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Transcriptional Regulation of Topoisomerase IIα at Confluence and Pharmacological Modulation of Expression by *bis*-Benzimidazole Drugs

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ABSTRACT

Topoisomerase IIα is a critical gene involved in DNA replication and maintenance of genomic stability. Several chemotherapeutic agents target topoisomerase II and levels of expression are an important factor in chemosensitivity. Transcriptional regulation has been demonstrated to regulate topoisomerase IIα levels under several circumstances, including cellular confluence, heat shock, and expression of oncogenes including *ras* and *myb*. Expression of topoisomerase IIα is regulated by cellular proliferation; transcriptional down-regulation in confluent cells is modulated through sequences within the promoter. In this study, we examined DNA-protein interactions within the topoisomerase IIα promoter in exponential and confluent phase NIH3T3 cells. Using electrophoretic mobility shift assay and in vitro DNase I footprint experiments, the involvement of NF-Y in transcriptional regulation was established. Incubation of the DNA minor groove-binding agents Hoechst 33342 and Hoechst 33258 with nuclear extracts revealed drug binding to regions surrounding the inverted CCAAT boxes within the topoisomerase IIα promoter and displacement of proteins binding to these elements. Addition of both Hoechst 33342 and Hoechst 33258 to NIH3T3 cells at confluence resulted in increased expression of topoisomerase IIα. In addition, MTT cytotoxicity assays in confluent cells showed an additive effect of incubation with Hoechst 33342 and the topoisomerase IIα poison etoposide. Therefore, DNA binding drugs which block transcription factor activation of the promoter may deregulate topoisomerase IIα and this strategy may be of value in modifying gene expression and modulating chemosensitivity.

Eukaryotic topoisomerase II (topo II) is a gene involved in essential cellular processes including chromosomal segregation at mitosis. In human cells, there are two expressed isoforms of topo II, termed α and β, each located on a distinct genetic locus. The topo IIα form has a molecular mass of 170 kDa, maps to chromosome 17q21–22 (Wang, 1996); expression is increased in proliferating cells and correlates with cellular S-phase fraction (Holden et al., 1992; Sandri et al., 1996a). There is increased expression of topo IIα in tumor cells compared with nonmalignant tissue (Kim et al., 1991; Hasegawa et al., 1993). However, there is marked heterogeneity of topo II expression within a population of cancer cells (Turley et al., 1997). Furthermore, there is evidence that several oncogenes, including *ras* (Chen et al., 1999), *myb* (Brandt et al., 1997), and p53 (Sandri et al., 1996b; Brandt et al., 1997) interact with sequences within the topo IIα promoter. Topo IIα is the target of several important anticancer agents such as doxorubicin and etoposide. The enzyme can pass an intact DNA helix through a transient double-stranded break that it generates in a separate strand (Burden and Osheroff, 1998). Topo IIα poisons can stabilize the cleavable complex, resulting in an increase in double-stranded breaks that ultimately results in cell death.

Several studies (Davies et al., 1988; Fry et al., 1991) have indicated that up-regulation of topo IIα within tumor cells may result in increased sensitivity to doxorubicin and etoposide. The transcriptional expression of topo IIα is reduced under conditions of cellular confluence (Sullivan et al., 1987). The reduction in topo IIα levels probably contributes to the increased resistance to topo II poisons in confluent cells. There is increasing evidence that experimental models using cells cultured under conditions of confluence may be better models than logarithmically growing cultures (Desoize and Jardillier, 2000; Padrón et al., 2000). Although depletion of growth factors does not seem to be a significant factor in the reduction of topo IIα levels at confluence (Isaacs et al., 1996), intercellular contact in confluent models may more closely approximate the situation within tumors than cells in exponential phase of growth.

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ABBREVIATIONS: topo, topoisomerase; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (thiazolyl blue); bp, base pair(s); DTT, dithiothreitol; BFB, bromphenol blue; EMSA, electrophoretic mobility shift assay; ICB, inverted CCAAT box; MGB, minor groove binder.

The sequences within the topo IIα promoter influencing confluence-induced down-regulation have been identified. Using a series of stable transfectants, including the topo IIα promoter linked to a growth hormone reporter in the Swiss 3T3 cell line, the inverted CCAAT box 2 (ICB2) at position −108 to −104 relative to the transcription start site was shown to be required for confluence induced down-regulation (Isaacs et al., 1996). Experiments using a gel shift assay identified NF-Y, a heterotrimeric transcription factor, as a component of the complex binding this sequence. There is reduced expression of topo II in areas of confluence and hypoxia (Tomida and Tsuruo, 1999) and this may decrease the therapeutic efficacy of topo II poisons. The significance of topo II as a target for several important anticancer agents such as etoposide and doxorubicin further increases the importance of understanding factors affecting the regulation of this gene.

This study aimed to analyze the protein-DNA interactions responsible for transcriptional repression at confluence and investigate strategies to modulate topo IIα expression. We show that DNA-binding drugs with affinity for adenine-thymine (AT)-rich sequences are able to bind these elements and thereby modulate topo IIα transcription. In addition, Hoechst 33342 induced up-regulation of topo IIα increases sensitivity of confluent-phase cells to etoposide.

