α-Inhibin Gene Expression Occurs in the Ovine Adrenal Cortex, and is Regulated by Adrenocorticotropin

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Inhibin is a glycoprotein hormone composed of two nonidentical subunits. It is produced by the ovary and testis and plays a vital role in gonadal function by inhibiting the secretion of FSH. More recently, additional activities associated with inhibin peptides have been identified. Inhibin heterodimers (α-β) are reported to act directly on ovarian granulosa cells and inhibit estrogen production induced by FSH. Furthermore, homodimers of β-inhibin subunits stimulate the secretion of FSH, an activity that is directly opposite to that of inhibin. Each of these inhibin-related activities are concerned with the hypothalamic-pituitary-gonadal axis. We have investigated further the complexity of inhibin activity by determining whether inhibin genes are expressed in nongonadal tissue. RNA hybridization experiments demonstrate that the α-inhibin gene is expressed in the sheep adrenal cortex and hybridization histochemistry shows that this gene is expressed in each of the functional zones within the cortex. Dot blot analysis showed that the level of αmRNA within the adrenal is influenced by ACTH, one of the major regulators of adrenal cortex function. These observations imply that there are inhibin-related peptides not directly associated with the gonads. β-Inhibin gene expression was not clearly detected in the adrenal and we conclude that if expression occurs then it does so at extremely low levels. (Molecular Endocrinology 1: 699–706, 1987)

INTRODUCTION

Inhibin, first identified in testicular extracts (1–3) and more recently in follicular fluid (4–6) is a peptide that specifically inhibits the secretion of FSH from the anterior pituitary. Its recent purification (7–9) has led to elucidation of its complete structure by recombinant DNA technology (10–12).

Both the structure and activities of inhibin are more complex than originally apparent. First, two forms (A and B) of inhibin were identified (10, 11). Each form is composed of two nonidentical subunits (termed α and β) linked by disulfide bridges. The α-subunits of A and B forms are identical while the βA and βB subunits differ. The β-subunits of inhibin are related by sequence homology to transforming growth factor β (10) and Mullerian-inhibitory substance (13), both of which are homodimeric peptides. Recently dimers of β inhibin subunits (βA-βA and βA-βB) were isolated from follicular fluid (14, 15), further extending the structural relationship between these peptides. Second, β-inhibin dimers were found to act as potent stimulators of FSH secretion (14, 15), in a manner similar to that exerted by transforming growth factor β (16). This activity is the direct opposite to that of the inhibin heterodimers (α-β), although its physiological significance is not clear. For example, release of β-inhibin dimers from the gonads into the peripheral circulation has yet to be reported. Recently, inhibins were shown also to act directly on rat granulosa cells and modulate estrogen secretion mediated by FSH (17), indicating that inhibin may be capable of exerting local effects, in addition to its well recognized inhibition of the gonadotroph cells in the pituitary gland.

Our studies are based on a long-standing interest in ovine reproductive physiology and a continuing interest in ovine inhibin (18). We were prompted by the complexity of inhibin peptides and their associated activities to determine whether inhibin genes are expressed in tissues other than the gonads. We report that the α-gene is expressed in the adrenal cortex and that the level of αmRNA within the adrenal is modulated by ACTH.

RESULTS

Southern Analysis of α- and β-Inhibin Genes in the Sheep Genome

The observation that two related β-inhibin genes are expressed in porcine and human ovaries (10, 12) raises the possibility that the mammalian genome may pos-

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sess larger α- or β-inhibin gene families. We have investigated this by using probes encoding α and β_A inhibin subunits in Southern analysis of ovine genomic DNA.

Stringent hybridization of *Eco*RI, *Bam*HI, or *Hind*III-cleaved sheep genomic DNA with the cloned αcDNA, gave single bands at 13.5, 15, and 6.4 kilobases (kb), respectively (Fig. 1A). Lowering the stringency of hybridization or washing gave a much higher background with no extra distinct bands visible. The same result was obtained when the overlapping α oligodeoxyribonucleotides were used as a probe.

