Articles

Expression of Human Erythropoietin Gene in the Mammary Gland of a Transgenic Mouse

(erythropoietin / recombinant protein / mammary gland / transgenic mouse)

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Abstract. WAP is being recognized as the principal milk protein expressed in pregnant or lactating females of several mammalian species. Recently, it has been shown that the 6.3-kb 5' untranslated region of the *rWAP* gene is able to control, and almost completely restrict, the expression of the transgene into the mammary gland of the transgenic animal. We cloned the genomic fragment carrying the *rWAP* gene locus from the rabbit phage genomic library and used the 8.5-kb long 5' untranslated part of the *rWAP* gene to target the expression of *hEPO*, cloned from the human phage genomic library, into the mammary gland of the mouse. The vectors, carrying either the *hEPO* gene or the *rWAP-hEPO* hybrid gene, were injected into the mouse ova, and 12 transgenic animals were identified by PCR and Southern blot from the progeny of 168 tested littermates. Transgenic mice were viable, fertile and displayed a normal development. Recombinant human erythropoietin was produced in the milk of a transgenic mouse female at a secretion level of 5.3 mIU/ml, as detected by ELISA. Despite the low production of the transgenic glycoprotein in the milk we demonstrate that the hybrid gene can be expressed in the mammary gland of the host animal. Thus, *WAP*-based recombinant vectors, with additional optimizing modifications, can be useful for production of therapeutic proteins in the transgenic mammals.

Introduction of foreign genes into mammals opens the possibility to produce recombinant proteins in transgenic animals (Hogan et al., 1994). Production of a recombinant therapeutic protein in the mammary gland of transgenic dairy animals is currently being tested as an alternative to the plasma fractionation for the manufacture of a number of blood factors (Massoud et al., 1991; Sohn et al., 1999), growth factors (Devinoy et al., 1994) and recombinant antibodies (Pollock et al., 1999; van Kuik-Romeijn et al., 2000).

Currently, a major concern with transgenic recombinant glycoprotein production is to balance the correct glycosylation make-up, allowing full function of the product, with the appropriate glycosylation pattern avoiding a possible immunogenic effect in the human body. However, this problem can be partially overcome, or even minimized, by selection of the glycoprotein of interest with an understanding regarding the particular type of oligosacharide chains and by selection of a suitable mammalian species, or even a particular host strain, with a defined mammary gland glycosyltransferase expression profile. A significant attention has been paid in the past years to studies of mammalian glycosyltransferase functions *in vivo* using gene-deficient mouse models (Thall et al., 1995; Malý et al., 1996). Further studies using transgenic and gene-ablation techniques will accelerate the progress in this field.

Erythropoetin (EPO) is a glycoprotein hormone that is associated with the differentiation and proliferation of erythroid cells (Jelkmann, 1992) and seems to be a suitable candidate for its production in transgenic animals. It consists of 166 amino acids and its sequence is highly conserved in various species (Jacobs et al., 1985; Lin et al., 1985). During the foetal period, EPO is synthesized mainly in the liver and after the birth, in the kidney (Zanjani et al., 1974; Lacombe et al., 1988).

Among species so far used for the introduction of foreign genes into the mammary gland, rabbits represent a promising animal candidate because of their relatively short reproductive interval and quantity of the milk produced. In addition, they can be reared under specific pathogen-free conditions (Castro et al., 1999). On the other hand, significant differences exist in the glycosylation pattern among particular rabbit strains, typically in the content of sialylated carbohydrate linkages (Richard D. Cummings, P. Malý, personal communication). Therefore, a suitable strain needs to be found.

Received November 11, 2000. Accepted August 8, 2001.

This research was supported by grant PP-Z1/09/96 of the Ministry of Industry and Trade of the Czech Republic.

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Abbreviations: EPO – erythropoietin, *hEPO* – human erythropoietin gene, rWAP – rabbit whey acidic protein, WAP – whey acidic protein.

Whey acidic protein (WAP) is the principal milk protein in the mouse, rat (Hennighausen et al., 1982) and rabbit (Grabowski et al., 1991). The expression of *WAP* is confined to the mammary glands of pregnant and lactating but not virgin female mice (Piletz et al., 1981; Dandekar et al., 1982). *WAP* gene expression is induced by prolactin, inhibited by progesterone and strongly amplified by glucocorticoids (Devinoy et al., 1988; Grabowski et al., 1991; Li and Rosen, 1994). The *WAP* gene (Thepot et al., 1990) thus possesses hormonal and tissue-specific regulatory elements and is sufficient to target foreign gene expression, to a high degree, into the mammary gland of lactating transgenic animals (Devinoy et al., 1991). The *WAP* promoter contains a negative regulatory element (NRE) that represses expression of the coding region of the gene by interaction with a nuclear protein. This nuclear protein occurs in tissues where the *WAP* gene is not expressed. However, in the lactating mammary gland, where WAP is normally produced, it appears only poorly or not at all (Kolb et al., 1994; Kolb et al., 1995).

