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Expression of Human Factor IX and Its Subfragments in *Escherichia coli* and Generation of Antibodies to the Subfragments†

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ABSTRACT: A cDNA clone encoding the entire human blood clotting factor IX (amino acids −3 to 415) has been placed under control of transcription and translation signals from bacteriophage T7 and expressed in *Escherichia coli*. The full-length cDNA and 13 different subfragments (which together cover the entire coding sequence of mature factor IX plus amino acids −40 to −19 of the prepro leader sequence) have each been joined to the coding sequence for the major capsid protein of T7 after the 326th codon and expressed as fusion proteins. All of the fusion proteins were insoluble, which facilitated their purification. A goat polyclonal antiserum against human factor IX reacted to different extents with the different fusion proteins, and rabbit polyclonal antibodies raised against the purified fusion proteins recognize the factor IX molecule, as demonstrated by immunoblotting techniques. Antibodies against at least one of the fusion proteins can also inhibit the biological activity of purified factor IX in a one-stage partial thromboplastin time bioassay. We expect these fusion proteins and the antibodies against them to be useful in studying the structure and function of factor IX.

Factor IX (Christmas factor) is a plasma glycoprotein essential for normal hemostasis. A defect in, or absence of, the factor IX molecule results in hemophilia B, an X-linked hereditary bleeding disorder. Purified human factor IX has a molecular weight of about 57 000 and contains approximately 17% carbohydrate (Fujikawa et al., 1973; Osterud & Flengsrud, 1975; DiScipio et al., 1978). The first 12 NH_2-terminal glutamic acid residues of the protein are posttranslationally modified to γ-carboxyglutamic acids (Gla) in a vitamin K dependent reaction (Fryklund et al., 1976; Olsen

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& Suttie, 1978; Gallop et al., 1980). These 12 Gla residues are thought to be responsible for calcium-mediated binding of factor IX to phospholipid (Jackson & Nemerson, 1980) and are required for biological activity. Aspartic acid residue 64 is also posttranslationally modified to β-hydroxyaspartic acid (McMullen et al., 1983b; Fernlund & Stenflo, 1983) which is thought to be a strong calcium binding site (Morita et al., 1984); however, the exact function of this modified residue is unknown. Factor IX circulates as an inactive zymogen which is activated by factor XIa and calcium (DiScipio et al., 1978; Fujikawa et al., 1974) as well as factor VIIa–tissue factor (Jesty & Silverberg, 1979) to generate an activation peptide of approximately 11 000 daltons and activated factor IX (factor IXa). Activated factor IX is a serine protease consisting of light and heavy chains joined by a disulfide bond(s). Factor IXa, together with its cofactors (factor VIIIa, phospholipid, and calcium), activates factor X to factor Xa [for reviews, see Davie and Fujikawa (1975), Jackson and Nemerson (1980), and Nemerson and Furie (1980)].

The normal gene for factor IX is about 35 kilobases (kb) and consists of eight exons and seven introns (Anson et al., 1984; Yoshitake et al., 1985). The full-length cDNA is about 2.8 kb and includes approximately 1.3 kb of 3′ nontranslated sequence (Kurachi & Davie, 1982; Jaye et al., 1983; McGraw et al., 1985). As derived from the DNA sequences, the amino acid sequence of the factor IX precursor molecule includes a prepro leader peptide of 46 residues and a zymogen of 415 residues.

The factor IX polypeptide can be divided into several functional domains at least some of which seem to correlate well with the exon structure of factor IX (Katayama et al., 1979; Anson et al., 1984; Yoshitake et al., 1985). The most easily definable domains (from the amino terminus to the carboxy terminus) are the prepro leader peptide region, the Gla domain containing the γ-carboxyglutamic acid residues, two epidermal growth factor domains, an activation peptide region, and the catalytic domain.

One approach to studying the structure and function of the factor IX molecule has been to make antibodies to different domains of factor IX. Our goal is to create antibodies which allow us to map the surface of the factor IX molecule and to probe its interaction with the other components of the coagulation system. We report here that we have expressed factor IX and several subfragments of the protein as fusion proteins in *Escherichia coli*; the fusion proteins have been used to elicit antibodies to different regions of the factor IX molecule.

Experimental Procedures

Materials. Synthetic oligonucleotide *Bam*HI linkers (8, 10, or 12 nucleotides in length) were purchased from New England Biolabs. Restriction enzymes, *E. coli* DNA polymerase I (Klenow fragment), calf intestine alkaline phosphatase, polynucleotide kinase, and T4 DNA ligase were obtained from either Bethesda Research Laboratory (BRL), New England Biolabs, or Boehringer Mannheim. ^{125}I-Labeled protein A, [^{35}S]methionine, [^{35}S]dATP, and [α-^{32}P]- or [γ-^{32}P]dATP were purchased from Amersham Corp. Purified human factor IX was a gift from Dr. Rogier M. Bertina or purchased from R.Q.P. Laboratories Inc. Goat anti-human factor IX antibody to native factor IX purified from plasma was kindly provided by Dr. Howard M. Reisner (Department of Pathology, The University of North Carolina). Freund's complete or incomplete adjuvants, isopropyl β-D-thiogalactoside (IPTG), and factor IX deficient plasma were obtained from Sigma. "Platelin Plus Activator" was purchased from General Diagnostics. All other reagents were the highest purity commercially available.

