Characterization of the human topoisomerase IIβ (*TOP2B*) promoter activity: essential roles of the nuclear factor-Y (NF-Y)- and specificity protein-1 (Sp1)-binding sites

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Eukaryotic topoisomerase II (topo II) catalyses topological genomic changes essential for chromosome segregation, chromatin reorganization, DNA replication and transcription. Mammalian topo II exists as two isoforms, designated α and β. Human topo IIα is an important cancer drug target, and an established determinant of drug sensitivity and resistance. Human topo IIβ is also the target of anticancer drugs but its role in drug resistance is less clear. The two human topo II proteins are encoded by the *TOP2A* and *TOP2B* genes, respectively, which despite their highly conserved structural organization, are subject to distinctly different modes of regulation. In the present study, we have cloned and characterized the human *TOP2B* promoter containing a 1.3 kb fragment of the 5′-flanking and untranslated region (-1067 to $+193$). We found that the promoter activity of this *TOP2B* fragment was constant throughout the cell cycle, in contrast to the activity of the proximal promoter of *TOP2A* which was low in resting cells and enhanced during proliferation. Analyses of 5′-serially and internally deleted luciferase reporter constructs revealed that 80% of the *TOP2B* promoter activity could be attributed to the region between -533 and -481. Mutational analyses of putative regulatory elements indicated that two inverted CCAAT boxes (ICBs) within this region were essential for *TOP2B* promoter activity and gel mobility-shift assays indicated these sites bound the transcription factor nuclear factor-Y (NF-Y). Co-transfection experiments using a dominant-negative form of subunit A of NF-Y suggested that *TOP2B* promoter activity required direct interaction of NF-Y with the ICBs. In addition, a specificity protein-1 (Sp1)-binding GC box located just upstream of the ICBs was shown to contribute to *TOP2B* promoter activity in a synergistic manner with the ICBs. Our results suggest that the binding sites for NF-Y and Sp1 are critical for *TOP2B* transcription.

Key words: gene regulation, promoter analysis.

INTRODUCTION

DNA topoisomerase II (topo II; human gene symbol *TOP2*) is a nuclear enzyme that alters the topology of DNA. It is essential for a number of nuclear processes, including chromosome condensation, chromatid separation and relief of torsional stress that occurs during DNA replication and transcription (reviewed in [1]). Two isoforms of the mammalian enzyme exist, designated topo IIα and topo IIβ, that are encoded by the *TOP2A* and *TOP2B* genes located on chromosomes 17q21–22 and 3p24, respectively [2–4]. The high degree of sequence similarity and conservation of the intron/exon organization of the two genes indicates that they have most likely arisen by duplication of an ancestral gene [5]. The two isoforms share similar catalytic properties [6], but the regulation of their activities and patterns of expression in normal tissues are distinctly different. For example, topo IIα expression is tightly regulated in a cell-cycle- and proliferation-dependent manner [7,8], and is believed to play a critical role in mitosis [8,9]. In contrast, topo IIβ expression is relatively independent of growth status and the cell cycle [7,10] and its cellular function is less certain. *In vivo*, topo IIα is expressed predominantly in proliferating compartments of tissues whereas topo IIβ is more widely expressed in both proliferating and differentiated tissues [11,12]. Although cell lines lacking topo IIβ expression can be established [13–15], mice with targeted disruption of *TOP2B* fail to develop appropriate neural innervation of skeletal muscle and die shortly after birth due to a breathing impairment, suggesting that this isoform is essential for mammalian neural development [16].

Topo IIα is a well-characterized determinant of drug sensitivity [17,18] and is known to be the cellular target for several widely used anticancer drugs whose mechanism of action involves either inhibition of the enzyme's catalytic activities or stabilization of topo II–DNA cleavable complexes that leads to DNA damage [17]. Unfortunately, resistance often emerges after exposure of tumour cells to topo II-targeting drugs or is inherently present depending on the type of tumour. Mechanisms of drug resistance involving topo IIα include altered enzyme activity, aberrant localization of the enzyme to the nucleus and reduced levels of protein expression, all of which result in decreased DNA damage in tumour cells exposed to topo II-targeting drugs [15,18–20]. Numerous studies of the regulatory mechanisms governing *TOP2A* gene expression have provided insight as to how this gene may be down-regulated in drug resistance as well as furthering our understanding of the biological function(s) of this isoenzyme [18,21].

Topo IIβ is also known to be a drug target, and several selective inhibitors have been described [22]. Moreover, there is some evidence that reduced topo IIβ expression is associated with the development of drug resistance [13,14,22,23]. However, in contrast to topo IIα, little is known about the regulatory mechanisms governing expression of this enzyme. As an initial step towards understanding the factors controlling the expression of *TOP2B*, we have isolated a 1.3 kb genomic fragment

Abbreviations used: DOTAP/DOPE, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate/dioleoyl phosphatidylethanolamine; GMSA, gel mobility-shift assay; ICB, inverted CCAAT box; NF-Y, nuclear factor-Y; Sp1, specificity protein-1; SV, simian virus; topo II, topoisomerase II.

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corresponding to the 5′-flanking and untranslated regions of *TOP2B* and investigated some of the functional elements and transcription factors involved in the ability of this region to drive expression of a reporter gene in transfected cells. Our data indicate that two inverted CCAAT boxes (ICBs), which bind the nuclear factor-Y (NF-Y) transcription factor, and a GC box, which binds the specificity protein-1 (Sp1) transcription factor, are important for *TOP2B* promoter activity.

MATERIALS AND METHODS

Cell culture and reagents

HeLa cells and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Monoclonal antibodies against NF-YA (sc-7712) and polyclonal antiserum against Sp1 (sc-59) were products of Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Wild-type NF-YA and dominant-negative mutant NF-YA cDNA expression vectors were kindly provided by Dr Roberto Mantovani (University of Modenae Reggio, Milano, Italy). Oligonucleotides were synthesized by Cortec (Kingston, Ontario, Canada) or by ACGT Corporation (Toronto, Ontario, Canada).

Cloning of the 5′-flanking and untranslated regions of *TOP2B* and construction of reporter plasmids

TOP2B genomic clones (109D5 and 263H17) were isolated by screening a human BAC library using a probe derived from *TOP2B* cDNA (+72 to +1370 of the coding sequence) by the Canadian Institutes of Health Research Genome Research Facility (Hospital for Sick Children, Toronto, Ontario, Canada). Positive clones were digested with *Hin*dIII and subcloned into the pcDNA3.1(−)® vector (Promega, Madison, WI, U.S.A.) to create pcDNA3.1(−)-TOP2B. The resulting clones were further screened with a ^{32}P-labelled oligonucleotide probe derived from *TOP2B* exon 1 (5′-CCGGGGACCAGCCACTTACCACCC-AGG-3′) using standard filter-hybridization techniques [24]. One of the positive clones isolated contained a 4.3 kb fragment that was determined to include a 1.3 kb sequence upstream of the translation-initiation site. A 1.4 kb *Hin*dIII/*Eco*RI fragment from this clone containing the entire 5′-flanking sequence, the first exon and part of the first intron of *TOP2B* was inserted into pGEM3Z® (Promega) to create pGEM3Z-TOP2B for further subcloning purposes.

