Stability and Telomere Structure of Chromosomal Fragments in Two Different Mosaic Strains of the Silkworm, Bombyx mori

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Stability and Telomere Structure of Chromosomal Fragments in Two Different Mosaic Strains of the Silkworm, *Bombyx mori*.

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ABSTRACT—Mottled mosaic strains in the silkworm, induced by X-ray irradiation, contain chromosomal fragments carrying the larval body marking genes that are lost occasionally during larval development. In one of the mosaic strains, mottled zebra (Ze^m), the somatic loss of the chromosomal fragment is presumed to cause the mosaic pattern, but the fragment has not yet been identified. Here, we showed that Ze^m individuals have an extra small chromosomal fragment (*Ze* fragment) using genetical and cytological methods. The rate of loss of the *Ze* fragment, calculated based on the data of segregation distortion, is higher than one from a different mottled strain, mottled striped (p^{Sm}). Fluorescent *in situ* hybridization with the *Bombyx* telomeric sequence $(TTAGG)_n$ as a probe demonstrated that the *Ze* fragment has a telomeric repeats at only one chromosomal end, although the fragment of p^{Sm} (p^S fragment) has the repeats at both ends. These data show that the broken ends of chromosomal fragments generated due to X-ray irradiation could be basically healed by *de novo* addition of the telomeric repeats and the structural difference of telomere may be related to the stability of chromosomal fragments.

INTRODUCTION

Mottling is one type of heritable mosaic found in *Bombyx mori*. Concerned with markings and translucency of the larval integument, it exhibits an intricate pattern of small white patches of two allelic characteristics in the same individual (Tazima, 1964). Starting with several marking mutants, many mottled types of mosaic called “madara” have been produced by X-ray irradiation and studied in detail (mottled od, Aruga, 1942; mottled striped, Tanaka, 1935; mottled os, Morohoshi, 1938). The mosaic characters of the X-ray induced mutants are inherited stably through many generations. The somatic loss of chromosomal fragments carrying the gene responsible for body marking is presumed to cause the mosaic pattern (Fujiwara *et al*., 1994).

We have previously shown that one of the mosaic strains, mottled striped (p^{Sm}), carries a small chromosomal fragment of about 2.3–2.5 Mb (p^S fragment) separated by pulsed field gel electrophoresis (Fujiwara *et al*., 1991). Another different mottled mosaic, mottled zebra (Ze^m or Zmt, Aruga, 1940; Kitahara, 1952), was established independently and also shows a mosaic character very similar to those of p^{Sm}. The similarity leads us to think that the Ze^m strain also includes a chromosomal fragment carrying another larval body marking gene *Ze* (zebra) located originally in the middle portion of the 3rd chromosome (3–20.8) (Doira, 1983). However, the chromosomal fragment in Ze^m (*Ze* fragment) has not yet been demonstrated clearly, and we have no information regarding what kind of extra chromosome could be included in this strain.

The chromosomal fragment in mottled strains provides a great opportunity to study the features of insect chromosomes. It is noteworthy that some insects such as hemipteran and lepidopteran orders, including the silkworm, are believed to have a diffuse type of centromere (Pimpinelli and Goday, 1989; Murakami and Imai, 1974). Such chromosomes with full-length kinetochores are not sensitive to breakage, induced either spontaneously or by X-ray irradiation, so that chromosome fragments are maintained through cell division (Blackman, 1987). Broken ends of fragmented chromosome are healed by telomerase in some organisms (Kipling, 1995) or by telom-

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ere-associated retrotransposons in *Drosophila* (Biessmann *et al.*, 1992; Levis *et al.*, 1993). Therefore, in insects that have diffuse type of centromere, chromosome fragmentation and *de novo* telomere formation at the breakage site may generate new chromosomes. To study karyotype evolution in insects, it is of interest to know structural features of chromosomal fragments, especially for telomeres and centromeres, in mottled mosaic strains.

In this study, we demonstrated, for the first time, a chromosomal fragment in mottled zebra (*Ze* fragment) using genetical and cytological methods. Further, to study the relationship between the genetic stability of chromosomal fragments and their structure, the telomere regions of the fragments in two different mosaic strains have been analyzed.

MATERIALS AND METHODS

Strains

Silkworm stocks, Ze^m (mottled zebra) strain 782 and p^{Sm} (mottled striped) strain 788, were obtained from the National Institute of Sericultural and Entomological Science, Kobuchizawa, Japan. The Ze^m strain had been induced by X-ray irradiation and established more than 40 years ago (Kitahara, 1952). The p^{Sm} 788 had been established by X-ray irradiation between 1960 and 1963 by Drs. Tazima and Takasaki (Fujiwara *et al.*, 1994). Silkworms were reared on artificial diets and mosaic animals were obtained by sibmating mottled mosaic individuals (Tazima 1978).

Chromosome preparation

Chromosomal samples were prepared from testes dissected from the larvae of day 0 to day 2 of 5th instar. Spermatocytes were collected by centrifugation at 1800 rpm (180g) for 5min and washed several times in 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate). They were suspended in 500 μl of .075 M KCl at room temperature for 15 min. Then, 200 μl of fixation solution (ethanol : acetic acid = 3 : 1, v/v) was added, mixed well, and centrifuged at 5000 rpm (1400g) for 5 min. The pellet was suspended in 500 μl of fixation solution and centrifuged again. The same step was repeated 2–3 times. In the final step, the pellet was suspended in a small volume of fixation solution and stored at –20°C before use. The chromosomal sample was dropped on a slide glass that is pre-chilled in 10°C water, and dried in the air.

