

Expression and Characterization of Functional Recombinant Bovine Follicle-Stimulating Hormone (boFSH α/β) Produced in the Milk of Transgenic Rabbits

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ABSTRACT Bovine follicle-stimulating hormone (boFSH) is a heterodimeric glycoprotein that belongs to the pituitary gonadotropins. Bioactive FSH is composed of α and β subunits which require extensive *N*-glycosylation and sialylation. The mammary gland of transgenic livestock is an attractive source for the synthesis of post-translationally modified proteins. Two mammary gland-specific gene constructs with the cDNA for the boFSH alpha (boFSH α) and beta (boFSH β) subunits controlled by bovine alpha-s1 casein regulatory sequences were co-microinjected into fertilized rabbit oocytes. Two FSH α /FSH β double transgenic rabbit lines were established. The transgene expression was strictly lactation and mammary gland specific. Protein analysis revealed the presence of the boFSH heterodimer in the milk of transgenic rabbits showing a molecular weight similar to that of purified pituitary gland derived boFSH (boFSH-P). Subunit specific antibodies detected both polypeptides with the expected molecular sizes. Biochemical characterization demonstrated the expected isoelectric points of the recombinant boFSH. The presence of the post-translationally added terminal sialic acid residues was indicated by wheat germ agglutinin (WGA) lectin Western blotting. The biological activity of the recombinant mammary gland produced boFSH was determined using a FSH-dependent reporter cell line. The bioactivity of the recombinant boFSH was comparable to that of purified boFSH-P. *Mol. Reprod. Dev.* 63: 300–308, 2002. © 2002 Wiley-Liss, Inc.

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(LH), thyroid-stimulating hormone (TSH), and the placenta derived chorionic gonadotropin (CG) or pregnant mare gonadotropin (PMSG). These hormones are heterodimeric with an α subunit common to all glycoprotein hormones and a hormone-specific β subunit (Pierce and Parsons, 1981). The subunits are noncovalently associated. Each subunit forms intrachain disulfide bridges and carries two *N*-linked oligosaccharides. These modifications are required for proper folding, subunit assembly and secretion of the gonadotropins (Suganuma et al., 1989; Feng et al., 1995). FSH and LH act essential but antagonistic for gonadal function and are synthesized within the same gonadotropic cells of the anterior pituitary (Pierce and Parsons, 1981; Kumar et al., 1997).

Gonadotropic hormones are required for reproductive biotechnology, i.e., superovulation in farm animals (Driancourt, 2001). Currently, the pre-dominant sources for FSH in animal biotechnology are purification from ruminant or porcine pituitaries. The pituitary gland derived FSH (FSH-P) is expensive and known to have a variable bioactivity in vivo. This is mainly caused by varying LH contamination in the FSH-P lots, which in turn are due to the preparation of FSH-P from slaughter animals of unknown reproductive status (Ulloa-Aguirre et al., 1995; Braileanu et al., 1998). Native FSH-P also bears the danger of contamination with infectious agents, an issue that gained in importance since the BSE crisis of the mid 80s. Recombinant DNA technology therefore provides an attractive alternative for the production of FSH without any hormonal, viral, or prion contaminants.

INTRODUCTION

The gonadotropins comprise a glycoprotein hormone family, which includes the pituitary gland derived follicle-stimulating hormone (FSH), luteinizing hormone

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Post-translational modifications, in particular *N*-glycosylation and sialylation are crucial for the *in vivo* bioactivity and biostability of FSH (Morell et al., 1971; Sairam and Bhargavi, 1985; Baenziger and Green, 1988). Mammalian cells (Chappel et al., 1988; Greenberg et al., 1991) were shown to be capable of performing the required post-translational modifications. Therefore they are discussed to be superior to other recombinant production systems including insect (van de Wiel et al., 1998), yeast (Samaddar et al., 1997), and plant (Dirnberger et al., 2001). The mammary gland of transgenic livestock is able to produce glycosylated and phosphorylated recombinant proteins (for a review see Ziomek, 1998; Larrick and Thomas, 2001). Rabbit is considered as a species to produce recombinant proteins in sufficient quantity for commercial use (Besenfelder et al., 1998; Houdebine, 2000).

Here, we used two gene constructs for bovine FSH-alpha (boFSH α) and FSH-beta (boFSH β) subunits, each under the control of bovine alpha-s1 casein regulatory sequences. We generated transgenic rabbits, which secrete bioactive recombinant boFSH α/β into their milk.

MATERIALS AND METHODS

Generation and Analysis of Transgenic Rabbits

The α s1-casein regulatory sequences based mammary gland expression cassette has been described previously (Brem et al., 1994). cDNAs for the coding regions of boFSH α (GeneBank Access. No. X00050, Erwin et al., 1983; Nilson et al., 1983) and boFSH β (GeneBank Access. No. M14853, Maurer and Beck, 1986; GeneBank Access. No. M13383, Esch et al., 1986) were fused into the expression cassette. The gene constructs pFA200 (boFSH α) and pFB200 (boFSH β) were co-microinjected into fertilized oocytes as described previously (Besenfelder and Brem, 1993; Besenfelder et al., 1998) to generate double transgenic rabbits. For the detection of transgenic animals, PCR analysis of genomic DNA extracted from ear biopsies was performed as described (Coulibaly et al., 1999). Primers were designed from the bovine α s1-sequences (GeneBank Access. No. X59856, (Koczan et al., 1991): Oligo248 (5'-GATGCTTCTCTATTCTCTG-3', pos. no. 3,266–3,285) and Oligo249 (5'-GACCATAACTGTGGAGTC-CC-3', pos. no. 18,442–18,461). Transgenesis for FSH α and β chains was analyzed by a combination of α s1-casein specific primers (GeneBank Access. No. X59856, Koczan et al., 1991) Oligo4 (5'-GATCATCAACCCAGCT-TGCTC-3', pos. no. 2,140–2,161, exon1) or Oligo6 (5'-CAGTGGCCTTTA-TACCAGCAT-3', pos. no. 19,460–19,481, exon 19) with OligoFSH α (5'-ACAGCATGTAGCTTCCGAGGTG-3', pos. no. 321–341, GeneBank Access. No. X00050, Erwin et al., 1983) or OligoFSH β (5'-AGTCTGTCCAGT-TCTGTTTC-3', pos. no. 51–70, GeneBank Access. No. M14853, Maurer and Beck, 1986). The expected length of PCR products upon amplification of genomic DNA with Oligo4/OligoFSH α and OligoFSH β /Oligo6 is 1,757 and 1,400 bp, respectively.

