Molecular Cloning of cDNA for Equine Ovarian Inhibin/Activin β A Subunit

Shintarou YOSHIDA, Keitaro YAMANOUCHI, Telhisa HASEGAWA¹⁾, Akihiro IKEDA, Masatoshi SUZUKI, Kyu-Tae CHANG, Shigemi MATSUYAMA, Masugi NISHIHARA, and Michio TAKAHASHI*

Department of Veterinary Physiology, Veterinary Medical Science, The University of Tokyo, Tokyo 113 and ¹⁾Laboratory of Molecular and Cellular Biology, Equine Research Institute, Japan Racing Association, Tokyo 154, Japan

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ABSTRACT. cDNAs encoding equine inhibin/activin β A subunit were isolated from an equine follicle cDNA library and characterized. Using primers based on the rat inhibin/activin β A subunit cDNA sequence, a RT-PCR was performed to generate the probe for screening. Four positive clones were isolated. Analysis of the nucleotide sequence of these clones revealed that two pairs of identical clones were present, Eq- β A-1 (0.9 kb) and Eq- β A-2 (1.5 kb). Eq- β A-2 clone contained a complete open reading frame encoding 426 amino acids. The deduced amino acid sequence of equine inhibin/activin β A subunit showed high similarity (>90%) to those of five other mammalian species. Northern blot analysis revealed that placenta from mare on day 180 of pregnancy contained a 1.5 kb inhibin/activin β A subunit mRNA.—**KEY WORDS:** activin, cDNA cloning, equine, inhibin, ovary.

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Inhibin and activin were originally discovered as polypeptides that are capable of suppressing and enhancing follicle stimulating hormone (FSH) release from the anterior pituitary [2], respectively. Inhibin is a heterodimer linked by disulfide bonds, consisting of α and β subunits (β A or β B), while activin is a homodimer of β subunits, known as activin A (β A β A), activin AB (β A β B) and activin B (β B β B) [19]. Recent evidence indicates that inhibin/activin are members of the transforming growth factor- β (TGF- β) family [19] and have many biological functions such as erythroid cell differentiation [4], regulation of embryonic development in mice [8–10], induction of axial mesoderm in *Xenopus* [18] and gonadotroph differentiation [7]. Recently, immunoreactive inhibin levels in the plasma and follicular fluid of mares were measured by means of double antibody RIA [15] and bioactive equine inhibin was characterized for the first time [13]. In addition, it was shown that equine granulosa-theca cell tumors express inhibin α - and β A-subunit mRNAs and their proteins [14]. To further understand inhibin/activin gene expression in the mare, it is of interest to isolate and characterize cDNA clones encoding each subunit of equine inhibin/activin. Because the β A subunit is a polypeptide that composes both inhibin and activin molecules, we attempted initially to isolate a cDNA encoding inhibin/activin β A subunit from an equine follicle cDNA library.

MATERIALS AND METHODS

Animals and tissue preparation: Ovaries were removed from one thoroughbred mare (non-pregnant) at anestrus under anesthesia. Only follicles were collected and frozen in liquid nitrogen. Placenta was collected from one

pregnant thoroughbred mare under anesthesia on day 180 of pregnancy. Total RNA was extracted using the cesium chloride ultracentrifugation method [16].

Synthesis of DNA probe for screening of equine inhibin β A subunit: A DNA probe for screening of equine inhibin β A subunit was prepared by RT-PCR using total RNA extracted from mare follicles as a template. Primers were designed [10] based on the sequence reported previously [3], and these sequences have no homology to other members of the TGF- β family. The forward primer, 5'-GGTCAACATTTGCTGTAAG-3' (rat 1047–1065, 19 bp) and the reverse primer, 3'-GGAAACGGTTGGAA-TTC-5' (rat 1244–1260, 17 bp), were synthesized with a DNA synthesizer Cyclone Plus (Milligen Bioresarch) apparatus. Reverse transcription of RNA to cDNA was carried out with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. Subsequent PCR was performed in a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Cetus Instruments) for 50 cycles at 94°C for 2 min, 55°C for 2 min and 72°C for 2 min. The final volume of 100 μ l contained the cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 200 μ M dNTP, each primer at 0.15 μ M and 2.5 units of *ampli*Taq^R DNA polymerase. A PCR product of approximately 200 bp was obtained, and this was labelled with [α -³²P]dCTP (3000 Ci/mmol, Amersham) by random primers.