Materials and Methods

Cell Culture and Drug Treatment. The NIH3T3 murine cell line was cultured at 37°C under an atmosphere of 10% CO_2 in Dulbecco's modified Eagle medium (Autogen Bioclear, Calne, Wiltshire, UK) supplemented with 10% newborn calf serum (Life Technologies, Paisley, Scotland), 4.5 g/l glucose and 2 mM L-glutamine. The human keratinocyte K1, CaCo2 colon cancer, and HeLa cell lines were grown as the NIH3T3 line, except 10% fetal bovine serum (Autogen Bioclear) and 5% CO_2 atmosphere were used. Drug treatment was carried out with exponential growing NIH3T3 cells or cells that were kept for 48 h at confluence. Hoechst 33258 or 33342 (Sigma, St. Louis, MO) at various concentrations or no drug as a control, was added in fresh, prewarmed media and the cells were subsequently incubated for 24 h at 37°C. AR-1–144 was kindly donated by Dr. Moses Lee (Dept. of Chemistry, Furman University, Greenville, SC) and distamycin A was obtained from Sigma.

Cytotoxicity Assays. Exponential or confluent phase cells were incubated for 8 h with Hoechst 33342, washed with prewarmed (37°C) complete medium, and subsequently incubated overnight with etoposide (Sigma). The treated cells were washed with prewarmed (37°C) complete medium and incubated for a further 36 h. At this point, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT; Sigma) was added to 0.5 mg/ml and, after a further 2-h incubation, the medium was removed, the cells were lysed, and the dye dissolved by adding dimethyl sulfoxide (BDH, Poole, Dorset, UK). The reduction of MTT was quantified by measuring the absorbance at 540 nm.

Reporter Constructs, Site Directed Mutagenesis, and Transient Transfections. To determine the effect of Hoechst 33342 and the dominant negative NF-YA mutant NF-Y29 (construct pNF-Y29; Mantovani et al., 1994) on topo IIα promoter activity, the 557-bp promoter fragment of pCAT557 (Hochhauser et al., 1992) was subcloned upstream of the promoterless luciferase reporter gene in pGL3-basic (Promega, Madison, WI), designated pT2WT. A topo IIα construct carrying a polymerase chain reaction-generated mutation in ICB2 (primers ICB2 MFW, sense, 5′-GGCAAGCTACGTTTCCTTCTTCTGGACG-3′; ICB2 MAS, antisense, 5′-CGTCCAGAAGAAGGAAACGTAGCTTGCC-3′) but otherwise identical to pT2WT was designated pT2 MT. The mutation in ICB2 was verified with a BESS Mutascan mutation characterization kit as described by the manufacturer (Epicentre Technologies, Madison, WI). Subsequently, the insert of the final construct was single stranded sequenced using the dideoxy termination method using an automated sequencing system (MWG-Biotech) and compared with the wild-type promoter sequence. The pGL3-promoter vector (Promega) was used to study the effects of Hoechst 33342 on a topo IIα unrelated promoter (i.e., without an inverted CCAAT box).

The constructs were transfected using Superfect transfection reagent (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Briefly, 2 μg of plasmid DNA was mixed with 100 μl of serum-free medium and incubated at room temperature for 15 min. Subsequently, 600 μl of complete medium was added and the mixture was layered on NIH3T3 cells (70–80% confluent). After 16 h, the mixture was replaced with fresh media and cells were grown to and kept for another 2 days at confluence. pCDNA3 (Promega) and pGL3-basic were the empty vector controls for pNF-Y29 and pT2WT, respectively. Transfections were normalized by cotransfection with 0.1 μg of pCH110 (Pharmacia, Peapack, NJ) and assaying for β-galactosidase activity. Luciferase and β-galactosidase activity were determined as described by the manufacturer (Promega).

Preparation of Nuclear Extracts. Nuclear extracts were essentially prepared as described previously (Firth et al., 1994) and all steps were performed at 4°C in the presence of a protease inhibitor mix (Complete; Roche Molecular Biochemicals, Mannheim, Germany). Briefly, cells were rinsed with ice-cold phosphate-buffered saline, scraped from the surface, and collected by centrifugation. The cells were washed with 5 equivolumes of hypotonic buffer containing 10 mM K-HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT; Sigma). Subsequently, the cells were resuspended in 3 equivolumes of hypotonic buffer, incubated on ice for 10 min, subjected to 20 strokes of a Dounce homogenizer, and the nuclei were collected by centrifugation. The nuclear pellet was resuspended in 0.5 equivolumes of low-salt buffer containing 20 mM K-HEPES, pH 7.9, 0.2 mM K-EDTA, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, and 0.5 mM DTT. While stirring, 0.5 equivolumes of high-salt buffer (as low-salt buffer, but containing 1.4 M KCl) was added and the nuclei were extracted for 30 min. Subsequently, the mixture was centrifuged for 30 min at 14,000 rpm in an Eppendorf centrifuge and the supernatant was dialysed in tubing with a 12-kDa cut off (Sigma) for 1 h in a 100× excess of dialysis buffer containing 20 mM K-HEPES, pH 7.9, 0.2 mM K-EDTA, 20% glycerol, 100 mM KCl, and 0.5 mM DTT. The dialysed fraction was centrifuged for 30 min at 14,000 rpm in an Eppendorf centrifuge and the supernatant was snap frozen in an ethanol dry ice bath and stored at −80°C. The protein concentration of the nuclear extract was assayed using a Bio-Rad micro protein assay kit (Bio-Rad, Hercules, CA).

Western Blot Analysis. For Western blot analysis, 5 μg of nuclear extract was denaturated by heating for 3 min at 95°C in sample buffer containing 100 mM Tris-Cl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.02% bromphenol blue (BFB). Bio-Rad high range SDS-polyacrylamide gel electrophoresis molecular mass standards were used as a reference. Proteins were separated on a 7% SDS-polyacrylamide mini gel (Mini Protean II system; Bio-Rad) and subsequently transferred (Trans Blot Cell; Bio-Rad) to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Western blot analysis was performed with the IHIC8 rabbit polyclonal topoisomerase IIα antibody (kindly provided by Dr. I. D. Hickson, Institute for Molecular Medicine, Oxford, UK) at a 1:5000 dilution using a enhanced chemiluminescence Western blot detection kit and protocol (Amersham Pharmacia Biotech, Piscataway, NJ) using 1% blot qualified bovine serum albumin (Promega) as blocking reagents and Tris-buffered saline plus 0.5% Tween 20 (BDH) as a buffer. The chemiluminescent signal was visualized by exposing the blots to Kodak X-Omat-LS film (Kodak, Rochester, NY).

