Expression of active human erythropoietin in the mammary gland of lactating transgenic mice and rabbits

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Transgenic mice and rabbits were generated using a chimeric gene comprising the human erythropoietin (hEPO) cDNA under the 5' and 3' regulatory sequences of the rabbit whey acidic protein gene. Transgenic mice expressed hEPO at levels of 0.01 mg/l in the milk of lactating females showing that the genetic construct was functional. Reverse transcriptase polymerase chain reaction with RNA from various tissues showed that this transgene was expressed mainly in the ovary and mammary gland. In rabbits, we demonstrated the germ line transmission of the transgene. The hEPO was obtained in the milk of lactating females at levels of up to 0.0003 mg/l. Although the expression levels were low, biologically active hEPO was obtained in the milk of transgenic rabbits without any apparent detrimental effect for the animals. ***In vitro****, the specific activity of the rabbit-derived hEPO was higher than that reported for the natural hEPO, thus suggesting differences in the glycosylation pattern in at least part of the molecules secreted by the mammary gland of transgenic rabbits.*

Key terms: *erythropoietin, rabbit, transgenic, whey acidic protein.*

INTRODUCTION

One of the most valuable advances of the modern biotechnology is the introduction of foreign genes into mammalian embryos (Hogan *et al*, 1986). When the DNA construct integrates into the host genome, the resulting transgenic animals may demonstrate a variety of new phenotypes, through the expression of the exogenous DNA molecule which can be transmitted to future generations by normal breeding methods.

Many important mammalian biomedical proteins such as human erythropoietin (hEPO) have post-translational modifications that are essential for their function. These modifications are not performed by microorganisms, such as bacteria or yeast, and functional recombinant proteins can only be produced by expression in mammalian cells. Transgenic animals may provide an alternative mean for the production of recombinant proteins (Clark *et al*, 1987; Van Brunt, 1988; Whitelaw & Clark, 1989). The approach generally taken has been to target expression to the mammary gland and to produce the desired protein in the milk (Gordon *et al*, 1987; Simons *et al*, 1987; Clark *et al*, 1989; Bayna and Rosen, 1990; Buhler *et al*, 1990; Riego *et al*, 1993; de la Fuente *et al*, 1994).

The whey acidic protein (WAP) gene encodes the major milk whey protein in mice, rats and rabbits. WAP regulatory sequences have been shown to direct the

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synthesis of heterologous proteins in the mammary gland of lactating mice (Gordon *et al*, 1987; Pittius *et al*, 1988), rabbits (de la Fuente *et al*, 1994) and pigs (Drohan *et al*, 1991). Proteins produced by this route are biologically active suggesting that the mammary gland is capable of carrying out the appropriate post-translational modifications (Yu *et al*, 1989; Meade *et al*, 1990; Wright *et al*, 1991; Velander *et al*, 1992; Riego *et al*, 1993).

The interest in hEPO has been documented since the beginning of this century. EPO is the principal hormone involved in the regulation and maintenance of a physiological level of circulating erythrocyte mass by the stimulation of progenitor cells to differentiate into mature erythrocytes (Goldwasser, 1975; Graber and Krantz, 1978). The hormone is produced primarily under conditions of hypoxia by the kidney in the adult and by the liver during the fetal life (Jacobson *et al*, 1957).

The progressive destruction of kidney mass, such as in chronic renal failure, results in an anemia due to a decreased production of EPO (Erslev *et al*, 1980). Thus, a therapeutic role for hEPO is clear in the treatment of anemia associated with renal failure. Relatively low serum levels of the hEPO may also be associated with malignancy (Mitler *et al*, 1990), acquired immunodeficiency syndrome (AIDS) (Spivak *et al*, 1989), long-standing rheumatoid arthritis (Baer *et al*, 1987) and the anemia seen in premature infants (Brown *et al*, 1984). These patients usually respond to exogenous hEPO administration.

The hEPO was first purified in a small amount from urine of aplastic anemia patients (Miyake *et al*, 1977). This hormone is heavily glycosylated and important roles of the carbohydrate moiety in the solubility, biosynthesis and biological activity have been reported. Desialylation of hEPO causes a complete loss of its hormonal activity *in vivo* but increases its activity *in vitro* through a more easy binding to the cell receptor (Goldwasser *et al*, 1974; Dordal *et al*, 1985; Goto *et al*, 1988; Dube *et al*, 1988).

A cDNA and a gene coding for hEPO have been cloned and expressed in several animal cell lines (Jacobs *et al*, 1985; Lin *et al*, 1985; Powell *et al*, 1986; Sasaki *et al*, 1987; Davis *et al*, 1987; Tsuda *et al*, 1988; Yanagi *et al*, 1989). It is well established that terminal glycosylation sequences differ among different tissues and from cell types within each tissue. Such cell type-specific variation in structure may prove to be an important consideration in the choice of eukaryotic cell lines used to produce recombinant glycoproteins.

At present, the commercially available recombinant hEPO for administration to humans is produced in CHO cells. In this paper, we present evidence showing that the mammary gland of transgenic rabbits can secrete complex active human proteins and may be another option to produce hEPO. We report on the generation of transgenic mice and rabbits with a chimeric gene comprising the hEPO cDNA under the 5' and 3' regulatory sequences of the rabbit WAP (rWAP) gene and the germ line transmission of the transgene in transgenic rabbits. Transgenic mice expressed the hEPO at levels of 0.01 mg/l of milk proving that our genetic construction was functional. In rabbits, biologically active hEPO was obtained in the milk of lactating females at levels of up to 0.0003 mg/l. Although the expression levels were low, biologically active hEPO was obtained in the milk of transgenic rabbits without any apparent detrimental effect for the animals. *In vitro*, the specific activity of the rabbit-derived hEPO was higher than that reported for the natural hEPO, thus suggesting differences in the glycosylation pattern, in at least, part of the molecules secreted by the mammary gland of transgenic rabbits.

