Transcriptional Control of the Human Thromboxane Synthase Gene in Vivo and in Vitro*

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Thromboxane A₂, a potent mediator of vasoconstriction and platelet aggregation, is synthesized from prostaglandin H₂ by thromboxane synthase (TXAS). We report here on promoter analyses of human TXAS using in vitro transcription and in vivo transfection methods. The 39-bp core promoter, containing both TATA and initiator elements, accurately initiates transcription in an orientation-dependent manner in a cell-free transcription system. Mutation of either TATA or initiator abolished transcriptional activity, but the upstream sequence had no effect on TXAS promoter activities in vitro suggesting that the core promoter is sufficient for transcriptional activity from a naked DNA template. In contrast, mutation of both elements drastically decreased the promoter activity in vivo. Furthermore, TXAS proximal promoter containing the nucleotides −90 to −56 relative to the transcriptional start site was necessary for promoter transactivation in vitro. Transcriptional activities from 5′-deletion mutants indicated that the effects of the nucleotides −90/−56 were more pronounced in stably transfected cells (a 150-fold difference) than in the transiently transfected cells (an 8-fold difference), reflecting the effects of TXAS transcriptional activation from replicating and nonreplicating DNA templates. Partial micrococcal nuclease digestion indicated that the sequence −90/−56, where the NF-E2 site is located, is associated with alterations of nucleosomal structure at the regions of promoter and reporter gene but not the region further downstream. Mutagenesis and forced expression studies demonstrated a critical role of p45 NF-E2 in controlling TXAS expression under native chromatin conditions. Band shifting and chromatin immunoprecipitation assays indicated that p45 NF-E2 was bound to the TXAS promoter in vitro and in vivo. Indirect end labeling and ligation-mediated PCR analyses further demonstrated that the occupation of TXAS promoter NF-E2 site was associated with disruption of nucleosomal structure. Collectively, these results indicate that binding of NF-E2 is critical both for alteration of the nucleosomal structure and for activation of the TXAS promoter, whereas the TATA and initiator elements are essential for transcription.

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†† The abbreviations used are: TXAS, thromboxane synthase; Inr, initiator; HEL, human erythroleukemia cell; MNase, micrococcal nuclease; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; LM-PCR, ligation-mediated polymerase chain reaction.
ing and is unlikely to be assembled into organized nucleosomes (Ref. 32 and references therein). Stably transduced DNA templates, however, represent replicating chromatin and are assembled into repeated arrays of nucleosomes. Considering that transcriptional activities may depend on the template state, we examined TXAS promoter activities from naked DNA, nonreplicating chromatin, and replicating chromatin templates. Our results led us to propose a model in which NF-E2 induces nucleosomal alteration in the vicinity of the TXAS promoter, thereby allowing the basic transcriptional machinery to bind to the core promoter.

**EXPERIMENTAL PROCEDURES**

**Materials**—The G-f free cassette vectors, pC AT and pMLC AT190, were kindly provided by Drs. Michele Sawadogo and Ming-Jer Tsai (33, 34). Luciferase reporter vectors, pGL3-Basic (promoterless), pGL3-Control (luciferase reporter gene driven by SV40 promoter/enhancer), and the luciferase assay kit were from Promega (Madison, WI). The β-galactosidase reporter vector pCMV-β-Gal (β-galactosidase reporter gene driven by cytomegalovirus immediate early promoter/enhancer) and the luminescent β-galactosidase assay kits were from CLONTECH (Palo Alto, CA). p220.2, an Epstein-Barr virus containing oriP and the human IgG1 constant region gene, was a generous gift from Dr. Bill Sugden. pCMV-β-Gal reporter reagent, PLUS reagent, OptiMEM, pGL3-TXAS reporter vector DNA, and 1 μg of pcMV-β-Gal in 2 ml of OptiMEM. For HeLa cells, 1 × 10^6 cells were transfected by 0.5 ml of OptiMEM containing 2 μg of pGL3-TXAS reporter vector DNA, 1 μg of pcMV-β-Gal, 20 μg of Lipofectin reagent, and 10 μl of PLUS reagent after washing with serum-free medium. The media were changed into the medium containing 10% fetal bovine serum for HeLa cells. After a 48-h incubation without changing the medium, the cells were harvested and lysed in reporter lysis buffer (Promega). Luciferase assays were performed with 20 μl of lysate and 100 μl of luciferase assay reagent using a MiniLumat Luminometer LB9506 (Berthold, Wildbad, Germany). β-Galactosidase activities were determined using a chemiluminescence assay kit.

**Stable Transfection**—Stable transfection of HEL cells (1 × 10^6 cells in 2 ml) and HeLa cells (1 × 10^6 cells in 2 ml) was carried out using 6 μg of pGL466 and 2 μg of the minichromosome DNA. One day after transfection, cells were subcultured into 20 wells of 96-well plate (HEL) or a 100-mm dish (HeLa) and selected with 400 μg/ml of hygromycin B. Several clones of each construct were obtained. Copy numbers of each clone were determined by Southern blot analysis.