The *TOP2B* luciferase reporter plasmids were constructed by subcloning the *TOP2B* sequence −1067 to +193 (where +1 denotes the major transcription start site) [25] from pcDNA3.1(−)-TOP2B into the *Hin*dIII/*Nco*I sites of the pGL3-Basic luciferase vector (Promega) to create −1067TOP2B-pGL3. Various 5′-serially deleted *TOP2B* luciferase constructs (see Figure 1 and Figure 3A below) were prepared by standard PCR cloning techniques. PCR-amplified products spanning progressively shorter regions of *TOP2B* promoter and all ending at +193 were prepared using the appropriate *Kpn*I-tagged forward primers with the *Nco*I-containing reverse primer (+193; 5′-TGGCGTCTT*CCATGG*CGAGTGCCTCC-3′; *Nco*I site in italics) and −1067TOP2B-pGL3 as template. The resulting amplified fragments were then subcloned into the *Kpn*I and *Nco*I sites of pGL3-Basic. Additional luciferase constructs with 5′-ends at positions −385, −125 and −37 were prepared by restriction-enzyme digestion of the −1067TOP2B-pGL3 construct and subsequent re-ligation of blunt-ended DNA as follows: −385TOP2B-pGL3, *Hin*dIII/*Bst*EII; −125TOP2B-pGL3, *Hin*dIII/*Pst*I; and −37TOP2B-pGL3, *Hin*dIII/*Xma*I.

The internally deleted constructs Δ1-TOP2B, Δ2-TOP2B and Δ3-TOP2B (see Figure 3C below) were prepared by first removing the *Bbs*I/*Ppu*MI, *Ppu*MI/*Not*I and *Not*I/*Bst*EII fragments, respectively, from pGEM3Z-TOP2B by restriction digestion followed by recircularization. The *Hin*dIII (−1067)/*Nco*I (+193) fragments from the resulting plasmids were then transferred into pGL3-Basic. The Δ4-TOP2B reporter construct was prepared by subcloning a *Bst*EII-tagged PCR fragment from −309 to +193 (*Nco*I) into a *Bst*EII/*Nco*I digested vector fragment of −1067TOP2B-pGL3. The Δ5-TOP2B and Δ6-TOP2B constructs were prepared by removing *Pst*I-digested or *Sma*I-digested fragments, respectively, from −1067TOP2B-pGL3 followed by recircularization.

Mutations of several potential regulatory elements (see Figure 4 below) were introduced into the −1067TOP2B-pGL3 reporter construct by PCR using the oligonucleotides listed in Table 1. Briefly, two individual amplification reactions were performed on the wild-type −1067TOP2B-pGL3 template using the following primers that have restriction sites introduced into the mutated elements (Table 1) and primers flanking the elements −1067 (5′-CTATCGATAGGTACC*AAGCTT*TTC-ATA-3′, *Hin*dIII site in italics) and −385 (5′-GCCGCCA-C*GGTCACC*TCCCTCTTGTCC-3′, *Bst*EII site in italics) to facilitate identification of mutants and subcloning. The two PCR fragments obtained were digested with the appropriate restriction enzymes and subcloned into the *Hin*dIII and *Bst*EII sites of −1067TOP2B-pGL3.

For construction of the *TOP2A* promoter reporter plasmid 620TOP2A-pGL3, a *TOP2A* fragment (−620 to +90) was isolated from genomic DNA from human peripheral blood mononuclear cells by PCR using the primer pairs, 5′-GG*GG-TACC*TCCAGCCACCGCACACAGCCTA-3′ [−620; tagged with a *Kpn*I-recognition site (italics)] and 5′-GA*AGATCT*GGT-GACGGTCGTGAAGGGGCTCA-3′ [+90; tagged with a *Bgl*II-recognition site (italics)]. The resulting amplified fragments were then subcloned into the *Kpn*I and *Bgl*II sites, respectively, of pGL3-Basic. The fidelity of all constructs was confirmed by sequencing (Canadian Molecular Research Services, Ottawa, Ontario, Canada).

Transfections, reporter assays and flow cytometry

HeLa cells were seeded at 1×10^5 cells/well in a 24-well plate the day before transfection with the luciferase reporter plasmids. For each transfection, a total of 0.3 μg of plasmid DNA was mixed with 2 μg of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate/dioleoyl phosphatidylethanolamine (DOTAP/DOPE) liposome (Avanti Polar Lipids, Alabaster, AL, U.S.A.) in 40 μl of serum-free Dulbecco's modified Eagle's medium and incubated at room temperature for 15 min. The DNA/lipid mixtures were then added to cells in 250 μl of complete medium. After a 4 h incubation, the transfection medium was replaced with fresh medium and the cells further incubated for 20 h. For co-transfection experiments with NF-YA cDNA expression vectors, 250 ng of *TOP2B* reporter plasmid DNA were transfected together with 50 ng of NF-YA cDNA or empty vector DNA (pSG5; Stratagene, La Jolla, CA, U.S.A.). *Renilla* luciferase internal control expression plasmids (10–30 ng of pRL-TK, pRL-CMV or pRL-SV40; Promega) were also co-transfected as indicated in the figure legends. Unless otherwise stated, the activities of pRL internal control plasmids chosen for particular experiments were not affected by co-transfection or post-transfection treatment and hence mainly reflect variations in transfection efficiencies or cell harvesting between samples.

Table 1 Oligonucleotides used for gel mobility-shift assays and mutagenesis of putative regulatory elements in the *TOP2B* promoter

Putative regulatory elements are shown in bold. The mutated nucleotides are underlined and the resulting restriction sites used for facilitating subcloning are indicated. Shown are the upper strands of the oligonucleotides.

Oligonucleotide	Sequence	Position	Restriction site introduced
TOP2B ICB1	5′-ACA AGG CCC GG**A TTG G**AC AGC ATG GCG-3′	−501 to −475	
TOP2B mICB1	5′-ACA AGG CCC GTC TAG AAC AGC ATG GCG-3′		*Xba*I
TOP2B ICB2	5′-GGA ATT TTG GG**A TTG G**CC GAG AGG CTG-3′	−533 to −507	
TOP2B mICB2	5′-GGA ATT TTG GAG ATC TCC GAG AGG CTG-3′		*Bgl*II
MHC Y (prototypical CCAAT box)	5′-ATA TTT TTC TG**A TTG G**CC AAA GAG TAA-3′		
TOP2B GC	5′-CTC GGG T**CC CGC CCC** TCC AGG-3′	−561 to −541	
TOP2B mGC	5′-CTC GGG TCG AGC TCC TCC AGG-3′		*Sac*I
Sp1 (prototypical)	5′-ATT CGA TC**G GGG CGG GG**C GAG C-3′		

Luciferase activities were determined using the Dual-Luciferase® Reporter Assay System according to the manufacturer's instructions (Promega) using a MicroLumat Plus® microplate luminometer (EG & G Berthold, Bad Wildbad, Germany). Results are reported as relative luciferase units.

To follow the effect of cell proliferation on *TOP2B* and *TOP2A* promoter activities, NIH3T3 cells were seeded at 1×10^5 cells/well in 24-well plates and cells transfected the following day with 300 ng of −620TOP2A-pGL3 or −1067TOP2B-pGL3 together with 10 ng of pRL-CMV using DOTAP/DOPE liposomes as before. After 24 h, cells were growth-arrested by serum starvation in medium containing 0.5% serum for 48 h. Cells were then stimulated to proliferate by replacing the medium with medium containing 10% serum. At the specified time periods, cells were harvested and luciferase activities measured as before. For estimation of cell-cycle distribution, cells were set up in parallel in 25 cm^2 tissue-culture flasks, subjected to the same growth-arrest/re-stimulation treatments and harvested at specified intervals for nuclear staining with propidium iodide. Samples were assayed with an EPICS® ALTRA™ flow cytometer (488 nm excitation wavelength, 595 nm emission wavelength) and the data analysed with Expo32™ software (Beckman Coulter, Mississauga, Ontario, Canada).

