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Homogeneously Staining Regions (HSR) in Chromosome 1 of the House Mouse: Synapsis and Recombination at Meiosis

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Abstract
Amplified sequences constitute a large part of mammalian genomes. A chromosome 1 containing 2 large (up to 50 Mb) homogeneously staining regions (HSRs) separated by a small inverted euchromatic region is present in many natural populations of the house mouse (*Mus musculus musculus*). The HSRs are composed of a long-range repeat cluster, *Sp100-rs*, with a repeat length of 100 kb. In order to understand the organization and function of HSRs in meiotic chromosomes, we examined synapsis and recombination in male mice hetero- and homozygous for the HSR-carrying chromosome using FISH with an HSR-specific DNA probe and immunolocalization of the key meiotic proteins. In all homozygous and heterozygous pachytene nuclei, we observed fully synapsed linear homomorphic bivalents 1 marked by the HSR FISH probe. The synaptic adjustment in the heterozygotes was bilateral: the HSR-carrying homolog was shortened and the wild-type homolog was elongated. The adjustment was reversible: desynapsis at diplotene was accompanied by elongation of the HSRs. Immunolocalization of H3K9me2/3 indicated that the HSRs in the meiotic chromosome retained the epigenetic modification typical for C-heterochromatin in somatic cells. MLH1 foci, marking mature recombination nodules, were detected in the proximal HSR band in heterozygotes and in both HSR bands of homozygotes. Unequal crossing over within the long-range repeat cluster can cause variation in size of the HSRs, which has been detected in the natural populations of the house mouse.

Introduction

Amplified sequences comprise a large part of mammalian genomes [de Koning et al., 2011]. They vary in adaptive significance of their template, copy number, and genome distribution [Santarius et al., 2010]. Homogeneously staining regions (HSRs) present a textbook example of a massive amplification of genes that provide a strong selective advantage to their carriers [Gollin, 2013]. HSRs usually occur during cancer progression and in cell cultures as chromosome segments of various length that show uniform staining after GTG- or C-banding and FISH with a DNA probe to the amplified sequence [Storlazzi et al., 2010; L'Abbate et al., 2014]. Occurrence and stable inheritance of HSRs containing amplifications of several insecticide resistance genes has also been de-

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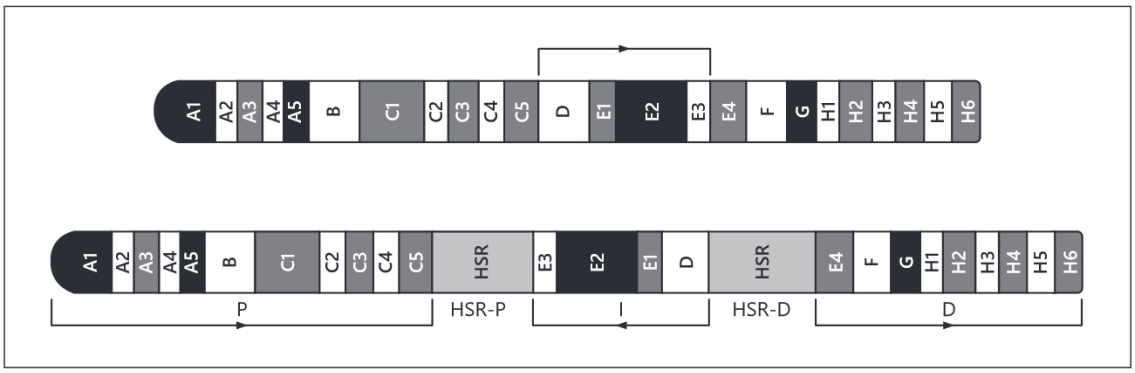


Fig. 1. Idiograms of the standard (top) and rearranged (bottom) chromosome 1 of *Mus musculus musculus*. Letters inside the idiograms show GTG-bands and homogeneously staining regions (HSRs), letters below show the regions measured at synaptonemal complex spreads. P, proximal euchromatic region; HSR-P, proximal HSR; I, inverted euchromatic region; HSR-D; distal HSR; D, distal euchromatic region. Arrows indicate the orientation of the regions (wild type vs. inverted).

scribed in natural populations of mosquitos [Hemingway et al., 2004].

The adaptive significance of large HSRs (50–100 Mb) widespread in many natural populations of the house mouse (*Mus musculus*) remains unclear. They are localized in the chromosome 1 and comprise 30–60% of its length [Traut et al., 1984; Borodin et al., 1990b; Winking et al., 1991; Agulnik et al., 1993a]. The HSRs are composed of multiple partial copies of the *Ifi75* gene (interferon-induced protein 75, which is likely involved in innate immunity) and a chimeric *Sp100-rs* gene that resulted from fusion of a truncated copy of the *Sp100* gene (interferon-stimulated antigen) with the *Csprs* gene of unknown function [Weichenhan et al., 1998].

