Rat Inhibin: Molecular Cloning of α- and β-Subunit Complementary Deoxyribonucleic Acids and Expression in the Ovary

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Inhibin is a gonadal protein hormone that suppresses the secretion of FSH from pituitary gonadotrophs. It has previously been characterized as a heterodimer of two dissimilar subunits (α, 18 kilodaltons and β, 14 kilodaltons) the smaller of which exists in two forms (β_A and β_B) and can form dimers that stimulate the secretion of FSH. In the present work, cDNA clones encoding the inhibin α- and β_A-subunits have been isolated from rat ovary and characterized. The α-inhibin cDNA predicts a precursor protein of 366 amino acids containing the 133 amino acid mature α-subunit at its COOH-terminus. The β_A-inhibin cDNA predicts a precursor protein of 424 amino acids containing the 116 amino acid β_A-subunit at its COOH-terminus. Analysis of rat ovarian RNA indicates that α-inhibin mRNA levels are stimulated by PMSG treatment *in vivo*. In cultured granulosa cells, FSH also stimulates α-inhibin mRNA, and the FSH effect is suppressed by cotreatment with GnRH. Hybridization *in situ* to rat ovarian tissue demonstrates that both the α-inhibin and β_A-inhibin mRNAs are specifically expressed in granulosa cells of the developing follicles. (Molecular Endocrinology 1: 561–568, 1987)

INTRODUCTION

The mammalian reproductive cycle is exquisitely regulated through the actions and interactions of hormones secreted from the hypothalamus, pituitary, and gonads. The pituitary gonadotropins, FSH and LH, affect a spectrum of gonadal functions including germ-cell production and steroidogenesis. The secretion of these hormones is positively regulated by the hypothalamic decapeptide GnRH (1, 2). In addition, the observation that secretion of the gonadotropins FSH and LH can be dissociated in a variety of circumstances provided an impetus for seeking specific regulators of FSH or LH distinct from GnRH (3, 4). This led to the identification and characterization of inhibin, a gonadal protein able to specifically suppress FSH secretion (5–8).

Several laboratories have recently described the isolation of inhibin from porcine and bovine follicular fluids (9–12). The porcine inhibin has been characterized as a 32 kilodalton (kDa) protein comprised of disulfide-bound polypeptide chains of 18 kDa, designated α, and 14 kDa, designated β. Two highly related forms of the smaller subunit, designated β_A and β_B, were described in the pig (11). Subsequently, two laboratories (13, 14) have described an FSH-releasing protein from ovarian sources, and characterized this molecule as a dimer (β_A-β_A or β_A-β_B) of the 14 kDa inhibin subunit. The existence of this second activity, termed either FSH-releasing protein (13) or activin (14), suggests that complex mechanisms for the control of FSH secretion via the regulated expression and/or dimerization of the α- and β-subunits of inhibin/FSH-releasing protein are likely to be operative.

Several groups have isolated cDNA clones encoding the inhibin subunits from porcine, bovine, and human sources (15–18). Analysis of these nucleic acid sequences indicates that each of the mature inhibin subunits resides at the carboxyl-terminus of a larger precursor protein. The two inhibin β-subunit precursor proteins show structural and sequence homologies to an emerging family of hormones possessing growth or

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differentiation-regulating properties, including transforming growth factor type β (19), Mullerian-inhibiting substance (20), and the *Drosophila* pattern formation gene product decapentaplegic (DPP) (21). In addition, the β_A-subunit of inhibin is probably identical to an erythroid differentiation factor recently isolated from a human leukemia cell line (22).

To pursue questions concerning the function of inhibin and inhibin-related hormones in reproductive processes, their regulation during the reproductive cycle, and their potential role in development of the gonads, we have chosen to use the rat as the animal model of choice because of its well known reproductive endocrinology. We describe here the molecular characterization of cDNAs encoding the α-inhibin and β_A-inhibin subunits from rat ovary.

