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# Fixing human factor IX (fIX): Correction of a cryptic RNA splice enables the production of biologically active fIX in the mammary gland of transgenic mice

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**ABSTRACT** **Transgenic mice and sheep secrete only low levels of human factor IX in their milk because of an aberrant splicing of the transgene RNA in the mammary gland. Removal of the cryptic 3′ splice site prevents this splicing and leads to the production of relatively high levels of factor IX. The purified protein is fully active showing that the mammary gland is capable of the efficient post-translational modification of this protein and that transgenic animals are a suitable means of its production.**

Transgenic animals are being used for the production of valuable proteins. The strategy is to target expression to the mammary gland and harvest the product from milk (1). A number of workers have demonstrated the production of human proteins, including $\alpha_1$-antitrypsin (2) and tissue plasminogen activator (3), by this route in transgenic mice. Commercial production requires that the approach is realized in large animals and this has been accomplished for a variety of products including $\alpha_1$-antitrypsin in sheep (4) and protein C in pigs (5).

High levels of foreign protein production can be achieved; one of the sheep described in ref. 4 exhibited an $\alpha_1$-antitrypsin concentration in milk of ≈35 g/liter. The protein was fully active and appeared to be correctly glycosylated. The demonstration that similar animals stably transmit the high-expression phenotype to generate producer flocks (6) demonstrates the efficacy of this route for the production of large amounts of recombinant proteins.

Efficient expression in the mammary gland has been difficult to achieve in some cases. This is despite the fact that strong promoter elements from a variety of milk protein genes have been well characterized (7, 8). In some instances additional regulatory elements may be required for maximal expression. In others the segment of DNA encoding the target protein may not be compatible with efficient expression or sequences at the chromosomal site of integration may suppress expression. Attempts have been made to address these problems—for example, by using introns (9) or manipulating the site of transgene integration (10).

Human factor IX (fIX), $M_r$ 56,000, is an essential component of the intrinsic pathway of the blood coagulation cascade. Deficiencies in fIX are sex-linked and result in hemophilia B occurring in about 1/30,000 males (11). Its normal site of synthesis is the liver where it undergoes several post-translational modifications required for activity including propeptide cleavage, β-hydroxylation, and γ-carboxylation. The production of recombinant fIX in cell culture has proved problematical in the past because many mammalian cell lines are unable to carry out these modifications effectively (12–14).

This laboratory has described transgenic sheep (15) and transgenic mice (10) expressing fIX constructs, but the level of protein in the milk has been very low or undetectable. In this report we show that these low levels are due to the aberrant splicing of the transgene RNA. Removal of the cryptic 3′ splice site eliminates this problem, enabling the production of relatively high levels of fIX in the milk of transgenic mice. The purified protein exhibits clotting activity comparable to plasma-derived fIX demonstrating that the mammary gland is capable of the complex post-translational modifications required for biological activity.

## MATERIALS AND METHODS

**RNA and cDNA Preparation.** Total RNA was prepared by using RNasol and fractionated on 1% formaldehyde/agarose gels for Northern blots. For reverse-transcribed PCRs (RT–PCRs), first-strand cDNA synthesis was carried out by using a Moloney murine leukemia virus reverse transcriptase kit (Pharmacia). Fifteen microliters containing 5 μg of total RNA was incubated with 1 μl of enzyme for 30 min.

**PCRs.** cDNA and plasmid sequences were amplified in a 100-μl reaction mixture [10 mM Tris/50 mM $KCl_2$, pH 8.3/all four dNTPs (each at 1.25 mM)/0.5 unit of *Taq* I polymerase/each primer at 0.1 μM]. Normally 1 μl of cDNA reaction mixture or 10–100 ng of plasmid DNA was added to the reaction mixture and amplified for 30 cycles. Primers were supplied by Oswell DNA Service.

**DNA Constructs.** The unmodified bovine β-lactoglobulin (BLG) construct, AATD, and FIXD have been described (9, 16). pRT-FIX was constructed by amplifying cDNA from mammary gland RNA from transgenic mouse BIX 33.1, which expresses high levels of a ≈1450-nt FIXD transcript (10). FIXDΔ3′ was constructed by amplifying a segment of DNA from FIXD containing 5′ BLG sequences, the fIX coding sequences, and a short stretch of fIX 3′ untranslated region (UTR) lacking the cryptic 3′ acceptor site present in FIXD (see Fig. 3*a*). The amplified fragment was cleaved with *Sph* I and *Sma* I, cloned into *Sph* I/*Eco*RV-cleaved pBJ41 (pBJ41 contains the same flanking and exonic BLG sequences as FIXD), and then sequenced.

