Multiple factors regulating the expression of human thromboxane synthase gene

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Characterization of the 5.5 kb promoter of human thromboxane synthase (TS) gene revealed a proximal positive regulatory sequence (PPRS, –90 to –25 bp) and several distal repressive elements. The maximal promoter activity was found to reside within the first 285 bp, ~75%, of which was contributed by the PPRS. The sequence between –365 and –665 bp exerted a strong repressive effect (~55%) on reporter gene expression independent of orientation and position, consistent with properties expected for a silencer. The sequence upstream of –665 bp to –5.5 kb contains mainly repressive elements which further reduce the promoter activity by 30%. The 65 bp PPRS worked in an orientation-independent, but position-dependent, manner and could be further divided into two independent elements, PPRS1 (–90 to –50 bp) and PPRS2 (–50 to –25 bp). While similar nuclear factor(s) from different cell types interact with PPRS1, those interacting with PPRS2 exhibit cell specificity. Internal sequence deletion and oligonucleotide competition established that a binding sequence for NF-E2 in PPRS1, (–60 tctgattcat –50) was important for enhancing TS promoter activity in HL-60 cells. The presence of NF-E2 mRNA in HL-60 cells was demonstrated by reverse-transcription PCR amplification of the cDNA and Northern blot analysis. A 9-fold transactivation of luciferase (luc) reporter gene expression had been detected when NF-E2 cDNA was co-expressed with a TS promoter/luc construct. Despite the fact that NF-E2 and the cis-elements could alter the efficiency of TS transcription, they were not sufficient for restricting cell-specific TS expression. Analysis of the methylation status at the TS promoter in several human cell lines reveals cell-specific patterns of methylation that might correlate with TS expression. Taken together, these results suggest that the expression of human TS gene is modulated by multiple factors including cis-elements, trans-activator(s), and possibly genomic methylation.

INTRODUCTION

Thromboxane synthase (TS) is a bifunctional, microsomal enzyme catalysing the synthesis of both thromboxane A2 (TXA2) and 12-hydroxyheptadecatrienoic acid (HHT) [1]. While the importance of TXA2 as a vasoconstrictor and inducer of platelet aggregation, in haemostasis has long been recognized, the physiological role of HHT remains poorly defined. Clinically, a deficiency of platelet TS [2,3] or mutations in the TXA2 receptor gene [4] were shown to result in bleeding disorders, while an deficiency of platelet TS [2,3] or mutations in the TXA2 receptor are associated with the 12-hydroxyheptadecatrienoic acid deficiency of human TS gene [14,15]. Although both of these regulatory events operate at different levels, they could be regarded as post-transcriptional. To date, little is known about the mechanism(s) or factor(s) controlling TS gene transcription. We have recently reported the genomic structure and CA repeat polymorphism associated with the ≥150 kb human TS gene [16]. Our studies also indicated that transcription of human TS gene is initiated from multiple start sites and mainly TATA-independent [16,17]. In this report, we describe the characterization of cis-regulatory elements within the 5.5 kb human TS gene promoter and provide evidence suggesting that TS gene expression is transactivated by NF-E2 and potentially modulated by cell-specific methylation.

MATERIALS AND METHODS

Plasmid constructions

The phTS5500/luc reporter vector containing the 5.5 kb (–5500 to +30 bp, relative to the major transcription start site [17]) human TS gene promoter was constructed by cloning the promoter fragment into the HindIII site of the promoterless pGL-Basic luciferase (luc) vector (Promega, Madison, WI, USA).
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**Figure 1** Luc gene expression in HL-60 cells controlled by various lengths of human TS gene promoter

The restriction sites and the relative positions of some used for expression vector construction are indicated schematically at the top. An arrow indicates the major transcription start sites [16,17]. Transfection of HL-60 cells and normalization for transfection efficiency were carried out as described in the Materials and methods section. All promoter fragments used contain the same 3' end which is 30 bp downstream from the 5' most nucleotide of the major transcription start sites [17]. The number next to each fragment indicates the length (bp) of the promoter. Triplicate plates of cells were transfected with each construct in each experiment. The data represent means ± S.D. (n = 4) of the relative luc activities, as compared with that obtained with the 285 bp promoter which was taken as 100%. SV2, SV40 early promoter/enhancer; SV0, promoterless.