RESULTS

Cloning of Inhibin cDNAs

To obtain rat inhibin cDNAs, a cDNA library was constructed using adult rat ovarian RNA as the template. Screening of this library (6×10^5 plaques) with pig or human-specific inhibin probes yielded 12 candidate α-inhibin cDNAs, but no β-inhibin cDNAs. To enrich for inhibin mRNA in the template material, RNA for the second library was derived from ovaries of immature rats that had been treated with estrogen and FSH. Screening of this library (1×10^7 plaques) identified 40 candidate β-inhibin cDNAs. Ten of these were plaque-purified and one end of the cDNA insert was sequenced; all 10 corresponded to the β_A-subunit of inhibin, as judged by homology to the porcine and human β_A sequences.

Structure of the Rat Inhibin Subunits

Potential α-inhibin and β_A-inhibin cDNAs with the largest inserts [each about 1.6 kilobases (kb)] were subcloned into plasmid vectors; these are designated rINA-13 (α-subunit) and rINB-5 (β_A subunit). Restriction enzyme maps were determined for both inserts, and the complete nucleotide sequences of these two cDNAs inserts determined. Figure 1 indicates the restriction map determined and sequencing strategy used for clone rINA-13 (Fig. 1A), and clone rINB-5 (Fig. 1B). Figure 2 shows the nucleotide sequence determined and amino acid sequence deduced for rINA-13; the 1578 nucleotide cDNA contains a single large open reading frame encoding a predicted protein of 366 amino acids. Figure 3 shows the same sequence information for rINB-5; this cDNA is 1553 nucleotides and it encodes a predicted protein of 424 amino acids.

The structural features of the predicted inhibin α- and β-subunit proteins are summarized in the schematic diagrams in Fig. 4. Consistent with the structures predicted from pig and human inhibin cDNAs, the mature rat α- and β_A-subunits reside in the carboxy-terminal third of larger precursor proteins, and are preceded by

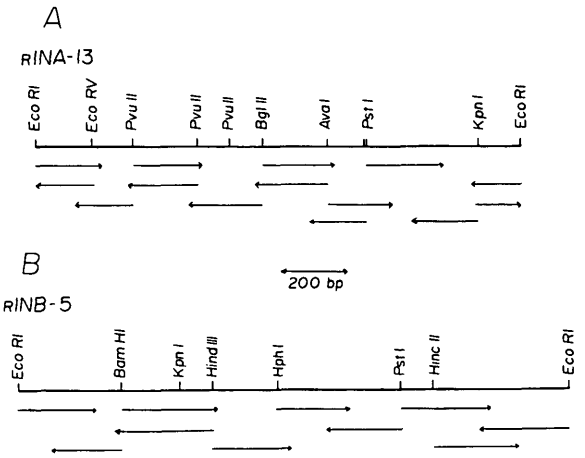

Fig. 1. Restriction Maps and Sequencing Strategies for Rat Inhibin cDNA Clones

A corresponds to the α-inhibin cDNA clone rINA-13, while B corresponds to the β_A-inhibin cDNA clone rINB-5. Each shows a restriction enzyme map of the *Eco*RI insert, and the strategy used for DNA sequencing. *Arrows* indicate the direction and extent of sequence information obtained.

a processing signal composed of multiple basic amino acid residues. Each precursor also includes an amino-terminal stretch of hydrophobic amino acids that is likely to function as a signal sequence for secretion of the prohormone. The mature rat α-subunit includes a single site for potential N-linked glycosylation (Asn-Ile-Ser, residues 269-271). Rat α-inhibin is a 133 amino acid peptide included in a 366 amino acid protein (Fig. 4A), while rat β_A-inhibin is a 116 amino acid peptide included in a 424 amino acid protein (Fig. 4B). Amino acid sequence homologies between the predicted rat α-inhibin and β_A-inhibin subunits and their porcine, bovine, and human counterparts are approximately 80% for the α-subunit and 95% for the β_A-subunit; the extreme conservation of the inhibin β-subunits seen in other species clearly extends to rat.

