Initial results of genomic scans for ovulation rate in a cattle population selected for increased twinning rate[1]

S. M. Kappes[2], G. L. Bennett, J. W. Keele, S. E. Echternkamp, K. E. Gregory, and R. M. Thallman

U.S. Meat Animal Research Center, USDA, ARS, Clay Center, NE 68933

ABSTRACT: Genomic scans were conducted with 273 markers on 181 sires from a cattle population selected for increased twinning rate to identify chromosomal regions containing genes that influence ovulation rate. Criteria used for selecting markers were number of alleles, ease of scoring, and relative position within linkage group. Markers were multiplexed or multiple-loaded on the gels to reduce the costs and labor required to obtain genotypic data. This approach reduced the number of gels by 45% when compared with running each marker independently. Male animals selected for the genomic scan sired the majority of the population. A modified interval analysis was used in a granddaughter design to compare effects of each allele within sire for 10 different sire families. The midparent deviation of the son's estimated breeding value for ovulation rate was used as the phenotype. Forty-one potential peaks were identified with a nominal significance level ≤ 0.05. The 10 peaks with the highest significance levels ($P <$ 0.02) were selected for further analysis. Markers were genotyped across daughters of the sire where nominal significance was found for each of the 10 peaks. One peak (BTA5, relative position 40 cM) was found to be nominally significant in the daughters. The nominal significance levels were $P = 0.01$ for the sons (n = 32) and $P = 0.02$ for the daughters (n = 94) of sire 784403. A combined genomewide significance value ($P = 0.07$) was calculated that accounted for the 10 analyses with sons and the 10 analyses with daughters. These results strongly suggest that this region contains a gene(s) that is involved in the follicular recruitment and development process.

Key Words: Cattle, Gene Mapping, Ovulation, Twinning

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Introduction

Reproductive performance is a major determinant of profitability for cattle producers. Melton (1995) found reproductive traits to be more than twice as economically important as production traits for commercial cow-calf producers. Selection response for reproductive performance has been limited because of the long generation interval for progeny testing and low heritability. If loci affecting reproductive performance traits can be identified, then DNA markers can be used to select genetically superior animals and improve the response to selection. Bovine linkage maps with moderate marker density have recently been published with 703 (Barendse et al., 1997) and 1,250 (Kappes et al., 1997) markers. These maps provide the basis for scanning the genome to identify chromosomal regions that affect any economically important trait.

A cattle population (Gregory et al., 1990, 1997) developed and maintained at the USDA-ARS, U.S. Meat Animal Research Center (**MARC**) has been selected for increased twinning rate since 1981. The current rate of multiple ovulation is approximately 25% in 12- to 18-mo old heifers, and the current twinning rate exceeds 35% (Echternkamp and Gregory, 1999). The MARC twinning population was used to initiate a **QTL** project designed to identify ovulation rate and twinning rate loci and subsequently identify critical components of ovulation, conception, and embryonal/fetal survival.

The objective of this research was to identify chromosomal regions containing genes affecting ovulation rate. Two hundred seventy-three markers were used to scan the genome in 181 sires from the MARC twinning population. Ten chromosomal regions with the highest probability of being real were identified from the genomic scan and were further evaluated in daughters of sires where nominally significant effects were found. It is

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[2]Current address and correspondence: USDA, ARS, NPS, 5601 Sunnyside Ave., Rm 4-2164, George Washington Carver Bldg., Beltsville, MD 20705-5138 (phone: 301-504-4736; fax: 301-504-5467; E-mail: kappes@ars.usda.gov).

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expected that genes influencing ovulation rate would be involved in follicular recruitment and(or) development.

