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 PPRS₂, those interacting with PPRS₁ exhibit cell specificity. Internal sequence deletion and oligonucleotide competition es-

tablished that a binding sequence for NF-E2 in PPRS₁ (–60 tgctgattcat –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, 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 elevated level of TxA₂ has been postulated to play a crucial role in cardiovascular and renal diseases [5,6]. Recent studies suggest that TS may also play an important role in the immune system. Supports for this notion are the findings that significant amounts of the TS transcript are expressed in spleen, thymus and myeloid cells [7–10], and that TxA₂ causes apoptotic DNA fragmentation of immature thymocytes via the TxA₂ receptor [11]. It is believed that TS activity *in vivo* needs to be delicately controlled in order to maintain haemostasis as well as other TxA₂-associated functions. Although the detailed molecular mechanisms remain largely unknown, studies in the past suggested that TS is subject to multiple-level regulation *in vivo*. One of the better studied aspects of TS regulation is ‘suicide inactivation’, in which TS loses its activity during catalysis as a result of covalent modification by its substrate prostaglandin endoperoxide H₂ (PGH₂) [12,13]. This catalytic inactivation presumably serves as a safeguard mechanism to prevent overproduction of the potent autacoid. A second control mechanism at work might be the

recently described ‘alternative splicing’ of the TS transcript. A truncated TS mRNA, lacking the entire 12th exon encoding the haem-binding domain of the enzyme, has been reported in several human cell lines [14]. The encoded protein of this truncated transcript, however, exhibits no enzymic activity for the formation of either TxA₂ or HHT [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 pH5500/*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 *Hind*III site of the promoterless pGL-Basic luciferase (*luc*) vector (Promega, Madison, WI,

Abbreviations used: β-gal, β-galactosidase; HHT, 12-hydroxyheptadecatrienoic acid; *luc*, luciferase; PGH₂, prostaglandin endoperoxide H₂; PPRS, proximal positive regulatory sequence; RLU, relative light units; RT-PCR, reverse-transcription PCR; PMA, phorbol 12-myristate 13-acetate; TS, thromboxane synthase; TxA₂, thromboxane A₂; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SV40, simian virus 40; X-gal, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside.

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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 $\pm$ 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 *SmaI* (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 *KpnI* 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 μ g/ml streptomycin and 50 I.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 GH₃ (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, 10⁷ cells were electroporated in 1 ml of buffer containing 20 μ g of a TS/luc construct, 5 μ g of pCMV- β -gal and 5 μ g of pRSV-SEAP using a Bio-Rad Gene Pulser set at 960 μ F and 300 V. PLC/PRF/5, CHO and GH₃ 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 Phospha-Light 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 μ l 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 μ l of the lysis buffer. For luc assay, 20 μ l of the extract were mixed with 100 μ l of the luc assay reagent (470 μ M luciferin, 270 μ M coenzyme A and 530 μ M 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) $\times 10^8$] 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 μ M leupeptin]. After incubation for at least 15 min on ice, cells were disrupted in a Dounce homogenizer (20 strokes, pestle A) and 100 μ l 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 Eppendorff 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 μ M leupeptin] and centrifuged at 130000 *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 μ g of poly(dI·dC)·poly(dI·dC) (Pharmacia), 10 μ g of nuclear extracts, and 2 $\times 10^4$ c.p.m. of the probe in a final volume of 20 μ l. Samples were electrophoresed in 5 % polyacrylamide gels (60:1 acrylamide/bisacrylamide ratio) at 15 V/cm. Gels were dried and exposed to XAR-5 films with one intensifying screen at –80 °C. For competition studies, the labelled probe was mixed with various amounts of an unlabelled competitor oligonucleotide prior to the addition of nuclear extracts. DNA–protein complexes were resolved as described above. The sequences of the oligonucleotides used are listed below with the recognition sites underlined. P1, 5'-tattgacagtgcagtcataaggaataaagtgtgctgattcattctt-3'; P2, 5'-attcattctttacactgaaccccttgttg-3'; AP-1, 5'-cgcttgatgactgagccggaa-3'; mutant AP-1, 5'-cgcttgatgacttgccggaa-3'; NF-E2, 5'-tggggaaacctgtgctgagtcactggag-3'; and mutant NF-E2, 5'-tggggaaacctgtgctgagtcactggag-3'.

