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Fish as Bioreactors: Transgene Expression of Human Coagulation Factor VII in Fish Embryos

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Abstract: A plasmid containing human coagulation factor VII (hFVII) complementary DNA regulated by a cytomegalovirus promoter was microinjected into fertilized eggs of zebrafish, African catfish, and tilapia. The active form of hFVll was detected in the fish embryos by various assays. This positive expression of human therapeutic protein in fish embryos demonstrates the possibility of exploitation of transgenic fish as bioreactors.

Key words: human coagulation factor VII, transgenic fish, bioreactor.

INTRODUCTION

The human coagulation factor VII (hFVII) is a vitamin K–dependent glycoprotein participating in the blood coagulation cascade. It is synthesized in the liver and secreted into the blood as a single-chain polypeptide that is activated to the 2-chain hFVIIa in the presence of thrombin and FXa. This activation is carried out by cleavage of an internal peptide bond located at Arg-152 and Ile-153 to generate a light chain containing 10 γ-carboxyglutamic acid residues and a heavy chain containing the serine protease domain. The hFVIIa initiates the extrinsic pathway of blood coagulation in the presence of Ca^{2+} and tissue factor, which is released from damaged tissue cells (Davie et al., 1979; Morrill et al., 2002). The complete nucleotide sequence of hFVII containing 9 exons and the putative promoter region was reported by O'Hara et al. (1987). An abnormal blood clotting syndrome is caused by an inherited deficiency of hFVII that occurs in approximately 1 in 500,000 humans. This disorder can be controlled with normal plasma-concentrates containing hFVII, or with recombinant human FVII (rhFVII), which is also used for patients with hemophilia A and B, severe thrombocytopenia, or a severe internal bleeding problem such as occurs with splenic rupture or liver transplantation (Jurlander et al., 2001; Gerotziafas et al., 2002; Hedner and Erhardtsen, 2002).

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During bleeding episodes frequent treatment is required because the life span of FVIIa is short.

Traditionally this protein was supplied to patients by a plasma-derived product. Two drawbacks to the use of this product are that it can transmit human blood-borne pathogenic virus and the availability of human blood is limitated. Hence rhFVII has been supplied as an alternative (Hedner and Erhardtsen, 2002). Bacterial cells harboring recombinant DNA could be a tool to produce rhFVII, but the complex eukaryotic posttranslational modifications of proteins are not performed in prokaryotic cells. Production of pharmaceutical proteins in mammalian cells or tissue culture is an alternative way to approach these problems. Recombinant hFVII is currently produced by baby hamster kidney (BHK) cell culture following transfection of the hFVII gene construct (Berkner, 1993). However, culture of mammalian cells is expensive, so large amounts of recombinant proteins cannot be produced at low cost by this mean (Houdebine, 2000).

An alternative to these strategies is the production of therapeutic protein by the introduction and expression of cloned DNA sequences in whole animal systems. One way to produce heterologous proteins that is less expensive than using cell cultures is to produce the protein in the milk of transgenic cows or sheep. There are many reports of successful production for pharmaceutical purposes of useful human proteins in milk of transgenic animals used as bioreactors (Wright et al., 1991; Ebert et al., 1991, 1994; Paleyanda et al., 1997; Van Cott et al., 2001). However, this system also has potentially serious problems. Milk commonly contains lytic enzymes that can hydrolyze the heterologous proteins unless they are highly resistant to degradation. Further, large mammals such as cows and sheep require extensive care, and high cost is involved in making them transgenic. Also, as some prion diseases are shared between nonhuman mammals and humans, there may be a danger that recombinant products produced in mammals will harbor pathogenic viruses or prions.

