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RAT FOLLISTATIN: GONADAL AND EXTRAGONADAL EXPRESSION AND EVIDENCE FOR ALTERNATIVE SPLICING

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Follistatin (FS) or FSH-suppressing protein is a polypeptide which exists in multiple forms and has inhibin-like activity. We investigated the distribution of tissues expressing FS mRNA. A segment of the rat FS mRNA corresponding to the last 157 nucleotides of exon 5 and the first 71 nucleotides of exon 6 was prepared by PCR and subcloned in plasmid GEM4Z (pGEM4Z) to produce a radiolabeled RNA probe. S1-Nuclease analysis of RNA extracted from rat tissues indicated alternative splicing of the rat FS gene and gonadal as well as extragonadal expression. In addition to the ovary and testis, the FS mRNA was detected in cerebral cortex, pituitary, adrenal, thymus, pancreas, gut, kidney, heart, uterus, skeletal muscle and lung. Treatment of female rats with combined Pregnant Mare Serum Gonadotropin (PMSG) and human Chorionic Gonadotropin (hCG) increased expression of FS mRNA in the ovary. Our results show that the mRNA for the precursor of the larger known form of FS (FS 344) is much more abundant than the mRNA for the smaller molecule (less than 5 %) and that stimulation of FS transcription by PMSG and hCG in the ovary does not change this relationship.

In 1985, different groups independently purified from bovine and porcine ovarian follicular fluid (FF) dimeric proteins which inhibit pituitary follicle stimulating hormone (FSH) secretion (1,2,3,4). According to McCullagh's concept (5) these factors, which are composed of two heterologues subunits, were named inhibin. Throughout the isolation of inhibin, side fractions which did not contain inhibin but also inhibited pituitary FSH secretion were observed, and the purification of the active material led to the discovery of follistatin (FS), also named FSH-Suppressing Protein (FSP)(6,7). Although FS has no structural similarities with inhibin, both hormones appear to act through the same mechanisms on pituitary gonadotrophs (8,9). The gene structure of porcine (10) and human (11) FS revealed two forms of a single chain protein which are generated by alternative splicing. The smaller molecular weight form (FS 317) represents a carboxy-truncated form of the larger precursor (FS 344) and a comparison between human, porcine and rat FS showed highly conserved amino acid sequences (12). The 3' end exon of FS 344 contains an extremely high proportion of acidic amino acids which is missing in the carboxy-truncated form of FS 317. Although the two forms of FS do not differ in potency to inhibit pituitary FSH release (8), they might differ in other as yet unknown functions which depend on the presence or absence of the acidic 3' tail.

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Nakamura et al. (13) have recently shown that FS is a binding protein of activin (or EDF) a dimer of inhibin beta subunits. Besides the stimulation of pituitary FSH synthesis and secretion(14,15), activin also plays roles in a number of other tissues. This roles include the regulation of erythropoiesis (16), as a nerve cell survival molecule (17), in regulating growth hormone secretion and biosynthesis in somatotrophs (18,19) and participation in oocyte maturation (20) and folliculogenesis (21). Therefore the tissue expression of FS may be an important regulatory mechanism for regulating the action of activin or EDF in these tissues.

In the present study, a part of the 3' end of the rat FS mRNA, spanning 157 nucleotides of exon 5 and 71 nucleotides of exon 6, was cloned using the Polymerase Chain Reaction (PCR) and further subcloned in plasmid GEM4Z (pGEM4Z). A radiolabeled RNA transcript from this subclone was used as a specific probe for FS in an S1-Nuclease protection assay to examine the gonadal and extragonadal expression of both forms of FS.

MATERIALS AND METHODS

RNA preparation. Tissues were obtained from normal adult female rats, either untreated or PMSG/hCG-stimulated (48 h 40 iU PMSG per rat followed by 48 h 10 iU hCG per rat), and from untreated adult male rats (testis only). Total RNA was prepared by the conventional guanidine isothiocyanate method (22) and the RNA concentration was estimated by measuring the OD prior to S1-Nuclease analysis. Poly(A)+ RNA was purified from the total RNA from PMSG- and hCG-stimulated ovaries with oligo(dT)-cellulose. The animal experiments were approved by the local institutional ethics committee and conformed to the code of practice on animal experimentation endorsed by the National Health and Medical Research Council of Australia.

