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**Isolation of a human anti-haemophilic factor IX cDNA clone using a unique 52-base synthetic oligonucleotide probe deduced from the amino acid sequence of bovine factor IX**

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**ABSTRACT**

A unique 52mer oligonucleotide deduced from the amino acid sequence of bovine Factor IX was synthesized and used as a probe to screen a human liver cDNA bank. The Factor IX clone isolated shows 5 differences in nucleotide and deduced amino acid sequence as compared to a previously isolated clone. In addition, precisely one codon has been deleted.

**INTRODUCTION**

Factor IX (Christmas Factor) is a coagulation factor of the intrinsic pathway of blood clotting. It is synthesized as a zymogen, and is modified post-translationally both by the addition of carbohydrate and by the conversion of 12 residues of glutamic acid to  $\gamma$ -carboxyglutamic acid, the latter modification being a vitamin K-dependent process (1). By analogy with other coagulation factors and serum proteins, the site of synthesis of factor IX is assumed to be the liver. Factor IX deficiency (Hemophilia B or Christmas Disease) is manifested by dangerously prolonged bleeding times, and is transmitted as a sex-linked recessive trait (2). The molecular nature of Factor IX deficiency is unknown.

We wished to obtain a cDNA clone of Human Factor IX, while at the same time attempting a novel method of screening cDNA banks for specific sequences. Based on the known amino-acid sequence of bovine Factor IX, we attempted to design a unique oligonucleotide to be used as a probe for the corresponding cDNA clone. In designing this unique oligonucleotide, the following variables were considered: 1) the segment of the Factor IX amino acid sequence with the least ambiguity in the corresponding codons, 2) codon usage in already sequenced bovine cDNAs corresponding to proteins synthesized and secreted by the liver, 3) the relative stability of G:T versus G:A mismatches while screening the cDNA bank with the oligonucleotide by colony hybridization, and 4) the sequence of the oligonucleotide probe permitting

minimum predictable secondary structure in the oligonucleotide. This strategy differs from other strategies currently employed in our laboratory to screen cDNA banks for unique sequences since one long oligonucleotide is used as specific probe, rather than mixtures of shorter oligonucleotides which cover all possible arrangements of codons. The latter strategy has recently been used to obtain Factor IX cDNA clones by Choo et al. (3) and Kurachi and Davie (4).

### MATERIALS AND METHODS

#### Isolation of liver poly-A-containing RNA and cDNA synthesis.

Livers were obtained post mortem and quickly frozen in liquid nitrogen. RNA was prepared from 5 gram of liver using the 8 M guanidium hydrochloride extraction procedure, essentially as described by Tolstoshev et al. (5). The RNA thus obtained was chromatographed on a polyU-Sepharose (Pharmacia) column, as described by the manufacturer to enrich for poly-A-containing RNA which served as a template for reverse transcription using oligo(dT) as a primer. cDNA was synthesized in a 100  $\mu$ l reaction containing 100 mM Tris-HCl, pH 8.3, 10 mM MgCl<sub>2</sub>, 50 mM KCl, 30 mM  $\beta$ -mercaptoethanol, 10 ug/ml oligo(dT)<sub>12-18</sub>, 50 ug/ml poly-A-RNA, 0.5 mM each of dATP, dGTP, dTTP, and dCTP, 80 units of avian myoblastosis virus reverse transcriptase (Life Sciences Inc., St Petersburg, Florida). After 45 minutes at 42°C, the reaction was terminated and cDNA-template complexes simultaneously denatured by heating at 105°C for 3 minutes followed by rapid transfer to an ice bath.

For the synthesis of the second DNA strand, the above reaction was diluted 5-fold and adjusted to final concentrations of 100 mM HEPES-KOH, pH 6.9, 100 mM KCl, 200  $\mu$ M each of dATP, dGTP, dTTP, and <sup>32</sup>P- $\alpha$ -dCTP (specific activity 0.5 Ci/mMole). 10 units of E. coli DNA polymerase (Klenow fragment, Boehringer Mannheim) was then added, and the incubation held at 25°C for 2 hours.

