The Role of an Inverted CCAAT Element in Transcriptional Activation of the Human DNA Topoisomerase IIα Gene by Heat Shock

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Expression of the DNA topoisomerase IIα (topoIIα) gene is highly sensitive to various environmental stimuli including heat shock. The amount of topoIIα mRNA was increased 1.5–3-fold 6–24 h after exposure of T24 human urinary bladder cancer cells to heat shock stress at 43 °C for 1 h. The effect of heat shock on the transcriptional activity of the human topoIIα gene promoter was investigated by transient transfection of T24 cells with luciferase reporter plasmids containing various lengths of the promoter sequence. The transcriptional activity of the full-length promoter (nucleotides (nt) −295 to +85) and of three deletion constructs (nt −197 to +85, −154 to +85, and −74 to +85) was increased ~3-fold 24 h after heat shock stress. In contrast, the transcriptional activity of the minimal promoter (nt −20 to +85), which lacks the first inverted CCAAT element (ICE1), the GC box, and the heat shock element located between nt −74 and −21, was not increased by heat shock. Furthermore, the transcriptional activity of promoter constructs containing mutations in the GC box or heat shock element, but not that of a construct containing mutations in ICE1, was significantly increased by heat shock. Electrophoretic mobility shift assays revealed reduced binding of a nuclear factor to an oligonucleotide containing ICE1 when nuclear extracts were derived from cells cultured for 2–24 h after heat shock. No such change in factor binding was apparent with an oligonucleotide containing the heat shock element of the topoIIα gene promoter. Finally, in vivo footprint analysis of the topoIIα gene promoter revealed that two G residues of ICE1 that were protected in control cells became sensitive to dimethyl sulfate modification after heat shock. These results suggest that transcriptional activation of the topoIIα gene by heat shock requires the release of a negative regulatory factor from ICE1.

DNA topoisomerases are essential enzymes that participate in the segregation of newly replicated chromosome pairs, in chromosome condensation, and in modification of the superhelical content of DNA (1–3). Human topoisomerase II (topoII) functions as a homodimer by cleaving and opening one DNA duplex, passing a second duplex through the opening, and then rescaling the break (4–6). Two topoII isoforms have been identified in mammals: 170-kDa topoIIα and 180-kDa topoIIβ (7). Although both enzymes are closely related in structure, they differ in important biochemical and pharmacological properties, including sensitivity to topoII-targeting drugs, cellular localization, and regulation by the cell cycle (8). Whereas the amount of topoIIβ remains relatively constant throughout the cell cycle, topoIIα expression is coupled to the cell cycle (9, 10). TopoIIα is of particular importance because of its association with DNA replication, mitosis, and cell proliferation.

Expression of topoIIα is highly susceptible to environmental stimuli, and such regulation is thought to be mediated at both the transcriptional and post-transcriptional levels. The promoter region of the topoIIα gene contains various regulatory elements, including five inverted CCAAT elements (ICEs), one GC box, and one heat shock element (HSE) (11). Exposure of human colon cancer cells to glucosamine induces down-regulation of topoIIα, resulting in the development of resistance to the topoIIα-targeting epipodophyllotoxin, etoposide (12). Development of resistance to such topoIIα-targeting agents is often associated with down-regulation of topoIIα in various mammalian cell lines (13, 14). In one etoposide-resistant cell line derived from human head and neck cancer KB cells (15, 16), the transcription factor Sp3 was implicated in the down-regulation of topoIIα (17).

Introduction of the wild-type p53 tumor suppressor gene into murine cells results in reduced expression of the topoIIα gene, and this effect appears to be mediated by one of the ICEs in the topoIIα gene promoter (18). Apoptosis induced by adenovirus E1A protein in human KB cells is associated with a marked decrease in the amount of topoIIα that is due to accelerated degradation of topoIIα by the ubiquitin proteolysis pathway (19, 20). The amount of topoIIα mRNA in late S phase is ~15 times that during the G2 phase of the cell cycle in human HeLa cells, apparently because of increased mRNA stability in S phase (10). These observations indicate that topoIIα expression is regulated by multiple mechanisms that operate at the levels of transcription, mRNA stability, and protein degradation.

