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Structural and functional basis of the developmental regulation of human coagulation factor IX gene: Factor IX Leyden

(hemophilia B-Leyden/puberty/DNA-binding protein)

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ABSTRACT **Hemophilia B Leyden is characterized by unusual developmental regulation of factor IX synthesis in affected individuals. One family affected with the hemophilia B Leyden phenotype was found to have a specific single-base mutation (G→A) at nucleotide −6 of the factor IX gene. The mutation site was found in a small region of the 5′-untranslated sequence designated the Leyden-specific region (LS region). This region, ≈40 base pairs in length, contains the unique mutation sites of all the known factor IX Leyden genes (five families) analyzed to date. This fact strongly suggests that the LS region is directly or indirectly involved in the developmental regulation of factor IX biosynthesis. Base changes at nucleotide −20 as well as at nucleotide −6 and deletions of the 3′ half of the LS region reduced expression activity of the factor IX gene to ≈15–31% that of the normal control, as assessed in a cultured cell (HepG2) expression system. The LS region binds at least two proteins. Androgen significantly increased the transcriptional activities of both mutant and normal factor IX genes in a concentration-dependent manner.**

Factor IX, along with a large cofactor protein, factor VIII, is involved in the middle phase of the blood coagulation cascade (1). A deficiency of factor IX causes an abnormal bleeding disorder called hemophilia B. The molecular mechanism underlying hemophilia B is very heterogeneous (2). The human factor IX gene, which has been completely sequenced, is located at the Xq27 region and is expressed in hepatocytes with high tissue specificity (3, 4). At present, little is known about the mechanism responsible for the developmental regulation of the factor IX gene. Normally, factor IX gene expression increases during the perinatal stage and reaches its subadult level in a few weeks after birth (5–7). The factor IX genes of the hemophilia B Leyden trait, however, do not express until the onset of puberty (8, 9). After onset of puberty, the factor IX level in the peripheral blood of patients with this phenotype gradually rises to a subnormal or normal level at a rate of ≈5% per year. Recently, the specific molecular defects in five known factor IX Leyden genes have been reported (10–13). However, no evidence of any direct correlation between the mutations and functional defects of these genes has been analyzed.

In the present report, we analyze the structure of a hemophilia B Leyden gene.¶ Experimental evidence correlates the defects found in the 5′-untranslated sequence of factor IX Leyden genes and regulation of these genes. We also describe specific protein binding to the region.

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MATERIALS AND METHODS

Materials. Restriction enzymes and DNA modification enzymes were obtained from Boehringer Mannheim. (α-^{35}S)-labeled dATP, [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{14}C]chloramphenicol were obtained from Amersham. Silica gel for thin layer procedures was from Baker. The DNA sequencing kit that uses Sequenase was obtained from United States Biochemical. *Thermus aquaticus* (*Taq*) DNA polymerase with AmpliTaq kit used for polymerase chain reactions (PCR) was obtained from Perkin–Elmer/Cetus. The Mutagene *in vitro* mutagenesis kit was obtained from Bio-Rad. β-Galactosidase expression plasmid vector (pCH110) and acetyl-CoA were obtained from Pharmacia P-L Biochemical. The ELISA amplification system was purchased from Bethesda Research Laboratories. Synthetic oligonucleotides were obtained from the Center for Molecular Genetics core facility on the Michigan campus. pUMSVOCAT is a low-background expression vector with the chloramphenicol acetyltransferase (CAT) gene (14). Human androgen receptor cDNA and pD5 (an expression vector with adenovirus major late promoter) were provided by C. Chang (University of Chicago) and K. Berkner (Zymogenetics), respectively. pD5hAR, an androgen receptor-expressing vector, was constructed by inserting the human androgen receptor cDNA into pD5.

