Characterization of the Human Blood Coagulation Factor X Promoter*

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Blood coagulation Factor X is a serine protease required for both the intrinsic and extrinsic pathways of coagulation. The gene for Factor X spans 27 kilobases and is located on chromosome 13, in close proximity to the gene encoding Factor VII. Expression of Factor X is restricted to the liver. We have characterized the human Factor X promoter by mapping the start sites of transcription and carrying out a functional analysis of the promoter. The first 279 base pairs (bp) of 5'-flanking sequence upstream from the first AUG are sufficient to confer maximal promoter activity in HepG2 cells. Protein-binding sites within the 279-bp fragment are defined using gel mobility shift assays. Mutagenesis of two specific sequences within the 279-bp fragment (CCAAT at -120 to -116, and ACTTTG at -96 to -51), results in loss of ability to bind proteins from a HepG2 nuclear extract, and profound reduction in promoter activity of the 279-bp fragment. We conclude that these two protein-binding sites are critical for the activity of the Factor X promoter.

The vitamin K-dependent procoagulant proteins, Factors II, VII, IX, and X, are the major enzymes of the coagulation cascade. Like the cofactors V and VIII, their expression is restricted to the liver, i.e. is tissue-specific (1). Recently, a number of liver-specific transcription factors have been isolated and characterized (2-7), but the interactions of these elements with the genes for the vitamin K-dependent clotting factors remain largely unexplored. Whether there is any degree of coordinate regulation of the vitamin K-dependent factors is also unknown. The human Factor IX and Factor II promoters have been characterized to some extent (8-10), and for the IX promoter, Crossley and Brownlee (10) have shown a binding site for the transcription factor C/EBP. The Factor X promoter has not been previously characterized; indeed, very little 5'-flanking sequence has been published to date. This paper, we report the functional characterization of the human Factor X promoter, including the sequence of 745 bp of flanking DNA, the start site of transcription, activity of the promoter using a reporter gene assay, and delineation of specific protein-binding domains within the promoter. One of these protein-binding regions, conserved in the promoters of Factors IX and VII, may be involved in coordinate regulation of the genes encoding the vitamin K-dependent clotting factors. Mutagenesis of the protein-binding domains described here is associated with loss of the protein-binding property and loss of promoter activity as well.

MATERIALS AND METHODS

Anchored PCR

Anchored PCR was carried out using the procedure of Loh et al. (13) with modifications. Using 0.1 µg of human liver poly(A) RNA (gift of Dr. D. W. Stafford, University of North Carolina at Chapel Hill) as starting material, first strand cDNA was synthesized using a Factor X-specific primer (5'-CCAGAATTCATTCTGTCTTCGCT GTCCTC-3', located 244 bp downstream from the first AUG) and 200 units of Moloney murine leukemia virus H- reverse transcriptase (Bethesda Research Laboratories), which lacks RNase H activity. 20 units of RNasin was included in this reaction. Excess primer and unincorporated deoxynucleotides were removed by two passages through Sephadex G-50, and first strand cDNA was then tailed with poly(G) using terminal deoxynucleotidyl transferase (Boehringer Mannheim). The tailed FX cDNA was amplified using a second Factor X-specific primer (5'-TGCGTCTCCTGGCAGATAC AGACTT-3', located 95 bp downstream from the first AUG) and a mixture of nonspecific primers ANC (5'-GCAAGCTTGAATTC CGATCCCTCCCCGCCCGG-3') and AN (5'-GCAAGCTTGTTGAA CTTCGGATCC-3') in a ratio of 1:10 for the poly(G) end of the cDNA. After amplification, the PCR product was gel-purified and subcloned into an M13 vector by blunt-end ligation for sequence analysis (14).

S1 Nuclease Analysis

S1 nuclease analysis was carried out using the procedure of Sharp et al. (15). A 357-base single-stranded DNA probe, beginning 279 bases upstream from the first AUG and continuing through exon 1, was 5'-end labeled and hybridized to 12.5 µg of total human liver RNA. After treatment with 50 units of S1 nuclease (Bethesda Research Laboratories) at 30 °C, the DNA-RNA heteroduplexes were electrophoresed on a 6% denaturing acrylamide gel. Nucleotide sequence was run on the same gel as a size marker. Hybridization of the probe to yeast tRNA (Boehringer Mannheim) served as a negative control.