Gel mobility-shift assays (GMSAs)

Extracts of nuclear proteins were prepared from cells by salt extraction of isolated nuclei to a final protein concentration of 2 μg/μl as described in [26]. Oligonucleotides were 5′-end-labelled with [γ-^{32}P]ATP using T4 DNA kinase to a specific activity of 500–1000 c.p.m./fmol and annealed with a 2-fold excess of complementary oligonucleotides. DNA–protein binding reactions (20 μl) containing 10 mM Tris/HCl (pH 7.8), 1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 1 μg of poly(dI-dC) · poly(dI-dC) and 5–10 μg of nuclear extract protein were incubated at room temperature for 10 min. ^{32}P-Labelled oligonucleotides (20 fmol) were then added to each reaction for a further 30 min. For competition or supershift GMSAs, a 100-fold excess of unlabelled competitor oligonucleotide or 1 μg of the appropriate antisera were preincubated with the nuclear extracts for 15 min at room temperature before adding the ^{32}P-labelled probes. The DNA–protein complexes were resolved on 6% polyacrylamide gels in 0.25 × Tris/borate/EDTA buffer and then exposed to Kodak X-ray film at −70 °C.

Immunoblot analysis

Expression levels of wild-type and dominant-negative NF-YA in transfected HeLa cells were determined by immunoblot analysis. Transfected cells were washed and whole-cell lysates prepared in buffer containing 20 mM Hepes, 450 mM NaC1, 0.5 mM EDTA, 25% glycerol and 0.5 mM PMSF. Protein extracts (10 μg) were resolved by 10% polyacrylamide gel and transferred to a nylon membrane. Blots were incubated with a monoclonal antibody against NF-YA and antibody binding detected with horseradish peroxidase-conjugated goat anti-mouse secondary antibody, followed by chemiluminescence detection (Perkin Elmer Life Sciences).

RESULTS

Isolation of the human *TOP2B* promoter

A genomic *TOP2B* clone isolated from a human BAC library was shown to contain 5′-untranslated and flanking regions of *TOP2B* extending more than 1.3 kb upstream of the translational initiation start codon (Figure 1). Sequencing of this 5′ region showed that it bears minimal sequence similarity with the human *TOP2A* promoter but, like *TOP2A*, lacks a TATA box [25,27]. The *TOP2B* promoter is very GC-rich with a 90% GC content in the proximal promoter and 5′-untranslated region. This region also has a high number of clustered CpG or GpC dinucleotides with features characteristic of a CpG island. In addition, two short dinucleotide repeats, $(GT)_5$ at −420 and $(GT)_6$ at −338, and one short trinucleotide repeat, $(GGC)_6$ at −377, were identified. The entire isolated sequence (−1067 to +193) is in exact agreement with a human genomic clone deposited in GenBank under accession no. AC093416. It is also identical to a short *TOP2B* proximal promoter sequence (−70 to +193) deposited by Ng et al. [25] (accession no. U65315), except that a CG is noted as GC at +160 to +161.

For initial characterization of *TOP2B* promoter activity, the 1.3 kb fragment upstream of the start codon (−1067 to +193) was subcloned into a promoterless luciferase reporter plasmid such that it was contiguous with the start codon of the luciferase coding region. This construct was transiently transfected into HeLa cells, and when assayed for luciferase activity was found to possess transcriptional activity comparable with that of control reporter plasmids driven by a simian virus (SV) 40 promoter (pGL3-control).

Comparison of *TOP2B* and *TOP2A* promoter activities

The *TOP2B* promoter activity was then compared with that of the *TOP2A* promoter, which is known to exhibit a moderate degree of cell-cycle-dependent and proliferation-stimulated transcriptional activity [28,29]. Thus *TOP2A* and *TOP2B* promoter reporter constructs (−620TOP2A-pGL3 and −1067TOP2B-

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      ... tatgta ttcatatgta gttttcttga aactgaccat atgctctttt
-1021 ggggtgaggg gaggagacgg aagaaggaga tggagaaggt ctgagatgat
-971  ttttttcgac agcagcgtcc acttaggccc ttggggaggc cgggtcacgg
-921  ggcctccttg caccagtggt ctacaacgtc ttgcctgcgg tttacaaccg
-871  ctcttttaaa ataatttccc tttgggacgg cccccggctt cttcctgcca
-821  cccccatccc caccccgccc ccatcctcat ccttagaggc cccaggcaaa
-771  cgcttcggat tcccggttag gtctcagagt aaacagcacg cttggaggat
-721  tacaatcgac ccgaggccaa ttcgaccctt tccctttgcc gccctcaatt
-671  gacccttgaa gcagcccctg ctctcccttc aaatggaaaa cccacagaca
-621  cacacacaaa caaaaacccc aagtcttcct ttcggttgct acccgcaatg
                          GC
-571  acgtttcccc cctcgggtcc cgcccctcca gggggcttgg aattttggga
      ICB2                                ICB1
-521  ttggccgaga ggctgtggcg acaaggcccg gattggacag catggcgctg
-471  actgacagcg ggggcggccg ccgcgccctc cctctctccc cggtgtgcaa
-421  atgtgtgtgt gcggtgttat gccggacaag agggaggtga ccgtggcggc
-371  ggcggcggcg gctctgttta ttgtccctct cggtgtgtgt gtgtgaggaa
-321  atcggggctg cagcgaggct aaggctgcct ttgaagcagc ggcggcgacc
-271  gggacgacta ctctggcgac tcgagtggct ggccttcgcg gagtgtgaga
-221  aggacaaggc acctctgcgt cctcgccacg tccgagcgcc tcgggctccc
-171  cggccgccct cgcggctcgc acgcccgggc ttcagcccgg cctgcagcgg
-121  cgcccgcggg cgggcgagaa ggcaacgccg ccgctcggcc gccgccggtc
 - 71  gctccctgct ttctcctcag ccgccgcgct aggcccgggc gacgcggacg
                           +1
 - 21  ccgcgcctcg agtttgaggg cAgccggcgg cgcggcctcc tcagcgggct
 + 30  cggctggacg tccgctccgg atcttcgcga tggggcgcgg gggtcggcgc
 + 80  ggctaggagt gcggcgagtg gagcggtggg tgcggagcgg cggggcccag
+130  cggcccgcag ggaggcggga gcggcggctg cggcctcagg gcctgtgagc
+180  tggaggcact cgcc ATG GCC AAG TCG TCG GGT GGC GCG GGA ..
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Figure 1 Sequence of the 5′-flanking and untranslated regions of human *TOP2B*

The transcriptional start site is denoted as +1 [25]. Putative transcriptional regulatory elements identified in this study are shown in bold, as is the ATG translational start codon. Nucleotide repeats are underlined.