Electrophoretic Mobility Shift Assay (EMSA). The following oligonucleotides (Genosys, Cambridgeshire, UK) containing ICBs

(underlined) were used in EMSAs: ICB1 sense, 5′-CGAGTCAGGGATTGGCTGGTCTGCTTC-3′; ICB1 antisense, 5′-GAAGCAGACCAGCCAATCCCTGACTCG-3′; ICB2 sense, 5′-GGCAAGCTACGATTGGTTCTTCTGGACG-3′; ICB2 antisense, 5′-CGTCCAGAAGAACC AATCGTAGCTTGCC-3′; ICB3 sense, 5′-CTCCCTAACCTGATTGGTTTATT CAAAC-3′; ICB3 antisense, 5′-GTTTGAATAAACCAATCAGGTTAGGGAG-3′; ICB4 sense, 5′-GAGCCCTTCTCATTGGCCAGATTCCCTG-3′; ICB4 antisense, 5′-CAGGGAATCTGGCCAATGAGAAGGGCTC-3′; ICB5 sense, 5′-GATCTTAAATAGATTGGCAGTTCCTGGAG-3′; ICB5 antisense, 5′-CTCCAG GAACTGCCAATCTATTTAAGATC-3′. Oligonucleotides containing mutated ICBs were used as specific competitors of similar sequence, except the wild-type ICB sequence was replaced by AAACC or GGTTT in sense and antisense oligonucleotides, respectively. Sense and antisense oligonucleotides were annealled in an equimolar ratio. Double-stranded oligonucleotides were 5′ end labeled with T4 kinase (New England Biolabs, Hitchin, Hertfordshire, UK) using [γ-^{32}P]ATP and subsequently purified on Bio-Gel P-6 columns (Bio-Rad). EMSAs were essentially performed as described previously (Firth et al., 1994). Briefly, 5 μg of nuclear extract in a total volume of 10 μl was incubated at 4°C for 30 min in a buffer containing 20 mM K-HEPES pH 7.9, 1 mM $MgCl_2$, 0.5 mM K-EDTA, 10% glycerol, 50 mM KCl, 0.5 mM DTT, and 0.5 μg poly(dI-dC). poly(dI-dC) (Pharmacia) and 1× protease inhibitor mix (Complete; Roche Molecular Biochemicals). For supershifts, antibodies against NF-YA or NF-YB (IgG fraction; Rockland, Gilbertsville, PA) were used and the preincubation on ice was extended for a total of 1.5 h. Upon addition of approximately 0.1 ng of radiolabeled probe, the incubation was continued for 30 min at room temperature. In competition experiments, radiolabeled probe and competitor were added simultaneously. Subsequently, 0.5 μl of loading buffer (25 mM Tris-Cl, pH 7.5, 0.02% BFB and 10% glycerol) was added and the samples were separated on a 4% polyacrylamide gel in 0.5× Tris/borate/EDTA buffer containing 2.5% glycerol at 4°C. After drying the gels, the radioactive signal was visualized by exposing the gels to Kodak X-Omat-LS film.

DNase I Footprinting. A radiolabeled probe of 479 bp corresponding to positions −489 through −10 relative to the transcriptional start site of the topo IIα promoter was generated as follows. Antisense oligonucleotide (4 pmol) 5′-GTCGGTTAGGAGAGCTCCACTTG-3′ was 5′ end-labeled with T4 kinase (New England Biolabs) using [γ-^{32}P]ATP in a 10-μl reaction, followed by heat inactivation for 20 min at 65°C. Subsequently, 4 pmol of sense oligonucleotide (5′-CTGTCCAGAAAGCCG GCACTCAG-3′), 2 μl of 10 mM dNTPs (Promega), 1 U of Red Hot DNA Polymerase (Abgene, Epsom, Surrey, UK), 2 μl of 25 mM $MgCl_2$, and 4.5 μl of 10× reaction buffer IV (Abgene) were added (in a final volume of 50 μl) and a polymerase chain reaction was performed consisting of: 3 min at 95°C and 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C for 35 cycles. The product was purified on a Bio-Gel P-6 column (Bio-Rad). DNase I footprint reactions were performed with 30 μg of nuclear extract in a 50-μl reaction in the same buffer as used for EMSA. After preincubation for 30 min at 4°C, approximately 0.1 ng of radiolabeled probe was added and the mixture was incubated at room temperature for another 30 min. Subsequently, 1 U of RQ1 DNase I (Promega) and up to 5 mM $MgCl_2$ and $CaCl_2$ were added. After exactly 3 min of digestion at room temperature, 1 volume of stop mix containing 30 mM K-EDTA, pH 8.0, 200 mM NaCl, and 1% SDS was added and samples were purified by phenol-chloroform treatment and alcohol precipitation. The resulting pellets were dried and resuspended in loading buffer (95% formamide, 20 mM K-EDTA, pH 8.0, 0.05% BFB, and 0.05% xylene cyanol). The sample was heat-denaturated for 3 min at 95°C and separated on a 6% denaturating polyacrylamide gel (Sequagel; National Diagnostics, Hessle Hull, UK). A 10-bp ladder (Life Technologies) labeled with ^{32}P by T4 kinase was used as a molecular mass standard. The dried gels were exposed to Kodak X-Omat-LS film with intensifying screens (Kodak) at −80°C.