Double stranded cDNA (dscDNA) was extracted with an equal volume of phenol : chloroform (50:50) saturated with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and precipitated with ethanol. Approximately 970 ng of dscDNA was obtained from 5  $\mu$ g poly-A-RNA. This was rendered blunt-ended by digestion with 5 units of S1 nuclease in a reaction volume of 0.1 ml containing 30 mM sodium acetate, pH 4.8, 300 mM NaCl, 3 mM ZnCl<sub>2</sub>. After 1 hour at 37°C, EDTA and SDS were added to final concentrations of 10 mM and 0.1 %, respectively and the reaction heated at 65°C for 5 minutes. The S1-digested dscDNA was then

applied to preformed 5-20 % sucrose gradient containing 100 mM Tris-HCl pH 7.5, 5 mM EDTA, 100 mM NaCl, and centrifuged at 30,000 rpm at 15°C for 16 hours in a SW60 Ti rotor. Fractions of 0.5 ml were collected. To determine the size of the cDNA, 1  $\mu$ l of each fraction was electrophoresed on neutral agarose gels and the migration of each fraction compared with appropriate molecular weight markers. Fractions containing cDNA larger than 1 kilobase were pooled and precipitated with 2 volumes of ethanol overnight in the presence of 5  $\mu$ g of E. coli tRNA as carrier. The pellet was resuspended in 20  $\mu$ l 0.1 x SSC (15 mM NaCl, 0.15 mM sodium citrate pH 7.0).

#### cDNA cloning.

Homopolymeric tails (approximately 15 dCMP residues) were added to the 3' ends of the cDNA using terminal deoxynucleotidyl transferase (6). Similarly, homopolymeric dGMP tails (approximately 13 dGMP residues) were added to the 3' extremities of plasmid pBR322 previously digested with the restriction nuclease Pst-I.

60 ng of dCMP tailed cDNA and 1  $\mu$ g of dCMP tailed vector were annealed for 2 hours at 42°C in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl. Recombinant plasmids were then transformed into E. coli strain 8739 (7), and transformants selected on LB-agar plates containing 15  $\mu$ g/ml tetracycline. 30,000 transformants were obtained, of which approximately 50 % contained cDNA inserts larger than 1 kilobase. All transformants were scraped from the plates into a minimal volume of LB, and after addition of an equal volume of glycerol, the "bank" was stored at - 20°C.

#### Selection and synthesis of oligonucleotide probe for Factor IX.

A total of 1755 nucleotides of coding sequence corresponding to bovine proteins synthesized by the liver (8, 9) were analyzed with regard to codon usage as described below and a sequence of 52 bases was chosen. This oligonucleotide was constructed as follows :

Oligonucleotides A d(CTCACACTGATCACCATCCACATACT),

B d(GCTTCCAGAACTCTGTAGTCTTCTCA),

and the complementary fragment C d(GGAAGCAGTATGT) were chemically synthesized by the phosphotriester method on an inorganic solid support as described previously (10) and purified by HPLC (11). 0.5 nmole

oligonucleotide B was incubated for 30 minutes at 37°C in a 10  $\mu$ l reaction containing 60 mM Tris-HCl pH 7.8, 6 mM MgCl<sub>2</sub>, 6 mM dithiothreitol, 0.1 mM ATP, 6 pmole <sup>32</sup>P- $\gamma$ -ATP (3000 Ci/mmole), 2 units T4 polynucleotide kinase.

The 5' phosphorylated oligomer B was mixed with 0.5 nmole oligonucleotides A

and C in 10 mM Tris HCl pH 7.5, 1 mM EDTA, heated to 100°C and the mixture annealed by slow cooling to 4°C. The annealed oligonucleotides A and B were ligated in a 50 µl reaction containing 66 mM Tris-HCl pH 7.5, 100 mM NaCl, 7.5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 0.5 mM spermidine, 0.2 mM EDTA, 4 units T4 DNA ligase at 4°C overnight. The resulting 52-mer was then purified from the ligation mixture by electrophoresis on a 20 % polyacrylamide gel.

### Screening of liver cDNA banks.

Approximately 10,000 colonies were grown overnight on LB agar plates containing 15 µg/ml tetracycline. The next day, the bacterial colonies were transferred to nitrocellulose filters and the filters prepared for colony hybridization essentially as described (12). The bacteria remaining on the original tetracycline plates were regrown for a few hours at 37°C.

