Molecular Cloning and Characterization of the Promoter for the Chinese Hamster DNA Topoisomerase IIα Gene*

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To investigate the mechanisms governing the expression of DNA topoisomerase IIa, the Chinese hamster topoisomerase IIα gene has been cloned and the promoter region analyzed. There are several transcriptional start sites clustered in a region of 30 base pairs, with the major one being 102 nucleotides upstream from the ATG translation initiation site. Sequencing data reveal one GC box and a total of five inverted CCAAT elements (ICEs) within a region of 530 base pairs upstream from the major transcription start site. Sequence comparison between the human and Chinese hamster topoisomerase IIα gene promoter regions shows a high degree of homology centered at the ICEs and GC box. In vitro DNase I footprinting results indicate protection by binding proteins at and around each ICE on both DNA strands. However, no obvious protection was observed for the GC box. Competition gel mobility shift assays with oligonucleotides containing either the wild-type or mutated ICE sequences suggest that identical or similar proteins specifically bind at each ICE, although with different affinities for individual ICE sequences. Chloramphenicol acetyltransferase assays employing nested 5′-deletions of the 5′-flanking sequence of the gene demonstrate that the sequence between −186 and +102, which contains three proximal ICEs, is sufficient for near wild-type level of promoter activity. When these three ICEs were gradually replaced with sequences which do not interact with the binding proteins, reducing promoter activity of the resultant constructs was observed. In conjunction with results from footprinting and gel mobility shift studies, the transient gene expression finding suggests that the ICEs are functionally important for the transcriptional regulation of the topoisomerase IIα gene.

Mammalian DNA topoisomerase II (Top II) is an essential nuclear enzyme which changes the topology of DNA by passing an intact helix through a transient double-stranded break made in a second helix followed by religation of the DNA break (reviewed in Refs. 1 and 2). The enzyme functions as a homodimer and in an ATP-dependent manner (3). A feature of Top II function is the covalent attachment of the enzyme to the 5′-termini of DNA breaks via a tyrosine-DNA phosphodiester linkage. Top II has been implicated in a number of cellular processes such as synthesis and transcription of DNA (4) and chromosomal segregation during mitosis (5). Top II enzyme also plays a structural role in organizing both mitotic chromosomes and interphase nuclei (6, 7). Use of specific antibodies has demonstrated that Top II is a major component of the mitotic chromosomes and the interphase nuclear-matrix fractions (7). Moreover, specific DNA scaffold-attachment sites have been found to contain the consensus cleavage sequence for Top II (8).

Top II is also the target of several classes of anti-cancer drugs such as anthracyclines, amsacrine, and epipodophyllotoxins. These drugs stabilize the cleavable complex formed between Top II protein and DNA, resulting in increased DNA scission and concomitant inhibition of the joining reaction (9). The drug-induced DNA breaks are reversible after drug removal. However, most of the cells are arrested in the G2 phase and eventually die (10).

Resistance to agents that target Top II is a major problem in cancer chemotherapy. In addition to the classical multidrug resistance, which is due to overexpression of the multidrug resistance transporter (mdr protein or P-glycoprotein) (11), atypical multidrug resistance (at-MDR) has been described and is associated with altered Top II activity that is due to either mutated enzyme or a decrease in the amount of the enzyme (11–13). It is likely that lower Top II levels result in fewer drug-induced DNA lesions and diminished cytotoxicity of Top II-targeting drugs (14, 15). A correlation between cellular expression of Top II and the in vitro sensitivity to Top II active anti-tumor drugs has been found in a VM-26-resistant human cancer KB cell line (16), the 9-hydroxyellipticine-resistant Chinese hamster lung fibroblast cell line DC3F/9-OHE (17), and in a panel of seven human lung cancer cell lines (18).

