

Chromosomal fragment responsible for genetic mosaicism in larval body marking of the silkworm, *Bombyx mori*

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Summary

Several genetic mosaics for larval body marking of the silkworm, *Bombyx mori*, have been induced by X-ray irradiation. It is hypothesized that the occasional loss of chromosomal fragments carrying the genes for body marking during development may give rise to this type of mosaicism. Using pulsed field gel electrophoresis (PFGE), we find that a DNA molecule of about 2.5 megabases (Mb) is present in one type of mosaic (mottled striped strains psm788 and psm872), and not in any other strain. This DNA fragment hybridizes strongly with some chorion genes which are less than 6.9 cM away from the p^s locus, and hence it corresponds to a chromosomal fragment containing genes for both striped marking (p^s) and the chorion. In the non-mottled p^s strain, the phenotype before X-ray irradiation, no band was detected either on a PFGE gel or after hybridization with the chorion probe. These results suggest that the mottled p^s strains carry short chromosome fragments which are lost differentially during cell divisions.

1. Introduction

Genetic mosaics provide a powerful tool for investigating various biological events. In the silkworm, *Bombyx mori*, mosaics can often be found at various stages of the life cycle and generated experimentally by several methods. Although most of these mosaics are not heritable, some are inherited by known mechanisms. In one of them, the recessive mutant gene *mo* generates abnormal fertilization by suppression of polar body elimination in homozygous females. Mosaics for sexual and somatic characters thus appear to be due to double fertilization (Ebinuma *et al.* 1988).

Mottling is another type of heritable mosaic. Concerned with markings and translucency of the larval integument, it exhibits an intricate pattern of small patches of two allelic characters in the same individual (Chikushi, 1972). Starting with several marking mutants, many mottled types of mosaic called 'madara' have been produced by X-ray

irradiation and studied in detail (Tanaka, 1935; Tazima, 1964; Virk, 1959 and 1960). The mosaic characters of the X-ray induced mutants are inherited stably through many generations. Genetic and cytological studies have suggested that they are probably not due to alterations in gene expressions; rather the somatic loss of chromosomal fragments carrying the genes responsible for body marking is presumed to cause the mosaic pattern. At the molecular level, however, the existence of such a chromosomal fragment has not been demonstrated.

The technique of pulsed field gel electrophoresis (PFGE) has advanced rapidly in recent years (Schwartz *et al.* 1984; Smith *et al.* 1987) and offers the possibility of identifying putative chromosomal fragments in these mosaic strains. Using the CHEF (contour-clamped homogeneous electric field) gel system (Chu *et al.* 1986), we have examined DNA migration patterns in two kinds of mosaic strains. In one of these we found evidence for a small chromosome fragment.

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2. Materials and Methods

(1) Strains

Silkworm stocks p^{sm788} (mottled striped), p^{sm872} (mottled striped), Ze^{m782} (mottled zebra) and the original p^s (striped) were obtained from Dr O. Ninaki at the National Institute of Sericultural and Entomological Science, Kobuchizawa, Japan. The practical strain (Kinshu × Showa) was purchased from Kanebo Silk Co. *Saccharomyces cerevisiae* (X2180-1A) and *Schizosaccharomyces pombe* (JY1) were used as sources of chromosomal DNA for markers.

(2) Preparation of intact chromosomal DNA

We used mainly the 5th-instar larvae of several *Bombyx* strains for PFGE. Testes were dissected from the larvae, and spermatocytes and sperm were collected by centrifugation and washed several times in $1 \times$ SSC. They were suspended at about 10^8 cells per ml with $1 \times$ SSC, added to an equal volume of 1% low melting agarose (BRL) in 125 mM EDTA and solidified. These agarose blocks were incubated at 50 °C for 1–2 days in 1 mg/ml proteinase K (Merck), 450 mM EDTA and 1% lauroyl sarcosinate. For size markers, yeast (*S. cerevisiae*) and fission yeast (*S. pombe*) chromosomal DNAs were prepared by published procedures (Schwartz *et al.* 1984; Smith *et al.* 1987). All agarose blocks were stored at 4 °C before use.

