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Expression of the genes encoding bovine LH in a line of Chinese hamster ovary cells

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Summary. Synthesis of biologically active LH is complex, due in part to its heterodimeric subunit structure and to the numerous post-translation modifications of each subunit. Through the use of mammalian expression vectors we have been able to introduce the bovine α subunit and LH-β genes into a Chinese hamster ovary cell line deficient in dihydrofolate reductase. The bovine genes are actively expressed and the Chinese hamster ovary cells secrete biologically active LH. The expression vector containing the bovine α subunit gene also contains a modified mouse gene encoding dihydrofolate reductase, permitting the use of methotrexate to amplify selectively the bovine α subunit gene after its integration into the genome of the Chinese hamster cells. This provides a novel means for assessing the importance of α subunit concentration with respect to assembly of the heterodimer. In addition, methotrexate selection leads to the over-production of LH (10 μg/10^6 cells/24 h). Finally, because the bovine LH produced in the Chinese hamster ovary cells is glycosylated, this transfection system can be used in conjunction with in-vitro mutagenesis to determine whether site-specific changes in glycosylation have an effect on subunit assembly and biological activity. This transfection approach therefore offers multiple avenues to explore further the molecular mechanisms underlying the complex biosynthetic pathway of bovine LH.

Introduction

Luteinizing hormone (LH) and chorionic gonadotrophin (CG) are two closely related heterodimeric glycoproteins synthesized in the pituitary and placenta, respectively (Pierce & Parsons, 1981; Chin, 1985). The α subunits of LH and CG are identical, a property of all glycoprotein hormones, whereas their β subunits are different, sharing an amino acid homology of 80%. Both hormones bind to the same gonadal receptor and stimulate steroidogenesis and gametogenesis, consistent with their structural homology (Pierce & Parsons, 1981).

Synthesis of biologically active LH and CG is complex, involving a number of post-translational events. The initial modification begins with the cleavage of the signal peptide sequence which occurs as the nascent α and β subunits cross the membrane of the endoplasmic reticulum (Chin *et al.*, 1978; Daniels-McQueen *et al.*, 1978). Cleavage of the signal sequence is followed immediately by the addition of *N*-linked, high mannose oligosaccharide cores (Bielinska & Boime, 1979; Magner & Weintraub, 1982; Hoshina & Boime, 1982). Non-covalent subunit combination occurs shortly thereafter. Following assembly, the heterodimers move through the Golgi complex where additional post-translational modifications take place, such as O-linked glycosylation and sulphation (Parsons *et al.*, 1983; Green *et al.*, 1985). Fully modified LH is sequestered in secretory granules where its release is regulated by hypothalamic-releasing hormones, gonadal steroids and other known secretagogues (Goverman *et al.*, 1982). In contrast, hCG is released constitutively after its synthesis due to the lack of secretory granules in placental cells (Hussa, 1980).

Characteristically, intracellular levels of free α subunit exceed the levels of dimeric hormone

(Daniels-McQueen *et al.*, 1978; Workewych & Cheng, 1979) whereas free β subunit is often not detectable (Workewych & Cheng, 1979). A common interpretation of this imbalance is that the extent of heterodimer formation is limited by the concentration of β subunit. However, Peters *et al.* (1984) indicate that almost 50% of both the α and CGβ subunits fail to combine in a human placental cell line. Both free α and β subunits of CG can be detected in the culture medium. Similar results have been reported for the assembly of α and β subunits of TSH in mouse pituitary tumour cells (Weintraub *et al.*, 1980), suggesting that extent of heterodimer formation may not be limited solely by the concentration of the β subunit. If extent of assembly is dependent on the concentration of both subunits, then changes in the extent of assembly should be proportional to the product of the change in concentration of both subunits. Thus, a small change in the concentration of each subunit will lead to a larger or multiplicative change in the concentration of heterodimer. This is an intriguing possibility and might explain the biological significance of the steadily emerging evidence that expression of the α and β subunit genes of gonadotrophin rise and fall together (Nilson *et al.*, 1983; Chin, 1985; Milsted *et al.*, 1985).

