Proceedings of the

4-7 july **2000** – Valencia Spain

These proceedings were printed as a special issue of WORLD RABBIT SCIENCE, the journal of the World Rabbit Science Association, Volume 8, supplement 1

ISSN reference of this on line version is 2308-1910
(ISSN for all the on-line versions of the proceedings of the successive World Rabbit Congresses)

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Volume A, pages 425-429

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ABSTRACT

Congenital X-linked deficiency or abnormality of coagulation factor VIII (FVIII) causes a bleeding disorder called haemophilia A. Treatment of patients suffering haemophilia A involves administration of human FVIII concentrates purified from human plasma, or recombinant protein prepared from mammalian cell culture. The cost of these products is the major limiting factor in haemophilia therapy. An alternative approach to this problem is to use transgenic animals expressing recombinant protein in the mammary gland.

Here we describe the generation of a line of transgenic rabbits bearing a gene construct with the human blood clotting factor VIII cDNA plus introns of the murine metallothionein gene under the control of the mammary gland specific murine whey acidic protein promoter. The founder male was fertile and transmitted the transgene to its progeny.

INTRODUCTION

Human antihaemophilic factor VIII is a plasma glycoprotein which plays an important role in blood coagulation. FVIII functions in the pathway of coagulation as a cofactor in the activation of factor X by factor IX. Human FVIII is synthetised as a 2351 length preprotein containing a 19 amino acid signal peptide (TOOLE et.al. 1984). The protein is proteolytically processed to a heavy chain and a light chain and circulates in the plasma closely associated with von Willebrand factor. The hFVIII gene consists of 26 exons and 25 introns with a total size of 186 kb. The gene encodes a 9 kb mRNA (GITSCHIER et.al. 1984). Haemophilia A is one of the most common human bleeding disorders affecting approximately one of 5-10000 males (TUDDENHAM et.al. 1991).

The haemophiliacs were once treated with whole plasma but more recently with highly concentrated and purified human plasma. This treatment allows the administration of effective but lower volumes to the patients. There are risk factors of this therapy such as contamination and antigenic responses. Between 1977 and 1985 half of the patients were infected with human immunodeficiency virus. Plasma concentrates are now screened for HIV virus however the potential to transmit diseases such as Creutzfeldt-Jakob disease or recently described hepatitis forms is still present (PALEYANDA et.al. 1997).

Human cell-culture production was shown to have a good safety profile so this was introduced into the therapy in 1993. 200 ng/ml protein per day was produced by this way in

10^8 cells. It is 2-3 magnitude lower than in the case of other recombinant proteins (EHMANN et.al. 1995). This fact could be due to the large size of the gene and the very complex posttranslational modifications containing proteolytic processing, N- and O-linked glycosylation and tyrosine sulfation. Due to the negative feedback in gene the FVIII mRNA is rather unstable. Additionally the protein is very susceptible to proteolytic degradation. These factors all contribute to the cost of the therapy (5000 US$/patient).

Expressing therapeutic proteins in the milk of transgenic animal is clearly a superior method in terms of yield and cost compared with mammalian cell culture. The choice of transgenic species depends on the actual aim of the experiment. It has been reported recently that the hFVIII cDNA-Mt-I, under the control of ovine β-lactoglobulin promoter could be expressed in the milk of transgenic ewes at 4-6ng/ml (NIEMANN et.al. 1999). Since the ultimate aim is to increase the quantity of active hFVIII factor in the milk of transgenic animals, in the present study the ovine β-lactoglobulin promoter was replaced by the mouse whey acidic protein promoter and transgenic rabbits were produced by microinjecting the fusion gene mWAP/hFVIII-Mt-I. The advantage of transgenic rabbit is that the rate of producing transgenic founder is higher than in larger livestock animals and the time required to reach sexual maturity and achieve pregnancy and lactation is much shorter than in sheep with still sufficiently high production levels.

MATERIALS AND METHODS

Cloning procedures

The hFVIII gene construct with and without the murine Mt-I gene has been described previously (HALTER et.al. 1993). We used the construct WAP/hFVIII-Mt-I for these experiments.

Generation of transgenic rabbits

The animals used in this study were sexually mature New Zealand White females which were superovulated by intramuscular administration of follicle stimulating hormone to enhance the yield of ova (20 I.U. PMSG/kg body weight), followed 3 days later by intravenous delivery of human chorionic gonadotropin (180 I.U HCG/ animal). The animals were mated to fertile males or artificially inseminated. The typical yield was 20-30 zygotes flushed from the oviducts 20 hours after the HCG injection. The efficiency of embryo production were increased by keeping the females for one week at long day length (18 hours of light) before embryo collection, following an extended period at short day length (8hours of light). The large male pronucleus of the rabbit zygote was microinjected with the solution of linearized DNA (2 ng/µl) in PBS+20% FCS media and then incubated at 38.5 °C for 2 hours. A total of twelve injected zygotes were implanted into each oviduct of pseudopregnant recipient females, which had been hormonally induced 24 hours before (120 I.U. HCG per recipient).