T7 Vectors. Four vectors for placing cloned DNA under control of a promoter for T7 RNA polymerase were used, pET-1, pET-2b, pET-3b (Rosenberg et al., 1987), and pAR3070. They are all derived from pBR322 by inserting fragments of T7 DNA containing the *ϕ10* promoter (the promoter for gene *10* which specifies the major capsid protein of T7) into the *Bam*HI site so as to direct transcription counterclockwise (opposing the tetracycline promoter). Each plasmid has a unique *Bam*HI cloning site downstream from the promoter. Plasmid pET-1 lacks translational initiation signals between the *ϕ10* promoter and the cloning site; therefore, translation is dependent on signals resident within the cloned fragment. In plasmid pET-2b, the promoter is followed by the translational start site and the first 11 codons of the gene *10* protein; proper insertion of a DNA fragment into the unique *Bam*HI cloning site will generate a recombinant whose translation product is a fusion protein containing, from its amino terminus, the first 11 amino acids of gene *10*, 3 amino acids derived from the linker sequence, and the polypeptide specified by the fragment. Plasmid pET-3b is a derivative of pET-2b in which the transcription terminator for T7 RNA polymerase, Tϕ, has been inserted just downstream of the *Bam*HI cloning site. In addition, the translation termination codons are present in front of Tϕ in three different reading frames. Plasmid pAR3070 is identical with pET-3b except that the unique *Bam*HI cloning site is preceded by the first 326 codons of the open reading of gene *10*. The fragments expressed in this vector will contain, from its amino terminus, 326 amino acids of gene *10*, 3 amino acids from the linker, the polypeptide specified by the fragment, and, depending on the fragment, 5, 21, or 23 amino acids of carboxy-terminal sequences derived from the vector. Factor IX subfragment i contains its own termination codon.

Construction of Factor IX Expression Plasmids. The cDNA plasmid containing the entire factor IX coding sequence (McGraw et al., 1985) was purified by using standard techniques. Subfragments were generated by digestion with the appropriate restriction enzymes. Prior to cloning, the termini of the fragments were rendered flush by incubation with the Klenow fragment of *E. coli* DNA polymerase I in the presence of all four dNTPs. The appropriate length *Bam*HI linker was added to ensure proper fusion of the open reading frame to the beginning of gene *10* when the fragment was inserted into the T7 expression vectors. There is no *Bam*HI site within the factor IX cDNA. Standard techniques (Maniatis et al., 1982) were used to clone individual fragments and identify the recombinant clones having the factor IX sequences inserted in the proper orientation relative to the T7 genetic elements. All recombinant plasmids were subjected to DNA sequence analysis (Maxam & Gilbert, 1980; Sanger et al., 1977) through the vector–factor IX junctions to confirm that the proper open reading frames were retained during construction. *E coli* HMS 174 [rK12$^-$mK12$^+$*recA* rif^R (Campbell et al., 1978)] was used as the initial host for recombinant plasmid strains.

Cell Growth and Expression of Fusion Proteins. To direct expression of the factor IX target genes, the recombinant plasmids used in this study were moved into *E. coli* BL21-(DE3), a λ lysogen of BL21 (rB$^-$mB$^-$rif^S) in which the prophage carries a copy of the gene for T7 RNA polymerase under control of the *lac*UV5 promoter (Studier & Moffatt, 1986). The resulting strains were grown with shaking at 37 °C in M9TB medium (Studier, 1969) supplemented with 100

μg/mL ampicillin. The *lac*UV5 promoter was induced by adding IPTG to a final concentration of 0.5 mM when the cultures reached OD_{600} = 0.5. After an additional 30–60 min of shaking at 37 °C, portions of the cultures were harvested by centrifugation and the proteins subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). SDS–polyacrylamide gel electrophoresis was performed as described (Studier, 1973), using a 10–20% gradient of acrylamide and a 5% stacking gel. Following electrophoresis, the proteins were visualized by staining with Coomassie brilliant blue or subjected to immunoanalysis.

Western Blotting. Proteins were subjected to SDS–polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose paper (Tombin et al., 1979). After transfer, the nitrocellulose blots were blocked with 8% bovine serum albumin (BSA) for a minimum of 2 h and reacted with the desired antibody at room temperature overnight. The unbound antibody was removed by several washes of 0.01 M sodium phosphate/0.15 M sodium chloride, pH 7.2 (PBS). The blots were incubated in PBS, 0.5% Tween 20, and ^{125}I-labeled protein A (1 μCi/30 mL reaction buffer) for 1.5 h, washed in the same buffer without isotope, air-dried, and autoradiographed.

Preparation of Fusion Proteins for Use as Antigens. One hundred milliliter cultures of BL21(DE3) harboring a recombinant factor IX plasmid were grown and incubated with IPTG as above. One hour after induction, cells were sedimented, washed, and resuspended in 4 mL of lysis buffer [50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), pH 8, 2 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM NaCl]. The cells were lysed with the addition of lysozyme to a final concentration of 1 mg/mL and sodium deoxycholate to a final concentration of 0.04%. The viscosity was reduced by sonication (Heat Systems Model W220F, Ultrasonics Inc.) with two, 1-min bursts (maximal setting) of a microprobe. This lysate was centrifuged at 10000 rpm for 15 min in a Sorvall SS-34 rotor, and the pellet, which contained the insoluble fusion protein, was resuspended in 4 mL of lysis buffer. An aliquot was subjected to SDS–polyacrylamide gel electrophoresis. The band containing the fusion protein was visualized by staining a strip from the edge of the gel, and the fusion protein was excised and macerated in 1 mL of PBS.

Immunization of Animals. New Zealand White rabbits, 5–8 lb, were immunized with emulsions made by mixing equal volumes of macerated acrylamide in PBS (containing about 500 μg of the fusion protein) and Freund's adjuvants (complete adjuvant for the first injection and incomplete adjuvant for booster injections). The animals were injected at four sites subcutaneously (hips and shoulders). Each fusion protein was injected into two rabbits. The initial injection was followed by two or three booster injections 2 weeks apart. Animals were bled 9 or 10 days after each booster injection, and serum was isolated and heated to 56 °C for 30 min. To remove the vitamin K dependent coagulation factors, the antisera were made 0.01 M in sodium oxalate (pH 7.3) and adsorbed with $BaSO_4$ (100 mg/mL). This suspension was then incubated at room temperature for 30 min, followed by centrifugation at 10000 rpm (Sorvall SS-34 rotor) for 20 min. The final serum supernatant was stored at −20 °C.