Construction of cDNA library: Poly(A)⁺RNA was selected with Dynabeads Oligo (dT)₂₅ (Dyna). Five micrograms of poly(A)⁺RNA was dissolved in water. First and second strand cDNA were synthesized by Amersham cDNA Synthesis System Plus (Amersham). Double stranded cDNA was ligated with EcoRI-NotI-BamHI adapter (Takara) and then with EcoRI predigested lambdaZapII phage DNA arms (Stratagene) after 5'-phosphorylation (T4 polynucleotide kinase, Boehringer Mannheim). Ligated DNA was packaged into phage particle by Gigapack II Gold Packaging Extract (Stratagene).

* CORRESPONDENCE TO: TAKAHASHI, M., Department of Veterinary Physiology, Veterinary Medical Science, The University of Tokyo, Tokyo 113, Japan.

Screening of lambda-phage library: 15×10^5 recombinants were obtained, and approximately 3×10^5 plaques of the library were plated and transferred to nylon filters (Hybond-N; Amersham). They were hybridized with the [α - 32 P]dCTP-labelled probe for 20 hr at 42°C in hybridization mix ($5 \times$ SSC, $2.5 \times$ Denhardt's reagent, 5 mM EDTA, 0.1% SDS, 10% dextran sulfate, 100 μ g/ml salmon sperm DNA). After washing with $0.5 \times$ SSC/0.1% SDS for 30 min at 65°C once and $0.1 \times$ SSC/0.1% SDS for 30 min at 65°C twice, the filters were dried and exposed to X-ray film at -80°C. Four positive clones were obtained, and inserted cDNAs were subcloned into EcoRI-digested pUC118 for sequencing.

DNA sequencing and sequence analysis: Sequencing was performed by the dideoxy chain-termination method using an AutoRead sequencing kit and A.L.F. DNA sequences (Pharmacia Biotech). The sequences were obtained for both strands with all restriction sites overlapped. Sequence analysis was performed with MacMollyTetra computer software (Soft Gene).

Northern blot analysis: Five micrograms of poly(A)⁺RNA extracted from mare placenta obtained on day 180 of pregnancy was separated by electrophoresis in 1.0% agarose MOPS-formamide-formaldehyde gels [16], and transferred to nylon membrane (BIODYNE, Pall Biosupport) and hybridized using [α - 32 P]dCTP-labelled cDNA fragment from Eq- β A-2 (see RESULTS AND DISCUSSION) as a probe.

RESULTS AND DISCUSSION

RT-PCR was performed to detect mRNA of inhibin/activin β A subunit in mare follicles. Primers in the present study were designed according to the sequence of rat inhibin/activin β A subunit cDNA [3]. When rat cDNA was used as a template, the predicted size of PCR product was 214 bp, and this amplified sequence also was expected to be conserved in the equine. In the present study, approximately 200 bp of a single PCR product was

Fig. 1. Structure and sequencing strategies for equine inhibin/activin β A subunit cDNA clones. Four restriction enzyme sites are shown. The dark box indicates the open reading frame. Each clone was sequenced according to the strategies shown.

obtained as expected when cDNA from equine follicles was used. This PCR product was labelled with [α - 32 P]dCTP and used as a probe for screening of equine inhibin/activin β A subunit cDNA.

Out of 3×10^5 plaques screened, four positive clones were obtained, and each insert from these four clones was subcloned into pUC118 vector. EcoRI-digestion of the recombinant plasmid released approximately 0.9 kb inserts from two clones and 1.5 kb inserts from the other two clones. Sequencing of these four clones demonstrated that each pair of clones (0.9 kb and 1.5 kb) was identical, namely Eq- β A-1 (0.9 kb) and Eq- β A-2 (1.5 kb).

