Analysis of the 5′-Flanking Regions of Rat Inhibin α- and β-B-Subunit Genes Suggests Two Different Regulatory Mechanisms

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The genes encoding rat inhibin α- and β-B-subunits were isolated and characterized. Both genes contain one intron that interrupts the region coding for the precursor portion of the α- and β-B-subunits. The transcription start sites of α- and β-B-subunit genes were determined by primer extension and nuclease mapping assay using mRNA from rat ovary and testis. Transcription of the α-subunit gene initiates predominantly at three adjacent sites with similar intensity. Several potential transcription start sites of β-B-subunit gene are spread over 150 nucleotides upstream from translation initiation site. Neither of these two genes contains obvious TATA or CCAAT boxes. The α-subunit gene contains many GA clusters in the promoter region, while β-B-subunit gene is highly GC rich. Several GGGCGG repeats and their inverted sequences, which are the potential binding sites for transcription factor Spl, were observed at the 5′-end as well as at the coding region of the β-B-subunit gene. The potential cAMP-responsive element CTGCGTCAG was identified in α- but not β-B-subunit gene. This sequence is identical to the cAMP- and phorbol ester-inducible DNA fragment found in human preproenkephalin gene. The different structure of the promoter region of rat α- and β-B-subunit genes and the presence of a potential cAMP-inducible DNA sequence in α- but not β-B-subunit gene is consistent with the hypothesis that transcription of α- and β-B-subunit genes in rat is regulated by different mechanisms. (Molecular Endocrinology 3: 1914–1925, 1989)

INTRODUCTION

Inhibin, a gonadal glycopeptide hormone that inhibits FSH secretion, was recently isolated from porcine (1–3) and bovine (4, 5) follicular fluid and ovine rete testis fluid (6, 7). In all these species inhibin is comprised of an α-subunit [18 kilodalton (kDa)] and a β-subunit (14 kDa) which are joined by disulfide bonds. Two highly related inhibin β-subunits, β-A and β-B, were identified. Both inhibin A (αβ-A) and inhibin B (αβ-B) inhibit FSH secretion. Activins, which are dimers of inhibin β-subunits (β-Aβ-A and β-Aβ-B), were also isolated from porcine follicular fluid based upon their ability to stimulate pituitary FSH secretion (8–10).

The cDNAs coding for inhibin subunits have been isolated from libraries prepared from porcine (11), bovine (12), human (13, 14), and rat ovaries (15, 16), as well as from human testis (17, 18). The complete amino acid sequences of inhibin subunits were deduced from the cloned cDNAs. These studies showed that the mature α- and β-subunits are derived from the C-termini of the large precursors. Both subunits have similar cysteine distributions and sequence homology around these residues, suggesting that the two inhibin subunits may have arisen from one ancestral gene.

The expression of three subunit genes was tissue-specific. For instance, α- and β-B-subunits were the predominant forms found in rat testis (15, 17, 18). The expression of these inhibin subunit genes in rat testis was also differentially regulated (17, 18). Hypophysectomy reduced α- but elevated β-B-subunit mRNA levels. FSH markedly stimulated α- but not β-subunit mRNA accumulation. In order to study the mechanisms by which the differential accumulation of testicular inhibin α- and β-B-subunit mRNAs are effected, we thought it pertinent to isolate and characterize the genes encoding these subunits and compare the structures of their 5′-flanking regions.

RESULTS

Isolation of Inhibin Subunit Genes from Rat Genomic Library

A genomic library was constructed in λ-phage (Charon 4A) from partially *Eco*RI digested rat liver DNA. Approximately 5×10^5 phage plaques were screened for inhibin α- and β-B-subunit genes using three human testicular

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cDNA clones as hybridization probes; human testicular αH67-2 [1.3 kilobase (kb)] cDNA clone contains nearly full-length cDNA encoding α-subunit precursor (17) and the combined nucleotide sequences of β-B H190-1 and β-B H195-1 clones correspond to almost all of the complete coding sequence (minus one amino acid at N-terminus) for human inhibin β-B-subunit precursor and 1.6 kb of 3′-untranslated region (18). Three α-subunit and five β-B-subunit positive plaques were obtained. These genomic fragments were isolated, subcloned in pGEM-3Z plasmid vector, and characterized by restriction enzyme mapping. All three inhibin α-subunit genomic clones contained approximately 12 kb inserts. When these fragments were digested with *Eco*RI, all contained one *Eco*RI fragment of 5.5 kb which was hybridized to full-length human testicular α-subunit cDNA (αH67-2) (17); the other fragment of 7 kb was not hybridized (Fig. 1). All five β-B-subunit genomic clones contained approximately 15 kb inserts. Two of these clones were characterized and were shown to be identical. One *Bam*HI fragment of approximately 8.2 kb (Fig. 2) was hybridized to human testicular cDNA clones, β-BH195-1 and β-BH190-1.

Genomic Structure of α- and β-B-Subunit Genes

The 5.5. kb *Eco*RI and the 8.2 kb *Bam*HI fragments of the respective α- and β-B-subunit genes were characterized by restriction endonuclease mapping and by Southern blot analysis using different portions of the human testicular inhibin cDNAs as hybridization probes and compared to the maps generated from the known nucleotide sequences for rat inhibin cDNAs (15, 16). The regions of the isolated genes, such as intron-exon junctions, coding regions, and certain 5′- and 3′-flanking regions were further characterized by nucleotide sequencing analysis. The intron-exon boundaries were identified by comparison of the nucleotide sequences of the isolated genes with those of the cDNAs isolated from rat ovary (15, 16).

The schematic structure and nucleotide sequence of inhibin α-subunit gene are shown in Figs. 1 and 3, respectively. The 5.5 kb *Eco*RI fragment of α-subunit genomic clone contained the entire coding region of the α-subunit precursor (Fig. 1). The restriction endonuclease cleavage sites and the nucleotide sequence of the coding region of the rat α-subunit gene are in good agreement with those of rat ovarian α-subunit cDNA (15, 16). One intron of approximately 1.5 kb was found to interrupt the coding sequence for glycine at amino acid 91 (Figs. 1 and 3) and was flanked by the consensus splice sequence (GT/AG). This genomic clone also contains approximately 2.1 kb DNA upstream from the translation initiation site (ATG) and 0.5 kb downstream from a poly(A) addition signal (AATAAA).