The use of transgenic fish as a bioreactor system could therefore be an alternative for producing rhFVII. The attractions of fish, particularly tilapia, in this regard are (1) the generation time is short; (2) the cost is comparatively low; (3) maintenance is easy; (4) germline-transmitted transgenes can be reproduced in large numbers of progeny fish; and (5) no known viruses or prions infect both humans and fish, so transmission of disease is highly unlikely (Maclean et al., 2002). Transgenic fish technology is well established in different species and subject areas by many research groups (Iyengar et al., 1996; Maclean, 1998). However, the use of transgenic fish as bioreactors for pharmaceutical proteins has not been previously reported. Here we report the production of bioactive rhFVII in fish embryos by gene transfer and demonstrate that transgenic fish can be used as bioreactors for therapeutically important proteins.

Materials and Methods

Transgenes Used in This Study

The hFVII coding gene cDNA was isolated by *Hin*dIII/*Bam*HI double digestion from the plasmid containing the hFVII sequence flanked by rat myosin heavy chain promoter and enhancers (pMyHC/FVII, FVII cDNA kindly provided by G. Kemball-Cook, MRC Clinical Science Centre, Royal Postgraduate Medical School, London, and G. Miller, Haemophilia Centre, Royal Free and UCL Medical School, London). The 2.62-kb hFVII cDNA fragment and the 0.12-kb SV40 poly(A) signal sequences were ligated into the pcDNAI expression vector (Invitrogene) within the *Hin*dIII/*Bam*HI sites. The resulting plasmid, pCMV/FVII (Figure 1), was checked for appropriate insertion and orientation by restriction mapping. The vector contains the CMV virus E1 early promoter and enhancer 5′ to the FVII sequences. The plasmid, prepared using conventional molecular techniques (Sambrook et al., 1989), was used for microinjection into fertilized fish eggs in circular plasmid form.

Production of Fish Eggs

Zebrafish (*Danio rerio*) embryos were obtained from natural spawnings of broodstock kept in a cycle of 14 hours light and 10 hours dark at 28°C. Eggs were laid into spawning boxes and microinjected at the one-cell or two-cell stage (Westerfield, 1995).

Embryos of African catfish (*Clarias gariepinus*) were produced by artificial stripping of eggs from broodstock. The females were injected with 3 mg/kg body weight carp pituitary homogenate dissolved in phosphate-buffered saline (PBS) solution 12 hours before ovulation. The eggs were stripped from the females and fertilized by addition of sperm derived from testis removed surgically from males. Single-cell embryos were used for microinjection. Nile tilapia (*Oreochromis niloticus*) eggs were obtained by stripping from mature females that showed spawning behavior and fertilized with sperm from mature male fish.

