1-3-2000

PARTIAL SEQUENCE OF THE cDNA FOR THE ?-SUBUNIT OF THE INHIBIN HORMONE IN JAPANESE QUAIL

Tristan Timothy Sands

PARTIAL SEQUENCE OF THE cDNA FOR THE α-SUBUNIT OF THE INHIBIN HORMONE IN JAPANESE QUAIL

A Thesis

Submitted to the faculty of
Louisiana State University and
Agricultural & Mechanical College
in partial fulfillment of the
requirements for graduation with
College Honors

in

Animal, Dairy, and Poultry Sciences

by

Tristan Timothy Sands
Honors 3992
January 3, 2000

Partial Sequence of a cDNA encoding the α–Subunit of the Inhibin Hormone in Japanese Quail

TRISTAN T. SANDS and GARY G. CADD

Department of Poultry Science, Honors College, Louisiana State University, Baton Rouge, Louisiana 70803

ABSTRACT An experiment was conducted to sequence a portion of a cDNA encoding the α-subunit of the inhibin hormone in Japanese quail for the purpose of comparison with the chicken sequence. Messenger RNA was isolated from granulosa tissue and RT-PCR was used to convert it into cDNA. A *Pst*I fragment was selectively amplified by PCR using primers developed from the chicken sequence.

Results indicate almost perfect conservation with the chicken fragment. The one difference between the two sequences lies in the 3' UTR and, along with other evidence, lends support to the likelihood that the published chicken sequence is incorrect at one base pair.

INTRODUCTION

Inhibin and activin are dimeric glycoprotein hormones that are produced by the ovary and testis. Inhibin is composed of an α– and β-subunit, the latter of which can be found in two forms, β_A and β_B, for which it is named inhibin-A or inhibin-B. Activin, in contrast, is a homo- or heterodimer of β-subunits. In both inhibin and activin, the subunits are linked via disulfide bonds (Ling *et al.*, 1986; Rivier *et al.*, 1987; Ying, 1987; de Jong, 1988; Ling *et al.*, 1988; Vale *et al.*, 1988; Ying, 1988; Risbridger *et al.*, 1990). Though they share subunits, inhibin and activin have opposite effects on the secretion of follicle-stimulating hormone (FSH) from the pituitary. As the names of the hormones suggest, inhibin has been implicated in the depression of FSH secretion, whereas activin elevates FSH secretion (Vale *et al.*, 1988).

Inhibin regulates FSH secretion via negative feedback (Johnson *et al.*, 1993a; Vanmontfort *et al.*, 1994; Johnson and Brooks, 1996), but also seems to have local effects (Woodruff *et al.*, 1990; Baly *et al.*, 1993; Findlay1993; Vanmontfort, 1996). In addition, the free α-subunit may act as a competitor for FSH receptor binding, thereby modulating its activity (Schneyer *et al.*, 1991). Although there is a correlation between avian laying rate and the expression of the inhibin α-subunit (Wang and Johnson, 1993a), it remains unknown whether or not the observed control over ovulation occurs by regulation of FSH secretion (Akashiba *et al.*, 1988; Tsonis *et al.*, 1988; Johnson *et al.*, 1993; Wang and Johnson, 1993a).

Research has shown inhibin to be produced in large amounts by the granulosa cell layer of the developing follicle (Akashiba *et al.*, 1988; Tsonis *et al.*, 1988). However, results vary as to whether inhibin production is highest in the most or the least developed follicles (Akashiba *et al.*, 1988; Vanmontfort *et al.*, 1992; Johnson *et al.*, 1993b), perhaps due to different experimental approaches.

Additionally, in the chick embryo, it has been suggested that inhibin plays a differential role in sexual development through local control over androgen secretion (Rombauts *et al.*, 1992; Rombauts *et al.*, 1996). There is also evidence that the α-subunit may play a role in gonadal tumor suppression (Matzuk *et al.*, 1992).

