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Expression of biologically active bovine luteinizing hormone in Chinese hamster ovary cells

(mammalian expression vectors/unlinked genes/gonadotropin subunit assembly)

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ABSTRACT **Biologically active bovine luteinizing hormone (LH) has been obtained through expression of the α- and LH β-subunit genes in stably transformed clones of DUXB11, a Chinese hamster ovary cell line deficient in dihydrofolate reductase (DHFR). Expression of α- and LH β-subunit mRNAs of the expected sizes (≈910 and 770 nucleotides, respectively) were revealed by blot analysis after electrophoresis of total cellular RNA. Furthermore, presence or absence of the gonadotropin mRNAs in several clonal lines was directly correlated with the appearance of one or both bovine LH subunits in the culture medium. Media from three clones secreting significant immunoreactive levels of both subunits also stimulated the release of progesterone in ovine luteal cells, suggesting that the secreted LH was assembled into a biologically active and glycosylated dimer. Immunoprecipitation and NaDodSO$_4$/PAGE of [^{35}S]methionine-labeled proteins secreted from one of the clones, CHODLH20, further confirmed the presence of an α/β dimer with apparent subunit molecular weights of 20,500 and 16,000, only slightly higher than those of pituitary α and LH β subunits.**

Luteinizing hormone (lutropin, or LH) is a member of the glycoprotein hormone family, which consists of the pituitary gonadotropins, LH and follicle-stimulating hormone (FSH), chorionic gonadotropin (CG), and thyroid-stimulating hormone (thyrotropin, or TSH). The hormones are structurally similar, consisting of an α subunit, common to all four proteins, and a unique, noncovalently associated β subunit which confers receptor specificity and biological activity (1).

Regulation of synthesis and secretion of the glycoprotein hormones, including the gonadotropins, is among the most complex for any polypeptide hormone (1). Even though the genes encoding the α and LH β subunits are unlinked, recent evidence suggests that their expression is coordinately regulated (2, 3). After translation from separate mRNAs, the gonadotropin subunits rapidly combine in the endoplasmic reticulum and undergo several posttranslational modifications prior to transit through the Golgi apparatus and subsequent storage in secretory granules (4, 5). Secretion of the mature gonadotropins is regulated by gonadal steroids and gonadotropin-releasing hormone. To begin to explore the requirements for assembly and regulated secretion of LH in more detail, we have placed transcriptionally functional copies of the bovine α- and LH β-subunit genes [16.5 and 1.1 kilobase pairs (kbp), respectively (6, 7)] into a Chinese hamster ovary cell line by DNA-mediated gene transfer (8, 9). Clones have been isolated that synthesize and simultaneously secrete both α and LH β subunits. Medium in which these cells have been cultured has the capacity to stimulate release of progesterone in ovine luteal cells, a feature consistent with assembly of the α and β subunits into hormonally active heterodimers.

MATERIALS AND METHODS

Cell Culture. A Chinese hamster ovary cell line, DUXB11, deficient in dihydrofolate reductase (DHFR) (10), was grown in low-glucose (1 g/liter) Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum, nonessential amino acids, 2 mM L-glutamine, 50 units of penicillin and 50 μg of streptomycin per ml, 100 μm hypoxanthine, and 30 μM thymidine. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

DNA Transfections and Selection of Transformed Cells. All transfections were performed by the calcium phosphate microprecipitation method (8), as modified by Wigler *et al.* (9). To obtain stable transformed cell lines, DUXB11 cells (10^6 per 60-mm dish) were transfected with 2 μg of vector DNA (*Pvu* I-treated) containing a truncated bovine α-subunit gene and a mouse DHFR minigene (pDSVα, Fig. 1) plus 10 μg of a construct (*Bam*HI-treated) containing the bovine LH β-subunit gene (pSV2LHβ, Fig. 1). After 20 hr, medium containing the calcium phosphate/DNA precipitate was removed and replaced with complete medium. Forty-eight hours later, the cells were divided into five 100-mm dishes per original 60-mm dish and fed with selective medium (DUXB11 complete medium, minus hypoxanthine and thymidine, with dialyzed fetal bovine serum), in which only DHFR$^+$ transformants should grow (11). Fresh selective medium was applied three times within the first week of selection. Cell death occurred within 3–10 days, with surviving colonies appearing at 5–10 days (1–5 colonies per dish). During the second and third week after transfection, growing colonies were fed with fresh medium once weekly. When colony diameter approached 1 mm (14–21 days after initiation of selection), the cells were treated with 2 mM Na_3EDTA in Dulbecco's phosphate-buffered saline at pH 7.4 (2 ml per dish, at 37°C for 15 min) and then transferred in a maximum of 50 μl of buffer, with a flame-narrowed Pasteur pipette, to 16-mm wells in a 24-well microtiter plate. Cells were maintained in selective medium (1.5 ml per well) and progressively were expanded into 35-mm, 60-mm, and 100-mm plates.

