Liver-specific Expression of the Gene Coding for Human Factor X, a Blood Coagulation Factor*

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Factor X is a vitamin K-dependent glycoprotein that plays an essential role in both the intrinsic and extrinsic pathways of blood coagulation. Studies on a recombinant λ phage containing the 5' flanking region of the human factor X gene showed that the factor X gene was linked to and was located at the 3' end of the factor VII gene: the initiation codon of the factor X gene was 2823 base pairs (bp) downstream from the polyadenylation site of the factor VII gene. This 2.8 kilobase intergenic region, and progressively deleted fragments of it, was fused to the chloramphenicol acetyltransferase gene, and transient expressions in HepG2 cells, human fibroblasts, and Chinese hamster ovary cells were measured. A liver-specific promoter element, FXP1-binding site, essential for hepatocyte-specific transcription was identified. This promoter sequence, further localized to -63 to -42 bp in DNase I footprint studies, was homologous to LF-A1 or hepatic nuclear factor-4 recognition sequence and was equally functional in the normal and inverse orientations. FXP1 site bound to nuclear protein(s) from HepG2 cells and complex formation was partially abolished by the presence of duplex oligonucleotides containing liver factor-A1 or hepatic nuclear factor-4 binding sequences. Two additional positive elements located upstream of the promoter region, spanning from -215 to -149 bp (FXP2 site), and -457 to -351 bp (FXP3 site), were also established by reporter gene assays.

Factor X is a vitamin K-dependent glycoprotein that participates in the middle phase of the blood coagulation cascade (Davie et al., 1991). It is synthesized as a single-chain precursor in the liver where it undergoes a number of covalent processing steps before secretion into the blood. These processing steps include removal of the signal peptide (23 amino acids) by signal peptidase (Fung et al., 1986; Leytus et al., 1986) and the propeptide (17 amino acids) by a processing enzyme (Blanchart et al., 1985), proteolytic conversion of the single-chain precursor polypeptide into a two-chain zymogen linked by a disulfide bond (Leytus et al., 1984), vitamin K-dependent carboxylation of the first 11 amino-terminal glutamic acid residues to form γ-carboxyglutamic acids, hydroxylated Asp63 in the first growth factor domain to hydroxylysine and Asp67 in the second domain to hydroxylysine, which was originally called Stuart factor X deficiency, results in a serious bleeding disorder. It is characterized by a prolonged prothrombin time and partial thromboplastin time. Factor X deficiency is inherited as an autosomal recessive trait (Lechler et al., 1965). The gene for factor X is approximately 25 kb in length and is located on chromosome 13q34-qter (Pfeiffer et al., 1982). Studies on chromosome 13 abnormalities showed that the structural gene for factor VII was also located in the same region as the factor X gene (Gilgenkrantz et al., 1986). The genes for the vitamin K-dependent proteins that participate in blood coagulation share significant organizational similarity and have evolved from a common ancestral gene (Leytus et al., 1986). However, significant differences in the steady-state mRNA levels and concentrations of these proteins in plasma suggest that mechanisms that govern their regulation are different. Thus far, gene regulation of two vitamin K-dependent proteins has been studied. The factor IX gene, which is located on Xq26-q27, is apparently regulated by the presence of liver-specific cis-acting elements that interact with the liver-specific transcription factors CCAAT enhancer-binding protein (C/EBP) and nuclear factor 1-like liver-specific protein (NF1-L). The C/EBP-binding site is located unusually close to the transcription initiation site, spanning from 1 to 18 bp whereas the NF1-L-binding site is located in the proximal promoter region, spanning from -99 to -76 bp (Crosley and Brownlee, 1990). Furthermore, the factor IX gene may be hormonal regulated since the deficiency in hemophilia B Leyden, which is caused by mutations in the regulatory region of the factor IX gene, can be partially overcome following puberty or testosterone administration (Briet et al., 1985). Reporter gene studies also showed that androgen significantly increased factor IX-regulated transcriptional activities (Hirosawa et al., 1990). The prothrombin gene, on the other hand, is located on chromosome 11p11-q12 (Royle et al., 1987). It contains a weak promoter immediately before the transcription initiation site and a liver-specific enhancer sequence located 860-940 nucleotides from the transcription initiation site. The latter region apparently interacts with hepatic nuclear factor-1 (HNF-1) (Chow et al., 1991; Bancroft et al., 1990; MacGillivray and Chow, 1991).