Fluorescent *in situ* hybridization (FISH)

Chromosome spreads were denatured by immersion in 70% formamide in 2 x SSC at 70°C for 3 min and immediately dehydrated through a cold ethanol series. Prior to incubation, DNA probe was denatured at 95°C for 10 min. The hybridization solution consisted of 0.2 μg of biotin labeled probe for ml, 50% formamide, 10% dextran sulfate in 2 x SSC. Fifty microliters of the solution was put on a slide, the surface was covered with a parafilm, and the slide was then incubated in a moist chamber at 37°C for 12–14 hr. After hybridization, slides were rinsed in three changes of 50% formamide in 2 x SSC for 3 min each time at 37°C, in three times in 2 x SSC at 37°C for 3 min each time. Each slide was added 50 μl of blocking buffer containing 3% bovine serum albumin (BSA), 0.1 % Tween 20, and 10 mM $MgSO_4$ in 4 x SSC; slides were covered with a coverslip and incubated for 3 min. After blocking, 50 μl of FITC (fluorescein isothiocyanate) - streptoavidin (Vector Lab. Burlingame, Calif.) diluted 1/50 in blocking solution was added on the slides. The slides were incubated in a moist chamber at 37°C for 30 min and rinsed five times in washing solution containing 0.1 % Tween 20 and 10 mM $MgSO_4$ in 4 x SSC. To amplify the fluorescent signals, 50 μl of FITC-anti-streptoavidin goat serum (Vector Lab.) diluted 1/50 in blocking solution was applied on the slides. The slides were incubated in a moist chamber at 37°C for 30 min, and rinsed in 5 changes of washing solution. Slides were observed under a fluorescence microscope after mounting in a fluorescence antifade solution including DABCO (Sigma) and DNA counterstain (PI, propidium iodide).

Biotin labeling of the probes for FISH

To obtain a labeled probe of the *Bombyx* telomeric repetitive sequence $(TTAGG)_{25}$, the repetitive region in pBT1-HK was specifically amplified by polymerase chain reaction with biotynylated dUTP (Bio-16 dUTP, Boehringer) (Okazaki *et al.*, 1993). The reaction mixture contained 400 nM dATP, dGTP and dCTP, 100 nM dTTP, 60 nM biotin-16- dUTP, 20 pmole each of primer set. The primer pair used for PCR was 5'-GAGGACCACGGCAGACTGGG-3' plus 5'-AAAAAAAAAACCTAACCTAAC-3'. The plasmid template was denatured at 94°C for 5 min. The cycle for PCR was repeated 30 times under the conditions; 1 min at 52°C for primer annealing, 2 min at 72°C for DNA extension and 1 min at 94°C for denaturation.

The telomeric repeat associated sequence (TRAS1, accession number D38414) was labeled by the PCR method mentioned above with a minor modification (Okazaki *et al.*, 1995). The partial region of TRAS1 was amplified with a primer set, 5'-CAAAGCGGCACTCC-TCACAG-3' and 5'-TTCTCTGCAAGGGTGCAAG-3'.

The plasmids containing chorion genes used for probe were pChΔNot, m5000, 2574, 2774 and 1911, which were kind gifts from Dr. Y. Suzuki and Dr. M. R. Goldsmith (Eickbush and Kafatos, 1982; Fujiwara and Maekawa, 1994). All chorion plasmids mentioned above were mixed together and labeled in the presence of biotin-16-dUTP by the nick translation method (nick translation kit, Boehringer).

RESULTS

Stability of chromosomal fragments in mosaic strains based on genetic data

The dominant allele Zebra (*Ze*), at the locus which resides in the middle of chromosome 3 (3-20.8) (Fig. 2), shows a narrow black stripe at the anterior margin of each segment (Fig. 1A). The recessive allele ($+^{Ze}$) at the *Ze* locus shows no pigmentation on the larval skin, as long as no other larval body marking gene is expressed. Small white patches are observed in the black stripe of the mottled zebra (Ze^m) (Fig. 1B and C). Occasional loss of the chromosomal fragment carrying the *Ze* gene during development of larval skin may produce these patches.

Previously, we postulated a schematic model for induction and establishment of mottled striped strains (p^{Sm}) (Fujiwara *et al.*, 1991). By analogy with p^{Sm}, mottled zebra is speculated to be induced by a similar mechanism (Fig. 2). Due to X-ray irradiation, one or two chromosomal breakage might have occurred near the *Ze* locus. When only one breakage occurred, a larger chromosomal fragment including the *Ze* locus might have been produced. The resultant chromosomal fragment with diffuse type of centromere is inherited in a mottled zebra silkworm among many individuals of the expected type of a subsequent generation crossed with $+^{Ze}/+^{Ze}$, a strain with no pigmentation on the skin. To determine the existence of this putative chromosomal fragment carrying the *Ze* gene (*Ze* fragment), we first examined the distribution pattern of the Ze phenotype in genetic crosses.

