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Role of an Inverted CCAAT Element in Human Topoisomerase IIα Gene Expression in ICRF-187-Sensitive and -Resistant CEM Leukemic Cells

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ABSTRACT

DNA topoisomerase (topo) IIα gene expression or activity is altered in tumor cells selected for resistance to inhibitors of topoII. To better understand the mechanisms by which topoIIα expression levels are modulated, we examined topoIIα transcriptional regulation in ICRF-187-sensitive and ICRF-187-resistant human leukemic cell lines that express an increased amount of topoIIα protein and mRNA. Transient transfections of luciferase reporter plasmids containing either the full-length human topoIIα promoter or fragments of it revealed that topoIIα transcriptional activity was significantly increased in the drug-resistant CEM/ICRF-8 cells, compared with CEM cells. Specifically, the transcriptional activity of the full-length topoIIα promoter (nucleotides −557 to +90) was doubled in CEM/ICRF-8 compared with CEM cells. Serial deletion of the topoIIα promoter permitted localization of the region responsible for its up-regulation in the drug-resistant cells between nucleotides −557 and −162, which includes the last three inverted CCAAT elements (ICE) 3 to 5. Note that construction of a point mutation in ICE3 resulted in a significant increase in transcriptional activity of the topoIIα promoter in the drug-sensitive CEM cells. In addition, by electrophoretic mobility shift assay, ICE3 was recognized by a protein complex containing NF-YB that was present at reduced levels in the topoIIα-overexpressing CEM/ICRF-8 extracts, suggesting that ICE3 plays a negative regulatory role in human topoIIα gene expression. This is the first study to show that topoIIα transcriptional up-regulation in ICRF-187-resistant cells is mediated in part by altered regulation of the third inverted CCAAT box in the topoIIα promoter.

DNA topoisomerase II (topoII) is an essential nuclear enzyme that alters DNA topology during DNA replication, transcription, recombination, and sister chromatid segregation by the cleavage and religation of double-stranded DNA (Champoux, 1990). Two topoII isoforms exist in mammalian cells [topoIIα (170 kDa) and topoIIβ (180 kDa)] that are encoded by different genes (Tsai-Pflugfelder et al., 1988; Jenkins et al., 1992). The α isoform plays a role in DNA replication, mitosis, and cell proliferation (Drake et al., 1989; Adachi et al., 1991) whereas the β isoform has been shown to have a critical role in neural development and serves as an important target for the cytotoxic effects of acridines and mitoxantrone (Errington et al., 1999; Yang et al., 2000). DNA topoII serves as a target for many anticancer agents, such as the epipodophyllotoxins (VP-16, VM-26), the anthracyclines (daunorubicin, doxorubicin), and the bisdioxopiperazines (ICRF-187 and ICRF-193) (Osheroff et al., 1994; Sehested and Jensen, 1996). The epipodophyllotoxins and the anthracyclines stabilize cleaved DNA-topoII complexes (Chen et al., 1984), whereas the second class of topoII inhibitors, the bisdioxopiperazines, serve as catalytic inhibitors of topoII by locking topoII on the DNA, thereby preventing its cleavage (Andoh, 1998). Recently, it was suggested that this closed-clamp form of topoII induces cell death through a novel mechanism involving impediment of DNA metabolic events (Jensen et al., 2000). Both classes of topoII inhibitors were also recently found to induce SUMO-1 conjugation (Mao et al., 2000). Because the sensitivity of human tumor cells to these topoII-targeting agents correlates with alterations in topoII expression levels (Beck et al., 1994, 1999; Nitiss and Beck, 1996), understanding the mechanisms that regulate topoII gene expression may ultimately lead to novel strategies for overcoming antitopoisomerase drug resistance.

TopoIIα gene expression levels are regulated through the cell cycle and can be affected by external stimuli such as heat shock and drug treatments or by cell density-induced growth

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ABBREVIATIONS: topoII, topoisomerase II; ICE, inverted CCAAT element; nt, nucleotide; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay.

arrest. For example, in terms of cell cycle, topoIIα levels accumulate during the S and G_2/M phases, with maximal levels at mitosis, and decrease during the G_0/G_1 phase (Heck et al., 1988; Woessner et al., 1991). Cell cycle-dependent topoIIα transcription is believed to be mediated in part by *cis*-acting elements in the topoIIα promoter that either activate or repress topoIIα transcription. One study revealed that an inverted CCAAT element (ICE) (nt −108 to −104) has a stimulatory role in topoIIα promoter activity in proliferating cells (Isaacs et al., 1996), characterized by increased binding of a proliferation-induced protein complex to this ICE. In contrast, another ICE (nt −68 to −64) was found to have a role in topoIIα transcriptional repression (Falck et al., 1999), because enhanced protein binding to this element correlated with topoIIα down-regulation in serum-starved cells. This same ICE has also been associated with both p53-mediated transcriptional repression (Wang et al., 1997b) and heat shock-mediated transcriptional activation (Furukawa et al., 1998) of the topoIIα promoter. The transcription factor c-MYB was also found to *trans*-activate the topoIIα promoter and to play an essential role in the regulation of topoIIα expression during maturation of hematopoietic cell lines (Fraser et al., 1995; Brandt et al., 1997).

In contrast to these observations, however, the mechanisms by which topoIIα expression levels are altered during the development of drug resistance have not been studied as thoroughly. Several factors that can directly affect gene expression include mutations, altered methylation patterns in gene promoters, and altered regulation of *trans*-acting factors. Studies from one laboratory implicated the overexpression of the Sp3 transcriptional repressor in the down-regulation of topoIIα mRNA levels of an etoposide-resistant KB cell line (Kubo et al., 1995; Takano et al., 1999), whereas work from this laboratory reported that reduced expression of Sp3 contributed to decreased topoIIα expression in a merbarone-resistant CEM cell line (Mo et al., 1997). Moreover, decreased activity of the transcription factor, CP-1 (NF-Y) resulted in transcriptional down-regulation of topoIIα in a cell line selected for resistance to doxorubicin (Wang et al., 1997a).