Comparison of the 5' flanking sequences of the factor X gene with those of the genes for factor IX and prothrombin

1 The abbreviations used are: kb, kilobase; CAT, chloramphenicol acetyltransferase; HF, human fibroblast; CHO, Chinese hamster ovary; HNF-1, hepatic nuclear factor-1; HNF-4, hepatic nuclear factor-4; C/EBP, CCAAT-enhancer binding protein; NF1-L, nuclear factor 1-like specific; bp, base pair; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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showed significant sequence divergence. As a first step in understanding the regulation of the factor X gene, reporter gene assays, DNase I protection, and gel retardation assays have been employed to identify sequences in the 5′ region of the factor X gene that are involved in transcription. Three regions in the 5′-flanking region have been identified to participate in liver-specific expression and regulation of the gene.

EXPERIMENTAL PROCEDURES

Isolation and Sequencing of Recombinant Phage Clone—A human genomic library constructed in EMBL3 (Clontech Laboratories) was screened using a radiolabeled human factor X cDNA (Leytus et al., 1983) as an hybridization probe. Southern blot analysis, restriction mapping, and DNA sequence analyses showed that a 3.5-kb BamHI segment of the recombinant phage λ-CTFX5 contained the 5′-flanking sequence of the factor X gene. This segment was further fragmented by a combination of partial restriction digestion, sequential Bal31 deletion, and sonication. The fragments were cloned into a M13 bacteriophage vector (Messing, 1985) and sequenced by Sanger’s dye- terminator method (Sanger et al., 1977). Overlaps were established by sequencing with specific synthetic oligonucleotides as primers. The DNA sequence was verified by sequencing the opposite strand.

Construction of Plasmids—Plasmid pCAT-0 was purchased from Promega Co and pSV3 plasmid from Clontech Laboratories, Inc. A 275-bp fragment containing the SV40 early region promoters was amplified from SV40 DNA (Bethesda Research Laboratories) by the polymerase chain reaction (Saiki et al., 1985; Mullis and Foosona, 1987), and inserted into pCAT-0 at the 5′ end of the CAT gene at a XbaI site to yield plasmid pSV2-CAT. Six fragments spanning from −2800, −2000, −1000, −457, −282, and −127 to −1 bp of the factor X gene were obtained by amplification of λ-CTFX5 using the polymerase chain reaction technique. The resulting DNA fragments were cloned into the XbaI site of pCAT-0, generating FX-CAT constructs containing inserts in either natural or inverse orientations. The orientation and sequence of these constructs were verified by dideoxy sequencing. pCAT-457 was further subjected to sequential deletions by ExoIII. The plasmid was linearized by digestion with Sall and SphI and was deleted unidirectionally with ExoIII and mung bean nuclease for varying amount of time according to a protocol from Stratagene. The deleted plasmids were religated to give constructs pCAT-357, pCAT-215, pCAT-149, and pCAT-47.

Cell Culture and Transfections—Human hepatoma cells (HepG2) and Chinese hamster ovary (CHO) cells were cultured in Ham’s F-12 medium, human kidney cells (293), and human fibroblast cells (HF) in Dulbecco’s modified Eagle medium in a 5% CO2 atmosphere at 37°C. Both media were supplemented with L-glutamine, antibiotics (penicillin, streptomycin, and neomycin), and 5% fetal calf serum (except CHO with 10% serum).

