Chromosome Research (2007) 15:1015–1022
DOI: 10.1007/s10577-007-1176-4

Analysis of heterochromatic epigenetic markers in the holocentric chromosomes of the aphid *Acyrthosiphon pisum*

Mauro Mandrioli* & Federica Borsatti
Dipartimento di Biologia Animale, Università di Modena e Reggio Emilia, Via Campi 213/D, 41100 Modena, Italy; Tel: +39-59-2055544; Fax: +39-59-2055548; E-mail: mandrioli.mauro@unimo.it, mandriol@unimo.it
**Correspondence*

Received 6 July 2007. Received in revised form and accepted for publication by Pat Heslop-Harrison 7 September 2007

Key words: *Acyrthosiphon*, aphid, chromatin, heterochromatin, histone, HP1, methylation

Abstract

Monomethylated-K9 H3 histones (Me9H3) and heterochromatin protein 1 (HP1) are reported as heterochromatin markers in several eukaryotes possessing monocentric chromosomes. In order to confirm that these epigenetic markers are evolutionarily conserved, we sequenced the HP1 cDNA and verified the distribution of Me9H3 histones and HP1 in the holocentric chromosomes of the aphid *Acyrthosiphon pisum*. Sequencing indicates that *A. pisum* HP1 cDNA (called *Ap*HP1) is 1623 bp long, including a 170 bp long 5′UTR and a 688 bp long 3′UTR. The *Ap*HP1 protein consists of 254 amino acidic residues, has a predicted molecular mass of 28 kDa and a net negative charge. At the structural level, it shows an N terminal chromo domain and a chromo shadow domain at the C terminus linked by a short hinge region. At the cytogenetic level, *Ap*HP1 is located exclusively in the heterochromatic regions of the chromosomes. The same heterochromatic regions were labelled after immunostaining with antibodies against Me9H3 histones, confirming that Hp1 and Me9H3 co-localize at heterochromatic chromosomal areas. Surprisingly, aphid heterochromatin lacks DNA methylation and methylated cytosine residues were mainly spread at euchromatic regions. Finally, the absence of DNA methylation is observed also in aphid rDNA genes that have been repeatedly described as mosaic of methylated and unmethylated units in vertebrates.

Introduction

Aphids represent an interesting experimental model in cytogenetics because they have holocentric chromosomes possessing centromeric activity spread along the whole chromosomal axis (Hughes-Schrader & Schrader 1961, Blackman 1987). These chromosomes are also termed holokinetic because, during mitotic anaphase, the spindle attachment is not localized and chromatids move apart in parallel and do not form the classical V shaped figures typically observed during the movement of monocentric ones (Blackman 1987).

In recent years, an extensive survey of the structure of heterochromatin was carried out in several aphid species (for a review see Manicardi *et al.* 2002). Nevertheless, the occurrence of epigenetic chromatin modifications in aphid heterochromatin has been neglected. The same is also true for most of the other insect species possessing holocentric chromosomes, since at present the occurrence and functions of heterochromatic epigenetic markers have been studied only in the coccid *Planococcus citri* and in the moth *Mamestra brassicae* (Field *et al.* 2004, Borsatti & Mandrioli 2005).

Two typical heterochromatic epigenetic markers observed in monocentric chromosomes consist of the methylation of H3 histone tails and in the methylation of DNA at cytosine residues. In particular, several papers reported that histones and their post-translational modifications play a pivotal role in the assembly of

heterochromatin from yeast to vertebrates (Rea *et al.* 2000, Jenuwein & Allis 2001).

Current theories suggest that in addition to the direct effects of these modifications on nucleosome structure, the modified histones also act as recognition motifs that mediate interactions with transcription factors and chromatin-binding proteins. In particular, several experiments indicated that methylated-K9 H3 residues constitute a binding target for the heterochromatin protein 1 (HP1) (Lachner *et al.* 2001, Eskeland *et al.* 2007). The HP1 class of proteins consists of polypeptides involved in organizing higher-order structure of chromatin by linking or anchoring chromatin subunits (James & Elgin 1986, Epstein *et al.* 1992, Eissenberg & Elgin 2000, Kellum 2003). The possible functions of these complexes are wide-ranging and include roles in transcription repression, transgene silencing, chromosome segregation and sex chromosome inactivation (Jones *et al.* 2000, Wang *et al.* 2000).

