]Cells ectopically expressing estrogen receptor α (ER-α) were treated with estrogen. [30]E2 increases c-Myc expression in MCF-7. [31]Estrogen receptor α-positive. [32]Estrogen receptor α-negative. [33]Progesterone receptor. [34]Progesterone induces transient expression (12 h) and reduces the estrogen mediated increase (48 h). Both effects are blocked by an inhibitor of MEK. [35]Retinoic acid receptor. [36]Constitutively active intracellular portion of Notch1. [37]MT: HPV16E6-8S/9A/10T, defective in p53 degradation. [38]Effect correlates with E6 binding to E6AP. [39]Kaposi's sarcoma associated herpesvirus latency-associated nuclear antigen.

3 DETECTION OF PROMOTER ACTIVITY BY FLOW CYTOMETRIC ANALYSIS OF GFP REPORTER EXPRESSION[3]

3.1 ABSTRACT

Low efficiency of transfection is often the limiting factor for acquiring conclusive data in reporter assays. It is especially difficult to efficiently transfect and characterize promoters in primary human cells. To overcome this problem we have developed a system in which reporter gene expression is quantified by flow cytometry. In this system GFP reporter constructs are co-transfected with a reference plasmid that codes for the mouse cell surface antigen Thy-1.1 and serves to determine transfection efficiency. Comparison of mean GFP expression of the total transfected cell population with the activity of an analogous luciferase reporter showed that the sensitivity of the two reporter systems is similar. However, because GFP expression can be analyzed at the single-cell level and in the same cells the expression of the reference plasmid can be monitored by two-color fluorescence, the GFP reporter system is in fact more sensitive, particularly in cells which can only be transfected with a low efficiency.

3.2 INTRODUCTION

Understanding the mechanisms controlling transcription of a gene requires the identification and characterization of its cis-acting regulatory elements. In mammalian cells transient transfection of plasmids in which a reporter gene is expressed under the control of a fragment of the gene to be analyzed is widely used for this purpose. Following transfer of the reporter construct into cells, the expression of the reporter gene is monitored by measuring the amount of reporter mRNA, of the reporter protein itself or its enzymatic activity. The commonly used reporters include chloramphenicol acetyltransferase (CAT), β-galactosidase, firefly or renilla luciferase, alkaline phosphatase (AP) or green fluorescent protein (GFP). GFP protein is unique in that the GFP fluorophore spontaneously forms intracellularly without added cofactors (Heim *et al.*, 1994). Therefore, the emitted fluorescence intensity provides a direct readout of GFP expression (Cheng *et al.*, 1996) that can be measured at the single-cell level without any processing steps. Flow cytometry analysis of GFP was used for monitoring expression of inducible reporters (Anderson *et al.*, 1996) and for detecting time dependent IκB degradation (Li *et al.*, 1999). Recently the combination of enhanced intensity

of GFP fluorescence (Yang *et al.*, 1996) with destabilization of the GFP protein (Li *et al.*, 1998) improved the detection reliability of GFP fluorophore principally in induction studies.

Most efforts to map cis-acting regulatory elements have made use of cell lines that can be transiently transfected with a sufficiently high efficiency to permit the use of the standard reporter systems. Since cell lines are never completely normal, the results obtained are always subject to some reservations. The most important of these could be avoided if normal cells were used as recipients. But the transfection efficiency of most normal cell types is not sufficiently high, even when more recently developed transfection reagents are used. Here we describe a system that overcomes this problem by permitting the quantification of the expression of reporter constructs as well as that of a reference plasmid at the single-cell level. With this approach we can reliably measure the activity of weak promoters in primary human lung fibroblasts.

[3] This chapter is in press in Nucleic Acid Research Online as a method article with the same title and the following authors: Anne-Lyse Ducrest, Mario Amacker, Joachim Lingner and Markus Nabholz. Mario Amacker constructed the GFP reporters used in this study.

3.3 MATERIALS AND METHODS

3.3.1 Cells

Primary human embryonic lung fibroblasts (HLF, a generous gift of Urs Ziegler, University Hospital, Zürich), a fibrosarcoma-derived line (HT1080, kindly provided by Ian Kerr, ICRF, London) and Phoenix cells, packaging cells derived from the 293 cell line (a gift from a Garry Nolan, Stanford University, CA) were maintained in high glucose DMEM with 10% fetal calf serum.

3.3.2 Plasmids

pSV2Thy-1.1 expresses the mouse Thy-1.1 allele under the control of the SV40 enhancer and early promoter (Wilson *et al.*, 1990). The luciferase reporters pGL3 Basic, pGL3 Promoter, pGL3 Promoter and Enhancer, pRL-SV40 were purchased from Promega. PRL-SV40 contains the renilla gene under the control of the SV40 early promoter and enhancer. To generate the GFP reporter vectors pd2G (basic vector), pSVd2G (promoter vector) and pSVEd2G (promoter/enhancer vector), we replaced the *Hind*III/*Xba*I fragment containing the luciferase gene of pGL3 by the *Hind*III/*Xba*I fragment of pEGFP-N1 (Clontech) containing the EGFP gene. The GFP gene was destabilized by adding the degradation domain of MODC as described by Clontech (Li *et al.*, 1998). The half-lives of the GFP and luciferase proteins were 2h and 3h, respectively (Li *et al.*, 1998; Thompson *et al.*, 1991).

3.3.3 Transfections

Transient transfections with calcium-phosphate precipitates were performed according to Jordan (Jordan *et al.*, 1996). In the standard protocol, cells were co-transfected with 1 µg pSV2-Thy-1.1 as reference plasmid and 1 to 5 µg of GFP-reporters. To compare the GFP with the luciferase system, cells were co-transfected with 0.3 µg of pRL-SV40 and 1 to 5 µg of pGL3 promoter constructs. The total amount of plasmid DNA was kept constant (6 µg) by adding pUC19.

3.3.4 Determination of reporter expression.

Reporter expression was determined 40 h after transfection. For GFP and Thy-1.1 assays, cells were harvested by trypsinization, incubated for 30 min with a saturating concentration of allophycocyanin (APC) labeled anti-Thy-1.1 antibody III-5 (MacDonald *et al.*, 1985), kindly prepared by Céline Maréchal, and washed once. We analyzed the cells on a FACScalibur microflow cytometer (Becton Dickinson, Franklin Lakes, NJ). Using forward and side scatter

parameters we eliminated dead cells and debris from the analysis. GFP was excited by an argon laser and fluorescence was detected using a 530/30 nm bandpass filter in the FL1 channel. Allophycocyanin was excited by a red diode laser and fluorescence emission was detected using a 661/16 nm bandpass filter in the FL4 channel. For Dual luciferase-renilla assays, cells were lysed in the Passive Lysis Buffer (Promega). The assay was performed on a Luminometer (Lumac, Biocounter M2500, MWG) as described by Promega.

3.4 RESULTS AND DICUSSION

3.4.1 Flow cytometry analysis of the GFP-reporter system.

Our reporter system consists of two plasmids: a GFP reporter that is used to test regulatory role of segments of the gene to be analyzed, and a plasmid (pSV2Thy-1.1) that encodes the murine Thy-1.1 cell surface marker. This antigen is resistant to the trypsin concentrations used to detach the cells (data not shown) and its expression can be quantified by labeling the cells with allophycocyanin (APC) coupled to anti-Thy-1.1 antibody.

Thy-1.1 expression is used to measure transfection efficiency. To determine whether GFP could be used as a reporter gene, we co-transfected GFP and pSV2-Thy-1.1 reporters into Phoenix cells and analyzed the GFP and Thy-1.1 expression by two-color flow cytometry 40 hours after transfection. Forward and side scatter signals were used to restrict the analysis to viable cells. GFP fluorescence intensity (FL1, X-axis) was plotted on a log scale against the fluorescence intensity (FL4, Y-axis) due to APC-coupled anti-Thy-1.1 antibody (Fig. 1). Signal amplification was set so that background fluorescence of non-expressing cells was below 10 (3 for GFP and to 7 for Thy-1.1) (Fig. 1). Thus, for both reporter plasmids 1000 fold differences in expression levels of over background could be measured. Fig. 1 shows that the GFP and APC signals can be separated over the entire range of signal intensity. In preliminary experiments we used a phycoerythryn (PE) labeled anti-Thy-1 antibody, but we found that it was impossible to compensate completely for the spill-over of the GFP fluorescence into the FL2 channel used to detect the PE-signal. This problem could be avoided by switching to an APC-anti-Thy-1.1 conjugate.

When co-transfected with both plasmids, most cells emit GFP and APC fluorescence; they appear in the upper right quadrant (Fig. 1). With lower amounts of pSVEd2G GFP positive cells were found preferentially among the population expressing high levels of Thy-1.1. These results are expected for transfection with calcium-phosphate precipitates, but the percentage of cells expressing both plasmids was similar when other, liposome-based methods (Fugene 6 (Roche), Lipofectamin 2000 (GIBCO), Effectene (Qiagen)) were tested (data not shown). The distribution of APC fluorescence intensity is not influenced by co-transfection of the GFP reporter into the same cells, indicating that the pSV2-Thy-1.1 promoter activity was not affecting by pSVEd2G (Fig. 1, 3). Thus, there is no evidence for competition for transcription factors between the two SV40-based promoters.

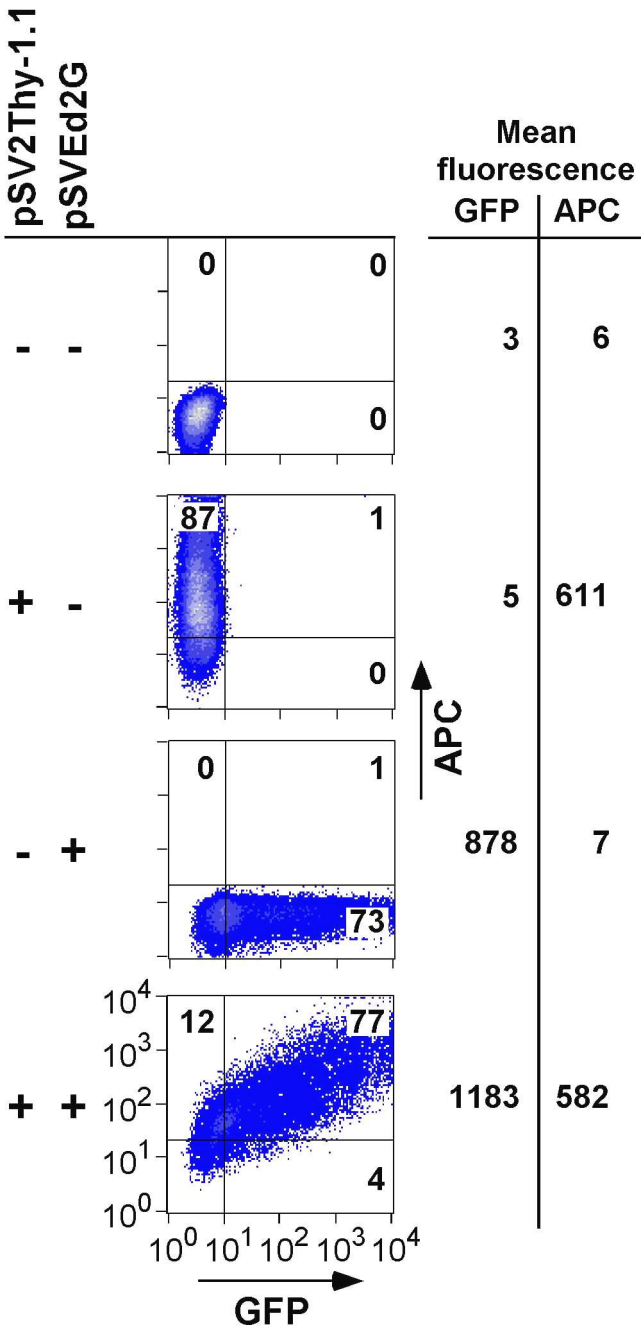


Figure 1: Two-color flow cytometry analysis of GFP reporter expression. Density plots of phoenix cells transfected with pUC19 alone, pSV2Thy-1.1 alone, pSVEd2G alone, or co-transfected with pSVEd2G and pSV2Thy-1.1. Reporter expression was analyzed 40h later. GFP fluorescence (X-axis) and Thy-1.1 surface expression, detected by an APC-labeled anti-Thy-1 antibody (Y-axis), were analysed by two-color flow cytometry. The numbers in the quadrants indicate the percentages of viable cells expressing pSV2-Thy-1.1 alone, pSVEd2G alone, or pSVEd2G and Thy-1.1. The mean GFP and APC fluorescence intensities of the entire cell populations are indicated on the right.

3.4.2 Comparison of GFP and luciferase reporter systems

To directly compare the luciferase and GFP reporter systems expression vectors containing the same backbone but coding either for GFP (pSVd2G) or luciferase (pGL3-Promoter) was transfected into the fibrosarcoma cell line HT1080. GFP and luciferase have similar half-lives of approximately 2 to 3 h (Li *et al.*, 1998; Thompson *et al.*, 1991). For the luciferase reporter assay, HT1080 cells were co-transfected with the firefly luciferase reporter pGL3-Promoter and pRL-SV40 as reference for transfection efficiency. pRL-SV40 contains the renilla gene under the control of the promoter and early enhancer of SV40, and is thus comparable to pSV2-Thy-1.1. 40 h after transfection cells were lysed and enzymatic activity measured using the dual luciferase assay (Promega). Background activity, measured in cells expressing only renilla luciferase, was subtracted from the firefly luciferase activity of each transfected sample. To normalize for transfection efficiency, this value was divided by the renilla luciferase value of the same sample. The values obtained from the flow cytometry analysis of cells co-transfected with pSVd2G and pSV2-Thy-1.1 were subjected to analogous operations, i.e. we subtracted from the arithmetic mean of GFP expression the background obtained with cells transfected only with pSV2-Thy-1.1. To normalize for transfection efficiency, this value was divided by the equivalent measure for Thy-1.1 expression in the same cells. Because 3.3 times less renilla plasmid was used, the GFP/APC ratio was multiplied by 3.3 in the plot shown in figure 2. Reporter gene expression increased linearly and with the same rate when between one and 4 µg of pSVd2G or pGL3 Promoter were transfected (Fig. 2). Comparing of several experiments, we found that beyond 4 µg of plasmid the increase in reporter expression was no more a function of plasmid concentration using either system. These results indicate that in HT1080 cells the GFP system monitors promoter activity with a similar sensitivity as the dual luciferase system.

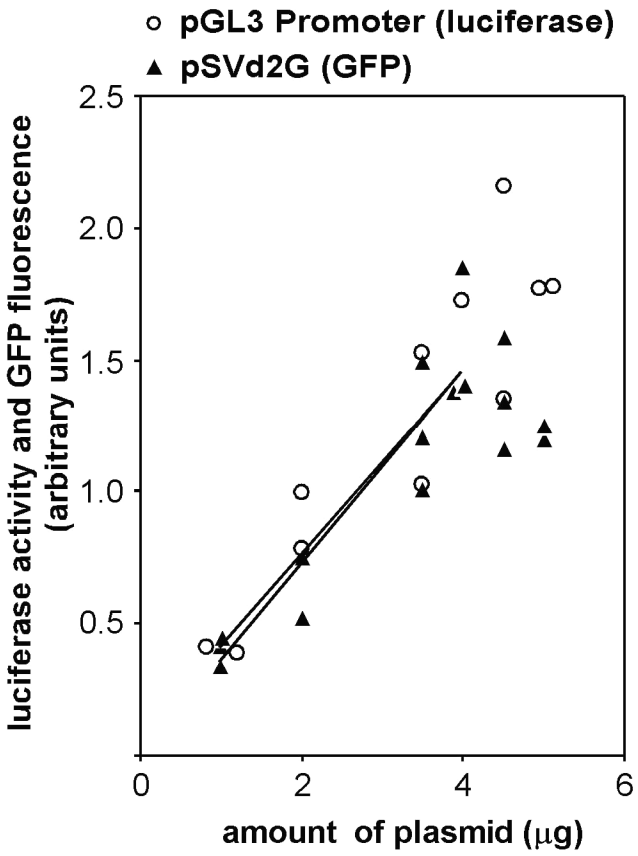


Figure 2: Comparison of GFP-Thy-1.1 and luciferase-renilla reporter systems. HT1080 were transfected with 1 to 5 μg of pSV2G (GFP) and 1 μg of pSV2Thy-1.1 or with 1 to 5 μg of pGL3 Promoter (Luciferase) and 0.3 μg of pRL-SV40. The expression of pSVd2G and of pGL3 Promoter reporters (F), normalized to that of their respective control plasmids (pSV2Thy-1.1 and pRL-SV40) is plotted against the amount of plasmid (A) used for transfection. The regression lines and correlation coefficients obtained when 1 to 4 μg of plasmid DNA were used are: F=0.037A + 0.0022 (R^2: 0.84); F=0.036A + 0.0049 (R^2: 0.74) for pSVd2G and for pGL3 Promoter, respectively.