The hybridization probe was prepared by kination of 100 ng 52-mer with 50 µCi of <sup>32</sup>P-γ-ATP. Hybridization was performed at 48°C overnight in 40 ml of 6 x SSC, 1 x Denhardt's solution, 0.1 % SDS, 50 µg/ml E. coli tRNA. The next day, filters were washed at 48°C with several changes of 6 x SSC, 0.1 % SDS. Filters were then dried, and autoradiographed overnight.

## RESULTS

### Design of the oligonucleotide probe.

The amino acid sequence of bovine Factor IX (13) was analyzed to find the longest region corresponding to the least codon ambiguity. We reasoned that if regions of amino acid sequence containing amino acids encoded by 6 codons were avoided in designing the oligonucleotide probe, the degeneracy of codons would be limited to the third position and thus the probe would be at least 66 % homologous. The probe was thus designed to correspond to amino acids 35 to 52 of bovine Factor IX, which consists of 5 amino acids encoded by 4 codons (thr, val, gly), 12 amino acids encoded by 2 codons (gln, lys, phe, gln, tyr, asp, cys), and one amino acid encoded by 1 codon (trp) (Fig. 1).

Analysis of 585 codons of bovine prothrombin and fibrinogen revealed that the valine codon GTG is used in 21/38 instances. Similarly, TTC and TGT are used 2 times more frequently than the other degenerate codon in the case of amino acids phe and cys, respectively. No other clear preferences were obvious, however certain nucleotides were infrequently seen in the third position of certain codons, for example G in the case of both glycine and threonine.

Next, we considered that a G:T mismatch, although less stable than a G:C



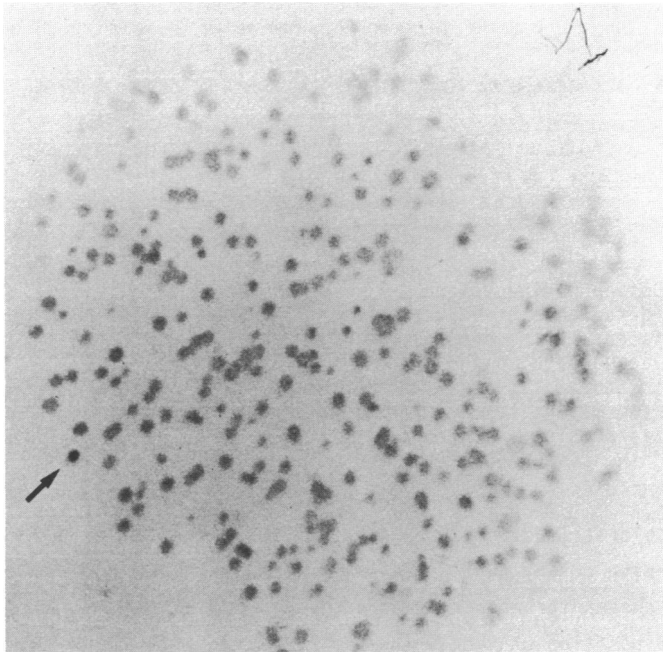


Figure 2 : Autoradiograph of Human Liver cDNA Bank Screened with Kinated 52-mer Probe. Hybridization and washing conditions are described in Materials and Methods. The arrow indicates the Factor IX cDNA clone obtained.

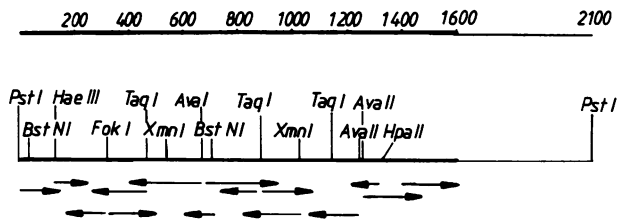


Figure 3 : Restriction Enzyme Map of cDNA Insert of pTG397, and Sequencing Strategy. Nucleotide sequences deriving from the PstI site at position 0, the XmnI site at position 1029, and the HpaII site at position 1334 were determined using dideoxynucleotides as chain terminators (ref. 16) after subcloning of the appropriate restriction fragments into bacteriophages M13mp8 or M13mp9 (ref. 15). All other sequence was performed as described by Maxam and Gilbert (17). Only the HaeIII site actually used in the sequencing strategy is indicated. Nucleotides 1640-2100, which have not been sequenced, are indicated by a thin line.

one colony was obtained which gave a strong signal under the conditions employed (Fig. 2). Since our objective was to obtain a human Factor IX cDNA clone, no further analysis was performed upon the bovine clones and we focused our effort on the human clone.