Materials and Methods

Animals and Tissue Collection. Blood or ear tissue was collected from almost all females (n = 2,582) born in the MARC twinning population since 1992 and some of the females born in previous years that were still available in 1994. The twinning project was established in 1981 and very few tissue samples were collected in the earlier generations. Semen samples (n = 293) were available from a large number of older sires and essentially all of the younger sires used in the population. Ear notches and semen samples were used as the primary source of DNA because contaminating alleles from the co-twin are detected in blood from twin-born calves (Plante et al., 1992; Schellander et al., 1992). Leucochimerism is found in bovine twins because anastomoses occur between the chorioallantoic vessels of the two placentas (Owen 1945; Ohno et al., 1962). More than 90% of multiple pregnancies lead to development of chorioallantoic anastomoses (Lillie, 1916; Horton et al., 1980).

DNA Extraction. Genomic DNA was extracted from semen of 181 sires that were progeny tested or in the process of being progeny tested and had semen available. The DNA from the 181 sires was used in a genomic scan. Results from the genomic scan were used to select female progeny to genotype in a locus scan of daughters. Genomic DNA used in the locus scan was extracted from primarily ear notches of 174 females that were progeny of selected sires.

Genomic DNA was extracted from semen with a modified phenol/chloroform method that provided improved yields relative to the standard method. Semen (0.25 mL) was washed with 1 mL of TNE (10 m*M* TrisCl, pH 8.0; 0.1 *M* NaCl, and 1 m*M* EDTA, pH 8.0) three times, resuspended in 500 μL of TNE, 25 μL of 20% SDS, 25 μL of proteinase K (20 mg/mL), and 25 μL of 1 *M* DTT, and incubated overnight at 37°C. The digested protein was removed with phenol/chloroform and the DNA was precipitated with 5 *M* NaCl and 95% EtOH. Genomic DNA was extracted from blood by the salt extraction method (Miller et al., 1988) and from ear tissue by a modified salt extraction method. The modified method was used because DNA extracted from some ear tissue by standard methods would not amplify with PCR. Most of the hair was removed from the ear tissue and the tissue was minced, placed in a conical tube with 3 mL of digestion buffer (10 m*M* NH_4Cl, 400 m*M* NaCl, 2% SDS, and 50 m*M* Na_2EDTA, pH 8.0) and 70 μL of trypsin (5 mg/mL; Gibco BRL, Grand Island, NY), and incubated for 1 h at room temperature. Proteinase K (50 μL of 50 mg/mL; Sigma, St. Louis, MO) was added to the conical tube and the solution was incubated in a shaking water bath at 50°C until the tissue was digested. Additional proteinase K was added if the tissue was not completely digested after 4 to 6 h. One-third volume of 5 *M* NaCl (1 mL) was added to the digested tissue and mixed gently, followed by centrifugation and removal of the supernate. Two and one-half volumes of 95% EtOH were added to the supernate and the DNA pellet was removed and washed in 70% EtOH.

Genotype and Marker Information. Marker data were collected, verified, and entered into a relational database (Keele et al., 1994) according to Kappes et al. (1997). Marker information and PCR conditions for multiplexing DNA markers used in the genomic scan and subsequent genotyping are presented on the Web site http://sol.marc.usda.gov. The PCR reaction mixture was the same as Kappes et al. (1997) except where the $MgCl_2$ or primer concentrations are indicated on the Web site. Markers were labeled by random incorporation of [α-^{32}P]dATP, except for six markers that were end-labeled with [γ-^{32}P]dATP. The PCR primer sequences and related marker information can also be found at http://sol.marc.usda.gov/genome/cattle/cattle.html. The heterozygosity, allele number, minimum and maximum allele size, and unamplified allele information were obtained from genotypic data from the entire twinning population.

Markers were either multiplexed (more than one marker per PCR reaction) or multiple-loaded (markers amplified in separate PCR reactions but loaded on the same gel) when possible to minimize reagent cost and labor. Amplification results from the MARC mapping population (Bishop et al., 1994; Kappes et al., 1997) were used to determine which markers could be multiplexed or multiple-loaded. Some of the markers that had excellent amplification results in the mapping population were tried as multiplex PCR reactions. For the genomic scan, 6 markers were triplexed, 143 duplexed, 3 markers triple loaded, 94 double loaded, and 27 loaded as singles, resulting in a 45% reduction in the number of gels required.

