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Production, purification, and characterization of recombinant hFSH glycoforms for functional studies

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Summary

Previously, our laboratory demonstrated the existence of a β-subunit glycosylation-deficient human FSH glycoform, $hFSH^{21}$. A third variant, $hFSH^{18}$, has recently been detected in FSH glycoforms isolated from purified pituitary hLH preparations. Human FSH^{21} abundance in individual female pituitaries progressively decreased with increasing age. Hypo-glycosylated glycoform preparations are significantly more active than fully-glycosylated hFSH preparations. The purpose of this study was to produce, purify and chemically characterize both glycoform variants expressed by a mammalian cell line. Recombinant hFSH was expressed in a stable GH_3 cell line and isolated from serum-free cell culture medium by sequential, hydrophobic and immunoaffinity chromatography. FSH glycoform fractions were separated by Superdex 75 gel-filtration. Western blot analysis revealed the presence of both $hFSH^{18}$ and $hFSH^{21}$ glycoforms in the low molecular weight fraction, however, their electrophoretic mobilities differed from those associated with the corresponding pituitary hFSH variants. Edman degradation of $FSH^{21/18}$ - derived β-subunit before and after peptide-N-glycanase F digestion confirmed that it possessed a mixture of both mono-glycosylated FSHβ subunits, as both Asn^{7} and Asn^{24} were partially glycosylated. FSH receptor-binding assays confirmed our previous observations that $hFSH^{21/18}$ exhibits greater receptor-binding affinity and occupies more FSH binding sites when compared to fully-glycosylated $hFSH^{24}$. Thus, the age-related reduction in hypo-glycosylated hFSH significantly reduces circulating levels of FSH biological activity that may further compromise reproductive function. Taken together, the ability to express and isolate recombinant hFSH glycoforms opens the way to study functional differences between them both *in vivo* and *in vitro*.

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Keywords

follicle-stimulating hormone; glycosylation; oligosaccharides; mass spectrometry

1. Introduction

Human pituitary FSH consists of three or four major glycoforms that differ in glycosylation of the hormone-specific β–subunit (Bousfield et al., 2008, Bousfield et al., 2014a, Bousfield et al., 2007, Walton et al., 2001). Fully glycosylated FSHβ subunit is detected in FSHβ-specific Western blots as a 24,000 M_r band, while two glycan-deficient forms have been described, one that appears as a 21,000 M_r band (Walton et al., 2001) and another that is characterized by an 18,000 M_r band (Bousfield et al., 2014a). A non-glycosylated hFSHβ was detected by mass spectrometry (Bousfield et al., 2008, Bousfield et al., 2007, Walton et al., 2001), but in Western blots the 15,000 M_r hFSHβ band has only been observed after peptide-N-glycanase F (PNGase F) treatment of the other glycoforms (Bousfield et al., 2014a). Most pituitary and urinary hFSH preparations we have analyzed possess both M_r 24,000 ($FSH\beta_{24}$) and Mr 21,000 ($FSH\beta^{21}$) β-subunit variants, although the ratios may differ (Bousfield et al., 2008, Bousfield et al., 2014a, Bousfield et al., 2007, Walton et al., 2001). For simplicity, the hFSH glycoform with $FSH\beta^{24}$ will be designated as $hFSH^{24}$, the one with $FSH\beta^{21}$ as $hFSH^{21}$, and the one with $FSH\beta^{18}$ as $hFSH^{18}$. Mixtures of glycoforms, such as pituitary hFSH, which possesses more $FSH\beta^{24}$ than $FSH\beta^{21}$, will be designated as $hFSH^{24/21}$, with the first number indicating the more abundant variant. In our previous publications, we referred to $hFSH^{21}$ as “di-glycosylated”, as mass spectrometry and Edman degradation independently demonstrated that the original $FSH\beta^{21}$ subunit preparation lacked both N-glycans (Walton et al., 2001). Thus, only the α subunit possessed N-glycans. Human FSH^{24} was termed “tetra-glycosylated” hFSH to indicate dual N-glycosylation of both α and β subunits (Bousfield et al., 2008, Bousfield et al., 2014a, Bousfield et al., 2007, Walton et al., 2001). Transgenic mice also express a non-glycosylated $FSH\beta^{15}$, which can heterodimerize with the mouse α-subunit to form $hFSH^{15}$, however, it appears to be retained by pituitary gonadotropes (Davis et al., 2014).

The relative abundance of $hFSH^{21}$ in individual female pituitaries appears to be dependent on the age of the woman and to decrease over reproductive life. The ratio of $hFSH^{21}$/ $hFSH^{24}$ in the pituitary changed from $hFSH^{21}$ -dominant in 21- to 24-year old women to roughly equivalent in 39- to 41-year old women to $hFSH^{24}$ -dominant in 55- to 81-year old women (Bousfield et al., 2014b). Increased abundance of high molecular weight forms of pituitary FSH followed ovariectomy in both rhesus macaque and rat females (Bogdanove, Campbell and Peckham, 1974, Peckham et al., 1973). Sephadex G-100 chromatograms indicated 56% ovariectomized rhesus pituitary hFSH was the high molecular weight variant (Peckham and Knobil, 1976). Western blots of individual ovariectomized rhesus pituitary FSH samples revealed 57% was FSH^{24} (Bousfield et al., 2007). Estrogen replacement in ovariectomized females reduced the abundance of high molecular weight FSH (Bogdanove et al., 1974, Peckham and Knobil, 1976). Neuraminidase digestion of rhesus FSH from ovariectomized females also reduced the abundance of high molecular weight FSH, which suggested the increase in size was due to sialic acid (Peckham and Knobil, 1976). It is not

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yet clear what ovarian factor(s) regulate human FSH glycoform abundance. For example, the shift from $hFSH^{21}$-dominant to $hFSH^{21}$/$hFSH^{24}$ equivalent ratios preceded the age at which circulating estrogen is known to decrease (Randolph et al., 2011).

Since both glycoforms were also present in the urine of women (Bousfield et al., 2014b), they were secreted by the pituitary into the circulation, where they can influence ovarian activity. Possibly, the glycoform ratios in the blood change over time and exacerbate declining function of the aging ovary by limited cellular activation via the FSH receptor. We recently reported that pituitary $hFSH^{21/18}$ exhibited a 9- to 20-fold higher hFSH receptor-binding activity and occupied twice as many receptors as $hFSH^{24}$ (Bousfield et al., 2014a). Urinary hFSH preparations used in assisted reproduction represent mostly $hFSH^{24}$ (Bousfield et al., 2007), since they are purified from postmenopausal urine. Most of the recombinant hFSH preparations commercially available for use in ovarian stimulation for assisted reproduction, such as Gonal F, consist largely of the $hFSH^{24}$ glycoform (see below). Therefore, it would be interesting to see if the $hFSH^{21}$ or $hFSH^{18}$ glycoforms are beneficial for assisted reproduction procedures.

A randomized, open-label clinical study performed on 188 infertile couples reported that two types of hFSH preparations with different glycosylation patterns had different impacts on oocyte quality and clinical outcome (Selman H, 2010). A sequential combined protocol using both acidic and less-acidic hFSH preparations for ovarian stimulation improved oocyte maturity, implantation, and pregnancy rates (Selman H, 2010). Another possible practical application is a development of a monoclonal antibody specific only to $hFSH^{21}$ or $hFSH^{18}$ glycoforms (in progress in our laboratory).

The aim of this study was to produce, isolate and structurally characterize recombinant hFSH glycoforms, to be used in the future for further *in vitro* and *in vivo* characterization of FSH action.

2. Materials and Methods

2.1 Hormone Preparations

Recombinant hFSH preparations, Follistim and GonalF were obtained from Organon and Serono, respectively. Purified pituitary hFSH preparations AFP-4161, AFP-5720D, and AFP-7298A were obtained from the National Hormone and Pituitary Program. Urinary hFSH was purchased from ProSpec, East Brunswick, NJ. Human pituitary FSH glycoforms were prepared as described previously (Bousfield et al., 2014a). Recombinant GH_3-$hFSH^{24/21}$ was purified from small samples of conditioned medium by the same procedure used to isolate pituitary $hFSH^{21/18}$; monoclonal antibody 46.3H6.B7 immunoaffinity chromatography followed by Superdex 75 gel filtration (Bousfield et al., 2014a). Antibodies used in this study are listed in supplement Table 1.

2.2 Analytical Procedures

Details of all procedures can be found in the supplement to this article. SDS-PAGE (Laemmli, 1970) was carried out using a Bio-Rad (Hercules, CA) Protean III mini-gel apparatus (Bousfield et al., 2007). Conventional Western blots of PVDF membranes were

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carried out as previously described (Bousfield et al., 2014a). Automated Western blot procedures were carried out using a ProteinSimple (Santa Clara, CA) Simon following the manufacturer's recommendations. Nano-electrospray ionization mass spectrometry was carried out as recently described for pituitary and urinary hFSH samples (Bousfield et al., 2014b). Carbohydrate composition analysis was carried out on 4 N TFA hydrolysates (Bousfield et al., 2000) using a Thermo Scientific Dionex (Sunnyvale, CA) ISC-5000 carbohydrate analyzer. FSHβ glycosylation sites were analyzed by a combination of PNGaseF digestion and automated Edman degradation. Glycosyltransferase expression was detected by RT-PCR.