The schematic structure and nucleotide sequence of inhibin β-B-subunit gene are shown in Figs. 2 and 4, respectively. The 8.2 kb *Bam*HI fragment of the genomic clone contained the entire coding region of the β-B-subunit precursor (Fig. 2). One intron of approximately 3.0 kb, which was observed to interrupt the coding sequence of amino acid 154, aspartic acid (Fig. 4A), was also flanked by the consensus splices sequence (GT/AG). Since the amino acid sequence for the N-terminal 130 residues of the rat β-B-subunit precursor had not been identified, the nucleotide sequence for the 5′-untranslated region and the N-terminal 130 amino acids were thus analyzed (Fig. 4A). Both nucleotides and deduced amino acids at this region shared high sequence similarity with those of human testicular

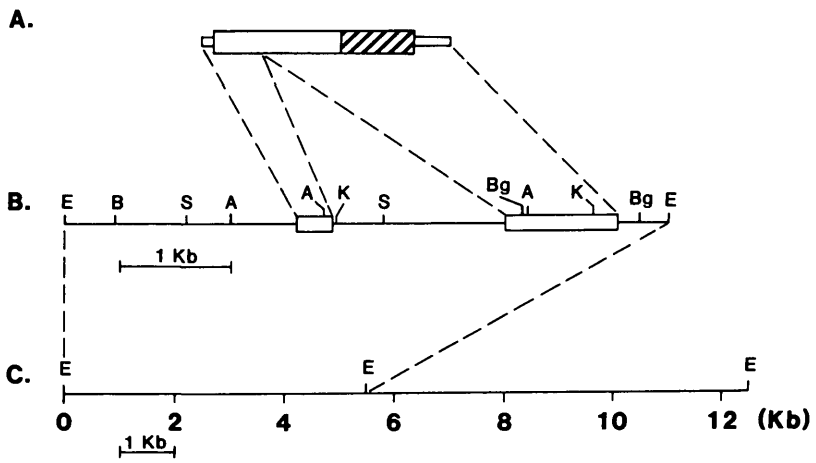


Fig. 1. Structure of the Rat Inhibin α-Subunit Gene

The structure of α-subunit mRNA (A) is schematically represented together with the structure of the rat inhibin α-subunit gene (B). The DNA fragment coding for α-subunit gene initially isolated from rat liver genomic DNA library is shown in C. In A, the schematic representation of rat α-subunit mRNA was generated from the sequence of the isolated α-subunit gene (Fig. 3) and from the ovarian cDNA (15,16). The untranslated sequence of the α-subunit mRNA is represented by a *thin open box*, the coding region for the pro-sequence is by a *thick open box*, and for the mature inhibin α-subunit is by a *shaded box*. In B, the exons are indicated as *open boxes*. The restriction endonuclease cleavage sites are indicated as follows: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; and S, *Sac*I.

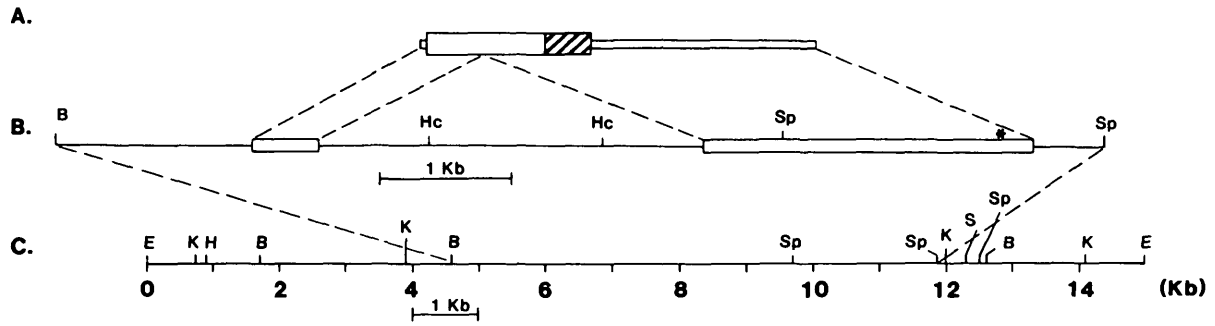


Fig. 2. Structure of the Rat Inhibin β-B-Subunit Gene

The structure of β-B-Subunit mRNA (A) is schematically represented together with the structure of the rat inhibin β-B-subunit gene (B). The DNA fragment for β-B-subunit gene initially isolated from rat liver genomic DNA library is shown in C. In A, the schematic representation of rat β-B-subunit mRNA was generated from the sequence of the isolated gene (Fig. 4) and of the ovarian cDNA (15). The untranslated sequence of the β-B-subunit mRNA is represented by a *thin open box*, the coding region for the pro-sequence is by a *thick open box*, and for the mature β-B-subunit is by a *shaded box*. In B, the exons are indicated as *open boxes*. The restriction endonuclease cleavage sites indicated in B and C are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; K, *Kpn*I; S, *Sal*I; and Sp, *Sph*I. The *asterisk in B* indicates the *Pst*I site which the *Pst*I-*Sph*I fragment of the 3′-untranslated region was isolated and the nucleotide sequence of this fragment was determined as shown in Fig. 4B.

(18) and ovarian (13, 19) cDNAs. There are four additional amino acids (glycine) found in rat β-B-subunit precursor (amino acid 61-64) as compared to those in human. Two differences in amino acid sequence (positions 92 and 167) were also observed at the N-terminal 173 amino acids analyzed. There are many proline residues at the N-terminus of rat β-B-subunit precursor as is the case for the previously reported sequence for human β-B-subunit (18, 19). The restriction endonuclease cleavage sites for the coding region and part of the 3′-untranslated region (1.7 kb) were identical to those of rat ovarian cDNA (15). However, differences were observed at 1707 nucleotides downstream from the translation stop site (TGA) (Fig. 4B). The nucleotide sequence for this region in the genomic clone was analyzed (Fig. 4B) and was shown to contain completely different sequences as compared to those of published rat ovarian β-B-subunit cDNA (15). Using this latter DNA fragment isolated from rat gene as a hybridization probe, we have detected two species of β-B-subunit mRNA (4.4 and 3.3 kb) as reported previously (18) in rat ovary and testis (data not shown). Three poly(A) addition signals (AATAAA) were found in the newly identified 3′-untranslated region of the rat β-B-subunit gene (Fig. 4B).

Identification of the Transcription Start Sites

The initiation sites of the transcription of rat α- and β-B-subunit genes in the testis and ovary were analyzed by primer extension and nuclease mapping assay (Figs. 5 and 6). For extension studies, radiolabeled single-stranded cDNAs were hybridized with rat testicular or ovarian poly(A) RNA and extended using AMV reverse transcriptase. The extended cDNA product was analyzed on a sequencing gel with DNA sequence reactions as size markers which were obtained from the same inhibin DNA subclones with the same primers. Thus, the positions of the extended DNA products could be precisely mapped.