Inhibition of Factor IX Activity. One-stage partial thromboplastin time assays were performed as described (DiScipio & Davie, 1979; Bajaj et al., 1985). All assays were carried out in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% BSA. To determine the appropriate amount of commercial factor IX to use in the assay, serial dilutions of factor IX were preincubated with 100 μL of factor IX deficient plasma at 37 °C for 2 min; 100 μL of platelin plus activator [platelet factor 3 reagent plus particulate activator (Celite)] was added and incubated for 5 min; 100 μL of 25 mM $CaCl_2$ was added; and the time for clot formation was observed. A log–log plot of the data demonstrated the linear dependence of the clotting time on the factor IX concentration. For the determination of the ability of the rabbit antisera to neutralize factor IX activity, 20 μL of factor IX (0.5 μg/mL) was incubated with serial dilutions of the $BaSO_4$-adsorbed rabbit antisera or the inert control (Tris/NaCl/albumin, or $BaSO_4$-adsorbed normal rabbit serum) at room temperature for 1 h. Aliquots were assayed for factor IX clotting activity.

Results

Synthesis of Factor IX in E. coli. *Hae*III digestion of the cDNA coding for human blood clotting factor IX releases a 1.3 kb fragment containing all 415 codons of the mature factor IX protein plus 3 additional codons from the upstream prepro leader region (Figure 1). To investigate the synthesis of factor IX in *E. coli*, we placed this entire factor IX coding sequence under control of transcription and translation signals from bacteriophage T7. The resulting plasmids are shown diagrammatically in Figure 1. In each case, synthesis of factor IX mRNA can be initiated from the *ϕ10* promoter for T7 RNA polymerase. Plasmid pFIX1 lacks a consensus ribosome binding site between the start of transcription and the beginning of the factor IX open reading frame and is therefore expected to be unable to initiate protein synthesis at the beginning of the factor IX coding sequence. In plasmid pFIX2, the factor IX coding sequence is fused in frame to the beginning of the gene *10* protein of T7, after the 11th codon. The mRNA produced from this plasmid will contain all of the upstream translation signals of the very actively translated gene *10* mRNA, signals that would presumably direct translation of the fusion mRNA. Plasmid pFIX3 is identical with pFIX2 except that it contains the transcription terminator for T7 RNA polymerase, Tϕ, following the factor IX open reading frame. In the absence of Tϕ, most transcription would proceed completely around the plasmid (Studier & Moffatt, 1986). Both pFIX2 and pFIX3 are expected to code for a fusion protein of 432 amino acids. Plasmid pFIX4 is similar to pFIX3 except that it contains 326 codons of gene *10* ahead of the factor IX sequence and is expected to code for a fusion protein of 747 amino acids.

To direct expression in vivo, the recombinant plasmids were transformed into BL21(DE3), a host strain carrying a chromosomal copy of the gene for T7 RNA polymerase under control of the inducible *lac*UV5 promoter (Studier & Moffatt, 1986). In the absence of induction, only small amounts of proteins having the electrophoretic mobilities expected for factor IX or the gene *10*–factor IX fusion proteins were detected (Figure 2A). However, induction generated significant new protein bands having the expected mobilities (Figure 2B). To verify that these bands contained factor IX sequences, the proteins were transferred to nitrocellulose and reacted with goat anti-human factor IX antibody. After being washed to remove unbound antibody, the immunoreactive proteins were visualized by their ability to bind ^{125}I-labeled protein A. As shown in Figure 2C, each of the proteins expected to contain full-length factor IX reacted with the goat anti-human factor IX antibody. In most cases, some smaller protein(s) reacted as well. Whether these come from internal starts, premature termination, proteolysis, or some type of modification has not been determined, but these smaller immunoreactive proteins