In the case of β-inhibin genes, the ovine β_A cDNA probe also gave single bands at 7.2, 12, and 3.8 kb, after digestion of genomic DNA with *Eco*RI, *Bam*HI, and *Hind*III, respectively (Fig. 1B). As shown in lane 4, this result was not altered by a reduction in the stringency of washing. We obtained a different result, however, using the overlapping β_A oligodeoxyribonucleotides which cover a region of maximum homology between the porcine or human β_A and β_B genes (10, 12). For *Eco*RI-digested sheep DNA (Fig. 1B, lane 5), we obtained the strong band at 7.2 kb corresponding to the β_A gene, moderate bands at 18 and 2.9 kb, and additional weak bands at 6.6, 5.2, 4.8 and 4.2 kb. We suggest that either the 18 kb or the 2.9-kb fragment corresponds to the β_B gene. It is possible that the remaining bands represent unidentified coding sequences distantly related to β_A and β_B genes. Several hybridizing bands were also observed when this probe was used in similar experiments with human, mouse, and porcine genomic DNA (unpublished results).

In summary, these Southern experiments indicate that the ovine genome contains a single α-inhibin gene and provide initial evidence for a family of β-inhibin related genes.

Inhibin Gene Expression in the Adrenal Cortex

The possibility that inhibin-related peptides might exert physiological activities beyond the hypothalamic-pituitary-gonadal axis prompted us to investigate whether inhibin genes are expressed in tissues other than the gonads. Accordingly, Northern analysis was used to probe poly(A) RNA from a range of ovine tissues with ^{32}P-labeled sheep α and β_A inhibin cDNAs.

Inhibin mRNA sequences were readily detected in sheep gonadal tissue. The α-inhibin probe hybridized to a 1.5-kb mRNA species in ovarian and testicular RNA (Fig. 2) identical in size to porcine α-inhibin mRNA (10). The β_A probe also hybridized to ovary and testis poly(A) RNA, in both Northern (data not shown), and dot blot experiments (Fig. 3).

Hybridization of nongonadal poly(A) RNA with α-inhibin cDNA demonstrated clearly that the α-gene is expressed in ovine adrenals during several different stages of development. One and a half kilobase α-inhibin mRNA was detected by Northern analysis in

