Conservation of HP1 and methylated H3 histones as heterochromatic epigenetic markers in the holocentric chromosomes of the cabbage moth, *Mamestra brassicae* (Lepidoptera)

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Abstract. The methylated H3 histone and heterochromatin protein 1 (HP1) are markers of heterochromatin in several eukaryotes possessing monocentric chromosomes. In order to confirm that these epigenetic markers of heterochromatin are evolutionary conserved, the distribution of methylated H3 histones and HP1 homologues on the holocentric chromosomes of the cabbage moth *Mamestra brassicae* (Lepidoptera) were studied. In particular, PCR experiments with degenerated primers identified a HP1 homologue (called *MbHP1*) in the *M. brassicae* genome. Sequencing showed that the *MbHP1* gene is 737 bp long including a 102 bp 5'UTR and a 635 bp coding portion (comprising an 80 bp intron). The MbHP1 peptide consisted of 184 amino acids, had a 20 kDa molecular mass and a net negative charge. At the structural level, it showed an N terminal chromo-domain and a chromo-shadow-domain at the C terminus linked by a short hinge region. At the cytogenetic level, MbHP1 was located exclusively in the heterochromatic regions of the chromosomes. The same heterochromatic regions became labelled after immuno-staining with antibodies against H3 histone methylated at lysine 9, reinforcing the hypothesis that this modified histone is essential for HP1 binding. Our data, as a whole, confirm that heterochromatic components and markers are evolutionary conserved both in mono- and holocentric chromosomes despite the difference in the distribution of heterochromatin on chromosomes.

INTRODUCTION

Studies on organisms ranging from yeast to vertebrates strongly suggest that histones and their post-translational modifications play a pivotal role in the assembly of heterochromatin. In particular, a major advance in the understanding of how heterochromatin is formed came with the observation by Jenuwein and colleagues that the *Su(Var)39H* gene, which was detected originally as a mutant allele that suppresses position-effect variegation in *Drosophila*, encodes a histone methyltransferase (HMT) protein (Rea et al., 2000). The specificity of the *Su(Var)39* HMT for lysine 9 of histone H3 provided the first evidence that methylation of this residue is directly involved in heterochromatin formation.

Several papers indicate that methylation is just one type of post-translational modification that occurs in histones and in according to the "histone code" hypothesis such covalent modifications may generate a "code", which can be "deciphered" by chromosomal proteins and thereby regulate tissue-specific patterns of gene expression (Strahl & Allis, 2000; Turner, 2000; Nakayama et al., 2001; Zhang & Reinberg, 2001). In particular, methylation at lysine 9 in histone H3 (Me9H3) represents one of the most robust histone modifications (Jenuwein & Allis, 2001).

Current theories suggest that in addition to the direct effects of these modifications on nucleosome structure, the modified residues also act as recognition motifs that mediate interactions with transcription factors and chromatin binding proteins. In particular, several experiments indicate that methylated H3 lysine 9 residues constitute a specific binding target for heterochromatin protein 1 (HP1) (Lachner et al., 2001).

The HP1 class of chromobox genes encodes 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 (Wang et al., 2000; Jones et al., 2000).

Studies on the structure and epigenetic markers of heterochromatin were performed mainly on organisms with monocentric chromosomes, whereas organisms possessing holocentric chromosomes have been almost neglected. Moreover, the available data derive from few species, which makes the analysis of this topic in a larger range of species more interesting (Cowell et al., 2002). In the present paper, the presence of methylated H3 histones and HP1 homologue was determined in order to verify if these two heterochromatic markers are evolutionary conserved in the heterochromatin of lepidopteran holokinetic chromosomes.

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MATERIAL AND METHODS

Cabbage moth in vitro culture and chromosome preparation

The IZD-MB-0503 cell line of *M. brassicae* (ATCC number: CRL-8003) was used for all the experiments. Cells were cultured in Ex-Cell 405 medium (JRH Biosciences, Lenexa, KS, USA) at 26°C.

CRL-8003 cells were spread following a modified version of the method described by Mandrioli (2002). Briefly, 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 (formaldehyde 4% in PBS) was successively added to the pellet, which was made to flow up and down for 1 min in a needle of a 1 ml hypodermic syringe. Finally, the pellet was resuspended in 200 µl of fresh methanol and 20 µl of this suspension was dropped on clean slides and air-dried.

In situ HP1 immunolocalization and C-banding

In situ immunostaining experiments were performed with an anti-*Drosophila melanogaster* HP1 antibody diluted 1:500 (a kind gift from R. Kellum) (Huang et al., 1998) and an anti-histone H3 methylated at lysine 9 (Me9H3) (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.