METHODS

Cloning of the hEPO cDNA and expression in CHO cells

The hEPO cDNA was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR; Rappolee *et al*, 1988) of total RNA extracted from a 20-week-old human fetal liver (Jacobs *et al*, 1985). To clone the PCR product, the 5' and 3'amplification primers included EcoRI and HindIII restriction sites,

respectively. For sequence analysis (Sanger *et al*, 1977), the amplified fragment was cloned into pM13mp18 and pM13mp19 generating the plasmids pmp18EPO and pmp19EPO, respectively. The functionality of the hEPO cDNA obtained was assayed by transient expression in CHO cells. The hEPO cDNA was extracted from pmp19EPO by digestion with HindIII and EcoRI and the resulting fragment containing the hEPO cDNA was cloned into the expression vector pAd30 (Lleonart *et al*, 1991). CHO cells were maintained in DMEM and transfected with the resulting plasmid (pAdEPOcDNA) by the standard protocol of DEAE- Dextran (Maniatis *et al*, 1989). Twenty four hours after transfection, the cells were cultured in media without serum for 24 h. Human EPO levels were analyzed in the media using a commercial sandwich-type enzyme-linked immunoassay (ELISA; Boehringer Mannheim, Germany) as recommended by the manufacturer.

Cloning and modification of the chromosomal rWAP gene

A rabbit genomic library was constructed in EMBL3 (Hernández *et al*, 1989) and screened with an oligonucleotide (5'-AGTTGAGGCCTCG-CCAAC-3') homologous to the -84 to -67 sequence of the rWAP promoter (Thepot *et al*, 1990). A clone containing a fragment comprising the rWAP gene was digested with BamH I and the resulting fragment (≈ 12.3 Kb) was cloned into the plasmid polyIII-I (Lathe *et al*, 1987) to generate the plasmid pWAPc (Fig 1). To direct the expression of heterologous genes employing the rWAP regulatory

