Genes encoding the α and β chains of follicle-stimulating hormone are not sites for the Booroola (Fec^B) mutation in sheep

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Summary. Bovine cDNA probes for the β -subunit of follicle-stimulating hormone ($FSH\beta$) and the α -subunit of the glycoprotein hormones identify genetic variation (polymorphic restriction fragments) near these genes in sheep. The inheritance of the polymorphic restriction fragments was studied in half-sibling pedigrees generated by mating heterozygous ($B+$) rams to non-carrier ($++$) ewes so that the co-inheritance or genetic linkage to the Booroola (Fec^B) locus and the α - and β -subunits of FSH could be analysed. Genetic recombination was observed between the $FSH\beta$ locus and the Fec^B locus in all five families studied and between the α -subunit and the Fec^B locus in the two families studied. We conclude that the Fec^B mutation does not lie within the FSH β - or α -subunit genes encoding the heterodimeric hormone FSH, and that the high concentrations of FSH observed in carrier ewes must result from indirect actions of the Fec^B mutation on the synthesis, processing, storage, release or metabolism of FSH.

Keywords: $FSH\beta$; α -subunit; Fec^B gene; sheep; Booroola; RFLP analysis; genetic linkage

Introduction

The Booroola Merino breed carries a major autosomal mutation that increases ovulation rate (Davis *et al.*, 1982; Piper & Bindon, 1982). The Committee on Genetic Nomenclature of Sheep and Goats (COGNOSAG) has named the locus Fec^B . Ewes are classified as homozygous (BB), heterozygous ($B+$) and non-carriers ($++$) at the Fec^B locus on the basis of their maximum ovulation rate record (Davis *et al.*, 1982).

The DNA alteration or mutation responsible for the increased ovulation rate has not been characterized or mapped. Consistent differences have been reported in circulating concentrations of follicle-stimulating hormone (FSH) in ewes carrying the Fec^B mutation (McNatty *et al.*, 1991). Concentrations of FSH are higher in BB compared with $++$ ewe lambs at 6 weeks of age (Montgomery *et al.*, 1989), throughout the oestrous cycle (McNatty *et al.*, 1987), during anoestrus (McNatty *et al.*, 1987) and after ovariectomy (McNatty *et al.*, 1989). Concentrations of FSH in $B+$ ewes are intermediate. Differences in FSH concentrations can be reproduced by gonadotrophin-releasing hormone (GnRH) treatment of hypothalamic–pituitary disconnected ovariectomized ewes (McNatty *et al.*, 1991). Increased FSH concentrations in ewes carrying the Fec^B mutation do not appear to result from alterations in the concentrations of GnRH in the hypothalamus (Gale *et al.*, 1988) or the binding characteristics of pituitary GnRH receptors (Fleming *et al.*, 1990). The Fec^B mutation could therefore lie close to the genes encoding FSH, altering the expression of these genes.

FSH is produced in the anterior pituitary gland and the FSH gene is part of a gene family including pituitary luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and placental chorionic gonadotrophin (CG). The hormones are all heterodimers consisting of a specific β -subunit, noncovalently linked to an α -subunit (Pierce & Parsons, 1981). The α -subunit is identical

for the glycoprotein hormones and is encoded by a single α -subunit gene (Pierce & Parsons, 1981). The gene for the β -subunit of FSH has been mapped to chromosome 11p in humans (Watkins *et al.*, 1987) and chromosome 15 in sheep (Hediger *et al.*, 1990). The α -subunit has been mapped to chromosome 6 in humans (McGill *et al.*, 1987), but the chromosome location in sheep is unknown.

The hypothesis that the site of the Fec^B mutation lies within the genes encoding the α and β subunits of FSH can be tested directly by examining genetic linkage or co-inheritance of the gene with the Fec^B mutation in suitable pedigrees (Botstein *et al.*, 1980). The FSH β locus in sheep contains an insertion/deletion polymorphism downstream from the third exon, and this polymorphism can be typed in animals for genetic linkage analysis (Montgomery *et al.*, 1990). Recombination between the Fec^B locus and the insertion/deletion polymorphism was detected in two half-sibling families. The results suggested that the FSH β gene is not the site of the Fec^B mutation, but require confirmation in a larger set of pedigrees to exclude genetic linkage to this locus since weak positive log of the odds for linkage (lod) scores were observed in one small family. A bovine cDNA probe for the α -subunit detects restriction fragment length polymorphisms (RFLPs) in sheep DNA digested with the restriction enzymes *Bst* EII and *Bam* HI (Penty *et al.*, 1991). We now report genetic recombination between the FSH β and Fec^B loci in an expanded set of pedigrees, and between α -subunit and the Fec^B loci.