3.4.3 Measuring reporter expression in cells transfected with low efficiency

Flow cytometry allows quantification of reporter gene expression in every cell of the transfected population. When cells are co-transfected with a reference plasmid, such as pSV2Thy-1.1, analysis of reporter gene expression can be restricted to cells that express the reference plasmid and are therefore likely to have been successfully transfected also with the reporter plasmid. This is particularly useful when transfection efficiency is low. Only 5 to 10% of HLF cells co-transfected with pSVEd2G and pSV2Thy-1.1, expressed the reference plasmid (Fig. 3), and the mean GFP fluorescence intensity of the entire population was close to background. When analysis of GFP expression was restricted to Thy-1.1 positive cells, mean GFP intensity was not only much higher but also directly proportional to the amount of

transfected pSVEd2G plasmid. As shown in Fig. 3, between one and 3 µg of pSVEd2G the increase in GFP fluorescence intensity was linearly related to the amount of transfected plasmid. Thus, the GFP reporter system allows the analysis of reporters even in cells with a very low transfection efficiency, provided enough cells are analyzed to accumulate statistically significant data.

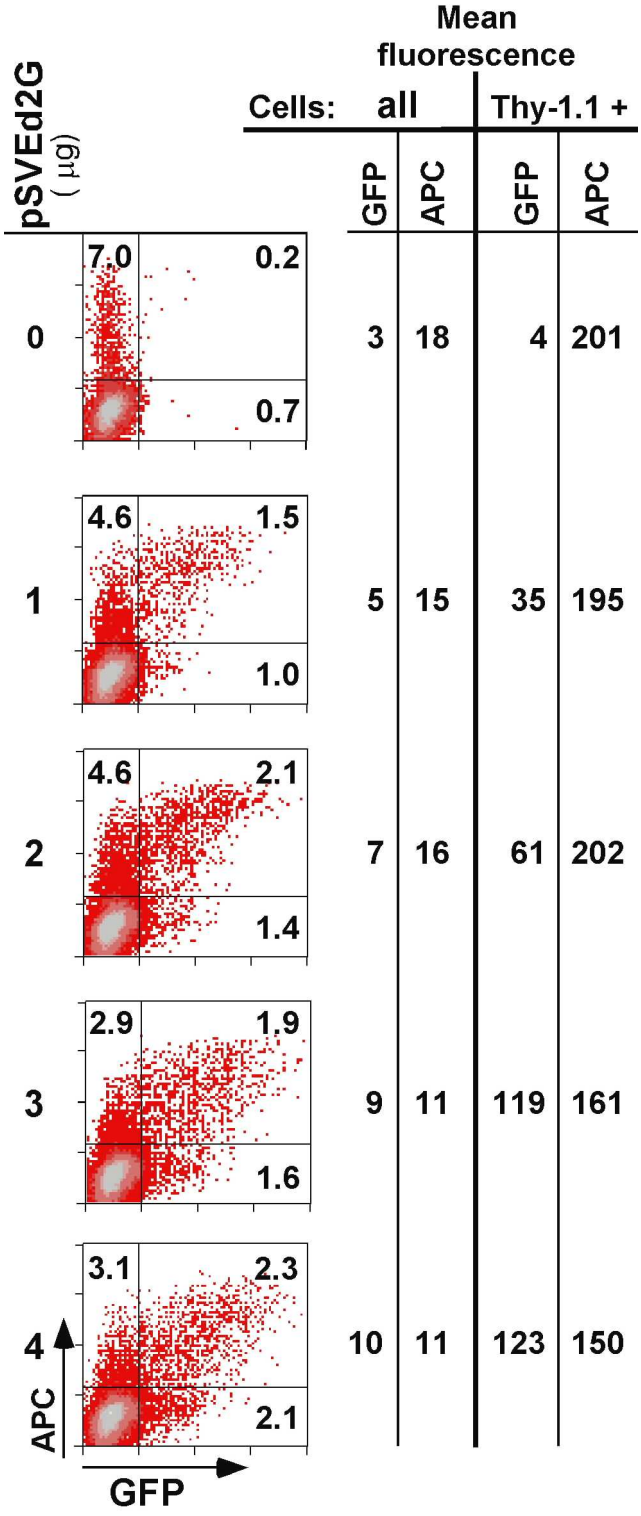


Figure 3: Analysis of cells (normal human lung fibroblasts) that cannot be transfected with high efficiency. From 50'000 to 100'000 cells of each sample were passed through the flow cytometer. The horizontal line separates the Thy-1.1 expressing cells from the negative cells. The numbers in the quadrants indicate the

percentages of viable cells expressing pSV2-Thy-1.1 alone, pSVEd2G alone, or pSVEd2G and Thy-1.1. The mean GFP and APC fluorescence intensities of the entire populations (all) and of the pSV2Thy-1.1 positive (Thy-1.1 +) cells are indicated on the right.

In summary, our result show that the GFP-based reporter system described here has a similar sensitivity than the luciferase system (Fig. 2). The GFP reporter system can be used for monitoring transcription like an enzyme-based system. But, because GFP expression can be quantified by flow cytometry in single cells, the system allows monitoring of transfection efficiency as well as of heterogeneity of the levels of reporter expression (Fig. 1). We show that by restricting analysis of reporter expression to cells that express a reference plasmid, reporter expression can be reliably quantified even in normal cells that cannot be transfected with high efficiency. Since flow cytometry can be combined with cell sorting, the system has other interesting applications, e.g. in situations in which one wants to measure the effect of a transiently transfected plasmid on a resident cellular gene or on a co-transfected reporter.

4 REGULATION OF HUMAN TELOMERASE ACTIVITY: REPRESSION BY NORMAL CHROMOSOME 3 ABOLISHES NUCLEAR hTERT TRANSCRIPTS BUT DOES NOT AFFECT c-Myc ACTIVITY[4]

4.1 ABSTRACT

Telomerase is required for the complete replication of chromosomal ends. In tumors, the telomerase reverse transcriptase subunit (hTERT) is up-regulated thereby removing a critical barrier for unlimited cell proliferation. To understand more about hTERT regulation, we measured hTERT RNA levels by quantitative reverse transcriptase (RT)-PCR. Telomerase-positive cell lines were found to contain between 0.2 and 6 molecules of spliced hTERT RNA per cell, whereas in telomerase-negative cells the number of molecules was below the sensitivity of the assay (<0.004 molecules/cell). Intron-containing, immature hTERT RNA was observed only in nuclei of telomerase-positive cells suggesting that hTERT RNA levels are transcriptionally regulated. Microcell transfer of a normal chromosome 3 into the human breast carcinoma cell line (21NT) abolishes telomerase activity and induces senescence. Endogenous hTERT transcripts were undetectable in the nuclei of 21NT-chromosome 3 hybrids, even in cells permanently expressing a transfected hTERT cDNA. However, chromosome 3 transfer did not affect the expression of GFP reporter constructs driven by up to 7.4 kb of non-coding DNA flanking the 5' end of the hTERT gene. Because direct up-regulation of hTERT through c-Myc overexpression had previously been reported, we investigated whether chromosome 3 transfer affected c-Myc activity. An at least 30 fold reduction of immature intron-containing hTERT RNA was observed following the introduction of a normal chromosome 3, but expression levels of c-Myc, Mad1 and other c-Myc target genes were unchanged. Our results suggest that telomerase is regulated primarily at the level of hTERT transcription by complex mechanisms involving regulatory elements distant from the 5' flanking region, and that the putative hTERT repressor on chromosome 3 does not regulate the expression of hTERT through c-Myc or one of its co-regulators.

[4] This chapter was published in Cancer Research, 2001, 61, 7594-7602. The authors are Anne-Lyse Ducrest, Mario Amacker, Yves Mathieu, Andrew Cuthbert, Deborah Trott, Robert Newbold, Markus Nabholz and Joachim Lingner. The main contribution of Mario Amacker is the construction of the hTERT GFP reporter constructs used in this study. Yves Mathieu started during his diploma work the setting of the real time RT-PCR.

4.2 INTRODUCTION

Telomeres are specialized DNA-protein complexes at the end of eukaryotic chromosomes that protect chromosome ends from fusion and degradation (Blackburn, 2000; Counter *et al.*, 1992; McClintock, 1941; van Steensel *et al.*, 1998). The complete replication of telomeric DNA requires a specialized reverse transcriptase, telomerase (Lingner and Cech, 1998; Nugent and Lundblad, 1998). Most normal somatic human cells lack this enzyme (Kim *et al.*, 1994) and their telomeres shrink with each replication cycle by approximately 30 to 100 bp (Counter *et al.*, 1992; Harley *et al.*, 1990; Huffman *et al.*, 2000). Since short telomeres induce cellular senescence in tissue culture (Bodnar *et al.*, 1998), it has been proposed that telomere shortening may limit the replicative potential of normal cells providing a powerful tumor-suppressive mechanism (Wright and Shay, 2001). Cells of the germline and certain stem cells, as well as 85% of tumor-derived immortal cells contain telomerase, and their telomere length is stabilized (Kim *et al.*, 1994). In a minority of tumor cells, however, an alternative non-telomerase dependent mechanism (ALT) is responsible for telomere stabilization (Bryan *et al.*, 1997).

Telomerase is a ribonucleoprotein (RNP) enzyme that consists of an RNA moiety and several protein subunits. Of these, the RNA moiety and the catalytic subunit are essential for telomerase activity in vitro. The RNA subunit contains a short segment that serves as the template for telomeric repeat synthesis (Chen *et al.*, 2000; Feng *et al.*, 1995; Greider and Blackburn, 1989; Yu *et al.*, 1990). The catalytic protein subunit (hTERT) is related structurally and functionally to reverse transcriptases (Harrington *et al.*, 1997; Lingner *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Nakayama *et al.*, 1998). Among the number of telomerase-positive and negative cells thus far examined, the presence of hTERT mRNA is related to the presence of telomerase activity (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). In contrast, the telomerase RNA subunit and other components implicated in telomere maintenance are present in both telomerase-positive and negative cells. Furthermore, ectopic expression of hTERT in telomerase-negative fibroblasts or endothelial cells is sufficient to restore telomerase activity and to stabilize telomere length (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998; Yang *et al.*, 1999), whereas over-expression of dominant negative mutants of hTERT in tumor cells can inhibit telomerase activity and induce growth arrest (Hahn *et al.*, 1999; Zhang *et al.*, 1999).

Andrew Cuthbert, Deborah Trott were working in the group of Robert Newbold, they kindly provide us with the

The mechanisms that control hTERT gene expression may involve transcriptional regulation, RNA stability, processing and/or export to the cytoplasm. To date a number of regulators of hTERT expression have been identified including the Wilms' tumor suppressor gene (WT1) product that reduces hTERT RNA levels in 293 kidney cells (Oh *et al.*, 1999). Retinoids were shown to down-regulate hTERT RNA in acute promyelocytic leukemia (Pendino *et al.*, 2001). Several activators of hTERT expression have also been identified. Estrogen induces hTERT RNA in estrogen receptor-positive cells (Kyo *et al.*, 1999; Misiti *et al.*, 2000). The E6 oncoprotein of human papillomavirus type 16 induces telomerase activity in epithelial, but not in fibroblasts (Kiyono *et al.*, 1998; Klingelhutz *et al.*, 1996). c-Myc directly acts on the hTERT gene inducing hTERT expression (Greenberg *et al.*, 1999; Wu *et al.*, 1999), whereas the c-Myc antagonist Mad down-regulates its expression (Gunes *et al.*, 2000; Oh *et al.*, 2000). hTERT regulation involves histone acetylation since treatment of telomerase negative cells with trichostatin A activates telomerase (Cong and Bacchetti, 2000).

In cell hybridization experiments the telomerase negative state behaves like a dominant trait, given that hybrids between telomerase-positive and negative cells are telomerase negative (Bryan *et al.*, 1995). Microcell transfer of human chromosomes 2, 7, 11 induced cellular senescence in some tumor-derived cells. However, telomere length and telomerase activity are retained in these cells, implying that several inducers of senescence function independently of telomerase (Tanaka *et al.*, 1999). By microcell transfer of human chromosomes into breast and kidney tumor cell lines a factor that directly or indirectly down-regulates telomerase activity has been mapped to a region on chromosome 3p (Cuthbert *et al.*, 1999; Horikawa *et al.*, 1998; Tanaka *et al.*, 1998). Recently, it was shown that transfer of human chromosome 6 into a HPV16-immortalized keratinocyte cell line (FK16A) and into a HPV16-containing cervical cancer cell line (SiHa) reduced hTERT RNA levels (Nishimoto *et al.*, 2001). Similar results were obtained after introduction of a fragment of human chromosome 10p into hepatocellular carcinoma cells (Li7HM) (Steenbergen *et al.*, 2001). It was also shown that transfer of human chromosomes 3 or 4 into HeLa cells abolished telomerase activity (Backsch *et al.*, 2001).

RNA processing has also been implicated in the regulation of hTERT. Several splice-variants of hTERT RNA which encode enzymatically inactive telomerases are expressed during embryonic development and are also detectable in some immortalized cells (Kilian *et al.*, 1997; Ulaner *et al.*, 1998; Wick *et al.*, 1999).

In order to study hTERT expression we developed a quantitative RT-PCR assay and measured spliced and unspliced hTERT RNA levels in primary cells and immortal cell lines.

21NT, 21NT-hTERT and 21NT-hTERT-chromosome 3 cells.

We found that low levels of hTERT RNA are expressed in tumor cells, and that the level of immature nuclear hTERT RNA correlates with telomerase activity, suggesting a regulation of hTERT RNA levels in the nucleus. In addition, we demonstrate that reporters containing up to 7.4 kb of 5'flanking region do not faithfully mimic expression of the endogenous hTERT gene. We show that transfer of a normal chromosome 3 into the human breast cancer cell line 21NT results in complete silencing of endogenous hTERT (indicated by an absence of immature nuclear hTERT RNA) even in cells that are rescued from senescence by ectopic expression of hTERT cDNA construct. Moreover we characterized the mechanism by which chromosome 3 represses hTERT RNA expression in the breast cancer cell line 21NT. We provide evidence that the repressor does not act on regulatory elements in the immediate 5' flanking region of the gene, and is independent of c-Myc or its co-regulators.

4.3 MATERIALS AND METHODS

4.3.1 Cells

Human embryonic lung fibroblasts (HLF, passage 6) were a gift from Urs Ziegler, Institute of Anatomy, University of Zuerich. The HT1080 fibrosarcoma-derived line was a gift from Ian Kerr, ICRF, London. SV40-transformed telomerase-positive human fibroblasts GM639 were obtained from the Coriell Institute for Medical Research, Camden, NJ. SV40-transformed telomerase-negative human fibroblasts GM847 were obtained from Roger Reddel, Children's Medical Research Institute, Sydney. HeLa cells were obtained from Beatrice Bentele, ISREC. SW480, a colon adenocarcinoma cell line, was obtained from Richard Iggo, ISREC. The above cells were maintained in high glucose DMEM with 10% fetal calf serum (FCS). The breast carcinoma cell line 21NT and its derivatives 21NT pCineohTERT (parental) and 21NT pCineohTERT HyTkchromosome 3 (21NT-chromosome 3 hybrids) were cultured as described (Cuthbert *et al.*, 1999). EREB 2-5 were obtained from Georg W. Bornkamm, GSF, Munich, and were cultured as described (Kempkes *et al.*, 1995). HaCaT human adult skin keratinocytes (Boukamp *et al.*, 1988) were obtained from Stephanie Lation, ISREC, and were maintained undifferentiated, in medium A (1:3 DMEM to HAM-F12) containing 0.6 mM $CaCl_2$, 5 % FCS, 8.3 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenine, 0.5 μg/ml hydrocortisone and 10 ng/ml EGF. After growth to confluence, the cells were induced to differentiate in medium A containing 1.2 mM $CaCl_2$, 20 % FCS, 8.3 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenine and 0.5 μg/ml hydrocortisone for 14 days. For measuring RNA stability, HT1080 cells were treated with 2 μg/ml of Actinomycin D for 0.5 to 8 h. HLF-hTERT cells were generated by infection of HLF cells with pMSCV-puromycin-hTERT (Migliaccio *et al.*, 2000). HLF-c-Myc cells were similarly generated using pBabe-puromycin-c-Myc obtained from Bruno Amati (Alevizopoulos *et al.*, 1997). Infections were performed as described previously (Migliaccio *et al.*, 2000).

