Role of the Liver-Enriched Transcription Factor Hepatocyte Nuclear Factor 1 in Transcriptional Regulation of the Factor VIII Gene

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Coagulation factor VIII is an essential cofactor required for normal hemostatic function. A deficiency in factor VIII results in the bleeding disorder hemophilia A. Despite the fact that the factor VIII gene was cloned a decade ago, the mechanisms which control its transcription remain unresolved. In our studies, we have characterized 12 protein binding sites within the factor VIII promoter by DNase I protection assays performed with rat liver nuclear extracts. Three of these elements (sites 1 to 3) are situated within the 5′ untranslated region of the gene, while three other sites (sites 4 to 6) lie within the first 100 bp upstream of the transcriptional start site. We have identified an additional site (site 7) ~300 bp upstream from site 6, as well as a cluster of five sites in a 250-bp region which terminates ~1 kb from the transcriptional start site. Seven of these binding sites (sites 2, 3, 4, 6, 7, 9, and 10) bind members of the C/EBP family of transcription factors. DBP also binds to five of these sites (sites 3, 4, 6, 7, and 9). Utilizing transient transfection studies in HepG2 cells, we have shown that deletion of the factor VIII promoter sequences distal to nucleotide −44 results in a significant but small increase in promoter activity. The activity of each of the various 5′ deletion constructs is significantly enhanced by cotransfection of C/EBPα and D-site-binding protein expression plasmids, while cotransfection of both C/EBPα and C/EBPβ plasmids resulted in a further enhancement of transactivation. These studies also provide evidence of a repressor element located between nucleotides −740 and −1002. Since the minimal promoter sequence (~44 to +148) maintains the transcriptional activity of the full-length promoter sequence, we proceeded to identify additional factors binding to sites 1 to 4. Competition studies revealed that a ubiquitous transcription factor, NF-Y, binds to site 4, while the liver-enriched transcription factor hepatocyte nuclear factor 1 (HNF-1) binds to site 1. Mutation analysis of the minimal promoter demonstrated that HNF-1 is critical for activating transcription of the factor VIII gene in vitro. Our results also suggest that the multiple upstream elements that we have identified may act as a backup regulatory region in the event of disruption of the HNF-1 element in the 5′ untranslated region.

Factor VIII is a large plasma glycoprotein that circulates in a noncovalent complex with von Willebrand factor (20). When factor VIII is released from von Willebrand factor and activated by thrombin, factor VIII, participates as a cofactor in the activation of factor X by factor IXa. Deficiency of factor VIII results in hemophilia A, an X-linked bleeding disorder which affects 1 in 5,000 males. The gene for factor VIII was cloned and characterized in 1984 (14, 53). It spans 186 kb on the long arm of the X chromosome at position Xq28. The gene encodes a mature mRNA of 9 kb which is expressed in a number of different tissues (58).

Wion et al. (58) have shown, using RNase protection studies, that factor VIII is expressed in whole liver and isolated hepatocytes as well as in the spleen and in lymph nodes. Factor VIII mRNA was also found at low levels in the pancreas, kidney, and muscle. The clinical evidence that supports the liver as a major site of factor VIII synthesis is the finding that liver transplantation in hemophiliacs corrects their bleeding disorder (3, 24).

In normal individuals, factor VIII circulates in the plasma in trace amounts (~100 to 200 ng/ml). The biosynthesis and secretion of factor VIII have been extensively studied (22, 42, 55, 59). It has been suggested that the low circulating levels of factor VIII may be attributed to mRNA accumulation and inefficiencies in the processing and secretion of the factor VIII protein (8, 9, 21). Thus, factor VIII synthesis may be regulated at the posttranscriptional level as well as at the transcriptional level.

The mutations responsible for the hemophilia A phenotype consist of a variety of single base substitutions, deletions, and insertions that have been found throughout the gene (54). To date, no mutations have been identified in the factor VIII promoter.