(3) Pulsed field gel electrophoresis and Southern blotting

Chromosome separation was performed in 1% agarose, $0.25 \times$ TBE gel electrophoresed using the CHEF system at 2 V/cm for more than 120 h with a pulse time of 5400 sec (Chu *et al.* 1986). Following electrophoresis and staining in 5 µg/ml of ethidium bromide, the gels were soaked in 0.25 M HCl for 30 min before transfer to a nitrocellulose filter as described by Southern (Southern, 1975). Hybridization was performed in $6 \times$ NET (0.9 M NaCl, 90 mM Tris-HCl (pH 7.9), 6 mM EDTA), 1.6% skim milk and 0.5% SDS, using pChΔNot, a plasmid clone containing a middle A/B chorion gene pair labelled by random priming, as a hybridization probe (see Fig. 4c). This plasmid was a gift from Dr Y. Suzuki of the National Institute of Basic Biology.

3. Results and Discussion

(1) Mottled genetic mosaics for larval body marking

The larval skin of the silkworm normally exhibits characteristic markings on three dorsal segments (Fig. 1a, $+^p$ normal marking). Numerous phenotypes of the larval body marking have been found and analysed genetically (Chikushi, 1972; Tazima, 1978). The p locus, which has more than 10 alleles, for instance, maps to the end of chromosome 2 (2-0-0) (Doira,

1983). In the dominant allele p^s (Striped), a black stripe marks each segment, whereas there is almost no marking in a strain carrying the recessive allele, p (plain) (Fig. 1a). At a second locus Zebra (Ze) which resides in the middle of chromosome 3 (3-20-8), the dominant allele shows a narrow black stripe at the anterior margin of each segment. Figure 1 shows two mottled mosaics, p^{sm} and Ze^m respectively, in which many small white patches are observed against their black stripes. It is believed that these white patches are formed by cells carrying only null or recessive alleles (such as p , $+^p$ and $+^{ze}$), while the black regions are formed by cells which have the dominant alleles (such as p^s and Ze).

Figure 2 illustrates a model for X-ray induction of mottled p^s and formation of the mosaics described above. Due to X-ray irradiation, a chromosomal breakage might have occurred near the end of chromosome 2, which contains the striped gene p^s (A–B). Thereafter, the resultant chromosomal fragment is inherited in a mottled striped silkworm among many individuals of the expected type of a subsequent generation (C). One of the p^{sm} strains was discovered among the F1 of a cross between irradiation striped (p^s) and non-irradiated plain (p) more than 50 years ago (Tanaka, 1935). In the same way, mottled strains used in this study were established at least 20 years ago and maintained in partial trisomic (D) or tetrasomic form through many generations. We don't know how the chromosomal fragment is distributed stably during gametogenesis in the trisomic or tetrasomic individuals. In the silkworm, however, it is known that three marked chromosomes in a complete trisomy for chromosome 2 are divided into gametes without loss (Takasaki & Tazima, 1944). This observation suggests that a fragment of the chromosome 2 can be also divided into a gamete, probably with a normal chromosome (C–D). If the p^s fragment is destabilized and lost during various developmental stages (D–E), the recessive alleles are expressed in those cells and thereby mottled patterns appear on the larval skin. We believe that the extra chromosomal fragments are retained because *B. mori* chromosomes have diffuse centromeres (Murakami & Imai, 1974).

As shown in Fig. 1b, the loss of the chromosomal fragment seems to occur at any stage of development. When the p^s fragment is lost in an early developmental stage, a larger white patch is obtained. The bottom one represents an extreme mottled pattern, probably due to the loss of the chromosomal fragment during an earlier developmental stage than the ones above. As an extreme case, when it occurs in the very early stage, a half mosaic is produced (Fig. 1c).