Isolation and Characterization of 5'-Flanking Region of Human Factor X

Previously published data on the 5'-flanking region of human Factor X included only 281 bp upstream from the first AUG (11). This sequence was amplified from human genomic DNA using primers based on the published sequence. To obtain additional 5'-flanking sequences, 2.8 × 105 cosmids clones from a human genomic

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† The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; hGH, human growth hormone; SEAP, secreted alkaline phosphatase plasmid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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placenta library (Stratagene, La Jolla, CA) were screened using the available 5'-flanking sequence (~279 bp → +12) as a probe. One positive cosmid clone was obtained. Based on restriction analysis and PCR amplification of downstream exons within the clone (exon 1 was present, exon 2 was not), the clone appears to contain at least 10 kb of 5'-flanking sequence. A 3.5-kb BamHI fragment, spanning the region from 2.8 kb upstream from the first AUG to the first intron, was subcloned into pBR322, sequenced, and used as starting material for preparing transfection constructs. The sequencing strategy consisted of synthesizing a sequencing primer based on the known 5'-flanking sequence, and preparing subsequent primers based on data from each run to “walk” further upstream.

**Tissue Culture**

HepG2 cells (16) (originally from Wistar Institute) and HeLa cells (17) were obtained from the Tissue Culture Facility in the Lineberger Cancer Research Center at the University of North Carolina-Chapel Hill. The following materials were also obtained through the Tissue Culture Facility in the Lineberger Cancer Research Center at UNC-CH: fetal bovine serum (HyClone Laboratories, Inc.), Eagle’s minimal essential medium (containing Eagle’s salt and L-Glutamine), phosphate-buffered saline (pH 7.5), 0.4% trypan blue stain (Bethesda Research Laboratories), 0.05% trypsin, 0.53 mM EDTA-4Na (Bethesda Research Laboratories), sterile H2O (endotoxin-free), dimethyl sulfoxide (Sigma), and CaCl2·2H2O (cell culture grade, Sigma). HepG2 cells were grown in Eagle’s minimal essential medium supplemented with 10% fetal calf serum, 0.01% penicillin, and 0.01% streptomycin. HeLa cells were grown in Dulbecco’s minimal essential medium–H with 10% fetal calf serum, and 0.025% kanamycin.

**Reporter Gene Assays**

**Preparation of Constructs in pOGH Vector**—The pOGH vector (18) employs insertion of hGH into the medium as an assay for eukaryotic promoter strength. The vector contains hGH structural sequences cloned into the BamHI-EcoRI sites of pUC12. For the assays for Factor X promoter activity, varying lengths of Factor X 5’-flanking sequence were cloned into the HindIII-XbaI site of pOGH by directional cloning (see Fig. 1). The flanking sequence fragments were generated by PCR and deleted at the 5’ end using convenient restriction sites. The 3’ amplifying oligonucleotide contains an engineered XbaI site within the translated sequence, used for subcloning into pOGH (see Fig. 2). All inserts contain the entire 5’-untranslated sequence. The PCR-generated fragments were sequenced to verify that no errors had been introduced in the amplification step.

**hGH Assay**—Levels of hGH in the medium were measured with a solid-phase two-site radioimmunoassay kit under the conditions recommended by the manufacturer (Nichols Insitute Diagnostics, CA). The lower limit of detection of the assay is 0.5 ng of hGH/ml; the assay is linear in the range of 0.5–50 ng/ml.