2.3 Large-scale Recombinant hFSH Purification

Details of recombinant GH_3-hFSH expression and glycoform purification can be found in the supplement. A rat pituitary tumor GH_3 cell line, stably transfected with hFSH α- and β-subunits (Muyan, Ryzmkiewicz and Boime, 1994), was the generous gift of Dr. I. Boime (Washington University Medical School, St. Louis, MO). Culture medium conditioned by these cells was the source of recombinant hFSH. The hormone was captured from 10.4 L serum-free culture medium by Octyl-Sepharose chromatography, then immunopurified with immobilized monoclonal antibody 4882 (SPD Development Co., Ltd., Bedford, UK.), which recognizes an α-subunit epitope and captures all human glycoprotein hormones. Immunopurified hFSH was fractionated by gel filtration using three, 10 X 300 mm Superdex 75 (GE Healthcare, Piscataway, NJ) columns, connected in series. Relative glycoform abundance was determined by Western blot and the appropriate fractions pooled.

2.4 FSH receptor-binding assays

Animal procedures were approved by an institutional animal care and use committee. Competitive binding assays were carried out as described previously (Butnev et al., 1996). Saturation binding assays were carried out as described previously (Bousfield et al., 2014a) except bovine (Dias, Huston and Reichert, 1981) and human (Butnev et al., 1998) FSH receptor preparations were included.

3. Results

3.1 GH_3-hFSH glycoform abundance

Western blot analysis of samples from small-scale expression experiments suggested recombinant hFSH expressed by GH_3 cells might provide a more abundant source of partially glycosylated FSH glycoforms than pituitary extracts and commercially available recombinant hFSH preparations (Fig. 1). The relative abundance of the FSHβ^{21} band averaged 55% in mAb 46.3H6.B7 immunoaffinity/Superdex 75 gel filtration-purified, recombinant hFSH samples recovered from GH_3 cells grown in 100 mm culture dishes (Fig. 1, lanes 2 and 3). This was greater than the 20–27% abundance found in pituitary and recombinant hFSH preparations (Fig. 1, lanes 4 and 7–10). GonalF revealed a triplet of immunoreactive bands in the FSHβ^{21} region of the gel, instead of the broad, glycoprotein-like band observed in pituitary FSH preparations. All the pituitary FSHα subunit bands migrated faster than commercially produced recombinant hFSHα subunits (compare lower panel lanes 4–6 with 7–10).

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3.2 Characterization of total GH_3-hFSH glycans by nano-spray mass spectrometry

Pituitary and recombinant hFSH oligosaccharide microheterogeneity was compared to identify differences in N-glycan populations that might impact biological activity. PNGaseF digestion quantitatively released oligosaccharides from reduced, carboxymethylated GH_3-hFSH, as indicated by a shift to later retention time for the PNGaseF-digested hormone sample (data not shown). The PNGaseF-released oligosaccharides were analyzed by nano-spray mass spectrometry as described in supplementary materials. The spectra (supplement Fig. 2) were relatively weak compared with those from the pituitary hFSH and hFSH glycoform samples analyzed at the same time (Bousfield et al., 2014a); consequently, some minor glycans may have been missed. The glycans were predominantly bi- and tri-antennary complex-type oligosaccharides (supplement Fig. 3 and supplement Table 3), with the bi-antennary more abundant than the tri-antennary (Fig. 2A), the opposite of what was encountered with the pituitary hFSH preparation and with reports in the literature (Green and Baenziger, 1988a, b, Renwick et al., 1987). A small amount of tetra-antennary structures may have been present but fragmentation spectra of these minor ions (supplement Table 3) were not obtained and, consequently, the possibility that these ions were from tri-antennary glycans with *N*-acetyl-lactosamine extensions cannot be excluded. Unlike pituitary hFSH samples, the tri-antennary glycans possessed 2 branches on the 6-antenna instead of the 3-antenna (Harvey et al., 2008). Both neutral and acidic glycans were present; the acidic groups were all Neu5Ac, no sulfate was detected. The glycans also contained a substantial amount of fucose on the antennae, unlike the glycans from pituitary hFSH.

Of the four most abundant pituitary glycan families, based on neutral core oligosaccharide structure, two were triantennary, two were biantennary, and together they accounted for almost 40% of the glycan families (Fig. 2A and B), while three of the 4 most abundant recombinant hFSH glycans were biantennary, only one was triantennary. Together, these added up to just over 50%. The three most abundant individual glycan structures in both preparations were di-sialylated biantennary glycans (Fig. 2C and D), while the fourth was tri-sialylated, triantennary for pituitary hFSH and fucosylated, tri-sialylated, triantennary for recombinant hFSH. The four most abundant glycans accounted for 30% and 36% of the pituitary and recombinant hFSH glycans, respectively. Overall, recombinant hFSH glycans were less diverse than in pituitary hFSH, possessed relatively fewer large glycans of the triantennary class, and appeared to completely lack the tetraantennary class. Biantennary glycans were the largest glycan class present and this may have contributed to the faster migration of recombinant GH_3-hFSH bands during SDS-PAGE. Nevertheless, the apparent greater abundance of hypo-glycosylated glycoform as compared with other pituitary and recombinant hFSH preparations indicated it was worthwhile to proceed to large-scale expression.

3.3 GH_3-hFSH isolation

Large-scale GH_3 cell expression of hFSH produced milligram quantities of immunoreactive recombinant hFSH in both serum-containing and serum-free conditioned media, which were processed separately. The following describes isolation of hFSH glycoforms from 10.4 liters of serum-free medium containing 21.2 mg hFSH immunoactivity. A total of 690 mg protein was eluted from Octyl-Sepharose with 30% ethanol. The FSH recovery was 95% of the

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initial immunoreactivity. The dialyzed and lyophilized protein was dissolved in 0.1 M sodium phosphate, pH 7.0, with 0.15 M NaCl, and GH_3-hFSH captured by immobilized anti-α monoclonal antibody 4882. GH_3-hFSH and subunits were eluted from this resin with 0.1 M glycine-HCl, 0.5 M NaCl buffer, pH 2.7. The recovery of recombinant GH_3-$hFSH^{24/21}$ was 14.5 mg (68%) based on size exclusion chromatography (SEC) peak area. Separation of the FSH glycoforms was achieved by high performance gel filtration using three Superdex 75 columns connected in series. The resulting chromatogram consisted of a large heterodimer peak and a smaller free subunit peak (Fig. 3). Seven, 300 μL column fractions associated with the heterodimer peak were dried individually, dissolved in 600 μL water, the FSH concentration determined by SEC, and representative chromatograms shown (Fig. 3, inset I).

3.4 GH_3-hFSH glycoform characterization

Each FSH and subunit fraction was evaluated by Western blot analysis (Fig. 3 inset II) and pooled glycoform preparations were characterized by SDS-PAGE (inset III). Comparison of the SEC chromatogram for fraction 1, at the beginning of the heterodimer peak, with that of fraction 7, near the end, revealed that the lower molecular weight fraction 7 exhibited a longer retention time and 71% increased peak width at half-height. The longer retention time indicated reduced molecular mass due, at least in part, to the absence of one or more FSHβ N-glycans, while greater peak width was consistent with the presence of two FSHβ bands in the Western blot of fraction 7 and only one in fraction 1. Similar results were obtained during analysis of fractions 2 and 6 (not shown). Carbohydrate composition analysis of fraction 1 and 2 hydrolysates indicated higher GlcNAc and Gal content relative to 3 Man residues than was found in fraction 6 and 7 hydrolysates (supplement Table 4). Greater relative abundance of both residues was consistent with fraction 1 and 2 glycans possessing largely triantennary glycans, which would possess 3 Gal residues/3 Man residues (actual values 2.9 and 2.8 for fractions 1 and 2, respectively). The Gal content of 1.4 residues/3 Man residues in fraction 6 and 7 hydrolysates suggests more 1- and 2-branch glycans, while the 5.6 and 5.7 GlcNAc/3 Man content was consistent with initiation of the second and third glycan branches, but failure to extend them. Such glycans were detected in mass spectrometric analysis of total recombinant GH_3-hFSH glycans (supplement Figs. 2 and 3). Sialic acid content was lower than the Gal content for all four hydrolysates indicating the presence of uncapped glycans, which were also observed in the glycan mass spectra. The same characteristics, enrichment for triantennary glycans in pituitary $hFSH^{24}$ and greater abundance of biantennary glycans in $hFSH^{21}$, along with partial sialylation of most glycans was observed following mass spectrometric analysis of pituitary hFSH glycoform glycans (Bousfield et al., 2014b).