While it may be intuitively obvious that extent of heterodimer assembly depends on the concentration of both subunits, there is no direct experimental evidence *in vivo* to support this notion. We have reported that the genes encoding the bovine α and β subunits of LH can be transferred stably into a line of Chinese hamster (*Cricetulus griseus*) ovary cells (Kaetzel *et al.*, 1985). The bovine genes are expressed and the resulting subunits assemble and are secreted as biologically active hormone. By exploiting the properties of the DNA transfection system, we have begun to develop an approach that permits the concentration of one of the gonadotrophin subunits to be changed, while the other remains constant. Thus, by monitoring the extent of assembly within the cell, we can test whether changes in the concentration of α subunit affect assembly of the heterodimer. Such an approach is a prelude to future studies designed to test the importance of both subunits in assembly of the heterodimer, and to address further the structural requirements for assembly and secretion of biologically active LH.

Here we shall review the structural characteristics of the gonadotrophin genes and the properties of the DNA transfection system used to establish permanent cell lines of Chinese hamster ovary cells harbouring functional copies of the bovine LH genes. Then, we shall present preliminary evidence indicating that a selective change in the concentration of α subunit alters the extent of heterodimer assembly—the first step in determining whether assembly is a function of the concentration of both subunits.

Structural features of the genes for α and β subunits of bovine LH

We have isolated and characterized three overlapping lambda clones from a bovine genomic library that contain portions of the α subunit gene (Goodwin *et al.*, 1983). The gene contains 4 exons and 3 introns and spans 16 kilobase pairs (kbp), even though the mature mRNA is only 730 nucleotides long (Fig. 1). The length of the bovine α subunit gene is due primarily to the first intron which is 13 kbp long and is positioned in the 5′-untranslated region, only 90 bp from the start-site of transcription. The other two introns are much smaller and interrupt the coding sequence.

The α subunit gene is present as a single copy in the bovine (Goodwin *et al.*, 1983) and human (Fiddes & Goodman, 1981; Boothby *et al.*, 1981) genome. While expression of the bovine gene is restricted to the pituitary, the human α subunit is expressed in the pituitary and placenta (Pierce & Parsons, 1981; Chin, 1985). The lack of α subunit gene expression in the bovine placenta is consistent with the absence of gonadotrophins in the placenta of ruminants (Pierce & Parsons, 1981).

In contrast to the bovine α subunit gene which spans 16 kbp, the gene subunit for the LH-β subunit spans less than 1·1 kbp (Fig. 1; Virgin *et al.*, 1985). We have determined the nucleotide sequence for the entire gene and 776 bp of 5′-flanking sequence, confirming that it encodes an authentic LH-β

subunit (Virgin *et al.*, 1985). The bovine LH-β gene contains three exons and encodes an mRNA of 550 nucleotides, excluding the poly A tail. The mRNA cap site and polyadenylation site have been mapped by primer extension and S1 nuclease protection, respectively (Virgin *et al.*, 1985). Surprisingly, the 5′-untranslated region is only 6–11 nucleotides long. This is unusually short for a eukaryotic mRNA, and stands in contrast to the 5′-untranslated region of the closely related human CG-β gene which is 350 nucleotides long (Talmadge *et al.*, 1984). The functional significance of this difference is unknown.

Gene quantitation studies reveal that the bovine LH-β gene is unique and that there are no other closely related genes in the genome of cattle (Virgin *et al.*, 1985). A similar result has also been reported for the rat (Jameson *et al.*, 1984). Additional studies indicate that the bovine LH-β gene is not expressed in the placenta. The unique LH-β gene found in cattle and rats contrasts to the human gene family which consists of 7 CG-β genes and 1 LH-β gene sharing greater than 90% homology in nucleotide sequence (Fiddes & Talmadge, 1984). The high level of nucleotide sequence homology between the human LH-β and CG-β genes, and the lack of CG-β genes in the cow and rat, suggest that the CG-β genes have evolved recently from an ancestral LH-β gene via a series of duplications (Fiddes & Talmadge, 1984).