A partial restriction enzyme map and sequencing strategy are shown in Fig. 1. Eq- β A-2 cDNA contained a complete open reading frame initiated from ATG and terminating at TAG but lacked 46 bp of the 3' untranslated region. On the other hand, Eq- β A-1 cDNA lacked

1	GGGGGGGAGGAAAGCAGGCGCTTTTAAAGGCAATCACAACCACTTTGCTGCC
57	AGG ATG CCC TTG CTT TGG CTG AGA GGA TTT TTG TGG GCG AGT TGC TGG ATT ATA GTG AAG
117	AGT TCC CCC ACC CCA GGA TCC GAG GGG CAC AGC GCA GCC CCC AAC TGC CCG TCC TGT GCG
177	CTG GCC ACC CTC CCA AAG GAT GTA CCC AAT GCT CAG CCG GAG ATG GTG GAG GCC GTC AAG
237	AAG CAC ATT TTA AAC ATC CTG CAC TTG AAG AAG AGA CCC GAT GTC ACC CAG CCT GTG CCC
297	AAG GCG GCG CTT CTG AAC GCT ATC AGA AAG CTT CAT CTC GGC AAA GTG GGG GAG AAC GGG
357	TAT CTG GAG ATA GAG GAT GAC ATC GGC AGG AGG GCA GAA ATG AAT GAA CTT ATG GAG CAG
417	ACC TCA GAG ATC ATC ATC TTC GCG GAA TCA GCG ACA GCC AGG AAG ACG CTG CAC TTT GAG
477	ATT TCC AAA GAA GCG AGT GAC CTG GTG GTG GAG GGT GCA GAA GTC TGG CTC TTC CTC
537	AAA CTC CCC AAG GCC AAC AGG ACC AGG TCC AAA GTC ACC ATC GGT CTC TTG CAG CAG CAG
597	AAG CAC CCG CAG GCG AGC TCG GAC AGC AGG GAG GAG CCG GAG GAA CCG GAC TTA ATC CAG
657	GAG AGG AGT GAA CAG TTG ATA TCG GAG AAG GTG GTG GAT GCT CCG AAG ACC ACC TGG CAC
717	ATC TTC CCT GTC TCC AGC AGC ATC CAG CCG TTG CTG CAG GAG GCG AAG ACC TCC CTG GAT
777	ATT CGG ATT GCC TGT GAT CAG TGT CAT GAG ACC GGT GCC ACC CTT GTC CTC CTG GGG AAG
837	AAG AAG AAG AAG GAA GAG CAG GCG GAG GGG AAG AAG AAG GAG GGA GAA GCA GGG GCA
897	GGG GTT GAG GAG AAG GAG CAG TCC CAC AGA CCT TTC CTC ATG CTG CAG GCC AGG CAG
957	TCT GAA GAT CAC CCT CAT CGC CGG AGG CGG CGC GGC CTG GAG TGT GAC GGC AAG GTC AAC
1017	ATC TGC TGT AAG AAA CAG TTC TTT GTT AGT TTC AAG CAG ATT GGC TGG AAT GAC TGG ATC
1077	ATC GCT CCC TCT GGC TAC CAC GCC AAC TAC TGC GAG GGT GAG TGC CCA AGC CAT ATA GCA
1137	GCC ACC TCG GGG TCT CTG CTG TCC TTT CAC TCC ACG GTC ATC AAC CAA TAC CCG TTG CCG
1197	GTT CAT AAC CCC TTC GCC AAC CTC AAG TCG TCG TGT GTG CCC ACC AAG CTG AGA CCT ATG
1257	ATG TTG TAC TAT GAT GAT GGG CAA AAC ATC ATC AAA AAG AAG ATT CAG AAC ATG ATA
1317	GTS GAG GAG TGT GGC TGC TCA TAG AGTGCTGGCCCGGGGAGGAGCAAGAGTTGTCCAGAGAAGACA
1388	ATGGCAAAATGAAGAAATTTTAAAGATTCTGAGTTAAGCAATCAGAAAATAAATAAATAAATAAATAAATAA
1467	AAGCAAAACCTGA