In human and probably in other mammals, Top II occurs in two isoforms, the 170-kDa α form and the 180-kDa β form, which are encoded by two discrete genes (19, 20). These isoforms have different in vitro sensitivities to antineoplastic agents, different cleavage sites, thermal stability, and inhibition by AT-rich oligonucleotides (21). Recent work has demonstrated that the expression of the 170-kDa form is quantitatively cell cycle-regulated and cell proliferation-related (21, 22). The level of expression peaks in the late G2 to M phases and is greater in rapidly proliferating cells. In proliferating granulocyte precursors, the levels of 170-kDa in vivo were 2–3-fold higher than mature cells and approached the levels in neoplastic cell lines of the same lineage (22). In ras-transformed cells, the proportion of 170-kDa Top II is higher and depends less on growth state than in untransformed cells (23). The ras-transformed cells were also more sensitive to the cytotoxic effects of teniposide and merbarone, drugs which selectively inhibit the...
170-kDa form of Top II, indicating a possible link between drug sensitivity and expression of the 170-kDa form (23). The changes in amount of the mRNA coding for the 170-kDa enzyme were similar to the changes in the 170-kDa enzyme levels, suggesting that the regulation might be mainly at the transcriptional level (23).

In order to investigate the cell cycle-regulated expression of the Top IIα gene and the mechanisms of altered top II expression in drug-resistant cells, genomic clones for the Top IIα gene of Chinese hamster were isolated, and the 5′-flanking region of the gene was analyzed. These studies have identified and characterized a group of inverted CCAAT elements, which are present in the proximal promoters of both human and Chinese hamster top IIα genes, and are functionally important for the transcriptional regulation of the Top IIα gene.

MATERIALS AND METHODS

Cell Culture

Wild-type Chinese hamster ovary cells (CHO) were maintained in α-minimal essential media, supplemented with 10% fetal bovine serum at 37°C in the presence of 5% CO2.

Isolation of CHO Genomic Clones

The Chinese hamster ovary genomic library was purchased from Stratagene, which was prepared by cloning CHO-K1 genomic DNA in Lambda-Fix™ II vector. The phages were propagated in host bacteria P2-392. The transformation and plating procedures were according to the recommendation of the manufacturer. 105 plaques were screened under stringent conditions as described elsewhere (24) with the CHO Top IIα DNA probes, pC431 (5′-end probe) or pC42 (middle and 3′-end probe) (25). The CDNA probes were multiprimed labeled with the Klenow fragment of DNA polymerase I in the presence of [α-32P]dCTP (24). Filter hybridization, recovery of recombinant phase DNA, restriction mapping, as well as the subcloning of genomic fragments into pBlue-script and M13 vectors (Stratagene) were as previously described (24). Sequencing was performed on both strands of DNA by using a Sequenase kit (U.S. Biochemical Corp.). Sequencing data were analyzed with MacVector and GCG (Genetics Computer Group) sequence analysis programs.

Determination of Transcriptional Start Site

Primer Extension—A 21-mer oligonucleotide with sequence 5′-CTCAGTGATCCGGAAGCGGAC-3′, which is complementary to the cDNA sequence 25–40 base pairs upstream from the ATG codon, was labeled by T4 polynucleotide kinase and [α-32P]ATP. 5 × 106 cpm of labeled primer were hybridized to 2 μg of CHO poly(A)+ RNA or 5 μg of control yeast RNA. The annealed primers were extended by Superscript reverse transcriptase (Life Technologies, Inc.), and the extended products were analyzed on a denaturing polyacrylamide gel. 

RNA Protection Assay—The 4.0-kb HindIII-SalI genomic fragment (the Sall site was from the vector) was cloned into the pBlue-script plasmid. This plasmid was used in the polymerase chain reaction with a pair of primers containing HindIII cloning sites, 5′-CGCTGAGTCCGGAAGCGGAC-3′ (the forward primer), and 5′-CGCTAGCTTGGGACGCTCTGTAAGG-3′ (the reverse primer), to amplify the 849-bp proximal fragment upstream from the ATG site. This fragment corresponds to the transcription start site (as determined by nuclease protection assays) and contains 93 nucleotides both 5′- and 3′-flanking sequences. This plasmid was labeled at the BamH I site using Klenow fragment of DNA polymerase I and [α-32P]dCTP (24). The Gel Mobility Shift Assay—Gel Mobility Shift Assays were performed according to Godin et al. (29). The DNAase I Footprinting—DNAase I footprinting experiments were performed according to Godin et al. (29). The DNase I digestion of the genomic DNA was performed with the same labeled DNA by the Maxam and Gilbert method (30).
Fig. 1. The 5'-genomic region of the CHO top IIα gene. A, the partial restriction maps of the two genomic clones, Top II-21 and Top II-93, are shown. The restriction map of the 5' genomic region is expanded in the middle (the SalI site is from the vector). B, Boxes represent noncoding sequences, while the stippled boxes show the coding regions of the first, second, and third exons in this region. The arrow on top of the NcoI site represents the translation start and the orientation of the gene. Beneath the 4.0-kb HindIII-SalI fragment are the three fragments (open boxes) employed for the DNase I footprinting and gel mobility shift assays, as well as the region of DNA sequence (solid box) shown in B. Abbreviations: B, BamHI; Bs, BstEII; E, EcoRI; H, HindIII; N, NcoI; P, PstI; S, SalI; B, DNA sequence of the 5'-portion (solid box in A) of the CHO top IIα gene. The coding sequence of the first exon is shown in boldface. The arrow indicates the minor transcriptional start site (+1). The five inverted CCAAT sequences are boxed, and both the GC box and the TATA-like sequence are underlined.