Hemophilia B Leyden Family. A young male family member (now 6 years old) was first referred to the Portland Hemophilia Center for an abnormal bleeding episode when he was circumcised at the age of 15 days; at this time he received a blood transfusion. Five months later, his factor IX activity level was determined at 5% normal, whereas his factor VIII:C level was normal. At 1 year, his factor IX activity level was also 5% normal. Since then he has been rather mildly affected, and no factor IX concentrate infusion has been required after the first bleeding episode. His factor IX activity level had slightly increased to 10% when he was recently assayed (December 13, 1989) at the same hemophilia center. One of his uncles also had similar abnormal bleeding when he was circumcised and had shown a mild bleeding tendency until pubertal stage, but this bleeding has since ameliorated. Five more distant, maternal, male relatives have had similar bleeding tendencies. Blood samples (10–20 ml) were freshly drawn into a 0.1 vol of 3.8% sodium citrate after written consent from these individuals of the hemophilia B

Abbreviations: CAT, chloramphenicol acetyltransferase; LS region, Leyden-specific region; PCR, polymerase chain reactions; nt, nucleotides.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. K02402).

family. The samples were centrifuged for 10 min at 3000 rpm to separate plasma and cell fractions. The plasma fraction was used for determining factor IX activity by a one-stage clotting assay with human factor IX-deficient plasma and antigen levels by a Laurel rocket immunoelectrophoresis method by employing polyclonal factor IX antibodies (Wisconsin Blood Center, Milwaukee). The antigen level reproducibly detected by this method was >6% normal control. The factor IX antigen level of the affected member and his mother were also determined by ELISA assays in our laboratory by using the polyclonal antibodies and ELISA amplification system (BRL). The lowest antigen level reproducibly detected by this assay was ≈1% normal control. Pooled plasma of 30 normal healthy individuals was used as control for these assays. Cell fractions were used to prepare total genomic DNA by the Triton X-100 method (15).

Amplification of Genomic DNA and Nucleotide Sequencing. Various regions of the factor IX gene of family members were directly amplified by PCR according to instructions provided by Perkins–Elmer/Cetus. The amplified DNA fragments were purified on low-melting-point agarose gel electrophoresis and subjected to direct nucleotide sequencing or subcloned into a plasmid vector (pUC18) before sequencing. Sites in the factor IX gene sequence employed for synthesizing PCR primers were as follows [see ref. 3 for nucleotide (nt) numbering]: exon 1 (−1274 to −1245; 166–195C); exons 2 and 3 (6217–6236; 6797–6814C); exon 4 (10227–10251; 10713–10737C); exon 5 (17518–17542; 17987–18011C); exon 6 (20178–20208; 20702–20726C); exon 7 (29813–29837; 30415–30439C); and exon 8 (30731–30753; 31490–31514C; 31420–31444; 32835–32859C; 32271–32295C; and 32550–32574); C at end of the numbering indicates the complementary sequence.

Construction of Expression Vectors. Expression vectors, p-416/29CAT and p-416/29(−6:A)CAT, were prepared by ligating a 445-base pair (bp) fragment (nt −416 through +29 of the normal factor IX gene) that was PCR amplified from normal genomic DNA and factor IX Leyden genomic DNA, respectively, to pUMSVOCAT at the *Sma* I site after adding *Sma* I linkers to the amplified DNAs. p-416/4CAT was constructed in a similar way, except that a 420-bp fragment (nt −416 to +4) was employed as described (4). Expression vectors p-416/4(−6:A)CAT, p-416/4(−20:A)CAT were prepared by site-directed mutagenesis of a DNA fragment spanning nt −416 to +4 by using an *in vitro* mutagenesis kit (Bio-Rad) (16). Synthetic oligonucleotides (5′-TGGTAAGGTTGATTAGTTGT-3′ and 5′-TAGTTGTACCTAAGTACAAG-3′, respectively) were used as mutagens in these experiments. All mutagenized DNA fragments were sequenced to confirm the specific base changes. Deletion constructs p-416/−3CAT, p-416/−13CAT, p-416/−23CAT, and p-416/−50CAT were prepared as follows: p-416/4CAT was first digested with *Eco*RI, followed by Bal-31 nuclease for various time periods. The digested DNAs were then incubated with *Sma* I. The resultant 5′-end fragments of factor IX gene with various deletions at the 3′ end were ligated to pUMSVOCAT at the *Sma* I site. The extent of the deletions in the expression plasmid were all confirmed by actual nucleotide sequencing.