S1-Nuclease Probe. Poly(A)+ mRNA extracted from the total RNA from PMSG/hCG-stimulated ovaries was primed with a specific 20-mer complementary to a part of the translated 3' end of exon 6 and a part of the untranslated 3' tail (5'TGCTGGACAGTTTACCTCTC3') from FS 344. cDNA synthesis was accomplished with reverse transcriptase from avian myeloblastosis virus (Boehringer, FRG). The first cDNA strand was used as template in a PCR reaction. A 26-mer containing an artificially introduced SacI restriction enzyme site (5'AGGTTGGCAGAGCTCGCTG CTCTCTC3') was used as an upstream primer (complementary to the first cDNA strand), whereas the 20-mer of the first cDNA synthesis (containing a XBaI restriction enzyme site) was used as a downstream primer. After purification of the amplified segment in a 1% agarose gel, the DNA was cut with restriction enzymes and ligated into an XBaI/SacI restriction enzyme site of pGEM4Z and subcloned in E. coli (fig.1). The subclone was sequenced in both directions by the dideoxy chain-termination method (23) with synthetic primers complementary to the SP6 and T7 promotor of pGEM4Z; the sequence was identical to the published sequence of Shimasaki et al. (12).

S1-Nuclease Analysis. RNA:RNA hybridization was performed in 30 ul solution containing 30 ug of total RNA or yeast tRNA (negative control), 80% formamide and 20% 5-fold hybridization buffer (2.5 M NaCl; 200 mM PIPES, pH 6.4; and 1 mM EDTA) at 50 ^{o}C for 12-14 h with an excess of radiolabeled probe; samples were digested with 750 U S1-Nuclease (Boehringer, FRG) in 500 ul final volume of digestion buffer (50 mM NaCl; 33 mM Na-acetate pH 4.6; 4 mM $ZnCl_2$ and 50 ug salmon sperm DNA) for 50 minutes at 42 ^{o}C. The digestion was stopped with 100 ul 4 M amonium acetate/0.1 M EDTA. After phenol/chloroform extraction, each sample was precipitated with 1 volume of isopropanol and 20 ug yeast tRNA. Each sample was washed with 70 % ethanol, dried and resuspended in 4 ul loading buffer (80 % formamide; 10 mM EDTA pH 8; 1 mg / ml xylene cyanol FF; 1 mg / ml bromophenol