Analysis of mRNA Expression

Total RNA was extracted with Trizol[®] (Gibco BRL, LifeTechnologies, Austria) from fresh milk samples, mammary gland biopsies and different tissues (ovary, cerebellum, cerebrum, liver, lung, spleen, kidney, pancreas, muscle, heart, bladder, mammary gland) from lactating rabbits. For RT-PCR first strand cDNA was synthesized by using oligodT in the presence of M-MLV reverse transcriptase (Gibco BRL, LifeTechnologies) as described (Coulibaly et al., 1999). mRNA expression was monitored by combining primers Oligo4 and OligoFSH α , and OligoFSH β , and Oligo6 (see above). The expected length of PCR products upon amplification of cDNA with Oligo4/OligoFSH α and OligoFSH β /Oligo6 is 385 and 612 bp, respectively. For an amplification control, the expression of rabbit α -casein (RK1, 5'-TAGGACACCT-GAAACTCACTC-3' and RK2, 5'-TCCCGGAGTATAG-TACCAAGG-3', pos. nos. 4,678–4,698 and 15,169–15,149, GeneBank Access. No. M77195; Jolivet et al., 1992) and rabbit α -actin (5'-TGTGACATCGACATC-AGGAAGG-3' and 5'-TAGGTAATGAGTCAGAGCTTT-GG-3' pos. nos. 917–938 and 1,277–1,299, GeneBank Access. No. X60732, Harris et al., 1992) was analyzed as described (Coulibaly et al., 1999).

Analysis of Recombinant Protein

Milk samples were collected as described previously (Zinovieva et al., 1998). Milk was defatted by centrifugation at 3,000g (for 10 min at 4°C) and analyzed immediately or stored at –80°C. Prior to analysis, defatted milk was centrifuged again (at 20,000g for 20 min at 4°C). For the detection of the native heterodimeric protein, clear supernatants (1.5 μ l) were mixed with 2 \times nonreducing Laemmli sample buffer and left unheated. For the detection of the FSH subunits 2 \times reducing Laemmli sample buffer and heating at 95°C for 5 min was used. Subsequently samples were fractionated on a 15% SDS-PAGE (Laemmli, 1970). Transfer of the proteins to nitrocellulose membranes (Immobilon NC pure, Millipore, Austria) and probing of the Western blots was performed as described (Coulibaly et al., 1999). Detection of the recombinant boFSH α/β and the boFSH α and β subunits was carried out with polyclonal anti-boFSH (AFP 271990), anti-rat LH-alpha (AFP 5351791GP), and anti-boFSH-beta (AFP 879691GP) antibodies (A.F. Parlow, National Hormone & Pituitary Program), respectively. Immunocomplex detection was performed with a secondary antibody anti-Guinea Pig IgG peroxidase conjugate (Sigma Immuno Chemical) and chemiluminescence (ECL, Amersham, UK) according to the manufacturer's protocols. Purified boFSH-P (AFP 5310B, biopotency 1,693 IU/mg in terms of 2nd IRP-HMG), boFSH α (AFP 5966B), and boFSH β (AFP 5968C) (A.F. Parlow, National Hormone & Pituitary Program) from bovine pituitary gland were applied as a standard control.

Detection of sialic acid residues was performed with boFSH containing isoelectric focusing fractions (see below). Proteins were separated by SDS-PAGE under

reducing conditions, Western blots were probed with conjugated lectin (wheat germ agglutinin, WGA, peroxidase conjugated, Sigma, diluted 1:10,000) and stained with chemiluminescence (ECL). Blots were scanned on a Sharp JX-330 scanner and analyzed with the software Quantity One (Amersham Biosciences, Uppsala, Sweden) run on a Sun SPARCstation 4 (Sun Microsystems, Mountain View).

Preparative Isoelectric Focusing

Isoelectric focusing was performed in flat-bed granulated gels (5% Sephadex G-75, Amersham Biosciences) according to Radola (1973). The gel was prepared on a glass-frame (26 × 12.5 × 0.5 cm, LKB, Bromma, Sweden) fitting in an LKB 2117 Multiphor II Electrophoresis System (LKB), as described by the manufacturer. Clear supernatants (5 ml, see above) from transgenic rabbit milk (line #5446) were applied cathodically. Servalyt T4-9 (Serva, Heidelberg, Germany) was used as an ampholyte to establish the pH-gradient, glutamic, and aspartic acid (25 mM each, Serva) served as anolyte, 1 M sodium hydroxide as catholyte. Electrofocusing was performed over an electrode distance of 24 cm overnight at 500 V, 16 mA, 8 W, and 13°C, afterwards raising the voltage to 1,000 V for 5 hr. After completion of the run, a metal grid was pressed into the gel, creating 30 fractions that could be collected individually. Approximately 1 cm of gel from each section was eluted with deionized distilled water, and the pH determined with a microelectrode (Schott, Hofheim am Taunus, Germany). The remainder of each gel section was eluted with phosphate buffered saline (PBS) for further studies (see below).

The protein content of each fraction was determined as described by Bradford (1976). Protein containing fractions (6–25) were analyzed by SDS-PAGE with Coomassie staining. For the immunological detection of recombinant boFSH each fraction (6–25) was analyzed by SDS-PAGE and Western blot (see above). Blots were scanned on a Sharp JX-330 scanner and analyzed with the software Quantity One (Amersham Biosciences) run on a Sun SPARCstation 4 (Sun Microsystems).