The cDNA coding for the α-subunit of chicken inhibin has been sequenced (Wang and Johnson, 1993b). Among mammals the α-subunit cDNA sequence is highly conserved (80%). The chicken sequence shares about 48% homology with pig and rat sequences. Most notable among conserved features are the number and position of cystein residues, believed to be involved in the disulfide bonds that are thought to play a role within the subunit, as well as in linking it to a β-subunit (Ying, 1987; Johnson and Wang, 1993; Wang and Johnson, 1993b). Sequencing revealed that the subunit is synthesized as a precursor and is subsequently processed. The cDNA sequence (GenBank Accession #U42377) begins with a ~ 35 bp signal sequence for secretion and is followed by another sequence (~ 200 bp) of unknown function. This Pro sequence is followed, in turn, by a region coding for an α-N peptide that is not part of the mature α-subunit. α-N is about 500 bp long and precedes the α-C peptide, which is 350 bp. α-C is the peptide that makes up the mature α-subunit. The entire cDNA, including the untranslated region (UTR), is approximately 1600 bp. The portions of the cDNA delineated above are separated within the macromolecule by double arginine residues, recognition cut sites, except for the α-C, which is separated from the UTR by a stop codon.

Immunoneutralization of inhibin is correlated with increased rate of ovulation in many mammalian species (Forage *et al.*, 1987; Findlay *et al.*, 1989; Rivier and Vale, 1989; Brown *et al.*, 1990; King *et al.*, 1990; Mizumachi *et al.*, 1990; Wrathall *et al.*, 1990; Glencross *et al.*, 1992; Scanlon *et al.*, 1993). This is likely the consequence of increase in the level of plasma FSH (Culler and Negro-Villar, 1988; Rivier *et al.*, 1988; Vale *et al.*, 1988; Martin *et al.*, 1991). Because of its associated increase in ovulation rate, immunoneuralization of avian inhibin has been a goal of the Applied Animal Biotechnology Laboratories under Dr. Cadd and Dr. Satterlee in the Department of Poultry Science. They have utilized a fusion protein, made of bacterial maltose binding protein (MBP) and a truncated form of chicken α-inhibin ($cINA_{521}$) to actively immunize poultry against native inhibin. This portion of the α-subunit is a 521 bp *Pst*I fragment of the cDNA. It was cloned into the pMAL-c2 vector and expressed as the MBP fusion protein in *Escherichia coli*.

Because the immune response is dependent upon antigenicity, the question of sequence similarity of the coding region for α-inhibin in Japanese quail is of practical concern to this project. For, if there are sequence differences between the two species, which result in amino acid changes, these differences could be exploited to increase the antigenicity of the fusion protein. However, the knowledge has value in its own right as part of the further elucidation of the evolutionary relationship among avians.

Therefore, herein the *Pst*I fragment of the cDNA from Japanese quail was amplified using reverse transcription and the polymerase chain reaction (RT-PCR) of the messenger RNA isolated from the granulosa cells of developing ova.

MATERIALS AND METHODS

Granulosa tissue was isolated from the primary and secondary follicles of female Japanese quail (*Coturnix japonica*) at roughly six months of age. Granulosa tissue

was chosen due to the high level of α-subunit expression. The ova were removed and cut along the stigma, allowing the yolks to drop into deionized water. Then, the granulosa tissue was manually teased away from the yolk transferred to a metal block cooled by dry ice and stored at –80˚C.

The messenger RNA was isolated using Stratagene's Messenger RNA Isolation Kit (Catalog #200347). The tissue was denatured and homogenized by sonication with a Branson Sonifier 450 in buffered β-mercaptoethanol solution. The proteins were precipitated from the solution by centrifugation. An oligo(dT) cellulose slurry was used to selectively bind the poly(A) RNA through several high and low salt washes. Finally, the mRNA was eluted through a filtered syringe.

