Expression of the hTERT Gene Is Regulated at the Level of Transcriptional Initiation and Repressed by Mad1

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Abstract

Telomerase, an enzymatic activity responsible for the replication of chromosome end structures, is strongly upregulated in most human cancers. In contrast, most differentiated tissues are telomerase negative. The rate-limiting step for telomerase activity seems to be the expression of the catalytic subunit of the enzyme, encoded by the human telomerase reverse transcriptase (hTERT) gene. The precise mechanism of how hTERT expression is regulated has not been elucidated yet. We show here that the down-regulation of hTERT mRNA during 12-0-tetradecanoylphorbol-13-acetate-induced differentiation of human U937 cells is a consequence of a fast decrease in the rate of transcription rather than changes in its half-life. The only transcription factor that has so far been implicated in the regulation of hTERT expression is the c-Myc oncoprotein. Our analysis shows that another member of the myc/max/mad network, mad1, encoding a transcriptional repressor that is significantly increased by 12-0-tetradecanoylphorbol-13-acetate treatment, represses hTERT promoter-driven reporter gene activity in transient transfection assays. This effect is dependent on the NH2-terminal domain of Mad1, which mediates the association with the transcriptional corepressor mSin3. Our findings suggest the involvement of an additional transcription factor in the regulation of hTERT expression and may provide a model for how hTERT activity is controlled during the differentiation process in human somatic tissues.

Introduction

Telomerase is a ribonucleoprotein complex that is responsible for the complete replication of chromosomal ends. These end structures, named telomeres, serve as protective caps and consist of short tandemly repeated DNA sequences. In humans, this sequence is TTAGGG, and the average telomere length is 5–15 kb (1, 2). Upon each cell division the chromosomal ends shorten at a rate of ~50–200 bp (3). This molecular erosion sets a physical limit to the potential number of cell divisions and serves as a “mitotic clock” defining the lifespan of somatic cells. One mechanism to escape this limitation is the activation of telomerase. Because telomerase can reset the mitotic clock, it has been linked to the processes of tumorigenesis and aging.

To date, three components associated with telomerase activity in humans have been identified. The RNA component of telomerase is encoded by the hTR gene (Refs. 4 and 5; now called hTERT) and functions as the template for elongation of the telomeric repeat units. hTERT is constitutively expressed in all cells and is therefore not likely to be involved in the regulation of telomerase activity (6). The same is true for another protein that is associated with telomerase, the product of the TP1/TLP1 gene (now called hTERTP1), which is also expressed ubiquitously (7, 8). Recently, the catalytic subunit of human telomerase has been cloned and named hTERT2 because it possesses the activity of an RNA-dependent DNA polymerase (9–11). Only hTERT and hTERT are required for the reconstitution of telomerase activity in vitro and therefore represent the minimal catalytic core of telomerase in humans (12).

Transfection of telomerase-negative cells with an hTERT cDNA has demonstrated that hTERT expression is rate-limiting for telomerase activity (13–15). Moreover, cells that ectopically express hTERT overcome crisis and have an extended life span, supporting the causal relationship between the shortening of telomeres and cellular senescence (16, 17). Telomerase activity has been shown to be associated with proliferation (18, 19); therefore, it is not surprising that almost all immortal and cancer cells display significant telomerase activity and express hTERT (20, 21). These observations and the finding that the myc oncogene can activate hTERT expression (22–25) indicate that this gene may play a critical role in tumorigenesis.

While telomerase becomes activated during neoplastic transformation, telomerase activity decreases during differentiation processes, which are accompanied by loss of proliferative potential. This has been studied in embryonic stem cells that were differentiated by the removal of leukemia inhibitory factor from the culture medium and in F9 teratocarcinoma after retinoic acid-induced differentiation (26). Loss of telomerase activity upon differentiation was also demonstrated in K-562 human erythroid leukemia cells as well as in HL-60 human promyelocytic leukemia cells (26, 27). In the latter, telomerase down-regulation was demonstrated to be an early event of the differentiation process and not its consequence (28). Repression of telomerase activity was subsequently demonstrated to be associated with the down-regulation of hTERT expression upon differentiation of HL-60 cells with TPA (10, 29). It is, however, not yet clear at which level of gene expression this down-regulation occurs.

