Cloning and Characterization of the 5′-Flanking Region for the Human Topoisomerase III Gene*

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The human DNA topoisomerase III (hTOP3) gene encodes a topoisomerase homologous to the Escherichia coli DNA topoisomerase I subfamily. To understand the mechanisms responsible for regulating hTOP3 expression, we have cloned the 5′-flanking region of the gene coding for the hTOP3 and analyzed its promoter activity. The presence of a single transcription initiation site was suggested by primer extension analysis. The hTOP3 gene promoter is moderately high in GC content and lacks a canonical TATA box, suggesting that hTOP3 promoter has overall similarity to promoters of a number of housekeeping genes. Examination of the promoter sequence indicated the presence of four Sp-1 consensus binding sequences and a putative initiator element surrounding the transcription initiation site. Transient expression of a luciferase reporter gene under the control of serially deleted 5′-flanking sequences revealed that the 52-base pair region from −326 to −275 upstream of the transcription initiation site includes a positive cis-acting element(s) for the efficient expression of hTOP3 gene. On the basis of gel mobility shift and supershift assays, we demonstrated that both YY1 and USF1 transcription factors can bind to the 52-base pair region. When HeLa cells were transiently transfected with a mutant construct which had disabled both YY1- and USF1-binding sites, the luciferase activity was greatly reduced, suggesting that these binding elements play a functional role in the basal activation of the hTOP3 promoter. Transfection studies with mutations that selectively impaired YY1 or USF1 binding suggested that both YY1 and USF1 function as activators in the hTOP3 promoter. DNA topoisomerases are nuclear enzymes that are able to break and reseal the sugar-phosphate backbone bonds of DNA and thereby adjust the topological states of DNA (1–4). The existence of multiple topoisomerases in both prokaryotes and eukaryotes has been documented. Eukaryotic DNA topoisomerases was originally identified from a hyper-recombination mutant that causes an increase in recombination between repetitive DNA elements (10). Null mutation of the topoisomerase III (TOP3) gene displays its pleiotropic phenotypes including slow growth, hyper-recombination, and a defect in sporulation. cDNA sequence of the yeast TOP3 gene revealed that it encodes a DNA topoisomerase homologous to Escherichia coli DNA topoisomerase I (10). In vitro biochemical studies with purified yeast TOP3 have shown that it partially relaxes negative but not positive supercoils, and its binding activity has a strong preference for single-stranded DNA (11). Recently, it was shown that a mutation of yeast SGS1 (slow growth suppressor) gene suppresses growth defect as well as increased genomic instability of TOP3 mutant (12). Sequence analysis of the SGS1 gene revealed that the SGS1 protein is highly homologous to the E. coli RecQ DNA helicase (12, 13). Two-hybrid analysis revealed that SGS1 protein physically interacts with TOP3, indicating that these proteins may function as a complex (12). This action mechanism is similar to that proposed for the Sulfolobus acidocaldarius reverse gyrase, which contains both a helicase-like domain and a bacterial DNA topoisomerase I-like domain combined in one polypeptide (14). Kim et al. (15) recently reported that the yeast TOP3 gene and EST1 gene, encoding a putative telomerase, are positioned head-to-head on chromosome XII. This arrangement suggests that the two genes may be co-regulated and functionally related. Yeast cell lacking TOP3 shows a shortening of telomeric repeats and a high frequency of loss of the subtelomeric sequences (15).

Human TOP3 (hTOP3) cDNA has recently been cloned and shown to consist of 976 amino acids (16). This gene locus has been mapped to chromosome 17p11.2–12. An alignment of the amino acid sequences of the hTOP3 with yeast TOP3, and E. coli topoisomerase I and III, revealed that for the region present in all four polypeptides, the human protein resembles yeast protein more than the two E. coli enzymes. Fritz et al. (17) demonstrated that the CAT4.5 human cDNA partially suppresses multiple aspects of the ataxia telangiectasia (A-T) phenotype upon transfection into A-T fibroblast. The CAT4.5 insert is an intron fragment fused to a nearly full-length cDNA.

