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Isolation and characterization of cDNA clones for α- and β-subunits of ovine luteinizing hormone

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Abstract. A cDNA library of ovine pituitary DNA in plasmid pBR322 has been constructed by conventional methods with certain modifications. The library was screened using partial cDNAs for rat α-subunit and LHβ. We have isolated cDNA clones for ovine α-subunit and LHβ. The identification of these clones was confirmed by partial sequencing. The clones bear about 80% sequence homology with the respective rat cDNAs in the sequenced regions and hybridize with the rat clones in 5 X SSC at 55°C. The ovine LHβ clone has an insert of about 650 bp and selects an RNA of about 750 bases in a northern blot. The α-subunit cDNA clone has an insert of about 550 bp; it has two internal *Pst* I sites and thus shows restriction-based differences from rat α-subunit cDNA, which does not have any *Pst* I site.

Keywords. Ovine LH; cDNA synthesis; molecular cloning; pBR322; southern and northern blots; DNA/RNA sequences.

Introduction

Luteinizing hormone (LH) is one of the pituitary gonadotropins. It is glycoprotein in nature and is produced and secreted by the gonadotrope cells of the anterior pituitary. Its synthesis is regulated in a very complex manner by the balance between hypothalmic and sex hormones. LH plays an important role in the regulation of reproductive functions and pregnancy. The bioactive hormone consists of two subunits α and β. The α-subunit is common to LH and the other pituitary glycoprotein hormones, namely follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and the placental chorionic gonadotropin (CG). Thus the specificity of hormone action is conferred by β-subunit. While a great deal of information is available about the structure, function and mechanism of action of gonadotropins, a lot remains to be understood (Pierce *et al.,* 1976; Vaitukaitis *et al.,* 1976; Pierce and Parsons, 1981)

The presence of different genes for the subunits raises the important question as to how the two genes have evolved. It is also of interest to understand how the two genes are expressed in a mutually coordinated manner. There is varying degree of homology between the β-subunits of different pituitary hormones (Acher, 1980). For example the β-subunits of human LH and human chorionic gonadotropin (hCG) have about 80% sequence homology. This sequence homology led to the belief that

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Abbreviations used: LH, Luteinizing hormone; FSH, follicle stimulating hormone; TSH, thyroid stimulating hormone; CG, chorionic gonadotropin; hCG, human CG; oLH, ovine LH; MLV, murine leukemia virus; AMV, avian myloblastosis virus.

the *β*-subunit genes of different hormones may have arisen from a common *β*-subunit gene. Further, a certain degree of homology has been observed between the *α*- and *β*-subunits within the same species. The two subunits may have diversified from an ancestral gene (Stewart and Stewart, 1977). Probably the divergence of these *α*- and *β*-subunit genes have occurred earlier than the divergence of the *β*-subunit genes of these hormones.

The *α*-subunits of human (Fiddes and Goodman, 1979), bovine (Nilson *et al.,* 1983; Erwin *et al.,* 1983), mouse (Chin *et al.,* 1981) and rat (Godine *et al.,* 1982) hormones have been cloned. The sequences of cDNA for human (Talmadge *et al.,* 1984) and rat (Chin *et al.,* 1983) LH*β* have been reported. No clones for ovine LH (oLH) are available. We have shown that the immunogenicity of hCG-*β* increases significantly when complexed with ovine *α*-subunit (Sharma *et al.,* 1986). An antifertility vaccine is made with hCG-*β* and ovine LH*β* linked to carriers (Talwar *et al.,* 1986). It was therefore pertinent to clone the genes for ovine *α*-subunit and LH*β*. In this communication, we report the isolation and characterization of cDNA clones for α- and *β*-subunits of oLH.