To better understand the mechanisms involved in the regulation of topoIIα gene expression, we examined topoIIα expression levels, topoIIα promoter activity, and nuclear protein binding to the topoIIα promoter in our leukemic CEM cell line selected for resistance to ICRF-187, CEM/ICRF-8. The CEM/ICRF-8 cell line expresses increased topoIIα protein at the transcriptional level compared with CEM (Morgan et al., 2000). Analysis of the topoIIα promoter elements in these ICRF-187-sensitive and -resistant cells, described herein, revealed that the third inverted CCAAT element (ICE3) has a key regulatory role in the basal transcriptional activity of topoIIα that is differentially recognized by a member of the family of NF-Y transcription factors in the parental CEM compared with our ICRF-187-resistant CEM cell line.

Materials and Methods

Cell Lines. Human leukemic CEM cells and the ICRF-187-resistant subline CEM/ICRF-8 were cultured as described (Morgan et al., 2000). All cell lines were incubated at 37°C in a humidified chamber containing 5% CO_2/95% air.

Western Blot Analysis. Nuclear extracts were prepared from logarithmically growing cells (5×10^5 cells/ml) as described previously (Mo and Beck, 1997). For analysis of NF-YA, NF-YB, and YB-1 transcription factors, proteins (100 μg/well) were separated on a 10% SDS-polyacrylamide gel, electrophoretically transferred onto nitrocellulose, and incubated with either purified mouse anti-NF-YA monoclonal antibody (PharMingen, SanDiego, CA), rabbit anti-NF-YB polyclonal antibody (Biodesign International, Kennebunk, ME), or rabbit anti-YB-1 antibody (generously provided by Dr. Manfred Dietel, Humbolt University, Berlin, Germany). Bound antibody was detected using the enhanced chemiluminescence (ECL) detection method (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions. Autoradiographic signals were quantified by densitometric scanning using a GS-700 Imaging Densitometer and Molecular Analyst Software (Bio-Rad, Hercules, CA). Equal loading of total cellular protein was determined by stripping and reblotting the membrane with anti-β-actin antibody (Oncogene Science, Cambridge, MA).

Construction of TopoIIα Promoter-Luciferase Plasmids for Transient Expression. The recombinant plasmid p557 that contains the full-length topoIIα promoter (nt −557 to +90) and recombinant plasmids containing serial deletions of the full-length promoter: p382 (nt −382 to +90), p252 (nt −252 to +90), p182 (nt −182 to +90), p162 (nt −162 to +90), and p90 (nt −90 to +90) were provided by Dr. Q. Wang (Fibrogen, Inc., San Francisco, CA; Wang et al., 1997b). Bases are numbered with respect to the major transcription start site (designated +1) (Hochhauser et al., 1992).

The topoIIα promoter constructs with mutations in specific ICE sites were generated using PCR overlap extension. PCR amplification of each half of the mutated promoter constructs was performed separately, followed by joining of the halves in a third PCR. Conditions for PCR were as described previously (Wang et al., 1997b). The topoIIα promoter with a mutation in ICE5 was generated using the following primers: the first half of the mutant ICE5 promoter was PCR-amplified with the forward primer 5′-GGATCGGTACCGGGGTTGAGGCAGATGCCAG-3′ (**f-557**) (nt −561 to −542), which contains a *Kpn*I restriction site, and the reverse primer 5′-CCAGGAACTGTCCAGCTATT-3′ (**r-mICE5**) (nt −398 to −379), which alters the ICE sequence from CCAAT to TCCAG (underlined). The second half was generated with the forward primer 5′-AATAGCTGGACAGTTCCTGG-3′ (**f-mICE5**) (nt −398 to −379), and the reverse primer 5′-GATCAGATCTGGTGACGGTCGTGAAGGGGC-3′ (**r-557**)(nt +71 to +90), which contains a *Bgl*II restriction site. Primer r-mICE5 contains nt complementary to the f-mICE5 primer. Each half of the mutant ICE4 topoIIα promoter construct was generated using the following primers: the first half was PCR-amplified with the forward primer 5′-GGATCGGTACCGTTCCTGGAGAATAAACATC-3′ (**f-382**)(nt −386 to −367), which contains a *Kpn*I restriction site, and the reverse primer 5′-AGGGAATCTGGACTCTGAGA-3′ (**r-mICE4**) (nt −263 to −244), which alters the ICE sequence from CCAAT to ACTCT (underlined). The second half of the mutant ICE4 topoIIα promoter was generated using the forward primer 5′-TCTCAGAGTCCAGATTCCCT-3′ (**f-mICE4**) (nt −263 to −244), and the reverse primer **r-557**. Primer r-mICE4 contains nt complementary to the f-mICE4 primer. Each half of the mutant ICE3 topoIIα promoter construct was generated using the following primers: the first half was PCR-amplified with the forward primer **f-557**, and the reverse primer 5′-GTTTGAATAAACTACTCAGG-3′ (**r-mICE3**) (nt −179 to −160), which alters the ICE sequence from CCAAT to CTACT (underlined). The second half was generated with the forward primer 5′-CCTGAGTAGTTTATTCAAAC-3′ (**f-mICE3**) (nt −179 to −160), and the reverse primer **r-557**. Primer r-mICE3 contains nt complementary to the f-mICE3 primer.

Each half of the mutated ICE3, ICE4, or ICE5 PCR-amplified DNA fragments containing the overlapping, complementary sequences were gel purified (Geneclean III kit; Bio 101, Vista, CA), mixed together with its complementary half (1 μg each), and subjected to a second PCR amplification using a pair of the corresponding external primers as described above. Primers f-557, r-mICE5 and f-mICE5, r-557 were used to construct a topoIIα promoter containing

a mutation in ICE5 (named p557-mt ICE5). Primers f-382, r-mICE4 and f-mICE4, r-557 were used to construct a topoIIα promoter containing a mutation in ICE4 (p382-mt ICE4). Primers f-557, r-mICE3 and f-mICE3, r-557 were used to construct a topoIIα promoter containing a mutation in ICE3 (p557-mt ICE3). All constructs were subcloned into the pGL2-Basic vector upstream of the luciferase reporter gene (PROMEGA, Madison, WI) and all mutations were verified by DNA sequencing.

Transient Transfections and Luciferase Reporter Assays. DNA was introduced into the cells by electroporation using the Gene Pulser II apparatus with an extender (Bio-Rad), according to the manufacturer's instructions. Each luciferase plasmid was cotransfected with pSV-β-galactosidase control vector (Promega) into CEM and CEM/ICRF-8 cells and cell extracts were prepared after electroporation as described previously (Morgan et al., 2000). Luciferase activity was measured by a luminometer with an auto-injector (Model TD-20/20; Turner Designs, Sunnyvale, CA). Luciferase activities were normalized to β-galactosidase activities.