Table 1 represents the results of crosses between two heterozygous offspring (Ze^m) of the mottled zebra strain

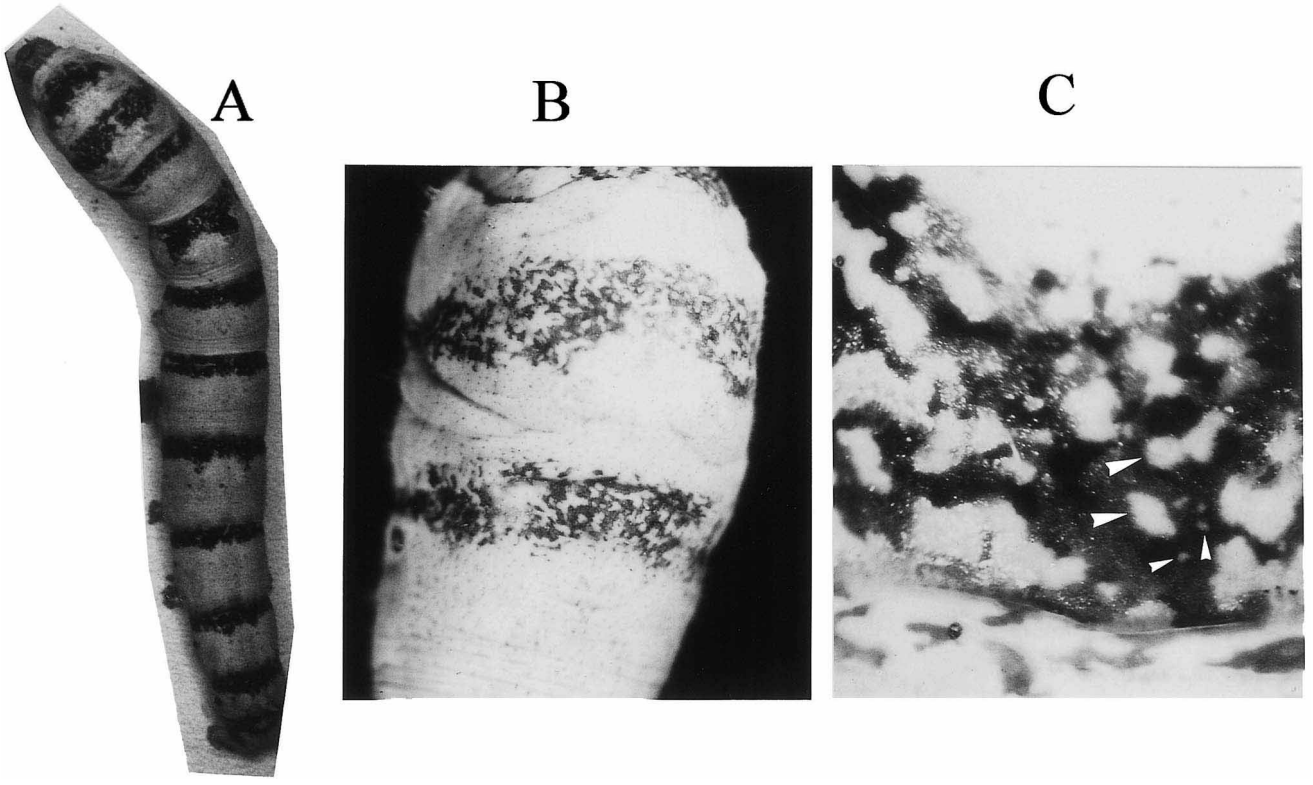

Fig. 1. Marking characteristics of 5th instar larva of mottled zebra (Ze^m) strain. (A) Dorsal aspects of larva. (B and C) Small white patches (white arrowheads in C) are observed in the black stripes.

