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Gene mapping of sperm quality parameters in recombinant inbred strains of mice

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ABSTRACT The aim of this study was to map chromosomal regions containing hypothetical genes responsible for the following parameters of mouse semen quality: (1) the percentage of sperm with abnormal head morphology, (2) the level of dead spermatozoa, (3) the percentage of sperm tails with residual cytoplasmic droplets, and (4) the percentage of sperm with impaired sperm tail membrane integrity. We also analyzed any possible correlations between these parameters. The most appropriate animal model for mapping genes controlling quantitative traits (QTL, quantitative trait locus) is a set of recombinant inbred (RI) strains. The set of RI strains used in this study was derived from crosses between two inbred mouse strains, KE and CBA/Kw, which differ significantly in fertility parameters and gamete quality. We analyzed the four parameters of sperm quality in male mice from two parental strains and from 12 RI strains. The strain distribution pattern (SDP) of 187 polymorphic microsatellite markers was prepared for 20 chromosomes of the mouse genome in 12 RI strains. We correlated the SDP of these markers with the values of sperm quality parameters, using MapManager QTX software (ver. b18). The mapping procedure indicated that the percentage of sperm with abnormal head morphology is controlled by gene(s) located in chromosomal regions 11q24, 11q31 and 6q15.6. There was also a strong correlation between male body weight and the hypothetical gene(s) in chromosomal region 18q47. A detailed analysis of the genes located in these regions enabled us to prepare a list of candidate genes. We discuss the basis of the correlation between the measured parameters.

KEY WORDS: *gene mapping, mouse, sperm quality*

Introduction

About 13-25% of human couples cannot conceive (Matzuk and Lamb, 2002; Olds-Clarke, 2003; Frey and Patel, 2004; Nayernia *et al*., 2004; Rajeev and Reddy, 2004), and approximately half of these cases are due to male fertility problems (Brugh and Lipshultz, 2004; Nayernia *et al*., 2004; Nikpoor *et al*., 2004). Spermatogenesis in mammals is regulated by genes located on the Y chromosome (Krzanowska, 1969; Burgoyne, 1998), and by autosomal genes such as *CFTR* (Foppiani *et al*., 2004) or genes coding for androgens, estrogens and their receptors (Robertson *et al*., 1999; Henderson and Robaire, 2005; Huhtaniemi 2006). Genetic causes are considered the main factor responsible for as much as 30% of human infertility (Nayernia *et al*., 2003), but knowledge of the genes controlling human sperm quality is poor (Nikpoor *et al*., 2004; Vogt, 2004).

Recombinant inbred (RI) strains of mice (Bailey, 1971; Taylor, 1978) allow mapping of the genes controlling quantitative traits (QT) (Silver, 1995; Williams *et al*., 2001; Broman, 2005). In our department we maintain KE and CBA/Kw strains of mice, which differ in gamete quality and fertility parameters (Krzanowska, 1970; Krzanowska, 1976; Kaleta,1977; Styrna and Krzanowska, 1995). A set of 12 RI strains was developed from these two progenitor strains. We used this animal model to map chromosomal regions containing genes coding for four mouse semen quality parameters that are also evaluated during semen analysis in humans: percentage of sperm head abnormalities, level of dead spermatozoa, percentage of sperm with residual cytoplasmic droplets (which determines the degree of sperm cell maturity), and the integrity of the sperm tail cytoplasmic membrane.

To obtain the complete marker map in 12 RI strains we analyzed polymorphic microsatellite sequences. We used MapManager QTX (ver. b18; Manly *et al*., 2001) to search for the

Abbreviations used in this paper: HOS, hypoosmotic (test), QTL, quantitative trait locus; RI, recombinant inbred (strain); SDP, strain distribution pattern.

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similarities between the strain distribution patterns (SDP) of these markers and the distribution of the measured parameters' values. This procedure enabled us to determine the most probable location of the QTL (quantitative trait locus) controlling these parameters. Further work is required to determine whether and how the candidate genes located in the mapped regions regulate gamete quality. The correlations between the analyzed parameters are discussed here. These are new results on genetic control of semen quality parameters in mice. The model should be useful in gaining an understanding of genetically based human infertility.