Results

Investigation of Effects of Confluence on the Topo IIα Promoter. To verify down-regulation of topo IIα at confluence in NIH3T3 cells, nuclear extracts from exponential and confluent phase cells were immunoblotted with an antibody for topo IIα (Fig. 1a). As previously demonstrated, there is dramatic down-regulation of topo IIα expression at confluence; this has been shown to be caused by alterations in transcriptional regulation (Isaacs et al., 1996). Similar patterns were seen in extracts from the human K1 keratinocyte and CaCo2 colon cancer cell lines in which down-regulation of topo IIα at confluence was also demonstrated. In contrast, the cervical cancer cell line HeLa showed little alteration of topo IIα expression in confluent cells.

The topo IIα promoter includes five inverted CCAAT boxes in which ICB2 was implicated in confluence-induced arrest (Fig. 1b). EMSA was performed with labeled probes of the ICB1–5 with extracts from exponential and confluent cells and revealed binding of complexes to each ICB (data not shown). In addition, a supershift was demonstrated after incubation with antibodies to NF-YA and NF-YB for ICB1–4 (data not shown). This is shown for ICB2 in Fig. 2a. Changes in the pattern of the supershift in extracts from confluent cells suggest that there may be post-translational alteration in the protein complex binding to the DNA.

To further investigate protein-DNA interactions in the topo IIα promoter, in vitro DNase I footprinting was carried out using a radiolabeled probe containing sequences including ICB1 and ICB2 (Fig. 2b). Clear protected regions were demonstrated over ICB1 and ICB2. There was increased intensity of the footprint demonstrated in extracts from confluent cells suggesting that increased binding of this complex may be inhibitory to topo IIα transcriptional expression. The apparent discrepancy between this result and that obtained from the EMSA may have been caused by the additional flanking sequence required by the complex expressed in confluent cells, which is present on the probe used in footprinting experiments. Furthermore, there is probably a secondary structure present on the probe used in the footprint experiments that may affect protein binding. Experiments using a probe with a mutation of the ICB2 (ATTGG → AAACC) resulted in loss of the footprint (Fig. 2c), confirming the

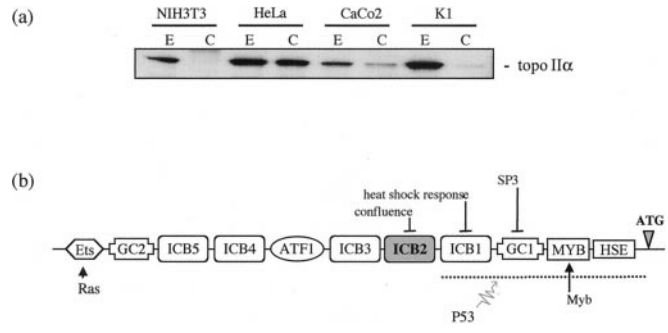


Fig. 1. Down-regulation of topo IIα at confluence and organization of the human topo IIα promoter. a, Western blot analysis of nuclear extracts isolated from NIH3T3, Hela, CaCo2, and K1 cells. The blot was probed with antibodies against topo IIα. E, exponential phase; C, confluent phase. b, relative position of putative *cis*-acting elements in the topo IIα promoter. GC, GC box; ICB1–5, inverted CCAAT boxes 1 to 5; ATF, activating transcription factor binding site; ATG, topo IIα translation start codon; HSE, heat-shock element; shaded box, ICB involved in topo IIα down-regulation at confluence.

critical nature of the intact CCAAT sequence for NF-Y binding, because this factor requires all five nucleotides (Bi et al., 1997). In footprinting experiments using extracts of HeLa cells, in which no down-regulation of topo IIα at confluence was demonstrated, there was no change in the pattern of the protected regions over ICB1 and ICB2 in extracts from log and confluent phase cells (data not shown).

Reporter assays were performed using a construct of the full-length topo IIα promoter (Hochhauser et al., 1992) linked to a luciferase reporter plasmid (pGL3-basic) designated pT2WT (Fig. 2d). There was marked reduction in luciferase expression using extracts from cells in confluent phase compared with extracts from cells in exponential phase; this corresponds to transcriptional down-regulation occurring at confluence. After cotransfection of the reporter containing the topo IIα promoter and a plasmid expressing a dominant negative NF-YA (pNF-Y29), which allows association with NF-YB but abolishes binding of the NF-Y complex to DNA (Mantovani et al., 1994), there was a marked increase in luciferase expression. In contrast, cotransfection with the vector alone (pCDNA3) resulted in no alteration in expression. This suggests that the NF-Y complex acts to repress topo IIα promoter activity. The ICB2 motif has been specifically implicated in control of repression of topo IIα at confluence (Isaacs et al., 1996). However, experiments using a mutated ICB2 did not demonstrate increased reporter expression. This may be because the stimulatory transcription factors are unable to bind to this motif. (Fig. 2d). The cotransfection of pT2 MT and pNF-Y29 also increased luciferase expression, but to a lower extent than pT2WT plus pNF-Y29, suggesting that ICB2 is one, but not the sole, promoter element involved in confluence induced down-regulation.