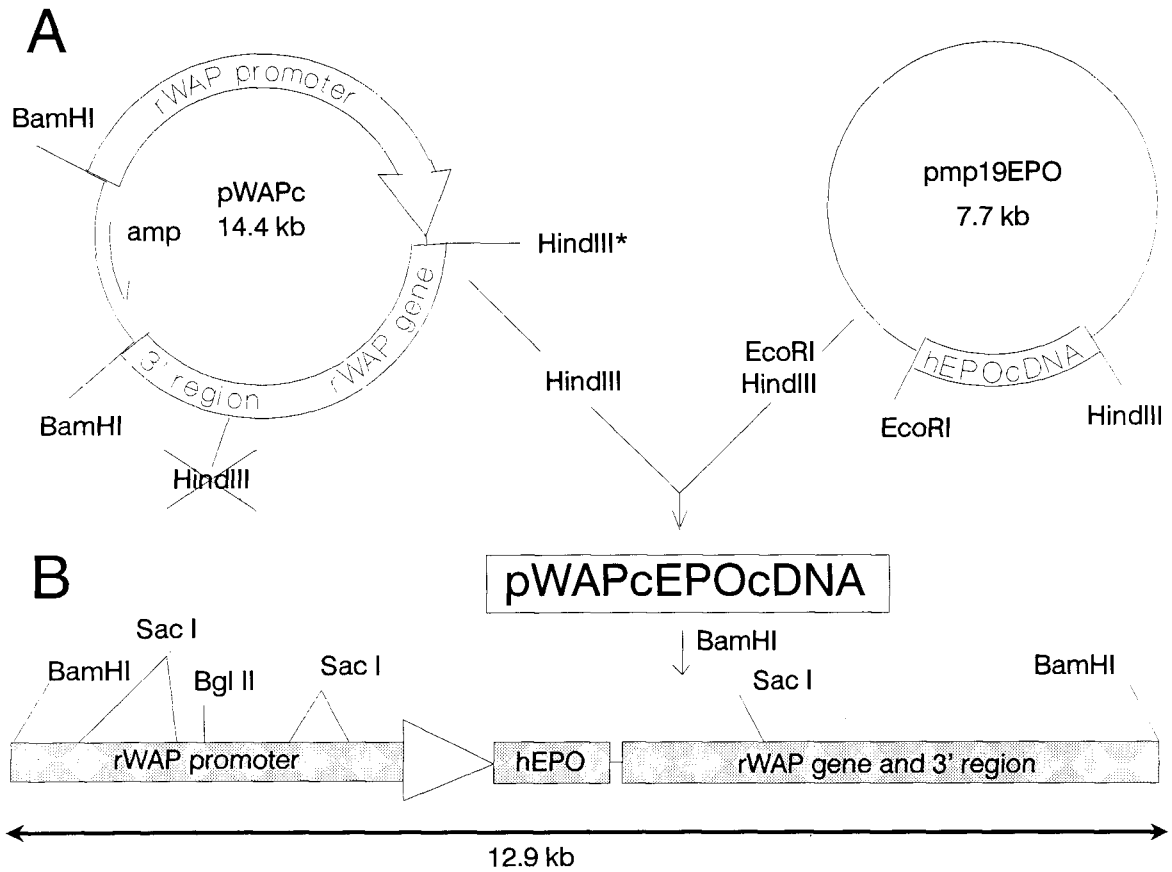

Fig 1. Construction of the chimeric gene. **A**. The rWAP gene was cloned into the BamHI site of the plasmid p-polyIII-I and was modified by eliminating a HindIII site in the 3' non-coding region and creating a new HindIII site (cloning site:*) in the 5' untranslated region, to generate the plasmid pWAPc. The hEPO cDNA was extracted from pmp19EPO by digestion with HindIII and EcoRI restriction enzymes and after a treatment with the Klenow fragment of the DNA polymerase I, it was cloned into the blunt-ended-HindIII site of pWAPc resulting in the plasmid pWAPcEPOcDNA. **B**. The rWAP-EPO chimeric gene was obtained by digestion of pWAPcEPOcDNA with BamHI and was used to generate transgenic animals.

sequences, the cloned rWAP fragment was modified as previously reported (Houdebine *et al*, 1992) by the elimination of a Hind III site in the 3' non-coding region and creating a new Hind III site (cloning site) in the 5' untranslated region (Fig 1).

Construction of the rWAP-hEPO chimeric gene

The rWAP-hEPO chimeric gene was constructed by subcloning the hEPO cDNA extracted from pmp19EPO into pWAPc, resulting in the plasmid pWAPcEPOcDNA (Fig 1). The rWAP-hEPO transgene was obtained by digestion of pWAPcEPOcDNA with BamH I (Fig 1) resulting in a fragment of ≈ 12.9 Kb which was used to generate the transgenic animals.

Generation of transgenic mice and rabbits

Mice and rabbits were purchased from CENPALAB (Havana, Cuba) and were kept in conventional facilities at 20 to 22°C and 60 to 65% humidity under 14 hour-light: 10 hour-dark photoperiod. Commercial feed (CENPALAB, Havana, Cuba) and tap water were available at libitum. B6D2F1 female mice and F1 albino rabbits (New Zealand White x Semigigante Blanco) were used as donor of embryos. All the manipulations of both mice and rabbit embryos were conducted in the Hepes-buffered M-2 medium. Transgenic mice and rabbits were generated using standard techniques (Hogan *et al*, 1986; Castro and Aguilar, 1989; Riego *et al*, 1993).

Detection of transgenic animals

Genomic DNA was extracted with phenol-chloroform from muscle samples (Hammer *et al*, 1985).The preliminary screening of F0 and F1 animals was performed by DNA dot blot analysis using 10 µg of DNA (Kafatos *et al*, 1972). Detailed studies of transgene integration and estimation of transgene copy number were performed by Southern blot analysis (Southern, 1975) using 10 µg of digested DNA.

The hEPOcDNA was employed as a probe and was labeled with random primers (Feinberg and Vogetstein, 1984) and [^{32}P]-αdATP (3000 Ci/mmol; Amersham, UK). Blots were performed on nylon membranes (Hybond N, Amershan, UK). Prehybridization and hybridization were carried out at 42°C in solutions containing 50% formamide, sodium citrate buffer (0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.2% SDS, 5X Denhardt and 200 mg/ml of heat-denatured yeast tRNA. Radioactive labeled DNA probe (108 cpm/µg) was added for hybridization. Membranes were washed twice with 1X SSC and 0.1% SDS at room temperature and at 42°C for 15 minutes each, and twice with 0.2X SSC and 0.1% SDS at 42°C and 65°C for 15 minutes each. Membranes were finally exposed to X-ray films with amplifying screens at -70°C for a week.

Analysis of transgene expression

The tissue-specificity of hEPO expression in transgenic mice was analyzed by RT-PCR (Rappolee *et al*, 1988). RNA from mammary gland, ovary, liver, spleen, kidney, heart and brain was prepared employing the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynsky and Sacchi, 1987). Each of these RNAs was used for the first strand synthesis of cDNA and amplification by PCR using 5' (5'-GTGCACGAATGTCCTGC-3') and 3' (5'-CTGTGTACAGCTTCAGC-3') primers corresponding to the hEPO coding region (Lin *et al*, 1985). PCR products were transferred to a nylon membrane and hybridized under previously described conditions.

Assay of hEPO in the milk

Milk from transgenic and control mice was collected on different days of lactation starting on the 3rd day. Females were separated from their pups for at least 3 hours and were injected intraperitoneally with 0.25 IU of oxytocin (Spofa, Praha, Czechoslovakia). One hour later, they were anesthetized and milk was collected into capillary tubes manually by gentle hand massage of the teats and subsequently expelled into Eppendorf tubes.

Transgenic and nontransgenic lactating rabbits were milked by hand after stimulation

with 3 IU of oxytocin. Milk was collected directly into Eppendorf tubes during the first twenty days of lactation starting on the 3rd day.

Fractionation of the milk from mice and rabbits was performed by a cold (4°C) centrifugation (10 000 x g) for 30 min to separate fat from whey and pellet fractions. Samples of defated milk fractions (50 µl) were assay for EPO by a commercial ELISA (Boehringer Mannheim, Germany) as recommended by the manufacturer. As controls, we used kit's positive controls diluted with defated milk fractions obtained by the same procedure from non related lactating rabbit females.

