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The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter

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Telomerase, the enzyme that synthesizes telomeric DNA, is not expressed in most human somatic cells but is activated with *in vitro* immortalization and during tumorigenesis, and repressed by cell differentiation. Of the two components of the core enzyme, the catalytic protein hTERT is limiting for activity. To investigate mechanisms of *hTERT* gene regulation, we have cloned genomic sequences encompassing the complete *hTERT* transcription unit. The *hTERT* gene consists of 16 exons and 15 introns spanning ~35 kb. Transient transfections of immortal human cells with potential regulatory 5′ sequences linked to a reporter, combined with deletion analysis of these sequences, indicated that elements responsible for promoter activity are contained within a region extending from 330 bp upstream of the ATG to the second exon of the gene. Assays in different cell types have shown that the *hTERT* promoter is inactive in normal and in transformed pre-immortal cells, but, like telomerase, it is activated with cell immortalization. Sequence analysis revealed that the *hTERT* promoter is GC-rich, lacks TATA and CAAT boxes but contains binding sites for several transcription factors that may be involved in its regulation. The abundance of these sites suggests the possibility that *hTERT* expression may be subject to multiple levels of control and be regulated by different factors in different cellular contexts.

INTRODUCTION

Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA (reviewed in ref. 1). The core enzyme consists of an essential structural RNA (hTER in human cells), with a region complementary to telomeric DNA that serves as template for *de novo* addition of deoxynucleotides to the G-rich strand of telomeres, and of a catalytic protein (hTERT in human cells) with reverse transcriptase activity (2–8). The absence of telomerase activity in human somatic cells prevents compensation for the loss of telomeric DNA ensuing from the inability of conventional DNA polymerases to fully replicate linear DNA molecules (9,10). The resulting shortening of the telomeres limits the cell proliferative lifespan, as recently demonstrated by the finding that restoration of telomerase activity to somatic cells leads to bypass of cell senescence (11,12). Cell immortalization *in vitro* and tumour progression are associated with, and may depend on, activation of telomerase (13–15), while cell differentiation results in down-regulation of the enzyme (16–19). Thus, telomerase is intimately involved in the regulation of the proliferative potential of both normal and malignant cells. In all cases, expression of *hTERT* mRNA parallels that of telomerase activity pointing to transcriptional mechanisms of enzyme regulation (2–4). Furthermore, ectopic expression of hTERT in somatic cells is sufficient to restore enzymatic activity (6,8,11,12,20,21). Together with the invariant expression of hTER (22) and of the telomerase-associated protein TEP1 (23,24), this observation demonstrates that hTERT is the key regulator of enzymatic activity.

Because the *hTERT* cDNA has only recently been cloned (2–5), little is known about the factors that control its expression. To date a single study has addressed this aspect and has demonstrated induction of *hTERT* mRNA expression and of telomerase activity by myc (25). Whether the effects of myc are direct or mediated by other proteins is presently unknown. As a first essential step in the study of *hTERT* regulation, we have cloned genomic sequences encompassing the *hTERT* transcription unit. We describe here the organization of the *hTERT* gene and the identification and analysis of upstream sequences responsible for its transcriptional activation.

RESULTS AND DISCUSSION

Cloning and organization of the *hTERT* gene

Screening of a human genomic BAC library with an *hTERT* probe yielded a positive clone from which we derived subfragments that hybridized to full-length *hTERT* cDNA. These were cloned into pBlueScript II SK and ordered along the cDNA by hybridization with *hTERT*-specific oligonucleotides. Exon/intron boundaries within the *hTERT* gene were mapped by sequencing using primers derived from the cDNA. This analysis demonstrated that