Preparation of Nuclear Extracts. Nuclear extracts were prepared from logarithmically growing cells (5×10^5 cells/ml). Cells were resuspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 6 mM $MgCl_2$), incubated on ice for 10 min and centrifuged (1,200*g*, 5 min at 4°C). The supernatant was removed and the pellet was incubated in buffer A containing 0.5% Nonidet P-40. After 5 min on ice, the nuclei were sedimented (3,300*g*, 15 min at 4°C) and the nuclear pellets were lysed by the addition of ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2.5 μg/ml pepstatin, 10 μg/ml trypsin inhibitor, and 1 mM benzamidine). The nuclear extracts were incubated on ice for 30 min with occasional vortexing, centrifuged (15,000*g*, 15 min at 4°C), and the cleared supernatant was transferred to a new tube.

Electrophoretic Mobility Shift Assays (EMSAs). Nuclear extracts (15 μg of protein/assay) were incubated for 30 min at room temperature in a 20-μl reaction mixture containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 8% glycerol, 1 μg poly(dI-dC), 20 μg bovine serum albumin, and ^{32}P-labeled double-stranded oligonucleotide probe (0.2 pmol). The oligonucleotides were labeled with ^{32}P using Klenow polymerase. Competition for protein binding activity was carried out in the presence of a 50-fold molar excess of unlabeled wild-type or mutant oligonucleotide probe. DNA-protein complexes and free DNA were applied to 4% nondenaturing polyacrylamide gels and separated by electrophoresis at 100 V for 2.5 h in a buffer containing 25 mM Tris, 190 mM glycine, and 1 mM EDTA. The gels were dried and exposed to X-ray film with intensifying screens. For supershift assays, approximately 2 μg of a rabbit anti-NF-YB antibody (Biodesign International) were preincubated with nuclear extract for 1 h at room temperature before the addition of ^{32}P-labeled oligonucleotide probe. The following DNA oligonucleotides, along with their complementary strands were synthesized (Life Technologies, Gaithersburg, MD) and used for EMSA analysis: ICE3 (5′-CCTCCCTAACCTGATTGGTTTATTCAAACAAACC-3′, nt −189 to −156 of topoIIα promoter); mutant ICE3 (5′-CCTCCCTAACCTGAgTaGTTTATTCA AACA AACC-3′, nt −189 to −156); ICE4 (5′-GGTGAGCCCTTCTCATTGG CCAGATTCCC-3′, nt −273 to–245); and ICE5 (5′- GGGATCTTA-AATAGATTGGCAGTTCCTGGAG-3′, nt −407 to −377). The criteria for assigning bands as nonspecific versus specific ICE-protein complexes is based on two controls. First, a DNA-protein complex is specific if it is eliminated by the addition of a molar excess of unlabeled competitor DNA of the same sequence but not by the addition of a molar excess of the same sequence containing mutation(s). Second, the specificity of the band shift is further confirmed when no DNA-complex is formed when the mutated ^{32}P-labeled oligonucleotide alone is used in the EMSA.

Results

TopoIIα Promoter Activity Is Increased in the ICRF-187-Resistant CEM/ICRF-8 Cells. We have recently demonstrated that the levels of topoIIα protein in the CEM/ICRF-8 cells are increased ~ 5-fold compared with the parental CEM cells (Morgan et al., 2000). Similarly, we have also demonstrated by Northern blot analysis that CEM/ICRF-8 cells express greater than 2-fold increase in topoIIα mRNA (Morgan et al., 2000). To determine whether the increased expression levels of topoIIα are caused by transcriptional up-regulation, basal transcriptional activity of the full-length topoIIα promoter was measured by transiently transfecting into drug-sensitive and -resistant cells a construct containing the wild-type topoIIα promoter (p557) fused to the luciferase reporter gene. These experiments revealed that CEM/ICRF-8 expressed approximately 2.3-fold more promoter activity than the parental CEM cells (Table 1, plasmid p557). These results are consistent with our previous data (Morgan et al., 2000) and suggest that the up-regulation of topoIIα in the drug-resistant cells occurs in part at the transcriptional level. Importantly, because sequence analysis of the full-length topoIIα promoter from CEM/ICRF-8 compared with CEM revealed no mutations (data not shown), we suggest that the observed transcriptional up-regulation of the topoIIα gene in the drug-resistant cells may be caused by alterations in *trans*-acting factors of the transcriptional machinery.

Determination of the Promoter Sequences Responsible for Mediating TopoIIα Up-Regulation in ICRF-187-Resistant Cells. To identify the promoter sequences responsible for enhanced topoIIα transcriptional activity in the drug-resistant cells, we measured basal luciferase activity in CEM and CEM/ICRF-8 cells transiently transfected with a series of topoIIα promoter-luciferase deletion constructs (Fig. 1). Sequence analysis of the topoIIα promoter has revealed the presence of five ICEs (Hochhauser et al., 1992), numbered 1 through 5. The CCAAT sequence is a common *cis*-regulatory element; mutation of this motif has been shown to alter the transcriptional activity of eukaryotic genes (Santoro et al., 1988; Hochhauser et al., 1992). Stepwise deletion of the 5′ topoIIα promoter sequences, which includes stepwise deletion of each ICE (Fig. 1), resulted in a concomitant decrease in luciferase activity in both the CEM and CEM/ICRF-8 cells (Table 1). This overall pattern of basal promoter activity in Table 1 is consistent with previous reports (Hochhauser et al., 1992; Wang et al., 1997b) and confirms that ICEs play an important role in the transcriptional regulation of topoIIα expression.

TABLE 1

Percentage of luciferase activity of topoIIα promoter deletion constructs in CEM and CEM/ICRF-8 cells. All data are derived from Fig. 3B. See legend to Fig. 3 for details.

Plasmid	CEM	CEM/ICRF-8	Fold-increase Relative to CEM
p557 (ICE 5,4,3,2,1)	100.0 ± 15.9[a]	233.1 ± 29.4[b]	2.3
p382 (ICE 4,3,2,1)	63.2 ± 10.3	131.1 ± 28.7[b]	2.1
p252 (ICE 3,2,1)	18.7 ± 3.5	53.5 ± 13.8[b]	2.9
p182 (ICE 3,2,1)	36.3 ± 2.3	128.6 ± 7.5[b]	3.5
p162 (ICE 2,1)	21.4 ± 7.0	30.0 ± 6.4	1.4
p90 (ICE 1)	1.7 ± 0.05	2.7 ± 1.0	1.5

[a] The mean of absolute luciferase units in five experiments using p557 was 1475.0 ± 221.0.
[b] $P \leq 0.05$ by Student's *t* test.