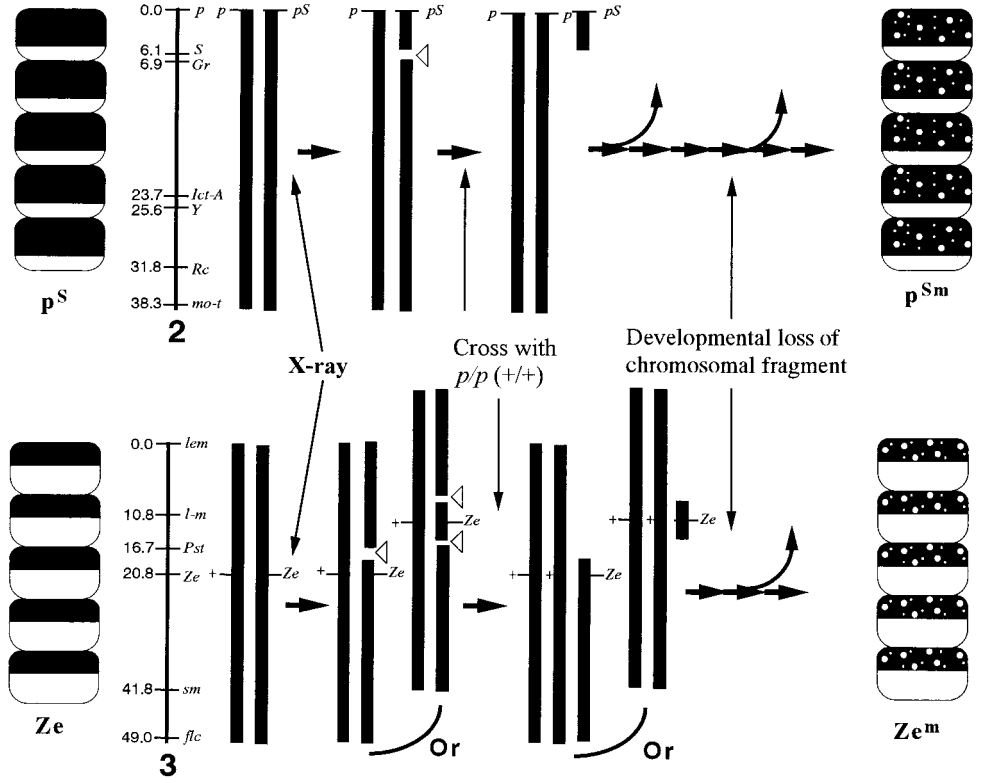

Fig. 2. Schematic illustration of the mosaic induction of mottled strains. The linkage maps of chromosome 2 and 3 of *Bombyx mori* are shown on the left. After chromosomal breakage occurred due to X-ray irradiation, subsequent generations containing the dominant marking genes (p^S or *Ze*) were obtained by crossing with double recessive homozygotes (p/p or $+^{Ze}/+^{Ze}$). Occasional loss of the chromosomal fragment carrying the dominant marking gene during development of individual p^{Sm} or Ze^m causes small white patches in black striped markings.

Ze^m782. The chi-squared test (χ^2) for each batch showed distortion of the expected segregation ratio (1:2:1). This abnormal distribution does not seem to be due to a high lethality of the Ze^m homozygote because its hatchability was essentially normal. Homozygotes and heterozygotes (hemizygotes) of Ze^m were discriminated each other based on the density of pigmentation. In all crosses, the number of offspring having the *Ze* gene tended to decrease. This indicates that the chromosomal fragment carrying the *Ze* gene is lost during gametogenesis, thus generating the segregation distortion. Based on these genetic studies, we speculate that the heterozygotes of Ze^m is a partial trisomic offspring ($+^{Ze}/+^{Ze}$/Dp(3;f), *Ze*) carrying an extra copy of the *Ze* gene on a chromosomal fragment, in addition to the recessive alleles ($+^{Ze}/+^{Ze}$) on two normal autosomes (Fig. 2).

To know the stability of the putative chromosomal fragment in Ze^m (*Ze* fragment), the percentage of gametes which lost the fragment during gametogenesis (L %) was calculated based on genetic data (Table 1). When two heterozygous individuals ($Ze^m/+^{Ze}$) were crossed, three phenotypes are expected to appear in the next generation; dominant homozygote (Ze^m/Ze^m, A in Table 1) with two *Ze* fragments, heterozygote ($Ze^m/+^{Ze}$, B in Table 1) with one *Ze* fragment and recessive homozygote ($+^{Ze}/+^{Ze}$) without the *Ze* fragment. The total number of gametes in parents which participated in fertilization should be 2 x N (the number of hatched eggs, Table 1). If all *Ze* genes on the *Ze* fragment in a parent ($Ze^m/+^{Ze}$) gametes are transmitted to offspring without loss, the number of gametes including *Ze* gene in parents which participated in fertilization, that is N, should be equal to the number of 2xA (Ze^m/Ze^m) + B ($Ze^m/+^{Ze}$). According to this hypothesis, the number of gametes including the *Ze* gene which was lost during gametogenesis corresponds to N - (2×A+B). Thus the percentage of gametes losing the *Ze* gene (L%) can be calculated by (N-2A-B)/N x 100. Values of L (%) in Tables 1 and 2 were estimated by this calculation. It is speculated that 56% of the gametes in the Ze^m parents lost the chromosomal fragment during gametogenesis (Table1). Further, this value of mottled zebra was compared with those in other mottled strains (Table 2). The highest score, 56%, in mottled zebra indicates the lowest stability of the chromosomal fragment.

Table 1. Results of crosses in a mottled zebra strain (Ze^m-782)
Progeny of each cross are listed by single pair matings between $Ze^m/+^{Ze}$ heterozygous parents ($Ze/+^{Ze}+^{Ze}$) (batch). The rate of loss of the chromosomal fragment fragment L (%) during gametogenesis was calculated by the equation (N-2A-B)/N (see text). The four independent crosses are combined and shown in the lower section. P (probability)and χ^2 for deviation from the expected segregation ratio of 1:2:1 (0.25:0.5:0.25) at the *Ze* locus are given.

