Transcriptional Regulation of Cell Type-specific Expression of the TATA-less A Subunit Gene for Human Coagulation Factor XIII*

(Masafumi Kida, Masayoshi Souri, Masayuki Yamamoto, Hidehiko Saito, and Akitada Ichinose)

From the Department of Molecular Pathological Biochemistry and Biology, Yamagata University School of Medicine, Yamagata 990-9585, the Department of Molecular Developmental Biology, Tsukuba University School of Medicine, Tsukuba 305, and the Department of Medicine I, Nagoya University School of Medicine, Nagoya 466, Japan

To study the mechanism of gene regulation for coagulation factor XIII A subunit (FXIIIA), we characterized its 5'-flanking region using a monocytoid (U937), a megakaryocytoid (MEG-01), and other cells. Our results confirmed that U937 and MEG-01 contained FXIIIA mRNA. A tentative transcription start site was determined to be 76 bases upstream from the first exon/intron boundary. Reporter gene assays revealed that a 5'-fragment (-2331 to +75) was sufficient to support basal expression in U937 and MEG-01 but not in the other cells. Deletion analysis confined a minimal promoter sequence from -114 to +75. DNase footprinting, electrophoretic mobility shift, and reporter gene assays demonstrated that promoter elements for a myeloid-enhanced transcription factor (MZF-1-like protein) and two ubiquitous transcription factors (NF-1 and SP-1) in this region were important for the basal FXIII expression. It was also revealed that an upstream region (-806 to -290) had enhancer activity in MEG-01 but silenced activity in U937. DNA sequences for binding of myeloid-enhanced factors (GATA-1 and Ets-1) were recognized in this region, and the GATA-1 element was found to be responsible for the enhancer activity. These transcription factors play a major role in the cell type-specific expression of FXIIIA, which differs from other transglutaminases.

Human coagulation factor XIII (FXIII), fibrin-stabilizing factor, is a plasma transglutaminase (TGase) and circulates in blood as a heterotetramer consisting of two catalytic A (FXIIIA) and two noncatalytic B (FXIIIB) subunits (A2B2) (1). It is a proenzyme that is activated by thrombin that is generated in the final stage of the blood coagulation cascade. The enzyme promotes clot stability by forming covalent bonds between fibrin molecules and also by cross-linking fibrin with other proteins including fibronectin, α2-plasmin inhibitor, and collagen (2, 3). These reactions lead to an increase in the mechanical strength, elasticity, and resistance to degradation by plasmin of fibrin clots and promotion of wound healing. Congenital FXIII deficiency is a severe bleeding disorder associated with impaired wound healing and an increased risk of spontaneous abortion in women (2). In most cases the disorder is due to FXIIIA deficiency, and only a few cases with FXIIIB deficiency have been reported (3). The FXIIIA gene is located on chromosome 6p24-25 (4) and spans more than 160 kb of sequence and contains 15 exons (5).

In addition to its presence in plasma, FXIII exists inside monocytes and macrophages (6–11), megakaryocytes/platelets (9, 12), and in placenta (13). It is not present in erythrocytes, granulocytes, or lymphocytes (6). Although the intracellular form of FXIII is a homodimer of FXIIIA alone without FXIIIB, it has functional characteristics identical to those of plasma factor XIII. Genetic studies have confirmed that plasma and intracellular FXIIIA are the products of the same gene (4, 5, 14). It is evident that FXIIIA is actively synthesized in circulating monocytes (10, 11), and it may also be synthesized in megakaryocytes (15). In contrast, the possible biosynthesis of FXIIIA in human liver has been debated (16–19). Transplantations of bone marrow have presented indirect evidence that bone marrow cells are a source of plasma FXIIIA (9).

FXIIIA belongs to a family of TGases, members of which are expressed in various organs, and is regulated by diverse mechanisms. Both keratinocyte TGase and seminal plasma TGase are expressed in highly specialized tissues. The former is expressed prominently in differentiating keratinocytes (20); the latter is synthesized by prostatic epithelial cells (21). Tissue TGase is ubiquitously expressed in many cells and tissues (22). Recent studies revealed transcriptional regulatory mechanisms for these genes. The expression of tissue TGase may prove to be regulated by DNA methylation (23), and retinoid response elements were found in the mouse tissue TGase promoter (24). In the human TGase 3 gene, SP-1- and Ets-1-like motifs were required for the function of its promoter (25). Analysis of the keratinocyte TGase promoter indicated a region containing three AP-2-like motifs essential for its expression (26). In contrast, mechanisms regulating the expression of the FXIIIA gene have not been reported. The mechanism of release of FXIIIA from the synthesizing cells in vivo also remains unknown to date (27).

In order to investigate the transcriptional regulatory mechanisms of the FXIIIA promoter, we have characterized cis-acting sequences and trans-acting factors responsible for the activation of transcription and for the tissue-specific expression of the TATA-less FXIIIA gene in the present study.