4.3.2 Plasmids

pGRN121 contains hTERT cDNA (Nakamura *et al.*, 1997) and was obtained from Geron Corporation, Menlo Park, CA. pNSV4 contains a genomic hTERT insert (Accession number AF114847) encompassing 7.4 kb of the 5'flanking region upstream of the hTERT translation start site, the first two exons and part of the second intron (Wu *et al.*, 1999). pSV2-Thy-1 expresses the mouse Thy-1.1 allele under the control of the SV40 enhancer and early promoter (Wilson *et al.*, 1990). We constructed hTERT-GFP plasmids using the following procedures. To generate the GFP reporter vectors: pG (basic vector), pSVG (promoter vector)

and pSVEG (promoter/enhancer vector), we replaced the HindIII/XbaI fragment containing the luciferase gene of pGL3 (Promega) by the HindIII/XbaI fragment of pEGFP-N1 (Clontech) containing the EGFP gene. phTERT.1.3G contains a 1.3 kb fragment upstream of the translation start site of the hTERT gene. The 1.3 kb fragment was amplified from pNSV4 by PCR using oligonucleotides P1328f and P1r (see below) and subcloned into the NheI/BglII sites of the promoterless GFP vector. phTERT.5.1G, containing 5.1 kb of upstream sequence, was generated by cloning a 3.8 kb SacI/NheI fragment of pNSV4 into the SacI/NheI sites of phTERT.1.3G. phTERT.7.4G was generated by cloning a 2.3 kb SacI fragment of pNSV4 into the SacI site of phTERT.5.1G. phTERT.4.8G was generated by religation of phTERT.7.4G after digestion with SpeI, deleting a 2.6 kb fragment from the 5’ end of the hTERT promoter. phTERT.3.3G was generated by subcloning a 3.3 kb XhoI/HindIII fragment of phTERT.5.1G into pG. phTERT.4.4G was obtained by cloning of a 1.1 kb SacI/XhoI fragment generated by PCR with primers P3061f and P4183r into the SacI/XhoI sites of phTERT.3.3G. We generated phTERT.0.9G, phTERT.0.6G and phTERT.0.3G like phTERT.1.3G except that oligonucleotides P951f, P602f and P314f, respectively, were used as forward primers. To generate phTERT.1.3Δ0.1G and phTERT.1.3Δ0.3G 108 bp and 160 bp, respectively, of the 3' end of the 1.3 kb insert of phTERT.1.3G were removed by PCR using oligonucleotides P1328f, and P108r or P260r, respectively.

4.3.3 DNA oligonucleotides

The following DNA oligonucleotides were purchased from Microsynth (Balgach, Switzerland) and used for hTERT reporter constructs.

P1r: 5'-GGAACTAGTAGATCTCGCGGGGGTGGCCGGGG-3’;
P108r: 5'-GGAACTAGTAGATCTGGGAGGCCCGGAGGGG-3';
P260r: 5'-GGAACTAGTAGATCTGTGCCCGCGAATCCACTG-3';
P314f: 5'-GGAGGATCCGCTAGCAGCTGCGCTGTCGGGG-3';
P602f: 5'-GGAGGATCCGCTAGCGCCTTCGTCCTCCCCTTC-3';
P951f: 5'-GGAGGATCCGCTAGCGGGCGGGATGTGACCAG-3';
P1328f: 5'-GGAGGATCCAGGGAGGGTGCGAGGCC-3');
P3061f: 5’-CATTTCCAGGAGCTCCCCGTCTC-3’;
P4181r: 5’-TTGCAGGCCTGGGCTCGAGGC-3’

4.3.4 Transfections

Transient transfections with calcium-phosphate precipitates were performed according to the protocol described by Jordan (Jordan *et al.*, 1996). Cells were co-transfected with 1 µg

pSV2-Thy-1.1 as reference plasmid and equimolar amounts of GFP-reporters. The total amount of plasmid was kept constant (6 µg) by adding pUC19.

4.3.5 Determination of reporter expression

Transfected cells were harvested 48 h after transfection by trypsinization and incubated for 30 min with a saturating concentration of either monoclonal phycoerythryn (PE)-labeled anti-mouse CD90.1 (Thy-1.1) antibody OX-7 (Pharmingen, San Diego, CA) or allophycocyanin (APC) labeled anti-Thy-1 antibody III-5 (MacDonald *et al.*, 1985) kindly prepared by Céline Maréchal, and washed once. We analyzed the cells on a FACS-scan or FACScalibur microflow cytometer (Becton Dickinson, Franklin Lakes, NJ). hTERT reporter gene expression was quantified by calculating the equivalent to the value used for enzyme reporter systems. For this we considered the arithmetic mean of GFP expression and Thy-1 fluorescence. We subtracted from the arithmetic mean of GFP expression the GFP background, obtained with cells transfected only with pSV2-Thy-1. To correct for transfection efficiency, this value was divided by the equivalent measure for Thy-1 expression in the same cells. The GFP expression of the reporter constructs was normalized to that of a plasmid containing the SV40 minimal promoter driving GFP expression (pSVG). The GFP-reporter assay will be described in detail elsewhere (manuscript in preparation).

4.3.6 Quantitative RT-PCR analysis

Total RNA was extracted from different cell lines using the RNAeasy mini-kit (Qiagen). The quality of the RNA was determined on agarose gel electrophoresis. RNA was quantified with spectrophotometry at 260 and 280 nm ($1OD_{260}$ ~40 µg/ml). To perform RT-PCR with primer pairs that are not located in different exons or to quantify intron 2-containing hTERT RNA, a DNase I treatment was performed prior to reverse transcription. Four µg total RNA was incubated in 20 µl with 10 U DNase I (Roche Diagnostics Ltd) in 10 mM Tris-HCl pH 8.0, 0.5 mM $MgCl_2$, 1 mM dithiotreitol, 0.2 U/µl RNasin (Roche Diagnostics Ltd) for 1 h at 37 °C, followed by 10 min at 65° C to inactivate the enzyme. We reverse-transcribed 100 ng of RNA in 20 µl using 100 ng of random hexamer primers and with 20 U of MMLV-RT (Gibco-BRL) according to the manufacturer's protocol. To quantify intron 2 containing hTERT RNA, 1 µg of DNase I treated RNA was reverse transcribed as above using 10 pmol of primers 13156rv (E2-I2) and 10 pmol of primers 3407rv (GAPDH), respectively (Table 1). Quantitative PCR was performed using an ABI Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). For each PCR run, a master mix was prepared with 1x TaqMan master mix or 1x SYBERGreen master mix (5.5

mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 0.01 U/µl AmpErase UNG and 0.025 U/µl AmpliTaq Gold DNA Polymerase ± SYBR Green I dye) (Perkin-Elmer Applied Biosystem), 0.3 µM of each primer and 0.1 µM TaqMan probe. 2.5 µl of the reverse transcriptase reaction was added to 22.5 µl of master mix. The thermal cycling conditions included an initial denaturation step at 95°C for 10 min followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. Primers and probes for all RT-PCR were chosen with the assistance of the computer program Primer express (Perkin-Elmer Applied Biosystems). BLASTN searches were used to check the gene specificity of the nucleotide sequences chosen for the primers and probes. PCR products were fractionated on agarose gel to confirm that their size corresponded to the expected length. Primers were purchased from Microsynth (Balgach, Switzerland), and TaqMan probes were from Eurogentec (Les Ulis Cedex, Belgium). To test the efficiency of the PCR primers, we carried out reactions with different concentrations of the appropriate template hTERT DNA (pGRN121, pNSV4) or GAPDH cDNA and plotted the cycle number at which the PCR signal raises above background (Ct) against the logarithm of the number of template molecules (L). The regression lines and correlation coefficients obtained were Ct=-3.29L+42.20, R^2=0.997; Ct=-3.21L+39.59, R^2=0.998; Ct=-3.62L+42.23, R^2=0.997; Ct=-3.33L+35.40, R^2=0.991 for hTERT E4-5, E9-10, E2-I2 and GAPDH primers, respectively. To test the efficiency of hTERT cDNA synthesis we used an in vitro transcript as template. Different quantities of this synthetic template were mixed with total RNA of telomerase negative HLF cells and reverse-transcribed. Comparison of Ct values obtained upon RT-PCR of the synthetic hTERT RNA with the Ct values obtained with known numbers of plasmid molecules showed that the efficiency of cDNA synthesis was 25%. The amount of total RNAs obtained from different cells was measured by alkaline hydrolysis as described (Brandhorst and McConkey, 1974). Per million cells the following µg amounts of total RNA was present: HLF and HLF-c-Myc: 23 to 25 µg; GM847: 20 µg; HT1080: 35 µg; 21NT and 21NT-chromosome 3 hybrids: 21 to 23 µg; HeLa: 35 µg; SW480: 20 µg; EREB: 30 µg.

4.3.7 Preparation of nuclear and cytoplasmic extract

Nuclear and cytoplasmic extracts were either prepared by hypotonic swelling according to Schreiber (Schreiber *et al.*, 1989) or by dounce homogenization as described by Mirkovitch (Mirkovitch *et al.*, 1992). The cytoplasmic fractions were then mixed (1:1) with the lysis buffer from the RNAeasy minikit (Qiagen) and the nuclear pellets resuspended in the same lysis buffer.

4.3.8 Cell cycle analysis

Live HT1080 cells were stained with 10 μg/ml DAPI (Fluka) for 30 min at 4°C in PBS containing 0.05 % Triton X-100, and washed with PBS. Cells were sorted according to DNA content on a FACS-sorter microflow cytometer (BECTON DICKINSON, Franklin Lakes, NJ) and collected in lysis buffer from the RNAeasy mini-kit (Qiagen).

4.3.9 Immunoblots

Total protein from four independent cultures of subconfluent 21NT parental and 21NT-chromosome 3 hybrids were extracted with 8M urea, 0.5% Triton X-100 and 0.5% NP40. Fifty μg of protein was resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. c-Myc protein was detected with a mouse monoclonal IgG against human c-Myc (9E10, 1/1000, Santa Cruz), Mad 1 with a rabbit polyclonal antibody (C-19, 1/200; Santa Cruz) and actin with a goat polyclonal antibody (I-19, 1/200, Santa Cruz). Western blots were developed using the enhanced chemiluminescence system (ECL, Amersham) for actin and the SuperSignal West Pico kit (Pierce) for c-Myc and Mad1.

4.4 RESULTS

4.4.1 hTERT RNA quantification and correlation with telomerase activity

To measure the amount of hTERT RNA in tumor-derived cell lines and primary cells, we developed a quantitative RT-PCR assay using three different hTERT primer pairs (Table 1). Two primer pairs, E4-5 and E9-10, spanned the boundary between exons 4 and 5, and between 9 and 10, respectively, and only amplified cDNA from spliced RNA lacking intron 4 and/or 9, whereas the third primer pair, E2-I2 amplified cDNA from immature hTERT RNA containing the end of exon 2 and 256 nt of intron 2. For comparison, we determined the amount of intron free GAPDH RNA. The number of cDNA molecules present in a sample was calculated by plotting the corresponding Ct value onto the regression line of the Ct values obtained when graded amounts of precisely quantified hTERT plasmid or GAPDH PCR product were amplified. As the efficiency of cDNA synthesis from an in vitro transcript of hTERT in the presence of total RNA of telomerase negative cells was 25 % (data not shown), the number of intron free hTERT RNA molecules was assumed to correspond to the quadruple of the estimated number of cDNA molecules. We obtained the same estimates of hTERT RNA molecule numbers using either the E4-5 or the E9-10 primer pairs (data not shown).

Telomerase-positive tumor-derived cell lines contained between 0.2 and 6 spliced hTERT RNA molecules/cell (Fig. 1A). No signal above the detection limit of 0.004 molecules/cell was obtained in telomerase-negative primary human lung fibroblasts or in the telomerase-negative cell line GM847 (Fig. 1A). Therefore, if telomerase-negative cells express any spliced hTERT RNA at all, its level is at least 50-1500 times lower than that of telomerase-positive cells. Primary fibroblasts that were transduced with an hTERT-retroviral construct expressed at least 100 times more spliced hTERT RNA than tumor-derived cells but their telomerase activity was not higher than tumor-derived cells (data not shown). Fibroblasts expressing c-Myc contained spliced hTERT RNA at levels comparable to those in some telomerase-positive tumor cells (0.2 molecule/cell) (Fig. 1A). Quantification of GAPDH RNA demonstrated that each cell contained between 700 and 15’000 molecules (Fig. 1A). Thus, by comparison to GAPDH, hTERT is a very rare RNA species, detectable exclusively in telomerase-positive cells.

TABLE 1 *Synthetic oligonucleotides used as primers for RT-PCR.*

Name	Primers and TQ[a] probes	Localization exon/intron (E/I)	Sequences 5'-3'	Primer length (nt)	Amplicon length (bp)
E2-I2	12896fw	E2	GAGCTGACGTGGAAGATGAGC	21	260
	13156rv	I2	GGTGAACCTCGTAAGTTTATGCAA	24	
	13095TQ	I2	CACGGTGATCTCTGCCTCTGCTCTCC	26	
E9-10	2600fw	E9	ATGGAGAACAAGCTGTTTGCG	21	80
	2680rv	E10	AGGTGTCACCAACAAGAAATCATC	24	
	2635TQ	E9/E10	CGGGCTGCTCCTGCGTTTGG	20	
E4-5	1949fw	E4	TGCGGCCGATTGTGAAC	17	98
	2046rv	E5	GAACAGTGCCTTCACCCTCG	20	
hTER	F3b	45	TCTAACCCTAACTGAGAAGGGCGTAG	26	125
	R3c	170	GTTTGCTCTAGAATGAACGGTGGAAG	26	
GAPDH	1457fw	E1	GAAGGTGAAGGTCGGAGT	18	226
	3407rv	E3	GAAGATGGTGATGGGATTTC	20	
β2-M[c]	531fw	531[b]	TCTACTTTGAGTGCTGTCTCCATGT	25	76
	606rv	606[b]	TTGCCAGCCCTCCTAGAGC	19	
c-MYC	5189fw	E2	GCTCTCCTCGACGGAGTCC	19	134
	6689rv	E3	CCACAGAAACAACATCGATTTCTT	24	
MAD	356fw	356[b]	TCGACCAGCTTCAGCGAGA	19	91
	446rv	446[b]	GTGGAGCCGATGCTGTCC	18	
CAD	329fw	329[b]	CAGGTTTGCCAGCTGAGGA	19	116
	444rv	425[b]	TGCCTGTCTCGGTACTGGTG	20	
ODC	ODCfw	591[b]	TGTAGGAAGCGGCTGTAC	18	228
	ODCrv	798[b]	GCTATGATTCTCACTCCAGAG	21	
GADD45	149fw	149[b]	ACCCCGATAACGTGGTGTTG	20	91
	239rv	239[b]	GCCTGGATCAGGGTGAAGTG	20	
EIF4E	388fw	388[b]	TGGCTAGAGACACTTCTGTGC	22	91
	468rv	468[b]	AACATTAACAACAGCGCCACAT	22	
LDHA	196	196[b]	CAACATGGCAGCCTTTTCCT	20	91
	286	286[b]	CCGTGATAATGACCAGCTTGG	21	
U3	U3f	178[b]	ACCACGAGGAAGAGAAGTAGCG	22	64
	U3r	225[b]	GCCAAGCAACGCCAGAA	17	

a) TQ = TaqMan probe
b) Position in the mature RNA
c) β2-microglobuline

4.4.2 Regulation of hTERT RNA levels in the nucleus

While it is generally assumed that hTERT expression is regulated primarily at the level of transcription there is little direct evidence for this. In support of this notion overexpression of c-Myc can directly induce hTERT expression (Greenberg *et al.*, 1999; Wu *et al.*, 1999).

Furthermore, hTERT run-on transcription signals (Gunes *et al.*, 2000) changed during differentiation of human hematopoietic U937 cells. We have been unable to detect run-on transcription signals from the hTERT gene with cells used in this study (A-L. D. and J. Mirkovitch, unpublished data) which contain only 0.2 - 6 spliced hTERT RNA molecules/cell. To substantiate the assumption that hTERT expression is regulated in the nucleus, we compared the amounts of various spliced and unspliced hTERT RNAs in the nucleus and the cytoplasm. Nuclear and cytoplasmic fractions of HT1080 cells were prepared according to two different protocols (Mirkovitch *et al.*, 1992; Schreiber *et al.*, 1989). Both methods produced very similar results for hTERT RNAs as well as for control RNAs (GAPDH and U3 snRNA; Fig. 1B). The two controls were included to monitor contamination between nuclear and cytoplasmic fractions. Such contamination was low as only 4 % of GAPDH RNA was found in the nuclear fraction, while 93 to 98 % of the U3 snRNA appeared in the nuclear fraction (Fig. 1B). However, 20 - 30 % of the telomerase RNA compound hTER, which was previously thought to be mostly nuclear, was also detected in the cytoplasm. On the other hand, we found a considerable fraction (35 - 75 %) of hTERT RNAs in the nucleus. This included transcripts still retaining intron 2 as well as molecules lacking intron 4 and/or intron 9. However, hTERT negative cells lacked both intron-containing and intron-less hTERT RNA (Fig. 1A).

We also analyzed the stability of spliced and unspliced hTERT RNA in HT1080 cells treated with Actinomycin D. Half-lives of intron 9-less and intron 2-retaining hTERT RNAs were 2 h and 2.5 h, respectively (data not shown). A slightly shorter half-life of 50 min for hTERT mRNA was found in human hematopoietic U937 cells (Gunes *et al.*, 2000). Since no form of hTERT RNA was detected in telomerase-negative cells, we conclude that hTERT regulation occurs in the nucleus.

hTERT RNA levels do not change during the cell cycle but decrease upon cell cycle exit and terminal differentiation. To determine whether RNA levels of hTERT fluctuate during the cell cycle, exponentially growing HT1080 cells were stained with DAPI and sorted by fluorescence flow cytometry (FACS) according to their DNA content (Fig. 2A). The fluorescence-activated cell sorter sorting gates were set sufficiently narrow to minimize cross-contamination of cells from different phases of the cell cycle. Total RNA was extracted and hTERT RNA levels were measured by quantitative RT-PCR. We found no significant differences between spliced hTERT RNA levels in the different phases of the cell cycle (Fig. 2B).