A single HSR located at the proximal half of chromosome 1 is characteristic of *M. musculus domesticus* [Traut et al., 1984; Winking et al., 1991; Hübner et al., 1994]. The aberrant chromosome 1 of *M. m. musculus* contains 2 HSRs separated by an inverted euchromatic region (Fig. 1) [Agulnik et al., 1988; Borodin et al., 1990b; Winking et al., 1991; Traut et al., 1999]. Comparative analysis of GTG-banding of the rearranged chromosome 1 between subspecies suggests that the double-band HSR chromosome could arise from the single-band HSR chromosome via a single inversion with a proximal breakpoint inside the HSR and a distal one between bands E3 and E4 [Agulnik et al., 1988, 1990b]. Both subspecies show a wide variation in the HSR length [Agulnik et al., 1993a; Yakimenko and Korobitsyna, 2007]. Strong female meiotic drive in favor of the rearranged chromosome has been detected in some populations of *M. m. domesticus* and *M. m. musculus* [Agulnik et al., 1990a, 1993c; Weichenhan et al., 1996].

The aberrant chromosomes are widely distributed all over the areas of both subspecies: in Europe and America in *M. m. domesticus*, and in Northern Asia in *M. m. musculus* [Agulnik et al., 1988, 1993b]. The frequency of the chromosome in local populations varies from 0.04 to 0.81 in *M. m. domesticus* and from 0.04 to 0.51 in *M. m. musculus*. The frequency of homozygotes in the populations studied was always lower than expected under Hardy-Weinberg assumption [Agulnik et al., 1993b]. The deficiency of the homozygotes was apparently due to their low fertility and reduced viability [Agulnik et al., 1993c, d; Sabantsev et al., 1993].

Analysis of organization and function of HSRs in meiotic chromosomes is important for understanding their transmission across generations. Meiosis in heterozygotes and homozygotes for both variants of the rearranged chromosome has been studied previously using conventional light and electron microscopy [Borodin et al., 1990b].

It has been shown that the HSR-carrying chromosome usually undergoes a synaptic adjustment in almost all spermatocytes and in most oocytes of the heterozygous carriers. In mid-pachytene cells, both lateral elements of the synaptonemal complex (SC) were of the same length and formed linear homomorphic bivalents, while at the earlier stages of meiotic prophase (late zygotene to early pachytene), a fraction of germ cells contained heteromorphic bivalents with D-loops, which apparently involved HSR chromatin [Borodin et al., 1990a, b; Winking et al., 1993]. However, the methods used in the early studies did not allow reliable identification of the chromosome 1 in the SC spreads. Borodin et al. [1990a, b] assumed that the largest SC in the set was the SC1, although a correspon-