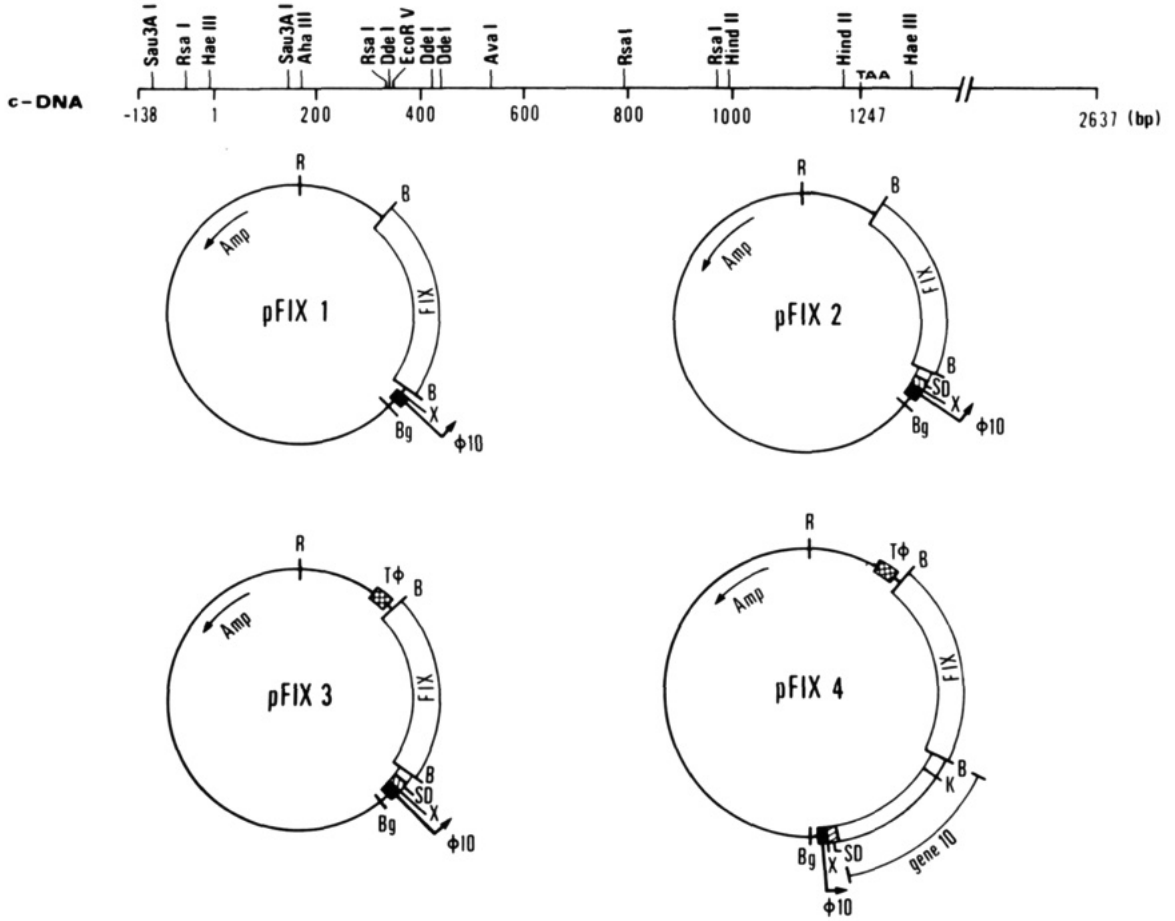


FIGURE 1: Plasmids for synthesizing factor IX protein. Relevant restriction sites in the factor IX cDNA are shown at the top of the figure. The 1.3 kb *Hae*III fragment of the factor IX cDNA was placed under control of the *ϕ10* promoter for T7 RNA polymerase by inserting into the unique *Bam*HI site of four different plasmid vectors (see Experimental Procedures): pFIX1 is derived from pET-1; pFIX2 from pET-2b; pFIX3 from pET-3b; and pFIX4 from pAR3070. Plasmid pFIX1 contains no translation signals between the *ϕ10* promoter and the factor IX sequence, but the other plasmids contain the ribosome binding site (SD) and translational start site for T7 gene *10*. In pFIX2 and pFIX3, the factor IX cDNA is fused in frame after the 11th codon of the gene *10* protein, and in pFIX4 after the 326th codon. Plasmids pFIX3 and pFIX4 also contain Tϕ, the transcription termination signal for T7 RNA polymerase. Restriction sites indicated in the plasmids are as follows: B = *Bam*HI, Bg = *Bgl*II, K = *Kpn*I, R = *Eco*RI, and X = *Xba*I.

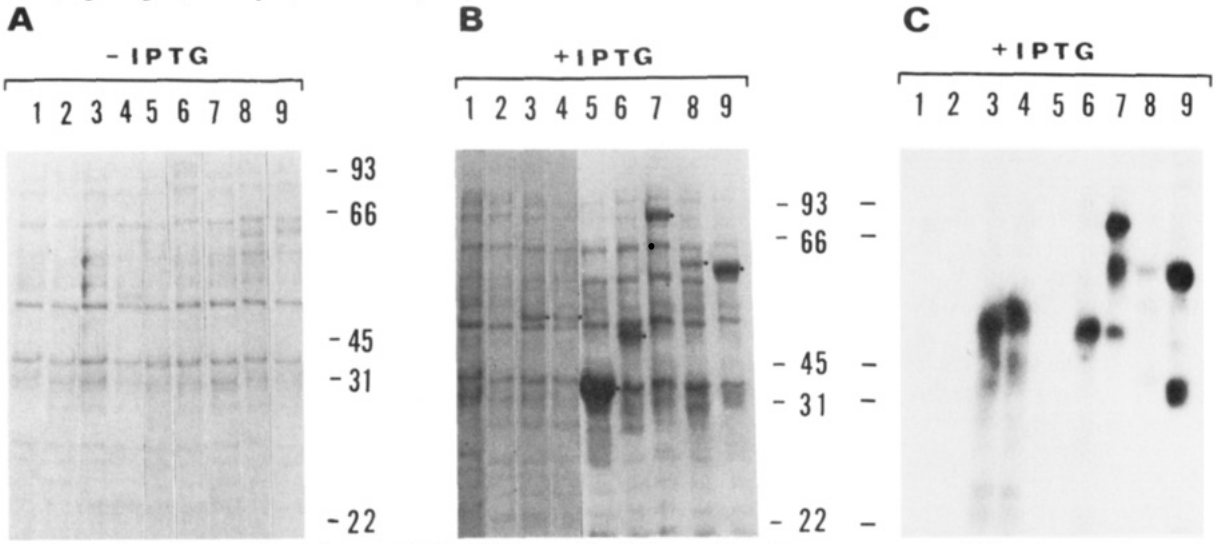
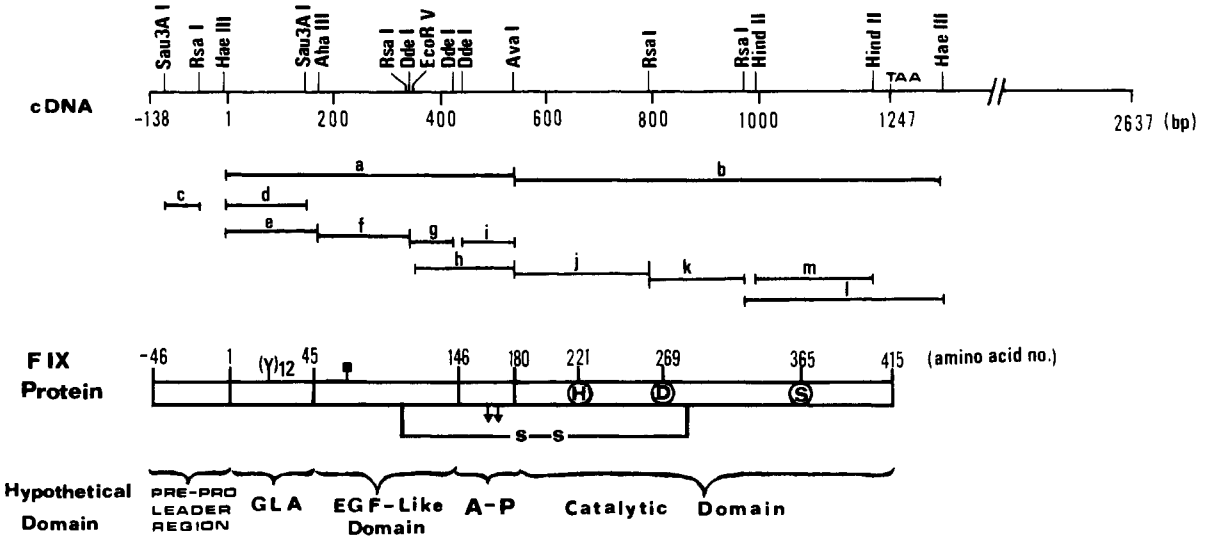