Heat shock stress also affects the abundance of topoIIα mRNA in mammalian cells. Exposure of human head and neck...
or colon cancer cells to high nonpermissive temperatures results in an increase in expression of the topoIIα gene, apparent 6–12 h later, and consequent sensitization to the cytotoxic effect of etoposide (21, 22). The same heat shock stress markedly increases the abundance of the heat shock protein HSP70 and induces a transient decrease in the amount of topoIIα mRNA and protein immediately after exposure to hyperthermia (10, 22, 23). Whereas this early effect of heat shock stress on topoIIα expression appears to be mediated by increased degradation of topoIIα mRNA (10), the later up-regulation of topoIIα gene expression appears to be due to transcriptional activation (22). We have now investigated which elements in the 5′-flanking region of the human topoIIα gene are responsible for the heat shock-induced activation of transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and other nucleic acid-modifying enzymes and reagents were obtained from Promega (Madison, WI), Life Technologies, Inc., or Takara Shuzo (Kyoto, Japan), unless indicated otherwise. Both [α-32P]dCTP and γ-[32P]ATP were from NEN Life Science Products. Human topoI cDNA was kindly provided by T. Andoh (Sokka University, Tokyo, Japan), and human topoIIα cDNA (pBS-hTOP2) was provided by J. C. Wang (Harvard University, Boston, MA). Human HSP70 cDNA was kindly given by R. T. N. Tjian (University of California, Berkeley, CA). All cDNA fragments were separated from vector DNA by agarose gel electrophoresis and labeled by random primer DNA synthesis.

**Cell Culture and Heat Shock Conditions**—The T24 cell line, established from human transitional cell carcinoma of the urinary bladder (24), was cultured at 37 °C under a humidified atmosphere of 5% CO2 in Iscove’s modified Dulbecco’s medium (Gibco/BRL) supplemented with 10% newborn calf serum (SeraLab, Sussex, United Kingdom), 1 mg/ml Bacto-peptone (Difco), 0.292 mg/ml l-glutamine, 100 units/ml penicillin, and 100 μg/ml kanamycin. For heat shock, culture plates were sealed with parafilm and immersed in a water bath at 43 °C for 1 h.

**Northern Blot Analysis**—Northern blot analysis was performed as described previously (17). Briefly, total RNA was extracted from T24 cells with the use of guanidine isothiocyanate (25), subjected (15 μg lane) to electrophoresis on a 1% agarose gel containing formaldehyde, and transferred to a Hybond N+ membrane (Amersham International, Buckinghamshire, United Kingdom). The membranes were exposed to 32P-labeled cDNA probes for 18 h and washed twice at 42 °C in 2× SSC containing 0.1% SDS and twice at 42 °C in 0.2× SSC containing 0.1% SDS. Radioactivity was detected with a Fujix BAS 2000 image analyzer (Fuji Film, Tokyo, Japan).

**Construction of topoIIα Plasmids**—We used the polymerase chain reaction (PCR) to amplify the human topoIIα gene promoter (nt −295 to +85, relative to the major transcription start site) as described previously (17). The 3′ end of all inserts was nt +85, 10 base pairs upstream of the translation initiation site. For the construction of other deletion constructs, HindIII fragments (nt −295 to +85) of the pTIIα295 plasmid were digested with BfaI (pTIIα197), SccFI (pTIIα154), BhpI (pTIIα74), and SacI (pTIIα20). The digestion products were blunt-ended with the Klenow fragment of DNA polymerase I, ligated, and cloned into the HindIII links and cloned into the HindIII site of the pG2-Base vector (Promega).