Materials and methods

Construction of cDNA library

The cDNA library was constructed by conventional methods (Retzel *et al.,* 1980; Maniatis *et al.,* 1982) with certain modifications. The pituitary RNA was isolated by homogenizing the tissue in 4 M guanidinium isothiocyanate, 50 mM sodium citrate, pH 7·0, 0·1 M mercaptoethanol and 0·5% (w/v) sarkosyl, and centrifuging the homogenate through 5·7 M CsCl (Ullrich *et al.,* 1977). Total RNA was purified by repeated precipitation with ethanol and poly $(A)^+$ RNA was isolated by affinity chromatography through oligo-(dT)-cellulose (type T_3, Collaborative Research, Waltham, USA) columns. The double-standard cDNA was synthesized against this mRNA using the cDNA synthesis kit of Amersham Corporation. Amongst other modifications, murine leukemia virus (MLV) reverse transcriptase (RTase) was used in place of commonly used avian myloblastosis virus (AMV) RTase. The second strand was synthesized with RNase H and DNA polymerase I (Gubler and Hoffman, 1983). Following blunt-ending with T_4 DNA polymerase, the cDNA was 'C-tailed' with terminal deoxy nucleotidyl transferase (New England Biolabs) and annealed to 'G-tailed' pBR322 (Bethesda Research Labs) at the *Pst* I site. The recombinants were used to transform *Escherichia coli* MC1061 cells pretreated with $CaCl_2$ to make the cells competent (Mandel and Higa, 1980). The transformants were selected by resistance to tetracycline. The library was screened by colony hybridization (Maniatis *et al.,* 1982) using nick-translated (Rigby *et al.,* 1977) partial length cDNAs for rat *α*-subunit and LH*β*.

Southern blot analysis

Plasmid DNA was isolated from overnight cultures by alkaline lysis (Birnboim and Doly, 1979) and purified by banding in CsCl (Yamamoto *et al.,* 1980). The DNA was digested with *Pst* I (New England Biolabs) and separated by electrophoresis on 1 % agarose gel in IXTBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8·3).

Following electrophoresis, the gels were denatured with 0·5 N NaOH/l·5 M NaCl, neutralized with 1 M Tris/1·5 M NaCl and equilibrated with 10 X SSC. The DNA in the gel was transferred to nitro-cellulose paper (Schleicher and Schuell) by capillary action (Southern, 1975). The blots were washed with 10 X SSC, air dried and baked at 80°C for 2 h. These were then prehybridized with 5 X SSC, 0·2% sodium dodecyl sulphate, 100 μg/ml sonicated salmon sperm DNA and 2X Denhardt's solution (Denhardt, 1966) for 4 h and hybridized for 16 h with nick-translated cDNA probe (2 x 10^8 cpm/μg DNA, 1 × 10^6 cpm/ml) in the same buffer. Following hybridization, the blots were washed with 5 X SSC at 55°C and autoradiographed with an intensifying screen (Dupont).

Northern blot analysis

The poly $(A)^+$ mRNA of ovine pituitaries was denatured with formamide and separated in 1% agarose gel containing formaldehyde (Lehrach *et al.,* 1977). The RNA was transferred to nitrocellulose, baked, prehybridized and hybridized with nick-translated cDNA probes as described for southern blots.

Sequencing

Restriction fragments of the cDNA inserts were subcloned in bacteriophage M 13 (Messing *et al.,* 1981) and sequenced by the dideoxy chain-termination method of Sanger *et al.* (1977).

Results and discussion

Construction and screening of library

For the first strand synthesis MLV RTase was used. While RTase is often contaminated with some exonuclease activity, it is reported that MLV RTase has much less exonuclease activity than AMV RTase (Gerard, 1985). Similarly RNase H and DNA polymerase I were used together (Gubler and Hoffman, 1983) for the synthesis of the second strand in place of the klenow enzyme (Retzel *et al.,* 1980) which is more commonly used. This way, we could avoid the use of S_1 nuclease, a step which may cause nicks and also may degrade the cDNA. The choice of these methods resulted in better yields of full length cDNAs. The size of the cDNA was 0·2–2 Kilobases with 0·5–1 kb as the predominant size class. Transformation of *E. coli* with the recombinant molecules produced a cDNA library of about 5 × 10^4 tetracycline resistant colonies. The screening of 350 colonies with rat α-subunit probe (3′ end, 150 base pairs in length) gave one positive clone. Similarly, the screening of 2000 colonies with the partial rat LHβ cDNA gave one positive clone. Before rat probes were used to screen the library, a northern blot of total ovine pituitary RNA was hybridized with these probes. In each case, the probe selected a single species of RNA. Further the sizes of these bands were similar to the sizes of the respective mRNAs for rat LH (Godine *et al.,* 1982; Chin *et al.,* 1983). This experiment confirmed our belief that rat probes would be useful for selecting the oLH cDNA clones.