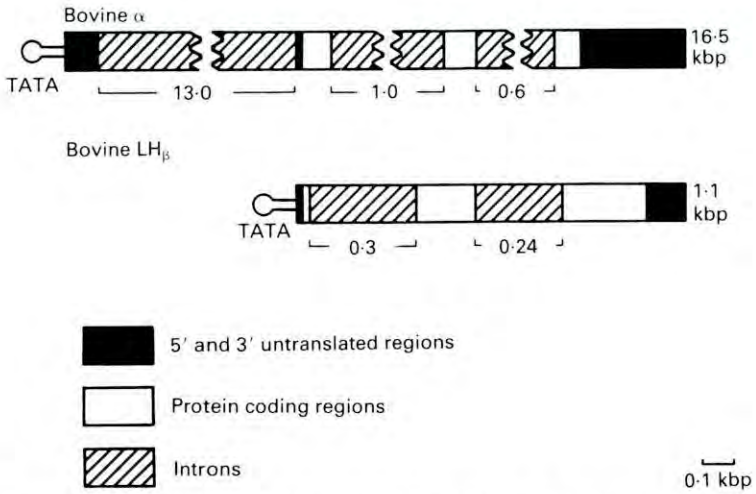

Fig. 1. Schematic comparison of the structure of the bovine α subunit and LH-β genes (see Goodwin *et al.* (1983) and Virgin *et al.* (1985) for further details).

Expression of bovine α subunit and LH-β genes in Chinese hamster ovary cells

Genes for the α and β subunits of bovine LH can be transferred to a line of Chinese hamster ovary cells deficient in dihydrofolate reductase (DHFR−) via DNA-mediated gene transfer (Kaetzel *et al.*, 1985). Because DHFR is required for de-novo synthesis of purines and pyrimidines, Chinese hamster ovary (DHFR−) cells require media supplemented with nucleosides (Kaufman *et al.*, 1985). If the bovine genes are co-transfected with a vector containing a DHFR gene, then clonal cell lines containing the DHFR genes and bovine genes can be selected by growing the transfected cells in media lacking nucleosides.

We have constructed two expression vectors, each containing a gene encoding one of the subunits of bovine LH (Fig. 2). Both vectors (pDSVα and pSV2LHβ) contain an α or LH-β gene linked to a strong viral promoter (SV40 late and SV40 early, respectively). This is intended to maximize expression of the bovine genes in non-pituitary cells, such as Chinese hamster ovary cells. Due to

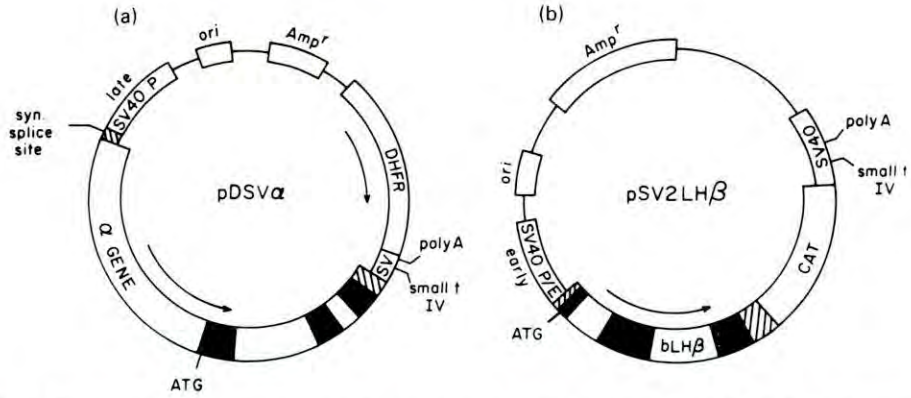

Fig. 2. Construction of two expression vectors containing the bovine α and LH-β subunit genes. **(a)** The 8·7 kbp α subunit gene fragment (intron sequences in white, exons in black, 5′ and 3′ untranslated regions hatched) is represented, with its initiator methionine (ATG) codon and direction of transcription indicated. Also shown are the late SV40 promoter element (SV40 P); a synthetic (syn) fragment containing a consensus splice–donor sequence; a SV40 small tumour antigen (t)-gene intron (IV) and polyadenylation signal, and the mouse DHFR minigene with its direction of transcription (arrow). ori, Origin of replication; Amp^r, ampicillin-resistance gene. **(b)** A 1·8 kbp Pst I genomic fragment containing the entire LH-β subunit gene was incorporated into pSV2LHβ. Still remaining in the final construct, at the 3′ end of the LH-β gene, is the bacterial chloramphenicol acetyltransferase (CAT) gene from the parent vector, pSV2CAT. P/E, promoter/enhancer. Reprinted from Kaetzel *et al.* (1985) with permission of the publisher.