**Electrophoretic Mobility Shift Assay (EMSA)—** Nuclear extracts of HEL cells were prepared by the method described previously (37) and stored at −70 °C until use. EMSA was performed based on a previously described method (38). Briefly, 5 μg of nuclear extracts were incubated with a 2^10^−end-labeled probe (TXAS promoter nucleotides −121 to +5) in a binding reaction buffer containing 20 mM HEPS, pH 7.9, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 100 mM NaCl, 15 mM MgCl2, and 1 mM dithiothreitol for 20 min at room temperature. After supershift assay, nuclear extracts were incubated with antibodies for 30 min before the labeled probe was added. The mixtures were electrophoresed in a 4.5% polyacrylamide gel and autoradiographed overnight at −70 °C.

**Chromatin Immunoprecipitation (ChIP) Assay—**—ChIP and duplex PCR were carried out as previously described (39). HEL cells (2.4 × 10^6) maintaining minichromosome pMC−280/137 were fixed with 1% formaldehyde for 10 min. Formaldehyde was neutralized by incubation with 125 mM glycine for 10 min and then washed twice with ice-cold PBS. Cells were lysed for 5 min on ice in 500 μl of buffer containing 50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol supplemented with protease inhibitors. Nuclei were centrifuged at 3000 rpm for 3 min and reuspended in 200 μl of 50 mM Tris, pH 8.0, 5 mM EDTA, 1% SDS, and then sonicated five times for 5 s each time. The nuclear preparations were cleared by centrifugation and diluted 10 times in 50 mM Tris, pH 8.0, 0.5 mM EDTA, 0.5% Nonidet P-40, 0.2% NaCl. Extracts were precleared for 2 h with 60 μl of a 50% suspension of protein A-Sepharose 4B beads (Sigma), 40 μg of sonicated salmon sperm DNA, and 80 μg of bovine serum albumin. An aliquot of precleared chromatin was removed, heat-treated to reverse protein-DNA interaction, and precipitated with isopropl alcohol (referred to as input) and used in the subsequent duplex PCR analysis. The remainder of chromatin sample was then separated into two tubes for different antibody treatments. Immunoprecipitations were carried out at 4 °C for 6 h by adding 10 μl of p65 NFR-B antibody or irrelevant rabbit serum with 50 μl of 50% protein A-Sepharose 4B beads with salmon sperm DNA. The beads were washed five times with 1 ml of wash buffer (20 mM Tris, pH 8.0, 0.1% SDS, 2 mM EDTA, 1% Nonidet P-40, 150 mM NaCl) and three times with 10 mM Tris, pH 8.0, 1 mM EDTA. Protein-DNA complexes were eluted by three successive treatments with 150 μl of 1% SDS, 100 mM NaHCO3. NaCl was then adjusted to 0.5 M, and 1 μl of RNase A (10 mg/ml) was added. Protein-DNA complexes were eluted by three successive treatments with 150 μl of 1% SDS, 100 mM NaHCO3. NaCl was then adjusted to 0.5 M, and 1 μl of RNase A (10 mg/ml) was added.
DNA cross-links were reversed by heating at 65 °C overnight, and DNA was extracted by adding 20 μl of 1× Tris, pH 7.5, 10 μl of 0.5× EDTA, and 2 μl of proteinase K (20 mg/ml) at 50 °C for 30 min. After phenol/chloroform extraction and isopropyl alcohol co-precipitation with 30 μg of glycogen, the pellet was resuspended in 30 μl of H2O. For the duplex PCR, 2 μl of ChiP DNA was used as template in a total volume of 10 μl of PCR buffer containing 0.5 μM each of the primer pair for TXAS promoter (corresponding to the promoter sequences –90/–72 and +115/+137) and 0.5 μM each of the primer pair for the TXAS 3′-untranslated region (corresponding to the cDNA sequences 1581/1600 and 1901/1920 downstream from the translational start site), 7.5 μCi of [α-32P]dCTP, 0.2 mM dNTP and Taq polymerase. After amplification, PCR products were separated by a 4.5% polyacrylamide gel before autoradiography.

*Chromatin-DNA Cross-linking, Preparation of Nuclei, and MNase-coupled Southern Blot Analysis—Nuclei from HEL cell clones were prepared according to a modification of published procedures (30). Briefly, 3 × 10^7 cells were washed with phosphate-buffered saline, and chromatin-DNA was cross-linked by incubation of cells with 1% formaldehyde at room temperature for 20 min (40) before being washed twice with ice-cold phosphate-buffered saline and resuspended in 6 ml of suspension buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 15 mM NaCl, 50 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine) containing 0.2% Nonidet P-40 and 5% sucrose. After a 5-min incubation on ice, the suspension was centrifuged (15 min, 150 °C) through a 3.5-ml cushion of suspension buffer containing 10% sucrose. Nuclei were gently resuspended in 3 ml of suspension buffer containing 5.5% sucrose, and aliquots of 1 ml were digested with 10, 50, or 150 units of MNase in the presence of 1 mM CaCl2 for 7 min at room temperature. The reactions were stopped by adding stop buffer at final concentrations of 0.5 mM NaCl, 10 mM EDTA, 1% SDS, and 0.1 mg/ml proteinase K and then incubated at 37 °C overnight. The cross-linking of DNA-protein was reversed by heating at 60 °C for 5 hr (41). The DNA was purified by multiple phenol/chloroform extractions, analyzed by gel electrophoresis, and subjected to Southern blot analysis using probes that were localized by random primers.