Analysis of Inhibin mRNAs

Various rat tissues were examined by Northern RNA analysis for expression of the inhibin α- and β_A-mRNAs. The results of this experiment are shown in Fig. 5. Figure 5A demonstrates that the inhibin α-mRNA is about 1500 nucleotides in length, is highly expressed in the ovary but not in liver or brain, and is induced 2- to 3-fold by treatment of the animal with a gonadotropin (PMSG). Figure 5B indicates that the major species of inhibin β_A-mRNA is about 6800 nucleotides in length, and is also specifically expressed in the ovary. β_A-mRNA levels do not appear to change substantially after gonadotropin treatment, but heterogeneity in the RNA samples due to the large size and low abundance of this mRNA makes quantification difficult. Comparative Northern blots indicate that the α subunit mRNA is at least 10-fold more abundant than the β_A-subunit mRNA. Upon longer exposure of the β_A-probed blot, diffuse bands of hybridization corresponding to sizes of 3100 and 1500 nucleotides are also seen (data not shown).

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        .       .      30       .       .      60       .       .      90
GGACACTAGAATGCTGTGTTGTTAGAGGAGTGGAGAGAGGAAGATGTGCTAAGTGTAGCAGTACACACCTATAATCCTAGCACTTGAGAG

        .       .     120       .       .     150       .       .     180
GTTGAAGGCAGGAGGATGAGACATTCAGGTCATTCTTAGCTACATGAAGAGTTTAAGGCCAGCACGGATTACAGGATATCTGTTTCTGGG

        .       .     210       .       .     240       .       .     270
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                                                                MetValIleGlnPro

        .       .     300       .       .     330       .       .     360
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        .       .     480       .       .     510       .       .     540
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        .       .     570       .       .     600       .       .     630
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        .       .     660       .       .     690       .       .     720
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GlnValThrSerAlaGlnLeuTrpPheHisThrGlyLeuAspArgLysSerThrAlaAlaSerAsnSerSerArgProLeuLeuAspLeu

        .       .     750       .       .     780       .       .     810
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        .       .     840       .       .     870       .       .     900
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AlaPheProLeuLeuThrHisProIleLeuValLeuLeuLeuArgCysProLeuCysSerCysSerGlyArgProGluThrThrProPhe

        .       .     930       .       .  ┌─α─→  .       .     990
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        .       .    1020       .       .    1050       .∇      .    1080
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        .       .    1110       .       .    1140       .       .    1170
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        .       .    1200       .       .    1230       .       .    1260
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        .       .    1290       .       .    1320       .       .    1350
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        .       .    1380       .       .    1410       .       .    1440
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Ile

        .       .    1470       .       .    1500       .       .    1530
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        .       .    1560       .
CCTGTGGATAACAATAAAGAAGGAAGTGTGTAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide Sequence of cDNA Clone rINA-13.

