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Sperm mitochondria diaphorase activity – a gene mapping study of recombinant inbred strains of mice

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ABSTRACT In order to study the genetic control of semen quality parameters, we derived a set of recombinant inbred (RI) mice from crosses between two inbred strains, KE and CBA/Kw, which differ significantly in gamete quality and fertility parameters. In this work, we used male mice from the two parental strains and from ten RI strains to map genes controlling quantitative traits such as sperm mitochondrial diaphorase activity, and assessed the correlation between this trait, sperm motility and *in vivo* fertilization efficiency. We analyzed sperm mitochondrial dehydrogenase (diaphorase) activity (NADH-dependent NBT assay) cytochemically by means of computerized image densitometry and obtained values for four parameters: 1) integrated optical density (IOD) for all pixels of the midpiece, 2) mean optical density (MOD) for the midpiece pixels, 3) length of sperm midpiece and 4) area of sperm midpiece. Polymorphic microsatellite marker profiles were prepared for 20 mouse chromosomes in the ten RI strains. We used Map Manager QTX software to correlate the strain distribution patterns (SDPs) of the four measured parameters with the SDPs of the analyzed markers. Hypothetical genes modifying diaphorase activity were mapped to chromosomal region 19q43-19q47, containing, for example, *Poll, Sfxn2, Cyp17a1* and *Usmg5* genes. Chromosomal regions 18q44 and 18q49-18q80 also showed correlation with the SDPs of diaphorase activity. *Katnal2, Me2* and *Stard6* candidate genes were proposed from this region. Diaphorase activity in the mouse sperm midpiece did not correlate with *in vivo* fertilization efficiency, but was negatively correlated with the linearity and straightness of sperm movement.

KEY WORDS: *mouse sperm mitochondria, diaphorase activity, gene mapping*

Introduction

One of the method used to determine the function of a gene, especially when we are interested in a qualitative trait, is knock-out technique. Quantitative traits (QTs) controlled by multiple genes require the development of a model population, such as an F2 generation or recombinant inbred (RI) strains. Quantitative trait loci (QTL) controlling, for example, testis weight (Zidek *et al*. 1998; Bennett *et al*. 2005), obesity (Cheverud *et al*. 2004) or alcohol metabolism (Grisel *et al*. 2002) have been mapped with the use of RI strains of mice. Our department maintains KE and CBA/Kw inbred strains of mice, which differ in gamete quality and fertility parameters (Krzanowska 1970; Krzanowska 1976; Kaleta 1977; Styrna 1986; Krzanowska *et al*. 1995; Styrna and Krzanowska 1995; Golas *et al*. 2004). A set of RI strains has been developed from these progenitor strains to map genes that control QTs such as sperm motility parameters (Golas *et al*. 2004), body mass and sperm head morphology (Golas *et al*. 2008). In this work we used both parental and RI strains of mice to analyze sperm mitochondrial diaphorase (NADH-dependent dehydrogenase) activity.

Diaphorase controls the ratio between reduced and oxidized forms of NADH; thus it takes part in the sperm energy balance (Atanassov *et al*. 1987). This oxidoreductase is integrated with the mitochondrial respiratory chain and generates superoxide anions, affecting the level of reactive oxygen species (ROS) (Fraczek and Kurpisz 2005). ROS have been shown to regulate DNA condensation during spermatogenesis, the acrosome reaction, and acquisition of hyperactivated motility by mature

Abbreviations used in this paper: IOD, integrated optical density; MOD, mean optical density; QT, quantitative trait; QTL, quantitative trait locus; RI, recombinant inbred strain; SDP, strain distribution pattern.

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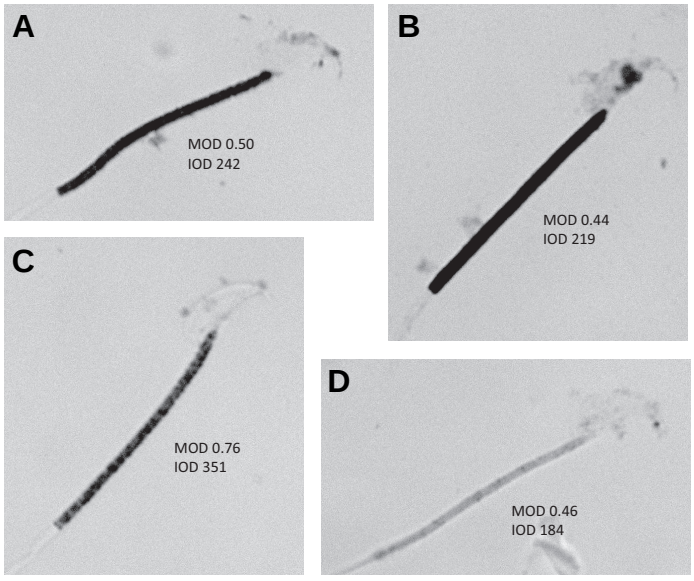

