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.

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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