FSH radioligand assay (Fig. 4A) indicated fractions 6 and 7 possessed the same receptor-binding activity, which was 6.4-fold greater than that of fraction 2 and 15-fold greater than that of fraction 1 (Table 1). There was no significant difference between $hFSH^{21/18}$ fractions 6 and 7 ($p = 0.37$), but $hFSH^{24}$ fraction 1 was significantly less active than fraction 2 ($p < 0.0001$). Both low molecular weight $hFSH^{21/18}$ fractions were significantly more active than both high molecular weight $hFSH^{24}$ fractions ($p < 0.0001$).

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SDS-PAGE revealed a single, broad Coomassie Blue stained band for each glycoform preparation, which is typical for FSH preparations that display a high degree of microheterogeneity in the glycan moieties attached to both subunits (Fig. 3, inset III). The faster mobility of the broad GH_3-$hFSH^{21/18}$ band suggested both subunits were smaller than those associated with GH_3-$hFSH^{24}$. Three high molecular weight protein contaminants appeared in the $hFSH^{24}$ lane, but were not observed in the SEC chromatogram (inset I), nor were they detected in Western blots.

Both glycoform preparations were analyzed by conventional, SDS-PAGE/electrotransfer to PVDF and probe, as well as automated, capillary electrophoresis Western blot procedures (Fig. 5). The pooled $hFSH^{24}$ glycoform preparation possessed 79% $FSH\beta^{24}$ along with 21% $FSH\beta^{21}$ (Fig. 5A, lane 1). The latter was only 11% by automated Western blotting (Fig. 5B, lanes 3 and 4). The $hFSH^{21/18}$ preparation appeared to possess a mixture of 54% $FSH\beta^{21}$ and 46% $FSH\beta^{18}$ in the conventional blot and 66% and 34%, respectively, in the automated blot. The $hFSH\beta^{21}$ band partially overlapped with the $FSH\beta^{24}$ band in the $hFSH^{24}$ preparation in both Western blot systems. The GH_3-$hFSH^{21}$ fraction resembled pituitary $hFSH^{21/18}$ isolated from hLH preparations, as both partially glycosylated FSHβ subunit variants were present (Bousfield et al., 2014a). It is likely the narrow detection range for our FSHβ Western blots was largely responsible for GH_3-$hFSH^{18}$ not being detected in the unfractionated GH_3-hFSH. The difference in mobility between GH_3-$FSH\beta^{24}$ and GH_3-$FSH\beta^{21}$ was not as large as expected from the individual fraction Western blot in Fig. 3.

Automated Western blot analysis involved separation of reduced samples by capillary electrophoresis under conditions that approximated 10% polyacrylamide gels, according to the manufacturer. Migration of FSH subunits differed from what we had become used to for conventional Western blots (Fig. 5B and C). Comparison with pituitary hFSH samples revealed GH_3-$hFSH^{24}$ β-subunit migrated as a single band, as in the conventional Western blot. While the separation of the GH_3-$hFSH^{21/18}$ bands was improved over polyacrylamide gels, migration of the $FSH\beta^{21}$ band was closer to that of the $FSH\beta^{24}$ band. Although the pituitary hFSH preparation possessed only $FSH\beta^{21}$, when subunits were immunopurified, its migration during capillary electrophoresis was like that of GH_3-$hFSH^{18}$. Migration of the FSHα subunits was similar for pituitary $hFSH^{24/21}$ and GH_3-$hFSH^{24}$, which contrasted with the slower migration of FSHα in the GonalF and Follistim preparations in a conventional Western blot (Fig. 1, lanes 7–10). Migration of GH_3-$hFSH^{21/18}$ α-subunit produced a band as broad as pituitary FSHα, attended by a somewhat faster mobility.

3.5 Determination of glycosylation site occupancy by Edman degradation

In case of the $hFSH\beta^{24}$ there was virtually no PhNCS-Asn detected at cycles 5 and 7, which correspond to Asn^7 in the truncated and full-length hFSHβ primary structures (supplement Figs. 6A and B). After PNGase F digestion, significant amounts of PhNCS-Asp were observed at Edman cycles corresponding to Asn^7 in $hFSH\beta^{24}$ (supplement Figs. 6C and D). The results of sequencing PNGase F-digested $hFSH\beta^{21/18}$ demonstrated 23% of the Asn^7 and 71% of the Asn^{24} residues were not glycosylated (supplement Fig. 7). This was consistent with the greater abundance of $hFSH^{21}$ (lacks Asn^{24} glycan) than $hFSH^{18}$ (lacks Asn^7 glycan) in GH_3-$hFSH^{21/18}$ Western blots.

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3.6 Comparison of GH_3-hFSH glycoform receptor-binding activities

In a homologous competition assay, the activity of recombinant GH_3-$hFSH^{24}$ was 20844 IU/mg, compared with highly purified pituitary hFSH reference preparation (Fig. 4B and Table 2). The activity of the recombinant GH_3-$hFSH^{21/18}$ preparation was 2.8-fold greater, 57942 IU/mg. The reduced difference in the pooled glycoform preparation activities remained significantly different from each other ($p < 0.0001$) and from pituitary $hFSH^{24}$ ($p < 0.0001$). It was unlikely that it was the use of human FSH tracer and hFSHR, as a 2-fold difference was observed in the rat/^{125}I-eFSH RLA for the same glycoform preparations (data not shown).

Leveling off of FSHR binding sites by both recombinant and pituitary $hFSH^{24}$ tracer occurred at a lower concentration than that for GH_3-$hFSH^{21/18}$ tracer in three species, rat, bovine and human (Fig. 6). The difference in binding ranged from almost 2-fold in calf testis membranes to 3-fold in the CHO-hFSHR cell line (Table 3). An almost 6-fold difference was observed in the rat, however, this was due to the last 3 concentrations, which abruptly diverged from the pattern established by the first 6 concentrations (Fig. 6A inset). While not consistently observed in every experiment, this phenomenon occurs frequently.

3.7 Glycosyltransferase expression in GH_3 cells

The majority of the glycans in GH_3-hFSH were bi-antennary and these were more abundant than their counterparts in pituitary hFSH (supplement Fig. 2 and Table 2). A striking difference between pituitary and GH_3 recombinant hFSH glycan populations was the absence of GlcNAc on the 4-position of the 3-antenna in tri-antennary glycans from the latter and the absence of GlcNAc on the 6-position of the 6-antenna in tri-antennary glycans from the former. As GlcNAc residues are attached to N-linked glycoproteins by specific GlcNAc transferases, we evaluated GH_3 cell mRNA for GlcNAc transferase IV and V isozymes. The former adds GlcNAc in β1–4 linkage to Man α(1–3)Man in the pentasaccharide core, while the latter adds GlcNAcβ1–6 to Man α(1–6)Man. Since pituitary hFSH possesses tetra-antennary glycans, at least one isoform of each transferase must function in gonadotropes. Both GlcNAc transferase IVa and IVb, but not IVc, were expressed in GH_3 cells, however, no GlcNAcβ(1–4)Man was detected in GH_3-hFSH glycans. Only GlcNAc transferase V, but not Vb, was detected (Fig. 7A). The former appeared to be active as, all tri-antennary glycans possessed GlcNAcβ(1–6)Man. The abundance of tri-antennary glycans was much higher in pituitary hFSH glycans than in GH_3-hFSH glycans, suggesting the activity of GH_3 GlcNAc transferase V was lower than that of pituitary GlcNAc transferase isozymes.

Antenna-linked Fuc residues were common in GH_3-hFSH glycans and relatively rare in pituitary hFSH oligosaccharides (Green and Baenziger, 1988a, b, Renwick et al., 1987). GH_3 cells expressed fucosyltransferase isozymes 1, 11, 10, 7, and 2, in decreasing order of PCR product abundance (Fig. 7B). The transferase isozyme responsible for antenna-linked fucose, Fut8, was undetectable in our experiments. Nevertheless GH_3-hFSH glycans included more antenna-linked fucose than those derived from pituitary hFSH or hFSH glycoforms.

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In FSH, Neu5Ac is added in either α2–3 or α2–6 linkage. Each linkage is the result of a separate sialyltransferase isoform (Weinstein, de Souza-e-Silva and Paulson, 1982). RT PCR identified two α2–3 sialyltransferase isoform and one α2–6 sialyltransferase isoform messages (Fig. 7C). While no experiments were performed to evaluate the relative abundance of these two linkages, it is reasonable to expect more α2–3-linked Neu5Ac in GH_3-hFSH preparations. This will be tested when additional recombinant GH_3-hFSH glycoform preparations become available.