CAT Assay. CAT activity expressed in HepG2 cells was assayed as described by Gorman *et al.* (17) with minor modifications. For each CAT assay, 20 μg of DNA consisting of a CAT construct (12 μg), β-galactosidase expression vector (1.5 μg of pCH110, an internal control), and herring sperm DNA (6.5 μg) were cotransfected to HepG2 cells at 60–70% confluency in 10-cm dishes in Eagle's minimal essential medium (GIBCO) supplemented with 10% fetal calf serum. After 4 hr, cells were treated with 15% (vol/vol) glycerol for 30 sec and incubated for another 48 hr at 37°C under 5% CO_2. The conversion in CAT assays for full promoter activity was kept within 20–40% and not >40%. Assay conditions used for assessing androgen effects were the same as above, except 5 μg of pD5hAR was cotransfected to the cells. After 16–20 hr, the medium was supplemented with various amounts of 5α-androstan-17β-0L-3-one (Sigma). Cells were then incubated another 24–30 hr before harvesting. pD5hAR was constructed by ligating the human androgen receptor cDNA (18) to the adenovirus major late promoter of pD5 at the 3′-downstream region. Cotransfection of this vector was done to ensure a high level of androgen receptor molecules in the cells, although HepG2 cells were found to contain the level of androgen receptor required in this experiment.

Gel-Retardation Assay. *(i) Preparation of DNA fragments.* The 420-bp DNA fragment spanning nt −416 to +4 of factor IX gene was prepared from plasmid p-416/4CAT by digestion with *Sma* I. The fragment was labeled with ^{32}P at the 5′ end with T4 polynucleotide kinase to a specific activity of 2×10^7 cpm/μg. In each gel-retardation experiment, 0.9 ng of the radiolabeled fragment (2×10^4 cpm) was used. A pUC18 fragment (501 bp) was prepared from pUC18 with *Hpa* II. A double-stranded 40-bp oligonucleotide spanning nt −32 to +8 of factor IX gene was prepared by mixing chemically synthesized complementary single-stranded oligonucleotides. *(ii) Preparation of nuclear extracts of HepG2 cells.* Nuclear extracts of HepG2 cells (≈1×10^8) were prepared according to Dignam *et al.* (19), as modified by Osborn *et al.* (20). *(iii) Gel retardation experiment.* The procedure is adapted from Fried and Crothers (21). The radiolabeled fragment spanning nt −416 to +4 (420 bp) and poly(dI·dC) (Pharmacia) were mixed with nuclear extracts in a 20 μl of reaction buffer containing 4% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, and bovine serum albumin (nuclease free) at 0.1 mg/ml. The mixture was incubated at room temperature for 20 min before loading onto a 4% polyacrylamide gel (acrylamide/bisacrylamide, 30:1) with 0.2 mM dithiothreitol. The gel was electrophoresed in Tris·glycine buffer (50 mM Tris·HCl, pH 8.6/0.38 M glycine/2 mM EDTA) at 20 mA for 4 hr followed by drying and autoradiography.

RESULTS AND DISCUSSION

The pedigree of the hemophilia B family, designated as factor IX Leyden-Portland, is shown in Fig. 1*A*. Member II-3, now 40 years old, was affected before reaching puberty but became phenotypically normal as he grew older. The factor IX antigen and activity levels (67% and 62%, respectively) of II-3 are now within the normal adult levels, whereas member II-1 (42 years old), who has never been affected, maintains normal levels of both antigen and activity. Plasma sample prepared from the blood of III-4 (6 years old, affected) showed low factor IX antigen and activity levels (9.1% and 11.3% of normal, respectively). These data agree well with the definition of the factor IX Leyden trait (8). This family is affected with only mild hemophilia B Leyden phenotype, whereas all other known Leyden phenotype families are severely to moderately affected (10, 11, 13). I-2 shows normal activity and antigen levels (89% and 115% of control normals, respectively), whereas II-4 has significantly reduced levels for activity and antigen (35% and 39%). This comparison suggests an interesting possibility that hormonal changes at menopause significantly affect the expression of factor IX gene in heterozygous females.