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).

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, USA) and using the Spot software supplied with the camera and processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

DNA and RNA extraction

DNA extraction from cultured CRL-8003 cells was performed using the standard protocol cited in Mandrioli (2002). RNA extraction was performed with the SV Total RNA Isolation System (Promega Corporation, Madison, USA) following the supplier's instructions.

HP1 amplification, sequencing and cloning

Amplification of *HP1* gene from the cabbage moth genome was performed with primers designed using the *D. melanogaster HP1* coding sequence (sequence access code *DROHP1*) as a reference sequence. However, the designed primers were degenerated according to the alignment results of *DROHP1* with other HP1 genes available in Genbank in order to improve primers ability to amplify a HP1 gene in Lepidoptera. The designed primer sequences are: F1$_{HP1}$ (5'-CCGTGGAAAARATMATSGAC -3') starting at nucleotide 74 and R1$_{HP1}$ (5'-ACCATTTCHGCTTVGTCRAC-3') starting at nucleotide 530 in the *DROHP1* sequence. The same primers were used in RT-PCR experiments that were made with the Access RT-PCR System (Promega), according to the supplier's instructions.

The complete gene sequence of *M. brassicae* HP1 homologue (*MbHP1*) was obtained by Rapid Amplification of cDNA Ends (RACE), according to Frohman (1990), using the same primers as previously used for PCR and RT-PCR. *M. brassicae* HP1 sequence was confirmed by inverse PCR (Borsatti et al., 2004) that also allowed the identification of introns and exons within the amplified gene.
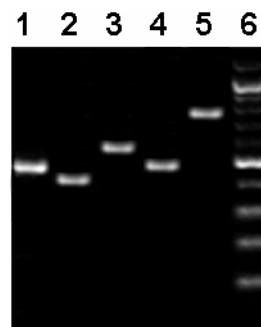

Fig. 1. PCR amplification of an internal fragment of the HP1 gene from the *M. brassicae* genome obtained by using direct PCR (lane 1) and RT-PCR (lane 2). HP1 gene sequence was completed by 5' and 3' RACE (lane 3 and 4) and confirmed by inverse PCR (lane 5). The molecular weight of the amplified fragments was evaluated using the 100 bp ladder DNA marker (lane 6).

The *MbHP1* was cloned using the "pGEM-T-easy" cloning kit (Promega Corporation, Madison, USA) according to the manufacturer's protocol and sequenced at the "BMR-University of Padova". Sequence analysis was performed using GCG software (GCG Computer Group, Madison, USA). Sequence phosphorylation was evaluated using the NetPhos 2.0 server that produces neural network predictions for serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins (Blom et al., 1999).