**In Vitro Synthesis Conditions**—Optimal transfection conditions were determined for each cell line using a secreted alkaline phosphatase plasmid (SEAP) (19). For assays of promoter activity, cells were cotransfected with pOGH constructs and the SEAP plasmid; levels of SEAP activity were used to normalize transfection efficiency. For HepG2 cells, transfection efficiency varied over a 3-fold range and for HeLa cells over as much as 5-fold. For HepG2 cells, 106 cells in 60-mm tissue culture dishes were transfected with 2 μg of SEAP and 2 μg of pOGH FX5’ by the CaPO4 precipitation method (20). After 5 h of exposure to DNA precipitates, the cells were washed with phosphate-buffered saline and fresh medium was added. Forty-eight hours after transfection, the medium was harvested to perform SEAP and hGH assays. For HeLa cells, optimal transfection conditions are slightly different. 5 × 105 cells in 60-mm tissue culture dishes were transfected with 6 μg of pOGH FX5’ and 2 μg of SEAP using the CaPO4 method. Sixteen hours later the cells were washed with phosphate-buffered saline and fresh medium supplemented with 5 mM sodium butyrate was added. Seventy-two hours after transfection, the medium is assayed for hGH and SEAP.

**Preparation of Nuclear Extracts**

Approximately 1 × 106 HepG2 cells were used to prepare the nuclear extract. The cells were grown to 50% confluence and procedures were performed as previously described (21–23). All steps were carried out at 0–4°C. Following gentle lysis of nuclear proteins in the presence of protease inhibitors, extracts are dialyzed, divided into 100-μl aliquots, frozen in liquid N2, and stored at −70°C.

**Fig. 1.** Constructs used in reporter gene assays. Varying lengths of Factor X promoter were subcloned into the HindIII-XbaI site of pOGH by directional cloning. The HindIII site in the vector was blunt-ended with Klenow fragment. The Factor X promoter fragments were generated using PCR; the 3’-oligonucleotide contained an XbaI site located within exon 1. The creation of the XbaI site does not alter any sequences within the Factor X promoter. The 5’ ends were generated by restriction enzymes and filled in with Klenow fragment if necessary. The constructs were sequenced through the regions of the inserts to insure that no errors had been introduced through cloning or PCR.

**In Vitro Synthesis of C/EBP Protein**

The cDNA of rat C/EBP (24) was the gift of Professor S. McKnight (Carnegie Institution of Washington). The cDNA was released from pMSV and subcloned into pSP6/T3 (BRL) in the same orientation as the T3 promoter. Following transcription with T3 RNA polymerase, in vitro translation was performed using a rabbit reticulocyte lysate system as described by the manufacturer (Promega). Purified C/EBP fragment containing the 86 COOH-terminal amino acid residues was the gift of Dr. Z. Cao and Prof. S. McKnight of Carnegie Institute of Washington, Baltimore, MD.

**Gel Mobility Shift Assays**

The basic procedure is that of Chodosh et al. (25). Oligonucleotide probes were prepared on an oligonucleotide synthesizer (Millipore), annealed, and 5’-end labeled using T4 polynucleotide kinase. Probes included the following: Factor X promoter sequence from −1135 to −103 (CCAAAT) same probe except CCAAT sequence changed to AGCTA (mCCAAT); promoter sequence from −68 to −39 (ACTTTG); same probe except ACTTTG has been changed to GACAA (mACTTTG); and a probe spanning the same region of the promoter and containing a single nucleotide change from wild-type, T→A at −62 (M→A). These were used for both the binding and competition assays. Binding conditions were optimized by varying the ionic strength of the binding buffer and the amount of nonspecific DNA or poly(dI-dC) added. For binding reactions with HepG2 nuclear extract, the DNA probe was incubated with 10 μg of the extract in 24 μl of 10 mM HEPES (pH 7.6), 40 mM KCl, 3 mM MgCl2, 4% Ficoll, 0.5 mM dithiothreitol, and 2 μg of dl-dC for 15 min at room temperature. For binding reactions with purified protein (either native protein or translation product), the DNA probe was incubated with either 5 ng of purified C/EBP fragment or 1/25 (2 μl) of the translation product from 1 μg of C/EBP. The transcript was produced in 20 μl of 10 mM HEPES (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 1 mg/ml bovine serum albumin, and 0.5–1.0 μg of dl-
dc at room temperature for 15 min. Varying amounts of DI-dC (0.1-2.0 μg) or salmon sperm DNA (0.2-2.0 μg) were tested to determine optimal binding conditions. In all cases, the whole reaction was loaded onto a 5% nondenaturing acrylamide gel (bis-acrylamide = 1:30) containing 0.5 × TBE and 5% glycerol and run in 0.4 × TBE at 300 volts at 4 °C. When retarded bands were observed, 15-150-fold concentration of unlabeled oligonucleotide probes were included in the reaction for the competition. A double stranded DNA fragment containing the C/EBP-binding site, spanning the sequence from −178 to +53 of the factor IX promoter, was included as a positive control for C/EBP binding (10).

