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Implication of the exon region in the regulation of the human telomerase reverse transcriptase gene promoter

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Abstract

The expression of the catalytic subunit (hTERT) represents the limiting factor for telomerase activity. In transfection studies, high level of activity of *hTERT* promoter is found, whereas low copy numbers of *hTERT* mRNA are detected in vivo. To explain this discrepancy, a series of vectors containing the *hTERT* promoter and gene were transiently transfected into HeLa cells. Four important regions were identified. First, the core promoter has bidirectional activity. Second, the distal upstream region (−1821 to −811 bp) involved in the splicing of the first intron and could be a key of splicing specificity. Third, the intermediate promoter region (−800 to −300 bp) could play an important role in silencing the reverse promoter activity. Fourth, the structural gene (up to +1077) strongly reduced *hTERT* promoter activity. These results provide the first evidence that the first two exons play a major role in the down-regulation of the *hTERT* promoter in telomerase-positive cells.

Keywords: Telomerase; hTERT; Transient transfection; Promoter; Splicing; Repressor

Telomerase is a ribonucleoprotein responsible for maintaining the ends of chromosomes, named telomeres, composed of tandem repeats of the sequence TTAGGG [1]. Possible functions of this non-coding DNA include prevention of chromosome degradation, end-to-end fusions, rearrangements, and chromosome loss [2]. Since DNA polymerase cannot completely replicate chromosome ends, telomerase is needed to add telomeric repeats to the chromosomes, thereby preventing the loss of telomeric DNA. Indeed, in normal cells, lacking telomerase activity, each division is associated with telomere shortening [3]. Telomerase activity is detectable during embryogenesis in a variety of fetal tissues [4]. In adults, telomerase activity is not detectable in most human somatic cells [5]. On the contrary, highly proliferative cells such as germ cells and stem cells express telomerase, as well as 85–95% of cancer cells [6].

Studies of the human telomerase complex reveal the presence of two major subunits: a RNA component (*hTERC*) that serves as template for the polymerase activity and a catalytic subunit with reverse transcriptase activity (hTERT) [7,8]. The presence of both is sufficient to reconstitute telomerase activity in vitro [9], but other telomerase-associated proteins have been identified in vivo [10,11]. The *hTERT* gene seems to be highly regulated and its expression correlates with telomerase activity [12,13]. Many studies have demonstrated that ectopic expression of *hTERT* was sufficient to restore telomerase activity in telomerase-negative cells [14–16].

Many studies have suggested that expression of *hTERT* represents the limiting factor for telomerase activity, and that the regulation of *hTERT* should mainly occur at the transcriptional level. Genomic organization and promoter characterization of the *hTERT* gene have been described by several groups [17–19]. These investigations demonstrate that a region named core promoter, encompassing the proximal 283 bp region upstream of the initiation ATG codon, is essential for transcriptional activation. Several groups have found specific sites for activators and inhibitors of the transcription in the *hTERT* promoter sequence [20–28]. Apparently complex mechanisms involving regulatory elements distant from the 5′ flanking region of the