In addition to cloning the factor VIII coding region in their initial studies, Gitschier et al. (14) sequenced 1.2 kb of the 5′ flanking region of the gene. They also identified the transcriptional start site by RNase protection to be 170 bp 5′ of the initiation methionine codon. There is a GATAAA sequence 30 bp 5′ of the transcriptional start site which is thought to represent an alternative TATA box, but other details concerning the cis-acting sequences which regulate transcription of the gene remain unknown.

In this study, we demonstrate that members of the basic leucine zipper (b/ZIP) family of proteins, the CCAAT/enhancer-binding proteins (C/EBPα and C/EBPβ) and D-site-binding protein (DBP), bind to several cis-acting elements in the factor VIII promoter and are important for transactivating the expression of the factor VIII gene. In addition, we show evidence of a possible repressor element approximately 1 kb 5′ of the gene. Furthermore, our studies show that hepatocyte nuclear factor 1 (HNF-1) binding to its cognate sequence element in
the 5′ untranslated region (UTR) plays an important role in sustaining the transcriptional activity of this promoter.

**RESULTS**

PCR and DNA sequencing of the factor VIII promoter. Following the conventional, the transcriptional start site is referred to as nucleotide (nt) +1. A region of 1,150 bp of the factor VIII gene corresponding to nt +148 to −1002 was amplified by PCR from genomic DNA, cloned, and sequenced. The sequencing data confirmed the presence of an extra cytosine nucleotide (underlined) at +16 in the untranslated region (GCAATC instead of GCAATO) missing from the original published sequence (14, 53). This sequence change has been documented previously by one other study (57).

Twelve DNase I-protected sites occur in the 5′ flanking region of the factor VIII promoter. To examine the extent and location of nuclear protein interactions, DNase I footprint analysis of the factor VIII promoter was performed with rat liver nuclear extracts. The results show that the factor VIII proximal promoter fragment (nt −277 to +148) shown in Fig. 1a and b, three regions of protein-DNA interactions occur in the 5′ UTR (sites 1 to 3). Site 1 is located between nt +118 and +148 and has 10- to 13-nmol homology to HNF-1 consensus sequence (5). Site 2 is located between nt +68 and +85 and has some similarity to the consensus sequence 5′-ATTNG CAAT-3′ of the C/EBP family of transcription factors (30). A large protected region which extends from nt +23 to the GATAA sequence at nt −30 was revealed (Fig. 1b). We hypothesize that this is a composite of two contiguous sites, given the relatively large size of the protected area. Evidence for delineation of the two sites is provided by footprint analysis of the 3′-labeled coding strand, which shows a separation between site 3 (+1 to +23) and site 4 (−1 to −30) (data not shown). Factor VIII promoter sites (F8 sites) 3 and 4 exhibit sequence similarity to the C/EBP consensus sequence. The fifth site is located between nt −45 and −62, and site 6 is located between nt −79 and −109. The factors binding to these sites are still unknown.

The remaining six sites are found in the distal region of the factor VIII promoter. The seventh site (nt −401 to −418) shown in Fig. 1c lies an additional 300 nt upstream of site 6 and also shows sequence similarity to the C/EBP consensus sequence. The five remaining sites are clustered in a 250-bp region which terminates −1 kb from the transcriptional start site: site 8 (nt −705 to −722) and site 9 (nt −800 to −822) (Fig. 1d), site 10 (nt −852 to −869) and site 11 (nt −889 to −913) (Fig. 1e), and site 12 (nt −946 to −963) (Fig. 1f). These five sites have no apparent homology to known consensus sequences for transcription factor binding sites. The locations of these binding sites in the 5′ flanking sequence of the factor VIII gene are summarized in Fig. 1g.