Tissue Culture and FSH Bioassay

The Chinese hamster ovary (CHO) cell line expressing the human FSH receptor (FSH-R) and a reporter gene (luciferase) driven by the human glycoprotein hormone alpha gene (CHO/hFSH-R/Luc) was described previously (Albanese et al., 1994) and was kindly provided by Dr. J.L. Jameson (Division of Endocrinology, Metabolism and Molecular Medicine, Northwestern University Medical School, Chicago). Cells were cultured in supplemented α MEM in the presence of 400 μ g/ml geneticin as described (Albanese et al., 1994). For the FSH bioassay, 2×10^5 cells/well were cultured in a 24-well culture plate at 37°C overnight. Cells were washed with PBS and cultured in 300 μ l fresh medium containing 0.25 mM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma Immuno Chemical). Cells were treated with different concentrations (100, 250, and 500 ng corresponding to

0.17, 0.425, and 0.850 IU in terms of 2nd IRP-HMG) of purified boFSH-P (AFP5310B, National Hormone & Pituitary Program) mixed with nontransgenic rabbit milk as standard control. Clear supernatant from defatted and centrifuged transgenic milk (line #5446) containing 100, 250, and 500 ng recombinant boFSH (according to Western blot) were added to the cells. Luciferase activity was measured using Luciferase Assay Systems (Promega Corp, Germany/Austria, Mannheim). After 6 hr incubation at 37°C, the cells were washed with PBS and lysed in 80 μ l of $1 \times$ Passive Lysis Buffer. Assay buffer (1 M Tris, pH 7.4, 1 M MgSO₄, 0.2 M ATP) and injection buffer (1 M Tris, pH 7.4, 10 mM luciferin) were prepared. Luciferase activity was measured by injection of 100 μ l of the assay buffer and the injection buffer into the individual tube containing 20 μ l of each cell lysate in a Luminometer (Lumat LB 9507, EG&G, Berthold). Light emittance was measured during the first 10 sec of each reaction. The experiments for each concentration of milk containing boFSH α/β and the controls (medium, milk from nontransgenic rabbit and boFSH-P) were performed in triplicate.

RESULTS

Generation of boFSH Transgenic Rabbits

Co-injection of bovine boFSH α and β encoding mammary gland specific expression cassettes resulted in boFSH α/β double transgenic founder animals. Two stably transmitting boFSH α/β transgenic lines (#5446 and #5497) were established. Integration of intact transgene copies was confirmed by Southern blotting and PCR analyses (data not shown). Female F1 animals were investigated for transgene expression on RNA and protein level.

RNA Expression Profile

Mammary gland biopsies of lactating transgenic rabbits were analyzed by RT-PCR for boFSH α and β transgene expression and for endogenous α -casein expression as an amplification control (Fig. 1). F1 females of both transgenic lines expressed the co-integrated gene constructs in a lactation specific manner. Next we screened various organs of lactating transgenic rabbits for ectopic expression of boFSH α or β using α -actin as an endogenous control (Fig. 2). Transgene-specific PCR products could be detected in no tissues other than the mammary gland. Analysis of a nonlactating mammary gland of a transgenic doe (line #5497) revealed no detectable transgene expression (data not shown). Therefore, the transcriptional activity of the α s1-casein controlled gene constructs was strictly limited to the mammary gland and controlled by lactogenic hormones. This has been demonstrated for the mammary gland expression cassette previously (Zinovieva et al., 1998).

Production of Recombinant boFSH α/β

For the detection of the boFSH α/β heterodimer we performed Western analysis under nonreducing conditions. Milk was cleared from cells, debris, and fat by

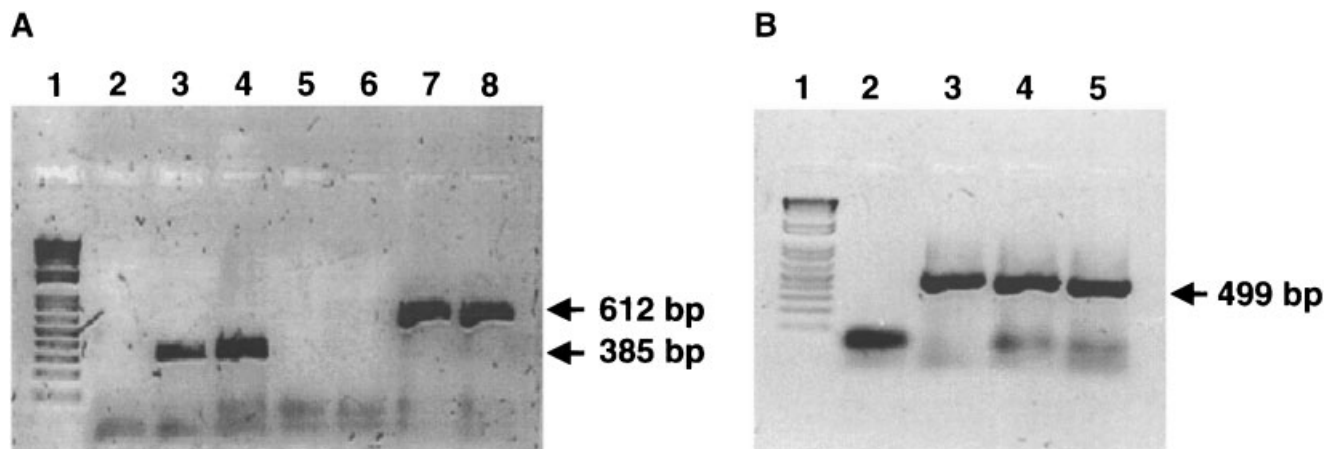


Fig. 1. Mammary gland specific transgene expression. Total RNA was extracted from biopsies of lactating nontransgenic and transgenic rabbits (lines #5446 and #5497). RT-PCR was performed with primers specific for boFSH (A) and rabbit α s1-casein (B). Amplification products are shown on 1.5% agarose gel stained with dimidium bromide (0.5 μ g/ml). **Lane 1**, molecular size marker (100 bp ladder); **lane 2**, non-template control. (A) **Lanes 3–5**, RT-PCR for boFSH α (expected length of PCR product 385 bp); lane 3, #5446-011; lane 4, #5497-014; lane 5,

nontransgenic rabbit; **lanes 6–8**, RT-PCR for boFSH β (expected length of PCR product 612 bp); lane 6, nontransgenic rabbit; lane 7, #5446-011; lane 8, #5497-014; in the case of amplification of genomic DNA the PCR products would be 1,757 bp (boFSH α) and 1,400 bp (boFSH β) in length (data not shown). (B) RT-PCR control for rabbit α s1-casein (expected length of PCR product 499 bp); lane 3, nontransgenic rabbit; lane 4, #5446-011; lane 5, #5497-14.