Isolation and Characterization of Gonadotropin mRNA. Techniques for the preparation of total cellular RNA and poly(A)$^+$ RNA, ^{32}P-labeled cDNA probes, RNA electrophoresis, blot-transfer, and hybridization have been described (3).

Radioimmunoassay of Bovine LH. Culture medium was analyzed by radioimmunoassay with two rabbit antisera

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Abbreviations: LH, luteinizing hormone; DHFR, dihydrofolate reductase; kbp, kilobase pair(s).

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(courtesy of J. Pierce, UCLA School of Medicine) directed against the native bovine α and LH β subunits. Each tube contained 20,000 cpm of ^{125}I-labeled α or β subunit (12) in 10 μl of assay buffer (10 mM Tris·HCl/1 mM Na_3EDTA/150 mM NaCl/0.1% bovine serum albumin/0.4% Nonidet P-40 at pH 7.5), up to 300 μl of culture medium, and 20 μl of diluted antiserum (1:50,000 for anti-α subunit and 1:200,000 for anti-LH β subunit). After a 21-hr incubation at 37°C, antibody–antigen complexes were precipitated by addition of 10 μl of a 10% (vol/vol) suspension of fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem) followed by a 40-min incubation at 25°C. The detection limits for the α- and β-subunit RIAs were 330 and 170 pg/ml (100 and 50 pg per assay tube), respectively. The crossreactivities of the antisera were assessed by the level of unlabeled antigen required to displace 50% of the ^{125}I-labeled antigen (B_{50}) in each assay (expressed as a percentage of the highest-affinity antigen for each antiserum). Crossreactivities with anti-bovine α subunit were as follows: α subunit, 100%; LH β subunit, 1.4%; LH, 27%. Crossreactivities with anti-LH β subunit were 100%, 0.3%, and 1%, respectively, for LH β subunit, α subunit, and LH.

Electrophoretic Analysis of Secreted Gonadotropin. To detect newly synthesized and secreted LH, transformed DUXB11 cells were incubated for 18 hr with 100 μCi of [^{35}S]methionine (1400 Ci/mmol; 1 Ci = 37 GBq) in 1.7 ml of methionine-deficient MEM [minimal essential medium (Eagle)] supplemented with 5% fetal bovine serum, 2 mM glutamine, nonessential amino acids, hypoxanthine, thymidine, and unlabeled methionine at 1.5 μg/ml (1/10th of the usual MEM level). Immunoprecipitation of the medium was performed by a 4-hr incubation at 37°C with 4 μl of antiserum followed by addition of 10 μl of Pansorbin cells (10%, vol/vol). Pellets were resuspended and denatured in sample buffer containing 8 M urea, 10% (vol/vol) glycerol, 1% $NaDodSO_4$, 1% (vol/vol) 2-mercaptoethanol, 50 mM Tris·HCl (pH 7.5), and 0.02% bromphenol blue, boiled for 5 min, and subjected to electrophoresis in 20% polyacrylamide gels containing 0.1% $NaDodSO_4$ (13). Radioactive proteins were subsequently detected after impregnation of gels with EN^{3}HANCE (New England Nuclear).