The messenger RNA was concentrated to approximately 0.15μg/μL using an Eppendorf Vacufuge. Messenger RNA was reverse transcribed into cDNA with Stratagene's cDNA Synthesis Kit (Catalog #200400). The cDNA encodes the same information as the mRNA, from which it is made, in a more stable form. First the mRNA was converted to a DNA-RNA heteroduplex using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) in the presence of deoxyribonucleotide triphosphates (dNTP's) of adenine, cytosine, guanine, and thymine, and primers at 37˚C. The primers were designed with poly(dT) tails, so as to prime the first strand synthesis from the poly(A) tails of the mRNA molecules. RNAse-H was used to nick the RNA of the heteroduplex, creating fragments to prime DNA polymerase I for the synthesis of the second strands at 16˚C.

From this cDNA, a portion of the α-subunit of inhibin was selectively amplified by polymerase chain reaction (PCR). The DNA polymerase from *Thermus aquaticus* is heat stable, allowing it to perform under the stressful conditions of thermal cycling. Thermal cycling is the alternate melting and reannealing of nucleic acid strands through raising and lowering of temperature, respectively. PCR is the process by which DNA is amplified by *Taq* polymerase through thermal cycling with an abundance of primers and dNTP's. On each cycle, each DNA strand is separated from its complementary strand, reannealed to a primer, and converted into a duplex. Thus, PCR is a way to amplify DNA exponentially. Using a Stratagene Robocycler and primers developed from a *Pst*I fragment internal to the domestic chicken (*Gallus domesticus*) α-subunit cDNA, the analogous fragment in Japanese quail (*Coturnix japonica*) was amplified. The amount of starting material and of MgCl (50mM) was varied so as to optimize amplification (Table 1).

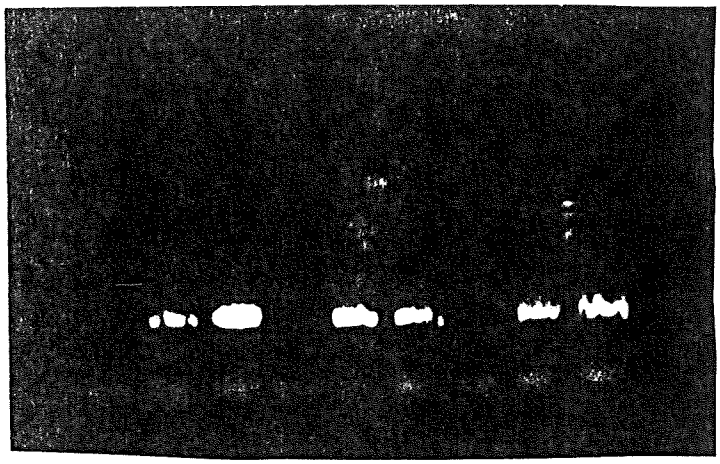
Table 1: PCR Variations

	10X Buffer	Primers	DNTP's	MgCl	cDNA	H20
1	5λ	4λ	1λ	1λ	1λ	38λ
2	5λ	4λ	1λ	2λ	1λ	37λ
3	5λ	4λ	1λ	3λ	1λ	36λ
4	5λ	4λ	1λ	1λ	2λ	37λ
5	5λ	4λ	1λ	2λ	2λ	36λ
6	5λ	4λ	1λ	3λ	2λ	35λ
7	5λ	4λ	1λ	1λ	3λ	36λ
8	5λ	4λ	1λ	2λ	3λ	35λ
9	5λ	4λ	1λ	3λ	3λ	34λ

The amount of MgCl proved to be crucial, as amplification did not occur in tubes one, four, and seven. The samples were run on a 1.1% agarose electrophoretic gel. The agarose in the gel impedes the progress of the negatively charged DNA fragments towards the positively charged cathode in an electric field. Larger molecules take longer to migrate

proportional to the log of their molecular weight. Thus, gel electrophoresis is essentially a type of chromatography useful for separating DNA by size. The DNA on the electrophoresed gel was stained and visualized with ethidium bromide, an intercalating agent that fluoresces under ultraviolet (UV) radiation. The DNA was run with 1kb ladders as referential size markers.