The expression of transgenes in mammary glands is also dependent on the interaction of regulatory elements located in the 5' flanking promoter sequence with intragenic sequences found in introns or 3' untranslated region. A deletion of conserved rat *WAP* 3' untranslated region led to the expression dependent on the site of integration. This suggests that elements within the gene body or 3' untranslated or flanking regions could be involved in high-level expression (Dale et al., 1992). The presence of enhancers in the introns and in flanking regions can result in a novel tissue specificity (Brem et al., 1991; Gunzburg et al., 1991) and can change the level of expression of the transgene when compared to the endogenous expression. Many cases show that the levels of gene expression obtained with cDNA-based constructs are often dramatically lower than those obtained when genomic sequences, including introns and exons, are used (Brinster et al., 1988). An addition of heterologous introns into the cDNA-based constructs can give sizable increases in expression levels without altering the tissue specificity of expression (Choi et al., 1991; Palmiter et al., 1991; Palmiter et al., 1993).

Different sizes of the promoter upstream sequences of the *WAP* gene have been used to direct the tissue-specific expression of several foreign proteins into the mammary gland of transgenic mice. In previous studies it has been shown that expression constructs containing short parts of the *WAP* promoter sequence, 949 bp upstream of the rat *WAP* gene (Bayna and Rosen, 1990) or 2.6 kb upstream of the mouse *WAP* gene (Burdon et al., 1991) can direct mammary gland-specific expression of the transgene when fused with the entire structural *WAP* gene. However, when the same DNA fragment containing the *WAP* upstream region was linked to heterologous structural genes, expression of foreign genes was generally weak (Andres et al., 1987; Gordon et al., 1987; Pittius et al.,1988; Tomasetto et al., 1989; Yu et al., 1989).

The promoter region located within 6.3 kb upstream of the *rWAP* gene is sufficient to control expression of a heterologous gene in the mammary gland of mice (Gordon et al., 1987; Pittius et al., 1988; Tomasetto et al., 1989; Devinoy et al., 1991; Gunzburg et al., 1991; Velander et al., 1992; Devinoy et al., 1994; Limonta et al., 1995a) and rabbits (Bischoff et al.1992; Limonta et al., 1995b; Rodriguez et al., 1995; Thepot et al., 1995; Massoud et al., 1996; Aguirre et al., 1998). In order to obtain hEPO from the milk of mice or rabbits (Rodriguez et al., 1995; Massoud et al., 1996; Aguirre et al., 1998), similar constructs, carrying the chimaeric gene comprising the 5' flanking promoter and 3' untranslated region of the *rWAP* gene linked to cDNA of the *hEPO* gene, have previously been used. Although principal hormonal regulatory elements were located 6.3 kb upstream of the *rWAP* gene (Devinoy et al., 1991), there were other regulatory elements located further upstream or within the gene and its 3'untranslated region (Bayna and Rosen, 1990; Burdon et al., 1991; Bischoff et al., 1992; Dale et al. 1992).

Here we report the generation of transgenic mice by pronuclear injections with chimaeric *rWAP/hEPO* hybrid DNA. The construct, carrying the 8.5-kb fragment of the 5' flanking region of the *rWAP* gene, controls the expression of human erythropoietin in the mammary gland of the host animal.

Material and Methods

Construction of chimaeric rWAP/hEPO gene

The genomic sequences corresponding to the *hEPO* gene and the *rWAP* promoter region were isolated as a separate phage λFIXII clone from the human genomic library (Stratagene, Amsterdam, The Netherlands) or λDASHII clone from the rabbit genomic library (Stratagene), respectively. Both genomic fragments were released from the phage sequences by restiction enzyme digestion and further subcloned into the pBluescript II SK (pBS) vector (Stratagene). The resulting plasmid phEBS-HB carrying the coding region of *hEPO* (Fig. 1A) and plasmid prW5'BS-KE carrying the upstream 5' flanking region of the *rWAP* gene were used for construction of the chimeric *hEPO/rWAP* gene.