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TCCTCTGAT-3' and its complementary strand. The second ICE-containing oligonucleotides were 5'-AGACCCGGTCAGATGTTTACTCTCTCAAA-3' and its complementary strand. The third ICE-containing oligonucleotides were 5'-CCTCTTTACCTAAATGGTTTACCTGACAG-3' and reverse strand 5'-TTCTGAATGAAAGCTATCTTCTGAGG-3'. The fourth ICE-containing oligonucleotides were 5'-ACACGAATAGCATTGGCTGCTCTCCTGAAGAG-3' and reverse strand 5'-TTGGCCTTTCTCTTCTGAGG-3'. The fifth ICE-containing oligonucleotides were 5'-TGGGCGCTTTCTACTGGGACAG-3' and reverse strand 5'-GGAAATCTGCGAATGGAGAGGGGCTAT-3'. The mutated ICE-containing oligonucleotides are the first ICE-containing oligonucleotide with the core 5'-ATTGG-3' sequence mutated to 5'-CTGGA-3' and its complementary sequence.

RESULTS

Cloning of the 5'-Flanking Region of the Chinese Hamster top IIα gene—The Chinese hamster genomic library was screened under stringent conditions with either the pC431 (5'-end of the cDNA) probe, or the pC42 (middle and 3'-end) probe, of the CHO top IIα cDNA (25). A total of six overlapping genomic clones were isolated. (The structure of the top IIα gene will be described in detail elsewhere.) The clone Top II-93 is the only clone which hybridized to the 5'-end cDNA probe and not to the pC42 probe. This and another overlapping clone, Top II-21, were further characterized. The 5'-end of the cDNA was mapped to a 4-kb HindIII-SalI fragment of Top II-93. This fragment was subsequently subcloned into pBluescript vector and analyzed. Sequencing data revealed the location of the first, second, and third exons and the 5'-flanking region of the top IIα gene (Fig. 1). The ATG translation initiation codon of the cDNA is located at the NcoI site of the genomic fragment. The coding sequence ends abruptly after 21 nucleotides at the PstI site and is followed by the 1.01-kb first intron. The second and the third exons are 153 and 91 bp, respectively. Sequencing of the 0.87-kb EcoRI and the 4.5-kb EcoRI fragments from the clone Top II-21 confirmed the Top II-93 sequence.

Determination of the Transcriptional Start Site of the Top IIα Gene—To locate the transcriptional start site and confirm the translation initiation site, primer extension was performed. A 32P-labeled 21-mer oligonucleotide complementary to the cDNA sequence 20-40 base pairs upstream of ATG was employed in the experiment. Several extension products were observed (Fig. 3A). The major transcript, as deduced from the predominant extension product, is being initiated from the cytosine 102 nucleotides upstream from the ATG codon. Other minor transcripts are initiated at adenine 99, cytosine 110, and thymine 119 and 129, respectively. To confirm the primer extension result, an RNase protection assay was carried out. A chimeric clone containing the genomic sequence upstream of the gene was constructed and analyzed for the presence of the major transcript (Fig. 3B).
the ATG site of the cDNA sequence was subcloned into pBlue-script vector (see "Materials and Methods"), and 32P-labeled RNA in antisense orientation was synthesized. Fig. 2 shows that several protected RNA fragments with sizes ranging from 100 to 130 bases were detected. The pattern of the RNase protected probes was similar to the primer extension pattern, and the sizes of the protected bands agree with the primer extension data, considering that there is a slight difference in the electrophoretic mobility of the RNA probes and the DNA marker. Downstream of the transcriptional start sites, the ATG initiation codon deduced from the cDNA sequence (25) is the first ATG codon in the genomic sequence, and the sequence, ACCATGG, is a perfect match to the optimal sequence for initiation by eukaryotic ribosomes as suggested by Kozak (31). The major transcriptional start site is designated hereafter as 1 unless otherwise stated.