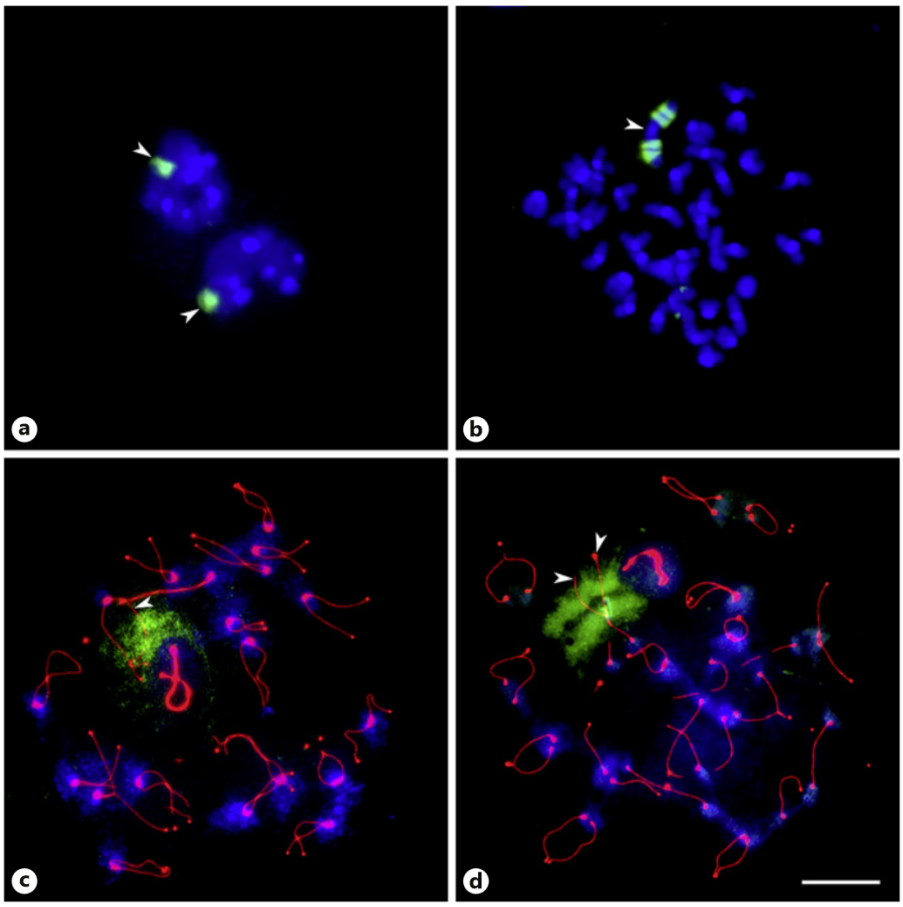


Fig. 2. Identification of the rearranged chromosome 1 in blood smear (**a**), bone marrow metaphase spread (**b**), and synaptonemal complex spreads at diplotene (**c**, **d**) in males heterozygous (**a**–**c**) and homozygous (**d**) for the homogeneously staining region (HSR) after FISH with DNA probe to HSRs (green), DAPI staining (blue), and immunolocalization of SYCP3 (red). Arrowheads point at the rearranged chromosome 1. Scale bar, 10 μm.

dence between the relative length of mitotic chromosomes and the SC is rather vague [Froenicke et al., 2002]. Winking et al. [1993] marked the chromosome 1 by robertsonian translocation, which might modify the initiation and completion of synapsis in the fused chromosome. Recombination in the rearranged chromosome was examined by linkage analysis [Borodin et al., 1990b] and chiasma mapping [Gorlov et al., 1993]. The former approach focused on narrow genomic regions and could not estimate the recombination landscape of the whole chromosome, the latter was unable to estimate the exact location of the recombination events in relation to HSRs.

In this study, we re-examined the meiotic behavior of the rearranged chromosome in heterozygous and homozygous male mice using FISH with an HSR-specific DNA probe and immunolocalization of the key meiotic proteins. The FISH probe enabled unequivocal identification of the HSR in chromosome 1 at all stages of the cell cycle (Fig. 2). Immunolabeling with SYCP3 (the main protein of the lateral element of the SC) visualized meiotic chromosome axes. Immunolocalization of MLH1 (a marker of mature recombination nodules) enabled precise mapping of recombination events along the SC1 and exact identification of the pachytene stage. Antibodies to histone H3K9me2/3 were used to detect transcriptionally inactive heterochromatin.

Here, we addressed the following questions: How does synaptic adjustment affect hetero- and euchromatic regions of the HSR chromosome in heterozygotes? Does it also involve elongation of the euchromatic regions of the wild-type partner? Is recombination possible within HSRs in homozygotes and heterozygotes?

Materials and Methods

Animals

The male founder of the stock was trapped in the village Kolyvanskoe (Altai district, 53°02′22.6″N; 82°52′11.6″E) and crossed to C57BL/6J females. Karyotyping of their progeny indicated that the male was heterozygous for the double-band HSR chromosome,

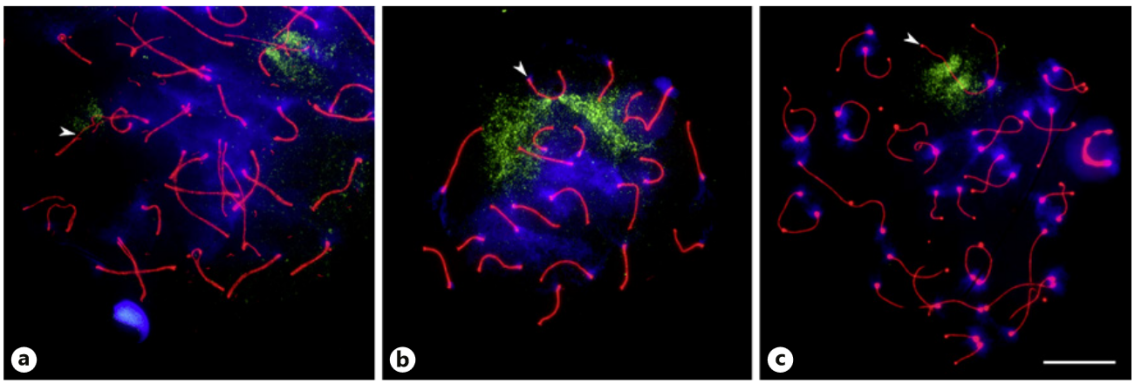


Fig. 3. Synaptonemal complexes at different stages of meiotic prophase in a male heterozygous for the rearranged chromosome 1 after immunolocalization of SYCP3 (red), DAPI staining for centromere heterochromatin (blue), and FISH with a DNA probe to the homogeneously staining regions (HSRs; diffuse green). **a** Zygotene. **b** Pachytene. **c** Diplotene. Scale bar, 10 μm.