For indirect end-label analysis, the procedures for sample preparation including nuclei preparation, chromatin-DNA cross-linking, MNase digestion, and DNA isolation, were the same as described above except that 0, 10, and 30 units of MNase were used. Purified DNAs were digested with XbaI and BclI, separated by gel electrophoresis after phenol/chloroform extraction, and analyzed by Southern blotting using the 5′-end of the luciferase cDNA (cut with HindIII and BclI from the pGL3-basic) as the probe.

*MNase-coupled Ligation-mediated Polymerase Chain Reaction (LM-PCR) Analysis—MNase-treated nuclei were prepared, and total DNAs were isolated as described above. LM-PCR was carried out following a standard method (36). Briefly, the DNA sample (5 μg) was phosphorylated with polynucleotide kinase (New England Biolabs) in the presence of 1 mM ATP and ligated to the unidirectional linker (prepared by annealing the two oligonucleotides, linker 1 (5′-GCGGTGACCCGG-GAGATCTGGAATTT-3′) and linker 2 (5′-GAATTCAGATC-3′)) with T4 DNA ligase (Invitrogen). The linker-ligated DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA samples (0.3–0.9 μg; based on the copy number of minichromosomes) were used as templates for 22 cycles of PCR amplification. The PCR mixture (40 μl) contained 0.4 μl of Taq DNA polymerase, 0.4 μl of 10× Expand HF PCR buffer with 15 mM MgCl2 (Roche Molecular Biochemicals), 0.8 μl of 10 mM dNTP, and 1.6 μl each of 10 μM linker 1 oligonucleotide and TXAS-specific primer P1. The reaction was carried out with the following conditions: first cycle, denaturation for 5 min at 95 °C, with the remaining cycles for 1 min at 95 °C, 2 min at optimal annealing temperature (54–56 °C, empirically determined for each primer set), 5 min at 72 °C, and an additional 5-min extension at 72 °C. The PCR product (5 μl) was added to 15 μl of a fresh PCR mixture containing 0.1 μl of Taq DNA polymerase, 1.5 μl of 10× Expand HF PCR buffer with 15 mM MgCl2, 0.4 μl of 10 mM dNTP solution, and 1.0 μl of 1 μM 32P-5′-end labeled TXAS-specific primer P2. The second amplification was carried out for 11–18 cycles (empirically determined for each primer set) of linear PCR amplification consisting of denaturation for 5 min at 95 °C for the first cycle, with the remaining cycles for 1 min at 95 °C, 2 min at optimal annealing temperature (56–58 °C, empirically determined for each primer set), 5 min at 72 °C, and an additional 5-min extension at 72 °C. After the second amplification, the reaction products were separated on a 6% polyacrylamide, 7 M urea-sequencing gel. The sequence of the TXAS-specific oligonucleotides was as follows: 5′ side promoter primers, P1 (5′-TCCTCTGAGACCGAGGTTG-3′) and P2 (5′-GATCTCGGCGCCCTTATGGGAGG-3′); 3′ side promoter primers, P1 (5′-GTCATGCTGGTTTCAACC-3′) and P2 (5′-GTCTATCTACAAACATGGGGGAAG-3′). The locations of the primers in the TXAS promoter are shown in Fig. 1.
RESULTS

In Vitro Transcription of the Human TXAS Gene Promoter—In initial experiments, we used the core promoter (nucleotides −36 and +3) of the TXAS gene linked to a 377-bp G-free cassette vector, pC2AT, as a template (pC 2AT-TX36(+)) to examine TXAS gene expression. Transcription of this template was carried out in HeLa nuclear extracts as described under “Experimental Procedures.” The test template yielded a 380-nucleotide transcript (Fig. 2 A, lane 1), consistent with the predicted length of transcript accurately initiated from the TXAS transcriptional start site. The promoter sequence from the adenovirus major late gene (−400 to +10) inserted into a 180-bp G-free pC2AT vector (pMLC2AT190) was used as control template and resulted in correct initiation and a 190-nucleotide transcript as well as “read-through” transcripts giving a 200-nucleotide transcript after digestion by the G-specific ribonuclease T1 (Fig. 2 A, lane 2). The reverse orientation of the TXAS core promoter inserted into the pC 2AT vector (pC 2AT-TX36(−)) showed no promoter activity above that for the internal promoter template pMLC2AT190 (Fig. 2 A, lane 3).

We conclude that the 39 bp of TXAS core promoter is sufficient to activate correct initiation of transcription in a direction-dependent manner in the cell-free system.

To optimize concentrations of nuclear extract, templates, KCl, and MgCl₂ for the TXAS in vitro transcription, we carried out assays at different final concentrations of these components. Increasing the amount of nuclear extract from 1 to 4 μl (the protein concentration was ~12 mg/ml) gave a linear increase in correctly initiated transcripts from both pC2AT-TX36(+) and pMLC2AT190 templates (data not shown).
thus used 3 μl of HeLa nuclear extract for the subsequent studies. Template levels of 1.0 μg for pC2AT-TX36(+) and 0.5 μg for pMLC2AT190, a KCl concentration of 45 mM, and a MgCl2 level of 3 mM were also found to maximize the in vitro transcription activities (data not shown). These conditions are similar to those used for analyzing other native and artificial promoters (42, 43).