Ovine α-subunit cDNA

Ovine α-subunit mRNA is one of the predominant species of pituitary mRNA, its abundance is about 0·3–0·4%. The cDNA clone has an insert of about 550 bp. Upon digestion with *Pst* I, it gives two bands of about 350 and 150 bp (figure 1A). When the southern blot was probed with rat *α*-subunit probe, only the smaller insert band hybridized and no hybridization with the larger band was seen (figure 1B; the hybridization signal obtained with 4·3 kb band in the figure represents undigested plasmid DNA). This suggested that the smaller fragment represents the 3′ end of the *α*-subunit gene, while the larger fragment is the 5′ end, the two fragments are separated by an internal *Pst* I site. To confirm that the two bands were part of a single insert, the clone was digested with limited amount of *Pst* I for 35 min to produce a partial digest. This gave a band of about 550 bp along with the two previously described bands of 350 and 150 bp (figure 1C). The 550 bp band also hybridized with the partial rat *α*-subunit cDNA, confirming that we had a pure clone and the insert had an internal *Pst* I site. The clone was characterized by digestion with several restriction enzymes. A partial restriction map is given in figure 2.

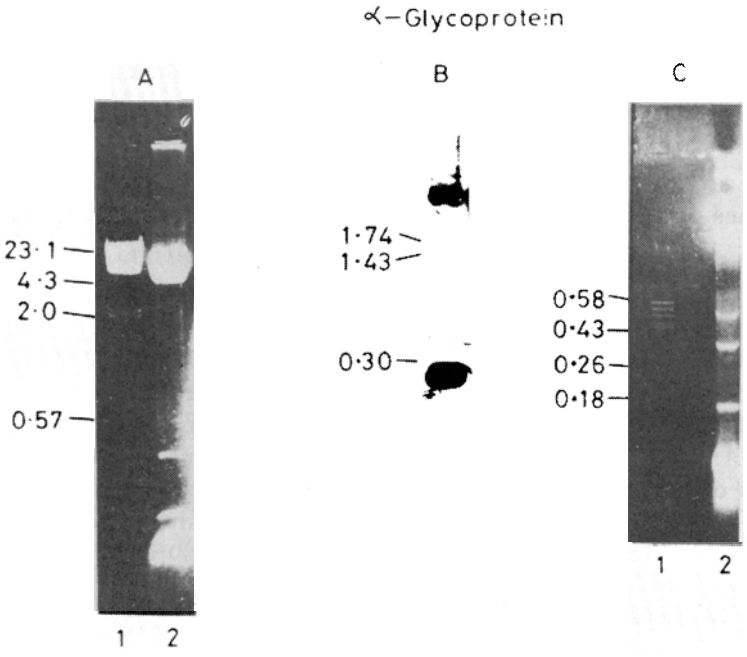

Figure 1. Ovine *α*-subunit cDNA clone.

The plasmid DNA was digested with *Pst* I for 2 h and separated on 1 % agarose gel. **A.** Ethidium bromide stained gel visualised under UV. Lane 1, Hind III digest of *λ*-DNA run as marker, the sizes of the bands are given in kb pairs (kbp). Lane 2, the *α*-subunit cDNA clone digested with *Pst* I. **B.** The southern blot of *Pst* I digest of ovine *α*-subunit cDNA clone hybridized with nick-translated rat *α*-subunit cDNA (see text for details). The sizes of marker bands of Avail digest of pBR322 in kbp are given on the left hand side. **C.** Lane 1, *Hae* III digest of pBR 322, with sizes of the bands in kbp; lane 2, *Pst* I partial digest of ovine *α*-subunit cDNA clone.

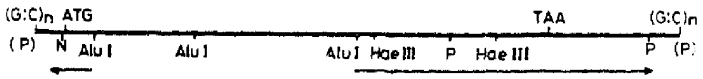

Figure 2. The restriction map of ovine α-subunit cDNA.

The sequenced regions are shown by arrow. The restriction sites are N, *Ncol,* P, *Pst* I. The *Pst* I sites at both ends of the cDNA have been enclosed in parentheses to show that these sites were created by G: C tailing. No restriction sites for *E*coRI, *Bam* H1, *Hind* III and *Bgl* II are present.

