Expression of active human blood clotting factor IX in transgenic mice: use of a cDNA with complete mRNA sequence

K.H.Choo*, K.Raphael⁺, W.McAdam and M.G.Peterson

Murdoch Institute, Royal Children's Hospital, Flemington Road, Parkville 3052, and ⁺CSIRO, Division of Animal Production, Great Western Highway, Blacktown 2148, Australia

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ABSTRACT

Haemophilia B is a bleeding disorder caused by a functional deficiency of the clotting factor IX (1). A full length human factor IX complementary DNA clone containing all the natural mRNA sequences plus some flanking intron sequences was constructed with a metallothionein promoter and introduced into transgenic mice by microinjection into the pronuclei of fertilised eggs. The transgenic mice expressed high levels of messenger RNA, γ -carboxylated and glycosylated protein, and biological clotting activity that are indistinguishable from normal human plasma factor IX. This study demonstrates the feasibility of expressing highly complex heterologous proteins in transgenic mice. It also provides the groundwork for the production of large amounts of human factor IX in larger transgenic livestock for therapeutic use, and the investigation of alternative genetic therapies for haemophilia B.

INTRODUCTION

Factor IX is a plasma serine protease which plays an essential role in the intrinsic blood clotting pathway (2). During blood coagulation, it is converted to an active form, IXa, which interacts with factor VIIla, phospholipid and calcium ions to form a complex which converts factor X to Xa. Factor IX is synthesised in the liver and requires four different post-translational modifications before it is secreted into the bloodstream:- the vitamin K-dependent γ -carboxylation of 12 glutamic acid residues (2,3), the addition of several carbohydrate residues (4), the hydroxylation of an aspartic acid residue at position 64 (ref. 5 and 6) and the proteolytic cleavage of the signal peptide.

A functional deficiency of factor IX leads to the severe bleeding disorder of haemophilia B or Christmas disease (1). This is an X-linked recessive condition which occurs at a frequency of about 1 in 30,000 males. Patients are currently treated by injection of factor IX concentrate prepared from pooled plasma from normal blood donors. This treatment is complicated by the risk of infection by blood-borne viruses such as those

responsible for hepatitis and AIDS (acquired immune deficiency syndrome). The cloning of the gene for human factor IX (7-9) opened up an alternative means of producing this factor and the prospect of gene therapy for haemophilia B using recombinant DNA technique. In the present investigation, we explored the feasibility of these approaches by demonstrating the expression of functional human factor IX in transgenic mice. Furthermore, since human factor IX is extensively post-translationally modified, this study also explored the fidelity of the expression of probably the most complex heterologous protein in transgenic animals to date.

Several groups have already demonstrated the expression of active human factor IX from recombinant DNA clones in transformed cell lines derived from rat hepatoma (10), human hepatoma (11), baby hamster kidney (12) and chinese hamster ovary (13). However, Palmiter and Brinster (14) have reported nonexpression of human factor IX in many transgenic mice despite demonstration that their constructs were functional when transfected into tissue culture cells. cDNA constructs containing the factor IX coding region but very little of the natural noncoding sequences were used in their studies (R. Palmiter, personal communication). In an attempt to circumvent and explore potential problems associated with specific sequence requirement for *in vivo* expression, we constructed a full-length human factor IX cDNA which included all of the nucleotide sequences of the normal mRNA. We report here the expression of this cDNA in transgenic mice.

MATERIALS AND METHODS

Construction of the pMT-IX/NEO/DHFR expression plasmid.

