Nucleotide Sequence of the Random
Amplified Polymorphic DNA (RAPD)
on the W Chromosome of the Silkworm,
Bombyx mori (Lepidoptera: Bombycidae)¹

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The sex chromosomes of the silkworm, *Bombyx mori*, are designated ZW (equivalent to XY) for the female and ZZ (XX) for the male (TANAKA, 1921). Femaleness is determined by the presence of a single W chromosome irrespective of the number of autosomes or of Z chromosomes as shown by the observation that even ZZW and ZZZW individuals in the triploid and tetraploid states, respectively, are normal females (HASIMOTO, 1933). Although 400 or more visible mutations have been placed on linkage maps in *B. mori* (DOIRA, 1992; GOLDSMITH, 1995), no gene for a morphological character has so far been found on the normal W chromosome; however, chromosomal fragments containing autosomal markers have been translocated to the W (HASIMOTO, 1948; KIMURA et al., 1971; TAZIMA, 1941, 1944, 1948; TAZIMA et al., 1955 a, b).

In order to analyze the mechanism of sex determination in *B. mori*, it is necessary to clone the presumptive/putative *Fem* gene on the W chromosome. But it is difficult to identify the W chromosome cytologically because chromosomes of the silkworm are generally small, holocentric, numerous ($2n = 56$) and indistinguishable, as in most Lepidoptera. Moreover, W-specific DNA sequences have not yet been

found. Therefore, finding a DNA sequence on the W chromosome would be an important breakthrough for further analysis of the W chromosome. Recently we identified a random amplified polymorphic DNA (RAPD) that is specific to the female (Female-218 RAPD) on a translocation-carrying W chromosome in the strain C137 (T(W;2) +^p) (ABE et al., 1995).

In this paper we report the nucleotide sequence of this female-specific RAPD and the polymerase chain reaction (PCR) primers for amplification of the RAPD locus from translocation-specific females.

MATERIALS AND METHODS

Insects. J137 (+^p/+^p) and C137 (Dp(2;W) +^p, *p/p*) are highly inbred strains and which have been maintained by sister-brother matings in the Tokyo University of Agriculture and Technology. +^p is the normal larval marking gene and *p* (plain) is a recessive allele; the *p/p* larva is all white. The TW + P strain, which is congenic to the J137 strain for the W chromosome, was constructed by crossing a C137 female with a male of the J137 strain and backcrossing the resulting F₁ females to J137 males. The TW + P strain has been maintained by repeated backcrossing of the females (ZT(W;2) +^p) to males of the J137 strain. The C108 and p50 strains were the same as described by PROMBOON et al. (1995). Both C137 and TW + P have the T(W;2) +^p chromosome; other strains have a normal W chromosome. These silkworms were fed on fresh mulberry leaves.

DNA extraction. We used about 20 individuals, including both males and females for each strain. Genomic DNA was extracted individually from larval posterior silk glands by the method of BENDER et al. (1983). It was further purified by extraction with phenol/chloroform, precipitated by ethanol and resuspended in 0.1 × TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

Cloning, sequencing and homology search. The Female-218 RAPD was recovered from an ethidium bromide-stained agarose gel by cutting out and using EASYTRAP Ver. 2 (TaKaRa Shuzo Co. Ltd., Kyoto, Japan) according to the manufacturer's instructions. The DNA ends were blunted using T4 DNA polymerase in a DNA blunting kit (TaKaRa Shuzo Co. Ltd.) according to the manufacturer's instructions. The blunted DNA was extracted with phenol/chloroform and recovered by ethanol precipita-

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Fig. 1. Nucleotide sequence of the Female-218 RAPD. The numbers to the left denote adjacent nucleotide positions. Arrows above and below the nucleotide sequence indicate location of primers used in this study. This nucleotide sequence data will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number of D86623.

tion. The plasmid, pBluescript II SK + (Stratagene Cloning System, La Jolla, U.S.A.), was digested with the endonuclease *Sma*I (Nippon Gene Co. Ltd., Tokyo, Japan), ligated with the blunted PCR product, and used to transform *Escherichia coli* MV1190. Plasmids were isolated from the transformants by using the Alkali-SDS method (SAMBROOK et al., 1989). Double-stranded plasmids were denatured with alkali and sequenced by the dideoxy chain termination method of SANGER et al. (1977) using the Sequence Version 2.0 DNA Sequencing Kit (United States Biochemical Corp., Cleveland, U.S.A.) and commercial primers. Homologies were searched for in Genbank database using the BLAST program (ALTSCHUL et al., 1990).