Results

Microsatellite sequence analysis

Of the 323 microsatellite sequences analyzed in 20 chromosomes of the mouse genome, 187 showed differences in PCR product length between the two progenitor inbred mouse strains, KE and CBA/Kw. These polymorphic sequences were further analyzed in order to prepare SDPs for 12 RI strains (RI 5, 6, 17, 21, 29, 40, 41, 42, 43, 44, 47, 49). The average distance between two consecutive markers on the chromosomes was 7.6 cM.

Analysis of mouse semen quality parameters

Table 1, 2 and 3 show the average values of the measured parameters, standard deviations (+/-SD), and the statistical significance ($p<0.05$) of between-strain differences.

The body mass of the tested males showed little variation in the analyzed strains. The body mass of three RI strains (RI 21, 29, 43) was significantly lower than that of both progenitor strains. Testis weights of KE and CBA/Kw males differed significantly ($p<0.05$), and this parameter showed segregation in all 12 RI tested. The percentage of live spermatozoa was low in all analyzed strains. The males of only two RI strains (RI 41 and RI 47) had significantly less live sperm than both parental strains (Table 1).

The values of all five types of sperm head morphology significantly differed between the two progenitor strains. The total percentage of sperm head abnormalities was higher in KE males. These parameters revealed strong segregation in all 12 RI strains tested (Table 2).

TABLE 1

Inbred strains	Body mass (g) (+/-SD)	Testis weight (g) (+/-SD)	Alive spermatozoa in eosin test (%) (+/-SD)
KE	27.9+/-2.9	0.207+/-0.024 [b]	32.7+/-5.7
CBA/Kw	28.1+/-2.8	0.140+/-0.015 [a]	33.3+/-6.5
RI 5	28.2+/-2.1	0.120+/-0.007 [a b]	36.2+/-2.7
RI 6	27.0+/-3.8	0.159+/-0.027 [a]	29.4+/-4.8
RI 17	26.6+/-3.3	0.184+/-0.017 [a b]	34.9+/-7.6
RI 21	21.2+/-2.5 [a b]	0.178+/-0.044 [b]	33.0+/-7.3
RI 29	21.7+/-2.1 [a b]	0.141+/-0.018 [a]	35.1+/-3.2
RI 40	30.1+/-3.4	0.161+/-0.009 [a b]	32.5+/-3.5
RI 41	26.8+/-1.8	0.164+/-0.013 [a b]	23.0+/-6.1 [a b]
RI 42	27.8+/-2.1	0.161+/-0.01 [a b]	29.5+/-4.1
RI 43	23.8+/-3.0 [a b]	0.175+/-0.024 [a b]	30.2+/-4.6
RI 44	29.2+/-4.1	0.129+/-0.012 [a]	35.2+/-13.4
RI 47	26.4+/-4.9	0.123+/-0.021 [a]	21.0+/-7.2 [a b]
RI 49	26.2+/-3.1	0.168+/-0.012 [a b]	30.1+/-7.8

The results of body mass, testis weight and eosin test analysis in both parental strains (KE and CBA/Kw) and in 12 RI strains of mice. The average values and the standard deviation (SD) values are presented. Statistically significant differences between tested strain and (a) KE strain and (b) CBA/Kw strain are also indicated.

The KE and CBA/Kw strains did not differ significantly in the percentage of sperm with a distal droplet or proximal droplet, nor in the number of sperm without residual droplets. These parameters showed weak segregation in the analyzed RI males, with proximal droplet level being the most varied characteristic (Table 3). Hypoosmotic tests showed the two progenitor strains to differ significantly in the percentage of sperm with broken tails and sperm with slightly bent tails. Males of the RI strains showed moderate segregation of four classes of sperm tail membrane integrity (Table 3).

Pearson's correlation coefficient (r) was statistically significant for the percentage of sperm with abnormal head morphology and the percentage of live sperm ($p<0.05$, $r=-0.57$). The percentage of sperm with slightly bent tails in the HOS test ($r=0.56$) and testis weight ($r=0.55$) were also correlated with the level of sperm head abnormality. The percentage of live sperm and the number of