In order to combine the *rWAP* promoter with the structural part of the *hEPO* gene, the PCR approach was used (Sambrook et al., 1989). The resulting plasmid was named prWhEBS-BNX. Sequencing of the PCR-generated *EPO* fragment revealed a possible point mutation compared to the sequence from Genebank, leading to the amino acid change in position 40 (Gln-Glu, 40Q-E). However, further detailed analysis of this sequence difference revelead that there is an allelic

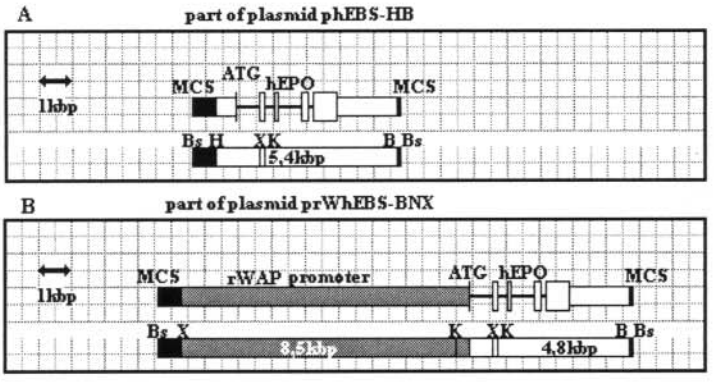

Fig. 1. Structure of the *hEPO* gene digested with *Bss*HII from plasmid phEBS-HB (A) and the *rWAP/hEPO* chimaeric gene derived from prWhEBS-BNX after digestion with *Bss*HII (B). The dark box represents the upstream fragment (8.5-kb) of the rabbit *WAP* gene. The white big boxes indicate *hEPO* exons and the thin line indicates introns of *hEPO*. The main restriction sites are represented as follows: B, *Bam*HI; Bs, *Bss*HII; H, *Hind*III; K, *Kpn*I; X, *Xba*I. Position of the translation initiation is indicated (ATG).

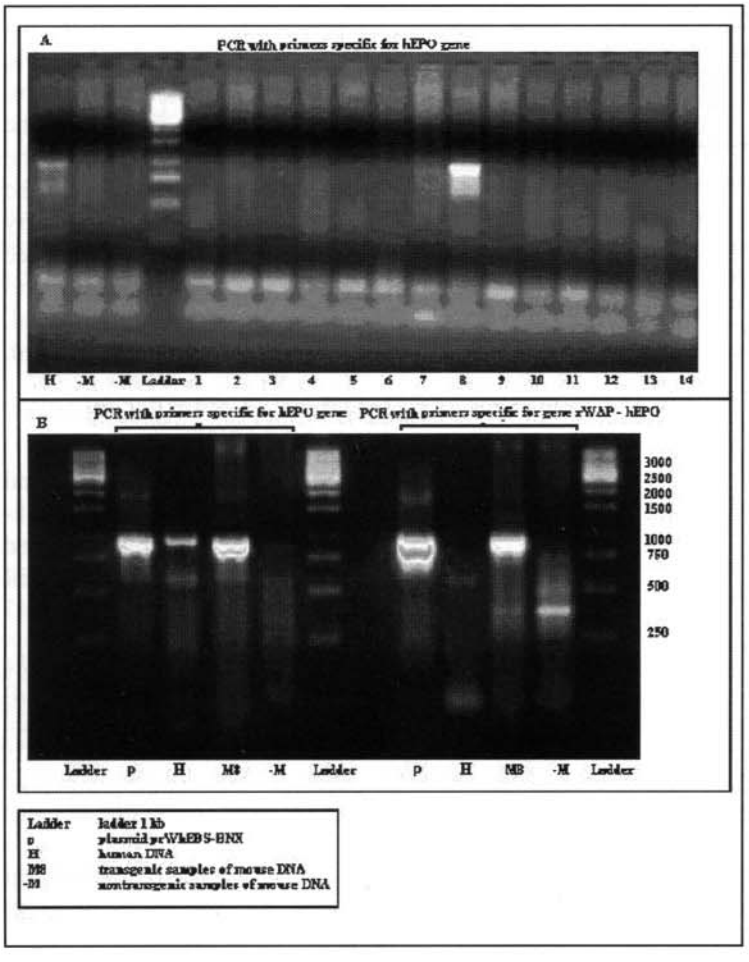

Fig. 2. Samples of the DNA isolated from the mouse tails in PCR reaction with primers specific for human *EPO* (Fig. 2A) and verifying of positive sample no. 8 with primers specific for the sequence of plasmid vector prWhEBS-BNX (Fig. 2B).

difference between Caucasians and Blacks in amino acid 40. In our case, this change matches the recently presented *EPO-EST* sequence cloned from a black woman (Jacobs et al., 1985). As we cloned our gene from the Stratagene genomic library constructed from the human material of a black woman, our point mutation seems to be the result of a polymorphic difference in the *EPO* allele rather then a PCR error. For the purpose of DNA microinjections, the *rWAP/hEPO* transgene (Fig. 1B) was released from the vector by digestion with the *Bss*HII restriction enzyme.