A

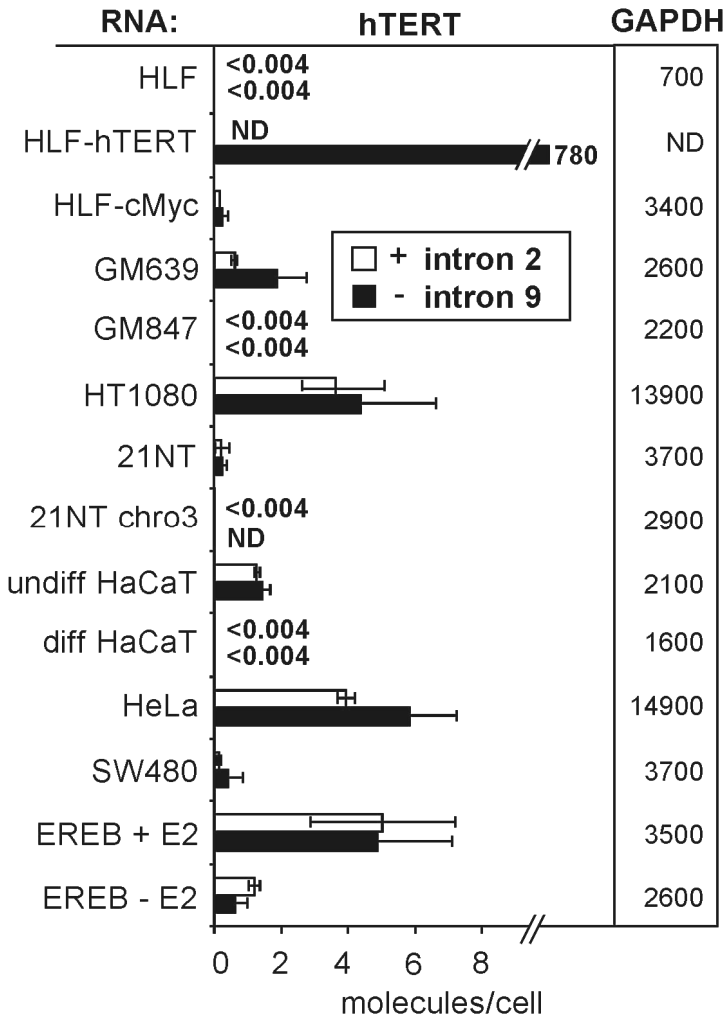


Figure 1: hTERT RNA quantification and subcellular localization. (**A**) Quantification of intron 9-less (black bars) and intron 2-retaining (white bars) hTERT RNA and GAPDH RNA (right panel). RNA was extracted from cells, reverse transcribed and analyzed by quantitative PCR with hTERT primer pairs E9-10 and E2-I2 and primers for intron-less GAPDH mRNA. Results represent the average (± range) of 1 to 6 different RNA extractions and RT-PCR experiments. HLF, primary human lung fibroblasts; HLF-hTERT, HLF transduced with MSCV-hTERT retrovirus; HLF-c-Myc, HLF transduced with pBabe-c-Myc; 21NT chro3, 21NT chromosome 3-hybrids. undiff HaCaT, undifferentiated HaCaT; diff HaCaT, differentiated HaCaT; EREB + E2, proliferating EREB; EREB – E2, starved EREB; ND, not determined.

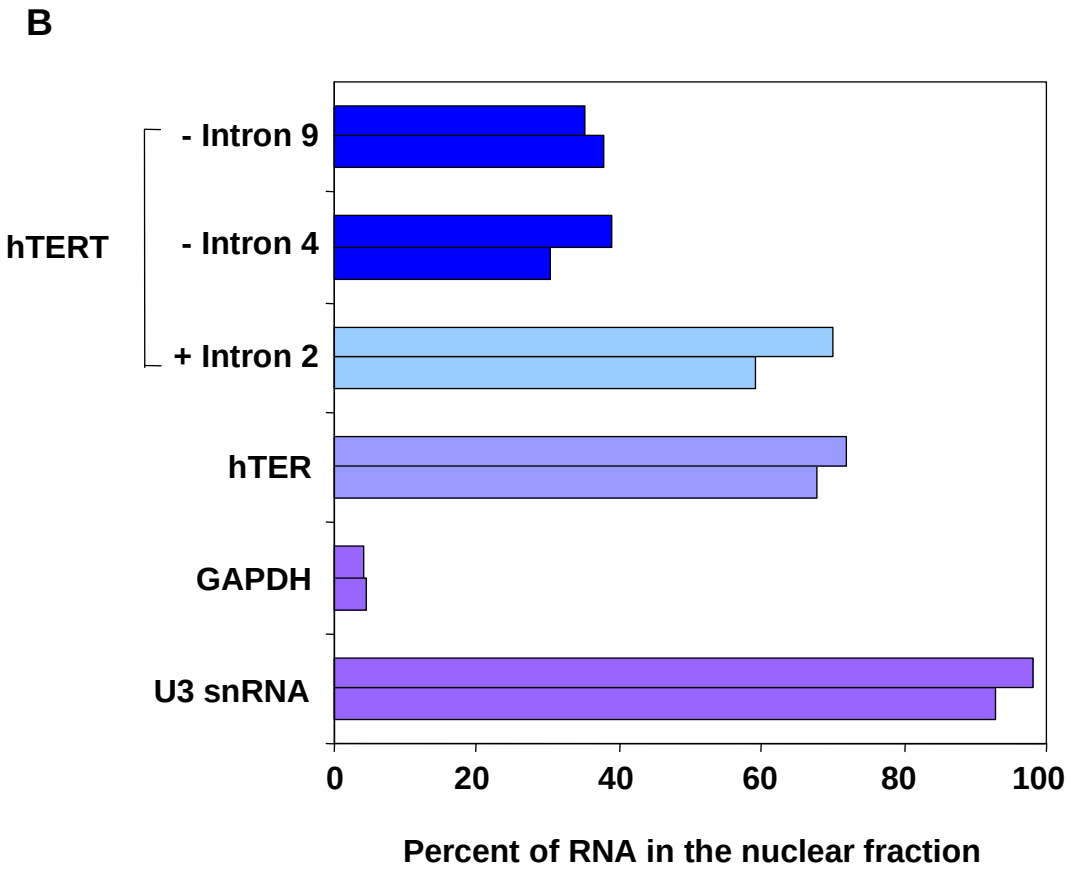


Figure 1: hTERT RNA quantification and subcellular localization. (**B**) Subcellular distribution of intron 4-less, intron 9-less and intron 2-containing hTERT RNA. Nuclear and cytoplasmic fractions of HT1080 were prepared according to two different methods, and treated with DNase I prior to the RT reaction. Bars represent the average of percentage of RNA in the nuclear fraction, obtained from 2 to 4 independent experiments. U3 served as a control for the contamination of cytoplasmic fraction with nuclear RNA. GAPDH served as a control for the contamination of nuclear with cytoplasmic RNA.

Using EREB cells, we investigated whether the proliferative state of the cells affected hTERT RNA levels. EREB cells are EBNA2-immortalized B-lymphocytes in which the EBNA2 gene is expressed as a chimeric fusion with the hormone-binding domain of the estrogen receptor. Thus, proliferation of the cells depends on the presence of estrogen (Kempkes *et al.*, 1995). As expected, the number of cells in G1 increased when EREB cells were cultured for 24 h in the absence of estrogen (from 60 to 90%; not shown). Estrogen deprival lead also to a five-fold reduction of spliced and intron-containing hTERT RNAs (Fig. 1A). A very strong decrease in hTERT RNA levels was seen upon terminal differentiation of immortalized human skin keratinocytes (HaCaT). HaCaT cells remain undifferentiated when cultured in low calcium. The addition of calcium to confluent cells is sufficient to induce cell differentiation. After six days into high calcium medium, spliced and unspliced hTERT RNA levels had dropped at least 300-fold compared to undifferentiated HaCaT cells (Fig. 1A). These results demonstrate that while hTERT RNA levels do not vary during different stages of the cell cycle in tumor-derived cells, the levels of hTERT RNA are significantly reduced

by cell cycle arrest and/or terminal differentiation. Diminution of spliced hTERT RNA during cell differentiation has been previously reported for U937 and HL-60 hematopoietic cells (Gunes *et al.*, 2000; Meyerson *et al.*, 1997).

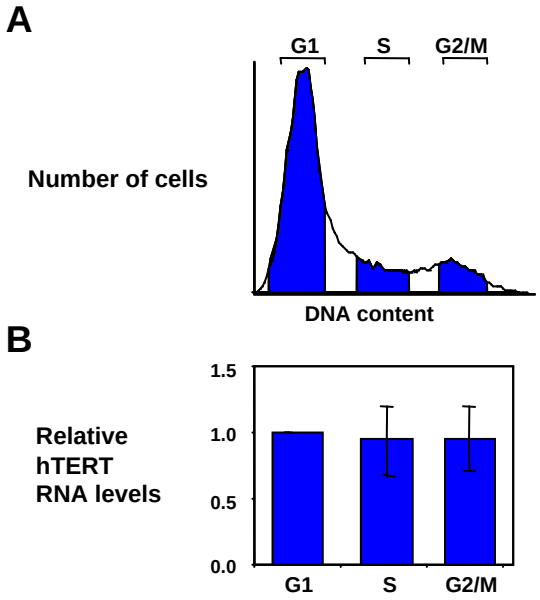


Figure 2: hTERT RNA levels during the cell cycle. (**A**) Subconfluent HT1080 cells were stained with DAPI and sorted by flow cytometry (FACS) according to their DNA content. The sorted cells were directly lysed and total RNA was prepared. The grey boxes represent the gates used to sort G1, S and G2/M phase cells, respectively. (**B**) Intron 9-less hTERT RNA level was quantified for each cell cycle phase. It was normalized to intron-less GAPDH and expressed relative to the amount in G1 phase cell. Results represent the average (± range) of 2 independent experiments.

4.4.3 Characterization of the 5' region of the hTERT gene

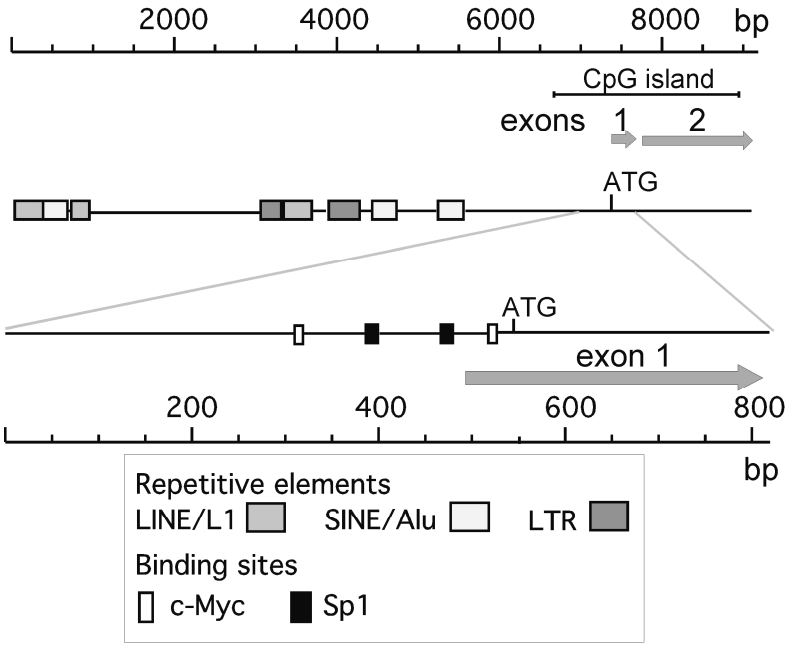
The results above are consistent with the hypothesis that hTERT RNA levels are regulated by transcription. To examine the regulatory role of the hTERT 5' flanking region, DNA fragments upstream of the start codon were fused to the GFP gene (Fig. 3A). Expression of these reporter constructs was quantified by two-color fluorescence flow cytometry (FACS) (see Materials and methods). As shown in figure 3B, reporters containing up to 7.4 kb of hTERT 5' flanking region expressed GFP in HT1080 cells. In telomerase-negative HLF cells, the reporters containing either the 1.3 kb or the 3.3 kb of 5'flanking region expressed low GFP fluorescence, while the other constructs did not express GFP at levels that were significantly above that of the background observed with a promoter-less construct (pG) (data not shown). Removal of 260 nt immediately upstream of the start codon (phTERT.1.3Δ0.3G) completely abolished the weak GFP expression in HLF cells, and strongly reduced it in HT1080 cells. Similar results were obtained using telomerase-positive HeLa cells (data not

shown). Thus, as expected, the hTERT-GFP reporters activated GFP expression in tumor-derived cell lines but not in telomerase-negative primary human lung fibroblasts.

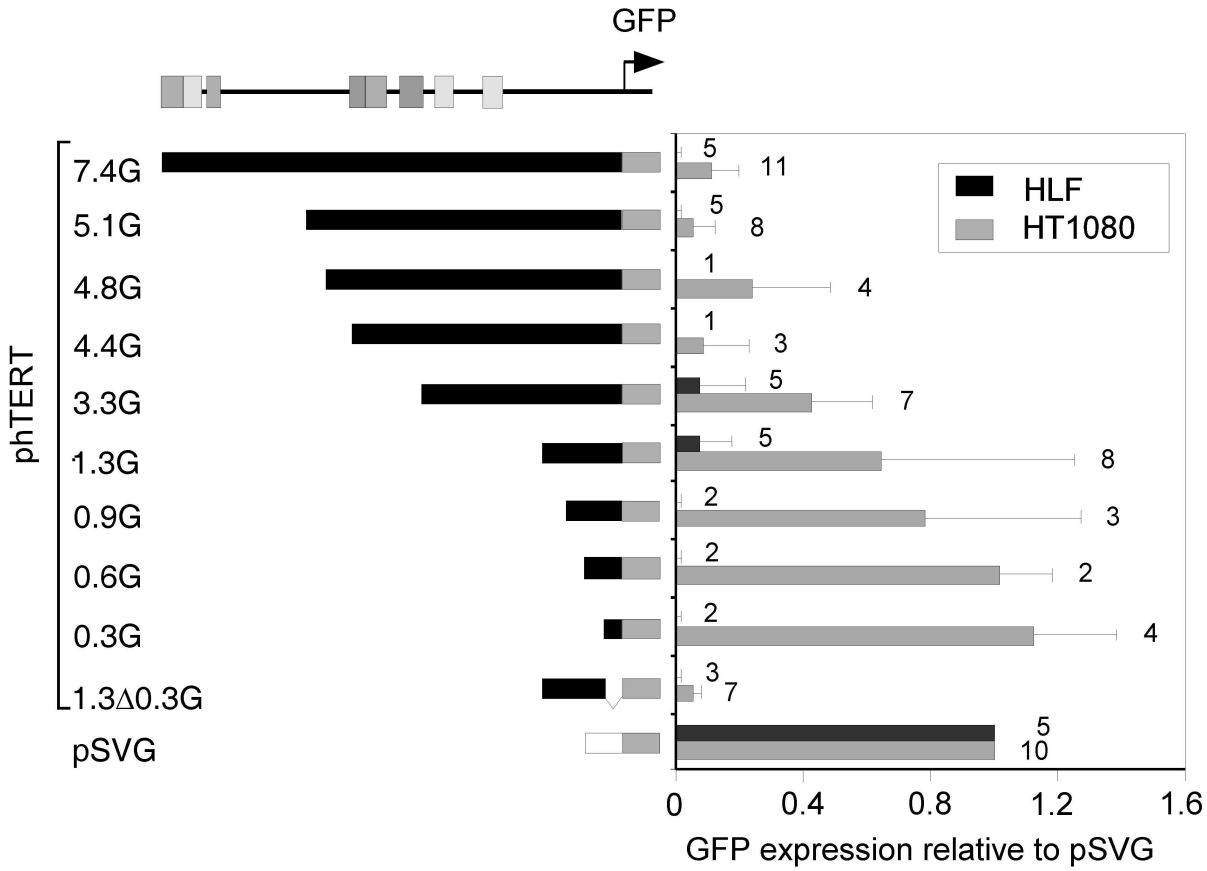
The reporters also induced GFP expression to similar levels in two SV40-transformed fibroblast lines (Fig. 3C). Of these, GM639 is telomerase-positive and contains detectable hTERT RNA (Fig. 1A). GM847, on the other hand, is telomerase-negative and contains neither intron 2-containing or intron 9-less hTERT RNA (Fig. 1A). Thus, the hTERT-GFP reporter activity in these fibroblast lines did not mimic hTERT RNA expression.

We investigated whether the hTERT-GFP reporter constructs could be used to identify regulatory elements in a breast carcinoma cell line, 21NT. Introduction of a normal human chromosome 3 in these cells, by microcell fusion, represses telomerase activity (Cuthbert *et al.*, 1999). This indicates that cis-acting targets of repression in the 21NT hTERT genes are intact. Repression of telomerase activity by chromosome 3 transfer is mediated by down-regulation of the hTERT RNA (Fig. 1A). We tested whether the hTERT-GFP reporter constructs would also be repressed by chromosome 3. To avoid cell senescence as a result of hTERT extinction (Cuthbert *et al.*, 1999), hTERT was ectopically expressed in the parental and in the hybrids cells. Fig. 3D shows representative results for 4 independent hybrids out of the 10 tested. As expected, we detected no intron 2-containing hTERT RNA in the 21NT-chromosome 3 hybrids. In contrast, GFP expression in the hybrids was the same as in the parental cells. We obtained similar results after stable transfection of the reporter constructs (data not shown). Therefore, it appears that the regulatory elements required for repression of hTERT in GM847 cells and 21NT-chromosome 3 hybrids are not contained within the 7.4 kb region upstream of the hTERT start codon or that they do not function properly when removed from their endogenous location.