U.S.A.). 5'-Deletion mutants containing the promoter from −4500 to −665 bp were obtained by double digestion of the phTS5500/luc construct with *Sac*I (a unique site in the multiple cloning sites) and an appropriate restriction enzyme which cut the promoter, as shown in Figure 1. The ends were filled-in by Klenow DNA polymerase and re-ligated. Other mutants containing the promoter from −365 to −5 bp were obtained by unidirectional deletion of the promoter, using exonuclease III/S1 nuclease (Promega), or by PCR amplification of the desired fragments. The end sequences of these constructs were verified by dideoxy sequencing analysis [18]. Internal deletion of the promoter was achieved by joining the upstream and downstream fragments, each amplified by a pair of synthetic primers. For the ease of cloning, the primers were designed so that a *Kpn*I site was generated at the junction of the amplified fragments.

**Cell culture and transfections**

All media were supplemented with 10% (v/v) fetal-calf serum, 50 µg/ml streptomycin and 50 U/ml penicillin (Gibco BRL). Cells were maintained at 37 °C under 95% humidity and 5% CO₂. All the cell lines used were purchased from the American Type Culture Collection (A.T.C.C.). HL-60 (human promyelocytic leukaemia), K562 and HEL (both human erythroid leukaemia), and IC-21 (murine macrophage) cells were cultured in RPMI 1640 medium, PLC/PRF/5 (human hepatoma) and CHO (Chinese hamster ovary) cells in Dulbecco’s modified Eagle’s medium (DMEM), and GH₄ (rat pituitary) cells in Minimum Essential Medium (MEM; Biofluids, Inc., MD, U.S.A.). Plasmid DNA for transfection was prepared using the Maxi plasmid kit (Qiagen Inc., CA, U.S.A.). Two plasmids, pCMV-β-gal (Clontech) and pRSV-SEAP (an expression vector producing the secretory form of alkaline phosphatase, Tropix, Inc., Bedford, MA, U.S.A.), were used as control vectors for transfection efficiency. HL-60 and K562 cells were transfected by electroporation as previously described [17]. Briefly, 10⁶ cells were electroporated in 1 ml of buffer containing 20 µg of a TS/luc construct, 5 µg of pCMV-β-gal and 5 µg of pRSV-SEAP using a Bio-Rad Gene Pulser set at 960 µF and 300 V. PLC/PRF/5, CHO and GH₄ cells, were transfected using Lipofectamine (Gibco BRL) as the DNA carrier according to the manufacturer’s protocols.

**Alkaline phosphatase, luc and β-galactosidase (β-gal) activity assays**

Alkaline phosphatase activity was measured 24 h after transfection using the protocols and buffers provided in the PhosphaLight reporter gene assay kit (Tropix, Inc.). The culture medium was diluted with an equal volume of a dilution buffer and incubated at 65 °C for 30 min before the addition of 100 µl of assay buffer. After 5 min at room temperature, a reaction buffer was added and the samples were incubated for an additional 20 min at room temperature. Chemiluminescence was determined in a luminometer (Turner Designs Model 20E, Sunnyvale, CA, U.S.A.). Luc and β-gal activities were determined by chemiluminescence and spectrophotometry, respectively, using a commercial kit (Promega). Cell extracts were prepared in 400 µl of the lysis buffer. For luc assay, 20 µl of the extract were mixed with 100 µl of the luc assay reagent (470 µM luciferin, 270 µM coenzyme A and 530 µM ATP) and chemiluminescence was determined. To determine β-gal activity, cell extracts were
incubated with an equal volume of the double-strength assay buffer (0.67 mg of O-nitrophenyl-β-d-galactopyranoside/ml) at 37 °C for 1 h or until the yellow colour developed. The absorbance at 415 nm was determined using a microplate reader (Bio-Rad). Luc activity was normalized with either the β-gal or alkaline phosphatase activity and expressed as relative light units (RLU). When luc activity was compared between two cell types, the transfection efficiency was directly determined from the percentage of cells stained blue with 5-bromo-4-chloroindol-3-yl β-d-galactopyranoside (X-gal) [19].

