Expression of biologically active heterodimeric bovine follicle-stimulating hormone in milk of transgenic mice

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Communicated by Neal L. First, June 6, 1991 (received for review April 15, 1991)

ABSTRACT Follicle-stimulating hormone (FSH; follicitropin) is a pituitary glycoprotein composed of two post-translationally modified subunits, which must properly assemble to be biologically active. FSH has been difficult to purify and to obtain in quantities sufficient for detailed biochemical studies. We have targeted FSH expression to the mammary gland of transgenic mice by using cDNAs encoding the bovine α and FSHβ subunits and a modified rat β-casein gene-based expression system. Lines of bigenic mice expressing both subunits have been generated either by coinjection of the subunit transgenes or by mating mice that acquired and expressed transgenes encoding an individual subunit. Up to 60 international units (15 μg) of biologically active FSH per ml was detected in the milk of the bigenic mice. These lines provide a model system for studying the post-transcriptional mechanisms that effect the expression and secretion of this heterodimeric hormone.

Follicle-stimulating hormone (FSH; follicitropin) is a member of the glycoprotein family of pituitary hormones, which includes thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and chorionic gonadotropin (CG). Like LH and CG, FSH is a gonadotropin and is composed of a common α subunit that is noncovalently linked to a hormone-specific β subunit (1, 2). FSH has been difficult to purify and to obtain in quantities for detailed biochemical studies (for a review, see ref. 3). The α and FSHβ subunits are post-translationally modified, and the nature and extent of such modifications can exert a profound effect on subunit assembly, secretion, and stability (4-6). Only heterodimers with appropriately glycosylated subunits exhibit significant biological and receptor-binding activity (5, 7, 8). Targeting FSH to the mammary gland of transgenic animals would, therefore, serve as a model system in which to study glycoprotein processing and secretion as well as a means to produce large quantities of FSH. A standardized source of recombinant FSH would be useful to both human and livestock fertility programs to achieve the reproducible development of ovarian follicles.

Several different milk protein-based constructs have been employed to express diverse heterologous proteins in the milk of a variety of transgenic animals (for reviews, see refs. 9-11). We have demonstrated previously that a -524/+490 minimal rat β-casein promoter fragment can direct the expression of chloramphenicol acetyltransferase to the mammary gland (12). To determine whether the mammary gland could be used to secrete large quantities of a biologically active heterodimeric protein into milk, we have used a modified rat β-casein-based vector to target and express bovine FSH (bFSH) to the mammary gland and into the milk of transgenic mice.

MATERIALS AND METHODS

Construction of the Transgenes. The FSH subunit cDNAs were obtained from Genzyme; α as a 730-base-pair (bp) EcoRI fragment and FSHβ as a 560-bp EcoRI/BamHI fragment. The cDNA fragments were inserted into pUC19 (13) with the rat β-casein -524/+490 fragment (12) and an 850-bp EcoRI fragment carrying the simian virus 40 small tumor antigen intron with transcript cleavage and polyadenylation signals (kindly provided by S. Berget, Baylor College of Medicine). A 408-bp HindIII fragment of the mouse mammary tumor virus long terminal repeat (LTR) carrying four glucocorticoid response element (GRE) sequences (kindly provided by M. Parker, Imperial Cancer Research Fund Laboratories) was placed at -330 in the rat β-casein fragment of the α construct.

Production and Screening of Transgenic Mice. Transgenic mice were generated and mouse tail DNA was isolated as described previously (12). The polymerase chain reaction (PCR) was employed to screen for positive transgenic mice. The sequences of the synthetic oligonucleotides used in PCR reactions were as follows (5′ → 3′): 1, GAGCTTCATCCTCCCTCTCCGCT; 2, ACAGAGACAAATGGCCGAATGAC; 3, GCCTTAATTCTGCTCTTATCTCT; 4, TCTCTGAGTTAGTTGCTCAAATA; 5, AGGCCATTCCACCACTGCTCCATCATC; 6, AAAAGGAAAGAAGACTGGACAAGAAGCAGACT; and 7, TACTGACCTGCTCCTCCGAGGAT.

Transgene cointegration was analyzed by Southern blotting tail DNA (10 μg) digested with EcoRI. Blots were hybridized with 32P-labeled α- or FSHβ-specific probes prepared by random oligonucleotide labeling.