Figure 1: Gel Electrophoresis of PCR



cDNA from tube three was digested with *Pst*I restriction enzyme and electrophoresed in NuSieve GTG agarose, stained and visualized, and then destained. The gel band containing the correct size fragment was excised and remelted. The cDNA was inserted into a PGEM3Z vector (Promega), using T4 ligase.

The sequence of the fragment was determined via fluorescent dideoxy sequencing, in which dNTP's are supplemented with ddNTP's, which lead to a termination of synthesis due to the lack of a 3' hydroxyl for phosphoesterification. The ddNTP's each fluoresce at a different wavelength. Since termination of synthesis occurs with a fluorescing ddNTP, the sequence can be constructed by placing the synthesized fragments in order by size and measuring fluorescence.

RESULTS AND DISCUSSION

Although both strands of the cloned cDNA were sequenced, only one had good fidelity. Alignment showed the good strand to be identical to the sequence in the chicken α-subunit, except in two base pairs (Figure 3).The first difference is residue 1094 in the chicken sequence, ten base pairs prior to the stop codon. The quail sequence appears to lack a corresponding cytosine. However, upon inspection of the fluorescence signal, between the peaks at 299 for a guanine and at 300 for an adenine, there is a small-undetected peak for a cytosine residue (Figure 2). The quail sequence is therefore most likely identical to the chicken sequence at that base pair, especially since the deletion of this base pair would cause a frameshift in the coding region for α-C. The conservation of this portion of the coding region implies that the amino acid sequence of the α-subunit is also conserved between the species.

The other difference in the sequences appears at residue 1316 in the chicken sequence, one base pair prior to the distal *Pst*I site within the 3' UTR. The chicken α-subunit cDNA reportedly has an adenine at that site, whereas the quail sequence shows a guanine. Interestingly, there had been previous evidence that the published chicken sequence may be incorrect at that base pair (G. Cadd, unpublished data). Specifically, it was questioned whether the residue might not in fact be a guanine. This difference with the published chicken sequence of the otherwise perfectly conserved *Pst*I fragment in quail provides further evidence that the published sequence for chicken may be flawed.

Thus, the *Pst*I fragments of cDNAs encoding the α-subunit in chicken and quail, as well as ostrich (G. Kousoulas, personal communication) probably have been perfectly conserved through the evolution of the species.