Generation of transgenic mice

As donors of ova, F1 hybrid mice (CBA/CaOla x C57B6) were used. Four–six weeks old females were superovulated with intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (Sergon, Bioveta, Czech Republic); followed 46 h latter i.p. injection of 5 IU of human chorionic gonadotropin (Praedin, Léčiva a.s., Czech Republic). Microinjection of DNA into the pronucleus of ova was done according to the procedure formerly published (Hogan et al., 1994). Two linear constructs, carrying the *hEPO* gene (digested from phEBS-HB with enzymes *Hind*III and *Bam*HI) or hybrid *rWAP-hEPO* gene (digested from prWhEBS-BNX with enzyme *Bss*HII) were microinjected into the male pronucleus. The released DNA inserts were separated on agarose gel and clean DNA was diluted in injection buffer. Enlargement of pronuclei during microinjection indicated a successful transfer of approximately 500 copies of DNA molecules into ova. Two-cell-stage embryos, developed from injected ova, were transferred into the oviducts of BALB/c/Ola pseudo-pregnant mouse recipients.

Screening of transgenic mice by PCR and Southern blot

Two sets of primers were used in PCR to identify positive transgenic mice. The first set of primers, specific for *hEPO* gene amplification, were called hE+1 (forward primer 5'ATG GGG GTG CAC GGT GAG TAC TCG CGG 3') and hE+998 (reverse primer 5'CAA GCT GCA GTG TTC AGC ACA GCC 3'). The second primer set specific for chimaeric *rWAP-hEPO* included rW-26 (forward primer 5'CCA CCA CCA GCC TAC CAG CGG CCG CCA) and hE+998 (the same as above). Positive DNA

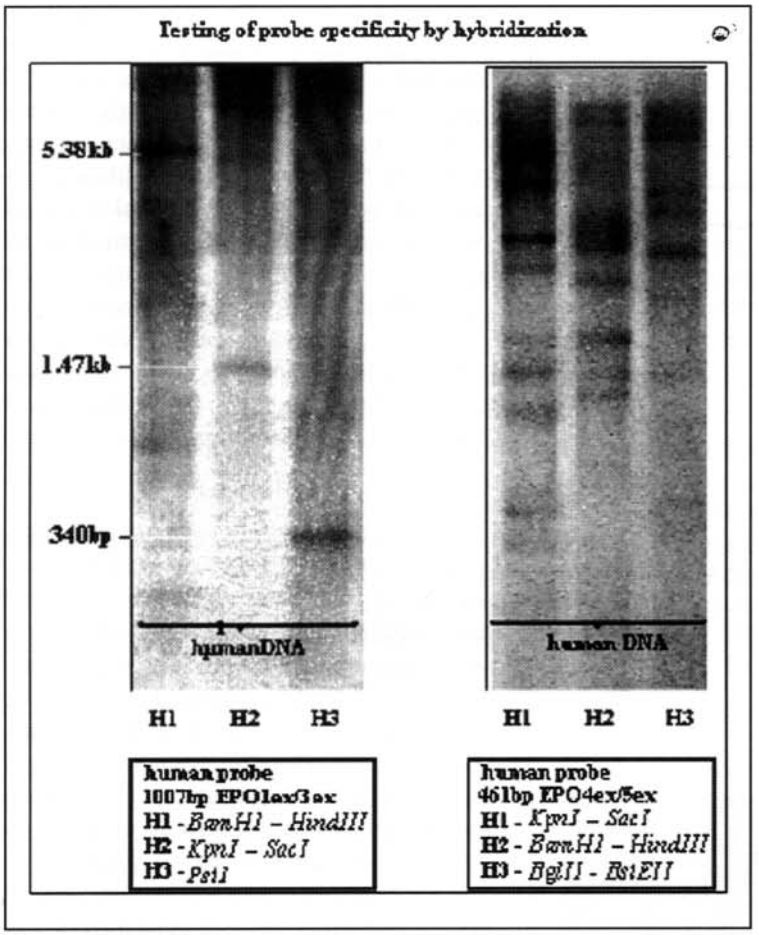

Fig. 3. In order to choose a suitable probe for identification of transgenic animals carrying the hybrid gene *rWAP/hEPO* we tested diferent parts of the *hEPO* gene in hybridization experiments for their specifity. The region encompassing the first two introns and second exon shows 55% homology with the mouse *EPO* gene and is able to distinguish the human gene for hEPO.