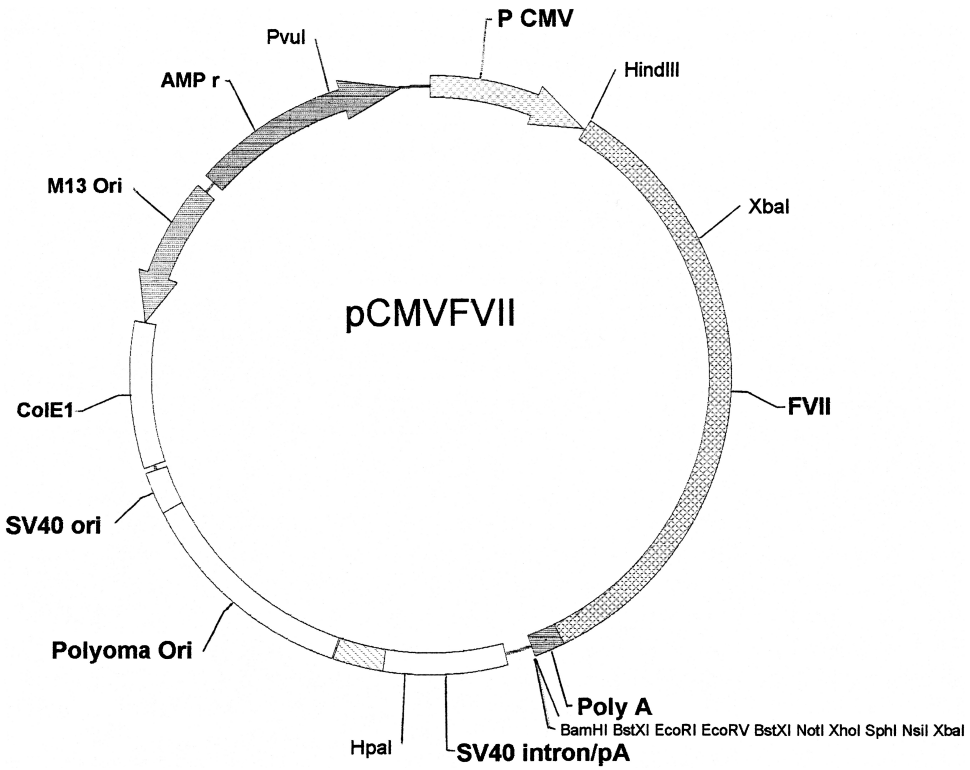

Figure 1. Expression vector into which the FVII cDNA is inserted. The map is not to scale. P CMV indicates CMV promoter; FVII, hFVII cDNA.

Microinjection of Transgene Constructs and Embryo Culture

Plasmids pMyHC/FVII and pCMV/FVII were separately microinjected into distinct batches of zebrafish embryos at the one-cell or two-cells stage, approximately 10^6 copies per egg, using a pressure-driven picoinjector (PLI-100, Medical System Corp., U.S.A.) as described in Williams et al. (1996).

The embryos of catfish were placed in petri dishes, where the naturally sticky eggs remained attached. The injection was done by hand using glass microcapillary needles filled with the appropriate DNA solution containing blue food dye for localization of the injection drop. The injection in the case of both species was targeted into the cytoplasmic region or the boundary between the cytoplasmic and yolk regions of the egg. The microinjected embryos were cultured for 1 day at 28°C in Holtfreter's solution until reaching the primordium-5 stage (Kimmel et al., 1995). The microinjected and noninjected control embryos were collected into microcentrifuge tubes and homogenized by plastic homogenizer. The tubes containing up to 100 homogenized embryos (approximately 20 µl) were snap frozen in liquid nitrogen and stored at −70°C until analysis.

DNA was injected into fertilized tilapia eggs as described in Rahman and Maclean (1992) using a picoinjector. Ten embryos at 3 days after injection (at approximately the primordium-22 equivalent stage of the developmental of classification (Kimmel et al., 1995) were pooled in a microcentrifuge tube and homogenized in 100 µl of PBS with bovine serum albumin (BSA) added (100 µg/ml) and centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatants were snap frozen in liquid nitrogen and stored at −70°C until analysis.

Human FVII Assay on Fish Embryo Injected

Immunochemical Detection

Enzyme-linked immunosorbent assay (ELISA) was carried out using a commercial kit based on polyclonal antisera to hFVII (Asserachrom VII: Ag, Diagnostica Stago, through Shield Diagnostics). Human FVII standard was derived from a donor plasma pool of 20 individuals with no history of coagulation or thrombophilic disorder. Standards were made by diluting pooled normal plasma 1:10, 1:30, 1:100, 1:300, and 1:1000. Standards, fish embryo homogenates, dilutions of protein, and appropriate negative control samples were loaded into the wells and incubated for 1 hour at room temperature. After 5 washes, FVII detection antibody conjugated with horseradish peroxidase was then added and incubated for 1 hour at room temperature. After addition

and incubation with the color reaction buffer, the samples were read in a conventional microtiter plate photometer.

Photometric Activity Assay

Enzymatic activity of the rhFVII produced in the fish embryos was measured by photometric assay (Coaset FVII, Quadratech). The assay was carried out in a microtiter plate that had been precoated with a 1% solution of BSA in PBS to prevent nonspecific protein binding during the assay. A standard curve was made from 20 normal plasma samples in the dilution range of 200 to 12.5 U/dl by doubling dilutions from 1:500. Plasma samples were diluted 1:1000.