Protein purification and western blotting

In vitro production and purification of *M. brassicae* HP1 protein were performed using the PinPoint System (Promega) according to manufacturer's protocol. Whole protein extraction

```
atgggggaagaagatccaaaattcctctaaggtaagcgatgtgcct
M  G  K  K  I  Q  N  S  S  K  V  S  D  V  P
gctgaggaagaggagtatgtagtcgaaaaaatattggatcgtcgg
A  E  E  E  E  Y  V  V  E  K  I  L  D  R  R
gtcgttaaaggaaag GTTAGCGCTGACAGCTGACAGATGACAGC
V  V  K  G  K
TCGATAGCTGACAGATATAGACAGACATAAAGACATGATTCG
TATAGATAG      gtggaatatttactgaagtggaagggctac
                V  E  Y  L  L  K  W  K  G  Y
agtgaagaggataatacgtgggagccggaagaaaatctcgattgt
S  E  E  D  N  T  W  E  P  E  E  N  L  D  C
ccagatcttatccagcaatatgcctcacgcaaagatgagaagaaa
P  D  L  I  Q  Q  Y  A  S  R  K  D  E  K  K
gatagacctagctcgagcgcagaaggaagagccacatctaccgca
D  R  P  S  S  S  A  E  G  R  A  T  S  T  A
tccaaaagaaatcggaggagagtggtaacaagtcaaaacgaact
S  K  R  K  S  E  E  S  G  N  K  S  K  R  T
acagatgctgagcaggacaccataccagttagtggatcttcagga
T  D  A  E  Q  D  T  I  P  V  S  G  S  S  G
tttgacagaggtctggtcccggaaagaattctaggagcaactgac
F  D  R  G  L  V  P  E  R  I  L  G  A  T  D
gttagtggcgagttgatgtttctaatgaaattcaaaggggtagac
V  S  G  E  L  M  F  L  M  K  F  K  G  V  D
gaagcggacttagtaccctccaaagaagcaaacgtgaagtgcccc
E  A  D  L  V  P  S  K  E  A  N  V  K  C  P
caactcgtaattgcgttctacgaagagagacttacgtggcattcg
Q  L  V  I  A  F  Y  E  E  R  L  T  W  H  S
gacccagaagactaa
D  P  E  D  *
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Fig. 2. *M. brassicae* HP1 coding sequence consisted of a 635 coding portion constituted by two exons and an 80 bp long intron. Intron sequence is underlined.

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(A)  HP1_termop     1  ------------------------------------------------------------
(B)  swi6_S._po    10  RRSSTSKRSVIDDDSEPELPSMTKEAIASHKADSGSSDNEVESDHESKSSSKKLKENAKE
(C)  MbHp1_mame     1  ------------------------------------------MGKKIQN-----SSKVSDVPAE
(D)  Hp1_dros_m     1  ------------------------------------------MGKKIDNPE--SSAKVSDAEEE
(E)  Hp1_dros_v     1  ------------------------------------------MGKKTDNPE--TNNASSGAEEE
(F)  pchet1         1  ---------------------------------------------MSGSGSE
(G)  pchet2         1  --------------------------------------------MSDELVPAVEE
(H)  HP1_al_mus     1  -----------------------------------------MGKK---TKRTADSSSS
(I)  Hp1_bet_mu     1  -----------------------------------------MGKK---QNKKKVEEVLE
(J)  Hp1_gam_mu    10  YKALL--IQYIKTNLKYELHMPGKGLILGKYILRGVCKGQSEITGAN--NAQQLSEEAEI
(K)  CHCB1_gall     1  -----------------------------------------MGKK---QNKKKVEEVLE
(L)  X-Hp1_xeno     1  -------------------------------------EQN---GKGKKVEEAEP
(M)  HP1_Danio      1  --------------MSQPTETVSDAPSVTEEVSPDSKLAATAGKK--QTKKKVEDVVE
(N)  Hp1_alpha      1  -----------------------------------------MGKK---TKRTADSSSS
(O)  Hp1_beta_h     1  -----------------------------------------MGKK---QNKKKVEEVLE
(P)  Hp1_gamma    121  QRCCR--RWWIALKFQTTQPFPISSFFIPVKKQLNTLQGTLSLDDEN--NIFKVVEKRHQ


(A)  HP1_termop     1  -----------------------------MTKVYEVENIIGHRKK--GSQIEYHIQWKGY
(B)  swi6_S._po    70  EEG-------GEEEEEDE---------------YVVEKVLKHRMARKGGGYEYLLKWEGY
(C)  MbHp1_mame    18  EEE-------------------------------YVVEKILDRRVV--KGKVEYLLKWKGY
(D)  Hp1_dros_m    21  EEE-------------------------------YAVEKIIDRRVR--KGKVEYYLKWKGY
(E)  Hp1_dros_v    21  EEE-------------------------------YAVEKILDRRVR--KGKVEYYLKWKGY
(F)  pchet1         8  EE----------E-------------------YVVEKIIDKRTV--NGKVQYFLKWKGY
(G)  pchet2        12  EF-------------------------------IVEKILDKRTEP-EGSVRYLLKWKGY
(H)  HP1_al_mus    15  EDE----------EE-----------------YVVEKVLDRRMV--KGQVEYLLKWKGF
(I)  Hp1_bet_mu    16  EEE----------EE-----------------YVVEKVLDRRVV--KGKVEYLLKWKGF
(J)  Hp1_gam_mu    66  EVRIQRITGSADNRQQQP-------SRPLYTIFKNEYPYGHVGEV--LLWVEYFLKWKGF
(K)  CHCB1_gall    16  EEE----------EE-----------------YVVEKVLDRRVV--KGKVEYLLKWKGF
(L)  X-Hp1_xeno    15  EE-----------------------------FVVEKVMDRRVV--NGKVEYYLKWKGF
(M)  HP1_Danio     43  EEE----------EE-----------------YVVEKVLDRRVV--KGRVEYLLKWKGF
(N)  Hp1_alpha     15  EDE----------EE-----------------YVVEKVLDRRVV--KGQVEYLLKWKGF
(O)  Hp1_beta_h    16  EEE----------EE-----------------YVVEKVLDRRVV--KGKVEYLLKWKGF
(P)  Hp1_gamma    177  NEQSVIFSATKMLSSEYNGKTLGYFSRAESLCLEHIFLILDRRVV--NGKVEYFLKWKGF


(A)  HP1_termop    30  SL-KQATYEPAKNIL-DKNMLKKYQQKHLLSNDKPQ--------TPIKKSIKKGASTHQT
(B)  swi6_S._po   108  DDPSDNTWSSEADCSGCKQLIEAYWNEHGGRPEPSKRKRTARPKKPEAKEPSPKSRKTDE
(C)  MbHp1_mame    46  SE-EDNTWEPEENLD-CPDLIQQYAS-RKDE---KKD----------RPSSSAEG-RAT-
(D)  Hp1_dros_m    49  PE-TENTWEPENNLD-CQDLIQQYEASRKDEEKSAAS------KK-DRPSSSAKA-KETQ
(E)  Hp1_dros_v    49  AE-TENTWEPEGNLD-CQDLIQQYELSRKDEANAAASSSSSSSKK-ERPGSSTKV-KETG
(F)  pchet1        36  DE-SENTWEPHENLE-CPELIAEFERKWEKKQE-----------------EKK-NARK
(G)  pchet2        39  GD-EDNTWEPPENMD-CEDLLEELQKKLSKPKKRRKR-----------IIEPDD-DGDS
(H)  HP1_al_mus    45  SE-EHNTWEPEKNLD-CPELISEFMKKYKKMKEGENN-----------KPREKS-EGNK
(I)  Hp1_bet_mu    46  SD-EDNTWEPEENLD-CPDLIAEFLQSQKTAHE------------------TDKS-EGGK
(J)  Hp1_gam_mu   117  TD-ADNTWEPEGNLD-CLELIEAFLNSQKADS-------------------SG------
(K)  CHCB1_gall    46  SD-EDNTWEPEENLD-CPDLIAEFLQSQKTAHE------------------SEKS-EGSK
(L)  X-Hp1_xeno    42  TD-SDNTWEPEENLD-CPELIEAFLNSQKAGK-------------------EKP-DSNK
(M)  HP1_Danio     73  SE-EDNTWEPEDNLD-CPDLIAEYMTKHKIND-------------------DKK-ESSA
(N)  Hp1_alpha     45  SE-EHNTWEPEKNLD-CPELISEFMKKYKKMKEGENN-----------KPREKS-ESNK
(O)  Hp1_beta_h    46  SD-EDNTWEPEENLD-CPDLIAEFLQSQKTAHE------------------TDKS-EGGK
(P)  Hp1_gamma    235  TD-ADNTWESEENLH-CPELIEAFLNSQKAGK-------------------EK--VGTK
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Fig. 3. Alignment of MbHP1 amino acid sequence (C) with the HP1 homologues reported in GenBank: *T. thermophila* heterochromatin-associated protein 1-like (AF079405) (A), *S. pombe* swi6 (SPSWI6) (B), *D. melanogaster* HP1 (DROHP1) (D), *D. virilis* HP1-like (DROHTCHRPI) (E), *P. citri* pchet1 (F) and pchet2 (G), *M. musculus* HP1 alpha (AF216290) (H), beta (CBX1) (I) and gamma (LOC333074) (J), *G. gallus* mRNA for chromobox protein HP1-like (AB005617) (K), *X. laevis* HP1 alpha (AY168927) (L), *D. rerio* HP1-like gene (BC045443) (M) and human HP1 alpha (CBX5) (N), beta (CBX1) (O) and gamma (LOC341009) (P).