RNA Isolation and Analysis. Total RNA was isolated from mouse mammary gland tissue by the method of Chirgwin et al. (14). For Northern blots, RNA (20 μg) was fractionated in agarose gels containing formaldehyde (15). For slot blots, RNA (1, 2, or 4 μg) was applied to ZetaProbe membrane (Bio-Rad) and compared to known amounts of the α or FSHβ cDNAs included as standards. Quantitation was performed by scanning with an LKB laser densitometer.

Collection of Mouse Milk. Mice were anesthetized with 1 ml of Avertin (20 mg/ml) administered i.p. immediately prior to milking, and 0.5 ml of oxytocin [200 international units (IU)/ml; Sigma] was administered i.p. before milk samples were harvested by gentle suction into tubes at 4°C. The whey fraction was prepared by centrifugation of skim milk at 16,000 × g for 15 min at 4°C.

Abbreviations: FSH, follicle-stimulating hormone (follicitropin); bFSH, bovine FSH; rFSH, recombinant FSH; oFSH, ovine FSH; GRE, glucocorticoid response element; IU, international units.

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Characterization of FSH in Mouse Milk. A heterologous double-antibody radioimmunoassay (RIA) was performed as described (16). For immunoblot analysis, whey protein (300 μg) was fractionated by SDS/PAGE at room temperature (15). Samples were not heated and did not contain 2-mercaptoethanol. Ovine FSH (oFSH; NIADDK-oFSH-16; 20 National Institutes of Health units/mg) was added to nontransgenic mouse milk for positive controls. A sample of recombinant bFSH (rbFSH) made in Chinese hamster ovary (CHO) cells (a gift of Genzyme) was used to assess the cross-reactivity of the antibody in this assay. Blots were probed with the JAD-17-689 antiserum (16:1:5000), kindly provided by J. Dias (State of New York Department of Health), and developed with a goat anti-rabbit IgG–horseradish peroxidase enhanced chemiluminescence (ECL) detection scheme (Amersham).

For radioreceptor assays, samples were initially diluted with an equal volume of assay buffer (100 mM Tris-HCl/100 mM sucrose/5 mM MgCl₂/0.1% bovine serum albumin, pH 7.4) and incubated with a chicken testis receptor preparation (17). The standard was NIH-FSH-S9 (18). Data analysis was by the ALLFIT(FLEXIFIT) program, version 2.6 (Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development). Calculation of the ng of rbFSH was based on the specific activity of bFSH (19). Calculation of FSH activity was from the ED₅₀ of the assay.

Acid-dissociation radioreceptor assay experiments (20) measured FSH activity in 50 μl of milk surviving 1 M propionic acid treatment for 1 hr at 37°C. Precipitated casein and other milk proteins were removed by centrifugation. The granulosa cell bioassay and chromatofocusing analysis were performed as described (5, 21).

RESULTS AND DISCUSSION

Characterization of the α and FSHβ Transgenes. The cDNAs encoding α or FSHβ were placed into a rat β-casein expression vector (Fig. 1). Lines of transgenic mice were generated by either individual or coinjection (12, 22) of α and FSHβ constructs. A construct carrying four copies of a GRE from the mouse mammary tumor virus promoter (Fig. 1B) was also employed to direct high-level α-subunit expression, since in the normal pituitary α is expressed in excess over the dimeric hormone (23–27). Screening by PCR identified founder animals carrying either the α or the FSHβ construct or both (Fig. 1D and E).

Southern blot experiments were used to characterize the architecture of the integrated transgenes. Since the transgenes carry a single EcoRI site, the detection of strongly hybridizing species in the 2- to 3-kb range (Fig. 2A and B) is diagnostic for transgene cointegration. When the α or FSHβ transgenes were coinjected, multiple copies were found to be cointegrated in >85% of the positive lines. Only a few lines carried individual transgenes [e.g., line 7905 carries a single α transgene (Fig. 2A and B, lane 1)]. Some head-to-head and tail-to-tail cointegration events occurred. Divergent PCR confirmed the head-to-tail orientation (Fig. 2C).