DNA methylation at cytosine residues is the second modification typically occurring at heterochromatin of monocentric chromosomes (Adams 1996). However, even if its involvement in heterochromatin condensation is well documented in vertebrates, there are few data regarding its occurrence in insect heterochromatin and in particular in species with holocentric chromosomes.

In the study reported in the present paper we verified the presence of monomethylated-K9 H3 histones and HP1 in aphid heterochromatin and fully sequenced the HP1 cDNA. Finally, we analysed the methylation status of heterochromatin in order to verify whether aphid heterochromatin contains methylated sequences.

Material and methods

Acyrthosiphon pisum specimens were collected on *Pisum sativum* plants near Modena and maintained at 19 °C with 16 h of light and 8 h of darkness on *Vicia faba* plants.

Chromosome preparations of parthenogenetic females were made by spreading embryo cells, as previously described (Mandrioli *et al.* 1999). C-banding treatment was performed according to Sumner's technique (1972). After the treatments, slides were stained with chromomycin A_3 (CMA_3) according to Schweizer (1976) and with 4′,6′-diamidino-2-phenylindole (DAPI), as described by Donlon & Magenis (1983).

In situ nick translation (NT) experiments were carried out on fresh chromosome spreads digested with 0.6 U/μl of *Msp*I in the appropriate buffer for 1 h at 37°C. NT was carried out for 30 min according to Sumner *et al.* 1990, except that extravidin-FITC was used as a label, in place of streptavidin alkaline phosphatase, to evidence the incorporation of biotin-dUTP.

In situ immunostaining experiments were performed with a mouse anti-*Drosophilamelanogaster* HP1a antibody diluted 1:500 (kindly furnished by Rebecca Kellum) (Huang *et al.* 1998) and a rabbit anti-monomethylated-K9 histone H3 (Me9H3) (cat. no. 07–212, Upstate, Cambridge, UK) diluted 1:250 following the protocol of Bongiorni *et al.* (2001), but with the difference that a FITC-conjugated secondary antibody (diluted 1:500) was used instead of an Alexa-conjugated one.

In situ immunodetection of 5-methylcytosine residues was carried out using a mouse monoclonal antibody anti-5-methylcytosine (cat. no. A-1014, Epigentek Inc., Brooklyn, NY, USA) following the protocol reported by Pfarr *et al.* (2005).

Immunofluorescent preparations were observed using a Zeiss Axioplan epifluorescence microscope equipped with a 100 W mercury light source. Photographs of the fluorescent images were taken using a CCD camera (Spot, Digital Instrument, Madison, WI, USA) and using the Spot software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

Whole-protein extractions from *A. pisum*, *M. brassicae* and *D. melanogaster* cells were performed in 125 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol and 1% β-mercaptoethanol. Electophoresis on SDS-PAGE gel and blotting on polyvinylidene difluoride membrane were carried out following Sambrook *et al.* (1989). Immunoblotting with the mouse anti-*D. melanogaster* HP1a antibody was performed according to Cowell *et al.* (2002).

RNA extraction was performed with the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) following the supplier's instructions. The extracted RNA samples were quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

DNA extraction was performed using a standard protocol given in Mandrioli *et al.* (1999). Southern

hybridization and restriction enzyme digestion were carried out as described in Bizzaro *et al*. (2000).

The 28S rDNA probe was obtained by PCR amplification of a 400 bp long fragment of the 28S rDNA gene using the two primers, F (5′-AACAAAC AACCGATACGTTCCG) and R (5′-CTCTGTC CGTTTACAACCGAGC), designed according to the coding gene sequences of insect 28S rRNA available in GenBank. The amplification mix contained 100 ng genomic DNA, 1 μM of each primer, 200 μM dNTPs and 2 U of DyNAZyme II polymerase (Finnzymes Oy). The amplification was performed with a Hybaid thermal-cycler at an annealing temperature of 60°C for 1 min and making extension at 72°C for 1 min.