Transgene detection

DNA from ear biopsy or blood samples was prepared by extraction with phenol/chloroform and isopropanol precipitation (LAIRD et.al. 1991). For rapid screening of founder animals a Polymerase Chain Reaction (PCR) assay was performed using the primers

5’GCC TCT CAG AGT CAC CAC TTC CTC TGT TGT 3’

5’ AAG ACG CTG GGT TGG TCC GAT ACT ATT TAC 3’

which define a 1020 bp region of the human VIII cDNA. The reaction mix contains 10 mM Tris HCl pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 5% DMSO; each primer was used at a concentration of 0.1 mM and 1.0 unit of Taq polymerase per reaction. A total of 30 cycles of PCR were performed incubating at 95°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72 °C for 1min (extension).
Aliquots were analysed in ethidium-bromide containing 2% agarose gels.

RESULTS AND DISCUSSION

In total, 627 microinjected zygotes were transferred to 29 recipients and 14 females gave birth to 51 pups. Screening by PCR identified three founder animals carrying the transgene. Fig 1 shows the result of the mWAP/hFVIII-Mt-I gene transfer. The total efficiency of gene transfer (transgenic rabbits per embryos transferred) was 0.5% with an integration rate (transgenic rabbit per analysed offspring) of 5.9% (Table1)

Table 1: Relative efficiencies of producing transgenic rabbits.

Injected construct	mWAP/hFVIII-Mt-I
Number of donors per recipient	1.43
Eggs injected and transferred	627
Integration rate efficiency %	5.88
Transgenic per egg transferred %	0.5
Pregnancy rate (%)	48%

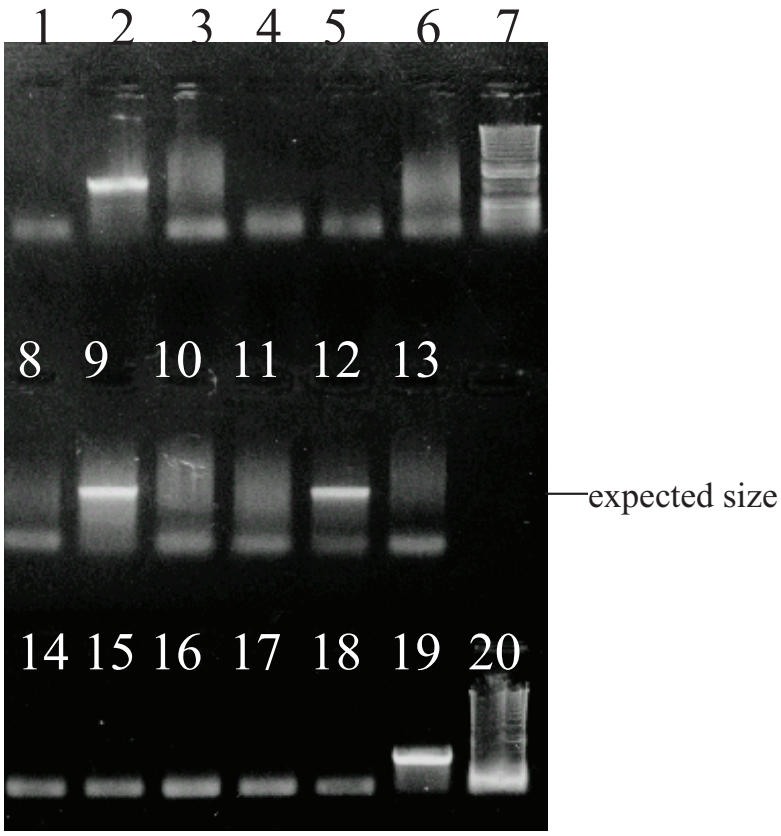

Fig1: PCR analysis of transgenic founder animals Lane1: 1 negative control rabbit DNA; lane 2:positive injected control lane 7 and 20 1 kb ladder (GIBCO), lane 9,12 19 represent the three founder animals (2;5;358) respectively.

Unfortunately due to unrelated health problems independent of the gene transfer experiment, propagation of the transgene could be performed with only one of the founders.
Stable transmission of the transgene was detected in DNA samples of two different tissues from F1 litters born after mating founder 358 male with NZW females (Fig 2.).

