ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Experimental and Molecular Pathology 75 (2003) 238–247

Experimental and Molecular Pathology

www.elsevier.com/locate/yexmp

Role of Ets/Id proteins for telomerase regulation in human cancer cells

Xiaodong Xiao,[a,*] Meropi Athanasiou,[b,c] Igor A. Sidorov,[a] Izumi Horikawa,[d] Gina Cremona,[a] Donald Blair,[b] J. Carl Barret,[d] and Dimiter S. Dimitrov[a,*]

[a] *Laboratory of Experimental and Computational Biology, NCI–Frederick, NIH, Miller Drive, Frederick, MD 21702–1201, USA*
[b] *Basic Research Laboratory, NCI–Frederick, CCR, NIH Frederick, MD 21702–1201, USA*
[c] *Basic Research Program, SAIC–Frederick, Inc., NCI-Frederick, Frederick, MD 21702, USA*
[d] *Laboratory of Biosystems and Cancer, CCR, NCI, NIH, Bethesda, MD 20892, USA*

Received 2 June 2003, and in revised form 30 June 2003

Abstract

Most human cancers express telomerase but its activity is highly variable and regulated by complex mechanisms. Recently, we have proposed that Ets proteins may be important for regulation of telomerase activity in leukemic cells. Here we provide further evidence for the role of Ets family members and related Id proteins in telomerase regulation and characterize the underlying molecular mechanisms. By using PCR-based and gel shift assays we demonstrated specific binding to a core hTERT promoter of Ets2, Fli1, Id2, c-Myc, Mad1, and Sp1 in lysates from subclones of U937 cells. Further analysis of binding of purified proteins and various mutants of the hTERT promoter suggested the existence of a trimolecular Ets–Id2–DNA complex, and Ets inhibitory activity mediated by c-Myc and the Ets binding site on the core hTERT promoter at −293 bp from the transcription initiation site as well as a positive Ets regulatory effect mediate through another Ets binding site at −36 bp. This analysis provided evidence for the existence of negative and positive Ets regulatory site and suggested a complex interplay between Ets/Id family members and c-Myc that may be an important determinant of the diversity of telomerase activity in leukemia and other cancers.

Keywords: Telomerase regulation; Ets; Id2; c-Myc; Sp1

Introduction

Telomerase is a RNA-dependent ribonucleoprotein polymerase that elongates telomeric repeats at the chromosome ends and may have other functions that are currently under investigation (Fu et al., 2002; Greider and Blackburn, 1989). Most human cancers express telomerase but its activity is highly variable and regulated by complex mechanisms (Cong et al., 2002; Ducrest et al., 2002; Kyo and Inoue, 2002; Shay and Bacchetti, 1997). The transcriptional regulation of the expression of the human telomerase catalytic subunit with reverse transcriptase activity (hTERT) appears to be a major determinant of the telomerase activity regulation (Ducrest et al., 2001; Kyo and Inoue, 2002). The hTERT promoter does not contain a TATA box, and the 200- to 400-bp region proximal to the transcription initiation site is responsible for most of its transcriptional activity (Cong et al., 1999; Horikawa et al., 1999; Takakura et al., 1999). Multiple E-boxes, Sp1, Ets, and other binding sites for transcription factors are located in this core promoter region. c-Myc binds to these E-boxes through heterodimer formation with Max proteins and directly activates the hTERT transcription (Greenberg et al., 1999; Wu et al., 1999). Binding of the c-Myc antagonists, Mad proteins, as Mad/Max complexes decreases the activity of the hTERT promoter (Gunes et al., 2000; Kyo et al., 2000; Xu et al., 2001). Sp1 also binds to the core promoter and activates hTERT transcription (Kyo et al., 2000). Although overexpression of c-Myc is frequently observed in a wide variety of tumor types, and in some cases expression levels of

* Corresponding authors. Laboratory of Experimental and Computational Biology, NCI-Frederick, NIH, Bldg 469, Rm 246, Rm 139, P.O. Box B, Miller Drive, Frederick, MD 21702–1201. Fax: +1-301-846-5598.

E-mail addresses: dimitrov@ncifcrf.gov (D.S. Dimitrov), xiaox@ncifcrf.gov (X. Xiao).

0014-4800/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0014-4800(03)00092-3

c-Myc and Sp1 correlate with the levels of telomerase activity at different stages of transformation (Kyo et al., 2000), some tumors lack c-Myc overexpression despite the presence of telomerase activity. Sp1 protein is abundant in some types of normal cells that do not have high telomerase activity. Thus, the wide divergence of telomerase activity in cancer cells and the cancer-specific telomerase activation may require additional factors yet to be discovered.

Recently, we have found that Ets2 binds specifically to the hTERT promoter and proposed that these proteins and their partners, Id proteins, can play a role in the regulation of telomerase activity (Xiao et al., 2002). Independently, Maida et al. (2002) found that Ets proteins are important for telomerase regulation—they can enhance epidermal growth factor–mediated telomerase up-regulation in cancer cells. The Ets family of transcriptional factors, which is defined by a highly conserved 85-amino acid Ets DNA binding domain (EtsDBD), has more than 30 members and is involved in many important cellular functions, including differentiation, senescence, apoptosis, and tumor suppression (Maroulakou and Bowe, 2000; Sharrocks, 2001; Suzuki et al., 1995). Ets family members have more than 200 targets identified, and the number is increasing. Id is a family of proteins that function as positive regulators of cell growth and inhibitors of cell differentiation (Lasorella et al., 2001; Zebedee and Hara, 2001). They form heterodimers with other helix–loop–helix transcription factors, which drive cell differentiation and lineage commitment upon expression, thus interfering with their DNA binding ability. Id2 was shown to be the oncogenic effector of N-Myc in human neuroblastoma (Lasorella et al., 2000, 2002). Myc expression resulted in increased levels of Id2 that can neutralize the function of Rb to block cell cycle progression. Id1 inhibited the senescence induced by high expression of $p16^{INK4a}$, which was transcriptionally up- regulated by Ets1,2; the inhibitory effect of Id1 on Ets1,2-induced senescence was through direct Id1/Ets association (Ohtani et al., 2001).

The complex interplay of Ets/Id proteins and Myc proteins in the regulation of processes such as cell proliferation, differentiation, and senescence, which correlates to certain extent with telomerase activity, the presence of multiple Ets consensus binding sequences -GGAA- in the hTERT promoter, and recent data (Maida et al., 2002; Xiao et al., 2002) indicating a role of Ets proteins in telomerase regulation prompted us to extensively characterize the role of Ets/Id, Myc, and Sp1 proteins in telomerase regulation. Here we provide evidence that Ets family members and their antagonistic Id proteins are directly involved in negative and positive telomerase transcriptional regulation, suggesting the existence of a complex regulatory network controlling telomerase activity in diverse cancer cells.