The complete gene sequence of *A. pisum* HP1 homologue (*Ap*HP1) was obtained by rapid amplification of cDNA ends (RACE), according to Frohman (1990), using the primers HP1 – 5′RACE (5′-CAGCG AAAACACCTGGGAG) and HP1 – 3′RACE (5′-GAC CGCGCTCAAAGCCGATCA). The two primers were designed using the *D. melanogaster* HP1a coding sequence (DROHP1) as a reference. Successively, two additional primers were designed in order to eliminate the internal RACE primer sequences from the full-length *A. pisum* HP1 cDNA. In particular, primers HP1F (5′-TCCACTGGTCACGCACTTCAA) and HP1R (5′-CAGGGGCATGCCATGTTAGTCT) were used in RT-PCR experiments performed using the one step RT-PCR kit (Promega).

Sequencing was performed at CRIBI sequencing unit (University of Padua), whereas sequence analyses were performed using the software VectorNT (Invitrogen).

Results

A. pisum HP1 cDNA search was performed by amplifying two overlapping fragments using RACE (Figures 1A,B). The sequence of the central region of HP1 cDNA was subsequently confirmed using two external primers (Figure 1C).

Sequencing indicated that *A. pisum* HP1 cDNA (termed *Ap*HP1) was 1623 bp long, including a 170 bp 5′UTR and a 688 bp 3′UTR and coded for a protein consisting of 254 amino acid residues with a predicted molecular mass of 28 kDa and a net negative charge.

The effective amplification of the HP1 cDNA was confirmed by BLAST comparison in GenBank and by CLUSTALW alignment with the HP1s identified in different eukaryotes (Figure 2). The overall identity of *Ap*HP1 peptide with other HP1s ranged from 54% to 63%, whereas it ranged from 84% to 96% if made with the chromo-domain (CD) sequence only. A similar range of sequence similarity was found when the chromo-shadow domains (CSDs) were aligned.

Before performing experiments to localize HP1 proteins on aphid chromosomes, western blotting experiments with the mouse anti-*D. melanogaster* HP1a antibody were performed as a preliminary step

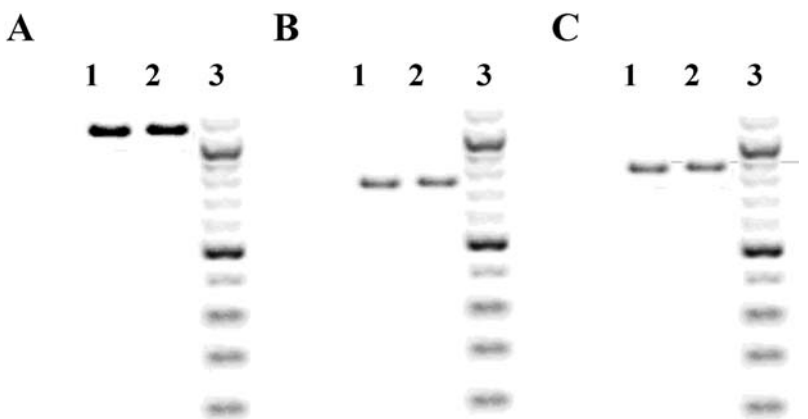

Figure 1. PCR amplification of two overlapping *Ap*HP1 fragments by 5′ and 3′ RACE respectively (A, B) and standard PCR for the internal *Ap*HP1 portion (C).