**Transgenic Mouse and Sheep Samples.** The production of BLG+FIXD (BIX) transgenic mice and the transgenic sheep have been described (10, 15). Transgenic sheep 10022, carrying the JFIXAi construct, was generated by the same methods; it secretes a low level of fIX (≈1 μg/ml) in milk (I.C., unpublished observations). *BIXΔ3′* mice were produced by coinjection of BLG and FIXDΔ3′ in a 3:1 molar ratio.

**Protein Analysis.** fIX was quantitated by sandwich ELISA using a polyclonal antibody (Dako) and purified fIX as standard. Western blot analysis was performed, after reduction and electrophoresis on 10% polyacrylamide gels, with

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Abbreviations: fIX, human factor IX; BLG, ovine β-lactoglobulin; RT–PCR, reverse-transcribed polymerase chain reaction; UTR, untranslated region.

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polyclonal antibody conjugated to horseradish peroxidase (Dako). Protein bands were visualized with chemiluminescence. fIX was purified from pooled mouse milks from line BIXΔ3′31 by immunoaffinity chromatography. Monoclonal antibody A7, which binds to the $Ca^{2+}$ binding fIX Gla domain (17), was from Charles Lutsch (Pasteur Merieux, L'Etoile, France). The antibody was coupled to cyanogen bromide-activated Sepharose. Diluted milk was incubated overnight with antibody-conjugated Sepharose in 50 mM Tris/150 mM NaCl, pH 7.5 (TBS) containing 50 mM $CaCl_2$ at 4°C. Bound protein was eluted isocratically with TBS/25 mM EDTA, pH 7.5. Coagulation activity was measured by ball oscillation with an ST4 analyzer (Diagnostico Stago, Asnieres, France), fIX-deficient plasma (Diagnostic Reagents, Oxon, U.K.), and APTT reagent (Sigma), and the reaction was initiated by addition of $Ca^{2+}$. Normal human plasma (fIX at 4 μg/ml) was used as standard.

## RESULTS

**Low Levels of fIX Are Caused by Aberrant Splicing.** FIXD (Figs. 1 and 2) contains fIX cDNA sequences fused to 5′ and 3′ BLG sequences. This construct failed to express in nine independently derived transgenic mouse lines (9). When cointegrated with the sheep BLG gene, however, its expression was "rescued" and high levels of fIX mRNA were detected in the mammary gland in a number of transgenic lines (10). In these experiments high levels of fIX mRNA in the mammary gland yielded only very low levels of fIX in milk. Analysis of the transcripts on Northern blots revealed that they were ≈450-nt shorter than expected. To investigate this problem further FIXD transcripts were cloned from one of the BIX lines by RT–PCR.

Primers to the 5′ fIX and 3′ BLG sequences of FIXD (Fig. 1) amplified a 1309-bp fragment from BIX mammary gland RNA, which was cloned and sequenced (Fig. 1). The sequence shows that BIX mRNA is 462 nt shorter than predicted due to an internal deletion extending from nt 1085 to 1547 of the previously described fIX mRNA (18). This deletion encompasses the sequences encoding the C-terminal 109 amino acids,