Materials and methods

Cells and viruses

Frozen cell pellets and cell cultures from the NCI 60 cancer cell line panel were obtained from the Biological Test Branch, Developmental Therapeutics Program (DTP), NCI. The human promonocytic histiocytic lymphoma U937 line (Sundstrom and Nilsson, 1976) clones 10 (plus clone) and 17 (minus clone) were provided by H. Moriuchi (National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, MD) and propagated in RPMI 1640 with 10% FCS (Moriuchi et al., 1997). The human myeloid erythroleukemia line K562 cells (Lozzio and Lozzio, 1975) were purchased from the American Type Culture Collection and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum plus antibiotics. Retroviral expression constructs expressing FLI-1 (Athanasiou et al., 1996), ETS-1 and ETS-2 (Clausen et al., 1997), and ERF and FLI-1-ERF hybrid (Athanasiou et al., 2000) were used to generate helper free virus stocks. High-titer viruses were prepared from PA317 packaging cells (Miller and Buttimore, 1986) infected with supernatant from ecotropic CRE packaging cells (Danos and Mulligan, 1988). K562 cells were infected with the retroviruses and selected by growth in media containing 0.8 mg/ml Geneticin (Gemini, Calabasas, CA) starting 24–48 h after infection.

Antibodies and plasmids

Antibodies to CKR2 (C-20), Id1 (C-20), Id2 (C-20), Id3 (C-20), Ets-1 (N-276), Ets-2 (C-20), Fli (C-19), c-Myc (N-262), Max (C-124), Mad1 (F1-221), and Sp1 (IC6) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CTCF C-terminus antibody (06-917) was purchased from Upstate Biotechnology (Lake Placid, NY). For quantitative telomerase activity assay, the $TRAP_{EZE}$XL kit was purchased from Intergen (Purchase, NY). Genomic DNA was isolated using the Puregene DNA isolation kit from Gentra (Research Triangle, NC). Luciferase reporter gene constructs carrying different length of hTERT promoter were reported previously (Horikawa et al., 1999) and used in this study to generate a probe of different length in the EMSA and PCR-based DNA/protein interaction protocol. We used the same numbering system on the nucleotides within the hTERT promoter as in our original publication (Horikawa et al., 1999).

Telomerase activity assay

Cells were washed once with ice-cold PBS and their numbers counted. The cell pellet was either lysed immediately or frozen at −80°C until use. Telomerase activity was determined quantitatively by using the fluorescence-based $TRAP_{EZE}$ XL Telomerase Detection Kit from Intergen. All

experimental steps, calibration, and quantitations were carried out according to the manufacture's protocol. The fluorescence signal was captured using the CytoFlour series 4000 fluorocytometer from PerSeptive Biosystems.

PCR based DNA–protein interaction assay and EMSA

Antibodies (1 μg each) against various transcriptional factors were blocked to the bottom of Poly-Sorb NUNC 96-well plates (Nalgene Nunc International). Cell lysate from 2×10^6 cells prepared for telomerase activity assay was added to each well and incubated overnight at 4°C. Each well was then washed twice with 200 μl of the same lysis buffer used to prepare the cell lysate. The wells were equilibrated with a DNA–protein interaction buffer (DPIB) containing 10 mM Tris–HCl (pH 7.4), 50 mM KCl, 5% glycerol, 0.25 mM EDTA, and 2.5 μg/50 μl of salmon sperm DNA, at 4°C for 30 min. After the equilibration buffer was removed, 50 μl of the DPIB containing 20 μg of the 408-bp hTERT promoter DNA was added to each well and incubated at room temperature for 30 min. Each well was then washed three times with the DPIB without hTERT promoter DNA. NaOAc buffer (300 mM/0.1 ml) was then added to each well to dissociate the DNA bound to the protein. The DNA was then precipitated by adding 80 μl of isopropyl alcohol to the DNA solution and centrifuged at 14,000 rpm in a refrigerated Eppendorf centrifuge for 30 min. The pellet was washed with ice-cold 70% ethanol and air dried. Double-distilled water (50 μl) was added to the DNA pellet and 5 μl was used for PCR analysis with primers: 5′ GCG CTT CCC ACG TGG 3′ for upstream and 5′ CTC GCG CCG CGA GG 3′ for downstream. The PCR was performed at 94°C for 30 s, at 52°C for 30 s, and at 72°C for 45 s for a total of 28 cycles, followed by a 72°C, 5-min step. A DNA fragment of 200 bp was generated by this procedure. For EMSA, 149-, 208-, and 408-bp fragments of the hTERT promoter DNA (Horikawa et al., 1999) were end-labled with α-^{32} p-dATP and 10^5 cpm equivalent of each probe was used in each EMSA reaction. The EMSA buffer was the same as that used in the PCR-based assay. The DNA probe and the purified protein (or nuclear extract) were incubated in the EMSA buffer at room temperature for 25 min before being loaded to a 4% nondenaturing polyacrylamide gel and run in 0.5% TBE buffer. The gel was dried and exposed to X-ray film.

Transient transfection and luciferase activity assay

K562 cells (2×10^6) were transfected with 4.5 μg total DNA using D-MRIE (Life Technologies) according to the manufacturer's instructions. The control plasmid pGL3 expression vector, which lacks an eukaryotic promoter, was utilized as a negative control. All plates also received 0.5 μg of RSV-gal, as a control for transfection efficiency. Forty hours after transfection, cells were lysed in Passive Lysis Buffer (Promega, Madison, WI), and luciferase and β-galactosidase (β-gal) activities were analyzed using the Promega Luciferase Assay System (Promega) and Tropix Galacto-Light Plus Kit (Tropix, Bedford, MA), respectively. Relative luciferase activities were determined for duplicate samples in three to five separate experiments and were normalized by setting all β-gal levels to be equal. Point mutations within the hTERT promoter were generated using the QuickChange Site- directed Mutagenesis Kit from Stratagene.

