High-level expression of biologically active recombinant bovine follicle stimulating hormone in a baculovirus system

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ABSTRACT

Superovulation treatment of cows can benefit from the application of very pure recombinant bovine FSH (rbFSH), which is produced in non-mammalian cells. rbFSH is completely free of LH, and therefore can possibly reduce the variability in the results of superovulation. Furthermore, it does not contain brain-tissue-derived proteins and, when produced under serum-free conditions, it is free of other mammalian substances or potentially infectious material. We have produced rbFSH in insect cells, with the ultimate aim of inducing superovulation in cattle. Sf21 insect cells were coinfecting with two recombinant baculoviruses, containing the cDNAs of bovine FSH α - and β -subunits respectively. High levels of production of bioactive rbFSH were obtained after cloning cDNA that contained a major part of the 3' untranslated region of the bFSH β gene. Maximum production of rbFSH 1–5 μ g/ml (as measured by immunoassay) was obtained 70–90 h after infection. The recombinant material was highly potent in two *in vitro* bioassays, giving biological activities of 13 IU/ml (Y_1 cell rounding assay), 22 IU/ml (Y_1 cell cAMP assay), and 23 IU/ml (bovine

oocyte maturation inhibition assay), and had a lower but significant activity of 6 IU/ml in the rat Sertoli cell assay. rbFSH was purified by immunoaffinity chromatography, using a monoclonal antibody directed against the human FSH β -subunit. The purified heterodimer appeared to be homogeneous by SDS-PAGE, whereas the free β -subunit appeared as a doublet, possibly indicating differently glycosylated forms. Intact heterodimer and both subunits were further identified by western blot analysis, and showed apparent molecular masses of 20 kDa (α -subunit), 23 kDa (β -subunit) and 32.5 kDa (heterodimer). This insect-cell-produced rbFSH did not bind to wheat germ agglutinin, thus indicating that glycosidic side-chains may not contain terminal sialic acid. The relevance of a large 3' untranslated region in bFSH β cDNA to the level of production of rbFSH, and the possible implications of the pattern of glycosylation for the biological activity of the recombinant hormone are discussed.

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INTRODUCTION

Follicle stimulating hormone (FSH) belongs to the family of glycoprotein hormones which are produced either in the pituitary (luteinizing hormone, LH; thyroid stimulating hormone, TSH) or in the placenta (human chorionic gonadotrophin, hCG; pregnant mare serum gonadotrophin, PMSG).

Within a species, each of these hormones consists of a common α -subunit which is non-covalently bound to a hormone-specific β -subunit. Purified FSH, administered alone or in combination with LH, is used widely to induce a superovulatory response in many species, including cattle.

A problem in the cow is that bovine FSH (bFSH) is difficult to purify in substantial quantities from

bovine pituitaries (Wu *et al.* 1993). For this reason, FSH of ovine (oFSH), porcine (pFSH) or equine (eFSH, PMSG) origin is commonly used for superovulation treatment of cows (Staigmiller *et al.* 1992). However, application of brain-tissue-derived material is not free of risk, because of the possible presence of prion proteins, which can cause bovine spongiform encephalopathy in cows, and possibly a variant of Creutzfeldt–Jacob disease in humans (Lasmézas *et al.* 1996). Placenta-derived material, such as PMSG, has the disadvantage of a very long biological half-life, which necessitates its neutralization by the injection of specific antibodies (Bouters *et al.* 1983). Furthermore, stimulation of the target animal should preferably be with a homologous hormone preparation, as indicated by Hakola *et al.* (1997). Finally, all currently used FSH preparations do contain some LH activity, which is considered responsible, at least in part, for the observed large variation in the results of superovulation (Kelly *et al.* 1997).

For these reasons, it seems likely that superovulation treatment of cows would benefit from the homologous application of very pure recombinant bFSH (rbFSH). Recombinant FSH has been produced in mammalian systems such as chinese hamster ovary (CHO) cells for the human (Keene *et al.* 1989, Van Wezenbeek *et al.* 1990, Cerpa-Poljak *et al.* 1993, Roth *et al.* 1993), ovine (Mountford *et al.* 1994) and rat (Hakola *et al.* 1997) species, whereas for the bovine species it has been produced in mouse epitheloid cells (Chappel *et al.* 1988), CHO cells and transgenic mice (Greenberg *et al.* 1991).

rbFSH as produced in a mammalian cell system has been used to produce superovulation in cattle (Looney *et al.* 1988, Wilson *et al.* 1993). Mammalian cells have the advantage that they possess the apparatus for addition of complex-type glycosidic side-chains; however, expression of recombinant proteins is low compared with that in insect cells (Grossmann *et al.* 1997).

Recombinant FSH in insect cells has been produced for the human (Lindau-Shepard *et al.* 1994) and porcine species (Sato *et al.* 1993). Recently, it has been shown that rpFSH as produced by insect cells is biologically active *in vivo* (Inaba *et al.* 1997). For the bovine species, recombinant FSH subunits have been produced in insect cells by Sharma *et al.* (1993) (α - and β -subunit) and by Grotjan *et al.* (1996) (β -subunit), but no data have been published about intact bovine heterodimer. The purpose of our study was to produce rbFSH in the baculovirus expression system in amounts sufficient to permit its application in cows, and to evaluate some of its biochemical, immunological and biological characteristics.