The only transcription factor that has thus far been implicated in the regulation of hTERT is encoded by the c-myc oncogene (22–25). The ability of c-Myc to function as a transcription factor has been shown to depend upon its dimerization with the protein Max (reviewed in Ref. 30). In addition to the formation of stable complexes with c-Myc, Max also heterodimerizes with proteins of the Mad(Mxi1) family (30–32). These Mad/Max complexes act in an antagonistic manner to c-Myc/Max-induced transactivation and result in potent repression of gene expression. How these additional members of the Myc/Max/Mad network affect hTERT expression has, however, not been studied yet.

We have used the differentiation of human hematopoietic U937 cells by phorbol ester as a model to study the regulation of hTERT expression. We demonstrate here that the half-life of the hTERT mRNA is not altered by TPA treatment. Nuclear run-off analysis shows that the down-regulation of hTERT is a consequence of a decrease in transcriptional initiation.

2 The abbreviations used are: hTERT, human telomerase reverse transcriptase; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRAP, telomeric repeat amplification protocol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RA, retinoic acid; RT-PCR, reverse transcription-PCR; ODC, ornithine decarboxylase; FACS, fluorescence-activated cell sorter.
tion of the gene. Furthermore, the decrease in hTERT activity is not preceded by a down-regulation of the oncoprotein c-Myc but is paralleled by an increase in levels of mad1. In transient transfection assays, Mad1 was able to repress hTERT promoter activity, an effect that was dependent on an E-box consensus sequence. The repressive effect of Mad1 also required an intact NH2 terminal domain, which mediates the interaction with the corepressor mSin3.

Materials and Methods

Cell Culture, Differentiation, and Immunoprecipitations. U937 cells were obtained from the European Collection of Cell Cultures and were kept in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. For differentiation, cells were seeded at a density of 1 × 10⁶/ml and TPA (10 ng/ml in 0.1% DMSO) or retinoic acid (5 × 10⁻⁶ M in ethanol) was added to the medium. The transcripational inhibitor actinomycin D was used at 5 μg/ml. Differentiation was assessed by expression of the monocytic markers CD11a and CD11c as well as CD4, which is expressed on immature U937 cells. Analysis was done using FACS assay with the respective monoclonal antibodies (PharMingen).

Metabolic labeling followed by high stringency immunoprecipitation was done as described (33). For the detection of Mad1, a polyclonal antiserum directed against the COOH terminus of human Mad1 was used (Santa Cruz Biotechnology).

Constructs, Transfections, and Reporter Gene Assays. Generation of the hTERT promoter constructs pGRN150 and pGRN261 has been described (22). pGRN176 has been generated by digestion of pGRN150 with PmlI and subsequent religation. Construct pGRN316 contains nucleotides +1 to −246 from the hTERT genomic sequence, i.e., sequences immediately upstream of the ATG codon, and has the same 3′ configuration as pGRN261 (cloned into the EcoRI site of pSEAP2-Basic).

U937 cells were transfected in six-well dishes using SuperFect (Qiagen) with 0.6 μg of reporter plasmid, 1.2 μg of expression plasmid, and 0.2 μg of internal control plasmid (pGL3 Control; Promega). Reporter gene activity was determined by the SEAP reporter gene assay (Roche).