Results

Specific interactions between Ets family members, Id2, and a core hTERT promoter

We have previously shown that Ets1 and Ets2 bind to a core hTERT promoter by using a sensitive PCR-based assay for detection of protein–DNA interaction that are below the sensitivity of the gel shift assay. By using the same assay we found that another Ets family member, Fli1, and Id2 also bind specifically to a core hTERT promoter in cell lysates from two subclones of U937 cells (Fig. 1a). The relative amount of bound promoter DNA was proportional to the amount of Fli1 or Id2 in the cell lysates from minus and plus U937 cells as measured by Western blotting. Id2 lacks DNA binding domain (DBD) and its binding is likely mediated through another protein. We also found that c-Myc and Sp1 bind the hTERT promoter in lysates from plus cells but not from minus cells while Mad1 binds the hTERT promoter in lysates from minus cells that is consistent with their levels of expression in these cells (data not shown). Except the Sp1 binding to the hTERT promoter, all these protein–DNA interactions were not detectable in cell lysates by the gel shift assay (data not shown).

To further characterize the interactions between Ets proteins, Id2, and a core hTERT promoter, we expressed a protein containing the EtsDBD and the full-length Id2 as His-tagged fusion proteins in *Escherichia coli* and tested their binding to the core hTERT promoter by the gel shift assay. The EtsDBD bound to the hTERT promoter (Fig. 1b). Mutation of sites A and B (see Fig. 1) simultaneously or the use of shorter promoter DNA sequences that do not contain sites A and B significantly reduced but did not eliminate the EtsDBD binding (Fig. 1c). Mutation of Ets binding sites C and one of the two -GGAA- sequences in D did not lead to a significant further decrease of the Ets–DNA complex mobility (data not shown). Mutation of site A alone resulted in the highest level of binding inhibition (data not shown), suggesting its dominant role in the EtsDBD interaction with the hTERT promoter. Id2 protein expressed and purified through exactly the same procedure did not bind to the core hTERT promoter and did not inhibit the interaction of purified EtsDBD with the hTERT promoter in the gel shift

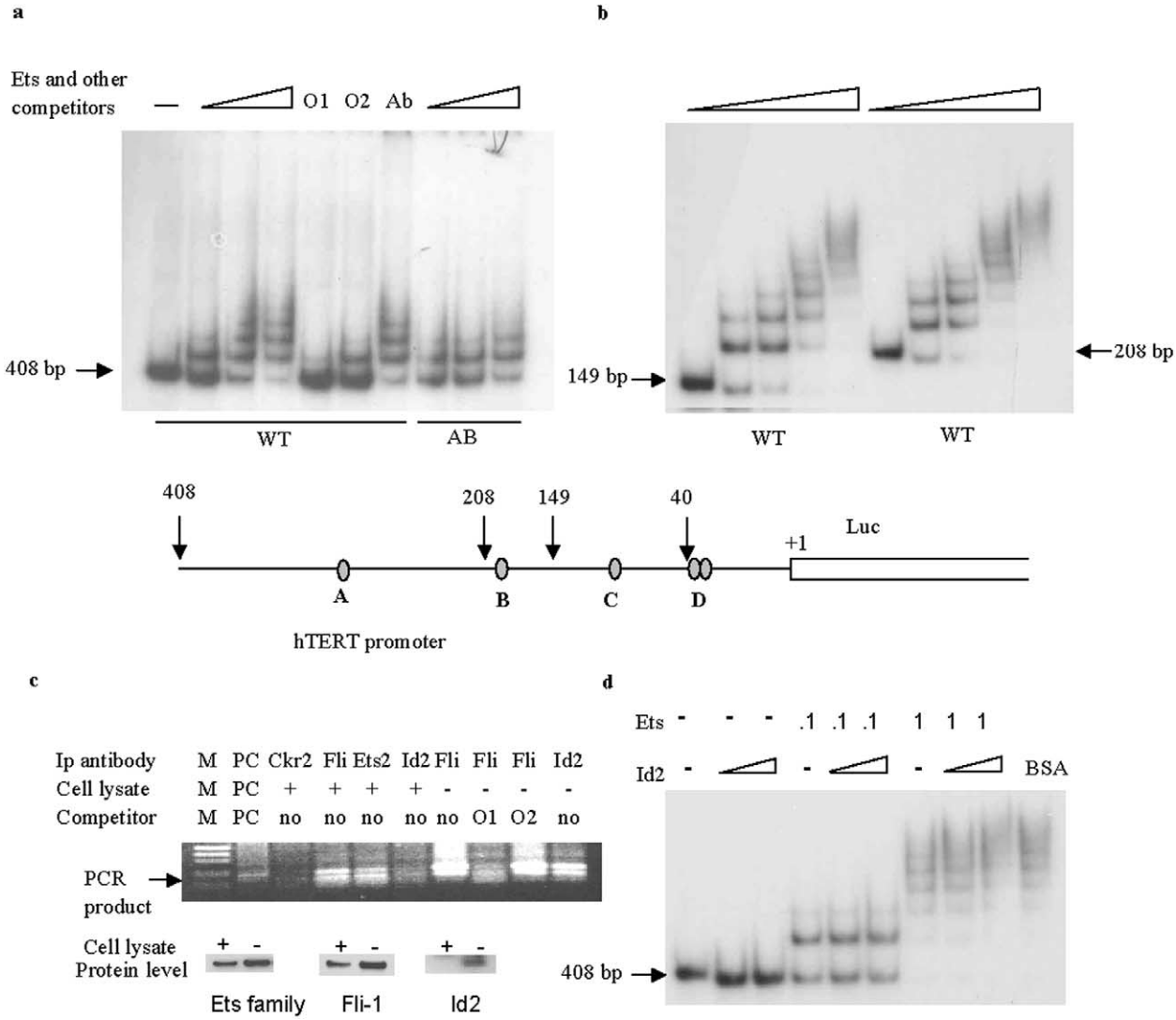

Fig. 1. Specific binding of Ets family members to hTERT promoter. Shown in the center is the schematic representation of the hTERT core promoter controlling the expression of the luciferase reporter gene. Ovals represent potential Ets binding sites containing -GGAA- core sequences. Arrows mark the positions of nucleotides corresponding to the numbers derived from the original publication describing the characterization of the hTERT promoter. (a) Specific binding of ETS-DBD to hTERT promoter. ETS-DBD expressed in *E. coli* was shown to bind to hTERT promoter specifically. The full-length core promoter with 408 bp was used in EMSA assay. Those lanes underlined and marked with WT are EMSA with wild-type promoter and the rest marked with AB are EMSA with the same promoter but sites A and B mutated to -TTAA-. For both WT and AB promoter, the same increasing amount of ETS-DBD was used in EMSA. For WT promoter, 80 ng of specific competitors O1 and O2 were also used in EMSA, with O1 containing -GGAA- sequence and O2 having -GGAA- changed to -AGAA-. Ab indicates that ETS-DBD antibody was included in EMSA assay. −, promoter control. The same amount of ETS-DBD was used in lanes 2, 5, and 6. (b), fragments of hTERT promoter were also recognized by ETS-DBD. HTERT fragments in the length of 149 and 208 bp were recognized by ETS-BDB. The same increasing amount of ETS-DBD was used in EMSA assay with both fragments. (c) Specific binding of ETS factors from cell lysate to hTERT promoter. Antibodies used in the capture of various factors, cell lysate, and specific competitors were indicated. + and −, Plus and minus cells. The amount of O1 and O2 used was the same as in EMSA. (d) Lack of inhibition of ETS-DBD DNA binding by Id2. Same amount of increasing concentration of ID2 was added to 408-bp promoter in the absence of or in different amount of ETS-DBD.