Shown is the DNA sequence and the deduced amino acid sequence of the inhibin α-subunit precursor. The presumed site for proteolytic processing at the amino-terminus of the mature α-subunit is indicated with an *arrow*, and the presumed polyadenylation signal is underlined. ∇, Potential site for N-linked glycosylation in the mature α-subunit.

```
         .         .        30         .         .        60         .         .        90
CTCTGACCTCATGAGACAAGAGCCGGCTGGCAAAACAGAAGGGACCCGAAAGAGAATTTGCTGAAGAGGAGAAGGAAAAAAGTCCAAAAA

         .         .       120         .         .       150         .         .       180
ACCTGTACGTGAGGGGTGGGGAGGAAAAGCAGGGCCTTTAAAGAAGGCAACCACACGACTTTTGCTGCCAGGATGCCCTTGCTTTGGCTG
                                                                    MetProLeuLeuTrpLeu

         .         .       210         .         .       240         .         .       270
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         .         .       300         .         .       330         .         .       360
TCCTGTGCGCTGGCCACCCTTCCGAAGGATGGACCTAACTCTCAGCCAGAGATGGTAGAGGCTGTCAAGAAGCACATCTTAAACATGCTG
SerCysAlaLeuAlaThrLeuProLysAspGlyProAsnSerGlnProGluMetValGluAlaValLysLysHisIleLeuAsnMetLeu

         .         .       390         .         .       420         .         .       450
CACTTGAAGAAGAGACCCGATGTCACCCAGCCGGTACCCAAGGCGGCGCTTCTCAACGCGATCAGAAAGCTTCATGTGGGTAAAGTGGGG
HisLeuLysLysArgProAspValThrGlnProValProLysAlaAlaLeuLeuAsnAlaIleArgLysLeuHisValGlyLysValGly

         .         .       480         .         .       510         .         .       540
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GluAsnGlyTyrValGluIleGluAspAspIleGlyArgArgAlaGluMetAsnGluLeuMetGluGlnThrSerGluIleIleThrPhe

         .         .       570         .         .       600         .         .       630
GCCGAGTCAGGCACAGCCAGGAAGACACTGCATTTTGAGATTTCCAAGGAAGGCAGTGACCTGTCAGTCGTGGAGCGTGCAGAAGTCTGG
AlaGluSerGlyThrAlaArgLysThrLeuHisPheGluIleSerLysGluGlySerAspLeuSerValValGluArgAlaGluValTrp

         .         .       660         .         .       690         .         .       720
CTCTTCCTGAAAGTCCCCAAGGCCAACAGGACCAGGACCAAAGTCACCATCCGTCTGTTTCAGCAGCAGAAGCATCCACAGGGCAGCTTG
LeuPheLeuLysValProLysAlaAsnArgThrArgThrLysValThrIleArgLeuPheGlnGlnGlnLysHisProGlnGlySerLeu

         .         .       750         .         .       780         .         .       810
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         .         .       840         .         .       870         .         .       900
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ThrTrpHisIlePheProValSerSerSerIleGlnArgLeuLeuAspGlnGlyLysSerSerLeuAspValArgIleAlaCysGluGln

         .         .       930         .         .       960         .         .       990
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CysGlnGluSerGlyAlaSerLeuValLeuLeuGlyLysLysLysLysLysGluValAspGlyAspGlyLysLysLysAspGlySerAsp

         .         .      1020         .         .      1050         .         .      1080
GGAGGGCTGGAAGAGGAAAAAGAACAGTCACACAGACCTTTCCTCATGCTGCAGGCTAGGCAGTCTGAAGACCATCCTCACCGCAGGCGT
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         ┌─βA─→    .      1110         .         .      1140         .         .      1170
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ArgArgGlyLeuGluCysAspGlyLysValAsnIleCysCysLysLysGlnPhePheValSerPheLysAspIleGlyTrpAsnAspTrp

         .         .      1200         .         .      1230         .         .      1260
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IleIleAlaProSerGlyTyrHisAlaAsnTyrCysGluGlyGluCysProSerHisIleAlaGlyThrSerGlySerSerLeuSerPhe

         .         .      1290         .         .      1320         .         .      1350
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HisSerThrValIleAsnHisTyrArgMetArgGlyHisSerProPheAlaAsnLeuLysSerCysCysValProThrLysLeuArgPro

         .         .      1380         .         .      1410         .         .      1440
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         .         .      1470         .         .      1500         .         .      1530
TGCCAGGTCCCAGAGCAAATGGATCTAGGGTGTCCAGGAAAAGACAGTGGCAAATGAAGAAAAATATATAAGATTTCTGCCTAAACAAGA

         .         .
CAACCAGAAAAATAAAAAAAAAA
```

Fig. 3. Nucleotide Sequence of cDNA Clone rINB-5

Shown is the DNA sequence and deduced amino acid sequence of the inhibin β_A-subunit precursor. The presumed site for proteolytic processing at the amino-terminus of the mature β_A-subunit is indicated with an *arrow*, and two potential polyadenylation signals are underlined.

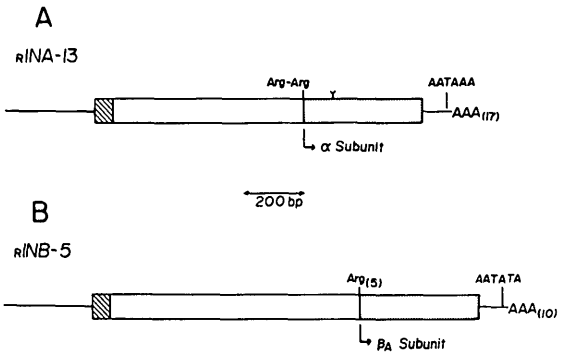

Fig. 4. Structural Features of the Rat Inhibin α- and β_A-subunit cDNAs

A, Structure of clone rINA-13 schematically; B, structure of clone rINB-5 schematically. □, Protein coding region; ▨, putative signal sequence; □, mature α- or β_A-inhibin subunit. Y, Potential site for N-linked glycosylation. Arginine residues presumed to signal proteolytic processing of the mature peptide from the precursor are indicated.