Figure 2: Relevant portion of fluorescence peaks in the cDNA sequencing

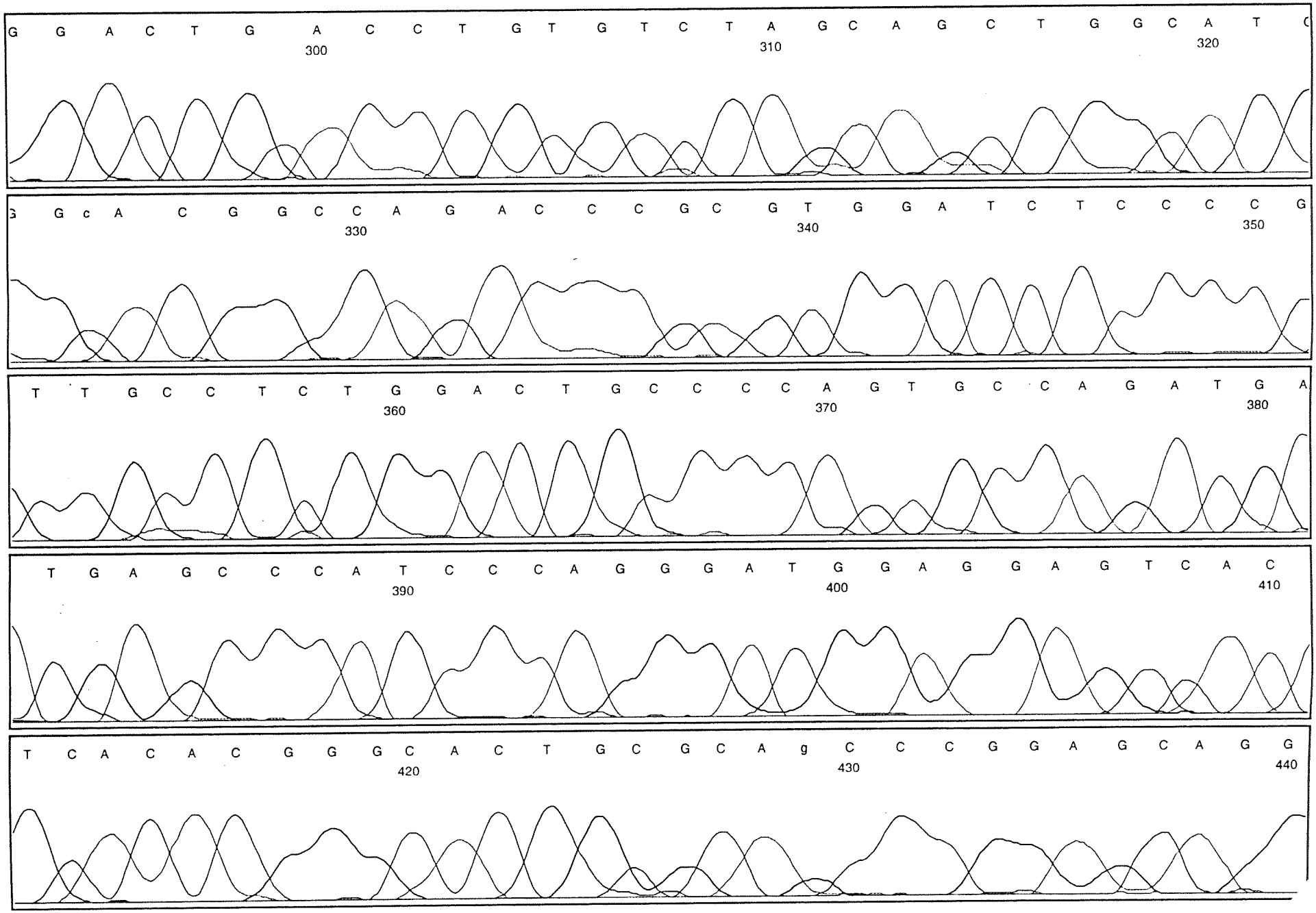
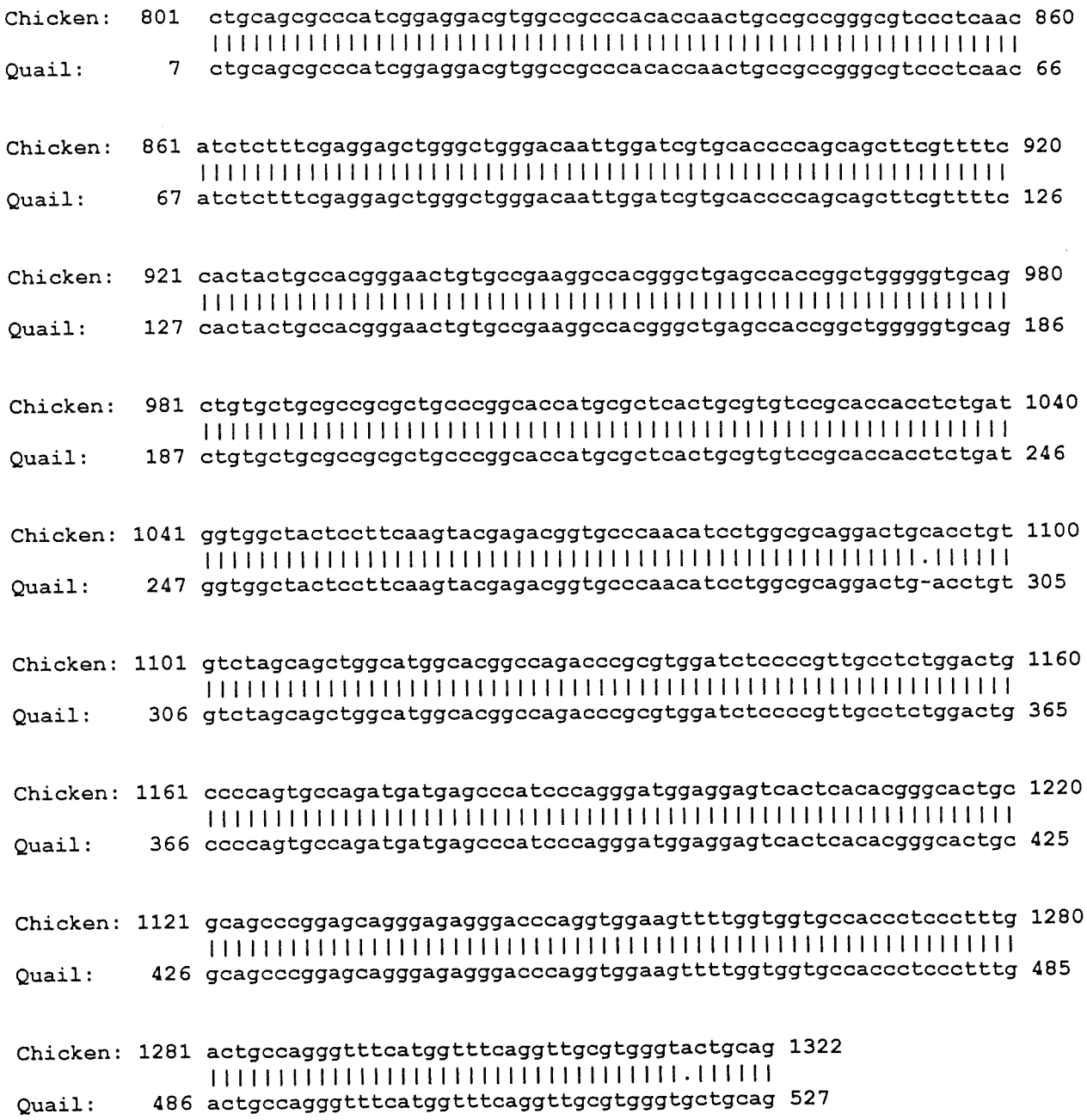


