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# Expression of the Human Follicle-Stimulating Hormone Receptor in the Baculovirus System

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Human follitropin receptor (hFSHR) cDNA was inserted into the genome of *Autographa californica* nuclear polyhedrosis virus under the control of the polyhedrin promoter. *Spodoptera frugiperda* (Sf9) insect cells expressed the hFSHR at the membrane level, 2-3 days after infection. Ligand binding analysis demonstrated saturable, high-affinity binding of $^{125}$I-labeled recombinant hFSH ($K_d$ = 0.17 nM) as well as binding specifity for both human and equine FSH. Interestingly, a highly purified preparation of equine choriogonadotropin was found to compete with the binding of $^{125}$I-rhFSH. Finally, Western-blot analysis, using a monoclonal antipeptide antibody directed to the 178-206 region of the hFSHR, revealed two bands migrating at $M_r$ 77 and 130 kDa. 

Follitropin (FSH) is a pituitary gonadotropin which belongs to the glycoprotein hormone family, including the lutropin (LH), the thyrotropin (TSH) and the placental choriogonadotropin (CG) (1). FSH binds to specific receptors present on the gonads to initiate and regulate functions essential for ovulation and spermatogenesis (2). The recent cloning and sequencing of the FSH receptor (FSHR) in various species (3-5) indicated that this receptor belongs to the G protein-coupled receptor family, characterized by seven hydrophobic α-helices that constitute a putative transmembrane domain. Furthermore, the FSHR presents a typical large, glycosylated extracellular (EC) N-terminal domain which constitutes the high affinity binding site for hormone (6). The elucidation of the molecular mechanisms involved in human FSH (hFSH) action has been impaired for a long time by the limited availability of human gonadal tissues as well as the paucity of gonadal FSH-binding sites (7). Thus, the recent availability of the recombinant hFSHR makes it useful for a variety of structural and functional experiments.

The baculovirus (*Autographa californica*) expression system (8) constitutes a promising approach for the production of a number of biologically active peptidic

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ligands and receptors. It has been recently and successfully used for the production of glycoprotein hormones, particularly the human CG (9). In contrast, several attempts to express the N-terminal half of the LH/CG porcine receptor and the entire and truncated TSH receptor failed or led to the production of uncorrectly folded protein resulting in non functional receptors (10-12). In the present study, we reported that the infection of insect *Spodoptera frugiperda* (Sf9) cells with a recombinant baculovirus carrying the full-length human FSHR gene under the control of the strong polyhedrin promoter (BV-hFSHR) results in the expression of a functional recombinant receptor in terms of binding properties. The rhFSHR was also identified by a monoclonal antipeptide antibody directed to the 178-206 region of the hFSH receptor.

## Materials and Methods

*Cell Culture and Reagents* - The Sf9 insect cell line was grown in TC100 insect medium (Gibco-BRL Laboratories, UK) containing 4% heat inactivated fetal calf serum (FCS) (IBF biotechnics, France). Highly purified hCG (CR-127) and recombinant hFSH (rhFSH) were generously supplied by the National Hormone and Pituitary program (NIDDK, NIH, MD) and by AAT-Serono (Randolph, MA), respectively. *Xho*I adaptor oligonucleotides were obtained from GENSET (Paris, France).

*Construction of a Baculoviral Transfer Vector Containing the hFSHR cDNA Sequence (pAC/hFSHR)* - The plasmid pUC18 containing the human FSH receptor cDNA, with an open reading frame of 2085 nucleotides, was digested with *Xho*I endonuclease (13). The vector pAcCL29.1 (kindly supplied by Dr. G. Allée) was digested at the unique *Sma* I restriction site and ligated to *Xho*I adaptor. The *Xho*I fragment (2.1 kb), containing the full-length coding region flanked by 18 bp upstream and 7 bp downstream noncoding sequences was isolated by agarose gel electrophoresis. This fragment was then inserted into the *Xho*I site of vector pAcCL29.1 downstream from the polyhedrin promoter to produce the pAC/hFSHR (Figure 1). A recombinant plasmid containing a single copy of the sense-oriented 2.1 kb recombinant hFSHR cDNA fragment was identified by restriction endonuclease mapping.