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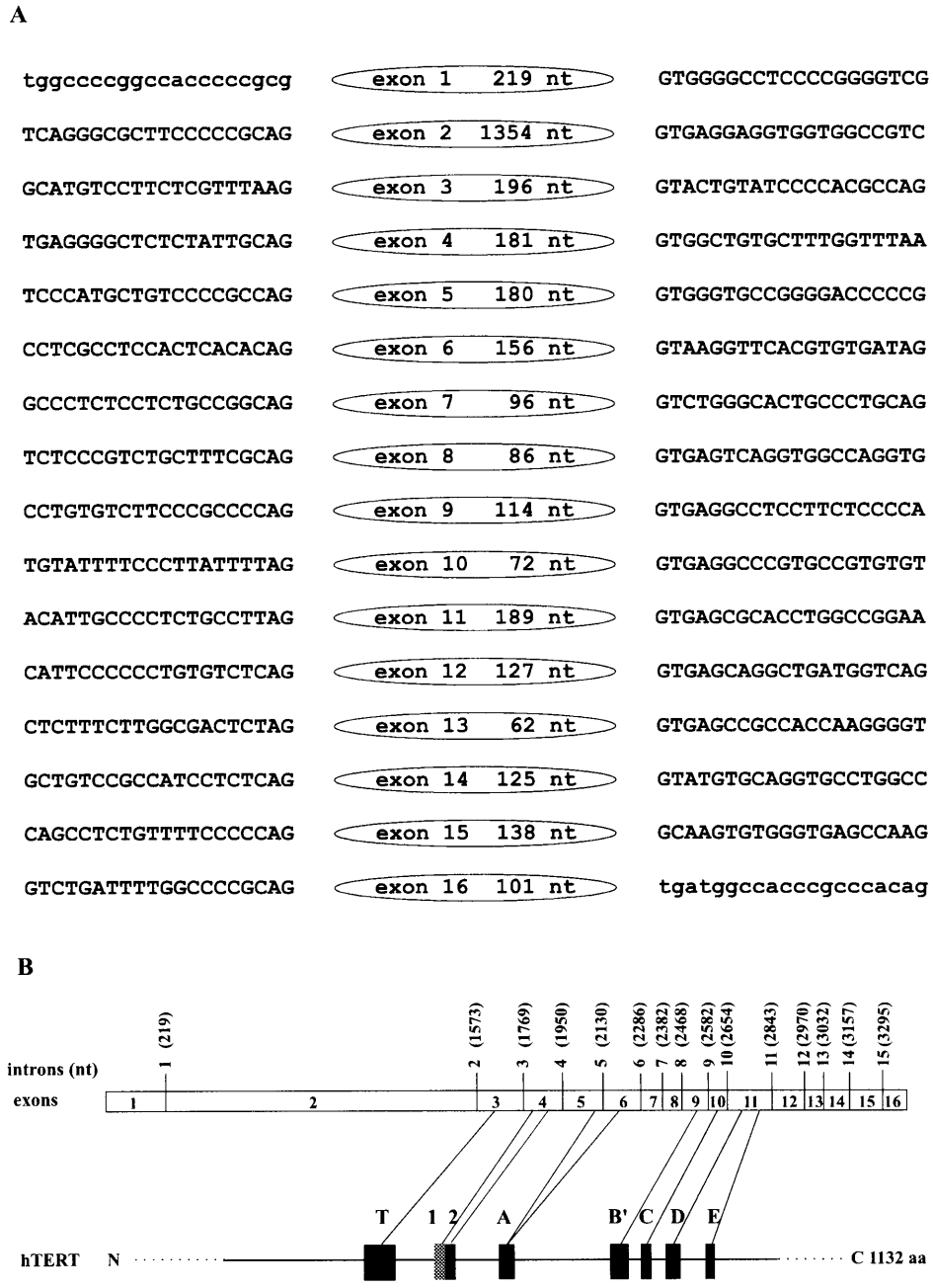

Figure 1. Organization of the *hTERT* gene. (**A**) The *hTERT* gene comprises 1132 amino acids which are distributed in 16 exons. The size in nucleotides of each exon is indicated. Intronic sequences flanking each exon (20 nt for both 3′-acceptor and 5′-donor side) are shown in upper case letters. Flanking sequences upstream of exon 1 and downstream of exon 16 are shown in lower case letters. (**B**) Localization of introns and exons on the *hTERT* cDNA: numbers in brackets indicate the nucleotide position of the intron, with nt 1 being the A of the ATG. hTERT contains a telomerase-specific (T) motif and seven motifs conserved in reverse transcriptases. The location of these motifs within the hTERT protein and their assignment to the exons encoding them are also shown.

the entire *hTERT* gene was contained within the BAC clone, extending over ~35 kb and comprising 16 exons and 15 introns (Fig. 1). A similar organization has recently been reported for the *TERT* genes from *Tetrahymena termophila* and *Schizosaccharomyces pombe*, which contain 18 and 15 introns respectively (26,27), while the *Oxytricha trifallax*, *Euplotes aediculatus* and *Saccharomyces cerevisiae TERT* genes contain no introns (26). All of the exon/intron splice junctions in *hTERT*, with the exception of the donor site of the last intron, conform to the GT/AG consensus sequence. Telomerases belong to a group of reverse transcriptases (RTs) that, in addition to the seven functional motifs that are conserved among RTs, contain a telomerase-specific T motif (1,3). Mutagenesis of these motifs in human telomerase has demonstrated their role in enzymatic activity (6–8). The exon assignment of all eight motifs of hTERT is shown in Figure 1B.