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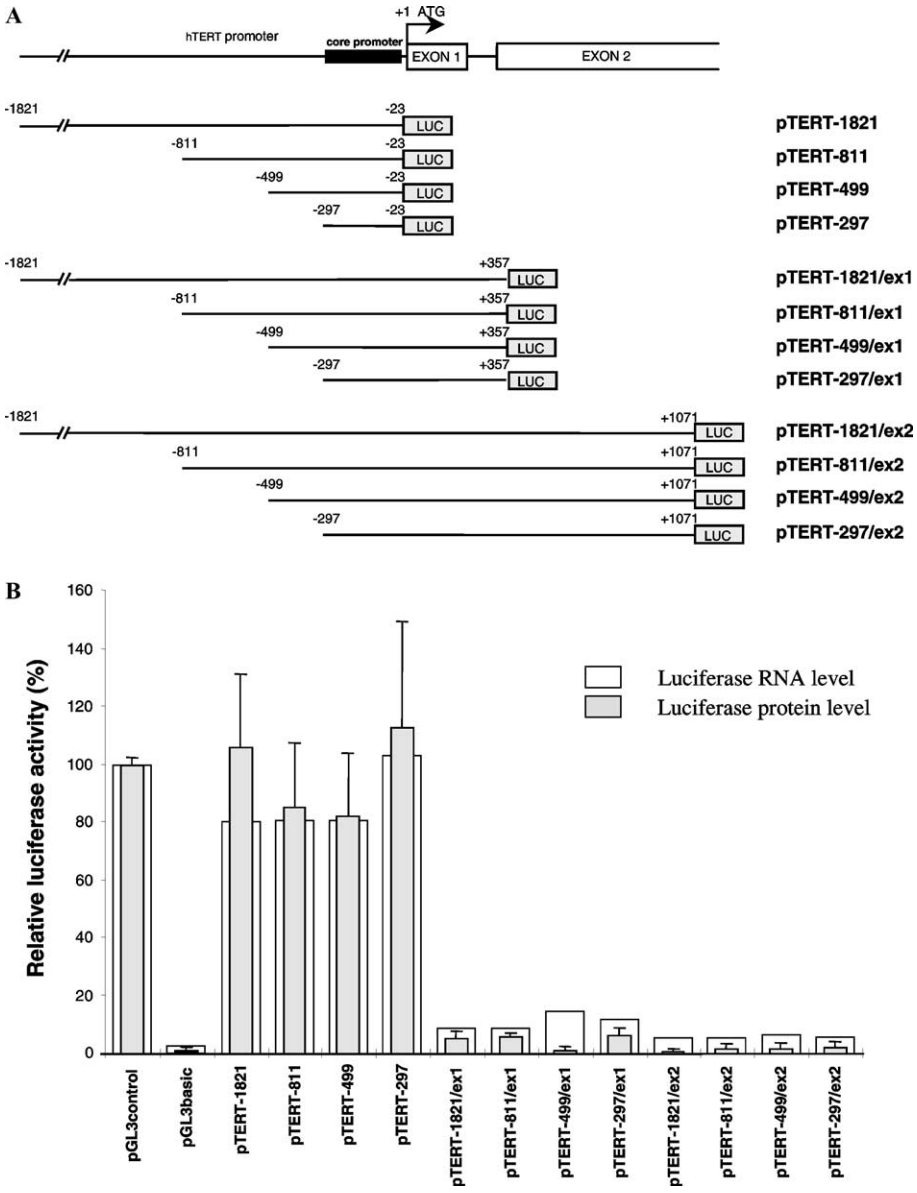

Fig. 1. Transcriptional activity of the *hTERT* promoter. (A) A schematic representation of the reporter plasmids. The fragments of *hTERT* promoter and structural gene are cloned into the luciferase (LUC) reporter vector pGL3-basic in the sense orientation. The 280 bp core promoter is shown as a black bold line. Numbers refer to the number of bases upstream (−) or downstream (+) of the ATG initiation codon of the *hTERT* gene. (B) Luciferase reporter plasmids containing fragments of *hTERT* promoter with or without structural gene were transfected in HeLa cells. White and gray bars indicate luciferase mRNA and protein levels, respectively. PGL3-basic, which lacks the enhancer/promoter, was used as a negative control; pGL3-control containing the SV40 enhancer/promoter was used as a positive control. The transcriptional activities in each reporter plasmid are indicated as relative luciferase activities. The values for the positive control are indicated as 100%.

hTERT promoter are also implicated in the regulation of *hTERT* expression [29]. Moreover, studies of RNA processing revealed complex splicing patterns in different cell types [12] and a potential regulation of *hTERT* expression by alternative splicing [30].