Fig. 2. Sequences of nucleotides and deduced amino acids of equine inhibin/activin prepro- β A subunit cDNA. The deduced amino acid sequence is shown under the nucleotide sequence of equine inhibin/activin prepro- β A subunit. Nucleotides are numbered left of the lines and amino acids are numbered from the first methionine (ATG initiation codon). Equine inhibin/activin prepro- β A subunit cDNA contains 1278 nucleotides encoding 426 amino acids. Putative proteolytic cleavage sites (Ser²⁰-Ser²⁰ and Arg³¹⁰-Gly³¹¹) and polyadenylation signal (AATAAA) are underlined. Sequences corresponding to the primers are double-underlined. The coding sequence for mature β A subunit is italicized.

555 bp of the 5' end including 496 bp of the open reading frame. Where these two clones overlapped, the sequence was completely identical.

In Fig. 2, the complete nucleotide and deduced amino acid sequence are shown. The full 1479 bp of the nucleotide sequence consisted of 59 bp of 5' flanking untranslated sequence, 1278 bp of open reading frame coding 426 amino acids, and a putative poly-adenylation signal "AATAAA" at 113 bp downstream of the stop codon. From the deduced amino acid sequence, the molecular weight of equine inhibin β A subunit precursor is 47756 daltons. To produce a mature form of β A subunit, the putative sites of signal peptide cleavage and proteolytic cleavage were at Ser²⁰-Ser²¹ and Arg³¹⁰-Gly³¹¹, respectively and this appeared consistent with a previous report

[17]. Proteolytic cleavage might result in two forms of β A subunit of approximately 13 kDa and 45 kDa. This is consistent with the findings by Piquette *et al.* [14]. When they used antisera directed against porcine inhibin/activin β A peptide⁸¹⁻¹¹³, both 13 kDa and 44 kDa forms of β A protein were present in equine granulosa-theca cell tumor-conditioned media.

The deduced amino acid sequence of equine prepro- β A subunit was compared and aligned with those of five other mammalian species [1, 3, 5, 6, 11] (Fig. 3); however, 257 amino acids were lacking in the bovine N-terminus [6]. As shown in Table 1, similarities between the amino acid sequence of equine inhibin/activin prepro- β A subunit and other five mammalian species were greater than 90%. A potential N-glycosylation site was found at Asn¹⁶⁵ in all species (Fig. 3; bold letter) except for the bovine. The number and position of cysteine residues in equine prepro- β A subunit completely matched with the other species (excluding bovine) except for ovine where an extra cysteine residue is present at Cys²²⁶ [19]. The number of amino acids in equine prepro- β A subunit is 426, while in the other species, one to two of the amino acids are lacking. This characteristic of the equine prepro- β A protein is unique from the view point of evolutionary biology.

The sequence of mature (after proteolytic processing) β A subunit shows almost 100% homology between the bovine, porcine, ovine, rat, and mouse with only one

Table 1. Amino acid sequence similarity of equine inhibin/activin prepro- β A subunit to other mammalian species

	Similarity (%)	Amino Acid Number in Fig. 3
Bovine	96.4	257-426
Porcine	91.6	1-426
Ovine	93.2	1-426
Rat	92.0	1-426
Mouse	92.2	1-426