*Transfection of Insect Sf9 Cells, Isolation of the Recombinant Baculovirus (BV-hFSHR) and Expression of the rhFSHR Protein in Sf9 Cells* - The permissive host Sf9 cell line was seeded at a density of 2 x $10^6$ cells/T25 flask (Falcon, NJ) in supplemented Grace's insect medium and cotransfected with cesium chloride-purified pAC/hFSHR (2μg) and wild-type (*wt*) AcNPV viral DNA (1μg) (Invitrogen Corporation, San Diego, CA), using the calcium phosphate precipitation technique. Five days later, viral pools were screened by dot blot hybridization performed on infected Sf9 cells. After 5-7 days culture, cells were lysed and blotted lysates were screened with 5'[$\alpha^{32}$P] dCTP-labeled hFSHR cDNA as a probe. Membranes were then washed and exposed for autoradiography. Supernatants from positive wells were submitted to four rounds of subsequent screening in order to eliminate *wt* virus. A positive recombinant viral clone, designated BV-hFSHR, was selected. Sf9 cells were infected at a multiplicity of infection (MOI) of 5-10 PFU per cell with either *wt* AcNPV or BV-hFSHR virus, in monolayers for virus amplification or in a shaker flask for expression (8).

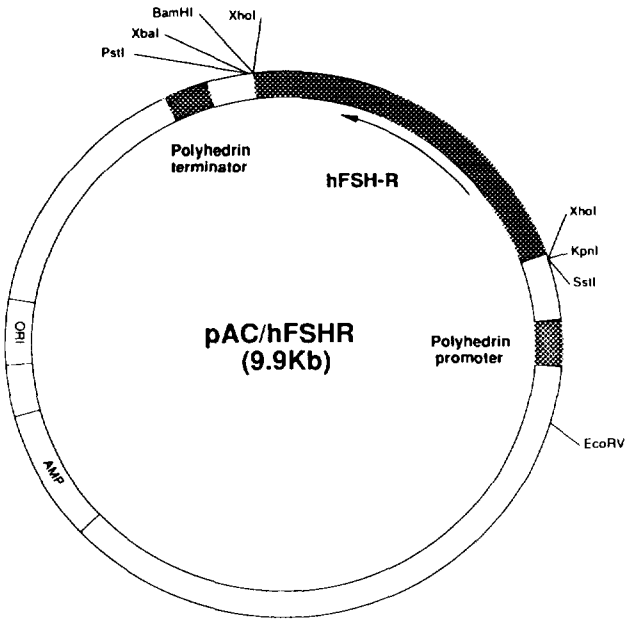



***Figure 1.*** Partial restriction map of baculovirus expression pAC/hFSHR plasmid. The black region represents the human FSHR cDNA. The grey regions represent AcNPV polyhedrin promoter and terminator, and the white region represents pAcCL29.1 vector DNA. The direction of transcription of hFSHR cDNA is indicated by the arrow.

*Binding Studies* - Infected Sf9 cells were harvested at various times post-infection by centrifugation at 1,500 rpm and washed three times with 10 mM phosphate buffer pH 7.4 containing 150 mM NaCl (Pi/NaCl). Pellets were then resuspended in the appropriate volume of binding buffer (Tris-HCl 10 mM pH 7.5, $MgCl_2$ 5mM, $CaCl_2$ 5mM) containing 1% bovine serum albumin (BSA). rhFSH was radioiodinated with $^{125}I$ (Commissariat à l'Energie Atomique, Saclay, France) to a specific activity of 65-70 mCi/mg of protein using the IODO-GEN procedure (14). In saturation binding assays, various concentrations of $^{125}I$-rhFSH were used for binding to $10^6$ cells. For the generation of displacement curves, a fixed amount of $^{125}I$-rhFSH (5 x $10^4$ cpm) was used for binding to $10^6$ cells in the presence of different concentrations of either unlabeled rhFSH, hCG, or the equine hormones eFSH and eCG. Samples were incubated overnight at 4°C in a final volume of 500 µl and 1 ml of binding buffer plus 0.1% BSA was then added. After centrifugation, cell pellets were washed once with 1 ml of binding buffer plus 0.1 % BSA and the bound radioactivity was measured.