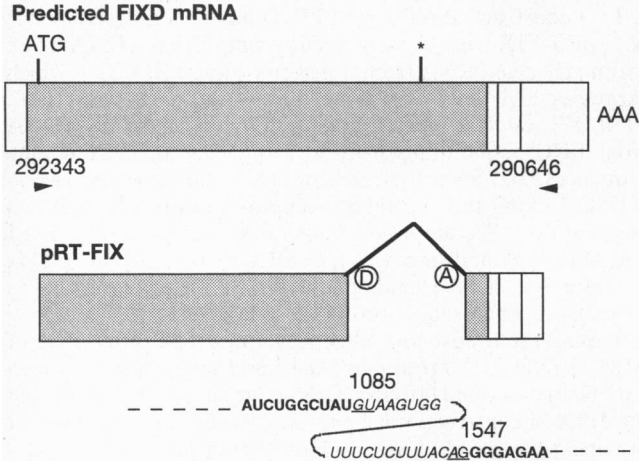


FIG. 1. Analysis of FIXD mRNA in BIX mice. The predicted size of mRNA transcribed from FIXD is 1813 nt excluding poly(A). Primers 292343/290646 were used to amplify FIXD mRNA from BIX mice. The amplified segment was cloned to generate pRT-FIX. Open box, BLG sequence; shaded box, fIX sequence. Sequencing revealed a 462-bp deletion within the fIX sequence spanning nt 1085–1547 of the published sequence (18) encompassing the C-terminal 109 amino acids, the stop codon, and 135 nt of the 3′ UTR. The sequences at the 5′ and 3′ breakpoints of the deletion are shown in boldface type. The 5′ and 3′ ends of the deletion are shown in italic type: these are consensus donor (D) and acceptor (A) splice sites and conform to the GU/AG rule (19).

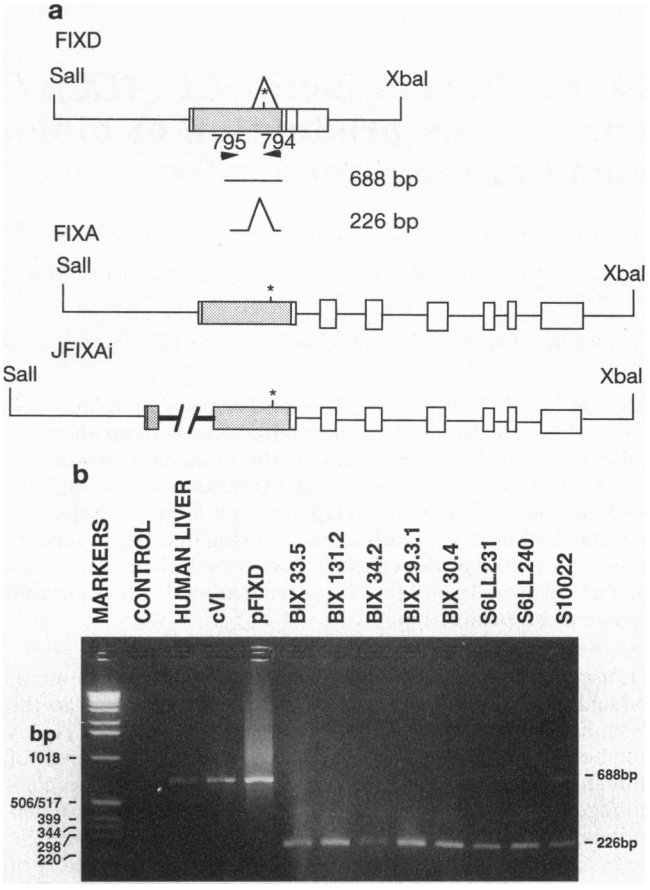


FIG. 2. Analysis of aberrant splicing in transgenic mice and sheep. (*a*) Constructs and diagnostic primers. FIXD contains a 1552-bp fIX cDNA segment fused to 5′ and 3′ BLG sequences (9, 10). FIXA contains the same fIX segment inserted in exon I of the otherwise unmodified BLG gene (15). JFIXAi is similar to FIXA except that the fIX cDNA contains intron 1 from the fIX gene. Thin line, BLG 5′, 3′, and intron sequences; open box, BLG exon sequence; shaded box, fIX sequence; thick line, fIX intron; *, fIX stop codon; ∧, deletion due to aberrant splicing. Primers 795/794 will amplify a 688-bp fragment from nonspliced fIX mRNA and a 226-bp fragment from aberrantly spliced transcripts. (*b*) Electrophoresis of PCR products. Products amplified with the 795/794 primer pair from the DNA or RNA sources indicated were analyzed by gel electrophoresis. cVI is the original fIX cDNA plasmid used to make the fIX constructs; it contains a rearrangement overlapping the first AUG and translational initiation must take place at one or other of two in-frame AUGs immediately downstream (15). BIX33.5, 131.2, etc., mammary gland RNA samples from different FIXD-expressing BIX lines; S6LL231 and S6LL240, mammary RNA samples from transgenic sheep expressing FIXA (15); S10022, mammary RNA from a JFIXAi-expressing sheep.

the stop codon, and 141 nt of the 3′ UTR. No other deletions or mutations were observed. Inspection of the sequences at the 5′ and 3′ junctions of this deletion showed that they corresponded to consensus donor and acceptor splice sites (Fig. 1). The shortened FIXD mRNA in the mammary gland of these transgenic mice and the low levels of fIX observed in the milk is, thus, almost certainly due to the aberrant splicing of FIXD transcripts.