DNA transfections were performed by the calcium phosphate precipitation method as described by Graham and van der Eb (1973). Cultured cells (~50% confluence) in 10-cm tissue culture dishes were cotransfected with 5 μg of plasmid DNA and 10 μg of pSV3 used as an internal control. The calcium phosphate-DNA precipitates were removed 4 h later, and the cells were exposed to 1.5 ml of 15% glycerol in Tris-buffered saline (25 mM Tris base, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl2, 0.5 mM MgCl2, 0.6 mM Na2HPO4) for 1 min. The cells were cultured in fresh medium for 60 h. The cells were then washed and harvested. Cell extracts were prepared according to the method described by Kaushansky (1989) for measurements of enzyme activity.

RESULTS

Transcriptional Control of the Human Factor X Gene—In order to characterize sequences responsible for transcriptional regulation of the human factor X gene, a 2.8-kb segment from the 5′ end of the gene was linked to a promoterless CAT reporter gene in plasmid pCAT-0. The resulting construct, pCAT-2.8kb, was transfected into human hepatoma HepG2, HF, and CHO cells. Transient gene expression was monitored by measuring CAT activity in the cell extracts. To correct for differences in DNA transfection efficiency, the cells were transiently cotransfected with a reference plasmid carrying the β-galactosidase gene under the control of the SV40 early promoter-enhancer. After transfection, CAT enzyme activities were measured and normalized to the β-galactosidase activity. The parent promoterless plasmid pCAT-0, which does not express CAT activity in HepG2, HF, or CHO cells, was employed as a negative control. The plasmid pSV2-CAT, which contains the SV40 early promoter-enhancer sequences inserted upstream of the CAT gene, was used as a positive control. CAT activity for this plasmid was expressed at high levels in all three cell lines. When plasmid pCAT-2.8kb, which contains the 5′ region (~800 to −1 bp) of the factor X gene, was transfected into HepG2 cells, the cell extract gave CAT activity.
To further locate the cis-acting elements present in promoterless plasmid pCAT-0 (Fig. 2). Furthermore, the pCAT-2.8kb construct exhibited 10- and 20-fold higher CAT percentages relative to that of the most active construct pCAT-457 containing varying lengths of the factor gene (-2800 to -1 bp) of the gene coding for factor X confers high level and liver-specific expression.

Identification of the Regulatory Elements in the 5'-Flanking Region—To further locate the cis-acting elements present in the FX 5'-flanking region, constructs containing progressive deletions of the 2.8-kb fragment were tested in HepG2 cells. As shown in Fig. 2, deletions of the sequences from -2.8 kb to -127 kb, -127 kb to -37 kb, and -37 kb to -14 bp did not significantly affect reporter gene expression. However, deletion from -457 bp to -351 bp resulted in a 1.7-fold decrease in activity. Further deletion from -351 bp to -215 bp led to an additional 1.7-fold decrease in CAT activity. Finally, a precipitous reduction in expression occurred upon deletion of sequences between -127 bp and -47 bp.

Properties of the Regulatory Elements—Four constructs containing one, two, or all three regulatory elements were tested for CAT expression efficiency in the normal and inverse orientations (Fig. 2). pCAT-127(+) and pCAT-127(-), which was inactive in HepG2 cells, was used as a negative control while pSV2-CAT was used as a positive control. The CAT activity of pCAT-857 was 35% of pSV2-CAT. All CAT assay experiments represented averages of three or more independent experiments. * orientation of 5'-flanking region; ND, not determined.

Fig. 2. Transient expression of CAT activities by deletion constructs in HepG2 cells. A series of FX-CAT fusion constructs containing varying lengths of the factor X 5'-flanking sequence were transfected into HepG2 cells. CAT activities were expressed as relative CAT activity. ND, not determined.
tation was reduced to very low levels compared to those in the normal orientation. These data implied that there was a possible negative element located between −457 and −351 bp which blocked transcription in the inverse orientation.