To identify the molecular defect(s) in the factor IX gene of this family we have sequenced genomic DNAs isolated from peripheral blood cells of I-2, II-1, II-3, II-4, and III-4 (Fig. 1*B*). In these experiments, we found that the guanine base present at position −6 in II-1 and a normal control was replaced by adenine in II-3 and III-4. I-2 and II-4 were clearly shown to be heterozygous at this position with both adenine

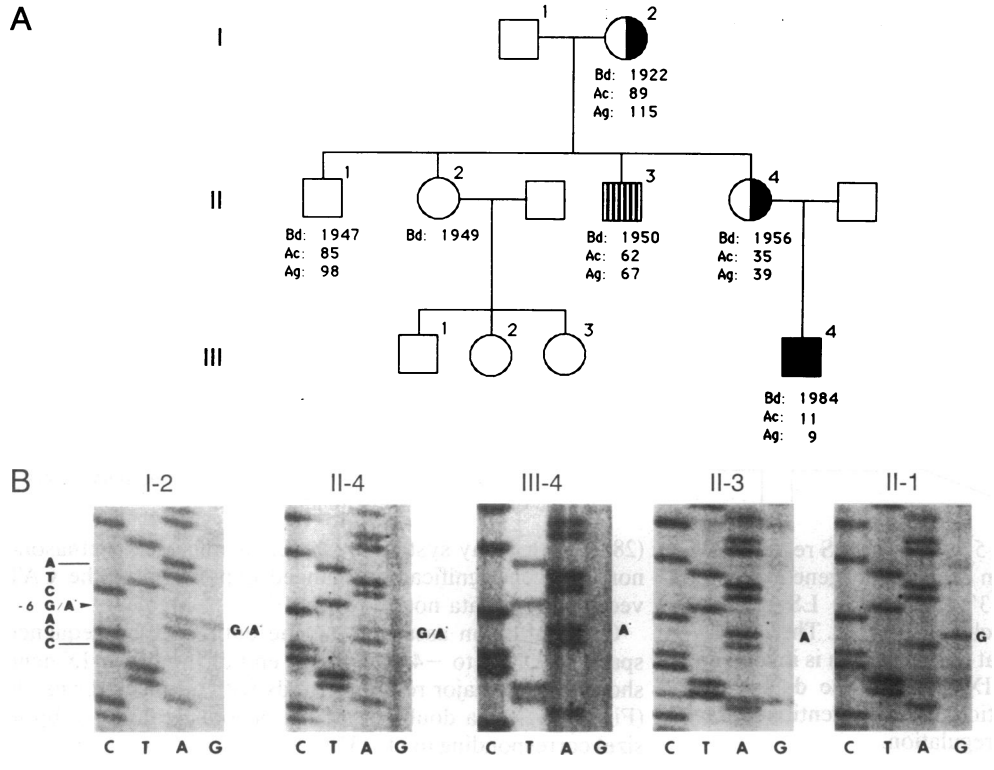

FIG. 1. (*A*) Pedigree of factor IX Leyden-Portland family. Three generations of the family are shown with year of birth (Bd), factor IX antigen level in % normal adult level (Ag), and factor IX activity level in % normal adult level (Ac). Numbers represent mean values of triplicated assays. ■, Affected member; ■, affected member in prepubertal age; ◑, carrier. (*B*) Molecular defect in factor IX Leyden-Portland gene. Autoradiograms of the nucleotide sequences of relevant regions are shown. Factor IX gene of III-4 was completely sequenced for all exons and splice junction regions as well as the 5′-end flanking sequence up to nt −1244. The same 5′-end regions in addition to other various portions of the genes of I-2, II-1, II-3, II-4, and of an unrelated normal male were also sequenced.

and guanine present at position −6. The segregation of the single-base mutation, G→A at position −6, agrees well with plasma data (Fig. 1*A*). All coding regions of factor IX and the splicing junctions as well as the 5′-end flanking region to nt -1244 were analyzed in III-4 (affected member). The mutation found in this family may be from a C→T change at the CpG sequence on the complementary strand. The base change, incidentally, resulted in loss of a *Taq* I site in the normal gene. This gene was also found to have the codon for threonine at the third position of its activation peptide, a known dimorphic residue, and a 1.8-kilobase (kb) *Taq* I polymorphic fragment (3). In this study, we also corrected our original sequence reported for the normal gene as follows: nt −1096, adenine; nt −597, cytosine; nt −598, thymine; and nt −423, cytosine.