Sequence Analysis of the 5'-Flanking Region of the CHO top IIα Gene—The region between the transcriptional and translational start sites and the 200-bp region immediately upstream of the transcriptional start site have a moderately high GC content of 64 and 49%, respectively. There is no canonical TATA box sequence, although an imperfect sequence of AAT-GAA was located 26 bp upstream of the predominant transcriptional start site. Further upstream at the −122 position, there is a TATA-like sequence (AATAAA). The 541-bp 5'-flanking sequence from the first upstream EcoRI site to the translation initiation site was searched for potential binding sites for transcription factors. The most prominent sequence motifs in this immediate upstream region are one GC box with potential for Sp1 binding on the coding strand and five CCAAT sequences on the opposite strand (refer to Fig. 1B). These five inverted CCAAT elements (ICEs) are designated one to five according to their proximities to the ATG start codon. There are also two sequences at (60 to 66) and at (−339 to −333), which are a one-nucleotide mismatch to a canonical Ap1 sequence T(T/G)AGTCA. A pair of perfect direct repeat sequences of AGAGCTGAG are located at positions −327 to −319 and −317 to −309. Downstream from them there is a pair of single mismatched inverted repeats at positions −300 to −294 and −293 to −287. The 560-bp 5'-flanking and first exon sequence was submitted to a search for homologous sequences in the GenBank™ data base. The Chinese hamster sequence shares extensive homology with the human top IIα promoter sequence (32) and the 5'-end of the mouse top IIα cDNA sequence (33) (Fig. 3A). The most homologous parts between human and Chinese hamster promoters are the region around the transcriptional start site (78% identity from 1 to 47 of the Chinese hamster sequence), the region immediately upstream of the ATG codon (85% from 59 to 106), and the region around the fifth ICE (80% from 260 to 215). The Chinese hamster and the human promoter sequences were aligned by the Bestfit program and are presented in Fig. 3B. The GC box, TATA-like element, and the first three ICEs of the hamster sequence can be aligned to the corresponding elements of the human sequence. However, the fourth hamster ICE element is positioned at a sequence having one mismatch with the human gene. The
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**Fig. 4. DNase I footprinting analysis of the 5′-flanking region of the CHO top IIα gene.** Experiments were performed as described under “Materials and Methods” with either the coding strand (A) or the noncoding strand (B) labeled. For all panels, Maxam and Gilbert (A-G) sequence ladders are electrophoresed on the side, with the positions of the sequence elements indicated: numbers 1-5 for the inverted CCAAT sequences, GC for the GC box, and TA for the TATA-like sequence. On the right side of the footprinting lanes, the footprints at each ICE and the juxtaposed positions are grouped together and marked with romanic numerals I-V. For A, the footprinting reactions with the 535-bp EcoRI-Ncol fragment labeled at the Ncol site (a) and the 285-bp EcoRI-BstBI DNA labeled at the BstBI site (b) are shown. The reactions were performed without nuclear extracts (lane 1), with 0.3 μl (lane 2), 3 μl (lane 3), and 10 μl (lane 4) of the nuclear extracts, respectively. For B, the footprinting reactions with the 535-bp EcoRI-Ncol fragment labeled at the EcoRI site (a) and the 383-bp BstBI-BamHI DNA at the BstBI site (b) are shown. Electrophoresis of the 535-bp EcoRI-Ncol reactions for both short and longer time are shown to present the footprints at the sequence elements. The reactions were carried out without nuclear extracts (lane 1), with 3 μl (lane 2), and 10 μl (lane 3) of the nuclear extracts, respectively.

fifth hamster ICE is aligned with the fourth ICE of the human sequence, whereas the fifth human ICE does not have a homologous counterpart in the hamster sequence. Except for the fourth hamster ICE, regions around the aforementioned sequence elements share a relatively high homology between the hamster and human genes. The overall similarity between the human and Chinese hamster promoter sequences is 67%. The sequence similarity diminishes upstream of the Chinese hamster fifth ICE. Interestingly, the translation start site sequence including part of the first exon of the Chinese hamster top IIα gene shows a 21/23 identity with the translation start site of the gene for human IgM heavy chain (Fig. 3A). It is not known though if the translational regulation of both genes have any similarity.