**Different fIX Constructs Exhibit the Same Cryptic Splice.** Primers were designed to detect the aberrant fIX splicing (Fig. 2*a*). These amplify a 688-bp fragment from wild-type fIX mRNA or cDNA and a 226-bp fragment from aberrantly spliced fIX mRNA. The 226-bp fragment was detected in samples from all BIX lines showing that they carry out the aberrant splicing in the mammary gland (Fig. 2*b*). A faint ≈350-bp fragment was also detected. This is probably an artefact since there was no evidence of other aberrantly spliced

forms when other primers were used (Fig. 3 *c* and *d*). The absence of the 688-bp fragment confirms that there is little unspliced FIXD mRNA in these samples. In contrast, only the 688-bp fragment is amplified from human liver RNA, demonstrating that this splicing event does not occur in the normal fIX-producing tissue.

This laboratory has reported (15) transgenic sheep expressing low amounts of fIX in milk. The construct used in these studies FIXA (Fig. 2*a*) contains the fIX cDNA segment inserted into the first exon of the BLG gene. The amplification of the 288-bp fragment from mammary gland RNA samples from these sheep shows that the FIXA RNA transcripts are also aberrantly spliced at the same cryptic donor and acceptor sites. A third fIX construct JFIXAi (Fig. 2*a*) has also been introduced into sheep (I.C., unpublished observations). This construct is similar to FIXA but contains intron I from the fIX gene. Mammary gland transcripts from this gene exhibit the 288-bp fragment in RT–PCR experiments but the 688-bp fragment characteristic of unspliced RNA is also detectable, although we cannot exclude genomic contamination as the cause of this. Thus, the aberrant splicing between nt 1085 and 1547 appears to be a general property of fIX cDNA constructs expressed in the mammary gland.

**Correction of Aberrant fIX Splicing.** To correct the aberrant splicing, a 145-bp segment of the 3′ UTR of FIXD, containing the aberrant 3′ acceptor splice site, was removed. FIXDΔ3′ (Fig. 3*a*) was coinjected with the BLG gene and nine transgenic lines were established. These lines contain both BLG and FIXDΔ3′ transgenes, which cosegregate and are almost certainly cointegrated (10).

Expression of the FIXDΔ3′ transgene in the BIXΔ3′ lines was examined by Northern blot and RT–PCR analyses. Mammary gland RNA from five out of the nine lines of mice exhibited detectable FIXDΔ3′ transcripts on Northern blots. The BIXΔ3′ transcripts were larger than the BIX transcripts (Fig. 3*b*) and were sized to be ≈1700 nt. The size expected from full-length FIXDΔ3′ mRNA is 1668 nt, indicating that these transcripts are not spliced.

Correction of aberrant splicing was confirmed by RT–PCR. Priming from a 5′ fIX site upstream of the cryptic donor site to a site encompassing the fIX stop codon, present in both FIXD and FIXDΔ3′, amplified a 559-bp fragment from