Materials and Methods

Animals

The inheritance of the polymorphic restriction fragments was studied in large half-sibling pedigrees generated by mating heterozygous (B+) rams to non-carrier (++) ewes so that the co-inheritance or genetic linkage of the Booroola (Fec^B) locus and the α - and β -subunits of FSH could be analysed. Blood samples were collected from thirteen rams and all the available daughters (median 27, range 9–43 per sire) for each family. Dams mated with the progeny-test rams were never individually identified and no samples could be obtained from these ewes. Ovulation rates of the daughters within the flocks were measured by laparoscopy after sedation and local anaesthesia (Montgomery & Hawker, 1987).

RFLP analysis

DNA was extracted from blood samples collected using heparin as an anticoagulant (Montgomery & Sise, 1990). DNA, 3–10 μ g, was digested using 5 units of the restriction enzymes *Msp*I or *Bst* EII (Boehringer Mannheim, Tutzing, Germany) per μ g of DNA under the manufacturer's recommended conditions. The restricted DNA was run in a 0.8% (w/v) agarose gel in TAE buffer (pH 7.8, 5 mmol Tris-acetate l^{-1} , 1 mmol EDTA l^{-1}) at 25 V/40 mA for 16–20 h. The gels were photographed under UV light, and transferred to Hybond N+ (alkaline transfer, Amersham International plc, Amersham, Bucks, UK) according to the manufacturer's recommended procedures.

Bovine cDNA probes for FSH β - (Maurer & Beck, 1986) and α -subunit (Erwin *et al.*, 1983) were verified by restriction mapping. Isolated insert was labelled with [32 P]-dCTP by nick translation (Rigby *et al.*, 1977). The filters were pre-hybridized for at least 30 min, hybridized overnight at 63°C after addition of the labelled probe and washed to a final stringency of $0.5 \times$ SSC (150 mmol NaCl l^{-1} , 15 mmol sodium citrate l^{-1}) at 63°C for 10 min (Montgomery & Sise, 1990).

Data analysis

Daughters of the progeny test rams were assigned as B+ or ++ at the Fec^B locus from at least three observations of ovulation rate recorded over two years. Ewes with a maximum ovulation rate of three or more were defined as B+ and ewes with ovulation rates of one or two were defined as ++ (Davis *et al.*, 1982). The rams for all families were scored for the polymorphisms at each locus. Only daughters of sires heterozygous at a particular locus were scored at that locus, since only the families of heterozygous rams are informative for linkage. Data were analysed only for daughters that were homozygous at the test loci. The allele inherited from the sire was scored directly in these offspring and the lod scores at different recombination fractions were calculated for pedigrees where the phase is unknown (Ott, 1985).

Quantitative and environmental effects influence ovulation rate and the criteria for assigning phenotypes will vary with the genetic background in which the Fec^B gene is expressed (Piper *et al.*, 1985). Contemporary control flocks were generated as part of the progeny tests for two half-sibling families. Five and seven per cent of these non-carrier ewes

had a single ovulation rate record of three, but no ewes had two records of three. Recombination for both loci was therefore also analysed using restricted definitions to minimize uncertainties in assigning Fec^B phenotypes. For this analysis, heterozygous (B+) ewes were defined as ewes with two ovulation rate records of three or more and homozygous non-carrier (++) ewes were defined as ewes with at least three observations of ovulation rate of one or two. Other ewes were excluded from the analysis.

The different restriction fragments at either the α or β FSH subunit loci define different alleles that may be associated with genetic variation (other than the Fec^B mutation) altering the expression of these genes and consequent physiological functions. The ovulation rates of ewes carrying different alleles at either the α or β FSH subunit loci were analysed by fitting separate least-squares models, including ovulation rate, Fec^B phenotype, and subunit genotype (different alleles) for ewes scored at each locus. The interactions between subunit genotype and Fec^B phenotype were investigated and found not to be significant.