EXPERIMENTAL PROCEDURES

Human Cell Culture—The megakaryoblastic leukemia cell lines MEG-01 and MEG-J and the histiocyte lymphoma cell line U937 were maintained in RPMI 1640 medium with 10% fetal bovine serum and...
antibiotics (penicillin, streptomycin, and neomycin) at 37 °C in 5% CO₂. The hepatoma cell line HepG2 and the cervical carcinoma cell line HeLa were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and the antibiotics. Finally, the transformed embryonic kidney cell line 293 was maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated horse serum and the same antibiotics as mentioned above. HepG2 and HeLa were obtained from the Japanese Cancer Research Resources Bank and 293 from the Health Science Research Resources Bank. U937 and MEG-J were kind gifts from Drs. S. Yokoyama and H. Mizoguchi, respectively, and MEG-01 was provided by H. Saito.

Northern Blot Analysis—RNA was isolated from a variety of cells including U937, MEG-01, MEG-J, HepG2, 293, and HeLa by the acid guanidinium thiocyanate method. Five μg of total RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde, transferred to a nylon membrane (Zeta-Probe, Bio-Rad), and hybridized with the DIG™-RNA probe (Boehringer Mannheim, Germany) made from the cDNA for FXIIIA (14). Detection was performed using a DIG™-nucleic acid detection kit following the manufacturer's instructions. The membrane was exposed to Kodak XAR autoradiography film, and the film was subjected to semi-quantitation of mRNA bands by Densitograph 3.01 Imaging Analyzer (Atto Inc., Tokyo, Japan). The fluorescence intensity of the band for FXIIIA was normalized to that for β-actin.

Rapid Amplification of 5'-cDNA Ends—To determine the transcription initiation site of the FXIIIA gene, rapid amplification of 5'-cDNA ends (5'-RACE) was performed using a 5'-RACE System, version 2.0 (Life Technologies, Inc.), and total RNA from U937, MEG-01, and HepG2. The first synthesis from 500 ng of total RNA using Superscript™ II reverse transcriptase (Life Technologies, Inc.) and an antisense primer (5'CATATAGAAAGACTGC-3' designed for exon III of FXIIIA) at 42 °C for 60 min. The cDNA purified by a GlassMax spin cartridge (Life Technologies, Inc.) was used in terminal dideoxynucleotide transferase (TdT) tailing, and PCR was then performed with an internal antisense primer (5'-AGCTCTCTTCTGCTGCTCACA-3' designed for exon II of FXIIIA) and a 5'-sense primer termed Abridged Anchor Primer. This was followed by a nested PCR using a nested gene-specific antisense primer (5'-GGGAATTCGACCATTTTTAGCTTTACAG-3' designed for exon II of FXIIIA) and a 5'-sense primer termed Abridged Primer. This was followed by a nested PCR using a nested gene-specific antisense primer (5'-GGGAATTCGACCATTTTTAGCTTTACAG-3' designed for exon II of FXIIIA) and a nested adapter primer termed Abridged Universal Amplification Primer. PCR products were analyzed by 2% agarose gel electrophoresis and by the dideoxy sequencing method both directly and after subcloning into pBluescript II vectors (Invitrogen, Carlsbad, CA).

The 5'-RACE experiment was repeated by employing a separate antisense primer for the PCR (5'-GACCTTTACAGGTCCTGCAGG-3' designed for exon II) and fresh mRNA samples.

1. **Nucleosome Mapping**—A chimeric DNA fragment containing the 5'-flanking region, exon 1, and exon II of the FXIIIA gene was prepared by ligation of a 5-μg genomic DNA fragment into a pBluescript II KS+ (50). A probe for S1 nuclease mapping was produced by the single-strand PCR method using Tag DNA polymerase, an antisense primer designed from the sequence in exon II (GSP2, 5'-GCTCTGGGCTTTCTTCC-3'), and the chimeric DNA plasmid as a template. Poly(A)+ mRNAs were purified from MEG-01 and U937 cells using an oligo(dT)-cellulose column. The probe was hybridized with 2 μg of the mRNA sample in 12.5 ml HEPES, pH 6.4, and 0.4 M NaCl at 60 °C for 8 h and then treated with 500–2000 units S1 nuclease (Life Technologies, Inc.) in 30 mm sodium acetate, pH 4.6, 1 mM zinc acetate, 0.2 M NaCl, and 5% glycerol at 37 °C for 60 min. 10 μg of yeast tRNA was added to the reaction mixture, and then the protected DNA was recovered by precipitation with ethanol. The samples were electrophoresed on a 6% polyacrylamide gel containing 8 M urea, together with a sequencing reaction mixture produced by employing the same antisense primer and template chimeric DNA as used in the generation of the probe. DNAs were transferred to a nylon membrane and hybridized with a sense strand digoxigenin (DIG)-labeled RNA probe, which was generated by 20 units of T7 RNA polymerase (Boehringer Mannheim) and the chimeric DNA plasmid as a template. Detection of the DNA was achieved using a DIG nucleic acid detection kit (Boehringer Mannheim).

**Estimation of Intron Excision—** mRNA was subjected to semi-quantitation of mRNA bands by Densitograph 3.01 Imaging Analyzer (Atto Inc., Tokyo, Japan). The fluorescence intensity of the band for FXIIIA was normalized to that for β-actin.