Fig. 1. Cytochemical reaction in mouse sperm midpiece. *IOD (integrated optical density) and MOD (mean optical density) values are presented. Sperms from* **(A)** *CBA/Kw males,* **(B)** *KE males,* **(C)** *RI 17 males (note strong NBT assay) and* **(D)** *RI 40 males (note weak NBT assay).*

sperms (de Lamirande *et al*. 1997; Garrido *et al*. 2004; O'Brien and Zini 2005). Diaphorase activity has been described in the midpiece mitochondrial sheath of boar (Gaczarzewicz *et al*. 2003), rat (Laszczynska *et al*. 1999) and human spermatozoa (Piasecka and Kawiak 2003).

We tested diaphorase activity in mouse spermatozoa by a cytochemical reaction. In this NADH-dependent NBT assay, exogenous NADH serves as a donor of hydrogen, which is translocated to the artificial acceptor, NBT (yellow; nitro blue tetrazolium), by the active NADH-dependent dehydrogenase (diaphorase). This leads to the production of reduced tetrazolium salt deposits (dark blue; diformazan) (Fahimi and Karnovsky 1966). The intensity of this reaction depends on diaphorase activity, and can be assessed with a computerized image densitometry analyzer (Quantimet 600 S, Leica, UK) measuring the integrated optical density (IOD) of whole pixels of the midpiece, the mean optical density (MOD) of midpiece pixels (Fig. 1), and the length and the area of the midpiece. These four parameters were evaluated in sperm midpieces of mice from two parental strains, KE and CBA/Kw, and from 10 RI strains.

The strain distribution patterns (SDPs) of 195 polymorphic microsatellite sequences were prepared in 10 RI strains. We used Map Manager QTX software (ver. b18; Manly *et al*. 2001) to correlate microsatellite SDPs with the values of the measured parameters. Two mapped chromosomal regions (one on chromosome 18 and one on chromosome 19) contain genes deemed to modify diaphorase activity, but to support these results further work using the newly developed RI strains is needed. We also wanted to see whether diaphorase activity in the sperm midpiece correlates with the sperm motility parameters and fertilization efficiency. These results are also presented.

Results

Microsatellite sequence analysis

Of the 344 microsatellite sequences analyzed in the mouse genome, 195 revealed PCR product length differences between the two progenitor inbred mouse strains, KE and CBA/Kw. We prepared the SDPs of these polymorphic sequences for 10 RI strains (RI 5, 6, 17, 21, 40, 41, 42, 43, 44 and 49). The average distance between two following markers on the chromosomes was 6.4cM.

Sperm mitochondrial diaphorase activity

Table 1 gives the results for the cytochemically measured parameters. Average, minimum and maximum values for the four measured parameters are shown in Table 1. The values of the four sperm mitochondria parameters differed significantly between the two progenitor mouse strains, KE and CBA/Kw. Since the IOD and MOD values were not normally distributed, histograms of the IOD distribution in RI 40 and RI 49 sperms are also presented (Fig. 2 A,B).

KE sperms showed a longer mitochondrial sheath but lower midpiece area than CBA/Kw sperms. Thus the mitochondrial sheath of KE sperms is thinner that of CBA/Kw sperms. Midpiece length varied little in the analyzed strains: 22.2 μm at minimum (RI 21 males) and 23.5 μm at maximum (RI 43 males). Mitochondrial sheath area ranged from 16.0 μm^2 in RI 40 sperms to 22.0 μm^2 in RI 43 sperms.