Analysis of the Proximal cis Elements in the 5′-Flanking Region by in Vitro DNase I Footprinting—The 535-bp EcoRI-Ncol fragment encompassing the GC box and all of the five ICes was labeled at either the EcoRI or Ncol ends and subjected to in vitro DNase I footprinting. To resolve the footprints away from the labeled ends, two smaller fragments, the 285-bp EcoRI-BstBI fragment and the 383-bp BstBI-BamHI fragment, were also labeled, respectively, at the BstBI site for the footprinting analysis of coding and noncoding strands. Analysis on both coding and noncoding strands of the 535-bp region is presented in Fig. 4. Footprints are observed at all the inverted CCAAT sequences and the regions juxtaposed to them on both coding and noncoding strands. The footprints of some flanking sequences, for example, around the fifth ICE on the coding strand, are more pronounced than the inverted CCAAT sequences. The fourth ICE has a lesser extent of protection than the others. Whereas there were no footprints observed at the TATA-like element and the GC box of the coding strand, analysis of the noncoding strand demonstrated a marked footprint at the TATA-like element and a small footprint at the 3′-end of the GC box (Fig. 4B). There are some enhanced cleavages with nuclear extracts observed at positions upstream of the fifth ICE and the TATA-like sequence, respectively, as well as downstream of the third ICE region on the noncoding strand footprinting. This may suggest protein-induced DNA bending at these regions.

**Fig. 5. Gel mobility shift assays of DNA fragments from the 5′-flanking region of the top IIα gene.** The reactions with the labeled proximal fragment, the 383-bp BstBI-BamHI DNA (lanes 1-5), and with the labeled distal fragment, the 285-bp EcoRI-BstBI DNA (lanes 6-10) are presented. The labeled DNAs were incubated without nuclear extracts (lanes 1 and 6) or with 5 μg of nuclear extracts (lanes 2-5 and 7-10). The binding of proteins to the labeled DNAs was competed with a 50-fold molar excess of proximal fragment DNA (lanes 3 and 8), distal fragment DNA (lanes 4 and 9), or the multicloning region of pBluescript DNA (lanes 5 and 10). The free labeled DNAs (F) and the nucleoprotein complexes are indicated.

Analysis of the Binding Activities to the Inverted CCAAT Elements by Gel Mobility Shift Assay—The binding of protein
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Fig. 6. Competition gel mobility shift assays with various ICE-containing oligonucleotides. A, the reactions with the labeled proximal fragment (lanes 1–9) and the distal fragment (lanes 10–20) are shown. The incubations were carried out with 5 µg of nuclear extracts. The binding was competed with 50-fold (lanes 2, 4, 6, 8, 11, 13, 15, 17, and 19) or 500-fold (lanes 3, 5, 7, 9, 12, 14, 16, 18, and 20) molar excess of competitor DNAs. The competitor DNAs used were the first (lanes 2, 3, 17, and 18), second (lanes 4 and 5), third (lanes 6, 7, 11, and 12), fourth (lanes 13 and 14), fifth (lanes 15 and 16), and the mutated (lanes 8, 9, 19, and 20) ICE-containing oligonucleotides. B, the sequences of the five ICE-containing oligonucleotides and the mutant ICE-containing oligonucleotide are aligned together. Identical nucleotides which are present in the same position of four or more aligned sequences are shown in boldface.