Site-directed mutagenesis of ICE1, the GC box, and the heat shock element was performed by a PCR-based method. The promoter sequences were amplified first with Pfu polymerase (Stratagene, La Jolla, CA), the 5′-primer +85 (5′-CGCTGCTGAAGGGGGCTCAAG-3′), and 5′-primers that introduce specific mutations into the target elements; m5 (5′-CAGGGAATAAATCTGGTCTTCGGCCGCTAAGGAGGT-3′) for mutation of ICE1, m6 (5′-CAGGAGTTGGCTGCTTGCTTACAAAGGATGGAGCT-3′) for mutation of the HSE (mutated nucleotides are underlined). A second PCR was performed with Taq polymerase, the first PCR products, and the 5′-primer −295 (corresponding to the normal promoter sequence with a 5′-end at nt −295). This second PCR products were digested with HindIII and ligated into pG2-Base. The mutations introduced into these clones were confirmed by DNA sequencing.

**Transient Transfection**—T24 cells (1 × 106) were transfected to 60-mm dishes, incubated at 37 °C for 48 h, and transfected with luciferase plasmid DNA (2.5 μg) by calcium phosphate precipitation as described previously (26). Four hours after transfection, the cells were washed, incubated at 37 °C for 24 h in fresh medium, and exposed to 43 °C for 1 h. The treated cells were then harvested immediately (0 h) or after further incubation at 37 °C for 1, 6, 12, or 24 h for determination of luciferase activity.

**Luciferase Assays**—Cells were lysed in 200 μl of 25 mm Tris-phosphate buffer (pH 7.5) containing 1% Triton X-100 and subjected to centrifugation at 14,000 × g for 15 s. The resulting supernatants were assayed for luciferase activity with the use of a Picogene kit (Toyoink, Tokyo); light intensity was measured for 15 s with a luminometer (Model TD-20/20, Promega). Cells were cotransfected with pSV2-β-gal and pRSV-neo for transfection control, and β-galactosidase activity was measured with an Aurora GAL-XE kit (ICN, Costa Mesa, CA).

**In Vivo Footprint Analysis**—Heat-treated or control T24 cells were exposed to dimethyl sulfoxide, and genomic DNA was then extracted and cleaved as described (27, 28). Ligation-mediated PCR was performed as described (27). Primer 1 (5′-CAGGAggcaGccccGccg-3′, nt +46 to +31) was used for first-strand synthesis; primer 2 (5′-CCGGAcCaGGcCGCGCTCTCACGAGCCG-3′, nt +21 to −7), which was labeled at the 5′-end with γ-[32P]ATP and T4 polynucleotide kinase, was used for final detection of the DNA ladder. Samples were analyzed on a 6% polyacrylamide sequencing gel.

**Isolation of Stable Transfectants**—T24 cells (5 × 105) were transfected with a luciferase reporter vector containing the topoIIα gene as described (29, 197). Four hours after transfection, the cells were transfected with pBabe-puro (6). Transfected cells were selected in 0.4 μg/ml of puromycin for 4 to 5 days. Cells were then washed, incubated at 37 °C for 24 h in fresh medium, and exposed to 43 °C for 1 h. The treated cells were then harvested immediately (0 h) or after further incubation at 37 °C for 1, 6, 12, or 24 h for determination of luciferase activity.

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared as described previously (17). Briefly, T24 cells (4 × 106), subjected or not to heat shock at 43 °C for 1 h, were collected by exposure to trypsin; resuspended in 200 μl of an ice-cold solution containing 10 mm Hepes-NaOH (pH 7.9), 10 mm KCl, 0.75 mm spermidine, 0.15 mm spermine, 0.2 mm EDTA, 0.2 mm EGTA, 0.5 mm dithiothreitol, and 0.5 mm phenylmethylsulfonyl fluoride; and incubated on ice for 15 min. The cells were then lysed by passing 10 times through a 25-gauge needle attached to a 1-ml syringe, and the lysates were centrifuged at 8,000 × g for 10 min at 4 °C. The resulting supernatant (nuclear extract) was stored at −70 °C, and its protein concentration was determined with a protein assay kit (Bio-Rad).