In vitro EPO activity assay

The defated milk fractions from transgenic and non-transgenic lactating rabbits were acidified to pH 4.8 with acetic acid to precipitate caseins and neutralized with NaOH (Riego *et al*, 1993). Samples of whey fractions (50 µl) were assayed *in vitro* for biological activity of hEPO by a microassay based on ^{3}H-thymidine incorporation into spleen cells from phenylhydrazine treated mice (Krystal,1983). Two to three month-old (C57BL/6J xCBA/CA)F1 hybrid mice (CENPALAB Havana, Cuba) weighing 25-35 g were made anemic by 2 consecutive daily intra-peritoneal injections of 60 mg/kg phenylhydrazine hydrochloride (Hara and Ogawa, 1976) prepared as a 6 mg/ml solution in 130 mM NaCl, 7.5 mM $MgCl_2$ and 5 mM KCl. Spleens were removed routinely 3 days after the second injection and homogenized through a 200 gauge mesh into alpha minimum essential medium without ribonucleosides and deoxyribonucleosides (α-MEM*) and 10% of dialyzed fetal bovine serum (FBS). Clumps were disrupted by gentle aspiration several times through a 21 gauge needle. Cells were counted and diluted to $8x10^6$ cells/ml. This mixture was aliquoted (0.05 ml/well) into U-shaped microtiter plate wells (Nunclon®, Denmark).

Samples (hEPO or test samples) were added to give final volumes of 0.1 ml/well. As controls, we used different concentrations (0; 10; 20; 30; 50; 100; 200; 400 mU/ml) of a commercial hEPO (EPOETIN ALFA EPOGENR, 4000 U/ml, Amgen, USA) using as diluent α-MEM* or milk whey fractions from non-related lactating rabbits. Cultures were incubated for 22 h at 37°C in a humidified atmosphere of 5% CO_2 + 95% air. To each well were then added 20 µl of a [methyl 1'2'-^{3}H] thymidine (1 µCi/ml in 2% ethanol, 124 Ci/mmol; Amersham, UK) stock containing 50 µCi/ml in α-MEM* to give a final concentration of 1 µCi/well. The assay plates were returned to the 37°C incubator for additional 2 hours and then the contents were harvested onto glass fiber filters using a microharvester (Nunc Cell Harvester 8, Denmark). For harvesting, the wells were rinsed with distilled water and the filters retaining ^{3}H-Thymidine labeled DNA, were placed in 2 ml of non- aqueous scintillation fluid (12 g PPO and 0.3 g POPOP in 3000 ml of toluene). Radioactivity in each scintillation vial was determined using a liquid scintillation counter (1214 Rackbeta, WALLAC, LKB; Sweden).

RESULTS AND DISCUSSION

Transient expression of hEPO cDNA in CHO cells

The cloned hEPO cDNA was assayed in CHO cells which were transiently transfected with an expression vector containing the hEPO cDNA driven by the adenovirus major late promoter (pAdEPOcDNA). Samples (50 µl) of culture media were assayed 48 hours after transfection by a commercial ELISA and the media of transfected cells contained more than 1 µg of hEPO per liter, thus showing that the cloned hEPO cDNA was able to direct the synthesis of hEPO.

Characterization of transgenic mice expressing hEPO in the milk

A total of 500 one cell embryos were microinjected with several hundred copies of the transgene. The survival rate was 50.8%. Twenty four females were transferred and 49 pups were obtained. Five of them were transgenic by dot blot analysis (three females and two males), to yield an efficiency of 10.2% with respect to the newborn pups and

1% regarding the total microinjected embryos. These frequency rates are similar to our previous reports (Castro and Aguilar, 1989; Hernández *et al*, 1990; Riego *et al*, 1993) and to the reports of others (Brinster *et al*, 1985; Hogan *et al*, 1986).

All transgenic female mice were mated to non-transgenic males to obtain milk samples for hEPO expression assay. Two of them gave offspring and milk samples were obtained. Defated milk samples (50 µl) were tested in the ELISA. Both animals had detectable levels of hEPO in their milk on the 8th day of lactation and F0-28 had hEPO on the 14th day too. The hEPO concentrations of the samples were calculated based on the standard curve of kit's positive controls in milk and were found to be around 1-10 ng/ml (Fig 2).

To address the tissue-specificity of the transgene transcription, RNA from mammary gland, ovary, spleen, heart, liver, kidney and brain from the F0-24 female were extracted at day 8 of lactation and transcripts for hEPO were found only in the mammary gland and ovary samples (Fig 3). These results agreed with those obtained in our laboratory for transgenic mice expressing human tissue-type plasminogen activator (htPA) under the control of the bovine αS1 casein promoter (de la Fuente *et al*, 1994) and for transgenic rabbits expressing hGH under the control of the mouse WAP promoter (de la Fuente *et al*, 1994). Previous reports have also shown transcription in salivary gland, tongue and sublingual gland of transgenes driven by mammary gland gene promoters in transgenic mice (Pittius *et al*, 1988). Our results might be explained by the fact that ovaries and mammary glands have similar developmental patterns regarding requirements of interactions between epithelial and mesenchymal tissues for proper duct formation to occur (Wall *et al*, 1991).