To analyse the mRNA(s) which codes for ovine α-subunit, a northern blot of total pituitary RNA was hybridized with nick-translated insert DNA (550 bp band in figure 1C). The probe selected a single mRNA band of about 800 bases (data not shown). The insert band was digested with *Alu* I and *Hae* III. The fragments were subcloned in M13 phage and sequenced. A part of the sequence from different regions of the clone is given in figure 3. The deduced amino acid sequence has been matched with the rat α-subunit sequence. As can be seen clearly from this comparison, region 1 represents the 5′ region of the clone. It shows an *Nco* I site (CCATGG) followed by an open reading frame which matches with the amino terminal region of the rat α-subunit (first 8 amino acids, excluding the initiation codon of the leader peptide). Region 2 represents the last 13 amino acids (84–96) of the carboxy-terminal end followed by the termination codon and 158 nucleotides of the 3′ non coding region.

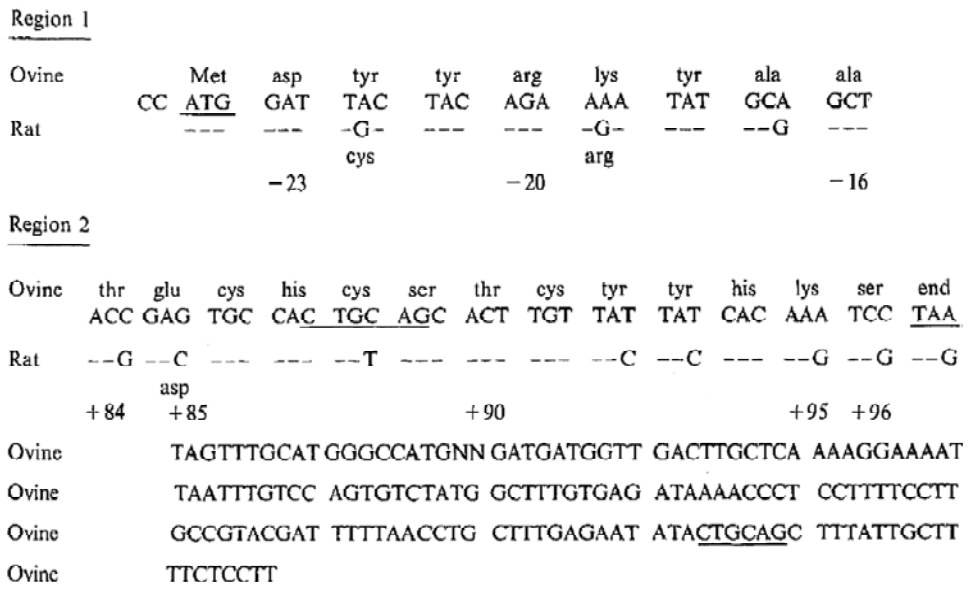

Figure 3. Partial nucleotide and deduced amino acid sequence of ovine α-subunit cDNA clone.

Sequencing was by the dideoxy chain-termination method. The sequence has been matched with that of rat α-subunit cDNA in the coding region. The rat sequence is given under the ovine sequence and amino acids are numbered for rat α-subunit. Region 1 matches with the 5′ end of the rat cDNA while region 2 matches with the 3′ end of the rat cDNA. The translational initiation and termination codons and the two *Pst* I sites have been underlined. The amino acids numbered negative in region 1 represent leader peptide.

The sequence analysis revealed that there are two *Pst* I sites (underlined in figure 3), one in the coding region and the other in the 3′ non-coding region downstream of the termination codon TAA. This should result in 3 fragments upon *Pst* I digestion of the cDNA. However, only two fragments are seen in figure 1A, suggesting that the third fragment is very small. The presence of two internal *Pst* I sites in ovine α-subunit cDNA is in contrast to rat α-subunit cDNA which does not have any *Pst* I site (Godine *et al.,* 1982). However, human α-subunit cDNA is known to have one *Pst* I site (Fidds and Goodman, 1979). Thus there is a restriction based polymorphism between rat, ovine and human α-subunit cDNAs. It is in agreement with the hypothesis that α-subunits of various species have certain remarkable differences from each other. Further, ovine α-subunit cDNA has more homology with human α-subunit cDNA than with the rat counterpart.

Ovine LH β cDNA

The βLH cDNA clone is present in the library at an abundance of about 0·05%; one out of 2000 colonies gave positive hybridization with the partial rat LHβ cDNA. The clone has an insert of about 650 bp (figure 4A), which can be excised as a single band upon *Pst* I digestion. The insert hybridizes with the rat LHβ cDNA (figure 4B).