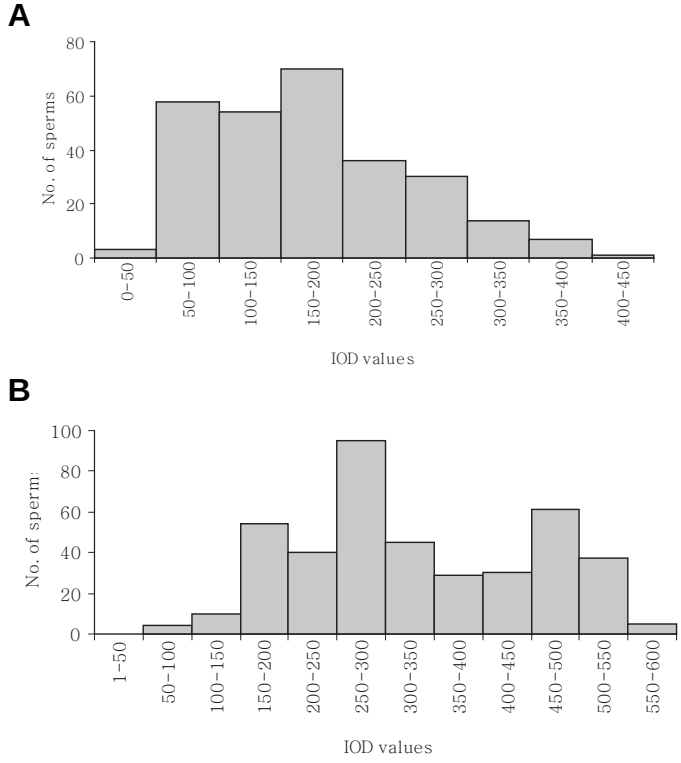

Fig. 2. Non-normal distribution of the integrated optical density (IOD) parameter values. *Histogram of IOD value distribution in sperms from* **(A)** *RI 40 males and* **(B)** *RI 49 males.*

TABLE 1

DIAPHORASE ACTIVITY FOR FOUR SPERM MIDPIECE PARAMETERS: MIDPIECE LENGTH, MIDPIECE AREA, IOD (INTEGRATED OPTICAL DENSITY) AND MOD (MEAN OPTICAL DENSITY)

Inbred strain	Cytochemical test results ($\bar{x}$ ±SD)				The SDP of D19Mit53 / D19Mit10 marker in RI strains
	midpiece length (μm)	midpiece area (μm^2)	IOD (integrated optical density)	MOD (mean optical density)	
KE	23.1+/-1.3 [b] (10.7-39.7)*	19.3+/-5.5 [b] (15.8-28.4)	226.0+/-98.4 [b] (61-578)	0.49+/-0.12 [b] (0.23-0.72)	-
CBA/Kw	22.3+/-1.1 [a] (8.5-34.0)	20.1+/-4.2 [a] (15.0-28.7)	257.5+/-84.1 [a] (59-499)	0.55+/-0.10 [a] (0.21-0.83)	-
RI 5	22.4+/-1.6 [a b] (7.7-32.0)	18.3+/-4.6 [b] (6.2-29.7)	189.3+/-82.1 [a b] (23-373)	0.43+/-0.11 [a b] (0.1-0.64)	CBA
RI 6	22.9+/-1.3 [a b] (8.8-39.7)	20.8+/-6.7 [a] (17.9-33.8)	241.7+/-147.7 [b] (40-644)	0.46+/-0.15 [a b] (0.17-0.82)	KE/CBA
RI 17	22.7+/-1.1 [a b] (10-34.9)	19.8+/-5.0 [a] (15.8-30.5)	396.6+/-186.2 [a b] (95-800)	0.82+/-0.22 [a b] (0.38-1.20)	KE
RI 21	22.2+/-1.0 [a b] (10.1-30.7)	19.4+/-4.2 [a b] (16.0-26.0)	287.9+/-117.7 [a b] (44-550)	0.63+/-0.20 [a b] (0.16-1.04)	KE
RI 40	23.2+/-1.8 [b] (6.0-23.9)	16.0+/-2.5 [a b] (5.8-29.5)	158.2+/-72.9 [a b] (33-423)	0.42+/-0.16 [a b] (0.14-0.9)	CBA
RI 41	23.1+/-1.6 [b] (2.1-34.5)	20.6+/-4.7 [a] (4.2-28.2)	232.3+/-112.8 [b] (19-668)	0.46+/-0.13 [a b] (0.2-1.03)	CBA
RI 42	22.6+/-1.5 [a b] (2.6-26.6)	17.4+/-3.8 [a b] (3.5-25.7)	195.6+/-120.7 [a b] (19-498)	0.46+/-0.20 [a b] (0.17-0.97)	CBA
RI 43	23.5+/-1.3 [a b] (2.7-31.0)	22.0+/-4.5 [a b] (2.5-28.4)	302.1+/-132.9 [a b] (26-672)	0.57+/-0.15 [a] (0.2-0.99)	KE
RI 44	23.0+/-1.2 [a b] (11.0-28.8)	18.5+/-3.6 [b] (17.0-27.6)	203.9+/-100.5 [a b] (59-530)	0.46+/-0.14 [a b] (0.15-0.94)	CBA
RI 49	23.1+/-1.4 [a b] (4.9-39.7)	19.2+/-3.4 [a b] (6.4-30.7)	311.5+/-116.4 [a b] (64-575)	0.69+/-0.17 [a b] (0.29-1.09)	KE