MATERIALS AND METHODS

Construction of transfectors and recombinant viruses

cDNA coding for bFSH α (Leung *et al.* 1987) and cDNA coding for bFSH β (Maurer & Beck 1986) were cloned separately into baculovector pARKh1, according to procedures described previously (Hulst *et al.* 1993). Briefly, plasmid pARKh1 (Fig. 1) was used for insertion of bFSH subunit genes. This vector is a derivative of transfectant pAcMo8 (J M Vlak & M M van Oers, personal communication), which is identical to vector pAcAs3 (Vlak *et al.* 1990) except for an additional T immediately preceding the BamHI site, and thereby introducing an ATG start codon. pARKh1 contains unique NcoI, StuI, XbaI and BamHI sites, with the NcoI site overlapping the ATG start codon. The unique StuI site allows for the exchange of vector DNA for bFSH β in two parts. The 5' part was obtained by PCR, and the 3' part by regular DNA isolation from miniprep plasmid DNA of BovFSHBeta-pGEM3. This strategy was chosen in order to minimize errors, which can be introduced by amplification via PCR.

DNA coding for bFSH β was obtained by amplification of the relevant region of BovFSHBeta-pGEM3 by PCR. A 39-cycle amplification (60 s at 94 °C/60 s at 55 °C/60 s at 72 °C) was performed with Vent DNA polymerase. The sequences of the synthetic oligonucleotides used in PCR are as follows (5'→3'):

1. CCTGAGAGATCTATCATGAAGTCTGTCCAGTCTCTG

BspHI
2. GAGGGATCCAGATCTAGAGGATTAGGTGACACTATA

XbaI
BglII

A BspHI/StuI-digested PCR product encoding the N-terminal part of the bFSH β -subunit was cloned into the NcoI/StuI sites of pARKh1, resulting in the transfectant, pDW-Beta-1.

Plasmid BovFSHBeta-pGEM3 was digested with StuI plus BglII. A 1106 bp fragment encoding the C-terminal part of the bFSH β -subunit was ligated into the StuI/BamHI sites of vector pDW-Beta-1, resulting in transfectant pDW-Beta-3.1. This plasmid contains a 1454 bp bFSH β fragment consisting of a 57 bp 5' fragment encoding the signal sequence, a 330 bp fragment coding for bFSH β and a 1067 bp 3' untranslated region (UTR), and it has an ATG codon exactly at the start of the signal sequence (Fig. 1).

FIGURE 1. Scheme of the construction of transfer vectors pDW-Alpha-9.1 and pDW-Beta-3.1. Arrows show the directions of transcription. Ac, AcNPV DNA; p10, p10 promoter; Phs, *Drosophila melanogaster* hsp promoter; SV₄₀, SV₄₀ transcription termination sequence; Lac Z, *E. coli* lac Z gene; PCR, polymerase chain reaction; Amp, ampicillin resistance gene. Vector DNA is exchanged for cDNA of bFSH β -subunit in two parts: first a BspHI site is introduced by PCR at the 3' end of the signal sequence of bFSH β cDNA. This site is used for exchanging a BspHI/StuI excised part of the gene for the NcoI/StuI excised vector DNA. This is followed by exchange of the remaining StuI/BglII part of the bFSH β cDNA for the StuI/BamHI excised vector DNA.

The entire BspHI/StuI fragment was sequenced, in order to check for possible errors introduced by PCR.

The cDNA coding for bFSH α was purified after double digestion of the plasmid BovFSHA α -pSP64 No. 1 with NcoI and XbaI. The DNA of 554 bp contains a signal sequence of 72 bp at the 5' end, and an untranslated region of 188 bp at the 3' end. It was cloned into the unique NcoI and XbaI sites of vector pARKh1. The NcoI site encompasses the ATG codon of the signal sequence of bFSH α . The constructed transfer vector is designated pDW-Alpha-9.1 (Fig. 1).

Construction and selection of baculovirus recombinants expressing rbFSH α or rbFSH β was

according to procedures published previously (Hulst *et al.* 1993). Briefly, the constructed plasmids were transfected into insect cells (Sf21) by the calcium phosphate precipitation technique. Recombinant viruses were selected by plaque purification based on LacZ staining; high-producing recombinants were selected on the basis of measurement of subunits in the culture medium by ELISA. After double infection with both a recombinant virus producing bFSH α and a recombinant virus producing bFSH β at a multiplicity of infection (MOI) of 5–10 50% tissue culture infective doses (TCID₅₀) per cell, media were collected, centrifuged at 3000 r.p.m. for 10 min, sterilized by filtration (0.2 μ m Acrodisc), and analysed for rbFSH heterodimer by

immunoassay or *in vitro* bioassay. For routine purposes, rbFSH-containing media were harvested 72 h post infection (p.i.).

DNA analysis, radiolabeling and radioimmunoprecipitation

The methods used were as described by Hulst *et al.* (1993). (See also the legend to Fig. 2).

Immunoaffinity chromatography

Recombinant bFSH was purified by immunoaffinity chromatography, using a monoclonal antibody to the human FSH β -subunit coupled to CNBr-activated Sepharose (Sepharose 4B, Pharmacia, Uppsala, Sweden) according to the procedures recommended by the manufacturer. rbFSH was eluted with 0.1 M glycine HCl-0.1 M NaCl buffer at pH 4.0 (6 ml), pH 3.5 (6 ml), pH 3.0 (7 ml), pH 2.5 (6 ml) and pH 2.0 (5 ml) and 1 ml fractions were collected on ice; the pH was immediately neutralized with 3 M Tris.

All fractions were stored at -20°C until required for assay, or used for SDS-PAGE.