				F1 phenotype			
Batch	Total	Hatch	%	Ze^m/Ze^m	$Ze^m/+$	+/+	L (%)
		N		A	B		
1	385	361	94	19	122	220	55.6
2	244	178	73	11	57	110	55.6
3	383	361	94	15	125	221	57.1
4	384	374	97	19	130	225	55.1

	F1 phenotype					
Total	Ze^m/Ze^m	$Ze^m/+$	+/+	χ^2	P	L (%)
1274	64	434	776	925.3	<0.0001	55.9
	0.05 :	0.34 :	0.61			
	(0.25 :	0.5 :	0.25) ex			

Table 2. Comparison of the rate of loss of chromosomal fragment (L %) during gametogenesis among several mottled mosaic strains
Parents used for crosses were heterozygotes at each locus of the larval body marking gene (*Ze*, p^S, *S*). Segregation ratios for three F1 phenotypes of offspring are shown in each cross of several mottled mosaic strains; 1, homozygotes (Ze^m/Ze^m, p^{Sm}/p^{Sm} , S^m/S^m); 2, heterozygotes ($Ze^m/+^{Ze}$, p^{Sm}/p, $p^{Sm}/+^p$, S^m/p); 3, double recessive homozygotes ($+^{Ze}/+^{Ze}$, p/p, $+^p/+^p$). L was calculated as in Table 1.

		F1 phenotype			
strains	Total	A/A^1	A/a^2	a/a^3	L (%)
Ze^m 782	1274	0.05	0.34	0.61	56
P^{sm} 788	625	0.14	0.47	0.39	25
P^{sm} 872	996	0.09	0.43	0.48	39
S^m 49IR	196	0.15	0.24	0.61	46

The telomeric structure of chromosomal fragments in two different mottled mosaic strains

Although the genetic data above are consistent with the notion that the mosaic phenotype of Ze^m may be due to the loss of a chromosomal fragment, there has been no molecular data supporting this hypothesis. In this study, to identify the chromosomal fragment, we prepared chromosome spreads from the 5th instar larva of mottled zebra and of $+^{Ze}$, stained with propidium iodide (PI), and observed chromosomal appearance in metaphase using fluorescence microscopy. *Bombyx mori* in Japan normally has 28 chromosomes per haploid genome (Kawaguchi, 1928). About 25 % of nuclei at metaphase or prometaphase of spermatocytes of Ze^m individuals showed an extra small chromosomal fragment in addition to 28 chromosomes (Fig. 3A and B), when more than 400 metaphase nuclei were observed. Probably due to the higher rate of loss (56%) of the *Ze* fragment during gametogenesis (Tables 1 and 2), we could see only 25 % of nuclei, retaining the fragment. In the contrast to Ze^m, no extra chromosome was ever observed among individual nuclei of $+^{Ze}$, the same as in normal *Bombyx* chromosomes. These observations demonstrated that the mottled zebra strain has a small chromosomal fragment, loss of which generates the mottling pattern.

To study the relationship between instability of the chromosomal fragments and their structure, we next examined the terminal structures of the broken ends of the *Ze* fragment by fluorescent *in situ* hybridization with telomeric short repeats $(TTAGG)_n$ as a probe. Although fluorescence signals were seen at all ends of the 28 normal chromosomes of prometaphase in a mottled zebra strain, only one signal was observed at one end of the *Ze* fragment (Fig. 3C and D). However, there was no chromosomal fragment with two signals at

