Cytogenetic analysis shows that the unusually large chromosome in the sex-limited $p^B$ silkworm (*Bombyx mori*) strain consists of three chromosomes.

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We have discovered an inordinately large chromosome pair at the pachytene stage in the oocyte of the sex-limited $p^B$ (black larval marking) silkworm (*Bombyx mori*) strain (TWPB). We have analyzed the composition and arrangement of this large chromosome. A genetic linkage analysis shows that the large chromosome is made up of the W chromosome, the second chromosome fragment ($p^F$ fragment), and the fifth chromosome (linkage group) containing at least the region from map position 0.0 to 40.8. We also observed a sex heterochromatin body (SB) that we deduced to be made up of condensed W chromosomes. The number of SBs in each female nucleus among the sucking stomach cells of the TWPB strain was variable. Evidently, the W chromosome of the TWPB strain is attached to another chromosome. The composition of the W chromosome, the second chromosome fragment, and the fifth chromosome was studied through linkage analysis for these three chromosomes. We used two strains derived from the TWPB strain, the sex-limited $p^M$ (moricaud larval marking)-like (TWPML) and the autosomal $p^M$-like (TSPML). The results show that the TWPML strain originates through a detachment of the fifth chromosome from the large chromosome of the TWPB strain, and the TSPML strain originates through a detachment of the W chromosome from that. Accordingly, the large chromosome of the TWPB strain is arranged in the order W chromosome—second chromosome fragment—fifth chromosome.

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Tanaka (1916) showed that females of the silkworm, *Bombyx mori*, are ZW, and males are ZZ. The female sex is determined through a single W chromosome. This was confirmed by the observation of polyploids (Hasimoto 1933; Kawaguchi 1934), a sex chromosome non-disjunction strain (Tanaka 1939), and sex-limited strains (Tazima 1944).

Chromosomal constitutive aberrations such as translocation, duplication, deletion, and attachment have been frequently found in *B. mori* (Takasaki 1952; Fujii et al. 1998). Strains that have an autosomal fragment having a dominant gene for egg color, larval marking, and blood color translocated on the W chromosome are useful because sex can easily be determined through the sex-limited expression of characteristics (Tazima 1941; Tazima et al. 1951; Hasimoto 1948; Kimura et al. 1971). Tazima (1941) found the sex-limited $p^{50}$ (sable larval marking) strain. This strain had a translocation between the W and the second chromosome having a mutant allele in the p locus, which controls larval markings. Tazima (1942, 1943, 1944) irradiated this sex-limited strain with X rays and obtained a sex-limited normal marking (+$p$) strain, which had eliminated the excessive part of the translocated second chromosome. The following strains originated spontaneously within these strains: ones with the translocated W chromosome, derived from the sex-limited +$p$ (normal larval marking), sex-limited $p^M$ (moricaud larval marking) (Tazima et al. 1955), sex-limited $p^B$ (black larval marking) (Sasaki 1955), and other sex-limited +$p$ strains (Sasaki 1958).

The chromosomes of *B. mori* are small, many (2$n=56$), and not available to banding techniques, so that each individual chromosome has not yet been cytologically identified. Spermatocytes have been in general used for observing chromosomes because it is relatively easy to obtain chromosome images in dividing cells. Chromosomes at the pachytene stage from the oocyte are more suitable for observing chromosome morphology, even though it is very difficult to make good chromosome preparations in the female pachytene stage. Female pachytene chromosomes are larger, and do not overlap one another as much as the male ones do (Rasmussen 1976; Traut 1976; Kawamura and Niino 1991). Recently, Kawamura and Niino (1991) and Sahara (1998) found an asymmetrical bivalent while observing the
pachytene chromosomes of oocytes in the sex-limited yellow blood strain. They have considered this bivalent to be the Z-W bivalent.

A sex heterochromatin body (SB), deduced to be condensed W chromosomes (Traut and Mosbacher 1968; Ennis 1976; Traut and Marec 1996), has been observed in the highly polyplod interphase nuclei of Lepidopteran females. It has been reported that the number of SBs in a cell nucleus of B. mori corresponds closely to that of the W chromosomes in polyplods (Ito 1977; Pan et al. 1986, 1987). Conclusive evidence for the relationship between the SB and the W chromosome has been obtained with W chromosome mutant strains of Ephesia kuehniella. Translocation or fusion of the W chromosome with an autosomal or with the Z chromosome is accompanied by fragmentation of the SB in polyplod cells (Traut and Rathjens 1973; Rathjens 1974; Traut and Scholz 1978; Traut et al. 1986; Marec and Traut 1994). The dispersed SBs in these cases were attributed to opposing tendencies during the polyplodization of the sticky heterochromatic segment (W chromosome) and adjacent non-sticky euchromatic chromosome segment (autosomes or Z chromosome) (Traut and Rathjens 1973; Rathjens 1974; Marec and Traut 1994).