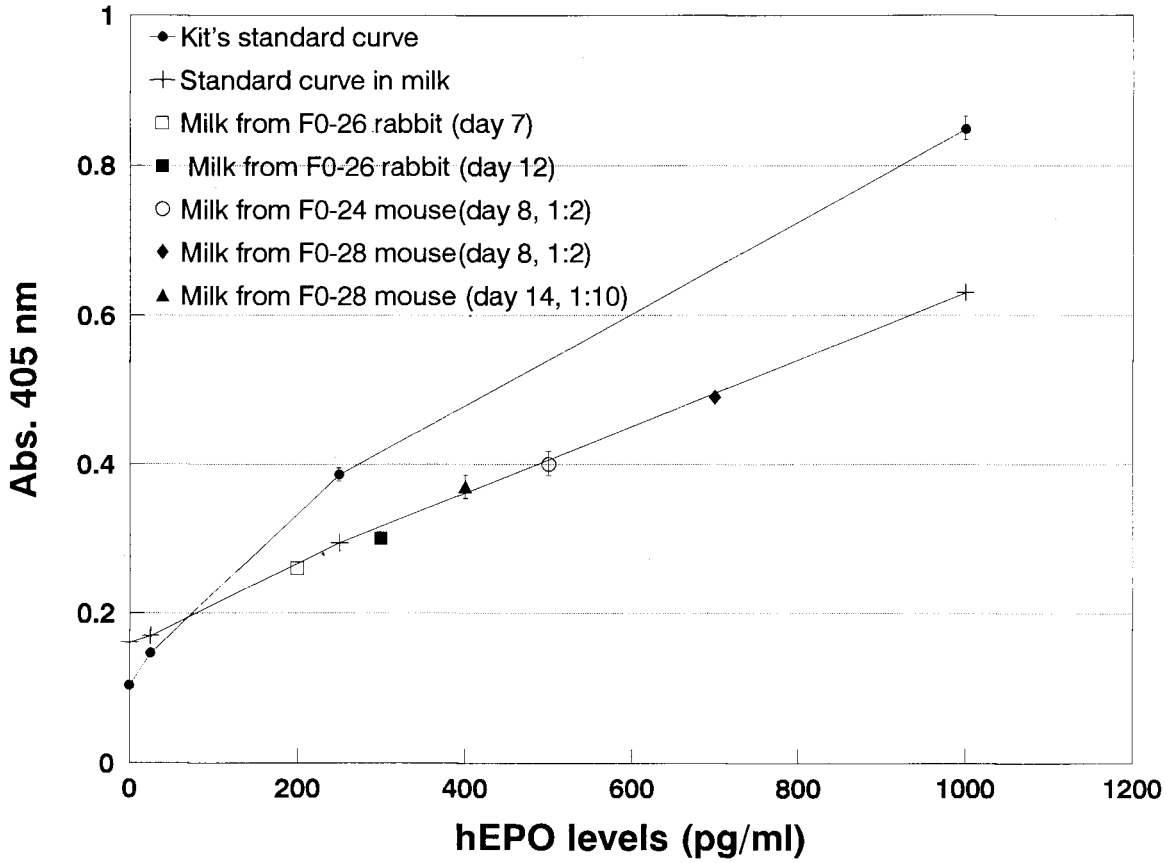

Fig 2. Determination of hEPO concentrations in the milk of transgenic mice and rabbits by a commercial ELISA (Boehringer Mannheim, Germany). Standard curve values from three independent determinations (mean ± SE) are shown. Sample values correspond to two independent determinations (mean ± SE).

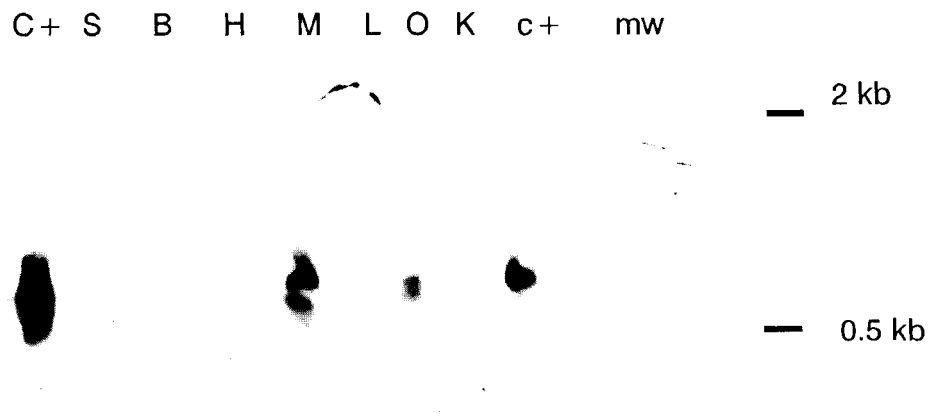

Fig 3. Southern blot of RT-PCR analysis of total RNAs extracted from the following organs of transgenic mice F0-24: S, spleen; B, brain; H, heart; M, mammary gland; L, liver; O, ovary; K, kidney. Oligonucleotides amplifying the hEPO cDNA were employed in the PCR. The hEPO cDNA was employed as a hybridization probe. Controls were included in the experiment (C+, RT-PCR of RNA extracted from the hEPO-producing CHO cell line; c+, PCR of DNA from plasmid pmp18EPO). As molecular weight marker (mw), HindIII-digested lambda DNA was used.

Despite the low level of expression of the hEPO cDNA in mice, we demonstrated that our transgene was active and was expressed in the appropriate tissue.

It has been shown that genetic background may dramatically influence transgene expression levels (Carver *et al*, 1993; de la Fuente *et al*, 1994). This is a question to keep in mind when using the mouse transgenic system as a predictive model regarding the genetic diversity of domestic livestock. Interestingly, cases of high expression levels of heterologous human proteins in the lactating mammary gland of livestock following poor expression or variation of expression level among different lines in mice have been reported (Ebert *et al*, 1991; Velander *et al*, 1992; Carver *et al*, 1993).

The mouse, although the most convenient, is not a totally suitable model for the evaluation of gene expression of foreign proteins in milk. However, it is certainly better than cell culture because there are no cell lines reported which support the expression of mammary gland specific promoters. Levels of expression observed in the milk of F0 founder female mice should be considered a baseline estimate of the potential expression of a given construct which will probably be surpassed in farm animals.