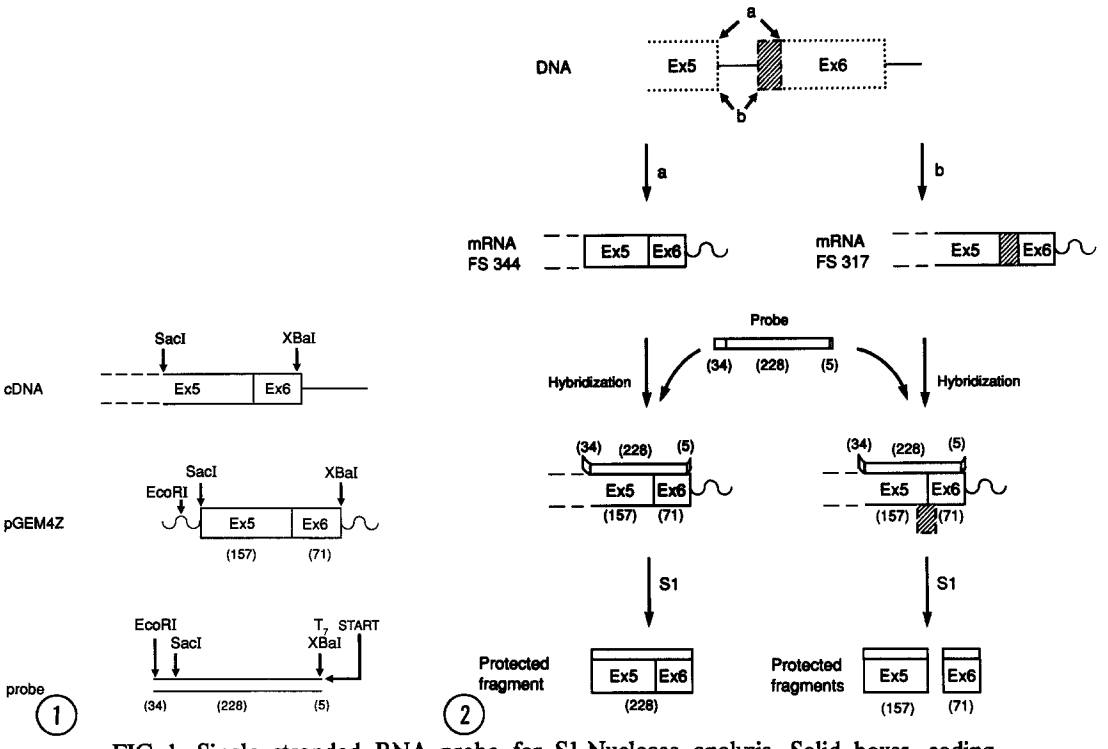

FIG. 1. Single stranded RNA probe for S1-Nuclease analysis. Solid boxes, coding regions for rat FS; solid black line, 3' untranslated sequences. Numbers in brackets, size of fragments of the probe: exon 5 (Ex 5) = 157, exon 6 (Ex 6) = 71; the 5' end of the vector = 34 and 3' sequences of the vector = 5 nucleotides. Arrows, restriction enzyme cut sides; wavy lines, sequences of pGEM4Z in which the cDNA fragment was subcloned. Double solid line, sequences of the full length probe transcribed from linearized plasmid.

FIG. 2. Predicted size of protected fragments of mRNA after S1-Nuclease analysis. Dotted boxes, coding regions for rat FS; solid lines, untranslated regions. Hatched box, region of alternative splicing to generate the two forms of FS (a -> FS 344; b -> FS 317). Solid boxes, translated sequences; wavy line, untranslated 3' end. Double solid line, radiolabeled RNA probe; numbers in brackets, size of fragments (in numbers of nucleotides).

blue) and 5 ul TE pH 8 (10 mM TRIS pH 8 and 1 mM EDTA pH 8). Samples were heated for 3 minutes to 90 °C and then kept for 3 minutes on ice before aliquots were loaded on a 6 % polyacrylamide / 8 M urea gel. A sequencing reaction was used as a molecular weight marker. To clearly visualize the FS mRNA in tissues with low FS gene expression half of each sample from each tissue and one-sixth of samples from ovary was loaded on the gel. Autoradiography was performed on X-ray film with an intensifying screen at -80 °C for 48 h. As shown in fig.2, S1-Nuclease digestion of a hybrid of radiolabeled probe and mRNA of FSP 344 should result in a protected fragment of 228 nucleotides in size, whereas a hybrid of probe and FS 317 mRNA should be digested into two fragments of 71 and 157 nucleotides.

RESULTS AND DISCUSSION

With the increased sensitivity of the S1-Nuclease technique, compared to Northern analysis, we detected transcribed FS message in rat ovary, testis, kidney, cerebral cortex, pituitary, adrenal, thymus, pancreas, heart, uterus, skeletal muscle and lung (fig. 3); in a second set of experiments, FS mRNA was also detected in the gut (data not shown).