assay (Fig. 1d). The protein samples of EtsDBD and Id2 used in the EMSA assay are shown in Fig 2.

The purified EtsDBD also bound to purified Id2 as demonstrated by two independent sets of experiments—coimmunoprecipitation by use of anti-Id2 and anti-EtsDBD antibodies (Fig. 2 and data not shown) and the use of Id2–GST fusion proteins that associated specifically with Ets factors (Fig. 3). The Id2–Ets interaction is likely determined by the balance of repulsive electrostatic and attractive hydrophobic interactions as demonstrated by their increased binding in solutions of increased ionic strength (Fig. 4). Overall these results demonstrate the possibility for existence of a trimolecular Ets–Id2–DNA complex in which Id2 binds to the EtsDBD but in a region that is distinct from the DNA binding site, and EtsDBD binds predominantly to the A site on the hTERT promoter.

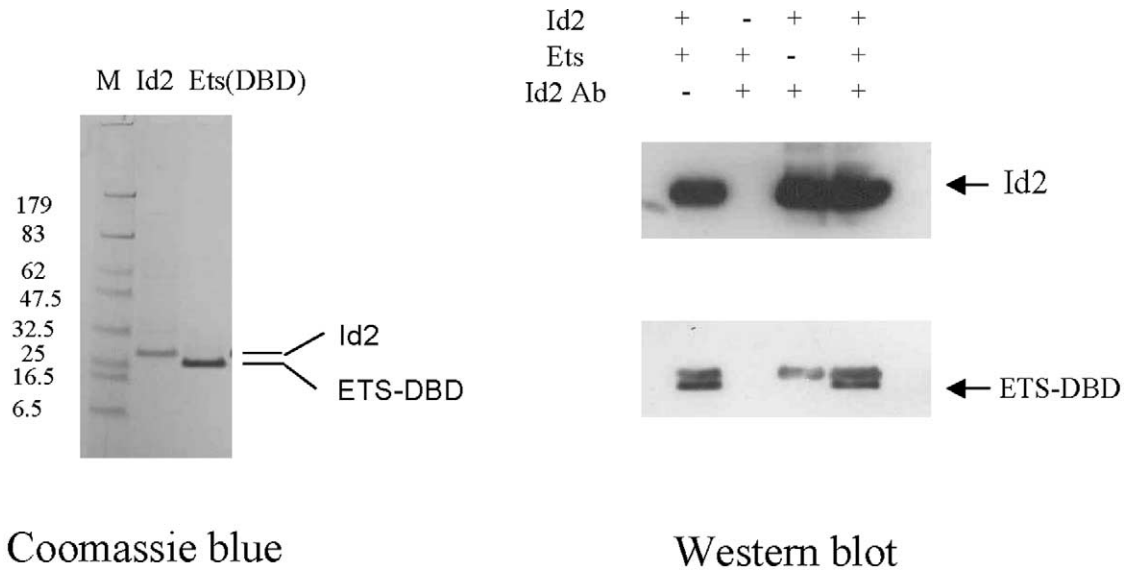

Fig. 2. Specific association between Id2 and ETS-DBD shown by coimmunoprecipitation. (Left) Id2 and ETS-DBD expressed in *E.coli* and purified using nickel ion column. (Right) Anti-Id2 antibody can immunoprecipitate ETS-DBD in the presence of Id2, but not when Id2 is absent.

Downregulation of telomerase activity by Ets and upregulation by Id2

To test the functional significance of Ets and Id2 interactions with the hTERT promoter, we expressed Ets proteins and Id2 and measured telomerase activity in cell lysates. Telomerase activity was decreased in K562 cells transiently or stably transfected either with Ets1 or Ets2 or with a hybrid of Fli1 and Erf but not significantly when transfected with Fli1 or Erf alone; in contrast Id2 transfection increased telomerase activity, although c-Myc and Sp1 levels did not change (Fig. 5 and data not shown). These results suggest the existence of an inhibitory effect on telomerase activity by Ets proteins, with Ets1 being of highest activity of those tested, and an up-regulatory effect by Id2.

Contrasting effects of Ets binding sites on the hTERT promoter activity—existence of a positive regulatory site

To find whether the hTERT promoter is the direct target of Ets and how various Ets binding sites on the promoter affect its activity, we used a 408-bp hTERT promoter controlling a reporter luciferase gene expression and mutated