**Primer Extension**—A DIG-labeled oligonucleotide (GSP2) was hybridized with 5 μg of poly(A)+ mRNA at 45 °C for 8 h in a solution containing 0.4 M NaCl and 12.5 mm HEPES, pH 6.4. After ethanol precipitation, the oligonucleotide was extended by Superscript II™ reverse transcriptase using a DIG nucleic acid detection kit (Boehringer Mannheim).

**Construction of CAT Plasmids**—Genomic clones of FXIIIA were obtained in the previous study (5). A 4.5 kb fragment containing the 5'-flanking region of FXIIIA was excised from a phage clone with HindIII and subcloned into a HindIII site of promoter-less pCAT-Basic and Enhancer vectors (Promega, Madison, WI). A fragment containing 290 to +75 relative to the transcription start site was isolated from the subcloned vector by digestion with PstI and inserted into the PstI site of promoter-less CAT vector (pCAT 0), generating pCAT–290/+75 (Fig. 1). A fragment spanning from −2331 to −115 was obtained by digestion of the first CAT construct containing the 4.5-kb fragment with HindIII and SacI and inserted into pCAT–290/+75 digested with HindIII and SacI, generating pCAT–2331/+75. The pCAT–2331/+75 was digested with SacI and XhoI and blunt-ended and circularized, generating pCAT–2331/-115. CAT constructs containing fragments spanning from positions −1593, −806, −114 to +75 were obtained by digestion of HindIII and StuI, SacI, or SacI of pCAT–2331/+75, respectively, and religation. The sequences of 5' and 3'-boundaries of generated CAT constructs were verified both by digestion with several restriction enzymes and by dideoxy sequencing. Large scale plasmid preparation was made using a plasmid purification kit (Qiagen, Valencia, CA).

A series of CAT plasmids harboring either a deletion or nucleotide substitutions that alter or abolish consensus sequences for protein-binding sites was also constructed by site-directed mutagenesis using an oligo-extension PCR protocol (28). Two separate PCR fragments for each half of a final hybrid product were generated employing mutagenesis primers (Table I) and outside primers (forward primer, 5'-CTTCTGAATAATCTCGCC-3'; reverse primer, 5'-CAGGAAACCGTGATGAC-3'). The two products were mixed, and a second PCR was performed using the two outside primers. The resulting products were digested with HindIII and XbaI and ligated into HindIII/XbaI sites of a

---

**Fig. 1.** Constructs for the 5'-flanking region of FXIIIA and promoter activity. Left panel, various lengths of the FXIIIA promoter region (thick solid lines) were inserted into the promoterless CAT plasmid (pCAT 0). Numbers above the constructs indicate positions relative to the transcription start site (+1). Right panels, CAT activity of a series of the deletion constructs was presented relative to that of the pCAT 0. The CAT activity was normalized to β-galactosidase activity. The data are presented as the mean ± S.D. for three independent experiments.
promoterless CAT plasmid. The inserts were then sequenced to confirm the intended mutations.

**TABLE I**

Sequences of the double-stranded oligonucleotides used in this study

| Oligonucleotide | Sequence |
|----------------|----------|
| Site A Wild type | TCCCTTTAGAAAGTATTTTAAAGGCCAACAGTCTTT |
| Mutant* | TCCCTTTAGAAAGTATTTTAAAGGCCAACAGTCTTT |
| Deletion S | CACGGGGGAGGGGGTGGGTCCTC |
| Deletion AS | CTAAACGCGTCTGGAAAGACAGAG |
| Site B Wild type | TGGAGAAAGGGGGAGGGAGAAACCGCTG |
| Mutant* | TGGAGAAATTTGAGGGAAACCGCTG |
| Site C Wild type | CAGATCTAGGGGGAGGGAG |
| Mutant | CAGATCTAGGGGGAGGGAG |
| Mutant 2 | CAGATCTAGGGGGAGGGAG |
| Mutant 3 | CAGATCTAGGGGGAGGGAG |
| Site D Wild type | CTGTTTGGGTTGGGGAGGGGACAGTCT |
| Mutant* | CTGTTTGGGTTGGGGAGGGGACAGTCT |
| XIIIA GATA-1 Wild type | TCACTCGAAAAAGAAGATGAAAG |
| Mutant* | TCACTCGAAAAAGAAGATGAAAG |
| XIIIA Ets-1a Wild type | CACATCTCTCCAGAAGCTTGTTG |
| Mutant* | CACATCTCTCCAGAAGCTTGTTG |
| XIIIA Ets-1b Wild type | TTAATGTGCGAGCCAGGGGACAGT |
| Mutant* | TTAATGTGCGAGCCAGGGGACAGT |
| Consensus Ets-1 | GCATTAGGAGGGAG |
| GATA-1 | GAGCTTCATCCTGATAGAAGAT |
| MZF-1 | GCCATCGGCTGATTGGAG |
| ZN1–4 | GGCATGAGGAGGGAG |
| ZN5–13 | GGCATGAGGAGGGAG |
| PEA-3 | CTGCCAGGAGGAAGGAG |
| NF-1 | TATTTGGTATGAGCAGGCACATAG |
| SP-1 | ATTCGATGGGGGGGGGGAG |

*The oligonucleotides used for mutagenesis.