Genomic Scan and Analysis. The genomic (whole-genome) scan consisted of genotyping 273 markers in the 181 progeny-tested sires. The 273 markers were selected from the USDA-MARC bovine linkage map (http://sol.marc.usda.gov/genome/cattle/cattle.html) with marker selection based on map location, number of alleles observed in the MARC reference population (Bishop et al., 1994), and ease of scoring. The objective was to cover all 29 autosomes and the X chromosome with moderate to highly informative markers at 10- to 20-cM intervals. Additional markers were added to regions containing markers with few alleles or in regions containing a potential locus for ovulation rate. The number of markers per chromosome used for the genomic scan and length of each linkage group are presented in Table 1.

The number and paternal relationship of the males used in the analysis of the genomic scan data are presented in Table 2. The statistical analysis was performed on 10 different sire families (Table 3), which included 150 of the 181 genotyped sires. The remaining 31 sires were from very small paternal families and

Table 1. Number of markers used in genomic scan and length of each linkage group

Chromosome	No. of markers	Length, cM
1	17	142
2	13	120
3	14	125
4	12	101
5	13	133
6	12	125
7	15	134
8	13	116
9	12	109
10	12	101
11	13	123
12	8	106
13	7	87
14	7	87
15	9	94
16	6	93
17	7	99
18	7	81
19	8	99
20	6	75
21	6	88
22	5	79
23	6	67
24	5	62
25	6	65
26	6	73
27	5	64
28	6	52
29	5	65
X	12	15

had very few daughters with ovulation rate records. The trait analyzed was the son's predicted breeding value (**PBV**) for ovulation rate minus the average of the sire's and dam's PBV. Because a son's PBV deviation from parental average is largely determined by the performance of his daughters, the genomic scan is similar to a granddaughter design.

The CHROMPIC option of CRI-MAP (Green et al., 1990) was used to determine which sons received putative QTL alleles from the paternal or maternal chromosome of a sire at a marker location. This procedure uses the relationships among sons and linked markers to establish grandparental origin. The interval method of Haley et al. (1994) was adapted to determine the probability of grandpaternal origin between marker locations and in cases in which markers were ambiguous. Marker order and interval distance was obtained from the MARC bovine map on the Web site.

The PBV deviation was regressed on the probability of grandpaternal origin for each sire family to estimate the slope and its standard error. The slope was divided by its standard error and the quotient squared to calculate an *F*-value with the numerator degree of freedom equal to 1 and denominator df equal to the number of sons − 2. A pooled *F*-value was calculated by adding the sums of squares for regression and the residual sums of squares from the 10 sire families of sons. The pooled *F*-value has a numerator df equal to 10 and a denominator df equal to 125. Calculations of individual family and pooled *F*-values were made for every 2 cM of the autosomal chromosomes.

Locus Scan and Analysis. The locus scan consisted of genotyping markers for 10 chromosomal regions (Table 4) that were previously identified in the genomic scan. Markers from a chromosomal region were only genotyped in daughters of the sire when nominally significant effects were found. The number and paternal relationship of the daughters used in the locus scan are presented in Table 2 and the number of markers genotyped for each chromosomal region are presented in Table 4.

The same method of analysis was used for the locus scan as was used for the genomic scan. The trait analyzed in the locus scan was the daughter's PBV for ovulation rate minus the average of the sire's and dam's PBV. The locus scan is similar to a daughter design because a daughter's PBV deviation from parental average is largely determined by her own ovulation rate. Calculations of *F*-values for the locus scan were made

Table 2. Paternal relationship of animals used in genomic scans and locus scans

Breed of grandsire[a]	Grandsire	Sire family	Sons in genomic scan	Daughters in locus scan[b]
Pinzgauer	—	784403	32	94
Norwegian Red	—	849801[c]	12	7
	—	849802[c]	11	20
Charolais	—	729809[c]	7	—
Simmental × Holstein	—	818827	8	24
	818827	849706	13	—
Swedish Friesian	300077[c]	839801	5	—
	300077[c]	839802[c]	11	29
	300077[c]	839803	36	—
	839802[c]	868554[cd]	10	—

[a]Breed of sire when grandsire is not listed.
[b]Daughters of animal listed in Sire column.
[c]DNA was not available for genomic scan.
[d]Maternal grandson of 784403.