4. Discussion

The goal of the current study was to isolate and characterize recombinant FSH glycoforms. We found the three variants we are interested in studying: FSH^{24}, FSH^{21}, and FSH^{18}. However, differences in electrophoretic mobility of the FSHβ bands required additional analysis to verify that GH_3 cells had indeed produced all three. Oligosaccharide microheterogeneity differed from pituitary hFSH, revealing a shift toward more biantennary glycans and addition of a third antenna on a more flexible position, which might have affected electrophoretic mobility. The pooled recombinant glycoform preparations exhibited a reduced difference in apparent affinity for the FSH receptor and it is not yet known if this reflects cross contamination of glycoforms or variations in the ratio of FSH^{21} to FSH^{18}. Furthermore, it is unknown if glycan structural differences from pituitary hFSH will be functionally significant. Results of both *in vitro* and *in vivo* functional studies using these recombinant glycoform analog preparations indicate that recombinant GH_3-$hFSH^{21/18}$ is more active than GH_3-$hFSH^{24}$ (Davis, J.S. and Kumar, T.R., unpublished data).

Mass spectrometry revealed many similarities and one major difference between pituitary hFSH and recombinant GH_3-hFSH oligosaccharide populations. Comparison on the basis of individual glycan structures suggested greater similarity in glycosylation patterns for both preparations than when the combination of structures sharing the same neutral core were compared. For example, the three most abundant glycan structures in both hormone preparations were all disialylated biantennary oligosaccharides, while the fourth most abundant structures were trisialylated and triantennary, but differed in the location of the third antenna. This antenna was located on the Man6-branch in GH_3-hFSH glycans, linked β1–6 to an exocyclic carbon atom in a mannose residue that was itself linked α1–6. The exocyclic linkages created the possibility of greater flexibility than is associated with triantennary pituitary FSH glycans, in which the third antenna was β1–4 linked to mannose ring carbon 4 in the Man3branch (Homans, Dwek and Rademacher, 1987). When glycan family abundance was compared, the greater abundance of triantennary glycans in pituitary hFSH was consistent with the fact that its two most abundant families both possessed triantennary glycan cores, as compared with biantennary cores for the two most abundant families in GH_3-hFSH. For GH_3-hFSH glycans, the third most abundant family was also biantennary, while only the fourth family was triantennary. The combination of smaller glycans and greater flexibility on the part of the GH_3-hFSH triantennary glycans may have contributed to the faster electrophoretic mobility for this preparation than pituitary hFSH in Western blots.

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The purified GH_3-hFSH Western blot revealed roughly equivalent amounts of $hFSH^{24}$ and $hFSH^{21}$, as compared with 20–27% $hFSH^{21}$ in pituitary and other recombinant hFSH preparations. The promise of increased yield of hypo-glycosylated hFSH was fulfilled, as purification yielded twice as much GH_3-$hFSH^{21/18}$ as GH_3-$hFSH^{24}$. All three glycoforms were present in GH_3-hFSH preparations, although $hFSH^{18}$ was not detected until the glycoform separation step, as previously reported for pituitary hFSH glycoforms (Bousfield et al., 2014a). Differentiating GH_3-$hFSH^{21}$ from GH_3-$hFSH^{24}$ by either conventional or automated Western blot experiments was not straightforward because of partial overlap in electrophoretic mobilities of the FSHβ and $FSH\beta^{21}$ subunit bands. This may have been due to greater biantennary glycan abundance (56%) and reduced abundance of tri-antennary glycans (30%) in GH_3-hFSH preparations than was observed in pituitary hFSH (38% and 41%, respectively). However, Gonal F preparations appear to possess even more (66%) biantennary glycans, with only 24% triantennary and 9% tetraantennary (Gervais et al., 2003), yet the electrophoretic mobilities of their β-subunits match those of pituitary hFSH. While glycans are generally considered to be very flexible molecules, one feature that exhibits the greatest flexibility is the antenna attached to the Man6-branch (Petrescu et al., 1999). Since GH_3-hFSH triantennary glycans almost exclusively possess Man6-linked third antennae, the increased flexibility may permit greater mobility during electrophoresis. Nevertheless, automated Edman degradation of the deglycosylated, hypo-glycosylated $FSH\beta^{21/18}$ preparation confirmed the presence of both mono-glycosylated β-subunits and the greater abundance of $FSH\beta^{21}$ as compared with $FSH\beta^{18}$.

While individual GH_3-$hFSH^{21/18}$ column fractions exhibited a 6- to 15-fold greater apparent affinity for the rat FSH receptor, the pooled GH_3-$hFSH^{21/18}$ preparation displayed only 2.8-fold greater affinity for both the rat and human FSH receptors. The reduced difference was caused by a leftward shift of the GH_3-$hFSH^{24}$ ID_{50}. It is possible that the 11–21% contamination with hypo-glycosylated hFSH, detected in Western blots, contributed to the increased receptor-binding activity of GH_3-$hFSH^{24}$. However, purified pituitary hFSH preparations often possess 20% $hFSH^{21}$, yet are only as active as most $hFSH^{24}$ preparations (Bousfield et al., 2014a, Bousfield et al., 2007). We were able to use ^{125}I-$hFSH^{21/18}$ to demonstrate that this glycoform could access at least twice as many FSH binding sites as $hFSH^{24}$ tracer in FSH receptor-binding experiments involving two additional species, bovine and human. This is consistent with a recent report from our laboratory that during competitive binding and association assays, that used the same concentration of ^{125}I-FSH tracer, or those experiments in which the tracer concentrations progressively increased, hypo-glycosylated pituitary FSH preparations bound more rat FSHR sites than fully-glycosylated FSH^{24} preparations (Bousfield et al., 2014a). The magnitude of the binding in the present study reflected the increasing numbers of FSH receptors found in rat and calf testicular membranes and in overexpressed human FSH receptors. Nevertheless, greater $hFSH^{21/18}$ binding was consistently observed, suggesting it is a general phenomenon.

When FSHRs were assumed to exist as single receptor molecules in target cell membranes, increased ligand binding associated with missing N-glycans would have been difficult to interpret because hFSH glycans do not appear to be close to the FSHR extracellular domain (supplement Fig. 8A). However, FSHR has been reported to exist as dimers, trimers or small oligomers in the membrane (Guan et al., 2010, Jiang et al., 2014, Thomas et al., 2007).

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Accordingly, binding of FSH to one ligand binding site may affect FSH binding to a second ligand binding site, and dissociation studies seemed to provide supporting evidence (Urizar et al., 2005). Moreover, FSH dissociation was incomplete, supporting the existence of monomeric as well as dimeric/oligomeric receptors. As the latter possess one occupied and at least one unoccupied FSH binding site, unlabeled hormone may bind the unoccupied site and subsequently displace bound FSH tracer. Dimeric/oligomeric FSHRs account for the ability of 1000-fold excess cold FSH to partially displace receptor-bound ^{125}I-FSH tracer. As monomeric FSH receptors lack unoccupied FSH binding sites, cold hormone cannot displace FSH tracer because the FSH-FSHR binding has been demonstrated to be stable for as long as 24 hours in the absence of cold FSH (Cheng, 1975). A recent review of FSH and FSHR structural biology pointed out that as FSH binding proceeds, the molecule exhibits less and less flexibility, which is consistent with the absence of spontaneous dissociation by FSH from its receptor (Jiang, Dias and He, 2013).

Qualitative differences in FSHR conformation or in the number of ligand binding sites occupied might alter signal transduction in gonadal target cells. Alternatively, it has been shown that some cellular responses to gonadotropin stimulation, such as cAMP accumulation, are directly proportional to the number of occupied receptors on the surface of target cells (Bhaskaran and Ascoli, 2005). The larger number of FSH binding sites available to hypo-glycosylated hFSH predicts that it will be more active than fully-glycosylated hFSH. Indeed, we have found this to be true both *in vitro* and *in vivo* (Davis et al., 2014). Despite some variation in the appearance of their β-subunit bands in Western blotting experiments, it seems likely that the mechanism responsible for the difference in receptor binding between hFSH$^{21/18}$ and hFSH^{24} is the same for both pituitary and recombinant FSH glycoform preparations.