Figure 3. DNA sequence comparison of chicken and Japanese quail inhibin α-subunit PstI fragments.



REFERENCES

Akashiba, H., Taya, K., Sasamoto, S., Goto, H., Kamiyoshi, M. and Tanaka, K. 1988. Secretion of inhibin by chicken granulosa cells *in vitro*. *Poultry Science*, **67**, 1625.

Baly, D.L., Allison, D.E., Krummen, L.A., Woodruff, T.K., Soules, M.R., Chen, S.A., Fendly, B.M., Bald, L.N., Mather, J.P. and Lucas, C. 1993. Development of a specific and sensitive two-site enzyme-linked immunosorbent assay for measurement of inhibin-A in serum. *Endocrinology*, **132**, 2099.

Brown, R. W., Hungerford, J.W., Greenwood, P.E., Bloor, R.J., Evans, D.F., Tsonis, C.G., and Forage, R.G. 1990. Immunization against recombinant inhibin alpha subunit causes increased ovulation rates in gilts. *J. Reprod. Fertil.*, **90**, 199.

Chen, C.C.,-L, 1993. Editorial: Inhibin and activin as panacrine/autocrine factors. *Endocrinology*, **132**, 4.

Culler, M.D., and Negro-Villar, A. 1988. Passive immunoneutralization of endogenous inhibin: sex-related differences in the role of inhibin during development. *Mol. Cell Endocrinol.*, **58**, 263.

de Jong, F.H. 1988. Inhibin. *Physiological Reviews*, **68**, 555.

Findlay, J.K., 1993. An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. *Biol. Reprod.*, **48**, 15.

Findlay, J.K., Doughton, B., Robertson, D.M., and Forage, R.G. 1989. Effects of immunization against recombinant bovine inhibin alpha subunit on circulating concentrations of gonadotrophins in ewes. *J. Endocrinol.*, **120**, 59.