Table 3. Number and location of nominally significant peaks identified by analysis of genomic scan

Family	$P \leq 0.05$	$P \leq 0.01$
729809	1	
784403	8	Chr 5
818827	7	
839801	0	
839802	5	Chr 11
839803	4	
849706	0	
849801	6	
849802	2	Chr 7, 19
868554	1	
Pooled	3	

every centimorgan of a targeted chromosomal region in a sire family.

A combined genomewide significance value was calculated that accounted for the 10 analyses with sons and the 10 analyses with daughters. The scan of breeding value deviations from midparent averages for sons was considered independent from the scan of daughters. The combined probability was calculated as the product of the probabilities of the genomic (son) scan and the locus (daughter) scan. The probability for the genomic scan (p_{son}) was calculated as described by Lander and Kruglyak (1995) for an entire genome scan in a single sire family and adjusted for the 10 independent sire families by subtracting $(1 - p_{son})^{10}$ from 1. The nominal probability of a locus scan (p_{dau}) that agreed in sign with the genomic scan was calculated from the *F*-value and divided by 2 to account for sign agreement. This probability was further adjusted for the total number of locus scans (N_{LS}) done similar to the genomic scan, $[1 - (1 - p_{dau})^{N_{LS}}]$.

Results and Discussion

The MARC twinning population (Gregory et al., 1990, 1997) has been selected for increased twinning rate since 1981. Ovulation rate and twinning rate data were used in a multitrait animal model to predict breeding values, which were used as a tool for selection (Van Vleck et al., 1991; Van Vleck and Gregory, 1996). Ovulation rate data are collected by ovarian palpation for six to eight estrous cycles per female. Twinning rate is calculated as the average number of calves born per parturition. The heritability estimates reported for this population for a single observation of ovulation rate and twinning rate were 0.10 and 0.09, respectively. The estimate of heritability was 0.35 for the mean ovulation rate from six estrous cycles (Gregory et al., 1997). The estimated genetic correlation between ovulation rate and twinning rate is 0.75. The current rate of multiple ovulation is approximately 25% in 12- to 18-mo-old heifers, and the current twinning rate exceeds 35% (Echternkamp and Gregory, 1999).

Results from the genomic scan analysis revealed 41 potential peaks exceeding a nominal significance value of 0.05 (Table 3). Here, a peak was defined as the location of the highest significance level for a chromosome within a sire. These peaks were identified in an analysis that compared the association of each chromosomal source within a sire with the midparent deviation of the PBV for ovulation rate in his sons. This procedure was applied to 10 different sires (Table 3) that had several progeny-tested sons. The 10 sires and their descendants actually represent six sire lines because two sires (849706, 868554) are sons of another two sires and three sires (839801, 839802, and 839803) are paternal half-sibs. The results from the pooled analysis on the

Table 4. Results from genomic and locus scans

	Genomic		Locus	
Family and chromosome	Relative position, cM	*P*	Number of markers	*P*
784403				
5	40	0.01	8	0.02
9	52	0.02	5	0.11[a]
25	0	0.02	4	0.03[a]
839802				
11	104	0.01	4	0.52
23	12	0.02	4	0.67
818827				
1	8	0.01	4	0.25
15	48	0.02	4	0.42[a]
849801				
10	88	0.02	5	0.38[a]
849802				
7	114	0.01	5	0.22
19	100	0.01	4	0.40[a]

[a]Indicates that the effect of the two alleles within a sire was reversed from the genomic scan to the locus scan.

genomic scan data suggested ($P < 0.05$) that potential peaks may exist on chromosomes 5, 13, and 15.