Effects of DNA Minor Groove Binding Drugs on the Topo IIα Promoter. Several DNA minor groove binding drugs (MGB) have been shown to bind preferentially to AT rich sequences in DNA. These include the oligopeptide antibiotic distamycin and the *bis*-benzimidazoles Hoechst 33342 and Hoechst 33258 (Nielsen, 1991). In view of the ubiquitous presence of CCAAT sequences within the topo IIα promoter region, EMSA experiments were carried out using labeled oligonucleotide probes containing sequences of the ICB2 found in the promoter (Fig. 3). The probes were incubated with cell extracts and increasing concentration of drug. There was inhibition of protein complex binding to the ICB sequence with increasing drug concentration for the MGB Hoechst 33342 and Hoechst 33258 (Fig. 3, a and b). In contrast, addition of Hoechst 33342 and Hoechst 33258 had no effect on a radiolabeled oligonucleotide containing the GC sequences implicated in Sp3 binding (Mo et al., 1997) (Fig. 3c). With distamycin, Hoechst 33342 and Hoechst 33258 abolition of binding was seen at 30 μM. Incubation with the agent AR-1–44, an imidazole derivative of distamycin targeting guanine and cytosine rich sequences (Lee et al., 1993), results in only a partial inhibition at drug concentrations above 100 μM (Fig. 3d).

To determine whether these agents are capable of binding specific sequences within the context of the topo IIα promoter, in vitro DNase I footprinting was carried out using a radiolabeled probe of the topo IIα promoter incubated with drug. Although there was clear protection over the ICB with

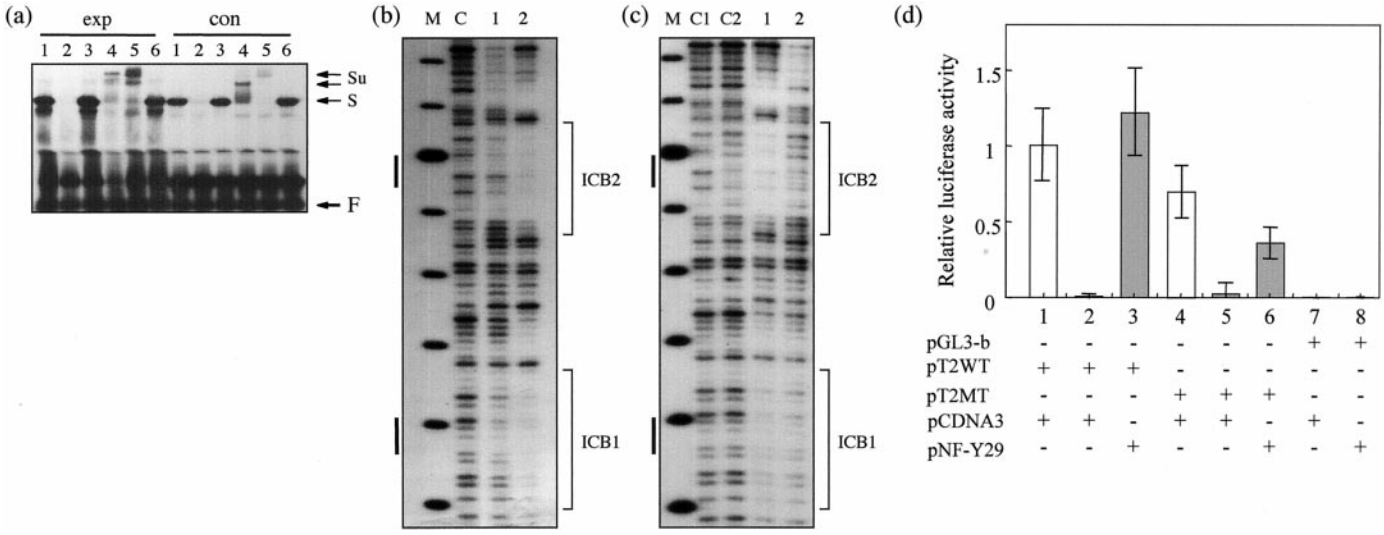


Fig. 2. EMSA, DNase I footprinting and luciferase reporter assay showing involvement of NF-Y in the complex that binds to ICB2. a, nuclear extracts prepared from NIH3T3 cells in exponential and confluent phase (lanes 1–6) were incubated with a radioactive oligonucleotide containing the ICB2 sequence of the topo IIα promoter. Lane 1, control lane containing probe and nuclear extracts only. Lanes 2 and 3 contain an unlabeled competitor oligonucleotide containing the wild-type or mutated NF-YA binding site, respectively. Lanes 4, 5, and 6 contain NF-YA, NF-YB, and (as a control) Sp1 antibodies, respectively. F, free probe; S, specific DNA/protein complex; Su, super shifted DNA/protein complex; Exp, nuclear extracts of exponential phase cells; Con, nuclear extracts of confluent phase cells. b, DNase I footprint of nuclear extracts isolated from NIH3T3 cells in exponential (lane 1) and confluent phase (lane 2). The position of ICB1 and 2 is indicated relative to the CCAAT sequence position (bars) and the actual footprint (bracket). M, molecular mass marker (increments of 10 bp); and C, control (no protein). c, DNase I footprint of nuclear extracts isolated from NIH3T3 cells in confluent phase with a probe containing the wild-type (lane 1) or mutated (lane 2) ICB2 sequence. The position of ICB1 and 2 is indicated relative to the CCAAT sequence position (bars) and the actual footprint (bracket). M, molecular mass marker (increments of 10 bp); C1, control lane (no protein) wild-type ICB2 sequence; C2, control lane (no protein) mutant ICB2 sequence. d, effect of dominant negative NF-YA on topo IIα promoter activity. Luciferase reporter assays were performed on extracts isolated from exponential (lanes 1, 4) and confluent (lanes 2, 3, 5–8) phase NIH3T3 cells. The combination of plasmids used is indicated below the figure. PT2WT, wild-type topo IIα promoter; pT2 MT, topo IIα promoter with mutated ICB2; pGL3-basic, empty vector control for pT2WT and pT2 MT; pNF-Y29, dominant negative NF-YA expression vector; pCDNA3, empty vector control of pNF-Y29.

lower doses of distamycin, with increased drug concentration there was extensive protection of sequences throughout the promoter (Fig. 4a). After incubation with Hoechst 33342, there were clear protected regions only over the CCAAT boxes and the nonfunctional TATAA sequence (Fig. 4b). However, in contrast to distamycin, there was little alteration in the pattern of footprint with increased concentrations of drug. Incubation with the agent AR-1–144 did not result in protection over the region of the ICB (data not shown).