Results

Five of the sires from the 13 progeny test families were heterozygous for the polymorphism. Genetic recombination between the FSH β and Fec^B loci was observed in all families (Table 1). The values for the lod scores were negative in the families studied, except for weak positive values in family 53. The combined lod score calculated from the five half-sibling families at a recombination fraction of 0.15 was -3.22 (Table 1).

Table 1. Analysis of linkage between the Fec^B locus and an insertion/deletion polymorphism at the FSH β locus: lod scores calculated for different recombination fractions for daughters of five progeny-tested B+ rams. The number of daughters analysed for each family is given in parentheses

Recombination fraction	Lod score					Combined estimate
	Family					
	03 (20)	04 (29)	05 (20)	06 (20)	53 (9)	
0.00	−∞	−∞	−∞	−∞	−∞	−∞
0.05	−2.63	−3.79	−2.63	−0.91	0.09	−9.87
0.10	−1.56	−2.20	−1.56	−0.42	0.28	−5.46
0.15	−1.00	−1.36	−1.00	−0.19	0.33	−3.22
0.20	−0.64	−0.84	−0.64	−0.07	0.32	−1.87
0.30	−0.24	−0.28	−0.24	0.02	0.22	−0.52
0.40	−0.05	−0.06	−0.05	0.01	0.08	−0.07

Three of the sires from the 13 progeny test families were heterozygous for the *Bst* EII RFLP at the α -subunit locus. Daughters of the two largest families were scored for the α -subunit RFLP. Results of the Southern blot for one of the families demonstrate that both B+ and ++ daughters inherited the same allele at the α -subunit locus from the sire (Fig. 1). Genetic recombination between the α -subunit and Fec^B loci was observed in both families (Table 2), and the combined lod score calculated from the two half-sibling families at a recombination fraction of 0.10 was -2.81 (Table 2).

Results of the linkage analyses were similar for both loci when the more restrictive definitions were used to assign phenotypes at the Fec^B locus. Genetic recombination was observed between the Fec^B locus and both test loci in all families and the lod scores were similar.

There was no evidence for any association between ovulation rate and the different alleles at either the α or β subunit loci. Least squares means for ovulation rate in ewes homozygous for either the insertion or deletion alleles at the FSH β locus were 2.09 and 2.01, respectively, and did not differ significantly between the genotypes (Table 3). Least squares means for ovulation rate in ewes homozygous for the A1 or A2 alleles at the α -subunit locus were 2.11 and 2.31, respectively, and not significantly different (Table 3).

Fig. 1. Segregation of alleles at the *Fec^B* locus (half filled symbols represent B+ individuals) and alleles (A1 = 10.1 kb, A2 = 8.4 kb) at the locus for the α -subunit of the gonadotrophins in a half-sibling pedigree. The ram was heterozygous for the two alleles at the α -subunit locus.

Table 2. Analysis of linkage between the *Fec^B* locus and a *Bst* EII polymorphism at the α -subunit locus: lod scores calculated for different recombination fractions for daughters of two progeny tested B+ rams

Recombination fraction	Lod score		Combined estimate
	01 (40) ^a	07 (20)	
0.00	−∞	−∞	−∞
0.05	−3.79	−1.35	−5.14
0.10	−2.20	−0.61	−2.81
0.15	−1.36	−0.26	−1.62
0.20	−0.84	−0.06	−0.90
0.30	−0.28	0.07	−0.21
0.40	−0.06	0.04	−0.02

^aThe number of daughters analysed for each family is given in parentheses.

Table 3. Least squares mean ovulation rates of ewes with different genotypes at either the FSH β - or α -subunit loci

Locus	Genotype	Mean ovulation rate	Standard error (SEM)
FSH β	A1A1	2.09	0.10
	A1A2	1.99	0.07
	A2A2	2.01	0.10
α -subunit	A1A1	2.11	0.24
	A1A2	2.41	0.10
	A2A2	2.31	0.13

Discussion

Genes encoding the proteins constituting the heterodimeric pituitary hormone FSH are potential candidates for the site of the Fec^B mutation since concentrations of FSH are increased in BB and B+ ewes (McNatty *et al.*, 1991) and concentrations of FSH are a major determinant of ovulation rate in sheep (see for example Henderson *et al.*, 1991). A single genetic recombination event should exclude a functional mutation in a candidate locus as the site of the Fec^B mutation (Edwards, 1990). False observations of recombination in all cases would result only from large numbers of errors in phenotypic assignments. The combined results from these genetic linkage studies demonstrate that the site for the Fec^B mutation does not lie near the genes for either the α or β subunits of FSH.