The *hTERT* promoter induced very high expression, similar to that of the SV40 early promoter, in transiently transfected telomerase-positive cell lines [31]. This is in stark contrast with the low *hTERT* mRNA levels detected in telomerase-positive cell lines which was found to be as low as 0.2–6 copies per cell [29]. These findings suggest a complex regulation of the expression of the *hTERT* gene.

In this study, we explore the implication of the structural gene itself, in the transcriptional regulation of the *hTERT* gene. A series of luciferase reporter constructs driven by the *hTERT* minimal promoter and distant sequences (from −1821 to +1071 bp) were constructed and transiently transfected into HeLa cells.

These constructs allowed the identification of three additional regions involved in the *hTERT* regulation.

Materials and methods

Cell culture. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovin serum (HI-FBS) and an antibiotics mixture (250,000 U penicillin, 25 mg streptomycin, and 2.5 μg fungizone) at 37 °C in a 5% CO_2 humidified incubator (all products from Invitrogen, Basel, Switzerland).

Plasmid construction. The structure of the different constructions is shown in Fig. 1A. Various lengths of DNA fragments upstream and downstream of the initiating ATG codon of the *hTERT* gene were amplified by PCR and inserted into a firefly luciferase reporter vector, pGL3-basic (Promega, Madison, WI), a promoterless and enhancerless vector. Plasmids were purified with Midiprep and Miniprep Kits (Qiagen, Hilden, Germany). Constructs containing only a fragment of the *hTERT* promoter were named pTERT-1821 (−1821 to −23), pTERT-811 (−811 to −23), pTERT-499 (−499 to −23), and pTERT-297 (−297 to −23). When constructs contained the 357 bp downstream of the ATG initiation codon, "ex1" was added to the name of each construction. This 357 bp downstream of the ATG corresponds to exon1, intron1, and the first 36 bp of exon2 of the *hTERT* gene. Constructs containing the 1071 bp downstream of the ATG initiation codon have the same name with addition of "ex2". This 1071 bp downstream of the ATG includes exon1, intron1, and a large part of exon2 of the *hTERT* gene (Fig. 1A). Fig. 2A shows three types of reporter plasmids derived from the "ex1" constructs. These constructs were generated by PCR using primers containing one or two additional nucleotides than the standard primer. These constructs allowed translation evaluation of the three open reading frames (ORFs). Each construct was confirmed by sequencing (Genome Express, Grenoble, France). As shown in Fig. 3, for the three reverse constructs, the plasmids have the same name as their homologs with "rev" at the end of the name. pTERT-811/ex1-rev contains, in reverse orientation, the 297 bp core promoter, the 357 bp sequence downstream of the core promoter, and 515 bp upstream of the core promoter corresponding to the sequence −297 to −811 bp of the *hTERT* promoter. pTERT-499/ex1-rev contains the same sequence as pTERT-811/ex1-rev except the sequence upstream of the core promoter which contains only 203 bp of the *hTERT* promoter (−297 to −499 bp of the *hTERT* promoter). The pTERT-297/ex1-rev construct contains only the core promoter and the reverse 357 bp sequence in reverse orientation.

Transient transfection and luciferase assay. Cells were seeded at a concentration of 100,000 cells/3.8 cm^2 and cultured overnight. Transient transfection of luciferase reporter plasmids was carried out in triplicate using lipofectin Plus Reagent (Invitrogen, Basel, Switzerland) according to the protocol recommended by the manufacturer. All experiments were performed at least three times. The *Renilla* luciferase reporter vector (Promega, Madison, WI) was co-transfected as an internal control for transfection efficiency. Briefly, cells were exposed to a transfection mixture containing 0.6 μg luciferase reporter plasmids and 0.5 μg internal control vector for 3 h at 37 °C. Then, 1 ml growth media supplemented with 15% HI-FBS was added to the cells. The cells were harvested 48 h after the transfection. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). To compare the results the mean values of relative luciferase activity were used. The levels of the different constructs were compared to the level of the pGL3-control vector containing the firefly luciferase gene under the control of the SV40 early promoter and to the level of the pGL3-basic vector, a promoterless and enhancerless luciferase vector.