Average values, standard deviations (SD) and ranges (*) are presented. Statistically significant differences (p<0.05) between tested strain and (a) KE strain and (b) CBA/Kw strain are indicated. The strain distribution patterns (SDPs) of D19Mit53 and D19Mit10 markers in 10 RI strains are added. Only one difference between these SDPs was noted (in RI 6). SDPs showed that the microsatellite marker derived from the KE strain correlates with increased IOD values.

TABLE 2

MARKER REGRESSION PROCEDURE: CORRELATIONS BETWEEN VALUES OF THE MEASURED PARAMETERS AND MICROSATELLITE SDPS

Marker regression analysis						Additive effect	Peak LRS value	Significance thresholds for peak LRS value		
Parameter	Marker	Locus	LRS	%	p			p=0.63	p=0.05	p=0.001
Midpiece area	D7Mit267	7q11	10.9	66	0.00098	1.59	12.5	10.1	15.6	21.7
	D8Mit104	8q37	13.7	70	0.00049	1.6	13.7	9.9	15.6	21.0
IOD	D18Mit33	18q44	14.8	77	0.00012	60.15	15.1	10.7	17.0	24.0
	D18Mit49	18q49	14.8	77	0.00012	60.15				
	D18Mit80	18q50	14.8	77	0.00012	67.93				
	D19Mit106	19q22	14.8	77	0.00012	67.93	16.6	10.6	17.0	23.0
	D19Mit13	19q33	13.8	75	0.0002	56.25				
	D19Mit53	19q43	13.8	75	0.0002	56.25				
	D19Mit10	19q47	14.8	77	0.00012	60.15				
MOD	D18Mit33	18q44	15.6	79	0.00008	0.1	15.7	10.3	20.4	35.8
	D18Mit49	18q49	15.6	79	0.00008	0.1				
	D18Mit80	18q50	14.5	77	0.00014	0.11				
	D19Mit106	19q22	14.5	77	0.00014	0.11	15.6	10.5	20.2	36.8
	D19Mit10	19q47	15.6	79	0.00008	0.1				

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

Both IOD (integrated optical density) and MOD (mean optical density) were higher in CBA/Kw than in KE males. For all tested inbred strains the correlation coefficient for IOD and MOD parameters was r=0.9 (p<0.05). Gametes from RI strains 17, 21 and 49 had higher IOD and MOD values than those of the two parental strains (p<0.05); sperms from RI strains 5, 40, 42 and 44 had lower IOD and MOD values than those of the two parental strains (p<0.05) (Table 1).

Mapping procedure

Correlations at p<0.001 obtained from the marker regression procedure are presented in Table 2. The interval mapping at 1cM