	10	20	30	40	50	60	70	80	90	100
EQUINE	MPLLWLGRFL	LASCWIIKVS	SPTPGSEGH	AAPNCPCAL	ATLPKDVNPA	QPEMVEAVKK	HILNMLHLKK	RPDVTQVPVK	AALLNAIRKL	HVGKVGNGY
BOVINE										
PORCINE	MPLLWLGRFL	LASCWIIKVS	SPTPGSEGH	AAPNCPCAL	ATLPKDVNPA	QPEMVEAVKK	HILNMLHLKK	RPDVTQVPVK	AALLNAIRKL	HVGKVGNGY
OVINE	MPLLWLGRFL	LASCWIIKVS	SPTPGSEGH	AAPNCPCAL	ATLPKDVNPA	QPEMVEAVKK	HILNMLHLKK	RPDVTQVPVK	AALLNAIRKL	HVGKVGNGY
RAT	MPLLWLGRFL	LASCWIIKVS	SPTPGSEGH	AAPNCPCAL	ATLPKDVNPA	QPEMVEAVKK	HILNMLHLKK	RPDVTQVPVK	AALLNAIRKL	HVGKVGNGY
MOUSE	MPLLWLGRFL	LASCWIIKVS	SPTPGSEGH	AAPNCPCAL	ATLPKDVNPA	QPEMVEAVKK	HILNMLHLKK	RPDVTQVPVK	AALLNAIRKL	HVGKVGNGY
	110	120	130	140	150	160	170	180	190	200
EQUINE	VEIEDDIGRR	AEMNELMEQT	SEIITFAESG	TARKTLHFEI	SKEGSDLSVV	ERAENVWFLK	VPKAMRTRSK	VTIRLLQQOK	HPQGSSDTRE	EAEEDLMEE
BOVINE										
PORCINE	VEIEDDIGRR	AEMNELMEQT	SEIITFAESG	TARKTLHFEI	SKEGSDLSVV	ERAENVWFLK	VPKAMRTRSK	VTIRLLQQOK	HPQGSSDTRE	EAEEDLMEE
OVINE	VEIEDDIGRR	AEMNELMEQT	SEIITFAESG	TARKTLHFEI	SKEGSDLSVV	ERAENVWFLK	VPKAMRTRSK	VTIRLLQQOK	HPQGSSDTRE	EAEEDLMEE
RAT	VEIEDDIGRR	AEMNELMEQT	SEIITFAESG	TARKTLHFEI	SKEGSDLSVV	ERAENVWFLK	VPKAMRTRSK	VTIRLLQQOK	HPQGSSDTRE	EAEEDLMEE
MOUSE	VEIEDDIGRR	AEMNELMEQT	SEIITFAESG	TARKTLHFEI	SKEGSDLSVV	ERAENVWFLK	VPKAMRTRSK	VTIRLLQQOK	HPQGSSDTRE	EAEEDLMEE
	210	220	230	240	250	260	270	280	290	300
EQUINE	RSEQLISEKV	VDARKSTWHI	FPVSSSIQRL	LDQKSSLDI	RIACDQCHET	GASLVLLGKK	KKKEEEGEGK	KKDGCEGAG	VDEEKEQSHR	PFLMLQARQS
BOVINE										
PORCINE	RSEQLISEKV	VDARKSTWHI	FPVSSSIQRL	LDQKSSLDI	RIACDQCHET	GASLVLLGKK	KKKEEEGEGK	KKDGCEGAG	VDEEKEQSHR	PFLMLQARQS
OVINE	RSEQLISEKV	VDARKSTWHI	FPVSSSIQRL	LDQKSSLDI	RIACDQCHET	GASLVLLGKK	KKKEEEGEGK	KKDGCEGAG	VDEEKEQSHR	PFLMLQARQS
RAT	RSEQLISEKV	VDARKSTWHI	FPVSSSIQRL	LDQKSSLDI	RIACDQCHET	GASLVLLGKK	KKKEEEGEGK	KKDGCEGAG	VDEEKEQSHR	PFLMLQARQS
MOUSE	RSEQLISEKV	VDARKSTWHI	FPVSSSIQRL	LDQKSSLDI	RIACDQCHET	GASLVLLGKK	KKKEEEGEGK	KKDGCEGAG	VDEEKEQSHR	PFLMLQARQS
	310	320	330	340	350	360	370	380	390	400
EQUINE	EDHPHRRRRR	GLECDGKVIN	CCKKQFFVSF	KDIGWNDWII	APSGYHANYC	EGECPSHIAG	TSGSSLSFHS	TVINQYRLRG	HNPANLKS	CVPTKLRPMS
BOVINE										
PORCINE	EDHPHRRRRR	GLECDGKVIN	CCKKQFFVSF	KDIGWNDWII	APSGYHANYC	EGECPSHIAG	TSGSSLSFHS	TVINQYRLRG	HNPANLKS	CVPTKLRPMS
OVINE	EDHPHRRRRR	GLECDGKVIN	CCKKQFFVSF	KDIGWNDWII	APSGYHANYC	EGECPSHIAG	TSGSSLSFHS	TVINQYRLRG	HNPANLKS	CVPTKLRPMS
RAT	EDHPHRRRRR	GLECDGKVIN	CCKKQFFVSF	KDIGWNDWII	APSGYHANYC	EGECPSHIAG	TSGSSLSFHS	TVINQYRLRG	HNPANLKS	CVPTKLRPMS
MOUSE	EDHPHRRRRR	GLECDGKVIN	CCKKQFFVSF	KDIGWNDWII	APSGYHANYC	EGECPSHIAG	TSGSSLSFHS	TVINQYRLRG	HNPANLKS	CVPTKLRPMS
	410	420								
EQUINE	MLYYDDGQNI	IKKDIQNMIV	EECGCS							
BOVINE										
PORCINE	MLYYDDGQNI	IKKDIQNMIV	EECGCS							
OVINE	MLYYDDGQNI	IKKDIQNMIV	EECGCS							
RAT	MLYYDDGQNI	IKKDIQNMIV	EECGCS							
MOUSE	MLYYDDGQNI	IKKDIQNMIV	EECGCS							