samples were digested with the *Sac*I restriction enzyme, fractionated on 0.9% agarose gels and transferred to nylon Hybon N+ membrane. The filters were hybridized at 42°C with radioactively labelled probes according to Sambrook et al. (1989). The filters were finally washed at 60°C for 20 min to the stringency of 0.2% SSC/0.5% (w/v) SDS and exposed to X-ray films for one week.

Milk collection and ELISA of hEPO

Milk samples from transgenic mice carrying the hybrid gene *rWAP/hEPO* or from control mice were obtained on the 14th day of lactation. Samples were isolated from the stomachs of sucking pups. Milk clots were weighed and resuspended in 4 volumes of sample buffer (100 mM NaCl, 10 mM Na2HPO4, 0.02% Tween 20, 0.1% lactose, pH 7.0). Fat and precipitate were removed by centrifugation (10 000 × *g*) for 20 min. Blood was diluted 10 times with the same buffer and centrifuged (10 000 × *g*) for 20 min. These samples (100 µl) were assayed for hEPO by commercial ELISA (R&D Systems Inc., Minneapolis, MN).

Results

Generation and identification of transgenic mice carrying the hEPO gene

A total of 1124 ova were microinjected with several hundred phEBS-BH fragments (Fig. 1A) and transferred into 146 suitable recipients. In sum, 133 mice were born. Introduction of the *hEPO* gene into the mouse genome was assayed in PCR with the primers specific for the *hEPO* gene. The presence of the foreign gene was verified by PCR in 121 mice and 11 transgenic mice were detected. These results correspond well with the results of Rodriguez et al. (1995), where a similar construct was injected.

Generation and identification of transgenic mice carrying the hybrid rWAP/hEPO gene

A total of 577 ova were microinjected with several hundred copies of the *rWAP/hEPO* chimaeric gene (12-kb DNA) released from prWhEBS-BNX by restriction enzyme digestion. A total of 445 surviving 2-cell embryos were transferred into 51 recipients. In sum, 47

Table 1. Determination of the hEPO activity in the milk and serum of a transgenic mouse female carrying the hybrid gene rWAP/hEPO and of a normal control mouse. The quantikine IVD Epo ELISA uses a monoclonal antibody and polyclonal antibody conjugate in sandwich ELISA. The minimum detectable dose is typically less than 0.6 mIU/ml.

Activity of hEPO in the milk and serum of a transgenic mouse carrying the hybrid gene *rWAP/hEPO*						
Technique of measurement	**Sandwich ELISA**					
	Levels of hEPO mIU.ml^{-1}				**Absorbance/450 nm**	
Detection limit	0.6 mIU.ml^{-1}					
Dilution factor of the sample	10x	1x	5x	1x	10x	5x
Sample	serum		milk		serum	milk
Transgenic mouse female	0.723	7.23*	1.068	5.34**	0.053	0.046
Normal control mouse female	0.0	–	0.0	–	0.037	0.029

*theoretical level of hEPO in the serum multiplied by dilution factor 10x
**theoretical level of hEPO in the milk multiplied by dilution factor 5x