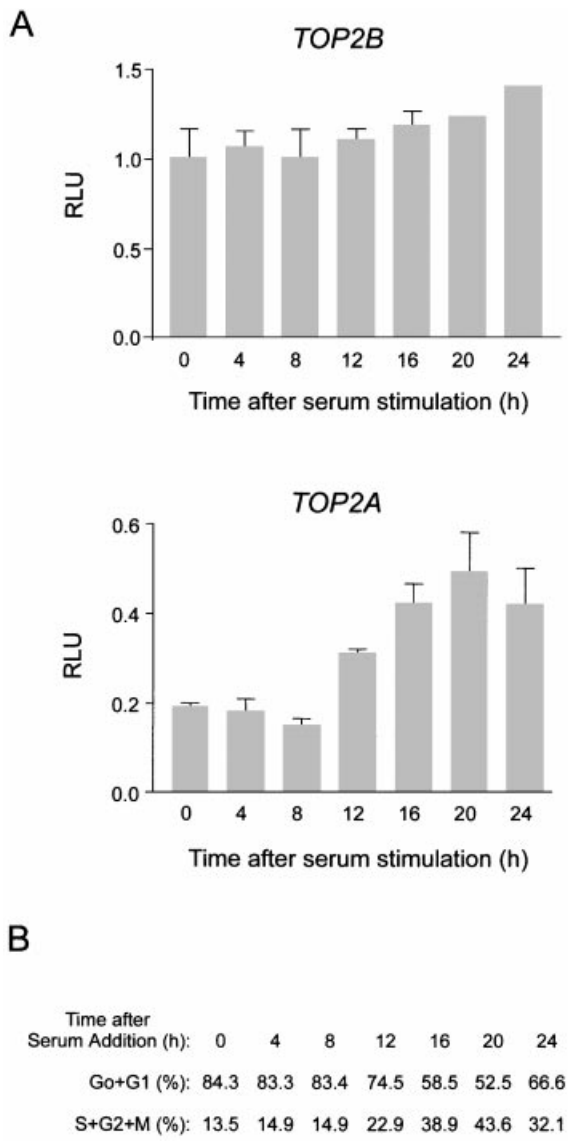

Figure 2 Comparison of *TOP2B* and *TOP2A* promoter regulation

(**A**) Effect of serum re-stimulated growth on *TOP2B* and *TOP2A* promoter reporter activities. NIH3T3 cells were transfected with reporter constructs of the *TOP2A* promoter (−620TOP2A-pGL3) or *TOP2B* (−1067TOP2B-pGL3) together with 10 ng of pRL-CMV as an internal control. Cells were serum-starved for 48 h and then stimulated to proliferate by replacing the medium with medium containing 10% serum. At the specified time periods, luciferase activities were measured using the dual luciferase assay system. Values shown are means ± S.E.M. from three determinations in a single experiment. Similar results were obtained in a second experiment. RLU, relative luciferase units. (**B**) Cell-cycle analysis of NIH3T3 cells. Parallel cultures were subjected to the same growth-arrest/re-stimulation treatments and harvested at specified intervals for flow cytometry analysis. The percentages of cells in resting ($G_0 + G_1$) and active growth ($S + G_2 + M$) phases are indicated.

pGL3) were transiently transfected into NIH3T3 cells. Transfected cells were then growth-arrested by serum starvation for 48 h followed by re-stimulation of proliferation by re-addition of 10% serum. Cell lysates were prepared at specified time intervals and luciferase activity measured (Figure 2A). The cell cycle status of cells subjected to the same serum starvation and replenishment procedures was assessed by flow cytometry (Figure 2B). Initial experiments indicated that the transfection procedures used had no effect on the subsequent growth arrest by serum deprivation and proliferation upon serum replenishment. As shown in Figure 2(A), the activity of the *TOP2B* promoter construct did not vary appreciably during serum-stimulated proliferation of NIH3T3 cells for 24 h. In contrast, *TOP2A* promoter activity was relatively low in the first 12 h when the cells were still in resting stage (G_0/G_1) and significantly increased 2–3-fold from 12 to 24 h when the cells entered the active phase of cell growth ($S + G_2 + M$), consistent with previous observations using a similar experimental system [28,29]. To further compare the properties of the *TOP2A* and *TOP2B* promoters, the effect of trichostatin A, a histone deacetylase inhibitor which is known to activate the *TOP2A* promoter, was also investigated. As demonstrated previously, we found that *TOP2A* promoter activity was markedly stimulated (> 7-fold) by exposure of cells to trichostatin A [30,31]. However, the *TOP2B* promoter reporter construct was stimulated only 2-fold, which is comparable with the increase observed for the internal control reporter plasmid (pRL-CMV; results not shown).