Bioassay of Bovine LH. Corpora lutea were collected from superovulated ewes on day 10 of the estrous cycle, dissociated into single cells, and elutriated to obtain a population of small (<22 μm) steroidogenic cells (14). The small luteal cells were plated (10^5 cells per plate) in Dulbecco's modified Eagle's medium containing 5% ram serum and then were incubated overnight at 37°C. The next day, 100 μl of the CHODLH clone medium samples (diluted to 3 ml with luteal cell medium) were applied to washed luteal cells for 3 hr. The incubation medium was collected and the quantity of progesterone secreted was determined by radioimmunoassay (15). Data were evaluated by analysis of variance. Differences between treatment means were determined by Tukey's "honestly significant difference" procedure.

RESULTS AND DISCUSSION

The plasmid vector pDSVα was constructed so that expression of a truncated bovine α-subunit gene was driven by the simian virus 40 (SV40) late promoter (Fig. 1). The α-subunit gene sequence employed was an 8.7-kbp genomic *Bam*HI fragment (3) that encompassed the 3′ half of the first intervening sequence, the ATG initiation codon, and all of the amino acid coding sequences (exons 2–4). To ensure correct processing of the 3′ half of the first intron, the vector contained a synthetic fragment carrying a consensus splice-donor site separated from the late SV40 polyadenylylation signal by a unique *Bam*HI cloning site; the α-subunit gene segment was inserted at this location in the plasmid (Fig. 1). The vector was also designed to contain the mouse DHFR minigene (11), isolated from the plasmid pMG1 (generously provided by R. Schimke), for use as a selectable marker. Following transfection into an appropriate DHFR$^-$ cell line such as DUXB11, only DHFR$^+$ transformants can grow in culture medium deficient in hypoxanthine and thymidine (11). Since the α gene was linked to the DHFR gene within the same plasmid, a high rate of DHFR$^+$, α-subunit$^+$ transformants was anticipated.

The bovine LH β-subunit expression vector, pSV2LHβ, contained the entire β-subunit gene (including the RNA cap site and TATAA sequence) located on a 1.8-kbp *Pst* I genomic fragment (7). The gene was ligated into a unique *Hin*dIII site in the parent vector pSV2CAT (kindly provided by B. Howard; see ref. 16). The final construct consisted of the LH β-subunit gene juxtaposed between the SV40 early promoter/origin of replication and the bacterial chloramphenicol acetyltransferase (CAT) gene (Fig. 1).

To obtain cell lines secreting biologically active bLH, we cotransfected DUXB11 cells with pDSVα and pSV2LHβ and

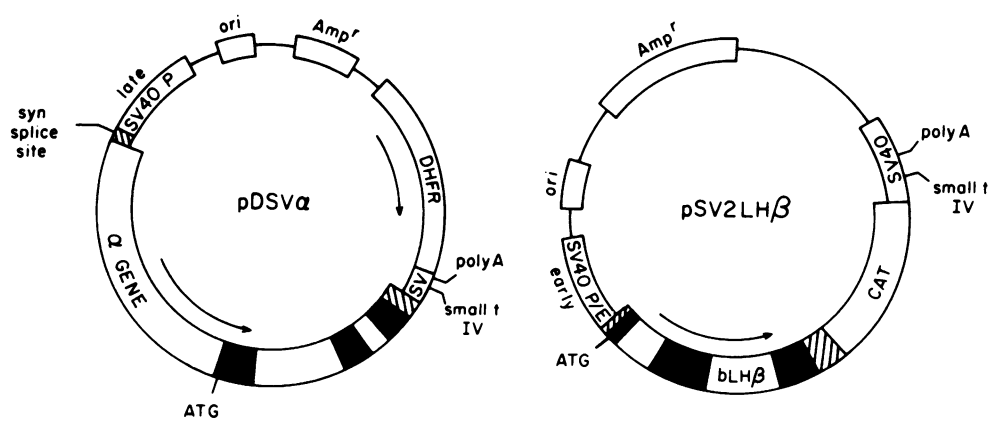

FIG. 1. Construction of two expression vectors containing the bovine α and LH β subunit genes. (*Left*) pDSVα. The 8.7-kbp α-subunit gene fragment (intron sequences in white, exons in black, 5′ and 3′ untranslated regions hatched) is represented, with its initiator methionine (ATG) codon and direction 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 tumor antigen (t)-gene intron (IV) and polyadenylylation signal, and the mouse DHFR minigene with its direction of transcription (arrow). ori, Origin of replication; Amp^r, ampicillin-resistance gene. (*Right*) pSV2LHβ. 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-subunit gene, is the bacterial chloramphenicol acetyltransferase (CAT) gene from the parent vector, pSV2CAT. P/E, promoter/enhancer. Other features of this construct are symbolized as in *A*.