RT-PCR assay. Three wells of transfected cells were pooled together. Two-third of the cells was used for RNA extraction and one-third was used for the luciferase assay to compare the protein level with the RNA level and to normalize the experiments. Total RNA of transfected cells was extracted using Trizol-LS (Invitrogen, Basel, Switzerland) according to manufacturer's protocol. A DNase I treatment was performed before the RT-PCR. Both cDNA synthesis and PCR were performed in a single tube using the SUPERSCRIPT

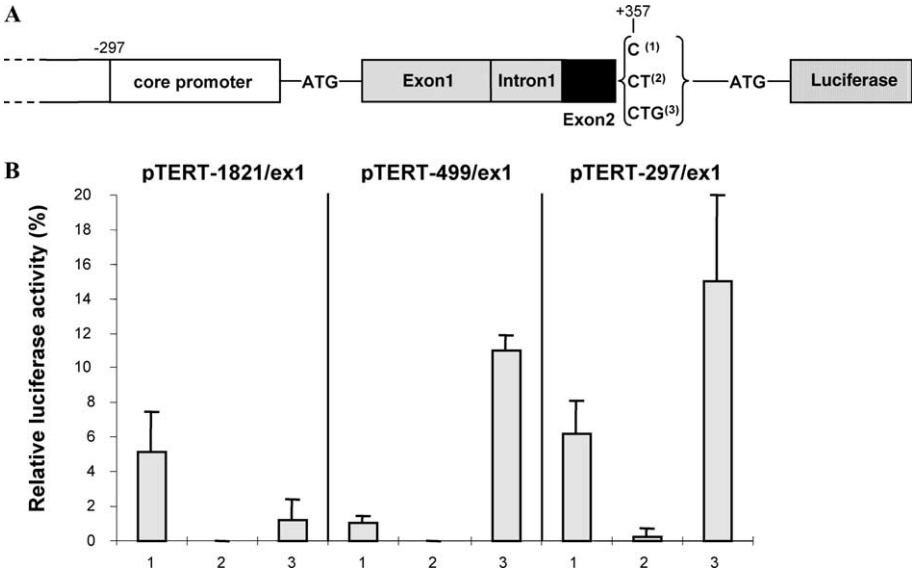

Fig. 2. The effect of the three reading frames on the transcriptional activity of the *hTERT* promoter. (A) Schematic representation of the reporter plasmids. Luciferase reporters contain a fragment of promoter, exon1, intron1, and beginning of exon 2 of the *hTERT* gene. For each reporter, three types of constructs were generated by the insertion of one or two nucleotides. (B) Reporters were transfected in HeLa cells and luciferase assays were performed. Numbers indicate the three possible reading frames; **1**, after splicing of the first intron, the two ATG of *hTERT* and luciferase gene are in phase; **2**, the two ATG are never in phase, with or without splicing of the first intron; **3**, the two ATG are in phase if the first intron is not spliced.