Effects of TXAS Gene Upstream Promoter Sequence on in Vitro Transcription—To test whether the upstream region is important in cell-free transcription, we linked TXAS promoter nucleotides between 248 and 3 to the 377-bp G-free cassette vector (pC2AT-TX248(+)) and compared its promoter strength with that of pC2AT-TX36(+). Both pC2AT-TX36(+) and pC2AT-TX248(+) showed strong signal intensity at the position of the expected 380-nucleotide transcript, with no significant difference between the two (Fig. 2B, lanes 3 and 4). In fact, the signal from pC2AT-TX36(+) was 30-fold higher than that from pC2AT-TX248(+) when normalized to signals from pMLC2AT190. As negative controls, constructs with reversed core promoter (pC2AT-TX36(−)) and upstream promoter (pC2AT-TX248(−)) were also examined. Both constructs produced very weak transcriptional activities (Fig. 2B, lanes 1 and 2). Because HeLa cells do not express TXAS (44), nuclear extracts of HEL cells (which constitutively express TXAS) were added to the HeLa nuclear extract. However, supplementation with HEL nuclear extract did not enhance the transcriptional activity of pC2AT-TX248(+) (data not shown). These results suggest that the core promoter of the TXAS gene, introducing as a naked DNA template in the cell-free system, is sufficient for transcription, and the upstream region is not critical for transcription.

Role of TATA Box and Initiator of TXAS Promoter in the in Vitro and in Vivo Transcription—We further examined the functional elements of TXAS core promoter that elicit transcriptional activity in vitro. Two putative elements, the TATA box and Inr, are present at nucleotides 29/−26 and −3/+3, respectively (Fig. 1). The sequence of the pyrimidine-rich TXAS Inr, CACA^TT, closely resembles a weak Inr consensus sequence, PyPyA^N(T/A)PyPy (A is the transcriptional start site and Py is pyrimidine) (45), and is similar to the adenovirus major late Inr (CTCACT) and the ß-globin Inr (CTTACA) (42). We first introduced single mutations in either the TATA box or Inr and a double mutation in pC2AT-TX248(+). As shown in Fig. 3A, a single mutation in either the TATA box (lane 2) or Inr (lane 3) and the double mutation (lane 4) each dramat-

![Diagram](http://www.jbc.org/)

**Fig. 4.** Transcriptional analyses of TXAS promoter in vivo. A, deletion analysis of TXAS promoter by transient transfection in MEG-01 cells. The diagram on the left illustrates the constructs, and numbers indicate the positions of the start and end points of the TXAS promoter fragments. The diagram on the right shows the promoter activities of each construct. B, promoter activity in stably transfected HEL cells harboring minichromosomes with various 5′-deletions of the TXAS gene. A schematic illustration of the constructs is on the left, and promoter activities monitored by luciferase activity are shown on the right (means ± S.D.). The promoter activity of the longest construct is assigned as 100%.
ically reduced the TXAS transcriptional activity, indicating that these elements are functional and crucial in directing the transcriptional activity in vitro. In a separate experiment, double-stranded oligonucleotide composed of wild-type TATA and the Inr region (−36/+3) was used as a competitor in the in vitro transcription assay. However, no competition was observed at
a 500-fold molar excess of competitor over the template pC2AT-TX36 (+) (data not shown). We suspect that the oligonucleotide competitor is too short for the basic transcription machinery to bind and thereby fails to compete with the template plasmid.

We then tested whether the TATA and Inr elements were also functional in vivo. As described below, the TXAS core promoter alone yielded very low transcriptional activity in vivo. We thus included the upstream promoter region and linked the nucleotides between −248 and +3 into the pGL3-Basic luciferase reporter vector as the wild-type construct. Mutations were then made in this construct at either TATA and/or Inr sites. These reporter vectors were transiently co-transfected into MEG-01 cells with pCMV-β-Gal serving as an internal control. In addition, we used pGL3-Control as a positive control and the promoterless pGL3-Basic as a negative control. Promoter activities were normalized to those with the pGL3-Control and promoter activities of TXAS gene promoter were found to grade-

**A**

1. **Figure 6.** Effect of NF-E2 on the TXAS promoter in the stably transfected cells. *A*, promoter activities of TXAS gene from HEL cells carrying wild type and NF-E2 mutated minichromosomes were stably transfected in HEL cells. Promoter activities were determined by normalization of luciferase activity, protein concentration, and copy number of minichromosome. Data represent means ± S.D. of the relative luciferase activities, as compared with that obtained from the wild type. *B*, stimulation of TXAS promoter activity in stably transfected HeLa cells by forced expression of p45 NF-E2. HeLa cells carrying wild type and NF-E2 mutated minichromosomes were transiently transfected with (+) or empty (−) p45 NF-E2 expression vector (pCMV-NF-E2). Data represent means ± S.D. of the relative luciferase activities, as compared with that obtained from the wild type transfected with p45 NF-E2 vector.