```
(A) HP1_termop   80  RKSSSTLKRSVKKS------VEKASNP---------------------KSK-LDKKALD
(B) swi6_S._po  168  DKHDKDSNEKIEDVNEKTIKFADKSQEEFNENGPPSGQPNGHIESDNESKSPSQKESNES
(C) MbHp1_mame   88  ----STAS----KR---------KSEE----SG---------------NKS--KRTTDAE
(D) Hp1_dros_m   99  GRASSSTSTAS-KR---------KSEEPTAPSG---------------NKS--KRTTDAE
(E) Hp1_dros_v  105  RTSTTASNSSGSKR---------KSEEPAGPAG---------------SKS--KRVESED
(F) pchet1       74  LKISSEDDAKEKKK---------KRST----EP---------------VKA--QPKVET-
(G) pchet2       84  RRQSNESESSIGSN---------TIAHQ---------------------RK--PKKSHG-
(H) HP1_al_mus   90  RK--SSFSNSADDI---------KS----------------------KK--KREQSN-
(I) Hp1_bet_mu   85  RKADSDSEDKGEES---------KP----------------------KK--KKEES--
(J) Hp1_gam_mu  149  ----------------------------------------------------
(K) CHCB1_gall   85  RKAESDTEDKGEES---------KP----------------------KK--KKEES--
(L) X-Hp1_xeno   79  RK--SVSDSESEDS---------KA----------------------KK--KRESV--
(M) HP1_Danio   110  KRKESDTDGGGEDS---------RP----------------------KK--RKDEQ--
(N) Hp1_alpha    90  RK--SNFSNSADDI---------KS----------------------KK--KREQSN-
(O) Hp1_beta_h   85  RKADSDSEDKGEES---------KP----------------------KK--KKEES--
(P) Hp1_gamma   271  RK--SLSDSESDDS---------KS----------------------KK--KREAA--