Using primer A-1 which includes sequences for amino acids from 13 to 44 of rat α-subunit gene (Fig. 5A), three major start sites (G, A, and A) with similar intensity for the transcription of α-subunit gene were observed both in rat testis and ovary. They are mapped to 72-74 bases upstream from translation initiation site (ATG). Under longer exposure, a minor initiation site of 103 bases upstream from ATG site was also detected. These observations were confirmed by using a synthetic 30 base-oligomer as primer (A-2), which contained 15 nucleotides of 5′-untranslated region and 15 nucleotides of coding region (Fig. 5B). The major extended products in rat testis and ovary were mapped to the same sites (G, A, and A) as those determined by primer A-1. A similar minor band at 103 bases upstream from translation initiation site was also observed. No similar results were obtained when rabbit liver tRNA or ribosomal RNA was used. The initiation of α-subunit gene transcription at three adjacent sites was further analyzed by S1 nuclease mapping assay (Fig. 5C). The probe S1 used contained 164 nucleotides upstream from translation initiation site (ATG) and 15 nucleotides of protein coding region. Three predominant fragments of 87-89 nucleotides and one minor fragment of 118 nucleotides were protected, both by ovarian (Fig. 5C) and testicular (data not shown) RNA. These fragments were mapped to the same positions as those determined previously by primer extension method (Fig. 5, A and B) for the initiation of transcription of α-subunit gene. In addition, no radioactive probe S1 could be detected after S1 nuclease digestion, suggesting that no α-subunit transcript initiates further upstream from the probe S1.

The start sites of β-B-subunit gene transcription in rat ovary and testis were next determined (Fig. 6). Using primer B, a synthetic 26 base-oligonucleotide which includes 13 nucleotides of 5′-untranslated region and

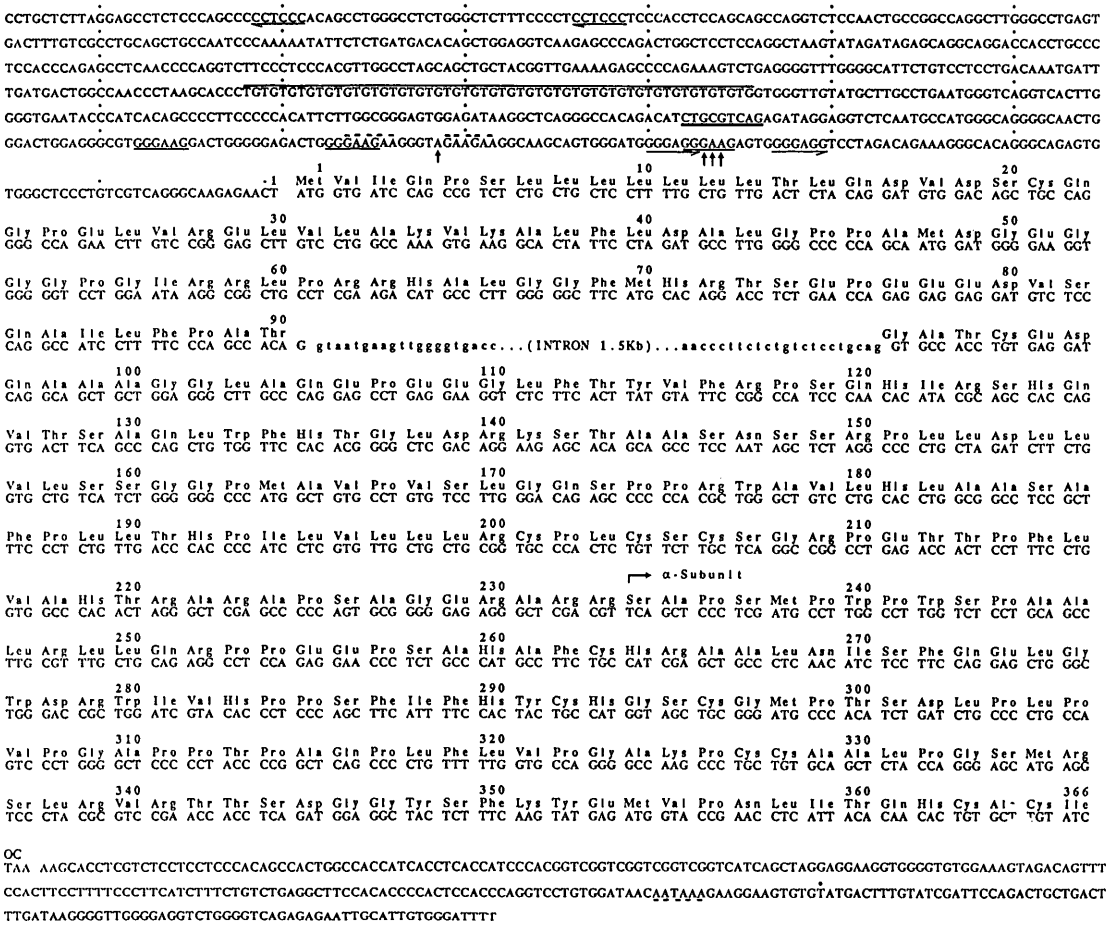


Fig. 3. Nucleotide and Predicted Amino Acid Sequences of the Rat Inhibin α-Subunit Gene

The sequencing strategy is described in *Materials and Methods*. The nucleotides of the 5′-flanking region are numbered from the translation initiation site (ATG) and are shown at the right end of each line. The transcription start sites determined by primer extension and SI nuclease mapping assay (Fig. 5) are indicated by the *arrows*. The amino acids are numbered throughout and the N-terminus of the mature α-subunit is indicated with a *horizontal arrow at 234*. The nucleotide sequences in the intron portion are written in lower case. The polyadenylation signal AATAAA is *underlined* by a *broken line* and the poly(A) addition site observed in ovarian cDNA (16) is marked with a *dot*. The TG sequence repeats 28 times at the 5′-flanking region are *overlined*. The GGGAAG repeats are *underlined* and the GAAGAA are *overlined* by *broken lines*. The pairs of GGGAGG and their invert repeated sequences are marked with *underlying arrows*. The potential cyclic AMP-responsive element is underlined with a *thick line*.

13 nucleotides of the coding region of β-B-subunit gene, several extended cDNA products were observed both in rat ovary and testis (Fig. 6A). These bands were not found in rabbit liver ribosomal RNA or tRNA. The several potential transcription start sites found specifically in rat ovary and testis are indicated in Figs. 4A and 6. They were spread over approximately 150 nucleotides upstream from its translation initiation site of β-B-subunit gene. The mapping of transcription start sites of β-B-subunit gene was further confirmed by ribonuclease protection assay (Fig. 6B). The RNA probe R used contained 450 nucleotides upstream from translation initiation site and 62 nucleotides of protein coding region. Several protected fragments were observed in rat ovary poly(A) RNA. Under longer exposure, identical observations were also found in testicular poly(A) RNA. No protected bands were detected in rabbit liver ribosomal RNA or tRNA. One most intense band located 125 nucleotides upstream of translation initiation site was observed in both ovarian and testicular RNA samples. The protected fragments (Fig. 6B) were mapped to the same positions as those determined by primer extension (Fig. 6A).