characteristic for *M. m. musculus*. The rearranged chromosome was transferred to C57BL/6J background by a series of backcrosses with heterozygotes for HSR. HSR carriers were identified by FISH on blood smears (Fig. 2a) or bone marrow spreads (Fig. 2b) with the probe Dist1 which had been prepared earlier by microdissection of the distal part of the rearranged chromosome 1 followed by DOP-PCR as described previously [Torgasheva et al., 2013]. The DNA fragments were labeled with TAMRA-dUTP in 17 additional PCR cycles, and FISH was carried out according to a standard protocol [Trifonov et al., 2017].

Six males of the mutant stock HSR/Icg (3 heterozygotes and 3 homozygotes for HSR) were used in this study. The animals were housed conventionally with a free access to food and water in the animal facility of the Institute of Cytology and Genetics.

SC Spreading and Immunostaining

Chromosome spreads were prepared from the testes of adult males by the drying-down method [Peters et al., 1997]. Immunostaining was carried out according to the protocol described by Anderson et al. [1999] using rabbit polyclonal anti-SYCP3 (1:500), mouse monoclonal anti-SYCP3 (1:100), mouse monoclonal anti-MLH1 (1:30), rabbit monoclonal H3K9me2/3 (1:100) primary antibodies (all from Abcam, Cambridge, UK) and human anti-centromere (ACA) primary antibodies (1:100; Antibodies Incorporated, Davis, CA, USA). The secondary antibodies used were Cy3-conjugated goat anti-rabbit (1:500), FITC-conjugated goat anti-mouse (1:30), and AMCA-conjugated donkey anti-human (1:40) antibodies (all from Jackson ImmunoResearch, West Grove, USA). Antibodies were diluted in PBT (3% bovine serum albumin and 0.05% Tween 20 in PBS). A solution of 10% PBT was used for blocking. The slides were incubated with primary antibodies overnight in a humid chamber at 37°C, and with secondary antibodies for 1 h at 37°C. Slides were then mounted in Vectashield antifade mounting medium (Vector Laboratories, Burlingame, USA) to reduce fluorescence fading. The preparations were visualized with an Axioplan 2 microscope (Carl Zeiss) equipped with a CCD camera (CV M300, JAI Corporation, Yokohama, Japan), CHROMA filter sets, and ISIS4 image-processing package (MetaSystems GmbH, Altlussheim, Germany). The location of each imaged immunolabeled spermatocyte spread was recorded so that it could be located on the slide after FISH. After acquisition of the immunofluorescence signals, the preparations were subjected to FISH with the probe Dist1 according to a standard protocol.

Measurements and Statistical Analyses

A total of 192 pachytene cells were examined (about 30 per mouse). We also analyzed 10 zygotene and 31 diplotene nuclei of heterozygous males. The centromeric ends of the SCs were identified as bright DAPI-positive clouds of AT-rich heterochromatin (Fig. 2c, d) or by AMCA-labeled secondary antibodies bound to ACA (Fig. 5). SC1 and HSR chromatin were identified by the probe Dist1 (Fig. 2c, d). At the diplotene stage, with partial desynapsis of the lateral elements, we could distinguish heterozygotes from homozygotes for HSR. The former displayed HSR FISH signal at one axis (Fig. 2c), the latter at the both of them (Fig. 2d). Using MicroMeasure3.3 (https://micromeasure.software.informer.com), we measured the total length of SC1 and its 5 regions (P, proximal euchromatic; HSR-P, proximal HSR; I, inverted euchromatic; HSR-D, distal HSR; and D, distal euchromatic) (Fig. 1) and estimated the frequency of MLH1 foci located in each region.

Statistica 6.0 software package (StatSoft, Tulsa, OK, USA) was used for descriptive (mean and standard deviation, SD) statistics and non-parametric statistics (Mann-Whitney U test and Wilcoxon signed rank test).