As shown above, the 5′-flanking sequence of the FX gene directed liver-specific expression of the gene. Tissue specificity was preserved in a series of deletion constructs (Fig. 3), except for the smallest construct (pCAT-47) where the CAT activity dropped to almost basal level. The construct containing the promoter element FXP1 site (pCAT-127) exhibited CAT expression 38-fold higher than the promoterless plasmid pCAT-0 in HepG2 cells, as compared to 5-fold in HF and CHO cells. These results demonstrated that the promoter element FXP1 directed liver-specific expression. Increases of 1.7-fold in CAT activities were observed in all three cell types with the pCAT-282 construct that contained the FXP1 site and FXP2 site. This nonspecific increase in all cell types was attributed to a general activation resulting from the interaction of FXP2 site with an ubiquitous transcription factor(s). Furthermore, the construct containing the element FXP3 site (pCAT-457) showed an additional 1.7-fold increase in promoter activity in HepG2 cells but not in HF or CHO cells. These results implied that both the promoter FXP1 site and the third element FXP3 site were responsible for liver-specific expression.

Characterization of the Factor X Promoter Region—To identify the sequences within the proximal promoter of the factor X gene that were involved in transcriptional regulation, DNase I footprints analyses of DNA fragments spanning the promoter region were performed (Fig. 4). An equivalent amount of nuclear protein from HepG2 and HF cells was used in these protection assays. The nuclear extract of HepG2 cells protected one distinct region from DNase I digestion, whereas the same region was not protected by the HF nuclear extract. The protected sequences extended from −64 to −42 bp on the sense strand (Fig. 4A, lane 3) and from −43 to −65 bp on the antisense strand (Fig. 4B, lane 3). Comparison of the nucleotide sequence of the protected site of the factor X promoter region with recognition sequences of known eukaryotic trans-acting factors revealed homology with the consensus recognition site described for the liver-specific transcription factor LF-A1 or HNF-4.

To further characterize the interaction of the promoter element with trans-acting factor(s), polyacrylamide gel mobility shift experiments were performed. A 32P-end-labeled duplex DNA fragment spanning the region from −65 to −36 bp was incubated with nuclear extracts from HepG2 and HF cells in the presence of nonspecific competitors of DNA-binding proteins, poly(dI-dC), and salmon sperm DNA. A protein-DNA complex was formed with HepG2 nuclear extract but not with HF nuclear extract (Fig. 5A). These results confirmed that the FXP1 site bound hepatocyte-specific transcription factor(s). To examine whether the FXP1 sequence bound LF-A1 or HNF-4 or other related factors, competition experiments were carried out in the presence of increasing amounts of competing oligonucleotides (Fig. 5B). The sequences of the oligonucleotides used as competitors are shown in Fig. 5C. The end-labeled factor X promoter element (−65 to −36 bp), when incubated with HepG2 cell nuclear extract, gave rise to a slow-migrating protein-DNA complex (Fig. 5B, lane 2) as compared with the free probe (lane 1). Addition of increasing amounts of unlabeled FXP1-binding oligonucleotide, which spanned the region protected from DNase I digestion in the factor X promoter sequence, abolished DNA-protein complex formation (Fig. 5B, lanes 3–5). However, the unlabeled LF-A1 oligonucleotide (10-, 50-, and 500-fold molar excess), which contained the well-characterized LF-A1-binding site sequence from the promoter of the α1-antitrypsin gene (De Simmone et al., 1987; Hardon et al., 1988; Monaci et al., 1988), partially competed with labeled FXP1-binding oligonucleotides (Fig. 5B, lanes 6–8). Furthermore, DNA-protein complex formation was not prevented by the addition of a 10- and 50-fold excess of unlabeled HNF-4 oligonucleotide, which contained the HNF-4-binding site in the promoter of the

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**Fig. 3.** Expression of CAT activities in HepG2, HF, and CHO cells. Deletion constructs containing varying lengths of the factor X 5′-flanking region were transfected into human hepatoma cells (HepG2), human fibroblast cells (HF), and Chinese hamster ovary cells (CHO). The plasmids pCAT-0 and pSV2-CAT were used as negative and positive controls, respectively. For comparison in the three different cell lines, CAT activities were calculated as percentages of pSV2-CAT activities (taken as 100%) in each cell line.