### Mutagenesis of Factor X Promoter at Potential Protein-binding Sites

Mutagenic oligonucleotides were synthesized in an oligonucleotide synthesizer by including the three non-wild-type precursors at each step of the synthesis, so that the sequence of the protein-binding region is replaced by randomly incorporated non-wild-type nucleotides. The oligonucleotide is designed such that both the 3' and the 5' ends contain 12 nucleotides of wild-type DNA sequence. Uracil-containing DNA templates were prepared by the method of Kunkel (26) and annealed to the primers described above. In vitro primer extension was carried out using 10 units of T4 DNA polymerase (New England Biolabs) and 800 units (2 μl) of T4 DNA ligase (New England Biolabs) and incubating at room temperature for 4 h. 1/50 to 1/20 of the reactions (10-25 ng of DNA) were used to transform *Escherichia coli* wild-type strain JM 103. Mutations were identified by sequence analysis. The mutated M13 templates were subjected to PCR, and the result DNA fragments were subcloned into pOGH plasmid vectors. Each individual clone was sequenced to insure that no mistakes were introduced into the promoter fragments during PCR and to verify the DNA sequences in the mutated regions.

### RESULTS

#### Start Site of Transcription

The start site of transcription for Factor X has not previously been determined. We have used both anchored PCR and S1 nuclease analysis to map the start sites of transcription. Briefly, anchored PCR involves reverse transcription of RNA, priming with a Factor X-specific primer and poly(C). The resulting PCR products are subcloned into M13 and sequenced to disclose transcription start sites. Human liver containing DNA from Factor X were cloned into the human growth hormone-containing plasmid pG0H (18) (Fig. 2) and introduced into HepG2 cells by calcium phosphate transfection. Deletion constructs were made using appropriate restriction sites or PCR fragments; pG0H, the growth hormone plasmid without any 5' flanking sequence, was used as a negative control. Optimal transfection conditions for HepG2 cells were first determined, and involved addition of 4 μg of DNA × 5 × h without glycerol shock. Transfection efficiency was measured using pBC12/CMV/SEAP (19) (gift of Dr. B. Cullen, Howard Hughes Medical Institute, Duke University) and the results, which represent the average of 4-10 transfection experiments, are shown in Fig. 3A. (Numbers of transfection experiments and standard deviations for each are shown in Table I.) The highest levels of promoter activity were present in the fragment containing 279 bp of DNA upstream from the first AUG. The addition to the construct of the next 109 bp upstream has little effect on promoter activity. Inclusion of additional 5' flanking sequences gradually diminishes activity, so that the 2.8-kb construct from Factor X were cloned into the human growth hormone-containing plasmid pG0H (18) (Fig. 2) and introduced into HepG2 cells by calcium phosphate transfection. Deletion constructs were made using appropriate restriction sites or PCR fragments; pG0H, the growth hormone plasmid without any 5' flanking sequence, was used as a negative control. Optimal transfection conditions for HepG2 cells were first determined, and involved addition of 4 μg of DNA × 5 × h without glycerol shock. Transfection efficiency was monitored using pBC12/CMV/SEAP (19) (gift of Dr. B. Cullen, Howard Hughes Medical Institute, Duke University) and the results, which represent the average of 4-10 transfection experiments, are shown in Fig. 3A. (Numbers of transfection experiments and standard deviations for each are shown in Table I.) The highest levels of promoter activity were present in the fragment containing 279 bp of DNA upstream from the first AUG. The addition to the construct of the next 109 bp upstream has little effect on promoter activity. Inclusion of additional 5' flanking sequences gradually diminishes activity, so that the 2.8-kb construct has only 55% of the maximal promoter activity observed with the 279-bp construct. For constructs shorter than the 279-bp fragment, the 209-bp fragment again has close to maximal activity. Further deletions result in substantial loss of activity, so that the 121-bp fragment has only 23% of maximal activity, and deletion of the next 13 bp (the 108-bp fragment) containing the CCAAT sequence (see below) results in a 4-fold further reduction in activity. Thus, the major elements required for Factor X promoter activity