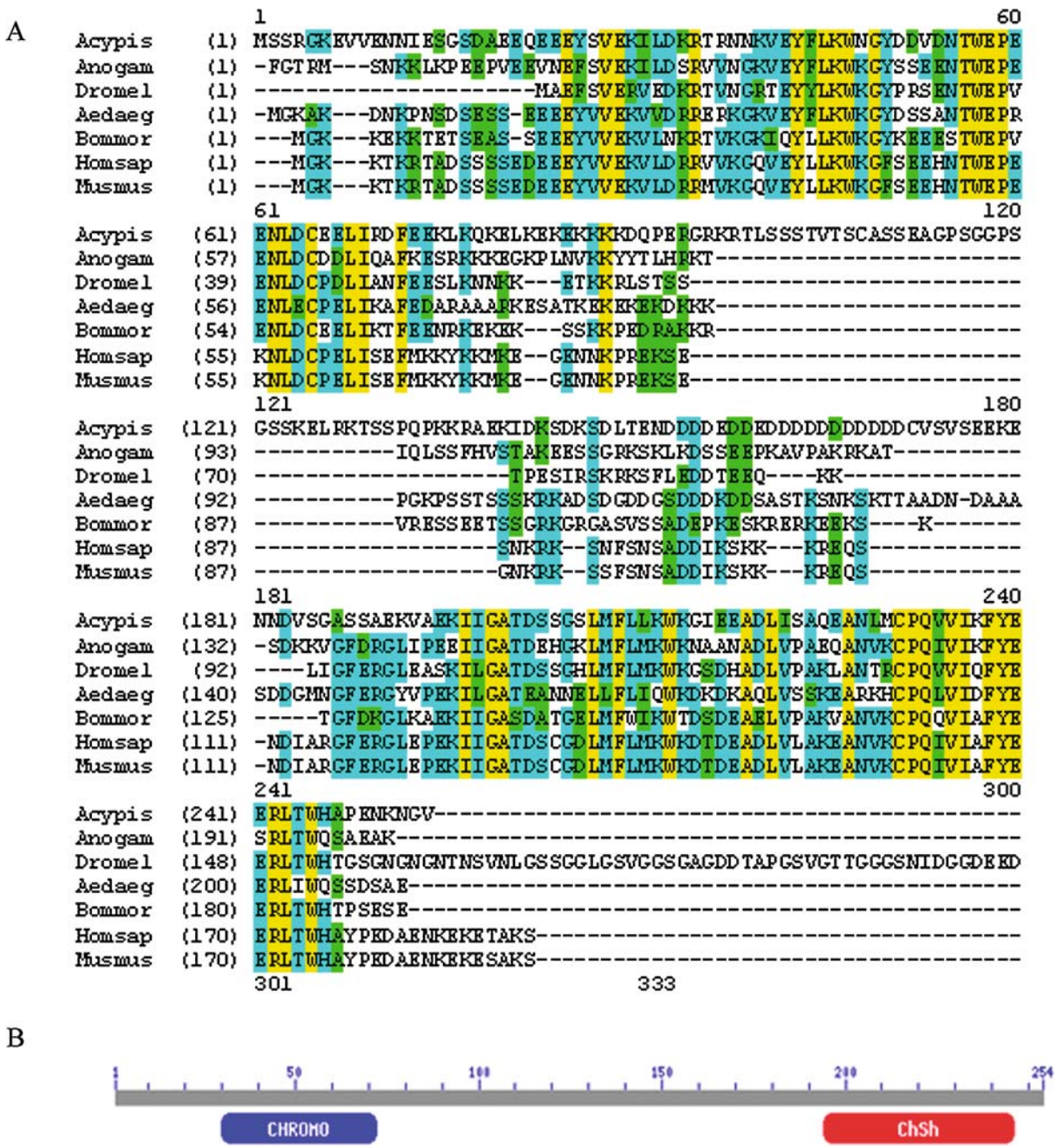

Figure 2. (A) The *Ap*HP1 amino acidic sequence was aligned with homologous sequences available in GenBank. Acypis, *A. pisum*; Anogam, *A. gambiae* (EAA13252); Dromel, *D. melanogaster* (DROHP1); Aedaeg, *A. aegypti* (EAT44114); Bommor, *B. mori* (ABF51450); Homsap, *H. sapiens* (AAH06821); Musmus, *M. musculus* (NP_031652). (B) Location of the conserved CD and CSD domains within *Ap*HP1.

to verify the antibody specificity (Figure 3). In the lane containing the protein extract from SL2 *Drosophila* cells, the HP1 antibody, as expected, recognized an antigen with an apparent molecular weight of 34 kDa (Eissenberg & Elgin 2000) (Figure 3, left). In the lane containing the whole-protein extract from *M. brassicae* cells, a single band of about 20 kDa was observed, as expected on the basis of the data reported by Borsatti & Mandrioli (2005) (Figure 3, centre). In the lane containing the protein