**Preparation of nuclear extracts**

Nuclear extracts from various cell types were prepared according to the published methods [20,21] with minor modifications. Cells [(2–3)×10⁶] were washed twice with PBS. The cell pellet (∼0.3 g) was resuspended in 2 vol. of buffer A [10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1000 k-units of aprotinin per ml and 10 μM leupeptin]. After incubation for at least 15 min on ice, cells were disrupted in a Dounce homogenizer (20 strokes, pestle A) and 100 μl of a sucrose solution (75% w/v in 5 mM MgCl₂) was added to the homogenate. The sample was quickly spun at full speed in an Eppendorf centrifuge for 1 min at 4 °C. The pelleted nuclei were resuspended in 1 ml of buffer B [6.8% sucrose (w/v) in buffer A] and centrifuged under the same conditions. The nuclear pellet was then resuspended in 1 ml of extraction buffer [10 mM Tris/HCl, pH 8.5, 1.5 mM EDTA, 10 mM DTT, 10% (v/v) glycerol, 0.8 M KCl, 1 mM PMSF, 1000 k-units of aprotinin per ml, and 10 μM leupeptin] and centrifuged at 13000 g for 30 min. Protein concentration was determined by the dye-binding method using the Bio-Rad protein assay kit. The extracts were stored at −80 °C until use.

**Electrophoretic mobility shift assays**

Oligonucleotide probes were end-labelled using [γ-32P]ATP and T4 polynucleotide kinase and purified twice through Bio-Gel P2 (Bio-Rad) spin columns. Binding reactions were carried out for 20 min at room temperature in a buffer containing 10 mM Hepes (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 5 mM DTT, 10% (v/v) glycerol, 1 mg/ml BSA, 2 μg of poly(dI·dC)·poly(dI·dC) (Pharmacia), 10 μg of nuclear extracts, and 2×10⁷ c.p.m. of the probe in a final volume of 20 μl. Samples were electrophoresed in 5% polyacrylamide gels (60:1 acrylamide/bisacrylamide ratio) at 120 V/cm. Gels were dried and exposed to XAR-5 films with one intensifying screen at −80 °C for 1 h or until the yellow colour developed. The absorbance at 415 nm was determined using a microplate reader (Bio-Rad). Luc activity was normalized with either the β-gal or alkaline phosphatase activity and expressed as relative light units (RLU). When luc activity was compared between two cell types, the transfection efficiency was directly determined from the percentage of cells stained blue with 5-bromo-4-chloroindol-3-yl β-d-galactopyranoside (X-gal) [19].

**Reverse-transcription PCR (RT-PCR) amplification of NF-E2 cDNA and Northern blot analysis of NF-E2 mRNA**

Total RNA was isolated according to the acid/guanidinium/thiocyanate procedure of Chomczynski and Sacchi [22]. RNA (5 μg) was reverse-transcribed in a reaction buffer (20 μl) containing 2.5 μl oligo(dT)₁₆, 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1 μM of each of the dNTPs, 10 units of RNase inhibitor and 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) at 42 °C for 1 h. The synthesized first-strand cDNA (4 μl) was then used as the template for PCR amplification of NF-E2 cDNA. The sequences of the forward (5'-tgactccagaggcttt-3') and reverse (5'-cttctgtagagaactca-3') primers correspond to −70 to −51 and +1211 to +1230, respectively, of the reported sequence of human NF-E2 cDNA [23]. The amplification was carried out in a final volume of 20 μl containing 200 nM dNTPs, 50 mM KCl, 10 mM Tris/HCl (pH 9.0), 1% (v/v) Triton X-100, 1.5 mM MgCl₂ and 1.25 units Taq DNA polymerase under the following conditions: melting at 94 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s for 30 cycles, followed by a final extension at 72 °C for 10 min. Amplified products were resolved in a 1.2% agarose gel. As an internal control, a ~300 bp product of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [24] was similarly amplified using two specific primers, 5'-cgtctcagcatgtggtgga-3' (360–379) and 5'-ggcctacagccagagt-3' (640–659). To detect NF-E2 mRNA, total RNA (15 μg) was fractionated by electrophoresis in a 1% agarose gel containing 3.3% formaldehyde and transferred on to a nylon membrane (Hybond, Amersham). The blot was subjected to Northern blot analysis as previously described [8], using NF-E2 cDNA as the probe.