The liver-enriched members of the bZIP family of transcription factors, C/EBPα, C/EBPβ, and DBP, have common binding sites in the factor VIII promoter. To study the proteins binding to each of these sites, double-stranded oligonucleotides which correspond to the 12 sites identified by DNase I footprint analysis were synthesized. Protein binding was assayed through gel mobility shift assays using rat liver nuclear extracts as a source of liver-enriched transcription factors. The banding patterns shown in Fig. 2a to c by F8 sites 2, 3, 4, 7, 9, and 10
show size heterogeneity of the protein-DNA complexes. These banding patterns, in turn, show a distinct similarity to that of the D site of the albumin promoter that has been reported to bind C/EBPα, C/EBPβ, and DBP (29, 34). The differential migration of the protein-DNA complexes observed is due to the bZIP structure of the C/EBP isoforms, which allows them to form homo- and heterodimers. We were able to confirm that C/EBPα can bind as a homodimer to at least six of the factor...
VIII sites, as a single protein-DNA complex is obtained with a recombinant C/EBP protein in lanes 3, 4, 6, 7, 9, and 10 of Fig. 2d. Furthermore, in competition studies, the D site of the albumin promoter (a well-characterized C/EBP binding site) was able to compete for the smear of complexes at site 2 (data not shown), suggesting that additional factors, including C/EBP, may also bind to this site. Supershift analysis with antibodies against various members of the C/EBP family confirms that both the C/EBPα and C/EBPβ isoforms bind to sites 3, 4, and 7 (data not shown; supershift studies were not performed on sites 6, 9, and 10). In addition, C/EBP and DBP have been shown to bind to common sites in the factor VIII promoter (Fig. 2c), a finding that has been documented previously for a number of other promoters of liver-specific genes (17, 23, 29, 34, 40, 52).

Transactivation of the factor VIII promoter by members of the C/EBP family and the PAR family. Since hepatocytes represent one of the cellular sites of synthesis of factor VIII, the human hepatocellular carcinoma cell line HepG2 was chosen as the cell line in which transfection of this gene might most appropriately be studied. Five constructs with progressive 5' deletions from the 1.2-kb factor VIII promoter sequence were assessed for the ability to direct transcription of a luciferase reporter gene in HepG2 cells. All of the constructs include 148 nt of the 5' UTR of factor VIII.

All transfection results have been reported with respect to the activity of the full-length construct. Analysis of a series of deletion constructs (Fig. 3a) indicates that removal of sequences distal to nt −44 results in a significant but small increase in promoter activity (at most ~2-fold). The construct containing sites 1 to 4 (pFVIII(−44/+148)-Luc) indicates that these four sites play a major role in regulating transcription of this locus.

The presence of multiple C/EBP and DBP sites in the proximal promoter indicates that members of the C/EBP and PAR domain family of transcription factors likely play a role in the activation of this gene. To test this hypothesis, HepG2 cells (which express only ~10% of the normal levels of C/EBPα and DBP [13, 35]) were cotransfected with expression plasmids for C/EBPα, C/EBPβ, and DBP (Fig. 3b). In these studies, cotransfection of the 5' deletion constructs with both C/EBPα and DBP resulted in strong transactivation of the factor VIII promoter with an overall additive effect. Similarly, cotransfection of the two C/EBP expression vectors, either alone or in combination, resulted in strong transactivation of the factor VIII promoter, particularly when both C/EBP isoforms were present. In addition, these experiments showed a significant enhancement of transactivation with the combined cotransfection of C/EBPα/DBP (~3-fold, P < 0.005) or C/EBPα/C/EBPβ (~10-fold, P < 0.001) when the region between nt −740 and −1002 was deleted, suggesting the presence of a repressor element within this region.

The liver-enriched transcription factor HNF-1 and a ubiquitous factor, NF-Y, bind to distinct sites in the minimal factor VIII promoter. Upon scanning of the protein binding sites in the factor VIII promoter for similarity to consensus sequences for transcription factors implicated in the regulation of other liver-specific genes, it became apparent that a sequence within site 1 had significant homology to the HNF-1 consensus sequence 5'-GTTAATNATTAAC-3' (5). HNF-1 is a member of the POU domain family of transcription factors. The two isoforms of HNF-1, HNF-1α and HNF-1β, are able to form homo- and heterodimers with each other and other related proteins (32). Competition studies with an HNF-1 oligonucleotide (Fig. 4a) demonstrated that a 10-fold molar excess of the HNF-1 competitor prevents the formation of complex 1, whereas a 100-fold excess of the competitor is required to compete for complex 2. The nature of the two complexes is not known since the expression patterns of HNF-1α and HNF-1β differ from those of the liver.
differ. Although HNF-1α and HNF-1β mRNA has been found in the liver, the HNF-1β protein is absent (32).