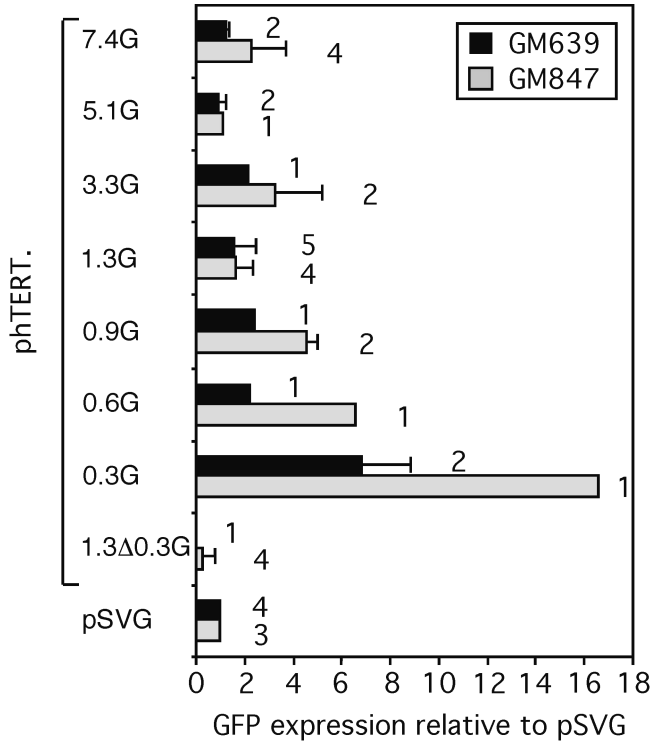
A


2000
4000
6000
8000
bp
CpG island
exons 1 2
ATG
ATG
exon 1
200
400
600
800
bp
Repetitive elements
LINE/L1
SINE/Alu
LTR
Binding sites
c-Myc
Sp1

B


GFP
phTERT
7.4G
5.1G
4.8G
4.4G
3.3G
1.3G
0.9G
0.6G
0.3G
1.3Δ0.3G
pSVG
HLF
HT1080
5
11
5
8
1
4
1
3
5
7
5
8
2
3
2
2
2
4
3
7
5
10
0
0.4
0.8
1.2
1.6
GFP expression relative to pSVG

C


GM639
GM847
phTERT.
7.4G
5.1G
3.3G
1.3G
0.9G
0.6G
0.3G
1.3Δ0.3G
pSVG
0 2 4 6 8 10 12 14 16 18
GFP expression relative to pSVG

D

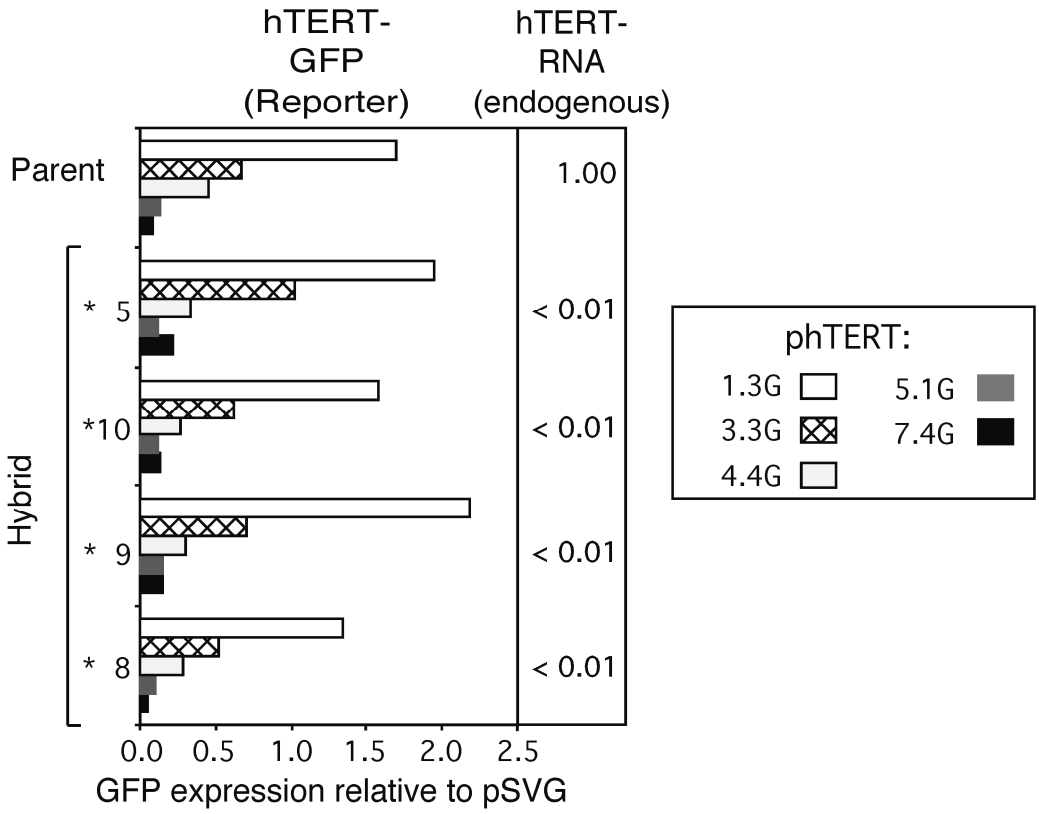
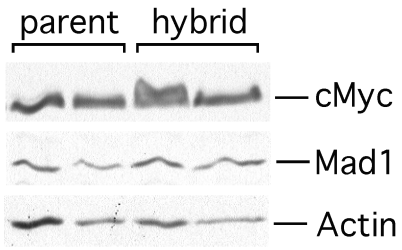

hTERT-
GFP
(Reporter)
hTERT-
RNA
(endogenous)
Parent
1.00
Hybrid
* 5
< 0.01
*10
< 0.01
* 9
< 0.01
* 8
< 0.01
0.0 0.5 1.0 1.5 2.0 2.5
GFP expression relative to pSVG
phTERT:
1.3G
3.3G
4.4G
5.1G
7.4G

Figure 3: hTERT-GFP reporter assay. (**A**) Features of the 7.4 kb 5' flanking region, the first two exons, and part of the second intron of the hTERT gene. Putative transcription factor binding sites in the promoter region of the hTERT gene are indicated. (**B**) Schematic representation of the hTERT-GFP reporter plasmids that were transfected into normal human fibroblasts (HLF) (black bars) and HT1080 fibrosarcoma cells (grey bars). Cells were co-transfected with the indicated plasmids plus pSV2Thy-1.1, harvested 48 h later, stained with an excess of allophycocyanin-coupled anti-Thy-1 antibody and analyzed by microflow cytometry. To correct for transfection efficiency, mean GFP fluorescence was corrected by the mean fluorescence for Thy-1. Results are expressed relative to the GFP expression of the control plasmid containing the SV40 promoter upstream of GFP (pSVG) and are average values (± standard deviations) of the number of independent transfection experiments shown in the figure. (**C**) hTERT-GFP reporter expressions in two SV40T-immortalized fibroblast cell lines: telomerase-positive GM639 (black bars) and telomerase-negative GM847 (grey bars). The experiment was performed as in *B*. (**D**) Expression of different hTERT-GFP reporters in parental breast cancer cells (21NT) and in four telomerase-negative 21NT chromosome 3-hybrids containing an extra normal human chromosome 3. The experiment was performed as in *B*. Numbers at the right indicate relative levels of endogenous hTERT transcripts. RT-PCR was performed with primer pairs E2-I2.

4.4.4 Chromosome 3-mediated hTERT down-regulation does not involve the c-Myc regulatory network

Previous studies showed that overexpression of c-Myc can induce hTERT expression in telomerase-negative cells (Greenberg *et al.*, 1999; Wang *et al.*, 1998; Wu *et al.*, 1999). Therefore, we tested whether hTERT RNA down-regulation in 21NT-chromosome 3 hybrids was associated with changes in the c-Myc pathway. Similar levels of c-Myc and Mad1 proteins were detected in parental and hybrid cells (Fig. 4A). Furthermore, using quantitative RT-PCR, we found that parental and hybrid cells expressed the same c-Myc and Mad RNA levels indicating that c-Myc is not a target of the putative repressor on chromosome 3 (Fig. 4B). To determine whether the chromosome 3-repressor would act on other genes or gene-products of the c-Myc regulatory network we measured the expression levels of five known c-Myc target genes: CAD, ODC, GADD45, eIF4E and LDHA (Dang, 1999). The RNA levels of CAD, ODC, GADD45, eIF4E and LDHA in the 21NT parental cells and chromosome 3 containing hybrids were very similar whereas hTERT RNA levels dropped at least 30 fold (Fig. 4B). We conclude that the repressor on chromosome 3 defines a regulatory pathway controlling hTERT expression that does not involve c-Myc.

A



B

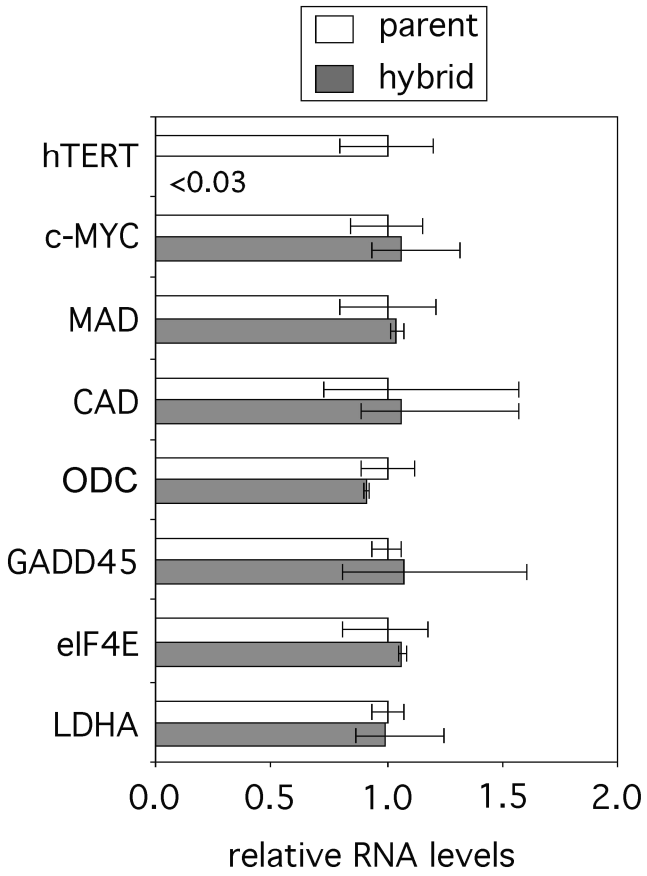


Figure 4: c-Myc levels in parental 21NT cells and 21NT-chromosome 3 hybrids. (**A**) c-Myc, Mad1 and actin protein levels in 21NT parental cells and 21NT-chromosome 3 hybrids. Total protein was extracted from two cultures of exponentially growing parental 21NT and two cultures of 21NT-chromosome 3 hybrid cells. c-Myc, Mad1 and actin levels were determined by Western analysis. (**B**) Relative levels of hTERT, c-Myc, Mad, CAD, ODC, GADD45, eIF4E and LDH RNAs in 21NT parental cells and 21NT-chromosome 3 hybrids. Total RNA was extracted from exponentially growing parental 21NT cells (white boxes) and of 21NT-chromosome 3 hybrids (grey boxes). To avoid genomic contamination, RNA was treated with DNAse I prior to reverse transcription. Real-time RT-PCR was performed with primer pairs hTERT E2-I2, MYC, MAD, CAD, ODC, GADD45, eIF4E, LDHA and β2M. RNA levels were normalized to β2M RNA and are expressed relative to the RNA level in parental 21NT cells. Results represent the average (± range) of two independent experiments with two parental 21NT cells and two independent 21NT-chromosome 3 hybrids.

4.5 DISCUSSION

In this paper, the transcripts of the gene coding for the catalytic subunit of human telomerase were quantified in different telomerase-positive and negative cells. Intron 9-less and intron 2-containing transcripts of hTERT were detected in telomerase-positive cell lines but not in telomerase-negative HLF and GM847 cells. These results provide support for the critical role of hTERT RNA regulation for telomerase activity. Our data indicate that on average, a telomerase-positive cell contains less than six spliced hTERT RNA molecules, whereas spliced and intron-containing hTERT RNA levels in telomerase-negative cells, if present, are below the limit of detection (0.004 molecule/cell). We found that intron 9-less and intron 4-less hTERT RNAs were predominantly cytoplasmic, whereas intron 2-containing hTERT RNA was mainly nuclear. The relative levels of both RNA species correlated well with each other in all telomerase-positive and negative cells examined. These data suggest that hTERT RNA levels are controlled mainly prior to exit from the nucleus, by changes either in the rate of transcription or in the stability of nuclear RNA.

The low hTERT RNA levels combined with the intermediate RNA stability suggest that the rate of hTERT transcription is low. Assuming a polymerization rate of 2000 nucleotides/min by RNA polymerase II (Jackson *et al.*, 2000) and a half-life for hTERT RNA of 2 h we estimate that 1-2 RNA polymerase complexes are transcribing the 40 kb gene (Wick *et al.*, 1999) at any given time. This low RNA polymerase II density on the hTERT gene is consistent with our inability to detect hTERT transcription by run-on analysis (not shown). However, successful run-on analysis was reported by Günes (Gunes *et al.*, 2000) using human myeloid leukemia U937 cells. Comparison of their data with ours suggests that the rate of hTERT transcription in U937 cells is much higher than in HT1080 fibrosarcoma cells.

In previous reports several hTERT RNA splice variants had been described which are differentially expressed during embryonic development and could also be detected in some immortal cell lines (Kilian *et al.*, 1997; Ulaner *et al.*, 1998; Wick *et al.*, 1999). The splice-variants cannot encode enzymatically active telomerase since critical regions in the RT-domain are missing. Since in the telomerase-negative cells tested here (HLF, GM847 and 21NT-chromosome 3 hybrids) no hTERT transcripts were detectable, telomerase-repression is likely to involve mechanism(s) preceding alternative splicing.

In arrested EREB cells and in terminally differentiated HaCaT cells we observed down-regulation of hTERT RNA, whereas no change was detected during the cell cycle in proliferating tumor cells. This is reminiscent of the RNA levels of other DNA polymerases (α, δ and ε) in proliferating cells (Huang *et al.*, 1999; Tuusa *et al.*, 1995). It is unclear what

factors increase hTERT RNA levels in proliferating cells and whether the same factors mediate hTERT up-regulation in tumors. c-Myc is known to trigger hTERT transcription when over-expressed, and is expressed in proliferating but not in arrested cells. Thus, c-Myc may contribute to the activation of hTERT transcription in proliferating EREB and HaCaT cells. However, the levels of c-Myc present in proliferating fibroblasts are not sufficient to induce hTERT expression. The c-Myc protein is expressed at higher levels in many tumors, and may contribute to the activation of hTERT expression. However, transfer of normal chromosome 3 into the breast cancer-derived cell line 21NT repressed hTERT expression without affecting c-Myc or Mad levels or expression of c-Myc target genes. This indicates that the gene(s) on chromosome 3 responsible for hTERT repression does (do) not act via changes in the Myc/Mad network. Genetic or epigenetic events other than changes in c-Myc levels must be required for hTERT activation in the tumor that gave rise to 21NT cells.

Our results strongly suggest that chromosome 3 acts to repress telomerase through transcriptional silencing of the gene encoding hTERT. In our previous study (Cuthbert *et al.*, 1999) we were unable to obtain definitive proof that replicative senescence induced by chromosome 3 was exclusively due to telomerase repression. In the present study we used, as recipients, 21NT cells that had previously been transfected with an hTERT cDNA expression construct in an attempt to prevent senescence resulting from repression of endogenous telomerase activity. The fact that chromosome 3 transfer did not induce senescence in these hTERT cDNA-transfected recipients, while endogenous hTERT immature RNA was down-regulated, clearly establishes that the effect of the repressor on chromosome 3 in inducing senescence is due entirely to a specific silencing effect on hTERT expression.