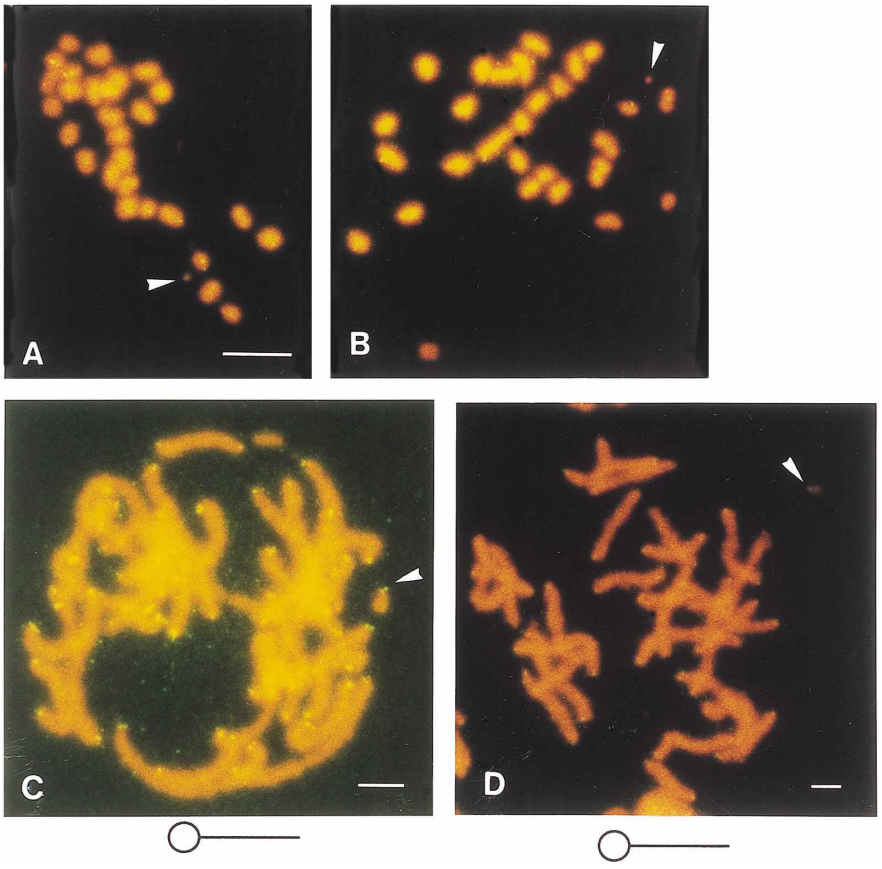

Fig. 3. Chromosomal fragment in mottled zebra. (A and B) Chromosome spreads at metaphase were prepared from testes of 5th instar larvae of Ze^{m}. White arrowhead represents extra chromosomal fragment. Chromosomes were observed with fluorescent microscopy after staining with DAPI (4', 6-diamino-2-phenylindole). White bar represents 10 µm. Two photographs have the same scale. (C and D) Fluorescent *in situ* hybridization of the *Ze* fragment with $(TTAGG)_n$ telomeric repeats. Two chromosome spreads at premetaphase of mottled zebra (Ze^{m}) were hybridized with biotin-labeled $(TTAGG)_{25}$. Hybridization signals were detected by FITC fluorescence (yellow), while the chromosomes were counterstained with propidium iodide (red). Arrowheads represent the FITC signals on one end of the *Ze* fragment. White bars represent 10µm.

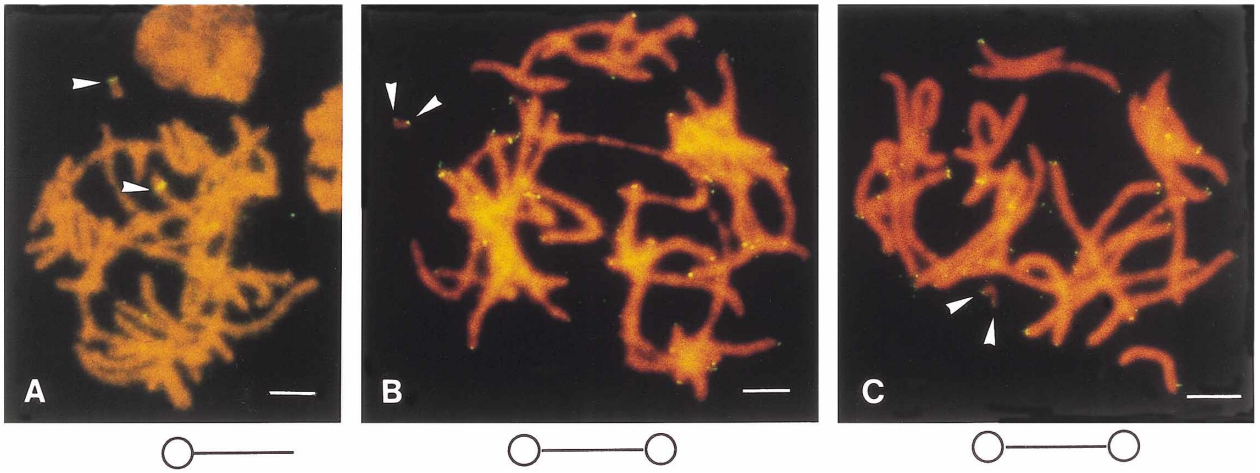

Fig. 4. FISH of the p^S fragment with the chorion (A) and $(TTAGG)_n$ (B and C) probes. The chromosome spreads of mottled striped (p^{Sm}788) were hybridized with two different probes. (A) Arrowheads represent the FITC signal for the chorion gene cluster on the p^S fragment and on the intact second chromosomes. (B, C) Arrowheads show two signals for $(TTAGG)_n$ on both ends of the p^S fragment. White bars represent 10µm.

both chromosomal tips in the mottled zebra strain.

Fig. 4 shows the results of FISH for the chromosomal fragment (p^S fragment) of mottled striped strain (p^{Sm}), with chorion genes (see Materials and methods) (A) and with the telomeric short repeats $(TTAGG)_n$ (B and C) as probes. The chorion genes were hybridized to two sites; one at an internal

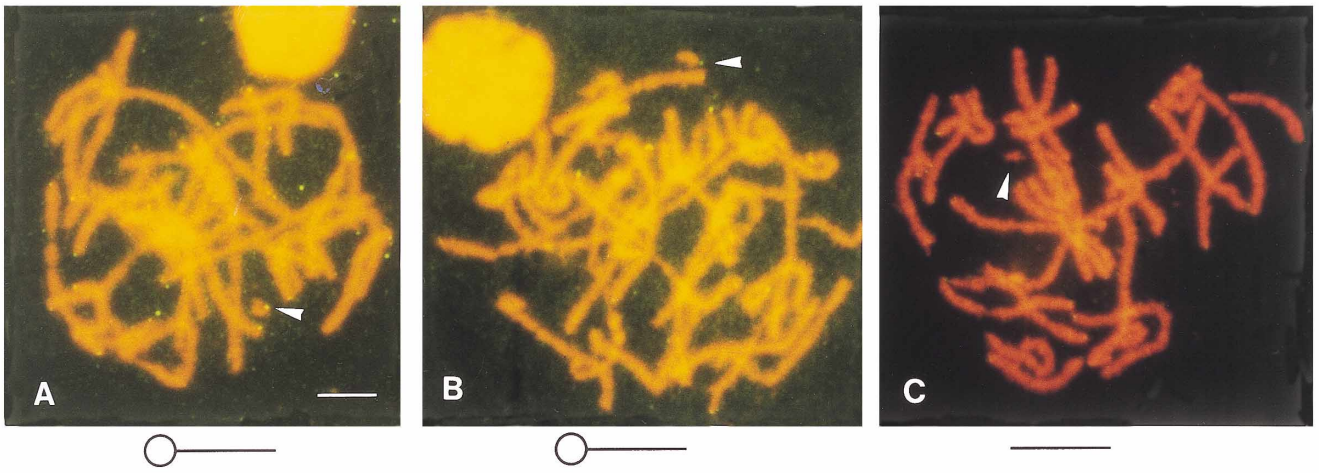

Fig. 5. FISH of the p^S fragment (A,B) and the *Ze* fragment (C) with the TRAS1 probe. The signal of TRAS1 was found on one end of the p^S fragment (A, B) while no signal on the *Ze* fragment (C). Three photographs have the same scale. White bars represent 10μm.