SDS-PAGE and western blot

Before electrophoresis (45 min at 200 V, stacking 3 min at 120 V), samples were concentrated either by Centricon 10 (Amicon, Beverly, MA, USA) filtration (3000 r.p.m. for 2 h at room temperature) or by precipitation with alcohol (addition of $10\times$ sample volume of ethanol, incubation for 10 min on ice, centrifugation at 13 000 r.p.m. for 15 min at room temperature). Centricon-filtrated samples were mixed with equal volumes of $2\times$ concentrated sample buffer according to Laemmli (1970), but without β -mercaptoethanol; alcohol precipitates were dissolved in half the original volume of sample buffer. Samples were applied onto SDS-PAGE (12%), without previous heating. This was done in order to preserve the heterodimer integrity of the FSH molecule. Silver staining was performed according to Morrissey (1981), except for the glutaraldehyde step. Western blotting onto 0.45 μm nitrocellulose paper was carried out according to established procedures (Laemmli 1970, Burnette 1981). Immunostaining after incubation with rabbit anti-guinea pig immunoglobulin peroxidase (DAKO, Glostrup, Denmark) was carried out using 3,3'-diaminobenzidine tetrahydrochloride (De Blas & Cherwinski 1983) as chromogen.

Hormones and antisera

Hormones

For immunoassays (ELISA, antigen capture assay, western blot) the following hormones were used as reference preparations: bovine LH α (J Closset/G Hennen, University of Liège, Belgium), or bLH α -AFP-3111A (A F Parlow, UCLA, CA, USA), bovine FSH β (USDA-bFSH- β -subunit; D J Bolt, USDA, Beltsville, MD, USA) and bovine FSH (i 058, UCB, Brussels, Belgium), or USDA-bFSH-I-2 (D J Bolt, USDA, Beltsville).

For *in vitro* bioassays (Y_1 cell assay, Sertoli cell assay) we used as reference preparation: bovine FSH [USDA-bFSH-I-2 (AFP 5318C)] with a biological potency of 854 IU/mg in terms of the second International Reference Preparation of human menopausal gonadotrophin (IRP-HMG; as determined by the hCG Augmentation Assay).

oFSH from Sigma (50 U/vial with reference to the National Institutes of Health standard NIH-FSH-S17) (Sigma Chemical Company, St Louis, MO, USA) was used as reference preparation in the oocyte maturation inhibition bioassay.

For conversion into IU (international units in terms of the second IRP-HMG), it was assumed that NIH-FSH-S17 is identical to NIADDK-oFSH-17, which has a biopotency of 20 U/mg in relation to NIH-FSH-S1; furthermore, it was extrapolated from the USDA-oFSH-19-SIAFP data sheet that 1 U(S1)=25 IU.

Antisera

For ELISA, radioimmunoprecipitation (RIP) and western blotting, antisera were used that were raised in guinea pigs, and which were specific for either bovine FSH α (No. 5551791, A F Parlow) or bovine FSH β (No. 899691, A F Parlow). RIP was also carried out with commercial monoclonal antibodies against either human FSH α or FSH β (clones ME.111 and ME.112 respectively; Maine Biotechnology Services, ME, USA).

In the antigen capture assay, the capturing antibody was commercial monoclonal antibody against human FSH β (ME.112), which has been shown to cross-react with both bovine FSH β and intact bovine FSH $\alpha\beta$ heterodimer. As a second antibody, we used guinea pig anti-bFSH α antisera (No. 5551791, A F Parlow).

ELISA and antigen capture assay

bFSH α - and bFSH β -subunits, expressed by recombinant viruses, were detected by specific ELISA systems. M96 plates (Costar, Cambridge, MA, USA) were coated (0.05 M carbonate buffer, pH 9.65 for 1 h at 37°C , or pH 9.65 overnight at

4 °C) with culture medium (maximally 10 µl/well) collected from Sf21 cells that had been infected with recombinant viruses containing either bFSH α or bFSH β cDNA. Coated wells were then incubated for 1 h at 37 °C with 1 : 1000 diluted polyclonal guinea pig bFSH α or bFSH β antisera. Bound immunoglobulins were detected by incubation with 1 : 500 diluted rabbit anti-guinea pig IgG coupled to horseradish peroxidase (RAGPPO, DAKO) for 1 h at 37 °C, followed by addition of substrate solution 100 µl/well (0.001% tetramethylbenzidine (wt/vol) + 0.005% H₂O₂ (vol/vol) in 0.1 M sodium acetate buffer, pH 6.0; 10 min shaking on a platemixer) and 100 µl 0.5 M H₂SO₄. Optical density was measured at 450 nm. Purified pituitary bFSH α and bFSH β were used as reference preparations (1, 10, 20, 40 or 80 ng/well) for quantitative measurement. Standards were pre-diluted in culture medium that had been collected from Sf21 cells infected with wild-type baculovirus (MO21).

Bovine FSH $\alpha\beta$ heterodimer expressed after double infection (at MOI 5–10) with bFSH α - and bFSH β -cDNA-containing recombinant viruses was detected by antigen capture assay (ACA). Briefly, monoclonal antibody against human FSH β was used as capture antibody at a dilution of 1 : 100 (1 µg/100 µl/well) by coating it on an M96 well (1 h at 37 °C). Culture medium (maximally 100 µl/well) harvested from doubly infected Sf21 cells was incubated in coated wells for 1 h at 37 °C and bound bFSH $\alpha\beta$ was detected by sequential incubation for 1 h at 37 °C with 1 : 1000 diluted polyclonal guinea pig anti-bFSH α and RAGPPO.