In Vivo Transcription of the TXAS Gene Promoter by Transient Transfection—To further dissect the cis-elements involved in TXAS expression, we made 5′- and 3′-deletion constructs of the TXAS promoter and linked them to the pGL3-Basic vector. The parent construct in this study (pGL3−280/+137) contained nucleotides between −280 and +137, which includes two putative GATA sites near nucleotide −250 (Fig. 1). For the 5′-deletion mutation analysis, DNA fragments containing nucleotides −190/+137, −140/+137, −90/+137, −56/+137, and −38/+137 were generated by PCR and linked to pGL3-Basic. These reporter constructs were transiently co-transfected into MEG-01 cells along with the pCMV-β-Gal plasmid. As shown in Fig. 4A, the promoter activity of pGL3−280/+137 was potent and was about 125% of that of pGL3-Control. Deletion of nucleotides between −280 and −90 did not much affect the promoter activity, since the pGL3−190/+137, −140/+137, and −90/+137 constructs showed similar levels of activity. However, the removal of nucleotides between −90 and −56 in pGL3−56/+137 reduced the promoter activity by 90% as compared with that of pGL3−280/+137, indicating the presence of important elements in this segment.

Many promoters contain functionally important sequences in the 5′-untranslated region, sequences that are referred to as downstream promoter elements (46, 47). Any such downstream promoter elements of TXAS cannot be studied by our in vitro transcriptional assay using the G-free cassette. To further examine the role of the 5′-untranslated region, a series of 3′-deletion mutants of TXAS promoter construct were made in the +1/+137 segment. Luciferase reporter vectors containing DNA fragments corresponding to TXAS sequences −280/+137, −280/+90, −280/+75, −280/+52, and −280/+3 (referred to as pGL3−280/+137, pGL3−280/+90, and so forth) were generated. A mutation at the putative Sp1 site near +80, the only recognizable site in the 5′-untranslated region, was also made (GGGCGGG to GAAAGGG) in the pGL3−280/+137 construct. These vectors were transiently co-transfected into MEG-01 cells with pCMV-β-Gal. Promoter activities were found to grad-
nuclear extracts and end-labeled TXAS promoter nucleotides. Antibodies against Nrf1, Nrf2, and p45 NF-E2 were incubated with nuclear extracts before the addition of the probe. The reaction mixtures were then separated by electrophoresis. The positions of NF-E2 DNA and H11032, and the other pair amplifies -untranslated region (3'UTR) described under "Experimental Procedures." One primer pair amplifies the TXAS 5'-untranslated region (3'-UTR), and the other pair amplifies the promoter region (Promoter). PCR templates from input DNA and ChIP DNAs treated with anti-p45 NF-E2, irrelevant serum, and no chromatin are indicated.

In vivo Transcriptional Studies of the TXAS Promoter Using Chromosomal Reporter Constructs—The transient transfection experiments indicated that the TXAS sequence between nucleotides −90 and −56 was important for transcriptional activity (Fig. 4A), but in vitro assay implied that this region was not critical for promoter activity (Fig. 2B). We suspected that the discrepancy was a result of differences in the template state; one is a naked DNA and the other an uncharacterized, nonreplicating nucleosomal DNA. To investigate the role of natural chromatin structure in TXAS transcription, we inserted various sizes of TXAS promoter/luciferase reporter constructs into a minichromosome containing the Epstein-Barr virus origin for self-replication. Initial attempts to develop MEG-01 cell lines harboring TXAS promoter/reporter constructs were unsuccessful. We subsequently transfected HEL cells, which constitutively express TXAS, with a series of 5'-deletion mutations corresponding to nucleotides −280/137, −190/137, −90/137, and −56/137 of the TXAS gene (designated as pMC−280/137, −190/137, −90/137, and −56/137, respectively). These episomal minichromosomes were stably maintained in HEL cells after selection by hygromycin B for 3–4 weeks. Quantitation of the expression levels of the minichromosomal reporter gene controlled by these TXAS promoter fragments revealed that cis-elements between −280/−190 and −90/−56 are important for transcription (Fig. 4B). These results are consistent with those obtained from transient transfection experiments except that the effects exerted by these elements are more pronounced in the stably transfected cells, more than a 150-fold difference between −90/137 and −56/137 in the stably transfected cells (Fig. 4B) as compared with an 8-fold difference between the corresponding constructs in the transient transfection (Fig. 4A). The results indicate that cis-element(s) between −90 and −56 is important in regulating TXAS promoter activity in the native chromosomal structure context.