(2) Genetic crosses between mottled striped strain (p^{sm})

Cross A in Table 1 presents the results of crosses between two trisomic ($p/p/Dp(2;f), p^s$) offspring of

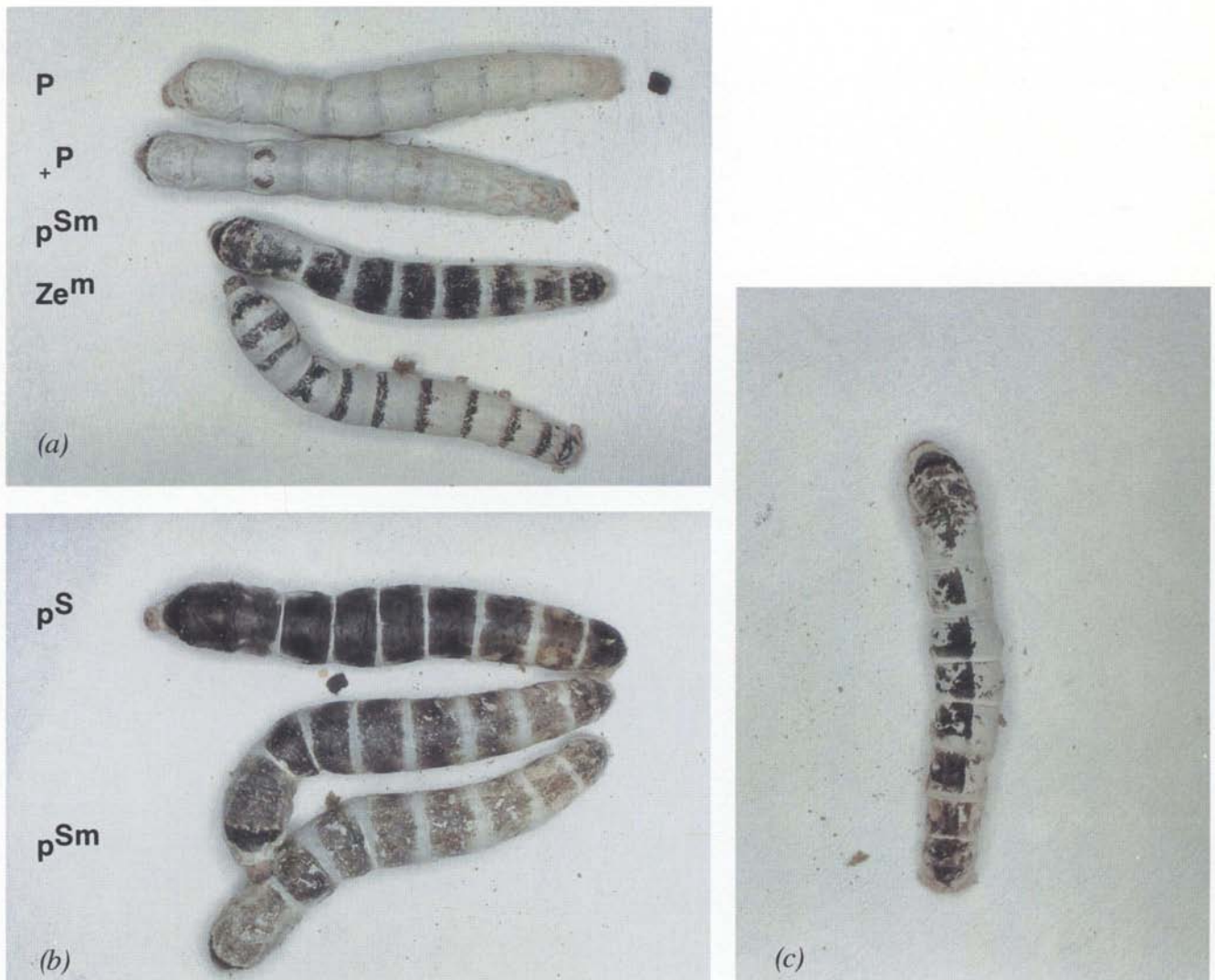


FIGURE 1. Marking characters and mottled genetic mosaics. (a) Several marking characters observed on the body surface of the 5th instar larva. Plain (p) is without marking. Normal marking ($+p$) is a typical phenotype of body marking showing several specific patterns on three dorsal segments of *Bombyx* larva: eye spots on the 2nd thoracic segment, semilunar markings on the 2nd abdominal segment, and star spots on the 5th abdominal segment. Mottled mosaics (mottled striped— p^{sm} and mottled zebra— Ze^m) have been established by X-ray irradiation from the original phenotypes. It should be considered that the difference of striped pattern between p^s and Ze lies only in the width of the black stripe. (b) Top of the figure represents a non-mottled phenotype which is nearly the same as the original striped p^s before X-ray irradiation. The lower two individuals from the same strain of p^{sm} show different mottling frequency (see text). (c) This half mosaic is one from the same p^{sm} strain as shown in b.