**RESULTS**

**Northern Blot Analysis**—Northern blot analysis employing total RNAs obtained from cultured cell lines showed a single mRNA band of about 4.0 kb both in U937 and MEG-01 cells, but the band was not detectable in MEG-J, HepG2 or 293 cells (Fig. 2), or HeLa cells (data not shown). Semi-quantitation of the bands by an image analyzer revealed that the FXIIIA mRNA level in U937 cells was 3-fold that in the MEG-01 cells, when normalized to β-actin mRNA levels.

**Determination of the Transcription Initiation Site**—Rapid amplification of the 5′-cDNA ends was performed to identify the transcription start site of the FXIIIA gene. A single band was observed in both U937 and MEG-01 cells (data not shown); a PCR product of the same size was visible in HepG2 cells only after PCR was performed extensively. Sequencing of 10 subclones of the resulting transcripts revealed that all 10 clones started at a G nucleotide located 76 nucleotides upstream from the first exon/intron boundary. This indicated the presence of a single major transcription initiation site for the FXIIIA gene. A single band fell upon an A nucleotide 218 bases upstream of the tentative transcription initiation site (indicated by an arrow in Fig. 3). These results were also confirmed by direct sequencing of the PCR products for U937 and MEG-01 cells (data not shown). When the 5′-RACE experiment was repeated by employing a new antisense primer and fresh mRNA samples, two major bands were observed: one band corresponded to the tentative transcription initiation site (+1), whereas another band fell upon an A nucleotide 218 bases upstream of the tentative initiation site (small asterisk in Fig. 3).

An S1 nuclease mapping analysis, employing a DNA fragment containing the 5′-flanking region, exon I, and exon II of the XIIIA gene, yielded two major bands for both MEG-01 and U937 (data not shown), which corresponded to an A nucleotide 24 bases and a C nucleotide 7 bases upstream from the first transcription initiation site.

**FIG. 2.** Northern blot analysis of the mRNA for FXIIIA in human cultured cell lines. Total RNA from various cell lines was hybridized to the DIG™-RNA probe for FXIIIA. A 4.0-kb band, as indicated by an arrow and labeled FXIIIA, corresponds to the FXIIIA transcript in U937 and MEG-01 cells (upper panel). β-Actin was used as a reference to permit normalization (lower panel).
exon/intron boundary (open triangles in Fig. 3). A primer extension experiment was also performed using the total RNA from U937, MEG-01, and HepG2 and the oligonucleotide GSP2 and yielded a single major fragment (data not shown) that corresponded to an A nucleotide 40 bases upstream from the tentative initiation site (+1), depicted with a large asterisk, was determined by the 5′-RACE method. Another possible transcription initiation site, found by a separate 5′-RACE study, is indicated by a small asterisk. Other candidates for transcription initiation sites determined by the S1 mapping experiment are shown by open triangles and that detected by the primer extension assay is indicated by a circle. Putative regulatory sites are indicated by horizontal arrows for GATA-1, Ets-1, SP-1, MZF-1 (myeloid zinc finger-1), PEA-3, and NF-1 (nuclear factor-1). Two upstream and downstream Ets-1 consensus sequences are designated as Ets-1a and -1b, respectively.

Promoter Activity of FXIIIA—Fig. 3 shows the nucleotide sequence of the FXIIIA promoter region. The four protected sites A–D (boxed) were determined by DNase I footprint analysis (Fig. 4). The tentative transcription initiation site (+1), depicted with a large asterisk, was determined by the 5′-RACE method. Another possible transcription initiation site, found by a separate 5′-RACE study, is indicated by a small asterisk. Other candidates for transcription initiation sites determined by the S1 mapping experiment are shown by open triangles and that detected by the primer extension assay is indicated by a circle. Putative regulatory sites are indicated by horizontal arrows for GATA-1, Ets-1, SP-1, MZF-1 (myeloid zinc finger-1), PEA-3, and NF-1 (nuclear factor-1). Two upstream and downstream Ets-1 consensus sequences are designated as Ets-1a and -1b, respectively.

Promoter Activity of FXIIIA—Fig. 3 shows the nucleotide sequence of the FXIIIA promoter region. The four protected sites A–D (boxed) were determined by DNase I footprint analysis (Fig. 4). The tentative transcription initiation site (+1), depicted with a large asterisk, was determined by the 5′-RACE method. Another possible transcription initiation site, found by a separate 5′-RACE study, is indicated by a small asterisk. Other candidates for transcription initiation sites determined by the S1 mapping experiment are shown by open triangles and that detected by the primer extension assay is indicated by a circle. Putative regulatory sites are indicated by horizontal arrows for GATA-1, Ets-1, SP-1, MZF-1 (myeloid zinc finger-1), PEA-3, and NF-1 (nuclear factor-1). Two upstream and downstream Ets-1 consensus sequences are designated as Ets-1a and -1b, respectively.

Promoter Activity of FXIIIA—Fig. 3 shows the nucleotide sequence of the FXIIIA promoter region. The four protected sites A–D (boxed) were determined by DNase I footprint analysis (Fig. 4). The tentative transcription initiation site (+1), depicted with a large asterisk, was determined by the 5′-RACE method. Another possible transcription initiation site, found by a separate 5′-RACE study, is indicated by a small asterisk. Other candidates for transcription initiation sites determined by the S1 mapping experiment are shown by open triangles and that detected by the primer extension assay is indicated by a circle. Putative regulatory sites are indicated by horizontal arrows for GATA-1, Ets-1, SP-1, MZF-1 (myeloid zinc finger-1), PEA-3, and NF-1 (nuclear factor-1). Two upstream and downstream Ets-1 consensus sequences are designated as Ets-1a and -1b, respectively.