To establish whether the colony selected from the human liver cDNA bank corresponded to a Factor IX clone, the recombinant plasmid was digested with PstI and the excised cDNA insert transferred to M13mp8 (15) and sequenced using dideoxynucleotides as chain terminators (16). The sequence obtained corresponded to nucleotides 1-180 of figure 3. The deduced amino acid sequence confirmed that the clone was a cDNA clone of human Factor IX. Further sequencing of the cDNA insert of this clone (pTG397) was performed both as described by Maxam and Gilbert (17) and by subcloning of several restriction fragments into M13mp9 (15). The sequencing strategy is presented in figure 3. The sequence obtained is shown in figure 4.

#### DISCUSSION

Employing a unique oligonucleotide probe, we have isolated a clone for human Factor IX containing an insert of 2.1 kb. When the 2.1 kb insert is used as a probe to rescreen the human liver cDNA bank, another Factor IX specific clone (pTG398), containing an insert of 2.6 kb, was isolated (data not shown). The 5' end of pTG398 is located at nucleotide 480 in figure 4. This suggests that the 3' non-translated end of pTG397 is incomplete, and that the complete 3' non-translated end has a length of approximately 1.7 kb.

The sequence of the entire coding region and 250 nucleotides of 3' non-coding region of pTG397 are presented in figure 4. Greater than 75 % of this sequence has been established on both strands. We observe several differences between our nucleotide sequence and that found by Kurachi and Davie (4). These differences involve transitions at nucleotides 38 (A→G), 581 (G→A), 611 (C→T), and 650 (G→A), one inversion at nucleotides 249-250 (AG→GA), and a deletion of an alanine codon (GCA) following position 895 in our sequence (Fig. 5). All but one of these differences (the first transition) have been confirmed on both strands. The first transition generates a Bst NI restriction site, which has been confirmed (data not shown). None of these differences are silent. They result in one change in the signal peptide (serine to glycine), two amino acid changes in the eventual activated Factor IX molecule (lysine to arginine, and a deletion of an alanine residue), and 3 amino acid changes (alanine to threonine, proline to serine, and glycine to serine) in the activation peptide region. These data suggest

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1      25      50      75
T ATG CAG CGC GTG AAC ATG ATC ATG GCA GAA TCA CCA GGC CTC ATC ACC ATC TGC CTT TTA GGA TAT CTA CTC AGT
  met gln val asn met ile met ala glu ser pro gly leu ile thr ile cys leu leu gly tyr leu leu ser

100      125      150
GCT GAA TGT ACA GTT TTT CTT GAT CAT GAA AAC GCC AAA AAT CTG AAT CGG CCA AAG AGG TAT AAT TCA GGT
  ala glu cys thr val phe leu asp his glu asn ala asn lys ile leu asn arg pro lys arg tyr asn ser gly

175      200      225
AAA TTG GAA GAG TTT GTT CAA GGC AAG CTT GAG AGA GAA TGT ATG GAA GAA AAG TGT AGT TTT GAA GAA CCA GCA
  lys leu glu glu phe val gln gly asn leu glu arg glu cys met glu glu lys cys ser phe gly glu ala arg

250      275      300
GAA GTT TTT GAA AAC ACT GAA AGA ACA ACT GAA TTT TGG AAG CAG TAT GTT GAT GAA GAT CAG TGT GAG TCC AAT
  glu val phe glu asn thr glu arg thr thr glu phe trp lys gln tyr tyr val asp gly asp gln cys glu ser asn

325      350      375
CCA TGT TTA AAT GGC GGC AGT TEC AAG GAT GAC ATT AAT TCC TAT GAA TGT TGG TGT CCC TTT GGA TTT GAA GGA
  pro cys leu asn gly gly ser cys lys asp asp ile asn ser tyr glu cys trp cys pro phe gly phe glu gly