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSAs were performed as described previously (29). Briefly, 6 μg of nuclear extract protein were incubated for 30 min at room temperature in a final volume of 20 μl containing 10 mm Tris-HCl (pH 7.5), 50 mm NaCl, 1 mm MgCl2, 1 mm EDTA, 8% glycerol, 1 mm dithiothreitol, 0.1 μg of poly(dI-dC), and 1 × 105 cpm of 32P-labeled oligonucleotide probe (1 ng) in the absence or presence of various competitors. The reaction mixtures were then applied to a nondenaturing 5% polyacrylamide gel and separated by electrophoresis at 100 V for 3 h in a buffer containing 50 mm Tris, 380 mm glycine, and 2 mm EDTA. The gel was exposed to x-ray film with intensifying screens. The following oligonucleotides were used for EMSA (nucleotides showing identity are underlined): m1 (5′-GGCTGCTGTCTGTTACAAAGGATGGAGCT-3′), m2 (5′-GGCTGCTGTCTGTTACAAAGGATGGAGCT-3′), and m3 (5′-GGCTGCTGTCTGTTACAAAGGATGGAGCT-3′). These probes were 5′′-end labeled with γ-[32P]ATP and T4 polynucleotide kinase. The labeled probe was used for DNA footprint analysis.
Regulation of Topoisomerase IIα Gene by Heat Shock

RESULTS

Effects of Heat Shock Stress on the Abundance of topoI, topoIIα, and HSP70 mRNAs—Consistent with our previous observations with human head and neck or colorectal cancer cells (22, 23), Northern blot analysis revealed that exposure of T24 cells to 43 °C for 1 h resulted in an initial small decrease in the amount of topoIIα mRNA, which was followed by an increase in transcript abundance that was maximal (3-fold) 24 h after heat treatment (Fig. 1). This experiment was repeated with two T24 cell lines stably transfected with the pTIIα–295 luciferase construct. Again, luciferase activity was decreased immediately after heat treatment, but then showed a time-dependent increase that was maximal (3–4-fold) after 24 h (data not shown).

To identify the promoter sequences responsible for conferring sensitivity to heat shock, we measured luciferase activity 24 h after exposure to 43 °C for 1 h of T24 cells transiently transfected with various topoIIα gene promoter constructs (Fig. 4). Heat shock increased luciferase activity ~3-fold in cells transfected with pTIIα–295, pTIIα–197, pTIIα–154, or pTIIα–74, but did not increase luciferase activity in cells transfected with pTIIα–20. The promoter sequence between nt −74 and −20, which contains ICE1, the GC box, and the HSE, thus appears to mediate transcriptional activation by heat shock.

Effects of Mutations in the topoIIα Gene Promoter on Heat Shock Sensitivity—The roles of ICE1, the GC box, and the HSE
in heat induction of topoIIα gene promoter activity were investigated in T24 cells transiently transfected with luciferase reporter plasmids containing promoter sequences with specific mutations in these elements: GGATTTGGCT in ICE1 was converted to GGAAAAACT (pTIIα−295m6), GGGCGGG in the GC box to AAAAAAG (pTIIα−295m6), and GGAAGGTTCAAGTG in the HSE to GAAAGGAAAAAATG (pTIIα−295m7) (Fig. 5A). The pTIIα−295m5 construct showed increased basal transcriptional activity, but luciferase activity was not increased further by heat shock (Fig. 5B). In contrast, heat shock increased the transcriptional activities of pTIIα−295m6 and pTIIα−295m7 ~3-fold; the transcriptional activities of these two plasmids were ~30 and 10%, respectively, of that of the wild-type plasmid. Thus, a factor that binds to ICE1 might negatively regulate basal promoter activity, and ICE1 appears to play a key role in heat-induced activation of the topoIIα gene promoter. Whereas the GC box and HSE appear to contribute to basal promoter activity, they do not appear to be directly responsible for heat-induced promoter activation.

EMSA Analysis—We next investigated the effects of heat shock on the ICE (Y-box) binding proteins and HSFs with the use of EMSAs. A marked decrease in Y-box binding activity was apparent 3, 6, 12, and 24 h after heat shock (Fig. 6A). Formation of the complex was inhibited in the presence of either excess unlabeled oligonucleotide.