Integration and characterization of the transgene in rabbits

To produce transgenic rabbits, 795 one-cell embryos were microinjected and 611 of them were transferred to 44 females. Thirteen resulted pregnant and 43 pups were obtained. The presence of the foreign gene was found in 7 animals by dot blot. Two of them died and the remaining 5 animals (F0-4, F0-5, F0-26, females; and F0-15, F0-16, males) were used for further characterization. The efficiency in the generation of transgenic rabbits was 0.9% regarding to microinjected embryos and 16% with respect to newborn pups. These frequency rates were similar to that previously obtained in our laboratory (Limonta *et al*, 1992; Riego *et al*, 1993) and in other studies (Hammer *et al*, 1985; Buhler *et al*, 1990; Brem *et al*, 1985).

The integration of the transgene in founder F0 rabbits was detected by Southern blot hybridization of Bgl II (Fig 1B-4A) and Sac I (Fig 1B-4B) digested DNA. The copy number of the transgene per genome was estimated by comparing the intensity of the hybridization signal with the intensity of loaded controls. Most of our founder animals detected by dot blot were mosaic because they had less than one copy of the transgene per cell. This result agreed with previous

reports showing that the mosaicism seems to occur in transgenic embryos at a relative high frequency (Castro and Aguilar, 1992; Whitelaw *et al*, 1993). We used the F0-26 female to assay the expression of hEPO in the milk because this animal had two copies of the transgene per genome as determined by Southern blot analysis (Fig 4).

In the hybridization analysis, we used the hEPO cDNA as a probe, thus expecting in the Southern blot analysis the recognition of a fragment produced by the internal cleavage of the rWAP Bgl II site and a cleavage outside the transgene (Fig 1). The signal obtained in the analysis of F0-26 DNA was at least 2 Kb smaller than the expected size. This indicated that the transgene suffered a deletion during the integration/replication process. To characterize the site of this deletion, we digested the F0-26 genomic DNA with the Sac I restriction enzyme, which cleaves twice within the transgene (Fig 1). A hybridizing band with the expected size, 4 Kb, was obtained (Fig 4B). This fact indicated that the deletion occurred in the 3' end of the transgene.

The female founder rabbit F0-26 was mated to a non-transgenic male to establish a transgenic F1 line. Offsprings were obtained and the transmission of the transgene was detected in 6 out of 11 pups tested by dot blot analysis of muscle DNA samples suggesting a Mendelian inheritance. The integration patterns were studied in F0-26 transgenic descendants by Southern blot analysis of DNA digested with Bgl II and