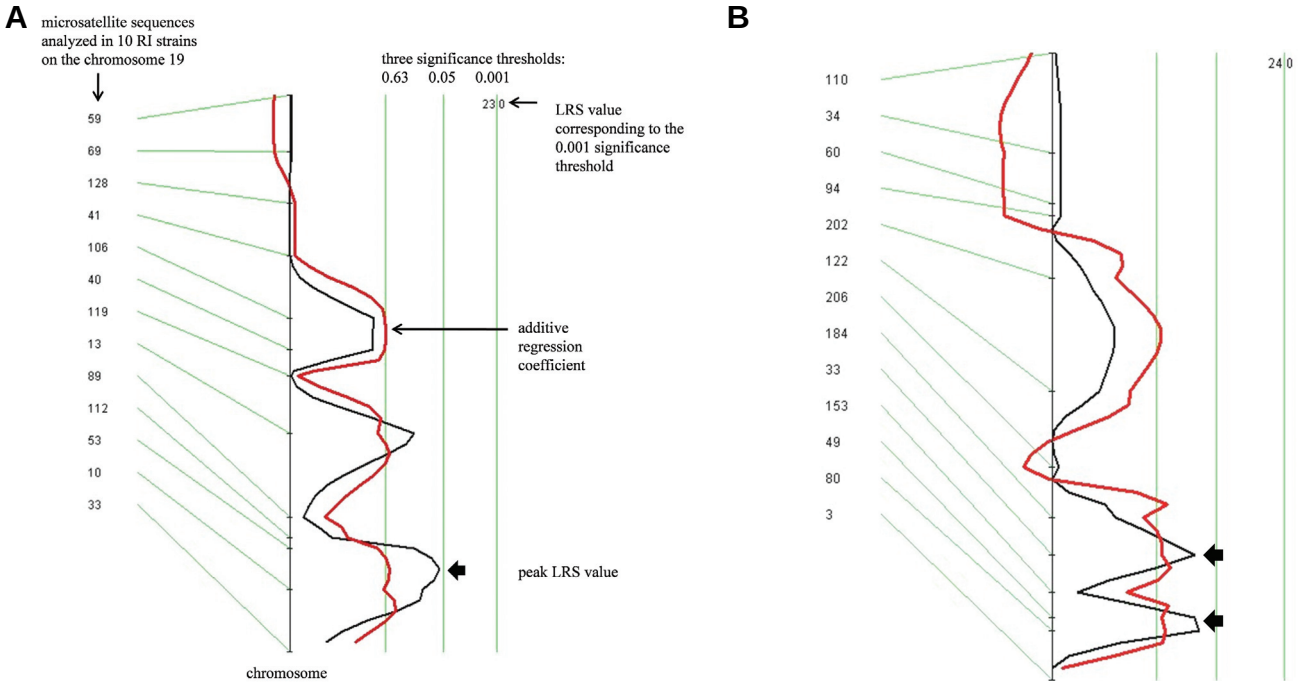

Fig. 3. The interval mapping procedure results of the gene(s) controlling the integrated optical density (IOD) parameter. *Suggestive correlations between IOD parameter and hypothetical QTL located on chromosomes* **(A)** *19 and* **(B)** *18 are shown (arrowheads).*

intervals showed suggestive correlation of sperm mitochondria diaphorase activity with one chromosomal region spanning 19q43 to 19q47 (45-47 Mbp; Fig. 3A) and two chromosomal regions on chromosome 18 (18q44, 69 Mbp and 18q49-18q50, 69-77 Mbp; Fig. 3B). Although the strength of these associations was suggestive only, the mapped chromosomal region was searched for candidate genes possibly regulating diaphorase activity.

In chromosomal region 19q43-19q47 a gene coding for polymerase lambda (*Poll*) is proposed, as *Poll* knockout male mice are infertile (Kobayashi *et al*. 2002). *Cyp17a1*, coding for the cytochrome P450 subunit, could also be functionally related to diaphorase activity. Two other genes, *Sfxn2* and *Usmg5*, coding for mitochondrial inner membrane proteins, could be related to diaphorase activity.

Chromosomal region 18q44 contains two genes possibly controlling diaphorase activity: *Stard6* (*StAR-related lipid transfer (START) domain containing 6*) expressed exclusively in male germ cells (Bose *et al*. 2008), and *Me2* (*malic enzyme 2, NAD(+)-dependent, mitochondrial*) coding for mitochondrial enzyme. *Katnal2* (*katanin p60 subunit A-like 2)* is the only candidate gene located in the 18q49-18q50 chromosomal region; it codes for a microtubule component protein showing ATPase activity.

TABLE 3

CORRELATIONS (r_S) BETWEEN SEVEN SPERM MOTILITY PARAMETERS AND FOUR CYTOCHEMICALLY MEASURED PARAMETERS

Parameter	lnVAP	lnVSL	lnVCL	lnALH	lnBCF	arcsinLIN	arcsinSTR
midpiece area	-0.34	-0.39	-0.34	-0.34	0.44	-0.18	0.06
midpiece length	0.28	0.34	0.28	0.23	0.23	0.05	-0.14
IOD	0.00	-0.18	0.00	0.08	0.15	**-0.71***	-0 54
MOD	0.00	-0.19	0.00	0.12	-0.09	**-0,78***	**-0.66***

* - correlation statistically significant at p<0.05.

Correlations between the diaphorase activity, sperm motility parameters and fertilization efficiency

The results for correlations between diaphorase activity and motility parameters are presented in Table 3. Mean optical density (MOD) correlated negatively with sperm movement linearity (LIN) and straightness (STR). Diaphorase activity did not correlate with *in vivo* fertilization efficiency, regardless of the cross type (Table 4). Of the seven motility parameters, four correlated positively with fertilization efficiency (VCL, VSL, VAP and ALH) only in crosses between females and males of the same strain (Table 4).