(A) HP1_termop  111  PVKEIHNYQKDVSKLLLKS--------------------DITSILHVFKDD-----GKS
(B) swi6_S._po  228  EDIQIAETPSNVTPKKKPSPEVPKLPDNRELTVKQVENYDSWEDLVSSIDTIERKDDGTL
(C) MbHp1_mame  110  QDTIPVSGSSGFDRGLVP-----------------------ERILGATDVS-----GEL
(D) Hp1_dros_m  132  QDTIPVSGSTGFDRGLEA-----------------------EKILGASDNN-----GRL
(E) Hp1_dros_v  139  TGDIVPAGGTGFDRGLEA-----------------------EKILGASDNN-----GRL
(F) pchet1      103  ------NKLNGFERGLKP-----------------------ERIIGATDTS-----GEL
(G) pchet2      111  ------SE-SRKSSTVSN-----------------------NCSSNVKVSD-----FDR
(H) HP1_al_mus  112  ------DIARGFERGLEP-----------------------EKIIGATDSC-----GDL
(I) Hp1_bet_mu  108  ------EKPRGFARGLEP-----------------------ERIIGATDSS-----GEL
(J) Hp1_gam_mu  149  ------------------------------------------------------EL
(K) CHCB1_gall  108  ------EKPRGFARGFEP-----------------------ERIIGATDSS-----GEL
(L) X-Hp1_xeno  100  ------DKPRGFARALDP-----------------------ERIIGATDSS-----GEL
(M) HP1_Danio   133  ------DKPRGFARGLDP-----------------------ERIIGATDSS-----GEL
(N) Hp1_alpha   112  ------DIARGFERGLEP-----------------------EKIIGATDSC-----GDL
(O) Hp1_beta_h  108  ------EKPRGFARGLEP-----------------------ERIIGATDSS-----GEL
(P) Hp1_gamma   292  ------DKPRGFARGRDP-----------------------ERVIGATDGS-----GEL


(A) HP1_termop  145  LYTVTIGKHPKQYLVSSSQIAKYASPDLIVRFYEKLIR--------------------
(B) swi6_S._po  288  EIYLTWKNGAISH--HPSTITNKKCPQKMLQFYESHLTFRENE----------------
(C) MbHp1_mame  141  MFLMKFKGVDEAD-LVPSKEANVKCPQLVIAFYEERLTWHSDPED--------------
(D) Hp1_dros_m  163  TFLIQFKGVDQAE-MVPSSVANEKIPRMVIHFYEERLSWYSDNE-D------------
(E) Hp1_dros_v  170  TFLIQFKGVDQAE-MVPSTVANVKIPQMVIRFYEERLSWYSDNED------------
(F) pchet1      128  MFLMKWEGTDEAD-LVRSVDARTKCPQLIIEFYEKHLTWNNASEDNK-----------
(G) pchet2      135  YVPSEILGVTKVGGSLLAKEANIVCPQLVIDYYERLQLFDPKMR-------------
(H) HP1_al_mus  137  MFLMKWKDTDEAD-LVLAKEANVKCPQIVIAFYEERLTWHAYPEDAENKEKESAK-S-
(I) Hp1_bet_mu  133  MFLMKWKNSDEAD-LVPAKEANVKCPQVVISFYEERLTWHSYPSEDDDKKDDK----N
(J) Hp1_gam_mu  151  MFLMKWKDSDEAD-LVLAKEVNMKYPQIVIAFYKERLTWHSCLQDEAQ----------
(K) CHCB1_gall  133  MFLMKWKNSDEAD-LVPAKEANIKCPQVVISFYEERLTWHSYPSEDDDKKEDKN----
(L) X-Hp1_xeno  125  MFLMKWKDSDEAD-LVPAKEANVKCPQVVIAFYEERLTWHSCPEDA-Q---------
(M) HP1_Danio   158  MFLMKWKNSDEAD-LVPAKEANVKCPQVVISFYEERLTWHSYPSEEEEKKDDKN----
(N) Hp1_alpha   137  MFLMKWKDTDEAD-LVLAKEANVKCPQIVIAFYEERLTWHAYPEDAENKEKETAKS--
(O) Hp1_beta_h  133  MFLMKWKNSDEAD-LVPAKEANVKCPQVVISFYEERLTWHSYPSEDDDKKDDKN----
(P) Hp1_gamma   317  MFLMKWKDSDEADSVLASKEANMKYLQIIIAFYEERLTWHACPEDEAQ----------
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Fig. 3 continued.

from *M. brassicae* cells and *D. melanogaster* SL2 cells was 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 (PVDF) membrane were carried out following Sambrook et al. (1989). Immunoblotting with the anti-HP1 was performed according to Cowell et al. (2002).