A factor IX genomic fragment containing exon h (nucleotides 31586 to 34082) was released from HIX-2 clone (15) by digestion with *Stu*I, purified by agarose gel electrophoresis and subcloned into pUC12 which had been digested with *Hinc*II. Following transformation of *E. coli* MC1061, an insert in the correct orientation was identified by restriction mapping. The nucleotides from 31586 to 32779 were removed by digestion with *Bam*HI (site in pUC12 5' to nucleotide 31586) and *Apa*I (nucleotide 32779), followed by 'filling-in' the *Bam*HI end using the Klenow fragment of DNA polymerase 1. The vector was purified by agarose gel electrophoresis and into this was subcloned a factor IX cDNA fragment consisting of nucleotides 25 to 1641 (ref 15). This fragment was prepared by excising it from cVI/VII clone, a

recombination of the cVI and cVII clones (15) (kindly provided by D.S. Anson and G.G. Brownlee), with ClaI (site in vector 5' to nucleotide 25) and ApaI (nucleotide 1641), followed by 'filling-in' the ClaI end and purification by agarose gel electrophoresis. A transformed *E.coli* MC1061 clone in which the BamHI site within the pUC12 vector 5' to the insert is reconstructed was identified by restriction mapping. Following linearization with BamHI and 'filling-in', a genomic fragment containing exon a (nucleotides 289 to approx. 1099, which is present within the first intron), was subcloned into the plasmid. This fragment was prepared from genomic subclone gXI (ref 15) by digestion with TaqI, followed by 'filling-in' and purification by agarose gel electrophoresis. A transformed *E.coli* MC1061 clone containing an insert in the correct orientation in which the BamHI sites at both ends are reconstructed, was identified by restriction mapping. Next, from this clone, a genomic fragment from nucleotides 363 to approx. 1099, which contains part of the first intron and a short stretch of the coding region which is repeated as a result of the previous subcloning step, was removed. This was achieved by digestion with StuI (2 sites within the insert) and religation, thus eliminating the fragment (along with an internal BamHI site). (In order to achieve digestion with StuI, it was necessary to passage the above construct into *E.coli* GM119 which is a *dcm*⁻ strain.) The religated DNA was transformed back into *E.coli* MC1061 and checked by restriction mapping. This plasmid was linearized by digestion with HindIII (within the vector 3' to the insert), followed by 'filling-in', and the entire insert containing the full-length factor IX cDNA was released by digestion with BamHI. This insert was then purified by agarose gel electrophoresis and subcloned into the expression vector pMT010 (16) at a position immediately downstream of the sheep MT-Ia promoter [Peterson and Mercer, unpublished]. The pMT010 vector was prepared for subcloning by digestion with SalI (6bp downstream of the BamHI site which was present within the MT promoter), followed by 'filling-in' and a second digestion with BamHI. The final construct was transformed into *E.coli* MC1061 and characterized by restriction analysis.

Transformation of cultured cells

Cultured human and rat hepatoma cells were transfected with pMT-IX/NEO/DHFR plasmid DNA using a modified calcium phosphate co-precipitation method as previously described (17). Transformed cells were selected in 400 $\mu\text{g ml}^{-1}$ G418 (Geneticin, Gibco Laboratories).

Production of transgenic mice

For the microinjection experiments, the MPr-FIX cDNA fragment (see Results) was excised from the pMT-IX/NEO/DHFR plasmid by Pst I digestion (refer to Fig. 1), followed by gel elution. This DNA was microinjected into the pronucleus of fertilised eggs from C57 x CBA F2 mice as previously described (18).

Northern analysis of RNA

Total liver RNA was prepared and analysed by Northern gel blotting as described by Thomas (19) and Dobner et al (20). For the hybridisation of the filter containing RNA from cultured hepatoma cells (Fig. 2A), a 2.5 kb factor IX cDNA fragment (excised by Bam HI/EcoRI double digestion; see Fig. 1) was used as a probe. For the hybridisation of the filter containing RNA from transgenic mice (Fig. 2B), a 0.45 kb probe (excised by EcoRI/Hind III double digestion; see Fig. 1) was used.

Western blot analysis

Plasma samples were collected from transgenic mice and constituted to a final concentration of 0.38% sodium citrate (which serves as an anticoagulant).