The transcription starting site of the gene was recently determined to be at nt −150 (4), placing the region containing the mutation in the 5′-untranslated sequence of the gene and close to the translation start site. The site of the mutation was found to be in a small general region where all other specific mutations (T→A substitution at −20, G→C substitution at −6, a base deletion or A→G substitution at +13) were identified in all five known families affected with this phenotype (10–13). We designate this general region of ≈40 bp in length the Leyden-specific region (LS region). Prepubertal members of families carrying mutations at nt +13 and −20 were severely to moderately affected (10, 11, 13). It is interesting to note that the family carrying a G→C mutation at nt −6 is rather severely affected (M. Vidaud, personal communication). A mutation of A→G at nt +13 in an unrelated hemophilia B patient was also reported (22). No additional family information of this patient, however, is available at present, but it is highly likely that this family is also affected with hemophilia B Leyden. These data strongly suggest that the LS region is involved in the developmental regulation of this gene because the chance of all unrelated Leyden families having random mutation in the LS region is extremely small.

We then carried out a series of functional assays to test the importance of the LS region in the regulation of this gene by using expression vectors derived from pUMSVOCAT (14) and HepG2 cells, which can express vitamin K-dependent proteins, including factor IX (23). The DNA fragments used in constructing these vectors contained the factor IX sequence spanning nt +4 to −416 as well as nt +29 to −416. In a recent gene dissection analysis, we demonstrated that fundamental cis-acting elements required for factor IX gene expression are contained within this immediate 5′-upstream region of the gene (4). In these vectors, the CAT gene was ligated to the 5′-end sequence of factor IX gene with or without the specific single-base changes at nt −20 (T→A) or nt −6 (G→A). Table 1 shows that these constructs were then used for expression in the human hepatoma cell line. Mutations at either nt −6 or nt −20 lowered the expression level to about one-third that of control. Although the mutations did not totally eliminate the expression, the decreases in CAT expression activity seen in the cultured cell system are very significant. The mutation at nt −20 consistently gave somewhat lower CAT activities compared with the mutation (G→A) at nt −6, which may, in part, reflect the difference in severity of symptoms. Whether the reduced activities are due to the effects of the mutations on the transcriptional level or on the translational level is yet undetermined.

Drastic decreases in expression activity were also seen when deletions were extended beyond position −3 toward the 5′-upstream region (Fig. 2). In this experiment, the residual activities, 26.1%, 16.7%, and 14.9%, were found for p-416/−13CAT, p-416/−23CAT, and p-416/−50CAT, respectively. These results indicate that the specific region

Table 1. Effects of mutations in LS region of factor IX Leyden genes on expression

Construct	Relative activity,* % (SD)	
	Exp. 1	Exp. 2
p-416/29CAT	100	
p-416/29(−6:A)CAT	38.5 (15.3)	
p-416/4CAT	104.6 (8.3)	100
p-416/4(−6:A)CAT		31.1 (10.6)
p-416/4(−20:A)CAT		28.1 (7.1)

*Expression activities (means of five independent experiments) relative to p-416/29CAT or p-416/4CAT.

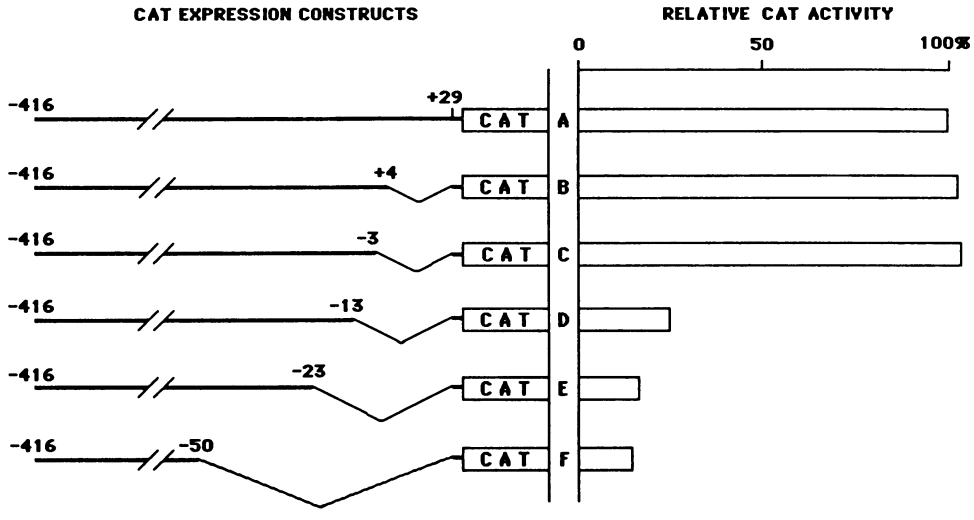

FIG. 2. CAT assays of constructs with deletions. CAT activities in % relative to that of construct A (p-416/29CAT) are shown (means of five independent experiments). Construct B, p-416/4CAT; construct C, p-416/−3CAT; construct D, p-416/−13CAT; construct E, p-416/−23CAT; and construct F, p-416/−50CAT. Conditions for CAT assays were the same as for those of Table 1. Control constructs that have factor IX sequences in reverse orientation showed only basal activity levels (data not shown).