Primers. The UBC-218 primer (5'-CTCAGCCCAG-3') was purchased from the University of British Columbia Nucleic Acid-Protein Service Unit (Vancouver, Canada), and used to amplify the Female-218 RAPD. To convert the RAPD into a SCAR marker (PARAN and MICHELMORE, 1993) which is amplified as a single band without accompanying any other PCR bands, four longer primers for PCR were designed based on the sequencing results and synthesized with a CYCLON DNA synthesizer (Millipore Corp., Bedford, U.S.A.). They are μ l as fol-

lows: W-1: 5'-CAGCCCAGAACTCAAAATCG-3' [3→22, forward]; W-1B: 5'-CTCAAAATCGAAGATTGCGA-3' [13→32, forward]; W-2: 5'-CTCAGCCCAGAGACGTGAGGTCT-3' [815→793, reverse]; W-2B: 5'-TCAATTGAATCGGGTCACTG-3' [787→768, reverse] synthesized with a CYCLON DNA synthesizer. Numbers indicate the corresponding bases in the Female-218 RAPD sequence.

PCR. In the PCR, each reaction was 30 μ l in volume and contained 150 ng template DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia-LKB Biotech.), 0.5 mM forward primer (W-1 or W-1B), 0.5 mM reverse primer (W-2 or W-2B), 0.68 units *Taq* polymerase (Wako Pure Chemical Industries, Osaka, Japan) and the manufacturer's reaction buffer. Each reaction was overlaid with 50 μ l of light mineral oil to prevent evaporation. We used a Zymoreactor II machine (ATTO Co. Ltd., Tokyo, Japan) for thermal cycling. The PCR consisted of 40 cycles performed as follows: at 94°C for 1 min, at 60°C for 2 min, at 72°C for 3 min, and a final extension of 10 min at 72°C. When we used UBC-218 primer for amplification of original Female-218 RAPD, the PCR protocol was the same as WILLIAMS et al. (1990). PCR products were analyzed on 2%

Fig. 2. Amplification patterns of genomic DNA from males and females of the C137 (A) and J137 (B) strains, by using four sets of primers. Each set of two lanes results from PCR amplification with a different primer combinations: W-1 + W-2, W-1 + W-2B, W-1B + W-2 and W-1B + W-2B. Lane M, molecular size markers, λ -Eco T14 digest. The number at the left indicate base pairs. The arrow indicate the Female-218 RAPD produced by UBC-218 primer in the C137 strain.

agarose gels.

Southern hybridization. DNA bands were blotted from an agarose gel to a nylon membrane (Hybond N+, Amersham), and hybridized to a 32 P labelled DNA probe by the standard procedure (SAMBROOK et al., 1989).

RESULTS

Nucleotide sequence and homology to known sequences in the database

The nucleotide sequence of the Female-218 RAPD is shown in Fig. 1. It consisted of 815 base pairs (bp), and did not include any long open reading frames. The computer search revealed that this sequence is partially homologous to four sequences already reported in *B. mori*. The first homology was found in the first intron of the fibroin light-chain gene (KIKUCHI et al., 1992; GenBank accession no. M76430); the sequences of 5074–5387 and 4937–5042 were 89% homologous to 308–621 and 93%

Fig. 3. Amplification patterns of genomic DNA from males and females of the TW + P strain, by using one set of primers, W-1 + W-2. Lane M, the numbers at the left, and the arrow are the same as Fig. 2.

homologous to 167–272 of the Female-218 RAPD, respectively. The second homology was found in the 5' upstream region of the silk protein p25 gene (CHEVILLARD et al., 1986; GenBank accession no. X04226), in which the sequence of 384–533 was 79% homologous to 319–170 of the Female-218 RAPD. The third homology was found in the first intron of the storage protein 2 (SP2) gene (FUJII et al., 1989; GenBank accession no. M24371), where the 120 bp sequence of 658–810 was 78% homologous to 158–310 of the Female-218 RAPD. The fourth homology was found in the Bombyxin gene (GenBank accession no. D10742), in which the sequences of 1075–1249 was 74% homologous to 141–315 of the Female-218 RAPD.