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1 The abbreviations used are: TOP3, topoisomerase III gene; A-T, ataxia telangiectasia; PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; USF, upstream stimulatory factor.
of the hTOP3 gene. Both the CAT4.5 vector and an antisense hTOP3 construct inhibited spontaneous and radiation-induced apoptosisin A-T cells, whereas overexpression of a full-length hTOP3 cDNA failed to suppress apoptosis. The results suggested that the hTOP3 may be deregulated in A-T cells and involved in maintaining genomic stability, perhaps in concert with the Bloom’s or Werner’s syndrome DNA helicase (18, 19). The recent study on targeted disruption of the mouse TOP3 gene revealed that this gene is essential in early embryogenesis (20). The requirement of mouse TOP3 for viability might be due to its plausible roles in DNA replication and its interaction with the RecQ/SGS1 family of DNA helicases (20). Although endogenous expression of the hTOP3 gene was found in multiple somatic tissues (17), the mechanisms of transcriptional regulation of the hTOP3 gene at the promoter level are not known.

In this report, we describe the structural organization and functional characterization of the hTOP3 gene promoter. Our data demonstrate that the hTOP3 gene contains a single transcription initiation site and lacks a canonical TATA box near the transcription initiation site, and high level expression of the hTOP3 gene. The functional significance of the cis-acting elements and their transcription factors is discussed.

**EXPERIMENTAL PROCEDURES**

**Cloning of the 5′-Flanking Region of the hTOP3 Gene**—Approximately 10 μg of HeLa cell genomic DNA was digested with BamHI and ligated to the BamHI-digested pBluescript according to standard protocols (21). A portion of the ligation mixture was used as a template for amplification with two primers: the T7 standard sequencing primer served as the vector-specific primer, and the second primer was specific for the hTOP3 gene-specific primer-1 (5′-CTTTCTTCGCTCATGCGGAGTTCGCTG-3′) complementary to nucleotide +115 through +89 from the translation initiation codon (16). The first PCR products were reamplified with the T7 primer and the nested gene-specific primer-2 (5′-TCTTGGGCGGCATCGACG-3′) complementary to nucleotide −23 through −47 from the translation initiation codon (see location of primers in Fig. 1B). The amplification products were analyzed on a 1.2% agarose gel in 1× TBE and visualized by ethidium bromide staining. The PCR amplification product was subcloned into the pGEM-T vector (Promega) and sequenced on both strands by the dideoxy sequencing method (U.S. Biochemical Corp.). In order to test further the specificity of amplification products, the above procedure was repeated with PstI-digested HeLa cell genomic DNA.

** Primer Extension Analysis**—A 20-nucleotide antisense primer with sequence 5′-CTTCCGCTCATGCGGAGTTCGCTG-3′ was synthesized corresponding to the cDNA region 222–203 bp upstream of the translation initiation codon (16). An oligonucleotide was radiolabeled at the 5′ end with T4 polynucleotide kinase and [γ-32P]ATP (3,000 Ci/mmol, Amer sham Pharmacia Biotech). The radiolabeled primer (1 ng) was added to 15 μg of total cellular RNA isolated from HeLa cells in 50 μl Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM dNTPs, and 0.5 mM spermidine. The reaction mixtures were heated at 65 °C for 15 min and incubated at 37 °C for 2 h. One unit of avian myeloblastosis virus reverse transcriptase (Promega) was added to the mixture, and the reaction was continued at 42 °C for 30 min. After completion of the reaction, samples were extracted with phenol/chloroform and precipitated with ethanol. The extension products were dissolved in a denaturing dye solution and analyzed on a 6% polyacrylamide/urea gel. The size was determined by comparison with a DNA sequencing ladder.

**Northern Hybridization Analysis**—Total cellular RNA was isolated from exponentially growing cells using Tri reagent (Molecular Research Center). RNA samples (10 μg/lane) were separated on an 1% formaldehyde-agarose gel and vacuum-transferred to Hybond N-1 membrane. Northern hybridization was performed using a random primer DNA labeling system—Amer sham Pharmacia Biotech. The blot was hybridized with the probe at 65 °C for 12–18 h and then washed twice in 2× SSC and 0.1% SDS for 15 min each in 1× SSC and 0.1% SDS for 15 min and once in 0.1× SSC and 0.1% SDS for 30 min. Control hybridization was carried out using labeled glyceraldehyde-3-phosphate dehydrogenase probe for the cell line Northern blot and using β-actin probe for the multiple tissue Northern blot.

