Cytogenetic and molecular characterization of the MBSAT1 satellite DNA in holokinetic chromosomes of the cabbage moth, *Mamestra brassicae* (Lepidoptera)

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Abstract

Digestion of *Mamestra brassicae* DNA with *Dra*I produced a prominent fragment of approximately 200 bp and a ladder of electrophoretic bands with molecular weights which are a multiple of 200 bp. Southern blotting revealed that this ladder is composed of DNA fragments that are multimers of the 200-bp *Dra*I band suggesting that *Dra*I isolated a satellite that has been called *Mamestra brassicae* satellite DNA 1 (MBSAT1). MBSAT1 is the first satellite DNA isolated in Lepidoptera. *In-situ* *Dra*I digestion of chromosome spreads, together with fluorescent *in-situ* hybridization, showed that MBSAT1 sequences are clustered in heterochromatin of the sex chromosomes, Z and W. MBSAT1 was 234 bp long with an AT content of 60.7%. The curvature–propensity plot suggested a curvature in the MBSAT1 structure.

Introduction

Most studies concerning satellite DNAs have been focused on organisms with monocentric chromosomes, whereas organisms possessing holokinetic (holocentric) chromosomes have been almost neglected. Only nematodes (Roth 1979, Naclerio *et al.* 1992, Grenier *et al.* 1997, Castagnone-Sereno *et al.* 1998a, 1998b), aphids (Bizzaro *et al.* 1996, Spence *et al.* 1998, Mandrioli *et al.* 1999a, 1999b, 1999c), a bug (Lagowsky *et al.* 1973) and a plant (Collet & Westerman 1987) have been studied in this regard.

Moths and butterflies, the Lepidoptera, have holokinetic chromosomes. Their chromosomes are

usually small and numerous, lack distinct primary constrictions (centromeres) and sister chromatids separate by parallel disjunction at mitotic metaphase (Murakami & Imai 1974). Some findings suggest that lepidopteran chromosomes are not truly holokinetic but exhibit a localized kinetochore that, in contrast to that in the typical monokinetic chromosomes, covers a relatively large portion of the chromosomal surface. These findings favour a chromosome type intermediate between the traditional holokinetic and monokinetic chromosomes (for a review, see Wolf 1996).

In accordance with the absence of distinct centromeres, lepidopteran chromosomes lack pericentric heterochromatin that is common in

monocentric chromosomes. In most species examined, heterochromatin was found only in sex chromosomes and, with a few exceptions, only in the heterogametic female sex. The Lepidoptera possess a WZ/ZZ (female/male) sex-chromosome system or its numerical variations (Traut & Marec 1997, Sharma & Sobti 2002). Like many Y chromosomes in the XY/XX system, the W chromosome often consists partly or largely of heterochromatin (Traut & Marec 1997). In addition, most species display one or more heterochromatin bodies in female somatic interphase nuclei but not in male nuclei. This female-specific heterochromatin (so-called 'W-chromatin' or 'sex-chromatin') is derived from the W chromosome (Traut & Marec 1996). Data on the DNA composition of W heterochromatin and repetitive DNAs in Lepidoptera are scarce and limited to a few studies reporting microsatellites (e.g. Reddy *et al.* 1999) and transposable elements (e.g., Abe *et al.* 1998, Ohbayashi *et al.* 1998) in *Bombyx mori*. To date, no published information about satellite DNAs is distributed in any lepidopteran species.

Here we present data on structure and chromosomal distribution of a satellite DNA identified in the cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae), a serious pest of agricultural crops.

Material and methods

We used the IZD-MB-0503 cell line of *Mamestra brassicae* (ATCC number: CRL-8003). The cells were cultured in Ex-Cell 405 medium (JRH Biosciences, KS, USA) at 26°C.

CRL-8003 cells were spread following the method described by Mandrioli (2002). Briefly, the cells were kept in a hypotonic solution (0.8% sodium citrate) for about 45 min, then transferred to minitubes and centrifuged at 350 g for 3 min. A fixative solution (methanol–acetic acid, 3:1) was successively added to the pellet, which was made to flow up and down for 1 min through a needle of a 1-ml hypodermic syringe. Finally, the pellet was resuspended in 200 µl of fresh fixative, and 20 µl of cellular suspension was dropped onto clean slides and air dried.

C-banding was performed according to the technique of Sumner (1972). After the treatment,

slides were stained with 4'-6'-diamidino-2-phenylindole (DAPI) as described by Donlon & Magenis (1983).

Fresh chromosome spreads were digested at 37°C with 0.6 U/µl of *DraI* and *ApaI* in the appropriate buffer solution, for time periods ranging from 10 min to 2 h. Nick translation was carried out for 20 min according to Mandrioli *et al.* (1999c).