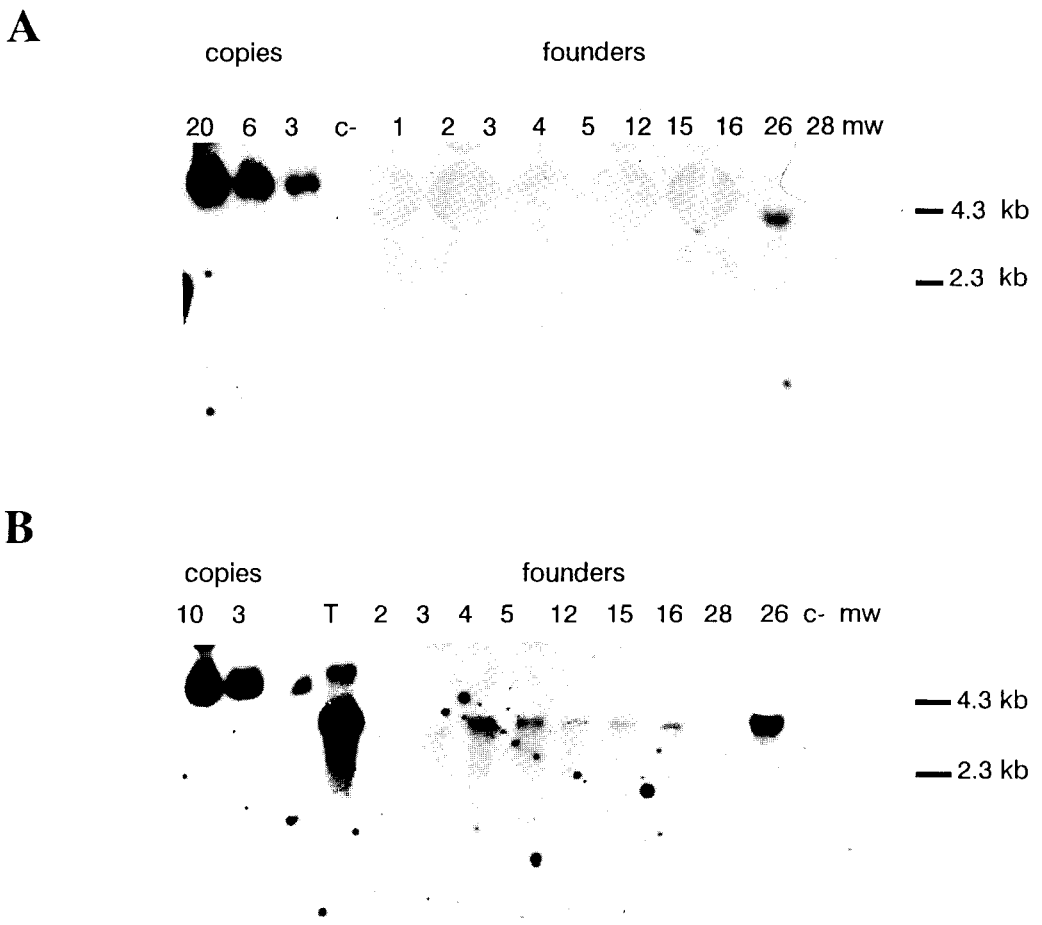

Fig 4. Southern blot analysis of muscle DNA samples obtained from founder rabbits. Ten µg DNA were digested with BglII (**A**) or SacI (**B**) and subjected to analysis employing the hEPO cDNA as a hybridization probe. Seventy eight, 156 and 520 pg (equivalent to 3, 6 and 20 copies/cell) of pmp18EPO DNA were loaded to estimate the transgene copy number. C-, DNA from a non-transgenic rabbit. T, transgene fragment digested with SacI. As molecular weight markers (mw), HindIII-digested lambda DNA was used.

Sac I restriction enzymes (Fig 5A and 5B, respectively). Patterns similar to those found in F0-26 were obtained. This fact pointed to the presence of only one integration site.

Expression of active hEPO in the milk of transgenic rabbits

Samples of defated milk from the F0-26 were assayed for the presence of hEPO by ELISA. We found hEPO levels of 0.2 and 0.3 ng/ml of milk on 7th and 12th days of lactation, respectively. Samples of defated milk from non transgenic animals were negative (Fig 2).

The expression levels of hEPO in the milk of the transgenic F0-26 animal were low. However, it could reflect a position-dependent expression for this founder animal.

It has been reported that expression of transgenes is improved when introns are included in the gene constructions (Brinster *et al*, 1988; Choi *et al*, 1991; Palmiter *et al*, 1991; Wright *et al*, 1991). As our levels might be influenced by the use of a cDNA in our construct, at present, we are studying several F0 transgenic animals generated with a construct with the chromosomal gene for hEPO (unpublished).

Whey fractions of defated milk from F0-26 were assayed to determine the biological activity of hEPO by the method described by Krystal in 1983. In our hands, the determination of hEPO *in vitro* biological activity by this method gave reproducible results diluting the hEPO both in cell culture media or in milk (Fig 6 A-B). However, values in milk (Fig 6B) were lower than those obtained in culture media (Fig 6A).

We found that the hEPO produced in the milk of F0-26 transgenic rabbit was active

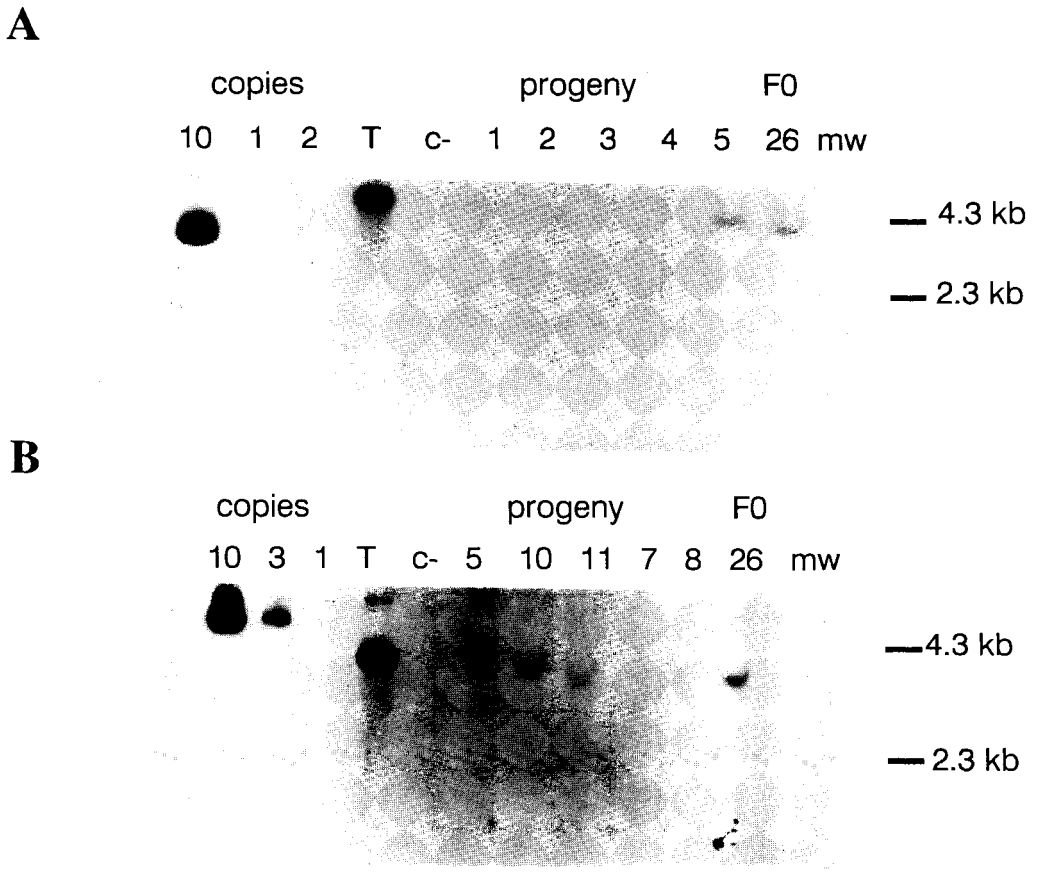

Fig 5. Southern blot analysis of muscle DNA samples obtained from F1 rabbits (F0-26 x non-transgenic male). Ten µg DNA were digested with BglII (**A**) or SacI (**B**) and subjected to analysis employing the hEPO cDNA as a hybridization probe. Twenty six, 78 and 260 pg (equivalent to 1, 3 and 10 copies/cell) of pmp18EPODNA were loaded to estimate the transgene copy number. C-, DNA from a non-transgenic rabbit. T, transgene fragment digested with BglII (in A) or SacI (in B). As molecular weight markers (mw), HindIII-digested lambda DNA was used.