**Fig. 4.** DNase I footprint analysis. A, sense strand of the factor X promoter region. A DNA fragment containing the factor X promoter (from −127 to −1 bp) was labeled at the 3′ end of the sense strand and was subjected to DNase I digestion in the absence (Control lane) and presence of nuclear extracts from HepG2 cells (HepG2 lane) and HF cells (HF lane). A purine-specific sequence marker (G+A lane) was obtained by Maxam-Gilbert sequencing of the end-labeled fragment. B, antisense strand. Brackets indicate regions that are protected from DNase I digestion. C, sequence of the protected regions identified in A and B.
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Fig. 5. Binding of a duplex oligonucleotide containing FXP1 sequences with nuclear protein extract. A, gel retardation assay. An end-labeled duplex FXP1 oligonucleotide was incubated with crude nuclear extracts, and the resulting complexes were electrophoresed on a nondenaturing polyacrylamide gel. Gel retardation peting oligonucleotides in the presence of HepG2 nuclear extract: even at 500-fold molar excess (Fig. 5B, lanes 9-11). Unrelated nucleotide sequences, e.g. C, did not compete with the labeled probe for complex formation (lane F, lane 11). Unrelated nucleotide sequences, e.g. C, did not compete with the labeled probe for complex formation (lane F, lane 11). Unrelated nucleotide sequences, e.g. C, did not compete with the labeled probe for complex formation (lane F, lane 11). Unrelated nucleotide sequences, e.g. C, did not compete with the labeled probe for complex formation (lane F, lane 11).

DISCUSSION

Studies presented in this report showed that the factor X gene was regulated by a promoter which exhibited inherent liver-specific properties. Reporter gene assays, DNase I protection, and oligonucleotide competition experiments showed that this element functions in an orientation- and position-independent manner and showed affinity for liver-specific transcription factor LF-A1 or HNF-4. These properties are similar to promoters of housekeeping genes which lack “TATA” and “CCAAT” sequences (Baarends et al., 1990; Buckingham et al., 1990; Hudson et al., 1990). In the factor X gene, an apparent CCAAT sequence is present at -116 to -120 bp. However, DNase I protection and competition in gel retardation experiments showed no evidence of this sequence interacting with C/EBP or related proteins (data not shown), implying that this sequence was not functional in factor X gene expression. In gel retardation experiments, oligonucleotides containing LF-A1 recognition sequences from the α1-antitrypsin gene and HNF-4 recognition sequence from the transthyretin gene can only partially compete with FXP1 in DNA-protein complex formation. These data suggested that the FXP1 site may bind to LF-A1 or HNF-4 with significantly higher affinity than the other two sequences. Alternatively, these results may mean that the FXP1 site was interacting with a different factor that closely resembles LF-A1 or HNF-4. Binding sites homologous to the LF-A1 site were also found in the regulatory regions of the apocIII gene (Reue et al., 1988), apolipoprotein A1 gene (Hardon et al., 1988), apolipoprotein A2 gene (Lucero et al., 1989), and pyruvate kinase L-type gene (Vaulont et al., 1989). A comparison of these sequences are shown in Table I. Comparing the FXP1 site with the consensus sequence derived from these homologous sequences, 82% identity was observed, demonstrating that FXP1 site was indeed interacting with LF-A1 or HNF-4 or related factors. It has been shown that more than one protein binds to the APFl site in the apoCIII gene and the HNF4P site in the transthyretin gene. Present evidence suggests that most DNA-binding proteins interact with DNA in the form of dimers or mixed oligomers (Johnson and McKnight, 1989). It is conceivable that the FXP1 site-binding protein was a complex hetero-oligomer of LF-A1 or HNF-4 with other transcription factors of the same family. HNF-4 belongs to the steroid hormone receptor superfamily which contains sequences homologous to the ligand-binding site of thyroid hormone receptor and estrogen receptor (Danielson et al., 1989; Mader et al., 1989; Umesono and Evans, 1989; Forman and Samuels, 1990). However, no known ligand has been identified for HNF-4 thus far. If in fact the factor X gene was regulated by HNF-4, this regulation might be further modulated by the binding of ligands.