DNA probes were labelled by random priming using the 'DIG high prime' (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's protocol. FISH was carried out as described by Mandrioli *et al.* (1999c) making the following stringency washes after hybridization: twice in $0.1 \times \text{SSC}$ for 10 min at room temperature and twice in $0.1 \times \text{SSC}$ at 45°C for 10 min.

DNA extraction from cultured CRL-8003 cells was performed using a standard protocol given in Mandrioli (2002). Southern hybridization, dot blotting and restriction enzyme digestion were carried out as described in Mandrioli *et al.* (1999c).

RNA extraction and RT-PCR were performed with the SV Total RNA Isolation System (Promega Corporation, Madison, USA) and with the Access RT-PCR System (Promega), respectively, according to the supplier's suggestions.

MBSAT1 fragments were cloned using the 'pGEM-T-easy' cloning kit (Promega Corporation, Madison, USA) according to manufacturer's protocol.

Sequence alignments and search for open reading frame and internal repeats were performed using the GCG software (GCG Computer Group, Madison, USA). The curvature–propensity plot was calculated with DNase I parameters of the bend.it server (http://www2.icgeb.trieste.it/~dna/bend_it.html) according to Gabrielian *et al.* (1996).

Results

In order to study *M. brassicae* heterochromatin at a molecular level, total genomic DNA was digested with different restriction endonucleases (*ApaI*, *AluI*, *DdeI*, *MspI*, *HpaII*, *DraI*) and separated in a 1.2% agarose gel by electrophoresis. Digestion with *AluI*, *DdeI*, *MspI*, and *HpaII*,

respectively, resulted in a diffuse smear on the gel. A faint electrophoretic band corresponding to a DNA fragment of 1800 bp was observed after digestion with *Apa*I, whereas *Dra*I produced a prominent band of approximately 200 bp and a ladder of electrophoretic bands that appeared as multiples of the 200-bp fragment (Figure 1a).

As the ladder observed is typical for a satellite DNA, the *Dra*I 200-bp fragment was isolated from the gel, cloned, and used as a probe for hybridization experiments. Southern blotting after *Dra*I digestion (Figure 1b, lane 1) revealed a regular ladder of bands composed of basic-length multimers. A ladder of bands was also present in *Dde*I and *Alu*I (Figure 1b, lanes 3 and 4) digested DNAs but, compared with the pattern obtained after *Dra*I digestion, *Dde*I lacked the first two multimers, whereas *Alu*I showed only three multimers. Based on its repetitive nature, the *Dra*I fragment was called *M. brassicae* satellite DNA 1 (MBSAT1). Comparison of hybridization patterns of MBSAT1 after *Msp*I and *Hpa*II digestion, respectively, showed

that MBSAT1 is not methylated since there was no difference between the two restriction patterns (Figure 1b, lanes 5 and 6).

By means of densitometric scanning of dot blots, obtained by hybridizing the MBSAT1 probe to the *M. brassicae* genomic DNA, we estimated that MBSAT1 accounts for $1.9 \pm 0.3\%$ of the genome.

In-situ digestion with restriction endonucleases (RE) followed by nick translation (NT) showed that *Dra*I restriction sites are clustered in C-positive segments of two chromosomes (Figure 2a) previously identified as the sex chromosomes, Z and W (Mandrioli 2002). *Apa*I targets were localized in the NOR-bearing telomeric segments of Z and W chromosomes (Figure 2b, c). GC-richness of the *Apa*I restriction target together with the results of silver staining after *Apa*I/NT indicated that the 1800-bp *Apa*I band (Figure 1a) contained rDNA genes.

FISH experiments, carried out on *M. brassicae* mitotic chromosomes using an MBSAT1 probe, confirmed the results of *in-situ* *Dra*I/NT. It was concluded that MBSAT1 sequences are clustered on Z and W chromosomes (Figure 2d) in chromosomal regions that are composed of heterochromatin as shown by C-banding and DAPI staining (Figure 2e).

Cloning and sequencing revealed that MBSAT1 length is 234 bp with an AT content of 60.7% (MBSAT1 Genbank accession number: AY-136944). A search for homology of MBSAT1 with other DNA sequences in GenBank and EMBL databases yielded negative results. Apart from short poly-A tracts, no significant direct or inverted repeats or open reading frames were revealed by sequence analysis.

The curvature-propensity plot that was calculated with DNase I parameters of the bend.it server revealed an MBSAT1 region, located between nucleotide 108 and 182, with a high value of curvature propensity (13.6). Its magnitude roughly corresponds to the value calculated for a highly curved motif described in *Columba risoria* DNA satellite (CRBENSAT) (Figure 3) indicating that MBSAT1 is curved.