Ten peaks (Table 4) with the highest significance value ($P < 0.02$) were selected for the locus scan and they were only found in five sires. All of the peaks were found in only one sire. Markers for the locus scan were selected from the 10 potential regions and genotyped only in the daughters from the specific sire where the peak was initially identified. The analysis for the locus scan was performed independently from the genomic scan analysis but still comparing chromosomal source within a sire.

Results of the locus scan (Table 4) indicated two peaks (BTA5 and 25) with nominal significance values of $P < 0.05$. However, effects of the two alleles for the chromosome 25 region in the daughters of sire 784403 were reversed relative to the genomic scan (sons). This result was interpreted as not supporting the results of the genomic scan analysis for this region and it was dropped as a potential peak.

The *F*-statistic profiles for the genomic and locus scans of chromosome 5 for the sons and daughters of sire 784403 are shown in Figures 1 and 2, respectively. The probability of obtaining one *F*-value at least as large as the 8.919 (Figure 1) found on chromosome 5 for the 32 sons of 784403 is 0.94. The probability increases to 1.0 for 10 independent scans. The probability of finding an *F*-value of 6.144 (Figure 2) in 94 daughters is 0.015. Adjusting this for agreement in sign with the independent sons and for 10 locus scans yields a probability of 0.07. Then, the significance level of the combined results is 0.07. This probability indicates that this result is only expected seven times out of 100 genome scans if no loci affecting ovulation rate were segregating in these 10 sires. This probability surpasses the genomewide suggestive level and almost reaches genomewide significance level (probability of 1 false-positive out of 20 genome scans) of Lander and Kruglyak (1995).

A large genotyping effort was required to identify this locus that is affecting ovulation rate in cattle. Multiplexing and multiple loading of markers has reduced the number of gels, cost, and labor required to collect the genotypic data. This process can be made more efficient with additional testing, especially with multiplexing markers. Markers that work in multiplex reactions can be used in different combinations; however, some specific combinations of primer pairs do not work together. Markers that were multiple-loaded will work in many different combinations as long as the allele sizes do not overlap. Markers that were amplified and loaded as a single generally have additional bands that would interfere with scoring another marker on the same gel. The multiplex and multiple loading results should not be considered conclusive but serve as a guide to design specific marker combinations to improve the efficiency of the genotyping process.

Blood samples are commonly used as the source of DNA for genotyping. However, leucochimerism, a product of anastomoses of the chorioallantoic vessels found between bovine twins early in gestation (Owen 1945; Ohno et al., 1962), has been observed in blood samples from twin-born cattle (Plante et al., 1992; Schellander et al., 1992). The freemartin syndrome in cattle is also the result of chorioallantoic anastomoses, and it is caused by the transfer of müllerian inhibiting sub-