**Construction of Promoter-Luciferase Constructs**—A series of plasmids containing various sizes of the 5′-flanking region of the hTOP3 gene were constructed by inserting DNA fragments between the KpnI and BglII sites of the vector pGL2 (Promega). Amplified DNA fragments were prepared by PCR reactions using the following synthetic oligonucleotides incorporating 5′-KpnI and 3′-BglII sites for the 5′-ends of the inserts, 5′-GGGTTACGGGGCGGATCTGAGGCGGGCCGC-3′ (−274 to −303 from the transcription start), 5′-GGGGTACTCCGCGGCGGATCTGAGGCGGGCCGC-3′ (−255 to −274), 5′-GGGGTACCCCGGGCCGGATCTGAGGCGGGCCGC-3′ (−125 to −103), 5′-GGGGTACCGGCGGCGGATCTGAGGCGGGCCGC-3′ (−74 to −53), and 5′-GGGTTACGCGGCGGCGGATCTGAGGCGGGCCGC-3′ (−28 to −49). Into the 3′-ends of the inserts, 5′-GAAGATCTCCGGCTTCCGCTGACTCTAC-3′ (12 to −9), 5′-GAAGATCTCCGGCTTCCGCTGACTCTAC-3′ (9 to −6), and 5′-GAAGATCTCCGGCTTCCGCTGACTCTAC-3′ (6 to −3) were ligated to the immediate upstream fragment of the luciferase reporter gene.

**Transfection and Luciferase Expression Assays**—Cells plated onto six-well plates were grown to 50–70% confluence prior to transfection. 2 μg of test constructs were cotransfected with 2 μg of β-galactosidase expression plasmid, pCH110 (Amer sham Pharmacia Biotech). Each plasmid containing hTOP3 promoter-luciferase fusion gene was diluted into 0.1 ml of Opti-MEM 1 (Life Technologies, Inc.), and 4 μl of LipofectAMINE was added to 0.1 ml of serum-free medium. Plasmid DNA and LipofectAMINE were then mixed together and incubated at room temperature for 30 min to form DNA-liposome complexes. The complexes were added to 0.8 ml of serum-free medium, mixed gently, and placed on top of the cells. Following incubation of the cells for 6 h at 37 °C in a 5% CO2 incubator, 1 ml of growth medium containing twice the normal concentration of serum was added. After an additional 48 h incubation, the cells were lysed in 0.15 ml of lysis buffer (Promega) and centrifuged at 10,000 g for 10 min to remove cell debris. The supernatants were assayed for luciferase and β-galactosidase activities using the luciferase and β-galactosidase assay systems according to the manufacturer’s recommendation (Promega). Amounts of lysates employed for the luciferase activity assays were normalized to the β-galactosidase activities.

**Nuclear Extracts and Gel Mobility Shift Assays**—HeLa cell nuclear extracts were prepared from 5 × 106 cells according to the method of Dignam et al. (22). Nuclear extracts were quantitated by the Bradford assay (Bio-Rad) and stored at −70 °C. The protein concentration was 6 mg/ml. For gel mobility shift assay, duplex probes were end-labeled by filling in with Klenow DNA polymerase and [γ-32P]CTP. Approximately 1 ng of the labeled probe was mixed with 2.4 μg of nuclear protein in a total of 20 μl of the binding buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM diethiothreitol, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) containing 1 μg of poly(dI-dC). After incubation in ice for 20 min, the reaction mixture was separated on a 5% nondenaturing polyacrylamide gel with 0.5× TBE buffer (1× = 0.5× TBE buffer and 1× EDTA). The gel was dried and subjected to autoradiography. For competition experiments, the molar excess of unlabelled competitor DNA was added prior to the addition of labeled probe as specified. For supershift experiments, 0.2 μg of antibody was added to the mixture 20 min prior to the addition of labeled probe, and the mixture was further incubated for 20 min at room temperature. All antibodies used in these experiments were purchased from Santa Cruz Biotechnology.