region of the original long 2nd chromosome and one on the tip of the p^S fragment. The broken end of the p^S fragment, thus, is shown to be very near the chorion gene cluster (Fujiwara and Maekawa, 1994). The fluorescence signals with the $(TTAGG)_n$ probe were observed and all ends of the chromosomes and also at both ends of the p^S fragment (Fig. 4B and C).

Next, we tested the location of one of the telomeric repeat associated sequences, TRAS1, in chromosomes of two mottled strains, Ze^m and pS^m. TRAS1 is a new class of retrotransposons in *Bombyx mori* which was recently found specifically integrated into the subtelomeric regions of dozen of chromosomes (Okazaki *et al.*, 1995). When the partial region of the TRAS1 was used as a probe, signals were shown in more than 10 sites of ends of autosomal chromosomes in two mosaic strains (Okazaki *et al.*, 1995). However, near the broken ends of the chromosomal fragment of the mottled zebra, no signal was observed with TRAS1 probe (Fig. 5C). In the mottled striped, one signal for TRAS1 was seen on one end of the chromosomal fragment (Fig. 5A and B).

Genomic Southern hybridization using TRAS1 probe for DNAs from the mottled striped strain 788, showed that there was no different RFLP signal between two phenotypes p and p^{Sm} (data not shown, Fujiwara and Maekawa, 1994). This indicates that the TRAS1 may not be newly integrated into the telomeric repeats at a broken end of the p^S fragment. Thus, we speculate that the one signal of TRAS1 on the p^S fragment locates on the intact chromosomal end, not on the broken end.

DISCUSSION

Frequency of loss of chromosomal fragment during gametogenesis

The chromosomal fragment in mottled mosaic strains is believed to be retained stably in the cell by the presence of a diffuse type of centromere, but to be lost randomly at a very low frequency, probably dependent on some intrinsic structural defects. Although the structural defects responsible for instability of the chromosomal fragment is still unknown at a molecular level, the loss of the fragment carrying the body marking genes generates the segregation distortion in germ cells (Tables 1 and 2) and mottled mosaic patterns in somatic cells (Figs. 1 and 2). The rate of loss of the chromosomal fragment during gametogenesis was calculated based on the data of genetic crosses and shown to be 25 to 56% in different mosaic strains (Table 2). Compared to somatic loss of the fragment, these rates during gametogenesis seem to be higher, as described below.

In a mottled striped strain 788, the average percentage of white area, where the chromosomal fragment was lost, in the black larval skin of the 5th instar larva was estimated as approximately 10% (Fujiwara *et al.* 1994). The total number of epidermal cells at an early stage of the final instar larva is approximately 2×10^6 (Kato and Oba, 1977), which are formed by undergoing 21 somatic cell divisions. On these assumptions, we estimated the frequency of chromosomal elimination (X) during each somatic cell division, to be 0.005 (0.5 %), according to the following equation:

$$(1-X)^{21}=1-0.1=0.9$$

The gametes are also formed by undergoing cell divisions during gametogenesis. Although we do not yet know the exact number of the cell division in *Bombyx*, the rate of loss of chromosomal fragment per cell division during gametogenesis is estimated to be more than 1% in a mottled striped strain 788 (25% in total, Table2) and should be higher than that (0.5%) of somatic loss. This implies that some process specific to meiosis, such as synapsis, may reduce the stability of the chromosomal fragment.

Stability and terminal structure of chromosomal fragments

The rate of loss of the *Ze* fragment (56%) during gameto-

genesis was higher than those of other mottled strains (25% in p^{Sm} 788 and 39% in p^{Sm} 872) (Table 2). This suggests that there are some structural differences among the chromosomal fragments of the mosaic strains. These fragments were originally obtained by X-ray irradiation and thus the telomeric structure at the broken end should affect their stability.

Fluorescent *in situ* hybridization showed that two ends of the p^S fragment and one end of the *Ze* fragment retain the telomeric short repeats. These findings indicate that the telomeric repeats were added on the broken ends of chromosomal fragments that had been generated by X-ray irradiation. The p^S fragment was derived from the terminal region of 2nd chromosome by one X-ray-induced break (Fig. 2). Therefore, one chromosomal tip of the fragment is an intact end and another is a broken one that had the telomeric repeats after the scission events. On the other hand, the corresponding area for the *Ze* fragment is the middle region of the 3rd chromosome. Judging from its lower stability and small chromosomal size on microscopy, we speculate that the *Ze* fragment was generated by two X-ray-induced scission events at two internal sites on the 3rd chromosome near the *Ze* gene (Fig. 2). The $(TTAGG)_n$ repeat, therefore, was added on one broken end but not on another end of the *Ze* fragment.