In higher eukaryotes, regulation of gene expression in a tissue-specific or development-dependent manner often involves

alternative splicing of mRNAs (28). A number of variant *hTERT* transcripts that are differentially expressed in different cells or during development have been described (4,29) but their biological relevance and the functional properties of their protein derivatives are presently unknown. The organization of the *hTERT* gene described here should be helpful for the detailed characterization of these alternative mRNAs and proteins.

Cloning and characterization of the *hTERT* promoter

Approximately 4 kb of sequences upstream of the *hTERT* ATG were present in an *Eco*RI fragment that hybridized with the 5′ end of the *hTERT* cDNA. A subfragment containing 3996 bp of sequences upstream of the ATG (with A being position 1) plus exon 1 (219 bp), intron 1 (104 bp) and 37 bp of exon 2 of hTERT was generated by 3′ deletion and cloned upstream of the luciferase gene in the pGL2-Enhancer vector (P-3996 construct). The ability of this construct to direct expression of the luciferase reporter was assayed by transient transfections of telomerase-positive and -negative human cells. Transfections with the pGL2-Enhancer vector were carried out as a control. Each transfection mixture included phβA-lacZ, as internal control for transfection efficiency based on β-galactosidase activity. As shown in Figure 2A, transfection of telomerase-negative WI-38 and MRC5 human fibroblasts or human embryonic kidney (HEK) cells with P-3996 resulted only in background levels of luciferase activity, whereas substantial levels of activity were produced by the same construct in immortal telomerase-positive HeLa and 293 cells. Moreover, promoter activation was concomitant with cell immortalization (Fig. 2B). HA1 cells are SV40-transformed HEK cells that do not express telomerase during their extended life-span but become hTERT- and telomerase-positive after proliferative crisis, i.e. at immortalization (3,13). Transfection with the P-3996 construct did not produce luciferase activity in pre-crisis cells (PD 15) but did so in cells soon after crisis (PD 104). Promoter activity therefore coincides with telomerase activity. This finding suggests that regulation of telomerase is primarily at the transcriptional level, in support of models invoking transcriptional mechanisms for repression and activation of the enzyme (2–4). Interestingly, in comparison with HeLa or 293 cells, immortal HA1 cells express much lower levels of luciferase activity. This correlates well with the relatively low amount of endogenous telomerase activity in these cells [~10% of that of 293 cells (22)] and suggests that amounts of the enzyme are strictly dependent on amounts of the hTERT protein.

To better define the *cis*-acting sequences responsible for transcriptional activation of *hTERT*, smaller promoter constructs were generated from P-3996 by 5′ deletions of *hTERT* upstream sequences. These were again assayed for their ability to produce luciferase activity in transient transfections of telomerase-positive 293 cells and telomerase-negative HEK cells (Fig. 3). Amounts of luciferase activity comparable to those induced by P-3996 were obtained following transfection of 293 cells with constructs containing upstream sequences ranging from 3216 to 330 bp, while significantly reduced activity was produced by a construct containing 145 bp upstream of the ATG. Lower but still detectable activity was produced even by a construct (P+228) containing only intron 1 and the first 37 bp of exon 2, suggesting that elements within this region are capable of limited promoter function at least in transient transfection assays. None of the constructs directed synthesis of luciferase following transfection into HEK cells. These results indicate that sequences responsible for promoter regulation are contained within a core region encompassing 330 bp upstream of the ATG and 37 bp of exon 2 of the gene. The differential activity of this region in mortal and