Analysis of the 5′-Flanking Region of Inhibin Subunit Genes

The nucleotide sequence of the 5′-flanking region of α- and β-B-subunit genes was determined as shown in Figs. 3 and 4, respectively. Since multiple transcription start sites were observed, the nucleotides were numbered as the initiation site of translation, +1. Neither α- nor β-B-subunit gene contains TATA box or CAAT box elements which may appear at 20-30 and 40-100 nucleotides, respectively, upstream from the transcription start site of many protein coding genes (20). A TATA

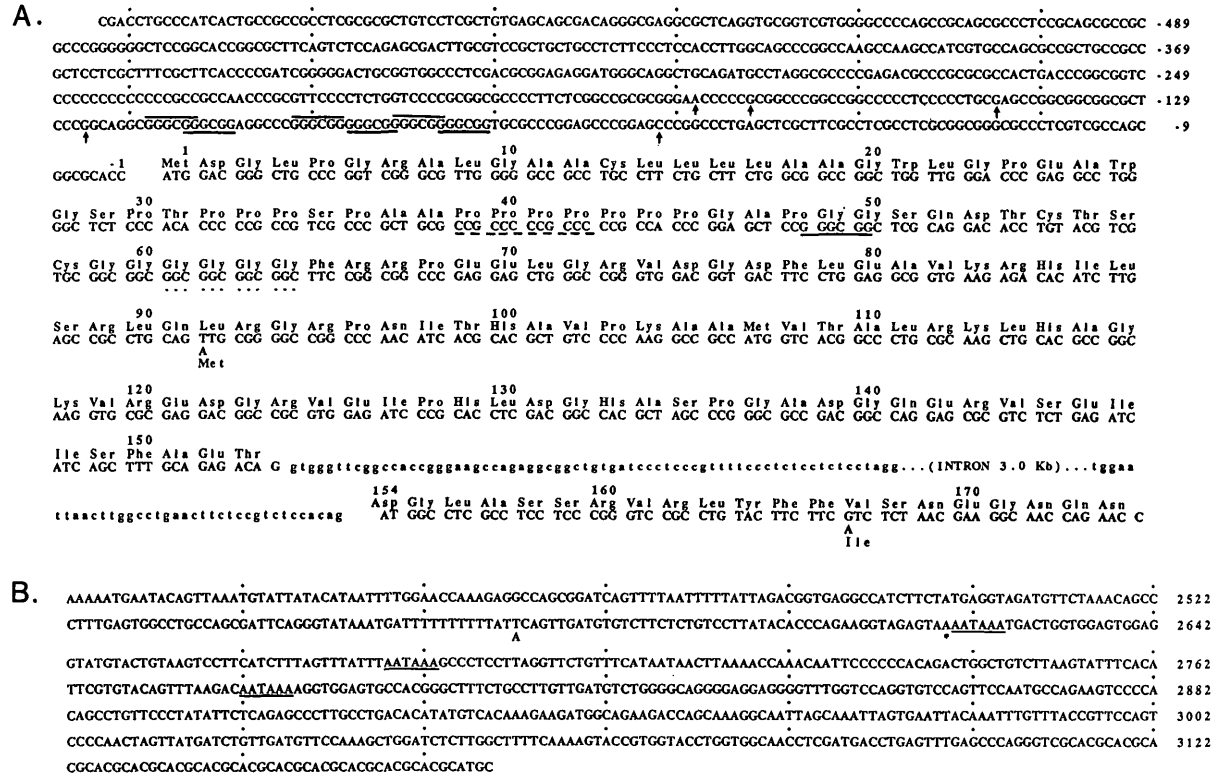


Fig. 4. Nucleotides and Predicted Amino Acid Sequences of the Rat Inhibin β-B-Subunit Gene

The sequence for the 5′-end of the rat β-B-subunit gene is shown in A, and the sequence for part of the 3′-untranslated region which is different from that of the reported ovarian cDNA (15) is shown in B. In A, the nucleotides of the 5′-flanking region are numbered from the translation initiation site (ATG) and are shown at the *right end of each line*. The potential transcription start sites determined by primer extension and RNase protection assay (Fig. 6) are marked by *arrows*. The GGGCGG repeats are either *underlined* or *overlined*, and their inverted repeat sequence CCGCCC are underlined with a *broken line*. The amino acids are numbered throughout. The sequences in the intron are written in lower case. Under the rat sequence are the differences found in human β-B-subunit. In B, the *Pst*I-*Sph*I fragment of the 3′-end of rat β-B-subunit gene (Fig. 2) was sequenced. The nucleotides are numbered from the translation initiation site (+1) to the 3′-untranslated region of the two exons (intron not included), and are shown at the *right end of each line*. The nucleotide 2403 (the first nucleotide shown in B) is equivalent to nucleotide 2331 in reported ovarian cDNA (15). The sequence for nucleotides 2403 to 2618 in B is identical to that in ovarian cDNA (15). Completely different sequence was observed from nucleotide 2619 (marked by an *asterisk*) which is 1707 nucleotides downstream from the translation stop site (TGA). The polyadenylation signals AATAAA are *underlined*.

and CCAAT sequence were identified at −540 to −537 and −610 to −606, respectively, of the α-subunit gene. However, results from primer extension and S1 nuclease mapping (Fig. 5) indicate that no transcription of α-subunit gene initiated at 20-30 and 40-100 nucleotides, respectively, downstream from these sequences, suggesting that they did not serve as promoters for the transcription of α-subunit gene.

Other sequences which may have promoter functions were next sought. The nucleotide sequences upstream from transcription start sites of α- and β-B-subunit genes were quite different. There are many GA clusters at the promoter region of α-subunit gene. For instance, three GGGAAG repeats were identified; one was within the major transcription start site. Two GAAGAA repeats were found at −102 to −97, and −113 to −108 regions upstream from translation initiation sites (ATG), which are 25-41 nucleotides upstream from the major transcription start sites. Two pairs of GGGAGG were also observed near the transcription start sites, and the invert repeated sequences of GGGAGG were also found at the region 670 nucleotides upstream from the major transcription start sites of α-subunit gene. In addition, there are TG sequence repeated 28 times at the region of −364 to −309 of the α-subunit gene. The promoter region of β-B-subunit gene did not contain GA clusters. Instead, there are many C residues and GC clusters throughout the 5′-flanking region as well as the coding region of β-B-subunit gene. It contains six pairs of GGGCGG repeats at the region of −118 to −81 upstream from translation initiation site. In addition, one pair of GGGCGG repeat at amino acid 48-50, and two pairs of invert repeated sequence CCGCCC at amino acid 39-40 and 41-42 were identified within the protein coding region of β-B-subunit precursor.