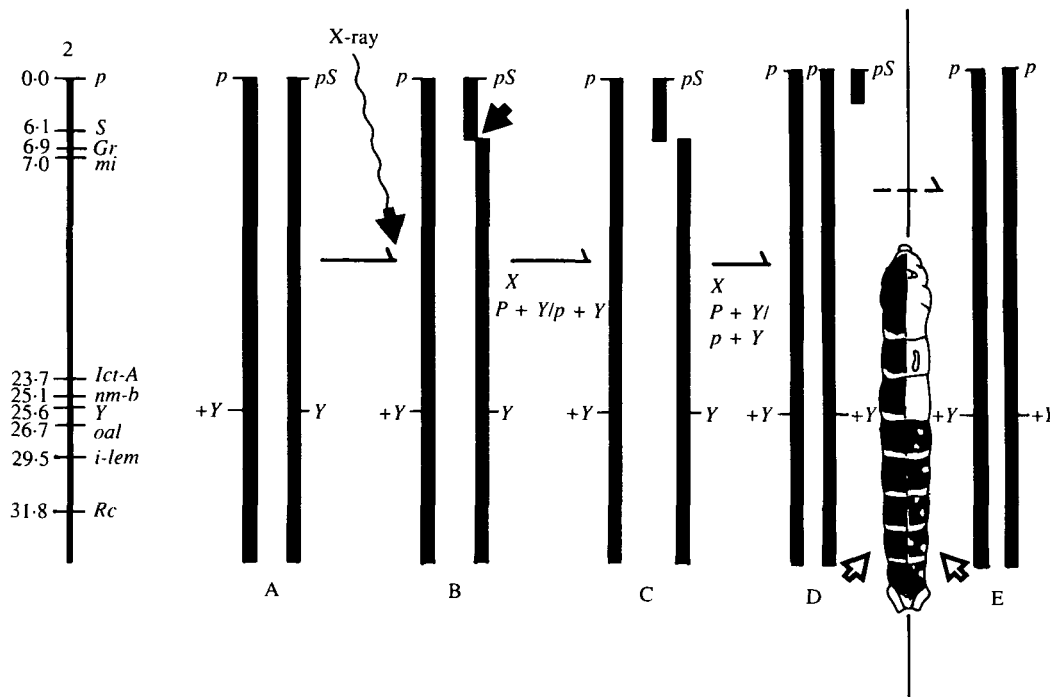


Fig. 2. Schematic illustration of the mosaic induction of mottled striped strain (p^{sm}). The linkage map of chromosome 2 of *B. mori* is shown on the left. (B–C) After a chromosomal breakage occurred due to X-ray irradiation, subsequent generations containing the p^s gene were obtained by crossing with double recessive

homozygotes (p^{+y}/p^{+y}). (C–D) The results of segregation of four types in F1 (p^sY ; p^s+^y ; pY ; $p+^y$) showed independence of two linked dominant markers, p^s and Y , indicating that a breakage actually occurred between them (Tazima, 1964). Phenotype of (D) is p^s+^y . Y ; Yellow blood, a dominant marker.

Table 1. Results of crosses in a mottled striped strain (p^{sm-788})

Cross/Batch	Offspring ($p^s/p^s/p$) ^a	Genotype		χ^2	P
		($p^s/p/p$) ^b	(p/p)		
A					
1	55	177	150	50.4	< 0.0001
2	25	90	76	27.6	< 0.0001
3	10	72	56	32.2	< 0.0001
	(1	: 2	: 1) ^c		
B	0	56	95	10.0	0.0015
	(0	: 1	: 1) ^c		
C	1	168	20	—	—
	(0	: 1	: 0) ^c		

Cross A, ($p^s/p/p$) × ($p^s/p/p$); Cross B, ($p^s/p/p$) × (p/p); Cross C, ($p^s/p^s/p/p$) × (p/p). We can discriminate between the hemizygote ($p^s/p/p$) and homozygote ($p^s/p^s/p/p$) according to intensity of the black colour of dorsal stripes. Progeny of each cross are listed by single pair matings (batch), except that batch 1 of cross A is the combined result from two crosses. χ^2 and P (probability) for the expected segregation ratio (^c) of 1:2:1 (Cross A), 0:1:1 (Cross B) and 0:1:0 (Cross C) at the p locus are given.