RESULTS

Identification of a HP1 homologue in the cabbage moth

Amplification performed with primers F1$_{HP1}$ (5'-CCGTGGAAAAARATMATSGAC-3') and R1$_{HP1}$ (5'-ACCATTTCHGCTTVGTCRAC-3') revealed a 480 bp long *HP1* fragment in the cabbage moth genome (Fig. 1, lane 1). RT-PCR experiments, using the same primers, showed a 400 bp long fragment (Fig. 1, lane 2) assessing the presence of an intron sequence.

In order to obtain a full-length fragment of *M. brassicae HP1* gene coding sequence (called *MbHP1*), RACE experiments were performed: amplification of the 5' terminal revealed a 566 bp long fragment (Fig. 1, lane 3), whereas amplification of the 3' terminal indicated the presence of a 491 bp long fragment (Fig. 1, lane 4). *MbHP1* sequence was confirmed by inverse PCR amplification (Fig. 1, lane 5). In particular, inverse PCR allowed us to verify the presence of an intron within the *HP1* gene.

Sequencing of all the fragments showed that *MbHP1* is a 737 bp long gene including a 102 bp long 5'UTR and a 635 bp coding portion (constituted by two exons and an 80 bp long intron). No 3' UTR was observed (Fig. 2).

M. brassicae HP1 coding sequence was aligned with *HP1*-like sequences available in Genbank, including those of *Schizosaccharomyces pombe swi6* (SPSWI6), *Tetrahymena thermophila heterochromatin-associated protein 1*-like (AF079405), *Drosophila melanogaster HP1* (DROHP1), *Drosophila virilis HP1*-like (DROHTCHRPI), *Planococcus citri pchet1* and *pchet2*, *Homo sapiens HP1* alpha (CBX5), beta (CBX1) and gamma (LOC341009), *Mus musculus HP1* alpha (AF216290), beta (CBX1) and gamma (LOC333074), *Xenopus laevis HP1* alpha (AY168927), *Gallus gallus* mRNA for chromobox protein *HP1*-like (AB005617) and *Danio rerio HP1*-like gene (BC045443). Alignment showed a sequence similarity ranging from 45.4 to 64.5% if performed using the total gene length, 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.
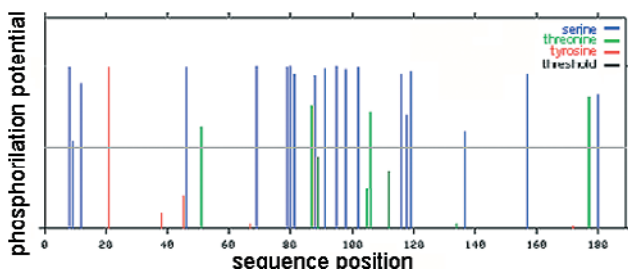
The putative MbHP1 peptide consisted of 184 amino acids and contained a high proportion of negatively charged amino acids that resulted in a the net negative charge of -9. The predicted molecular mass is 20 kDa, which is in accordance to the range reported for HP1-like proteins (Eissenberg & Elgin, 2000). The overall similarity of MbHP1 peptide with other HP1 peptides in the GenBank ranged from 37 to 85% (Fig. 3) and was as high as 97% in both the CD and CSD sequence. In contrast to that reported for the *P. citri* HP1 peptide, MbHP1 contained no internal repeat sequences.

Bioinformatic analysis of the *M. brassicae* HP1 amino acid sequence indicated the presence of multiple sites with a high serine and threonine phosphorylation potential and just one with a high tyrosine phosphorylation potential (Fig. 4).