FIGURE 2: Expression of factor IX. Cultures of BL21(DE3) carrying different plasmids were grown to mid log phase, and a portion from each was induced with IPTG as described in the text. Thirty minutes later, 20-μL samples were removed for analysis by electrophoresis on SDS–polyacrylamide gels. In panels A (uninduced cultures) and B (induced cultures), the proteins were visualized by staining with Coomassie brilliant blue. In panel C, another 20-μL portion of each induced culture was subjected to Western blot analysis, using goat anti-human factor IX antibody and treatment with ^{125}I-labeled protein A, followed by autoradiography. The lanes represent cultures harboring the following plasmids (see Figures 1 and 3): (1) no plasmid; (2) vector plasmid pET-3b; (3) pFIX2; (4) pFIX3; (5) vector plasmid pAR3070, containing the first 326 codons of gene *10*; (6) pFIX1; (7) pFIX4; (8) pFIX4b, containing codons for amino acids 180–415 of factor IX; (9) pFIX4a, containing codons for amino acids −3 to 180 of factor IX. The positions of gene *10* and factor IX related proteins expressed from the plasmids under control of T7 RNA polymerase are marked by dots in panel B. The positions of molecular weight markers (93K, 66K, 45K, 31K, and 22K) are shown.



cDNA Fragment	Plasmid Designation	Human FIX Contribution (a.a. no.)	Total Residues Predicted	cDNA Fragment	Plasmid Designation	Human FIX Contribution (a.a. no.)	Total Residues Predicted
a	pFIX4a	-3 to 180	536	h	pFIX 4h	116 to 180	403
b	pFIX4b	180 to 415	567	i	pFIX 4i	147 to 180	387
c	pFIX4c	-40 to -19	375	j	pFIX 4j	180 to 265	441
d	pFIλ4d	-3 to 49	408	k	pFIX 4k	266 to 324	412
e	pFIX4e	-3 to 57	413	l	pFIX 4l	325 to 415	422
f	pFIX 4f	50 to 111	400	m	pFIX 4m	332 to 405	411
g	pFIX 4g	112 to 142	386				

FIGURE 3: Schematic representation of the factor IX cDNA restriction fragments and their relationship to hypothetical domains in the protein. The locations of the restriction enzyme sites used to generate fragments a–m of the factor IX cDNA are indicated. *Bam*HI linkers were ligated to each fragment which was subsequently cloned into pAR3070 to generate the plasmids designated pFIX4a–pFIX4m, listed at the bottom of the figure. Also indicated are the amino acid residues of factor IX coded for by each plasmid and the predicted total residues of each fusion protein. The hypothetical domains of human factor IX (FIX protein) are shown, as are the positions of histidine-221 (H), aspartic acid-269 (D), and serine-365 (S), comprising the catalytic triad; the 12 γ-carboxyglutamic acids, $(\gamma)_{12}$; β-hydroxyaspartic acid (■); activation peptide (A-P); and the carbohydrate groups (↓).

were also observed when fusion proteins containing smaller fragments of factor IX were expressed (Figure 2, lane 9, and Figure 4).

It was somewhat surprising to observe synthesis of factor IX related protein directed by pFIX1, since the factor IX mRNA is not preceded by a recognizable translation initiation signal. However, the factor IX protein produced from this plasmid is appreciably smaller than that produced when the open reading frame is fused to the first 11 codons of gene *10* (pFIX2 and pFIX3), suggesting that translation initiates internally at a codon located near the beginning of the factor IX open reading frame. A good candidate is the methionine codon at position +19 of factor IX, which is separated by six nucleotides from the plausible ribosomal binding sequence GAGAA. Nearly equal amounts of factor IX protein appear to have been produced from pFIX2 and pFIX3, indicating that the presence of the T7 transcriptional termination signal had little effect on the overall level of factor IX expression.