**RESULTS**

**Cloning and Characterization of the hTOP3 Promoter Region**—The 5′-flanking region of the hTOP3 gene was cloned using a gene-specific primer based on the known sequence of hTOP3 cDNA (23). BamHI-digested HeLa cell genomic DNA was ligated to the linear pBluescript treated with the same restriction endonuclease. The ligation mixture was subjected to PCR amplification using a specific primer-1 annealing to the fragment of the hTOP3 cDNA was labeled with [α-32P]dCTP (Amer sham Pharmacia Biotech) to a specific activity of 1 × 108 cpm/μg using random primer DNA labeling system (Amer sham Pharmacia Biotech). The blots were hybridized with the probe at 65 °C for 12–18 h and then washed twice in 2× SSC and 0.1% SDS for 15 min each in 1× SSC and 0.1% SDS for 15 min and once in 0.1× SSC and 0.1% SDS for 30 min. Control hybridization was carried out using labeled glyceraldehyde-3-phosphate dehydrogenase probe for the cell line Northern blot and using β-actin probe for the multiple tissue Northern blot.
known 5′-end sequence of the hTOP3 cDNA and the standard sequencing primer T7 annealing to the unknown end. To increase the specificity of the amplification, the first PCR products were reamplified using the T7 primer and the nested gene-specific primer-2 (see location of primers in Fig. 1). The amplification products were analyzed on a 1.2% agarose gel and visualized by ethidium bromide staining (data not shown). Southern blot analysis of the same gel using a 32P-labeled 5′-untranslated region of hTOP3 cDNA further confirmed the specificity of the amplification products (data not shown). By using this method, a 376-bp region upstream of the known 5′-end of the hTOP3 cDNA previously published (16) was obtained (Fig. 1). The same process was performed with PstI-digested HeLa cell genomic DNA, and an additional 938-bp upstream region was obtained and sequenced (Fig. 1).

**Determination of the Transcription Initiation Site**—To determine the transcription initiation sites of the hTOP3 gene, primer extension was performed as described under “Experimental Procedures.” A radiolabeled antisense primer was hybridized to total RNA isolated from HeLa cells, and the extension products were analyzed on a sequencing gel (Fig. 2). As deduced from the extension products, the major transcript is being initiated from the guanine residue located 304 bp upstream of the ATG codon. Accordingly, this base was designated hereafter as +1 bp unless otherwise stated and extended the 5′-end of hTOP3 cDNA previously reported by 52 bases (16). The same primer extension product was obtained using total RNA isolated from other human cell lines (data not shown).

**Constitutive Expression of the hTOP3 mRNA in Multiple Somatic Tissues and Various Cell Types**—The identification of the transcription initiation site revealed that the 5′-untranslated region of hTOP3 mRNA is 304 bp. By adding this region to the 2,928 bp of the open reading frame and to the 575 bp of 3′-untranslated sequence (16), the size of hTOP3 mRNA was estimated as 3.8 kb. This was further confirmed by Northern analysis of the transcription product of the hTOP3 gene. Blot containing total RNAs from five human cell lines was hybridized with a probe consisting of a 1.1-kb PstI fragment of the hTOP3 cDNA. One distinct hTOP3 transcript of about 3.8 kb was detected, and the level of hTOP3 mRNA was approximately identical in all cell lines examined (Fig. 3 A). Control hybridizations with a glyceraldehyde-3-phosphate dehydrogenase probe confirmed nearly equal mRNA amounts in each lane. Northern analysis of hTOP3 transcript in multiple normal somatic tissues showed that hTOP3 mRNA is present as a 3.8-kb transcript and is expressed to a similar level in all tissues except for the weak levels of expression in lung and kidney (Fig. 3 B). The fast migrating band in the skeletal muscle is known to be an artifact. (While our paper was being prepared, Fritz et al. (17) reported the Northern analysis of hTOP3 mRNA in multiple tissues that overlaps with the result of Fig. 3 B in this study.)

**Transient Expression Analysis of the hTOP3 Promoter**—In order to determine the promoter activity and localize important regulatory regions within the 5′-flanking sequence of the hTOP3 gene, a series of deleted promoter region-luciferase fusion plasmids were constructed (Fig. 4 A). These plasmids were transfected into HeLa cells, and the luciferase activities were measured from the cell lysates. As shown in Fig. 4 A, both...
the \(-1,262/\text{+}82\) and \(-326/\text{+}82\) fusion constructs promoted high levels of luciferase expression. In contrast, \(5\)'-deletions of various lengths from \(-274\) to \(-74\) resulted in a reduction of promoter activity to about 30–43% that of the \(-326/\text{+}82\) construct. These results clearly indicate that a positive regulatory element(s) is located between \(-326\) and \(-275\) and that this region is essential for a high level expression of the hTOP3 gene. Transfection of the \(-26/\text{+}82\) fusion construct resulted in a lower level of luciferase activity (30%) as compared with that of the \(-74/\text{+}82\) construct. This suggested the presence of another positive regulatory element(s) between \(-74\) and \(-27\). The \(-74/\text{+}12\) construct showed a much lower luciferase activity when compared with that of the \(-74/\text{+}82\) construct, whereas the \(-26/\text{+}12\) construct did not have any measurable promoter activity. Based on these results, the Sp-1-binding site located downstream of the transcription initiation site may be essential for the minimal promoter activity of the hTOP3 gene (see Fig. 1 for location of downstream Sp-1-binding site).