We have found that the sex-limited pA silkworm strain (TWPB) has 27 chromosome pairs including an inordinately large chromosome pair in the pachytene stage of an oocyte, while a normal strain has 28 bivalents. In addition, we have obtained two new strains, sex-limited and autosomal pM-like strains (TWPML and TSPML strains, respectively), derived from the TWPB strain (unpublished). Here, we analyze the large chromosome by using the TWPB, TWPML, and TSPML strains. We have observed the female pachytene chromosomes and the SBs in the somatic cell nuclei and have made a linkage analysis using chromosomal marker genes. The results show the composition and the arrangement of the large chromosome of the TWPB strain and the chromosomal constitution of the TWPB-derived strains.

MATERIALS AND METHODS

We have used the strains J137, N21, TWPB, and the TWPB-derived strain, i.e., the sex-limited pM-like strain (TWPMLA, TWPML) and the autosomal pM-like strain (TSPML). The TWPB strain, which is expected to be congeneric to the J137 strain (+ pF/+ p) for the W chromosome, was constructed by back-crossing more than 15 times a female of the sex-limited pF strain from SASAKI (1955) with a male of the J137 strain (Ohbayashi et al. 1996). + p is the normal larval marking gene, and pA is a dominant allele of it. In the TWPB strain, the females have black larval marking, and the males have normal larval marking. Individuals of the N21 strain are homozygous for both pe (pink-eyed white egg, 5-0.0) and oc (chinese translucent, 5-0.8). This strain was used as a marker for the fifth chromosome (linkage group) in the linkage analysis. Both pe and oc are recessive alleles.

The linkage analysis was based on the circumstance that female silkworms do not have crossing over (Tanaka 1913). Linkage analysis for the inordinately large chromosome of the TWPB strain was carried out between the W chromosome with the second chromosome fragment and the Z (sch), second (Y), third (lem Ze), fifth (pe re oc), sixth (Ekp), tenth (w2), 13th (ch), or 18th (min) chromosomes. When a dominant marker gene was used, the segregation in the F2 was examined. When a recessive marker gene was used, the segregation in the BF1 was examined.

Pachytene chromosome preparations from oocytes were made by following Tsuchida et al. (1997). Ovaries were excised from fifth instar larvae on the third day and immersed in a hypotonic KCl solution (0.5%) for 60 to 180 minutes. Oviducts were dissected from the ovaries with forceps in the hypotonic solution and, after washing, transferred to a 1.5 ml microtube with the KCl solution. The oviducts were slowly fixed with an MA solution (Carnoy's fluid) (methanol 3: acetic acid 1) and then squashed with a polytron homogenizer (KINEMATICA). The contents were dropped on slides in a humid box, and the slides were left overnight. The tissues were then stained with DAPI at room temperature for 10 min and observed under a Zeiss Axioskop microscope equipped with epifluorescent optics.

The sex heterochromatin body (SB) was observed in the highly polyplod nuclei of sucking stomachs. In this material SB is easily detected in female individuals (Pan et al. 1987). Excised sucking stomachs were dissected on slides with forceps. The tissue was then stained by dropping acetic orcein (3%), covered with coverslips, pressed after 10 min, and observed by light microscopy.

RESULTS

The large chromosome of the sex-limited pA silkworm strain (TWPB)

In the pachytene chromosomes from oocytes, the J137 strain of a chromosomally normal type had 28 bivalents (Fig. 1a), while the TWPB strain had 27 chromosome pairs (Fig. 1b) including one inordinately large chromosome pair (see arrowhead). The large chromosome pair was about 1.3 times longer
than the second longest bivalent, and that shape was symmetrical and smooth. Since the TWPB strain used here was constructed by backcrossing a sex-limited $p^g$ silkworm strain with a male of the J137 strain ($n = 28$), one would expect that the large chromosome of the TWPB strain would be formed by an attachment between the W chromosome with the second chromosome fragment ($p^g$ fragment) and another chromosome.