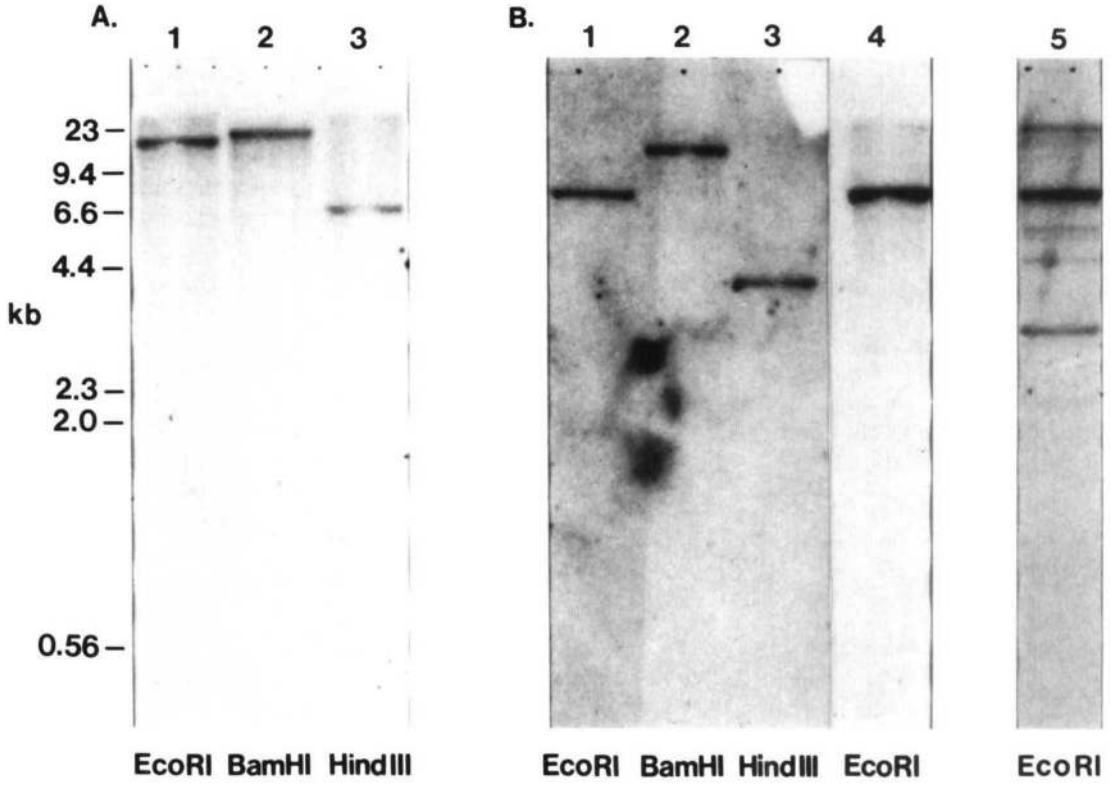

Fig. 1. Hybridization of Ovine DNA with ^{32}P-Labeled Inhibin Probes

Ovine DNA was isolated from liver, digested with restriction enzymes (15 μg/lane), transfered to nitrocellulose (28), and hybridized with ^{32}P-labeled inhibin probes. Lanes 1–3 in A and B were washed at 60 C in 0.075 M NaCl-7.5 mM Na Citrate. Lanes 4 and 5 (B) were washed at 60 C in 0.3 M NaCl-30 mM Na Citrate. A, Ovine α-inhibin cDNA. B, Lanes 1–4, ovine β_A inhibin cDNA; 5, synthetic porcine β_A inhibin oligodeoxyribonucleotide.

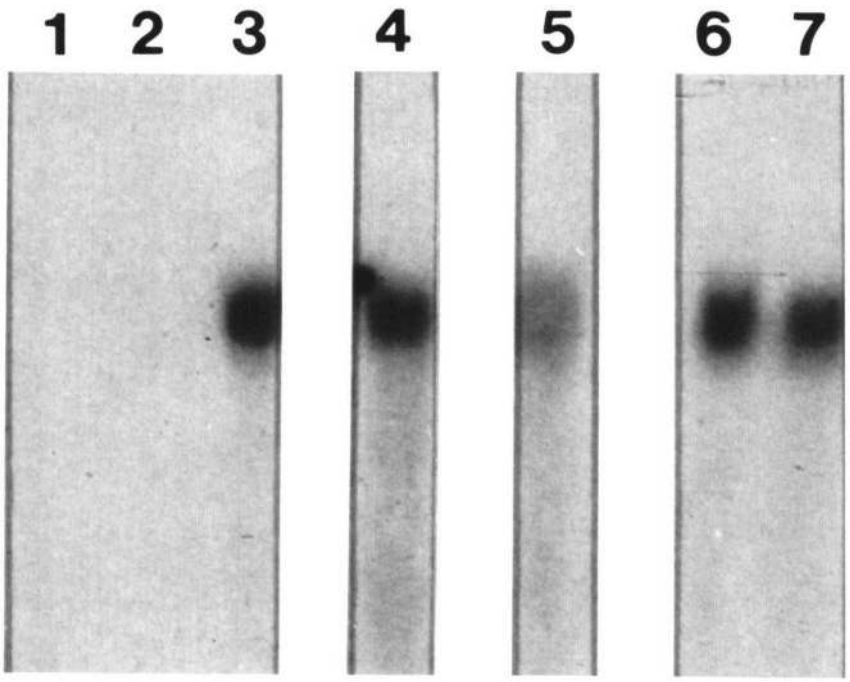

Fig. 2. α-Inhibin mRNA Sequences in Ovine Poly(A) RNA

Poly(A) RNA from several ovine tissues were probed with ^{32}P-labeled ovine α inhibin cDNA using Northern transfer techniques (26) Lanes: 1, kidney; 2, pituitary; 3, ovary; 4, testis; 5, adult adrenal; 6, adrenal from 4 day old lamb; 7, adrenal from fetal lamb (105 days post fertilization).