Amplification patterns from the newly designed primers

To find a set of primers that amplify the Female-218 RAPD locus specifically as a single band, we compared four sets of primers, using W-1 or W-1B as a forward primer, and W-2 or W-2B as a reverse primer. Figure 2A shows the results of amplification using four combinations of these primers in the C137 strain. Although the Female-218 RAPD (815 bp) was amplified in females and not in males with primer UBC-218 alone, the new primers produced a single band of approximately 800 bp both in females and males despite the use of a relatively higher annealing temperature. Intensity of the band in the female was more than in male in every combination, indicating that the band included both the Female-218 RAPD and a non-sex-specific band. When used as a probe for Southern blot analysis, the cloned Female-218 RAPD fragment hybridized to these bands in both sexes (data not shown). These results indicate that the newly designed primers appeared to match and am-

plify multiple regions of the genome in the C137 strain, and that the female band originated from at least two loci which had similar nucleotide sequences. This 800 bp DNA band was amplified not only from C137 but also from strains C108 or p50 (data not shown). In the J137 strain, when using only one of the four sets of primers, W-1 and W-2, no band was amplified both in either females or males. However, using other sets of primers, minor bands were seen in both sexes. Figure 2B shows the amplification patterns of only one female and male. Amplification patterns of minor bands varied a little among the 20 J137 individuals sampled (data not shown).

In the TW + P strain, a major band of approximately 800 bp was amplified from female DNAs only when using W-1 and W-2. Moreover, this combination of W-1 and W-2 did not amplify the 800 bp band from males. To confirm this result, many template DNA samples were extracted from the TW + P strain individually and tested. A major band was obtained from all females but not from males (Fig. 3). These results suggest that the 800 bp band in the TW + P strain is a "pure" Female-218 RAPD which was originated from the W chromosome in C137.

DISCUSSION

We determined the nucleotide sequence of the Female-218 RAPD amplified from the T(W;2) +^b chromosome in C137. This sequence is the first report of a sequence from the W chromosome in *B. mori*. The result of a homology search, however, indicated that it contains several elements which are homologous to autosomal DNA sequences. The PCR studies using new primer sets also showed that the males of strains C137, C108 and p50 have the Female-218 RAPD, suggesting that an autosome(s) or the Z chromosome in these strains carries a very similar sequence. Since the W chromosome of C137 carries a region originating from the +^b locus on the second chromosome, further studies are needed to determine the relationship between the Female-218 RAPD and the +^b locus.

The TW + P strain which we established is useful for further analyses of the W chromosome because the W-1 and W-2 primer combination can specifically amplify a DNA segment, region on the W chromosome in this strain. This PCR strategy might be used to obtain a longer DNA sequence from a genomic library of the TW + P strain using yeast artificial chromosomes (YAC), bacterial artificial chromosomes (BAC) or P1 phage as a vector. It

might also be possible to identify the W chromosome cytologically by using an *in situ* labelling method (JUAN and GOSALVEZ, 1994; KOCH et al., 1989).

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REFERENCES

- ABE, H., T. SHIMADA, T. YOKOYAMA, T. OSHIKI and M. KOBAYASHI (1995) *J. Seric. Sci. Jpn.* **64**: 19–22 (in Japanese with English summary).
- ALTSCHUL, S.F., W. GISH, W. MILLER, E.W. MYERS and D.J. LIPMAN (1990) *J. Mol. Biol.* **215**: 403–410.
- BENDER, W., P. SPIERER and D.S. HOGNESS (1983) *J. Mol. Biol.* **168**: 17–33.
- CHEVILLARD, M., P. COUBLE and J.C. PRUDHOMME (1986) *Nucleic Acids Res.* **14**: 6341–6342.
- DOIRA, H. (1992) *Genetical Stocks and Mutations of Bombyx mori: Important Genetic Resources* (H. DOIRA, ed.). Silkworm Genetic Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University, Fukuoka, pp. 1–73.
- FUJII, T., H. SAKURAI, S. IZUMI and S. TOMINO (1989) *J. Biol. Chem.* **264**: 11020–11025.
- GOLDSMITH, M.R. (1995) Genetics of the silkworm: revisiting an ancient model system. In *Molecular Model Systems in the Lepidoptera* (M.R. GOLDSMITH and A.S. WILKINS, eds.). Cambridge University Press, New York, pp. 21–76.
- HASIMOTO, H. (1933) *Jpn. J. Genet.* **8**: 245–247 (in Japanese).
- HASIMOTO, H. (1948) *J. Seric. Sci. Jpn.* **16**: 62–64 (in Japanese).
- JUAN, C. and J. GOSALVEZ (1994) *Genome* **37**: 173–175.
- KIKUCHI, Y., K. MORI, S. SUZUKI, K. YAMAGUCHI and S. MIZUNO (1992) *Gene* **110**: 151–158.
- KIMURA, K., C. HARADA and H. AOKI (1971) *Jpn. J. Breed.* **21**: 199–203 (in Japanese).
- KOCH, J., S. KOLVRAA, K.B. PETERSEN, N. GREGERSEN and L. BOLUND (1989) *Chromosoma* **98**: 259–265.
- PARAN, I. and R.W. MICHELMORE (1993) *Theor. Appl. Genet.* **85**: 985–993.
- PROMBOON, A., T. SHIMADA, H. FUJIWARA and M. KOBAYASHI (1995) *Genet. Res. Camb.* **66**: 1–7.