![Fig. 2. 5' Flanking sequence of human Factor X gene. The sequence reported was determined from a cosmid clone from a human placental library. The 281-bp immediately upstream from the translation start site had been previously reported (11). The nucleotides in this sequence which differ from the previous report are marked with an asterisk. Lower case denotes translated sequence. Transcription start sites are marked with an arrow. The location of the engineered XbaI site is denoted by a diamond. The areas that were mutated for the gel shift and reporter gene assays are underlined. The fragments used as probes in the gel shift assays are enclosed in brackets.](image-url)
the presence of the sequence ACTTTG, which is conserved putative protein-binding site CCAAT (29) and the other by a 30-mer which is identical except that ACTTTG has been changed.

Results with the CCAAT-containing probe are shown in Fig. 2 for location within the promoter) are shown in Fig. 4b. In HepG2 cells, the fragment between 209 and 388 bp upstream from the first AUG confers maximal promoter activity; in HeLa cells, however, maximal promoter activity is seen with the 209-bp fragment, and inclusion of the next 179 bp of sequence ACTTTG has been altered by random mutagenesis. There are perhaps a very modest decrease in the intensity of the slowest moving band at 150-fold excess. When the mutated sequence is used as probe (lanes 8–10), the slowest moving band is faintly detected (lane 9), but the other bands are not detected at all. This band is no longer detected after the addition of a 150-fold excess of the mutant probe. Thus the slowest moving band may result from binding at a site other than CCAAT within the 30-base sequence used as probe.

Similar studies with the ACTTTG-containing probe (see Fig. 2 for location within the promoter) are shown in Fig. 4b. Lane 1 contains probe alone and lane 2 probe and HepG2 nuclear extract. Two bands are apparent in close proximity, with the slower moving band being more intense. Addition of 15-fold excess of unlabeled competitor abolishes the faster moving band and greatly reduces the intensity of the slower moving band; addition of a 60-fold excess of competitor abolishes this band as well. When mutant 30-mers, in which the sequence ACTTTG has been altered by random mutagenesis to GACCTA, are used as unlabeled competitors (lanes 5–7, designated mACTTTG) no reduction in either band is seen, even at 150-fold excess of competitor. When the mutant oligonucleotides are used as probe (lanes 8–10), no retarded bands are seen, confirming that alteration of the ACTTTG site results in loss of the characteristic protein-binding property of the DNA sequence.

The location within the promoter of the 30-base oligonucleotide probe (designated CCAAT) is shown on Fig. 2. Lane 1 contains oligonucleotide probe alone; lane 2, the probe has been incubated with 10 μg of HepG2 nuclear extract.

Three slower moving bands, representing protein-DNA complexes, are seen. Addition of a 15-fold excess of unlabeled competitor (unlabeled oligonucleotide, lane 3) markedly reduces the intensity of these bands and a 60-fold excess (lane 4) eliminates them, suggesting that the binding is specific for the fragment used.

In lanes 5–7, the same 30-mer has been used as probe, but a mutated 30-mer (mCCAAT), in which the sequence CCAAT has been changed to AGCTA by random mutagenesis, is used as unlabeled competitor. Note that even at 150-fold excess, the mutated sequence does not compete well with the wild-type sequence for binding of proteins in the HepG2 nuclear extract. There is perhaps a very modest decrease in the intensity of the slowest moving band at 150-fold excess. When the mutated sequence is used as probe (lanes 8–10), the slowest moving band is faintly detected (lane 9), but the other bands are not detected at all. This band is no longer detected after the addition of a 150-fold excess of the mutant probe. Thus the slowest moving band may result from binding at a site other than CCAAT within the 30-base sequence used as probe.