We prepared sucking stomachs of the J137 and the TWPB strains, and inspected in detail for the presence and shape of SBs in their highly polyploid nuclei. In the J137 strain, a single SB was regularly observed in each nucleus of the female moths (Fig. 2a), while no SB was detected in nuclei of the male moths (Fig. 2b). On the other hand, in the TWPB strain, several SBs in each nucleus (four in Fig. 2c and at least five in Fig. 2d) were observed in the female moths, SBs were absent from the nuclei of the male moths. The proportion of nuclei displaying dispersed SBs in the TWPB females was clearly higher than that in the J137 females. The SB in the TWPB strain was similar to that in W chromosome mutant strains of *Ephestia kuehniella*. These have a fused chromosome formed with the W chromosome and another chromosome. Evidently, the W chromosome with the second chromosome fragment of the TWPB strain is attached to an unknown chromosome.

To determine the composition of the large chromosome, we crossed the females of the TWPB strain with males of strains having marker genes on various chromosomes, and examined the segregation in BF$_1$ and/or F$_2$ progeny. Consequently, in the BF$_1$ progeny obtained by crossing the female of the TWPB strain with the male of N21 strain homozygous for both $pe$ and $oc$ genes on the fifth chromosome, individuals hatched from normal pigmented eggs [$+^v$] were females with black larval marking [$p^g$] except for one individual, and individuals hatched from white eggs [$pe$] were males with normal larval marking [$+^v$] except for two individuals (Table 1). Accordingly, it was evident that the large chromosome of the TWPB strain is composed of the W chromosome, the second chromosome fragment and the fifth chromosome. In addition, in the F$_1$ progeny obtained by crossing the females of the TWPB strain with the males homozygous for $pe$ (5--0.0) and $oc$ (5--40.8), or $re$ (5--31.7), individuals for these three kinds of recessive genes failed to appear. This means that the fifth chromosome of the large chromosome in the TWPB strain has $+^v$, $+^e$, and $+^c$ genes.

![Fig. 1. Pachytene chromosomes of oocytes. a Normal strain (J137). Twenty-eight bivalents are observed. b Sex-limited $p^g$ silkworm strain (TWPB). Twenty-seven chromosome pairs are observed. Arrowhead indicates a large chromosome pair. Bar = 10 μm.](image)

The strain derived from the exceptional individuals obtained for linkage analysis on the TWPB strain (TWPMLA)

In the TWPB strain there arise individuals with moricaud-like ($p^M$-like) larval marking and detachment of the translocated second chromosome fragment from the W chromosome, though at very low frequency (unpublished). Therefore, we expected that the exceptional individuals (Table 1) would be like the $p^M$-like males hatched from [$+^v$], which arose as a result of detachment of the W chromosome from the large chromosome, and $p^M$-like females hatched from $[pe]$ arose as a result of detachment of the fifth chromosome from the large chromosome. As for the two females with $p^M$-like larval marking that hatched from the [pe], we wanted to confirm the expected detachment. We investigated the filial segregation of characteristics by crossing the above two females with $pe/pe$ males of the N21 strain (Table 2).
The results showed that all offspring from the two females had the \([pe]\) and the \([oc]\), and the linkage between the second chromosome fragment and the W chromosome was maintained, except for six females with the \([+P']\). Consequently, the above \(p^M\)-like females arose as a result of the detachment of the fifth chromosome from the large chromosome. Among the female pachytene chromosomes derived from batch “b” in Table 2, 27 chromosome pairs including one large chromosome pair were observed (Fig. 3a). Furthermore, as for the SBs in each nucleus for these females, several SBs in each nucleus were found in the fashion of the TWPB strain (Fig. 3b). The above results suggested that the females with the sex-limited \(p^M\)-like larval marking arose as a result of the detachment of the fifth chromosome from the large chromosome, and by the subsequent attachment with another chromosome.

We attempted to select from the batches for sex-limited \(p^M\)-like silkworms with \([pe]\) and \([oc]\), and we called the batches, which had a stable expression of \(p^M\)-like larval marking, the TWPMLA strain. We made a linkage analysis for the large chromosome of the TWPMLA strain. This analysis suggests that an unknown chromosome attached with the W chromosome having the second chromosome fragment is not a Z chromosome.