- SAMBROOK, J., E.F. FRISCH, and T. MANIATIS (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York. pp. 9.31–10.17.
- SANGER, F., S. MIKLEN and A.R. COULSON (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**: 5463–5467.
- TANAKA, Y. (1921) *Bull. Seric. Exp. Stn.* **6**: 1–33 (in Japanese).
- TAZIMA, Y. (1941) *J. Seric. Sci. Jpn.* **12**: 184–188 (in Japanese).
- TAZIMA, Y. (1944) *Bull. Seric. Exp. Stn.* **12**: 109–181 (in Japanese with English summary).
- TAZIMA, Y. (1948) *Rep. Silk Sci. Res. Inst.* No. 2: 27–29 (in Japanese).
- TAZIMA, Y., T. KUME and M. KAMIOKA (1955 a) *Rep. Silk Sci. Res. Inst.* No. 5: 5–24 (in Japanese).
- TAZIMA, Y., C. HARADA, N. OHTA, Y. KOBAYASHI and S. URABE (1955 b) *Rep. Silk Sci. Res. Inst.* No. 5: 24–47 (in Japanese).
- WILLIAMS, J.G.K., A.R. KUBELIK, J.L. KENNETH, J.A. RAFALSKI and S.V. TINGEY (1990) *Nucleic Acids Res.* **18**: 6531–6535.

Replication of a Small Isometric Virus in Cultured *Lymantria dispar* (Lepidoptera: Lymantriidae) Cells¹

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Small RNA viruses isolated from insects belong to three virus families (MURPHY et al., 1995). Some of the viruses replicate in cultured insect cells. These are cricket paralysis virus (*Picomaviridae*), *Drosophila* C virus (*Picomaviridae*), black beetle virus (*Nodaviridae*), flock house virus (*Nodaviridae*), and *Heliothis armigera* stunt virus (*Tetraviridae*).

QUIOT (1976) and GOODWIN et al. (1978) independently established a cell line from the ovarian sheath tissue of the gypsy moth, *Lymantria dispar*. We obtained two cell lines, one (SCLD 135) from Dr. S. BELLONCIK of Université du Québec in 1982 and the other (IPLB-LD-652Y) from Dr. Y. TANADA of the University of California in 1985, and maintained

them by serial cell passages.

GRIGNON (1982) observed progressive degeneration of the QUIOT cell line (SCLD) due to the multiplication of a densovirus which persisted in a latent state during the first 100 cell passages. Although we initiated the present study to confirm the above observation, a different virus was isolated from the same cell line. We herein report the virus properties and the cytopathology.

MATERIALS AND METHODS

Cell culture. Two *Lymantria dispar* cell lines, SCLD 135 and IPLB-LD-652Y, were cultured at 26°C in GRACE's medium (pH 6.2) supplemented with 5–10% heat-inactivated fetal bovine serum (JRH). Inocula were prepared from apparently healthy SCLD cells or infected IPLB-LD cells by disrupting the cells in GRACE's medium with the use of a glass homogenizer or an ultrasonicator (Branson Ultrasonics Corp.). IPLB-LD cell cultures were infected by the addition of 1/10 volume of the homogenate suspension.

Virus purification. A total of 400 ml of IPLB-LD cultures (8.0×10^5 cells/ml) was infected with the virus which had been passed more than 10 times within the same cell line. Infected cells were sedimented by low-speed centrifugation, suspended in 50 mM Tris-HCl buffered saline solution (pH 7.4, 0.5 M NaCl), and ultrasonicated at 110 W for 15 s. The cell debris was removed by centrifugation at $9,000 \times g$ for 20 min. The supernatant containing virions was combined with 55% (final conc.) ammonium sulfate to salt out the protein. The precipitate

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