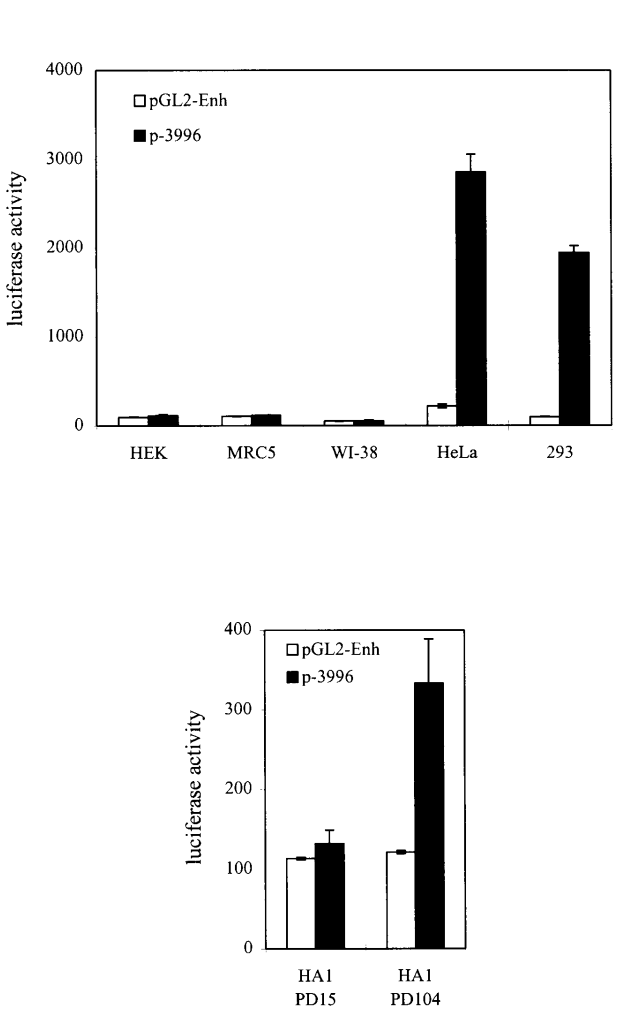

Figure 2. Expression of luciferase activity under control of the *hTERT* promoter. (**A**) Detection of promoter activity in mortal and immortal human cells. HEK, MRC5 and WI-38 are normal mortal cells; HeLa and 293 are immortal cell lines. The P-3996 construct contains 3996 bp of flanking sequences upstream of the ATG (with A being position 1) plus exon 1 (219 bp), intron 1 (104 bp) and 37 bp of exon 2 of *hTERT* cloned into the pGL2-Enhancer vector upstream of the firefly luciferase reporter. Cells were transiently transfected with P-3996 or the control pGL2-Enhancer vector, in each case together with phβA-lacZ. Cells were transfected in triplicate and luciferase activity was normalized to β-galactosidase activity, produced by phβA-lacZ, to control for transfection efficiency. Mean and standard deviation of normalized luciferase activity produced by each construct are shown. (**B**) Upregulation of the *hTERT* promoter with cell immortalization. HA1 are SV-40-transformed HEK cells that do not express telomerase activity prior to immortalization (PD15) but become telomerase positive when immortal (PD 104). Transient transfection, enzymatic assays and normalization of luciferase activity are as in (A).

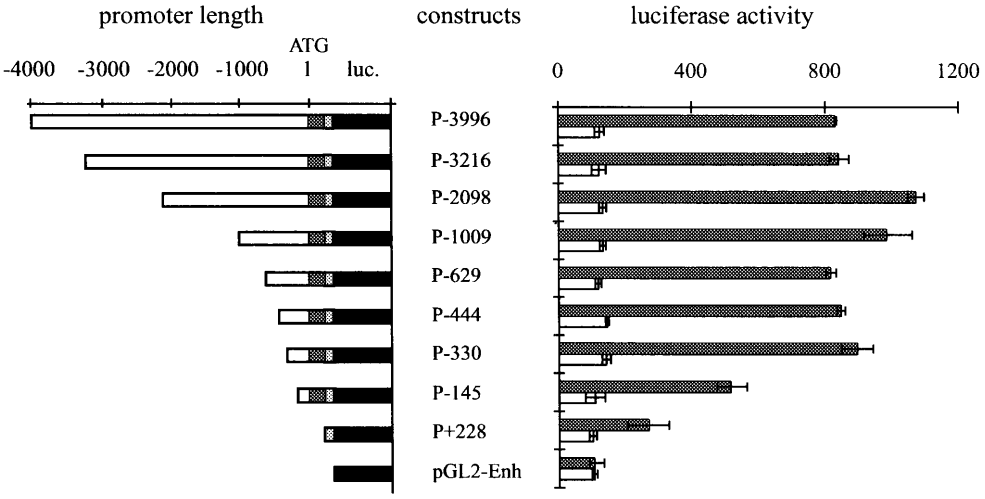

Figure 3. Deletion analysis of the *hTERT* promoter. For each construct the length of sequences upstream of the *hTERT* ATG (with A being position 1) is shown to the left and reflected in the name of the construct, and the corresponding luciferase activity is shown to the right. All constructs contain the *hTERT* first exon (dark grey) and first intron plus 37 bp of exon 2 (light grey), except for P+228 which contains only intron 1 and the portion of exon 2. Immortal 293 cells (grey histograms) and mortal HEK cells (white histograms) were transiently transfected and assayed as in Figure 2. Values of normalized luciferase activity were also obtained as described in Figure 2.

immortal cells opens the possibility of utilizing this promoter for the selective expression of toxic genes in cancer cells.