The potential hormone regulatory elements at the 5′-flanking region of α- and β-B-subunit gene were also sought. A putative cAMP-responsive element CTGCGTCAG was identified at the position of −196 to −188, which was 114-122 nucleotides upstream from

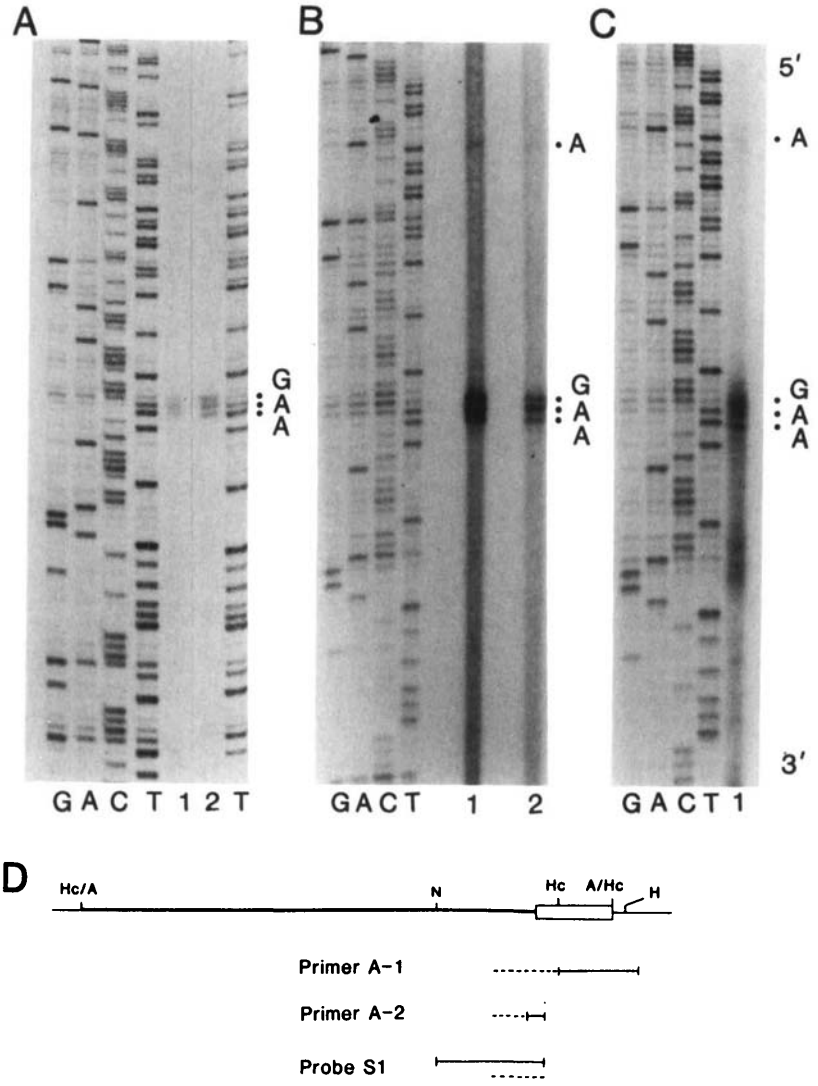


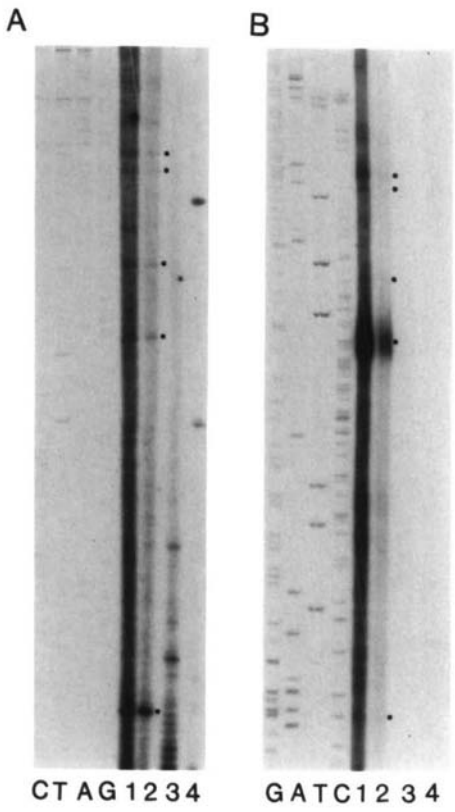
Fig. 5. Determination of the Transcription Start Sites of Rat α-Subunit Gene

Rat ovarian (5 μg, lane 1) and testicular (10 μg, lane 2) poly(A) RNA were hybridized with ^{32}P-labeled primer A-1 (A) or Primer A-2 (B), and the primer was extended with AMV reverse transcriptase as described in *Materials and Methods*. The extended products were analyzed on denaturing 5% polyacrylamide gels. In order to precisely map the start sites, DNA sequencing reactions were performed on the same α-subunit gene containing plasmid DNA using the same primers, and were electrophoresed in the adjacent lanes. The 5′-end of α-subunit mRNA was also determined by SI nuclease mapping (C). Five micrograms of rat ovarian poly(A) RNA was hybridized to Probe SI, the SI nuclease protected fragment was analyzed as described. The three major and one minor initiation sites are marked by *dots* and the nucleotide sequences for the start sites are indicated at the *right*. In A, under longer exposure the minor start site can also be detected at the same position as that in B. The structure of the probes used is illustrated in D. The α-subunit genomic fragment subcloned in pGEM-3Z plasmid was used as a template for preparing radioactive Primer A-1 and Probe S1. The coding region is indicated by an *open box* and the rest of the α-subunit gene fragment is by a *thick line*. The pGEM-3Z plasmid DNA is shown by a *thin line*. The Primer A-1 and A-2 are indicated by *solid lines* and the major extended products by these primers are shown by *broken lines*. Probe SI is indicated by a *solid line* and the major SI nuclease protected fragment is a *broken line beneath*. The restriction endonuclease cleavage sites in D are A, *Apa*I; H, *Hin*dIII; Hc, *Hin*cII; and N, *Nco*I.

the major transcription start sites of α-subunit gene (Fig. 2). No such element was present at the 5′-flanking region of the β-B-subunit gene.

Single α- and β-B-subunit Gene in Rat

The copy number of rat inhibin α- and β-B-subunit gene was analyzed in rat liver DNA by Southern blot analysis (Fig. 7). The rat liver DNA was digested with *Bam*HI, *Eco*RI, or *Hin*dIII. In all cases, only one band was hybridized to α- or β-B-subunit DNA probes. These observations suggest there are single copies of α- and β-B-subunit genes in the rat. One 5.5 kb *Eco*RI fragment (Fig. 7A, lane 2), and one 8.2 kb *Bam*HI fragment (Fig. 7B, lane 1) in rat liver DNA were found to contain α- and β-B-subunit gene, respectively. These observations



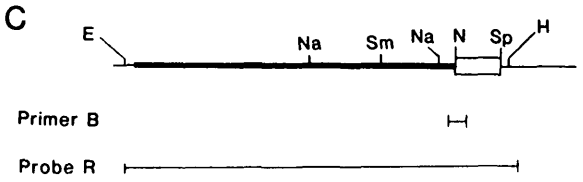


Fig. 6. Determination of the Transcription Start Sites of Rat β-B-Subunit Gene

Rat ovarian (5 μg, lane 1) and testicular (10 μg, lane 2) poly(A) RNA, and rabbit liver tRNA (10 μg, lane 3) and ribosomal RNA (10 μg, lane 4) were annealed with ^{32}P-labeled primer B and the primer was extended with reverse transcriptase (A), or hybridized with ^{32}P-labeled RNA probe R and the ribonuclease protected fragment analyzed (B). The extended products were analyzed by gel electrophoresis along with the DNA sequencing reactions in the adjacent lanes. Under longer exposure, identical results were detected in testicular and ovarian samples. The potential start sites of transcription of β-B-subunit gene are marked by *dots* and nucleotide sequences for the possible start sites are indicated in Fig. 4A. The structure of the primer B and probe R used is illustrated in C. The β-B-subunit genomic fragment subcloned in pGEM-3Z was used as a DNA template. The coding region of β-B-subunit is indicated by an *open box*, and the rest of the gene is by a *thick line*. The pGEM-3Z DNA is marked by a *thin line*. Primer B and probe R are shown by *solid lines*. The restriction endonuclease cleavage sites indicated in C are H, *Hin*dIII; N, *Nco*I; Na, *Nar*I; Sm, *Sma*I; and Sp, *Sph*I.