Forage, R.G., Brown, R.W., Oliver, K.J., Atrache, B.T., Devine, P.L., Hudson, G.C., Goss, N.H., Bertram, K.C., Tolstoshev, P., Robertson, D.M., de Kretser, D.M., Doughton, B., Burger, H.G., and Findlay, J.K. 1987. Immunization against an inhibin subunit produced by recombinant DNA techniques results in increased ovulation in sheep. *J. Endocrinolol.* **114**, R1.

Glencross, R.G., Bleach, E.C.L., McLeod, B.J., Beard, A.J., and Knight, P.G. 1992. Effect of active immunization of heifers against inhibin on plasma FSH concentrations, ovarian follicular development and ovulation rate. *J. Endocrinol.*, **134**, 11.

Johnson, P.A., 1993. Inhibin in the hen. *Poultry Sci.*, **72**, 955.

Johnson, P.A. and Brooks, C. 1996. Developmental profile of plasma inhibin and gonadotropins from hatch to sexual maturity in male and female chickens. *Gen. Comp. Endocrinol.*, **102**, 56.

Johnson, P.A., Brooks, C., Wang, S.-Y. and Chen., C.-C. 1993a. Plasma concentrations of immunoreactive inhibin and gonadotropins following removal of ovarian follicles in the domestic hen. *Biol. Reprod.*, **49**, 1026.

Johnson, P.A. and Wang, S.-Y. 1993. Characterization and quantification of mRNA for the inhibin α-subunit in the granulosa layer of the domestic hen. *Gen. Comp. Endocrinol.*, **90**, 43.

Johnson, P.A., Wang, S.-Y. and Brooks, C. 1993b. Characterization of a source and levels of plasma immunoreactive inhibin during the ovulatory cycle of the domestic hen. *Biol. Reprod.*, **48**, 262.

King, B.F., Sesti, L.A.C., Britt, J.H., Esbenshade, K.L., Flowers, B. and Ireland, J.J. 1990. Enhancement of ovulation rate by immunization of gilts against inhibin. *J. Anim. Sci.*, **89**(Suppl. 1), 437. (Abstr.)

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R. 1986. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. *Nature*, **321**, 779.

Ling, N., Ueno, N., Ying, S.-Y., Esch, F., Shimasaki, S., Hotta, M. Cuevas, P. and Guillemin, R. 1988. Inhibins and activins. *Vitamins and Hormones*, **44**, 1.

Martin, T.L., Williams, G.L., Lunstra, D.D. and Ireland, J.J. 1991. Immunoneutralization of inhibin modifies hormone secretion and sperm production in bulls. *Biol. Reprod.*, **45**, 73.

Matzuk, M.M., Finegold, M.J., Su, J.-G.J., Hsueh, A.J.W. and Bradley, A. 1992. α-Inhibin is a tumor-suppressor gene with gonadal specificity in mice. *Nature*, **360**, 313.

Mizumachi, M., Voglmayr, J.K., Washington, D.W., Chen, C.L.C. and Bardin, C.W. 1990. Superovulation of ewes immunized against the human recombinant inhibin alpha-subunit associated with increased pre- and postovulatory follicle-stimulating hormone levels. *Endocrinol.*, **126**, 1058.

Risbridger, G.P., Robertson, D.M. and de Krester, D.M. 1990. Current perspectives of inhibin biology. *Acta. Endocrinologica* (Copenh), **122**, 673.

Rivier, C., Cajander, S., Vaughan, J., Hsueh, A.J.W. and Valre, W. 1988. Age-dependent changes in physiological action, content, and immunostaining of inhibin in male rats. *Endocrinol.*, **123**, 120.

Rivier, C. and Vale, W. 1989. Immunoneutralization of endogenous inhibin modifies hormone secretion and ovulation rate in the rat. *Endocrinology*, **125**, 152.

Rivier, C., Vale, W. and Rivier, J. 1987. Studies of the inhibin family of hormones: A review. *Horm. Res.*, **28**, 104.