TABLE 2

	The level of the five classes of the abnormal sperm head morphology in all inbred strains testes (%) (+/- SD)					Total abnormal sperm
Inbred strains	class 1	class 2	class 3	class 4	polyploid cells	heads level (%) (+/-SD)
KE	1.01+/-1.23 [b]	11.12+/-3.15 [b]	5.89+/-1.82 [b]	2.66+/-1.38 [b]	0.00 [b]	20.3+/-4.0 [b]
CBA/Kw	0.31+/-0.48 [a]	2.19+/-0.93 [a]	2.84+/-1.21 [a]	1.13+/-0.66 [a]	0.15+/-0.23 [a]	6.6+/-1.9 [a]
RI 5	0.21+/-0.49 [a]	2.58+/-1.05 [a]	1.56+/-0.80 [a b]	1.66+/-0.84 [a b]	0.03+/-0.11 [b]	6.0+/-1.9 [a]
RI 6	0.20+/-0.26 [a]	3.87+/-1.48 [a b]	4.01+/-1.04 [a]	3.13+/-0.94 [b]	1.07+/-0.71 [a b]	12.3+/-1.9 [a b]
RI 17	0.92+/-1.85	4.62+/-2.52 [a b]	4.43+/-2.37 [a b]	1.72+/-1.21 [a]	0.00 [b]	11.7+/-3.1 [a b]
RI 21	2.03+/-1.86 [b]	2.21+/-1.35 [b]	7.36+/-3.40 [b]	2.37+/-1.22 [b]	0.00 [b]	13.3+/-3.2 [a b]
RI 29	0.20+/-0.19	2.39+/-1.01 [a]	3.51+/-1.33 [a]	1.51+/-0.93 [a]	0.21+/-0.34 [a]	7.8+/-2.4 [a]
RI 40	0.25+/-0.41 [a]	3.26+/-1.07 [a b]	3.21+/-1.57 [a]	1.10+/-0.64 [a]	0.08+/-0.22	7.9+/-2.2 [a]
RI 41	0.25+/-0.62 [a]	2.74+/-1.02 [a]	2.11+/-0.94 [a b]	2.10+/-1.00 [b]	1.03+/-0.81 [a b]	8.2+/-1.8 [a b]
RI 42	0.40+/-0.52	3.59+/-1.52 [a b]	4.29+/-1.44 [a b]	0.91+/-0.44 [b]	0.12+/-0.32	9.3+/-2.4 [a b]
RI 43	0.33+/-0.51 [a]	5.98+/-1.85 [a b]	4.25+/-1.58 [a b]	3.16+/-1.78 [b]	0.04+/-0.12	13.8+/-3.1 [a b]
RI 44	0.38+/-0.47	1.88+/-0.92 [a]	3.42+/-0.84 [a]	1.20+/-0.75 [a]	0.20+/-0.34 [a]	7.1+/-1.6 [a]
RI 47	0.51+/-0.38	4.90+/-1.78 [a b]	6.29+/-1.63 [b]	1.63+/-0.99 [a]	1.31+/-0.88 [a b]	14.6+/-2.9 [b]
RI 49	0.68+/-0.60	4.74+/-1.64 [a b]	4.67+/-1.77 [b]	1.77+/-0.80 [a b]	0.15+/-0.22 [a]	12.0+/-3.1 [a b]

The results of five sperm head morphology abnormality classes and total percentage of abnormal spermatozoa analysis. The standard deviation (SD) values and statistically significant differences between tested strain and KE strain (a) and CBA/Kw strain (b) are also indicated.