To investigate if the displacement of the complex containing CCAAT binding proteins by MGB could be demonstrated within the topo IIα promoter, in vitro DN*ase* I footprinting was carried out on nuclear extracts incubated with radiolabeled probe in the presence of increasing amounts of drug (Fig. 4c). The pattern of footprint over the ICB was altered with increasing concentration of drug, resulting in a pattern of protection identical to that found when only drug and DNA were incubated together. This indicates that an effect of incubation with Hoechst 33342 (and Hoechst 33258, data not shown) is to displace the normal CCAAT-binding complex by occupying the binding site.

Effect of Minor Groove Binding Drugs on Topo IIα Expression. The result of the EMSA and footprinting experiments indicated that incubation with Hoechst 33342 and Hoechst 33258 could prevent the binding of proteins inhibiting topo IIα expression. Furthermore, these results suggested that the affinity of these drugs for the CCAAT sequence is high and the pattern of binding does not alter with increasing drug concentration. To demonstrate the effect of drug incubation on topo IIα expression, NIH3T3 cells were grown to confluence and incubated with the various drugs. Exposure of cells to Hoechst 33342 resulted in a time- and concentration-dependent increased expression of topo IIα compared with cells grown in drug-free medium as demonstrated by immunoblotting (Fig. 5a). The increase in expression was to approximately the level of exponentially growing cells. A similar result was obtained with Hoechst 33258 (data not shown). This increased expression occurred within six h of treatment and was concentration-dependent (Fig. 5, b and c).

To confirm the stimulatory effect of drug treatment on the topo IIα promoter, cells were transfected with a topo IIα (pT2WT) and simian virus-40 control (pGL3-Promoter) promoter/reporter construct (which lacks CCAAT sequences) as well as with the empty vector (pGL3-basic). These cells were grown to confluent phase and treated with Hoechst 33342. There was a dose-dependent increase in luciferase expression with increased doses of drug confirming an effect on the topo IIα promoter (Fig. 5d). The effect of Hoechst 33342 on luciferase expression via the SV40 promoter/reporter and the empty vector was minimal. The lower threshold for effects on the promoter as compared with cellular gene expression may be due to the greater accessibility of plasmid sequences compared with nuclear DNA.

MTT cytotoxicity assays in confluent cells were performed to investigate the relation between successive incubation of Hoechst 33342 and the topoisomerase IIα poison etoposide. In contrast to the IC_{50} value of etoposide in exponential phase cells (5–10 μM) the IC_{50} value in confluent phase cells is high (>250 μM). However, preincubation of confluent phase cells with 15 to 25 μM Hoechst 33342 significantly lowered the IC_{50} value of etoposide (Fig. 5e). The IC_{50} value of Hoechst 33342 alone in confluent phase cells was 50 μM.

Therefore, DNA binding drugs that block transcription factor activation of the promoter may deregulate topoisomerase IIα; this strategy may be of value in modifying gene expression and modulating chemosensitivity. These experiments demonstrate that MGBs that target AT-rich sequences can up-regulate topo IIα in the presence of confluence-induced transcriptional repression.

Discussion

There is evidence that transcriptional control of topo IIα regulation is important in regulating gene expression at confluence (Isaacs et al., 1996), and under heat-shock conditions (Furukawa et al., 1998). A recent study indicated that transcriptional elements may regulate the cell cycle control of topo IIα expression (Falck et al., 1999). Furthermore, p53 may directly repress the promoter (Sandri et al., 1996b;

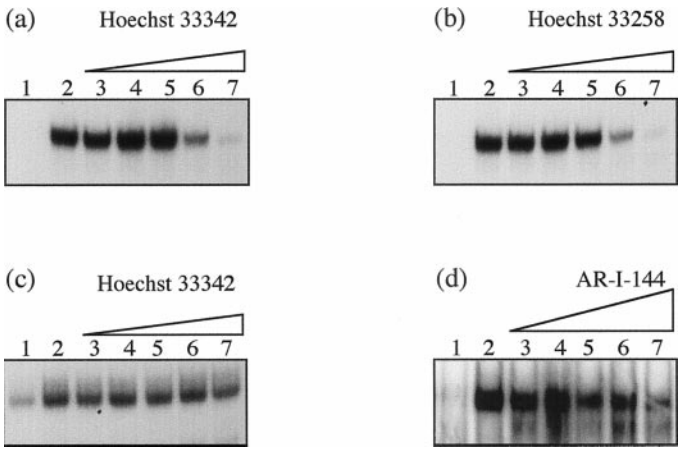


Fig. 3. Inhibition of protein complex binding to ICB2 by AT specific minor groove binders. Effect of minor groove binders on EMSA of nuclear extracts isolated from NIH3T3 cells in exponential phase. Nuclear extracts were incubated with a radiolabeled probe containing the ICB2 wild-type binding site (a, b, d) or a GC-box sequence (c). In each case, lane 1 contains unlabeled competitor probe and lane 2 contains mutated probe. Lanes 3 to 7 contain increasing concentrations of minor groove binding agent as indicated at concentrations of 10, 20, 30, 40, and 50 μM (Hoechst 33342 and Hoechst 33258) or 20, 40, 60, 80, and 100 μM (AR-1–144).