Expression of α and FSHβ mRNAs in Mammary Glands of Lactating Transgenic Mice. Northern blot analysis indicated the presence of major 1519-nucleotide α and 1340-nucleotide FSHβ mRNA species (Fig. 3) corresponding to the expected transcript sizes. The smaller α mRNA species (Fig. 3A, lane 3) may arise from cleavage and polyadenylation of sequences within the 3' untranslated region of the α cDNA (28, 29). Examination of the transcripts by reverse transcriptase-mediated PCR indicated that most α and FSHβ mRNA species encode unit-length proteins (N.M.G. and J.M.R., unpublished results).

When Northern blots were rehybridized with a mouse β-casein exon 7 probe, the β-casein mRNA level was found to be ~5- to 10-fold greater than that observed for the α-subunit mRNA (data not shown). Since β-casein mRNA has been estimated to make up ~20% of the total mRNA at day 10 of lactation, the level of the α-subunit mRNA should correspond, therefore, to ~2% of the total mRNA, in agreement with the quantitative slot blot determination (see below).

Two-thirds of the mice carrying the GRE-enhanced α construct expressed the transgene, whereas only one-sixth of those lacking the GRE expressed α. Of mice carrying minimal α and FSHβ constructs, 3 of 11 (27%) expressed both transgenes, while 6 of 10 (60%) expressed the cointegrated GREα and FSHβ constructs. Lines of transgenic mice carrying the GRE-enhanced constructs expressed more frequently (30, 31) and at higher levels (see below). Line 7905 (single copy of GREα) has been bred to line 7502 (4 to 6 copies of a tandemly arranged FSHβ) to establish line 2038 (Fig. 1D and E), which expressed both independent loci (Fig. 3).

Secretion of rbFSH into Mouse Milk. rbFSH was detected in milk collected at lactation (Fig. 4A), but not in milk from nontransgenic littersmates, by using a heterologous double-
antibody RIA. Proteins present in normal mouse milk did not interfere with the assay.

The species of FSH present in milk were further characterized by immunoblotting (Fig. 4B). Preparations of pituitary oFSH (lanes B, C, and D in Fig. 4B) and rbFSH prepared from transfected CHO cells (lane 1 in Fig. 4B) were included as controls. The antiserum to oFSH detected a species of ~38 kDa in the milk from bigenic mouse 8942 (lanes E and K in Fig. 4B). The 38-kDa species corresponds in size to the species detected in both the oFSH and rbFSH standards. Some microheterogeneity in the post-translational modifications of the FSH may explain the broad bands observed (see chromatofocusing results) (32, 33). One microgram of the oFSH standard (lane B) gave a much stronger signal at 38 kDa than an equivalent amount of the CHO rbFSH protein (lane I), reflecting that the antiserum was raised against oFSH rather than bFSH.

A strongly immunoreactive species with a mass of 18 kDa was detected in the milk from bigenic mice, as well as from mice expressing only the α-subunit mRNA and may be free α subunit. This was not detected in the control milk sample. The immunoblot and RIA results suggest the polyclonal anti-oFSH antiserum can crossreact with both heterodimer and free α subunit, and it may contain species recognizing free bovine α and heterodimer, but with different affinities. Therefore, the immunoblot could not be used to quantitatively determine the relative abundance of α and rbFSH.

Steady-state α and FSHβ mRNAs were quantitated by slot blot hybridization analysis. Summarized in Table 1, α mRNA levels were consistently higher, 7- to 17-fold, than those for FSHβ mRNA. Levels of both mRNAs were independent of transgene copy number; line 7905 carries a single GREα transgene yet expresses high levels of α mRNA. Consistent with previous results, expression appeared to be highly dependent on the site of integration (11, 12, 31), and the level of mRNA was observed to vary as much as 3-fold between littermates (e.g., α-FSH mRNA, line 7919). The relatively high α and low FSHβ mRNA levels suggest that post-transcriptional mechanisms influence their steady-state levels, supporting the hypothesis that the 3' untranslated region of FSHβ mRNA may impart instability (34).