MNase-coupled Southern Blot Analyses of TXAS Promoter/Reporter Minichromosomes—To investigate how the transcriptional activities of pMC-TXAS promoter constructs were affected by changes in nucleosomal structures, we chose the two clones carrying pMC−90/137 or pMC−56/137 for MNase-coupled Southern blot analysis, because they exhibited the largest difference in transcriptional activities (Fig. 4B). Partial digestion of chromatin by MNase, which cleaves linker DNA between nucleosome cores, produces an array of DNA fragments corresponding to various multiples of the length of nucleosomal DNA monomer. Ethidium bromide-stained total nuclear DNA digested by different concentrations of MNase showed the expected laddering pattern for bulk DNA (Fig. 5B). The positions of probes used for structural analysis in the TXAS and downstream regions of the minichromosomes are indicated in Fig. 5A. When Southern blotting was performed using TXAS promoter nucleotides −90/137 as a probe, a ladder of fragments with a repeat of about 150 bp indicated that this part of the minichromosome of pMC−56/137 was assembled into nucleosomes (Fig. 5C). On the other hand, a less distinct ladder and poorer hybridization were observed for the same region of the pMC−90/137 minichromosome, particularly at the highest MNase concentration. The results indicated that the promoter region of pMC−90/137 was less compact and protected than that of pMC−56/137. We next examined the nucleosomal structure of luciferase gene by hybridizing the same membrane with a probe containing a segment of the luciferase gene. As shown in Fig. 5D, the luciferase segment in pMC−56/137 adopts a nucleosomal structure, and its MNase digestion pattern is very similar to that observed for the TXAS promoter region (cf. Fig. 5, C and D). However, the luciferase
FIG. 8. Nucleosome structure analysis of TXAS promoter by MNase-coupled LM-PCR. Nuclei from HEL cells carrying stably transfected minichromosomes were digested by 30 or 60 units/ml MNase as indicated by the wedges. A, LM-PCR analysis using 3'-P1 and 3'-P2 primer set from the nuclei stably transfected with pMC-90/+137 or pMC-56/+137. The thick vertical line and thin line indicate TXAS promoter and vector DNA, respectively, and X indicates the NF-E2 site. The positions of MNase cleavage sites are shown by the arrows. The oval at the right and the dotted oval at the left represent nucleosome 1 (N1) and remodeled nucleosome N1, respectively. L1 and L2 indicate linker regions and are bracketed. B, LM-PCR analysis using the 3'-P1 and 3'-P2 primer set from nuclei of cells stably transfected with pMC-280/+137 (wild type) and pMC-280/+137-mNF-E2 (mutant). The bent arrow indicates the transcription start site. C, LM-PCR analysis 5'-P1 and 5'-P2 primer set from nuclei of cells stably transfected with pMC-280/+137 (wild type) and pMC-280/+137-mNF-E2 (mutant). N, naked DNA; M, HpaII-digested pBR322 size marker.
gene region in pMC−90/+137 reveals less nucleosomal structure (Fig. 5C), similar to that seen in the promoter region (Fig. 5F), again suggesting a disrupted nucleosomal structure. Finally, the DNA region downstream from the luciferase gene was examined by rehybridizing the same membrane with a probe of p220.2 DNA fragment. Both pMC−90/+137 and pMC−56/+137 showed a similar nucleosomal ladder in this downstream region (Fig. 5F). It should be emphasized that mononucleosomes can be clearly seen in both pMC−90/+137 and pMC−56/+137, suggesting that the region downstream from the reporter gene is well protected in both minichromosomes.Taken together, these results indicate that the disruption of nucleosomes occurs at the promoter and luciferase gene region in the minichromosome and TXAS is also actively transcribed when nucleotides −90/−56 are present. In contrast, the minichromosome maintains its intact nucleosomal structure and loses its promoter activity when the −90/−56 is deleted. These results suggest that the cis-element(s) located in the −90/−56 region of the TXAS promoter is involved, at least in part, in the disruption of nucleosomes.

Role of NF-E2 Site in TXAS Gene Transcription in a Native Chromatin Context—In transient transfection experiments, the −300/−40 TXAS upstream promoter sequence, particularly at the NF-E2 site between −83 and −77, is crucial for transcriptional activity in several types of cells (15, 17, 18, 20). To test whether NF-E2 activates the TXAS gene in a natural chromatin context, we generated a mutation at the NF-E2 site in the pMC−280/+137 minichromosome (pMC−280/+137-mNF-E2) and stably transfected it into HEL cells. Cells harboring the wild-type TXAS promoter exhibited 14-fold higher luciferase activity than those harboring mutant NF-E2 promoter (Fig. 6A), indicating that an intact NF-E2 site dramatically increases TXAS transcriptional activity under the native chromosomal conditions.

Next, we asked whether p45 NF-E2 could activate TXAS gene expression in HeLa cells, which do not express TXAS. We first carried out transient transfection experiments using the same series of 5′-deletion reporter plasmids described in the legend to Fig. 4A. As expected, the promoter activities of all constructs transfected into HeLa cells were very low, reaching at most only −2% of the pGL3-Control (data not shown). No significant change was observed when the −90/−56 region was deleted. We also compared the promoter activities of stably transfected pMC−280/+137 and pMC−280/+137-mNF-E2 in HeLa cells. Luciferase activities were again very low from both wild type and mutant constructs. However, transient transfection with a p45 NF-E2 expression vector strongly stimulated luciferase activity in the cells containing pMC−280/+137 (150-fold increase) as compared with those containing pMC−280/+137-mNF-E2 (20-fold increase) (Fig. 6B). These results clearly demonstrated an important role for the NF-E2 site in controlling the TXAS gene expression under native chromosomal conditions.

In Vitro and in Vivo Binding of p45 NF-E2 to the TXAS Promoter—To further demonstrate that p45 NF-E2 is involved in the binding of TXAS promoter, we carried out EMSA and ChIP assay. EMSA results indicated that the nuclear extracts from HEL cells contained factor(s) capable of binding DNA fragments corresponding to the TXAS promoter sequences −121/+5 (Fig. 7A). In a separate experiment, we performed reverse transcription-PCR experiments and found out that HEL cells expressed Nrf1, Nrf2, and Nrf3 as well as p45 NF-E2 (data not shown). Supershift analyses were therefore conducted with antibodies against p45 NF-E2, Nrf1, and Nrf2. As shown in Fig. 7A, a supershift of protein-DNA complex was observed with the addition of antibodies against p45 NF-E2 but not with Nrf1 or Nrf2 antibodies. We also carried out competition experiments using a 20-bp oligonucleotide encompassing the NF-E2 site. The shift band was completely diminished by the addition of a 10-fold molar excess of wild type oligonucleotide but was not affected by even a 100-fold molar excess of oligonucleotide with mutated NF-E2 (data not shown).