In the zinc induction experiments, the mice were injected intraperitoneally with a solution of ZnSO₄ at 5 mg zinc per kilogram body weight. For the 26 h sample, two injections were given, the first at 0 h followed by a second at 11 h. Plasma samples were immunoprecipitated using a rabbit anti-human factor IX polyclonal antibody (Calbiochem.-Behring) as described previously (21). The immunoprecipitated samples were resolved on a 12% SDS-polyacrylamide gel (21), followed by electrophoretic transfer onto a nitrocellulose filter paper according to the method of Burnette (22). Factor IX polypeptide was detected by probing the nitrocellulose paper with the rabbit antibody, followed by ¹²⁵I-protein A (23) (Amersham International).

Enzyme-linked immunosorbent assay (ELISA)

The amount of human factor IX polypeptide was determined by ELISA as described before (10) using a mouse anti-human factor IX monoclonal antibody 3A6 (ref. 24) as the first antibody, and rabbit anti-human factor IX polyclonal antibody as the second antibody. Sensitivity of the ELISA was ~ 2 ng ml⁻¹.

Clotting activity assay

The level of clotting activity was measured using the one-stage clotting assay (25). This assay is based on the ability of a sample

containing factor IX to reduce the blood clotting time of congenital factor IX-deficient plasma (General Diagnostics). The inhibitory effect of the monoclonal antibody 3A6 on clotting activity was assayed by preincubation of the plasma samples with a final concentration of 2.5% 3A6 ascites fluid (+Ab), or PBS (-Ab), for 20 min. at 4°C. Clotting activity due specifically to human factor IX was derived by subtracting the +Ab value from the -Ab value. Factor IX polypeptide (as quantitated by ELISA above) and clotting activity were both expressed as a percentage of that in pooled normal human plasma and then converted to $\mu\text{g ml}^{-1}$ using a standard value of 5 $\mu\text{g ml}^{-1}$ (ref. 1) of factor IX in normal plasma.

RESULTS

Structure of the factor IX expression plasmid

First, we prepared a factor IX expression plasmid (Fig. 1) which was suitable for initial testing in cultured mammalian cell lines. The full-length human factor IX cDNA was generated by utilising a series of human genomic and cDNA subclones. The resulting construct contained 6 nucleotides upstream of the mRNA start point in the factor IX gene, the entire 5' noncoding, coding and 3' noncoding region, and 140 nucleotides downstream of the poly(A) addition site in the factor IX gene. This cDNA construct was then placed under the transcriptional control of the sheep metallothionein-Ia gene promoter. Neomycin (NEO) and DHFR markers were incorporated into the plasmid (17) to allow dominant selection for transformants in cultured mammalian cells, and potential amplification of the factor IX cDNA by stepwise methotrexate selection, respectively.

Expression in cultured cells

In preliminary experiments, we tested the expression plasmid pMT-IX/NEO/DHFR by transfection into a human hepatoma (H-H1) (26) and a rat hepatoma (H₄-II-E-C₃) (27) cell line, both of which do not normally express factor IX. Each of these cell lines yielded a number of transformant colonies which expressed a factor IX-specific mRNA (Fig. 2A). The size of the factor IX mRNA produced in these cells was identical to that found in total human liver mRNA. Treatment with zinc induced a significantly higher level (10-20 fold) of the mRNA. Similar results were obtained with the rat hepatoma transformants (not shown). Thus, the MT promoter-factor IX cDNA construct (designated MTpr-FIX) proved functional in producing a human factor IX mRNA.

Fig.1. Structure of the expression plasmid pMT-IX/NEO/DHFR. The factor IX transcription unit, from 5' to 3', consists of the metallothionein promoter (MTpr; solid box), sequences upstream of the mRNA start point in the factor IX gene (crossed box), factor IX 5'-untranslated region (open box), factor IX coding region (hatched box), factor IX 3'-untranslated region (stippled box), and sequences downstream of the poly(A) addition site in the factor IX gene (cross-hatched box). The full-length human factor IX cDNA was reconstructed from various genomic and cDNA segments, followed by cloning into the pMT010 vector (denoted by broken line), as described in Materials and Methods. Indicated on the map are the mouse dihydrofolate reductase (DHFR) and bacterial NEO genes, the pBR322 origin of replication (Ori) and B-lactamase (Amp^R) regions. The direction of transcription is indicated by the arrows. Restriction sites for PstI, EcoRI, BamHI, StuI, ApaI and HindIII are denoted by P, E, B, S, A and H respectively.