The substrate reaction was as described for the ELISA. Purified pituitary bFSH $\alpha\beta$ prediluted in MO21 culture medium was used as reference preparation (1–80 ng/well) for quantitative measurements.

(It should be noted that measurement of bFSH in this system may lead to underestimation because of possible blocking of capture antibody by free bFSH β -subunits.)

Lectin ELISA

Affinity-purified rbFSH fractions (10 × concentrated by Centricon filtration) were analysed for carbohydrate structure by lectin ELISA according to the method of Rafferty *et al.* (1995), except that proteins to be analysed were coated directly onto M96 wells. Briefly, 1–20 µl of test solution were incubated for 1 h at 37 °C with 90 µl of coating buffer (0.05 M sodium carbonate, pH 9.6) in an M96 well (Costar). After being washed with PBS–0.05% Tween-80, the wells were incubated for

2 h at 37 °C with biotinylated wheat germ lectin (WGA; Sigma) 1 µg/well in 20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 0.05% Tween-20, 0.2% BSA (Langeveld *et al.* 1991). The plates were washed and incubated for 1 h at 37 °C with 100 µl 1 : 250 diluted streptavidin–horseradish peroxidase (DAKO). Plates were developed with 100 µl substrate solution, as described for ELISA and ACA.

Y₁-cell bioassay

Y₁ mouse adrenal cells, stably transfected with cDNA for the human FSH receptor (coupled to the gene for resistance to methotrexate) were kindly donated by ARES-Serono, Rome, Italy. Those cells respond to FSH stimulation by accumulation of cAMP, progesterone synthesis and a change in cell morphology. Unstimulated cells grow flat on the surface, but after addition of a cAMP-stimulating agent the cells round off. This change in cell morphology is maximal after 2–3 h and disappears after approximately 7 h. The rounding-off shows good correlation with cAMP accumulation (Westhoff *et al.* 1996). Cells were plated in M96 plates in Ham's F10 medium (Gibco BRL, Breda, The Netherlands) supplemented with 2 mM L-glutamine. The incubation with FSH was carried out in Ham's F10 medium, and after 2 h the rounding-off was determined by light microscopy. One hundred microlitre aliquots of media were then harvested for determination of cAMP (cAMP ³H assay systems, Amersham TRK 432, UK).

The minimal dose giving a significant response in the Y₁ cell assay was 2 ng/ml for bFSH (USDA-bFSH-I-2), 30 ng/ml for oFSH (NIADDK-oFSH-16, AFP-5592C) and 200 ng/ml for pFSH (NIH-FSH-P-1).

Rat Sertoli cell bioassay

The rat Sertoli cell bioassay was performed as described by Oonk & Grootegoed (1987), with modifications as described by Westhoff *et al.* (1996). Briefly, 3-week-old Wistar rats were killed by CO₂ inhalation and the testes were removed quickly, decapsulated and weighed, then treated with collagenase in PBS for 30 min at 37 °C and the tubuli isolated by treatment with DNase (2 min gravity sedimentation). After further treatment with collagenase and DNase, Sertoli cells were obtained by treatment with a dounce homogenizer and centrifugation (2 min at 100 g). Cells were plated in M24 wells and incubated for 3 days in the absence of hormones. After incubation with hormone for 1 h at 32 °C, culture media were harvested and analysed

for cAMP concentrations (cAMP ^3H assay systems, Amersham). 17β -Oestradiol (E_2) synthesis was measured after the cells had been incubated with 10^{-7} M testosterone and the culture media analysed for E_2 by RIA (DPC Laboratories, Los Angeles, CA, USA).

Oocyte maturation inhibition bioassay

Before birth, mammalian oocytes are arrested at the diplotene stage of prophase I. *In vitro*, meiotic maturation is mediated by the release of oocytes from their ovarian follicles into a suitable culture medium. In cattle, in contrast to the mouse, both protein synthesis and RNA synthesis are required for meiotic maturation. This maturational process can easily be observed by the disappearance or breakdown of the germinal vesicle (GVBD) of the oocyte. The specific inhibitor, α -amanitin, when added to an appropriate FSH-containing culture medium, can be used to block mRNA synthesis, and thus prevent GVBD. Bovine oocyte cumulus complexes were isolated from fresh slaughterhouse material and tested for maturation inhibition (i.e. absence of GVBD) by α -amanitin in the presence of FSH, according to the method of Hunter & Moor (1987) with modifications described by De Wit *et al.* (1996). As a positive control we used 2 or 10 μl oFSH (Sigma, 50 U(S1)/vial, 12.5 U/ml). Test samples of SF900 media containing bFSH α , bFSH β or bFSH $\alpha\beta$ were added at a ratio of 104 μl : 2.5 ml culture medium. As a negative control, culture medium without α -amanitin and FSH was used. Oocytes were cultured for 24 h in 100 μl drops (20 oocytes per drop) M199 with Earle's salts (Flow Laboratories, ICN, Zoetermeer, The Netherlands) supplemented with 10% fetal calf serum, 20 $\mu\text{g}/\text{ml}$ α -amanitin (Boehringer, Mannheim, Germany) and 0.05 U(S1)/ml oFSH (control) or baculoculture medium (test sample), under paraffin oil. Culture was carried out at 39 °C in a humidified atmosphere of 5% CO_2 in air. In order to assess nuclear stage, a group of oocyte cumulus complexes was denuded mechanically with the help of a finely drawn Pasteur pipette, and mounted on slides. The oocytes were fixed in acetic acid : ethanol (1 : 3), stained with 1% acetoorcein and examined under a phase-contrast microscope (400 $\times$) (Kastrop *et al.* 1991).