Discussion

The molecular background of sperm quality parameters has been studied in several animal models. One molecule used in such studies is sperm mitochondrial diaphorase. This protein is one of about 99% of mitochondrial proteins that are encoded by genomic DNA (Szibor and Holtz 2003). No relationship was found between diaphorase activity and diaphorase electrophoretic patterns (Gavella and Lipovac 1992); in this work we used recombinant inbred (RI) strains of mice to map genes controlling diaphorase (NADH-dependent dehydrogenase) activity. Diaphorase is active in the sperm mitochondrial sheath and can be evaluated cytochemically. Our previous works on the RI strains enabled us to correlate diaphorase activity with sperm motility characteristics and *in vivo* fertilization efficiency.

IOD was significantly higher in CBA/Kw that KE sperm, but was elevated in RI strains possessing D19Mit53 and D19Mit10 microsatellite markers derived from the KE strain (Table 1). High

additive coefficient values also suggested that the KE allele increased IOD (Table 2). The current genetic constitution of the RI strains apparently is a consequence of the crossing-over between microsatellite markers and gene(s) controlling the IOD parameter. After the recombination process at the very beginning of the breeding history of the RI strains, this new genetic composition was inherited by further generations and persist in the RI strains tested in our laboratory.

Male gamete mitochondria become ubiquitinated during spermatogenesis and are effectively eliminated in fertilized oocytes (Sutovsky *et al*. 2004). In our animal model, mice from RI strains inherited maternal mitochondria from CBA/Kw (RI 17 and RI 21) or KE females (the rest of the RI strains), depending on the type of cross between parental strains. The data we obtained here showed that sperm mitochondrial diaphorase activity is not strongly correlated with mitochondrial (and mtDNA) origin (Table 1). Thus, major genes controlling it seem to be located in the genomic DNA. We designed this study to map these genes and found that the distribution of the IOD and MOD values correlated with three chromosomal regions. Region 19q43-19q47 spans between 45Mbp and 47Mbp of 19 chromosome (i.e., between microsatellite markers D19Mit53 and D19Mit10), the other is located within 69Mbp (18q44, close to D18Mit33) and third one spans between 76-77Mbp (18q49-18q50, between D18Mit49 and D18Mit80) of chromosome 18.

Chromosomal region 19q43-19q47 contains several genes that could influence the activity of sperm mitochondria NADH-dependent dehydrogenase (diaphorase). Among them is *Poll*, *Cyp17a1*, *Sfxn2* and *Usmg5.* Three other genes on the chromosome 18 are proposed. Our results showed a correlation between diaphorase activity and sperm movement linearity and straightness. Thus, *Katnal2* (18q49-18q80) was listed due to its microtubule-severing ATPase activity. From chromosomal region 18q44, *Me2* and *Stard6* might also influence diaphorase activity.

Presented mapping results are based on segregating pattern similarities between chromosomal regions and sperm diaphorase activity values, but they are only suggestive. In our breeding department we have developed F13-F16 generations of eight new RI strains. We will analyze the microsatellite markers and sperm quality parameters in these additional strains, to improve the statistical significance of the correlations. To prepare denser marker profile in the RI strains we will also analyze other polymorphisms (e.g., SNP and RFLP).

TABLE 4

CORRELATIONS (r) BETWEEN ANALYZED SEMEN PARAMETERS AND *IN VIVO* FERTILIZATION EFFICIENCY IN RI STRAINS

Parameter	Sperm motility parameters and parameters measured cytochemically were correlated with *in vivo* fertilization efficiency using:	
	females and males from the same RI strain	outbred females and males from RI strains
lnVAP	**0.63***	0.27
lnVSL	**0.60***	0.21
lnVCL	**0.66***	0.28
lnALH	**0.71***	0.34
lnBCF	-0.18	-0.55
arcsinLIN	-0.20	-0.26
arcsinSTR	-0.25	-0.31
midpiece area	0.29	0.04
midpiece length	0.10	0.01
IOD	0.41	0.29
MOD	0.39	0.36

* - correlation statistically significant at p<0.05.

The cytochemical test we used here allowed us to analyze the length and the area of the spermatozoan midpieces. In human, the area of the midpiece is important for sperm motility (Piasecka *et al*. 2003, 2004). In mice we found no correlation between motility parameters and midpiece area or length (Table 3). Sperm midpiece length shows little variation in males of the same species (Beatty 1971, Olds-Clarke 2003), and our data support this observation (Table 1).