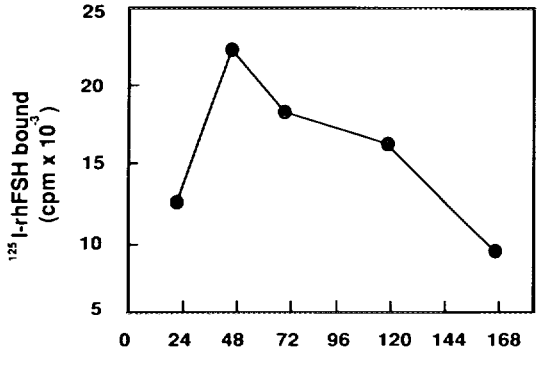
*Western-Immunoblotting of the rhFSHR* - Sf9 cells infected with either BV-hFSHR virus or *wt* AcNPV were harvested 48-96 h post-infection and washed twice in Pi/NaCl . After centrifugation for 10 min at 1,500 rpm, pellets were resuspended and lysed in Laemmli buffer containing 5% β-mercaptoethanol. Samples, representing about 14 x $10^4$ cells, were then submitted to SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Biorad, Ivry, France) at 4° C overnight at 50V, as described (15). After saturation with Pi/NaCl containing 5% nonfat milk for 1 h, the membranes were incubated for 4 h at room temperature with a 1:100 dilution of culture supernatant containing a monoclonal antipeptide antibody, designated FREC05 and raised against a synthetic peptide mimicking the

178-206 portion of the hFSHR[1]. Membranes were then washed with Pi/NaCl containing 0.2% Tween 20 and incubated with $^{125}$I-labeled sheep anti-mouse Ig F(ab')$_2$ (Amersham) for 2h at room temperature. After subsequent washing, membranes were autoradiographed.

## *Results and Discussion*

Expression of the recombinant hFSHR (rFSHR) protein was analyzed at different times post-infection by using the $^{125}$I-rhFSH binding assay. As shown in Figure 2, a maximal expression of rhFSHR was observed between 48-96 h after infection. The synthesis of rhFSHR then decreased as the virus entered in its lytic phase. This biosynthesis kinetic is typical for genes under the transcriptional control of the very late polyhedrin gene promoter. To determine whether the recombinant hFSHR expressed in insect cells exhibited similar binding properties to those presented by tissue purified receptors (16), binding studies were performed at 48 or 96 h post-infection.

A dose-dependent increase in specifically bound $^{125}$I-rhFSH was detected in BV-hFSHR-infected Sf9 cells incubated with increasing concentrations of labeled rhFSH (Figure 3), whereas no binding was observed on uninfected cells or cells infected with the *wt* virus (data not shown). This process was found to be saturable and the number of hFSH receptors calculated on the basis of the rhFSH binding capacity was about 7,000 receptors/cell at 48h post-infection. Recombinant hFSHR



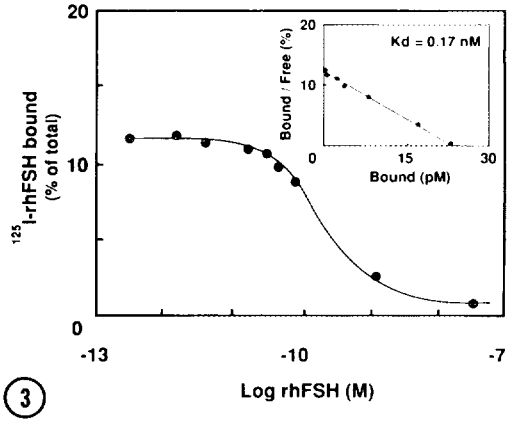



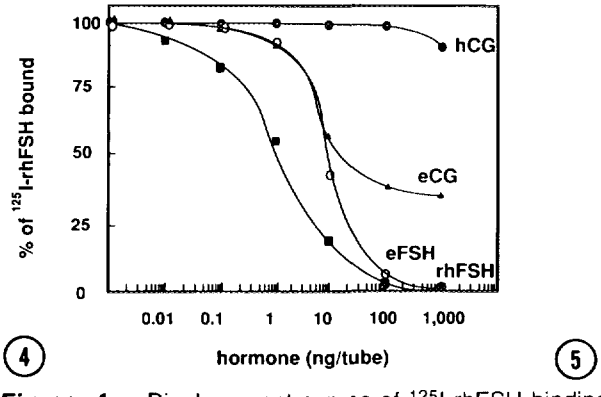

***Figure 2.*** Time course of hFSHR synthesis in BV-hFSHR-infected Sf9 cells as analyzed by radioreceptor assay.

***Figure 3.*** Scatchard plot analysis of $^{125}$I-rhFSH binding to BV-hFSHR-infected Sf9 cells. Results were analysed using the Ligand computer program (22).