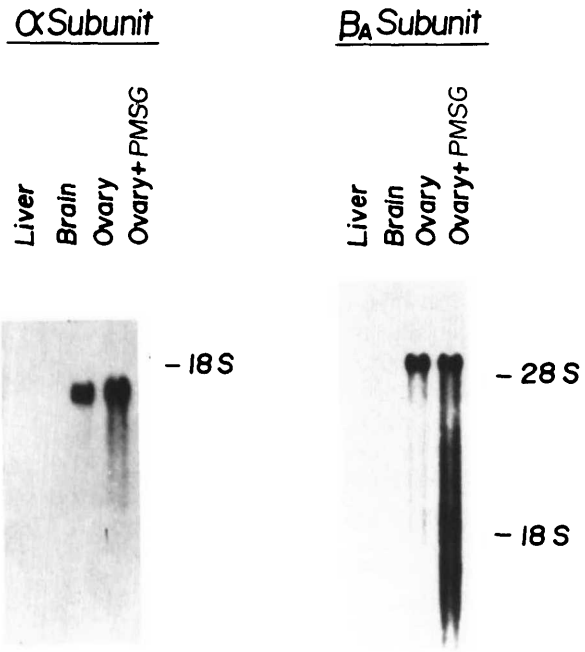

Fig. 5. Analysis of Rat Inhibin mRNAs

Poly(A)$^+$ RNA (10μg) from the indicated tissues was electrophoresed on denaturing agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized to rat inhibin α- or β_A-cDNA inserts labeled with ^{32}P-dCTP by nick-translation. A is probed with rINA-13; B with rINB-5. The migration of 18 S and 28 S ribosomal RNAs is indicated. +PMSG indicates a 24-h treatment of the animal with 20 IU PMSG.

In Situ Hybridizations

Because the ovary is a complex and heterogeneous tissue composed of many cell types at various developmental stages, *in situ* hybridization was used to examine expression of the inhibin α-and β_A-subunit mRNAs at the cellular level. Figure 6 shows ovarian sections from both a control animal and a gonadotropin-treated animal, hybridized with either α-inhibin or β_A-inhibin specific antisense riboprobes. Inhibin mRNAs are localized to individual ovarian follicles in a pattern consistent with specific expression in the granulosa cell population. Both mRNAs appear to be most highly expressed in large Graffian follicles, although hybridization to secondary follicles and even to the corpus luteum (α-subunit) is evident. In the PMSG-treated animal, follicular recruitment is evident, and increased amounts of the inhibin α-mRNA can be seen. Consistent with Northern RNA analysis (Fig. 5), PMSG appears to have a minimal effect on β_A-subunit mRNA levels. Analysis of adjacent sections by *in situ* hybridization (not shown) indicates that the same follicles are producing both the α- and β_A-subunit mRNAs.

Expression in Granulosa Cell Cultures

The localization of inhibin mRNAs to the granulosa cell, and the apparent ability of gonadotropin treatment to increase α-subunit mRNA levels, led us to more fully examine the FSH induction in a defined granulosa cell culture system (22). Figure 7 indicates that the inhibin α-subunit mRNA is induced 4-fold in cultured granulosa cells in response to treatment with 30 ng/ml FSH in the culture medium for 16 h. Treatment of the cell cultures with GnRH (100 nm) was also tested and found to have little effect on α-inhibin mRNA, but GnRH did substantially reduce the ability of FSH to induce α-subunit mRNA (2-fold induction).