The relationship between diaphorase activity and sperm motility seems to be species-dependent. A weak cytochemical reaction was frequently noted in sperms from patients with low sperm motility (Piasecka and Kawiak 2003). In boar, the mean diaphorase activity (MOD) parameter was shown to be significantly correlated with the percentage of spermatozoa with progressive motility (Gaczarzewicz *et al*. 2003). Our detailed analysis of sperm motility and of correlation between motility parameters and diaphorase activity revealed a significant negative correlation between mean diaphorase activity (MOD) and the linearity (LIN) and straightness (STR) of sperm motility. There was no connection between the MOD parameter and the velocity parameters (VCL, VSL and VAP) or beat cross frequency (BCF; Table 3).

Previously we showed that BCF is under the control of genes located in chromosomal region 7q11 (Golas *et al*. 2004). *Gapdhs* (glyceraldehyde-3-phosphate dehydrogenase, spermatogenic) is also located in this region of chromosome 7. Its function in sperm motility control was observed in knockout male mice (Miki *et al*. 2004). Sperms of *Gapdhs* mutants were motile but did not reveal progressive movement, and other CASA parameters were also decreased. Sperm metabolism is partially species-dependent, but the analysis of *Gapdhs* mutants showed that glycolysis, not oxidative phosphorylation, is the main source of the energy needed for sperm forward motility. Those findings suggest that different sperm motility parameters rely on different energy sources. Moreover, spermatozoan movement characteristics can be regulated by independent processes and genes. This could be a reason why only the linearity and straightness of sperm movement correlated with mitochondrial diaphorase activity in our study.

Human semen quality correlates with respiratory chain functioning in sperm mitochondria (Ruiz-Pesini *et al*. 2000). The *in vivo* fertilization efficiency of our mice was not correlated with diaphorase activity, regardless of which females were used; thus the function of this enzyme in sperm mitochondria is species-specific. Fertilization outcome correlated positively with the sperm velocity parameters (VCL, VSL and VAP) and the amplitude of lateral head displacement (ALH) in RI x RI crosses, but not in crosses between outbred females and RI males (Table 4). These differences suggested that oocytes origin (and quality) rather than sperm motility parameters are responsible for the observed correlations. It is not the first time such compensation of gamete quality was observed in our department (Wabik-Sliz *et al*. 2005).

To summarize, we showed that sperm mitochondrial diaphorase activity is under the control of genes located in chromosomal region 19q43-19q47, 18q44 and 18q49-18q80. These data need

further investigation with the use of newly developed RI strains and denser markers such as SNP or RFLP. Diaphorase activity in mouse sperm mitochondria was negatively correlated with the linearity and straightness of sperm movement, but neither diaphorase activity nor LIN and STR influenced *in vivo* fertilization efficiency. Velocity parameters and lateral sperm head amplitude correlated positively with *in vivo* fertilization efficiency.

Materials and Methods

Mice

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

Microsatellite sequence analysis

Genomic DNA was isolated from mouse tails using a standard phenol-chloroform protocol, and 344 microsatellite sequences in KE and CBA/Kw strains were scanned by PCR and electrophoresis. Primer sequences were derived from the Jackson Laboratory database (www.jax.org). PCR was carried out for 30-35 cycles, in total volume of 15 μl, under the following conditions (Biometra, T-gradient): 30 s at 94° C, 30 s of primer annealing at temperature depending on the primer pair used, and extension at 72° C with *Taq* polymerase for 30 s. PCR products were electrophoresed on 3.5% agarose gel stained with ethidium bromide.

To prepare marker maps for 20 chromosomes of the mouse genome, 195 sequences that were polymorphic in KE and CBA/Kw strains were further analyzed in 10 RI strains. The obtained polymorphisms between KE and CBA/Kw strains and the SDPs of these polymorphic microsatellites in RI strains were placed in the Mouse Genetics Database (www.informatics.jax.org; accession IDs: J:84237, J:90435, J:104037 and J:121994).

Cytochemistry

NADH-dependent NBT assay were done to access the activity of mouse sperm mitochondrial NADH-dependent dehydrogenases. Male mice were killed by cervical dislocation, both vas deferens were dissected, and their content was gently squeezed out into 100 μl PBS in 1.5 ml tubes. Sperms were allowed to disperse for 5 min and 400 μl PBS was added. Centrifugation at 1000 rpm for 4 min and washing the sperm pellet with 500μl PBS was done twice. The collected spermatozoa were resuspended in 100 μl PBS.