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 μ g) was reverse-transcribed in a reaction

buffer (20 μ l) containing 2.5 μ M oligo(dT)₁₆, 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1 mM 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 μ l) was then used as the template for PCR amplification of NF-E2 cDNA. The sequences of the forward (5'-tgactccaccaggtttct-3') and reverse (5'-cttctggtctagagaactca-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 μ l containing 200 μ M 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'-cgtcttcaccaccatggaga-3' (360–379) and 5'-cggccatcacgcccagttt-3' (640–659). To detect NF-E2 mRNA, total RNA (15 μ g) 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 μ g 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 +8 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 μ g) 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 μ g) was first digested with *Hind*III or *Nco*I enzyme followed by *Msp*I or *Hpa*II 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 *Pst*I fragment of the TS promoter as the probe (see Figure 9A). Hybridization was performed at 65 °C in a solution containing 4 $\times$ SSC, 50 mM NaH₂PO₄ (pH 7.0), 5 $\times$ Denhardt's solution, 100 mg/ml of heat-denatured salmon sperm DNA, 10 % (w/v) dextran sulphate and the heat-denatured probe (1 $\times 10^6$ c.p.m./ml) for 20 h (1 $\times$ SSC:0.15 M NaCl/0.015 M sodium citrate; 1 $\times$ 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 $\times$ SSC containing 0.1 % (w/v) SDS and exposed to XAR-5 films with one intensifying screen at –80 °C.

Figure 2 Position and orientation dependence of the major negative and positive regulatory sequences in the TS promoter

(A) Effect of the major repressive sequence (—665 to —365 bp) on luc expression controlled by the TS promoter and SV40 promoter/enhancer. (B) Effect of the PPRS (—90 to —25 bp) on luc expression. The test sequences were cloned upstream of the indicated promoter or downstream of the luc reporter gene in both orientations. Right and left arrows indicate the correct and reverse orientations, respectively. Constructs were transiently transfected into HL-60 cells in the presence of pCMV- β -gal. The normalized luc activity obtained with each parent construct is designated 1.00. The results represent the means $\pm$ S.D. ($n = 3$).

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 —365 and —665 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

Figure 3 Protein-DNA interactions between PPRS and HL-60 nuclear extracts

Double-stranded oligonucleotide probes P1 (—90 to —43 bp) and P2 (—54 to —24 bp) were end-labelled with T4 polynucleotide kinase and [γ - 32 P]ATP and incubated with nuclear extracts prepared from HL-60 cells in the absence and presence of the unlabelled probes (A and C) or poly(dI-dC) (B and D) as competitors. Binding reactions and competition were carried out as described in the Materials and methods section. Samples were subjected to 5% non-denaturing polyacrylamide gel electrophoresis. Abbreviation: N.E., nuclear extract.

vector and used to transfect 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 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

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

Electrophoretic mobility shift assays were performed using the same amount (10 μ g) 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.

divided into two regions, PPRS₁ (–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 PPRS₁ 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.

Figure 5 Effects of internal deletion of PPRS₁, 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. Luc activities were expressed relative to that obtained with the 365 bp promoter which was taken as 100%. The results are the means $\pm$ 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 μ g 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.

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 *tf sites dat* program of the Wisconsin Package, identified only an AP-1-like element (–56 tgattca –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 pHTS365/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 PPRS₁ 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 PPRS₂, 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.

NF-E2 modulating TS gene expression in HL-60 cells

The binding site for NF-E2, a transcription factor recognizing the locus control region of the globin genes, overlaps with that of AP-1 [26]. We had found that the PPRS₁ contained a sequence (–60 tgcgtgattcat –50) nearly identical to the consensus NF-E2 binding site (t/c)gctga(c/g)tca(t/c) [26]. To test if NF-E2 could be the factor interacting with the P1 probe, we carried out band-shift assays using a 20 bp oligonucleotide containing the NF-E2 recognition sequence as the competitor. We first demonstrated that the NF-E2 probe could interact with protein factors in HL-60 nuclear extracts, as shown in the left-hand panel of Figure 6. The competition of P1/protein complex by the wild-type versus