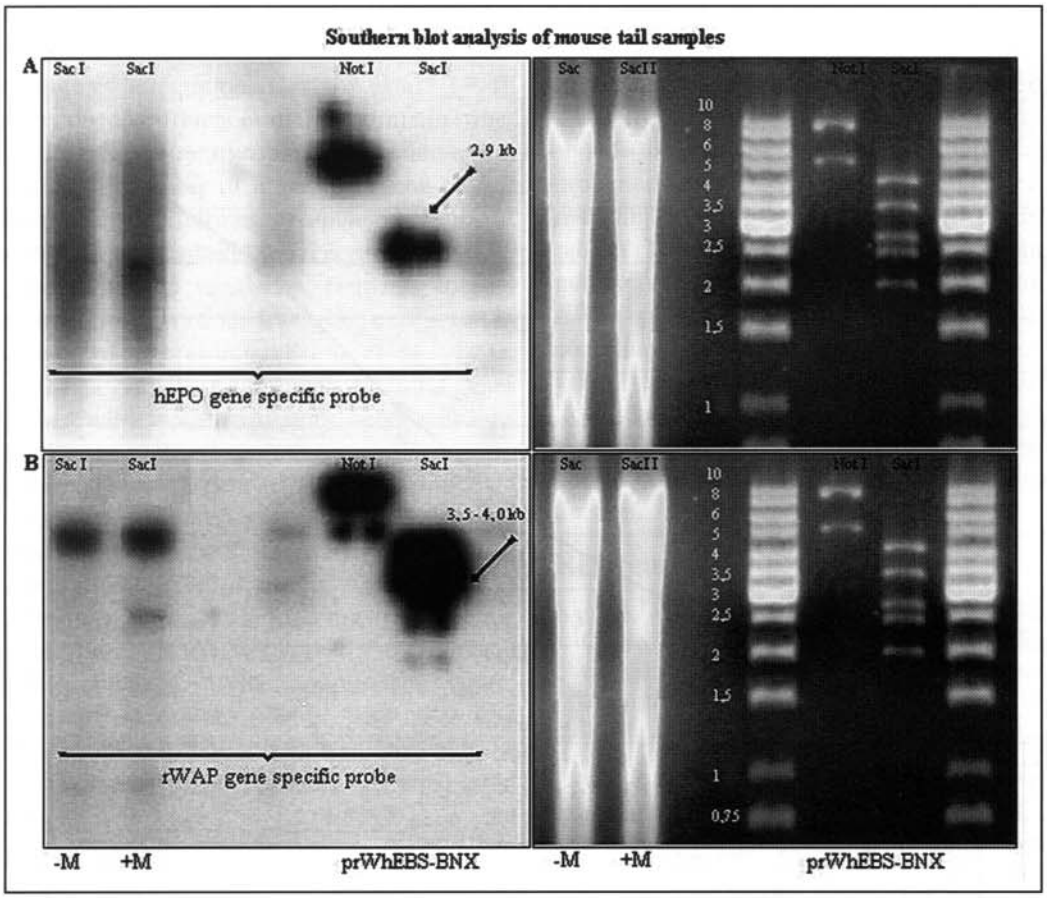

Fig. 4. Southern blot analysis of DNA from transgenic mice. DNA was isolated from tails of PCR-positive mice, digested with *Sac*I and subjected to Southern blot analysis with the fragment ATG-*Kpn*I of the *hEPO* gene (A) and promoter region of the *rWAP* gene (B), both as probes digested from vector prWhEBS-BNX. DNA from positive mouse no. 8 (lane M+) and DNA from non-transgenic mice were analysed. The last two lines correspond to DNA of vector prWhEBS-BNX digested with *Sac*I and *Not*I enzymes.

pups were born and the presence of the *rWAP/hEPO* foreign gene was detected in one female.

Introduction of linear hybrid *rWAP/hEPO* gene into the mouse genome was assayed by PCR with the primers specific for the *hEPO* gene (Fig. 2A) or with the primers specific for the chimaeric gene *rWAP/hEPO* (Fig. 2B).

In addition, the PCR-positive female was also tested by Southern hybridization using two different probes specific for the *hEPO* gene and the *rWAP* gene. First we tested two different fragments of the *hEPO* gene for specificity in hybridization experiments (Fig. 3). We found one specific part of the *hEPO* gene between the ATG start and *Kpn*I site. This fragment (hE1ex/3ex) is specific for the region placed between the first and the third exons. This part of the *hEPO* gene shows 50–60 % homology in the primary nucleotide structure with the mouse *EPO* gene.

The second probe suitable for identification of transgenic animals carrying the hybrid gene *rWAP/hEPO* is the fragment of the promoter of *rWAP* gene adjacent to an upstream-ATG regulatory sequence, called *Kpn*I-ATG probe.

Integration of the hybrid *rWAP/hEPO* gene into the genome of a transgenic mouse and transgenic rabbit was verified in other hybridization experiments (unpublished result) using a probe carrying the full-length coding region of the *hEPO* gene (Fig. 4).

Recombinant human erythropoietin is produced in the milk of transgenic animals

The actual concentration of hEPO samples was obtained from the calibration curve developed for the hEPO assay. Samples of the milk (diluted five times) and blood (diluted ten times) collected from a transgenic mouse carrying the hybrid gene *rWAP/hEPO* showed activities of 1.068 mIU/ml and 0.723 mIU/ml, respectively (Table 1).