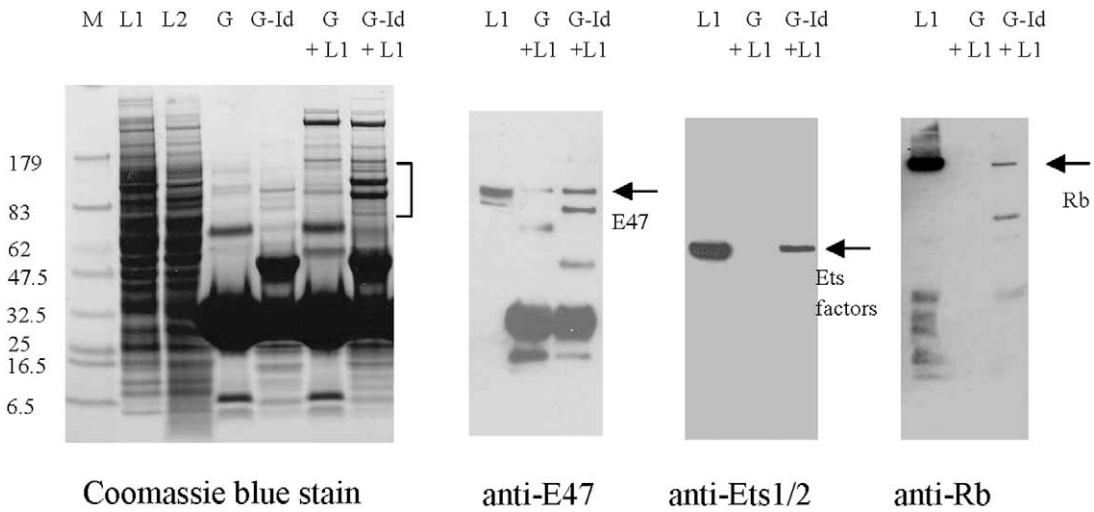

Fig. 3. Specific association between Id2 and Ets factors revealed by GST pull-down experiment. Id2 was expressed as a fusion protein with GST and used in the pull-down experiment with cell lysate from plus cells. (Left) Cell lysate from plus and minus cells, purified GST and GST-ID2, and protein pull down by GST and GST-ID2 on a Comassie blue-stained gel. (Right) Western blots with indicated antibodies on the samples shown also on left by Comassie blue stain. M, molecular weight marker shown in kDa; L1, lysate from plus cells; L2, lysate from minus cells; G, GST only; G–Id, GST–Id2.

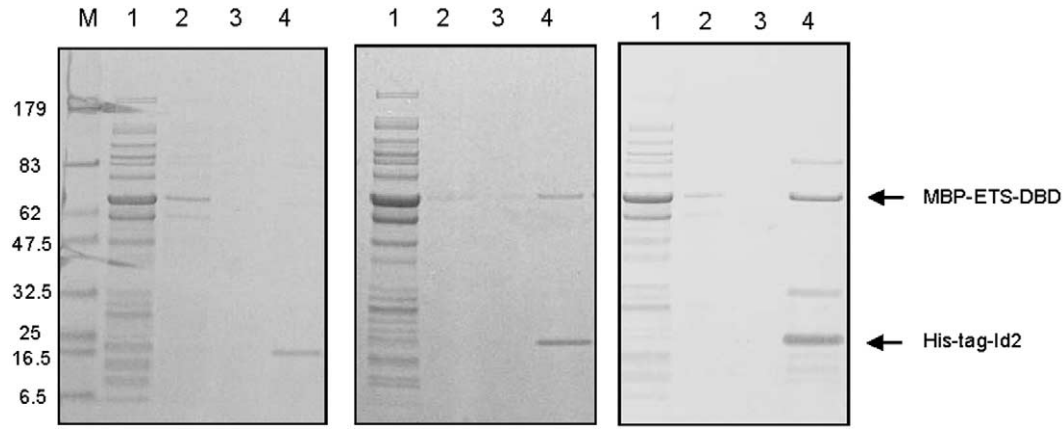

Fig. 4. Specific association between Id2 and ETS-DBD was increased by increased salt concentration. MBP–ETS-DBD and His-tag-Id2 were coexpressed in *E. coli* and subjected to purification by nickel ion column. In each panel, lanes 1–4 represent crude cell lysate, washed with loading buffer, eluted with washing buffer containing 50 mM imidazole, and eluted with washing buffer containing 500 mM imidazole. (Left) Washing buffer contained 25 mM Tris–HCl, pH 8.0, and 100 mM NaCl. (Middle) NaCl concentration was increased to 400 mM. (Right) NaCl concentration was increased to 600 mM. Everything else in the washing buffer remained the same. M, molecular weight marker in kDa.

Ets binding sites. Mutations of Ets binding sites on the core hTERT promoter at −293 bp (A), −190 bp (B), and −138 bp (C) from the transcription initiation site (see also Fig. 1) alone or in combination increased the telomerase activity to various extents, with the A site being most active, suggesting its dominant role for the Ets inhibitory activity (Fig. 6a and data not shown). Simultaneous mutations of site A and the c-Myc binding site resulted in telomerase activity that was decreased to the same level as for the c-Myc binding site mutation alone, suggesting that the Ets inhibitory effect through site A is mediated by c-Myc (Fig. 6b). Interestingly, a mutation of an Ets binding site at −36 bp (site D), alone or in combination with mutations in sites A, B, and C, reduced telomerase activity, suggesting the existence of a positive Ets regulatory effect mediated through D (Fig. 6c and data not shown). These results provide evidence for the existence of negative and positive Ets regulatory sites and for the complex interplay between Ets/Id family members and c-Myc.

Telomerase activity of diverse cancer cell lines—correlation with c-Myc and lack of correlation with Sp1 and Ets

The possibility for negative and positive regulatory effects of Ets proteins on telomerase activity and the interplay with c-Myc suggested the possibility that variability of telomerase activity in diverse cancer cell lines could be partially due to modulation of a dominant c-Myc effect by Ets/Id proteins or to Ets/Id interplay in the absence of c-Myc. To begin to exploit this hypothesis we performed preliminary studies with the NCI 60 cancer cell line panel. Telomerase activity of these cells was measured by the TRAP assay and the levels of c-Myc, Sp1, and Ets expression were measured by Western blotting. Telomerase activity and the levels of c-Myc and Sp1 expression were highly variable in the 58 cancer cell lines that were measured. The levels of c-Myc and Sp1 were below the assay sensitivity in 7 and 8 cell lines, respectively, while telomerase activity

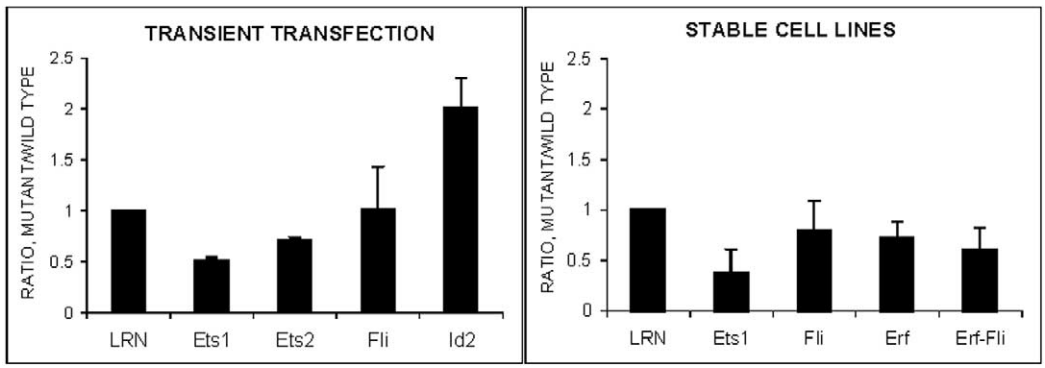

Fig. 5. Inhibitory effect of Ets factors on hTERT activity. (Left) Ets factors cloned into pLRN were transfected into K562 cells transiently and the telomerase activity of transfected cells was analyzed using the TRAP assay 48 h after transfection. The hTERT activity from control cells was set at one and the others were compared to the control. (Right) K562 cells stably transfected with various ETS factors as indicated were analyzed for their hTERT activity. Again, control cell activity was assigned as one.