**Transactivation**

NF-E2 cDNA was subcloned in both orientations into the eukaryotic expression vector pcDNA3 (Invitrogen) to produce pCMV/NF-E2(+)+ and pCMV/NF-E2(−), which contain NF-E2 cDNA in the correct and reverse orientation, respectively. Aliquots (15 μg each) of the resulting constructs were used to transfect CHO cells in the presence of the same amount of a luc reporter construct controlled by the TS, simian virus 40 (SV40) or BCKD-E2 [the 5'-flanking sequence (~290 to +48 bp) of murine branched-chain α-keto dehydrogenase E2 subunit gene, provided by Dr. Jeffrey Chinsky, University of Maryland School of Medicine] promoter. The latter two promoters contain no NF-E2-binding site and were used as negative controls. The pCMV-β-gal construct (5 μg) was included in all transfections for normalizing transfection efficiency.

**Genomic Southern blot for methylation analyses**

Genomic DNA was isolated from HL-60, K562, or PLC/PRF/5 cells using the Puregene DNA isolation kit (Gentra Systems, Inc., Research Triangle Park, NC, U.S.A.). The DNA (10 μg) was first digested with HindIII or Ncol enzyme followed by MspI or HpaII restriction digestion. The digested DNA was electrophoresed in a 1% agarose gel, transferred on to a nylon membrane (Hybond-N, Amersham), and subjected to Southern blot analysis using an 800 bp PvuI fragment of the TS promoter as the probe (see Figure 9A). Hybridization was performed at 65 °C in a solution containing 4× SSC, 50 mM NaH₂PO₄ (pH 7.0), 5× Denhardt’s solution, 100 μg/ml of heat-denatured salmon sperm DNA, 10% (w/v) dextran sulphate and the heat-denatured probe (1×10⁶ c.p.m./ml) for 20 h (1× SSC: 0.15 M NaCl/0.015 M sodium citrate; 1× Denhardt’s solution: 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA). The blots were washed for 30 min at 65 °C in 0.5× SSC containing 0.1% (w/v) SDS and exposed to XAR-5 films with one intensifying screen at −80 °C.
RESULTS

Identification of cis-elements within the 5.5 kb TS promoter

To reveal the cis-regulatory elements, a 5.5 kb human TS promoter was deleted from the 5′-end to generate a series of shorter promoters and their ability to direct expression of the luc reporter gene was analysed in the TS-expressing HL-60 cells. As shown in Figure 1, the highest reporter activity was obtained with the 285 bp promoter, which was more than 3-fold higher in promoter strength than the control SV40 promoter/enhancer. A significant decrease in the reporter activity was observed as the length of the promoter was increased beyond 285 bp. The major repressive effect (55%) was exerted by the sequence between 665 and 365 bp. An additional 30% decrease in the reporter activity was seen as the length of the promoter was increased from 755 bp to 5 ± 5 kb, suggesting the presence of multiple repressive elements. Deletion of the promoter from 285 bp to 90 bp reduced the promoter strength by ~20–25%. Further deletion of the promoter to 50 or 24 bp resulted in 2.5- or 10-fold, respectively, decreases in reporter activity, suggesting that the major positive regulatory sequence is located between 90 to 24 bp. No significant reporter activity was detectable when the cells were transfected with the shortest (5 bp) promoter.

The sequence between –665 and –365 bp functions as a silencer, while the proximal positive regulatory sequence (PPRS) located between –90 and –25 bp is enhancer-like

To determine the orientation and position dependence of the major negative and positive regulatory sequences, each was cloned in different orientations and positions within the reporter vector and used to transfected HL-60 cells. As shown in Figure 2, the major repressive sequence (–665 to –365 bp) functioned as a silencer, as it repressed the reporter expression independent of the orientation and position (Figure 2A). It also worked on the heterologous SV40 promoter, suggesting that its activity is independent of the presence of the proximal positive regulatory sequence (PPRS). The PPRS stimulated reporter gene expression in both correct and reverse orientations, although the stimulatory effect was more prominent in the correct orientation. No significant stimulation was observed when the fragment was cloned 3′ to the reporter gene (Figure 2B).