RT-PCR Analysis and TRAP Assay. First-strand cDNA synthesis was done using 2–5 μg of total RNA with SuperScript reverse transcriptase (Life Technologies, Inc.) in the presence of 100 ng of oligo-dT₁₇ primers in a volume of 20 μl. The cDNA was diluted 1:5 in water, and 5 μl of the reverse transcriptase reaction was used for PCR analysis in a total volume of 50 μl containing 0.2 μM specific primers, 10% DMSO, 1.5 mM MgCl₂, 0.2 mM deoxyadenosine triphosphates, and 1 unit of Taq polymerase (Life Technologies, Inc.). For radioactive PCR analysis, 2.5 μCi of [α-³²P]dCTP (3000 Ci/mmol; Amersham) was added to the reaction. Amplification products were analyzed on 5% nondenaturing polyacrylamide gel slab gels. PCR analysis was done using FACS assay with the respective monoclonal antibodies (PharMingen).

For the run-off assay, 200 μl of nuclei were mixed with 200 μl of reaction buffer [10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 300 mM KCl, 0.5 mM each of ATP, CTP, and GTP, and 100 μCi of [α-³²P]dUTP (800 Ci/mmol; Amersham)] and incubated with shaking for 30 min at 30°C. Subsequently, DNA was digested by the addition of 20 μl of Dnase I (1 mg/ml; RNase free) and incubation for 15 min at 30°C. Isolation of RNA was done using the TRIzol reagent (Life Technologies, Inc.) following the recommendations of the manufacturer.

Hybridization was performed with 1 × 10⁶ cpm labeled RNA per sample using 5 ml of the Rapid-hyb buffer (Amersham) according to the manufacturer’s recommendations. To enhance sensitivity, the hybridization was done overnight. To reduce background signals, filters were washed under stringent conditions: two times in 2 × SSC/0.1% SDS, two times in 0.2 × SSC/0.1% SDS, and two times in 0.1× SSC/0.1% SDS for 25 min at 65°C. For each gene, 1 μg of a specific fragment was immobilized on a nylon membrane: for hTERT and GAPDH, the 450-bp (position 3014–3464) or the 449-bp (position 586–1039) amplification products described above; for c-fos, a 1-kbp PstI fragment; and for c-jun, a 1.4-kbp Smal-HindIII fragment of the respective cDNAs was used.

Electrophoretic mobility shift assays were performed using a 26-bp oligonucleotide containing the consensus E-box (Santa Cruz Biotechnology) and nuclear extracts from U937 cells, which were prepared as described (35). Approximately 10 fmol of labeled oligonucleotide (25,000 cpm) was incubated with 3 μg of nuclear extract in the presence of 1 μg of poly(deoxyinosinosinic-deoxycytidylic acid) in 1× binding buffer (36) for 20 min at room temperature. Subsequently, 3 μg of a C-myc-specific antibody (N-262;Santa Cruz Biotechnology) or of an E2F-1-specific antibody (as a control) were added to the mixture, and after 10 min at room temperature, binding was allowed to occur overnight on ice. Complexes were separated on 5% nondenaturing polyacrylamide gels that were run at 4°C.

Northern Blot and Array Analysis. For Northern analysis, 20 μg of cytoplasmic RNA were immobilized on a filter and sequentially probed with specific fragments to detect the c-myc (2.4-kb) and mad1 (3.8 and 6.5-kb) mRNA without stripping the filter. To analyze the expression of mad1 in human tumors, the commercially available Matched Tumor/Normal Expression Array (Clontech) was used. Hybridization with a mad1-specific probe was done according to the manufacturer.