We found differences in optical density of samples obtained from the transgenic or non-transgenic animal. It shows a weak activity of the hybrid transgene *rWAP/hEPO* in cells of the mammary gland (Fig. 5).

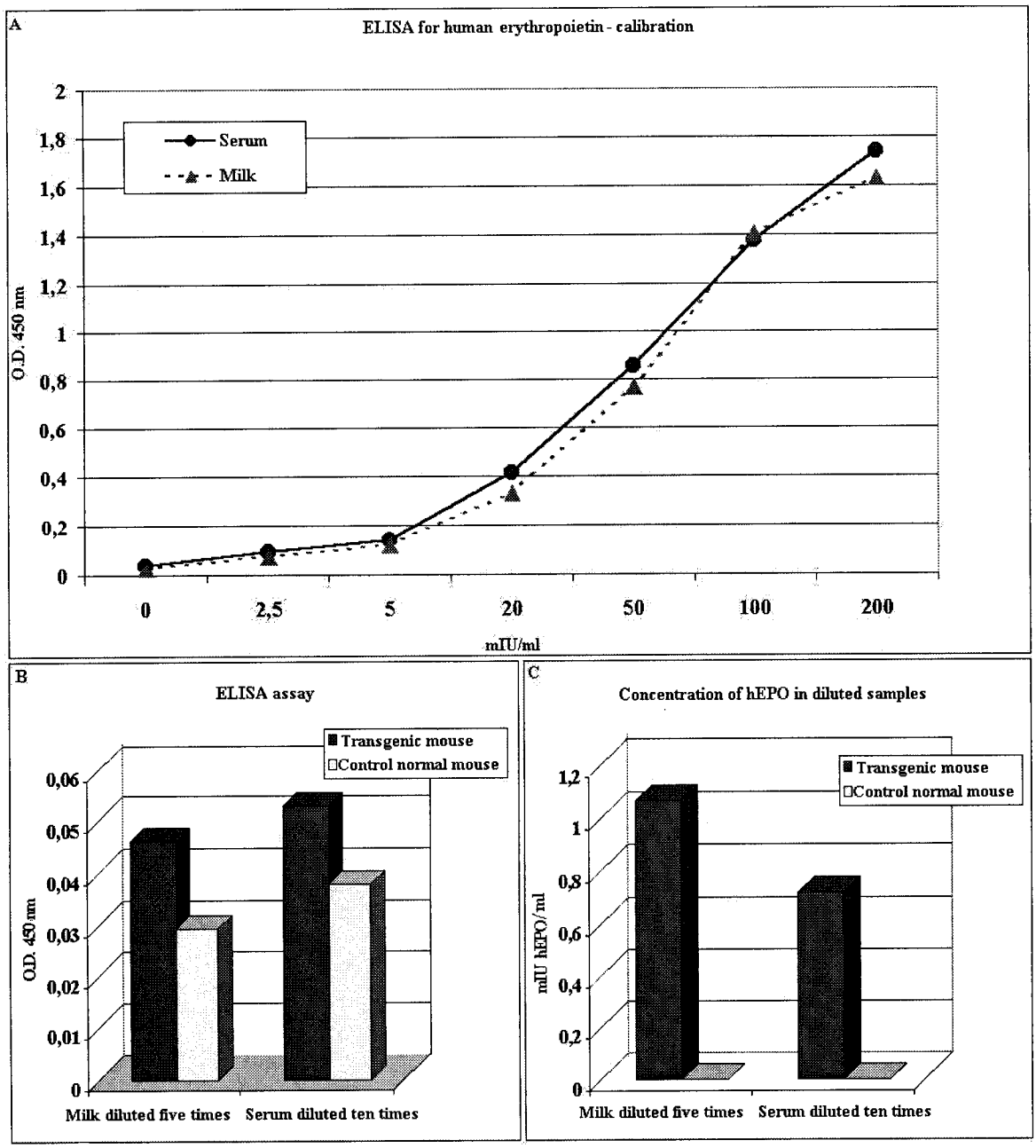

Fig. 5. Determination of hEPO concentration by ELISA testing in the milk and blood of a transgenic mouse carrying the hybrid gene *rWAP/hEPO*. A standard curve was obtained by adding hEPO into the mouse blood and milk. B: Signals of absorbance in diluted milk and blood samples of the transgenic mouse carrying the hybrid gene *rWAP/hEPO* and of a control normal mouse. C: Levels of hEPO in diluted samples of the milk and blood of the transgenic mouse carrying the hybrid gene *rWAP/hEPO*.