Expression in transgenic mice

The MTpr-FIX cDNA fragment (see Materials and Methods) which was used for the microinjection experiments contained in addition to the full-length factor IX transcription unit, approx. 400 bp of pBR322 sequences. This DNA was microinjected into the pronucleus of a total of 240 fertilised eggs from C57XCBA F2 mice. Of these, 158 survived the injection procedure. These were transferred into 7 recipient mothers, and 39 animals were born. Tail DNA was prepared from these animals and checked for the integration of the human factor IX cDNA using Southern analysis. Seven independent positive transgenic mice were identified. When plasma samples were collected from these animals following zinc injection (26h; 5 mg kg⁻¹ body weight), and analysed by ELISA (see Materials and Methods), four (designated FN7, 10, 17 and 34) were found to express a detectable amount

Fig. 2. Northern analysis of RNA. A, RNA from cultured human hepatoma H-H1 cells which have been transfected with the factor IX expression plasmid pMT-IX/NEO/DHFR. Except where indicated, 15 ug of RNA was applied onto each track. 1, 3 and 4 are three different transformed cell colonies before (-) and after (+) zinc treatment (final concentration of 10^{-4} M ZnSO_4 in the culture medium for 8 h). 2, untransfected parental cells. L, human liver RNA (3 ug). B, total liver RNA from control and transgenic mice. Control (nontransgenic) mice were injected intraperitoneally with 0.9% NaCl (sample 1), or with a solution of ZnSO_4 at 5 mg zinc per kilogram body weight, and their livers collected after 3 h (sample 2) or 26 h (sample 3). Samples 4-6 are transgenic mice similarly injected as 1-3, respectively. L, human liver RNA. 20 ug of RNA was applied onto each track. The rRNA size markers (28S and 18S) were visualised by staining the gel with ethidium bromide, and these correspond to mol. wt. of approx. 4.5 kb and 2.0 kb, respectively.

of human factor IX. The highest producer, FN7, was chosen for further characterisation. This transgenic mouse was shown to have incorporated 5-10 copies of the human factor IX cDNA by Southern blot hybridisation (data not shown). The following studies were performed on F1 heterozygous progeny mice derived from this female parent.

Expression of human factor IX mRNA

Total liver RNA was prepared from the FN7 mice and analysed on a Northern gel. Initial hybridisation experiments using a 2.5 kb near full-length factor IX cDNA probe (see Materials and Methods) did not allow discrimination between mouse and human mRNA even under high stringency

Fig. 3. Western blot analysis of human factor IX polypeptide in transgenic mice. Samples are : 1, 100 μ l phosphate buffered saline (PBS); 2, 4 and 6, 25 μ l, 100 μ l and 50 μ l, respectively, of transgenic mouse plasma after 26 h zinc treatment; 3, 100 μ l of transgenic mouse plasma prior to zinc treatment; 5, human plasma factor IX concentrate (Prothrombinex, CSL, Melbourne) containing approx. 0.125 μ g of factor IX; 7 and 8, 100 μ l of control, nontransgenic mouse plasma before and after (26 h) zinc treatment, respectively; 9, as 4 except the factor IX was precipitated with barium citrate (29). Solid arrow indicates position of human factor IX; open arrow indicates position of a putative degraded form of factor IX which is present in both the human plasma (sample 5) and transgenic mouse plasma (samples 2, 4 and 6). The inset shows a longer X-ray film exposure of lanes 3 and 4 to reveal the faint factor IX band in lane 3. The mol. wt. standards (Pharmacia) are phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soyabean trypsin inhibitor (20,000) and α -lactalbumin (14,400).

washing conditions. We have overcome this by using a 0.45 kb fragment from the 3' noncoding region of the human factor IX cDNA (See Materials and Methods). Evidently, sequences in the noncoding region have diverged significantly more between human and mouse compared to the rest of the sequences within the factor IX mRNA.