Fish embryo samples were measured undiluted and in doubling dilutions from 1:40. Results were multiplied by 1.28, to match the normal plasma dilution of 1:1000, and expressed in nanograms per milliliter on the basis that 100 U/dl is equivalent to the accepted normal plasma concentration of 500 ng/ml.

Blood Clotting Assay

Factor VII contains a polyglycine tail that binds to phospholipid and is essential for its physiologic function. The photometric assay detects the enzymatic site of FVII, but is insensitive to this essential binding site. We tested the samples in a FVII clotting assay (Brozovic et al., 1974), in which FVII physiologic activity is detected by interaction with normal proteins of the human coagulation cascade. FVII-deficient plasma, containing all other required coagulation proteins, was used for blood clotting assays derived from immunodepleted plasma (Diagnostic Reagents Ltd.). Thromboplastin and the appropriate samples were added, followed by the addition of $CaCl_2$. The time lapse of clotting reaction was measured in a Coagulometer (Amelung KC10). The time lapse results were compared with dilutions of normal plasma samples using logarithmic scale and expressed in the percentage activity of normal plasma, where normal indicates 100 U/dl FVII protein.

Results

Human FVII Transgene Expression in Zebrafish Embryos

We tested the expression of 2 constructs introduced into zebrafish embryos by ELISA. One construct consisted of a rat myosin heavy chain (MyHC) promoter spliced with hFVII cDNA, and the other construct was similar but driven by a CMV promoter. Two trials using the MyHC promoter construct failed to show expression of the hFVII transgene in zebrafish embryos but showed levels of activity similar to those from noninjected control embryos. However, with the second construct regulated by the CMV promoter, significant levels of rhFVII were detected in zebrafish embryos (Table 1).

In another experiment, in trials using a photometric activity assay, higher expression levels of rhFVII were detected than with the ELISA test. In this trial we analyzed 3 batches that had different numbers of zebrafish embryos grown from eggs injected with the pCMV/FVII construct. The level of rhFVII expression detected per embryo in various numbers of pooled injected embryos was higher than that detected by the EL1SA technique (Table 2).

Human FVII Transgene Expression in Catfish Embryos

The homogenates of catfish embryos grown from eggs microinjected with the pCMV/FVII construct were analyzed by the blood clotting activity assay. Because the MyHC/FVII did not produce foreign protein in zebrafish embryos, this construct was not tested in catfish. An average of 25% of the clotting activity of normal human plasma was detected (Table 3), indicating that the rhFVII produced in the fish embryos had gone through the necessary posttranslational modifications for blood clotting activity.

Human FVII Transgene Expression in Tilapia Embryos

The rhFVII expression in tilapia embryos microinjected with pCMV/FVII construct was measured by ELISA (Figure 2). The active form of rhFVII was detected in tilapia embryos grown from eggs injected with this construct. The highest value obtained was 293 ng/ml, and the lowest was 116 ng/ml. An average of 214.7 ng/ml of rhFVII was measured in homogenates of pooled tilapia embryos. Noninjected embryos showed slightly higher than baseline values. However, a significant difference was revealed between positive injected and noninjected embryos by *t*-test. The embryos grown from injected eggs that proved to have lower values than noninjected control embryos were assumed to be nonexpressing samples. Normal human plas-

Table 1. ELISA of Zebrafish Embryos for Detection of Human FVII Protein

Test	Number of embryos	FVII concentration (ng/ml)
Embryos microinjected with pMyHC/FVII	100	2.0
	100	8.0
Embryos microinjected with pCMV/FVII	100	4.5
	100	61.5
	119	84.2
Noninjected control embryos	100	7.5
FVII dilution series	FVII from standard human serum (ng/ml)	FVII concentration measured (ng/ml)
	500	508
	250	239
	125	109
	63	65
	16	14
	0	8

Table 2. Activity Assay for Recombinant hFVII Produced in Pooled Zebraflsh Embryos[a]

Embryos	Number of embryos	FVII concentration (ng/ml)
Microinjected with pCMV/FVII	14	20
	100	220
	50	135
Noninjected control	100	0

[a]The data shown were taken from the 1:1280 dilutions of the sample.