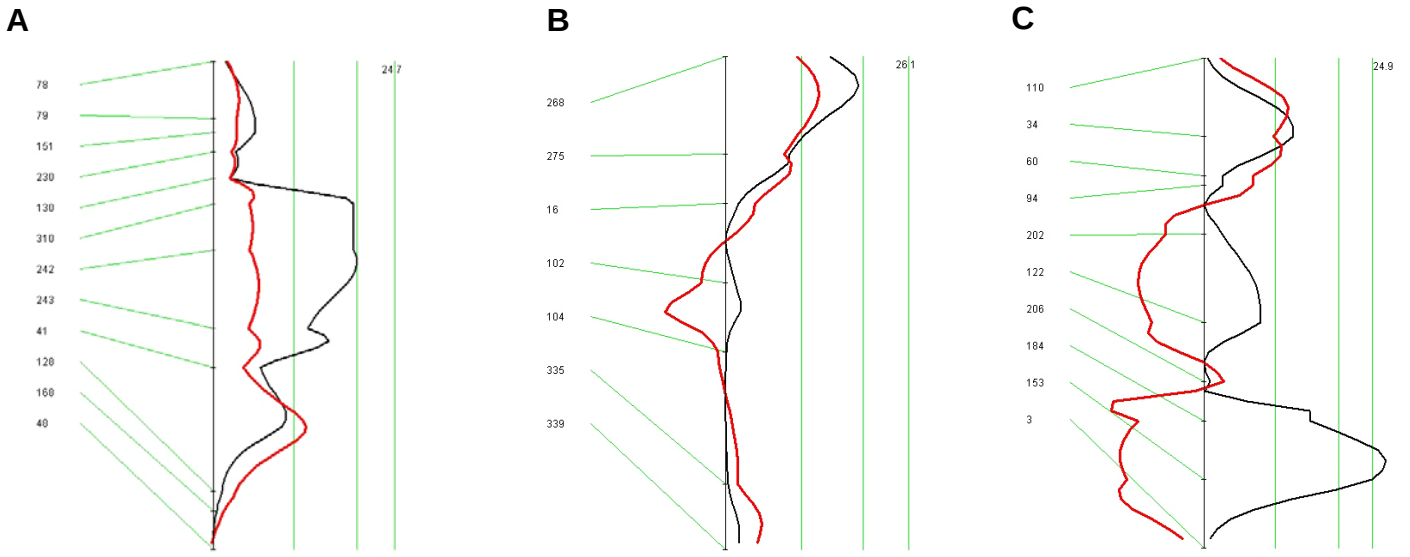

Fig. 1. The mapping results of sperm head abnormality percentage on chromosome 11(A) and on the chromosome 6 (B), and the results of male mice body mass gene mapping on chromosome 18 (C) are show.

sperm with swollen tails in the HOS test correlated positively (r=0.64).

The results of the mapping procedure are shown in Table 4, presenting the correlations obtained from the marker regression procedure that are statistically significant at p<0.001. The interval mapping procedure revealed two chromosomal regions that correlate significantly (p<0.05 for the whole genome scan) with the percentage of abnormal sperm head morphology, and one chromosomal region highly correlated with the body mass of the tested males. These chromosomal regions were searched for candidate genes possibly responsible for these two parameters.

Chromosomal region 11q24-11q31 (54-69Mpz; Fig. 1A) correlated with the percentage of abnormal sperm head morphology; it contains, for example, the *Sparc* gene controlling testis development. The products of *Kif3a* and *Tekt3* genes located in this region are known to be functionally related to microtubules. Other genes coding for histone 3 subunits (*Hist3h2ba*, *Hist3h2bb*, *Hist3h2a*), and the *Aurkb* gene which is expressed in diplotene spermatogonia, are also located in the 11q24-11q31 region. Abnormal sperm head morphology also correlated strongly with chromosomal region 6q15.6 (34-45Mpz; Fig. 1B). In this region are two genes (*Hipk2*, *Casp2*) related to *Trp53*-dependent apoptosis.

Chromosomal region 18q41-18q47 (66.9-74.9Mpz; Fig. 1C) correlated with the body mass of the analyzed males; it contains a number of genes related to metabolism. One of them is *Lipg* coding for lipase, and another is *Acaa2* whose protein product is responsible for lipid metabolism in mitochondria. The protein product of *Cidea* is related to elevated metabolism and lipolysis. Cholesterol binding and transport is mediated by the protein