Expression of Hypothetical Functional Domains of Factor IX. As shown in Figure 3, factor IX is thought to have at least five distinct domains. Fragments specifying an entire domain or a limited portion of a particular domain can be isolated following digestion of factor IX cDNA with appropriate restriction endonucleases. We have cloned 13 individual subfragments of the cDNA in frame after codon 326 of gene *10*, anticipating that the large fragment of gene *10* protein would both ensure efficient synthesis of the fusion proteins and perhaps stabilize the factor IX fragments against possible degradation in *E. coli*. A diagram of the cloned fragments and their plasmid designations is given in Figure 3.

Expression of each fusion protein was induced by adding IPTG to a growing culture of BL21(DE3) carrying the plasmid, and the accumulation of protein was analyzed by SDS–polyacrylamide gel electrophoresis. The protein patterns for cultures carrying pFIX4a and pFIX4b are shown in Figure 2, and those for pFIX4c through pFIX4m are shown in Figure 4. In each case, a prominent protein band corresponding in size to the predicted gene *10*–factor IX fusion protein (Figure 3) is observed. Comparable amounts of fusion protein were produced from most of the plasmids, except that significantly lower amounts were produced from pFIX4f and pFIX4j.

Considerable variation was seen in the ability of goat antibody directed against mature human factor IX to react with the different gene *10*–factor IX fusion proteins. If each fusion protein was transferred and bound to the nitrocellulose paper with approximately the same efficiency, then the relative intensity of the signal on the autoradiogram might be expected to reflect the relative reactivity of each fusion protein with the goat antibody. As expected, no reaction was seen with the fusion protein containing only the prepro leader region of factor IX (amino acids −40 to −19, specified by pFIX4c), since these amino acids are not retained in the mature factor IX protein. As might also be expected, the two fusion proteins containing the Gla region (pFIX4d and pFIX4e) react poorly with antibody. This may be because the glutamic acid residues in this region are not carboxylated in *E. coli* but could be due to other

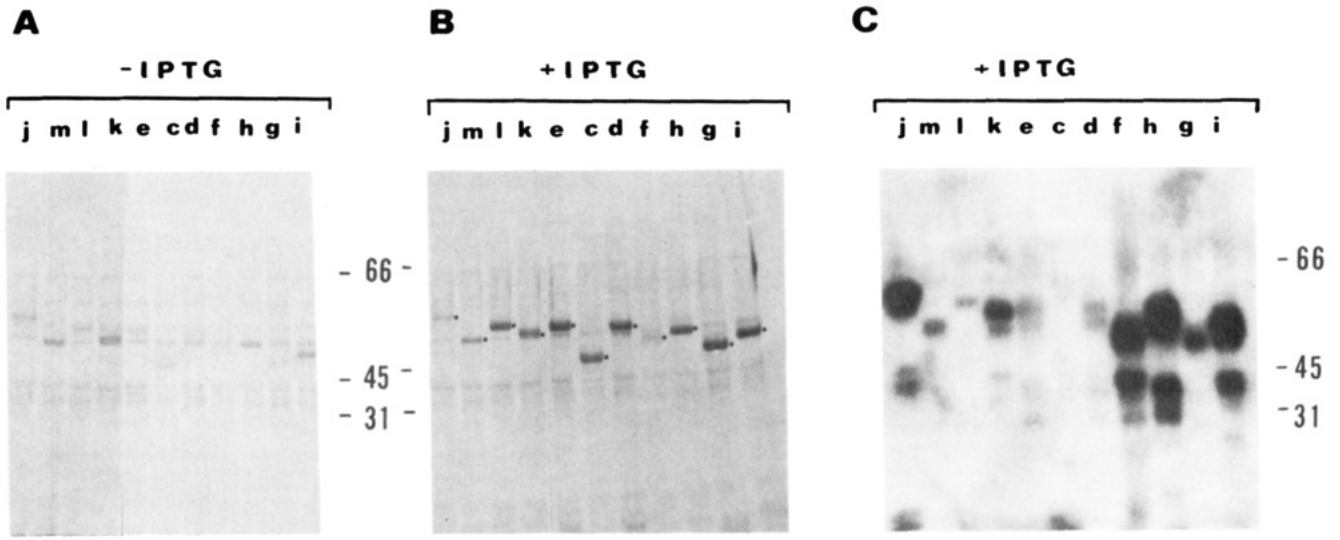


FIGURE 4: Gene *10*–factor IX fusion proteins expressed from pFIX4c to pFIX4m. BL21(DE3) cultures harboring individual plasmids were induced and the protein patterns analyzed as described in Figure 2: (A) uninduced cultures; (B) induced cultures; (C) Western blots of induced cultures. Samples were derived from the plasmids carrying fragments j, m, l, k, e, c, d, f, h, g, and i (see Figure 3), as indicated in the figure. The positions of 66K, 45K, and 31K molecular weight markers are indicated.

factors (see below). The fusion proteins carrying subfragments f–k, which come from the middle of the protein, react strongly with this antibody preparation, whereas subfragments l and m, from the carboxy terminus, react only weakly. Somewhat surprisingly, subfragment b, which carries the complete heavy chain of factor IX, reacts very poorly with the antibody (Figure 2C, lane 8), even though it contains the reactive subfragments j and k. Perhaps the pFIX4b fusion protein is folded in such a way that these reactive regions are masked. This result raises the possibility that poor reactivity of any particular subfragments of factor IX could be due to peculiarities of the fusion protein rather than to an intrinsic lack of reactivity of that part of the factor IX protein.