Results and Discussion

Down-Regulation of Telomerase Activity and hTERT Expression upon Differentiation of U937 Cells. To study the regulation of telomerase activity as well as hTERT mRNA expression upon cellular differentiation, we have chosen the human myeloid leukemic U937 cell line. RA as well as phorbol esters such as TPA induce U937 cells to differentiate along the monocytic and macrophage-like pathway. These cells are thus very similar to HL-60 cells, where telomerase activity has been studied (10, 26, 37) but are much easier to transfect and are therefore advantageous for the present study. In the first set of experiments, we analyzed whether the hTERT gene as well as telomerase activity is regulated in a similar manner in U937 cells as it has been reported for HL-60 cells. We performed RT-PCR analysis with hTERT- and hTEP1-specific primers at various time points after induction of differentiation of U937 cells with RA or TPA. As demonstrated in Fig. 1a, differentiation of these cells leads to a down-regulation of hTERT mRNA after 5 h and to its complete disappearance after 24 h. In contrast, the expression of hTEP1 was slightly increased after the addition of the differentiating agents, an observation that has been reported earlier for HL-60 cells (38). TRAP assays using extracts from differentiating U937 cells showed that the addition of RA or TPA leads to a decrease of telomerase activity (Fig. 1b), although with much slower kinetics when compared with the hTERT mRNA. After 24 h, a reduction of telomerase activity was visible, and after 72 h, all the activity had disappeared. These data confirm that expression of hTERT is the rate-limiting step for telomerase activity. To verify that the experimental conditions used here resulted in the differentiation of U937 cells, we have examined expression of the cell surface markers CD4 as well as CD11a and CD11c (Fig. 1c). Whereas expression of the monocytic antigens 

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CD11a and CD11c increased upon TPA treatment, CD4 surface expression, which is a hallmark for immature U937 cells, was lost rapidly. We conclude from these experiments that hTERT regulation in U937 cells is tightly controlled and that these cells therefore provide a useful model for studying the regulation of the hTERT gene upon differentiation. For all of the subsequent experiments, only TPA was used as a differentiating agent.

The Half-Life of the hTERT mRNA Is Not Altered by TPA. The rapid down-regulation of hTERT mRNA after TPA stimulation could result from a short half-life of the hTERT message. To test this and to examine whether TPA-mediated changes in hTERT mRNA stability contribute to its rapid disappearance, we performed semiquantitative RT-PCR analysis using DNA from actinomycin D-treated cells. In the presence of actinomycin D, a significant decrease of the hTERT mRNA could be observed as early as 1 h after addition of the drug (Fig. 2). After 4 h, hTERT message had almost completely disappeared. This regulation was specific because hTEP1 mRNA was affected to a much lesser extent by the inhibitor. Densitometric analysis revealed the half-life of hTERT in U937 cells to be ~50 min. The rate of decay of the hTERT mRNA was not affected by the addition of TPA, which suggests that the induction of differentiation did not change hTERT mRNA stability. The decrease of hTERT message induced by TPA was slower than that induced by actinomycin D alone or by both drugs, indicating that there is still residual transcription of the hTERT gene after 6 h of TPA treatment.

The Rate of hTERT Transcription Is Decreased by TPA Treatment. Because altered mRNA stability is not responsible for the down-regulation of the hTERT mRNA upon induction of differentiation, we analyzed the level of initiation of hTERT transcription upon TPA treatment. Before and at various time points after induction of

Fig. 1. Down-regulation of hTERT mRNA level and telomerase activity upon RA and TPA treatment of U937 cells. a, RT-PCR analysis with primers specific for hTERT, hTEP1, and GAPDH. Exponentially growing cells were induced with either RA or TPA for the times indicated. b, TRAP assay using extracts from U937 cells that were treated identically to the cells used in a. The positive control (pos. control) and the internal control (IC, reveals the absence of PCR inhibitors) are provided by the manufacturer of the kit (TRAPeze kit). For the negative controls (neg. control), extracts from untreated cells were used. M, 100-bp marker. c, differentiation of U937 cells. Cells were induced by TPA, and surface antigen expression was measured by FACS analysis using specific antibodies.