^a tetrasomy ($p/p/Dp(2; f)$, p^s/p^s).

^b trisomy ($p/p/Dp(2; f)$, p^s).

the mottled striped strain p^{sm788} . These trisomic individuals are thought to have an extra copy of p^s on a chromosomal fragment, in addition to the recessive alleles (p/p) on two normal autosomes (see Fig. 2D). The χ^2 test for each batch clearly shows a distortion of the expected segregation ratio (1:2:1). This distribution does not seem to be due to a high lethality of

the p^s homozygote because its hatchability is essentially normal (93% in batch 1 and the homozygous individuals grew normally). In order to confirm that discrimination between the hemizygote ($p^s/p/p$) and homozygote ($p^s/p^s/p/p$) can be performed correctly, respective individuals were crossed with the recessive homozygotes (p/p) (Table 1 Cross B and C). No

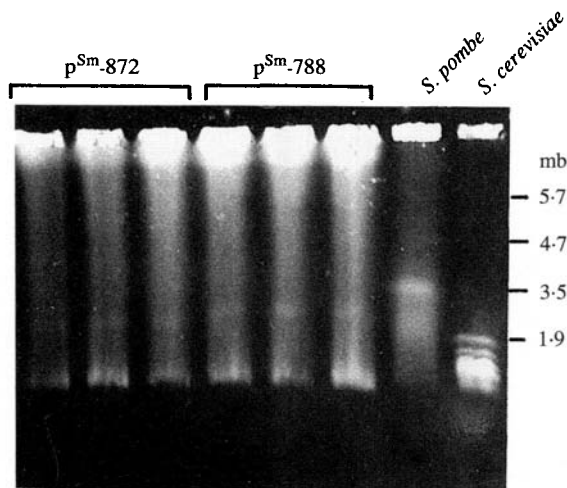


Fig. 3. Pulsed field gel electrophoresis of *B. mori* chromosomal DNAs isolated from two mottled striped strains. Chromosomal DNAs from different strains of mottled striped, p^{Sm} -788 and p^{Sm} -872, were run in three lanes, respectively. The chromosomes of *S. pombe* and *S. cerevisiae* were run alongside as size markers.

homozygote was observed except one in cross C and distribution tended toward the expected ratio in both cases, suggesting that discrimination can be successful. In all crosses above, number of offspring having p^S fragment shows a tendency to decrease. Thus it is possible that the loss of the fragment carrying the p^S gene generates the segregation distortion in germ cells and mottled mosaic pattern in somatic cells.

(3) Pulsed field gel electrophoresis and Southern blot analysis of chromosomal DNAs from mottled mosaics

Although the genetic data are consistent with the notion that the mosaic phenotype of p^{Sm} or Ze^m may be due to the loss of a chromosomal fragment, there are no molecular data supporting this hypothesis. The technique of pulsed field gel electrophoresis (PFGE) offers the possibility of identifying and characterizing the putative chromosomal fragments.

Figure 3 shows the EtBr staining pattern of chromosomal DNAs from sperm cells and spermatocytes of two mottled striped strains electrophoresed by CHEF (contour-clamped homogeneous electric fields) (Chu *et al.* 1986). As shown in the figure, each strain shows a discrete band which migrates between the smallest chromosome of *S. pombe* (3.5 Mb) and the largest one of *S. cerevisiae* (1.9 Mb), suggesting that the size of the DNA is about 2.5–3.0 Mb. The DNA of strain p^{Sm} 872, which has a higher degree of mottling, seems smaller than that of strain 788.