TABLE 3

	The level of sperms with/without the cytoplasmic residual droplet (%) (+/- SD)			The level of sperm tails revealing four different shapes in hypotonic suspension (%) (+/- SD)			
Inbred strain	**proximal droplet**	**distal droplet**	**without droplet**	**straight tails**	**slightly bent tails**	**broken tails**	**swollen tails**
KE	3.20+/-1.47	84.45+/-2.22	12.35+/-2.04	34.76+/-5.13	13.90+/-3.77 ^{b}	4.07+/-1.38 ^{b}	47.27+/-6.60
CBA/Kw	4.46+/-2.41	84.74+/-6.56	10.80+/-5.68	32.68+/-5.75	9.86+/-3.30 ^{a}	8.26+/-3.29 ^{a}	49.20+/-9.83
RI 5	1.48+/-0.63 $^{a\,b}$	84.60+/-2.13	13.92+/-2.23	21.54+/-6.29 $^{a\,b}$	7.22+/-3.68 ^{a}	16.53+/-5.92 $^{a\,b}$	54.70+/-7.38
RI 6	1.99+/-1.10 ^{b}	78.31+/-4.00	19.70+/-4.60 $^{a\,b}$	33.63+/-18.80	15.17+/-6.38	3.77+/-4.60	47.43+/-10.99 ^{b}
RI 17	2.26+/-1.43 ^{b}	85.28 +/-5.01	12.46+/-3.79	28.11+/-9.87	6.34+/-2.87 $^{a\,b}$	5.73+/-3.46	59.83+/-9.40 $^{a\,b}$
RI 21	3.63+/-1.53	82.63+/-3.00	13.74+/-2.73	24.55+/-5.02 $^{a\,b}$	11.35+/-4.02	7.49+/-6.21	56.61+/-11.56
RI 29	2.65+/-0.57	86.08+/-2.77	11.28+/-2.36	37.55+/-4.64	4.38+/-0.85 $^{a\,b}$	3.73+/-1.86 ^{b}	54.35+/-4.77
RI 40	10.65+/-3.98 $^{a\,b}$	81.88+/-5.64	7.48+/-2.07 ^{a}	38.59+/-8.45	4.64+/-3.07 $^{a\,b}$	2.43+/-1.37 $^{a\,b}$	54.35+/-9.06
RI 41	2.74+/-1.05 ^{b}	82.90+/-4.61	14.36+/-5.22	45.15+/-6.67 $^{a\,b}$	9.79+/-3.96	9.84+/-4.54 ^{a}	35.23+/-9.54 $^{a\,b}$
RI 42	10.09+/-4.74 $^{a\,b}$	74.95+/-6.35 $^{a\,b}$	14.96+/-7.04	31.05+/-7.56	10.53+/-4.61	8.94+/-6.57	49.49+/-11.83
RI 43	0.53+/-0.60 $^{a\,b}$	90.73+/-2.42 $^{a\,b}$	8.75+/-2.17 ^{a}	34.06+/-9.31	7.57+/-2.27 ^{a}	1.14+/-0.77 $^{a\,b}$	57.23+/-8.04 ^{a}
RI 44	1.47+/-1.01 $^{a\,b}$	90.87+/-3.34 $^{a\,b}$	7.67+/-3.07 ^{a}	25.03+/-8.71 ^{a}	11.81+/-5.68	7.80+/-7.00	55.36+/-14.02
RI 47	3.66+/-1.17	81.80+/-4.18	14.54+/-5.25	40.39+/-16.20	22.13+/-10.60 ^{b}	3.76+/-3.58 ^{b}	33.73+/-12.28 $^{a\,b}$
RI 49	2.05+/-1.04 ^{b}	89.27+/-3.23 ^{a}	8.68+/-3.23 ^{a}	26.02+/-7.82 ^{a}	7.83+/-5.37 ^{a}	13.16+/-7.30 ^{a}	52.99+/-14.16

The results of residual droplet location and HOS test analysis. The standard deviation (SD) values and statistically significant differences between tested strain and KE strain (a) and CBA/Kw strain (b) are also indicated.

product of *Stard6*, which is in chromosomal region 18q41-18q47. That region also contains *Mc4r*. Mice with a disrupted *Mc4r* gene show abnormal leptin function and obesity.

Discussion

Mapping the genes responsible for mouse sperm quality can aid our understanding of the molecular mechanisms underlying the regulation of human sperm quality. Reproductive development and physiology are conserved in mammal evolution; this makes mouse models useful in studies of human fertility. We hope our work leads to the development of simple molecular tests to analyze the genetic causes of human infertility.

The aim of this study was to map chromosomal regions controlling four parameters of mouse sperm quality: head morphology, the level of live spermatozoa, sperm maturity, and the integrity of the sperm tail cytoplasmic membrane. We also estimated the correlations between the analyzed parameters.