Different protein factors interact with distinctive regions of the PPRS

The deletion analysis (Figure 1) indicated that the PPRS could be
divided into two regions, PPRS, (−90 to −50 bp) and PPRS2 (−50 to −24 bp). To identify protein factors interacting with the PPRS, band-shift assays were carried out using two probes (−90 to −43 bp, and −54 to −24 bp; referred as P1 and P2, respectively) and the HL-60 nuclear extracts. The two probes were designed to overlap each other to assure that proteins binding at the junction (−50) of PPRS, and PPRS2 could be detected. As shown in Figure 3, specific protein–DNA interactions were observed with both probes, which were sensitive to competition by the unlabelled probe (Figures 3A and 3C), but not by the poly(dI–dC) competitor (Figures 3B and 3D), suggesting that the interactions were specific. We have confirmed that P1 and P2 could not cross-compete each other for their respective complex (results not shown), suggesting that different proteins interacted with these probes. To see if there were quantitative differences in the amounts of the PPRS-interacting proteins in different cell types, band-shift assays were carried out using nuclear extracts from six cell lines. As shown in Figure 4, while the amounts of protein factor(s) interacting with the P2 probe were similar in all the cell lines, only nuclear extracts from HL-60, HEL, and K562 cells interacted significantly with the P1 probe, suggesting that P1-binding protein(s) may be cell-specific.

The AP-1 binding site in the PPRS is important for TS gene expression

Computer searches for the recognition sites of transcription factors in the PPRS, using the tfsites.dat program of the Wisconsin Package, identified only an AP-1-like element (−56 tggactc −50, ref. [25]). To evaluate the importance of PPRS1, PPRS2, the AP-1 binding site, and the sequence near the transcription start site (−12 to −4 bp) on TS gene expression, these sequences, alone or in combination, were internally deleted from the pTS365/luc vector and the resulting plasmids used in transient assays. As shown in Figure 5, deletion of the PPRS (construct 2) reduced the promoter activity by ~75 %, which was almost equal to the sum of the losses in promoter activity caused by the deletion of PPRS1 (construct 3) and PPRS2 (construct 4). Interestingly, deletion of the AP-1 binding site (construct 5) decreased the reporter activity to a similar extent as though the entire PPRS, was deleted, indicating that the AP-1-binding site is crucial for enhancing TS promoter activity. Deletion of the sequence between −12 and −4 bp (construct 6) abolished 90 % of the promoter activity, suggesting that this sequence, rather than the PPRS, might be an integral portion of the basal promoter. The results prompted us to investigate the role of AP-1 in TS gene expression. However, three experiments failed to confirm its involvement, as will be discussed later.

**Figure 4 Distinctive transcription factor(s) interacting with P1 and P2**

Electrophoretic mobility shift assays were performed using the same amount (10 μg) of nuclear extracts prepared from various cell types as indicated. Probe labelling and DNA–protein complex analysis were as described in the legend to Figure 3.

**Figure 5 Effects of internal deletion of PPRS, PPRS2, AP-1-binding site, and the sequence −12 to −4 on the strength of TS promoter**

The construction of TS promoter/luc gene vectors with internal deletions has been described in the Materials and methods section. The plasmids were used to transfect HL-60 cells as described in the legend to Figure 1. Luciferase activities were expressed relative to that obtained with the 365 bp promoter which was taken as 100 %. The results are the means ± S.D. of two experiments using triplicate samples for each construct.

**Figure 6 Competition of protein/P1 complex by the wild-type or mutant NF-E2 oligonucleotide probe**

Left panel: protein–DNA complex formation between NF-E2 probe and HL-60 nuclear extracts. Middle and right panels: competition of complex formed between HL-60 nuclear extracts and P1 probe by the unlabelled wild-type (middle panel) or mutant (mt, right panel) NF-E2 probe. Probes (104 c.p.m.) were incubated with 0 or 10 μg of nuclear extracts in the presence of the indicated molar excess of the unlabelled probe and electrophoretic mobility assays were carried out as described in the legend to Figure 3. The sequences of the wt and mt NF-E2 oligonucleotides are as described in the Materials and methods section.
Figure 7 NF-E2 expression in HL-60 and other cultured cell lines

(A) RT-PCR amplification of NF-E2 cDNA. Total RNA (5 µg) was reverse-transcribed from RNA of the indicated cell lines and used for PCR amplification in the presence of two specific primers encompassing either the coding sequence of human NF-E2 cDNA [23] or a 300 bp fragment of the human GAPDH cDNA [24]. In the negative (−) control, no first-strand cDNA was used in the PCR reaction. The sequences of the primers used and the conditions for PCR reactions have been described in detail in the Materials and methods section. After 30 cycles of amplification, 5 µl of the products were resolved in a 1% agarose gel. (B) Northern blot detection of NF-E2 mRNA. Aliquots (15 µg) of total RNA from each of the indicated cell lines were electrophoresed in a 1% agarose gel, blotted on to a nylon membrane, and probed with the NF-E2 cDNA obtained by RT-PCR.