RESULTS

Construction and selection of recombinant viruses expressing bFSH α or bFSH β

Transfervectors pDW-Alpha-9.1 and pDW-Beta-3.1 were constructed as depicted in Fig. 1.

Sf21 cells were cotransfected with pDW-Alpha-9.1 or pDW-Beta-3.1 and wild-type (wt) AcNPV/MO21 DNA isolated from extracellular virus particles.

Polyhedrin-positive plaques expressing β -galactosidase were isolated and analysed for expression of bFSH α or bFSH β by ELISA of culture media. One plaque-purified bFSH α virus (AcNPV α 3.4) and one plaque-purified bFSH β virus (AcNPV β 1.4) were used to prepare virus stocks with TCID $_{50}$ values of approximately 10^7 and 10^8 respectively.

Expression and secretion of bFSH α and bFSH β

The α and β expression products were further analysed by RIP (Fig. 2A, B). bFSH α , which was precipitated from the culture medium, migrated as a single band with a molecular mass of approximately 18 kDa (Fig. 2A, lane 4). Cell lysates showed a variety of labelled bands, possibly because we used polyclonal instead of monoclonal antibodies (Fig. 2A, lane 3). A monoclonal antibody against hFSH (ME.111, MBS, Maine, USA) did not precipitate any bFSH α . This was expected, as this antibody did not cross-react with bovine α -subunit in the ELISA.

bFSH β , which was precipitated from the medium of Sf21 cells infected with AcNPV β 1.4, migrated as a doublet, with a molecular mass of 15–16 kDa, both with polyclonal antisera (Fig. 2B, lane 4) and with monoclonal antibody (Fig. 2B, lane 9).

In cell lysates, a doublet of slightly greater molecular mass was observed with both antibodies (lanes 3 and 8).

Production curves of rbFSH α , rbFSH β and rbFSH heterodimer

The levels of expression of rbFSH α , rbFSH β and rbFSH heterodimer in the medium of infected Sf21 cells were determined by taking aliquots of culture medium at different time intervals after infection; the levels in Sf21 cell lysates were determined at 162 h after infection. Recombinant glycoprotein hormone and subunits were analysed by specific ELISA systems and ACA (Fig. 3). Most of the rbFSH α , rbFSH β and rbFSH heterodimer was secreted into the medium, and only very small amounts were found in the cell lysates (results not shown). Concentrations of rbFSH α in medium were approximately 10 times greater than those of rbFSH β , whereas concentrations of rbFSH heterodimer were intermediate. The maximum concentration of rbFSH α was 1.1 $\mu\text{g}/10^6$ cells/0.5 ml at

FIGURE 2. Radioimmunoprecipitation with polyclonal bFSH α antiserum (anti b α) or polyclonal bFSH β antiserum (anti b β) (lanes 2–5), and with monoclonal antibody against hFSH α (anti h α) or hFSH β (anti h β) (lanes 7–10). Culture media (sup) and cell lysates (lys) of Sf21 cells were analysed after infection with AcNPV 3·4 (α), AcNPV β 1·4 (β) or AcNPV/MO21 (WT, wild-type control). Cells were labelled 42 h p.i. with 40 μ Ci/ml [35 S]methionine for 6 h. Immunoprecipitates were analysed by SDS-PAGE and visualized by autoradiography. A: bFSH α ; B: bFSH β . Lanes 1 and 6, molecular mass (MW) markers (rainbow trout); lanes 2 and 7, wild-type cell lysate; lanes 3 and 8, recombinant α or β cell lysate; lanes 4 and 9, recombinant α or β culture medium; 5 and 10, wild-type culture medium.

48 h p.i., for rbFSH β the maximum was 0·13 μ g/10⁶ cells/0·5 ml at 72 h p.i. and for rbFSH heterodimer the maximum was 0·65 μ g/10⁶ cells/0·5 ml at 92 h p.i. In cell lysates, rbFSH α and rbFSH β concentrations were below the limit of detection of the assay, and rbFSH heterodimer concentration was less than 0·01 μ g/10⁶ cells.

Purification of rbFSH by immunoaffinity chromatography

The majority of immunoaffinity-absorbed rbFSH, as measured by both immunoassay and *in vitro* bioassay, was eluted at pH 4·0 (Fig. 4). Additional material was eluted after application of buffers of pH 3·5 and 3·0 respectively.

FIGURE 3. Time-course of FSH and subunit production in Sf21 cells infected with AcNPVα3·4 (○) or AcNPVβ1·4 (△) alone, or with AcNPVα3·4 plus AcNPVβ1·4 (■). Concentrations of rbFSHα and rbFSHβ were determined by ELISAs, and concentrations of rbFSH heterodimer by ACA in culture media 18, 24, 41, 48, 65, 72, 92, 96 and 162 h p.i. Levels of production are expressed in µg/10⁶ cells, by comparison with reference preparations of purified pituitary hormone or subunits.