The results show that sperm head morphology is controlled by a gene or genes in chromosomal region 11q24-11q31. It has been suggested that abnormal organization of the cytoskeleton during sperm maturation is partially responsible for teratozoospermy (Tachibana *et al.*, 2005). One of the candidate genes from the mapped region 11q24-11q31 is a gene related to the function of microtubules in spindle formation (*Kif3*, kinesin family member 3A; Haraguchi *et al.*, 2006), and the other is *Tekt3* (tektin 3), coding for a microtubule-associated cytoskeletal protein expressed in mouse pachytene spermatocytes and early round spermatids (Roy *et al.*, 2004). Other genes found in this region, *Hist3h2ba*, *Hist3h2bb* and *Hist3h2a* (histone 3, H2ba, H2bb, H2a), are responsible for histone 3 activity and function. The protein product of *Aurkb*, aurora kinase B, which is active before histone phosphorylation (Parra *et al.*, 2003), influences chromosome segregation in male meiosis (Tang *et al.*, 2006) and is responsible for an increased level of abnormal sperm head morphology (Chemes and Rawe, 2003). Another candidate gene in region 11q24-11q31 is *Sparc*, coding for a secreted acidic cysteine-rich glycoprotein which may influence the formation of abnormal spermatozoa due to its role in the regulation of embryonic testis development (Wilson *et al.*, 2006).

It is believed that abnormalities in the shape of the sperm head have low specificity; that is, similar abnormalities (although occurring at different frequencies) can be found in normal (especially in prepubescent) males, and in males treated with mutagens. Such abnormalities may result either from the activity of a mutagen or as an effect of an adverse microenvironment in the gonad. The pleiotropic effects of most mutations indicate that they may be related to the function of genes involved in the production of proteins regulating the basic functions of the cell (housekeeping genes), which may be particularly important during spermatogenesis.

The mapping procedure we used allowed us to locate another chromosomal region, 6q15.6, related to the morphology of the sperm head. The protein products of two genes located in this region (*Hipk2* gene, homeodomain interacting protein kinase 2 and *Casp2* gene, caspase 2) control proliferation and apoptosis processes and thus may participate in the elimination of abnormal cells during spermatogenesis. Several genes located in the mapped region code for vomeronasal receptors. Male germ cells have been shown to express some of the genes active in olfactory pathways (Defer *et al.*, 1998). Possibly the role of olfactory receptors extends far beyond their well known function in sperm

TABLE 4

	Marker regression analysis					Additive	Peak	Significance thresholds for the peak LRS value		
Parameter	**Marker**	**Locus**	**LRS**	**%**	**p**	**effect**	**LRS value**	**p=0.63**	**p=0.05**	**p=0.001**
sperms with abnormal head morphology (%)	D11Mit310	11q24	19.1	80	**0.00001**	5.11	**19.5**	10.9	19.6	24.7
	D11Mit242	11q31	19.1	80	**0.00001**	5.11	**19.5**	10.9	19.6	24.7
	D13Mit3	13q10	14.9	71	0.00011	5.07	14.9	10.8	19.7	27.0
	D6Mit268	6q15.6	14.9	71	**0.00011**	5.07	**18.9**	10.8	19.5	26.1
	D8Mit289	8q11	12.8	66	0.00035	-4.95	12.8	10.8	19.4	25.1
sperms without cytoplasmic droplet (%)	D1Mit452	1q86.6	13.4	67	0.00025	-2.39	13.5	9.9	17.9	22.4
	D1Mit215	1q47	12.2	64	0.00047	-2.35	12.2	10.0	17.8	22.3
	D11Mit130	11q20	11.2	61	0.00083	2.28	11.2	10.0	17.8	22.4
sperms with slightly bent tails (%)	D15Mit252	15q15	11.3	61	0.00079	-4.21	12.1	9.1	22.5	33.7
sperms with broken tails (%)	D11Mit48	11q77	11.5	62	0.0007	4.52	10.5	10.1	16.3	22.4
	D7Mit37	7q49.8	12.4	64	0.00043	4.57	12.4	10.2	16.2	23.5
sperms with straight tails (%)	D13Mit147	13q49	12.7	65	0.00036	-3.8	12.7	10.0	17.7	26.2
	D1Mit296	1q8.3	15.0	71	0.00011	-3.75	15.0	10.0	17.5	23.1
	D6Mit102	6q38.5	14.8	71	0.00012	3.72	14.8	10.1	17.7	26.7
	D11Mit79	11q10.9	11.9	63	0.00057	-3.51	12.2	10.0	17.6	26.7
	D11Mit151	11q13	11.9	63	0.00057	-3.51	12.2	10.0	17.6	26.7
Body mass (g)	D18Mit153	18q47	23.0	85	**0.00000**	-2.77	**24.9**	9.8	18.4	23.0
	D19Mit10	19q47	12.2	64	0.00048	-2.2	14.1	9.8	18.2	24.5