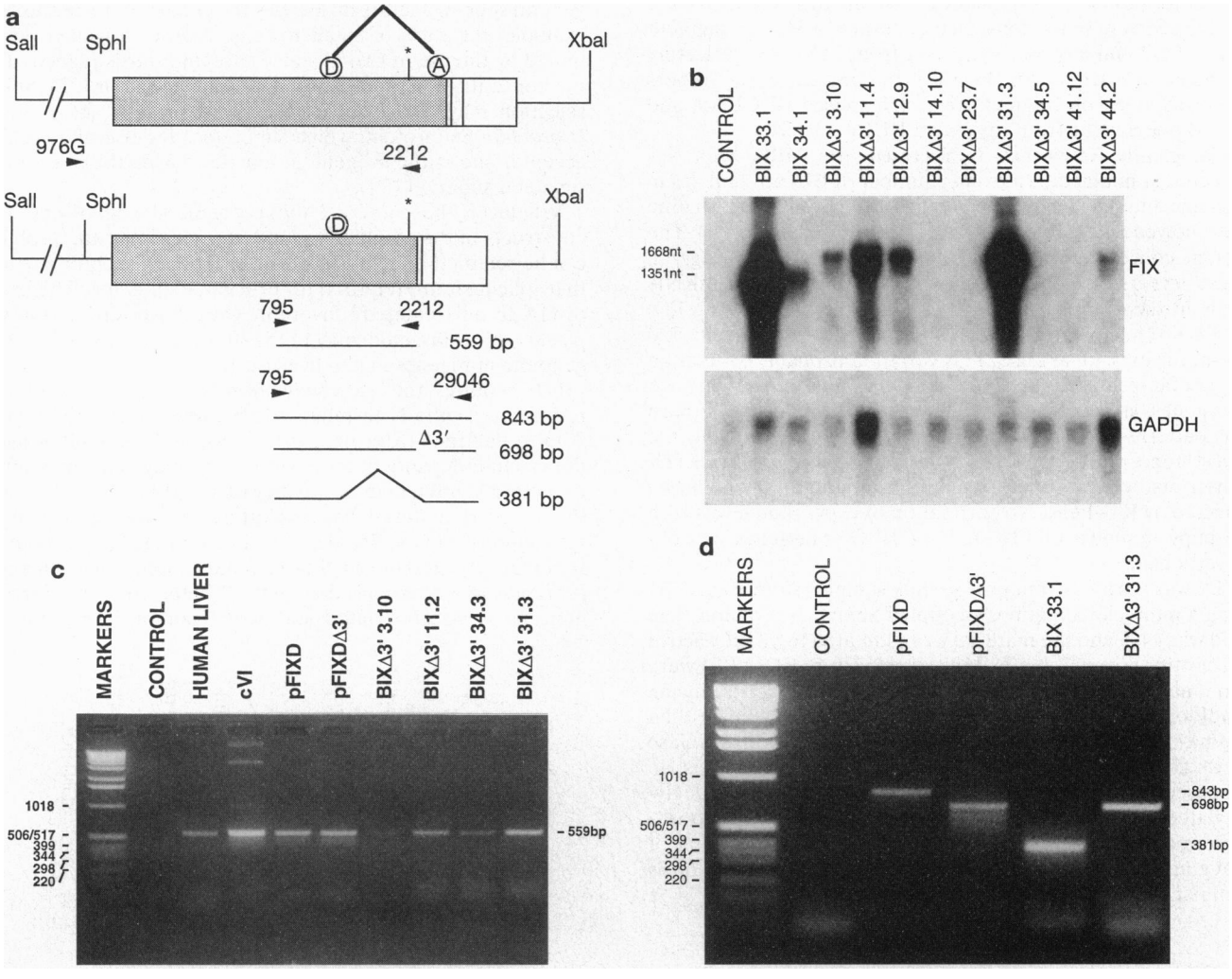


FIG. 3. Construction and expression of FIXDΔ3′. (*a*) Relationship between FIXD and FIXDΔ3′. Primers 976G and 2212 were used to amplify a segment of FIXD lacking 145 bp of 3′ UTR including the cryptic acceptor splice site. This segment was ligated to 5′ and 3′ BLG sequences to generate FIXDΔ3′. Primer pair 795/2212 amplifies a 559-bp fragment from FIXD, FIXDΔ3′, and nonaberrantly spliced RNA. Primer pair 795/29046 will amplify an 843-bp fragment from FIXD, a 381-bp fragment from spliced FIXD transcripts, and a 698-bp fragment from FIXDΔ3′ and nonspliced mRNA transcripts from this construct. (*b*) Northern blot analysis. Mammary gland RNAs from high and medium expressing BIX mice (33.1 and 34.1) were compared to samples from BIXΔ3′ transgenic mice (3.10–44.2). Blots were probed with labeled insert from cVI and then reprobed with a GAPDH probe. The sizes of the transcripts are indicated. (*c*) PCR products from 795/2212 primers. PCR products from DNA (cVI, pFIXD, or pFIXDΔ3′) or RNA samples from human liver or mouse mammary gland (control or BIXΔ3′ 3.10–31.3) were visualized by gel electrophoresis. (*d*) PCR products from 795/29046 primers.