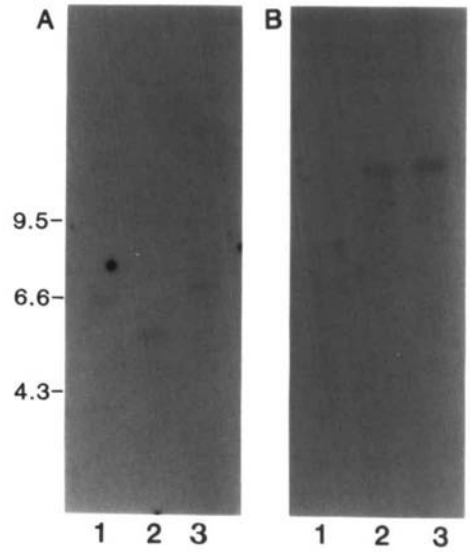


Fig. 7. Southern Blot Analysis of α- and β-B-Subunit Genes in Rat Liver DNA

Ten micrograms each of male rat liver DNA were digested with *Bam*HI (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3) restriction endonuclease, fractionated in 0.8% agarose gel, and transferred onto GeneScreen Plus membrane. The inhibin α-(A) and β-B- (B) subunit genes were identified by hybridization with human testicular cDNAs encoding α- and β-B-subunits, respectively. Similar observations were obtained when different portions of genomic fragments were used. λDNA digested with *Hin*dIII was used as size markers.

are in good agreement with the findings (Figs. 1 and 2) of the isolated genomic clones from rat genomic DNA library.

DISCUSSION

We have isolated and characterized inhibin α- and β-B-subunit genes of the rat. Both genes contain one intron interrupting the regions coding for the precursor portion of the subunits. Human inhibin subunit genes also contain only one intron (2 kb, and 2.8 kb) which interrupts at amino acid positions 89 and 149 of α- and β-B-subunit precursors, respectively (19, 21). Taken together with the available information of human inhibin genes and rat inhibin cDNAs (15, 16), the α- and β-B-subunit rat genomic fragments reported here contain most of the important information for the structure of these genes and their flanking regions.

The nucleotide sequences in the rat genomic fragment that corresponds to the mature mRNA for α-subunit, were identical to those from isolated cDNA of rat ovary (15, 16). The restriction endonuclease cleavage sites of the coding and part of 3′-untranslated regions of β-B-subunit gene were also identical between the isolated gene and the ovarian cDNA (15). However, completely different sequences were observed 1.7 kb

downstream from the translation termination site (Fig. 4B). The fact that this DNA could be used as a hybridization probe to identify inhibin β-B-subunit mRNA in rat ovary and testis (18, 22) suggests that this newly identified portion of the 3′-untranslated region of β-B-subunit gene codes for β-B-subunit mRNA.

We and others have reported two species of β-B-subunit mRNAs in rat testis (18, 22). Since there appears only one β-B-subunit gene in rat (Fig. 7) and the initiation sites for the transcription of this gene reside closely within 150 nucleotides (Fig. 6), the different size of β-B-subunit mRNA detected in rat testis may possible be the result of using different polyadenylation sites. Multiple polyadenylation signals (AATAAA) were found in the 3′-untranslated region of the isolated β-B-subunit gene (Fig. 4B). Further investigation of the 3′-end of the isolated β-B-subunit gene and of the mRNA will be necessary. Moreover, we cannot rule out the possibility that the heterogeneous β-B-subunit mRNA in rat testis is derived from altered splicing of nuclear precursor RNA to mRNA.

Neither inhibin α- nor β-B-subunit genes contain TATA element in the 5′-flanking region adjacent to the transcription start sites. The lack of obvious TATA promoter element has been found in several genes, including Müllerian inhibiting substance (23) and rat androgen-binding protein (ABP) which are also expressed in the Sertoli cells of the testis (24). The promoters for the genes lacking of TATA box have been divided into two classes. One class contains GC-rich promoters, which are found primarily in housekeeping and growth-related genes (25). These genes characteristically contain several transcription start sites spread over a fairly large region and several potential binding sites for the transcription factor Spl with the sequence GGGCGG (GC box) (26). The rat β-B-subunit gene promoter belongs to this class. The second class of promoters without TATA boxes is not GC rich. These promoters are usually not constitutively active but are regulated during differentiation or development. Many of these genes initiate transcription at one or a few tightly clustered sites (27). The α-subunit gene promoter belongs to this class of promoters.

The several pairs of GGGCGG and their inverted repeats are at the 5′-end as well as in the coding region of the rat β-B-subunit (Fig. 4A). Spl is believed to regulate the transcription of growth-control genes that contain the repeat GC box (28). These sequences are similar to the GC boxes in the 21-bp repeat region of the SV40 promoter and are recognized by transcription factor Spl (26). This factor enhances transcription by RNA polymerase II 10 to 50-fold from a select group of promoters that contains at least one properly positioned GC box. Spl binds to some, but not all, sequences that contain GC box and binding sites for Spl are functional in either orientation. Promoters that are Spl-responsive often contain multiple binding sites (29). The Spl-responsive promoters have been demonstrated in many genes including housekeeping genes, such as hypoxanthine guanine phosphoribosyltransferase (30), the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (31), as well as growth control genes such as c-Harvey ras gene (28), epidermal growth factor receptor (32), and nerve growth factor receptor (25) genes. Similar observation of high G+C content is found in the promoter region of the bovine MIS gene (23). Interestingly, the inhibin β-subunits also share marked homology at C-terminal protein domain with MIS (23), transforming growth factor-β (11, 33) and drosophila decapentaplegic peptide (34). These proteins also regulate cell growth. The similarities of the β-B-subunit to MIS of the presence of GC rich region at the 5′-end of the gene and the structure of C-terminal protein domain suggest that the β-B-subunit gene may also belong to a growth-related gene.