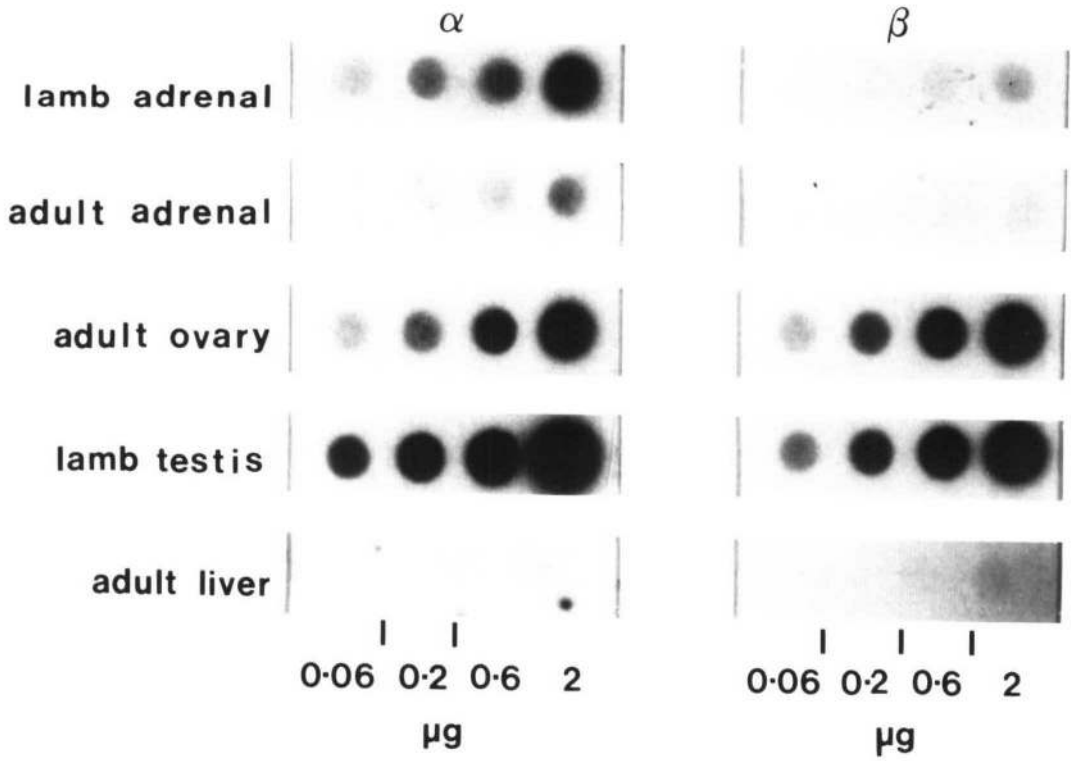

Fig. 3. Dot Blot Analysis of Inhibin mRNA Levels in Ovine Gonadal and Adrenal Tissues

Poly(A) samples were serially diluted 3-fold, bound to nitrocellulose, probed with ovine β_A inhibin cDNA, and exposed to x-ray film. The filter was then washed free of β_A probe and subsequently hybridized with ovine α-inhibin cDNA. Signal intensities indicate the relative levels of α or β_A inhibin mRNA in each tissue.

poly(A) RNA from adult and lamb (1 to 5 days old) as well as from fetal adrenals (105 days post fertilization) (Fig. 2, lanes 5, 6, and 7). Subsequently, several αcDNA clones were isolated from an adrenal cDNA library, confirming that α-inhibin gene expression occurs in this tissue. The nucleotide sequence of the adrenal cDNA clones was identical to the ovarian α cDNA sequence (manuscript in preparation).

Hybridizations with β_A cDNA suggested that if the adrenal contains β_A mRNA, then its abundance is very low. No hybridization was observed with RNA from adult adrenal (Fig. 3). We were also unable to detect β_A cDNA clones in both adult and lamb adrenal cDNA libraries. Similarly no hybridization between a synthetic porcine β_B probe and adrenal RNA was observed in dot blot experiments (data not shown).

RNA from ovine kidney, pituitary (Fig. 2), and liver (Fig. 3) did not hybridize with α-inhibin cDNAs, indicating that α-inhibin genes are not expressed at detectable levels in these tissues.

The relative levels of a mRNA in adult and lamb adrenal were determined by comparing autoradiograph intensities resulting from dot blot hybridizations. This analysis indicated that the level of αmRNA in lamb adrenal poly(A) RNA is about 9-fold greater than in adult adrenal and that the concentrations of αmRNA in lamb adrenal and adult ovary are similar (Fig. 3). Additional dot blot experiments did not detect any difference in adrenal αmRNA levels between male and female sheep (data not shown).

Subsequent Northern experiments were carried out to determine whether adrenal α-inhibin mRNA is synthesized in the cortex (the site of steroid biosynthesis) or the medulla (the region of catecholamine synthesis). Adrenal cortex and medulla were separated by surgical dissection and poly(A) RNA was isolated and probed with α cDNA. α-Inhibin mRNA was clearly detected in RNA from the adrenal cortex (Fig. 4). Low levels of αmRNA (about 200-fold less than in the cortex) were also detected in the medulla RNA preparation (Fig. 4). We believe that this hybridization results from contamination of medulla tissue with cortex cells during surgical dissection although low level α-inhibin gene expression within the medulla cannot be ruled out.