To extend the study of hTOP3 promoter activity to various human cell types, we transfected the \(-326/\text{+}82\) construct into SW-48 (colon) and MCF-7 (breast) cells. A similar level of luciferase activity (30%) as compared with that of the \(-326/\text{+}82\) construct showed a much lower luciferase activity when compared with that of the \(-74/\text{+}82\) construct, whereas the \(-26/\text{+}12\) construct did not have any measurable promoter activity.

Analysis of the Binding Activities to the Regulatory Elements by Gel Mobility Shift Assays—In order to demonstrate a nuclear protein factor(s) specific for binding to the 52-bp region extending from \(-326\) to \(-275\) in the hTOP3 promoter, the nuclear protein-DNA interaction was detected by reduced electrophoretic mobility on a native polyacrylamide gel. As shown in Fig. 5A, two major complexes (complexes A and B) and one weak complex (complex B') were formed with nuclear extracts from HeLa cells (lane 2) (see below for the explanation of the complex B' between complex B and free probe). The specificity of these complexes for the sequence was shown by a competition experiment, in which the complexes were completely abolished by competition with a 40- or an 80-fold excess of an unlabeled wild-type probe (lanes 3 and 4). In contrast, the same molar excess of a nonspecific DNA fragment failed to compete (lanes 5 and 6), indicating that the 52-bp region contains cis-elements for transcription factor binding. Computer analysis of the 52-bp cis-element using MatInspector program (version 2.1) revealed the presence of putative regulatory consensus sequences. In order to identify the nuclear proteins involved in the formation of the protein-DNA complexes, two mutated oligonucleotides were synthesized and used as competitors in the mobility shift assays. M1 oligonucleotide contained mutated sequences in the YY1 core-binding element (CCAT), and M2 oligonucleotide was mutated in the E box (CACATG) as shown in Fig. 5B. The complex A selectively decreased in the presence of the excess M1 oligonucleotide, and this inhibition was dose-dependent with respect to the amount of the M1 oligonucleotide used in the assay, but the complexes B and B' were not affected by the M1 oligonucleotide (Fig. 5C, lanes 3–6). Conversely, preincubation with the excess M2 oligonucleotide selectively eliminated complexes B and B' but did not affect complex A (lanes 7–10). In conjunction with supershift data using anti YY1 antibody (see below, Fig. 6B), these results indicated that the complex B was formed with YY1. The complex B' most likely contained proteolytic degradation products of YY1, as it was removed by a M2 oligonucleotide and supershifted by the anti YY1 antibody (Fig. 6B). YY1 has been previously reported.
to be susceptible to proteolytic degradation (24).

The Binding of YY1 and USF1 to the Regulatory Element—Since many helix-loop-helix proteins such as USF1 and c-Myc/Max are known to bind to CANNTG (E box) motif (25, 26), we next identified the transcription factor(s) which is involved in the formation of the complex A. The duplex oligonucleotides containing the binding consensus sequences of AP-1, CREBP, USF1, OCT1, and c-Myc/Max were prepared and used as competitors. In the competition experiments shown in Fig. 6A, the complex A was completely eliminated by the unlabeled authentic USF1-binding oligonucleotide (lanes 7 and 8) and c-Myc/Max-binding oligonucleotide (lanes 11 and 12). In contrast, the complexes B and B' were not affected by any of the competitor oligonucleotides used. Wild type oligonucleotide completely abolished the formation of all complexes (lanes 13 and 14). These results indicate that the 52-bp cis-element region could form two specific complexes as follows: complex A containing USF1 or c-Myc/Max and complex B containing YY1.