One exception, however, was the p182 construct, in which promoter activity in CEM and CEM/ICRF-8 transfected with p182 was increased approximately 2- and 2.4-fold, respectively, compared with luciferase activity derived from p252 (Table 1). This observation suggests that the 70 base pairs between nt −252 and −182 may be important for binding of transcription factors and topoIIα expression in our leukemic cell lines. There were statistically significant differences in luciferase activity in CEM and CEM/ICRF-8 transfected with p557, p382, p252, and p182 but not with p162 or p90, which is of importance to our study. The low level of promoter activity associated with the p162 and p90 constructs is because our data were expressed as a percentage of the corrected luciferase activity of control CEM cells (Table 1). For example, with respect to the p162 construct, the absolute luciferase activities were actually 509 units in CEM/ICRF-8 and 283 units in CEM, which are then normalized to control CEM and expressed as a percentage. Data from Table 1 suggest that promoter sequences between nt −557 and −162, which include ICE3, ICE4, and ICE5, seem to be involved in the up-regulation of topoIIα promoter activity in the drug-resistant CEM/ICRF-8 cells. Notably, luciferase activity of the p182 promoter construct was significantly increased (3.5-fold) in CEM/ICRF-8 relative to CEM; this was the greatest difference compared with any of the other topoIIα promoter-luciferase plasmids (Table 1). Therefore, one of the critical *cis*-acting promoter elements involved in topoIIα transcriptional up-regulation in the ICRF-187-resistant cells seems to include a 20-base-pair region (nt −182 to −162) that contains ICE3.

An Inverted CCAAT Element in the TopoIIα Promoter Plays a Role in the Down-Regulation of Promoter Activity in CEM and CEM/ICRF-8 Cells. To better determine the roles of ICE3, ICE4, and ICE5 in the regulation of topoIIα expression, CEM and CEM/ICRF-8 cells were transiently transfected separately. Each of the topoIIα promoter-luciferase constructs contained a mutated ICE3, ICE4, or ICE5. As shown in Fig. 2, point mutation of ICE3 in the context of the full-length topoIIα promoter resulted in an increase in topoIIα transcriptional activity (1.33-fold) in the drug-sensitive CEM cells. Although this increase seems small, it is statistically significant as determined by the Student's *t* test, suggesting that ICE3 may have an important role in topoIIα expression by negatively regulating basal promoter activity. In the case of the drug-resistant cells, CEM/ICRF-8 exhibited ~2-fold more wild-type topoIIα promoter activity compared with CEM. Importantly, this activity did not significantly change when ICE3 was mutated (Fig. 2). From these data, we conclude that (a) ICE3 may serve as a negative *cis*-acting element, and (b) because enhanced transcriptional activity in CEM/ICRF-8 is independent of a wild-type ICE3 sequence, then there may exist alterations in transcription factor binding to ICE3 in the drug-resistant cells.

Luciferase activity of the topoIIα promoter plasmids containing wild type ICE4 or ICE5 was significantly increased in CEM/ICRF-8 compared with CEM cells (Fig. 3A and B). Mutation of ICE4 or ICE5 contributed to a decrease in luciferase activity in both the CEM and CEM/ICRF-8 cells, although the decrease was not significant, suggesting that neither ICE4 nor ICE5 alone seems to contribute to topoIIα promoter activity in these cells.

Differential Binding of Protein Complexes to ICE3 in the TopoIIα Promoter from CEM and CEM/ICRF-8 Cell Lines. To further explore the role of ICE3 in the regulation of topoIIα gene expression, protein factor binding to this element was analyzed in CEM and CEM/ICRF-8 by EMSA using a radiolabeled double-stranded DNA oligonucle-

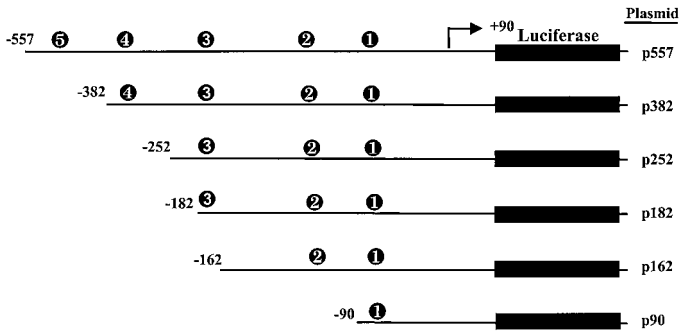

Fig. 1. Schematic map of the human topoIIα promoter-luciferase reporter constructs. Plasmids containing serial deletions from the 5′ end of the human topoIIα promoter were provided by Dr. Q. Wang (Wang et al., 1997). The sequence numbers depicted correspond to those published by Hochhauser et al. (1992), with the transcription start site set to +1 (indicated by arrow). The positions of the ICEs are indicated by the circles and are numbered (1–5) beginning with the ICE nearest to the +1 transcription start site. The full-length topoIIα promoter is represented by plasmid p557.