Different FN7 transgenic mice were treated with zinc to see if the

integrated Mfpr-FIX gene could be transcriptionally induced by the heavy metal. Fig. 2B shows that there was no detectable amount of human factor IX mRNA in control mice both before and after zinc treatment. In the transgenic animals, however, a trace amount of the human factor IX mRNA was seen prior to zinc injection. On induction with zinc for 3 h and 26 h, the mRNA level increased by approximately 5- and 20-fold, respectively. This mRNA had a mol.wt. of ~ 3.0 kb, which was indistinguishable from that of human liver RNA (Fig. 2B).

Analysis of human factor IX polypeptide

For the analysis of factor IX polypeptide, initially we collected plasma samples from the transgenic mice at 0 h, 3 h, 6 h, and 26 h following zinc injections. The plasma samples were immunoprecipitated and analysed by Western blotting. Only a small amount of human factor IX was detected before zinc treatment (0 h - Fig. 3), and a similar low amount at 3 h and 6 h after treatment (not shown), but the level increased dramatically by 26 h (Fig. 3). The observed discrepancy between the elevation in mRNA level (see Fig. 2B) which was not accompanied by a similar increase in the plasma protein at 3 h and 6 h suggested a lag period necessary for translation and/or post-translational modification prior to secretion. The protein detected in the transgenic mouse plasma had a mol.wt. of $\sim 70,000$ dalton, which is identical to that of control human plasma factor IX. These data suggest that the human factor IX produced by the transgenic mice is properly processed in terms of glycosylation since the unglycosylated protein has a much smaller mol.wt. of $\sim 50,000$ (ref. 28).

To determine whether the factor IX protein produced by the transgenic mice was γ -carboxylated, plasma samples were fractionated using barium citrate (29). This fractionation is based on the ability of γ -carboxylated proteins to adsorb to, and co-precipitate with, the insoluble salt of barium citrate, leaving any non-carboxylated proteins in solution. Fig. 3 shows that the human factor IX in the transgenic mouse plasma was adsorbed by barium citrate. The adsorbed molecules had a molecular weight which was identical to that of factor IX in normal human plasma. Direct quantitation of the barium citrate fractions using ELISA (see below) revealed that approximately 90% of the factor IX was found in the insoluble fraction (Table 1). These data indicate that most of the expressed protein were γ -carboxylated. [Since the γ -carboxylation step is vitamin K-dependent, we noted that the mice were maintained on a ration (Barastoc,

TABLE 1 Analysis of human factor IX polypeptide and clotting activity in transgenic mice

		ASSAY		CLOTTING		
		ELISA				
Treatment with Zinc	Human factor IX polypeptide		Total factor IX activity		Activity due to human factor IX	
	Whole plasma	Nonadsorbed to barium	Adsorbed to barium	-Ab	+Ab	
-	0.5	n.d.	n.d.	4.8	4.8	0
+	7.9	0.4 (9%)	4.3 (91%)	11.8	4.3	7.5

Table 1. Transgenic mice were injected with 0.9% NaCl (-), or with zinc (+), and the plasmas were collected after 26 h. (+Ab) designates preincubation of plasma sample with monoclonal antibody 3A6 before assaying for clotting activity. (-Ab) designates no preincubation with the antibody (Refer to Materials and Methods for details). Since the barium citrate precipitation procedure resulted in a substantial protein loss (during the dialysis step), the amount of human factor IX adsorbed or nonadsorbed to the barium salt was also expressed as a percent (values in brackets) of the total amount recovered in both the insoluble and soluble fractions. n.d. = not determined.

KMM Pty Ltd, Melbourne) containing a minimum vitamin K content of 25 g/tonnel.