Parameters, parameters revealing correlation with microsatellite markers at the p<0.001 threshold during marker regression procedure; Marker, the name of the microsatellite marker; LRS, *likelihood ratio statistic*, statistical test measuring the strength of the correlation between the SDP of analyzed parameter and the hypothetical QTL; %, the variance percentage that could be explained by the hypothetical QTL; p, significance threshold of the LRS value at the marker locus; Additive effect, the additive regression coefficient for the association between microsatellite marker and measured parameter; Peak LRS value, the highest value of the LRS test evaluated with the interval mapping procedure; Significance thresholds for the peak LRS values, statistical significance thresholds for the peak LRS value evaluated with the permutation test (for 5000 and with 1cM interval) in the whole genome scan.

chemotaxis in the female reproductive tract and in the migration of primordial germ cells towards the gonads (Goto *et al.*, 2001).

Mapping of the genes controlling body mass in mouse males showed that this parameter correlates with chromosomal region 18q41-18q47, which is especially rich in candidate genes whose protein products are involved in lipid metabolism. For example, *Lipg* coding for lipase which regulates the HDL level, and *Acaa2* encoding acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) which is connected with lipid metabolism in mitochondria, are both in this region. The protein product of another gene from this region (*Cidea*, cell death-inducing DNA fragmentation factor, alpha subunit-like effector A) plays a role in the regulation of the metabolism level in mitochondria and lipolysis, by regulating the function of the mitochondrial proton carrier protein (uncoupling protein 1) encoded by *Ucp1* (Zhou *et al.*, 2003). Region 18q41-18q47 also contains *Stard6* (StAR-related lipid transfer (START) domain containing 6), whose protein product binds lipids and is involved in cholesterol transport. *Stard6* knockout mice are resistant to obesity (Soccio *et al.*, 2002). The *Mc4r* gene (melanocortin 4 receptor) is also located in region 18q41-18q47. Obesity due to abnormal metabolic rate and food intake was observed in *Mc4r* knockout mice (Chen *et al.*, 2000).

Three measured parameters of semen quality – level of live spermatozoa, percentage of fully matured sperm, and sperm tail membrane integrity – showed no significant correlation with microsatellite markers. Because the differences between the parental strains are minimal for these traits, the genes controlling them do not segregate in the RI strains, and the mapping procedure cannot be applied. The parental strains differ significantly in testis weight, and segregation of this trait can be seen in all 12 RI strains, but most of them have testis weights similar to that of the CBA/Kw parental strain. In only one RI strain (RI 21), testis weight did not differ from that in the KE parental strain. This skewed trait segregation could have an effect on the mapping procedure. It is also possible that these four parameters are controlled by a large number of genes having weak influences on the parameters' values and that none of them can be mapped.

Pearson's correlation coefficients were calculated for the correlations between sperm quality parameters. A higher percentage of abnormal sperm head morphology correlated with higher testis weight. It may be that testis temperature increases with testis mass, impairing spermatogenesis. The level of sperm head abnormality correlated negatively with the percentage of live spermatozoa. This suggests the operation of processes eliminating some of the abnormal spermatozoa by apoptosis. There was a positive correlation between the level of live spermatozoa and the percentage of sperm with swollen tails (i.e., with proper tail membrane integrity) in the HOS test. This quality parameter might also be subject to processes that eliminate abnormal sperm.

Additional microsatellite sequence analyses of the mapped chromosomal regions are needed to obtain denser marker distributions. New RI strains are being developed in our department from KE and CBA/Kw parental strains. The parameters presented in this paper will be measured in these strains and added to the mapping procedure in order to decrease the chromosomal region intervals. These procedures should help shorten the candidate gene lists. The expression levels and sequencing of the chosen genes will be analyzed to confirm their role in regulating mouse sperm quality parameters. The results of this work should lead to the development of simple diagnostic molecular tests to determine human infertility causes, and new contraception methods for men.

Materials and Methods

Mice mice

The study used groups of 8-10 adult male mice (3-7 months) from two progenitor inbred mouse strains, KE and CBA/Kw, and from 12 RI strains maintained in our department. Three RI strains were developed from crosses between CBA/Kw females and KE males (CBXE strains 17, 21, 29), and nine RI strains were derived from reciprocal crosses between KE females and CBA/Kw males (EXCB strains 5, 6, 40, 41, 42, 43, 44, 47, 49). All animals were given a standard pelleted diet (Labofeed B, Kcynia) and water *ad libitum*, and were kept under a 12L:12D conditions.