Table 3. Results of Bioassay for Blood Clotting Activity of Recombinant hFVII Produced in Pooled Catfish Embryos Using hFVII-Deficient Blood Plasma

Test	Number of embryos	Activity[a](%)
Embryos microinjected with pCMV/FVII	100	21
	100	25
	100	28
	100	22
Nonfinjected control embryos	100	8
	10	8
Control blood plasma		100
Blank		8

[a]Blood clotting activity is measured as a percentage of the activity of normal human blood plasma containing normal levels of FVII (100 U/dl).

ma serving as a positive control gave values of the expected level (around 500 ng/ml). Note that some values for negative controls were higher than some positive values recorded in other fish species. We assume this resulted from variations in protein content of the homogenates.

Discussion

Although rhFVlI was detected in zebrafish embryos grown from eggs microinjected with pCMV/FVII construct by ELISA, only baseline expression was observed with the pMyHC/FVII construct (Table 1). This difference was probably caused by different capacities of the regulatory elements used in both constructs. Ubiquitous transgene expression driven by a viral CMV promoter has been reported in fish embryos and cell lines (Friedenreich and Schartl, 1990; Winkler et al., 1992). However, the MyHC promoter was utilized to try to provide tissue-specific expression or at least nonubiquitous expression (Maclean, 1998). It is unlikely that MyHC promoter can drive hFVII cDNA in fish embryos ubiquitously.

Levels of rhFVII expression in fish embryos varied within and between species. The fact that fish of different species were used in different assays was accepted to be nonideal. The work was carried out in this way as part of an ongoing search for an ideal species with which to pursue further work. The unequal distribution of transgenes in

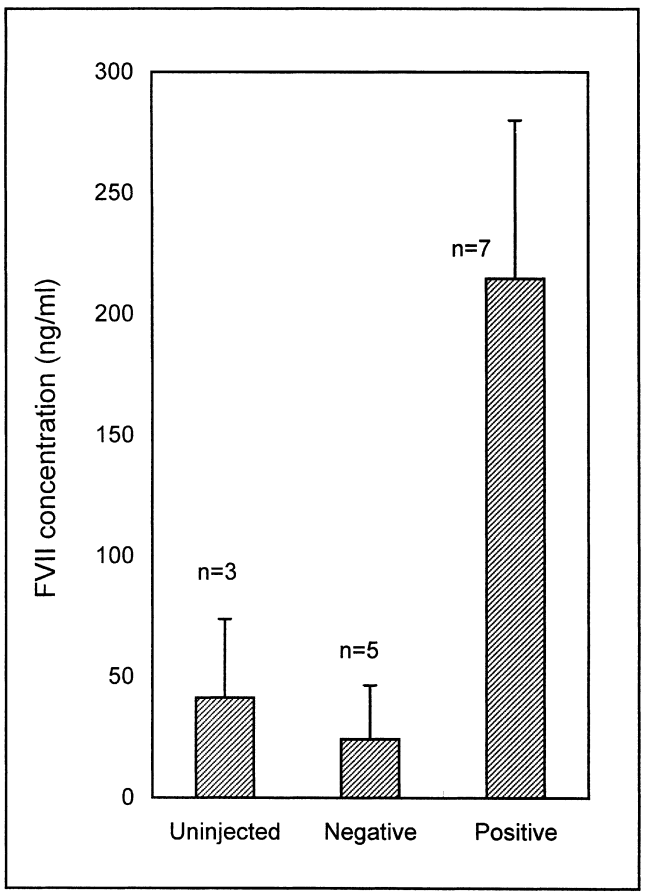

Figure 2. Expression level of hFVII in tilapia embryos developed from eggs injected with pCMV/FVII plasmid. Ten embryos were pooled in a microcentrifuge tube. Numbers on histograms indicate the number of tubes analyzed. The mean values from uninjected and negative embryos are not significantly different from one another ($P > 0.05$), but they are both significantly different from positive embryos ($P < 0.01$). Since a subset of unknown number out of the 10 pooled embryos in any one tube will express hFVII, the precise amount synthesized per embryo cannot be determined. It must be between 2.93 and 29.3 ng per embryo. Uninjected indicates control embryos not injected; negative, embryos injected but showing lower values than maximum value of control of assay treated as background activities.