Promoter Activity of FXIIIA—Fig. 3 shows the nucleotide sequence of the FXIIIA promoter region. The four protected sites A–D (boxed) were determined by DNase I footprint analysis (Fig. 4). The tentative transcription initiation site (+1), depicted with a large asterisk, was determined by the 5′-RACE method. Another possible transcription initiation site, found by a separate 5′-RACE study, is indicated by a small asterisk. Other candidates for transcription initiation sites determined by the S1 mapping experiment are shown by open triangles and that detected by the primer extension assay is indicated by a circle. Putative regulatory sites are indicated by horizontal arrows for GATA-1, Ets-1, SP-1, MZF-1 (myeloid zinc finger-1), PEA-3, and NF-1 (nuclear factor-1). Two upstream and downstream Ets-1 consensus sequences are designated as Ets-1a and -1b, respectively.

Promoter Activity of FXIIIA—Fig. 3 shows the nucleotide sequence of the FXIIIA promoter region. The four protected sites A–D (boxed) were determined by DNase I footprint analysis (Fig. 4). The tentative transcription initiation site (+1), depicted with a large asterisk, was determined by the 5′-RACE method. Another possible transcription initiation site, found by a separate 5′-RACE study, is indicated by a small asterisk. Other candidates for transcription initiation sites determined by the S1 mapping experiment are shown by open triangles and that detected by the primer extension assay is indicated by a circle. Putative regulatory sites are indicated by horizontal arrows for GATA-1, Ets-1, SP-1, MZF-1 (myeloid zinc finger-1), PEA-3, and NF-1 (nuclear factor-1). Two upstream and downstream Ets-1 consensus sequences are designated as Ets-1a and -1b, respectively.

Promoter Activity of FXIIIA—Fig. 3 shows the nucleotide sequence of the FXIIIA promoter region. The four protected sites A–D (boxed) were determined by DNase I footprint analysis (Fig. 4). The tentative transcription initiation site (+1), depicted with a large asterisk, was determined by the 5′-RACE method. Another possible transcription initiation site, found by a separate 5′-RACE study, is indicated by a small asterisk. Other candidates for transcription initiation sites determined by the S1 mapping experiment are shown by open triangles and that detected by the primer extension assay is indicated by a circle. Putative regulatory sites are indicated by horizontal arrows for GATA-1, Ets-1, SP-1, MZF-1 (myeloid zinc finger-1), PEA-3, and NF-1 (nuclear factor-1). Two upstream and downstream Ets-1 consensus sequences are designated as Ets-1a and -1b, respectively.
In EMSAs using mutated oligonucleotides as a probe for site C, mutant 1 with four base changes at the MZF-1 site failed to form a complex (Fig. 5C, right, lanes 16 and 19), as did mutant 3 with one base mutation at the same MZF-1 site (Fig. 5C, right, lanes 22 and 24). The mutation in mutant 3 is a G to A polymorphism found in the 5'-flanking region of two unrelated cases with FXIII deficiency (37, 38). EMSAs employing mutant 2, which has a typical SP-1-binding site generated by a T to C change, resulted in an increase in the slower migrating complex and an elimination of the faster migrating complex (Fig. 5C, right, lanes 17 and 20), suggesting that the slower migrating complex was formed with SP-1. The intensity of the slower migrating complex of U937 was much less than that of MEG-01, which is consistent with the fact that Western blotting detected a lesser amount of SP-1 in U937 than in MEG-01 (data not shown).

EMSAs for site D demonstrated essentially the same results as those for site B. There was a nonspecific complex in U937 cells (Fig. 5D, lane 2), whereas two specific complexes appeared to form with an MZF-1-like protein and an SP-1 protein in MEG-01 cells (Fig. 5D, lane 7). The slower migrating complex was prominently recognized in MEG-01 cells. This complex was competed out with unlabeled self, SP-1, and ZN5–13 oligonucleotides (Fig. 5D, left, lanes 8, 9 and 11), whereas it was not completely eliminated by ZN1–4 (Fig. 5D, left, lane 10). A mutant oligonucleotide with four bases changes at the MZF-1 site eliminated both complexes in MEG-01 (Fig. 5D, right, lane 19).

All the above EMSA experiments for sites A, B, C, and D were confirmed by cross-competitions, employing consensus sequences for transcription factors as probes and wild type oligonucleotides for four sites as competitors (Table I).

Finally, supershift assays were performed in order to identify a nuclear protein(s) for each of the above transcription factor-binding sites. When an antibody against NF-1 was added to the binding reaction, a supershifted band appeared for both U937 and MEG-01 cells (Fig. 6 left), indicating that the protein bound to site A was NF-1. An oSP-1 antibody competed away only the slower migrating complexes of sites B, C, and D (Fig. 6, middle), strongly suggesting that these complexes were made by SP-1. Since no antibody against human MZF-1 suitable for supershift assays is available at present, a protein forming the faster migrating complexes could not be determined in this study.