Fig. 7. Gel mobility shift assays of the radiolabeled ICE-containing oligonucleotides. The reactions with the 32P-labeled first (lanes 1, 2, and 13–15), second (lanes 3 and 4), third (lanes 5 and 6), fourth (lanes 7 and 8), fifth (lanes 9 and 10), and mutated (lanes 11 and 12) ICE-containing oligonucleotides are shown. The incubations were carried out without (lanes 1, 3, 5, 7, 9, and 11) or with 5 µg (lanes 2, 4, 6, 8, 10, and 12–15) of nuclear extracts. The binding of nuclear proteins to the labeled first ICE-containing oligonucleotide was competed with a 50-fold molar excess of the first (lane 13) and mutated (lane 14) ICE-containing oligonucleotides or the nonspecific multilonging region of pBluescript DNA (lane 15). The specific DNA-protein complexes are indicated by the arrow.
plexed A' and C' and partially the complex B'. The first ICE-containing oligonucleotide functioned as a competitor at least as well as the ICE-containing oligonucleotides derived from the distal fragment. The addition of 50-fold molar excess of ICE-containing oligonucleotide competitors resulted in low level complex formation of B' (Fig. 6A, lanes 11, 13, 15, and 17). However, the bands were not completely depleted even with a 500-fold excess of competitors (Fig. 6A, lanes 12, 14, 16, and 18), suggesting that a portion of B' was derived from complexes formed by non-CCAAT binding activity. These findings suggest that the complexes A, B, A', C', and partially B' were composed of proteins which recognized the common sequences of all the five ICE-containing oligonucleotide competitors. Since the sequence 5'-ATTGG-3' (its complementary sequence is 5'-CCAT-3') is the common sequence represented by all five ICEs, the binding proteins are likely to be CCAAT-binding proteins. The second (lanes 4 and 5) and fourth (lanes 13 and 14) ICE-containing oligonucleotide competitors were not as effective as other ICE-containing competitors in the competition. To confirm that the complexes are formed by CCAAT-binding proteins, an oligonucleotide duplex containing the same flanking sequences as the first ICE with the core ATTGG sequence mutated to CTGGGA was employed in the competition gel mobility shift assay (Fig. 6A, lanes 8, 9, 19, and 20). A 50-fold molar excess of the mutated ICE-containing competitor did not compete for complex formation, while large excess amounts (500-fold) of the mutated competitor could compete for the formation of complexes A and A'.

To analyze the CCAAT-binding activities to the ICEs, the six pairs of oligonucleotide duplexes were radiolabeled and employed in the gel mobility shift assay (Fig. 7). A DNA-protein complex was formed with each ICE-containing oligonucleotide duplex but not with the mutated ICE-containing oligonucleotide duplex (lane 12). The mutated ICE-containing oligonucleotide duplex was also not an effective competitor for the complex formation (lane 14). These are consistent with the previous results (Fig. 6) that the bindings to the ICE-containing oligonucleotides were by CCAAT-binding proteins. All of the ICE-specific complexes comigrated in the gel, suggesting that the same CCAAT-binding factors or proteins of similar electrophoretic properties were bound to the oligonucleotides. There was less complex formation with the fourth ICE-containing oligonucleotide duplex (lane 8), despite the fact that equal amounts of radiolabeled oligonucleotides were used. There were also some faster migrating complexes observed in the gel mobility shift assays, which might be due to protein degradation or some unknown protein bindings not specific to the ICEs.

Delineation of the 5'-End of the CHO top IIα Promoter and the Functional Analysis of the ICEs—To search for a DNA sequence important for in vivo promoter activity, constructs with nested 5'-deletions of the 5'-flanking sequence through the residue immediately upstream of the ATG codon were fused to the CAT coding sequence and transfected into CHO cells. CAT activities were assayed from the cell lysates (Fig. 8). pCAT-0 (construct with deletion of sequence upstream of the transcriptional start site) and pCAT-49 (construct with an in-
the available genomic sequence of the human top IIα gene (32) with the Chinese hamster gene suggests that the genomic structures of these two genes may be similar. In both genes, the first exon is comprised of about 90–102 nucleotides as an untranslated region, and 21 nucleotides as the coding sequence. The first and second introns are of similar sizes and are of the same class.² The promoters of these two genes share a high degree of homology (67% sequence identity). The highly homologous regions are centered around and between the transcription and translation initiation sites, and the ICE areas in the 5′-flanking region (Fig. 3). The genomic sequence for the mouse top IIα gene is not available, but comparison of the Chinese hamster sequence with the 5′-end of the mouse cDNA sequence demonstrates 86% sequence identity (Fig. 3). The 5′-flanking sequence, however, does not share any homology to the Drosophila and yeast sequences (data not shown). This suggests that mammalian top IIα genes may share the same transcriptional regulation machinery.