centrifugation, and proteins were fractionated by SDS-PAGE. Probing the Western blot with polyclonal anti-boFSH antibodies revealed a band with an apparent molecular mass of 38 kDa (Fig. 3A, lanes 2–6). The mammary gland produced boFSH co-migrated with pituitary gland derived boFSH-P mixed with milk of nontransgenic rabbits (Fig. 3A, lane 7). At high transgenic boFSH α / β levels a faint band of approximately 20 kDa in size was also observed (Fig. 3A, lanes 2–4). A similar band was detected subjecting high concentrations of native boFSH-P to Western analysis (data not shown). Further testing of the anti-boFSH antiserum revealed that the antibodies recognized boFSH α / β under nonreducing conditions and were specific for the 20 kDa boFSH α chain but did not recognize the boFSH β chain (data not shown). We therefore concluded that the band is specific for free boFSH α which may originate from dissociation of boFSH α from boFSH α / β during SDS-PAGE or alternatively from nonequimolar production of the FSH chains in the pituitary gland and the mammary gland. The synthesis of free FSH α in the bovine pituitary gland has been reported (Baenziger and Green, 1988).

Western blotting under reducing conditions and probing with anti-ratLH (cross-reacting with boFSH α but not boFSH β) and anti-boFSH β antibodies showed a band of 20 kDa (Fig. 3B) and a 21.5/19 kDa doublet (Fig. 3C), respectively. A similar electrophoretic mobility of denatured pituitary gland derived boFSH α and β mixed with rabbit milk was observed (Fig. 3B,C, lane 7). No cross-reaction of all used antisera with mammary gland proteins of nontransgenic rabbits could be detected (Fig. 3A, lane 1).

The concentration of boFSH in the milk of transgenic rabbits was determined by comparing Western blot signals of transgenic milk samples with known concentrations of boFSH-P mixed with nontransgenic milk.

Secreted boFSH levels in the milk of hemizygous transgenic rabbits varied between 50 and 100 mg/L (data not shown).

Biochemical Properties of Recombinant boFSH

The carbohydrate moiety of FSH is essential for its biopotency in vitro and in vivo (e.g., Creus et al., 2001). Variation in the carbohydrate complex may explain the heterogeneous forms of FSH and the variable biological activity. This is mainly determined by the sialic acid content, which correlates with the isoelectric properties of FSH isoforms. Therefore, we performed isoelectric focusing of transgenic milk in order to separate boFSH α / β isoforms according to their pH charge, and WGA lectin staining to demonstrate the presence of sialic acid residues.

Defatted transgenic milk was cleared again by centrifugation and 5 ml supernatant was subjected to preparative isoelectric focusing. Figure 4A shows the pH gradient and the total protein content of each fraction of transgenic milk. The majority of proteins were detected in fractions 6–19 (peak of 5,354 mg/ml in fraction 15) with pH values between 3.79 and 6.75 (Fig. 4A). Non-reducing SDS-PAGE and Western blot analysis of these fractions revealed the presence of boFSH α / β in fractions 11–18, predominantly in fractions 15 and 16 (Fig. 4B). Fractions (11–18) containing boFSH α / β were analyzed by SDS-PAGE Western blot and WGA lectin staining. Proteins with sialic acid residues were detected in all fractions with the highest peaks in fraction 13–16 (Fig. 4B). A FSH microheterogeneity caused by the presence of isoforms varying from fraction to fraction was also observed by others (Creus et al., 2001). WGA lectin staining was also observed in fractions 9, 10, and 19, in which boFSH was undetectable (data not shown). This