Table 1 Transactivation of luc gene expression by co-transfection of NF-E2 cDNA with TS promoter/luc constructs in CHO cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luciferase activity</th>
<th>Transactivation (fold)</th>
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<tbody>
<tr>
<td>phTS365/luc</td>
<td>329 ± 32</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>phTS365A(PPRS)/luc</td>
<td>39 ± 3</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>pSV/luc</td>
<td>1250 ± 210</td>
<td>1075 ± 230</td>
</tr>
<tr>
<td>pBCKD-E2/luc</td>
<td>270 ± 25</td>
<td>230 ± 40</td>
</tr>
</tbody>
</table>

For each cell type, triplicate samples were transfected, two for luc assay and one for X-gal staining. The data represent the averages of two independent assays.

amplification of NF-E2 cDNA from HL-60 RNA was therefore carried out, using two specific primers encompassing the coding sequence of human NF-E2 cDNA [23]. As controls, RNAs from the NF-E2-positive HEL and K562 cells and the NF-E2-negative PLC/PRF/5 cells [23] were used. As shown in Figure 7(A), significant amounts of a cDNA having the size expected for the coding sequence of NF-E2 cDNA (1.3 kb) were amplified from
Figure 9 Profiles of methylation at the TS promoter region in three human cell lines

(A) Southern blot detection of the methylation profiles within the 5.5 kb TS promoter in HL-60, PLC/PRF/5 and K562 cells. The line diagram shows three HpaII/MspI sites (I, II and III) and the HindIII and NcoI restriction maps within the promoter. The PstI fragment used as the probe is indicated by a thicker line. The numbers indicate the positions relative to the transcription start site. Genomic DNA (10 µg) from the indicated cell lines were digested by NcoI (left blot) or HindIII (right blot), followed by HpaII or MspI digestion. The digested DNA was subjected to Southern blot analysis using the 800 base PstI probe. (B) Schematic summary of the methylation patterns at the three HpaII/MspI sites of TS promoter in the three cell lines, as inferred from the results of Southern analyses in (A). Solid squares, methylated; open squares, non-methylated; hatched squares, partially methylated. (C) Effect of synchronization on the methylation profile of the TS promoter in HL-60 cells. HL-60 cells were synchronized by serum starvation or PMA-induced differentiation as described in the Materials and methods section. DNA from non-treated (control), serum starved (synch.), or PMA-differentiated (TPA) HL-60 cells was isolated, digested with NcoI/HpaII (left blot) or HindIII/HpaII (right blot), and subjected to Southern analyses, as described in (A).

RNA of HL-60 and the two erythroid cell lines, K562 and HEL. No product was detectable from the RNA of hepatoma cells, although similar amounts of the control GAPDH cDNA were amplified from RNA of all cell types. This result is consistent with that of Figure 4, indicating no significant interaction of P1 probe with the PLC/PRF/5 nuclear extracts. Following sub-cloning, the authenticity of the amplified cDNA as human NF-E2 cDNA was confirmed by end sequencing (∼200 bp from both ends) and restriction mapping with four (ApaI, HindIII, KpnI, XbaI) enzymes. The expression of NF-E2 mRNA in HL-60 cells was later verified by Northern blot analysis as shown in Figure 7(B).

To demonstrate that NF-E2 was able to enhance TS promoter activity, the effect of co-transfection of NF-E2 cDNA on the expression of luc gene controlled by TS or other promoters was investigated. CHO cells were chosen as host cells, since they have no endogenous NF-E2, and can be transfected with high efficiency (∼50%). The NF-E2 cDNA was cloned in the correct (+) and reverse (−) orientations under the control of the CMV promoter in pCMV/NF-E2(+) and pCMV/NF-E2(−), respectively. As shown in Table 1, the luc reporter activity controlled by the 365 bp promoter was 9.1-fold higher in cells co-transfected with pCMV/NF-E2(+) vector than those co-transfected with the pCMV/NF-E2(−) vector. The transactivation was mediated by the PPRSs, as deletion of the sequence (−90 to −50) from the phTS365/luc construct abolished the transactivation. As negative controls, no significant transactivation of the reporter activity by NF-E2 could be detected when the luc gene was controlled by the SV40 promoter/enhancer or the BCKD-E2 promoter.