There appear many clusters of GA-rich regions at the promoter as well as near the transcription start sites of the α-subunit gene. The three tightly clustered start sites, GAA, were within one of the GGGAAG repeats. Two GAAGAA repeats were found at the promoter region where a usual TATA element would be expected. In addition, two pairs of GGGAGG repeats and their invert repeats CCTCCC were identified. The presence of GA clusters at the promoter region was reported in rat ABP gene in which one of the GA clusters is also located 31 nucleotides upstream from the transcription start site. Multiple GAGA sequence motifs were found in *Ultrabithorax* homeotic gene (35). The factor that binds to GAGA sequence in the promoter was shown to activate the transcription from this promoter. In human $T_3\delta$ gene, which does not contain a TATA element, the purine-rich region in the promoter was postulated to play a role in defining the start position of RNA polymerase II (36).

The ability of cAMP to regulate transcription has been reported in many systems. Different cAMP-responsive sequence elements have been identified in the promoters of these genes. A number of cAMP-inducible genes, including rat somatostatin (37), human vasoactive intestinal peptide (VIP) (38), and human gonadotropin-α (39–41), are induced by cAMP through the palindrome TGACGTCA sequence, which is considered as the core sequence of CRE. The second cAMP-responsive element was found in the promoter of human preproenkephalin gene (42). The transcription of this gene is activated in response to two different signal transduction pathways, one involving cAMP-dependent protein kinase A and the other involving the phorbol-ester and diacylglycerol-activated protein kinase C. The DNA sequence required for regulation by both cAMP and phorbol ester mapped to the same 37-base pair region located −107 to −71 upstream from the start site of preproenkephalin gene (42). This sequence element is shown to contain a sequence motif that is highly homologous to the transcription factor AP-1 consensus binding site (43, 44). This potential cAMP- and phorbol ester-responsive element CTGCGTCAG was also identified in the 5′-flanking region of inhibin α-subunit gene

(Fig. 3) as well as in c-*fos* gene (−298 to −290) (45). Testicular inhibin α-subunit mRNA levels were dramatically elevated by FSH treatment both in the hypophysectomized animals and in Sertoli cell primary cultures (17). This increase can be mimicked by forskolin, an adenyl cyclase activator, or cAMP (manuscript in preparation) (46). Therefore, cAMP may through protein kinase A pathway directly activate inhibin α-subunit gene transcription at the potential inducible DNA element in the 5′-flanking region. Whether the transcription of α-subunit gene is also regulated by phorbol esters through protein kinase C pathway is not clear. FSH was shown to induce the expression of proto-oncogene c-*fos* and *Jun*/AP-1 in Sertoli cell primary cultures via cAMP (46, 47). The expression of these genes by cAMP may be required for the expression of inhibin α-subunit gene modulated by binding of AP1/jun-fos complex to the inducible DNA sequence of the α-subunit gene. The possible mechanisms involved in the activation of α-subunit gene transcription are currently under investigation in our laboratory.

In conclusion, FSH markedly stimulated α- but not β-subunit gene expression in rat testis (17, 18). Similarly, PMSG caused an apparent increase in ovarian α-subunit mRNA levels but only a minimal effect on β-A-subunit mRNA (16, 48). These observations suggest that the expression of three inhibin subunit genes may be under differential control. Analysis of the 5′-flanking regions of α- and β-B-subunit genes indicates that different DNA sequences are present in the promoter regions, and probably different transcription factors are involved in the activation of transcription of these genes. In addition, our findings of potential cAMP- and phorbol ester-inducible DNA sequence in α- but not β-B-subunit gene suggest that different regulatory mechanisms involved in the basal and hormonally modulated expression of these two genes.

MATERIALS AND METHODS

Isolation of Inhibin Subunit Genes

Genomic DNA library constructed in λ phage Charon 4A Vector used for screening was from partially *Eco*RI digested rat liver DNA (Clontech Laboratories, Inc., Palo Alto, CA). Approximately 5×10^5 phage plaques were screened for inhibin α- and β-B-subunit genes using *in situ* plaque hybridization technique (49). The nitrocellulose filters containing phage DNA were first hybridized with radiolabeled human testicular α-subunit cDNA probe (αH67-2) (17). After obtaining the positive clones for α-subunit gene, the filters were reused in screening for β-B-subunit gene. Both β-BH190-1 and β-BH195-1 cDNA clones isolated from human testis (18) were ^{32}P-labeled by nick translation and used for identifying rat β-B-subunit gene. The positive clones for α- and β-B-subunit genes were isolated and subcloned into pGEM-3Z vector (Promega Biotech, Madison, WI). The structure of the isolated genes was initially determined by restriction endonuclease mapping and Southern blot analysis (50). Restriction endonuclease digested DNA was fractionated on 0.8 or 1.2% agarose gel and transferred to nitrocellulose filters. The DNA fragments containing α- or β-B-subunit gene were identified by hybridization with nick-translated DNA probes from different portions of the inhibin α- or β-B-subunit cDNAs.

DNA Sequencing Analysis

The regions of interest in these genes were further characterized by DNA sequencing analysis. The 5.5 kb *Eco*RI fragment of α-subunit gene was first digested with *Kpn*I, *Apa*I, or *Bgl*II restriction endonuclease, and the 8.2 kb *Bam*HI fragment of β-B-subunit gene was digested with *Sph*I and *Hin*cII enzymes. Various restriction endonuclease-digested fragments of α- or β-B-subunit genes were isolated and subcloned in pGEM-3Z vector. The nucleotide sequence was analyzed directly from the isolated plasmid DNA, after complete denaturation with alkali (51). The sequence was then determined by dideoxynucleotide chain-termination method (52) from both orientations using [α-^{35}S]dATP and SP6 or T7 RNA polymerase promoter primer. After primer annealing, the sequencing reactions were carried out using Sequenase (U.S. Biochemical Corp., Cleveland, OH) and fractionated on polyacrylamide gels (53). The nucleotide sequence for the coding region and the intron-exon junction of α-subunit gene, and part of 3′-untranslated region of β-B-subunit gene (*Pst*I-*Sph*I fragment) were analyzed directly from these genomic DNA subclones.

Some of the genomic fragments were characterized by nucleotide sequencing analysis of a collection of subclones which were constructed by unidirectional deletions of the genomic DNAs in pGEM-3Z plasmid by exonuclease III digestion using Erase-a-Base System (Promega Biotech, Madison, WI). The exonuclease III-digested DNA fragments were religated to pGEM-3Z vector and used to transform *Escherichia* coli cells. The deletion subclones were also obtained by exonuclease III digestion of the genomic DNAs from opposite orientation. The nucleotide sequence was analyzed by dideoxynucleotide chain-termination method of the isolated plasmid as described above. the *Apa*I fragment (0.9 kb) of α-subunit gene containing 5′-flanking region and part of the first exon (Fig. 1), the *Bam*HI-*Hin*cII fragment (2.8 kb) containing 5′-flanking region, first exon and part of the intron of β-B-subunit gene, and the *Hin*cII-SphI fragment (1.25 kb) containing part of the intron and the second exon of β-B-subunit gene (Fig. 2) were characterized using the latter procedure. All the nucleotide sequences presented were generated from the sequence analysis on both strands.