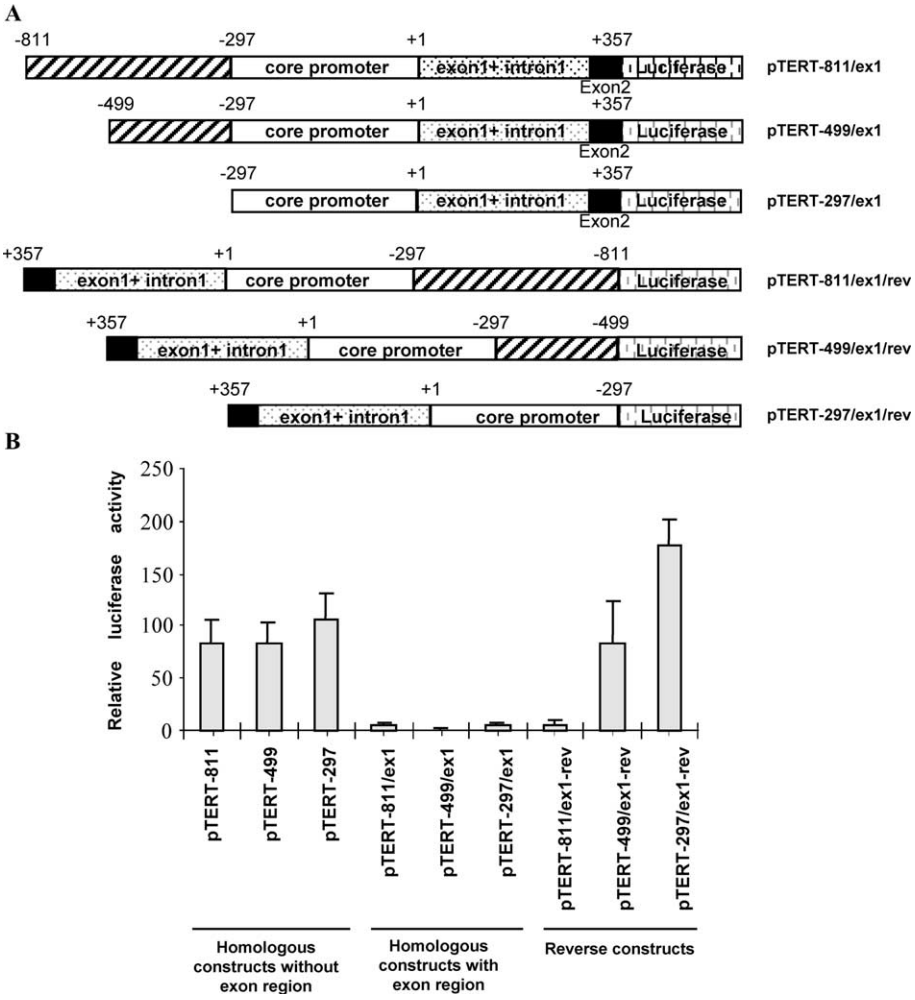

Fig. 3. The *hTERT* promoter is bidirectional. (A) Schematic representation of the reporter plasmids. The region of the *hTERT* promoter was reversed in the pTERT-297/ex1, pTERT-499/ex1, and pTERT-811/ex1 constructs. The new constructs were named pTERT-297/ex1-rev, pTERT-499/ex1-rev, and pTERT-811/ex1-rev. (B) Relative luciferase activity of these three reverse constructs versus no reverse constructs: pTERT-297/ex1, pTERT-499/ex1, and pTERT-811/ex1; and versus homologous constructs containing only the *hTERT* promoter: pTERT-297, pTERT-499, and pTERT-811. The transcriptional activity in each reporter plasmid was indicated as relative luciferase activities. One hundred percent corresponded to the value of the pGL3-control vector activity (data not shown).

one-step RT-PCR with Platinum Taq Kit (Invitrogen, Basel, Switzerland). Sense and antisense oligonucleotide primers amplified a region of 468 bp of the firefly luciferase cDNA (5′-AACCGCTGGA GAGCAACTGCATAAG-3′ and 5′-AAACCGGGAGGTAGATGA GATGTGA-3′). The reverse transcriptase step was performed at 55 °C during 30 min followed by 2 min at 94 °C to release the *Taq* polymerase. Then 25 cycles of PCR were performed, including denaturation at 94 °C for 2 min, annealing at 56 °C for 45 s, and extension at 72 °C for 50 s.