We were also interested in identifying the distinct protein-DNA complex above the smear in band shift analysis of F8 site 4. Analysis of this sequence showed that it contained an element which was similar to the C site of the albumin promoter.

It has been previously reported that the CCAAT-binding factor NF-Y binds to this site (25). The C site of the albumin promoter contains a CCAAT sequence preceded by GGAA, which is virtually identical to the site 4 sequence 5'-GGAAG-GCAAT-3'. Previous studies of the albumin promoter have shown that NF-Y is the major transcription factor binding to site C, whereas C/EBP binds only weakly to this site (60). When we used site C of the albumin promoter as a competitor (Fig. 4b), the prominent gel retarded band at site 2 disappeared, as did the heterogeneous complexes which have been previously attributed to be C/EBP binding complexes. Both polyclonal and monoclonal antibodies to rat NF-Y supershifted the distinct F8 site 4 complex (Fig. 4c). With the anti-NF-YB polyclonal antibodies, the supershifted complex has been retarded in the wells of gel or is more apparent by the lack of formation of the complex which is present in a normal band shift. With the anti-NF-YA monoclonal antibody, the supershifted complex appears as a doublet; this may be due to the fact that in rats, two isoforms of the NF-YA subunit, produced by the initiation of transcription at different start sites, have been found (56).

**Mutations which disrupt HNF-1 binding, but not C/EBP binding, to the factor VIII minimal promoter abolish transcriptional activity.** We extended our study of the transcription factors binding to sites 1 to 4 by modifying important nucleotides within the consensus sequences for HNF-1 and C/EBP by site-directed mutagenesis. The nucleotide substitutions which were made within F8 site 1 were identical to those which had been previously reported to inhibit HNF-1 binding in studies with the α2,6-sialyltransferase gene promoter (52). It is well known that C/EBP binds to a notoriously loose consensus (30). When we compared many of the reported C/EBP sequences, we noticed that they have in common a highly conserved adenine residue (underlined) within at least one of the GCAAT half sites. We thus decided to substitute the adenine residue at nucleotide position +17 within site 3 and at position −15 within site 4 to thymine (Fig. 5a). It was hoped that this transversion mutation would disrupt the binding of C/EBP to both site 3 and site 4. Additional evidence which supported our choice of mutation was that one of the naturally occurring mutations at site 1 of the factor IX promoter, which gives rise to the hemophilia B Leyden phenotype, was a transition at this residue of an A to G (nt +13), which disrupts C/EBP binding (1). In band shift studies, we have confirmed that the +17 mutation disrupts the binding of C/EBP, whether the source is rat liver nuclear extract or recombinant C/EBPα (Fig. 5b). The binding of DBP to this site is also reduced. The −15 mutation prevents the binding of C/EBP and DBP but not NF-Y (Fig. 5b). To confirm that NF-Y still binds to the site 4 mutant oligonucleotide, polyclonal and monoclonal antibodies to the A and B subunits of NF-Y were shown to supershift a protein-DNA complex which has previously been attributed as NF-Y binding to the wild-type site 4 oligonucleotide (Fig. 4c). The site 2 C/EBP binding mutant possesses four transversion mutations at positions +75, +79, +81, and +82. As expected, this
FIG. 5. Effects of mutations on the activity of the factor VIII minimal promoter. (a) Nucleotide sequence of the minimal promoter pFVIII(− 148)-Luc construct. The arrow indicates the transcriptional start site as well as the boundary which separates site 3 from site 4. Asterisks indicate the nucleotides modified by site-directed mutagenesis. The mutations in site 3 and site 4 are transversion mutations, A to T at nucleotides −15 and +17 (within the GCAAT sequence). The nucleotide changes in site 1 consist of transition and transversion mutations, which have been previously described to disrupt HNF-1 binding (52). (b) Double-stranded oligonucleotides corresponding to the wild-type F8 site 3 (WT), mutant F8 site 3 (M3), wild-type F8 site 4 (WT), and mutant F8 site 4 (M4) were analyzed in gel mobility shift assays for the ability to bind rat liver nuclear extracts (Liver), recombinant C/EBPα (rC/EBPα), and recombinant DBP (rDBP). These mutations were substituted in the factor VIII minimal promoter. The schematic diagrams in panel c illustrate the effects of the various promoter mutations on the binding of the transcription factors C/EBPα, DBP, NF-Y, and HNF-1. HepG2 cells were transfected with 5 μg of a pFVIII-Luc reporter plasmid under the control of the full-length [pFVIII(−1002/+148)] promoter, wild-type minimal promoter [pFVIII(−44/+148)], and mutant forms of the factor VIII promoter sequence. The pFVIII(−1002/+148)-Luc construct is the reference plasmid. The transcriptional activity obtained with this construct has been assigned a value of 1, with all other values shown relative to this. The first column represents the basal activity ± 1 standard deviation from four separate experiments with the factor VIII promoter constructs alone. The second column represents the fold increase in activity ± 1 standard deviation with cotransfection of C/EBPα and C/EBPβ.
sequence has been shown to disrupt C/EBP binding in a supershift assay using liver nuclear extracts (data not shown).