encompassing nt −20 to −6 of the 5′ half of the LS region has an important role in the regulation of factor IX gene expression, whereas the role of the 3′ half of the LS region containing the +13 position is not clear at present. These data further support the hypothesis that the LS region is involved in the regulation of the factor IX gene. These data also suggest the possibility of an additional, yet-unidentified element(s) that may be involved in regulation.

Table 2 shows that expression levels of the mutant factor IX gene sequences generally increased with androgen in a concentration-dependent manner. Androgen, however, also elevated expression of the normal control similarly. These results strongly suggest that induction of factor IX synthesis at the onset of puberty in hemophilia B Leyden patients is probably from the indirect, but not the direct, effects of testosterone on factor IX biosynthesis. A set of genes from hepatocytes may be more directly affected by testosterone, and products of these genes singly or collectively may induce expression of the factor IX Leyden gene but not of the normal factor IX gene. The mechanism of this effect, which is highly likely to involve the LS region of the gene, has yet to be determined.

Testosterone given in therapy increased factor IX activity in hemophilia B Leyden patients (24). This effect, however, appears more general and not specific for the factor IX Leyden gene because similar effects were also seen for other proteins, such as factor VIII (25–27). Our results obtained from the *in vitro* assay system agree well with this observation. Interestingly, sequence (GGTACAACTAATCG) significantly similar to a glucocorticoid responsive element is located between nt −20 and −6. Such sequences are responsive to various hormones, including androgens, glucocorticoids, and progesterones in mouse mammary tumor virus (28). In our assay system, however, neither dexamethasone nor estrogen significantly changed expression of the CAT vectors used (data not shown).

Table 2. Effect of androgen on expression of CAT constructs with normal and factor IX Leyden sequences

Construct	CAT activity,* %				
		Androgen			
	Control	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
Exp. 1					
p-416/4CAT	100	137	216	—	292
p-416/4(−6:A)CAT	29	46	72	71	77
p-416/4(−20:A)CAT	49	78	87	100	140
Exp. 2					
p-416/29CAT	100	89	79	110	157
p-416/29(−6:A)CAT	39	49	49	63	101

*Expression activities relative to p-416/4CAT (Exp. 1) or to p-416/29CAT (Exp. 2). A set of representative results is shown.

Gel-retardation assays using the entire 420-bp sequence spanning nt +4 to −416 of the 5′ end of the factor IX gene showed three major retarded bands with a few minor bands (Fig. 3). When a double-stranded oligonucleotide (40 bp in size) corresponding to nt −32 to +8 was used as a competitor (Fig. 4), the top two retarded bands (a and b) specifically disappeared, indicating that these proteins bind to the region containing the specific mutations. This intepretation was further supported in our recent observation that two oligonucleotide subsets (20 bp and 16 bp in sizes) that specifically cover the −20 region or the −6 region, respectively, also bound proteins (unpublished data). Binding of these proteins, designated LSP-1 and LSP-2, to the LS region of the gene may be important in regulating factor IX synthesis. These proteins may also bind to the LS region of factor IX mRNAs, affecting their processing, stability, or even its elongation. Recently, the tat protein of human immunodeficiency virus type 1 was reported to trans-activate the virus gene tran-