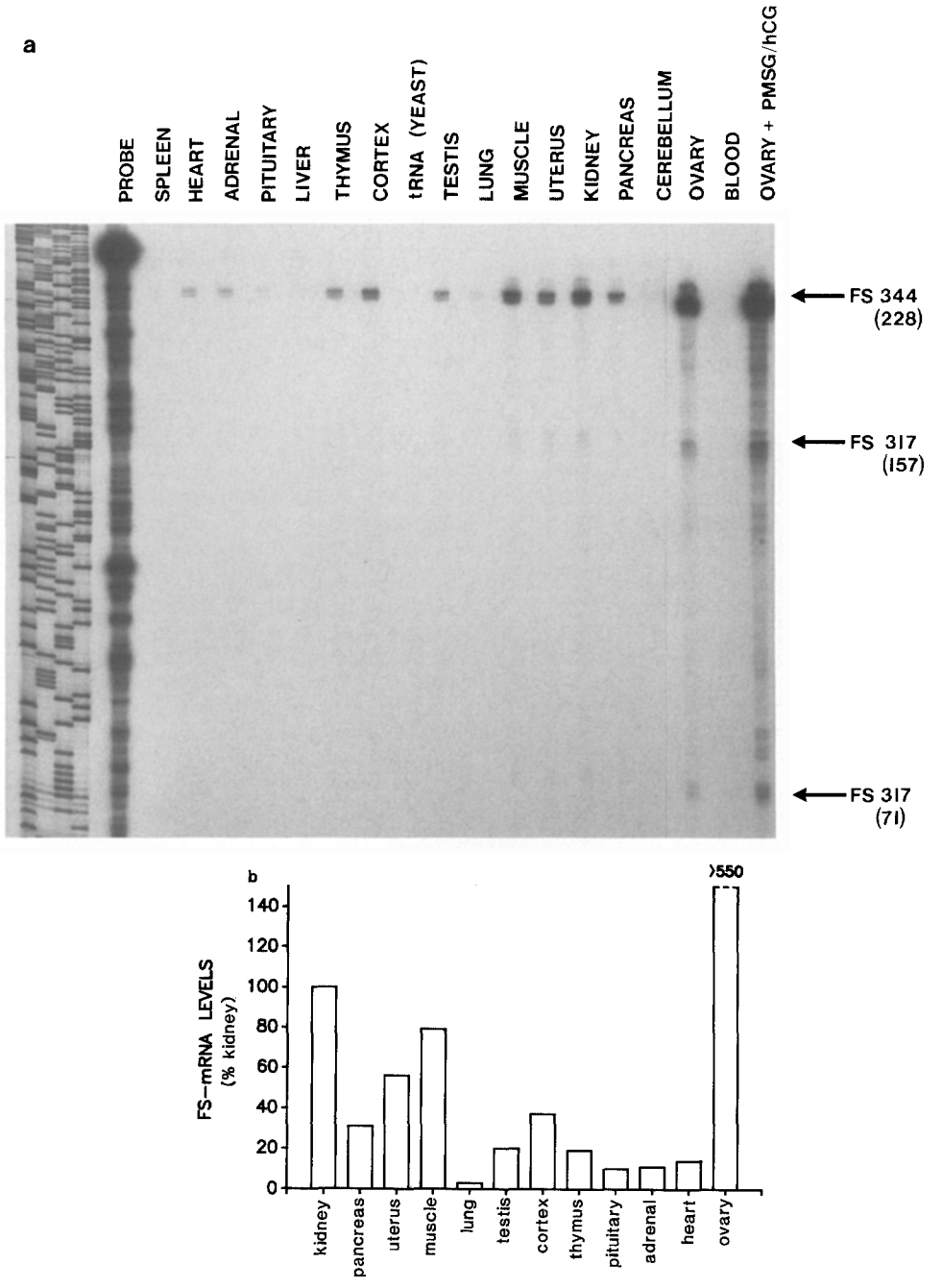

FIG. 3a. Levels of FS gene expression in various tissues of the rat. Total RNA was prepared from mature male (testis only), PMSG/hCG-stimulated mature females (ovary only) and untreated mature females (all other tissues, including ovary). The S1-Nuclease digest equivalent to 15 ug of total RNA (except stimulated and unstimulated ovary, equivalent to S1-Nuclease digest of 5 ug of total RNA) was analysed by gel electrophoresis and the autoradiograph shown is from a 48 h exposure. The sizes of the protected fragments are given in numbers of nucleotides at the right.

FIG. 3b. The band for FS 344 on the autoradiograph was scanned by densitometer, and integration values are expressed relative to the kidney (100 %). Liver, blood, spleen and cerebellum did not contribute signals above background level and, therefore, are not included in Fig. 3b.

Spleen, liver, cerebellum and blood cells did not show any signs of FS gene transcription. PMSG/hCG treatment of adult female rats caused a 5- to 6-fold stimulation in the relative levels of FS mRNA. Northern analysis has recently shown FS gene expression in rat ovary, kidney, cerebral cortex (12) and decidual tissue (24) and Gospodarowicz and Lau provided evidence for FS secretion from pituitary follicular cells (25).

Two forms of precursor are known for porcine and human FS (10, 11) and if alternative splicing of FS also occurs in the rat, RNA hybridized to our probe and subjected to S1-Nuclease analysis should result in 3 different sized fragments (fig.2). Our results in fig. 3a suggest the existence of two forms of FSP in the rat. Although the minor splicing event (represented by FS 317) was visible on the autoradiograph after 48 h exposure, the amount of signal from the major splicing event (FS 344) was already over exposed. For this reason we could not calculate the exact ratio of FS 344 to FS 317, but it was apparent that FS 317 mRNA was expressed at less than 5 % of FS 344 mRNA. Besides the ovary the FS message was most abundant in the kidney. Less expression was found in skeletal muscle, cerebral cortex, pancreas and uterus, whereas in testis, adrenal, heart, lung and thymus the level of expression was relatively low (Fig. 3). Although the message for FS 317 was below detection in most FS-expressing tissues, we could not detect any change in the ratio of FS 344 to FS 317 in those tissues where the message for FS 317 was visible. Furthermore, a stimulation of FS transcription in the ovary by PMSG/hCG did not alter the pattern of expression. Although we did not find any evidence for differential regulation or tissue-specific differences in the expression of the two forms of FS, we cannot exclude the possibility of different patterns of expression under circumstances which were not included in this study. There was a large difference in the abundance of FS in the ovary compared with the testis of mature rats. This reflects either a sex difference or a difference in the relative number of cells in each type of gonad responsible for FS production.