Like others we have developed hTERT constructs in which 5’ flanking segments of the hTERT gene drive expression of a reporter gene. Our data are in agreement with previous studies (Cong and Bacchetti, 2000; Cong *et al.*, 1999; Fujimoto *et al.*, 2000; Greenberg *et al.*, 1999; Gunes *et al.*, 2000; Kyo *et al.*, 1999; Kyo *et al.*, 2000; Oh *et al.*, 1999; Oh *et al.*, 1999; Oh *et al.*, 2000; Takakura *et al.*, 1999; Wick *et al.*, 1999; Wu *et al.*, 1999) in that the hTERT promoter is active in telomerase-positive immortal cell lines, but barely so in telomerase-negative primary cells. However, we also describe examples in which hTERT reporter expression does not mimic expression of the endogenous gene. Firstly, the reporters are as active in the telomerase-negative ALT cell line GM847 as in another telomerase-positive SV40-transformed fibroblast line, GM639. Secondly, in microcell hybrids in which chromosome 3 turns off expression of endogenous hTERT, the activity of the reporter constructs is not affected. In contrast, Horikawa (Horikawa *et al.*, 1999) found that in RCC23-chromosome 3 hybrids, luciferase expression was abrogated using a reporter containing 1.7 kb of hTERT upstream region. The discrepancy between the reporter analysis in RCC23-

chromosome 3 hybrids and in 21NT-chromosome 3 hybrids remains to be addressed. Endogenous hTERT RNA levels are influenced by the proliferative state of the cells (see Fig 1*A* EREB and HaCaT cells) (Gunes *et al.*, 2000; Pendino *et al.*, 2001; Tzukerman *et al.*, 2000, Xu *et al.*, 2001). Different growth rates were observed for RCC23 cells and for RCC23-chromosome 3 hybrids (Horikawa *et al.*, 1998), whereas 21NT and 21NT-chromosome 3 hybrids containing an hTERT transgene proliferated at the same rate (data not shown). Analyses of GFP reporters in 21NT-chromosome 3 hybrids and GM847 cells show that the region extending 7.4 kb upstream of the hTERT promoter is not sufficient to confer proper regulation outside its endogenous context.

The hTERT gene resides very close to the telomere of the short arm of chromosome 5 (Bryce *et al.*, 2000). Telomeric chromatin in yeast is transcriptionally silent (Gottschling *et al.*, 1990) and recent evidence indicates that telomeric repression exists also in human cells (Baur *et al.*, 2001). Thus, it is tempting to speculate that the chromatin structure near the telomere may play an important role in the repression of the hTERT gene in normal human somatic cells, and that the repressor gene on chromosome-3 may in part exert its effect through chromatin remodeling.

5 REGULATION OF HUMAN TERT BY NOTCH SIGNALING

5.1 ABSTRACT

Telomerase is a reverse transcriptase that maintains the ends of chromosomes. Its activity is limited by the expression of its catalytic subunit, hTERT. In human, hTERT RNA is detected in tumors, but not in most somatic cell types. During embryonic development hTERT expression is reduced in fetal tissues after 16 to 20 weeks of gestation. The Notch signaling pathway controls cell fate decisions during embryonic development and Notch expression is up-regulated in some cancers. Therefore we considered the possibility that Notch controls hTERT expression during development and tumors. Overexpression of the intracellular part of Notch1IC, which is the active form of Notch1, up-regulated hTERT RNA in a breast cancer-derived cell line (21NT), whereas it reduced hTERT RNA levels in HeLa and HLF-cMyc cells. We identified several putative binding sites for CBF1, a known downstream effector of Notch1IC, in the hTERT gene. Two of these binding sites overlap with E-boxes that had previously been implicated in hTERT regulation. However we have no evidence for a direct interaction between Notch1IC and the hTERT gene. Using conditional Notch1IC knockout mice we found that depletion of Noch1IC did not affect liver TERT RNA levels, suggesting that Notch1IC may not be involved in the regulation of the mouse TERT gene, which also lacks the putative binding sites for CBF1.

5.2 INTRODUCTION

Telomerase is a ribonucleoprotein complex required for the addition of telomeric repeats to the ends of linear chromosomes. The core of the telomerase consists of the catalytic subunit, a reverse transcriptase, hTERT, and an RNA moiety that contains the template region for telomere elongation (hTERC). hTERT mRNA expression is limiting for telomerase activity in telomerase negative cells (Bodnar *et al.*, 1998; Morales *et al.*, 1999; Vaziri and Benchimol, 1998; Yang *et al.*, 1999). In man telomerase is found in 85 % of tumor-derived cells (Kim *et al.*, 1994), whereas no telomerase activity is detectable in the majority of somatic cells. hTERT expression is developmentally regulated. Enzyme expression is restricted to germ-line tissues, blastocysts and fetal tissues up to 16 to 20 weeks of gestation (Ulaner and Giudice, 1997; Wright *et al.*, 1996). Proteins, which control cell differentiation and which are frequently up-regulated in cancer may be involved in hTERT regulation and Notch is such a potential regulator of hTERT expression. Indeed the Notch pathway controls cell fate decisions during development in organisms from *Drosophila* to humans (Artavanis-

Tsakonas *et al.*, 1999, Artavanis-Tsakonas, 1995 #419). Notch signaling influences distinct cellular processes such as differentiation, proliferation and apoptosis (Artavanis-Tsakonas *et al.*, 1999; Miele and Osborne, 1999; Milner and Bigas, 1999). In particular, Notch activation inhibits or delays cell differentiation in developmental pathways (Delfini *et al.*, 2000; Lam *et al.*, 2000; Milner *et al.*, 1996; Nofziger *et al.*, 1999; Shawber *et al.*, 1996). Thus, we speculated that Notch might prevent hTERT down-regulation in undifferentiated cells and in tumors.

Notch genes encode transmembrane receptors (Fig. 1). Four vertebrate *notch* genes denominated *notch1-4* have been identified. They are strongly related to each other and to *Drosophila notch* (Lardelli *et al.*, 1995; Sugaya *et al.*, 1997). Notch signaling is believed to mediate communication between neighboring cells, since Notch activation results from the binding of ligands expressed on adjacent cells. Ligand binding induces proteolytic processing of Notch that releases the intracellular portion of the receptor (NotchIC) from the plasma membrane. It is thought that NotchIC translocates to the nucleus where it interacts with effector molecules to alter gene expression (Jarriault *et al.*, 1995; Kopan *et al.*, 1996). One such effector molecule is the transcriptional regulator CBF1 (Grossman *et al.*, 1994), also called RBP-JK or RBP-2N (Dou *et al.*, 1994; Hamaguchi *et al.*, 1989). CBF1 is homologous to *Drosphila* Su(H) (Furukawa *et al.*, 1992) and to *C. elegans* Lag-1 (Christensen *et al.*, 1996). CBF1 directly interacts with Notch1IC (Hsieh *et al.*, 1996; Jarriault *et al.*, 1995) and recognizes the core DNA sequence GTGGGAA (Tun *et al.*, 1994). DNA-bound CBF1 may act as transcriptional repressor by recruiting a co-repressor complex. Components of the co-repressor complex identified by 2-hybrid screens, GST-pull down and transcriptional reporter assays include CIR, SMRT, SKIP, NCoR, SAP30, Sin3A, HDAC1 and HDAC2 and KyoT2 (Hsieh *et al.*, 1999; Kao *et al.*, 1998; Taniguchi *et al.*, 1998). Through interaction with NotchIC CBF1 is converted from a repressor of transcription into a transcriptional activator (Fortini and Artavanis-Tsakonas, 1994; Hsieh *et al.*, 1996; Jarriault *et al.*, 1995; Oswald *et al.*, 1998; Stifani *et al.*, 1992; Tamura *et al.*, 1995). To displace the co-repressor complex from CBF1, NotchIC may recruit co-activators such as Mastermind, p300, GCN4 and PCAF histone acetylases (Kurooka and Honjo, 2000; Oswald *et al.*, 2001; Wu *et al.*, 2000). The same region of CBF1 appears to interact with the co-repressors and the activators (Hsieh and Hayward, 1995; Hsieh *et al.*, 1996; Tani *et al.*, 2001), supporting a model of competition between co-repressors and co-activators for binding to CBF1.

To explore whether rodent and human TERT expression might be modulated by Notch signaling, we compared both 5' flanking region and searched for CBF1 binding sites in the TERT genes. We over-expressed Notch1IC in the breast cancer cell line 21NT, in the cervical carcinoma cell line HeLa, and in telomerase negative cells. We chose these cell lines because

the intracellular part of Notch 1 or Notch 4 are able to transform mouse mammary epithelial cells (Dievart *et al.*, 1999; Gallahan and Callahan, 1997; Gallahan *et al.*, 1987; Jhappan *et al.*, 1992) and because Notch1 and 2 are overexpressed in 100% of the cervical cancers analysed (Daniel *et al.*, 1997; Zagouras *et al.*, 1995). Using conditional Notch1IC knockout mice we investigated whether depletion of Noch1IC affects liver mTERT RNA levels.

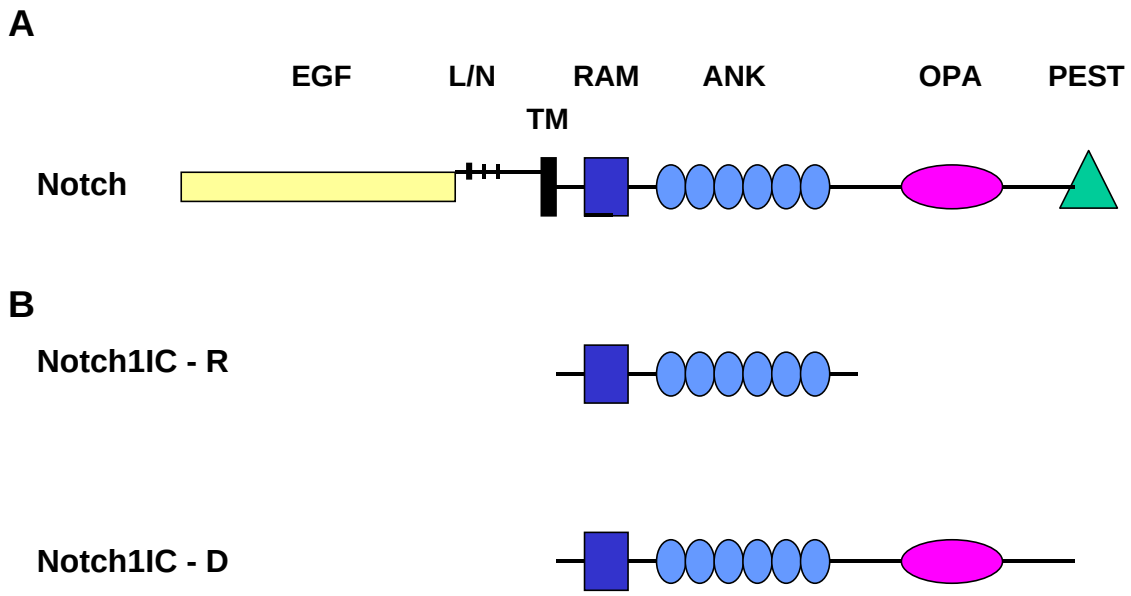


Figure 1: **A**) Notch receptors. The Notch receptor is a heterodimer of an extracellular domain (EGF, L/N), which is linked through a transmembrane domain (TM) to an intracellular domain (IC). The RAM domain adjacent to the transmembrane domain is the major docking site for the CBF1 protein (Aster *et al.*, 1997; Jarriault *et al.*, 1995; Tamura *et al.*, 1995). The ankyrin repeats (ANK) adjacent to the RAM domain mediate protein-protein interactions (Artavanis-Tsakonas *et al.*, 1999). The C-terminal region contains a polyglutamine region (OPA) and a proline, glutamic acid, serine and threonine rich region termed PEST. **B**) The two Notch1IC constructs used in this study are presented. Both constructs lack the extracellular and the transmembrane domains. In Notch1IC (R) the OPA and PEST motifs are deleted. In the Notch1IC (D) only the PEST sequence is missing.

5.3 MATERIALS AND METHODS

5.3.1 Cells

Primary human embryonic lung fibroblasts (HLF, a generous gift of Urs Ziegler, University Hospital, Zürich), c-Myc-immortalised HLF cells (HLF-c-Myc (Ducrest *et al.*, 2001), a cervical carcinoma derived-cell line (HeLa, obtained from Beatrice Bentele, ISREC) and Phoenix cells, packaging cells derived from the 293 cell line (a gift from a Garry Nolan, Stanford University, CA) were maintained in high glucose DMEM with 10% fetal calf serum. The breast carcinoma cell line 21NT and its derivative 21NT pCineohTERT HyTkchromosome 3 (21NT-chromosome 3 hybrid) were cultured as described (Cuthbert *et al.*, 1999).

5.3.2 Plasmids

The murine Notch1IC-R cDNA (nucleotide position 1751-2294 of the ORF) was isolated from pSKN1IC (obtained from F. Radtke) by digestion with *Hind*III and *Eco*RI. Upon ligation of *Eco*RI linkers (New England Biolabs) it was subcloned into the pBabe retrovirus. The pBabeNotch1IC-D construct (nucleotides position 1751-2444 of the ORF) was obtained from Kenneth Raj (ISREC). The GFP reporter vectors pd2G (basic vector), pSVd2G (promoter vector) and pSVEd2G (promoter/enhancer vector) were generated as described in chapter 3. The putative CBF1 binding sites were mutated from GTGG**GA**A to GTGG**CC**A for site (A) that lies 23 bp upstream of the translation start site, from CT**CC**CAC to CT**GG**CAC for site (B) at position -240 bp and from T**TC**CCAC to T**GG**CCAC for site (C) at position –820 bp. These mutations abolished CBF1 binding activity in gel shift assays (Tun *et al.*, 1994).The CBF1 binding site mutants were prepared from the phTERT.1.3G reporter construct (Ducrest *et al.*, 2001) by PCR with the primers described below. The phTERT.1.3d2G variants were obtained by cutting phTERT.1.3G with *Nhe*I and *Bgl*II and ligating the respective fragments into pd2G also cut with *Nhe*I and *Bgl*II. The mutation A was obtained by cloning a 1.3 kb *Nhe*I/*Bgl*II fragment generated by PCR with primers P1328f and CBF-A2r and with phTERT.1.3G as template into the *Nhe*I/*Bgl*II sites of pd2G. The mutation B was obtained in two steps. First a fragment of 0.25 kb was generated by PCR with primers CBF-B2fw and P1r and with phTERT.1.3G as template. Then this product was used as primer with P1328f in a second PCR with phTERT.1.3G as template. The final 1.3 kb product was cloned as described above. The mutation C was obtained in two steps. First a fragment of 0.5 kb was generated by PCR with primers CBF-Cr and P1328f and with phTERT.1.3G as template. Then this product was used as primer with P1r in a second PCR with phTERT.1.3G

as template. The final 1.3 kb product was cloned as described above. The combined mutation AB was obtained by generating by PCR amplification of a fragment of 0.2 kb with primers CBF-B2f and CBF-A2r and with phTERT.1.3G as template. This product was used as primer with P1328f and with phTERT.1.3G as template in a second PCR producing the final 1.3 kb fragment that was cloned as described above. The mutation AC was obtained by generating by PCR amplification of a fragment of 0.5 kb with primers P1328f and CBF-Cr and with phTERT.1.3G as template. This product was used as primer with CBF-A2r and with phTERT.1.3G as template in a second PCR producing the final 1.3 kb fragment that was cloned as described above. The mutation BC was obtained by generating by PCR amplification of a fragment of 0.5 kb with primers p1328f and CBF-Cr and with phTERT.1.3G as template. In a second PCR a 0.25 kb fragments was generated with primers CBF-B2f and P1r and with phTERT.1.3G as template. These two products were used as primers with phTERT.1.3G as template in a PCR producing the final 1.3 kb fragment that was cloned as described above. The mutation ABC was obtained by generating by PCR amplification of a fragment of 0.5 kb with primers p1328f and CBF-Cr and with phTERT.1.3G as template. In a second PCR a 0.2 kb fragments was generated with primers CBF-B2f and CBF-A2r and with phTERT.1.3G as template. These two products were used as primers with phTERT.1.3G as template in a PCR producing the final 1.3 kb fragment that was cloned as described above. All the plasmids were sequenced and mutations were also confirmed by restriction digests.

5.3.3 DNA oligonucleotides.

The following DNA oligonucleotides were purchased from Microsynth (Balgach, Switzerland) and used for hTERT reporter constructs:

P1r: 5'-GGAACTAGTAGATCTCGCGGGGGTGGCCGGGG-3';

P1328f: 5'-GGAGGAATCCAGGGAGGGTGCGAGGCC-3';

CBF-A2r: 5'-AGTAGATCTCGCGGGGGTGGCCGGGGCCAGGGCTGGCCACGTGCGCA-3';

CBF-B2f: 5'-AGGACCGCGCTGGCCACGTGGCGG-3';

CBF-Cr: 5'-GAGAAAGGGTGGCCAATGGAGCCAGG-3'.

5.3.4 Transient transfections

Transient transfections with calcium-phosphate precipitates were performed as described (Jordan *et al.*, 1996). Cells were co-transfected with 1 µg pSV2-Thy-1.1 as reference plasmid and equimolar amounts of GFP-reporter plasmids. The total amount of plasmid was kept constant (6 µg) by adding pUC19. GFP reporter expression was measured

as described in chapter 3, except that the GFP expression of the reporter was normalized to that obtained upon transfection of a plasmid containing the wild type hTERT fragment phTERT.1.3dG.

5.3.5 Infections.

Amphotrophic pBNotch1IC-R and pBNotch1IC-D retroviruses were generated by calcium-phosphate transfections of amphotropic Phoenix cells. 48 hours to 72 hours post transfection the viral supernatant was collected, filtered to remove packaging cells (0.45 μm filters, Millipore) and stored at –70°C in 1 ml aliquots. Target cells were infected with virus supplemented with 8 μg/ml polybrene (Sigma). Infected cells were selected 48 h after infection with the appropriate drugs (0.4 mg/ml hygromycin, 1 μg/ml puromycin; Calbiochem). Lentivirus pAT-1-Notch1IC-D containing Notch1IC-D under the control of the pGK promoter was kindly provided by Kenneth Raj (ISREC).