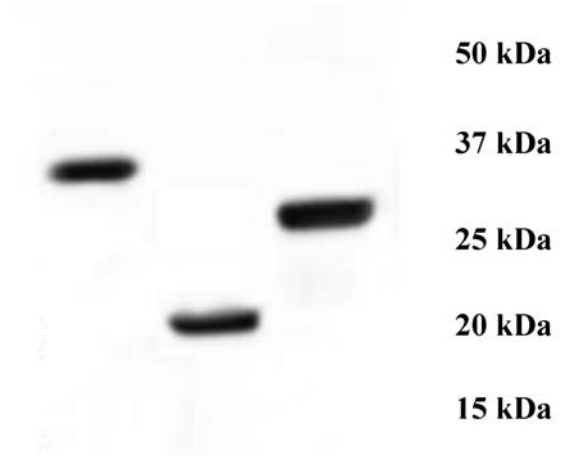

Figure 3. Western blot of protein extracts from *D. melanogaster* (lane 1), *M. brassicae* (lane 2) and *A. pisum* (lane 3) probed with mouse anti-*Drosophila melanogaster* HP1a antibody.

extract from aphid tissues, the antibody recognized a single protein with an approximate weight of 28 kDa that is coherent with the estimated weight of the predicted *Ap*HP1 peptide (Figure 3, right).

Western blotting therefore confirmed that the tested mouse anti-*D.melanogaster* HP1a antibody is able to specifically recognize HP1 peptides in aphids, making possible its use for immunostaining experiments on *A. pisum* chromosomes.

In situ immunodetection showed that HP1 proteins were not scattered along all *A. pisum* chromosomes but located on both the telomeres of the X chromosomes and on a telomeric region of the autosome pair 2 (Figure 4B). Previous C banding experiments identified these immunopositive areas as heterochromatic regions (Figure 4A) indicating a complete overlap between heterochromatin and HP1 proteins on aphid chromosomes.

Heterochromatic regions were also labelled after immunolocalization experiments with the rabbit anti-monomethylated-K9 histone H3 indicating that