Similarities in the tissue specific expression of inhibin beta subunit RNA (26) and FS (fig.3) are common: low or undetectable level of FS gene expression accompanies a low level of expression of inhibin beta mRNAs in the testis, pituitary, adrenal and spleen; the ovary with high expression of inhibin beta mRNAs also shows a high level of expression of the FS gene. However no inhibin beta mRNA is detected in the kidney and pancreas (26), whereas the FS gene is highly expressed in these tissues. Therefore, coexpression of FS and activin genes is not obligatory.

Our data demonstrate that FS is expressed at different levels in many tissues and that it, like inhibin and activin (26) is more than a gonadal hormone. Whether FS is secreted by all tissues exhibiting FS gene expression and what hormonal actions FS exerts in the corresponding target tissues remain to be investigated. Considering that FS is a binding protein of activin (EDF) (13), and activin itself can act as a growth and differentiation factor (see introduction), it is tempting to speculate that FS plays an important role in controlling activin action in many tissues.

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REFERENCES

1. Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L., and Guillemin, R. (1985) Proc. Natl. Acad. Sci. USA 82. 7217-7221.

2. Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H. (1985) Biochem. Biophys. Res. Commun. 129. 396-403.

3. Rivier, J., Spiess, J., McClintock, R., Vaughan, J., and Vale, W. (1985) Biochem. Biophys. Res. Comun. 133. 120-127.

4. Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H.,and DeKretser, D.M. (1985) Biochem. Biophys. Res. Commun. 126. 220-226.

5. McCullagh, D.R. (1932) Science 76. 19-20.

6. Ueno, N., Ling, N., Ying, S.-Y., Esch, F., Shimasaki, S., and Guillemin, R. (1987) Proc Natl. Acad Sci USA 84. 8282-8286.

7. Robertson, D.M., Klein, R., de Vos, F.L., McLachlan, R.I., Wettenhall, R.E., Hearn, M.T.W., Burger, H.G., and de Kretser, D.M. (1987) Biochem. Biophys. Res. Commun. 149. 744-749.

8. Ying, S.-Y., Becker, A., Swanson, G., Tan, P., Ling, N., Esch, F., Ueno, N., Shimasaki, S., and Guillemin, R. (1987) Biochem. Biophys. Res. Commun. 149. 133-139.

9. Robertson, D.M., Farnworth, P.G., Clarke, I., Jacobsen, J., Cahir, N.F., Burger, H.G., and de Kretser, D.M. (1990) J. Endocrinol. 124. 417-423.

10. Shimasaki, S., Koga, M., Esch, F., Mercada, M., Cooksey, K., Koba, A., and Ling, N. (1988) Biochem. Biophys. Res. Commun. 152. 717-723.

11. Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S.-Y., Ling, N., and Guillemin, R. (1988) Proc. Natl. Acad. Sci USA 85. 4218-4222.

12. Shimasaki, S., Koga, M., Buscaglia, M.L., Simmons, D.M., Bicsak, T.A., and Ling, N. (1989) Mol. Endocrinol. 3. 651-659.

13. Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugino, H. (1990) Science 247. 836-838.

14. Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin (1986) Nature 321, 779-782.

15. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986) Nature 321, 776-779.

16. Yu, J., Li-en, S., Vaughan, J., Vale, W., and Yu, A.L. (1989) Blood 73. 952-960.

17. Schubert, D., Kimura, H., La Corbiere, M., Vaughan, J., Karr, D., and Fischer, W.H. (1990) Nature 334. 868-870.

18. Bilezikjian, L.M., Corrigan, A.Z., and Vale, W. (1990) Endocrinology 126. 2369-2376.

19. Billestrup, N., Gonzalez-Manchon, C., Potter, E., and Vale, W. (1990) Mol. Endo. 4. 356-362.

20. Itoh, M., Igarashi, M., Yamada, K., Hasegawa, Y., Seki, M., Eto, Y., and Shibai, H. (1990) Biochem. Biophys. Res. Commun. 166. 1479-1484.

21. Xiao, S. Findlay, J.K., and Robertson, D.M. (1990) Mol. Cell. Endo. 69. 1-8.

22. Chirgwin, J., Przybyla, A., MacDonald, J., and Rutter, W. (1979) Biochemistry 18. 5294-5299.

23. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74. 5463-5467.

24. Kaiser, M., Gibori, G., and Mayo, K. (1990) Endocrinology 126. 2768-2770.

25. Gospodarowicz, D., and Lau, K. (1989) Biochem. Biophys. Res. Commun. 165. 292-298.

26. Meunier, H., Rivier, C., Evans, R., Vale, W. (1988) Proc. Natl. Acad. Sci. USA 85. 247-251.