Milk of bigenic mouse 8942 was capable of displacing an

\[ ^{125}\text{I}-\text{labeled purified porcine FSH preparation from chicken testis FSH receptors (Fig. 4C) (2, 17). No displacement was observed when milk from a nontransgenic littermate was used. The calculated competitive binding displacement (single point assay, Fig. 4C) of milk treated with 1 M propionic acid was equivalent to only 82 ng of FSH as compared to 2000 ng of FSH in 50 µl of untreated milk, representing 96% inactivation. \]
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Fig. 4. Characterization of rbFSH in milk of transgenic mice. (A) RIA using rabbit antiserum JAD-17-679. Standard curves: (●), USDA B5 standard; (▲), USDA B5 standard in nontransgenic milk. Sample inhibition curves are (values obtained for FSH in milk calculated from the dilution giving 50% inhibition in parentheses); (○), milk 8942 (2.4 mg/ml); (□), milk 8611 (2.5 mg/ml); (□), milk 1262 (0.65 mg/ml). (B) Immunoblot analysis of FSH in milk. Milk samples fractionated by SDS/PAGE were probed with JAD-17-679. Lanes A–D, normal mouse milk with 0, 1, 0.5, 0.25 µg of oFSH added, respectively. Lanes E-H are milk from mice (line number in parentheses) 8942 (7919), 9667 (7905), 9434 (7502), and 2038 (2038), respectively. Lane 1, CHO rbFSH (200 µg of total protein; 1.25 µg of FSH). Lanes J and K are 2-hr exposures of lanes D and E. Lanes L and M show lanes equivalent to D and E from a gel run in parallel and stained with Coomassie blue. (C) Competitive binding experiments for rbFSH in milk, using a chicken testis radioreceptor assay. Samples used were FSH (NIH-FSH-S9; ○), milk from transgenic mouse 8942 (●), and milk from a nontransgenic mouse (▲). The putative ng of rbFSH has been plotted in comparison with the ng of NIH-FSH-S9 used in the assay (to avoid the weight-to-dilution comparison). A comparable dilution for the control milk (●) is shown on the same scale. A sample of milk 8942 treated in 1 M propionic acid for 1 hr at 37°C (♦) was also analyzed. (D) Analysis of bioactive rbFSH by granulosa cell aromatase assay. Granulosa cell cultures were treated with increasing aliquots of milk whey protein fractions from transgenic and control mice. Symbols as in A; data are mean ± SEM.

These results, summarized in Table 1, indicate that the rbFSH secreted into milk can interact with FSH receptors.

To measure the biological activity of the rbFSH, rat granulosa cell in vitro bioassays were utilized (Fig. 4D). In granulosa cells, FSH stimulates both the conversion of cholesterol to pregnenolone and the aromatization of the estrogen precursor androstenedione (33). The results are summarized in Table 1. Milk samples from independent bigenic lines (mice 7994, 1262, 8611, and 8942) contained high levels of biologically active FSH. High FSH activity was detected in milk from line 2038, while milk from the parental lines contained no detectable bioactive FSH. Therefore,

<table>
<thead>
<tr>
<th>Line</th>
<th>Mouse</th>
<th>Construct</th>
<th>ng α mRNA/µg total RNA</th>
<th>ng FSHb mRNA/µg total RNA</th>
<th>A/B ratio</th>
<th>Radioreceptor assay</th>
<th>Granulosa bioassay</th>
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<tr>
<td>ICR</td>
<td>9667</td>
<td>GREα</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>ND</td>
<td>&lt;0.1 (n = 1)</td>
<td>&lt;0.1 (n = 1)</td>
</tr>
<tr>
<td>7905</td>
<td>9667</td>
<td>GREα</td>
<td>10.8</td>
<td>&lt;0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7502</td>
<td>9434</td>
<td>FSHβ</td>
<td>&lt;0.1</td>
<td>0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7398</td>
<td>7994</td>
<td>aFSHβ</td>
<td>3.8</td>
<td>0.2</td>
<td>17.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7485</td>
<td>1262</td>
<td>aFSHβ</td>
<td>1.4</td>
<td>0.2</td>
<td>7.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7667</td>
<td>8611</td>
<td>GREαFSHβ</td>
<td>2.8</td>
<td>0.2</td>
<td>12.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7919</td>
<td>7919</td>
<td>GREαFSHβ</td>
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<td>5.6</td>
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</tr>
<tr>
<td>7919</td>
<td>8941</td>
<td>GREαFSHβ</td>
<td>2.8</td>
<td>0.3</td>
<td>10.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7919</td>
<td>8942</td>
<td>GREαFSHβ</td>
<td>6.6</td>
<td>0.5</td>
<td>13.6</td>
<td>66.7 ± 1.5 (n = 6)</td>
<td>ND</td>
</tr>
<tr>
<td>2038</td>
<td>2038</td>
<td>GREα × FSHβ</td>
<td>8.2</td>
<td>0.7</td>
<td>11</td>
<td>ND</td>
<td>36 (n = 1)</td>
</tr>
</tbody>
</table>