Table 1. RNA and protein expression in BIXΔ3′ lines

| Line | Copy nos. (BLG/FIXDΔ3′) | RNA, ng/μg | Protein, μg/ml¶ |
|---|---|---|---|
| 3 | ND | + | 2.9 (2) |
| 11 | 8/2 | + (0.04) | 4.2 (3) |
| 12 | 15/2 | + (0.02) | 9.1 (8) |
| 14 | 14/3 | − | 0.3 (1) |
| 23 | 28/3 | − | 0.4 (2) |
| 31 | 6/2 | + (0.44) | 60.9 (18) |
| 34 | 9/1 | − | 0.38 (3) |
| 41 | 6/1 | − | <0.1 (2) |
| 44 | ND | + | 0.6 (3) |

Numbers of copies of BLG and FIXDΔ3′ were estimated by PhosphorImager analysis of Southern blots of tail DNA; these values are approximate. In some samples, the level of FIXDΔ3′ mRNA was estimated relative to an *in vitro*-transcribed fIX transcript; the estimated mRNA level is in parentheses. Protein was measured by ELISA; averaged from the number of mouse generation 1 or 2 samples is shown in parentheses. ND, not done.

BIXΔ3′ mammary RNA from a number of the transgenic lines identical in size to the fragment generated from the plasmids (Fig. 3*c*). Priming from the same 5′ fIX site to sequences in the 3′ BLG segment of the constructs generated a 843-bp fragment from FIXD and a 698-bp fragment from FIXDΔ3′ reflecting the deleted 3′ sequences (Fig. 3*d*). In contrast, these primers amplified a 381-bp fragment from the spliced BIX RNA and a 698-bp fragment from unspliced BIXΔ3′ RNA.

**Biologically Active fIX Is Secreted into Milk.** Milk was collected at midlactation from a number of BIXΔ3′ females in each line and the fIX levels were measured by ELISA. All nine lines showed detectable levels of fIX in the milk (Table 1). The average concentration varied from <100 ng/ml to ≈60 μg/ml. These levels correlate roughly with the steady-state mRNA levels in the mammary gland. In the highest expressing line (BIXΔ3′31), fIX levels varied between 30 μg/ml and 120 μg/ml for individual mice. This variation probably reflects an epigenetic instability of expression sometimes observed with transgenes, as mice within the line showed identical Southern blot patterns (data not shown). In the lines expressing the lowest concentrations of fIX, we were unable to detect fIX transcripts by Northern blot analysis, although they could be detected by RT–PCR. No correlation of expression levels with the copy numbers of FIXDΔ3′ or BLG transgenes was observed (Table 1).

fIX was purified from pooled milk samples from BIXΔ3′31 using a monoclonal antibody-coupled Sepharose column. The yield was 44% and the material was estimated to be fully active in a clotting assay (Table 2). Milk samples from BIXΔ3′31 were also analyzed on Western blots (Fig. 4). Under reducing conditions the mass of milk-derived fIX is slightly lower than plasma-derived human fIX: Deglycosylation with PNGase shows that the majority of the transgenic material has an increased mobility after digestion and comigrates with deglycosylated fIX standard (unpublished observations). The transgenic material does not exhibit noticeably high amounts of heavy and light chain species showing that fIX activation is minimal.

## DISCUSSION

Splice site selection may be determined by both cis- and trans-acting factors. Evidence that cis-acting sequences may contribute to splice-site selection comes from work showing that adjacent sequences can affect the accuracy and efficiency of selection (20) and that the activity of inserted splice sites depends upon their location within the pre-mRNA (21). Splice site context may be a critical determinant of activity and, in the present instance, could reflect differences between the higher-order structure of pre-mRNAs transcribed from cDNA vs. genomic sequences. However, the cryptic donor and acceptor sites are both located in the large terminal exon 8 of the fIX gene (18), so their activation cannot be due to juxtaposition. Furthermore, a full-length fIX cDNA appears to be correctly spliced when it is expressed in the liver (the normal site of fIX expression) of transgenic mice (22). Therefore, it seems more likely that the aberrant splicing reflects differences between trans-acting splicing factors in the mammary gland and liver. There are a number of examples in which the balance of general splicing factors influences the choice of splice site. For example, the ratio of small to large tumor antigen is determined by the use of two 5′ splice sites and this is regulated by the concentration of an alternative splicing factor (23). Splice selection may also involve specialized proteins such as the *Drosophila* Sxl protein, which suppresses the use of a specific acceptor site in the *tra* gene in females during the pathway of sex determination (24).