Fig. 3. Comparison of deduced amino acid sequences of inhibin/activin prepro- β A subunit among six mammalian species. The sequences are from Bovine [6], Porcine [11], Ovine [5], Rat [3], and Mouse [1] sources. Potential N-glycosylation sites at Asn¹⁶⁵ are indicated with bold letters.

amino acid substitution in the ovine βA subunit (Phe³²⁷ to Tyr³²⁷ in Fig. 3). In the mature equine βA subunit, three amino acid substitutions were identified, His³⁷⁵ to Gln³⁷⁵, Met³⁷⁸ to Leu³⁷⁸ and Ser³⁸² to Asn³⁸².

In preliminary experiment using twenty micrograms of total RNA from the follicles of a mare at anestrus, Northern blot analysis was performed. No detectable band was obtained. This might be due to the low amount of inhibin/activin βA mRNA present in our RNA preparation. Since placenta was shown to be a rich source of inhibin/activin βA subunit mRNA in the rat [12], we used five micrograms of poly(A)⁺RNA from mare placenta obtained on day 180 of pregnancy for Northern blot analysis. As shown in Fig. 4, a single major band of approximately 1.5 kb was detected and thus the size of the cDNA matched that of inhibin/activin βA subunit mRNA extracted from placental tissue.

In summary, we succeeded in cloning cDNA containing the complete amino acid encoding region of equine inhibin/activin βA subunit from an equine follicle cDNA library. Further study will be required to clarify regulation of this gene's expression and the distribution of βA subunit in mare tissues.