A recent study characterized hFSH isoforms, classified as either di- or tetra-glycosylated hFSH glycoforms (and corresponding to hFSH$^{21/18}$ and hFSH^{24} in the present study) in daily serum samples obtained from 79 healthy women during their menstrual cycles revealed very interesting observations (Wide and Eriksson, 2013). The pattern of the serum hFSH^{21} concentrations was characterized by a steep rise from day 27 of a previous cycle to days 3–6 of the next cycle and then a decrease in concentration lasting from day 7 to day 11 followed by a pronounced midcycle peak (Wide and Eriksson, 2013). After that there was a rapid decrease to the lowest level on days 17–19 (Wide and Eriksson, 2013). The hFSH^{24} glycoform concentration increased to a high plateau level lasting from day 3 to day 15 followed by a slow decrease with no midcycle peak (Wide and Eriksson, 2013). These findings suggest that FSH glycoforms have different physiological roles in ovarian stimulation and are consistent with clinical studies showing improved ovarian stimulation with mixtures of acidic and less acidic hFSH isoforms (Selman, Pacchiarotti and El-Danasouri, 2010). High and low sialic acid content FSH and Conanavalin A unbound and tightly bound FSH have recently been shown to elicit strikingly different patterns of gene expression in cultured KGN granulosa cell tumor cells (Loreti et al., 2013). The availability of recombinant hFSH glycoform preparations will make it possible to determine if similar effects can be obtained with fully- and hypo-glycosylated hFSH, as GH_3-hFSH$^{21/18}$

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glycoforms have been demonstrated to be more active than GH_3-$hFSH^{24}$ both *in vitro* and *in vivo* (Davis et al., 2014).

Transformed GH_3 cells expressed the three physiologically relevant hFSH glycoforms, FSH^{24}, FSH^{21}, and FSH^{18}, previously found in the pituitary gland. Glycan mass spectrometry revealed many of the glycan structures found in pituitary hFSH were also present in GH_3-hFSH. Amino acid sequencing of the hypo-glycosylated glycoform β-subunits revealed that the Asn^{24} oligosaccharide is missing from $hFSH^{21}$ and the Asn^{7} glycan is missing in $hFSH^{18}$. Hypo-glycosylated $hFSH^{21/18}$ preparations exhibited higher apparent affinity for the FSH receptor and occupied twice as many FSH binding sites as fully-glycosylated $hFSH^{24}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Recombinant hFSH glycoforms $hFSH^{24}$, $hFSH^{21}$, and $hFSH^{18}$ were expressed in transformed GH_3 cells.
- Recombinant hFSH showed a similar pattern of glycosylation macroheterogeneity as pituitary hFSH.
- Oligosaccharide microheterogeneity was similar for both pituitary and recombinant hFSH, the major qualitative difference was the branching pattern for triantennary glycans.
- Recombinant $hFSH^{21/18}$ occupied more FSH binding sites than pituitary $hFSH^{24}$ in binding assays involving rat, bovine, and recombinant human FSH receptor preparations.

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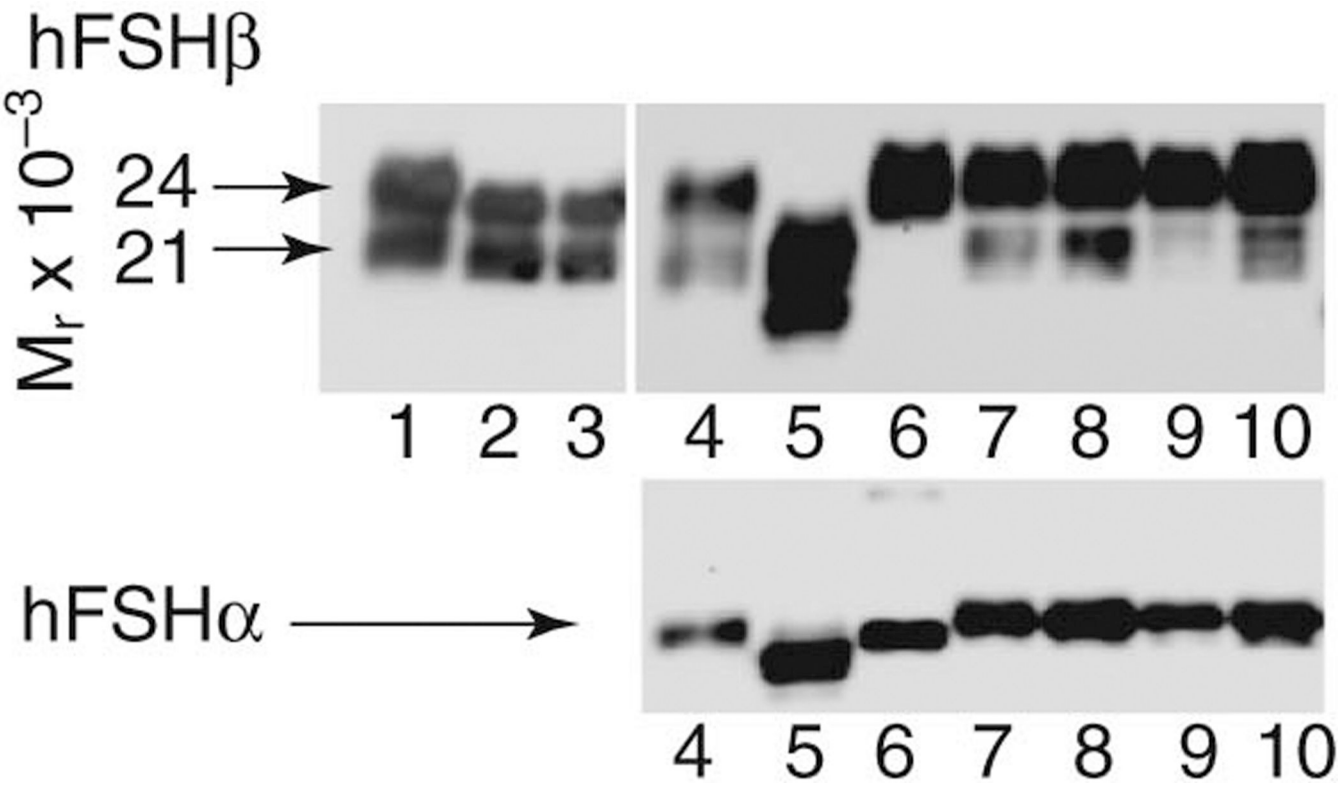

Figure 1. FSH subunit Western blot collage comparing several pituitary and recombinant hFSH glycoform preparations

Samples of reduced hFSH preparations were subjected to electrophoresis on 15% polyacrylamide gels, electroblotted to PVDF, and probed with anti-hFSHβ monoclonal antibody RFSH20 (upper panel) and anti-alpha antibody HT13 (lower panel). The arrows indicate the 24,000 and 21,000 M_r pituitary hFSHβ bands, as well as the FSHα subunit band. Lane 1, 1 μg pituitary hFSH AFP4161; lane 2, 1 μg GH_3-hFSH; lane 3, 0.5 μg GH_3-hFSH; lane 4, 1 μg hFSH AFP4161 (20% $FSH\beta_{21}$); lane 5, 1 μg pituitary $hFSH^{21/18}$ (60% $FSH\beta^{21}$, 40% $FSH\beta^{18}$); lane 6, 1 μg $hFSH^{24}$ ($FSH\beta^{21}$ below limits of detection); lanes 7 and 8, 0.5 and 1 μg samples Follistim recombinant hFSH (27% $FSH\beta^{21}$); lanes 9 and 10, 0.5 and 1 μg samples GonalF recombinant hFSH (21% $FSH\beta^{21}$). Lanes 1–3 and 4–10 are from separate Western blots.