400      425      450
AAG AAC TGT GAA TTA GAT GTA ACA TGT AAC ATT AAG AAT GGC AGA TGC GAG CAG TTT TGT AAA AAT AGT GCT AGT
  lys asn cys glu leu asp val thr cys asn ile lys asn gly arg cys glu gln phe cys lys asn ser ala asp

475      500      525
AAC AAG GTG GTT TGC TCC TGT ACT GAG GGA TAT CGA CTT GCA GAA AAC CAG AAG TCC TGT GAA CCA GCA GTG CCA
  asn lys val val cys ser cys thr glu gly tyr arg leu ala glu asn gln lys leu ala glu asn glu pro ala val pro

550      575      600
TTT CCA TGT GGA AGA GTT TCT GCT TCA CAA ACT TCT AAG CTC ACC GGT GCT GAG ACT GTT TTT GCT GAT GTG GAC
  phe pro cys gly arg val ser val ser gln thr ser lys leu thr arg ala glu thr val phe pro ser val asp

625      650      675
TAT GTA AAT TCT ACT GAA GCT GAA ACC ATT TTG GAT AAC ATC ACT CAA AGC ACC CAA TCA TTT AAT GAC TTC ACT
  tyr val asn ser thr glu ala glu thr ile leu asp asn ile thr gln ser thr gln ser phe asn asp phe thr

700      725      750
CGG GTT GTT GGT GGA GAA GAT GCC AAA CCA GGT CAA TCC CTT TGG CAG GTT GTT TTG AAT GGT AAA GTT GAT GCA
  arg val val gly gly val asp ala lys pro gly gln phe pro trp gln val val leu phe leu lys val asp ala

775      800      825
TTC TGT GGA GGC TCT ATC GTT AAT GAA AAA TGG ATT GTA ACT GCT GCC CAC TGT GTT GAA ACT GGT GTT AAA ATT
  phe cys gly gly ser ile val asn glu lys trp ile val thr ala ala his cys val glu thr gly val lys ile

850      875      900
ACA GTT GTC GCA GGT GAA CAT AAT ATT GAG GAG ACA GAA CAT ACA GAG CAA AAG CAA AAT GTG ATT CGA ATT ATT
  thr val val ala gly glu his asn ile glu glu thr glu his thr glu lys arg asn val ile arg ile ile

925      950      975
CCT CAC CAC AAC TAC AAT GCA GCT ATT AAT AAG TAC AAC CAT GAC ATT GCC CTT CTG GAA CTG GAC GAA CCC TTA
  pro his his asn tyr asn ala ala ile asn lys tyr asn his asp ile ala leu leu glu leu asp glu pro leu

1000      1025      1050
GTG CTA AAC AGC TAC GTT ACA CCT ATT TGC ATT GCT GAC AAG GAA TAC ACG AAC ATC TTC CTC AAA TTT GGA TCT
  val leu asn ser tyr val thr pro ile cys ile ala asp lys glu tyr thr asn ile phe leu lys phe gly ser

1075      1100      1125
GGC TAT GTA AGT GGC TGG GGA AGA GTC TTC CAC AAA GGG AGA TCA GCT TTA GTT CTT CAG TAC CTT AGA GTT CCA
  gly tyr val ser gly trp gly arg val phe his lys gly arg ser ala leu val leu gln tyr leu arg val pro

1150      1175      1200
CTT GTT GAC CGA GCC ACA TGT CTT GCA TCT ACA AAG TTC ACC ATC TAT AAC AAC ATG TTC TGT GGT GGC TTC CAT
  leu val asp arg ala thr cys leu arg ser thr lys phe thr ile tyr asn asn met phe cys ala gly phe his

1225      1250      1275
GAA GGA GGT AGA GAT TCA TGT CAA GGA GAT AGT GGG GGA CCC CAT GTT ACT GAA GTG GAA GGG ACC AGT TTC TTA
  glu gly gly arg asp ser cys gln gly asp ser gly gly pro his val thr glu val glu gly thr ser phe leu