The RT-PCR products were denatured at 100 °C for 10 min followed by 5 min on ice. Then 2 μl of each sample was loaded onto a nylon membrane and fixed with UV for 5 min. The DIG-labeled probe was synthesized by PCR on the pGL3-basic vector with the same primer set used for the RT-PCR. Hybridization was performed at 42 °C for 2 h with 30 ng of probe per ml of hybridization buffer. After washing and detection with CDP-star (Roche, Rotkreuz, Switzerland), the membrane was exposed to a X-Omat sensitive film (Eastman Kodak Company, NY) for 30 min. Blots are quantified by comparing its intensity to a reference range dilution of RNA extracted from pGL3-control transfected in HeLa cells.

Results

Identification of an inhibitory region downstream of the ATG initiation codon of the hTERT gene

To identify the regulatory region responsible for the repression of the *hTERT* gene expression, a series of luciferase reporter plasmids, containing various lengths of the promoter and structural gene, were prepared and tested in transient transfection assays in telomerase-positive HeLa cells. As shown in Fig. 1, the constructs, containing only a fragment of the *hTERT* promoter, show high levels of reporter gene expression, similar to that of the SV40 early promoter (pGL3-control). The proximal 297 bp region is defined as the core promoter of the *hTERT* gene (Fig. 1A, pTERT-297). The 5′ deletions of the promoter up to −811 bp weakly

reduced transcriptional activity (Fig. 1, pTERT-499 and pTERT-811). However, transcriptional activity increased with less extended truncation (Fig. 1, pTERT-1821). These findings suggest the existence of a minimal promoter, sufficient to exhibit a high transcriptional activity and the existence of a potential negative regulatory region between −800 and −300 bp of the promoter. These observations are in agreement with recent studies that defined the function of the proximal 181 bp region as a minimal core promoter [20] and a negative regulatory region between −776 and −378 bp [26].

Interestingly, a strong decrease of luciferase activity was observed if the constructs tested contained a part of the structural gene (Fig. 1). The three constructs that contain the exon1 and intron1 exhibited a transcriptional activity 10 times less than the homologous constructs without the downstream region of the ATG initiation codon. Furthermore, the addition of part of exon2 entailed up to a 100-fold decrease of the transcriptional activity of the *hTERT* promoter. These observations imply a potential role for the exon1, intron1, and exon2 in the repression of the *hTERT* gene expression.

A RT-PCR analysis of transfected cells showed levels of luciferase mRNA expression consistent with the respective enzyme activity (Fig. 1B). This result suggests that this strong down-regulation, due to the presence of a *hTERT* exon region, occurred at the mRNA level.

Presence of a splicing regulatory region upstream of the hTERT core promoter

Due to the presence of an intronic region in the constructs, it was necessary to confirm our results by testing the different reading frames. To create the different reading frames, one or two nucleotides were inserted in the standard reporter plasmids (Fig. 2A). The splicing sites in the first intron are defined in the literature [31]. For the first reading frame, luciferase activity is detected only when the first intron is spliced because, only in this condition will the two ATG start sites of the *hTERT* and luciferase genes be in phase. For the second reading frame, no activity should be observed because the ATG sites cannot be in phase (with or without splicing of *hTERT* intron1). For the third reading frame, an activity must be detected if the first intron was not spliced, only in this condition will the two ATG sites be in phase.

Fig. 2B shows the results obtained with these three possible reading frames for the three constructs pTERT-1821/ex1, pTERT-499/ex1, and pTERT-297/ex1 (Fig. 1). As expected, no transcriptional activity was observed with the second reading frame (Fig. 2B, lanes 2). In contrast, for the first reading frame, splicing of the first intron led to a weak luciferase activity for the three different constructions (Fig. 2B, lanes 1). The results obtained with the third reading frame were more surprising. Some luciferase activity was observed with all the constructs, which indicates that splicing of the first intron did not occur completely. Nevertheless, for the longest construct pTERT-1821/ex1, the activity was significantly lower than that with the first reading frame. In contrast to the construct containing the longest promoter sequence, the level of luciferase activity of pTERT-499/ex1 and pTERT-297/ex1 was higher with the third reading frame (Fig. 2B, lanes 3). Therefore, more efficient splicing of the first intron of the *hTERT* gene was obtained with increasing length of the *hTERT* promoter.