Sequencing of the *hTERT* promoter

Sequence analysis (Fig. 4) revealed that the *hTERT* promoter is GC-rich, has no detectable TATA or CAAT boxes but contains binding sites for several transcription factors. Notable among these are two binding sites for the myc oncogene which is known to induce *hTERT* expression (25). The presence of these sites is compatible with a direct effect of myc on *hTERT* expression. The abundance of binding sites for potential regulatory proteins within the *hTERT* minimal promoter further suggests that *hTERT* expression may be subject to multiple levels of control and regulation by different factors in different cellular contexts. It has been reported that protein kinase C is involved in the regulation of telomerase activity (30,31) and transcription factors may be differentially modified by this enzyme in different circumstances. Sequences upstream of the core promoter (Fig. 4) also contain a high concentration of binding sites for transcription factors as well as hormone response elements. The presence of binding sites for both estrogen (ER) and progesterone (PR) receptors is of particular interest since telomerase activity may be modulated by these hormones in human endometrial cells (32). We further note a continuous stretch of 66 bp (from –361 to –427) that is remarkably identical to sequences within the preS2/S gene of Hepatitis B virus [bp 307–372 (33)]. The significance of these sequences is presently unknown. Although potential *cis*-acting sequences located upstream of –330 bp are clearly not required for the activity of the *hTERT* minimal promoter in transient transfection assays, it seems plausible that at least some of them may contribute to the regulation of the endogenous *hTERT* promoter in different cells or growth conditions. The definition of the *hTERT* promoter will now allow detailed analysis of the *cis*-acting sequences by DNA footprinting and deletion mutagenesis, and of the effects of the cognate regulatory proteins following their overexpression in appropriate cells. These combined approaches should identify the key regulators of promoter activity and the pathways responsible for suppression or activation of the enzyme in different cells.

MATERIALS AND METHODS

Cells

Human fibroblasts WI-38 and MRC-5 and human embryonic kidney (HEK) cells are telomerase- and hTERT-negative normal cells (13,20,21). HeLa and 293 cells are immortal telomerase/hTERT-positive cells derived respectively from cervical carcinoma and from HEK cells following transformation with adenovirus DNA (34). HA1 cells are HEK cells transformed by SV40 T-antigen; they do not express telomerase activity or hTERT prior to crisis but express both on immortalization (13). With the exception of 293 cells which were grown in MEM-F11 plus 10% fetal calf serum, all cells were grown in α-MEM with 10% fetal calf serum.

Cloning and sequence analysis

The *hTERT* probe used for hybridization to the human genomic BAC library (35) consisted of the 5′ 2020 bp of the cDNA. Screening of the library yielded a positive clone. Restriction of this clone with *Eco*RI or *Bam*HI and hybridization with the full-length *hTERT* cDNA were used to identify positive subfragments. Of these, one *Eco*RI fragment of 23 kb and five *Bam*HI fragments ranging from 2.1 to 8 kb were cloned into pBluescript II SK (Stratagene), and hybridized with a series of oligonucleotides derived from the cDNA sequence to order them along the cDNA. Exon–intron junctions were sequenced using primers based on the cDNA sequence. For cloning of the *hTERT* promoter, the 23 kb *Eco*RI fragment that contained the 5′ region of the cDNA and upstream sequences was subjected to a series of 3′ deletions with *Pst*I. A candidate promoter fragment of 4355 bp, containing 3996 bp upstream of the ATG plus exon 1 (219 bp), intron 1 (104 bp) and 37 bp of exon 2, was sequenced on both