the large size of the α subunit gene, it was convenient to relocate only the portion of the gene which contained the complete coding sequence. As shown in Fig. 2, this fragment contains the 3′ half of the first intervening sequence and exons 2, 3 and 4; the ATG initiation codon is located in exon 2. To ensure the correct removal of the 3′ half of the first intron, pDSVα contains a synthetic fragment carrying a consensus splice donor site positioned between the SV40 late promoter and the truncated α subunit gene. The vector also contains a mini-gene encoding mouse dihydrofolate reductase (Kaufman & Sharp, 1982). Linkage of the DHFR and α subunit genes should increase the percentage of transfected cells capable of growth in selective media and that retain the α subunit gene.

The bovine LH-β expression vector, pSV2LHβ, contains the entire LH-β subunit gene (including the RNA cap site and TATAA sequence) located on a 1·8-kbp Pst I genomic fragment (Virgin *et al.*, 1985). The gene is juxtaposed between the SV40 early promoter/origin of replication and the bacterial chloramphenicol acetyltransferase (CAT) gene (Gorman *et al.*, 1982). The CAT gene is a remnant from the SV2CAT parent vector (Gorman *et al.*, 1982) and is non-functional. The LH-β expression vector can be transferred to Chinese hamster ovary cells (DHFR−) by co-transfection with the α subunit expression vector. Even though LH-β gene is not linked to the DHFR gene, a sufficient number of clones capable of growth in nucleoside-free media should contain the LH-β gene.

To date, we have obtained several clonal lines of Chinese hamster ovary cells capable of growth in nucleoside-free media by using the expression vectors described above. Analysis of these clonal lines by RNA blot hybridization, RIA, electrophoresis of immunoprecipitated ^{35}S-labelled proteins, and LH-specific bioassay, reveals that the clones can be divided into three classes: some synthesize and secrete only α subunit, others only LH-β, while others produce both subunits (Kaetzel *et al.*, 1985). Intact and biologically active LH is found only in the clones which synthesize both subunits (Kaetzel *et al.*, 1985). Together, these results suggest that the bovine α and LH-β genes can be expressed in Chinese hamster ovary cells and that the resulting subunits assemble and

are secreted as biologically active LH. These findings also suggest that the LH of Chinese hamster ovary cells is glycosylated because biological activity appears to depend on glycosylation (Pierce & Parsons, 1981).

Methotrexate selectively increases synthesis of α subunit and secretion of biologically active LH

When cells are selected for growth in the presence of increasing concentrations of methotrexate, resistant subpopulations arise which contain amplified copies of the endogenous DHFR gene (Alt *et al.*, 1978). Furthermore, if Chinese hamster ovary cells (DHFR−) are co-transfected with a DHFR gene and a non-selectable gene, then methotrexate selection commonly results in the co-amplification of both transfected genes (Kaufman *et al.*, 1985). Having established that Chinese hamster ovary cells support expression of the bovine gonadotrophin genes, we wanted to ascertain whether methotrexate selection can affect LH synthesis. For this purpose, we selected a cell line that expresses approximately equal amounts of α and LH-β mRNA (CHO-LH20 or simply LH20 cells; see Table 1). Initial selection was performed at a concentration of 3 nM-methotrexate. After about 3 weeks, the surviving subpopulation of cells was either maintained in 3 nM-methotrexate (LH20-3 cells), or exposed to a stepwise increase in methotrexate (10 nM) for an additional 3-week period. By repeatedly increasing the concentrations of methotrexate in the media, we have isolated a number of subpopulations of LH20 cells, each of which is resistant to a defined concentration of the drug.