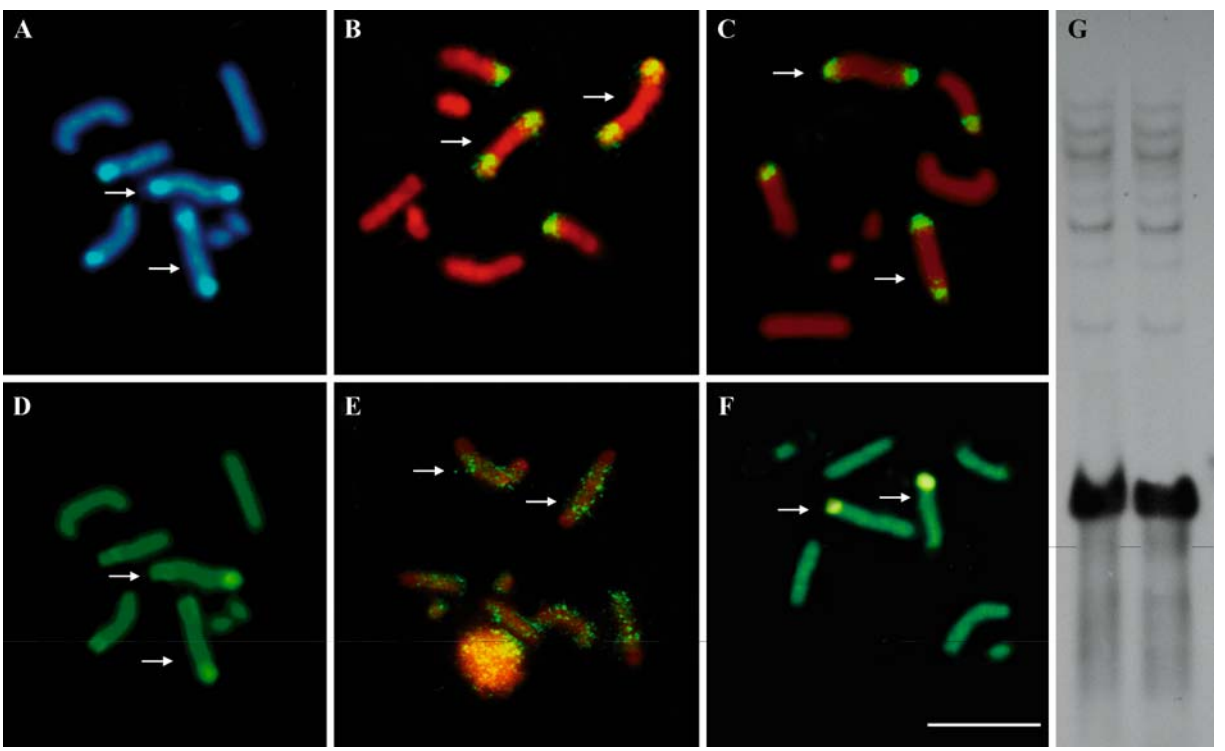

Figure 4. *A. pisum* chromosomes were analysed by C banding followed by DAPI (A) and CMA_3 staining (D), by immunostaining with antibodies against HP1 (B), monomethylated-K9 H3 histones (C) and methylcytosine residues (E), and by *in situ* NT with *Msp*I (F). The methylation status of 28S rDNA genes was evaluated by digesting *A. pisum* genomic DNAs with *Msp*I (lane 1) and *Hpa*II (lane 2) and blotting with a 28S rDNA probe (G). Arrows indicate sex chromosomes. Bar corresponds to 10 μm.

Me9H3 and HP1 are similarly located on aphid chromosomes (Figure 4C).

Surprisingly, aphid heterochromatin was not labelled after immunodetection of methylated cytosines using mouse anti-5-methylcytosine antibodies (Figure 4E). Indeed, aphid chromosomes presented a widespread occurrence of methyl-cytosine residues in euchromatin.

The absence of labelling with the anti-5-methyl-cytosine has also been observed at the constitutive heterochromatic GC-rich and CMA_3 fluorescent telomeres of the two X chromosomes (Figure 4D) that are known in aphids as the chromosomal sites for the genes coding for the major rDNA transcriptional units, suggesting that rDNA genes are not methylated in aphids.

In order to confirm these data, we performed a Southern blot experiment hybridizing a 28S rDNA probe on *A. pisum* genomic DNAs digested with the endonucleases *Msp*I and *Hpa*II. Considering that these two enzymes are isoschizomers that recognize the same target sequence 5′-CCGG-3′, the absence of differences in the restriction pattern of the two enzymes clearly indicated that methylation was not present at aphid rDNA genes (Figure 4G).

A final confirmation of our results was obtained by performing *in situ* NT experiments with *Msp*I in order to verify the presence of CpG targets at NORs (Figure 4F)]. NT experiments confirmed that NORs were CpG-rich, indicating that the absence of labelling with the anti-5-methylcytosine was not due to the absence of CpG targets but to the lack of DNA methylation in the cytosine residues located at these chromosomal regions.

Discussion

Previous studies on *A. pisum* chromosomes evidenced, in accordance to data reported in other aphid species, a tendency of constitutive heterochromatin to be concentrated on the X chromosomes (Bizzaro *et al.* 2000, Manicardi *et al.* 2002). Furthermore, *A. pisum* heterochromatin was essentially AT-rich, whereas CMA_3 staining induced a bright fluorescence limited to one telomere of each X chromosome. The different response to CMA_3 and DAPI staining after C-banding points out the heterogeneity of heterochromatic DNA composition in the *A. pisum* genome, making this pattern of constitutive heterochromatin heterogeneity a general characteristic of aphid chromatin since it has been described in all species investigated at cytogenetic level so far (Manicardi *et al.* 2002).

Despite this initial survey, at present a functional and structural characterization of *A. pisum* heterochromatin is still lacking, and we attempted to investigate this model by paying attention in particular to the occurrence of epigenetic chromatin modifications that have until now been neglected not only in *A. pisum*, but more generally in aphids.

Amplification and sequencing revealed that the *A. pisum* genome encodes for a HP1-like protein whose structural features are consistent with those reported for other HP1s. In particular, *Ap*HP1 showed an high similarity with *D. melanogaster* HP1a and with alpha isoforms of human and mouse HP1 that are reported to have heterochromatic rather than euchromatic localizations (Eissenberg *et al.* 1994), suggesting that *Ap*HP1 could have specific functions in constitutive heterochromatin assembly in aphids.