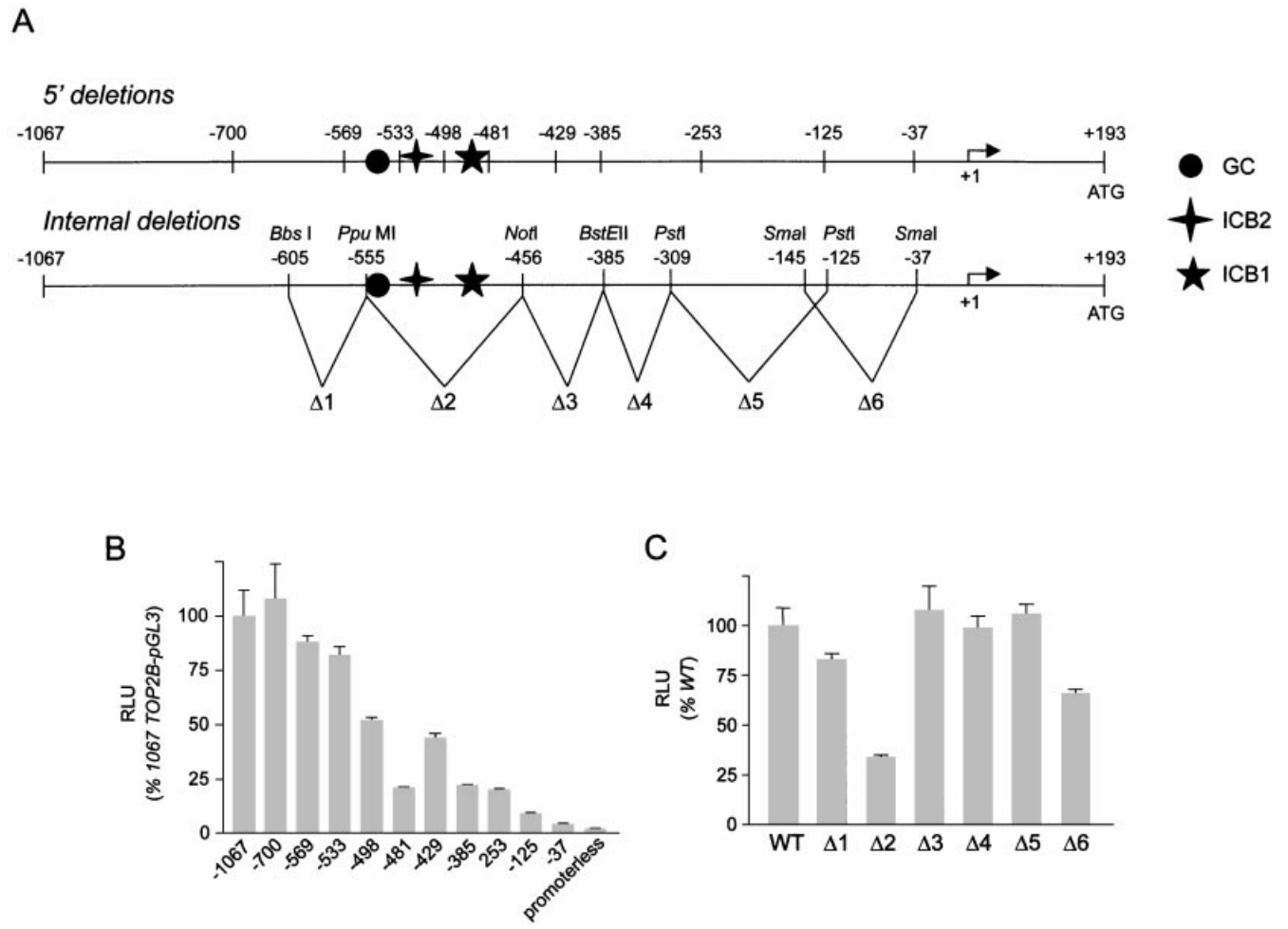

Figure 3 Deletion analysis of the *TOP2B* promoter

(**A**) Schematic diagram of fusion constructs of *TOP2B* promoter sequences linked to luciferase reporter gene used in this study. (**B** and **C**) Reporter gene assays of 5′-deletion constructs (**B**) and internal deletion constructs (**C**). HeLa cells were transfected with 300 ng of DNA of the indicated deletion mutants and 30 ng of pRL-TK for normalization of transfection efficiencies and processed for measurement of luciferase activities 24 h later. Values shown are means ± S.E.M. from three determinations in a single experiment and are expressed relative to the luciferase activity of the full-length promoter construct, −1067TOP2B-pGL3. Similar results were obtained in a second independent experiment. RLU, relative luciferase units.

Deletion analysis of the *TOP2B* promoter

A panel of 5′-serially deleted *TOP2B* reporter constructs generated from −1067TOP2B-pGL3 were then tested in transient transfection assays to determine the minimal sequence necessary to drive luciferase expression (Figure 3A). These experiments showed that deletion up to −533 did not appreciably affect *TOP2B* promoter activity while further deletions resulted in at least a 50% decrease in activity (Figure 3B). These findings suggested that the sequence immediately downstream of −533 contains elements required for maximal *TOP2B* promoter activity. Reporter constructs with deletions of several internal sequences were generated to further define critical regions in the *TOP2B* promoter. Deletion of *TOP2B* sequence between −555 and −456 (Δ2-TOP2B) resulted in a 70% decrease in luciferase activity, consistent with results with the 5′-serially deleted reporter constructs (Figure 3C). A 35% reduction in promoter activity was observed for the Δ6-TOP2B construct which was lacking −125 to −37, a region that has a relatively high GC content. Whether or not this decrease is significant is unknown and requires further investigation. Other internal deletions had either no effect on *TOP2B* promoter activity or decreased it by less than 20%.

Mutational analysis of GC box and ICBs

Computer-assisted analyses of the region between −555 and −456, shown to be critical for *TOP2B* promoter activity, were carried out using the TRANSFAC database and MatInspector search program (http://transfac.gbf.de/TRANSFAC). These analyses identified multiple putative regulatory elements, including a GC box (Sp1-binding site) at −553 as well as two ICBs, ICB1 and ICB2, at −522 and −490, respectively (Figures 1 and 4). CCAAT boxes are *cis*-acting elements recognized by transcription factors such as NF-Y, CCAAT transcription factor/nuclear factor-1 (CTF/NF-1) and CCAAT/enhancer-binding protein (C/EBP) [32], whereas GC boxes are potential binding sites for members of the Sp family of transcription factors [33]. To determine whether these sequences contributed to *TOP2B* promoter activity, mutations were introduced

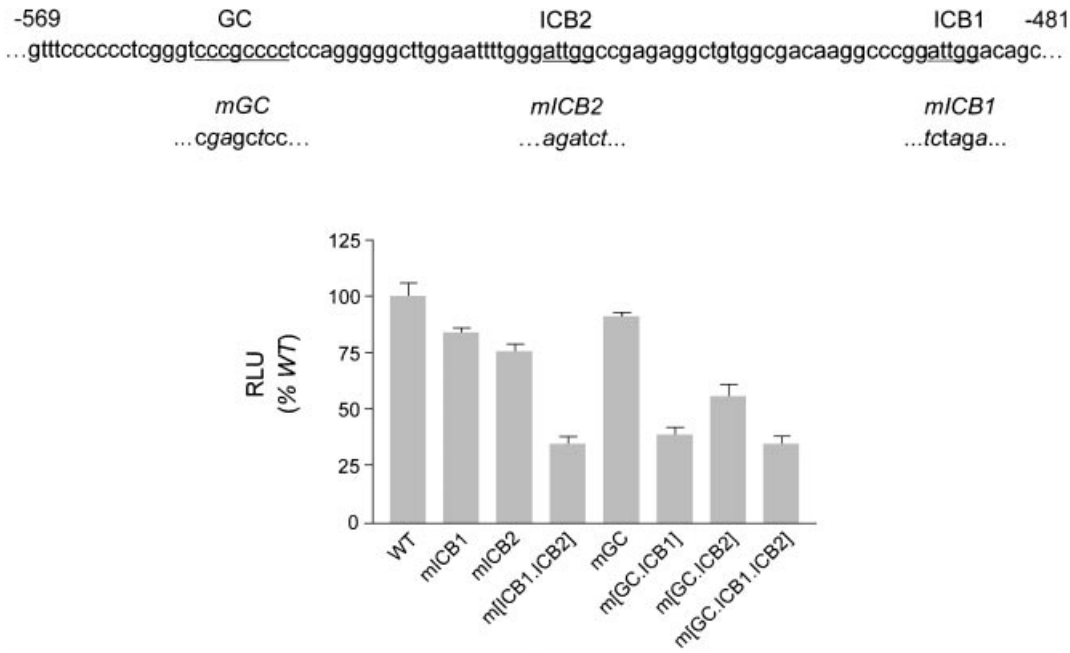

Figure 4 Mutational analysis of putative Sp1 and ICB regulatory elements in the *TOP2B* promoter

HeLa cells were co-transfected with 300 ng of wild-type or mutant −1067TOP2B-pGL3 reporter construct DNA and 30 ng of pRL-TK and luciferase activities measured 24 h later. Values shown are means ± S.E.M. from three determinations in a single experiment, and are expressed relative to the luciferase activity of the wild-type −1067TOP2B-pGL3 construct. Results shown are representative of three independent experiments. RLU, relative luciferase units.