SDS-PAGE revealed that immunoaffinity-purified rbFSH still contained free rbFSH β-subunit, at about eightfold the amount of rbFSH by densitometric scanning (Fig. 5A, #16, F). This was expected, because the antibody used for immunoaffinity chromatography also binds free β-subunit. Precipitation of rbFSH by ethanol resulted in partial denaturation, as can be seen from the appearance of free α-subunit in fraction 16 (Fig. 5A, #16, P). This was confirmed by western blot analysis of fractions 15 and 17 after ethanol precipitation, in which scarcely any intact rbFSH heterodimer was detectable (Fig. 5B, #15 and #17). Fraction 16, which was concentrated by Centricon filtration, was also shown to contain a small amount of free α-subunit (Fig. 5B, #16, lane α), although this could not be detected by silver staining. Comparison with high-molecular-mass markers gave apparent kDa values of 20, 23 and 32.5 for recombinant α-subunit, β-subunit and heterodimer respectively. These values are somewhat greater than those found after RIP, which may reflect differences in glycosylation arising from different times of harvesting (RIP 48 h, western blot 72 h p.i.).

Further analysis of immunoaffinity-purified rbFSH fractions by WGA lectin ELISA showed

that there was no binding with rbFSH, in contrast to results obtained with native hormones from both bovine and human origin (data not shown). This demonstrates that there is a difference between recombinant and native FSH with respect to terminal sialic acid and/or *N*-acetyl-D-glucosamine residues in glycosidic side-chains.

In vitro bioactivity of rbFSH

Y₁-cell *in vitro* bioassays were applied for analysis of SF900 media (serum-free) of Sf21 cells infected with either AcNPVα3·4 or AcNPVβ1·4, alone or in combination.

It appeared that media containing only rbFSHα or rbFSHβ did not induce a change in cell morphology, but media from cells infected with AcNPVα3·4 plus AcNPVβ1·4 showed very clear FSH-specific responses (up to a dilution of 1 : 4000), corresponding to biological activities of 8.5–17.0 IU/ml (results not shown). Direct comparison with a commercial oFSH preparation (OVAGEN, Immunochemical Products, Auckland, NZ; total dose recommended for one superovulation treatment: 180–360 U NIH-FSH-S1 as calibrated against NIADDK-oFSH-17) gave a bioactivity for rbFSH of 45 IU/ml (i.e. 1.8 U(S1)/ml).

FIGURE 4. Elution profile of rbFSH after affinity binding to monoclonal anti-hFSH β antibody coupled to CNBr-activated Sepharose (Sepharose 4B). rbFSH was measured by both heterodimer specific immunoassay (○) and Y₁ *in vitro* bioassay (△). Fractions with maximum activity in the immunoassay (fractions 15, 16 and 17) were taken for further analysis by electrophoresis (Fig. 5).

Media harvested from Y₁-cell cultures were analysed for cAMP. It appeared that Y₁-cells incubated with baculomedia from doubly infected Sf21 cells showed dose-dependent cAMP responses having a bioactivity of 19.1–23.9 IU/ml compared with that of a (freshly prepared) reference preparation of bFSH, whereas the bioactivity of media containing the single subunits was non-detectable (Fig. 6).

Bioactivity of rbFSH media determined in a rat Sertoli cell *in vitro* bioassay varied between 2.7 and 8.5 IU/ml; again, no bioactivity was detectable in media containing the single subunit (Fig. 7). Statistical analysis (one-way ANOVA) of cAMP concentrations revealed that there was a significant difference ($P=0.0012$) between baculomedia containing rbFSH heterodimer and media containing rbFSH subunit, at a dilution of 1 : 10; the difference at a dilution of 1 : 25 was not significant ($P=0.1126$). However, maximal stimulation of rbFSH was lower by a factor of 2–4 compared with USDA-bFSH-I-2 (see Discussion).

rbFSH culture media were also tested at a dilution of 1 : 25 in a bovine oocyte cumulus complex *in vitro* bioassay, with oFSH from Sigma as a reference preparation (Fig. 8). Although, in this particular experiment, the positive control sample had only 31% of oocytes remaining in the germinal vesicle (GV) stage, it was known from previous

experiments that the GV percentages vary with varying oFSH bioactivity (0.01–0.25 U(S1)/ml) according to a bell-shaped distribution, with a maximum of approximately 50% GV at 0.05 U(S1)/ml oFSH (A de Wit, personal communication). Therefore we assumed that the 53% GV observed with 104 μ l rbFSH $\alpha\beta$ -containing culture medium corresponded to an oFSH concentration of 0.05 U(S1)/ml. After correction for dilution factors and conversion into IU (see Materials and Methods, Hormones), an *in vitro* bioactivity of 31 IU/ml was calculated for rbFSH. In another experiment, the *in vitro* bioactivity was calculated as 15 IU/ml. For rbFSH α - and β -subunits, no bioactivity was observed (Fig. 8).

DISCUSSION

To the best of our knowledge, this is the first report of the synthesis of bFSH in insect cells.

The levels of production of rbFSH α - and β -subunits, in our system, are comparable to those of non-bovine gonadotrophin subunits obtained in the baculovirus system, published elsewhere (Chen *et al.* 1991, Huang *et al.* 1991, Nakhai *et al.* 1991, 1992, Jha *et al.* 1992, Sridhar *et al.* 1993, Dias *et al.* 1994, Hasnain *et al.* 1994, Lindau-Shepard *et al.* 1994, Delahaye *et al.* 1996). Levels of production