Several papers reported that H3 histones methylated at lysine 9 and HP1s are common epigenetic markers for heterochromatin in both vertebrates and invertebrates (e.g. Jacobs *et al.* 2001, Lachner *et al.* 2001). In view of this, immunodetection of HP1 and Me9H3 was performed, indicating that aphid constitutive heterochromatin is enriched both in H3 histone monomethylated at lysine 9 and in HP1 proteins. These epigenetic markers are, therefore, true heterochromatic markers not only for vertebrate chromosomes but also for insect heterochromatin, as previously reported in the dipteran *D. melanogaster* (Cowell *et al.* 2002), in the homopteran *P. citri* (Cowell *et al.* 2002) and in the lepidopteran *M. brassicae* (Borsatti & Mandrioli 2005).

Interestingly, data reported in aphid holocentric chromosomes argue for an important role of methylated histones also outside pericentromeric heterochromatin (that is not present in aphids) suggesting a possible involvement, for example, in euchromatic gene silencing.

In contrast to what is reported in vertebrates, aphid constitutive heterochromatin lacks DNA methylation since methylated cytosine residues are widespread in euchromatic chromosomal regions. These results are very interesting since, together with those reported in *P. citri* (Bongiorni & Prantera 2003), they indicate that insect heterochromatin is poor in DNA methylation, whereas actively transcribed euchromatic chromosomal areas can be highly methylated.

These data, even if apparently surprising in comparison with the canonical view of DNA methylation, are not unexpected in insects, where it has previously been suggested that methylated genes can be actively transcribed (Mandrioli & Volpi 2003, Field *et al*. 2004, Mandrioli 2004, Mandrioli & Borsatti 2006). In this regard, it has been suggested that DNA methylation has been recruited in insects not for silencing gene transcription but to avoid transcription initiation from spurious promoters (Mandrioli 2004). On the basis of this suggested function, in insects DNA methylation should be present in euchromatin rather than in heterochromatin and the data obtained in *A. pisum* fully support this hypothesis.

The absence of an overlapping between heterochromatin and methylated chromosomal domains, reported in *A. pisum* and in the coccid *P. citri* (Field *et al*. 2004), is therefore not related to the occurrence of holocentric chromosomes in both these species but to the presence of a similar function of DNA methylation. Indeed, as clearly shown in *D. melanogaster* and *Caenorhabditis elegans*, in invertebrates heterochromatin can be assembled and condensed also without any involvement of DNA methylation (Mandrioli 2004).

The absence of methylation has been also found in the aphid rDNA units that have been repeatedly described as mosaic of methylated and non-methylated repeats in vertebrates (Brock & Bird 1997, Tweedie *et al*. 1997).

The heterochromatin of aphid holocentric chromosomes thus shows some common features with monocentric chromosomes since both contain HP1 proteins and methylated-K9 H3 histones. Nevertheless, it is peculiar since it lacks a preferential accumulation of methylated cytosine residues.

The location of DNA methylation on aphid chromosomes, together with data previously reported regarding the peculiar effects of DNA methylation on gene expression (Field *et al*. 2004), make aphids an intriguing model for the study of DNA methylation in insects. It could be, therefore, very interesting to look for the presence of genes coding for DNA methyltransferases and methyl-binding proteins in order to better understand which genes are involved in the definition and functioning of the aphid genome. Finally, considering that heterochromatin is generally enriched in transposable elements, it would be interesting to verify the mechanisms involved in transposon control in the aphid genome in view of the absence of DNA methylation in heterochromatin.

Among aphids, *A. pisum* may prove to be a perfect model for these studies, considering also that its genome project is currently ongoing and that a large-scale gene discovery project has been already published (Sabater-Muñoz *et al*. 2006).

Acknowledgements

We are greatly indebted to Rebecca Kellum for providing the mouse anti-*Drosophila* HP1a antibody. This work is supported by a 'F.A.R.' grant from the University of Modena and Reggio Emilia (M.M.) and by a grant 'Experimental approach to the study of evolution' from the Department of Animal Biology of the University of Modena and Reggio Emilia (M.M.).

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