To determine whether methotrexate had an effect on LH synthesis, we incubated LH20 cells, and a stable population of LH20 cells resistant to 100 nM-methotrexate (LH20-100), for 18 h with 500 μCi [^{35}S]methionine/ml and 150 μCi [^{35}S]cysteine/ml. Media were collected and then subjected to quantitative immunoprecipitation with rabbit antiserum specific for bovine α or LH-β subunits (Kaetzel *et al.*, 1985). The immunoprecipitates were analysed by electrophoresis through SDS-polyacrylamide gels (Fetherston & Boime, 1982) followed by autoradiography. Two specific polypeptides were precipitated from LH20 and LH20-100 media samples by the LH-β-specific antibody (Fig. 3). Their molecular weights of 20 500 and 16 000 were slightly larger than those reported for bovine α and LH-β subunits (Pierce & Parsons, 1981; Kaetzel *et al.*, 1985). The specificity of

Table 1. Secretion of biologically active bovine LH after methotrexate selection of transfected Chinese hamster ovary cells

Cell line	Methotrexate (nM)	mRNA (relative level)* alpha	mRNA (relative level)* beta	LH bioassay (μg LH/10^6 cell/24 h)†
CHO (DHFR−)	0	0	0	<0·008
LH20	0	1	1·4	0·40 ± 0·01
LH20-3	3	1·3	2·2	0·62 ± 0·04
LH20-100	100	2·6	1·7	2·8 ± 0·03
LH20-1000	1000	5·8	1·6	10·0‡
LHβ25	0	0	0·60	<0·008
LHβ25-1000	1000	0	0·65	<0·008

*mRNA levels were determined by northern blot analysis followed by densitometry of the resulting autoradiographs. ^{32}P-labelled, single-stranded DNA probes specific for bovine α and LH-β mRNA were prepared as previously described (Nilson *et al.*, 1983).

†LH was calculated from the amount of progesterone secreted from luteal cells after treatment with 3 different dilutions of culture medium. Samples were assayed in triplicate. Values are expressed as mean ± s.d. of the dilution nearest the midpoint of the LH standard dose–response curve.

‡Single determination.

the antibody for the LH-β subunit was revealed by the addition of excessive amounts of non-radioactive α or LH-β subunits during immunoprecipitation because only LH-β displaced the radiolabelled bands. Both the presumptive α and LH-β bands were displaced by the unlabelled antigen, suggesting that the antiserum brings down intact heterodimer through recognition of the LH-β subunit. The possibility that the antibody was recognizing LH-β subunits with different molecular weights was ruled out because α-specific antiserum also precipitates the same two labelled proteins (data not shown). Furthermore, the molecular weights of the two labelled proteins were identical to the α and LH-β subunits secreted by other Chinese hamster ovary cell lines which have been transfected with only one of the two gonadotrophin subunit genes.

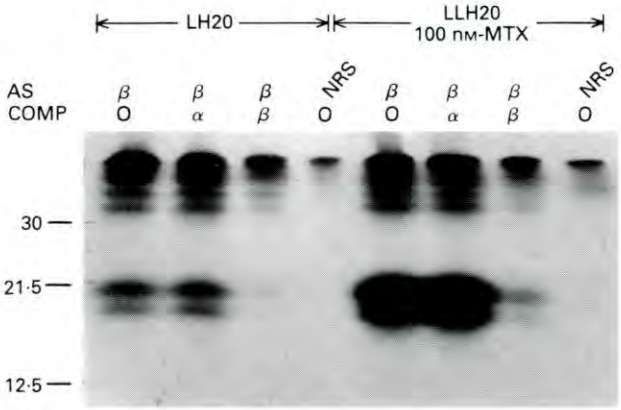

Fig. 3. Expression of bovine LH in Chinese hamster ovary cells. LH20 cells, and a subpopulation of LH20 cells selected for growth in the presence of 100 nM-methotrexate (LH20-100), were labelled with [^{35}S]methionine and [^{35}S]cysteine for about 18 h. Medium was subjected to immunoprecipitation, NaDodSO$_4$/PAGE, and autoradiography (Kaetzel *et al.*, 1985). Immunoprecipitation was carried out with 4 μl antiserum (AS) directed against the β subunit of LH, in the presence or absence of 10 μg unlabelled α subunit or LH-β competitor (COMP). NRS, normal rabbit serum. Numbers at left represent $M_r \times 10^{-3}$ of marker proteins run in parallel.