To characterize nuclear factors in the protein-DNA complexes further, we performed an extensive supershift analysis of the complexes by utilizing antibodies against various transcription factors having potential binding sites in the 52-bp cis-element region. As expected, preincubation with the anti-YY1 antibody reduced the level of complex B, but had no effect on the complex A (Fig. 6B, lane 3). Note that complex B' was also supershifted by the anti-YY1 antibody. Conversely, preincubation with the anti-USF1 antibody selectively inhibited the formation of complex A and generated a supershifted complex but did not affect the formation of complexes B and B' (lane 8). However, when anti-c-Myc or anti-Max antibody was preincubated with nuclear extracts, both complexes A and B still remained (lanes 6 and 7), indicating that the nuclear factor involved in the formation of complex A may be USF1 and not c-Myc/Max. Such control antibodies as anti-OCT1 and anti-SREBP did not affect the retarded complexes (lanes 4 and 5). Neither the retarded nor the supershifted complex was found when the antibodies were incubated with the probe in the absence of cell extracts (data not shown).

The identities of USF1 and YY1 complexes in cell extracts were further confirmed by gel mobility shift analyses using both oligonucleotide competition and antibody supershift assays as shown in Fig. 6C. USF1-retarded complex A and YY1-retarded complexes B and B' were selectively abolished in the presence of a 40-fold excess of M1 and M2 oligonucleotides, respectively (Fig. 6C, lanes 3 and 5). Preincubation with the anti-YY1 antibody selectively abolished complexes B and B' (lane 4), but preincubation of the anti-USF1 antibody had no effect on the formation of complexes B and B' (lane 5). Preincubation with the anti-YY1 or anti-USF1 antibody in the presence of M2 oligonucleotide further identified the complex A as a USF1 complex (lanes 7 and 8).

Mutational Analysis of the YY1- and USF1-binding Sites in the hTOP3 Promoter—Since the regulatory elements located in the region between −326 and −275 exhibited strong binding affinities for YY1 and USF1, we next analyzed the transcriptional regulatory role of this region. In order to measure the promoter activities of YY1- and USF1-binding sites, we mutated the hTOP3 promoter by site-directed mutagenesis at the YY1-binding site, USF1-binding site, or at both sites (Fig. 7A). Their promoter activities were examined by transient transfection...
tion of HeLa cells. As shown in Fig. 7B, the double mutation at YY1- and USF1-binding sites reduced the luciferase gene expression by 60%. This could suggest that either YY1 binding or USF1 binding or both are involved in maintaining the expression of wild-type promoter. A point mutation at the YY1 core-binding site, which permits only USF1 binding, showed a 13% reduction in the promoter activity, whereas a point mutation at the USF1 core-binding site, which permits only YY1 binding, reduced the promoter activity by 28%. Thus, both YY1 and USF1 can increase the activity of the hTOP3 promoter by binding to this region and potentially contribute to its basal activation in HeLa cells. These results clearly suggest that the YY1- and USF1-binding sites both function as positive cis-elements for the efficient expression of the hTOP3 gene. Although the degree to which YY1 and USF1 contribute may depend on the physiological state of the cell, the USF1 site has a higher enhancing activity of the hTOP3 promoter than the YY1 site in HeLa cells.

**DISCUSSION**

Recently, a human cDNA encoding the hTOP3 has been identified (16). This enzyme is probably present in many different cell types as manifested by Northern analysis of hTOP3 mRNA levels in multiple somatic tissues (17). However, mouse TOP3 was highly expressed in the testis in comparison with other tissues (27), suggesting that mouse TOP3 gene might be differentially regulated in a tissue-specific manner. In this work, to identify the transcriptional control region, we have isolated and characterized genomic clones containing the 5'-flanking region of the hTOP3 gene. The promoter region, which is sufficient to confer high level expression of a luciferase reporter gene, was mapped to a region between −2326 and −182. This appears to indicate that this region contains cis-acting regulatory elements responsible for the hTOP3 gene expression and that a number of different DNA binding factors are involved in the basal hTOP3 expression. The hTOP3 promoter
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region is moderately high in GC content and lacks a canonical TATA box, suggesting that hTOP3 promoter has overall similarity to promoters of a number of housekeeping genes (28). The dinucleotide CpG occurs approximately every 10 bp in the combined regions of the basal promoter and the 5'-untranslated sequence of the hTOP3 promoter, whereas CpG occurs every 50 to 100 bp on average in the major fraction of the mammalian genome (29, 30). Thus, methylation of these regions might be involved in regulation of hTOP3 gene expression.