selected for DHFR$^+$ transformants. Among the surviving clones, a few DHFR$^+$, α- and β-subunit$^+$ transformants were anticipated which would be capable of synthesis and assembly of the gonadotropin subunits into dimeric, biologically active hormone. A total of 41 DHFR$^+$ transformants were detected; 30 were successfully expanded as clones. Of these, 3 (CHODLH or LH1, LH20, and LH29) secreted significant levels of both α and LH β subunits, 14 secreted only β subunit, 3 produced α subunit alone, and 10 did not secrete detectable levels of either subunit as measured by RIA (Table 1). Because subtle differences in the presentation of the LH subunit epitopes (free subunit versus subunit present in dimer; recombinant bovine subunit from CHO cells versus bovine pituitary subunits) to the antisera used in these studies is a possibility, the data presented in Table 1 are qualitatively, but not necessarily quantitatively, valid.

To further verify that the bovine α- and LH β-subunit genes were transcribed, total cellular RNA was isolated from the α/β-subunit-cosecreting clones (LH1, LH20, and LH29), an α-subunit-secreting clone (LH28), and an LH β-subunit-secreting clone (LH25). The isolated RNA was subjected to electrophoresis and blot analysis with cDNAs specific for either the α or the β subunit of bovine LH (Fig. 2). A qualitative correlation was observed between the presence of α-subunit or β-subunit mRNA and secreted immunoreactive subunit protein; clones LH1, LH20, and LH29 contained both mRNAs, LH28 synthesized only α-subunit mRNA, and LH25 made detectable levels of only the LH β-subunit mRNA. The average sizes of the α and LH β transcripts were larger (910 and 770 nucleotides, respectively) than their respective counterparts found in steer pituitary extracts (830 and 675 nucleotides), an observation consistent with the lengths predicted on the basis of additional transcribed SV40 sequences.

Evidence of intact heterodimers of LH present in the culture medium of the LH20 cell line was obtained after incubating the cells for 18 hr with [^{35}S]methionine. Radiolabeled products in the medium were immunoprecipitated with antisera directed against native α and LH β subunits and analyzed by NaDodSO$_4$/PAGE. Under the conditions used for sample denaturation and NaDodSO$_4$/PAGE, dimeric LH protein dissociates into α and β subunits. Antiserum directed against bovine α subunit precipitated two proteins (M_r 20,500 and 16,000) that comigrated approximately with ^{125}I-labeled standards of bovine α and LH β subunits (M_r 17,000 and 15,000). Both ^{35}S-labeled polypeptides were completely displaced from the immunoprecipitate by an excess (10 μg) of unlabeled α subunit (Fig. 3, lane 2), but neither was displaced by the same amount of unlabeled β subunit (lane 3). This observation suggests that the α-subunit antiserum reacted either with two molecular weight forms of immunoreactive α subunit or with an α/β dimer through antigenic determinants on the α subunit. The latter hypothesis is more likely, since cell lines secreting only α subunit produce a protein that migrates as a single band at M_r 20,500. In addition, pSV2LHβ-transfected COS-1 cells and the cell lines LH6 and LH25 produce a single protein that is specifically precipitated with β-subunit (but not α-subunit)-directed antisera (data not shown) and that is of the same apparent M_r as the putative β subunit (16,000) found in the LH20 immunoprecipitate obtained with antiserum to α subunit.