Values in columns A and B are rounded off. The A/B ratio is accurate to two significant figures. FSH subunit mRNA levels are expressed as ng of specific mRNA per µg of total RNA per assay. To assay FSH in milk, samples harvested from lactating female mice were characterized by the radioreceptor and in vitro granulosa cell bioassays; results are given ± SEM. ND, not determined.
Fig. 5. Analysis of rbFSH by chromatofocusing. Samples of rbFSH from bigenic mouse milk or transfected CHO cells (Genzyme) were chromatographed on a PBE-94 column (Pharmacia). The pH (●) and ability to stimulate estrogen synthesis by 8942 milk (♦; 800 ng of FSH) and CHO FSH (▲; 2.5 µg of FSH) were determined from alternate fractions.

Production of estrogen in the granulosa cell assay was specifically related to the presence of heterodimeric FSH. The amounts of rbFSH in milk from mouse 8942 determined by the radioreceptor and granulosa cell assays were qualitatively similar. This value of 66 IU/ml corresponds to ~15.3 µg of FSH per ml, assuming 1 µg of FSH = 4 IU of FSH (19). No adverse reproductive consequences were observed in the expressing lines, and rbFSH was not detected in the serum collected from lactating animals (D. Bolt, personal communication), suggesting that rbFSH is secreted vectorially into milk. Normal patterns of transgene transmission and expression have been observed in several litters of offspring for all our expressing lines.

Since isoforms of FSH can be separated on the basis of their isoelectric properties, which are, in part, related to terminal sialic acid content (32, 33) chromatofocusing analysis was performed. Fractions from 8942 milk (4 IU total) and the CHO rbFSH (10 IU total) were collected, the pH was measured, and FSH activities were determined by granulosa cell bioassay. The 8942 rbFSH had one major peak of activity between pH values 6.1 and 4.2 (Fig. 5) and the CHO rbFSH profile exhibited a similar single peak (pH 5.2 to 4.0). Both profiles are consistent with the observations of Galway et al. (5) for FSH with appropriate N-linked carbohydrate structures. The broader peak observed for transgenic rbFSH probably reflects the capacity of the mammary gland to add terminal sialic acid residues to these proteins. The lack of terminal sialic acid residues does not affect FSH receptor binding or in vivo bioactivity but may, however, be related to enhanced clearance rates for FSH in blood plasma (5, 32).

By several independent criteria we have demonstrated that rbFSH can be produced in transgenic mice using a rat β-casein expression system. As post-transcriptional mechanisms are probably responsible for the differences observed in the relative levels of the α and FSHβ subunit mRNAs, it may be possible with appropriate engineering to express a more stable FSHβ mRNA, thereby increasing the levels of rbFSH in milk. For example, a “second generation” of transgenes has been constructed to determine whether higher steady-state levels of FSHβ mRNA will result from the precise exchange of FSHβ and α cDNA open reading frames. The lines of mice bearing the individual α, and FSHβ transgenes provide useful models for the study of the mechanisms regulating the post-translational processing of both the individual subunits and the heterodimer. Finally, these studies have demonstrated that the mammary gland can be used as a bioreactor to direct the high expression and vectorial secretion into milk of heterodimeric proteins requiring extensive post-translational modifications. Although the quantities of glycosylated hormone produced in mice are sufficient for further biochemical analysis, the introduction of such transgenes into livestock (9) will be required to provide sufficient quantities for both research and commercial purposes.

We thank T. Duffy, F. Farzam, C. Nichols, and Hyun Nahm for technical assistance. Hormone standards were gifts of the U.S. Department of Agriculture Animal Hormone Program. This work was supported in part by the U.S. Department of Agriculture (Grant 88-37266-3951 to J.M.R.) and Granada Biosciences (J.M.R.) and the National Institutes of Health (Grant DK-09901 to D.N.W.).