To know whether the band actually represents a strain-specific DNA fragment, we performed PFGE analysis for chromosomal DNAs from other *Bombyx* stocks. The alleles p and p^{Sm} shown in Fig. 4 segregated from the same parents of a mottled mosaic strain 788, while the $+^p$ and the Ze^m segregants were from

independent strains. As shown in Fig. 4a, the '2.5 Mb' band (indicated by arrow) occurs only in the p^{Sm} samples. This band is found specifically in the mottled mosaic larvae of p^S , but not in the p larvae of the same generation.

To provide additional evidence that the band in p^{Sm} -788 represents a chromosomal fragment which carries the genes for the p^S phenotype, we performed Southern blot hybridization using silkworm chorion sequences as hybridization probes. Previous studies indicate that chorion structural genes of *B. mori* map to chromosome 2, with the most proximal markers only 2.3 cM from the p (p^S) locus (Goldsmith & Clermont-Rattner, 1979; Goldsmith & Kafatos, 1984). Organization of the chorion gene clusters on chromosome 2 is shown in Fig. 4c. The chorion probe used in the experiment, pCh Δ Not, contains coding and intergenic regions of a middle chorion gene pairs, L12 A/B from the cluster Ch1-2 (Spoerel *et al.* 1989; Mitsialis & Kafatos, 1985; Eickbush & Kafatos, 1982; Y. Suzuki, personal communication), and expected to cross-hybridize with many chorion genes under our experimental condition. Thus, if the DNA fragment shown in Fig. 4a is actually as large as 2.5 Mb and contains the p^S genes, it is possible that the fragment may also contain at least a part of the chorion gene families. Indeed, Fig. 4b shows clearly that the 2.5 Mb DNA fragment in 'a' hybridizes with the chorion probe. This observation supports the notion that the '2.5 Mb' bands are derived from chromosome 2. Based on a genome size of 530 Mb and total map of about 990 cM of *B. mori*, we can estimate the minimum DNA length between p and Ch1-2 as around 1.2 Mb ($530/990 \times 2.3$) and the maximum needed to hybridize with the L12A/B probe as the distance between p and Gr , or around 3.7 Mb ($530/990 \times 6.9$) (see Fig. 4c). This estimation of DNA length of the fragment agrees reasonably well with our data shown on the gel.

Southern blot analysis was also performed using the non-mottled p^S which is the phenotype before X-ray irradiation, and with the two mottled striped strains (Fig. 5). When the chorion gene pair was used as a hybridization probe, specific bands of about 2.5 Mb were detected in p^{Sm} strains but not in the original p^S . These results suggest that the chromosomal fragments in the mottled p^S strains were apparently generated by X-ray irradiation.

Unlike the p^{Sm} strains, we could not detect any distinct DNA band in the mottled Ze strain (Ze^m) which exhibits a similar mosaic and distorted segregation pattern (data not shown). However, since a suitable probe for identifying a chromosome specific DNA fragment carrying the Ze genes is not yet available, we can't exclude the possibility that this strain may also harbour a small chromosomal fragment.

It is not clear how these fragments are maintained stably during cell divisions. In this respect, it should be emphasized that each chromosome of *B. mori*