The hTERT core promoter is bidirectional

Promoters that lack TATA boxes and initiator elements frequently display bidirectional activity [32]. To determine if this is also true for the *hTERT* promoter, we conceived three reverse constructs corresponding to pTERT-811/ex1, pTERT-499/ex1, and pTERT-297/ex1 (Fig. 3A). In the minimal construct, pTERT-297/ex1-rev, the core promoter and the exon region are reversed, which means that the exon region is now situated upstream of the promoter. This construction led to a 2-fold transcriptional activity compared to that of the minimal *hTERT* promoter activity in the correct orientation (Fig. 3B). This result seems to indicate that the inhibitory role of the region containing the exon1 and the intron1 is effective only if this region is situated downstream of the promoter. For the other inverted constructs, pTERT-499/ex1-rev and pTERT-811/ex1-rev, a 200 and a 500 bp reversed fragment of the *hTERT* promoter were, respectively, inserted between the reverse core promoter and the luciferase reporter gene. The 200 bp fragment corresponds to the −500 to −300 bp of the *hTERT* promoter and the 500 bp fragment corresponds to the −800 to −300 bp of the *hTERT* promoter. Fig. 3B shows that the transcriptional activities of the reverse constructs pTERT-297/ex1-rev and pTERT-499/ex1-rev are, respectively, 86- and 28-fold higher than activities of its homologous constructs in the correct orientation. In contrast, the activity of the pTERT-811/ex1-rev is not significantly different from the activity of its homologous construct in the correct orientation (pTERT-811/ex1). The difference between the two first constructs and the pTERT-811/ex1-rev is the 300 additional bp of the *hTERT* promoter corresponding to the −800 to −500 bp region of this promoter. Our experiments show that this region, containing multiple binding motifs for MZF-2 [26], downregulates the *hTERT* promoter transcription more strongly in the reverse orientation than in the correct one.

Discussion

The *hTERT* gene regulation is rather complex and not yet well understood. In this study, we addressed the

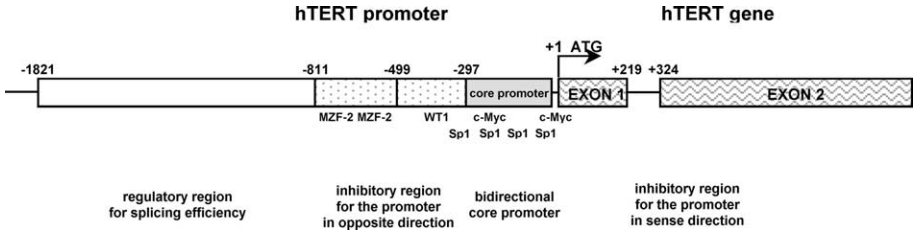

Fig. 4. Schematic representation of the four important regions involved in the *hTERT* gene regulation. The −297 to +1 bp region corresponding to the core promoter shows a bidirectional activity. The −811 to −499 region contains inhibitors which contribute to repress reverse transcription of the core promoter. The −1821 to −811 region is involved in mRNA splicing processing. Finally the +1 to +1077 bp region contains inhibitor of the *hTERT* gene transcription.

potential role of the proximal part of the structural gene as a negative regulatory element for the *hTERT* promoter. To answer this question, different parts of the *hTERT* promoter, with or without a part of the structural gene, were cloned in luciferase reporter vectors. Four important regions, involved in the transcriptional regulation of the *hTERT* gene, have been identified from −1821 to +1071 bp of the gene (Fig. 4).

The first region contains the core promoter, which gives the highest transcriptional activity. The result is consistent with other studies using *hTERT* promoter reporter vectors [17–19,31]. In addition, reverse constructs allowed us to conclude that this core promoter has the particularity of being bidirectional, and that, at least in HeLa cells, the expression in the reverse sense is 2-fold higher than the normal *hTERT* gene transcriptional level.