The stable population of LH20 cells capable of growth in 100 nM-methotrexate (LH20-100) secreted approximately 8-fold more LH than did the LH20 parent cell line as indicated by the increased intensity of both α and LH-β bands (Fig. 3). Presumably, methotrexate selection caused an increase in both subunits through gene amplification (Kaufman & Sharp, 1982). Alternatively, it is possible that only one gene encoding an LH subunit increased, but that this increase is sufficient to change the extent of assembly. Indirect evidence bearing on this latter point is presented below.

We have also examined the effects of methotrexate selection by subjecting media samples from several unlabelled cell lines to electrophoresis in SDS-polyacrylamide gels. For these determinations, β-mercaptoethanol was omitted from the sample buffer and electrophoresis was performed at 4°C. Several laboratories have reported that LH will not dissociate under these conditions (Chin *et al.*, 1981; Strickland & Puett, 1982; Strickland & Pierce, 1983). To visualize LH and any free subunit, the proteins were transferred from the gel to nitrocellulose by electrophoresis and then incubated successively with rabbit antiserum specific for bovine α subunit and goat anti-rabbit IgG

conjugated to alkaline phosphatase. Colour was developed by further incubation with a chromogenic alkaline phosphatase substrate. As judged from the reaction products formed with the standards, LH was clearly separated from free α subunit after electrophoresis (Fig. 4). From the differential staining intensity of the reaction products associated with equivalent amounts of the α subunit and LH standards, it is apparent that the antibody had more affinity for the free subunit rather than the intact heterodimer (Fig. 4). This was expected because purified α subunit was used to elicit antibody formation, and the finding is consistent with our previous estimates of cross-reactivity (Kaetzel *et al.*, 1985). It is difficult to determine whether the free α subunit band detected in LH standard is a contaminant or represents partial denaturation of the dimer. The same is true for α subunit detected in all of the samples from the subpopulations of LH20 cells capable of growth in increasing concentrations of methotrexate. However, the antibody and the gel system can be used to provide a minimum estimate of LH concentration in media samples. Therefore, the data from the subpopulations of LH20 cells suggests that resistance to increasing concentrations of methotrexate is strongly associated with increased secretion of intact LH.

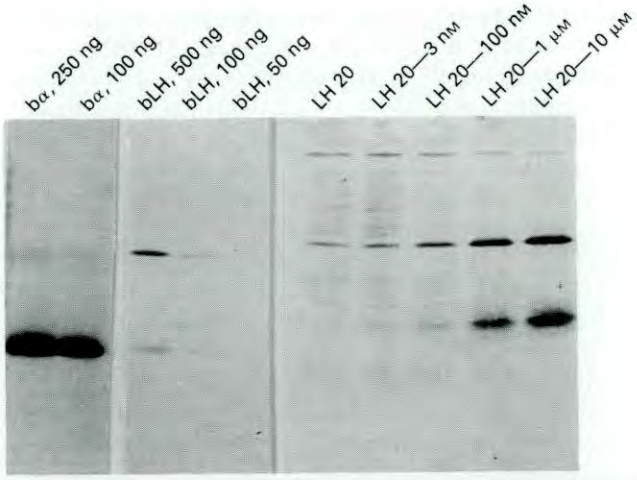

Fig. 4. Western blot analysis of secreted bovine LH and free α subunit from methotrexate-treated LH20 cells. Purified α subunit, LH, or media samples from LH20 cells and subpopulations of LH20 cells treated with the indicated concentrations of methotrexate were diluted with equal volumes of 2 × $NaDodSO_4$-PAGE sample buffer, except for the omission of β-mercaptoethanol and heat treatment (Strickland & Puett, 1982). Electrophoresis was performed at 4°C in 15% polyacrylamide gels containing 0·1% $NaDodSO_4$ (Strickland & Puett, 1982). After electrophoresis, the proteins were transferred from the gel to nitrocellulose and subjected to immunostaining as described in the Bio-Rad Immuno-blot manual (Bio-Rad, Richmond, CA).