Genetic recombination between the FSH β locus and the Fec^B mutation was observed in all five families studied. A lod score of -2 or less is considered significant evidence for non-linkage between loci (Ott, 1985). The combined lod score for all these families extends the exclusion distance between the Fec^B and FSH β loci from the previous estimate (Montgomery *et al.*, 1990) to a genetic distance of 15 cM (1 cM is the distance along the chromosome that gives a recombination fraction of 1%). Genetic recombination was also observed between the α -subunit gene and the Fec^B locus in the two families studied and linkage between the two loci was significantly excluded at 10 cM. The relationship between the recombination fraction and physical distance along the chromosomes has yet to be determined for sheep, but a genetic distance of 15 cM probably corresponds to a physical distance of some 15 million base pairs, far exceeding the distance between any control and coding sequences for these genes.

In humans, cattle and rats there is a single gene encoding the α -subunit and a single gene encoding the FSH β subunit (for review see Gharib *et al.*, 1990). Single bands on the Southern blots of sheep DNA samples suggest that a single gene encodes the sheep α -subunit locus. In contrast, the bovine cDNA for FSH β detects two genomic sequences in sheep (Montgomery *et al.*, 1990; Guzman *et al.*, 1991). Fragments of 15 kb and 10 kb, from digestion of sheep DNA with the restriction enzyme *Bgl* II, have recently been cloned. The 10 kb fragment encodes the gene for the FSH β subunit expressed *in vivo* (Guzman *et al.*, 1991). Comparison of the restriction map for the insertion/deletion polymorphism (Montgomery *et al.*, 1990) and the DNA sequence of the 10 kb fragment (Miller, pers. commun.; Guzman *et al.*, 1991) shows that the polymorphism is associated with the FSH β gene. Expression of the FSH β -like sequences on the 15 kb fragment have not been demonstrated (Guzman *et al.*, 1991), although expression of an FSH-related protein could influence the results from radioimmunoassays. Genetic polymorphisms were not observed in the related sequence while studying the insertion/deletion polymorphism of the FSH β sequence. Specific probes will have to be developed to map the sequence and identify polymorphisms for genetic linkage studies. The sequence would be excluded from genetic linkage to the Fec^B gene, if it was the result of a duplication event at the FSH β locus.

The site of the Fec^B mutation must be located elsewhere in the genome and have indirect actions responsible for the increased concentrations of FSH observed in BB and B+ ewes. The mutation could be located in genes directly involved in the synthesis, glycosylation, mRNA stability, release or degradation of FSH or in an indirect pathway influencing development or feedback regulation of the reproductive axis. The content of bioactive inhibin was lower in the ovaries of ewes of the Booroola strain compared with control Merinos (Cummins *et al.*, 1983) and evidence from Australian Booroola populations suggests that Booroola ewes have significantly lower plasma concentrations of inhibin (McNatty *et al.*, 1991). The identification of genetic polymorphisms associated with other candidate genes will allow a direct test of the presence of the Fec^B mutation near these genes.

The genetic polymorphisms identified at the α and β subunits of FSH identify different alleles that are passed on to successive generations. The insertion/deletion polymorphism previously reported near the FSH β gene is approximately 2 kb in size (Montgomery *et al.*, 1990) and located downstream from the third exon of the gene, but additional variation in the DNA may occur upstream in the gene sequence. Variation in the sequence of one of the alleles that influenced the expression of the gene or the gene product may alter physiological function. We therefore examined the ovulation rates of ewes carrying the different alleles at each locus. There was no evidence from the present studies for any differences in ovulation rate between ewes carrying either the insertion or deletion alleles at the FSH β locus or the different alleles at the α -subunit locus.

The site of the Fec^B mutation does not lie within or close to the genes for the α and β subunits of FSH and must be located elsewhere in the genome. The high concentrations of FSH observed in BB and B+ ewes must result from indirect actions of the mutation on the synthesis, processing, storage, release or metabolism of FSH.

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