5.3.6 Quantitative RT-PCR analysis.

Total RNA was extracted and RT-PCRs were performed as described in (Ducrest *et al.*, 2001). The following primers were used to detect HES1 RNA: HES1-102fw: 5'-CAGAAAGTCATCAAAGCCTATTATGG-3'; HES1-179rv: 5'-CTTTCTTCAGAGCATCCAAAATCA-3' (Microsynth, Switzerland). They spanned the boundary between exon 2 and exon 3 of hHES1 thus amplifying only cDNA derived from spliced RNA. RT-PCR of mouse RNA was performed as described (Ducrest *et al.*, 2001) with the following modifications. Total RNA of murine livers was treated with DNaseI prior to RT-PCR. Murine β2-Microglobulin RNA was used as endogenous control to normalize the amount of RNA of each reaction. The concentration of the mβ2M primer was 600 μM for the forward and 900 μM for the reverse primer. mTERT and mβ2M RNA were amplified with the following primer pairs:

mTERT2616fw: 5'-TTTCTGTTGGTGACGCCTCA-3';

mTERT2693rv: 5'-CCCATACTCAGGAACGCCAT-3;

mβ2Mfw: 5'-TCTGGTGCTTGTCTCACTGACC-3';

mβ2Mrv: 5'-CAGTATGTTCGGCTTCCCATT-3'.

5.3.7 Generation of mice with loxP-flanked Notch1 allele and activation of the Cre recombinase.

Notch1$^{lox/lox}$ (thereafter called WT) and Notch1$^{lox/lox}$ Mx-Cre (thereafter called KO) mice were generated as described previously (Radtke *et al.*, 1999). To induce the Cre-recombinase, adult mice received three intraperitoneal injections of 250 μg polyI-polyC (Sigma Chemical

Co.) at 2 day intervals (Radtke *et al.*, 1999). Fifteen days (2 WT and 3 KO) and 22 days (2 WT and 4 KO) after the last injection mice were killed. Total RNAs were prepared from their livers, washed in PBS and dounce homogenized. One third of homogenized livers were used for total RNA extraction.

5.3.8 Immunoblots.

Total protein from four livers of WT and KO mice was extracted with 8M urea, 0.5% Triton X-100 and 0.5% NP40. Seventy-five μg of protein/lane was resolved on a 6 % SDS-polyacrylamide gel and transferred to a protein nitrocellulose membrane. The membrane was stained with Ponceau to control for loading and transfer. mNotch1 was detected with a goat polyclonal antibody (M-20, dilution 1/100, Santa Cruz). Western blots were developed using the enhanced chemiluminescence system (ECL, Amersham).

5.4 RESULTS

5.4.1 Comparison of the 5'flanking region of human and rodent TERT genes

Conservation of binding sites in the 5'flanking regions of homologous genes suggests that the same transcription factors regulate these genes. In order to look for such binding sites in the TERT genes, the 4 kb fragment upstream of the translation start site of the mTERT gene (AF 121949) was compared to the 7.4 kb fragment upstream of the translation start site of the hTERT gene (AF114847), using Dotter ((Sonnhammer and Durbin, 1995). When both sequences were compared, only two regions showed high similarity (Fig. 2). These two fragments are located 5580 to 5360 bp and 1220 to 1420 bp upstream of the ATG of hTERT, respectively. Both regions do contain neither repetitive elements nor obvious transcription binding sites (TRANSFAC 4.0, Quandt *et al*., 1995 and PatSearch, Heinemeyer *et al*., 1998). Moreover, when the expression of GFP-reporter containing and deleted of these regions were compared, no significant difference in GFP expression was observed in telomerase positive or negative cells (data not shown). Thus it is not clear whether these two regions play a role in the regulation of the hTERT gene.

It is expected that in the most proximal 5' flanking region of the human and mouse TERT genes, where in both genes the transcription start sites and putative binding sites for c-Myc and SP1 are located, the sequences should be highly similar. In contrast to the expectation, only very weak similarity was observed. The absence of high conservation between the 5'flanking regions of rodent and human TERT could explain the differential expression between hTERT and mTERT genes. In rodents TERT expression is maintained during differentiation (Russo *et al.,* 1998), whereas hTERT expression is limited to stem cells and activated lymphocytes (Chiu *et al.,* 1996; Wright *et al.,* 1996).

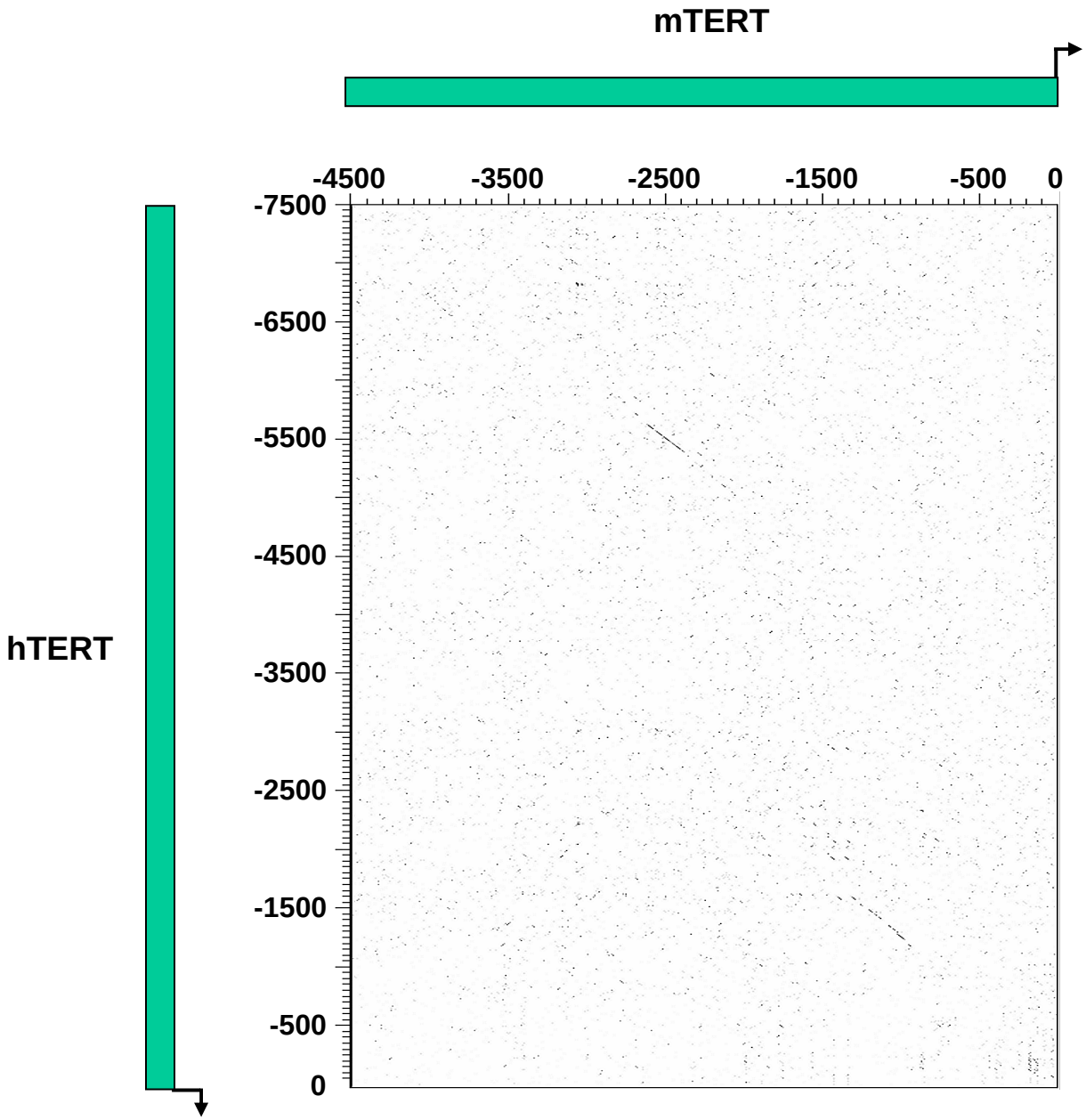


Figure 2: Comparison of the 5'UTR regions upstream of the translation start site of the hTERT and mTERT. The 7.4 kb of hTERT gene are plotted against the 4 kb of the mTERT gene. Good matches (matrix identity) are shown by a darker trait on the diagonal. Two regions show some similarity, that is 5450 bp and 1300 bp upstream of the hTERT gene upstream of the translation start site.

5.4.2 The proximal upstream region of the hTERT gene contains putative CBF1 binding sites

CBF1 recognizes the core DNA sequence GTGGGAA and interacts weakly with ACT and CG sequences flanking this heptanucleotide 5' and 3' (Tun *et al.*, 1994). CBF1 binding sites have been shown to play a role in the regulation of human Cyclin D1 (Ronchini and Capobianco, 2001), NF-κB (Oswald *et al.*, 1998; Palmieri *et al.*, 1999), IL-6 (Kannabiran *et al.*, 1997) genes, of mouse HES1 genes (Tun *et al.*, 1994), of Drosophila Enhancer of split gene complex (Bailey and Posakony, 1995), of the Epstein Barr virus genes such as EBNA2 (Ling *et al.*, 1993) or LMP1 (Ling *et al.*, 1994) and of the adenovirus pIX gene (Dou *et al.*, 1994). In these genes one to three 7 bp consensus or imperfect 6 bp CBF1 binding sites in the sense and antisense orientation are located in the proximal part of the promoter, between 500 to 150 bp upstream of the transcription start sites. The activity of the binding of CBF1 to the DNA was shown by reporter assays, EMSA and by purification of CBF1 from the binding site. CBF1 binding sites were also found in enhancer. In the human β-globin gene the CBF1 binding sites overlaps a putative E box in the hypersensitive site 2 (HS2) of the locus control region 10 to 50 kb upstream of the β-globin gene (Lam and Bresnick, 1998).

The hTERT gene contains putative CBF1 binding sites (Fig. 3). In the 5'flanking region one putative binding site overlaps with the putative E box located at position –31 bp upstream of the translation start site. A second site lies 826 bp upstream of the translation start site in the antisense orientation (Fig. 3). We also found perfect consensus sequences in the second, third and sixth introns of hTERT gene at positions 2057, 11660 and 14988 bp downstream of the translation start site, respectively (according to AF128893; (Wick *et al.*, 1999). In the 5'flanking region the second E box (-240 bp) overlaps with an imperfect 6 bp putative CBF1 binding site present in an antisense orientation (Fig. 3).

Since the first 800 bp upstream of the ATG are not conserved between mouse and human, it is suspected that the CBF1 binding sites are missing in the mouse. Perfect consensus binding sites are missing in the mouse TERT gene. But two less well-conserved consensi of 6 bp are found. One overlaps with the putative E box 23 bp upstream of the translation start site. A second imperfect CBF1 binding sites lies 115 bp upstream of the translation start site in the antisense orientation. These observations suggest that CBF1 may play a role in hTERT, but not in mTERT regulation.

```
-833  ccattttccca  ccctttctcg  acgggaccgc  cccggtgggt  gattaacaga
      tttggggtgg
.....................
-293  ggccgggctc  ccagtggatt  cgcgggcaca  gacgcccagg  accgcgctcc
      ccacgtggcg
-233  gagggactgg  ggacccgggc  acccgtcctg  ccccttcacc  ttccagctcc
      gcctcctccg
-173  cgcggacccc  gccccgtccc  gacccctccc  gggtccccgg  cccagccccc
      tccgggccct
-113  cccagccccc  cccttcctt  tccgcggccc  cgccctctcc  tcgcggcgcg
      agtttcaggc
 -53  agcgctgcgt  cctgctgcgc  acgtgggaag  ccctggcccc  ggccaccccc
      gcgatg
```

Figure 3: CBF1 binding sites in hTERT 5'flanking region. The putative CBF1 binding sites are shown in red and violet. The putative c-Myc binding sites are shown in violet and blue. Violet represents the overlapping part between the putative CBF1 and c-Myc binding sites. Negative numbers represent the position of the nucleotides relative to the translation start site. The major transcription start site lies between –55 and – 77 (MA unpublished data, (Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999).

5.4.3 Ectopic expression of Notch1IC increases hTERT RNA expression in 21NT scells

To determine whether RNA levels of hTERT are modulated by Notch, we over-expressed two different mouse Notch1IC constructs in tumor-derived cell lines and primary cells and monitored their ability to modulate hTERT and HES1 RNA levels by quantitative RT-PCR. Mouse Notch1IC has been shown to be able to activate target genes in human cell lines (Jarriault *et al.*, 1995). In one of the Notch1IC constructs, Notch1IC-R, the OPA and PEST sequences are missing (Fig. 1). Since it was recently described that the OPA sequences may be important for Notch1IC transactivation (Kurooka *et al.*, 1998) and for the interaction of Notch1IC with PCAF (Kurooka and Honjo, 2000) we also tested a construct, Notch1IC-D, in which only the PEST sequences are missing (Fig. 1) (Deftos *et al.*, 1998). In 21NT cells, expression of the Notch1IC-R increased hTERT and HES1 RNA levels 4 - 6 fold (Fig. 4), whereas GAPDH or β2-microglobulin were unchanged (data not shown). A 2 – 4 fold increase of hTERT and HES1 RNA was also detected with the Notch1IC-D (Table 1). A similar increase in nuclear, intron-containing hTERT RNA was observed (Table 1) (see chapter 4; (Ducrest *et al.*, 2001), suggesting that Notch1IC acts on hTERT transcription and not on hTERT splicing or hTERT nuclear export.

We also followed in a time course experiment the RNA levels of hTERT and HES1 in 21NT cells infected with lentivirus expressing Notch1IC-D (Fig. 5). Eight hours post-infection, HES1 RNA levels increased 2 fold, while an increase in hTERT RNA was detected

only after 16 hours. Both transcripts reached a maximal increase of 3 fold 16h post-infection upon which they decreased again. The observation that the increase in HES1 RNA preceded the one of hTERT sensibly suggests that the constitutive active form of Notch1 might have activated hTERT transcription indirectly.

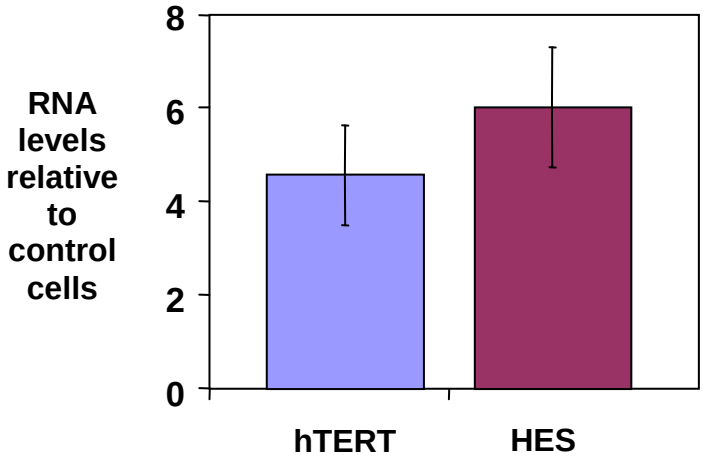


Figure 4: Relative hTERT and HES1 RNA levels in 21NT cells infected with pBabeNotch1IC retroviruses. Subconfluent 21NT cells were infected with pBP-NotchIC retroviruses and selected for 7 days with puromycin. Extracted RNA was reverse transcribed and analyzed by quantitative PCR. hTERT and HES1 RNA were normalized to GAPDH and expressed relative to the amount of RNA in the control cells infected with pBP. The average and standard deviations of hTERT (blue) and HES1 (violet) RNA measured in 2 different infections are represented.

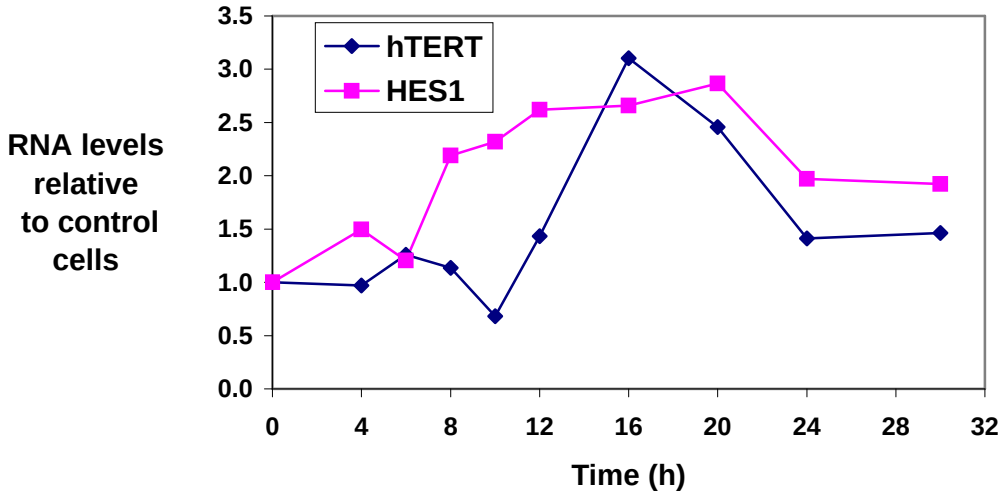


Figure 5: Delayed timing of hTERT induction in 21NT infected with Notch1IC-D lentivirus. Extracted RNA was processed and normalized as described in figure 1.