Fig. 2. Determination of the hTERT mRNA half-life. Top, semiquantitative RT-PCR analysis. Exponentially growing U937 cells were treated with 10 ng/ml TPA (T), 5 μg/ml actinomycin D (ActD), or with a combination of both (T+D) for the times indicated; total RNA was extracted, and RT-PCR analysis was performed with primers specific for hTERT and hTEP1. To stay within a linear amplification range, PCR was done for 25 cycles in the presence of radiolabeled dCTP. Bottom, quantitation of the hTERT mRNA levels in the top panel using the program NIH Image 1.61. The signal of the uninduced sample was set to 100%.
differentiation by the addition of TPA, nuclei were isolated, and nuclear run-off experiments were performed. As shown in Fig. 3, the level of transcription of hTERT started to decrease as early as 15 min and was significantly down-regulated after 8 h of induction. In contrast, GAPDH expression was only mildly altered by the addition of TPA. To verify the effect of TPA on gene expression, we have included the immediate early genes c-fos and c-jun in the experiment. The expected transient rise in the expression of both genes that can be observed after 15 min serves as a positive control. These results indicate that the down-regulation of hTERT mRNA upon differentiation of U937 cells is predominantly caused by a decrease of the transcriptional activity of the gene.

hTERT Down-Regulation Is Not Preceded by Loss of c-myc Activity. The c-myc oncogene has been implicated in the regulation of the hTERT gene (22–25). Using reporter constructs harboring fragments of the hTERT promoter, we could confirm that the overexpression of c-myc leads to activation of the hTERT promoter in transient transfection assays in U937 cells (Fig. 4f). We then wanted to investigate the level of endogenous c-myc expression in response to TPA treatment in our cell system. Surprisingly, RT-PCR (data not shown) and Northern analysis did not reveal significant changes in c-myc mRNA, whereas hTERT levels decreased after addition of the drug (Fig. 4a). To analyze possible posttranscriptional effects, we examined the level of C-Myc protein. The amount of C-Myc that could be supershifted from complexes binding to an E-box-containing fragment slightly decreased after TPA addition (Fig. 4b). This decrease, however, was not complete, and there was still significant C-Myc activity when hTERT expression had already disappeared. This observation represents an exception to the general observation that c-myc mRNA and protein levels are turned off in differentiating cells. A similar observation, however, has been made in a subline of U937 cells in which TPA treatment did not result in a decrease in c-myc levels (39). Moreover, a C-Myc target gene, ODC decreased rapidly, despite high levels of C-Myc, but the mechanism by which ODC expression was down-regulated remained undetermined. As in the case with ODC, our results imply that C-Myc is not the only determinant of hTERT activity in U937 cells.

Mad1 Can Repress the hTERT Promoter. Whereas c-myc and max mRNA levels (data not shown) remained constant after TPA treatment, the amount of mad1 mRNA and protein increased dramatically after induction of differentiation (Fig. 4a, d and h). This observation is in agreement with earlier reports (40) and prompted us to test the effect of Mad1 on the hTERT promoter. Overexpression of Mad1 in transient transfection assays in U937 cells resulted in a consistent and significant decrease of hTERT promoter activity (Fig. 4d). This repressive effect of Mad1 was dose dependent (Fig. 4e). The hTERT promoter contains two E-box consensus sites, one of which is located close to the translational initiation codon at position −29 to −34 (proximal E-box), whereas the second one is at position −238 to −243 with regard to the ATG (distal E-box; Fig. 4c; Ref. 22). The effect of Mad1 on the hTERT promoter was dependent on an intact proximal E-box. Deletion of this E-box led to reporter gene activity that was almost indistinguishable from a construct without an obvious E-box consensus. This observation was paralleled by the effect of c-Myc on the hTERT promoter, which was also mainly exerted via the evolutionarily conserved proximal E-box (Fig. 4f; Ref. 22). Thus, c-Myc and Mad1 exert their transcriptional effects via the same site in the hTERT promoter. A comparison of the c-Myc and Mad1 effects on the reporters pGRN261 and pGRN316 suggests that sequences upstream of both E-boxes appear to contribute to the repression of the hTERT promoter by Mad1 and its activation by c-Myc. Although there is no additional E-box in the 5′ region of pGRN261, the reporter harbors several additional sequence elements that deviate by only 1 bp from the canonical CACGTG. Whether c-Myc/Max and/or Mad1/Max complexes bind to these sites, however, remains to be shown. To exclude competition between Mad1 and c-Myc as the basis for the observed effect, we have used a mutant form of Mad1. The MadLAN mutant (41) is devoid of the mSin interaction domain (SID) and is therefore not able to actively repress transcription. As can be seen in Fig. 4d, cotransfection of MadLAN does not lead to repression of the hTERT promoter constructs. In contrast, the mutant led to a consistent increase in reporter gene activity. This effect could be explained by the displacement of endogenous Mad1, which is expressed at a low level in undifferentiated cells (Fig. 4b), bottom, by the overexpressed mutant form. Thus, repression of the hTERT promoter by Mad1 involves an active repression mechanism rather than simple competition with c-Myc.