Hybridization histochemistry was used to locate precisely the sites of α-inhibin gene expression within the adrenal cortex. Lamb adrenal sections rather than adult tissue were hybridized with ^{32}P-labeled α-inhibin oligodeoxyribonucleotide because of their higher concentration of αmRNA sequences (Fig. 5). These experiments indicated that cells containing α-inhibin mRNA were located in the zona glomerulosa, zona fasciculata, and reticularis. Hence, α-inhibin gene expression occurs throughout the cortex and is not concentrated in small isolated regions, although the higher hybridization intensity within the zona glomerulosa suggests that the concentration of αmRNA is greater in this region.

α-Inhibin mRNA Levels in the Adrenal are Influenced by ACTH

The anterior pituitary hormone ACTH is one of the major regulators of adrenal cortex function and growth. It acutely regulates steroid biosynthesis and secretion through transport and metabolic mechanisms (19) and its chronic action involves regulating genes encoding steroidogenic enzymes (20, 21). We have investigated whether adrenal inhibin gene expression is also regulated by ACTH.

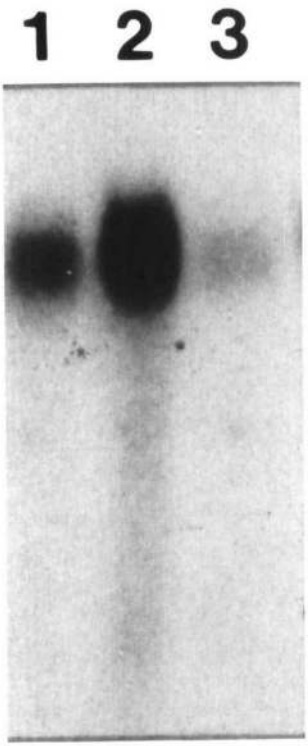

Fig. 4. α-Inhibin mRNA is Located in the Adrenal Cortex
Ovine adrenals were isolated and the cortex separated from the medulla by surgical dissection. Poly(A) RNA was prepared from each tissue fraction and probed with ^{32}P-labeled α-inhibin cDNA (26). Lanes: 1, whole adrenal; 2, adrenal cortex; 3, adrenal medulla.

ACTH was administered daily to two adult ewes for 2 days. This short-term treatment enabled the adrenals to be obtained before ACTH-induced adrenal growth occurred. Dexamethasone was administered to three additional ewes to determine whether suppression of ACTH secretion also influenced α-inhibin gene expression n the adrenal.

Poly(A) RNA was prepared from adrenals exposed to ACTH or dexamethasone treatment and the relative abundances of inhibin mRNAs were determined by dot blot analysis.

No β_A hybridization was observed with RNA from ACTH or dexamethasone-treated adrenals (data not shown).

The level of α-inhibin mRNA in adrenals from ACTH-treated sheep was about 4-fold greater than in normal animals and about 8-fold greater than in sheep treated with dexamethasone (Fig. 6, Table 1).

DISCUSSION

Although properties associated with inhibin peptides are more diverse than first apparent, all activities reported so far are associated the feedback regulation of the gonads and pituitary gonadotrophs (1–6, 14, 15, 17). Our aim was to establish whether inhibin genes are expressed in other tissues not directly associated with gonadal function.