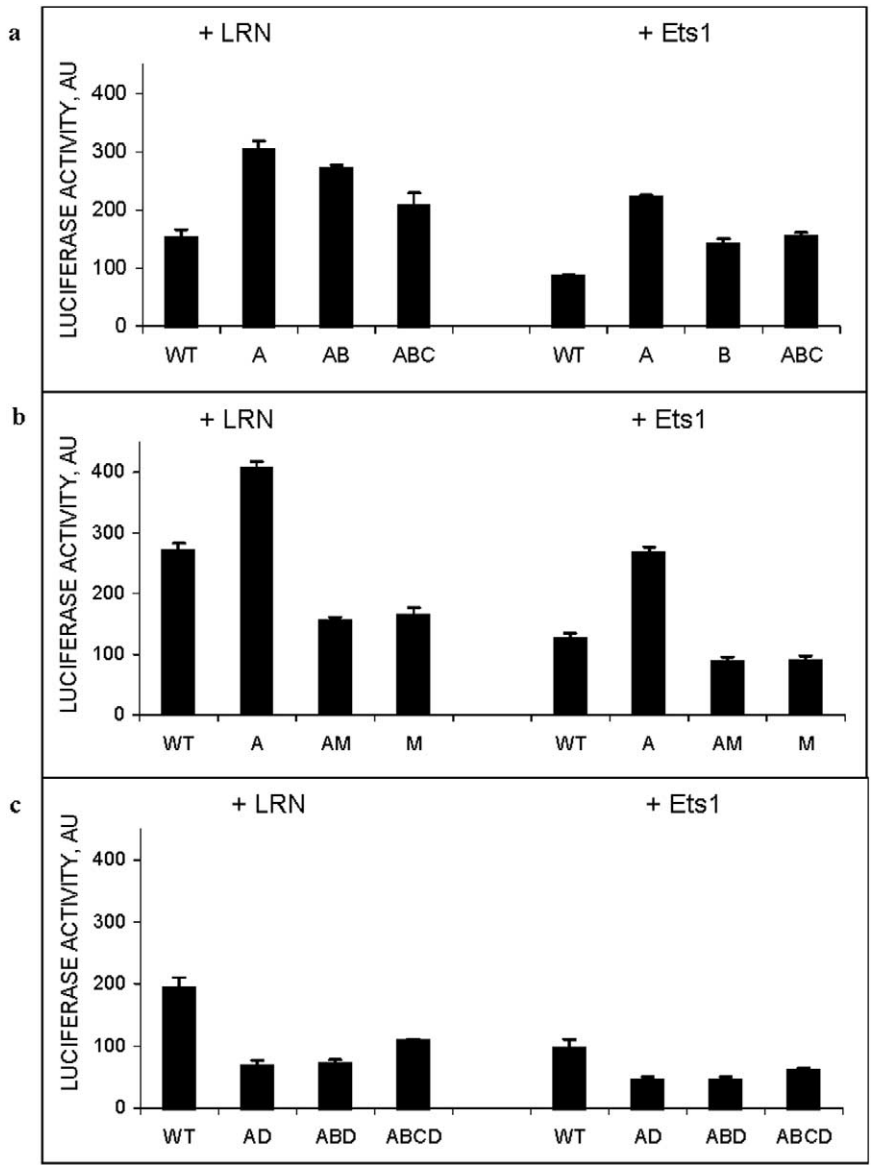

Fig. 6. The effect of Ets1 on the hTERT promoter activity. (a) Mutations of potential Ets binding sites as well as the Ets1 effect on the hTERT promoter was evaluated. WT, A, AB, and ABC, wild-type promoter or promoter with binding sites A, AB, or ABC mutated from -GGAA- to -TTAA-. (b) The inhibitory effect of Ets is through other positive regulators. Wt, wild-type promoter; A and AM, binding sites A and c-Myc changes from -GGAA- to -TTAA- and -CACGTG- to -CACGGA-. (c) Site D is a positive regulatory site of hTERT. AD, ABD, and ABCD, site changes from -GGAA- to -TTAA-. For site D, only half of the two -GGAA- sites was changed. In each panel, the left represents transfection of reporter construct with pLRN plasmid, and the right represents transfection of reporter construct with Ets1 cloned in pLRN.

was detected in all cell lines. Seven cancer cell lines representing prostate, breast, and ovarian cancers with highly variable telomerase activity were selected for measurements of the levels of Ets expression (by using an antibody to domain that is conserved among many Ets family members) and cell doubling time (Table 1). c-Myc levels correlated well ($r = 0.55$, $n = 58$, $p < 0.001$) with telomerase activity, indicating its dominant role for telomerase regulation in many (but not all) cancer cell lines; in contrast Sp1 and Ets levels did not correlate ($r = 0.15$, $n = 58$; $r = 0.16$, $n = 7$, $p > 0.05$). As expected there was very good negative correlation between telomerase activity and cell doubling time ($r = 0.79$, $n = 7$, $p = 0.039$). These results support the notion for a dominant role of c-Myc in telomerase regulation of cancer cells and the complex interplay with Sp1 and Ets proteins.