### Table 1. Linkage analysis of the large chromosome of the TWPB strain for the fifth chromosome

<table>
<thead>
<tr>
<th></th>
<th>(+pe)</th>
<th>(+oc)</th>
<th>(pe) (oc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(p^B)</td>
<td>224</td>
<td>0</td>
<td>2*</td>
</tr>
<tr>
<td>(+p)</td>
<td></td>
<td>0</td>
<td>212</td>
</tr>
</tbody>
</table>

* Body color shows \(p^M\)-like marking.
Table 2. Segregation of larval marking and sex in the cross of the pM-like females in Table 1 x pe oc males

<table>
<thead>
<tr>
<th></th>
<th>pM′ like</th>
<th>pM-like</th>
<th>+p</th>
</tr>
</thead>
<tbody>
<tr>
<td>♀</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>♂</td>
<td>23</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>0</td>
<td>31</td>
</tr>
</tbody>
</table>

Results of two batches and their sum are listed.

Sex-limited pM-like silkworm strain derived from the TWPB strain (TWPML)

We subjected females of the sex-limited pM-like silkworm strain (TWPML) derived from the TWPB strain to linkage analysis between the W chromosome with the second chromosome fragment (pM-like fragment) and the fifth chromosome (Table 3). The results of [pM-like]+/+=1:1 and [pM-like]/+=1:1 in individuals hatched from both [+p] and [+p] show that the W chromosome with the second chromosome fragment is not linked with the fifth chromosome. Also, in the female pachytene chromosomes of the TWPML strain, 28 bivalents were observed as in the normal strain and unlike the TWPB strain (Fig. 4a). Furthermore, in the female cell nuclei from the sucking stomach of the TWPML strain, a single SB in each nucleus was in general observed (Fig. 4b). These results suggest that the TWPML strain has originated through the detachment of the fifth chromosome from the large chromosome of the TWPB strain.

Autosomal pM-like silkworm strain derived from the TWPB strain (TSPML)

We subjected females of the autosomal pM-like silkworm strain (TSPML) derived from the TWPB strain to linkage analysis between the second chromosome fragment (pM-like fragment) and the fifth chromosome (Table 4). The results show that individuals hatched from the [+p] had the pM-like larval marking, except for four individuals, and those hatched from the [p] had the +p larval marking (except for four individuals). It is apparent that the second chromosome fragment is linked to the fifth chromosome. This means that in the large chromosome of the TWPB strain, the second chromosome fragment is attached to the fifth chromosome. Also, in the female pachytene chromosome of the TSPML strain, 28 bivalents were observed in the fashion of the normal strain (Fig. 5a). In the sucking stomach cells of the female moths derived from the individuals with pM-like larval marking of the TSPML strain, a single SB in each nucleus was in general detected (Fig. 5b). In the nuclei of the male moths derived from the individuals with pM-like larval marking of the

Table 3. Linkage analysis of the pM-like gene in the sex-limited pM-like strain (TWPML) derived from the TWPB strain for the fifth chromosome

<table>
<thead>
<tr>
<th></th>
<th>+p</th>
<th>+pM-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>♀</td>
<td>190</td>
<td>0</td>
</tr>
<tr>
<td>♂</td>
<td>210</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3. TWPMLA strain. a Pachytene chromosomes of an oocyte. Twenty seven chromosome pairs are observed. Arrowhead indicates a large chromosome pair. b Female nucleus of a sucking stomach cell with a string of SBSs (arrowhead). Bar = 10 pm.
DISCUSSION

The results on the TWPB, TWPML, and TSPML strains show that the large chromosome of the TWPB strain is arranged in order W chromosome—second chromosome fragment—fifth chromosome. Furthermore, this fifth chromosome contains the loci from $+p^r$ (0.0) to $+oc$ (40.8) at least. Also, since there is little difference in the growth rate between females with large chromosomes and males without large chromosomes, it is expected that the about complete fifth chromosome of the large chromosome is attached to the second chromosome fragment. Accordingly, we conclude that the large chromosome of the TWPB strain has originated from a reciprocal translocation with break points close to telomeres in both the second chromosome fragment linked with the W chromosome and the fifth chromosome. The chromosome constitutions of the chromosome mutant strains are illustrated as pachytene pairing configurations in female meiosis (Fig. 6). We made a crossing over experiment using the TSPML strain for the mapping of the $p^{M}$-like gene (data not shown). It seems likely that the fifth chromosome of the large chromosome in the TWPB strain is attached to the second chromosome fragment close to the terminal site in $+oc$ direction.