[1] Christophe *et al.*, manuscript in preparation.

also bound glycoprotein hormones with appropriate affinity and selectivity. Scatchard analysis provided an estimated $K_d$ value for rhFSH of 0.17 nM, quite similar to that measured on calf testis. To test the binding specificity of the expressed rhFSHR, infected Sf9 cells were incubated with $^{125}$I-rhFSH in the presence of increasing doses of the various competitors. As shown in Figure 4, rhFSH and eFSH displaced $^{125}$I-rhFSH binding with an $ED_{50}$ of 2 and 20 ng/ml, respectively, while hCG was not effective. In contrast, a highly purified preparation of eCG appeared to compete with the binding of $^{125}$I-rhFSH to its receptor, albeit being a 20-fold less potent than rhFSH. This result is in disagreement with the previous observation of Tilly *et al.* (17), indicating that only hFSH and rat FSH bound to the hFSHR expressed in human fetal kidney 293 cells. The reasons for such discrepancy are not clearly understood but differential post-translational processing, i.e. glycosylation pathways, in insect and human cells might be hypothesized. Taken together, these observations suggested that the rhFSH receptor expressed in insect Sf9 cells exhibited the correct conformation to ensure its hFSH binding capacities with appropriate affinity and selectivity.

Western immunoblotting analysis, using antipeptide mAb FREC05 and performed under reducing conditions, showed a major band migrating at an apparent $M_r$ of 77 kDa, consistent with the molecular size of the recombinant hFSHR predicted from the cDNA. A second band migrating at 130 kDa was observed, probably corresponding to a glycosylation variant of the receptor (17,18). No cross-



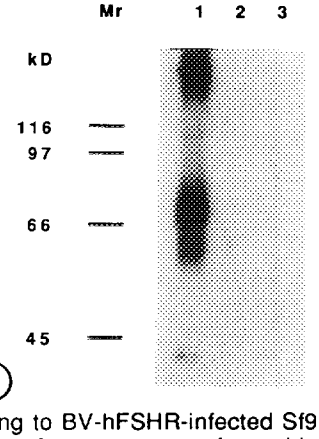




***Figure 4.*** Displacement curves of $^{125}$I-rhFSH binding to BV-hFSHR-infected Sf9 cells by human and equine gonadotropins. Assays were performed in triplicate in three separate experiments with different batches of infected cells.

***Figure 5.*** Western blot analysis of the rhFSHR. Extracts were prepared from BV-hFSHR- (lane 1) or AcNPV-infected Sf9 cells (lane 2) at 48 h post-infection, resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose, and immunostained using the FREC05 antibody. Binding of the antibody was inhibited by preincubation with $10^{-5}$ M of the corresponding synthetic peptide (lane 3).

reacting material was detected in uninfected cells or in cells infected with the *wt* virus. Moreover, immunostaining appeared to be specific as shown by the inhibition of FREC05 binding after preincubation with increasing concentrations of the corresponding peptide (Fig. 5). Interestingly, the two immunostained bands exhibit apparent molecular sizes similar to those displayed by the hFSHR expressed in the 293 cell line as analyzed by cross-linking studies (17). It has been reported that the mature form of the FSH receptor, isolated from bovine calf or rat testis, may be an oligomer of about 240 kDa as viewed by ligand blotting after SDS-PAGE under nonreducing conditions (16,19). Dimerization and/or aggregation of the 77 kDa form of the hFSHR, while not critical for hormone binding, are important for signal transduction and might explain these different observations. In addition, our results also suggest that, although four putative N-glycosylation sites are predicted from the cDNA sequence, the rhFSHR is poorly glycosylated. Several studies have indicated that most of the N-glycan chains on the CG/LH receptor are not critical for high affinity binding of the hormone (20).

To our knowledge, this is the first report of the expression in the baculovirus system of an entire receptor for the glycoprotein hormone family. Production of the EC domain of both porcine CG/LH receptor and human TSH receptor resulted in high levels of proteins that weakly bound their respective ligands (10,11). Using the entire hTSH receptor, Harfst *et al.* showed that, despite high mRNA levels, no protein was detectable (12). Although there is no clear explanation for this result, it might be possible that biochemical processing, required for the maturation of the entire receptor, is specifically present in some cells. Indeed, in contrast to the CG/LH and FSH receptors, a proteolytic post-translational maturation event appears to be necessary for the correct folding of the TSH receptor (21). Finally, the production of the hFSH receptor in the baculovirus expression system provides unlimited material for both structural and clinical studies, for the development of screening systems as well as for antibody production.

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