Discussion

Microinjection of foreign DNA into the pronuclei of mouse ova is the most common method for generation of transgenic animals. In our study, two constructs of different sizes, phEBS-HB, carrying the *hEPO* gene, and prWhEBS-BNX, carrying the *rWAP/hEPO* hybrid gene, were microinjected into the pronuclei of ova. At the beginning, we used vector phEBS-HB to optimize the techniques for generation of transgenic mice and also to test the specificity of primers and their capability to identify *hEPO* transgenic mice using PCR. Later, we used the chimaeric *rWAP/hEPO* gene construct to verify the ability of the *rWAP* 5' flanking region (8.5-kb) to direct the expression of *hEPO* into the mammary gland of the host animal. Under normal conditions, *WAP* gene expression is restricted to the mammary gland cells only. Although the upstream region of the *WAP* gene has been described to be a very efficient DNA regulatory element for targeted expression in the

mammary gland, it seems to be clear that the resulting level of transgenic expression remains to also be dependent on the site of integration (Devinoy et al., 1994). This is mainly due to differences in the chromatin structure surrounding the site of transgene integration and is also a result of the number of introduced gene copies. Mutual interactions of hormone responsive elements located within 5' untranslated sequences of the *WAP* gene together with the regulation elements located within the structural part of the transgene may result in a new variant of expression (Brem et al., 1991; Gunzburg et al., 1991). Thus, different structural genes fused together with *WAP* regulatory sequences can modify the tissue-specific expression pattern in comparison to the endogenous gene expression profile.

In previous works (Gordon et al., 1987; Pittius et al., 1988; Tomasetto et al., 1989; Devinoy et al., 1991a; Gunzburg et al., 1991; Bischoff et al., 1992; Velander et al., 1992; Devinoy et al., 1994; Limonta et al., 1995a; Limonta et al., 1995b; Rodriguez et al., 1995; Thepot et al., 1995; Massoud et al., 1996; Aguirre et al., 1998), the upstream region (6.3-kb) of the *rWAP* gene was found to be sufficient to target high transgene expression into the mammary gland. However, when the same DNA fragment was linked to the *hEPO* gene, the expression was currently low (Rodriguez et al., 1995; Massoud et al., 1996; Aguirre et al., 1998). Transgenes are known to be much better expressed when their structural part is a native gene, with their introns, rather than cDNA. However, the introns in the *hEPO* gene may contain silencers, which can reduce the expression in non-renal cells. In order to achieve specific and high expression of *hEPO* in the mammary gland of the mouse, we used the upstream promoter sequence (8.5-kb) of the *rWAP* gene, introduced into the prWhEBS-BNX construct, to direct expression of the *hEPO* gene.

Similar constructs, but with a shorter part of the *rWAP* promoter, have been recently used in mice and rabbits to express the *EPO* gene in the mammary gland (Rodriguez et al., 1995; Massoud et al., 1996; Aguirre et al., 1998). In our case, samples of the milk extract and blood serum of transgenic mice were assayed by commercial ELISA. The milk extract contained 5.3 mIU of the hEPO/ml, thus showing that the cloned *rWAP* promoter was able to direct synthesis of the *hEPO* gene into the mammary gland of the transgenic mouse.

The main goal of this study was to verify the function of the chimaeric gene *rWAP/hEPO in vivo* using the transgenic approach.

It is possible that increased expression of the *rWAP/hEPO* fusion gene, and recombinant hEPO production, will be achieved in transgenic rabbits, where rabbit-regulatory elements of the *WAP/hEPO* construct are believed to be more efficient to control and restrict expression of the transgene.

Our data also show that the expression of the *hEPO* transgene in a transgenic animal is not strictly limited to the mammary gland. We found ectopic expression of the transgenic *hEPO* product in the blood. This corresponds to previously obtained results where the *rWAP* promoter caused ectopic expression of the *hEPO* gene, resulting in a deleterious effect of *hEPO* in transgenic rabbits (Massoud et al., 1996). At this moment we cannot exclude the role of other genetic factors affecting *EPO* expression in the transgenic animal, including the combinatory effect of the integration site, *EPO* intragenic elements and the number of gene copies. An additional modification of the regulatory recombinant sequences can be performed to optimize the specificity and level of the desired therapeutic glycoprotein production.

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