The transcription initiation site determined by primer extension analysis showed a single major site at the guanine residue located 304 bp upstream from the ATG codon. Because the hTOP3 promoter contains no potential upstream TATA-like sequence, transcription initiation does not appear to be associated with TATA box, which would have the role of directing accurate transcription initiation through a mechanism that depends on its specific interaction with the TATA-binding protein (31). Some TATA-less promoters retain the ability to direct transcription initiation from a specific nucleotide, whereas others direct transcription initiation at multiple start sites (32). Many promoters also contain initiator sequences in the vicinity of transcription initiation that appear to be essential for the precise location of a transcription initiation site (32). The sequence surrounding transcription initiation site of the hTOP3 promoter is homologous to the murine terminal transferase initiator (33). Like many housekeeping genes, the promoter of hTOP3 gene contains four GC boxes around the transcription initiation site with a potential for binding of the transcription factor Sp1 (Fig. 1). Although the Sp1 sites are generally located upstream of the transcription initiation site, Sp1 can also increase promoter activity at downstream locations (34). One of the GC boxes (from position +7 to +15), located downstream of the transcription initiation site, is essential for the minimal promoter activity. The −74/+12 construct, which does not contain downstream GC box, exhibited much lower luciferase activity as compared with that of the −74/+22 construct (Fig. 4). The basal promoter fragment extending to −326 bp from the transcription initiation site directed higher levels of luciferase activity in transient transfection assays than that of the promoter fragment extending to −274 bp (Fig. 4). The result clearly demonstrated that an element(s) responsible for the promoter activity must be contained in the 52-bp region between −326 and −275. As manifested by combined gel mobility shift assays, competition experiments, and supershift assays, this region contains core CCAT and CACATG motifs for binding of YY1 and USF1 transcription factors, respectively. Transient transfection studies with mutations showed that this region is important for the expression of hTOP3 promoter and that both YY1 and USF1 factors function as transcriptional activators.

YY1 is a zinc finger transcription factor that contains structural similarity to the GLI-Krüppel protein (35, 36). Depending on the DNA sequence context, YY1 can function as an activator (37, 38), a repressor (24, 39, 40), or an initiator of transcription (41, 42). The YY1-binding element within the hTOP3 promoter matches well the consensus sequence proposed for the YY1 binding (43–45). Previously, a number of composite binding elements, in which YY1 competes for occupation by another transcription factor, have been described (24, 40, 46). In all the reported composite sites, YY1 acted as a repressor either by decreasing transcription through a repressor domain in its C terminus or by displacing an activator from the site (43). Unlike these composite YY1-binding sites, YY1 acted as an activator in the YY1/USF1-binding site of hTOP3 promoter (Fig. 7B). Recently, Furlong et al. (47) demonstrated that YY1 functions as an activator to increase human p53 promoter activity in a composite element that can bind both YY1 and NF1 in a mutually exclusive manner. Thus, these cases may reflect the general function of YY1 as an activator in the composite site.

A number of helix-loop-helix proteins bind as dimers to the hexanucleotide sequence known as an E box (25, 26, 48–50). Our supershift assay indicated that a transcription factor, which binds to an E box of the hTOP3 promoter, was USF1 (Fig. 6). USF1 plays an important role in the basal expression of many genes (51). Transient transfection of the wild-type fusion plasmid (−326/+82) into HeLa cells showed a significant luciferase gene expression (Fig. 7). This promoter activity was greatly reduced when neither YY1 nor USF1 binds to the 52-bp region, suggesting a functional role of this region in the basal activity of the hTOP3 promoter. A mutation in a USF1-only binding element or in a YY1-only binding element showed a reduction in luciferase gene expression as compared with that of wild-type construct but exhibited a higher level of luciferase activity than that for −274/+82 construct (Fig. 7). Although HeLa extracts contain both YY1 and USF1 in differing amounts, these results confirm that both YY1 and USF1 have a potential to contribute positively to the basal hTOP3 promoter activity.

In summary, we have isolated and characterized the hTOP3...
promoter region that drives expression of a luciferase reporter gene in HeLa cells. Our results clearly demonstrate that the 52-bp region between -326 and -275 containing consensus binding elements for YY1 and USF1 transcription factors is essential for the high level expression of the hTOP3 gene. From mutations that selectively impaired YY1 and USF1 binding, we concluded that both YY1 and USF1 function as activators for the hTOP3 expression (Fig. 7). Further study will be required to elucidate the physiological roles of other embedded elements in the promoter and the upstream regions that regulate the basal and cell cycle-dependent expression as well as tissue-specific expression of the hTOP3 gene.

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