**TS promoter activity in other cell types**

To evaluate the importance of cis-regulatory sequences in conferring cell-specific TS expression, selective constructs containing
TS promoters from 90 bp to 5.5 kb were transected into four non-TS-expressing cell lines, including two human (K562 and PLC/PRF/5) and two rodent (CHO and GH4) cell lines. As shown in Figure 8, expression of the reporter gene was detectable in all cell types tested, albeit at a much lower efficiency. As the length of the promoter increased, the promoter activity in non-TS-expressing cells became less significant.

**Cell-specific methylation at TS promoter**

An increasing number of reports indicate that methylation at the promoter region can influence gene expression [27]. We therefore analysed the profiles of methylation at the 5.5 kb TS promoter for three human cell lines, HL-60, K562 and PLC/PRF/5. Sequencing and genomic Southern blot analyses indicated that there are three potential sites of methylation (CCGG, referred as I, II and III) in the 5.5 kb promoter, as depicted in Figure 9(A). Genomic DNA from each cell line was first digested with *Hind*III or *Nco*I. The digested products were split into two aliquots, one further digested with *Hpa*II, which does not digest methylated CCGG (CC«GG), and the other with *Msp*I, which is insensitive to methylation of CCGG. The products were subjected to Southern blot analysis using a *Pst*I probe. As shown in Figure 9(A), each of the three cell lines exhibited a distinctive pattern of methylation at these three sites, which were independent of the restriction enzyme (*Hind*III or *Nco*I) chosen for initial genomic DNA digestion. A summary of the profiles of methylation derived from the Southern blot analyses was depicted in Figure 9(B). No methylation within the 5.5 kb promoter could be detected in the hepatoma cells, as an identical pattern was observed with either *Hpa*II or *Msp*I digestion. Neither site II or site III was methylated in K562 cells, while a partial methylation at site I was observed, as evidenced by the presence of a 2.0 kb fragment. In contrast, complete methylation at site I (lack of the 1.57 kb fragment) and partial methylation at the other two sites were seen in HL-60 cells.

**Partial methylation at TS promoter is cell cycle-independent**

Since partial methylation was observed in two of the cell lines used, we wondered whether methylation at the promoter was cell-cycle dependent. In order to address this question, DNA from HL-60 cells synchronized by either serum starvation or phorbol 12-myristate 13-acetate (PMA)-induced differentiation was analysed. In serum starvation, which arrests the cells at G0 phase [28], HL-60 cells were cultured in a serum-free medium for 60 h. For PMA-induced differentiation, HL-60 cells (8 × 10^6 cells/ml) were treated with PMA (Gibco BRL) at a final concentration of 33 nM for 36 h, which arrests the cells in G1 phase [29]. Within 4 h of PMA treatment, more than 95% of the cells became attached to the plates. As shown in Figure 9(C), the profiles of DNA methylation at the promoter were not affected by either method of synchronization, suggesting that partial methylation is probably not due to mixed populations of cells at different stages of the cell cycle.

**DISCUSSION**

Our promoter analysis revealed that TS gene expression is regulated by positive as well as negative cis-elements. The maximal promoter activity was localized within 285 bp, 75% of which could be accounted for by the 66 bp PPRS. The PPRS is enhancer-like, since its activity depends strictly on its position but much less on its orientation. One likely interpretation is that close proximity of the PPRS-binding proteins to those in the basal transcriptional machinery was required to achieve optimal interactions. The sequence upstream of –285 bp consists mostly of repressive elements. The major repressive sequence (–365 to –665) functions independent of orientation and position, and therefore conforms to the definition of a silencer. The silencer activity did not require the presence of the PPRS, and showed no cell specificity as it worked on the SV40 promoter/enhancer and in several cell types (Figure 8). Attempts to localize the core silencer sequence by 5‘ deletion from –665 bp resulted in gradient increases in the promoter strength, while gel-shift assays using the 300-bp silencer probe and HL-60 nuclear extracts failed to detect significant association of protein factors (results not shown). We suspect that the silencer is composed of multiple elements which either alter chromatin structure or bind to repressor proteins low in affinity and/or abundance. It has been demonstrated that some DNA sequences, which failed to bind protein factors, could alter chromatin structure and affect the efficacy of transcription [30]. Whether this silencer sequence works in a similar fashion remains to be determined. The sequence between –755 bp and –5.5 kb caused an additional ~30% reduction in reporter activity in HL-60 cells. The gradual decline of the promoter strength indicates that transcriptional control of the TS gene may rely on the coordinate activity of multiple but individually weak cis- and trans-acting factors. It has been shown that such multiple weak effectors can form an array capable of directing transcription with a high degree of specificity [31].