Rombauts, L., Vanmontfort, D., Verhoeven, G. and Decuypere, E. 1992. Immunoreactive inhibin in plasma, amniotic fluid, and gonadal tissue of male and female chick embryos. *Biol. Reprod.*, **46**, 1211.

Rombauts, L., Vanmontfort, D., Decuypere, E. and Verhoeven, G. 1996. Inhibin and activin have antagonistic panacrine effects on gonadal steroidogenesis during the development of the chicken embryo. *Biol. Reprod.*, **54**, 1229.

Scanlon, A.R., Sunderland, S.J., Martin, T.L., Goulding, D., O'Callaghan, D., Williams, D.H., Headon, D.R., Boland, M.P., Ireland, J.J., and Roche, J.F. 1993. Active immunization of heifers against a synthetic fragment of bovine inhibin. *J. Reprod. Fert.*, 97(1), 213.

Schneyer, A.L., Sluss, P.M., Whitcomb, R.W., Martin, K.A., Sprengel, R. and Crowley, Jr., W.F. 1991. Precursors of α-inhibin modulate follicle-stimulating hormone receptor binding and biological activity. *Endocrinology*, **129**, 1987.

Tsonis, C.G., Sharp, P.J. and McNeilly, A.S. 1988. Inhibin bioactivity and pituitary cell mitogenic activity from cultured chicken ovarian granulosa and thecal/stromal cells. J. *Endocrinol.*, **116**,293.

Vale, W., Rivier, C., Hsueh, A., Campen, C., Meunier, H., Bicsak, T., Vaughan, J., Corrigan, A., Bardin, W., Sawchenko, P. Petraglia, F., Yu, J., Plotsky, P., Spiess, J. and Rivier, J. 1988. Chemical and biological characterization of the inhibin family of protein hormones. *Recent Progress in Hormone Research*, **44**, 1.

Vanmontfort, D. 1996. Inhibin in the laying hen (*Gallus domesticus*): source, regulation and fuction, Ph.D. thesis, Catholic University, Leuven, Belgium.

Vanmontfort, D., Rombauts, L., Decuypere, E. and Verhoeven, G. 1992. Source of immunoreactive inhibin in the chicken ovary. *Biol. Reprod.*, **47**, 977.

Vanmontfort, D., Berghman, L.R., Rombauts, L., Verhoeven, G. and Decuypere, E. 1994. Changes of immunoreactive inhibin, follicle-stimulating hormone, luteinizing hormone, and progesterone in plasma after short-term food deprivation and during the ovulatory cycle of the domestic hen. *Gen. Comp. Endocrinol.*, **95**, 117.

Wang, S.-Y. and Johnson, P.A. 1993a. Increase in ovarian α-inhibin gene expression and plasma immunoreactive inhibin level is correlated with a decrease in ovulation rate in the domestic hen. *Gen. Comp. Endocrinol.*, **91**, 52.

Wang, S.-Y. and johnson, P. 1993b. Complementary deoxyribonucleic acid cloning and sequence analysis of the α-subunit of inhibin from chicken ovarian granulosa cells. *Biol. Reprod.*, **49**, 453.

Woodruff, T.K., Lyon, R.J., Hansen, S.E., Rice, G.C. and Mather, J.P. 1990. Inhibin and activin locally regulate rat ovarian folliculogenesis. *Endocrinology*, **127**, 3196.

Wrathall, J.H.M., McLeod, B.J., Glencross, R.G., Beard, A.J. and Knight, P.G. 1990. Inhibin immunoneutralization by antibodies raised against synthetic peptide sequences of inhibin alpha subunit: effects on gonadotropin concentrations and ovulation rate in sheep. *J. Endocrinol.* **124**, 167.

Ying, S.-Y. 1987. Inhibins and activins: chemical properties and biological activity. *Proc. Soc. Exper. Biol. Med.*, **186**, 253.

Ying, S. -Y. 1988. Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrinol. Rev.*, **9**, 267.