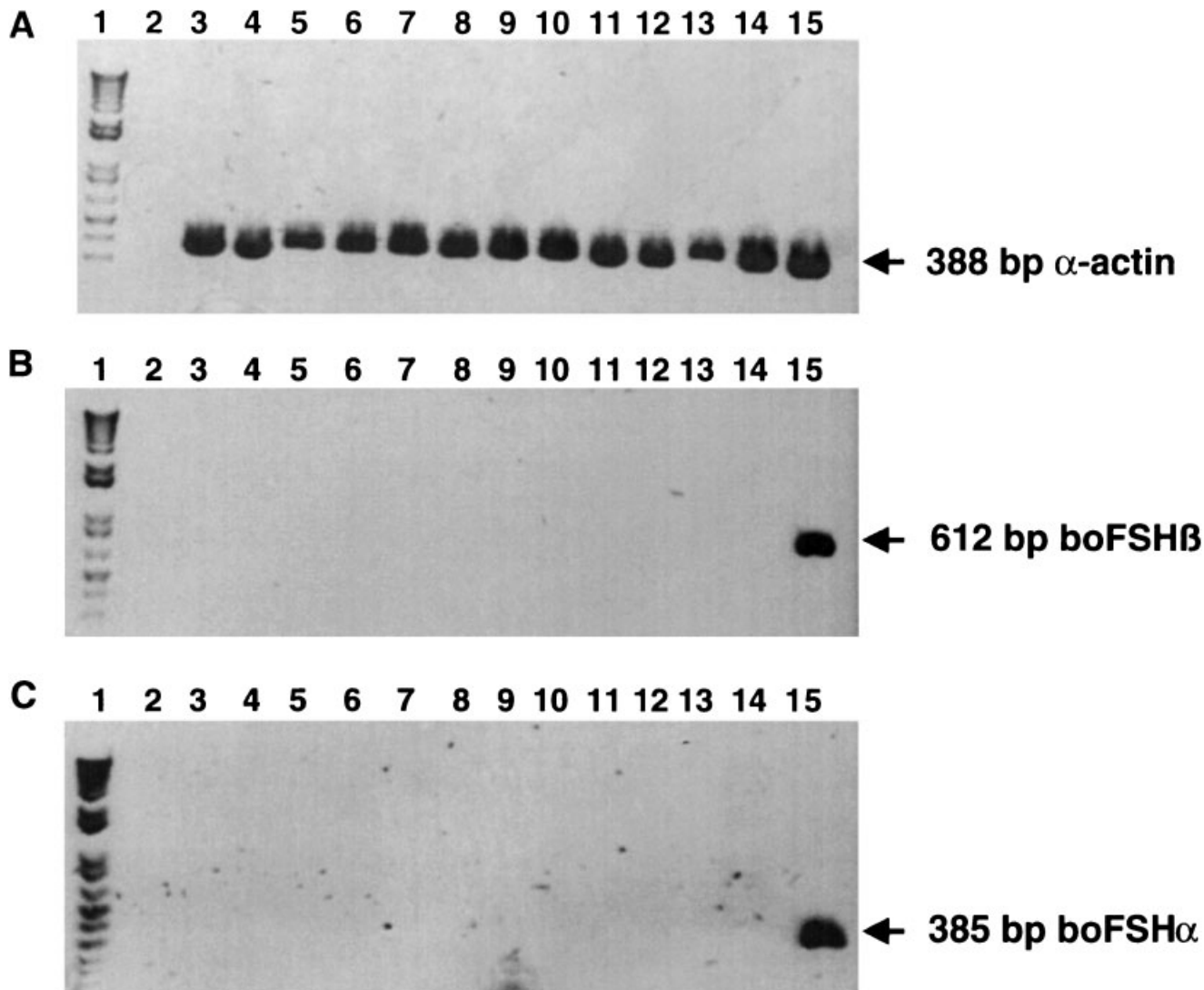


Fig. 2. Tissue-specificity of the mammary gland expression cassette. RNA was isolated from the different tissues (line #5446) and RT-PCR was performed for the detection of α -actin (A), boFSH β (B), and boFSH α (C). Lane 1, DNA ladder; lane 2, nontemplate control; lane 3, ovary;

lane 4, cerebellum; lane 5, cerebrum; lane 6, liver; lane 7, lung; lane 8, spleen; lane 9, kidney; lane 10, pancreas; lane 11, muscle; lane 12, heart; lane 13, bladder; lane 15, mammary gland; lane 14, mammary gland of nontransgenic rabbit.

could be caused by milk (glyco)proteins with terminal neuraminic acids.

Biological Activity of Recombinant boFSH

The biological activity of mammary gland produced recombinant boFSH α/β was assessed by employing a cell line (CHO) stably expressing the human FSH-R and harboring the luciferase reporter gene driven by a FSH-responsive promoter (Albanese et al., 1994). As a positive control different concentrations (100, 250, and 500 ng) of boFSH-P were mixed with milk of a nontransgenic rabbit. The transgenic milk samples were adjusted in their boFSH α/β concentrations by Western probing of dilutions of transgenic milk and comparison of the signals to that obtained with known amounts of native boFSH-P (data not shown). Incubation of the reporter cell line with increased concentrations of

recombinant and native boFSH showed a dose dependent luciferase activity. The estimated bioactivity in vitro of boFSH α/β was 1.7 mIU/ng according to the bioactivity of purified boFSH-P (AFP 5310B, biopotency 1,693 IU/mg in terms of 2nd IRP-HMG). The bioactivity of recombinant boFSH was comparable to that of native boFSH-P (Fig. 5).

DISCUSSION

We have used α s1-casein regulatory sequences fused to cDNAs encoding boFSH α and β to generate transgenic rabbits expressing the heterodimeric FSH in the mammary gland. Two double transgenic and stably transmitting lines were established and studied at the level of transcription and translation. In all studied transgenic rabbits (F1 females), RT-PCR demonstrated that the expression of boFSH α/β mRNA was strictly mammary