In order to determine whether the promoter element under study is adequate to confer tissue specificity, the Factor X promoter-containing hGH constructs were transfected into HeLa (17) cells, a malignant cell line which does not normally express Factor X. Direct comparison of results with HepG2 is difficult, since optimal transfection conditions differ in the two cell lines. However, maximal activity using optimal transfection conditions in HeLa cells was only 20% of the maximum observed with HepG2 cells. Comparison of relative activities in the two cell lines is also informative (see Fig. 3).

Identification of Protein-binding Sites within the Factor X Promoter—Within the X promoter, two regions were selected for further study, one characterized by the presence of the putative protein-binding site CCAAT (29) and the other by the presence of the sequence ACTTTG, which is conserved in the promoters of Factors VII, IX, and X (11).

As a first step, gel mobility shift assays using normal and mutant oligonucleotides from these regions were carried out. Results with the CCAAT-containing probe are shown in Fig. 4a. The location within the promoter of the 30-base oligonucleotide probe (designated CCAAT) is shown on Fig. 2. Lane 1 contains oligonucleotide probe alone; lane 2, the probe has been incubated with 10 μg of HepG2 nuclear extract. The results with the wild-type sequence are shown in Fig. 2. A, in HepG2 cells protein-binding activity is measured by quantitating growth hormone activity in the medium. Results reported are the average of 4–10 transfection experiments. The activity of the maximally active construct (279-bp fragment) is arbitrarily set at 100%, and other results are reported as a percent of the maximum. B, in HeLa cells maximal activity is seen with the 209-bp fragment, and other results are expressed as a percentage. All results are the average of at least two transfection experiments.

Table 1

| 5' Fragments | Relative activity ± S.D. |
|--------------|-------------------------|
| 2.8 kb       | 55.5 ± 13.6 n = 4       |
| 547 bp       | 75.2 ± 15.9 n = 10      |
| 474 bp       | 76.1 ± 7.6 n = 10       |
| 388 bp       | 99.8 ± 6.6 n = 4        |
| 279 bp       | 100                     |
| 209 bp       | 93.1 ± 19.5 n = 5       |
| 121 bp       | 22.8 ± 2.8 n = 7        |
| 108 bp       | 6.25 ± 1.3 n = 4        |

Standard deviation = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n - 1}}. n, number of independent transfection experiments; \bar{x}, relative activity of one experiment; \bar{x}, mean of relative activities of separate experiments.

Fig. 3. Promoter activity of deletion constructs. A, in HepG2 cells promoter activity is measured by quantitating growth hormone activity in the medium. Results reported are the average of 4–10 transfection experiments. The activity of the maximally active construct (279-bp fragment) is arbitrarily set at 100%, and other results are reported as a percent of the maximum. B, in HeLa cells maximal activity is seen with the 209-bp fragment, and other results are expressed as a percentage. All results are the average of at least two transfection experiments.
The transcription factor C/EBP does not bind to the CCAAT sequence within the Factor X promoter. The transcription factor C/EBP, present in liver and adipose tissue (4, 31), binds to a number of different DNA elements, some characterized by the presence of the sequence CCAAT. Crossley and Brownlee (10), using DNase footprinting studies, have shown that C/EBP binds to the Factor IX promoter. We tested the ability of C/EBP to bind to the CCAAT-containing fragment of the Factor X promoter in a gel mobility shift assay. The labeled probe was the 30-mer designated CCAAT; protein in the binding assay consisted of either C/EBP translation product (1/25 of translation product from 1 µg of C/EBP transcript), or 5 ng of purified C/EBP fragment (88 COOH-terminal amino acids capable of binding DNA). Under several different sets of binding conditions, neither the translation product nor the purified protein bound to the CCAAT oligonucleotide, whereas both bound to a positive control sequence (residues -178 to +53 from the Factor IX promoter), confirming as probe in lanes 1–7. mACTTTG is the same sequence except that ACTTTG has been changed to GACAAT by random mutagenesis. Again, lane 1 contains labeled probe alone, and lanes 2–7, probe and 10 µg of HepG2 nuclear extract. In lanes 3–7 unlabeled competitor is added as indicated. In lanes 8–10 unlabeled competitor is added as indicated, and lanes 8–10 contain mACTTTG as labeled probe. Two prominent bands are generated by addition of HepG2 nuclear extract (lane 2) which are competed specifically by unlabeled wild-type oligonucleotide (lanes 3 and 4), whereas binding to the probe is unaffected by the addition of excess mutant oligonucleotide (lanes 5–7), except for a modest decrease in the intensity of the slowest moving band. Lane 8 contains mutant oligonucleotide alone, and lane 9, mutant oligonucleotide and 10 µg of HepG2 nuclear extract. Only the slowest moving band is present, suggesting that the protein-binding site within the oligonucleotide is at a site other than the CCAAT sequence. Addition of unlabeled mCCAAT (lane 10) eliminates the band. b, the element ACTTTG in the Factor X promoter binds hepatic nuclear proteins. The labeled oligonucleotide 5'-GCAGGCTCTCGGTTCACAACGAGGCTTA-3' (designated ACTTTG) is used as probe in lanes 1–7.
Anchored PCR has not been extensively used previously to map start sites of transcription (13). Thus we confirmed the results using S1 nuclease analysis. As an additional check on the method, we used anchored PCR to determine the start site of transcription for human Factor IX. Using total human liver RNA as starting material, we identified two start sites, one at 26 bp upstream and the other at 10 bp upstream from the first AUG. These data are in close agreement with those of Anson et al. (28), who identified a start site 29 bp upstream, using S1 nuclease analysis.