Transcriptional activity of MBSAT1 was examined by Northern blotting and RT-PCR without producing any positive results. Thus, it was concluded that MBSAT1 is not transcribed in *M. brassicae* cells.

Figure 1. Genomic DNA of *Mamestra brassicae* digested with *Dra*I (lane 1), *Apa*I (lane 2), *Alu*I (lane 3), *Dde*I (lane 4), *Msp*I (lane 5), and *Hpa*II (lane 6), respectively. (a) Electrophoresis in a 1.2% agarose gel stained with ethidium bromide; (b) Southern hybridization with the MBSAT1 probe. Note a positive band of about 1800 bp (indicated by asterisk) corresponding to the *Apa*I digested DNA and a ladder of bands after digestion with *Dra*I. The ladder of bands is still evident in *Dra*I lane after hybridization with the MBSAT1 probe, whereas several bands are present in *Alu*I and *Dde*I lanes.

Figure 2. Mitotic metaphase chromosomes prepared from the *Mamestra brassicae* CRL-8003 cells. **(a–c)** *In-situ* RT/NT shows that *Dra*I cutting sites **(a)** are clustered in heterochromatic segments of both sex chromosomes, whereas *Apa*I cutting sites **(b)** are restricted to one telomeric end of each sex chromosome containing rDNA genes as shown by NOR silver staining **(c)**; **(d, e)** FISH with the MBSAT1 probe **(d)** demonstrates that the Z and W sex chromosomes contain each several copies of MBSAT1 located in heterochromatic regions as shown by C-banding followed by DAPI staining **(e)**. Arrows indicate sex chromosomes. Bar represents 10 μ m.

Discussion

Digestion of *M. brassicae* genomic DNA with *Dra*I showed a new DNA satellite, called MBSAT1. The satellite sequences are arrayed in tandem with some subsets containing more than five units, and represent about 1.9% of the genome.

By means of *in-situ* RE/NT and FISH, we showed here that the MBSAT1 repeats are strictly concentrated in heterochromatin of both sex chromosomes, Z and W. In addition, the MBSAT1 hybridization sites corresponded to DAPI-positive heterochromatic segments of Z and W, indicating a high AT content in MBSAT1. This was subsequently confirmed by sequence analysis. The fact

that the same satellite DNA was located in both sex chromosomes but absent in all autosomes is of particular interest. Taken together with the absence of recombination in lepidopteran females (for a review, see Marec 1996), it implies that the MBSAT1 was pre-existing in the chromosomes before they differentiated as sex chromosomes. The presence of NOR on both the sex chromosomes strongly suggests that *M. brassicae* possess neo-W and neo-Z chromosomes that have evolved by fusion of the original WZ pair with the autosome pair bearing the NOR. Similar neo-W/neo-Z sex chromosomes were reported in *Orgyia antiqua* and *O. thyellina* (Traut & Marec 1997). The karyotype of *M. brassicae* favours this

Figure 3. Curvature-propensity plot of MBSAT1 satellite DNA showing the presence of a strong curvature propensity in the region between nucleotides 108 and 182.

hypothesis. It shows a low number of chromosomes ($n=11$) that differ considerably in their sizes (Mandrioli 2002), thus indicating multiple chromosome rearrangements in the genome. This might have happened recently as a population-specific event, since the early study of Saitoh (1959), performed in a Japanese population, reported a completely different karyotype of *M. brassicae* consisting of 31 small and uniform chromosome pairs. Thus, it appears that *M. brassicae* could be an interesting model, not only for the study of sex chromosome differentiation, but also for karyotype evolution in Lepidoptera.

The present study clearly shows that the satellite DNA in *M. brassicae* is localized in heterochromatic chromosome segments and, in addition, spreads throughout the entire length of the segments. This suggests that the constitutive heterochromatin of holokinetic chromosomes is principally made of the satellite DNAs as in monocentric chromosomes (John & Miklos 1979, John 1988). However, the distribution of heterochromatin is not equilocal in holokinetic chromosomes. In other words, heterochromatin is not located at specific sites such as, for example, centromeres in monocentric chromosomes. Our findings in *M. brassicae* thus support a hypothesis of Schweizer & Loidl (1987), who stated that the theory of 'equilocal distribution of heterochromatin' is not valid for holokinetic chromosomes.

The MBSAT1 repeat, isolated from *M. brassicae*, is a short DNA satellite with a consensus sequence of 234 bp. It exhibits a high AT content (60.7%) which is a general feature of satellite DNA (Singer 1982). The MBSAT1 is the first satellite DNA identified in Lepidoptera.

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