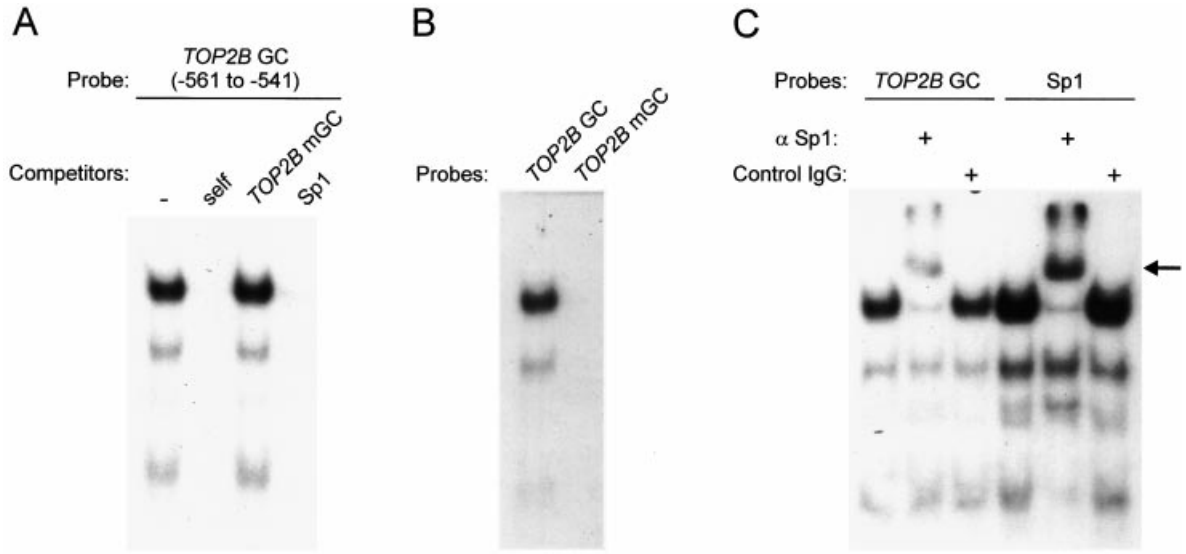

Figure 5 GMSAs of the GC box sequence in the *TOP2B* promoter

HeLa cell nuclear extracts (5 μg) and the indicated ^{32}P-labelled oligonucleotides (20 fmol) were incubated in a reaction volume of 20 μl at room temperature and then resolved on a 6% polyacrylamide gel. Oligonucleotide sequences are shown in Table 1. (**A**) Competition GMSA in the presence of 100-fold excess of the indicated unlabelled oligonucleotide competitors. (**B**) Comparison of binding activities of sequences corresponding to the wild-type and mutated *TOP2B* GC box. (**C**) Supershift assays using 1 μg of anti-Sp1 antibody; 1 μg of non-immune IgG was used as control. Supershifted complexes are indicated with an arrow.

into the wild-type −1067TOP2B-pGL3 construct to generate mutants with specific disruption of the respective elements (Table 1 and Figure 4). The resulting constructs were then transiently transfected into HeLa cells and dual luciferase activities measured as before. As shown in Figure 4, one of the most striking changes resulted from mutations of both ICB1 and ICB2 (m[ICB1.ICB2]). This double mutation reduced *TOP2B* promoter luciferase activity by 70–80%. In contrast, a single mutation of either the

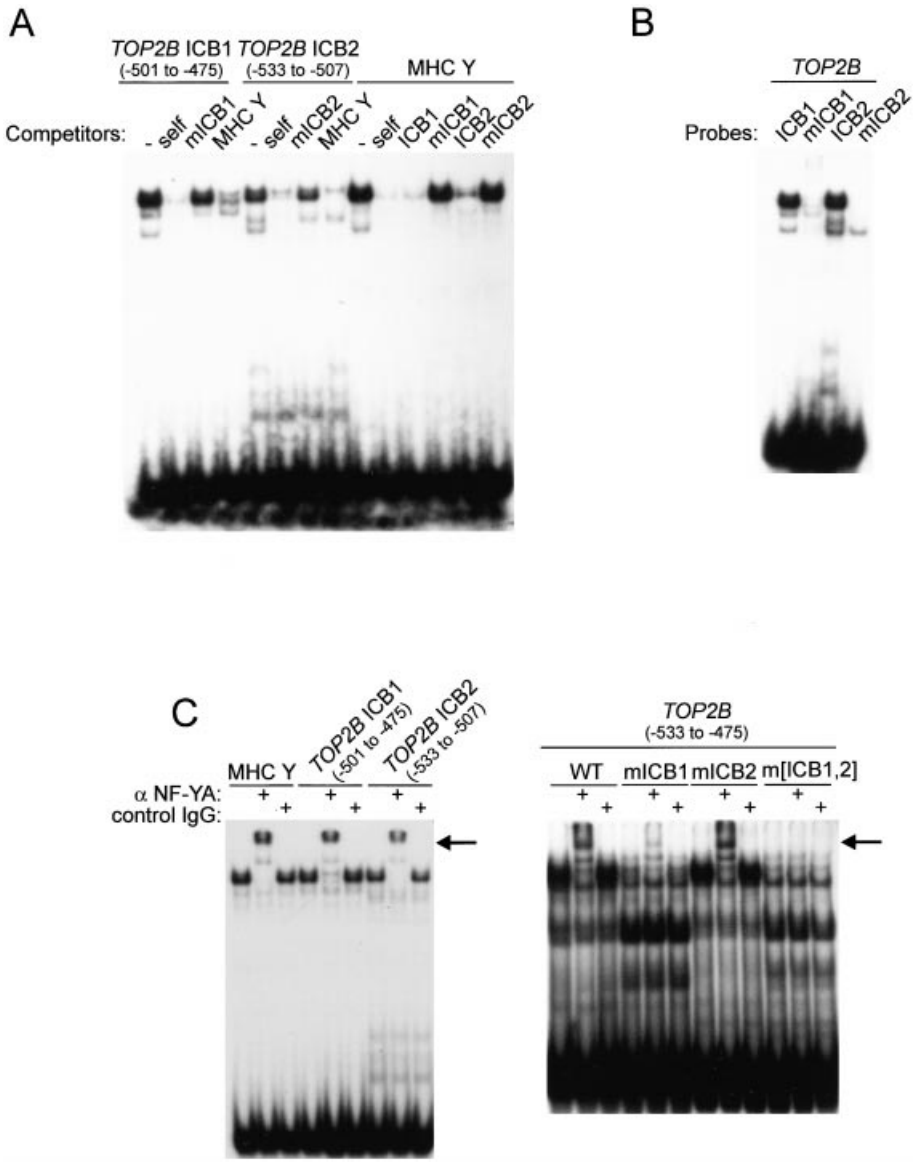

Figure 6 GMSAs of the ICBs in the *TOP2B* promoter

HeLa cell nuclear extracts (5 μg) and the indicated ^{32}P-labelled oligonucleotides or PCR-derived probe (*TOP2B* −533 to −475; 20 fmol) were incubated in a reaction volume of 20 μl at room temperature and then resolved on a 6% polyacrylamide gel. Oligonucleotide sequences are shown in Table 1. (**A**) Competition GMSA in the presence of 100-fold excess of the indicated unlabelled oligonucleotide competitors. (**B**) Comparsion of binding activities of sequences corresponding to wild-type and mutated *TOP2B* ICBs. (**C**) Supershift assays, using 1 μg of NF-YA monoclonal antibody, of the indicated oligonucleotides (left-hand panel) or the PCR-derived probe (*TOP2B* −553 to –475) with the indicated mutations (right-hand panel). Non-immune IgG (1 μg) was used as control. Supershifted complexes are indicated by arrows. WT, wild type.

ICB1 or the ICB2 site did not appreciably affect −1067TOP2B-pGL3 activity. These findings suggest that each ICB is sufficient on its own to support *TOP2B* promoter activity.