DISCUSSION

In the short time since the initial characterization of the inhibin protein, a great deal of structural information about these molecules has been accrued, largely through recombinant DNA means. The present work extends this structural information to the rat, an animal model ideally suited to studying the physiology of inhibin. The complete amino acid sequences of both the α- and β_A-subunits of rat inhibin have been deduced from the nucleotide sequences of the corresponding cDNAs, and indicate a high degree of homology between the rat hormones and those of other mammals. This homology is particularly striking for the β_A-inhibin subunit, and suggests a strong pressure for conservation of its structure. This is particularly intriguing in that the β-subunits of inhibin appear to belong to a family of growth and/or differentiation regulatory hormones including transforming growth factor type β, Mullerian-inhibiting substance, and decapentaplegic, and it suggests a possible action of the β-subunit of inhibin in regulating some aspect of gonadal development. The recent observation that a human erythroid differentiation factor has apparent identity with the β_A-homodimer of inhibin, suggests an additional nonreproductive role for this interesting hormone (22).

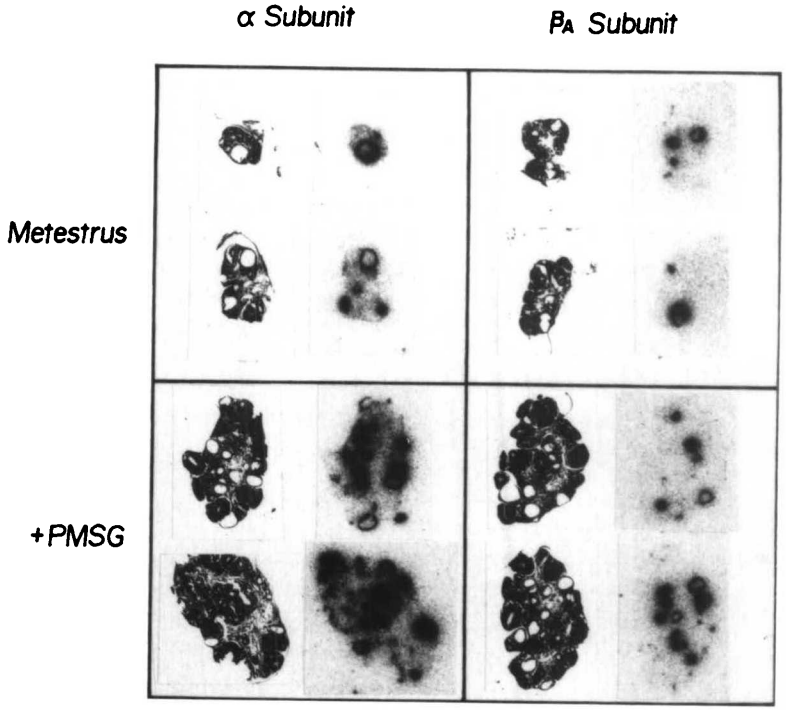

Fig. 6. *In Situ* Hybridization Detection of Rat Inhibin mRNAs

Ovarian sections are from either a control (metestrus) or PMSG-treated female, and are probed with either α-inhibin or β_A-inhibin specific probes as indicated. Within each panel, the photograph on the *left* is of a hematoxylin/eosin-stained ovarian section (20 μm), while the photograph on the *right* is the autoradiographic detection of hybridization in the same section. Two sections from different regions of the same ovary are shown in each case.

In the pig, the inhibin β_A-mRNA was found to exist in two forms, a 4.5 kb and a 7.2 kb transcript (15), but all β_A-cDNA clones described in the literature have been shorter than this (15, 17). We have made similar observations in the rat, in that the major transcript appears to be about 6.8 kb (with perhaps several smaller minor transcripts), but cDNA clone rINB-5 includes only about 1600 nucleotides. We have recently analyzed several additional rat β_A-cDNAs, and find that they are all about 1.6 kb but show substantial heterogeneity at their 3′-ends. The sequence analysis indicates that this region contains multiple short adenine tracts, and suggests that cDNA synthesis might be primed from this region during library construction. Alternatively, the use of multiple polyadenylation sites to generate different mRNA species is a possibility.

Complementary DNA clones corresponding to the β_B-subunit of inhibin were not identified in our initial screening of the libraries, even though we isolated many β_A-clones. Complementary DNAs corresponding to this subunit also were not identified from bovine sources (18), and analysis of the pig inhibin mRNAs suggests that the β_B-mRNA is the least abundant inhibin transcript (15). We have recently isolated two cDNAs that appear to encode the β_B-inhibin subunit and are currently characterizing these clones.