Results

Synapsis in Heterozygous and Homozygous Spermatocytes

In all examined heterozygous and homozygous pachytene nuclei (99 and 93, respectively), we observed a completely synapsed linear homomorphic SC1 marked by the HSR FISH probe. No heterozygous nuclei containing an

Table 1. Length of the SC1 regions in heterozygous and homozygous spermatocytes

Region	Pachytene, μm		Zygotene, μm	Diplotene, μm
	Heterozygotes (n = 99)	Homozygotes (n = 93)	Heterozygotes (HSR-carrying lateral element) (n = 10)	Heterozygotes (HSR-carrying lateral element) (n = 31)
P	4.5±1.1	4.9±1.1[a]	7.4±1.3[b]	5.1±0.8[c, d]
HSR-P	1.2±0.4	1.2±0.8	1.9±0.3[b]	1.8±0.4[c, d]
I	1.6±0.4	2.2±0.8[a]	2.2±0.4[b]	1.4±0.4[c]
HSR-D	1.1±0.4	1.6±0.6[a]	1.9±0.5[b]	1.8±0.4[c, d]
D	4.1±0.9	3.7±1.6[a]	5.7±0.8[b]	5.1±1.4[c]
Total	12.5±2.0	13.6±3.8	19.1±1.7[b]	15.2±1.0[c]

Data are presented as means ± SD. [a] Significant differences heterozygotes versus homozygotes at pachytene, Mann-Whitney U test, $p < 0.01$. [b] Significant differences heterozygotes at pachytene versus heterozygotes at zygotene, Mann-Whitney U test, $p < 0.01$. [c] Significant differences heterozygotes at pachytene versus heterozygotes at diplotene, Mann-Whitney U test, $p < 0.01$. [d] Significant differences heterozygotes at zygotene versus heterozygotes at diplotene, Mann-Whitney U test, $p < 0.01$.

SC1 with expected D-loop or inversion loop were observed (Fig. 3b). At this stage, the HSR chromatin painted by FISH probe appeared as a beam of DNA fibers diverging from 2 regions in the middle of SC1.

We did not detect significant differences in the total length of SC1 and in the length of the proximal HSR block between heterozygotes and homozygotes. However, the heterozygotes had a significantly shorter distal HSR block and proximal and inverted euchromatic regions and longer distal euchromatic region (Table 1).

We tried to trace the dynamics of synapsis and synaptic adjustment in the heterozygotes for HSR. Zygotene nuclei were rather rare in our SC preparations. In all 10 zygotenes in which we were able to trace the lateral element carrying HSRs and its standard partner, we observed an excessive asynapsis in HSRs. Other chromosomes also showed asynapsis at this substage (Fig. 3a). The lateral element carrying HSRs was significantly longer than its partner (19.1 ± 1.7 μm vs. 17.1 ± 2.2 μm; Wilcoxon signed rank test, $p < 0.01$). It was also significantly longer than SC1 at pachytene (Table 1). In our case, pachytene SC1 comprised 65% of the length of the zygotene HSR-carrying lateral element. The shortening of its separate regions was rather even: from 58 to 73% of their zygotene size.

In 31 heterozygous diplotenes examined, we observed an extensive desynapsis in the middle of SC1 (Fig. 3c). The lateral element carrying HSRs was significantly longer than the wild-type lateral element (15.2 ± 1.0 μm vs. 12.6 ± 1.2 μm; Wilcoxon signed rank test, $p < 0.01$). It was also significantly longer than SC1 at pachytene (Table 1). The elongation of its separate regions was uneven. It was higher in both HSRs (151 and 160% of pachytene length) than in the proximal and distal euchromatic regions (115 and 123%). The inverted euchromatic region was shorter at diplotene than at pachytene (90%).

These data indicate that synaptic adjustment between the HSR-carrying and wild-type chromosome 1 is bilateral. It involves both shortening of the HSRs and an elongation of the wild-type homolog. The adjustment is reversible. Desynapsis at diplotene is accompanied by higher elongation of the HSRs in the lateral element compared with its euchromatic regions.

Epigenetic Modification of HSR in Spermatocytes

We observed a heavy labeling of HSRs with antibodies to histone H3K9me2/3 at the meiotic chromosomes (Fig. 4). The signal at the HSR was as strong as at the pericentromeric heterochromatin. This indicates that the HSRs remain heterochromatic and transcriptionally inactive in the meiotic cells.