ChIP experiments were performed using the stably transfected HEL cells harboring −10 copies of pMC−280/+137. The enriched chromatin was cross-linked and sonicated to generate fragments averaging less than 500 bp. Antibodies specific for p45 NF-E2 were used to immunoprecipitate protein-DNA complexes, and a duplex PCR was carried out with one primer pair specific for the TXAS promoter sequence encompassing the NF-E2 site and a second pair specific for the TXAS 3′-untranslated region to generate the 227- and 340-bp PCR products, respectively. The 3′-untranslated region is 193 kb away from the TXAS promoter in the human chromosome 7 and does not contain the NF-E2 consensus sequence (15), thus serving as a negative control for NF-E2 binding. Duplex PCR assay was carried out under conditions of linear amplification for both products and results of 28 cycles of PCR were shown in Fig. 7B. Immunoprecipitation with anti-p45 NF-E2 antibody resulted in the recovery of considerably more promoter fragment than the irrelevant serum control or the control with no chromatin added to the protein-A-Sepharose beads. In contrast, the 3′-untranslated region, which lacks the NF-E2 binding site, was not detectable with anti-p45 NF-E2 antibody or control samples in the same experimental conditions. In comparison with input DNA sample, p45 NF-E2 binding to TXAS promoter was significantly enriched relative to an irrelevant locus, i.e. 3′-untranslated region. Taken together, these results demon-
strate that p45 NF-E2 binds TXAS promoter in vitro and in vivo.

Indirect End Label and MNase-coupled LM-PCR Analyses of TXAS Promoter/Reporter Minichromosomes—To examine the nucleosomal organization of the TXAS promoter encompassing the NF-E2 region in more detail, we carried out MNase-coupled LM-PCR using the minichromosomal constructs in stably transfected HEL cells. For the pMC–56/+137 minichromosome, MNase digestion revealed two prominent sites at nucleotide positions 130 and 280, indicating a 150-bp nucleosome core particle (Fig. 8A). A weaker band was also seen at nucleotide position 105, suggesting a 25-bp linker. MNase digestion of the pMC–90/+137 minichromosome, however, revealed several additional cleavage sites not observed with pMC–56/+137, namely at nucleotide positions 185, 180, and 147 (Fig. 8A). These results are consistent with the MNase-coupled Southern blot analysis in which intact nucleosomes were readily seen in the promoter of pMC–56/+137 but not in pMC–90/+137, indicating a different nucleosomal structure when nucleotides –90 to –56 were present.

To ascertain whether binding at the NF-E2 site was associated with the nucleosomal remodeling, we examined the nuclei harboring the pMC–280/+137 and pMC–280/+137-mNF-E2 minichromosomes by indirect end label analysis. The minichromosomes were partially digested with MNase, cleaved with XbaI and BclI to obtain the 1.03-kb parent fragment, and then subjected to Southern blot analysis using the 0.58-kb 5′-end luciferase gene as a probe (Fig. 9). MNase-sensitive sites were observed in the wild type minichromosome. The positions of MNase cleavage sites of the wild type minichromosome were mapped approximately between the size of 900 and 750 bp, corresponding to TXAS nucleotides –180 and –30. Mutation of NF-E2 site, however, resulted in loss of the MNase-hypersensitive sites (Fig. 9). We thus conclude that an intact NF-E2 site is associated with nucleosomal disruption.

To further map the MNase-hypersensitive sites in this region, MNase-coupled LM-PCR analysis using the nested primer set 3′-P1 and 3′-P2 (Fig. 1) was carried out. LM-PCR defined a nucleosome core particle for both wild type and mutant minichromosomes between nucleotide positions 130 to 280 and a linker at nucleotide positions 130 to 105 (Fig. 8B), which correspond to the TXAS promoter nucleotides –40 to –190 and –40 to –15, respectively. This position of the nucleosome particle identified by LM-PCR is consistent with that by indirect end label analysis (Fig. 9). In this nucleosome particle, MNase-hypersensitive sites at nucleotide positions 230, 184, and 172 (corresponding to TXAS promoter nucleotides –140, –98, –94, and –82, respectively) were found in pMC–280/+137 minichromosome but not in pMC–280/+137-mNF-E2 minichromosome or naked pMC–280/+137 (Fig. 8B). Furthermore, many MNase cleavage sites were observed near the transcriptional start site (nucleotide position 90 in Fig. 8B) of pMC–280/+137 and were absent in pMC–280/+137-mNF-E2. We subsequently examined the nucleosomal structure from the 5′-end of TXAS promoter using the nested primer set 5′-P1 and 5′-P2 (Fig. 1). As shown in Fig. 8C, MNase cleavage sites at nucleotide position 96 and the 68–74 region (corresponding to the TXAS promoter nucleotides –81 and the –103 to –108 region, respectively) were more pronounced in the wild type minichromosome than in the mutant. In contrast, two cleavage sites at nucleotide positions 152 and 135 were seen at similar levels of intensity for both wild type and mutant minichromosomes. These two sites corresponding to TXAS promoter nucleotides –25 and –42 define a linker region, in agreement with LM-PCR using the nested primer set 3′-P1 and 3′-P2. Moreover, these MNase cleavage sites were not found using the naked wild type minichromosome DNA (Fig. 8, B and C).