Figure 5c summarizes the functional effects of the factor VIII promoter mutations on the binding of HNF-1, C/EBP, DBP, and NF-Y to sites 1 to 4. These studies include cotransfection of both C/EBPα and C/EBPβ expression plasmids but do not include cotransfection of HNF-1, since HepG2 cells have been shown to express normal levels of HNF-1 (1). Disruption of C/EBP binding to sites 2 to 4 did not result in any significant change in basal promoter activity (Fig. 5c). However, the transactivation potential of C/EBPα and C/EBPβ was reduced by 40 to 60% (P < 0.025) for all of these mutant promoters. Furthermore, transactivation initiation appears to be unaffected when the GATAAA sequence is removed [pFVIII(−5/−148)mutS3-Luc], suggesting that an initiator (Inr) element at the transcriptional start site may be important in this construct. Mutational analysis of nt −2 and −1 in the putative Inr element [pFVIII(−5/−148)mutInr/S3-Luc] does not interfere with basal promoter activity; however, the ability of C/EBPα and C/EBPβ to transactivate the mutant Inr construct is significantly reduced by an additional −60% (P < 0.005). It is only upon mutation of the HNF-1 binding site [pFVIII(−5/−148)mutS1/S3-Luc] that basal promoter activity is significantly reduced by −75% (P < 0.005) and that the transactivation potential of C/EBPα and C/EBPβ is decreased by 95% (P < 0.005). These results suggest that HNF-1 binding to site 1 plays a critical role in the context of the activity of the minimal factor VIII promoter sequence. However, our results with a promoter construct including only sites 3 to 12 [pFVIII(−1002/+25)-Luc] also show that in the absence of HNF-1 binding, the multiple upstream sites maintain the activity attainable by the full-length factor VIII promoter. This finding suggests that the multiple upstream sites may play a compensatory role should HNF-1 binding be prohibited.