The second positive element identified in the factor X gene, FXP2 site, was located at -215 to -149 bp. The presence of this element elevated reporter gene expression approximately 1.7-fold in HepG2 cells. Although FXP1-directed expression in CHO and HF cells was very low, the presence of this element led to a small but reproducible 2-fold increase in reporter gene expression in non-hepatic cells suggesting that FXP2 was not tissue-specific. Inversion of the orientation of the FXP1 and FXP2 site as in the construct pCAT-282(-) showed similar activity indicating that both FXP1 and FXP2 site as in the construct pCAT-282(-) were orientation-independent. This general potentiation of promoter activity by FXP2 may be attributed to the presence of an inverted Sp1 recognition sequence (CCGCCC) located at -207 to -202 bp. Studies on genes expressed tissue specifically show that general elements that bind ubiquitous factors are usually present in the vicinity of tissue-specific elements to further enhance the activity of these elements (Maniatis et al., 1987; Paonessa et al., 1988; Wasylyk, 1988). The third element FXP3 located at -457 to -351 bp further elevated reporter gene expression 1.7-fold in HepG2 cells but not in HF or CHO cells indicating that this effect was also liver-specific. However, when the region containing all three elements was inverted as in the construct pCAT-457(-), reporter gene expression was reduced to background levels. Since the FXP1 and FXP2 sites function in either orientation, the complete abolition was attributed to the presence of a
negative element located upstream from the third element (Fig. 6). This negative element in its natural location between the factor X and factor VII gene will unidirectionally block the activity of the three positive elements and prevent activation and transcription of the adjacent factor VII gene. In this arrangement, the regulatory functions of the promoter FXP1, and the accompanying positive elements FXP2, and FXP3 are restricted exclusively to the factor X gene. It is also possible that this negative element, being close to the polyadenylation site of the factor VII gene, might be the actual transcription termination site of the factor VII gene.

Reporter gene experiments showed no additional functionally active elements in the region between −2.8 kb to −457 bp. However, this region contained a unique sequence, 28 bp in length (−1955 to −1928) that was conserved in the 5′-flanking regions of several other genes, particularly genes coding for vitamin K-dependent proteins. Although this sequence does not contribute to the tissue-specific regulation of the factor X gene, it may function as a regulatory element in embryonic development or in stimuli-modulated gene expression.

In other experiments (data not shown), the pCAT-2.8 kb and several of the deletion constructs have been transfected into human kidney 293 cells. Reporter gene assays indicate that the factor X liver-specific promoter was able to direct the expression of the CAT gene in kidney cells as efficiently as in HepG2 cells. A similar lack of tissue specificity of the promoter of the factor IX gene in baby hamster kidney cells has also been observed (Salier et al., 1990). This apparent lack of tissue specificity in kidney cells may be ascribed to a loss of differentiated properties after these cells have been established as a perpetually propagating cell line. Furthermore, HNF-4 and its mRNA have been detected in liver and kidney (Sladek et al., 1990; Xanthopoulos et al., 1991). These data suggest that tissue-specific expression is not accounted for solely by the presence of these known transcription factors. Identification of liver-specific positive acting factors is only the first step in explaining cell-specific transcription (Costa et al., 1990). The presence of negative acting factors (Hammer et al., 1987; Herbst et al., 1989; Yan et al., 1990) and adequate concentrations of positive acting factors at appropriate stages of development are critical for tissue-specific expression. Furthermore, tissue-specific methylation of selective regions of genes during development may also contribute to tissue-specific expression.

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