Antiserum directed against bovine LH β subunit also precipitated two proteins, which were of the same M_r as those in the anti-α-subunit immunoprecipitate (Fig. 3, lane 4) but

Table 1. Constitutive expression of bovine LH in transformed DUXB11 cells

Cell line	Culture medium immunoreactivity*		Bioassay	
	α subunit	LH β subunit	Progesterone,† ng/ml	% control‡
DUXB11	<0.3	<0.2	3.6§ ± 0.4	70
LH1	4.2	65	13.2¶ ± 0.3	260
LH20	6.5	43	21.7‖ ± 3.0	430
LH29	19.8	252	22.2‖ ± 2.4	430
LH28	30.0	<0.2	5.5§ ± 1.5	110
LH37	12.5	<0.2	6.0§ ± 0.6	110
LH6	<0.3	80	7.3§ ± 0.2	140
LH7	<0.3	50	6.3§ ± 0.4	120
LH26	<0.3	<0.2	6.6§ ± 0.9	130
LH31	<0.3	<0.2	5.3§ ± 0.6	100

*Radioimmunoassay data (duplicate determinations, at 20 and 200 μl of culture medium) were accumulated over 72–90 hr, with cells plated at an initial density of $\approx 10^6$ per 100-mm dish. Values represent an estimation of accumulation of immunoreactive subunit protein in ng (relative to bovine pituitary LH subunit protein standards) per ml of medium, normalized to 24 hr.

†Amount of progesterone secreted from ovarian luteal cells after treatment with 100 μl of the indicated culture medium. Samples were assayed in triplicate; results are expressed as mean ± SD.

‡Control: progesterone secretion of untreated ovarian luteal cells (5.1 ng/ml).

§¶‖Values with unlike superscripts are significantly different ($P < 0.01$).

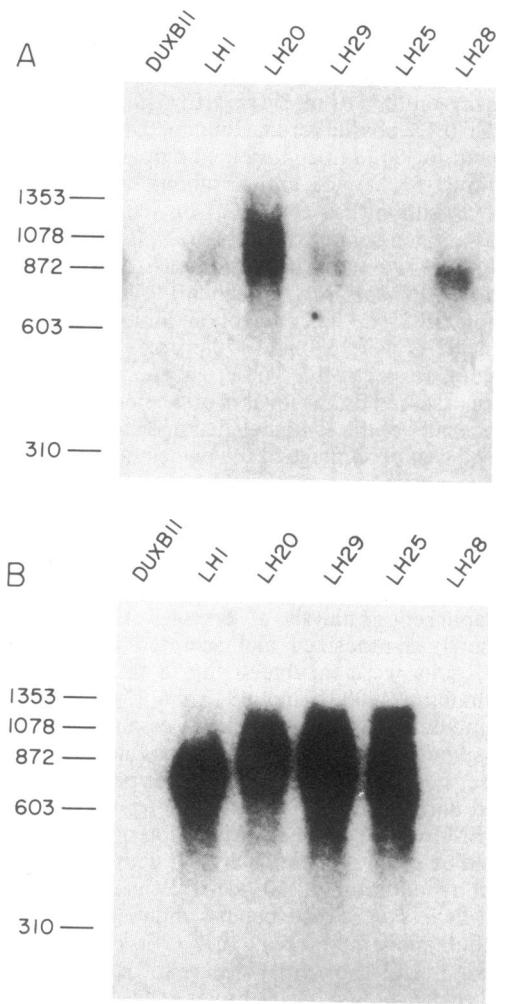

FIG. 2. Expression of bovine α- and LH β-subunit mRNAs in transformed DUXB11 clones. Total cellular RNA (10 μg) from DUXB11 and total cellular RNA (5 μg) from CHODLH clones 1, 20, 29, 25, and 28, respectively, were probed with cDNAs specific for the α subunit (*A*) or the LH β subunit (*B*). Autoradiograms were obtained with a 24-hr exposure at −70°C, using intensifying screens. At left, the positions and sizes (in nucleotides) of markers generated by *Hin*dIII restriction of ϕX174 phage DNA are shown.