1300      1325      1350
ACT CGA ATT ATT AGC TGG GGT GAA GAG TGT GCA ATG AAA GGC AAA TAT GGA ATA TAT ACC AAG GTA TCC CGS TAT
  thr gly ile ile ser trp gly glu glu cys ala met lys gly lys tyr gly ile tyr thr lys val ser arg tyr

1375      1400      1425
GTC AAC TGG ATT AAG GAA AAA ACA AAG CTC ACT TAA TGA AAG ATG GAT TTC CAA GGT TAA TTC ATT GGA ATT GAA
  val asn trp ile lys glu lys thr lys leu thr ----

1450      1475      1500
AAT TAA CAG GGC CTC TCA CTA ACT AAT CAC TTT CCC ATC TTT TGT TAG ATT TGA ATA TAT ACA TTC TAT GAT CAT

1525      1550      1575
TGC TTT TCT TCT TTA CAG GGG AGA ATT TCA TAT TTT ACC TGA GCA AAT TGA TTA GAA AAT GGA ACC ACT AGA GGA

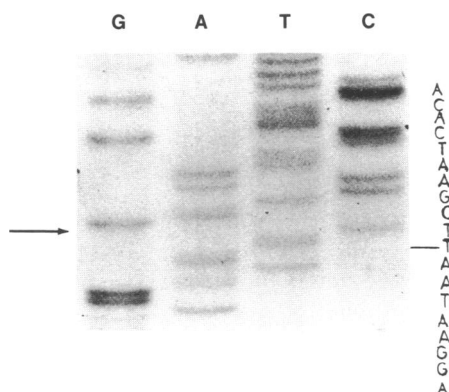
1600      1625
ATA TAA TGT GTT AGG AAA TTA CAG TCA TTT CTA AGG GCC CAG CCT TGA CAA ATT GTG AGT AAA .....

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Figure 4 : Nucleotide and Deduced Amino Acid Sequence of cDNA Insert of pTG397. ... represents nucleotides 1640-2100, which have not been sequenced. Asterisks indicate the positions of divergence between the nucleotide sequence of this clone and that previously published (reference 4). Δ represents the position of the missing alanine codon (see Discussion).

that, like other serum proteins (18) Factor IX is polymorphic. The G→A transition at nucleotide 581, from which we deduce threonine, rather than alanine in the connecting peptide, corresponds to the presence of threonine in this position, as determined by amino acid sequence (19). It is interest





**Figure 5 : Nucleotide Sequence around the Missing Alanine Codon**  
 The sequence read 5'...AGGAATAATTCGAATCACA...3' corresponds to the reverse, complementary sequence of nucleotides 886-904 of figure 4. The presence of the alanine codon GCA (reference 4) would cause this sequence to be read 5'...AGGAATAATGCTCGAATCACA...3'

ing that the greatest divergence in nucleotide and protein sequence is found in the activation peptide. This may be due to a lesser degree of selective pressure on this portion of the gene, which corresponds to a peptide which is removed from Factor IX upon activation.

The deletion of an alanine codon following nucleotide 895 in our sequence is reminiscent of the rat prolactin gene, wherein the deletion of one codon in one of two cDNA sequences can be explained by differential splicing (20). Differential splicing probably does not explain the deletion of the alanine codon in our sequence, since no differential splice sites which follow the GU.....AG rules proposed by Breathnach et al. (21) are apparent in the sequence containing the extra codon.

Consideration of codon use, G:T mismatch, and elimination of possible secondary structure within the oligonucleotide probe has enabled the design of a unique 52mer which has been successfully used as probe for a specific cloned sequence. The 52mer which was deduced from the bovine sequence is homologous with the cloned human sequence in 43/52 nucleotides, while 2 mismatches are G:T basepairs. This precisely reflects the 85 % homology in interspecies nucleotide sequence observed previously (3). We have not rigorously examined signal strength or specificity during colony hybridization as a function of hybridization and washing conditions. These conditions

would inevitably vary depending upon the degree of homology between probe and cloned sequence as well as actual position and nature of the mismatched bases. The general approach of utilizing a long unique oligonucleotide, rather than a mixture of shorter oligonucleotides, should find broad application in probing for unique sequences.

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