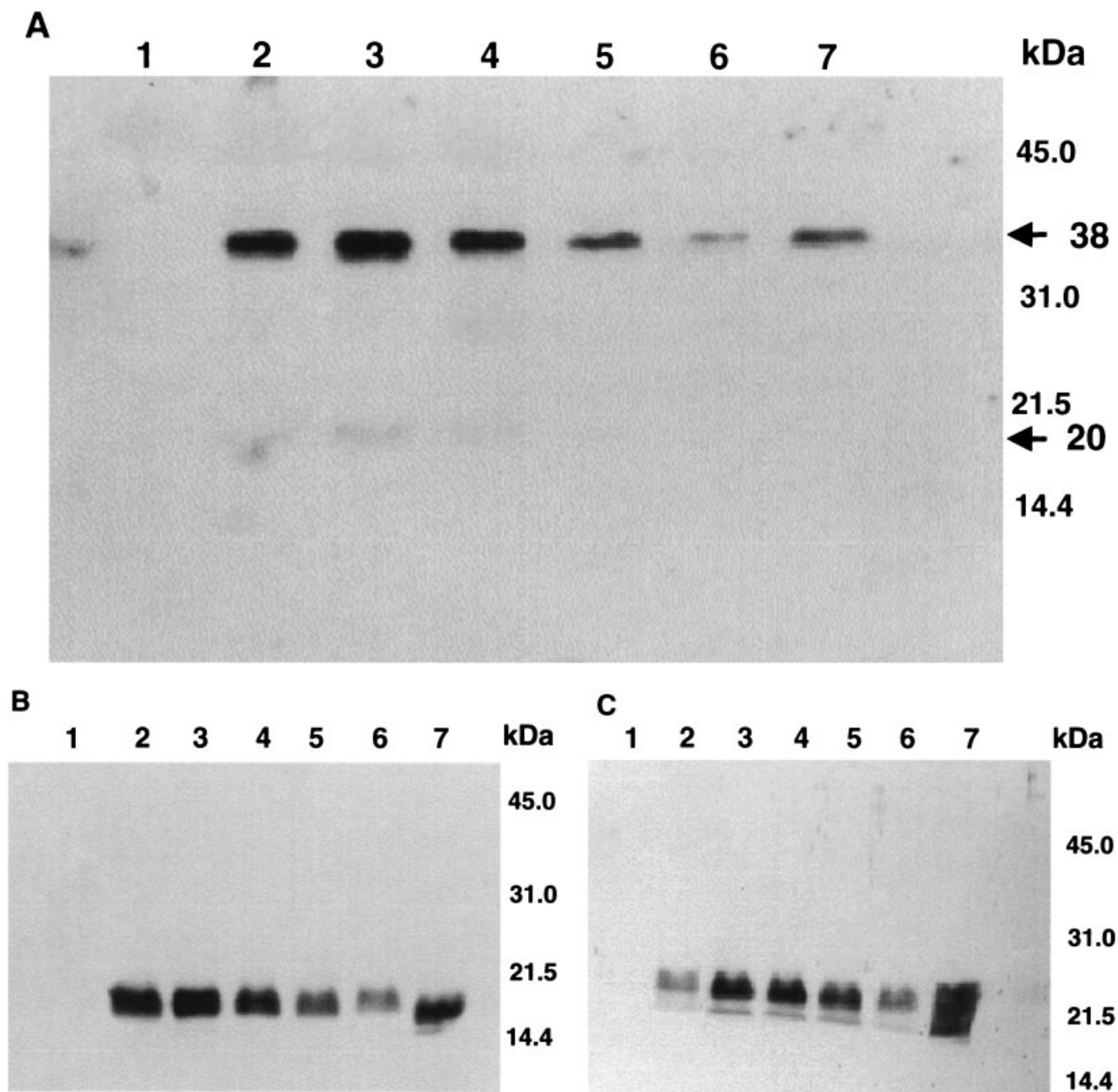


Fig. 3. Western blot analysis of mammary gland produced boFSH. Transgenic milk samples and controls were fractionated by SDS-PAGE and probed with the indicated antibodies. Purified boFSH-P, boFSH α and β (A.F. Parlow National Hormone and Pituitary Program) mixed with the milk of nontransgenic rabbit served as positive control. (A) Nonreducing conditions, probing with polyclonal anti-boFSH. (B) Reducing conditions (β -mercaptoethanol), probing with polyclonal

anti-rat LH α (crossreacting with boFSH α). (C) Reducing conditions (β -mercaptoethanol), probing with polyclonal anti-FSH β . (A-C) Lane 1, nontransgenic milk; lane 2, #5446-011; lane 3, #5446-6443; lane 4, #5497-7095; lane 5, #5794-6521; lane 6, #5497-014. Lane 7, nontransgenic milk mixed with 50 ng purified boFSH-P (A); 50 ng purified boFSH α (B); or 200 ng purified boFSH β (C).

gland specific. This spatial temporal expression pattern of the α s1-regulatory sequences in transgenic rabbits was known from previous studies (Brem et al., 1994; Zinovieva et al., 1998; Coulibaly et al., 1999; Van den Hout et al., 2001). The transgenic α s1-casein regulatory sequences used are therefore sufficient to provide the pattern of endogenous rabbit α s1-casein expression (Puissant et al., 1994). Recombinant boFSH was

secreted into the milk with an apparent molecular mass of 38 kDa, as shown by Western blot analysis using antiserum to native bovine FSH. Native boFSH purified from pituitary gland (boFSH-P) and mixed with nontransgenic milk was also detected with the similar molecular mass. The Western data are in consistence with those reported for transgenic mice producing FSH in their mammary gland (Greenberg et al., 1991).

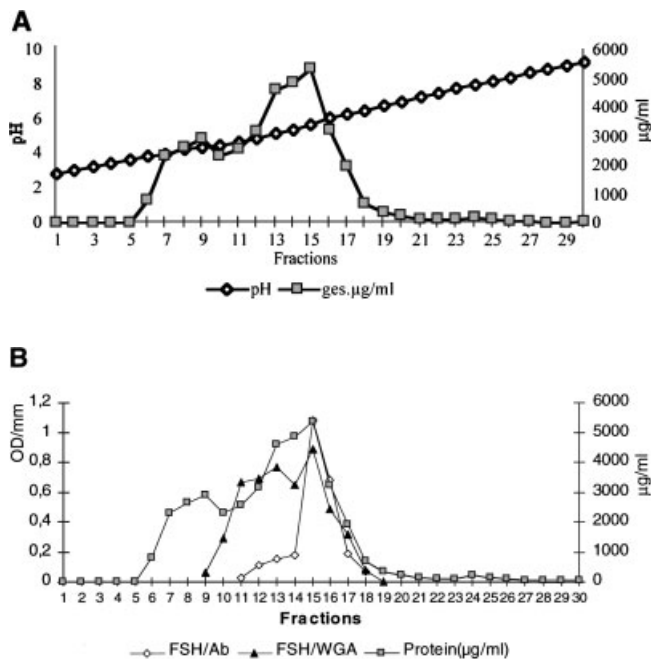


Fig. 4. Analysis of recombinant boFSH by preparative isoelectric focusing of transgenic milk was performed in bed-flat granulated gels. Fractions were analyzed for total protein content (A) and probed for the presence of boFSH and sialylated proteins (B). Recombinant protein was detected with polyclonal anti-boFSH antibodies. WGA lectin Western blotting indicated the presence of proteins with terminal sialic acid residues. Band intensity on the blots was evaluated with a densitometry imaging system and expressed as trace quantity (OD \times mm).

Gonadotropin isoforms can be separated according to their isoelectric point (pI), a property that is correlated with the carbohydrate residues of glycoproteins. Therefore, we have performed isoelectric focusing investigations of transgenic milk. Protein content of the fractions was determined and Western blot was used to detect the recombinant boFSH α/β . Only fractions with pH ranges between 4.65 and 6.28 showed the presence of recombinant boFSH α/β with a major peak at pH 5.7–6.05. Similar pH ranges were determined by chromatofocusing analysis of boFSH produced in transgenic mouse milk (pH 4.2–6.1) or in CHO cells (pH 4.0–5.2) (Greenberg et al., 1991).

WGA lectin staining indicated the presence of sialic acid residues in all fractions containing boFSH α/β . This clearly indicated that the mammary gland is capable of secreting glycosylated and sialylated proteins. It has been described that mammary epithelial cells possess several glycosyl-transferases including sialyl-transferase (Brokhausen et al., 1995). Glycosylation enzymes, however, may be species and tissue variable, resulting in carbohydrate moieties of transgenic mammary gland produced polypeptides with no full structural identity to the native homolog (Edmunds et al., 1998) (see below).