A northern blot of ovine pituitary RNA (figure 5A) was hybridized with nick-

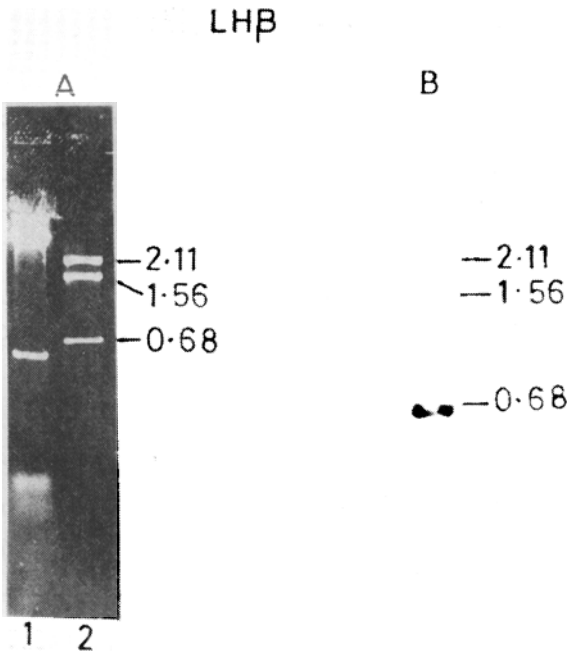

Figure 4. Restriction analysis of ovine LHβ cDNA clone.

Plasmid DNA was digested with *Pst* I and separated on 1 % agarose gel. **A.** Ethidium bromide stained gel. Lane 1, LHβ cDNA digested with *Pst* I (the lower band represents RNA present in plasmid DNA preparation). Lane 2, size markers. **B.** Southern blot of *Pst* I digest of LHβ cDNA hybridized with nick translated partial cDNA for rat LHβ. The sizes of *Rsa* I digest of pBR322 in kbp are given on the right hand side.

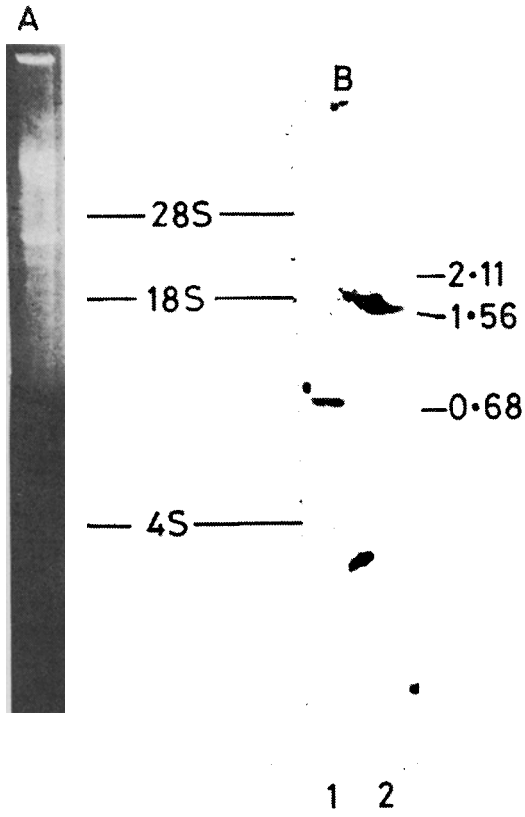

Figure 5. Northern blot analysis.
Total pituitary RNA was treated with formamid and separated on 1% agarose gel containing formaldehyde. **A.** Ethidium bromide stained gel visualised under UV. The positions of ribosomal and tRNA are marked. **B.** Lane 1, northern blot hybridized with nick-translated ovine LH*β* cDNA. Lane 2, *Rsa* I fragments of pBR322 as markers bands run as size marker.

translated LH*β* cDNA insert. The probe selected a single mRNA of about 750 bases (figure 5B). This size of mRNA for oLH*β* is very close to that of rat *β*LH mRNA.

Pst I and *Hae* III fragments of the cDNA were subcloned in bacteriophage M 13 and sequenced. The partial sequence is shown in figure 6. The deduced amino acid sequence was matched with the rat LH*β* sequence. In the region sequenced, the two clones have about 80% homology.