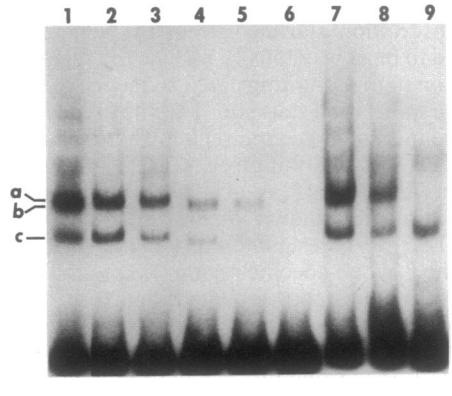

FIG. 3. Gel-retardation assay. DNA fragment used in this assay is the 5′-end region of the factor IX gene spanning nt −416 to +4 (420 bp). The ^{32}P-labeled 420-bp fragment (0.9 ng with 2 × 10^4 cpm) was used in each reaction. Lanes: 1, ^{32}P-labeled fragment (420 bp) and 2 μg of poly(dI·dC) incubated with 8 μg of HepG2 nuclear extract; 2 and 3, components in lane 1 plus 200 and 800 ng of the pUC18 fragment (516 bp, nonspecific competitor), respectively; 4–6, components in lane 1 plus 200, 400, and 800 ng of the nonradiolabeled 420-bp fragment (specific competitor), respectively; 7–9, components in lane 1 plus 32, 64, and 128 ng of double-stranded synthetic oligonucleotide (40 bp in size corresponding to region −32 to +8), respectively. Without nuclear extracts, the radiolabeled 420-bp fragment migrates as a single band that is seen at the bottom of each lane. a, b, and c, Retarded bands.

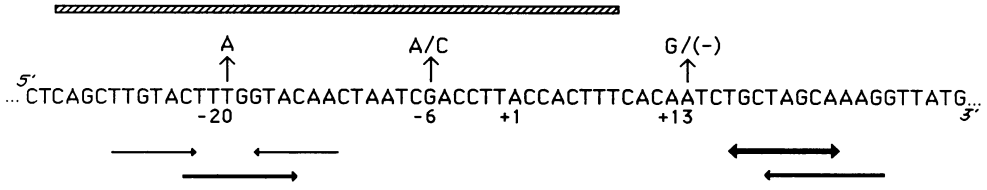

FIG. 4. LS region in the 5'-untranslated sequence of factor IX gene. Nucleotide sequence containing LS region and its neighboring regions is shown from left (5' end) to right (3' end). Mutations in factor IX Leyden genes so far identified (10, 11, 13), including a G→A change at nt −6 found in this study, are shown with small arrows. (−) at nt +13 indicates a base deletion. Our original numbering system (3) was used. According to the revised transcription start site that corresponds to nt −150 in the original numbering system (4), the LS region is contained in the 5'-untranslated region. Pairs of arrows facing each other as well as the thick arrow with two arrowheads show the location of pallindromic sequences. A tandem repeat of two regions (nt −31 through −6 and nt −5 through +21) is noted (11). Hatched bar region (40 bp) corresponds to the synthetic oligonucleotide used as specific DNA competitor in the gel-retardation assay (Fig. 3).

scription through a nascent RNA target (29, 30). Iron-responsive elements were also found in the 5'-untranslated region of human ferritin mRNA (31).

Various secondary structures are often found in regulatory elements of genes (32). The LS region has interesting palindromic structures as well as repeats (Fig. 4). These particular structures, although rather short, may be involved in binding of these protein factors. Although the role of the 3' half of the LS region is not clear at present, a fair tandem repeat of region −31 through −6 and region −5 through +21 (10) may support the possibility that the region containing nt +13 is also involved in regulating this gene through mechanisms yet unknown. The role of the region containing nt +13 in the regulation of this gene could be secondary to the region containing nt −20 and −6 or any other unidentified region(s). If so, the subtle defective effect of the mutations at nt +13 on expression may not be easily detected in the assay system used in our study.

Most mammalian genes are developmentally regulated. Among them, developmental regulation of the expression of hemoglobin genes (33), α-fetoprotein, as well as albumin have been extensively studied (34). Establishment of the structural and functional importance of LS region for regulating expression of factor IX gene will facilitate further studies on the developmental regulation of this gene in *in vivo* assay models, such as transgenic animals.

Note Added in Proof. Recently we observed that point mutations at +13 (G or base deletion) also lower the expression activity of p-416/29 (+13:G) or p-416/29 (+13:del) to 26% of that of p-416/29 (normal control). The data, together with those in Fig. 2, suggest a possibility that the 5' and 3' halves of the LS-region may not function cooperatively. Also, to observe the effect of mutations, the intact sequences surrounding +13 are required.

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