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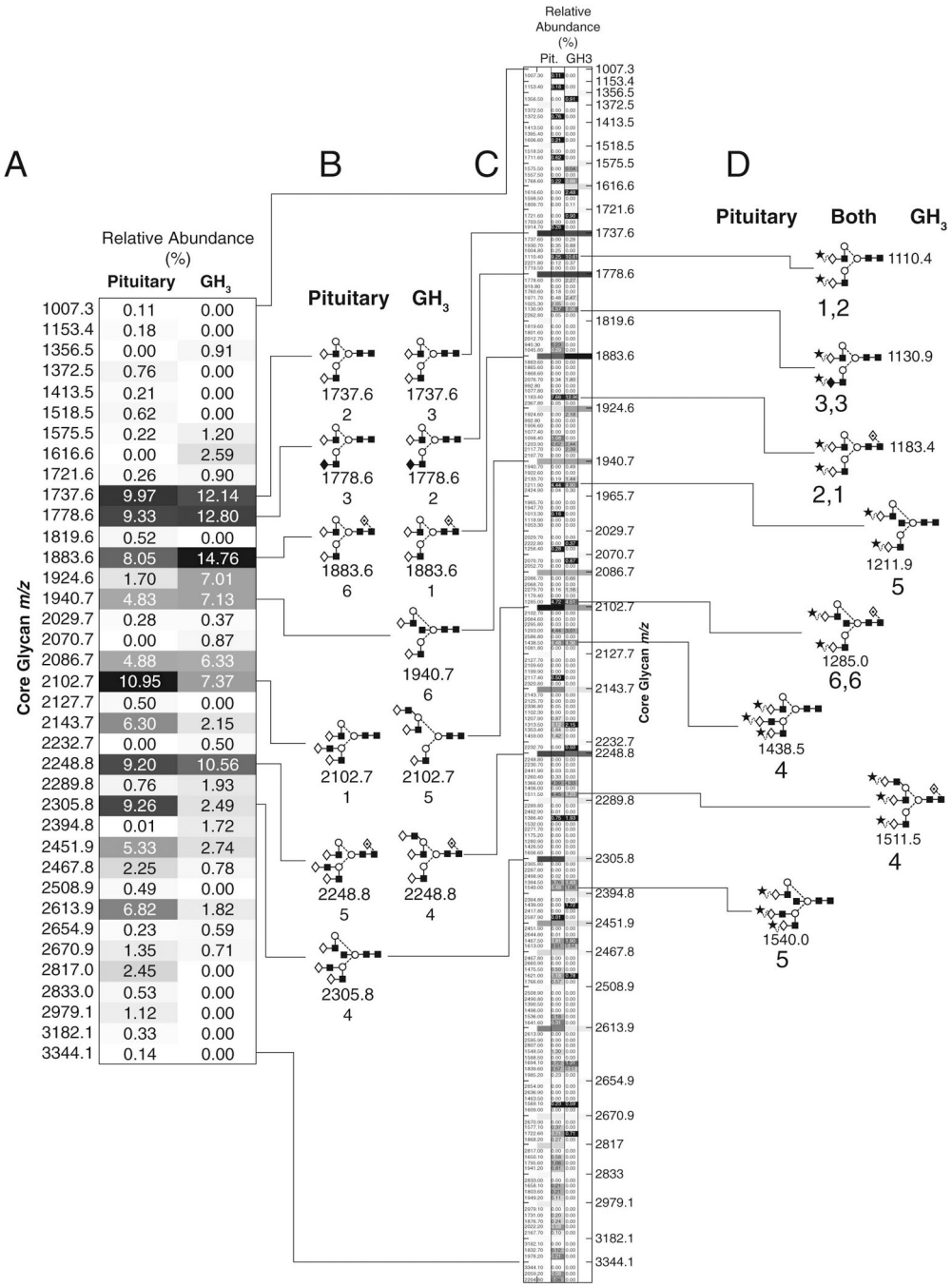

Figure 2. Comparison of pituitary hFSH with recombinant GH_3-hFSH glycans

The mass spectra, data tables, and glycan structure diagrams are shown in the supplement. A. Glycan abundance based on neutral core oligosaccharide structures, as indicated. B. Six most abundant neutral glycan cores for pituitary and recombinant hFSH, as indicated. C. Glycan variant abundance, grouped by neutral core structure. D. Six most abundant glycans in pituitary and recombinant hFSH, as indicated.

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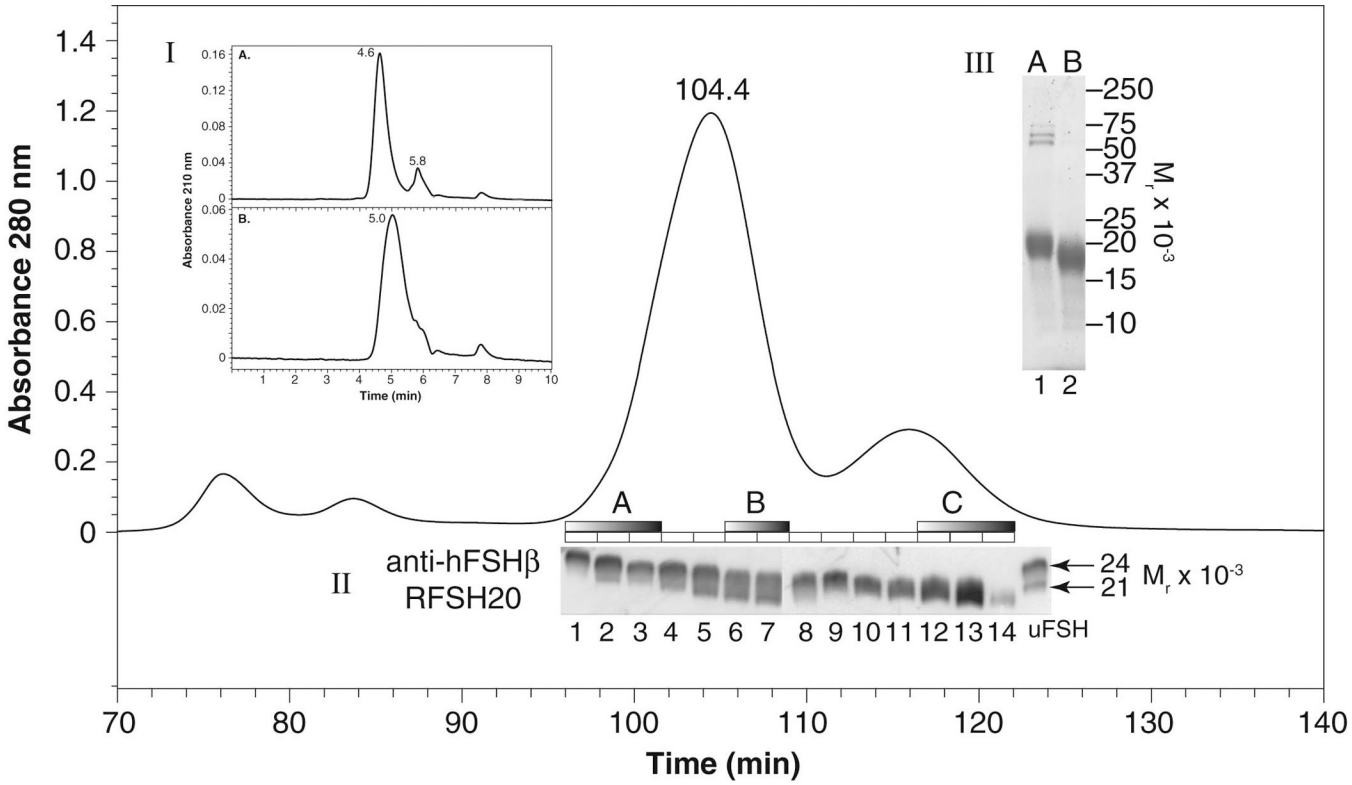

Figure 3. Superdex 75 chromatography of immunopurified recombinant, GH_3-hFSH

Representative chromatogram showing fractionation of hFSH heterodimer and subunit peaks. Fractions 1–3 were pooled, as indicated by shaded bar labeled, A, and yielded 514 μg GH_3-$hFSH^{24}$, while GH_3-$hFSH^{21/18}$ consisted of pooled fractions 6 and 7, which yielded 1058 μg lyophilized protein (B). The intermediate molecular weight, mixed glycoform preparation consisted of 1106 μg. Total GH_3-hFSH recovery was 2680 μg, 12.6% of the FSH immunoreactivity in conditioned medium. Inset II shows a collage of two Western blots used to identify the components of each fraction. The blots were aligned by the tops of the $FSH\beta^{24}$ bands in urinary $hFSH^{24/21}$ used as a reference. The bar indicating the portion of the free subunit peak evaluated in this study was labeled C. Inset I: analytical SEC of fractions 1 and 7 illustrating the differences in peak shape and retention time. Inset II: Anti-hFSHβ Western blot of fractions 1–14, as indicated. Inset III: SDS-PAGE followed by Coomassie Blue staining. Lane 1, GH_3-$hFSH^{24}$; lane 2, GH_3-$hFSH^{21/18}$. BioRad Precision Pre-stained MW marker positions are indicated.