EMSA performed with a typical HSE derived from the human HSP70.1 gene revealed the absence of a retarded signal in untreated cells (Fig. 6B). A retarded complex was detected with nuclear extracts of cells prepared immediately (0 h) after heat treatment; formation of this complex was inhibited in the presence of excess unlabeled oligonucleotide, and the complex was “supershifted” in the presence of antibodies to HSF1, but not in the presence of antibodies to HSF2. With the HSE of the topoIIα gene as probe, a retarded complex was observed with nuclear extracts prepared from untreated cells and from cells after heat shock (Fig. 6C). However, the amount of this retarded complex was not affected by heat stress. Formation of this complex was inhibited by excess unlabeled oligonucleotide, but was not affected by antibodies to HSF1 or HSF2.

In Vivo Genomic Footprint Analysis—We examined the effects of heat shock on the dimethyl sulfate methylation patterns in the promoter region of the topoIIα gene by in vivo genomic footprint analysis. Both G−64 and G−65 in ICE1 were protected in untreated cells, but protection was markedly reduced 3, 6, and 24 h after heat shock (Fig. 7). Methylation patterns of the GC box, HSE, and other elements in the topoIIα promoter region (nt −295 to +85) were not substantially affected by heat shock stress (data not shown).

DISCUSSION

We have previously shown that expression of the topoIIα gene is increased 6–24 h after exposure of human head and neck or colorectal cancer cells to heat shock stress (22, 23). In the present study, we have shown that heat stress also induced activation of topoIIα gene expression in human urinary bladder cancer cells. This heat-induced up-regulation of topoIIα gene expression appeared to be mediated through an ICE or Y-box located between nt −74 and −21 on the basis of the following results. (i) The luciferase activity of T24 cells transfected with reporter constructs containing pTIIα−295, pTIIα−197, pTIIα−154, or pTIIα−74 was increased ~3-fold by heat shock
stress, whereas that of cells transfected with a construct containing pTIIa–20 was not increased by heat treatment. (ii) Introduction of mutations into ICE1 of the topoIIa gene promoter virtually eliminated the heat shock-induced increase in transcriptional activity, whereas mutation of the GC box or HSE had no such effect. (iii) EMSA analysis with nuclear extracts revealed a marked decrease in ICE1-binding activity 3–24 h after heat shock, consistent with the time course of the heat shock-induced increase in promoter activity, whereas HSE-binding activity was not affected by heat stress. (iv) In vivo genomic footprint analysis revealed a specific change in the methylation pattern of ICE1 induced by heat shock stress.

Members of the ICE-binding (YB-1) family of proteins are expressed in a wide range of cell types and function as important regulators of growth-associated and other genes (31–34). The expression of genes encoding the epidermal growth factor receptor (35), proliferating cell nuclear antigen (36), DNA polymerase α (37), and thymidine kinase (38) is regulated in a positive manner by ICEs. In contrast, such elements mediate down-regulation of the expression of genes encoding serum albumin, estrogen-dependent very low density lipoprotein apo-

lipoprotein II, aldolase B, and class II major histocompatibility complex (39–41). In the present study, deletion or mutation of ICE1 in the promoter region between nt 295 and pTIIa–197. Further deletion of nt 154 to −75, containing ICE2, and of nt −74 to −21, containing ICE1, reduced basal promoter activity to ~10 and 2%, respectively, of that apparent with pTIIa–295. Consecutive deletion of the five ICEs from the topoIIa gene promoter was also previously shown to reduce basal promoter activity in a stepwise manner (11, 18). Thus, the ICEs in the promoter of the human topoIIa gene appear to play an important role in basal transcriptional activity.

Introduction of point mutations into ICE1 of the topoIIa gene promoter alleviated the inhibition of topoIIa gene expression by wild-type p53 (18). Fraser et al. (42) showed that the topoIIa gene promoter is activated at an early stage during monocytic differentiation of human leukemia cells induced by phorbol ester or sodium butyrate and that this sodium butyrate-de-

pendent up-regulation of topoIIa gene expression is mediated by the promoter region between nt −90 and +90, which contains ICE1. In contrast, inhibition of topoIIa gene promoter activity in confluence-arrested cells appears to be mediated through interaction of the CCAAT-binding factor CBF/NF-Y with ICE2 (43).