These observations except those on one end of the *Ze* fragment, suggest that the chromosomal breakage by X-ray irradiation can be healed basically by an activity of telomerase in *Bombyx mori*. This type of telomere formation has been reported in several organisms. In ciliates and *Ascaris*, *de novo* telomere addition on the fragmented chromosome ends generated by programmed genome rearrangements has been demonstrated (Muller *et al*., 1991; Kipling, 1995). However, these may be involved in special mechanisms to promote an interaction between telomerase and non-telomeric double strand breaks. Rather, several reports in humans, where spontaneous chromosome breakage was shown to be healed by *de novo* $(TTAGG)_n$ addition (Wilkie *et al*., 1990; Lamb *et al*., 1993), seem to resemble the telomere healing on the broken ends of chromosomal fragments in *Bombyx*.

Although the broken end of *Bombyx* chromosomes may be basically healed by telomerase, we do not know why one end of the *Ze* fragment could not be added with telomeric short repeats. One possible explanation for this is that the telomeric short repeats on the end of the *Ze* fragment is too short to be detected by FISH. Usually, the ends of *Bombyx* chromosomes are composed of long stretches of $(TTAGG)_n$ repeats, which are 6 to 8 kb long (Okazaki *et al*., 1993). However, in the *Ze* fragment, the addition of telomeric repeats may not have been sufficient, compared to that in the intact chromosomal ends. Another possibility is that one end of the *Ze* fragment actually lacks any short repetitive telomeric sequences. That end may have an unusual structure to block the telomerase activity or no signal involved in telomerase recognition. In this case, the fragment should be shortened by the incomplete replication at the DNA ends (Watson, 1972; Lundblad and Szostak, 1989). In *Drosophila*, such telomere shortening was observed in normal chromosomal ends at the rate of 75bp on average per generation (Levis *et al*., 1993). Mottled zebra is a long established strain induced more than 40 years ago (Kitahara, 1952) and thus the *Ze* fragment might have been shortened by several kilobases if the telomeric ends were lost at the similar rate as shown in *Drosophila* with no new sequences added. Since the area shortened is much shorter than total length of the chromosomal fragment, there seems to be no effect on the internal genes in the Ze fragment and shortening may still be continuing.

In *Drosophila*, sacrificial retrotransposons, named TART and HeT-A, are hypothesized to transfer the chromosomal ends and prevent the gradual loss of the chromosomes (Levis *et al*., 1993; Biessmann *et al*., 1990). We found that the *Bombyx* chromosomal ends contain insertions of huge numbers of retrotransposon groups named the TRAS family (Okazaki *et al*., 1995). To know the possibility that the TRAS family have transposed to the broken end of the chromosomal fragments, we examined FISH with a TRAS1 element, one of the TRAS family, as a probe (Fig. 5). We found one weak signal with a TRAS1 probe at one end of the p^S fragment, but no signal at any other ends of chromosomal fragments. Thus, TRAS1 may not be involved in healing of the chromosomal breakage directly. However, we found more several members of TRAS localized in the telomere or sub-telomere regions (Takahashi *et al*., 1997; Kubo and Fujiwara, in preparation) and thus further study will be necessary to determine whether some of the TRAS family other than TRAS1 are involved in healing of broken chromosomes.

The chromosomal fragments should have the dispersed centromeres (holocentric chromosomes) in *Bombyx mori* because they could be transmitted to the next generation through gametogenesis (Table 1) or to daughter cells of epidermis during larval development (Fujiwara *et al*., 1994). However, the occasional loss of the chromosomal fragment suggests some structural deficiency that may cause reduced stability compared to normal chromosomes. One possible candidate for the structural deficiency is incomplete telomeric structure on the broken ends. Telomeres have been proposed to play important roles in protecting chromosomes from fusion, degradation and incomplete replication (Blackburn, 1991; Gilson *et al*., 1993). The incomplete telomeric structure on the broken ends may cause instability of the chromosomal fragment. On this point, the *Ze* fragment that has only one end with the long telomeric repeats should be less stable compared to the p^S fragment with two “intact” telomeric repeats. Even the p^S fragment with two telomeric repeats seems to lack some structure involved in the stabilization of the chromosome. Thus, some structure other than telomeric repeats in the subtelomeric region may be necessary for complete transfer of chromosomes during cell divisions.

Alternatively, a smaller size of *Ze* fragment compared to p^S fragment can explain differences in stability of both chromosomal fragments. It is well known that yeast artificial chromosome (YAC) clones of *S. cerevisiae* containing more than 50kb insert of DNA are maintained as normal chromosomes. However, short YACs of 10–15kb in size are mis-segregated

at a much higher frequency than long YACs (Murray *et al*., 1986; Roy and Runge, 1999). Although the size-dependent loss of yeast mini-chromosomes is not yet understood well, a recent report suggests that trans-acting proteins can stabilize short YAC through interacting with telomeric proteins (Roy and Runge, 1999). Further studies on fine structure of telomere and sub-telomere regions of Ze and p^S fragments make it possible to answer these questions concerning the chromosomal stability.

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