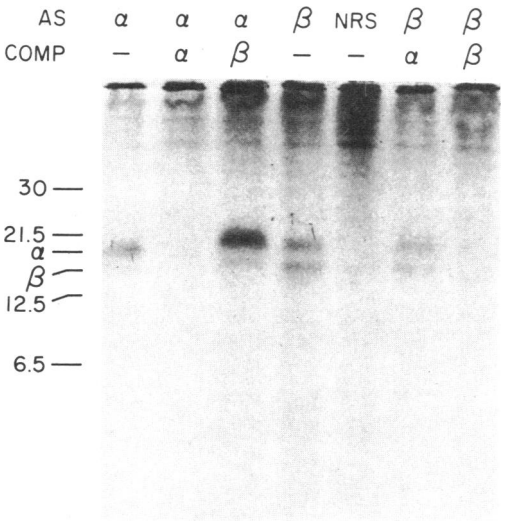

FIG. 3. Expression of LH in transformed DUXB11 cells. LH20 cells were labeled with [^{35}S]methionine and medium was subjected to immunoprecipitation, NaDodSO$_4$/PAGE, and autoradiography. Immunoprecipitation of 0.5 ml of medium was carried out with 4 μl of antiserum (AS) directed against either the α or the β subunit, in the presence or absence of 10 μg of unlabeled LH subunit competitor (COMP). NRS, normal rabbit serum. Numbers at left represent M_r × 10^{-3} of marker proteins run in parallel.

which were displaced only by unlabeled β subunit (lane 7) and not by α subunit (lane 6). These data are consistent with an interaction between the antiserum and antigenic determinants on the β subunit of the same α/β dimeric molecule seen in lanes 1 and 3 (Fig. 3). No specific immunoprecipitate was observed in the medium obtained from the nontransformed DUXB11 cells (data not shown). Although it is likely that assembly of the α/β heterodimers occurs intracellularly, we cannot presently rule out the possibility that assembly of the subunits occurs after their secretion into the culture medium. In addition, from the relative intensity of the α-subunit band with respect to the LH β-subunit band in lanes 1 and 3 of Fig. 3, it is tempting to infer that clone LH20 secretes an excess of α subunit. However, the immunoprecipitation data stand in contrast to the data obtained by RIA (Table 1) and RNA blot hybridization (Fig. 3), which indicate that LH β subunit is in excess of the α subunit.

The apparent increase in molecular weight of both subunits when expressed in the Chinese hamster ovary cell line, compared to those expressed in the bovine pituitary, may represent differences in carbohydrate modifications. Ramabhadran *et al.* (17) have reported the secretion of an unexpectedly larger human α-subunit protein after transfection of the gene into mouse cells. Furthermore, the pituitary gland has the unusual capacity to attach sulfate groups to the terminal *N*-acetylglucosamine residues of the N-linked oligosaccharides present in bovine LH (18); Chinese hamster ovary cells quite likely lack this capacity. Thus, the apparent difference in molecular weight between bovine LH synthesized in Chinese hamster ovary cells or in bovine pituitary may reflect differences in carbohydrate structures.

To determine whether the dimeric proteins produced by LH-secreting cell lines were biologically active, we analyzed samples of culture media for their ability to stimulate secretion of progesterone by ovine luteal cells. The three cell lines that elaborated measurable levels of both α and LH β subunits (LH1, LH20, and LH29) also displayed significant levels of LH biological activity in their culture media (Table 1). None of the other cell lines secreted biologically active LH (Table 1 and data not shown). These data suggest that the bovine LH synthesized in the hamster cells must be glycosylated, since deglycosylated LH (19–22) has been shown to lack biological activity. Subsequent analysis of proteins from cells metabolically labeled with [^{3}H]glucosamine has confirmed this conclusion (data not shown).

Others have reported expression of biologically active heterodimers after microinjection of mRNA prepared from a hybridoma cell line secreting a specific monoclonal antibody (23) and after transfer of immunoglobulin genes into various lymphoid cell lines (24, 25). In this study, heterologous cells have been used as recipients for two unlinked genes, whose expression resulted in the elaboration of a biologically active, heterodimeric polypeptide hormone.

After this work was completed, Reddy *et al.* (26) reported the expression of biologically active hormone after infection of monkey cells with a recombinant SV40 containing linked human chorionic gonadotropin genes.

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