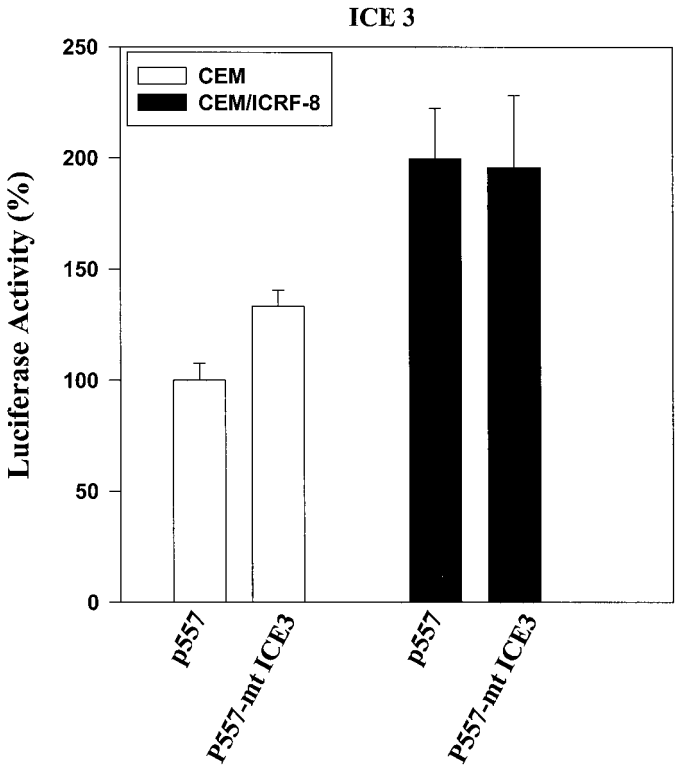

Fig. 2. ICE3 negatively regulates topoIIα promoter activity in CEM cells. CEM and CEM/ICRF-8 cells were transiently transfected with a wild-type, full-length topoIIα promoter-luciferase plasmid (p557) or with the same plasmid containing a mutated ICE3 (p557-mt ICE3), along with the pSV-β-galactosidase plasmid. Luciferase activity was assayed and normalized to β-gal activity 17 h after electroporation as described under *Materials and Methods*. Data are expressed as a percentage of the corrected luciferase activity of CEM cells transfected with p557, and are averages of three independent experiments (bars, SE). $P < 0.05$ for p557-mt ICE3 compared with p557 in CEM cells.

otide spanning ICE3 (nt −187 to −155). Nuclear extracts derived from CEM and CEM/ICRF-8 cells contained a factor that specifically bound to the ICE3 probe (Fig. 4, lanes 1 and 6). This DNA-protein complex was eliminated by the addition of 50-fold molar excess of unlabeled competitor DNA of identical sequence (Fig. 4, lanes 2 and 7) but not by the addition of 50-fold molar excess mutated ICE3 probe (Fig. 4, lanes 3 and 8). No DNA-protein complex was formed when the mutated ICE3 oligonucleotide alone was used in the EMSA (Fig. 4, lanes 4 and 9), confirming the specificity of this band shift. The addition of an antibody against NF-YB, a specific ICE-binding factor (Hooft van Huijsduijnen et al., 1990; Maity et al., 1992), resulted in both inhibition of complex formation and a supershift (Fig. 4, lanes 5 and 10). At a longer gel exposure time, the antibody supershift became more apparent in extracts derived from CEM/ICRF-8 (data not shown). The major nonspecific band seems to decrease in intensity with NF-YB antibody coincubation. Given that we are using a polyclonal antibody, it is possible that the anti-NFYB antibody cross-reacts nonspecifically with other protein in the prepared cell extracts, thus resulting in the inhibition of nonspecific DNA-protein complex formation. As a control, the addition of a nonspecific rabbit polyclonal antibody of the same isotype as anti-NF-YB gave no supershift (data not shown). These results suggest that NF-YB makes up at least one component of the ICE3 protein-binding complex. Our gel shift profiles showing ICE-protein complexes and NF-YB supershifts are in agreement with others (Isaacs et al., 1996; Herzog and Zwelling, 1997; Furukawa et al., 1998). Interestingly, although the result is qualitatively similar to that obtained using extracts from the parental CEM cells, the level of DNA-protein complexes formed was lower in the ICRF-187-resistant cell line (Fig. 4, compare lane 1 to lane 6). These quantitative differences are not caused by differences

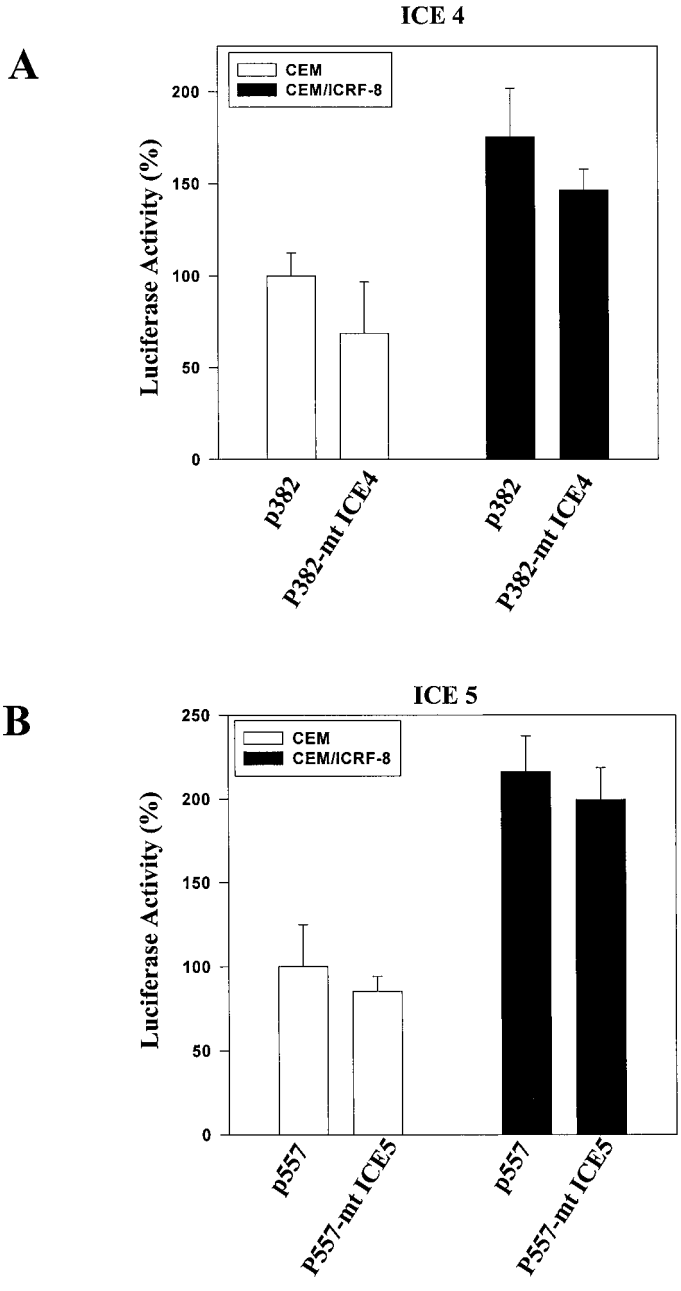

Fig. 3. Effects of mutations in ICE4 or ICE5 in the topoIIα gene promoter on transcriptional activity. A, CEM and CEM/ICRF-8 cells were transiently transfected with either the wild type luciferase reporter plasmid (p382) or with p382 containing a mutation in ICE4 (p382-mt ICE4), along with the pSV-β-galactosidase plasmid. B, CEM and CEM/ICRF-8 cells were transiently transfected with either the wild type luciferase reporter plasmid (p557) or the p557 containing a mutation in ICE5 (p557-mt ICE5), along with the pSV-β-galactosidase plasmid. In both A and B, luciferase activity was assayed and normalized to β-gal activity 17 h after electroporation as described under the *Materials and Methods*. Data are expressed as a percentage of the corrected luciferase activity of CEM cells transfected with wild type plasmid, and are averages of three independent experiments (bars, SE).