Significant amounts of gene *10*–factor IX fusion proteins can be produced from these plasmids, but much greater amounts of the truncated gene *10* protein are produced from the pAR3070 vector alone (Figure 2B, lane 5). This difference is unlikely to be due to a difference in stability of the proteins, since in pulse–chase experiments they all appear to have half-lives longer than 1 h (data not shown). A more likely explanation is that the presence of factor IX coding sequence as part of the gene *10* mRNA somehow reduces the translation rate of the fusion mRNA in *E. coli*. However, it seems that each part of the factor IX coding sequence has this effect. In a separate experiment (data not shown), we cloned codons −3 to 415 of factor IX into pAR3070 using a *Bam*HI linker that would place the factor IX codons in a different reading frame than the upstream 326 codons of gene *10*. In this mRNA, ribosomes would terminate translation very soon after they encountered the out of frame factor IX sequence. Upon induction, the amount of truncated gene *10* protein produced was essentially identical with the amount produced from pAR3070, consistent with the idea that translation through the factor IX sequence somehow limits the rate of expression of the fusion proteins in *E. coli*.

Preparation of the Antibody to Domains of Factor IX. To prepare sufficient amounts of fusion proteins to serve as specific antigens, 100-mL cultures of *E. coli* BL21(DE3) containing each of the plasmids pFIX4c to pFIX4m were induced for 1 h, and the cells were collected. After cell lysis and low-speed centrifugation, all of the fusion proteins were in the insoluble fraction. This result was not unexpected since we knew from previous experiments that the truncated gene *10* product from

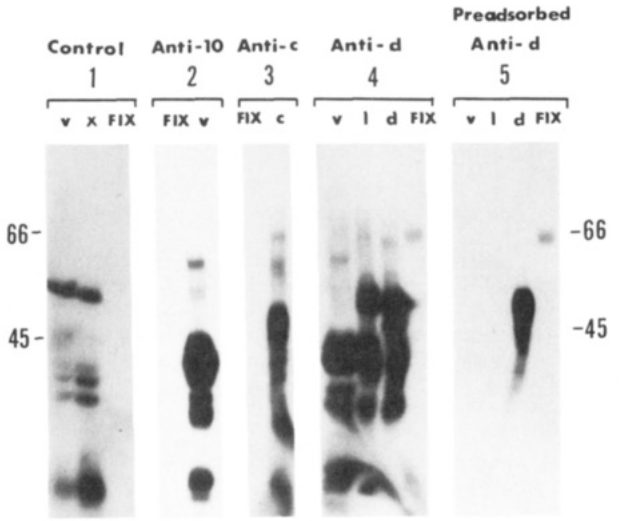


FIGURE 5: Specificity of antibodies elicited by gene *10*–factor IX fusion proteins. One microgram of purified factor IX (lanes FIX) and 20-μL samples of induced cultures of BL21(DE3) carrying different plasmids were subjected to Western blot analysis. The plasmids, described under Experimental Procedures or in Figure 3, were as follows: the vector pAR3070, which contains the first 326 codons of gene 10 protein (lanes v); a mixture (3 μL of each) of pFIX4c–pFIX4m (lane x); pFIX4c (lane c); pFIX41 (lanes l); and pFIX4d (lanes d). The antibody used to probe these blots was from normal rabbits (control) or was raised against purified proteins: the truncated gene *10* protein produced from pAR3070 (anti-*10*), or the gene *10*–factor IX fusion protein from pFIX4c (anti-c) or pFIX4d (anti-d). In the last set of samples (preadsorbed anti-d), serum from rabbits immunized against the fusion protein from pFIX4d was preadsorbed to a blot of an induced culture carrying the vector plasmid pAR3070, to remove antibodies reacting with the gene *10* portion of the fusion protein.

pAR3070 is itself insoluble. The pellet from each 100-mL culture contained about 12 mg of the fusion protein. After solubilization of the pellet in buffer containing 1% SDS, the proteins were fractionated on preparative SDS–polyacrylamide gels. The band for each fusion protein was excised, macerated, and injected into rabbits to elicit production of antibodies. Western blot analysis was used to show that the rabbits produced antibodies against the factor IX region of these fusion proteins. A typical result of these experiments is depicted in Figure 5, where antibodies generated against the pFIX4d fusion protein, which contains the first 49 amino acids of the

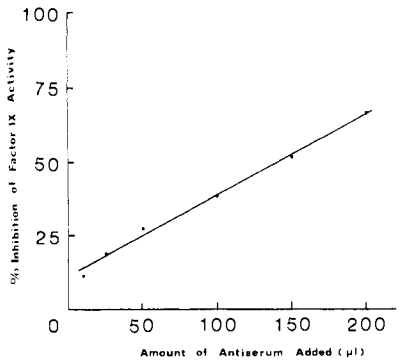


FIGURE 6: Neutralization of factor IX clotting activity by anti-FIX4d serum. Samples of purified human factor IX were preincubated with dilutions of $BaSO_4$-adsorbed serum containing antibodies against pFIX4d. The samples, final volume 250 μL, were incubated for 1 h at room temperature at which time a 100-μL portion was removed to measure remaining factor IX dependent clotting activity by using the one-stage partial thromboplastin time assay described under Experimental Procedures. Under the conditions used here, up to 200 μL of $BaSO_4$-treated normal rabbit serum as well as serum against pFIX4c (prepeptide) showed no inhibition of factor IX dependent clotting activity.

factor IX protein, were used as the probe.

As shown in Figure 5, panel 4, the immune serum contains antibodies that recognize not only the human factor IX protein (lanes FIX) but also the gene *10* protein and the gene *10*–factor IX fusion protein specified by pFIX4d. Antibodies against the gene *10* portion of the fusion protein, and any *E. coli* proteins that may have comigrated with the antigen during electrophoresis, can be removed from the serum by incubation with nitrocellulose paper coated with SDS-solubilized extracts from induced cultures carrying the vector plasmid pAR3070. After preadsorption, the remaining antibodies specifically recognize the mature factor IX protein as well as the fusion protein from pFIX4d and do not react with the truncated gene *10* protein from the vector or with the fusion protein from pFIX41, which contains amino acids 325–415 of the factor IX protein (Figure 5, panel 5). The polyclonal rabbit antibody made to the fusion protein from pFIX4d gives a much stronger signal with the fusion protein than with plasma factor IX. This is not surprising as the fusion protein was the immunizing reagent. As expected, preimmune rabbit sera or serum from rabbits immunized with truncated gene *10* protein or from the fusion protein containing the prepeptide leader sequence (specified by pFIX4c) did not react with authentic factor IX (panels 1, 2, and 3, respectively).