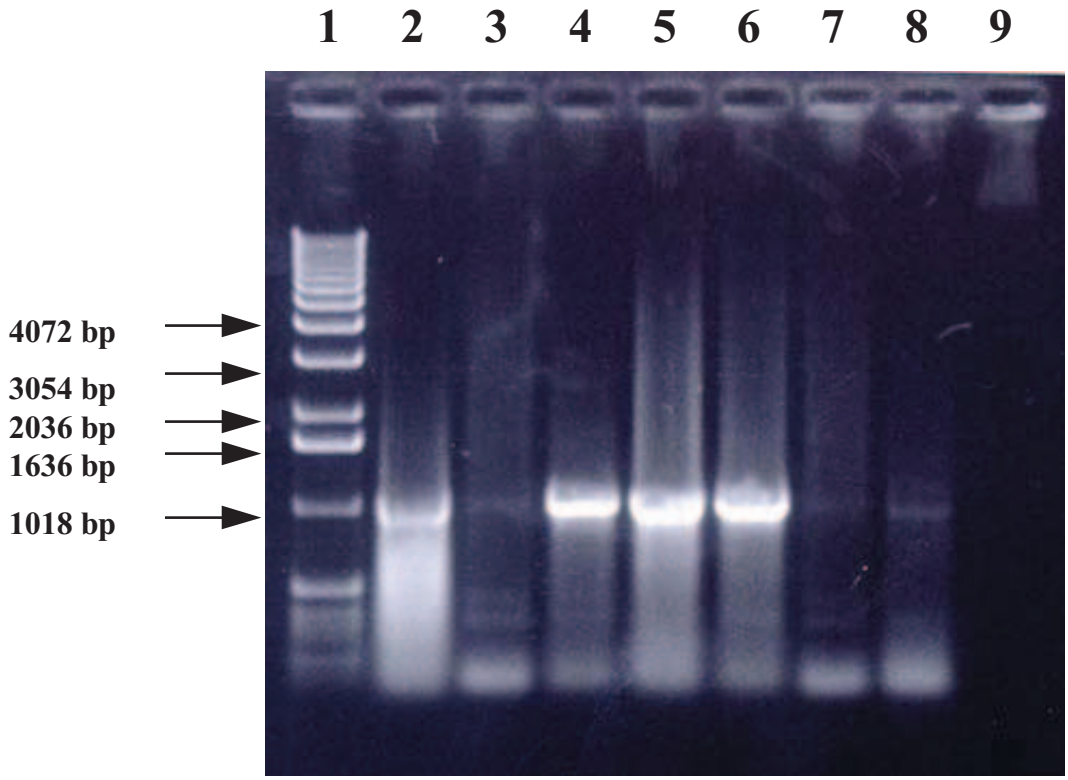

Fig 2. Lane 1: 1 kb ladder; lane 2: ear DNA founder 5; lane 3 ear DNA of litter 126; lane 4 blood DNA of litter 126; lane 5 ear DNA of litter 103; lane 6 blood DNA of litter 103; lane 7 ear DNA of founder 385; lane 8 blood DNA of founder 385; lane 9 ear DNA of control rabbit

None of the transgenic founder animals showed transgene related problems during their lifetimes, and no transgene related alterations could be detected in the F1 generation.
Expression analysis of the transgenic rabbit line 385 is in progress.

REFERENCES

EHMANN WC., EYSTER ME., ALEDORT L.M., GOEDERT JJ. 1995: Causes of death in haemophilia. *Nature* **378(6553):** 124

GITSCHIER J., WOOD WI., GORALKA TM., WION KL., CHEN EY., EATON DH., VEHAR GA., CAPON DJ., LAWN RM. 1992: Characterisation of the human factor VIII gene. 1984. *Biotechnology* **24:**288-92

HALTER R., CARNWATH JW., ESPANION G., HERRMANN D., LEMME E., NIEMANN H., PAUL D. 1993: Strategies to express factor VIII gene constructs in the ovine mammary gland. *Theriogenology* **39:** 137-149

LAIRD PW., ZIJDERVELD A., LINDERS K., RUDNICKI MA., JAENISCH R., BERNS, A.1991: Simplified mammalian DNA isolation procedure.*Nucleic Acids Res* **11;19(15):** 4293

NIEMANN H., HALTER R., CARNWATH JW., HERRMANN D., LEMME E., PAUL D. 1999: Expression of human blood clotting factor VIII in the mammary gland of transgenic sheep. *Transgenic Res* **8(3):**237-47

PALEYANDA RK., VELANDER WH., LEE TK., SCANDELLA DH., GWAZDAUSKAS FC., KNIGHT JW., HOYER LW., DROHAN WN., LUBON H. 1997: Transgenic pigs produce functional human factor VIII in milk. *Nat Biotechnol* **15(10):** 971-5

TOOLE JJ., KNOPF JL., WOZNEY JM., SULTZMAN LA., BUECKER JL., PITTMAN DD., KAUFMAN RJ., BROWN E., SHOEMAKER C., ORR EC. 1984: Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature* **312(5992):**342-7

TUDDENHAM EG., COOPER DN., GITSCHIER J., HIGUCHI M., HOYER LW., YOSHIOKA A., PEAKE IR., SCHWAAB R., OLEK K., KAZAZIAN HH. 1991: Haemophilia A: database of nucleotide substitutions, deletions, insertions and rearrangements of the factor VIII gene. *Nucleic Acids Res* **19(18):** 4821-33