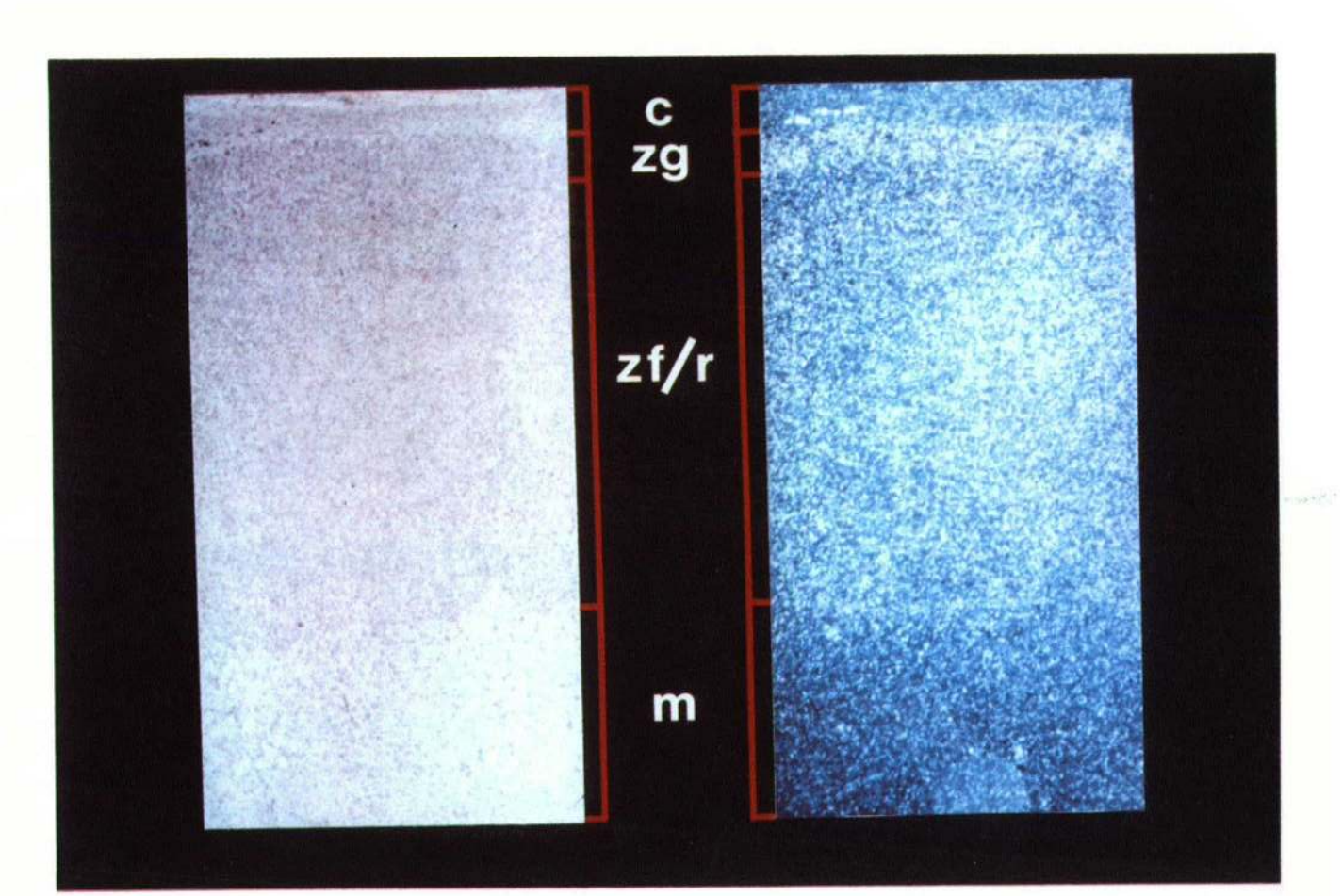

Fig. 5. Distribution of α-Inhibin mRNA within Lamb Adrenal

Sections of lamb adrenal were prepared as described (27). *Left panel*, Bright field micrograph of lamb adrenal detailing adrenal histology. The adrenal slice was stained with hematoxylin and eosin. *Right panel*, darkfield micrograph of same adrenal section after hybridization (27) with a ^{32}P-labeled synthetic porcine α-inhibin oligodeoxyribonucleotide and liquid emulsion autoradiography. c, Capsule; Zg; zona glomerulosa; Zf/r, zona fasciculata and zona reticularis; m, medulla. Bar represents 0.2 mm.