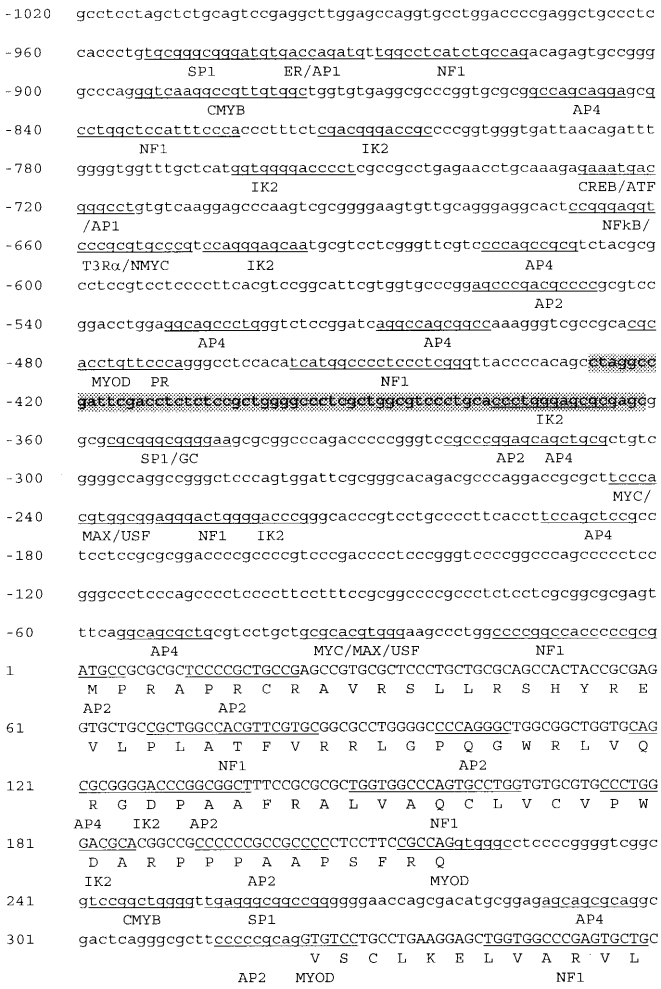

Figure 4. Nucleotide sequence of the 5′ flanking region of the *hTERT* gene. Numbers to the left indicate bases upstream and downstream of the ATG (position 1). The coding region of exon 1 and of a portion of exon 2 (separated by the first intron) are in upper case letters, with single letters below these sequences indicating amino acids. The core promoter region identified in this study extends from –330 to +361 bp. The shaded sequence was identified by BLAST search as a region of identity to the hepatitis B virus pre-S2/S gene (33). Putative binding sites for transcription factors and hormone response elements are underlined, and the name of the cognate proteins is listed below each site. AP1, AP2, AP4, activator protein 1, 2, 4; ATF, activating transcription factor; CREB, cAMP-responsive element binding protein; ER, estrogen receptor; GC, GC box element; IK2, Ikaros 2; MYOD, myoblast determining factor; NF1, nuclear factor 1; PR, progesterone receptor; SP1, stimulating protein 1; T3Rα, thyroid hormone receptor α; USF, upstream stimulating factor.

strands using T3 and T7 primers and subsequently primers derived from the obtained sequences (GenBank accession no. AF097365). All sequencing was performed by the Central Facility (Institute for Molecular Biology and Biotechnology, McMaster University) using BD-terminator chemistry with Taq-FS and cycle sequencing protocol on a Perkin Elmer-ABI 373A Stretch XL sequencer.

Sequence analysis was performed with the MatInspector V2.2 program using solution parameters corresponding to 100% similarity for the core region and 85% similarity for the flanking region of protein binding sites.

Promoter–luciferase constructs

A series of 5′ deletions of the 4355 bp candidate promoter was generated by partial digestion with *Pst*I. The resulting fragments were subcloned into the pGL2-Enhancer vector (Promega) upstream of the luciferase reporter gene to generate constructs P-3996, P-3216, P-1009, P-629 and P-444. Constructs P-330, P-145 and P+228 were obtained by partial digestion of P-3996 with *Xma*I and religation. The structure of all the constructs is shown in Figure 3.

Transfections and enzyme assays

Cells were seeded at a concentration of 3×10^5 cells/60 mm plate and cultured overnight. Transfections were carried out in triplicate using Lipofectamine (Gibco BRL), and were repeated three times. Plasmids used for transfection were purified on CsCl density gradients and DNA concentration was determined by fluorometry. Promoter–luciferase constructs and control pGL2-Enhancer vector were transfected together with phβA-lacZ, encoding the lacZ gene under control of the human β-actin promoter, as an internal control for transfection efficiency. Transfected cells were harvested after 60–70 h, and cell extracts were used in assays of luciferase and β-galactosidase activity using reagents and protocols from Promega. Luciferase activity of each extract was normalized to the β-galactosidase activity produced by the same extract and expressed in arbitrary units.

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