**Functional Analysis of Regulatory Sequences by Site-directed Mutagenesis**—In order to determine the functional significance of the protein-binding sites detected by footprinting and EMSAs, we constructed a series of substitution mutants and a deletion mutant that alter or abolish the consensus sequences by site-directed mutagenesis (Table I). The CAT activity of the mutants was compared with that of the wild type in transfection assays in U937 and MEG-01 cells (Fig. 7, middle four constructs). Mutations in either site B or C resulted in drastic decreases in CAT expression, whereas a mutation in site D had no significant effect in either cell. These results suggest that the MZF-1/SP-1 sequences in sites B and C are important for FXIIIA expression. In particular, the former sequence must be essential for FXIIIA expression, since the mutation in site B reduced the CAT activity of the minimal promoter to virtually background levels in both U937 and MEG-01 cells (Fig. 7, bottom).

A mutation in site A in the minimal promoter, as well as its complete deletion, also resulted in moderate and drastic reductions in activity, respectively, indicating that the NF-1 site is important for the FXIIIA expression, as well.

**Characterization of GATA-1 and Ets-1 Sites in the Enhancer**
Region Specific for MEG-01 Cells—Deletion analysis in the CAT assay revealed the presence of an enhancer region specific for MEG-01 which spans from −806 to −290 (Fig. 1). A computer-assisted search in the TRANSFAC 3.2 data base revealed that this region contained one GATA-1 and two Ets-1 consensus sequences (Fig. 5), which were reported as enhancers in the 5′-flanking regions of several megakaryocyte-specific genes (34, 39–42). These sites were examined first by EMSAs. For the GATA-1 site, there was one shifted band in U937 cells (Fig. 8, left, lane 1) and two in MEG-01 cells (lane 6). These complexes were competed away by an excess self-competitor (lanes 2 and 7). The faster migrating complex was competed by a mutant oligonucleotide for the GATA-1 site (lanes 3 and 8), and the slower migrating complex was abolished by a GATA-1 consensus oligonucleotide (lanes 4 and 9), although the labeled mutant oligonucleotide failed to form any complex (lanes 5 and 10). Thus, it was suggested that the slower migrating complex was formed by a GATA-1-like protein(s), and the faster one by a nonspecific protein. This assumption is confirmed by the fact that both antibodies against GATA-1 and -2 supershifted only the nonspecific complex. This result confirmed the correctness of the conclusion.
EMSAs for the putative Ets-1b site showed the same results as those for Ets-1a (Fig. 8, right). These results suggested that GATA-1 and Ets-1 might contribute to the expression of FXIIIA in MEG-01 cells.

In order to determine the functional significance of these protein-binding sites detected by EMSAs and the supershift assays, we performed functional analyses by employing substitution mutants that alter the consensus sequences by site-directed mutagenesis (Table I). A mutation of the GATA-1 site led to a great reduction in the CAT activity in MEG-01 cells, whereas only a mild decrease was observed in U937 cells (Fig. 7, top four). In contrast, mutations of both Ets-1a and -1b sites had no significant effect on the CAT activity. Thus, these results indicate that the GATA-1 site is responsible for the enhancer activity of this region for FXIIIA expression in MEG-01 cells, but the Est sites are not. Moreover, both αEts-1 and αEts-1/Ets-2 antibodies failed to supershift or compete away the complexes of Ets-1a and Ets-1b sites (data not shown), suggesting that these complexes might be formed with Ets-like proteins other than Est-1 and Est-2.

DISCUSSION

FXIIIA is present inside certain cells originating from the bone marrow including monocytes/macrophages (6–11) and megakaryocytes/platelets (9, 15), whereas FXIIIB is apparently produced in hepatocytes (43, 44). It was previously demonstrated that peripheral monocytes and U937 cells contained the mRNA for FXIIIA (10, 12). In the present study, we detected the FXIIIA mRNA not only in U937 cells but also in a megakaryocytoid cell line (MEG-01) by Northern blot analysis. These results are consistent with the microscopic and biochemical observations as mentioned in the Introduction.

In the present study, we have tentatively defined the transcription start site of FXIIIA by 5'-RACE using total RNAs obtained from both U937 and MEG-01 cells. Since no intronic sequence was found in the transcripts of these cell lines, it was concluded that there are no alternative promoter regions for the FXIIIA gene. The transcription initiation site is located 76 nucleotides upstream from the exon I/intron A boundary and only 13 nucleotides upstream from the longest cDNA (14).
Thus, exon I codes for only the 5'-untranslated region in the FXIII A gene (5).

This study revealed several cis-elements and trans-acting factors involved in the constitutive and cell type-specific expression of FXIIIA. These include hematopoietic- or myeloid-specific elements such as MZF-1, GATA-1, and Ets-1 as well as general elements such as NF-1 and SP-1.