The results for the Factor X transcript show the presence of multiple start sites of transcription, most clustered in a segment 13 to 33 bp upstream from the first AUG. The finding of multiple start sites is consistent with results reported for other TATA-less promoters (27). The cluster of start sites that we report here predicts a relatively short 5′-untranslated segment for human Factor X. This is similar to the findings for human Factor IX (Ref. 28 and this report), where the 5′- untranslated region appears to be short, <30 bp.

**Functional Characterization of the Human Factor X Promoter**—In HepG2 cells, maximal promoter activity is attained with a 279-bp fragment of 5′-flanking DNA (Fig. 3A). Inclusion of an additional 109 bp (388 bp fragment) results in virtually no change in promoter activity, while inclusion of additional 5′-flanking elements (beyond 388 bp) results in a gradual decrease in activity. These results are similar to those previously reported for the Factor IX promoter (8) where maximal activity is attained using a 303-bp promoter element (numbering from the first AUG), and inclusion of an additional 5′-flanking sequence, up to 445 bp upstream from the first AUG, results in little change in promoter activity.

In HeLa cells, which do not express Factor X, the pattern of expression is different, with maximal activity seen with the 209-bp fragment, and marked loss of promoter activity with inclusion of additional 5′-flanking sequence. The 388-bp construct has only 28.4% of the activity seen with the 209-bp construct. Although direct comparisons between cell lines are difficult, since optimal transfection conditions are different, it should be noted that maximal GH activity for the HepG2 cells was 5-fold higher than that observed for HeLa cells. The relative promoter activities, and the differing patterns of promoter activity with addition of the fragment from −209 to −388 (sustained maximal levels of expression in HepG2 versus reduced expression in HeLa cells) both point toward a possible tissue-specific regulatory element within this fragment.

Analysis of promoter activity in HepG2 cells reveals that deletion of the 88 bp between the 209- and 121-bp constructs results in a 4-fold decrease in promoter activity, and deletion of the next 13 bp has an additional 4-fold loss of activity. Thus critical promoter elements reside in the fragment from −209 to −108. Whether any of these elements are tissue-specific is unclear, but the difference in overall promoter activity in the two cell lines is consistent with the notion that these elements may also possess some degree of tissue specificity for hepatocytes.

We chose for further study two specific elements within the 5′-flanking region: the sequence from −133 to −103, containing the element CCAAT (at −120 to −116), and the sequence ACTTTG located −56 to −51 upstream from the first ATG. The former was chosen because of the marked decrease in promoter activity resulting from deletion of this element (compare −121 to −108 in Fig. 3A) and the latter because of its conservation in the promoter regions of FVII, IX, and X. Based on gel mobility shift assays (Fig. 4), both elements bind proteins contained in a HepG2 nuclear extract. In both cases specificity of binding is demonstrated by disappearance of

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**Table II**

| Mutant construct | Nucleotide position | Relative activity ± S.D. |
|------------------|---------------------|-------------------------|
| ACTTTG<br>GACAAT | −56 → −51          | 17.2 ± 0.9 n = 3*      |
| ACTTTG<br>ACAAT | −52                | 19.2 ± 6.6 n = 6       |
| GGGCGG<br>TCTATC | −65 → −60          | 90.2 ± 2.1 n = 2       |
| CCAAT<br>AGCTA  | −120 → −116         | 11.8 ± 6.2 n = 6       |

* n, number of independent transfection experiments.