Table 1: hTERT and HES1 RNA levels in 21NT expressing Notch1IC.

Constructs	hTERT[1]	SD[3]	HES1[1]	SD[3]	n[4]
pBP-NOTCH1IC-R	4.6	1.1	6.0	1.3	2

pBP-NOTCH1IC-R	4.0[2]		3.1		1
pBH-NOTCH1IC-R	2.0	0.3	2.4	0.5	2
pBP-NOTCH1IC-D	3.0	1.5	3.9	2.1	5

hTERT/HES1[1]:average RNA levels relative to control cells; Spliced hTERT RNA was detected with pimer pairs E9-10, except for 4.0[2]: detection of intron 2-containing hTERT RNA using primer pair E2-I2 (Ducrest *et al.*, 2001). SD[3]: standard deviation; n[4]: number of different infections.

5.4.4 Ectopic expression of Notch1IC did not affect endogenous hTERT RNA levels in telomerase negative cells

We also tested if Notch would be sufficient to induce hTERT expression in telomerase negative cells. For this experiment we used human lung fibroblasts (HLF), human mammary epithelial cells (HMEC) and 21NT-chromosome-3 hybrids, in which the transfer of a normal chromosome 3 turns off expression of endogenous hTERT (see chapter 3; (Ducrest *et al.*, 2001). The latter cells were rescued from senescence by ectopic expression of a hTERT cDNA construct. Following ectopic expression of Notch1IC-R in HLF cells, a 3 to 10 fold induction of HES1 RNA was detected while hTERT RNA could not be detected (Table 2). With the same viruses only a slight induction in HES1 transcripts was detected in HMEC and 21NT-chromosome-3 hybrid cells and no signal above the detection limit was detected for endogenous hTERT RNA (Table 2). Thus Notch1IC is not sufficient to induce hTERT RNA expression in the telomerase negative cells that were tested.

Whereas over-expression of Notch1IC induced hTERT in 21NT (see above), a different effect was observed in HLF-c-Myc and in HeLa cells. Here hTERT RNA levels decreased 2 to 3 fold, whereas HES1 RNA levels increased 5 and 2 fold, respectively (Table 2). This suggests that in HeLa and HLF-c-Myc, Notch1IC may induce repressors of hTERT or that Notch1IC may compete with activators of hTERT for hTERT promoter binding sites.

Table 2: hTERT and HES1 RNA levels in cells expressing Notch1IC-R.

Cells	**hTERT[1]**	**SD[2]**	**HES1[1]**	**SD[2]**	**n[3]**
21NT-chromosome-3 hybrids	BG[4]		1.8	0.5	4
HMEC	BG[4]		1.4	0.1	2
HLF	BG[4]		6.8	5.3	2
HLF-c-Myc	0.3	0.0	1.4	0.1	2
HeLa	0.5	0.3	4.6	3.1	3

hTERT/HES1[1]:average RNA levels relative to control cells, SD[2]: standard deviation, n[3]: number of different infections, BG[4]: RNA levels below the detection limit.

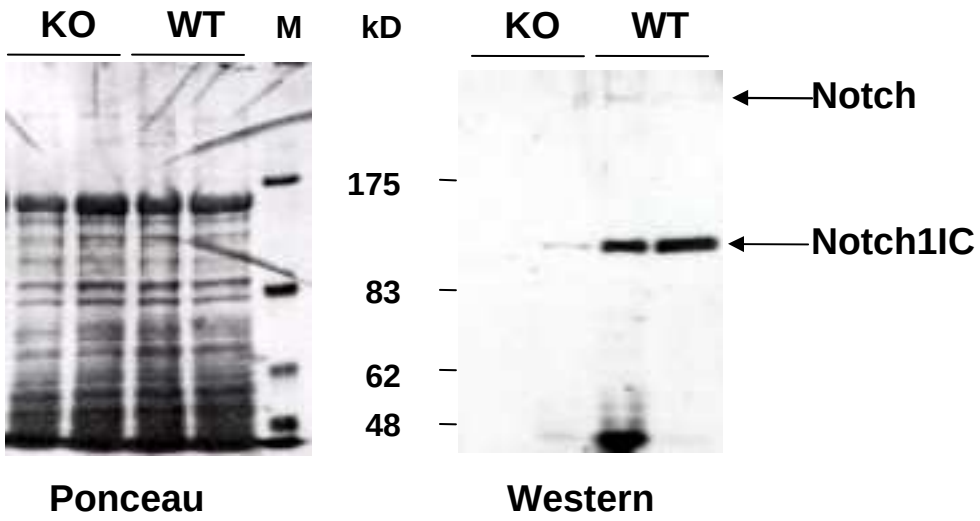
5.4.5 Mutations in putative CBF1 binding sites do not affect hTERT-GFP reporter expression

Competition between Notch1IC and c-Myc for binding in the hTERT promoter is an attractive hypothesis, since two of the CBF1 and c-Myc binding sites are overlapping (Fig. 2). To examine the role of the putative CBF1 binding sites on hTERT expression, point mutations affecting only the CBF1 binding sites were introduced in GFP-reporter constructs containing 1.3 kb of the hTERT upstream region (Fig. 3B, chapter 4; (Ducrest *et al.*, 2001). Their effects were tested in HeLa, 21NT, 21NT Notch1IC, 21NT-chromosome-3, HLF and HLF-c-Myc cells. The point mutations did not affect GFP expression in 21NT, 21NT-Notch1IC, 21NT-chromosome 3 and HeLa cells, even when combined in the same reporter construct (data no shown). In another study, the proximal putative CBF1 binding site was identified as a binding site (called MT box) for a DNA binding activity in gel shift assays (Braunstein *et al.*, 2001).

5.4.6 Notch1 knockout and wild-type mice have similar mTERT RNA levels

Mouse and human TERT are differently regulated since in contrast to hTERT, mTERT is expressed in the majority of somatic cells (Blasco *et al.*, 1995), (Sugaya *et al.*, 1997). To determine whether Notch1 controls mTERT expression, we monitored the mTERT RNA levels in wild type and conditional knockout mice. Five wild type and seven conditional knockout mice were sacrificed and RNA was extracted from their livers, in which high efficiency of Notch1 deletion was observed (Fig. 7A). In liver of adult man, Notch1 was shown to be expressed weakly in biliary epithelial cells and hepatocytes, and strongly in liver endothelial cells (Nijjar *et al.*, 2001). Therefore, Notch1 is expressed in the majority of the liver cells. The livers of knockout mice appear smoother and bigger than wild-type (data not shown), suggesting that knocking out the Notch1 gene has a broad effect on livers. Moreover patients suffering of the Alagille syndrome due to a nonsense mutation in the binding partner of Notch1, Jagged1, develop cholestatic liver and intrahepatic ductal paucity (Louis *et al.*, 1999). In contrast, the expression pattern of mTERT in liver cells is not precisely known. No difference in mTERT RNA levels between the knockout and wild type mice was detected (Fig.7B). This suggests that mTERT may not be regulated by Notch1. This result may be explained by the absence of putative CBF1 binding sites in the 5' flanking region of the mTERT gene. However a high expression of mTERT in liver cells that do not express Notch1 could also mask the effect of knocking out the Notch1 gene on mTERT.

A



B

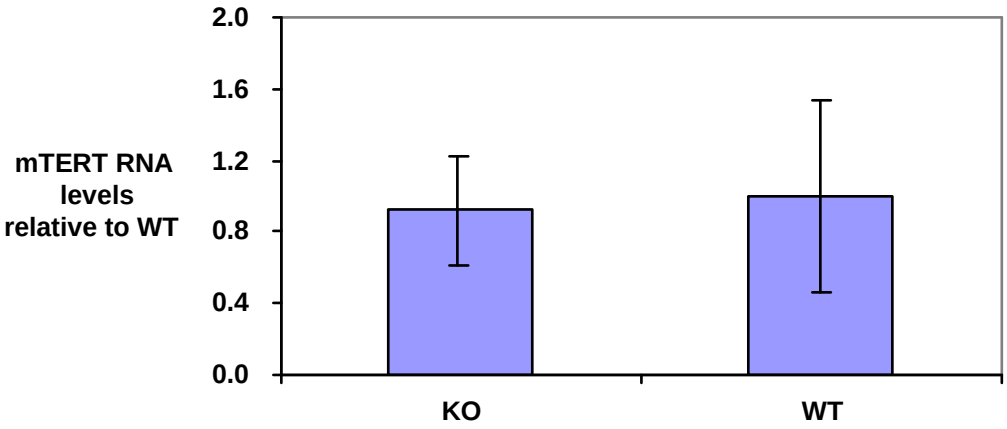


Figure 7: mTERT RNA levels are not affected by Notch1. **A**: Notch1 deletion was controlled in the liver of 2 wild-type and 2 KO mice by western blot (on the right) using antibodies specific for mNotch1. The arrows show the position of full length (300 kD) and intracellular Notch1 (120 kD). On the left a ponceau staining shows that equal amounts of proteins were loaded. **B**: Liver of Notch1IC KO and wild-type mice have the same levels of mTERT RNA. Average (±SD) of mTERT RNA levels in Notch1IC KO (KO) mice relative to wild-type (WT) mice are represented. Total RNA was extracted from the livers of 7 KO and 5 WT mice.

5.5 DISCUSSION

Our results show that Notch1IC can modulate hTERT RNA levels in some cell types whereas no effect on mTERT RNA was observed in conditional Notch1IC knockout mice. Ectopic Notch1IC increased in 21NT partially spliced hTERT RNA as well as nuclear, intron-containing immature hTERT RNA 2 - 4 folds. This suggests that Notch1IC activates hTERT transcription (Ducrest *et al.*, 2001). However Notch1IC over-expression was not sufficient to overcome hTERT repression in telomerase negative primary mammary epithelial cells (HMEC), primary lung fibroblasts (HLF) or 21NT-chromosome 3 hybrids. Why Notch1IC overexpression caused a decrease of hTERT RNA and an increase of HES1 RNA levels observed in HeLa cells and in HLF-c-Myc is not clear. It is possible that HES1, which can bind to E-boxes and acts as a repressor, competes with c-Myc for binding the hTERT gene. It is also possible that HES1 decreases endogenous Notch levels in a feedback loop or that ectopic Notch1IC may titrate out an activator of hTERT or activates a repressor of hTERT.

It is unclear if Notch1IC acts directly on the hTERT gene. Mutations of the putative CBF1 binding sites in hTERT-GFP reporters did not affect the expression of GFP in 21NT, 21NT-Notch1IC, 21NT-chromosome 3 and HeLa cells. However, since we showed that the reporters did not mimic endogenous hTERT expression, this results is not conclusive (Chapter 2 and 4; (Ducrest *et al.*, 2001; Ducrest *et al.*, 2002). In the time course experiment, hTERT induction upon Notch1IC over-expression was delayed relative to HES1, which is a direct target of Notch1. This result supports the notion that hTERT induction by Notch1IC may be indirect. To answer this question further chromatin immunoprecipitation (ChIP) experiments could be carried out.

The biological significance of Notch1 for hTERT regulation remains elusive. It could regulate hTERT expression during differentiation in embryonic cells. During embryonic development, Notch is expressed in undifferentiated cells and controls cell differentiation. In human, telomerase activity correlates with Notch expression being limited to germ-line tissues, blastocysts and to 16 to 20 week old fetal tissues (Ulaner and Giudice, 1997; Wright *et al.*, 1996). Notch signaling has been shown to accelerate progression through the G1 phase in HL60 promyelocytic leukemia (Carlesso *et al.*, 1999) and to activate CBF1-mediated transcription of the cyclin D1 gene (Ronchini and Capobianco, 2001), thus promoting S-phase entry. Levels of hTERT RNA are sensitive to the proliferative state and decrease in arrested cells (Chapter 4; (Ducrest *et al.*, 2001). Telomerase activity is detected in 85% of tumor-derived cells (Kim *et al.*, 1994). Inappropriate expression of Notch1 and Notch2 has been

observed in numerous human cancers of different origins (Aster *et al.*, 1994; Daniel *et al.*, 1997; Ellisen *et al.*, 1991; Zagouras *et al.*, 1995). Finally truncated forms of Notch1, Notch2 and Notch4/Int3 have been demonstrated to have transforming activity in several different systems (Dievart *et al.*, 1999; Gallahan *et al.*, 1987; Girard *et al.*, 1996; Pear *et al.*, 1996; Robbins *et al.*, 1992; Rohn *et al.*, 1996; Ronchini and Capobianco, 2000; Smith *et al.*, 1995).

6 CONCLUDING REMARKS

This work contributes to the understanding of the mechanisms that control hTERT expression. First, we have quantified hTERT RNA molecules in telomerase negative and positive cells. In all telomerase positive cells hTERT RNA levels were detectable but at a very low level (0.2 to 6 molecules/cell). In telomerase negative cells hTERT RNA could not be detected (<0.004 molecules/cell). We compared the levels of spliced cytoplasmic hTERT RNA with intron-containing nuclear hTERT RNA in telomerase positive and negative cells. This showed that intron-containing nuclear hTERT RNA is present only in telomerase positive cells. These results strongly suggest that hTERT RNA levels are controlled at the level of transcription. However, this does not exclude that regulation involves also changes in the efficiency of nuclear processing of primary transcripts.

Second, we demonstrated that the hTERT reporters containing up to 7.5 kb of the 5'flanking region do not faithfully mimic endogenous hTERT RNA expression. Comparing related telomerase positive and negative cells showed that hTERT-GFP reporters were expressed in certain cell lines that do not contain detectable levels of hTERT transcripts. These cell lines are a SV40-immortalized cell line that maintains its telomeres by the alternative pathway (ALT) and a breast cancer cell line (21NT), in which the transfer of an extra chromosome 3 extinguished hTERT RNA expression (21NT-chromosome 3). Thus endogenous hTERT expression may be controlled either by cis-acting elements located outside of the 5'flanking region analyzed, or by the chromatin structure at the endogenous hTERT locus. It may also be possible that telomeric silencing influences hTERT expression, since the gene is located near the telomere of chromosome 5p (Bryce *et al.*, 2000).

Third, we characterized candidate hTERT regulators. We assessed the role of c-Myc in 21NT-chromosome 3 hybrids and of the Notch signaling pathway for regulating hTERT in several tumor-derived and primary cells. c-Myc had been shown to directly activate hTERT expression in EBV-immortalized B lymphocytes and in embryonic lung fibroblasts (Greenberg *et al.*, 1999; Oh *et al.*, 2000; Wu *et al.*, 1999). Transfer of chromosome 3 decreased hTERT RNA levels 30 fold without altering the expressions of c-Myc and its target genes. This suggests that the putative repressor on chromosome 3 does not mediate its effect via c-Myc.

Notch is involved in controlling cell differentiation and is over-expressed in some cancers. Therefore Notch might also regulate hTERT expression. Indeed, over-expression of Notch1IC increased hTERT expression in the breast tumor-derived cell line from epithelial origin, 21NT, reduced hTERT transcripts in a cervical carcinoma cell line, HeLa, and in c-

Myc transformed primary fibroblasts. Ectopic Notch1IC expression had no detectable effect in hTERT RNA levels of telomerase negative cells tested. We also found putative CBF1 binding sites in the hTERT gene, supporting the notion that Notch1IC directly bind hTERT via CBF1. However, this has not been assed directly.

As mentioned above, there are a low number of hTERT RNA molecules in telomerase positive cells. We also found that the level of hTERT RNA does not vary during the cell cycle and that hTERT RNA has an intermediate stability with a half-life of 2h in a telomerase positive cell. This indicates that only one or two polymerases may transcribe hTERT gene at any given time. This low level of gene transcription is not too unusual, since in mammalian, cells the steady state levels of the majority of mRNAs are below 10 copies per cell (Jackson *et al.*, 2000). The low copy number of hTERT mRNA must be sufficient for the synthesis of enough hTERT protein molecules to stabilize the telomere length of 46 chromosomes (Hemann *et al.*, 2001). As each mRNA molecule can be translated many times, one single copy of hTERT mRNA might allow the synthesis of more than 200’000 protein molecules during one cell cycle, if one assumed a translation rate of 250 amino acids per minute and one initiation event per 0.4 minutes. Moreover hTERT protein is stable with a half-life of 24h (Holt *et al.*, 1997). It is also possible that besides maintaining telomere length, hTERT RNA expression is detrimental for some cell processes. However, ectopic expression of hTERT in telomerase negative cell restores telomerase activity and extends their lifespan (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998) and no cellular defect has been reported (Morales *et al.*, 1999). Since telomerase repression may have tumor suppressive function, telomerase may only be expressed in the cell types that undergo a high number of cell divisions during our lifespan. Thus regulation of hTERT expression is tissue-dependent. Therefore, multiple regulators of hTERT expression may be required (Chapter 2; (Ducrest *et al.*, 2002).

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