The Chinese hamster top IIα gene promoter has a moderately high GC content, no canonical TATA box sequence, and the transcriptional start sites are scattered in several discrete positions. These are the characteristics of promoters of genes that have housekeeping and growth-related functions (34). Unlike many housekeeping genes, the promoter of Chinese hamster top IIα gene contains a GC box with the potential for binding of the transcription factor Sp1. However, footprinting analysis of the top IIα promoter did not reveal any bona fide protection of the GC box. GC box elements that do not bind Sp1 are also observed in other promoters such as the herpesvirus immediate-early 3 (ICP-4) promoter (35).

Five ICEs were found scattered within the 400-bp proximal promoter region in the Chinese hamster top IIα gene. DNase I footprinting and gel mobility shift assays demonstrated the binding of sequence-specific proteins at and around the ICEs. The CCAAT sequence is a moderately conserved transcriptional regulatory element in many eukaryotic promoters, such as histone (36), albumin (37), globin (38), major histocompatibility complex class II (39), and viral gene promoters (40, 41), and has been shown to function in either orientation (40). Inverted CCAAT elements are important for the cell-cycle regulation of transcription in the human thymidine kinase gene (42, 43) and the serum induction of transcription from the human DNA polymerase α gene (44) and transcription from the long terminal repeat of Rous sarcoma virus (41). Several proteins that specifically recognize CCAAT elements have also been characterized (37, 39, 45). In the analysis of the ICEs in the top IIα promoter, competition with ICE-containing oligonucleotides in the gel mobility shift assays employing labeled proximal and distal fragments suggests that complexes A, B, C, and part of B′ are ICE-binding complexes (Fig. 6A). Although they may be formed by different proteins, the combined results of Figs. 6 and 7 suggest that the deplatable complexes are more likely formed by the same or very similar ICE-binding proteins to the ICEs with different affinities. For example, in the gel mobility shift assay with the proximal fragment, the ICE-binding protein would bind to the first ICE with greater affinity to form complex B. Additional binding of the ICE-binding protein to the second ICE of the proximal fragment with lower affinity produced the less intense complex A. In the competition assays, addition of the ICE-containing oligonucleotides would easily compete out the binding to sites with lower affinity and disrupt the formation of higher ordered complexes. The fourth and second ICEs have less affinity to the ICE-binding proteins since the fourth ICE-containing oligonu-

²S.-W. Ng, J. P. Eder, and L. E. Schnipper, unpublished data.
decoy formed less complex (Fig. 7), and the fourth and second ICE-containing oligonucleotide competitors were less effective in the competition gel mobility shift assays (Fig. 6A). This is consistent with the result of DNase I footprinting, in which the fourth ICE exhibited lesser protection from DNase I digestion (Fig. 4). The different affinities of the ICE-binding protein to the ICEs can be a function of the interaction with the flanking sequences around the core ATTGG sequence. This may also account for the partial competition of 500-fold molar excess of mutated ICE-containing oligonucleotide for the complex formation (Fig. 6A). All five ICEs are similar in that they have a pyrimidine-rich 3′-flanking sequence (Fig. 6B), and like many CCAAT elements, they are asymmetrical. However, alignments of the ICE sequences did not show any obvious flanking sequence residues which suggest distinction between the high affinity ICEs and the low affinity ICEs.

Transient gene expression assays have delineated the 5′-limit of the functional promoter to the region between 186 and 152 bp upstream of the major transcription site. 5′-Deletion beyond this limit significantly reduces the promoter activity. The three ICEs in this core promoter region were analyzed by site-directed mutagenesis and transient gene expression (Fig. 9). Mutations of the ICEs elicited reduction in basal promoter activity, although a residual promoter activity remained when all three ICEs were mutated. This suggests the activation role of ICEs in the transcriptional regulation of the top IIα gene and some other elements may be present in the core promoter for the residual activity. The reduction in basal promoter activity was additive for the first and third ICE mutations, whereas the mutation of the second ICE had a minimal effect on the decrease of promoter activity. Thus, the in vitro binding activity of the ICEs is likely consistent with their in vivo activation activity.

In summary, these studies have characterized the promoter region of the Chinese hamster topoisomerase IIα gene and the five protein-binding ICEs which have promoter activation function. Further study is required to characterize CHO topⅠα binding protein(s) and compare them to other CCAAT-binding proteins, as well as other embedded elements in the promoter and the upstream regions which regulate both the basal and cell cycle-regulated expression of the top Iα gene.

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