Mutation of the GC box at −553 (Figure 4) or elimination of this site, as in the −533TOP2B-pGL3 construct (Figure 3B), led to a modest (< 20%) decrease in luciferase activity. However, when the GC box was mutated together with one or other of the ICBs, a much more pronounced decrease in luciferase activity was observed. Thus simultaneous mutation of the GC box and the proximal ICB (m[GC.ICB2]) caused a 50% decrease in promoter activity (Figure 4), similar to the decrease observed with the −498TOP2B-pGL3 5′-deletion construct in which both the GC box and ICB2 site are eliminated (Figure 3B). The combination of mutations in the GC box and the ICB1 site (m[GC.ICB1]) resulted in a further decrease in promoter activity to 30% of wild-type −1067TOP2B-pGL3 activity, a level of activity similar to that observed when both ICB1 and ICB2 were mutated (m[ICB1.ICB2]; Figure 4). All of the mutant constructs above were also tested in a number of additional cell lines, including MCF-7, HEK-293, CALU-6 and NIH3T3. In all cases, comparable results were obtained. Thus the m[ICB1.ICB2], m[GC.ICB1] and m[GC.ICB1.ICB2] mutants were the most disruptive and conferred only 20–50% promoter activity (results not shown).

DNA-protein binding activities at the ICBs and GC box

To determine whether the ICB1, ICB2 and GC box in the *TOP2B* promoter are capable of protein binding, GMSAs were performed with HeLa cell nuclear extracts. As shown in Figure 5(A), an oligonucleotide (−561 to −541) containing the putative GC box of the *TOP2B* promoter sequence bound proteins with mobilities similar to those bound to an oligonucleotide containing a consensus Sp1 sequence. These binding complexes could be competed with by a 100-fold excess of unlabelled Sp1-binding sequence oligonucleotide but not by an excess of oligonucleotides harbouring a mutated Sp1 sequence (TOP2B mGC; Table 1), which was shown to be defective in protein binding (Figure 5B). Furthermore, the major complex was completely supershifted by an Sp1-specific antiserum (Figure 5C). Thus the GC box of the *TOP2B* promoter appears to be a binding site for Sp1.

The ICBs at −522 and −490 were investigated in a similar fashion. As shown in Figure 6(A), shifted complexes were detected using oligonucleotides corresponding to sequence containing the putative ICB1 (−501 to −475) or ICB2 (−533 to −507) as the labelled probes and the electrophoretic mobilities were similar to those detected using a prototypical CCAAT box sequence, the Y-box found in the MHC class II (MHC II) promoter [34]. These complexes could also be effectively abolished by a 100-fold excess of unlabelled oligonucleotides of the same sequence. However, they were not affected by an excess of unlabelled oligonucleotides containing a mutated CCAAT motif (*TOP2B* mICB1 or *TOP2B* mICB2; Table 1), which showed significantly less binding activity (Figure 6B). These observations indicate that the ICB sequences are the specific motif conferring the nuclear protein-binding activities. In addition, the complexes detected could be competed by a 100-fold excess of oligonucleotide corresponding to the MHC Y-box (MHC Y) sequence (Figure 6A). Thus the putative ICB1 and ICB2 sequences of *TOP2B* behave in a fashion similar to the MHC Y-box, which is recognized by the NF-Y transcription factor [34].

To further characterize the protein-binding activity of the *TOP2B* ICBs, antiserum raised against subunit A of NF-Y was included in the binding assays. As shown in Figure 6(C) (left-hand panel), the binding activity at both ICBs of the *TOP2B* promoter reacted with the anti-NF-YA antibody, but not non-immune IgG, to form a 'supershifted' complex in a manner similar to that observed in control reactions with the MHC Y probe. PCR-derived probes corresponding to the *TOP2B* promoter sequence −533 to −475 containing both ICB1 and ICB2 were also employed in a GMSA. As shown in Figure 6(C) (right-hand panel), binding complexes that reacted with the anti-NF-YA antibody were also detected using this probe. When either ICB1 or ICB2 was mutated (mICB1 or mICB2), the anti-NF-YA antibody still detected binding complexes. However, when both ICBs were disrupted (m[ICB1.ICB2]), no more supershifted activity was detected, indicating that the double mutant is defective for NF-Y binding. The ability of ICB2 to bind NF-Y appears to be somewhat weaker than that of ICB1 (Figure 6C), but whether or not this difference is significant requires further investigation.

Effects of expression of dominant-negative NF-YA on *TOP2B* promoter activity

To further investigate the role of NF-Y on *TOP2B* promoter activity, a cDNA expression vector encoding a dominant-negative mutant of the A subunit of NF-Y (NF-YA), which renders endogenous NF-Y unable to bind to a CCAAT box [32],

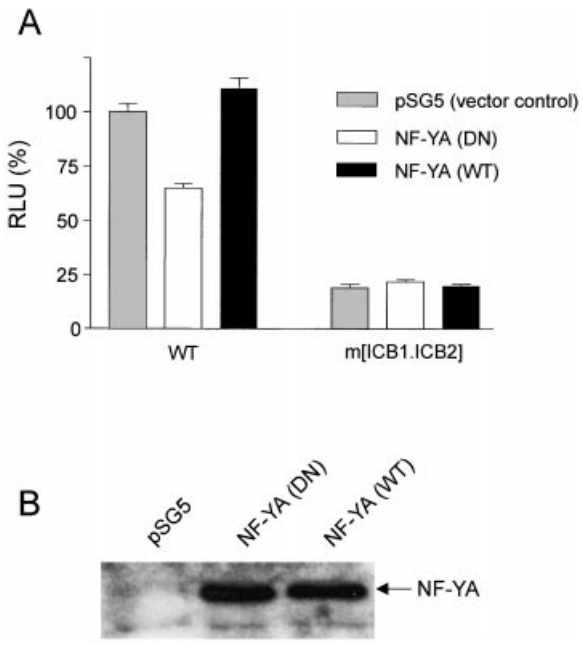

Figure 7 Effect of dominant-negative NF-YA expression on *TOP2B* promoter luciferase activities

(**A**) HeLa cells were transfected with 200 ng of wild-type −1067TOP2B-pGL3 reporter DNA (WT) or ICB mutated reporter DNA m[ICB1.ICB2] together with 100 ng of expression plasmid DNA for wild-type NF-YA (WT), dominant-negative NF-YA (DN) or control vector without insert (pSG5). Individual transfections were normalized by co-transfection with pRL-SV40. Results shown are representative of three independent experiments. Values shown are means ± S.E.M. from quadruplicate determinations and are expressed relative to the activity of the wild-type −1067TOP2B-pGL3 construct. RLU, relative luciferase units. (**B**) Confirmation of NF-YA expression by immunoblot analysis of lysates prepared from transfected HeLa cells.

or a cDNA encoding the wild-type NF-YA was co-transfected with wild-type and mutant −1067TOP2B-pGL3 plasmids into HeLa cells and promoter activity assayed 24 h later (Figure 7). Results were expressed relative to the activity observed in independent co-transfections of the empty vector (pSG5) with the −1067TOP2B-pGL3 construct (Figure 7A). The results show that ectopic co-expression of dominant-negative NF-YA significantly reduced wild-type −1067TOP2B-pGL3 promoter activity by approx. 40 % whereas co-expression of wild-type NF-YA had no effect. As shown in Figure 7(B), both the dominant-negative and wild-type NF-YA were expressed in the transfected HeLa cells at comparable levels. To confirm that the effect of dominant-negative NF-YA on *TOP2B* promoter activity was due to specific inhibition of NF-Y binding to the ICBs, co-transfection experiments were also performed using the −1067TOP2B-pGL3 construct with both ICBs (m[ICB1.ICB2]) disrupted. In these experiments, dominant-negative NF-YA had no effect on the luciferase activity of the mutated *TOP2B* reporter construct. The dominant-negative NF-YA also had no effect on promoter activity driven by the SV40 promoter, which has no CCAAT box (e.g. pRL-SV40 or pGL3-control), or on the background activity of pGL3-Basic (results not shown).