**DISCUSSION**

The 1.2-kb 5′ flanking region of the factor VIII gene was cloned in 1984 (14); in the same study, the transcriptional start site was mapped 170 bp upstream of the initiation methionine codon. In our initial experiments, we have shown that the regulation of factor VIII gene expression involves a complex array of transcription factors that mediate their function through both positive and negative cis-acting regulatory elements. We have identified 12 cis-acting binding sites in the factor VIII promoter by DNase I protection analysis (Fig. 6). There is a cluster of five distal elements within 250 bp that extends to −1 kb from the transcriptional start site. Within this region between nt −740 and −1002, there appears to be a negative regulatory element since the activity of the factor VIII promoter is increased upon removal of this region. Three proximal elements, while lie within 100 bp 5′ of the transcriptional start site, while an additional three elements lie downstream of the CAP site.

The structure of the factor VIII promoter suggests a number of parallels to the regulatory elements of the factor IX gene (6, 40, 46). In both cases, sites for DNA-binding proteins overlap the transcriptional start site. It has been postulated that in the factor IX promoter, these sites may be important in the stabilization of the preinitiation complex, since each of these sites in isolation has been reported to mediate only weak direct transcriptional activation of factor IX (41). In addition, the sites immediately downstream of the CAP site bind isoforms of C/EBP in both promoters. A naturally occurring mutation at position +13 of the factor IX gene results in the reduced binding of C/EBP to this site and produces a hemophilia B Leyden phenotype (6, 41). C/EBPβ binding to sites 1, 4, and 5 of the factor IX promoter mediates transcriptional activation, and the coexpression of C/EBPα with DBP is thought to play a role in the phenotypic recovery of patients with the hemophilia B Leyden phenotype following the onset of puberty (41). Similarly, there are at least seven C/EBP binding sites in the factor VIII promoter, five of which also bind DBP. Therefore, both of these b/ZIP proteins have been shown to be important factors that mediate the transcriptional regulation of these genes in vitro. An additional common feature of these two genes concerns the occurrence of several critical protein binding sites within their 5′ UTRs. In considering all of these similarities, one can suggest that factor VIII and factor IX (and perhaps other coagulation factors) have evolved some common mechanisms for their transcriptional regulation, thus facilitating their coordinate expression.

A fundamental difference between the factor VIII and factor IX genes is their patterns of tissue expression. Factor IX is expressed only in the liver, whereas factor VIII is expressed at moderate levels in the spleen, kidney, and lymph nodes, in addition to the liver (58). Although there are a number of liver-specific factors which regulate factor VIII expression, other ubiquitous and tissue-specific factors likely play a role in its expression in tissues other than the liver. The results from our studies suggest that HNF-1 may be one such factor that appears to be involved in determining the tissue-specific expression of factor VIII, while the relative abundance of the two isoforms of this protein, HNF-1α and HNF-1β, may regulate the magnitude of this response. HNF-1α and HNF-1β have a high degree of homology in their dimerization domains and homeodomains, which allows them to bind to similar sequences, but HNF-1β lacks an activation domain (32). HNF-1α is expressed in the same tissues as factor VIII (liver, spleen, kidney, and intestine), whereas HNF-1β has a more

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**FIG. 6.** Schematic diagram of the factor VIII promoter illustrating the 12 cis-acting DNA sequences and their coordinates within 1.2 kb of the 5′ flanking sequence of the factor VIII gene. Nucleotide numbering is relative to the transcriptional start site at position +1. Some of the trans-acting factors which bind to these sites are indicated above the sites. Site 1 (+118 to +148) binds HNF-1; site 2 (+68 to +85) binds C/EBP (heterodimeric forms); site 3 (+1 to +23) binds C/EBP and DBP; site 4 (−30 to −1) binds C/EBP, NF-Y, and DBP; site 7 (−418 to −401) binds C/EBP and DBP; site 9 (−822 to −800) binds C/EBP and DBP; site 10 (−869 to −852) binds C/EBP, site 5 (−62 to −45), site 6 (−109 to −79), site 8 (−722 to −705), site 11 (−913 to −889), and site 12 (−963 to −946) bind proteins that remain uncharacterized.
ubiquitous expression pattern. In addition, mammalian tissues contain different ratios of HNF-1α and HNF-1β, and although HNF-1β does not block the transcriptional activity of HNF-1α (32), exclusive expression of HNF-1β has been implicated in the repression of a number of hepatocyte-specific genes (32). In light of this evidence, we suggest that factor VIII is likely to be expressed in the same cells in which HNF-1α is synthesized, with its expression being enhanced in the liver because of the apparent absence of HNF-1β protein in this tissue (32) and the presence of other liver-enriched factors that bind to this promoter.