In the present study, deletion or mutation of ICE1 in the topoIIa gene promoter prevented the heat shock-induced increase in transcriptional activity. Moreover, both EMSA and in vivo genomic footprint analysis indicated that nuclear ICE1-binding activity was decreased after heat shock stress. These observations indicate that ICE1 negatively regulates the human topoIIa gene and that heat shock stress reverses this effect, possibly by inducing the dissociation of negative regulatory factors from ICE1. The Y-box binding protein YB-1 has been shown to inhibit interferon-γ-induced activation of class II major histocompatibility complex genes (41). In contrast, activation of the human MDR1 gene in response to heat shock, DNA-damaging anticancer agents, or ultraviolet light is mediated by interaction of a Y-box binding protein with an ICE in the promoter of this gene (29, 44–47). Expression of YB-1 is also increased in response to genotoxic stress, suggesting that the promoter of the YB-1 gene itself is also sensitive to cytotoxic environmental stimuli (32, 48). ICEs thus appear to mediate either negative or positive regulation of specific genes in response to exogenous stimuli. Brandt et al. (21) recently showed that c-Myb activated the human topoIIa gene promoter via a Myb-binding site at nt −16 to −11 in human leukemia cells. In the present study, the basal promoter activity of pTIIa–20 was only 1.6% of that of pTIIa–295, and heat shock did not increase the transcriptional activity of this construct. It is thus unlikely that the Myb-binding site at −16 to −11 plays an important role in the heat activation of promoter activity of the topoIIa gene.

Heat shock induces the expression of heat shock-related genes in mammalian cells, and this activation is mediated by HSFs (49–53). HSFs bind to HSEs, which consist of contiguous arrays of the pentanucleotide motif 5′-NGAAN-3′ present in

FIG. 6. EMSA analysis of the effects of heat shock on the binding activity of proteins that interact with the topoIIa gene promoter. A, EMSAs were performed with 32P-labeled topo-ICE1 oligonucleotide as probe, and nuclear extracts were prepared from untreated control (C) cells or from heat-treated (43 °C for 1 h) cells after incubation for the indicated times at 37 °C. The effect of a 100-fold excess of unlabeled topo-ICE1 oligonucleotide as a competitor (Competitor) is shown. Arrowheads indicate specific retarded complex (S), nonspecific complex (NS), and free labeled probe (Free). B, EMSAs were performed with 32P-labeled HSP70-HSE oligonucleotide as probe, and nuclear extracts were prepared from untreated control cells or from heat-treated cells after incubation for the indicated times at 37 °C. The effects of a 100-fold excess of unlabeled HSP70-HSE oligonucleotide (Competitor) and of antibodies to HSF1 (anti-HSF1) or HSF2 (anti-HSF2) are shown. Arrowheads indicate specific complexes (S), specific supershifted complex (SS), nonspecific complex (NS), and free probe (Free). C, EMSAs were performed with 32P-labeled topo-HSE oligonucleotide as probe, and nuclear extracts were prepared from control cells or from heat-treated cells after incubation for the indicated times at 37 °C. The effects of a 100-fold excess of unlabeled topo-HSE (Competitor) and of antibodies to HSF1 (anti-HSF1) or HSF2 (anti-HSF2) are shown. Arrowheads indicate specific complexes (S), nonspecific complex (NS), and free probe (Free).
alternating orientations in the promoter regions of heat shock genes. Most heat-inducible genes, including Hsp genes, contain an HSE consisting of four or more pentanucleotide motifs and respond to heat treatment within 1 h concomitant with marked fluctuations in nuclear HSF content (27, 30, 54, 55). Our data confirm that HSF1, but not HSF2, binds to the HSE of the human Hsp70 gene immediately after heat shock. However, the HSE of the topoII gene consists of only two pentanucleotide motifs, and heat shock-induced transcriptional activation of the topoII gene was not apparent until 6–24 h after heat treatment. Furthermore, no increase in the binding of nuclear factors to the HSE of the topoII gene after heat treatment was apparent by EMSA or in vivo footprint analysis. It is thus unlikely that the HSE in the topoII gene promoter is responsible for the heat-induced activation of this gene.

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