Inhibition of the Biological Activity of Factor IX. As a first attempt to use these antibodies to define the location of regions of the factor IX protein that are important for its biological activity, we tested the anti-pFIX4d serum for its ability to inhibit factor IX dependent blood clotting. The assay we used (described in detail under Experimental Procedures and the legend to Figure 6) is linearly dependent on the concentration of added factor IX. However, since endogenous factor IX present in rabbit serum also would be detected in this assay, we first incubated the serum with $BaSO_4$ to adsorb the rabbit factor IX. As shown in Figure 6, preincubation of purified factor IX with $BaSO_4$-treated anti-pFIX4d serum resulted in inhibition of the added factor IX's ability to promote clotting. $BaSO_4$-treated preimmune serum or serum against pFIX4c showed no inhibition of factor IX clotting activity in the same assay and over the same range of added serum. Thus, antibodies raised against the fusion protein containing the first 49 amino acids of the mature factor IX protein are able to inhibit the clotting activity of purified factor IX.

Discussion

A gene expression system based on T7 RNA polymerase (Studier & Moffatt, 1986) has been successful in producing substantial amounts of factor IX protein and fragments of it in *E. coli*. In most of the configurations we have used, the factor IX coding sequences have been fused in frame to the first 326 codons of the T7 gene *10* protein. This large carrier protein has the advantage of being actively synthesized in *E. coli*, and it may help to stabilize factor IX protein fragments against degradation. The gene *10* portion of the fusion protein is itself insoluble, as are all of the fusion proteins, which aids in their purification. Interestingly, addition of any of the factor IX coding sequences to the 3′ end of the gene *10* mRNA appears to substantially reduce the amount of the protein that is made, an effect we do not yet understand.

Fusion proteins containing different regions of the factor IX molecule react to different extents with a single preparation of goat anti-human factor IX antibody. In general, fragments from the middle of the molecule, amino acids 50–324, react much more strongly than those from either end. Several explanations are possible. The amino acid sequences near the ends of this molecule might be more conserved in evolution, and therefore be recognized as "self" by the immune system. It is known that the repertoire of antibodies raised in response to an antigen may differ depending on the animal used for injection (Benjamin et al., 1984). It is likely that a substantial proportion of the goat antibody population is specific for conformational determinants not present in the fusion protein because the factor IX portion of the protein fails to form the correct three-dimensional structure. Perhaps most of the goat antibodies that react with the fusion proteins are recognizing "sequence determinants" rather than "conformation determinants" of the factor IX molecule (Benjamin et al., 1984). The lack of modifications such as the γ-carboxyglutamic acids, β-hydroxyaspartic acid, or attached carbohydrate residues might also affect the ability to be recognized.

Another possible explanation for the differential reactivity of the fusion proteins with the goat antibody is that some of the sequences present in the more reactive fragments are normally found on the surface of the factor IX molecule whereas the less reactive fragments are mostly internal. In proteins whose crystal structures are known, the most immunogenic parts of the molecule appear to be surface protuberances or highly mobile regions (Tainer et al., 1985; Thornton et al., 1986). To date, crystal structure information about factor IX is not available, but the heavy chain is quite homologous to trypsin and chymotrypsin. Furie et al. (1982) have used computer modeling to generate a three-dimensional structural model for bovine factor IX heavy chain, based on this homology. This model should also apply to human factor IX, since the amino acid sequences of the human and bovine proteins are very similar (Katayama et al., 1979). According to this structure, much of fragments j and k (Figure 3) should be found at the surface of the molecule whereas most of fragments m and l should be in the interior, consistent with their relative reactivities with the goat antibody preparation (Figure 4).

We have used fusion proteins containing full-length factor IX or 13 different fragments of it to raise antibodies in rabbits. Using these fusion proteins as antigens has the advantage that different epitopes of the protein can be easily obtained in relatively pure form and the serum obtained will not contain any antibodies against potential contaminating antigens from human plasma. Small amounts of contaminating *E. coli* proteins are not a problem, since antibodies against *E. coli*

proteins are typically present in rabbit serum and can be removed by adsorption to a bacterial lysate (see Figure 5, panels 4 and 5).

We find that antibodies raised against each of the fusion proteins (with the exception of the fusion protein containing the prepro leader peptide) react with the factor IX zymogen on Western blots. It is clear that the antibody to the Gla region (fragment d) also reacts with factor IX in solution, because it inhibits the clotting activity of purified factor IX in an in vitro assay. The factor IX produced in *E. coli* seems unlikely to contain γ-carboxyglutamic acid residues, so this antibody is probably recognizing an epitope not dependent on carboxylation. It will be interesting to see which of the antibodies raised against the other segments of the factor IX protein are also able to inhibit clotting, information which may be helpful in trying to understand the clotting mechanism. These antibodies may also be useful in defining the surface of the factor IX protein molecule or as affinity reagents to purify proteolytic fragments of factor IX.

The fusion proteins themselves have other potential uses. We are currently using them to define the binding domains of monoclonal antibodies, and they may also be useful in assigning binding domains between factor IX and its cofactors, or in testing whether patients with "inhibitors" have antibodies that react with specific domains of the factor IX molecule.

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