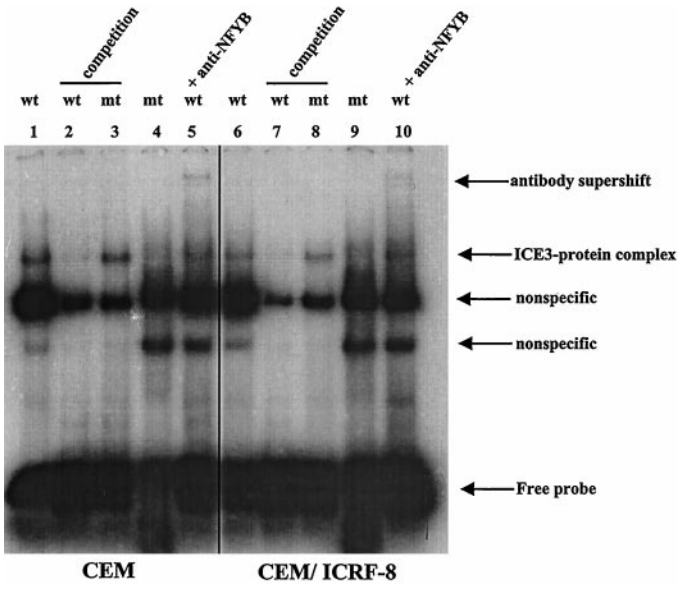

Fig. 4. Electrophoretic mobility shift assay using nuclear extracts derived from CEM and CEM/ICRF-8 cells. Nuclear extracts (15 μg) derived from CEM or CEM/ICRF-8 cells were incubated (30 min, 25°C) with a ^{32}P-labeled oligonucleotide that unless otherwise stated contained the wild-type ICE3 probe. The DNA-protein complexes and free DNA probe were separated on a 4% nondenaturing polyacrylamide gel and radioactivity was detected as described under *Materials and Methods*. Lanes 1 to 5 contained extract from CEM cells, whereas lanes 6 to 10 contained extract from CEM/ICRF-8 cells. Lanes 1 and 6, extract alone; lanes 2 and 7, extracts incubated with 50-fold molar excess of unlabeled ICE3; lanes 3 and 8, extracts incubated with 50-fold molar excess of unlabeled mutant ICE3; lanes 4 and 9, extracts incubated with the mutant ICE3 probe only; lanes 5 and 10, extracts incubated with an antibody (2 μg) against NF-YB. The positions of the free probe, nonspecific DNA-protein interactions, ICE3-protein complex, and antibody supershifts are shown on the right. See *Materials and Methods* for details. This EMSA is representative of three independent experiments.

in cell growth in culture or in extract preparation because only cells growing logarithmically were used for each independent experiment, and all have yielded the same results.

Nonetheless, to confirm these quantitative differences in protein-ICE3 complex formation between the drug-sensitive and -resistant cells, we incubated increasing pmol amounts of ICE3 probe with a constant amount (in micrograms) of nuclear extract derived from CEM and CEM/ICRF-8 cells and analyzed these ICE3-protein interactions by EMSA. Incubating increasing picomolar amounts of ICE3 probe with a constant microgram amount of nuclear extract derived from CEM resulted in a general increase in the formation of ICE3-protein complexes (Fig. 5A). Using ICE3 probe concentrations greater than 1.0 pmol interferes with DNA-protein complex formation. By contrast, incubating the same increasing amounts of ICE3 probe with a constant microgram amount of nuclear extract derived from the ICRF-187-resistant cells resulted in little or no increase in ICE3 binding (Fig. 5A). Importantly, although there is little or no increase in binding for the drug-resistant cells, the amount of nonspecific binding and free probe increased in both the drug-resistant and -sensitive cells alike. These results, quantified in Fig. 5B, demonstrate that at 1.0 pmol ICE3 substrate, up to 3-fold more DNA-protein complexes were formed in CEM compared with CEM/ICRF-8 cells (Fig. 5B).

Data from Figs. 4 and 5 indicate that ICE3 in the topoIIα promoter is recognized by a protein complex that contains NF-YB and that this complex is present at a reduced level in