Discussion

The regulation of telomerase activity is a multifactorial process that involves a number of transcriptional factors (Kyo and Inoue, 2002). This work adds another component to this complex mechanism—Ets proteins and their inter-

Table 1
Correlation between hTERT activity and cell doubling time and expression of critical regulatory factors

Cell/factor	c-Myc	Sp1	Ets	Telomerase activity (ΔF/ΔR)	Doubling time
BT549 breast cancer	+	+++	3500	0.27	54
MCF-7 breast cancer	+	+++	3930	1.48	25
MDAMB435 breast cancer	+++	++	3850	2.73	26
OVARCAR5 ovarian cancer	+	+	4750	0.14	49
OVARCAR8 ovarian cancer	+++	++	4410	2.13	26
DU145 prostate cancer	++	+	2280	0.31	32
PC3 prostate cancer	+++	+++	2830	1.72	27

Note. HTERT activity is described as F/R as instructed by the TRAP kit manufacturer. Doubling time is in hours and the expression of c-Myc, Sp1, and Ets is described in absolute units derived from phosphoimager quantification. +++, expression level comparable to that of PC3; +, level at the threshold of detection; ++, levels in between.

play with c-Myc and Id proteins. Not only do Ets proteins bind to the hTERT promoter but the activity of the different binding sites can inhibit or enhance in dependence on the position of the binding site and the presence of other transcription factors, specifically c-Myc. Expression of different Ets proteins also leads to different extents of telomerase inhibition. We have not tested all Ets protein family members but it seems plausible that they could exhibit a wide range of positive or negative regulatory effects on the telomerase activity. Similar considerations apply for the Id family proteins and different combinations with Ets family members. Because the Ets inhibitory effect is c-Myc dependent one can expect that variations in the c-Myc levels could further modulate the extent of Ets protein influence on telomerase activity. One can speculate that the Ets inhibitory effect on the up-regulation of telomerase activity by c-Myc is due to interference with c-Myc binding—experiments are in progress to test this possibility. If this is the case then the overall effect of Ets–Myc interplay could be determined by the protein–DNA affinities and the protein concentrations. A note of caution is that other potential Ets binding sites not analyzed in this work could modulate hTERT promoter activity without eliminating its responsiveness to the repressive effect of Ets. This possibility is supported by our finding that hTERT promoter containing mutations on sites A, B, C, and D was still recognized by the EtsDBD. In addition, potential Ets binding sites outside the core hTERT promoter could play a role. For example, one such potential site is 27 bp downstream from the transcription initiation site (Horikawa et al., 1999). Further studies are required to clarify the molecular details of Ets-mediated telomerase regulation, although it is clear that these effects are dependent on the interplay with other transcription factors, especially members of the Id protein family.

The dual effects of Ets on hTERT promoter activity could be important for differential effects of Ets factors on telomerase activity in normal and cancer cells. In normal cells Ets factors are expressed while c-Myc is not. It is possible that Ets proteins upregulate hTERT expression in normal cells since their inhibitory effect is c-Myc dependent, whereas in cancer cells-Ets effects might be positive or negative depending on the concentrations of Ets proteins and c-Myc. This is also consistent with the previous findings that Ets proteins can serve both as oncogenes and tumor suppressors and differentiation and apoptosis-inducing factors (Maroulakou and Bowe, 2000; Sharrocks, 2001; Suzuki et al., 1995). Interestingly, several E2F proteins exhibit somewhat similar opposing effects—repression of telomerase activity in tumor cells and its up-regulation in normal cells (Won et al., 2002).

Id proteins can induce cell proliferation and recent reports show that they are overexpressed in various cancer types, suggesting a role in carcinogenesis (Hasskarl and Munger, 2002; Lasorella et al., 2001). We have recently observed that Id2 is overexpressed in a U937 subclone (minus cells) with very low telomerase activity but rapid cell division comparable to that of another U937 subclone (plus cells) with very high telomerase activity and much lower (10- to 50-fold) Id2 expression (Xiao et al., 2002). This observation appeared to indicate a negative correlation of Id2 expression with telomerase activity. However, in view of the results reported here and data suggesting that high levels of Id2 inhibit the suppressor function of Rb (Lasorella et al., 2002), it seems likely that Id2 has a dual function. It up-regulates hTERT expression by inhibiting the suppressor effects of Ets proteins and drives rapid proliferation by inactivating Rb even at low concentrations of c-Myc. What could be an important consideration for diagnosis and therapies involving telomerase is that high concentrations of Id proteins appear to be able to drive cancer cell division at very low, barely detectable levels of telomerase activity. What upregulates Id2 at the low c-Myc concentrations in the minus U937 cells remains unclear. It has been suggested that blocking Id2 activity could be an important strategy for treatment of cancer because of the Id2 inactivating effect on Rb (Lasorella et al., 2002). Our results suggest that inactivating Id2 in cancer cells could have an additional beneficial effect of downregulating telomerase activity.

Previous studies have provided evidence that c-Myc and Sp1 are involved in hTERT transcriptional regulation (Kyo

and Inoue, 2002). However, in some cancer cells telomerase activity apparently can be regulated independently on c-Myc (Drissi et al., 2001). We found that in the 60 cancer cell line panel there was a general correlation between the c-Myc expression and telomerase activity. However, many cancer cell lines with undetectable c-Myc exhibited high telomerase activity and vice versa. This suggests that, although c-Myc plays an important role in the regulation of the telomerase activity in cancer cells, other factors could be equally important. Among them Sp1 certainly plays a role in the hTERT expression regulation although we did not find any correlation between its expression and telomerase activity for the NCI 60 cancer cell line panel. There was no correlation between the Ets concentration and telomerase activity for 7 cancer cell lines. Although the number of cell lines tested is relatively small to make any definite conclusions, it appears likely that the lack of correlation is valid for many cancer cells probably due to the ability of the Ets proteins to regulate telomerase activity both positively and negatively. In addition, because of the large number of the Ets family members (more than 30), it is likely that different members could regulate telomerase activity differently. Of note is that the antibody used for Western blotting of the 7 cancer cell lines does not distinguish between individual members of the Ets family. Experiments are in progress to examine the levels of different members of the Ets and Id families in the NCI panel of 60 cancer cell lines and how they correlate with telomerase activity.

In conclusion, our data further support the role of Id and Ets proteins for telomerase regulation in cancer cell lines and provide a molecular mechanism. While c-Myc is a major determinant of the telomerase regulation in many cancer cell lines, Id and Ets proteins could be critical for the fine regulation and diversity of telomerase activity through effects on the hTERT promoter.

Acknowledgments

We are grateful to Dr. Satoru Kyo for helpful comments. We thank Richard Camalier of Biological Test Branch, DTP, NCI for providing frozen pellets and cell cultures from the NCI 60 cell line panel, Dr. Sanjay Phogat for helping with the RT–PCR cloning of Id2, and Dr. David Waugh for constructing the ETS-DBD/ID2 coexpression vector. This work is supported by the NCI intramural program. The publication has been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract NO1-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

Athanasiou, M., Clausen, P.A., Mavrothalassitis, G.J., Zhang, X.K., Watson, D.K., Blair, D.G., 1996. Increased expression of the ETS-related transcription factor FLI-1/ERGB correlates with and can induce the megakaryocytic phenotype. Cell Growth Differ. 7, 1525–1534.