FIGURE 5 SDS-PAGE (12% acrylamide, non-reducing conditions) of immunoaffinity-purified rbFSH fractions 15, 16 and 17, followed by silverstaining (A) or western blotting (B). Before electrophoresis, samples were concentrated either by filtration (F) or precipitation (P). A: MW, high-molecular-mass standards (Sigma), which were separately alkylated; lanes F and P: rbFSH (fraction 16) concentrated by either filtration (F) or precipitation (P). Samples were not boiled and not reduced, and were silver stained. B: Electrophoresis was performed under the same conditions as in A. After blotting onto nitrocellulose filter, lanes were divided in three equal parts and were incubated with guinea pig control serum, anti-bFSH α antiserum, or anti-bFSH β antiserum, at a dilution of 1 : 1000. Lanes designated bFSH-I-2 contain 1 μ g purified pituitary bFSH (AFP-5318C, radioiodination grade), as a reference preparation. MW, alkylated high-molecular-mass standards (Sigma), which were silver stained separately.

of recombinant baculovirus-derived bFSH α - and β -subunits were reported as approximately 120 ng/ml by Sharma *et al.* (1993), but no mention was made of the production of bFSH heterodimer. Recently, baculovirus-derived bFSH β -subunit, when produced in High Five cells, was reported to reach a concentration of 8 μ g/ 10^6 cells; however, no data were given concerning the type of assay used or the ability of the subunit to combine with FSH α -subunit and form a bioactive heterodimer (Grotjan *et al.* 1996). To date, the only recombinant forms of FSH that have been produced in the baculovirus system are pFSH (Sato *et al.* 1993, Geshi *et al.* 1995, Inaba *et al.* 1997) and hFSH (Dias *et al.* 1994, Lindau-Shepard *et al.* 1994).

The cDNA that was used for hFSH or pFSH α -subunits in the above studies is very similar to the cDNA that we used. In contrast, the cDNA of both hFSH and pFSH β -subunits reportedly contained the minimal contiguous FSH β sequence, including the leader sequence, but virtually lacking UTRs at either the 5' or the 3' end. There is considerable evidence that the untranslated 3' region of the mRNA of the bFSH β -subunit that we used can influence stability of the mRNA, and may thus have contributed to the secretion of a high concentration of bioactive rbFSH into the medium. FSH β -subunit biosynthesis is probably a rate-limiting step in FSH heterodimer assembly and secretion (Greenberg *et al.* 1991). Furthermore, it has been suggested by Keene *et al.* (1989) that the unusually

FIGURE 6. *In vitro* bioactivity of rbFSH as measured by Y₁ cell assay. Two hundred microlitre aliquots of serially diluted insect cell culture medium containing rbFSHα (□), rbFSHβ (△) or rbFSH (○), or Y₁ cell culture medium containing bFSH-I-2 (*) were analysed in duplicate; mean cAMP values (expressed as pmol per 10⁵ cells) are used in the graphs. The assay was repeated twice, and appeared to be reproducible. Concentrations of FSH or subunits indicated on the abscissa are as determined by ELISA (rbFSHα, rbFSHβ), ACA (rbFSH) or weight (bFSH-I-2).

great length of the 3' UTR of the FSHβ gene, compared with that of the UTRs of other glycoprotein hormone β genes, may account for the hormonal control of the turnover rate for FSHβ mRNA. This 3' UTR of FSHβ mRNA, which is AU rich, may be unstable and may therefore form a site of negative regulation of FSH gene expression. This would agree with the observation made by Mountford *et al.* (1992), that removal of the 1100 bp 3' UTR enhances the expression of FSH β-subunit mRNA in transfected COS cells.

We hypothesize that, in our experiments, the inclusion of a major part of the 3' UTR of the bFSHβ gene, which contains four of the six AUUUA sequences found in the full-length UTR, has enabled high levels of expression and secretion of the bioactive hormone. It has been shown that overexpression of recombinant proteins in insect cells can compromise post-translational processing and secretion of the wanted protein (Sridhar *et al.* 1993); reduction of overexpression therefore can have a beneficial effect in the case

FIGURE 7 *In vitro* bioactivity of rbFSH as measured by rat Sertoli cell assay. Five hundred microlitre aliquots of serially diluted insect cell culture medium containing rbFSH α (□), rbFSH β (△) or rbFSH (○), or Sertoli cell culture medium containing bFSH-I-2 (*) were analysed in duplicate; mean cAMP values (expressed as pmol per 5×10^5 cells) are used in the graphs. The assay was repeated twice, with cAMP and oestradiol-17 β as the respective endpoints, and appeared to be reproducible. Concentrations of FSH or subunits indicated on the abscissa are as determined by ELISA (rbFSH α , rbFSH β), ACA (rbFSH) or weight (bFSH-I-2).

of heavily glycosylated proteins such as gonadotrophins.

The type of promoter can be another determinant of the levels of expression and secretion of hormones (Nakhai *et al.* 1992, Sridhar & Hasnain 1993, Sridhar *et al.* 1993). This has also been shown for the porcine LH receptor ectodomain, with which high levels of expression with a very late promoter such as the polyhedrin gene promoter lead to intracellular accumulation of inactive expression products (Pajot-Augy *et al.* 1995). When the p10 gene promoter, which is weaker and active at earlier times in the viral life cycle than the polyhedrin gene promoter, was used, recombinant pLH receptor ectodomain was secreted into the culture medium and was biologically active, probably because of a lower level of production (Bozon *et al.* 1995). In our experiments, in which we used the p10 gene promoter, we obtained high levels

of both expression and secretion of bioactive rbFSH.