Recombination in Heterozygous and Homozygous Spermatocytes

Homozygous and heterozygous spermatocytes did not differ from each other in the number of MLH1 foci at the SC1 (1.41 ± 0.50 and 1.53 ± 0.49, Mann-Whitney U test, $p = 0.2$). Both homozygotes and heterozygotes had peaks of MLH1 foci at distal and proximal euchromatic regions (Fig. 5a, b, 6). In the homozygotes, 10% of MLH1 foci

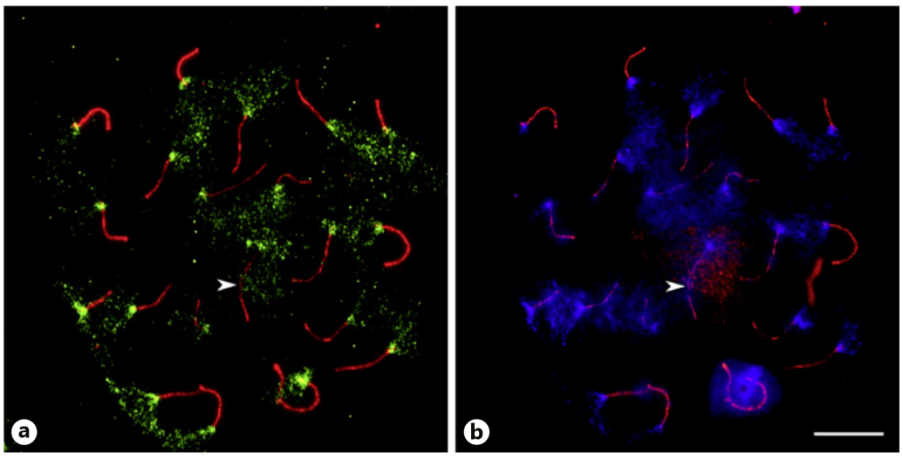


Fig. 4. Pachytene spermatocyte of a mouse heterozygous for the rearranged chromosome 1 after immunolocalization of histone H3K9me2/3 (green) and synaptonemal complexes (red) (**a**) and FISH with the DNA probe to the homogeneously staining regions (HSRs; diffuse red) and DAPI staining for centromere heterochromatin (blue) (**b**). Arrowhead points at the rearranged chromosome 1. Scale bar, 10 μm.

were located in the inverted region (Fig. 5c). This corroborates published data on chiasma number and distribution [Borodin et al., 1990b; Gorlov et al., 1993]. In the previous study, chiasmata in the inverted region had been found in homozygotes, but not in heterozygotes [Agulnik et al., 1993c]. Here, to our surprise, we detected 1 cell with MLH1 focus in the inverted region in the heterozygous spermatocyte as well (Fig. 5d). Moreover, we also observed unexpected MLH1 foci in the HSRs, both in homo- and heterozygotes (Fig. 5e). This indicates that the synaptic initiation and crossing over occur in these heterochromatic regions.