Several cases of spontaneous chromosome fusion have been reported in B. mori. Examples include fusions between the sixth and 14th chromosomes (ITIKAWA 1952; CHIKUSHI 1959), the sixth and seventh (SAKaida et al. 1996), the 23rd and 25th (DOIRA et al. 1987; BANNO et al. 1993), the fifth (lacking of the $+p'$ locus) and the normal W (ONIMARU and TAZIMA 1983), so far. Because of preparation difficulties, no cases of chromosome fusions have been observed at the pachytene stage. The images of fused chromosomes shown here are the first instances at the female pachytene stage of B. mori.

Body colors of mutant strains derived from the TWPB strain (TWPMLA, TWPML, and TSPML) changed from the $pB$ larval marking into the $p^{M}$-like larval marking, and the origin of three strains was accompanied by partial detachment of the fused chromosome of the TWPB strain (see the Fig. 6). Though all such detached individuals do not display body color variations, it is expected that the body color change from the $pB$ larval marking into the $p^{M}$-like larval marking shows a sign of detachment at the fused chromosome. We shall give a detailed evaluation of multiple expression of the larval marking due to the $p$ locus of the translocated second chromosome fragment elsewhere.

ITIKAWA (1952) irradiated individuals having attached sixth and 14th chromosomes and obtained 3.3% detached individuals. This detachment was confirmed through genetic analysis. The primary spermatocytes of these individuals had either 26 or 27 chromosome pairs. ITIKAWA (1952) inferred that this

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**Table 4.** Linkage analysis of the $p^{M}$-like gene in the autosomal $p^{M}$-like strain (TSPML) derived from the TWPB strain for the fifth chromosome

<table>
<thead>
<tr>
<th></th>
<th>$+p^r$</th>
<th>$+oc$</th>
<th>$+p'$</th>
<th>$p^{M}$-like</th>
<th>$+p'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varphi$</td>
<td>153</td>
<td>2</td>
<td>2</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>$\delta$</td>
<td>168</td>
<td>2</td>
<td>2</td>
<td>136</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5. TSPML strain. a Pachytene bivalents of an oocyte. Twenty-eight bivalents are observed. b Female nucleus of a sucking stomach cell with a normal SB (arrowhead). c Male nucleus of a sucking stomach cell without an SB. Bar = 10 μm.

Fig. 6. Female pachytene configuration of normal and mutant strains in the present study. (a) Normal strain (J137). (b) W chromosome–p$^W$ fragment–fifth chromosome fusion strain (TWPB). (c) W chromosome–p$^M$-like fragment–autosome fusion strain (TWPMLA). (d) W chromosome–p$^M$-like fragment translocation strain (TWPML). (e) fifth chromosome–p$^M$-like fragment translocation strain (TSPML). W: W chromosome, Z: Z chromosome, V: fifth chromosome, A: Autosome, (II): second chromosome fragment, *: The arrangement of the three chromosomes (components) was assumed with no confirmation.
was due to the reattachment of the detached chromosome(s) for other chromosomes. Our cytogenetic analysis shows that the TWPLMA strain has arisen through a detachment of the fifth chromosome from the fused chromosome of the TWPB strain and the W chromosome has been subsequently attached to an unknown autosome. This is analogous to the observation of ITIKAWA (1952). We have found an instance of breakage and subsequent reattachment for the fusion chromosome of the TWPB strain (TANAKA et al. 2000).

In a W chromosome-autosome fusion strain of E. kuehniella, TRAUT (1986) reported that an attached autosome was replaced by a different, non-homologous one. In conclusion, once a fusion chromosome is formed in Lepidoptera, and even if this fusion chromosome is detached, it becomes easy to form one (or two) fusion chromosome(s) involving the two detached chromosomes. One might, at least hypothetically, produce a series of sex-limited strains involving a fusion of W with another chromosome. Such sex-limited strains with probably complementary chromosomal constitutions are expected to have rather few individuals with growth abnormalities such as retarded growth of females in comparison to sex-limited strains that have arisen through translocation of the chromosomal fragment on the W chromosome. Strains of this kind might be useful for the improvement of commercial silkworms.

The TWPB strain used here originated from the sex-limited pB silkworm found by Sasaki (1955). An analysis of the W chromosomes in different sex-limited pB silkworm strains derived from the one described by Sasaki (1955) would indicate how the fusion chromosome of the TWPB strain had originated.

REFERENCES