Figure 7 NF-E2 expression in HL-60 and other cultured cell lines

(A) RT-PCR amplification of NF-E2 cDNA. Total RNA (5 μ g) 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 μ l of the products were resolved in a 1.2% agarose gel. (B) Northern blot detection of NF-E2 mRNA. Aliquots (15 μ g) 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

CHO cells were transfected with 15 μ g each of the indicated promoter/*luc* fusion constructs in the presence of the same amount of pCMV-NF-E2(+) or pCMV-NF-E2(—). The (+) and (—) signs indicate that the cDNA was cloned in the correct and reverse orientation, respectively. In the pTS365 Δ (PPRS₁)/*luc* construct, the PPRS₁ (—90 to —50) has been deleted from the 365 bp promoter. Neither the pSV₂/*luc* nor the pBCKD·E2/*luc* vector contains the NF-E2 recognition site in its promoter. The relative luciferase activity (RLU) was normalized with β -gal activity. Transactivation was expressed as the ratio of the *luc* activity obtained in the presence of pCMV-NF-E2(+) over that in the presence of pCMV-NF-E2(—). The data are the means $\pm$ S.D. of two experiments.

Construct	Co-transfection ...	Luciferase activity		Transactivation (fold) [NF-E2(+)/NF-E2(—)]
		NF-E2(+)	NF-E2(—)	
pTS365/ <i>luc</i>		329 $\pm$ 32	36 $\pm$ 3	9.1
pTS365 Δ (PPRS ₁)/ <i>luc</i>		39 $\pm$ 3	31 $\pm$ 4	1.3
pSV ₂ / <i>luc</i>		1250 $\pm$ 210	1075 $\pm$ 230	1.2
pBCKD·E2/ <i>luc</i>		270 $\pm$ 25	230 $\pm$ 40	1.2

mutant NF-E2 oligonucleotide was then compared, as shown in the middle and right-hand panels, respectively, of Figure 6. At 50 $\times$ molar excess, the wild-type NF-E2 oligonucleotide competed as efficiently as the authentic unlabelled P1 probe (see Figure 3), while the mutant NF-E2 probe, which differs from the wild-type probe only by a GA $\rightarrow$ AG transition in the 20 bp sequence, failed to compete, indicating that the competition by the wild-type probe was specific.

For NF-E2 to be responsible for the formation of the P1/protein complex (Figure 4) and to play any significant role in TS gene expression, it should be present in the TS-expressing HL-60 cells. Although NF-E2 has been shown to be expressed in erythrocytes, megakaryocytes and mast cells [26], its presence in HL-60 cells (a myeloid cell line) has not been reported. RT-PCR

Figure 8 Expression of *luc* activity under the control of various lengths of human TS promoter in different cell types

Cells were cultured in their respective media as specified in the Materials and methods section. Transfection was carried out by electroporation (HL-60 and K562) or lipofectamine (PLC/PRF/5, CHO and GH₃). *Luc* activity was normalized for the amount of protein used and for transfection efficiencies in each cell type, as determined by direct X-gal staining of the transfected cells [19]. 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 μ g) 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

($\sim 50\%$). 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 PPRS₁, 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 transfected into four non-TS-expressing cell lines, including two human (K562 and PLC/PRF/5) and two rodent (CHO and GH₃) 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^mGG), 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 × 10⁵ 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 PPRS₁ 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 PPRS₁-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 PPRS₁.

Our results strongly suggest that NF-E2 is responsible for the PPRS₁-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 PPRS₁ 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_2 , 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]. It has been shown that the sensitivity of a eukaryotic promoter to methylation depends primarily on the position and less on the absolute number of these methylation sites [38]. DNA methylation has been demonstrated to interfere, from a distance, with the activity of the thyroglobulin gene promoter [39]. In this regard, it is worth noting that all three sites of methylation in the 5.5 kb TS promoter are located in regions which exhibit a repressive effect on the reporter gene expression. We have recently found that the methylation pattern at the TS gene promoter of another TS-expressing cell line, HEL, is identical to that of HL-60 cells (results not shown). Further studies are required in order to elucidate whether TS gene expression is actually under epigenetic regulation by methylation and the mechanisms involved.

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