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REFERENCES

1. Albano, P. M., Groome, N., and Smith, J. C. 1993. Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation. *Development* 117: 711–723.
2. De Jong, F. 1988. Inhibin. *Physiol. Rev.* 68: 555–607.
3. Esch, F. S., Shimasaki, S., Cooksey, K., Mercado, M., Mason, A. J., Ying, S. Y., Ueno, N., and Ling, N. 1987. Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. *Mol. Endocrinol.* 1: 388–396.
4. Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y., and Shibai, H. 1990. Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. *Biochem. Biophys. Res. Commun.* 142: 1095–1103.
5. Fleming, J. S., Tisdall, D. J., Greenwood, P. J., Hudson, N. L., Heath, D. A., and McNatty, K. P. 1992. Expression of the genes for alpha inhibin, beta A inhibin and follistatin in the ovaries of Booroola ewes which were homozygotes or non-carriers of the fecundity gene FecB. *J. Mol. Endocrinol.* 8: 265–273.
6. Forage, R. G., Ring, J. M., Brown, R. W., McInerney, B. V., Cobon, G. S., Gregson, R. P., Robertson, D. M., Morgan, F. J., Hearn, M. T., Findlay, J. K., Wettenhall, R. E., Burger, H. G., and de Kretser, D. M. 1986. Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proc. Natl. Acad. Sci. U.S.A.* 83: 3091–3095.
7. Katayama, T., Shiota, K., and Takahashi, M. 1991. Effect of activin A on anterior pituitary cells fractionated by centrifugal elutriation. *Mol. Cel. Endocrinol.* 77: 167–173.
8. Lu, R. Z., Matsuyama, S., Nishihara, M., and Takahashi, M. 1993. Developmental expression of activin/inhibin βA , βB , and α subunits, and activin receptor-IIB genes in preimplantation mouse embryos. *Biol. Reprod.* 49: 1163–1169.
9. Lu, R. Z., Shiota, K., Tachi, C., and Takahashi, M. 1992. Histochemical demonstration of activin/inhibin βA , βB and α subunit in early embryos and oviducts of different strains of mice. *J. Reprod. Dev.* 38: 79–90.
10. Lu, R. Z., Shiota, K., Toyoda, Y., and Takahashi, M. 1990. Activin A (EDF) release the “two-cell block” of mouse embryos in culture. *Jpn. J. Anim. Reprod.* 36: 127–132.
11. Mason, A. J., Hayflick, J. S., Ling, N., Esch, F., Ueno, N., Ying, S. Y., Guillemin, R., Niall, H., and Seeburgh, P. H. 1985. Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor- β . *Nature (Lond.)* 318: 659–663.
12. Meunier, H., Rivier, C., Evans, R. M., and Vale, W. 1988. Gonadal and extragonadal expression of inhibin α , βA , and βB subunits in various tissues predicts diverse functions.

Fig. 4. Northern blot analysis of equine placental inhibin/activin βA subunit mRNA. Five micrograms of poly(A)⁺RNA was subjected to 1% agarose MOPS-formamide-formaldehyde gel electrophoresis and transferred to a nylon membrane. It was then hybridized with [α -³²P]dCTP-labelled equine inhibin/activin βA cDNA probe (Eq- βA -2). The sizes of RNA molecular weight markers are shown on the left and the size of the inhibin/activin βA subunit mRNA (1.5 kb) is indicated on the right by an arrow.

- Proc. Natl. Acad. Sci. U.S.A.* 85: 247–251.
13. Moore, K. H., Dunbar, B. S., Bousfield, G. R., and Ward, D. N. 1994. Initial characterization of equine inhibin. *Biol. Reprod.* 51: 63–71.
 14. Piquette, G. N., Kenney, R. M., Sertich, P. L., Yamoto, M., and Hsueh, A. J. W. 1990. Equine granulosa-theca cell tumors express inhibin α - and β A-subunit messenger ribonucleic acids and proteins. *Biol. Reprod.* 43: 1050–1057.
 15. Roser, J. F., McCue, P. M., and Hoye, E. 1994. Inhibin activity in the mare and stallion. *Domest. Anim. Endocrinol.* 11: 87–100.
 16. Sambrook, J., Fritsch, E. E., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
 17. Sugino, K., Nakamura, T., Takio, K., Miyamoto, K., Hasegawa, Y., Igarashi, M., Titani, K., and Sugino, H. 1992. Purification and characterization of high molecular weight forms of inhibin from bovine follicular fluid. *Endocrinology* 130: 789–796.
 18. Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., and Melton, D. A. 1990. Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. *Cell* 63: 485–493.
 19. Vale, W., Hsueh, A., Rivier, C., and Yu, J. 1991. The inhibin/activin family of hormones and growth factors. pp. 211–248. *In: Peptide Growth Factors and their Receptors II* (Sporn, M. B. and Roberts, A. B. eds.), Springer-Verlag New York Inc.