Is the effect of methotrexate on LH secretion caused by an increase in the amounts of both LH subunits? We have begun to address this question by quantitating the relative amounts of the mRNAs encoding the α and LH-β subunits by northern blot hybridization and scanning densitometry. In addition, we have measured the amount of biologically active LH through the use of an ovarian luteal cell bioassay (Hoyer *et al.*, 1984; Kaetzel *et al.*, 1985). Results from these experiments are summarized in Table 1. When LH20 cells were selected for growth against increasing concentrations of methotrexate, LH-β mRNAs remained relatively constant while α subunit

mRNAs increased with increasing concentrations of the drug. This suggests that the α subunit gene was amplified in response to methotrexate selection, while the LH-β gene remained unaffected. This is not unexpected because the α subunit gene was directly linked to the mouse DHFR gene in the vector. LH-β mRNAs remained unchanged even in the LH-β25-1000 cell line which was resistant to 1 μM-methotrexate. This selective effect of methotrexate is consistent with a report that amplification in response to methotrexate occurs along a gradient, with genes nearest the DHFR gene amplified to a greater extent than genes farther away (Kaufman *et al.*, 1985). Further verification of selective amplification in our system requires measurement of gene copy number by DNA 'dot-blot' hybridization (Kafatos *et al.*, 1979).

Bioassay measurements of media from the subpopulations of LH20 cells indicate that methotrexate selection correlated positively with an increase in secreted levels of LH, from a nadir of 0·4 μg/10^6 LH20 cells to a peak of 10 μg/10^6 LH20-1000 cells during a 24-h collection period. This increase correlated with similar increases in α subunit mRNA and suggests that heterodimer assembly is not complete in the parent LH20 cell line even though levels of α and LH-β mRNAs were essentially equal. Although preliminary, these results also suggest that extent of heterodimer assembly may be related to the concentrations of both subunits because an increase in α subunit leads to increased secretion of biologically active LH. Further substantiation will require measurement of intracellular levels of both α and LH-β proteins to confirm that α subunit protein levels indeed increase whereas LH-β protein levels remain constant. Because the methotrexate effect appears to be a function of linkage to the DHFR gene, the LH-β gene can be linked to the DHFR gene to test whether selective increases in LH-β protein levels also lead to an increase in secreted LH. Perhaps the most definitive test will be to link both LH genes to DHFR and determine whether the methotrexate-induced increase in LH synthesis and secretion is a function of the product of the concentration of both subunits.

The bioassay data indicate indirectly that LH produced by the Chinese hamster ovary cells is glycosylated because only glycosylated LH is hormonally active (Pierce & Parsons, 1981). We have been able to label CHO-LH by incubation of LH20 cells with [^{3}H]glucosamine and [^{3}H]mannose, further confirming that CHO-LH is glycosylated. Because the asparagine-linked oligosaccharides of CHO cells have been extensively characterized, CHO-LH is likely to contain a biantennary oligosaccharide structure with terminal sialic acid residues linked to galactose which in turn is linked to *N*-acetyl glucosamine (Hubbard & Ivatt, 1981). If verified, CHO-LH would have a different type of a complex Asn-linked oligosaccharide from that found normally in pituitary LH (Green *et al.*, 1985). Such a difference may indicate that the biological activity of LH is not strictly dependent on the type of complex oligosaccharide attached to the polypeptide backbone. The subpopulations of LH20 cells selected with methotrexate produce more than enough hormone to permit direct assessment of this possibility.

Conclusions

Bovine LH genes can be expressed in Chinese hamster ovary cells and their expression leads to the appearance of biologically active LH. Through the use of expression vectors which contain the bovine α subunit gene linked to a modified mouse DHFR gene, we have been able to use methotrexate selection to increase the concentration of α subunit while maintaining the concentration of LH-β. This approach provides a novel means to assess the importance of α subunit concentration with respect to assembly of the heterodimer. Our data indicate that heterodimer assembly is incomplete before methotrexate selection and that the amount of assembled LH can be increased by selectively increasing the concentration of α subunit. This suggests that the concentration of LH-β subunit may not be the sole determinant of the extent of heterodimer formation. The transfection approach described herein can be used to verify that changes in extent of assembly are a function of the change in the product of the concentration of both subunits. In addition, because

CHO-LH is glycosylated and biologically active, the transfection system can also be used with in-vitro mutagenesis to determine whether site-specific changes in glycosylation have an effect on subunit assembly and biological activity. The DHFR-based expression vector and the DHFR-deficient cell line from Chinese hamster ovaries offer several unique avenues to explore further the molecular mechanisms underlying the complex biosynthetic pathway of bovine LH.

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