We have shown that both in PMSG-treated rats and in FSH-treated cultured granulosa cells, gonadotropin increases steady state levels of the α-inhibin mRNA. This is consistent with the proposed regulatory role of FSH in inhibin biosynthesis (23), in that by increasing α-subunit availability (and hence α-β-dimer formation), inhibin activity would be made available to subsequently suppress the FSH. We have recently obtained similar results in normally cycling female rats in which α-inhibin mRNA levels change in concert with serum FSH levels (Woodruff, T. K. and K. E. Mayo, unpublished observations). The present data is consistent with a recent report showing the stimulatory effect of PMSG on α-inhibin gene expression in immature rats (24). These authors also identified α-inhibin mRNA in rat corpus luteum, in agreement with our *in situ* hybridization results.

In the granulosa cell culture system, GnRH was also tested for effects on α-inhibin mRNA accumulation. GnRH alone had little effect, but it did reduce the FSH-induced increase in α-subunit mRNA. These results are compatible with the finding that GnRH inhibits the FSH-stimulated production of inhibin in rat granulosa cells as measured by inhibin RIA (23). The effect of GnRH in this system is also consistent with its inhibitory effects on other FSH-induced granulosa cell activities, such as steroid production, LH and PRL receptor formation, and cAMP production (25).

Analysis of ovarian sections by *in situ* hybridization promises to be a powerful technique for examining the expression of the inhibin subunit genes during follicular development. We have shown that both the α- and β_A-subunit mRNAs can be localized to the granulosa cells of maturing follicles. In addition, an increase in hybridization intensity of the α-subunit probe after PMSG administration can be seen, in agreement with the more

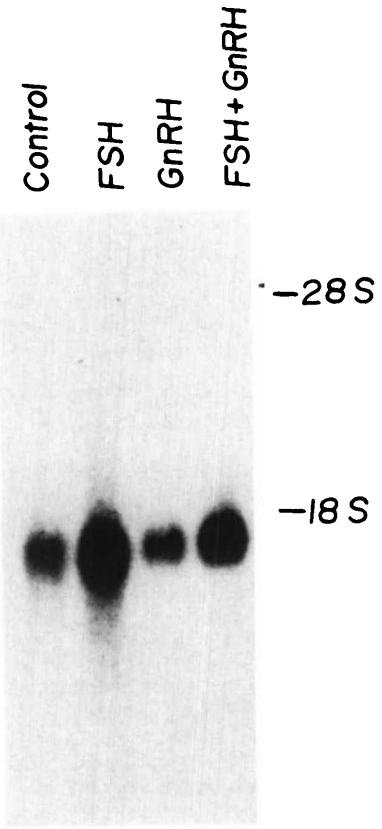

Fig. 7. Expression of the Rat α-Inhibin mRNA in Granulosa Cell Cultures

Total RNA from each culture (20 μg) was electrophoresed on denaturing agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized to radiolabeled insert from cDNA clone rINA-13. The migration of 18 S and 28 S ribosomal RNAs is indicated. Samples and their relative densitometric intensities are control cells (control, 3.1); 30 ng/ml FSH for 16 h (FSH, 12.6); 100 nm GnRH for 16 h (GnRH, 2.8); and FSH plus GnRH as above for 16h (FSH+GnRH, 8.0).

quantitative Northern RNA analysis. Although the inhibin mRNAs are clearly found in granulosa and luteal cells, we cannot rule out the possibility that that they are also made in other ovarian cells; for example, thecal or interstitial cells, due to the low resolution of the ^{32}P-RNA probes used here. We are currently exploring the use of ^{3}H-RNA probes and liquid emulsion autoradiography in this system, which should allow a detailed picture of the cellular expression of the inhibin mRNAs at each stage of follicular development to be obtained.

MATERIALS AND METHODS

Animals and Materials

Adult or immature female Sprague-Dawley rats were obtained from Charles River (Wilmington, MA) or Johnson Laboratories (Bridgeview, IL). Adult animals were maintained on a schedule of 14-h light, 10-h dark, and cycle stage was determined by daily examination of vaginal cytology. For granulosa cell preparation, immature (21 days old) female Sprague-Dawley rats were hypophysectomized and implanted with diethylstilbestrol-containing silastic capsules for 4–6 days (25). Chemicals and hormones were from Sigma (St. Louis, MO); radionucleotides were from New England Nuclear (Boston, MA), and recombinant DNA enzymes from either Bethesda Research Laboratories (Gaithersburg, MD) or Promega Biotech (Madison, WI).