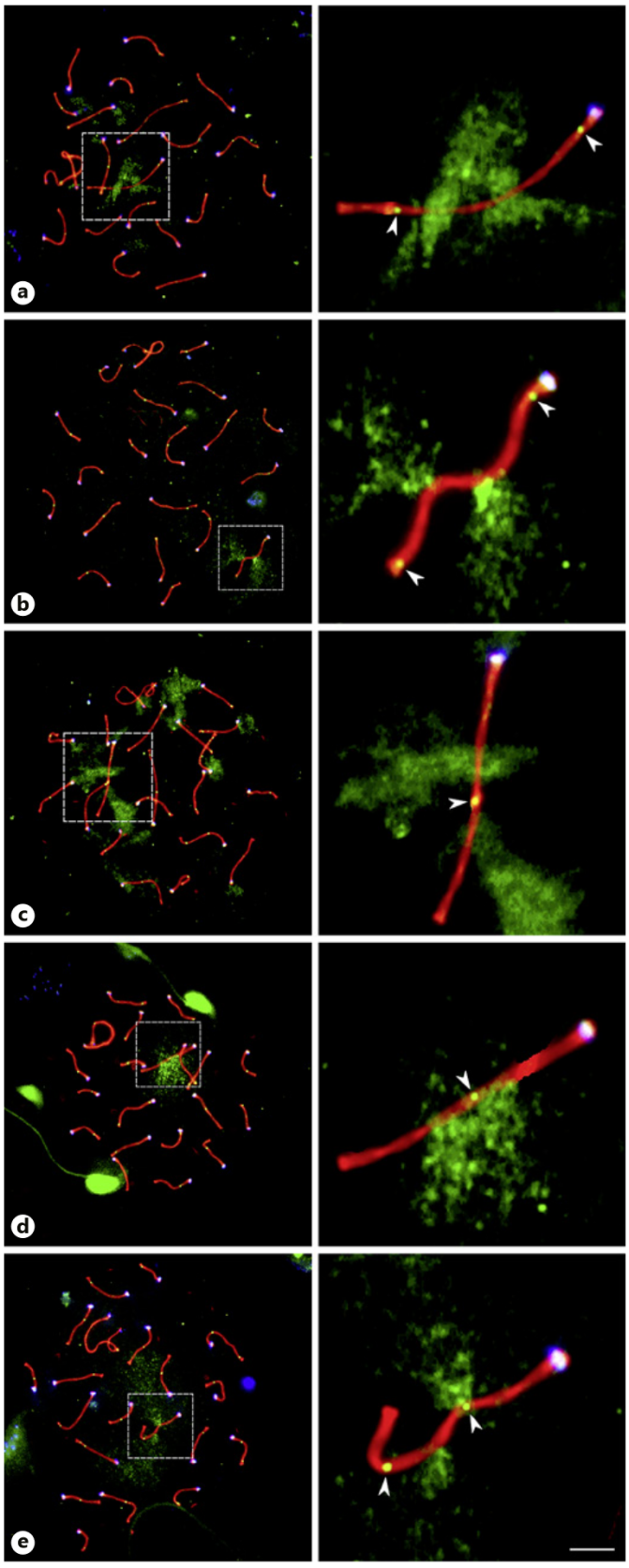
Discussion

Analysis of synapsis and recombination of the chromosome 1 in hetero- and homozygous male carriers of double-band HSRs resulted in several findings important for the understanding of the structure and function of highly amplified DNA sequences in the meiotic chromosomes.

We confirmed previous discoveries done by electron microscopic analysis of SCs in the HSR heterozygotes [Borodin et al., 1990a, b; Winking et al., 1993]. HSRs undergo synaptic adjustment in the heterozygous males. FISH with an HSR DNA probe enabled us to trace the dynamics of this process and revealed that synaptic adjustment in males is bilateral and reversible. It involves both shortening of the HSR-carrying lateral element and the elongation of the wild-type partner. Equalization of the chromosome axes was first described by Moses et al. [1985] in mice heterozygous for tandem duplication. Pronounced equalization has been observed at the ZW sex bivalent in female birds [Solari, 1992; Torgasheva and Borodin, 2017]. We found that the equalization is reversible. HSR-linked segments restore their original size after chromosome desynapsis at diplotene. A decrease of the SC length from zygotene to pachytene and its restoration at diplotene is a well-known phenomenon [Goetz et al., 1984]. Our results indicate that these changes are not uniform along the SC lateral elements. The degree of contraction-expansion depends on the genetic content of the chromatin linked to particular segments of the chromosome axes. This result corroborates the suggestion about unequal contribution of eu- and heterochromatin in SCs [Stack and Anderson, 2001; Belonogova and Borodin, 2010].

Our results on immunolocalization of H3K9me2/3 indicate that in the meiotic chromosome HSRs retain the epigenetic modification characterizing C-heterochromatin in somatic cells.

Surprisingly, the heterochromatic state of HSRs does not impede their involvement in homologous synapsis and recombination. Occurrence of MLH1 foci inside both HSRs in the homozygotes is a direct evidence of homologous recombination. Crossing over in the proximal HSR in the heterozygotes is especially interesting. The presence of multiple copies (60 to 200) of long-range repeat cluster *Sp100-rs* with a repeat length of 100 kb in the wild-type chromosome 1 makes such recombination possible. Recombination in these regions composed of multiple repeated sequences might lead to unequal crossing over, which in turn should produce deletions and duplications within the cluster, generating a variation in HSR size in the aberrant chromosomes and the number of copies of the repeat cluster in the wild-type chromosome.



Field studies demonstrated a wide variation in the size of HSRs in both subspecies. The size of the single-band HSR in *M. m. domesticus* varied from 6 to 30% and the size of the double-band HSRs in *M. m. musculus* from 30 to 70% of the wild-type chromosome 1 length [Traut et al; 1984; Winking et al; 1991; Agulnik et al; 1993b; Yakimenko and Korobitsyna, 2007]. The variation in the proximal HSR is more pronounced. It usually has more sub-blocks than the distal one [Yakimenko and Korobitsyna, 2007]. It has been suggested that such variation could be due to "unequal recombination either by crossing over or sister chromatid exchange" [Winking et al., 1991]. Our data indicate that crossing over does occur in both HSRs in the homozygotes and in the proximal HSR in heterozygotes.

Another surprise of this study was the detection of an MLH1 focus in the inverted region in SC1 in an HSR heterozygote. Because we observed only one such SC, this result should be considered with caution. It can be an artifact either of immunolabeling or of FISH (e.g., a lateral shift of HSR chromatin loops). The other possibility is that an inversion might have occurred in the inverted region, restoring collinearity and normal recombination in this region between HSR-carrying and wild-type chromosomes. This seems unlikely, because in this case recombination in this region would produce chromosomes with single HSRs, distal or proximal. Such chromosomes have never been observed in population or in laboratory studies.