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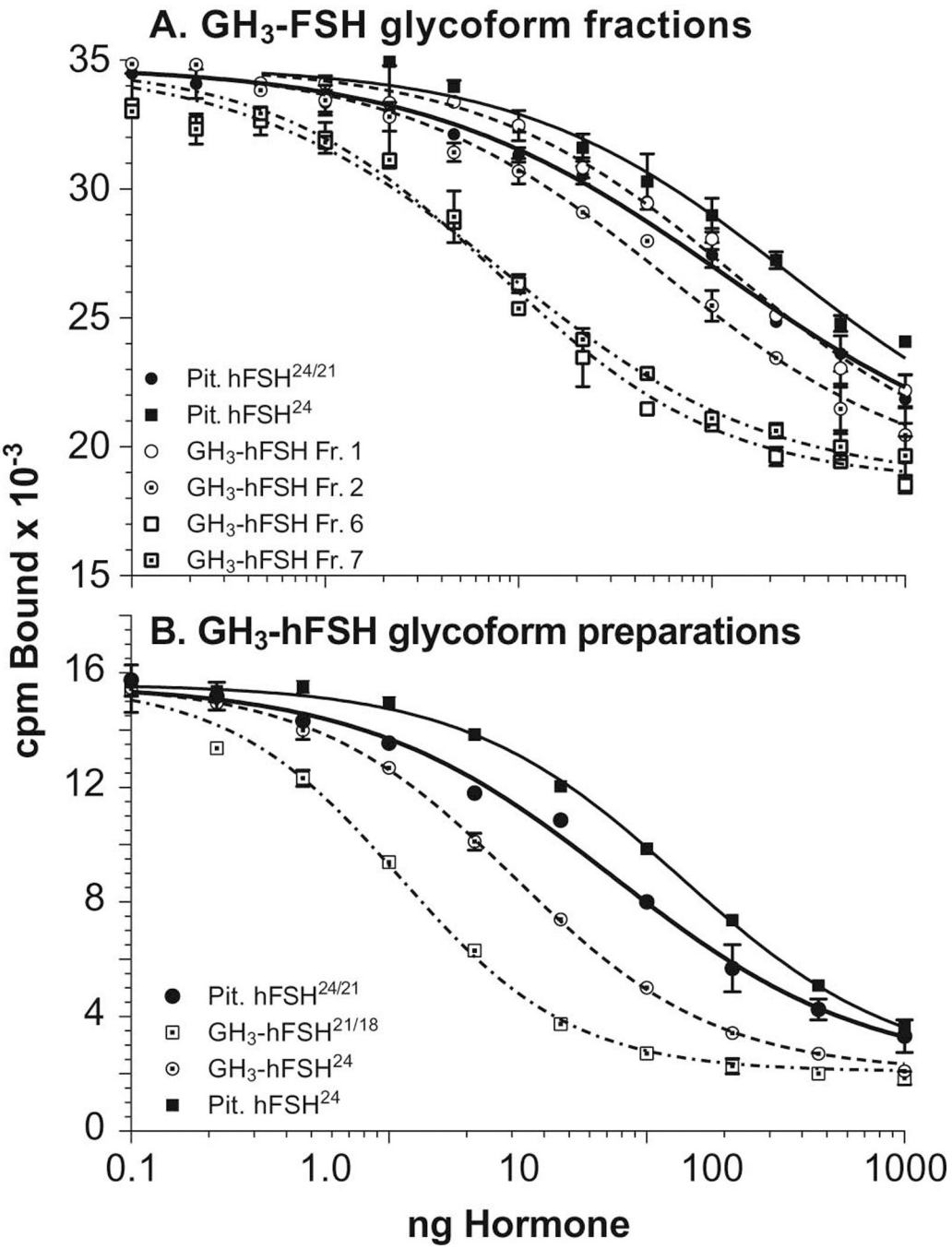

Figure 4. FSH radioligand assay of GH_3-hFSH fractions and glycoform preparations

A. Samples of fractions 1, 2, 6, and 7 from the chromatogram in Fig. 3 were serially diluted and used to compete for the binding of ^{125}I-eFSH to rat testis homogenate. Reference preparations included $hFSH^{24/21}$ (AFP7298A, closed circle) and pituitary $hFSH^{24}$ (closed square). Quantitative results are listed in Table 1. B. GH_3-hFSH glycoform preparations tested in a homologous FSH RLA, which employed ^{125}I-$hFSH^{21}$ as tracer and CHO cells expressing hFSH receptors as the receptor preparation. Quantitative results are listed in Table 2.

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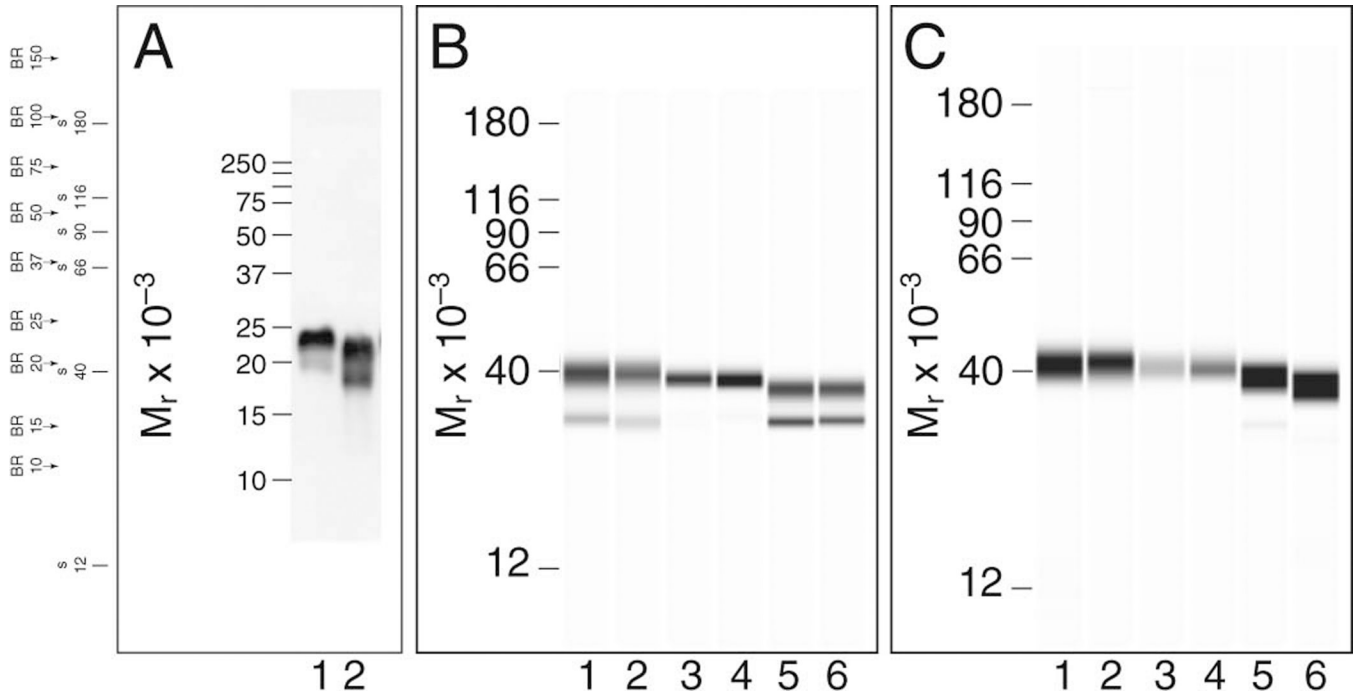

Figure 5. Conventional and automated Western blotting of GH_3-hFSH^{24} and –hFSH$^{21/18}$ preparations

A. Conventional Western blot probed with anti-hFSH/FSHβ antibody RFSH20. Lane 1, 1 μg GH_3-hFSH^{24}; lane 2, 1 μg GH_3-hFSH$^{21/18}$. **B.** Automated Western blot probed with anti-FSHβ antibody 15-1.C3C5 diluted 1:100. Lane 1, 40 ng pituitary hFSH AFP7298A; lane 2, 40 ng pituitary hFSH AFP7298A; lane 3, 40 ng GH_3-hFSH^{24}; lane 4, 40 ng GH_3-hFSH^{24}; lane 5, 40 ng GH_3-hFSH$^{21/18}$; lane 6, 40 ng GH_3-hFSH$^{21/18}$. **C.** Automated Western blot probed with anti-α antibody 15-2.C3.B8 diluted 1: 100. Samples are shown in the same order as in panel B.

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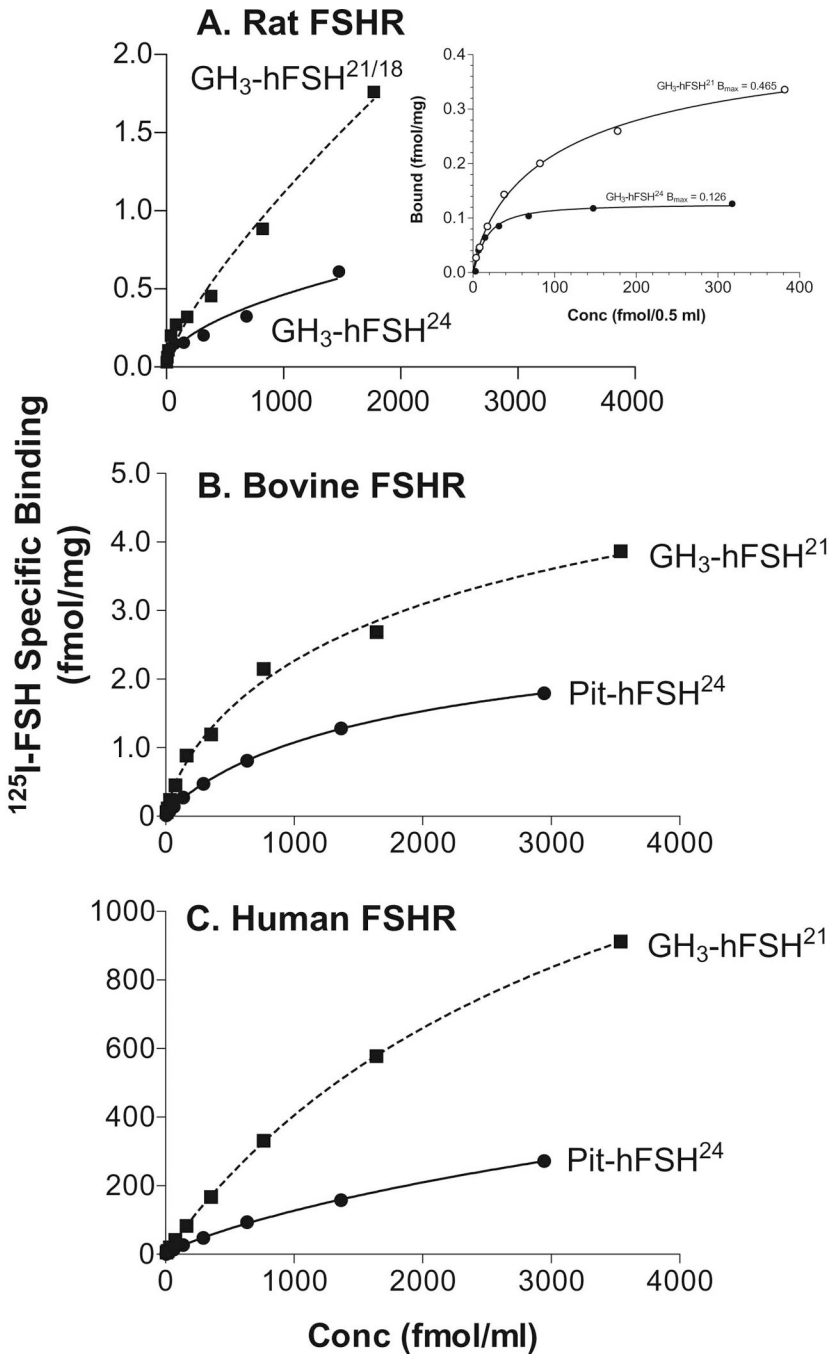