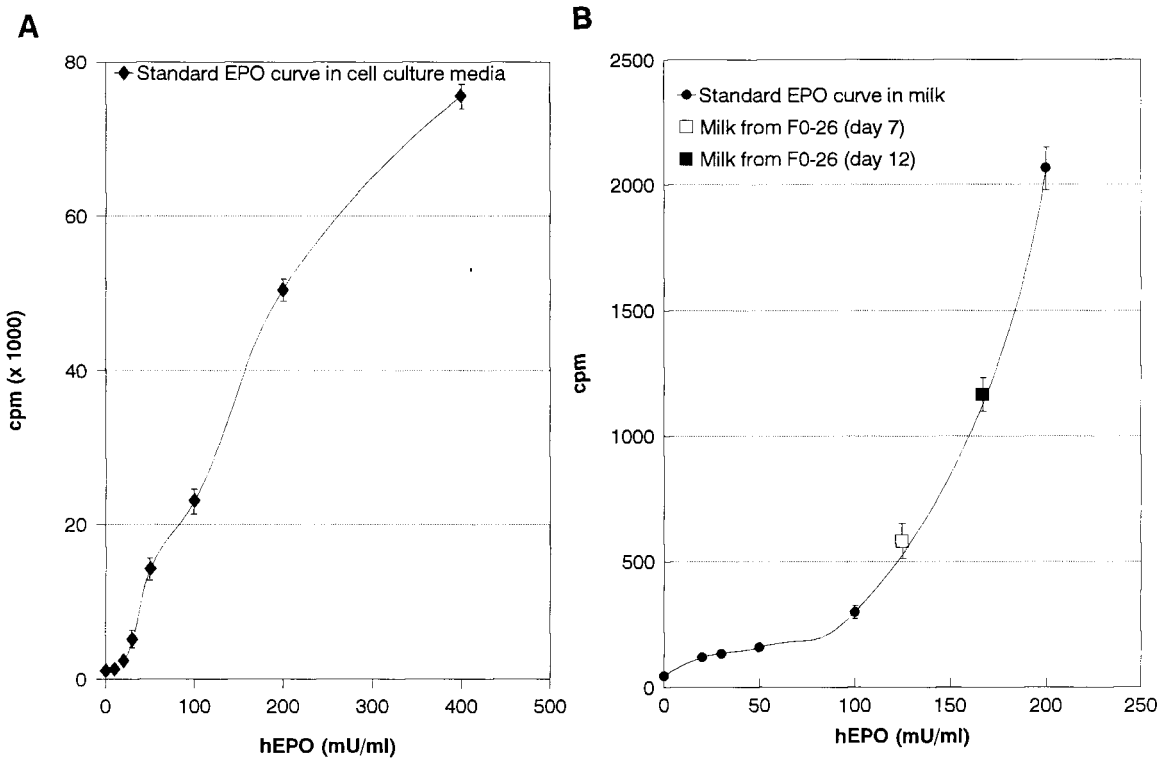

Fig 6. *In vitro* determination of hEPO biological activity by the method of Krystal (1983). **A.** Standard curve of hEPO (EPOETIN ALFA EPOGENR, 4000 U/ml, Amgen, USA) using as diluent α-MEM* cell culture media. Values from four independent determinations (mean ± SE). **B.** Standard curve of hEPO (EPOETIN ALFA EPOGEN®, 4000 U/ml, Amgen, USA) using as diluent milk whey fractions from a non-transgenic rabbit. Values for milk whey fractions of transgenic F0-26 rabbit are shown. Values from two independent determinations (mean ± SE).

(Fig 6B). The estimated specific activity was around 500 000 U/mg. This value is 3 times higher than the *in vitro* activity of the commercial recombinant human EPO (EPOETIN ALFA, EPOGENR, 4000 U/ml, Amgen, USA) and of the recombinant human EPO obtained in our laboratory from a stable clone of CHO cells expressing hEPO (unpublished results).

Several recombinant human EPOs produced in mammalian (Egrie *et al*, 1986; Goldberg *et al*, 1987; Recny *et al*, 1987; Tsuda *et al*, 1988; Goto *et al*, 1988) and non mammalian cells (Wojchowski *et al*, 1987) have recently become available but their biological activities differ among different cell lines. Since the structure of the polypeptide moieties is the same, such variations in activity was suspected as being due to the differences in the glycosylation.

Both urinary and recombinant human EPO contain about 40% of the molecular weight composed by carbohydrates in the form of three N-linked and one O-linked oligosaccharide chains. An unusual form of EPO (EPO-bi) in addition to the usual form (EPO-tetra) has been reported (Takeuchi *et al*, 1989). This EPO has only 1/7 of the *in vivo* activity, but 3 times the *in vitro* activity of the standard EPO.

One possible explanation for the higher *in vitro* activity observed for the hEPO secreted in the milk of transgenic founder rabbit F0-26, might be that, at least a fraction of the molecules, are EPO-bi. However, no definitive conclusions can be discussed until detailed characterization of the *in vivo* biological activity and carbohydrate composition analysis of this hEPO are done.

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