We have demonstrated that at least two protein factors in HL-60 cell nuclear extracts specifically interact with the PPRS. The one interacting with P1 at the NF-E2/AP-1-binding site is cell-specific, while that interacting with the P2 is probably ubiquitous. Three lines of evidence (results not shown) suggest that AP-1 is not involved in the PPRS-mediated transactivation. First, oligonucleotides containing either the consensus (tgactca) or the mutated (tgacttg) AP-1-binding site competed 20-fold less efficiently than the unlabelled authentic P1 probe. Secondly, antibodies against either c-Jun or c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) could not supershift the complex or inhibit its formation in band-shift assays. Thirdly, PMA, known to stimulate AP-1 activity in HL-60 cells [25], induced a 20-fold increase of luc expression in the SV40/luc-transfected HL-60 cells, but failed to stimulate luc activity in cells transfected with several TS promoter/luc constructs bearing the PPRS.

Our results strongly suggest that NF-E2 is responsible for the PPRS-dependent transactivation of luc activity. The 20 bp NF-E2 oligonucleotide used in the competition assays (Figure 6) shares only a 10 bp (the nearly consensus NF-E2-binding site) homology with the P1 sequence, yet competed efficiently for the entire complex formed between the authentic P1 sequence and HL-60 nuclear extracts (Figure 6). Furthermore, the transactivation by NF-E2 in CHO cells relied strictly on the presence of PPRS, in the vectors (Table 1). We have also found significant, though less (1.5- to 2-fold), transactivation of luc reporter expression by NF-E2 in HL-60 and another NF-E2-positive K562 cell line (results not shown). The reduction in transactivation is presumably due partly to the fact that NF-E2 protein is already abundant in these cell lines (Figure 7) and partly to the relatively lower efficiency of transfection (3–5%) achievable in these two haematopoietic cell lines. NF-E2 has been shown to function as an obligate heterodimer with p18, a ubiquitous protein which increases the binding specificity for the bases lying outside the AP-1 core of the 11 bp NF-E2 consensus sequence [26]. Since NF-E2 alone cannot directly bind DNA [23,32], it is likely that transactivation of the TS promoter is also mediated by the NF-E2/p18 complex.
In addition to the PPRS, several upstream repressive sequences have been localized within the 5.5 kb TS promoter. Since TS directly controls the production of the potent autacoid TxA<sub>2</sub>, it is likely that, in addition to ‘suicide inactivation’ [12,13] and alternative splicing of the TS transcript [14], transcriptional repression of the TS gene may provide an additional mechanism to curtail the level of TS. Although these repressors were not sufficient to confer cell-type specificity (Figure 8), the difference in the promoter activity between TS-expressing and non-TS-expressing cells became more significant as the length of the promoter was increased, suggesting that differences in the level of repressor proteins among cell lines may contribute, at least in part, to cell-specific expression of the TS gene.

We have demonstrated cell-specific patterns of methylation at the 5.5 kb TS promoter in human cell lines. The TS promoter was more extensively methylated at all three sites in HL-60 cells, as compared with that of the two non-TS-expressing cell lines. The methylation did not change in response to growth arrest by serum starvation or induced differentiation, suggesting that it is probably intrinsic and independent of cell cycles. Cell-specific partial methylation at promoters has also been reported for several other genes [33–35]. Hypermethylation, though generally associated with silent genes, has been shown to enhance gene expression. Both insulin-like growth factor II (igf2) and its receptor (igf2R) genes are expressed from the more methylated alleles [36,37]. DNA methylation has been demonstrated to interfere, in part, to cell-specific expression of the TS gene. This work was supported by a Grant-in-Aid (to R.-F.S.) from the American Heart Association and with funds contributed in part by the AHA, Maryland Affiliate.

REFERENCES


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