Western blotting and immunolocalization of HP1 on cabbage moth chromosomes

In order to detect HP1 protein in the cabbage moth, western blotting experiments with an antibody that recognize *Drosophila* HP1 protein were performed as a preliminary step. In particular, immunoblotting was done using a rabbit anti-HP1 antibody against *D. melanogaster* HP1 protein (Huang et al., 1998) (Fig. 5). 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) (Fig. 5, lane 2). In the lane containing the whole protein extract from *M. brassicae* cells a single band of about 20 kDa was observed (Fig. 5, lane 3). If the anti-HP1 antibody is incubated with the in vitro produced
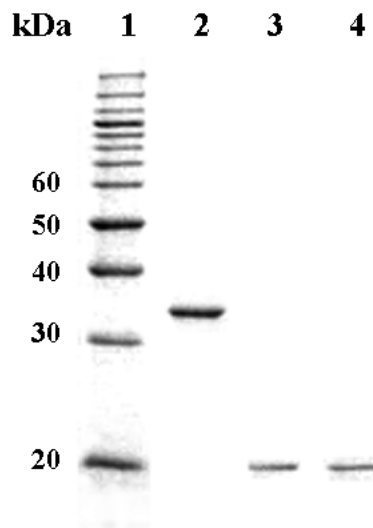

Fig. 5. Western blot of protein extracts from *D. melanogaster* (lane 2) and *M. brassicae* (lane 3) probed with fly anti-HP1 antibody as the primary one and goat anti-mouse IgG-HRP as secondary one. Note that the band detected in *D. melanogaster* migrates with an apparent MW of 34 kDa (as expected on the basis of previous papers) and the presence of a unique band of approximately 20 kDa in the cabbage moth extract. MbHP1 produced in vitro (lane 4) has the same MW and it is recognized by *Drosophila* anti-HP1 antibody. The MW of each protein was deduced according to the standard weight marker (lane 1).

Fig. 4. Neural network predictions of the serine, threonine and tyrosine phosphorylation sites in *M. brassicae* HP1 protein indicated the presence of several putative site for phosphorylation in the serine and threonine residues.
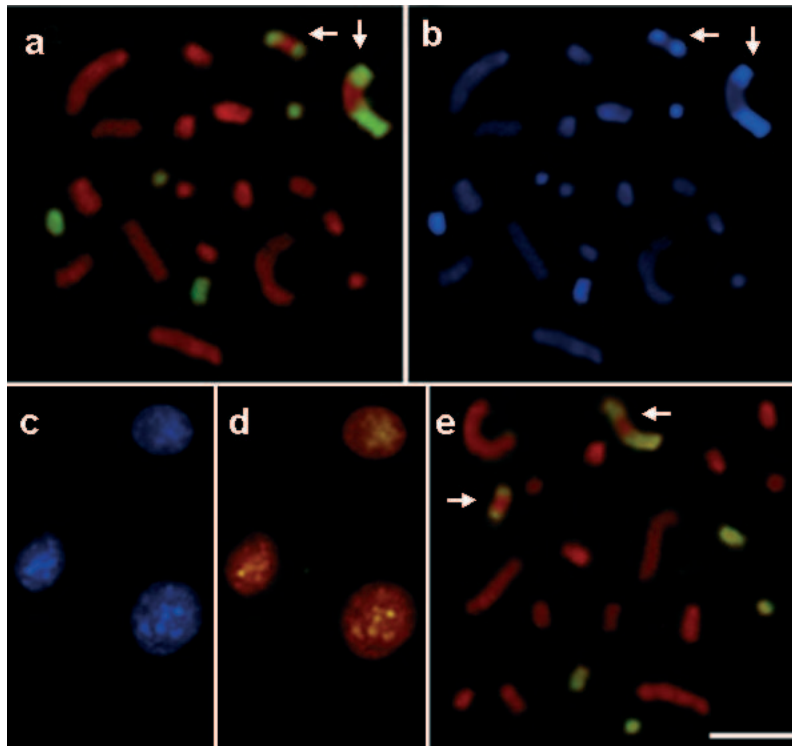
Fig. 6. In situ immunofluorescence of cabbage moth chromosomes (a, b and e) and nuclei (c, d) showed that HP1 is clustered on the heterochromatic regions of the sex chromosomes (indicated by arrows) and chromosome pairs 7 and 10 as deduced by C banding (b, c). The same regions resulted brightly fluorescent after immunostaining with anti-histone H3 methylated at lysine 9 (e).

HP-1 antibody before blotting (HP1 depletion), no signal was observed. These data indicate that anti-*Drosophila* HP1 specifically recognizes an HP1 protein in the cabbage moth extract.

To confirm this hypothesis, MbHP1 protein was produced in vitro from the cloned *MbHP1* gene and analysed on immunoblot (Fig. 5, lane 4). Western blotting confirmed that *Drosophila* anti-HP1 is able to recognize MbHP1 that was produced in vitro.