These studies are of considerable interest. They show that LH*β* and *α*-LH of different species are related with considerable sequence homology and the cDNA of one species can be used to isolate the corresponding cDNA of other species. The remarkable difference in the restriction analysis of rat and ovine *α*-subunit cDNAs and the greater similarity between ovine and human *α*-subunit are of much interest. It has been observed that the immunogenicity of hCG -*β* is enhanced if it is complexed with the *α*-subunit of oLH (Talwar *et al.,* 1986). These conjugates of gonadotropins linked to carriers are being proposed as antifertility vaccines and are currently undergoing clinical trials. The cloning of ovine *α*-subunit and LH*β* cDNAs will enable us to further understand the mechanism of action of these vaccines as well as produce substantial amounts of bioactive hormonal subunits which are necessary for our ongoing studies on these vaccines.

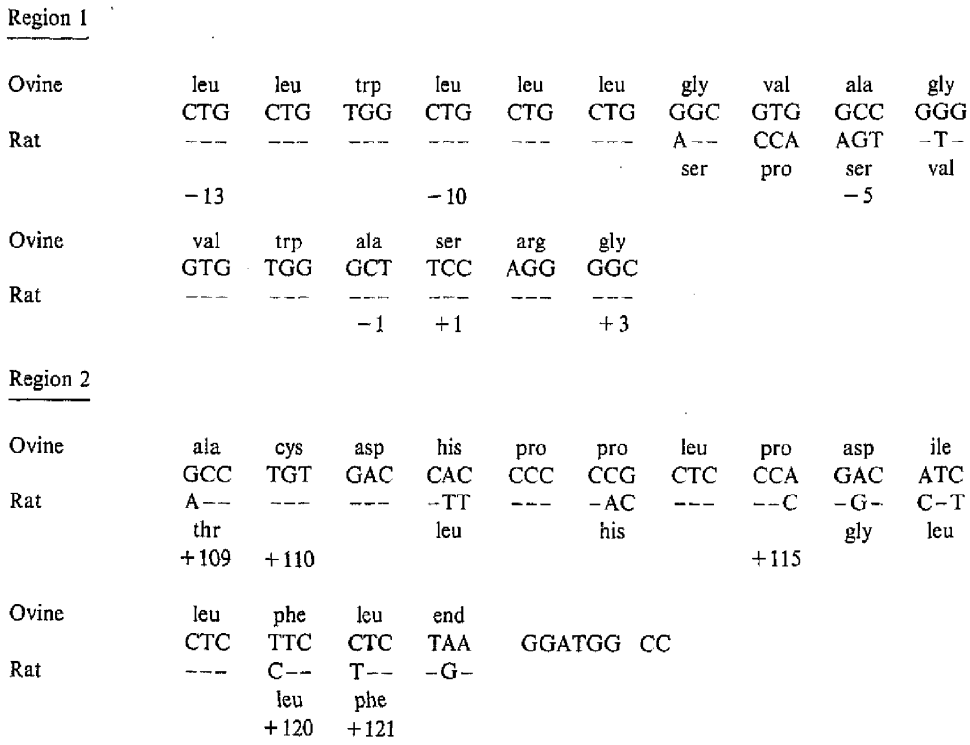

Figure 6. Comparison of nucleotide and deduced amino acid sequences of ovine and rat LHβ cDNAs.

The sequences of two different *Hae* III fragments of ovine LHβ cDNA are shown. The amino acid sequences deduced from the nucleotide sequence of these region are matched with rat LHβ cDNA sequences. The rat sequence is given below the ovine sequence. The amino acids are numbered according to published rat sequence (Chin *et al.,* 1983). The amino acids numbered negative represent leader peptide.

We are trying to express these cDNAs in an appropriate system to obtain substantial amounts of bioactive hormones. Constructs are being made in which the cDNA is cloned downstream of a metallothionein promoter. The transfection of mammalian cell lines with similar constructs has led to the expression of hCGβ (Khandekar P., unpublished results), and bovine gonadotropins (Kaetzel *et al.,* 1985). The LH cDNA has also been cloned in vaccinia virus downstream of a viral promoter (Chakraborti, S. and Jain, S. K., unpublished results). These clones will provide us with a valuable tool to study the regulation of LH gene expression. Besides, it will also open a new era in the development of antifertility vaccines.

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