Occurrence of MLH1 foci in an inverted region of the linear SC heterozygous for the large paracentric inversion in chromosome 1 of the house mouse has been demonstrated by Torgasheva et al. [2013]. They suggested that such configurations occur due to synaptic adjustment of the inversion loop with crossing over in the middle of the loop. These events are rather rare and will result in gametes carrying crossover chromosomes with the large deletions and duplications leading to nonviable embryos. Thus, recombination in the inverted region is most likely suppressed in the heterozygotes, but is possible in homozygotes. Nevertheless, the frequency of homozygotes in the natural populations of *M. m. musculus* is negligible.

Fig. 5. Recombination nodules detected at bivalent 1 in pachytene spermatocytes by immunolocalization of MLH1 (green), SYCP3 (red), ACA (blue), FISH with the DNA probe to the homogeneously staining region (HSR; diffuse green). **a**, **b** Recombination outside the rearranged regions in homozygotes (**a**), in heterozygotes (**b**), in the inverted region in homozygotes (**c**), in heterozygotes (**d**), and in the HSR in heterozygotes (**e**). Scale bar, 10 μm.

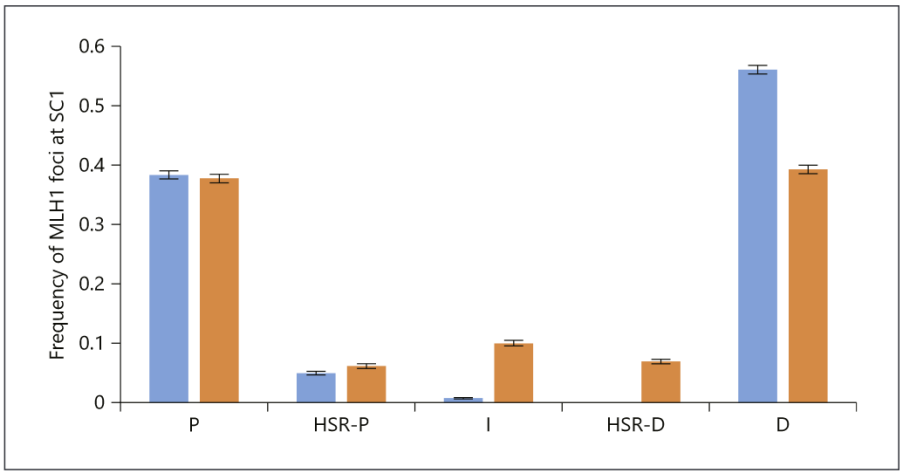


Fig. 6. Frequency of MLH1 foci in separate regions of SC1 in pachytene spermatocytes of heterozygotes (blue) and homozygotes (orange) for the rearranged chromosome 1. P, proximal euchromatic region; HSR-P, proximal homogeneously staining region; I, inverted euchromatic region; HSR-D; distal homogeneously staining region; D, distal euchromatic region.

In a wide population study, Yakimenko and Korobitsyna [2007] examined a total of about 700 mice from various populations and found only 4 homozygotes among them (about 0.5%). We estimated the probability of recombination in the inverted region as 0.1. This means that recombination in the inverted region is extremely rare. Therefore, this region should accumulate a genetic load via Muller's ratchet mechanism [Gabriel et al., 1993]. This in turn would lead to a reduced fitness of the HSR homozygotes. Indeed, a decreased fertility and viability of the homozygotes has been reported previously [Agulnik et al., 1993c, d; Sabantsev et al., 1993]. Direct comparative analysis of the genetic content of the inverted region in HSR homozygotes and its homolog in the wild-type chromosome 1 should shed some light on the dynamics of accumulation of genetic load in nonrecombining chromosome regions.

Acknowledgement

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Statement of Ethics

Capture, handling and euthanasia of the mice followed the protocols approved by the Animal Care and Use Committee of the Institute of Cytology and Genetics SD, RAS (protocol #45/2 from January 10, 2019). Experiments described in this manuscript were carried out in accordance with the approved national and international guidelines for the care and use of animals.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conceptualization: P.M.B.; investigation: N.Y.T., L.P.M., T.I.B., T.V.K.; resources: N.Y.T., E.A.K.; writing and original draft preparation: N.Y.T.; writing, review, and editing: N.Y.T., P.M.B.; visualization: N.Y.T., T.V.K., L.P.M., P.M.B.; supervision: P.M.B.; project administration: P.M.B.

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