Athanasiou, M., LeGallic, L., Watson, D.K., Blair, D.G., Mavrothalassitis, G., 2000. Suppression of the Ewing's sarcoma phenotype by FLI1/ERF repressor hybrids. Cancer Gene Ther. 7, 1188–1195.

Clausen, P.A., Athanasiou, M., Chen, Z., Dunn, K.J., Zhang, Q., Lautenberger, J.A., Mavrothalassitis, G., Blair, D.G., 1997. ETS-1 induces increased expression of erythroid markers in the pluripotent erythroleukemic cell lines K562 and HEL. Leukemia 11, 1224–1233.

Cong, Y.S., Wen, J., Bacchetti, S., 1999. The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. Hum. Mol. Genet. 8, 137–142.

Cong, Y.S., Wright, W.E., Shay, J.W., 2002. Human telomerase and its regulation. Microbiol. Mol. Biol. Rev. 66, 407–425, table.

Danos, O., Mulligan, R.C., 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA 85, 6460–6464.

Drissi, R., Zindy, F., Roussel, M.F., Cleveland, J.L., 2001. c-Myc-mediated regulation of telomerase activity is disabled in immortalized cells. J. Biol. Chem. 276, 29994–30001.

Ducrest, A.L., Amacker, M., Mathieu, Y.D., Cuthbert, A.P., Trott, D.A., Newbold, R.F., Nabholz, M., Lingner, J., 2001. Regulation of human telomerase activity: repression by normal chromosome 3 abolishes nuclear telomerase reverse transcriptase transcripts but does not affect c-Myc activity. Cancer Res. 61, 7594–7602.

Ducrest, A.L., Szutorisz, H., Lingner, J., Nabholz, M., 2002. Regulation of the human telomerase reverse transcriptase gene. Oncogene 21, 541–552.

Fu, W., Lu, C., Mattson, M.P., 2002. Telomerase mediates the cell survival-promoting actions of brain-derived neurotrophic factor and secreted amyloid precursor protein in developing hippocampal neurons. J. Neurosci. 22, 10710–10719.

Greenberg, R.A., O'Hagan, R.C., Deng, H., Xiao, Q., Hann, S.R., Adams, R.R., Lichtsteiner, S., Chin, L., Morin, G.B., DePinho, R.A., 1999. Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. Oncogene 18, 1219–1226.

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

Gunes, C., Lichtsteiner, S., Vasserot, A.P., Englert, C., 2000. Expression of the hTERT gene is regulated at the level of transcriptional initiation and repressed by Mad1. Cancer Res. 60, 2116–2121.

Hasskarl, J., Munger, K., 2002. Id proteins: tumor markers or oncogenes. Cancer Biol. Ther. 1, 91–96.

Horikawa, I., Cable, P.L., Afshari, C., Barrett, J.C., 1999. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res. 59, 826–830.

Kyo, S., Inoue, M., 2002. Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy. Oncogene 21, 688–697.

Kyo, S., Takakura, M., Taira, T., Kanaya, T., Itoh, H., Yutsudo, M., Ariga, H., Inoue, M., 2000. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). Nucleic Acids Res. 28, 669–677.

Lasorella, A., Boldrini, R., Dominici, C., Donfrancesco, A., Yokota, Y., Inserra, A., Iavarone, A., 2002. Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. Cancer Res. 62, 301–306.

Lasorella, A., Noseda, M., Beyna, M., Yokota, Y., Iavarone, A., 2000. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature 407, 592–598.

Lasorella, A., Uo, T., Iavarone, A., 2001. Id proteins at the cross-road of development and cancer. Oncogene 20, 8326–8333.

Lozzio, C.B., Lozzio, B.B., 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45, 321–334.

Maida, Y., Kyo, S., Kanaya, T., Wang, Z., Yatabe, N., Tanaka, M., Nakamura, M., Ohmichi, M., Gotoh, N., Murakami, S., Inoue, M., 2002. Direct activation of telomerase by EGF through Ets-mediated transactivation of TERT via MAP kinase signaling pathway. Oncogene 21, 4071–4079.

Maroulakou, I.G., Bowe, D.B., 2000. Expression and function of Ets transcription factors in mammalian development: a regulatory network. Oncogene 19, 6432–6442.

Miller, A.D., Buttimore, C., 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. 6, 2895–2902.

Moriuchi, H., Moriuchi, M., Arthos, J., Hoxie, J., Fauci, A.S., 1997. Promonocytic U937 subclones expressing CD4 and CXCR4 are resistant to infection with and cell-to-cell fusion by T-cell-tropic human immunodeficiency virus type 1. J. Virol. 71, 9664–9671.

Ohtani, N., Zebedee, Z., Huot, T.J., Stinson, J.A., Sugimoto, M., Ohashi, Y., Sharrocks, A.D., Peters, G., Hara, E., 2001. Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. Nature 409, 1067–1070.

Sharrocks, A.D., 2001. The ETS-domain transcription factor family. Nat. Rev. Mol. Cell. Biol. 2, 827–837.

Shay, J.W., Bacchetti, S., 1997. A survey of telomerase activity in human cancer. Eur. J. Cancer 33, 787–791.

Sundstrom, C., Nilsson, K., 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer 17, 565–577.

Suzuki, H., Romano-Spica, V., Papas, T.S., Bhat, N.K., 1995. ETS1 suppresses tumorigenicity of human colon cancer cells. Proc. Natl. Acad. Sci. USA 92, 4442–4446.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., Inoue, M., 1999. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res. 59, 551–557.

Won, J., Yim, J., Kim, T.K., 2002. Opposing regulatory roles of E2F in human telomerase reverse transcriptase (hTERT) gene expression in human tumor and normal somatic cells. FASEB J. 16, 1943–1945.

Wu, K.J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J., Dalla-Favera, R., 1999. Direct activation of TERT transcription by c-MYC. Nat. Genet. 21, 220–224.

Xiao, X., Phogat, S.K., Sidorov, I.A., Yang, J., Horikawa, I., Prieto, D., Adelesberger, J., Lempicki, R., Barrett, J.C., Dimitrov, D.S., 2002. Identification and characterization of rapidly dividing U937 clones with differential telomerase activity and gene expression profiles: role of c-Myc/Mad1 and Id/Ets proteins. Leukemia 16, 1877–1880.

Xu, D., Popov, N., Hou, M., Wang, Q., Bjorkholm, M., Gruber, A., Menkel, A.R., Henriksson, M., 2001. Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. Proc. Natl. Acad. Sci. USA 98, 3826–3831.

Zebedee, Z., Hara, E., 2001. Id proteins in cell cycle control and cellular senescence. Oncogene 20, 8317–8325.