Mad1 Expression Is Lost in Human Tumors. To analyze the expression of mad1 in human tumors, we have measured mad1 mRNA levels in an arrayed panel of human tumor and the respective normal tissue (Fig. 5). In a number of tissues, mad1 expression was too low to be analyzed; but in the particular case of colon cancers, as many as 91% (10 of 11) of the tumors showed decreased mad1 mRNA levels. In the combined 29 samples of tumors from colon, lung, stomach, and rectum, mad1 expression was lost or down-regulated in 69% (20 of 29) as compared with normal tissue from the same patient. Of note, several members of the Mad family have been discussed as tumor suppressors (42). On the basis of our observations, one can therefore speculate that the repression of the hTERT promoter by Mad1 limits the replicative potential of a cell and thereby contributes to the tumor suppressor phenotype.

In summary, we have demonstrated that the down-regulation of hTERT expression in differentiating U937 cells occurs at the level of transcriptional initiation. Furthermore we provide evidence that in
Fig. 4. Effects of c-Myc and Mad1 on hTERT expression. 

**a.** Analysis of hTERT mRNA levels by RT-PCR using RNA from cells treated with TPA for the times indicated (top). Middle, Northern Blot analysis of c-myc and mad1 expression. Bottom, the same filter was subsequently reprobed with a GAPDH probe as a loading control.

**b.** Electrophoretic mobility shift assay (top) using extracts from U937 cells treated with TPA for the times indicated. Note that the extracts for the EMSA experiment were made from the same cells that were used for the RNA preparation analyzed in a. The probe contained a consensus E-box sequence. c-Myc DNA binding activity was analyzed with a c-Myc-specific antibody. As a control for specificity, an E2F-1 antibody was used. The synthesis of Mad1 (bottom) during U937 differentiation was analyzed by metabolic labeling and subsequent immunoprecipitation using a Mad1-specific antibody.

**c.** Schematic representation of the genomic hTERT promoter fragments contained in the reporter constructs used in d, e, and f. Arrow, the transcriptional start site of hTERT.

**d.** Results of transient transfection assays in which a mad1 expression vector (Mad1-wt), a mad1DN expression vector (Mad1-mut), or the respective empty vector was cotransfected into U937 cells with different reporter plasmids. e. Dose dependence of Mad1-mediated repression. Increasing amounts of the mad1 expression vector (in μg) were cotransfected into U937 cells with a constant amount of the pGRN316 reporter construct.

**f.** Results of transient transfection assays in which a c-myc expression vector or the respective empty vector was cotransfected into U937 cells with different reporter plasmids. Results in d–f are given as relative activation/repression of the expression constructs as compared with the empty vector. Every experiment has been performed at least five times; in each case, a representative experiment is shown. Bars, SE.
addition to the c-Myc oncoprotein, the transcriptional repressor Mad1 regulates hTERT activity. To our knowledge, Mad1 is the first transcription factor identified that can repress the hTERT gene. This finding might also open up the possibility for novel therapeutic strategies with regard to telomerase inhibition. Finally, we think the cellular system used here is a useful model for the regulation of hTERT activity during the differentiation of somatic tissues.

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