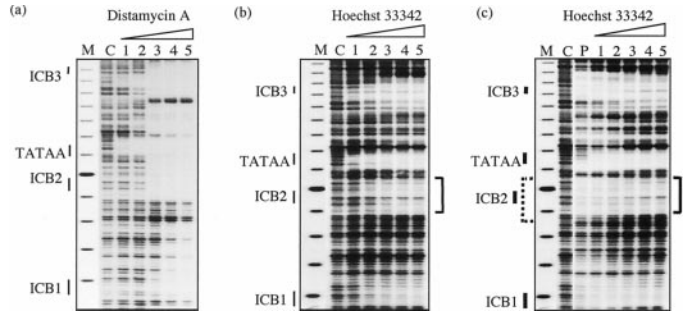


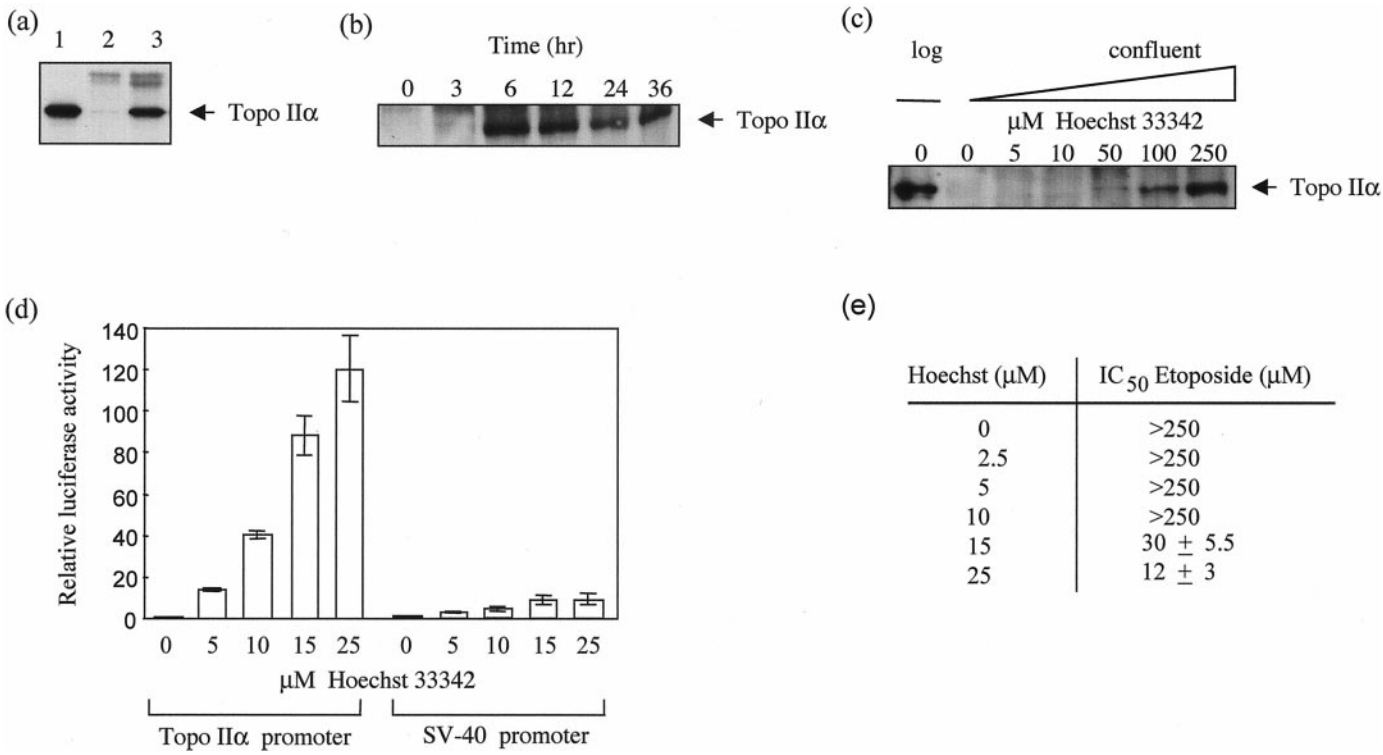
Fig. 4. Displacement of protein complexes from the topo IIα promoter by minor groove binders. Effect of distamycin A and Hoechst 33342 on DNase I footprinting in the absence (a, b) or presence (c) of nuclear extracts isolated from NIH3T3 cells in exponential phase. Extracts were incubated with MGBs and a radiolabeled probe of the topo IIα promoter. TATAA, nonfunctional TATAA-box; M, molecular mass marker (increments of 10 bp); C, control (no protein); solid and dashed brackets, Hoechst and nuclear extract footprints over the ICB2, respectively; bars, position of ICBs and TATAA-box relative to the promoter sequence; triangle, increasing amount of Hoechst 33342 (10, 20, 30, 40, and 50 μM).

Wang et al., 1997). The oncogenes *ras* and *myb* have also been demonstrated to act through specific sequences within the topo IIα promoter. Both heat-shock and cell cycle regulation have been demonstrated to act through the ICB1 in the promoter (Falck et al., 1999), whereas confluence-induced down-regulation is regulated through the ICB2. This study confirms that, unlike with cell cycle regulation, the transcription factor NF-Y forms part of the ICB binding complex. There is an alteration in the intensity of the in vitro footprint seen at confluence, although this is not associated with alteration in the amount of NF-YA or NF-YB expressed.