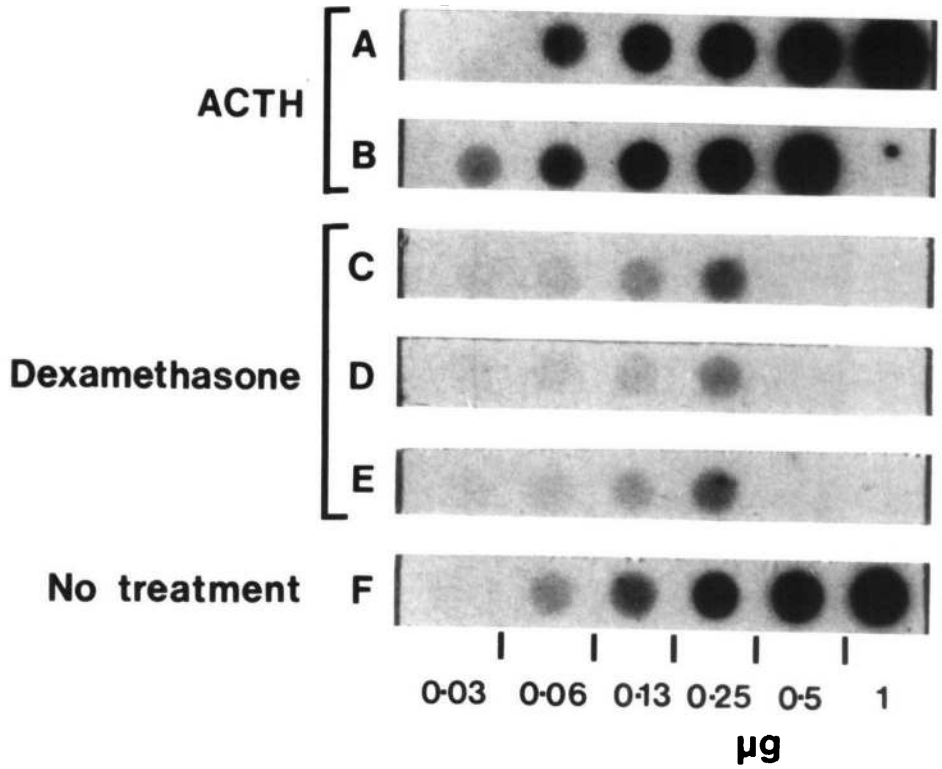

Fig. 6. Effect of ACTH and Dexamethasone on α-Inhibin mRNA Levels in Ovine Adrenals

Adult ewes were injected im with ACTH (25 μg/kg·day for 2 days) or dexamethasone (8 mgm/day for 7 days) and then killed. Adrenal poly(A) RNA was isolated, serially diluted 2-fold, bound to nitrocellulose (26), probed with ^{32}P-labeled α-inhibin cDNA, and exposed to x-ray film. Signal intensities indicate the relative levels of α-inhibin mRNA. Each panel shows α-inhibin mRNA levels in adrenals from separate sheep. No RNA from dexamethasone-treated sheep was loaded in the 1-μg and 0.5-μg panels.

Table 1. Relative αmRNA Levels in Adrenals from ACTH or Dexamethasone-Treated Sheep

Treatment[a]	Relative Level of α-Inhibin mRNA[b]
ACTH (n = 2)	8
Dexamethasone (n = 3)	1
No treatment (n = 1)	2

[a] ACTH or dexamethasone was administered as described in legend to Fig. 6.

[b] α-Inhibin mRNA levels were estimated from dot blot intensities shown in Fig. 6.

Inhibin-Related Sequences in the Ovine Genome

The presence of several genes within the sheep genome hybridizing to inhibin probes provides initial evidence for a family of inhibin-related peptides. Southern analysis of the sheep genome detected single major α and β_A DNA fragments providing evidence for one α and β_A gene. Additional sequences hybridizing weakly to a synthetic β_A probe were also evident suggesting there are about six other β_A-related genes within the ovine genome. One of these additional sequences is likely to be the ovine β_B gene. The precise nature of the other β-related sequences, and whether they are expressed has not yet been investigated. It is unlikely that they encode transforming growth factor β or Mullerian-inhibitory substance because the synthetic probe used in these hybridizations contains low sequence homology to these genes.

Expression of the α-Inhibin Gene in the Adrenal

Expression of the α-inhibin gene was detected clearly in the adrenal cortex by Northern analysis, hybridization histochemistry, and cDNA cloning experiments. These results imply that inhibin-related peptides are synthesized in the adrenal cortex and that their physiological influence is outside the hypothalamic-pituitary-gonadal axis.

The precise structure of adrenal inhibin peptides could not be deduced from these experiments because no clear evidence for adrenal β-inhibin gene expression was obtained. No β_A hybridization was detected in adult adrenal RNA although low level β_A hybridization was observed with RNA from lamb adrenal. If β_A mRNA is present in the adrenal, then its abundance is at least 50 times lower than in the ovary. Similarly, no adrenal β_B gene expression was detected. In contrast αmRNA levels in lamb adrenals are comparable to ovarian levels, providing the potential within the adrenal for the synthesis and secretion of α-subunits unlinked to β-chains. Alternatively, adrenal α-subunits may link with the product of a novel β-related subunit gene not detected by the β-probes. Purification of adrenal inhibin peptides will distinguish between these possibilities. α inhibin gene expression also occurs in the human placenta (22) but hybridization with β-probes was not reported. In the ovine placenta, α-subunits are most likely to associate with β_A subunits as we have detected both α and

β_A mRNAs in placental poly(A) RNA by Northern and dot blot analysis (unpublished data).