injected fish embryos is typical of other work (Iyengar et al., 1996) and is accompanied by extensive mosaic transgene expression in positive embryos. It is frequently observed that even with potentially ubiquitously expressing promoters reporter gene expression in injected fish embryos is mosaic, nonubiquitous, and varies greatly from embryo to embryo, with some tissues expressing strongly and some not. The transgene copy number present in each cell also strongly affects the level of transgene expression in transgenic fish (Rahman et al., 2000; Hwang et al., 2003). Even when germ line transmitting transgenic animals are established, the expression level of human proteins in a transgenic animal bioreactor can be influenced by position effects. Therefore the variability in temporal and spatial patterns and in the level of expression is quite broad (Lubon, 1998).

Higher expression of recombinant therapeutic proteins in fish could be expected if a regulatory system derived from the same fish species were used. It has been reported that the regulatory system originating from the target species drives much higher transgene expression than regulatory sequences from foreign species (Alam et al., 1996; Hwang et al., 2003).

We have demonstrated that it is possible to use fish to express heterologous mammalian proteins that have valuable pharmaceutical or biological properties. This technique potentially allows the production of properly glycosylated and folded proteins, thus avoiding the disadvantages of nonmammalian expression systems, and is relatively inexpensive when compared with the use of mammalian cell cultures or large mammalian hosts, both of which require a labor-intensive approach, albeit for different reasons. Proteins of interest can be expressed in and recovered from fish eggs, which may be fertilized and unfertilized, or from developing embryos and larvae. For example, eggs may be microinjected or transfected by other gene transfer protocols directly with an expression vector coding for polypeptide of interest, under the control of a suitable promoter. We have calculated that if the system with tilapia egg injection were made intensive, then in 3 months around 1800 eggs could be injected and 4.5 mg of rhFVII could be recovered. If the recent technique of co-injection with a meganuclease were used (Thermes et al., 2002), then much higher levels could probably be achieved.

There is evidence that, at least on occasion, the introduced transgene may multiply extrachromosomally by autonomous replication (Chong and Vielkind, 1989). Heterologous proteins are expressed at high levels owing to the high copy number of the introduced gene and the high metabolic activity of tissues such as the yolk syncytial layer (Williams et al., 1996). The advantage of this system is that it allows rapid transformation of a large number of embryos and fast recovery of proteins from the transfected embryos. Co-transfection and selection protocols could also allow flexible production of complex multimeric proteins. Alternatively stable lines of transgenic sexually mature fish could be produced that express the polypeptide

of interest, under the control of suitable promoter, in eggs. These eggs could then be removed, and the protein could be extracted. Assuming that levels of foreign protein produced in stable transgenic lines would be comparable to those synthesized in transiently expressing microinjected embryos, and calculating that several milliliters of FVII could be produced by embryos from a single tilapia or catfish female, this would allow the production of milligram quantities of rhFVII protein. This system has the advantage that no mature transgenic fish need be sacrificed to obtain the recombinant polypeptide. For this approach a promoter such as albumin would be required that is capable of directing expression of introduced genes in a fish egg.

The positive expression in catfish and tilapia embryos also gave promising results, and both species could be good candidates for transgenic fish bioreactor systems because of their size.

Consequently, these results demonstrate that fish can be a tool to produce recombinant human therapeutic proteins as a bioreactor by transgenesis. Our research group is currently producing transgenic tilapia fish lines harboring a construct in which a tilapia promoter is spliced with hFVII cDNA to ensure efficient expression of rhFVII in tilapia serum.

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