Table 2. Purification and biological activity of fIX from transgenic mouse milk

| Parameter | Pooled milk | Eluate |
|---|---|---|
| Total fIX, μg | 140 | 61.6 |
| Recovery, % | — | 44 |
| Concentration, μg/ml | — | 33.8 (2) |
| Activity, μg/ml | — | 33.7 (3) |

In the pooled milk, a number of samples from line BIXΔ3′31 were pooled. Total fIX and the concentration were measured by ELISA. Activity was measured by a clotting assay. Numbers in parentheses are the number of independent determinations.

Whatever the cause of the aberrant slicing of the fIX constructs in the mammary gland, it is clear that the problem can be rectified by removal of the cryptic 3′ acceptor site and that splicing is not required for efficient expression. The levels of fIX in milk compare favorably with those reported in the plasma of transgenic mice (15–40 μg/ml) expressing fIX genomic minigenes in the liver (25).

fIX requires the γ-carboxylation of a cluster of 12 Glu residues near the N terminus for full biological activity (11). Almost half the fIX protein was recovered from milk by using the calcium-dependent monoclonal antibody. This only binds to material with a high occupancy of γ-carboxylation (17) and the purified material has a clotting activity equivalent to plasma-derived fIX. We also conclude that the fIX propeptide is correctly processed in the mammary gland, since this is a prerequisite for biological activity. Whether the unrecovered material is γ-carboxylated and active cannot be determined

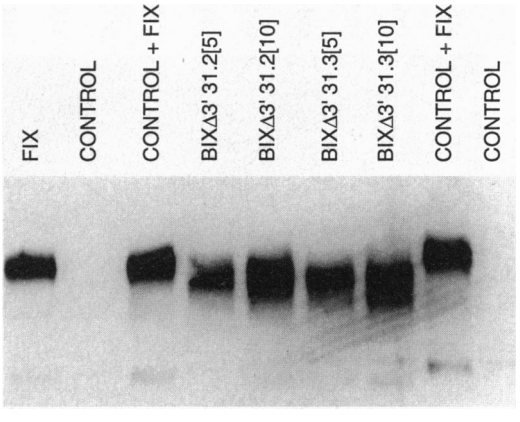


FIG. 4. Western blot analysis. Milk samples from two animals from line BIXΔ3′31 (31.2 and 31.3) were electrophoresed under reducing conditions. The samples were diluted 1:200 and either 5 μl or 10 μl was loaded. fIX, 10 ng of plasma-derived fIX; CM, control milk; CM+fIX, control milk + 10 ng of fIX.

easily but the purification yield is very good, considering the working scale, and so losses may be attributed to nonspecific absorption rather than failure to bind to the monoclonal antibody. The average expression level of biologically active fIX in the BIXΔ3′31 line is thus at least 27 μg/ml and probably higher in individual high-expressing animals.

Recombinant fIX produced from cultured mammalian cells is often partially γ-carboxylated and, consequently, has a reduced biological activity. fIX secreted from transfected CHO cells is partially γ-carboxylated and the propeptide is incompletely cleaved (13). Even in cell types such as BHK21, capable of synthesizing other vitamin K-dependent γ-carboxylated proteins, the fIX produced exhibited only partial activity (14), although human 293 cells have been reported to secrete active fIX at relatively modest levels (26).

The production of relatively high levels of fully active fIX by the mammary gland is gratifying, particularly as γ-carboxylation has not been reported for any natural milk proteins. Another γ-carboxylated protein, protein C, has been made in the milk of transgenic animals. In transgenic mice, propeptide cleavage and γ-carboxylation were incomplete and only traces of biological activity were detected in the purified protein (27). In transgenic pigs, protein C was present in milk at levels between 0.5 and 1.0 mg/ml but this material was only 30–60% active (5). Interestingly, preliminary results in transgenic sheep indicate that protein C expressed in milk at levels >0.1 mg/ml is fully active (I.C., unpublished observations).

Transgenic sheep carrying fIX can be anticipated now. Sheep produce up to 500 liters of milk per lactation. With levels of expression similar to those in mice, on average, a ewe could produce at least 13.5 g of biologically active protein per lactation. The estimated requirement for fIX worldwide is ≈3 kg per annum, and so with effective purification and downstream processing, a substantial fraction of this could be met by a relatively small flock of producer animals.

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