Core Promoter of the FXIII A Gene—It is common in many TATA-less promoters that transcription initiates from a cluster of nuclear protein-binding sites. Protein binding to the NF-1 element at site A was abundant in U937 and MEG-01 cells expressing the FXIIIA mRNA but essentially absent in HepG2, 293, HeLa, and MEG-J cells. These results are consistent with the fact that the latter cell lines did not show any transcript in the Northern blotting analysis. The mutation of site A led to a moderate loss of the promoter activity and a complete loss of the NF-1 binding to site A. Accordingly, the NF-1 element in site A is important for the expression of FXIIIA in U937 and MEG-01 cells. The NF-1 element at site A alone, however, is not sufficient to drive FXIIIA expression, since a DNA fragment containing site A alone did not demonstrate any CAT activity. The removal of site A (27 bases, −15 to −39) led to a nearly complete loss of the promoter activity, suggesting that proper space arrangement around the transcription start site may be required for FXIIIA gene expression.

The MZF-1/SP-1 element at site B (−92 to −73) appeared to be most essential for the expression of FXIIIA, since the removal or mutation of this site completely abolished the promoter activity in all cell lines. Both SP-1 and MZF-1-like proteins bind site B in MEG-01 cells, whereas only MZF-1-like protein binds site B in U937 cells. MZF-1 protein is preferentially expressed in myeloid leukemia cell lines and in myeloid progenitor cells from normal marrow (45). Accordingly, the MZF-1 element at site B may play a central role in FXIIIA transcription.

It is of note that the DNA-protein complex formed by SP-1 was present in MEG-01 but absent or in trace amounts only in U937 cells for sites C and D as well as for site B. The SP-1 protein may modulate but may not be indispensable for FXIIIA expression, since U937 cells contain a large amount of FXIIIA mRNA and demonstrate strong promoter activity despite the fact that U937 cells contain only a small amount of SP-1 protein.

Upstream Regulatory Sequences—cis-Acting elements located upstream from the promoter regions can also modulate the transcriptional activity. The MZF-1/SP-1 elements at sites C and D were present side by side in a region that acted as a weak enhancer in U937 cells, but as a silencer in MEG-01 cells. The MZF-1-like protein and SP-1 bound to these sites in MEG-01 cells, whereas the complex formed by SP-1 was not observed in U937 cells. Thus, it is possible that SP-1 may act as a negative trans-activator or repressor by interacting with the MZF-1-like protein at sites C and D in MEG-01 cells. MZF-1-like protein per se, in turn, is likely to be a weak trans-activator of basal expression in U937 cells.

The nuclear protein that bound to site C showed low affinity for MZF-1 consensus oligonucleotides despite the fact that the MZF-1 sequence of site C is exactly the same as that of site D (Fig. 3). Therefore, it is suggested that this MZF-1-like protein bound to site C may differ from that bound to sites B and D and could be a novel MZF-1 protein.

It is of interest that a G to A polymorphism was found in the 5'-flanking region of two patients with FXIII deficiency (37, 38). This polymorphism falls exactly upon site C and eliminates the complexes of site B; moreover, the site C mutant 3 did not form complexes with either SP-1 or the MZF-1-like protein. Accordingly, the replacement of G by A nucleotide may affect FXIII expression and its plasma levels in the population.

It has been reported that GATA-1 and Ets proteins play important roles in the expression of many genes in the megakaryocytic lineage (34–42). A GATA-1 and two Ets-1 motifs were found in an enhancer region of FXIIIA, and nuclear proteins bound to these sites. The mutation of the GATA-1, but not those of Ets elements, resulted in a great
reduction in the promoter activity in MEG-01 cells. Accordingly, it was concluded that the GATA-1 motif is involved in the megakaryocytic expression of the FXIIIA gene.

It is of interest that there is also a short tandem repeat (STR) polymorphism in the 5′-flanking region of FXIIIA (46). Since the GATA-1 site and STR polymorphism are adjacent to each other, the STR polymorphism may have implications in activation of expression by the GATA proteins and/or their binding affinity to DNA.

Possible interactions between these proteins (MZF-1-like protein, SP-1, and GATA-1 and GATA-2) and with FXIIIA promoter elements likely modulate FXIIIA expression, resulting in a complex pattern of tissue- and cell type-specific gene regulation. A precedent for this is found in the fact that MZF-1 represses the gene expression of CD34, a hematopoietic stem cell antigen, in non-hematopoietic cell lines such as NIH3T3 and 293, whereas it activates gene expression in hematopoietic cell lines such as K562 and Jurkat (47).

Gene Regulation of TGase Family—There are many other TGases (48). The genes for these TGases share significant similarity in their organization and have evolved from a common ancestral gene. Their nucleotide sequences in the 5′-flanking regions, however, are not homologous to each other, and mechanisms for their gene regulation seem to be diverse as well (25, 26, 49). For example, the FXIIIA gene has exon I encoding only a 5′-untranslated region, and its translation start site ATG is present in exon II, whereas other TGase genes have their translation start sites inside exon I adjacent to the transcription start sites (Fig. 9). The genes for keratinocyte and tissue TGases are regulated by TATA-like (CATA) and TATA promoters, whereas that for FXIIIA is not. Accordingly, these genes do not share common transcription regulatory elements (Fig. 9). Thus, the tissue-specific transcriptional regulation of each gene is controlled by differential interactions between positive and negative cis-acting elements and their corresponding transcription factors. In conclusion, the TATA-less promoter of FXIIIA is controlled by transcription factors expressed with tissue specificity, preferentially in myeloid cells, which distinguishes FXIIIA from other members of the TGase family.