Microsatellite sequence analysis

A standard phenol-chloroform protocol was used to isolate genomic DNA from mouse tails. Microsatellite sequences were analyzed by PCR and electrophoresis to prepare a marker map of the mouse genome. Primer sequences were derived from the Jackson Laboratory database (www.jax.org). PCR was carried out in 15 μl for 30-35 cycles with the following conditions (Biometra, T-gradient): 30 s at 94° C, primer annealing for 30 s at temperature appropriate for the primer used, and 30 s of extension at 72° C with *Taq* polymerase (Finnzymes, Finland). PCR products were electrophoresed on 3.5% agarose gel stained with ethidium bromide. Only sequences showing length differences between the two progenitor strains were used for further studies, and to prepare their SDPs for 20 chromosomes of 12 RI strains.

Mouse semen quality parameter analysis.

The animals were killed by cervical dislocation and weighed. Both testes were dissected and weighed. The content of both vas deferens was gently squeezed out directly into 100 μl of M2 medium (Sigma-Aldrich, Germany), placed in small Petri dishes, and allowed to disperse at room temperature for 5 minutes.

Sperm head morphology

Smears prepared from 10 μl of sperm suspension were air-dried, fixed in a mixture of ethanol and acetic acid (3:1, v/v), stained with 0.2% eosin Y (Sigma-Aldrich, Germany) for 30 minutes, and observed by LM under a 100x immersion objective. For each smear, 300 sperm were analyzed. We classified sperm head shape into one of four sperm head abnormality types as described by Krzanowska (1976). All sperm characterized by increased sperm head size (presumably polyploidy) were put into an additional fifth abnormality class.

Dead spermatozoa level (eosin test)

Twenty μl of sperm suspension was mixed with 20 μl 0.2% eosin Y and incubated for 10 minutes on a microscope stage warmer (Japan) at 37° C. Smears were air-dried and observed by LM under a 40x objective. For each smear, 350 sperm were analyzed. Sperm with red heads were classified as dead and those with green heads were classified as alive.

Integrity of sperm tail cytoplasmic membrane (hypoosmotic test, HOS)

Twenty μl of sperm suspension was mixed with 100 μl of sterile water (Sigma-Aldrich, Germany). Then 20 μl of the diluted semen was placed on a microscope slide and incubated at 37° C on a microscope stage warmer for 5 minutes under a cover slip. For each smear about 400 spermatozoa were observed by LM under a 40x objective, and classified according to tail shape in a hypotonic suspension as (a) straight, with defective integrity of the sperm cytoplasmic membrane, (b) slightly bent,

(c) broken, and (d) swollen, with proper integrity of the sperm cytoplasmic membrane.

Sperm tails with cytoplasmic residual droplets

Thirteen μl of sperm suspension was observed by LM with a 40x objective. About 300 living sperm of each sample were analyzed and classified as (a) completely matured, without cytoplasmic droplets, (b) with distal cytoplasmic droplets, that is, droplets located at the end of the mitochondrial sheath, and (c) with proximal cytoplasmic droplets, that is, droplets located on the tail close to the sperm head.

Statistical analysis

After angle transformation of the percentage values of four measured parameters, the Student test at $p<0.05$ (equal variances) was applied. We used Pearson's correlation coefficient ($p<0.05$) to find the relationships between the analyzed parameters.

The marker regression procedure was performed with MapManager QTX ver. b18 (Manly *et al*., 2001) to find microsatellite markers that are correlated (LRS test, likelihood ratio statistic) with the SDP of the analyzed parameters at $p<0.001$ using the Kosambi function (i.e., with the middle interference rate). Further analyses (interval mapping) were done to determine the most probable *locus* of the genes deemed to control these traits. Three statistical significance thresholds were estimated with a permutation test (5000 iterations) for the empirical probability of the relationship between the putative *locus* of the gene and the value of the trait: $p<0.63$ for suggestive correlation only, $p<0.05$ for a statistically significant correlation, and $p<0.001$ for a highly significant correlation (Chmielewicz and Manly, 2002; Li *et al*., 2005).

Chromosomal regions showing LRS test values above the $p<0.05$ statistical significance threshold were searched in the mouse genome database (www.ncbi.nih.gov) to choose candidate genes for further analysis needed to confirm their role in controlling gamete quality in male mice.

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