We have described several features of α-inhibin gene expression within the adrenal cortex that provide clues to the function of adrenal inhibin-like peptides. α-Inhibin mRNA is not localized to a discrete region of the cortex but occurs in each zone. This wide distribution throughout the adrenal cortex suggests that adrenal inhibin-like peptides might play a general role in adrenal cortex function and are not concerned with unique zonal activities. A general role is further suggested by the occurrence of adrenal α-gene expression during several stages of development from fetal through to adult life and a similar abundance of αmRNA in adrenals from male and female sheep.

It is intriguing that adrenal α-inhibin gene expression is stimulated by ACTH. Whether this response is direct or is due to elevated corticosteroid levels induced by ACTH action is not certain, although suppression of α-gene expression by dexamethasone treatment suggests a direct ACTH influence on α-inhibin mRNA levels. ACTH is a major regulator of steroid biosynthesis in the adrenal cortex and its long term (chronic) activity is characterized by an increase in the expression of genes coding for steroidogenic enzymes. It will be interesting to determine whether ACTH influences the expression of steroidogenic enzyme genes and the α-inhibin gene by a common mechanism.

The specific role of adrenal α-inhibin gene expression is not apparent, although it is possible that it influences corticosteroid production or secretion. The observation that α-inhibin mRNA levels are regulated by ACTH is consistent with this proposal. In this context it is worth noting that inhibin mRNAs have been detected in all major steroidogenic organs, including the ovary, testis, placenta (22), and adrenal cortex. Furthermore, inhibins influence steroid production in the ovary; they regulate FSH-stimulated estrogen production by inhibiting FSH secretion from the pituitary (see Ref. 6) as well as acting locally to inhibit FSH action (17). Whether inhibin-related peptides from the adrenal also influence local aspects of steroid production remains to be determined, although any effect is likely to be general rather than specific to mineralocorticoid, glucocorticoid, or androgen production because α-inhibin mRNA was detected in each zone. Purification of inhibin-like peptides from the adrenal will be important in determining their precise structure and physiological role.

MATERIALS AND METHODS

Synthesis of Oligodeoxyribonucleotides and ^{32}P-Labeling of Probes

Porcine α and β_A inhibin probes were synthesized by the solid-phase phosphoramidite procedure (23) and purified by preparative gel electrophoresis. Probes were designed to overlap by about 10 nucleotides leaving single-stranded 5′-extensions of about 70–75 nucleotides. labeling was carried out in end-fill reactions using [α^{32}P]dATP, [α^{32}P]dCTP, and Klenow fragment from *Escherichia coli* DNA polymerase I. The α-porcine probe covered the region from nucleotide 1017 to 1221 (10) and the β_A probe extended from nucleotide 1190 to 1303 (10). This region of porcine β_A cDNA sequence was chosen to maximize the possibility of cross-hybridization with β_B gene sequences.

Ovine inhibin cDNA inserts were ^{32}P-labeled using random primers (24). Ovine inhibin cDNAs were isolated from an ovarian cDNA library constructed in λgt10 vector. Both the αcDNA (about 450 base pairs) and the cDNA β_A cDNA (about 600 base pairs) included sequences encoding the 20 kilodalton α-subunit and the 15 kilodaltons β_A subunit (manuscript in preparation).

Analysis of Ovine RNA and Genomic DNA

Poly(A) RNA was isolated from ovine tissue using guanidinium thiocyanate (25) and oligo(dT) cellulose chromatography. Inhibin mRNA was detected by Northern analysis (26) after electrophoresis on formaldehyde gels or by dot blot techniques using serially diluted RNA samples. Relative inhibin mRNA concentrations were estimated from signal intensities of dot blot hybridizations. These intensities were normalized to total poly(A) RNA levels determined by similar hybridizations between ^{32}P-labeled oligodeoxythymidine and serially diluted dots containing 1–30 ng RNA. Inhibin genes in ovine genomic DNA were detected by Southern transfer analysis (27)

Hybridization Histochemistry

Sections of ovine adrenals (~5-μm thick) were probed with the ^{32}P-labeled synthetic porcine α-inhibin oligodeoxyribonucleotide and exposed to liquid emulsion autoradiography as described (28).

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