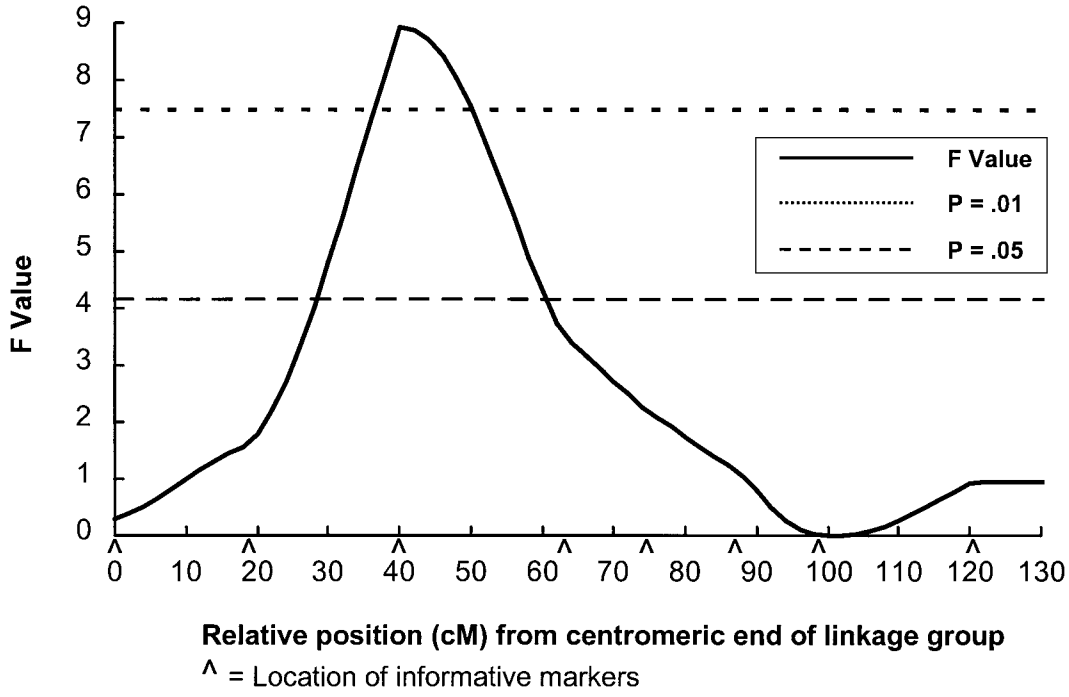

Figure 1. Ovulation rate *F*-statistic profile on chromosome 5 from genomic scan analysis of sons of 784403.

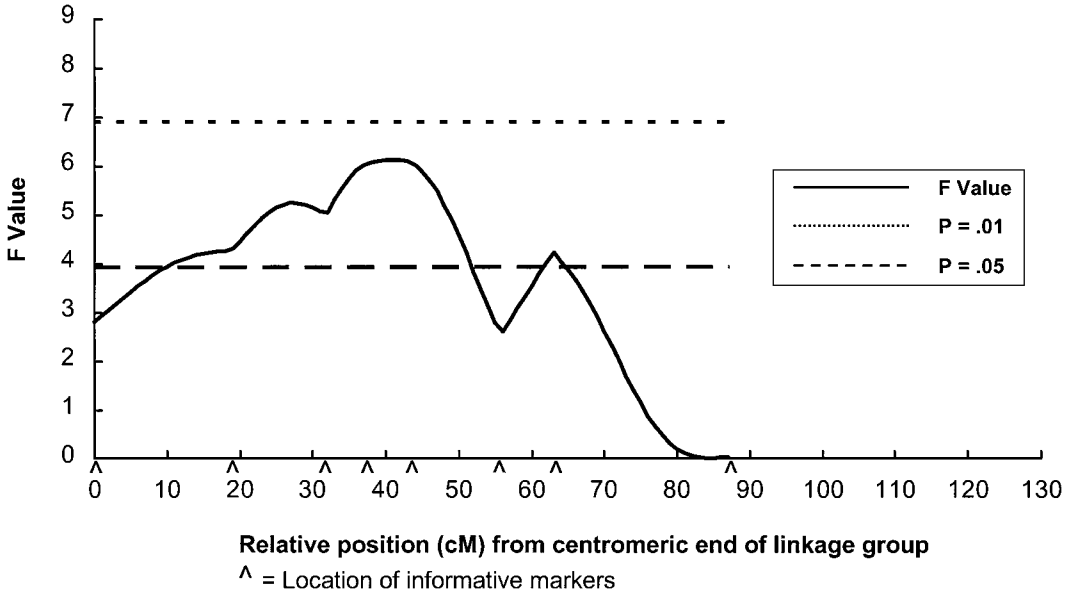

Figure 2. Ovulation rate *F*-statistic profile on chromosome 5 from secondary scan analysis of daughters of 784403.

stance from the male to female fetus, which suppress differentiation of the female reproductive tract. In spite of 96% of the females from heterosexual twins being freemartins (Gregory et al., 1996), evidence for a contaminating allele from the co-twin has not been observed in DNA extracted from >2,400 ear notches from females born in the twinning population. This result might be expected because the majority of skin grafts between leucochimeric cattle are eventually rejected despite the presence of a high proportion of leucocytes from the donor of the skin graft in the blood system of the recipient (Stone and Cragle, 1968).