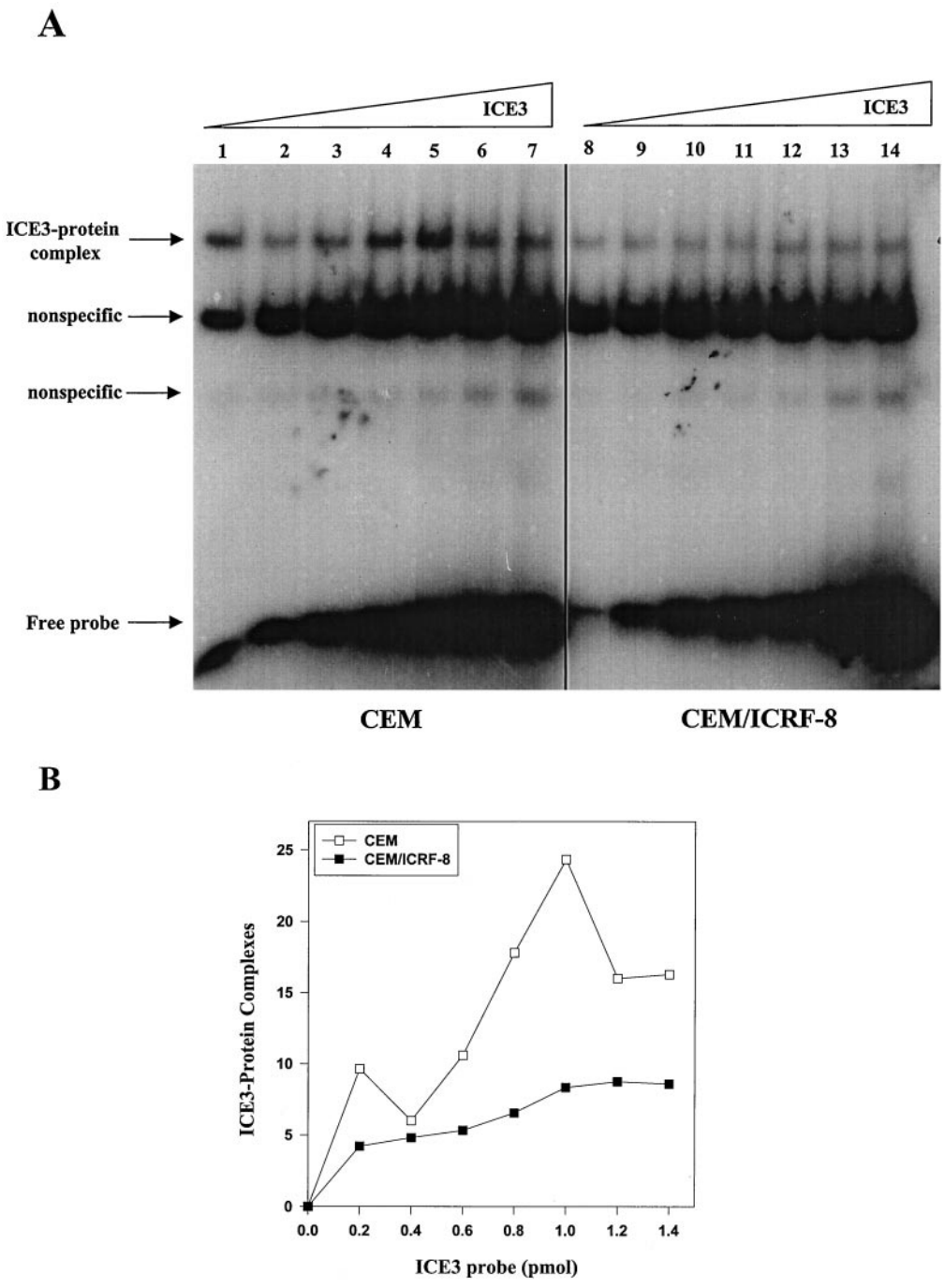

Fig. 5. Electrophoretic mobility shift assay using increasing molar amounts of ICE3 probe. A, nuclear extracts (15 μg) derived from CEM or CEM/ICRF-8 cells were incubated with 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, or 1.4 pmol of a ^{32}P-labeled ICE3 oligonucleotide (lanes 1–7 for CEM cells or lanes 8 to 14 for CEM/ICRF-8 cells, respectively). The DNA-protein complexes and free DNA probe were separated on a 4% nondenaturing polyacrylamide gel, and the gel was dried and exposed to X-ray film. The positions of the free probe, nonspecific DNA-protein interactions, and the ICE3-protein complexes are shown on the left. Shown is a representative of three independent experiments. See *Materials and Methods* for details. B, shown is the quantification by densitometric scanning of ICE3-protein complexes formed as derived from Fig. 5A.

the drug-resistant CEM/ICRF-8 cells. The observation that enhanced topoIIα promoter activity in the drug-resistant cells (Table 1) correlates with decreased protein complex binding to ICE3 suggests that the protein factors that specifically bind to ICE3 may negatively regulate basal topoIIα promoter activity.

Analysis of Protein Expression Levels of the CCAAT Box Binding Factors NF-YA, NF-YB, and YB-1 in CEM and CEM/ICRF-8 Cells. By Western blot analysis, the protein levels of the CCAAT box binding factors, NF-YB and NF-YA (Li et al., 1992; Mantovani et al., 1992), were similarly expressed in CEM and CEM/ICRF-8 cells (Fig. 6). The apparent sizes of the isoforms for NF-YB (~32 and 34 kDa) and for NF-YA (~42 and 44 kDa) in Fig. 6 are in agreement with previously published results (Li et al., 1992; Mantovani et al., 1992). Another known CCAAT binding factor includes a 35-kDa YB-1 protein (Didier et al., 1988), which was also present in equal amounts in the CEM and CEM/ICRF-8 cells (Fig. 6). Equal loading of nuclear protein was confirmed by blotting for β-actin (data not shown). Thus, our observations suggest that the transcriptional up-regulation of topoIIα in the drug-resistant cells is not caused by altered expression of these *trans*-acting factors, suggesting that such alterations as mutations in or post-translational modifications of NF-Y or other binding proteins in the complex may exist.

Discussion

We have shown recently that topoIIα expression is up-regulated at both the protein and mRNA levels in a leukemic CEM cell line (CEM/ICRF-8) selected for resistance to the topoII catalytic inhibitor, ICRF-187 (Morgan et al., 2000). Here, we demonstrate that this up-regulation of topoIIα occurs at the transcriptional level because topoIIα promoter activity was significantly increased in CEM/ICRF-8 compared with CEM. We have localized the region of the promoter (nt −557 to −162) that seems to be responsible for topoIIα transcriptional up-regulation in the drug-resistant cells. An ICE located between nt −182 and −162 (ICE3) may play a role as a negative *cis*-acting element in our leukemic cell lines. We base this conclusion on the following: 1) Using EMSA, ICE3 was recognized by a NF-YB-containing protein complex and this DNA-protein complex was present at a reduced level in the topoIIα-overexpressing ICRF-187-resistant cells compared with the drug-sensitive cells, and (2) point mutation of ICE3 resulted in a significant increase in topoIIα transcriptional activity in the drug-sensitive CEM cells.