Complementary DNA Library Construction and Screening

Two cDNA libraries were prepared. The first used poly(A)$^+$ RNA from randomly cycling rat ovary as template, the second used poly(A)$^+$ RNA from the ovaries of immature, estrogen-treated rats that had been treated with 5 μg ovine FSH twice daily for 1 day. Standard methods were used for oligo(dT)-primed cDNA synthesis; *Eco*RI linkers were ligated to the double-stranded cDNA, and the cDNAs were inserted into the phage expression vector λgt10 or λgt11 (26, 27). The adult rat ovarian library contained 6×10^5 recombinants; the immature rat ovarian library 2×10^7 recombinants, each with an average insert size of 1500 base pairs. To screen for α-inhibin cDNAs, a nick-translated insert from the pig inhibin cDNA clone pFS7 (16) was used. To screen for β-inhibin cDNAs, two kinase-labeled oligonucleotides (54mers) predicted from the human inhibin β_A- and β_B-cDNA sequences (17) were used.

DNA Sequence Analysis

Inserts from hybridization-positive phage were subcloned, restriction enzyme maps for the cDNAs were determined, and portions of the inserts subcloned again into the pGEM3/4 plasmid vector. Dideoxy chain termination sequencing was performed on denatured double-stranded plasmid DNA by extending primers hybridized to the SP6 or T7 promoters of the vector (28).

RNA Preparation and Hybridization Analysis

For *in vivo* work, adult rats were either randomly cycling or were injected ip with 20 IU PMSG 24 h before killing. Granulosa cell cultures were prepared by manual expression of the cells from follicles of immature, hypophysectomized, estrogen-treated rats as described previously (29), and hormones were added for 16 h on culture day 1. Total RNA was prepared from both sources by homogenization in guanidine isothiocyanate and centrifugation through CsCl cushions (30). Poly(A)$^+$ RNA was prepared by chromatography on an oligo(dT)-cellulose column. RNAs were electrophoresed on denaturing formaldehyde-agarose gels and the gels were stained with acridine orange to check for equivalent loading of RNA samples. The results of these controls showed that in each case all lanes were loaded with equal amounts of RNA (*c.f.* Figs. 5 and 7). RNA was transferred to nitrocellulose and hybridized using standard conditions to cDNA inserts radiolabeled by nick-translation. After washing and autoradiography, autoradiograms were scanned (Helena Labs Quick-Scan densitometer, Beaumont, TX) to quantify bands of hybridization.

In Situ Hybridization

Adult female rats were perfused intracardially with acetate-buffered paraformaldehyde (pH 6.5) followed by borate buffered paraformaldehyde (pH 9.5). Ovaries were removed, postfixed overnight in borate-buffered paraformaldehyde with 10% sucrose, and 20-μm frozen sections cut on a Reichert cryostat. Sections were mounted on microscope slides previously subbed with gelatin and polylysine, and treated with proteinase K followed by acetic anhydride (31). Slides were hybridized to [^{32}P]antisense RNA probes specific for α-inhibin or β_A-inhibin that were generated by transcription from the SP6 or T7 promoters of pGEM subclones. Hybridization was performed under mineral oil in 50% formamide, 6 × SSC, 10%

dextran sulfate, and 1×10^8 cpm/ml labeled probe RNA at 48 C for 16 h. Slides were washed free of oil, treated with RNAse A (10 μg/ml), washed in 0.2 × SSC at 48 C, and dehydrated through an ethanol series. Autoradiography was performed on Kodak XAR-5 film (without intensifying screen) for 3 h.

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Note Added in Proof

After acceptance of this manuscript, a similar report describing rat inhibin cDNA clones was published (Esch, F. S., S. Shimasaki, K. Cooksey, M. Mercado, A. J. Mason, S.-Y. Ying, and N. Ling 1987 Complimentary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. Mol Endocrinol 1:388–396).

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