**Fig. 5.** Mutations at specific elements in the Factor X promoter result in a marked loss of promoter activity. Specific mutations tested are shown beneath the bar graph. Results shown are the average of at least three transfection experiments. Mutations at the ACTTTG and CCAAT sites result in a marked decrease in promoter activity whereas a change at GGGCGG has virtually no effect.

**DISCUSSION**

**Start Site of Transcription**—A necessary prerequisite for the study of the Factor X promoter is definition of the start site of transcription. We used anchored PCR to determine the start site, and confirmed these data using S1 nuclease analysis. In both cases the starting material was RNA from normal human liver, so that potential problems related to the use of RNA from transformed cell lines (e.g. aberrant start sites) are avoided.

Anchored PCR is a technique which allows one to amplify a specific segment of DNA or RNA when sequence information is available for only one end of the segment. The method has general applicability but offers special advantages for mapping start sites in genes belonging to superfamilies, since the start site is provided as sequence data, which allows the identification and exclusion of extraneous sequence. This is in contrast to the potential pitfalls of S1 nuclease mapping or primer extension, where there is always the concern that signals on the autoradiograph may have arisen from hybridization with a closely related transcript rather than the transcript under study.
binding complexes following addition of unlabeled competitor. For the more 5′-element, alteration of the CCAAT sequence by random mutagenesis (to AGCTA) results in a sequence that cannot compete for binding to HepG2 nuclear proteins (lanes 5–7). When the altered sequence is used as a probe (lane 9) the two faster migrating complexes are entirely absent, indicating that they both require an intact CCAAT sequence for binding, whereas the slowest moving complex is still seen, suggesting that binding for this protein does not require the CCAAT sequence. Further evidence of the critical nature of the CCAAT sequence is seen in the promoter activity assays displayed in Fig. 5. Alteration of the sequence to AGCTA within the 279-bp fragment of 5′-flanking results to a 10-fold drop in promoter activity. Thus a change in the CCAAT element abolishes protein binding and profoundly reduces promoter activity of the Factor X promoter. Since the element CCAAT can serve as a recognition sequence for transcription factor C/EBP, which has been shown to bind to a site within the F.IX promoter (10), we wished to determine whether C/EBP bound to the 30-mer spanning -133 to -103. Results using both the purified protein and the translation product were negative, suggesting that the protein(s) binding to this element within the X promoter are distinct from this previously characterized liver transcription factor.

Results for analysis of the ACTTTG element were similar to those described for the CCAAT element. An oligonucleotide probe spanning the sequence from -68 to -39 and containing ACTTTG binds proteins from a HepG2 nuclear extract; the complexes are abolished by the addition of unlabeled competitor. Mutant sequences in which ACTTTG has been changed to GACAACT cannot compete for binding (lanes 5–7) nor can they bind protein from the nuclear extract (lane 9). The substitution of even a single nucleotide within this sequence (ACTTTG → ACTTAG) results in the complete loss of the faster moving band (lane 8). In the reporter gene assay (Fig. 5) both of these mutations result in a >5-fold loss of promoter activity compared to wild-type. Again loss of ability to bind a nuclear protein (or proteins) is associated with a dramatic decrease in promoter activity for the sequence under study.

The point mutation in the ACTTTG sequence is identical to a naturally occurring mutation described in the F.IX promoter (T→A at -20, within the conserved ACTTTG sequence) (32). This variant displays the Leyden phenotype, with absence of F.IX protein (antigen and activity) in affected children, but gradually increasing levels following puberty. The pathophysiology of the Leyden phenotype remains unexplained, but further characterization of the protein which binds at this site may shed light on this interesting problem.

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