DISCUSSION

As a first step towards better understanding of the regulation of human topo IIβ expression, we cloned and sequenced the 1.3 kb 5′-flanking region of the *TOP2B* gene. This *TOP2B* promoter

region is very GC-rich and contains clustered CpG or GpC dinucleotides with features characteristic of a CpG island that represent potential sites for methylation. Several short nucleotide repeats were also identified, but whether or not any of these features play a role in *TOP2B* gene regulation remains to be determined. Thus these findings add to our previous knowledge of the intron/exon organization of the *TOP2B* gene [5] and the partial sequence of its promoter [25].

We next analysed the ability of the *TOP2B* fragment from −1067 to +193 to drive expression of a luciferase reporter gene. Initially, we compared the properties of the *TOP2B* promoter with the relatively well characterized *TOP2A* proximal promoter [27]. In contrast to *TOP2A*, we found that the *TOP2B* promoter activity was less dependent on cell-proliferation status. These findings are consistent with previous studies comparing the relative expression levels of the two isozymes. In addition, unlike the *TOP2A* promoter [31], the *TOP2B* promoter was not significantly activated by exposure to an inhibitor of histone deacetylation (C. N. Lok and S. P. C. Cole, unpublished work). These results confirm that a fundamental difference exists between the transcriptional regulation of these two genes.

By deletion analysis, we identified a region in the *TOP2B* promoter between −533 and −481 that appears responsible for approx. 80% of promoter activity. By introducing specific mutations into the putative regulatory elements, two ICBs were identified that are critical for *TOP2B* promoter activity. DNA–protein-binding assays clearly demonstrated that these two putative ICBs bind nuclear factors. CCAAT boxes are found in the regulatory region of 30% of genes, including those which are so-called housekeeping genes as well as inducible and cell-cycle-regulated genes [32]. The role of CCAAT boxes as *cis*-acting promoter elements depends on their ability to interact with a variety of transcription factors. The most prevalent of these is NF-Y, a ubiquitously expressed heterotrimeric transcription factor composed of three subunits (NF-YA, NF-YB and NF-YC) [32]. All three subunits are necessary for constitution of a functional binding complex to the CCAAT box. Using specific NF-Y antisera in GMSAs, the ICBs of *TOP2B* were shown to bind NF-Y. Our observations using a dominant-negative form of NF-Y (A subunit) with mutations in the DNA-binding domain, previously demonstrated to abolish the interaction of NF-Y with CCAAT boxes [32], further confirm that NF-Y interacts with the *TOP2B* ICBs and is involved in *TOP2B* promoter activity.

An interesting finding of our study is the loss of *TOP2B* promoter activity upon simultaneous disruption of the two ICBs while mutation of a single ICB had little effect. These results suggest that each ICB by itself is sufficient to support *TOP2B* promoter activity. Multiple CCAAT boxes are found in a number of promoters and their spacing is often variable. The centre-to-centre distance between the two ICBs of the *TOP2B* promoter is 30 bp, which is very close to three multiples of a helical turn of DNA (10.4 bp) [35]. This suggests that protein factors such as NF-Y that bind to the two ICBs are likely to be situated on the same side of the helix and are thus located in very close proximity to one another in the *TOP2B* promoter. The similar spatial relationships of the DNA–protein interaction at the two ICBs may explain, at least in part, our observation that the two ICBs can be compensatory for one another with respect to their contribution to *TOP2B* promoter activity when either one is mutated. A similar redundancy of functional ICBs has also been found in the promoter regions of genes encoding human cyclin B1 and cdc25C [36,37].

The major protein bound to the *TOP2B* ICBs was identified as NF-Y. Although extensively studied, the mechanism by which NF-Y regulates gene expression is still not completely understood. However, one widely recognized property of NF-Y is its ability to induce distortion of the double helix upon binding to DNA *in vitro*, thereby influencing the DNA–protein interactions for gene transcription and hence serving as a 'promoter organizer' [32]. In the *TOP2B* promoter, the GC box/Sp1 site is located in close proximity to ICB2 and we have shown that Sp1 can bind to this site. We have also shown that mutation of this site, together with the mutation of either one of the ICBs, causes a significant loss of *TOP2B* promoter activity. These observations suggest that a functional synergy may exist between the ICBs and the GC box in the *TOP2B* promoter. Such a functional co-operation between NF-Y and Sp1 has previously been shown to be essential for the transcriptional activity of promoters of a number of genes, including the human MHC II-associated invariant chain (Ii) [38], human and rat fatty acid synthase (*FAS*) [39], human cathepsin L [40] and human P-glycoprotein (*ABCB1/MDR1*) [41]. In all cases, the Sp1- and NF-Y-binding sites are located in close proximity to each other in the promoter regions of these genes. Co-operation may occur in several different ways. Thus it has been demonstrated that NF-Y and Sp1 bind co-operatively to the *FAS* promoter [39]. In addition, interactions of Sp1 and NF-Y with the p300 co-activator [42,43], and a direct physical association of NF-Y and Sp1 [44], may also explain such co-operativity.

In summary, our results indicate that the binding sites for NF-Y and Sp1 are critical for *TOP2B* transcription and thus *TOP2B* expression may be modulated by the levels or activities of these transcription factors. Although NF-Y and Sp factors are considered to be ubiquitously expressed, their levels of expression or activities still vary depending on cell type and stage of cell growth and differentiation [32,33], as does *TOP2B* expression [11,12]. In this regard, it is of interest that *TOP2B* mRNA is not detectable in skeletal muscle [11] and NF-Y is not expressed in postmitotic differentiated skeletal muscle cells because of a deficiency of the A subunit [37]. Whether or not a broader correlation exists between the expression of NF-Y (and Sp1) and *TOP2B* expression in normal and/or malignant cells remains to be determined.

The *TOP2A* promoter also contains functional ICBs and GC boxes and the regulation of *TOP2A* promoter activity by these elements is known to be complex. All five ICBs in the *TOP2A* promoter have been shown to interact with nuclear factors, including NF-Y [28,30,45,46] as well as a novel CCAAT-box-binding factor, ICBP90 [47]. Interestingly, both stimulatory and repressive roles of the individual ICBs in basal, cell-proliferation-dependent, stress-regulated and drug-resistance-associated *TOP2A* promoter activity have been reported in various cellular backgrounds [28,30,46,48–50]. CCAAT boxes, through their interaction with NF-Y or other related factors, have also been shown to play a role in the transcriptional regulation of a number of cell-cycle-regulated genes, including *TOP2A*, that contain a bipartite motif, a so-called cell-cycle-dependent element (CDE) and a cell-cycle genes homology region (CHR) [51]. However, no similar motif is found in the *TOP2B* promoter. Whether this difference accounts for the differential regulation of *TOP2A* and *TOP2B* during cell growth requires further investigation. It is also likely that differences in the organization of the Sp1- and NF-Y-binding sites in these two promoters are important as well [52]. Finally, in view of the functional significance of the ICBs in the regulatory regions of both *TOP2* isoforms, it will be of interest to determine whether the emerging class of DNA-binding anticancer compounds that can target CCAAT boxes (or a structurally similar motif) can affect expression of *TOP2A* and *TOP2B* and hence drug sensitivity of malignant cells [53].

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