For each 100 μl of spermatozoa suspension, 400 μl incubation medium was prepared using the following substrates: NADH (2.64 mmol/L), NBT (1.5 mmol/L), BSA (essentially fatty acid free, fraction V; 0.125% w/v), $MgCl_2$ (6.25 mmol/L) and 1% Triton X-100 (0.025% v/v) diluted in PBS. Sperms were gently mixed with the medium and incubated in the dark for 60 min at 37° C. Two smears of each spermatozoa suspension were prepared and air-dried, then washed in PBS and fixed for 10 min in 2% glutaraldehyde (25%, Grade II, Sigma-Aldrich) in 0.1M cacodylate buffer. After washing in distilled water and air-drying, cover slides were mounted using transparent Entellan (Merck, Germany).

In the cytochemical reaction, active mouse sperm mitochondrial NADH-dependent dehydrogenase (diaphorase) translocates hydrogene from exogenous NADH to the artificial hydrogene acceptor NBT (nitro blue tetrazolium). Reduced NBT salt forms dark blue deposits (diformazans) in the sperm midpiece. The intensity of this reaction depends on diaphorase activity and can be assessed by an optical density computerized image analyzer (Quantimet 600 S, Leica, UK), with grades of grayness (range 0-255) replaced by optical density values (range 0-2) (Fig. 1). Each male mouse was represented by an analysis of 100 sperm midpieces under LM (40x objective). Four parameters were measured for a single midpiece: a) IOD (*integrated optical density*), the sum of the optical densities of pixels in the midpiece, b) MOD (*mean optical density*), the average optical density of the midpiece pixels, c) midpiece area, and d) midpiece length.

Correlations between diaphorase activity, sperm motility parameters and in vivo *fertilization efficiency*

The sperm motility of males from KE, CBA/Kw and RI strains was analyzed using computer-assisted semen analysis system (CASA, ver. 10, Hamilton Thorne Research, Beverly, Mass.). Seven parameters were calculated for each semen sample after 1.5h incubation at 37°C and 5% CO_2: curvilinear, straight line and average path velocity (VCL, VSL and VAP), lateral head displacement amplitude (ALH), beat cross frequency (BCF) and sperm movement linearity (LIN, VSL/VCL) and straightness (STR, VSL/VAP). The experimental conditions and data obtained are described in detail elsewhere (Golas *et al*. 2004).

In vivo fertilization efficiency was analyzed previously in our department, using matings between females and males from the same RI strain (pure mating) as well as between our outbred females and RI males (males testing). Briefly, one day after the copulation plug was found, the females were killed, the ova were flushed from oviducts, and two-cell embryos were counted. The experimental procedures and results were described by Wabik-Sliz *et al*. (2005).

Statistical analysis

For statistical purposes the data for four measured sperm mitochondria parameters were pooled by mouse strain. Nonparametric ANOVA was applied (Kruskal-Wallis test; Statistica 6.0) to test the significance of differences between strains. Differences were taken to be statistically significant at $p<0.05$.

The marker regression procedure was performed with Map Manager QTX ver. b18 software (Manly *et al*. 2001) to search for microsatellite markers that correlate (LRS test, likelihood ratio statistic) with the SDPs of four analyzed parameter values at $p<0.001$ using Kosambi function (i.e., with the middle interference rate). The interval mapping procedure was used to evaluate LRS values at 1cM intervals to determine the most possible loci of the hypothetical genes deemed to control measured traits. The additive coefficients were also evaluated. Three statistical significance thresholds were estimated with a permutation test (for 5000 iterations) for the whole-genome empirical probability of the relationship between the hypothetical locus of the gene and the value of the trait: $p<0.63$ for a suggestive correlation, $p<0.05$ for a statistically significant correlation, and $p<0.001$ for a highly significant correlation (Chmielewicz and Manly, 2002; Li *et al*. 2005).

The chromosomal locations of the markers correlating with the SDPs of the measured parameters were confirmed by their base pair positions in the www.ensembl.org database. Chromosomal regions revealing LRS test values above the $p<0.05$ statistical significance threshold were searched in the mouse genome database (www.ncbi.nih.gov) for candidate genes.

Motility parameter values were not normally distributed; before statistical analysis they were normalized by natural logarithms (VCL, VSL, VAP, ALH, BCF). Percentage values (LIN, STR) were transformed using arcsin function. Spearman's correlation coefficients (r_S) were calculated between the average values of normalized motility parameters and the average values of the four parameters. We applied Pearson's correlation coefficient (r) to correlate *in vivo* fertilization efficiency with motility parameters and the four parameters analyzed here. In both cases $p<0.05$ was taken as the level of statistical significance.

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