The amount of factor IX polypeptide in the plasma of the transgenic mice was quantitated using a more sensitive and accurate enzyme-linked immunosorbent assay (ELISA). The assay is based on a specific anti-human factor IX monoclonal antibody 3A6 (Table 1). Control experiments showed that under the conditions described in Table 1, the antibody does not cross-react significantly with the mouse enzyme (data not shown; see also Table 1). As shown in Table 1, before zinc treatment, the transgenic mice produced $\sim 0.5 \text{ ug ml}^{-1}$ of human factor IX polypeptide in their plasma. Following zinc induction, an approximately 16-fold increase in the polypeptide level was achieved. This corresponds to $\sim 160\%$ of the concentration of factor IX in normal human plasma. [Using this assay, the concentrations of human factor IX polypeptide in the zinc-induced plasmas of the other three transgenic mice (FN10, 17 and 34) were found to be less than 1% that of FN7 plasma].

Determination of clotting activity

The amount of biologically active human factor IX in F7 was determined using a one-stage clotting assay (see Materials and Methods). Plasma samples from the transgenic mice prior to zinc induction did not show any significant difference in clotting time compared to those of control, nontransgenic mice (data not shown). Presumably, the level of human factor IX in the uninduced animal is insufficient to contribute significantly to the clotting activity of the mouse plasma (see below). When plasma samples from zinc-induced transgenic mice were assayed, the clotting time was dramatically reduced (Table 1). Preincubation of the plasma with the specific anti-human factor IX monoclonal antibody reduced the clotting activity of the plasma to that of normal mouse plasma. The amount of active human factor IX was calculated to be approx. 7.5 ug ml^{-1} . This value correlates well with that of human factor IX antigen (7.9 ug ml^{-1}) obtained by ELISA suggesting that the protein produced and secreted into the plasma by these transgenic mice is fully active.

DISCUSSION

The above data indicate that the cDNA construct used in this study contained all the necessary sequence information for *in vivo* expression in transgenic animals. It would be interesting to compare our results with those of others who have attempted similar experiments. Palmiter and

Brinster (14) have generated many transgenic mice (43 in total) carrying a human factor IX cDNA fused to a MT or albumin promoter, but in none of these could expression be detected in the liver, although the constructs used were functional when introduced into tissue culture cells. Since these constructs contained most of the factor IX coding sequences, but lacked the 5' as well as most of the 1.5 kb 3' noncoding regions, (R. Palmiter, personal communication), we propose that the successful *in vivo* expression was related to the inclusion of critical sequences in the 5' and/or 3' noncoding regions of the natural mRNA in our construct. We are currently carrying out further experiments to test this, as well as to determine the frequency of expressors in a larger number of animals using the present construct.

In recent years, many recombinant genes have been introduced into transgenic mice. Although these studies provided the general impression that gene expression in transgenic mice quite frequently resembles the endogenous counterpart, the capacity of the system to faithfully express a highly complex heterologous protein has not been directly tested. In the present study, we carried out such a test using human factor IX, which, because of the multiple tissue-specific post-translational modification steps involved, probably represents the most complex heterologous molecule expressed in transgenic animals to date. We have demonstrated that the transgenic mice produced high levels of biologically active human factor IX which was indistinguishable from the protein found in normal human plasma. In addition, the study demonstrated that the human factor IX cDNA was stably incorporated into the germ line, making the phenotype heritable.

The expression of factor IX in transgenic mice represents a first step towards the use of larger transgenic livestock for the production of large amounts of a highly purified recombinant human factor IX protein. Production in transgenic animals is particularly important for complex proteins like factor IX which cannot be adequately processed in other expression systems using bacteria or yeast. Others (30) have already shown that foreign genes can be expressed in larger animals, such as rabbits and pigs. We are optimistic that this line of investigation will eventually allow haemophiliacs suffering from deficiencies of factor IX, and factor VIII, to be treated with improved virus-free recombinant products. Finally, successful expression of active human factor IX in transgenic animals provides the groundwork for the investigation of alternative therapies for haemophilia by gene replacement.

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*To whom correspondence should be addressed

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