The second region identified, located in the 5′ flanking region of the promoter between −1821 and −811 bp, was involved in the regulation of the mRNA splicing processing (Fig. 4). Since sequences subcloned contained the first intron, the possible reading frames were tested. The frequency of correct splicing of the first intron was significantly more efficient with constructs containing the largest part of the promoter. Indeed, spliced mRNA was four times more frequent than non-spliced mRNA if the construct contains the 1821 bp upstream of the ATG start codon. In contrast, if the transfected constructs contained a shorter promoter region, the level of non-spliced mRNA became significantly higher than the spliced one. It is well known that regulation of the *hTERT* gene also implies alternative splicing of mRNA [12,32]. Our results indicate that the 5′ flanking region of the *hTERT* promoter is involved in the splicing, at least, of the first intron of the *hTERT* gene. Hence, some regulatory factors of the RNA splicing process may be located in the 5′ flanking region of the promoter between −1821 and −811 bp. Other studies have also suggested that the distant 5′ flanking region of the *hTERT* gene is involved in the splicing regulation as several transcripts of *hTERT* mRNA could be detected. In this situation, the chromatin structure could be implied in this regulation; some factors could bind these sequences and could contribute to stabilize the spliceosome, allowing the correct splicing [29]. Moreover, various *hTERT* splicing patterns have been observed during human development [29,33,34]. This observation provided evidence that alternative splicing is non-random and could play a role in the regulation of telomerase activity. Nevertheless, other mechanisms could explain our results. First, variations of the different levels of the three reading frames could be due to a switching of the ORF during translation. However, the switch of the ORF had to lead to similar variation for the three constructs containing different parts of the *hTERT* promoter, which was not the case in our experiment. Furthermore, this event was observed in bacteria, virus, and yeast, but not in higher eukaryote. Secondly, the ATG of luciferase could be chosen as the initiated codon. Indeed, under rare conditions, the first ATG is not always selected as the exclusive translation initiation site [35].

The third and fourth regions were involved in repression of *hTERT* expression. The third is located within the promoter between −800 and −300 (Fig. 4). The three reverse constructs containing sequences situated in the intermediate promoter region (upstream to the core promoter) reduced *hTERT* transcriptional activity. With the −500 to −300 bp region of the promoter, the activity is decreased 2-fold. When the 300 bp of the promoter, corresponding to the MZF-2 binding site region [26], was added, there was almost no transcriptional activity detected. Therefore the inhibitory region, containing MZF-2 binding sites, might be important to reduce significantly or even to stop the reverse transcriptional activity of the *hTERT* promoter.

The fourth inhibitory region is located in the structural gene between the beginning of the exon1 and a large part of the exon2 (Fig. 4). This sequence could play a major role in the repression of the *hTERT* promoter activity. Indeed, the transcription level of constructs with a part of the structural gene was decreased up to 20- to 50-fold, compared with the transcription level of the homologous construct lacking structural gene components. If a longer part of the second exon was added, the transcription was decreased up to 100-fold. Moreover, reverse constructs showed that this region did not

inhibit the transcriptional activity of the *hTERT* gene when reversed and situated upstream of the core promoter. This region might be required for the binding of transcription repressor(s). It could explain why the *hTERT* core promoter, which has a transcriptional activity similar to that of the SV40 early promoter in vitro, induces a very low level of gene expression in vivo [29]. In our experiments, the luciferase mRNA expression was consistent with the respective enzyme activity. Nevertheless, it is important to consider that the low level of detected luciferase could also be the consequence of the mRNA instability generated by the fusion between *hTERT* and luciferase mRNA.

In conclusion, our results show that the first two exons of the *hTERT* gene play an important role in the regulation of its expression. We furthermore found the most distal upstream promoter region to play a role in the regulation of *hTERT* mRNA splicing. How these sequences regulate the expression of the *hTERT* gene merits to be further elucidated.

Acknowledgments

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