Further investigations are required to examine possible effects on the promoter activity of the polymorphisms including the nucleotide substitution and STR polymorphism in the 5′-flanking region of FXIIIA. In addition, the possibility of cooperative regulation in the transcription of the FXIIIA and FXIIIIB genes will be studied in the future, since plasma levels of both subunits are partially determined by the presence and absence of their counterparts (2, 3). Since U937 and MEG-01 cells are known to be stimulated and differentiated by phorbol ester, granulocyte/macrophage colony-stimulating factor, and retinoic acid, possible effects of these agents on the transcriptional activity of FXIIIA need to be examined, as well.

Acknowledgments—We thank Drs. S. Yokoyama and H. Mizoguchi for providing U937 and MEG-01 cell lines, respectively; Drs. Naoko Tanese and Dennis Watson for providing an αNF-1 and αEst-1 antibodies, respectively; Drs. T. Izumi and T. Yamazaki for helpful discussions; and L. Boba for help in preparation of the manuscript.

REFERENCES

1. Schwartz, M. L., Pizzo, S. V., Hill, R. L., and McKee, P. A. (1973) J. Biol. Chem. 248, 1395–1407
2. Lorand, L., Losowsky, M. S., and Miloszewski, K. J. M. (1980) Prog. Hemost. Thromb. 5, 245–290
3. Ichinose, A. (1994) in Haemostasis and Thrombosis (Bloom, A. L., Forbes, C. D., Thomas, D. P., and Tuddenham, E. G. D., eds) pp. 531–546, Churchill

FIG. 9. Schematic diagram of 5′-flanking regulatory sequences in FXIIIA and other TGases. The transcription and translation initiation sites were indicated by +1 and ATG, respectively. *+, the sequences of transcription factors in the 5′-flanking region of tissue TGase have not been experimentally determined by protein-binding experiments.
Transcriptional Regulation of the Human FXIII A Subunit Gene

Livingstone, Edinburgh

4. Board, P. G., Webb, G. C., McKee, J., and Ichinose, A. (1986) Cytogenet. Cell Genet. 48, 25–27
5. Ichinose, A., and Davie, E. W. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5829–5833
6. Henriksson, S., Becker, S., Lynch, G., and McDonagh, J. (1985) J. Clin. Invest. 75, 528–534
7. Muszbek, L., Adánya, R., Szegedi, G., Polgár, J., and Kávai, M. (1985) Thromb. Res. 37, 401–410
8. Adánya, R., Belkin, A., Vasilevskaya, T., and Muszbek, L. (1985) Eur. J. Cell Biol. 38, 171–173
9. Poon, M. C., Russell, J. A., Low, S., Sinclair, G. D., Jones, A. R., Blahey, W., Euscher, B. A., and Hoar, D. L. (1989) J. Clin. Invest. 84, 787–792
10. Weisberg, L. J., Shiu, D. T., Conkling, P. R., and Shuman, M. A. (1987) Blood 70, 579–582
11. Grundmann, U., Amann, E., Zettlmeissl, G., and Küpper, H. A. (1986) Br. J. Haematol. 59, 171–173
12. Trimble, C. L., Gray, M. H., and McNutt, N. S. (1992) J. Biol. Chem. 267, 14183–14187
13. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8024–8028
14. Ichinose, A., Hendrickson, L. E., Fujikawa, K., and Davie, E. W. (1986) Biochim. Biophys. Acta 1247, 127–134
15. Kesselbach, T. H., and Wagner, R. H. (1972) Ann. N. Y. Acad. Sci. 202, 318–328
16. Kaczmarek, E., Liu, Y., Berse, B., Chen, C. S., and McDonagh, J. (1995) Biochim. Biophys. Acta 1278, 267–272
17. Adánya, R., and Antal, M. (1996) Thromb. Haemostasis 76, 74–79
18. Grundmann, U., Amann, E., Zettlmeissl, G., and Küpper, H. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 579–582
19. Thacker, S. M., and Rice, R. H. (1985) Cell 20, 4355–4365
20. Thacker, S. M., and Rice, R. H. (1985) J. Biol. Chem. 260, 14183–14187
21. Polymoropoulo, M. H., Rath, D. S., Xiao, H., and Merril, C. R. (1991) Nucleic Acids Res. 19, 4306–4309
22. Morris, J. F., Rouschier, F. J., III, Davis, B., Krishna, M., Xu, D., Tenen, D., and Hromas, R. (1995) Blood 86, 3649–3647
23. Ichinose, A., Bottenus, R. E., and Davie, E. W. (1996) J. Biol. Chem. 271, 1272–1279
24. Ichinose, A., Belkin, A., Vasilevskaya, T., and Muszbek, L. (1985) Eur. J. Cell Biol. 38, 171–173
25. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8024–8028
26. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Biochemistry 25, 1786–1795
27. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
28. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
29. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
30. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
31. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
32. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
33. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
34. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
35. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
36. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
37. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
38. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
39. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
40. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
41. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
42. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
43. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
44. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
45. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
46. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
47. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
48. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
49. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489
50. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) Blood 68, 1475–1489