Levels of production can also be influenced by degradation of recombinant proteins intracellularly (Pajot-Augy *et al.* 1995), at the membrane level (Christophe *et al.* 1993), or after secretion into the medium (Bozon *et al.* 1995), although the last of these is not a general observation (Huang *et al.* 1991). In our study, degradation of recombinant protein in the medium was seen especially with the rbFSH α -subunit, from 50 h p.i. onward. Degradation of the rbFSH β -subunit and rbFSH heterodimer was also observed, but to a lesser degree, and starting much later (92 h p.i.) (Fig. 3). Intracellular degradation of the rbFSH β -subunit may be partly responsible for its much lower level of secretion compared with that of the rbFSH α -subunit. Interestingly, there is evidence that degradation rates within the cell can be reduced by the presence of chaperone molecules, as suggested by Pajot-Augy *et al.* (1995). When rhFSH was produced in CHO cells, it was observed that FSH β -subunit was secreted very slowly, but cotransfection of the FSH β - and α -subunit genes resulted in 'rescue' of the β -subunit, and rapid secretion of the dimer (Keene *et al.* 1989). Mountford *et al.* (1994) observed that recombinant oFSH β -subunit, as produced in CHO cells, was secreted inefficiently and displayed a short intracellular half-life in the absence of α -subunit; cotransfection of the α -subunit cDNA vector in conjunction with the β -subunit cDNA vector resulted in efficient secretion of oFSH heterodimer. This phenomenon has also been observed with the expression of rhCG in monkey cells that are infected with α - and β -subunit cDNA-containing simian virus 40 expression vector (Reddy *et al.* 1985). More recently, increased levels of production of soluble hFSH receptor in insect cells were reported to have been obtained by concomitant expression of hFSH β or hFSH $\alpha\beta$ heterodimer with truncated hFSH receptor cDNA (Stavis *et al.* 1996). Although more than one mechanism may be responsible for the increased stability and production of β -subunit when expressed in conjunction with α -subunit, as observed by us and others, we hypothesize that the FSH α -subunit acts as a chaperone molecule for the FSH β -subunit. This notion probably also extends to the interaction between subunits of other glycoproteins.

We also found that reassociation of separately produced rbFSH α - and β -subunits according to procedures described by Nakhai *et al.* (1992) resulted in bioactive rbFSH (Y_1 cell *in vitro* bioassay), but at a level of less than 1% compared with that obtained after coexpression of α - and

FIGURE 8. Effect of rbFSH or recombinant subunit on germinal vesicle breakdown (GVBD) of bovine oocyte cumulus complexes *in vitro*. Shaded bars indicate inhibition of GVBD (oocyte development is arrested in the germinal vesicle stage); cross-hatched bars indicate GVBD (oocyte development takes place). Numbers on top of the bars indicate numbers of oocytes tested. The assay was repeated once, with a similar result.

β -subunit genes (results not shown). This indicates that the association of recombinant α - and β -subunits takes place predominantly within the cell and, indeed, that the chaperone effect is already required at the stage of synthesis and secretion of the recombinant subunits. Early association of subunits therefore seems beneficial for their stability, and in this respect it may be of advantage to clone the cDNAs for the α - and β -subunits into one vector, as has been suggested by Reddy *et al.* (1985). Dual expression vectors containing cDNAs for both the FSH α - and β -subunits have been used for the production of rhFSH in CHO cells (Keene *et al.* 1989, Van Wezenbeek *et al.* 1990) and rpFSH in insect cells (Geshi *et al.* 1995), although no comparative data on the levels of production have been published.

With respect to the *in vitro* biological activity, we observed a large variation when we measured rbFSH in different *in vitro* bioassay systems. This may be explained partly by differences in the endpoints chosen (Padmanabhan *et al.* 1993), and possibly also partly by the presence of inhibitors of receptor binding in the conditioned media. This may have caused poor receptor–ligand interaction in the rat Sertoli cell assay, although such an effect was observed in neither the Y_1 cell assay nor the oocyte maturation inhibition assay. We consider it of

particular interest that the rbFSH that we produced showed high *in vitro* bioactivity in the bovine immature oocyte assay, which is a homologous assay system. The relevance of homology between hormone and target animal or cell system to the biological activity has recently been demonstrated by Hakola *et al.* (1997).

Another point of interest is the effect of glycosylation, and especially of the terminal sialic acid, on the bioactivity of FSH. In general, no sialic acid residues have been found in baculovirus-expressed recombinant proteins (King & Possee 1992, Kretzchmar *et al.* 1994, Jarvis & Finn 1995), although contradictory results have been published (Castellino *et al.* 1993). It is generally believed that the main function of the attachment of sialic acid to glycosidic side-chains is to protect glycoproteins from rapid clearance by the asialo-glycoprotein receptor in the liver (Ulloa-Aguirre *et al.* 1995, Grossmann *et al.* 1997). However, the site-specific roles of glycosidic side-chains in so-called rhFSH mutants indicate that the degree of sialylation is not the only determinant of metabolic half-life (Heikoop *et al.* 1996). Furthermore, it has recently been found that rpFSH as produced in the baculovirus system is biologically active *in vivo*, at least when injected into immature mice (increased uterine weight), hypophysectomized mice (increased ovulation rate)

and immature hypophysectomized rats (increased ovarian and uterine weight, increased number and size of follicles) (Inaba *et al.* 1997).

In conclusion, we have been the first to produce bFSH in insect cells. This baculovirus-expressed rbFSH is highly active in at least two *in vitro* bioassay systems, and shows lower but significant activity in the rat Sertoli cell assay, and is secreted into the culture medium in large amounts. The high levels of production and secretion of bioactive hormone are probably due to the cloning of a large AU-rich part of the bFSH β cDNA 3' untranslated region. This AU-rich region probably causes a reduction of overexpression, and therefore an improved post-translational processing of the hormone.

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