In situ immunolocalization of chromosomes showed that HP1 proteins were not scattered along all *M. brassicae* chromosomes but were located in several chromosomal regions of the Z and W sex chromosomes and chromosome pairs 7 and 10 (Fig. 6a). C banding performed on the slides previously used for HP1 immunolocalization (Fig. 6b) revealed that C-banded constitutive heterochromatin and HP1 positive chromosomal regions were overlapped in the cabbage moth complement and interphase nuclei (Fig. 6c, d).

Immunolocalization of Me9H3 on cabbage moth chromosomes

Immunolocalization with anti-Me9H3 was used to reveal the distribution of histone H3 methylated at lysine 9 on *M. brassicae* chromosomes. The Me9H3 was found to be located only in the heterochromatic regions of the two sex chromosomes (Z and W) and on chromosome pairs 7 and 10 (Fig. 6e). In view of the previous results obtained with the anti-HP1, Me9H3 and HP1 are similarly located on *M. brassicae* chromosomes.

DISCUSSION

Sequencing of the *M. brassicae HP1* gene (called *MbHP1*) showed that it was 737 bp long including a 102 bp long 5'UTR and a 635 bp coding portion (constituted by two exons and an 80 bp long intron). The MbHP1 protein had a N terminal chromo-domain and a chromo-shadow domain at the C terminus. A peculiarity of MbHP1 is that the hinge region is particularly short.

The putative MbHP1 peptide consisted of 184 amino acids, had a predicted molecular mass of 20 kDa and a net negative charge (-9). All these features are consistent with those reported for HP1-like proteins. The overall similarity of MbHP1 with the other HP1-polypeptides in the GenBank ranged from 37 to 85% and was particularly high (at least 78%) with *D. melanogaster* and *D. virilis* HP1s and with alpha and beta isoforms of human and mouse HP1. This is particularly interesting since all of these HP1 molecules are reported to have heterochromatic rather than euchromatic localizations (as reported for mammalian HP1γ) (Eissenberg et al., 1994) suggesting that HP1 has specific functions in heterochromatin assembly in Lepidoptera.

Drosophila HP1 is multiply phosphorylated by serine-threonine kinases and consensus CKII kinase target sites are found near the N- and C terminal ends and in the hinge domain (Eissenberg et al., 1994). The occurence of phosphorylation at different sites is thought to be functionally important since phosphorylation could regulate the assembly of HP1 into higher order chromatin struc-

ture (Koike et al., 2000). Bioinformatic analysis of *M. brassicae* HP1 amino acid sequence indicated the presence of multiple sites with a high serine phosphorylation potential in the hinge and in the chromo-domain, whereas the unique sequence with a high tyrosine phosphorylation potential is located within the chromo-domain. Sequences with high threonine phosphorylation potential were found within the hinge and in the chromo-shadow-domain. The general pattern and prevalence of serine and threonine residues observed in the cabbage moth HP1 are consistent with the consensus kinase target sites reported in *Drosophila* (Zhao et al., 2000).

Western blotting results showed that the anti-HP1 antibody raised against *D. melanogaster* HP1 could be effectively used to localize HP1 on *M. brassicae* chromosomes. The specificity of the antibody was verified not only by the presence of a unique band in the immunoblotting performed on the whole cabbage moth protein extract but also by the ability of *Drosophila* anti-HP1 to recognize the in vitro purified *M. brassicae* HP1 peptide and by the depletion of the antibody obtained by incubating anti-HP1 with *Mb*HP1 before western blotting. As a whole, western blotting experiments indicated that the anti-HP1 tested was useful for HP1 immunolocalization.

Several papers report that the binding of HP1 to heterochromatic regions is mediated by H3 histone methylated at lysine 9 in vertebrates (e.g. Lachner et al., 2001). In view of this, the immuno-fluorescence experiments were performed using an antibody specific for Me9H3, which indicate that cabbage moth constitutive heterochromatin is enriched in H3 histone methylated at lysine 9. This epigenetic mark is therefore a true heterochromatin marker not only for vertebrate but also for insect heterochromatin, as previously reported in the dipteran *D. melanogaster* and the homopteran *P. citri* (Cowell et al., 2002).

Our results, as a whole, indicate that HP1 and Me9H3 are both true markers of *M. brassicae* heterochromatin and confirmed that these two heterochromatic tags are evolutionary conserved in lepidopteran holocentric chromosomes. The heterochromatin of holocentric chromosomes, therefore, shows several common features with that of monocentric chromosomes since both contain AT-rich satellite DNAs, transposable elements, HP1 proteins and methylated histones (e.g. Ohbayashi et al., 1998; Mandrioli et al., 1999; Cowell et al., 2002; Mandrioli, 2002; Mandrioli, 2003a, b; Mandrioli et al., 2003).

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