A recent publication by Figueiredo and Brownlee (11) presents evidence indicating that the ubiquitous transcription factor NF-κB binds adjacent to the downstream C/EBP site (site 2). Our results show that a mutation within the HNF-1-binding site significantly reduces the C/EBP responsiveness of the minimal promoter sequence. This finding suggests the possibility of a functional interaction between C/EBP and HNF-1. Furthermore, there is previously published evidence of a physical and functional association between C/EBP and NF-κB (44, 51), suggesting that further study is required to elucidate the interactive role of these three factors in the 5′ UTR of the factor VIII gene.

The presence of many cis-acting sequences establishes binding of transcription factors at a location where they can interact with the transcription initiation complex that must assemble in order for the polymerase to initiate transcription. As an alternative to the TATA box, the GATAAA sequence at position −30 of the factor VIII gene is thought to be important for assembly of the preinitiation complex. We have shown that deletion of the alternative TATA sequence in factor VIII does not disrupt transcription in vitro (Fig. 5c; promoter construct pFVIII−5/+148muss3-Luc). Similarly, Figueiredo and Brownlee have shown that upon mutation analysis of the GATAAA sequence, no significant changes in promoter activity are found (11). There is a putative Inr element between nt −4 and +4 in the factor VIII gene promoter which has sequence homology (7 of 8 nt) to the terminal deoxynucleotidyltransferase Inr element (5′-PyPyPyPyPyPyPyPy-3′) (50), suggesting that this sequence may play a role in localizing the preinitiation complex at this locus. However, in this report, we have demonstrated that mutations within the Inr element do not result in a complete down-regulation of transcription but may reduce the frequency of initiation or allow for a variety of transcription initiation sites (48).

A number of examples in which mutations in the protein binding sites of promoters have had clinical consequences have been documented. It has been found that promoter mutations in factor IX (4, 7, 12, 19, 28, 39, 45, 47, 49), protein C (2), haptoglobin (16), and β-globin (10, 31, 36–38) lead to disruption of the binding of transcription factors, with a resulting clinically significant reduction of gene expression. Such a lesion might be expected to affect the level of expression of the factor VIII protein and give rise to a clinical phenotype.

In 1982, when the hemophilia B Leyden phenotype was first described, the additional discovery of variable factor VIII expression in rare patients was thought to represent a factor VIII form of the Leyden phenotype (4). These patients exhibited a mild form of hemophilia A with levels of factor VIII between 10 and 20% of normal, which gradually increased to 40 to 160% of normal (27, 43, 57). In a previous study of 127 unrelated patients with hemophilia A, no changes were reported in the promoter sequence extending 200 nt 5′ of the transcriptional start site (18). In addition, the full 1.2-kb factor VIII promoter has been sequenced in three other unrelated patients with a variable hemophilia A phenotype (26, 57). However, no regulatory mutation causing hemophilia A has been found. In our studies, we generated mutations at critical residues for HNF-1 and C/EBP binding in what was thought to be a minimal promoter. Our results suggest that even though HNF-1 binding is critical in maintaining the activity of the factor VIII promoter in the absence of upstream elements, it is not necessary in the native promoter context, as the multiple upstream sites seem to compensate for its loss. We postulate from these results that our inability to detect the down-regulation of factor VIII promoter activity in vitro parallels our inability to detect a disease-causing mutation which would give rise to a clinical phenotype. The role of the multiple upstream sites can be hypothesized as a safeguard or backup regulatory system in the instance of a potentially harmful mutation occurring in this very narrow region of the downstream HNF-1 binding site.

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