Recombinant boFSH produced by insect and plant cells lacks the terminal sialic acid residues (van de Wiel et al., 1998; Dirnberger et al., 2001). Recombinant FSH produced in these systems is therefore more adequate

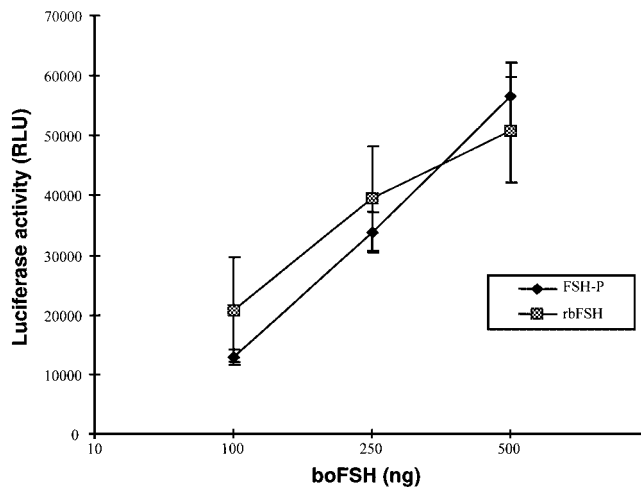


Fig. 5. In vitro bioactivity of boFSH. A FSH responsive reporter cell line expressing luciferase was used to assay the biopotency of the recombinant boFSH produced in the milk of transgenic rabbits. Luciferase activity was measured after stimulating cells for 6 hr with transgenic milk containing 100, 250, and 500 ng boFSH per milliliter. The bioactivity of the mammary gland produced boFSH was compared to that of purified boFSH-P mixed with milk of nontransgenic rabbits. Incubation of the reporter cell line with milk from nontransgenic rabbits did not result in detectable luciferase activity (data not shown). Experiments were done for each concentration in triplicate. Results (mean \pm SD) are expressed as relative light units.

for biochemical studies or bioassays in cell cultures, which presumably may not require fully glycosylated proteins (Sairam and Bhargavi, 1985; Galway et al., 1990; Grossmann et al., 1997). The sialic acid residues are crucial for the survival of glycoproteins in circulation (Morell et al., 1971; Galway et al., 1990). This may explain in part the poor or variable biological in vivo potency of insect or plant cell derived recombinant bovine FSH (Fieta et al., 1991; Ulloa-Aguirre et al., 1995; Dirnberger et al., 2001).

Stable transfected cell line (CHO/hFHS-R) expressing luciferase as a reporter gene showed that mammary gland produced boFSH is biologically active. A dose dependent luciferase activity was observed. The in vitro bioactivity of recombinant boFSH was comparable to that of purified boFSH-P. Moreover, in a preliminary superovulation scheme in cattle, defatted and ultracentrifuged transgenic milk was tested. A clear induction of superovulation was observed (Besenfelder et al., unpublished results).

Secreted boFSH α/β levels in the milk of transgenic rabbits varied between 50 and 100 mg/L. This yield exceeds the expression level of boFSH (1–3 mg/L) in insect cells using baculovirus (van de Wiel et al., 1998). Till date, the only commercialized gonadotropin is human FSH produced from CHO cells which is used in assisted human reproduction technology (Olijve et al., 1996). However, the yield of hormone production in CHO cells is low (0.5 mg/L) which makes the product unattractive for the large amounts required for animal breeding programs. Recently, the production of

a recombinant gonadotropin (chimeric equine LH/CG) in the mammary gland of rabbits was reported (Galet et al., 2000). Although the recombinant gonadotropin had full *in vitro* bioactivity no *in vivo* bioactivity could be detected. The lack of *in vivo* biological activity was suggested to be due to the short half-life of the recombinant protein which in turn could be caused by insufficient post-translational modifications.

In conclusion, we have produced recombinant bovine FSH in the mammary gland of transgenic rabbits, which had a full *in vitro* bioactivity. Biochemical studies indicated the presence of the glycoforms of FSH required for *in vivo* biopotency.