Figure 6. Binding of GH_3-hFSH glycoforms to rat, bovine, and human FSH receptors under conditions close to saturation

Specific binding of ^{125}I-GH_3-$hFSH^{24}$, ^{125}I-pituitary $hFSH^{24}$, or ^{125}I-GH^3-$hFSH^{21/18}$, as indicated, to FSH receptor preparations. A. Rat testicular homogenate 25 mg/tube. Inset. Binding at low tracer concentrations. B. Calf testis membranes 20 mg/tube. C. Human FSH receptors expressed in Chinese hamster ovarian cells, 250,000 cells/tube. Each panel, representative results from two experiments.

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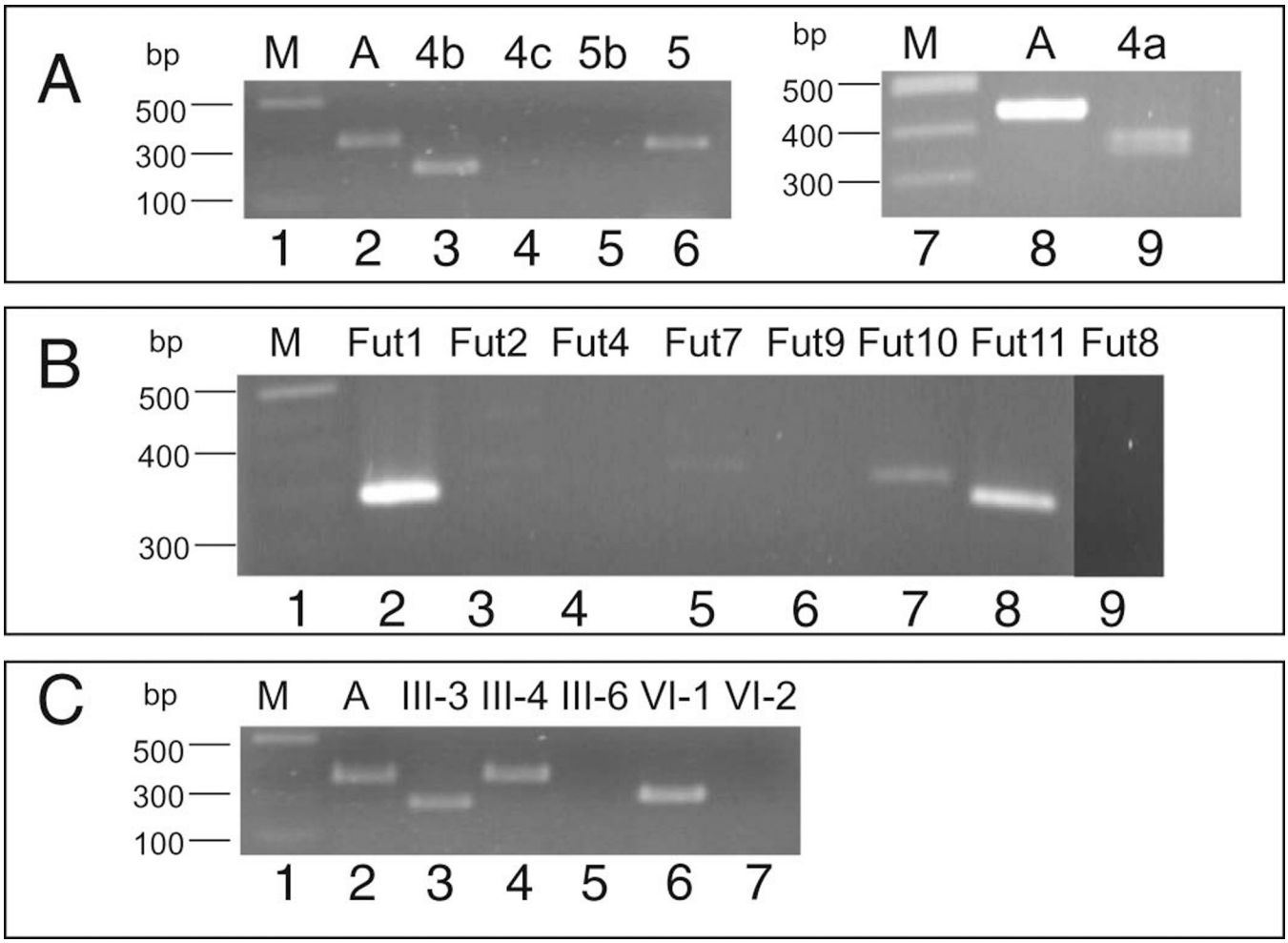

Figure 7. Glycosyltransferase expression in GH_3 cells

A. RT-PCR detection of glycosyltransferases in GH_3 cells expressing hFSH. B. Fucosyltransferase isoform expression in GH_3 cells expressing hFSH. C. Sialyltransferase isoform expression in GH_3 cells expressing hFSH.

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Table 1

FSH receptor-binding activities of GH3-hFSH glycoform fractions

Two high molecular weight and two low molecular weight fraction that were recovered from Superdex 75 gel filtration chromatography (Fig. 3) were tested in rat testis FSH RLA using ^{125}I-eFSH tracer.

FSH Preparation	ID_{50} (ng)	Relative Potency (IU/mg)	$FSH^{21/18}/FSH^{24}$
Pituitary $hFSH^{24/21}$	114	8560[a]	
Pituitary $hFSH^{24}$	253	3875	
GH3-hFSH Frxn 1	131	7498	15.0
GH3-hFSH Frxn 2	56	17518	6.4
GH3-hFSH Frxn 6	7.9	124335	
GH3-hFSH Frxn 7	8.7	112598	

[a] As provided by the National Hormone and Pituitary Program for AFP7298A.

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Table 2

FSH receptor-binding activities of GH3-hFSH glycoform preparations

The pooled GH_3-$hFSH^{21}$ and GH_3-$hFSH^{21/18}$ preparations (Fig. 3) were tested in CHO-hFSH receptor RLA using ^{125}I-$hFSH^{21}$ tracer.

FSH Preparation	ID_{50} (ng)	Relative Potency (IU/mg)	$FSH^{21/18}/FSH^{24}$ Activity Ratio
Pituitary $hFSH^{24/21}$	75	8560[a]	
Pituitary $hFSH^{24}$	136	4721	
GH3-$hFSH^{24}$	30.8	20844	
GH3-$hFSH^{21/18}$	11.1	57942	2.8

[a] As provided by the National Hormone and Pituitary Program for AFP7298A.

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Table 3

Ratio of $FSH^{21/18}$ to FSH^{24} binding to rat, bovine, and human FSH receptors in receptor binding studies under conditions close to saturation shown in Fig. 6.

FSH receptor preparation	Rat Testis Homogenate	Calf Testis Membranes	CHO-hFSHR
FSH^{24} Bmax	0.9972	2.609	600.8
FSH^{24} Kd	1062	685.0	1805
$FSH^{21/18}$ Bmax	5.859	4.721	1784
$FSH^{21/18}$ Kd	4198	484.9	1697
Ratio $FSH^{21/18}$/FSH^{24} Bmax	5.9	1.8	3.0