The results of LM-PCR thus place the NF-E2 site at one nucleosome particle (N1 in Fig. 8, A–C), TATA at a linker (L2), and Inr at another nucleosome particle (N2). When the NF-E2 site is occupied, both nucleosomes N1 and N2 are disrupted (Fig. 8, B and C). It should be noted that in Fig. 5C, significant nucleosomal changes at the promoter region were observed at 150 units of MNase but were less evident at 50 units of MNase. Because the MNase concentrations used in the LM-PCR and indirect end labeling assays were at the range of 10–60 units, gross nucleosomal changes were not seen, since the linker regions were still prominent (Fig. 8, A and B). However, the MNase-hypersensitive sites were clearly detected at this concentration of MNase. Taken together, these results demonstrated that NF-E2 binding was associated with the disruption of the nucleosomal structure of TXAS promoter, particularly in the NF-E2 region.

DISCUSSION

The strength of promoter and accuracy of initiation have been shown, in general, to largely depend on the sequence and relative distance of TATA and Inr elements in many genes driven by RNA polymerase II (Ref. 49 and references therein). Although the consensus Inr sequence is loosely defined, a pyrimidine at –1, an A at +1, and a T or A at +3 are the most critical nucleotides for determining the strength of an Inr (50). These critical nucleotides are conserved in the TXAS Inr, CA1−TTT. In addition, the distance of 25 bp between TATA and Inr, also the case in the TXAS core promoter, was found to give the highest level of in vitro transcription from the artificial templates (51). These factors may thus contribute to a strong TXAS core promoter activity in vitro. Lee et al. identified the transcription start site and TATA box of the TXAS gene at the positions corresponding to our nucleotides –98 and –127/–124, respectively (52), whereas Miyata et al. (53) identified the transcription start site corresponding to our nucleotide +2. However, no functional analysis was reported with respect to the Inr and TATA elements. We showed here that mutation of either element considerably reduced the promoter strength in the cell-free transcription but did not affect the accuracy of initiation, suggesting that the TATA and Inr elements played similar roles in determining the transcription initiation site and directing the transcription machinery to the TXAS promoter. In contrast to in vitro studies, mutation of either the TATA or Inr element in the presence of upstream sequence had little effect on promoter activity, whereas mutation of both elements abolished TXAS promoter activity. These findings suggested that the TATA and Inr elements of the TXAS promoter were functionally redundant in vivo. This is consistent with the notion that a preinitiation complex can be formed through either a TATA or an Inr element, followed by a common step leading to the gene activation (54).

Our results support the model that the upstream activator (i.e. NF-E2) alters the nucleosomal structure and opens up the core promoter. Because NF-E2 lacks the chromatin-modifying activity, NF-E2 activation upon TXAS promoter is probably aided by recruiting other proteins. In this aspect, evidence for NF-E2 involved in chromatin disruption has been extensively studied in the globin genes (30, 31). The mechanism by which NF-E2 alters nucleosomal structures was elucidated from several studies. In the glutathione S-transferase pull-down experiments, p45 NF-E2 was shown to interact directly with CBP/p300, a protein that possesses histone acetyltransferase activity (55). Transient transfection experiments showed that NF-E2 transactivation activity was enhanced by expression of CBP/p300 and was inhibited by E1A, an inhibitor of CBP/p300 (56). Interestingly, both subunits of NF-E2 were acetylated by
CBP, and the DNA binding and transcriptional activities were hence increased (57). It is noteworthy that, despite the evidence of direct interaction between NF-E2 and histone-modifying enzymes in the globin gene transactivation, no evidence of this direct interaction is presented for the TXAS promoter. Furthermore, although our hypothesis favors the model in which NF-E2 induces the nucleosomal changes of the TXAS promoter, it is also plausible that NF-E2 increases transcription of the TXAS gene by recruiting the transcriptional machinery before the nucleosomal structures are altered.

The importance of NF-E2 in TXAS expression can probably explain why TXAS is expressed at high levels in blood cells but much lower levels in the nonblood cells (58). It should be noted that considerable functional redundancy has been found among TXAS transcription factors, which may thus account for the diversity of TXAS expression in different cells (65). For example, Rat macrophage TXAS is suppressed by peroxisome proliferator-activated receptor y ligands through an interaction of Nrf2 and peroxisome proliferator-activated receptor binding at the NF-E2 site (20). p18 NF-E2 can form a heterodimer with Fos to act as a repressor for NF-E2 activity (66). Furthermore, the gene regulation can also be controlled by the localization of NF-E2 transcription factors. A recent work showed that p18 NF-E2 was localized in the centromeric heterochromat in compartment and bound globin gene as a homodimer in the repressed stage. Upon induction, p18 NF-E2 was relocated to the euchromatin compartment and formed a heterodimer with p45 NF-E2 (67).

We are currently studying the TXAS transcriptional activity in the nonblood cells. Piao et al. indicated that the NF-E2 site is also important for TXAS promoter activity in these cells. It is therefore plausible that through the NF-E2 site TXAS is regulated not only for certain activators or inhibitors but also for the cell preference expression.

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Transcriptional Control of the Human Thromboxane Synthase Gene \textit{in Vivo} and \textit{in Vitro}

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