Primer Extension

The primer A-1, a single-stranded cDNA (138 nucleotides, Fig. 5D) including small portion of pGEM-3Z (46 nucleotides) and the sequence for amino acids from 13 to 44 (92 nucleotides), was prepared from *Apa*I fragment (0.9 kb) of the 5′-end of rat α-subunit gene. The *Apa*I fragment was subcloned into the *Hin*cII site of pGEM-3Z and was uniformly radiolabeled on the pGEM-3Z plasmid using SP6 primer. The plasmid DNA was denatured by alkali, and SP6 primer was annealed to the DNA at 37 C for 30 min. The synthesis of radioactive primer was carried out at 15 C for 30 min in the presence of 20 μM (final concentration) each dNTP (without dCTP), 100 μci [α-^{32}P] dCTP and Klenow enzyme. Chase solution containing 80 μM each dNTP in final concentration was then added and the reaction was continued for 40 min at the same temperature. After digestion with *Hin*cII, the labeled strand of the 138 nucleotide-*Hin*cII fragment was gel purified. Primer A-2, a 30 base-synthetic oligonucleotide, is complementary to the sequence of rat α-subunit gene at the position starting 15 bases upstream from translation initiation site to 15 bases of the coding region (Fig. 5D). Primer B, a synthetic 26 base-oligonucleotide, is complementary to the sequence of rat β-B-subunit gene including 13 bases upstream from translation initiation site and 13 bases of the protein coding region (Fig. 6C). These primers were prepared using a DNA synthesizer from Applied Biosystem (380B, Foster City, CA) and were radiolabeled at the 5′-end with [γ-^{32}P]ATP using bacterio-

phage T4 polynucleotide kinase (New England Biolabs, Boston, MA) followed by gel purification.

Total RNA from testis and ovary was isolated by urea-lithium chloride precipitation (54) and poly(A) RNA was obtained by oligo(dT)-cellulose column chromatography (55). The radiolabeled primers 3.5×10^6 cpm were hybridized with 2.5–10 μg poly(A) RNA isolated from rat testis or ovary in 10 μl 0.4 M NaCl, 40 mM PIPES (piperazine-*N*,*N*′-bis [2-ethanesulfonic acid]), pH 6.4, and 1 mM EDTA (56, 57) at 85 C for 10 min followed by immediate transfer to 50–60 C for 3 h. For A-2 primer, the reaction mixtures were diluted to 100 μl by addition of 10 μl 10× reverse transcriptase buffer (0.5 M Tris, pH 8.3, 0.1M dithiotheitol, 0.6 M $MgCl_2$), 5 μl 2.5 mM each dNTP (without dCTP), 100 μci [α-^{32}P] dCTP, 40 U RNasin (Promega Biotech) and 20 U AMV reverse transcriptase (Life Sciences, St. Petersburg, FL). The mixtures were incubated at 42 C for 15 min followed by addition of 5 μl 2.5 mM each dNTP (dCTP included). The chase reactions were carried out at 42 C for 1 h. For A-1 and B primers, the reactions were carried out as described above for A-2 primer except that [α-^{32}P]dCTP was omitted and only chase solution (5 μl 2.5 mM each dNTP) was added to the reverse transcriptase buffers. The primer-extended products were extracted by phenol/chloroform, and precipitated with ethanol. Using this procedure, radiolabeled products with high specific activity were obtained. The reaction products were analyzed on 5% or 6% polyacrylamide denaturing gels. DNA sequence reactions were obtained from the same characterized DNA fragments subcloned in pGEM-3Z using the same primers as described above and were electrophoresed in adjacent lanes as markers. The gels were dried and exposed to x-ray films.

S1 Nuclease Analysis

Mapping of the 5′-end of α-subunit gene mRNA was also carried out by S1 nuclease analysis (56). The radiolabeled cDNA probe for α-subunit gene was first synthesized from *ApaI*/*Hin*cII fragment (0.9 kb) containing the 5′-end of α-subunit gene (Fig. 5D) by using A-2 primer. After denaturation by alkali of the plasmid DNA containing α-subunit gene, the A-2 primer was annealed to the DNA at 37 C for 30 min. The radioactive cDNA was synthesized using the similar procedure described for nucleotide sequencing analysis or by primer extension. After digestion with *Nco*I, a single-stranded radiolabeled cDNA (Probe S1, 179 nucleotides) was purified by gel electrophoresis. The probe S1 (1.2×10^6 cpm) was coprecipitated with 5 μg rat ovarian or testicular poly(A) RNA. The hybridization was carried out in 10 μl 0.4 M NaCl and 50 mM PIPES (pH 6.4) and 1 mM EDTA at 85 C for 10 min followed by immediate transfer to 55 C for 5 h for probe S1. The reaction mixture was then diluted to 100 μl containing S1 nuclease buffer (40 mM potassium acetate, pH 4.6, 340 mM NaCl, 1.5 mM $ZnSO_4$, and 6% glycerol), 5 μg denatured salmon sperm DNA, and 100 U S1 nuclease (Promega Biotech) and was incubated at 34 C for 30 min. The protected DNA fragment was analyzed on a 5% polyacrylamide denaturing gel. DNA sequence reaction obtained from the *ApaI*/*Hin*cII fragment of α-subunit gene in pGEM-3Z plasmid by using A-2 primer was electrophoresed in adjacent lanes as markers. The gels were dried and exposed to x-ray films.

Ribonuclease Protection Assay

The radiolabeled RNA probe used for RNase mapping assay was synthesized by *in vitro* transcription method (18, 56). The β-B-subunit *Eco*RI-*Hin*dIII genomic fragment in pGEM-3Z which contains 62 nucleotides of coding region and 0.45 kb upstream from translation initiation site (ATG) (Fig. 6C) was used as a template. After digestion with *Eco*RI, the linearized plasmid DNA containing *Eco*RI-*Hin*dIII fragment of β-B-subunit gene was used to prepare radiolabeled RNA probe using [α-^{32}P]CTP and SP6 RNA polymerase. After removal of DNA template with DNase I, the radiolabeled RNA (Probe R, ~510 nucleotides) was extracted by phenol-chloroform and precipitated with ethanol. The Probe R (1×10^6 cpm) was coprecipitated with 2.5–5 μg rat ovarian or testicular poly(A) RNA. The hybridization was carried out in 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA at 85 C for 5 min followed by immediate transfer to 55 C for 12–16 h. The reaction mixture was diluted to 350 μl ribonuclease digestion buffer containing 40 μg/ml RNase A and 1600 U/ml RNase T1 and was incubated at 30 C for 45 min. The protected RNA fragment was then incubated with proteinase K (1.25 mg/ml), phenol/chloroform extracted, ethanol precipitated, and analyzed on a 6% polyacrylamide denaturing gel. DNA sequence reaction obtained from the same genomic fragment using SP6 primer was used to determine the size of the protected fragments. The gels were dried and exposed to x-ray film.

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