Preliminary results from the genomic and locus scans indicate that it is extremely likely that a gene(s) in the chromosome 5 region influences ovulation rate in cattle. The low number of sons (5 to 36) per sire analyzed from the genomic scan data limits the ability to detect additional loci that influence ovulation rate. Because the genomic scan only serves to limit the regions that might be genotyped in the rest of the population (>2,800 DNA), a very relaxed statistical threshold (nominal significance < 0.05) is used to select potential peaks for the secondary scan. Only 10 peaks ($P < 0.02$) have been evaluated in locus scans. There are 31 remaining peaks with a nominal significance value of $P < 0.05$ that could be evaluated in daughters of the specific sire where the peak was identified. It is likely, with the current genotyping and analysis protocol, that a number of loci that are affecting ovulation rate were not detected or were detected in the genomic scan and supporting evidence was not found in the daughters because of the small number of daughters per sire (n = 7 to 94).

An earlier genomic analysis (Blattman et al., 1996) of progeny from three of the sires (784403, 839802, and 839803) indicated that regions on chromosomes 7 and 23 may affect ovulation rate. Our genomic scan analysis did detect peaks on chromosomes 23 and 7 but with some differences. We detected peaks on chromosome 23 in the paternal half-brother (839802; position 12 cM; $P = 0.02$) and in the same sire (839803; position 26 cM; $P = 0.03$) as identified in the Blattman analysis (position 31 cM on the MARC map); however, our locus scan on 839802 daughters did not support the genomic scan results. Blattman's initial analysis included fewer informative markers per sire (n = 44 and 45) with similar number of progeny (n = 20 and 41) for the 839802 and 839803 sires, respectively, when compared with our genomic analysis (201 and 188 informative markers and 11 and 36 progeny for 839802 and 839803, respectively). The animals used in the Blattman et al. (1996) analysis generally were born in earlier years than the animals used in the present study. Our locus scan analysis only included 29 daughters from the 839802 sire, whereas Blattman's secondary analysis included at least 136 progeny and grandprogeny.

Our chromosome 7 peak was found in a different and unrelated sire (849802; position 114 cM) and at a different location than the Blattman et al. (1996) results (839802; positions 0 and 60 cM on the MARC map). The results from our locus scan analysis on 20 daughters from 849802 did not support the genomic scan analysis.

The analyses used in the present study and in Blattman et al. (1996) used only small subsets of data to make comparisons between specific alleles and, therefore, utilized a very small amount of mapping information that is available for any locus in this population. This population is the result of a long-term selection project that represents a complex pedigree with many loops in the relationships among animals. The multiple generations in this pedigree provide a large amount of mapping information that was not captured in either

analysis. Computational requirements of current analytical programs limit the amount of pedigree information that can be analyzed at one time. We are developing an approximate method for calculating founding allele probabilities that will allow the entire population to be used in a QTL detection analysis. Analyzing the entire population with additional genotypes and combining the genotypic data sets from this study and Blattman et al. (1996) will resolve the differences in results between the two studies.

Implications

Genetic factors that affect reproduction are not well understood, and identifying these factors could lead to substantial improvements in these traits, primarily by genetic selection but also with pharmaceuticals to enhance reproduction. The MARC twinning population is well suited to identify genes that influence ovulation rate and twinning rate. Genes that influence ovulation rate are likely to be involved in follicular recruitment and development or ovulation. Genes that influence twinning rate and not ovulation rate are likely to be involved in fertilization, embryo survival, implantation, or other components of conception. Current results strongly suggest that a gene(s) exists on chromosome 5 that affects ovulation rate in cattle. Identification of this gene(s) and other genes affecting ovulation rate and twinning rate will increase the basic understanding of the reproductive process and may also be used to improve reproductive efficiency in cattle.

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