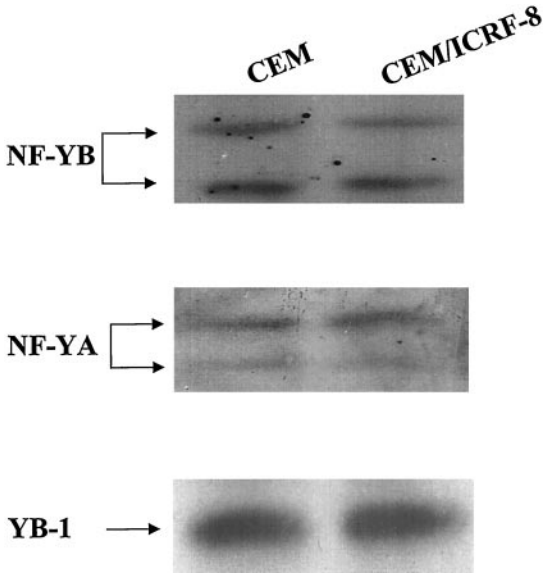

Fig. 6. Expression of CCAAT box binding factors in CEM and CEM/ICRF-8 cells. Nuclear extracts derived from CEM and CEM/ICRF-8 cells were electrophoresed in a 10% SDS-polyacrylamide gel. NF-YA, NF-YB, and YB-1 were detected by immunoblot using antibodies as described in *Materials and Methods*. Equal loading of nuclear protein was detected by staining the transferred membrane with Ponceau Red or by blotting for β-actin. Each panel is representative of three independent experiments.

ICEs are known to be involved in either up- or down-regulating topoIIα gene expression. For example, wild-type p53 represses topoIIα gene expression through functional interaction with ICE1 (Wang et al., 1997b). Moreover, ICE1 was involved in topoIIα transcriptional repression in serum-starved cells (Falck et al., 1999), and heat-shock activation of topoIIα required a decrease in nuclear binding activity to ICE1 (Furukawa et al., 1998). In contrast, ICE2 was identified as a positive regulatory element for topoIIα expression in proliferating cells (Isaacs et al., 1996).

Results here suggest that the up-regulation of topoIIα transcriptional activity in the ICR F-187-resistant cells may be caused in part by differential binding of the NF-Y family of transcription factors to a region of the topoIIα promoter that contains the third inverted CCAAT *cis*-acting element. The presence of ICE3 binding activity has been reported (Herzog and Zwelling, 1997; Wang et al., 1997a), but ours is the first study to demonstrate differential ICE3 binding activity as a function of drug-resistance. Accordingly, we offer the suggestion that transcription factor binding to ICE3 negatively regulates topoIIα expression in CEM cells, possibly by displacing positive regulatory factors flanking this region of the topoIIα promoter. In this regard, one study has shown that NF-Y DNA binding activity seems to induce bends in the DNA and the extent of bending depends upon the promoter sequences flanking the CCAAT box (Ronchi et al., 1995). The putative negative role of an ICE is further supported by the observation made by Takano et al. (1999) that binding of a nuclear protein factor to ICE1 down-regulates topoIIα gene expression in an etoposide-resistant cell line.

In terms of our ICRF-187-resistant cells, regulation of topoIIα expression seems to be attenuated by dissociation of these NF-Y-containing nuclear proteins from the ICE3 promoter region. Altered binding is not caused by mutations in the topoIIα promoter, because sequence analysis revealed a wild-type promoter in the ICRF-187-resistant cells, just as in the drug-sensitive CEM cells. Furthermore, by Western blot analysis, there were no apparent differences in the levels of the CCAAT box binding proteins, NF-YB, NF-YA, or YB-1 in drug-sensitive and -resistant cells that could account for changes seen in nuclear protein-ICE3 complex formation. These observations suggest that in CEM/ICRF-8, such alterations as mutations in or post-translational modifications of NF-Y or other binding proteins in the complex may exist. If this were the case, then protein-protein interactions and protein-DNA binding activity may all be affected. In the case of interactions between NF-Y and histones, a recent study has suggested a model in which NF-Y interacts/recruits histone acetyltransferases to the promoter, thereby stimulating histone acetylation and activating G_2/M-dependent tran-

scription of the topoIIα gene (Adachi et al., 2000). Studies are currently underway to determine whether differential phosphorylation and/or gene mutation in CCAAT box binding proteins, and differential histone acetylation, would alter transcription factor binding to the topoIIα promoter in our ICRF-187-resistant cells.

Although our data presented herein suggest that ICE3 is a negative regulator of topoIIα expression, it does not seem to be the sole contributor to the altered regulation of topoIIα expression in the ICRF-187-resistant cells, as suggested by our mutational analysis in Fig. 2. The topoIIα promoter fragment (nt -557 to -162) found responsible for topoIIα transcriptional up-regulation in the drug-resistant cells contains ICE3, ICE4, and ICE5. Although there were no differences in the amount of ICE4 or ICE5 protein-complexes formed between the drug-sensitive and -resistant cells as reported by EMSA (data not shown), the possibility exists that promoter elements further upstream or downstream of these ICEs may cooperate with these *cis*-acting elements to enhance topoIIα expression in the drug-resistant cells. Additional changes in chromatin structure and/or methylation status within specific regions of the topoIIα promoter may also contribute to the altered topoIIα expression observed here; studies examining some of these possibilities are currently underway.

We have also shown here that ICE1 and ICE2 are neither necessary nor sufficient for topoIIα up-regulation in CEM/ICRF-8 cells. It is not known what distinguishes one ICE from another, but the specific sequences and regulatory promoter elements flanking each ICE that contribute to the overall regulation of inverted CCAAT boxes may be involved. Indeed, by EMSA analysis, the ICE5 oligonucleotide seems to bind to a different nucleoprotein complex in cell extracts compared with the ICE3 and ICE4 oligonucleotides (Herzog and Zwelling, 1997; S.E.M. and W.T.B., unpublished observations). In addition to ICEs, other *cis*-acting promoter elements have been known to affect topoIIα expression (Fraser et al., 1995; Brandt et al., 1997). Larger DNA fragments of ~200 bp each, spanning the entire topoIIα promoter, were used in EMSAs to determine whether *cis*-acting promoter elements, independent of ICEs, play a role in topoIIα up-regulation in CEM/ICRF-8, but we have obtained no clear evidence of differential DNA-protein interactions outside of ICE3 in CEM or CEM/ICRF-8 cells (data not shown).

In the context of drug resistance, very little is known about the factors that regulate topoIIα expression at the transcriptional level. Our study is the first to show that topoIIα transcriptional up-regulation in ICRF-187-resistant cells is mediated in part by altered regulation of the third inverted CCAAT box in the topoIIα promoter. Furthermore, we have demonstrated that ICE3 seems to play a repressive role in topoIIα expression. Our present studies involve characterization of additional ICE3-binding proteins and *cis*-acting elements and delineation of differences in the overall chromatin structure of the topoIIα promoter that may account for topoIIα over-expression in our ICRF-187-resistant cells.

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