Previous studies have suggested that the inverted CCAAT boxes within the promoter are important in regulation of topo IIα transcription. Although NF-Y is significant in heat-shock response, a study on cell cycle regulation did not confirm NF-Y binding to the ICB1 site in the topo IIα promoter as being important in transcriptional repression (Falck et al., 1999). The effects of NF-Y are generally stimulatory on gene expression but NF-Y has also been shown to mediate inhibition of gene transcription of several genes including cdc2 (Yun et al., 1999). The role of other transcription factors in control of topo II expression has not been clarified. Although there is a clear footprint over the TATAA box the significance of this is unclear as the distance from the transcription initiation site suggests that it is nonfunctional (Hochhauser et al., 1992). Furthermore, expression of a novel 90-kDa CCAAT-box binding protein (ICBP90) that binds to the ICB2 consensus sequence coincides with that of topo IIα and is expressed in Hela cells at confluence (Hopfner et al., 2000).

The correlation between levels of topo IIα and chemosensitivity to topo II poisons has been shown in several studies, although other factors such as p53 status may also be important. Therefore, strategies to increase topo II expression may improve therapeutic effectiveness. The resistance to chemotherapeutic drugs seen in vitro at confluence is related in part to reduced expression of topo II (Dimanche-Boitrel et al., 1992), although altered membrane permeability to cytotoxic drugs may also play a role. It is unclear to what extent the reduced expression of topo II in tumors is caused by hypoxic down-regulation or by the confluence pathway investigated here. Further experiments with xenografts using animals treated with analogs of the agents used in this study should clarify this issue.

MGB agents have a variety of pharmacological effects. Hoechst 33258 binds to double-helical DNA (Bontemps et al., 1975) and has ready access into cells with minimal toxicity (Soderlind et al., 1999). It binds preferentially at AT-rich DNA sequences with a minimum of four consecutive AT base pairs. This may result in inhibition of transcription factor binding to DNA with consequent effect on gene regulation (Zewail-Foote and Hurley, 1999; White et al., 2000). These compounds have also been shown to act as topoisomerase I inhibitors (Chen et al., 1993). It has been demonstrated that exposure to Hoechst 33342 can directly alter gene expression by binding to promoter sequences within the c-fos promoter (White et al., 2000). Thus there are several mechanisms of action for these agents that are relatively sequence nonspe-



(e)

Hoechst (μM)	IC_{50} Etoposide (μM)
0	>250
2.5	>250
5	>250
10	>250
15	30 ± 5.5
25	12 ± 3

Fig. 5. Increased expression of topo IIα in confluent cells following exposure to the minor groove binder Hoechst 33342. a, Western blot analysis of nuclear extracts isolated from exponential (lane 1), confluent (lane 2), and confluent phase cells treated with 100 μM Hoechst 33258 for 24 h (lane 3). b, time course of the incubation of confluent NIH3T3 cells with 100 μM Hoechst 33342. c, dose response of the incubation of confluent NIH3T3 cells with Hoechst 33342 for 6 h. The blots were probed with antibodies against topo IIα. d, luciferase reporter assays of nuclear extracts isolated from confluent phase NIH3T3 cells transfected with the topo IIα (pT2WT) or SV-40 promoter (pGL3). Cells were maintained at confluence for 48 h before adding the drug Hoechst 33342. The activity relative to pT2WT without drugs was set to 1. e, effect of Hoechst on the IC_{50} value of etoposide at confluence. MTT cytotoxicity assays were performed after successive incubation of confluent phase NIH3T3 cells with Hoechst 33342 for 8 h and etoposide for 16 h. The IC_{50} value of Hoechst 33342 alone in confluent phase cells was 50 μM.

cific; the development of novel lexitropsins will allow more precise sequence-specific targeting.

There is considerable potential to exploit the DNA sequence-specific properties of MGBs in modulating gene expression (Zewail-Foote and Hurley, 1999). The ability to overcome transcription factor-induced repression is important in the case of topo IIα because increased expression of the gene would increase sensitivity to topo II poisons. A component of topo II down-regulation in tumors may be caused by hypoxia, which has been shown to decrease topo II transcription, although the regions of the promoter modulating this have not been identified. However, it is likely that reduction in expression may also be modulated through the confluence pathway analyzed in this study, which may occur concurrently with hypoxia-induced repression. Abrogation of this repression may also induce apoptosis, which occurs after enforced expression of topo IIα (McPherson and Goldenberg, 1998). Other factors modulating topo II transcription have been identified. The *trans*-activation of topo IIα by c-Myb may account for the bulk of topo IIα promoter activity in human leukemia cells (Brandt et al., 1997). Furthermore, the Sp3 transcription factor is increased in cell lines resistant to topo II poisons with reduced transcription of topo IIα (Mo et al., 1997). The Sp3 factor binds to the GC box, and use of MGBs that preferentially bind this motif may also be useful in increasing gene expression and consequently enhancing chemosensitivity.

The initial report on topo IIα promoter sequences modulating down-regulation at confluence identified the ICB2 as the critical sequence modulating this effect (Isaacs et al., 1996). The MGB sequence-specificity is such that drug binding can be demonstrated over several ICBs and these experiments do not therefore rule out a contribution from other ICB sequences within the promoter. Cotransfection of a dominant negative NF-Y plasmid with a reporter construct containing a mutant ICB2 did not increase luciferase levels to the same level as that with constructs of the wild-type sequence. This could be because the transcription factor complexes with stimulatory effects are unable to bind to the promoter. Although there is data implicating ICB2 alone in transcriptional repression at confluence, there is evidence from other studies that several ICBs may be implicated in control of expression (Adachi et al., 2000). However, our data confirm that NF-Y is critical for transcriptional repression at confluence and prevention of its interaction with the promoter will alter gene regulation.

In conclusion, the topo IIα promoter is a valuable tool in investigation of the potential modulation of gene expression by inhibition of transcription factor binding. Strategies that modulate the activity of these transcription factors may also have a significant effect on gene expression.

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