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REFERENCES

- Albanese C, Christin-Maitre S, Sluss PM, Crowley WF, Jameson JL. 1994. Development of bioassay using a recombinant human FSH receptor and a cAMP responsive luciferase reporter gene. *Mol Cell Endocrinol* 101:211–219.
- Baenziger J, Green E. 1988. Pituitary glycoprotein hormone oligosaccharides: Structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. *Biochim Biophys Acta* 947:287–306.
- Besenfelder U, Brem G. 1993. Laparoscopic embryo transfer in rabbits. *J Reprod Fertil* 99:53–56.
- Besenfelder U, Aigner B, Müller M, Brem G. 1998. Generation and application of transgenic rabbits. In: Cid-Arregui A, Garcia-Caranca A, editors. *Microinjection and transgenesis*. Berlin: Springer Verlag, 561–586.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Braileanu GT, Albanese C, Card C, Chedrese PJ. 1998. FSH bioactivity in commercial preparations of gonadotropins. *Theriogenology* 49:1031–1037.
- Brem G, Hartl P, Besenfelder U, Wolf E, Zinovieva N, Pfaller R. 1994. Expression of synthetic cDNA sequences encoding human insulin-like growth factor-1 (IGF-1) in the mammary gland of transgenic rabbits. *Gene* 149:351–355.
- Brokhausen I, Yang J, Burchell J, Whitehouse C, Taylor-Papadimitriou J. 1995. Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. *Eur J Biochem* 233:607–617.
- Chappel S, Loosney CR, Bondioli KR. 1988. Bovine FSH produced by recombinant DNA technology. *Theriogenology* 29:235.
- Coulibaly S, Besenfelder U, Fleischmann M, Zinovieva N, Grossmann A, Wozny M, Bartke I, Tögel M, Müller M, Brem G. 1999. Human nerve growth factor beta (hNGF- β): Mammary gland specific expression and production in transgenic rabbits. *FEBS Lett* 444:111–116.
- Creus S, Chaia Z, Pellizari EH, Cigorraga SB, Ulloa-Aguirre A, Campos S. 2001. Human FSH isoforms: Carbohydrate complexity as determinant of *in-vitro* bioactivity. *Mol Cell Endocrinol* 174:41–49.
- Dirnberger D, Steinkellner H, Abdennebi L, Remy JJ, van de Weil D. 2001. Secretion of biologically active glycoforms of bovine follicle stimulating hormone in plants. *Eur J Biochem* 268:4570–4579.
- Driancourt MA. 2001. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology* 55:1211–1239.
- Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson JM, Cole ES. 1998. Transgenically produced human antithrombin: Structural and functional comparison to human plasma-derived antithrombin. *Blood* 91:4561–4571.
- Erwin CR, Croyle ML, Donelson JE, Maurer R. 1983. Nucleotide sequence of cloned complementary deoxyribonucleic acid for the alpha subunit of bovine pituitary glycoprotein hormones. *Biochemistry* 22:4856–4860.
- Esch FS, Mason AJ, Cooksey K, Mercado M, Shimasaki S. 1986. Cloning and DNA sequence analysis of the cDNA for the precursor of the beta chain of bovine follicle stimulating hormone. *Proc Natl Acad Sci USA* 83:6618–6621.
- Feng W, Matzuk MM, Mountjoy K, Bedowsa E, Ruddon RW, Boime I. 1995. The asparagine-linked oligosaccharides of the human chorionic gonadotropin beta subunit facilitate correct disulfide bond pairing. *J Biol Chem* 270:11851–11859.
- Fieta D, Srivastava V, Hindsgaul O, Baenziger JU. 1991. A hepatic reticuloendothelial cell receptor specific for SO₄-4GalNAc beta₁, 4GlcNAc beta₁, 2Man₃ that mediated rapid clearance of lutropin. *Cell* 67:1103–1110.
- Galet C, Le Bourhis CM, Chopineau M, Le Griec G, Perrin A, Magallon T, Attal J, Viglietta C, Houdebine L-M, Guillou F. 2000. Expression of a single beta alpha chain protein of equine LH/CG in milk of transgenic rabbits and its biological activity. *Mol Cell Endocrinol* 174:31–40.
- Galway AB, Hsueh AJW, Keene JL, Yamato M, Fauser BCJM, Boime I. 1990. *In vitro* and *in vivo* bioactivity of recombinant human follicle-stimulating hormone and partially deglycosylated variants secreted by transfected eukaryotic cell lines. *Endocrinology* 127:93–100.
- Greenberg NM, Anderson JW, Hsueh AJW, Nishimori K, Reeves JJ, Deavila DM, Ward DN, Rosen JM. 1991. Expression of biologically active heterodimeric bovine follicle-stimulating hormone in milk of transgenic mice. *Proc Natl Acad Sci USA* 88:8327–8331.
- Grossmann M, Wong R, Teh NG, Tropea JE, Palmer EJ, Weintraub BD, Szkudlinski MW. 1997. Expression of biologically active human thyrotropin (hTSH) in a baculovirus system: Effect of cell glycosylation on hTSH activity *in vitro* and *in vivo*. *Endocrinology* 138:92–100.
- Harris DE, Warshaw DM, Perissamy M. 1992. Nucleotide sequences of the rabbit alpha-smooth-muscle and beta-non-muscle actin mRNAs. *Gene* 112:265–266.
- Houdebine L-M. 2000. Transgenic animal bioreactors. *Transgenic Res* 9:305–320.
- Jolivet G, Devinoy E, Fontaine ML, Houdebine LM. 1992. Structure of the gene encoding rabbit alpha-s-1-casein. *Gene* 113:257–262.
- Koczan D, Hobom G, Seyfert H. 1991. Genomic organization of the bovine alpha-S1 casein gene. *Nucleic Acids Res* 19:5591–5596.
- Kumar T, Wang Y, Lu N, Matzuk M. 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* 15:201–204.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Larrick JW, Thomas DW. 2001. Producing proteins in transgenic plants and animals. *Curr Opin Biotechnol* 12:411–418.
- Maurer RA, Beck A. 1986. Isolation and nucleotide sequence analysis of a cDNA encoding the beta-subunit of follicle-stimulating hormone. *DNA* 5:363–369.
- Morell AG, Gregoriadis G, Scheinberg IH, Hickman J, Ashwell G. 1971. The role of sialic acid in determining the survival of glycoproteins in the circulation. *J Biol Chem* 246:1461–1467.
- Nilson JH, Thomason AR, Cserbak MT, Moncman CL, Woychik RP. 1983. Nucleotide sequence of a cDNA for the common alpha subunit of the bovine pituitary glycoprotein hormones. Conservation of nucleotides in the 3'-untranslated region of bovine and human pre-alpha subunit mRNAs. *J Biol Chem* 258:4679–4682.
- Olijve W, de Boer W, Mulders JW, van Wezenbeek PM. 1996. Molecular biology and biochemistry of human recombinant follicle stimulation hormone (Puregon). *Mol Hum Reprod* 2:371–382.
- Pierce JG, Parsons TF. 1981. Glycoprotein hormones: Structure and function. *Annu Rev Biochem* 50:465–495.
- Puissant C, Bayat-Sarmadi M, Devinoy E, Houdebine L-M. 1994. Variation of transferrin mRNA concentration in the

- rabbit mammary gland during the pregnancy-lactation-weaning cycle and in cultured mammary cells. A comparison with the other milk protein mRNAs. *Eur J Endocrinol* 130:522–529.
- Radola B. 1973. Isoelectric focusing in layers of granulated gels. I. Thin-layer isoelectric focusing of proteins. *Biochim Biophys Acta* 295:412–428.
- Sairam MR, Bhargavi G. 1985. A role of glycosylation of the alpha subunit in transduction of biological signal in glycoprotein hormones. *Science* 229:65–67.
- Samaddar M, Catterall JM, Dighe RR. 1997. Expression of biologically active β subunit of bovine follicle-stimulating hormone in the methylotrophic yeast *Pichia pastoris*. *Protein Expr Purif* 10:345–355.
- Suganuma N, Matzuk MM, Boime I. 1989. Elimination of disulfide bonds affects assembly and secretion of the human chorionic gonadotropin beta subunit. *J Biol Chem* 264:19302–19307.
- Ulloa-Aguirre A, Midgley AR, Beitins IZ, Padmnabhan V. 1995. Follicle-stimulating isohormones: Characterization and physiological relevance. *Endocr Rev* 16:765–787.
- van de Wiel DFM, van Rijn PA, Melloen RH, Moormann RJM. 1998. High-level expression of biologically active recombinant bovine follicle-stimulating hormone in a baculovirus system. *J Mol Endocrinol* 20:83–98.
- Van den Hout JMP, Reuser AJJ, De Klerk JBC, Arts WF, Smeitink JAM, Van der Ploeg AT. 2001. Enzyme therapy for Pompe disease with recombinant human α -glucosidase from rabbit milk. *J Inher Metab Dis* 24:266–274.
- Zinovieva N, Lassnig C, Schams D, Besenfelder U, Wolf E, Müller S, Frenyo L, Seregi J, Müller M, Brem G. 1998. Stable production of human insulin-like growth factor 1 (IGF-1) in the milk of hemi- and homozygous transgenic rabbits over several generations. *Transgenic Res* 7:437–447.
- Ziomek C. 1998. Commercialisation of proteins produced in the mammary gland. *Theriogenology* 49:139–144.