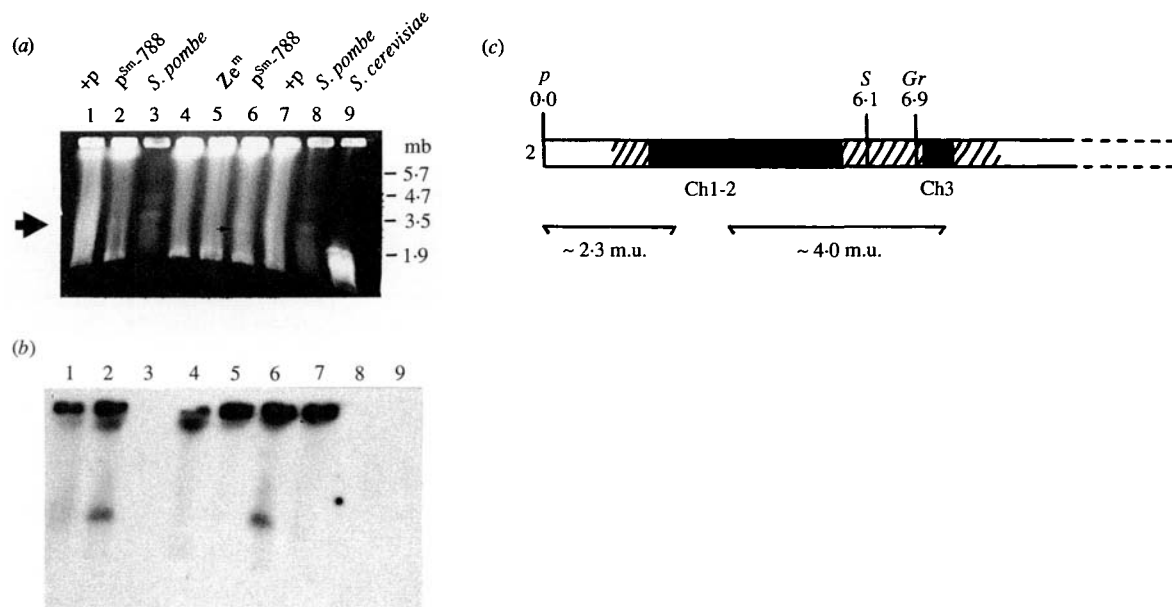


Fig. 4. Pulsed field gel electrophoresis and Southern blot analysis of *B. mori* chromosomal DNAs isolated from several strains and mosaic mutants: +^p (normal marking); p (plain); psm (mottled striped); Ze^m (mottled zebra). The phenotypes of p and psm used in the figure are segregated from the same parents of psm-788 strain.

(a) Ethidium bromide strain. (b) An autoradiogram of the gel in (a) hybridized with pChΔNot, a plasmid clone of the chorion gene. (c) Organization of chorion gene clusters around the *Gr*. Undefined border regions are indicated as oblique hatched areas.

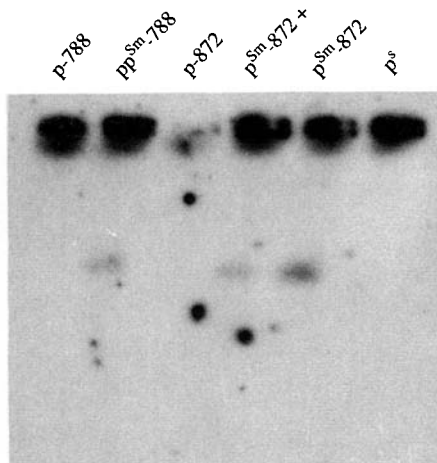


Fig. 5. Southern blot analysis of *B. mori* chromosomal DNAs separated by CHEF from several strains: p-788 and p-872 (plain); p^s (striped, non-irradiated strain); psm-788, psm-872 and psm-872⁺ (mottled striped). The phenotypes of p and psm used in the figure are segregated from the parents of mosaic strains 788 and 872, respectively. Hybridization probe is pChΔNot as used in Fig. 4b.

probably has a diffuse or polycentric (holocentric) centromere. Condensed metaphase chromosomes of this insect do not have primary constrictions, and microtubules attach all along their polar faces (Friedlander & Wahrman, 1970; Murakami & Imai, 1974). These observations suggest that the centromeres may distribute along their whole length. This raises the possibility that the chromosomal fragments in the mottled mosaics seen above may be kept stably in the cells due to presence of dispersed centromeres.

We also do not know how the unstable chromosomal fragment becomes established following irradiation and giving rise to the mosaic pattern. Based on studies of X-ray induced chromosomal rearrangements in *Drosophila melanogaster*, it has been generally accepted that a broken chromosome cannot survive without a telomere (Roberts, 1975). If the p^s fragment found in our studies has no telomeric sequences at the broken end, its stability may be decreased significantly. Recently, however, Levis reported viable deletions of a telomere from a *Drosophila* chromosome (Levis, 1989). This supports a notion that the p^s fragment can be maintained through numerous cell divisions even without a telomere. Further studies on the isolated fragment will make it possible to answer these questions concerning its structure and function.

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