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Interaction of Heterochromatin Protein 2 with HP1 defines a novel HP1 binding domain

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Abstract

Heterochromatin Protein 2 (HP2) is a nonhistone chromosomal protein from *Drosophila melanogaster* localized principally in the pericentric heterochromatin, telomeres, and fourth chromosome, all regions associated with HP1. Mutations in HP2 can suppress position effect variegation, indicating a role in gene silencing and heterochromatin formation [Shaffer, C.D. *et al.* (2002) *Proc. Natl. Acad. Sci.* 99, 14332-14337]. *In vitro* coimmunoprecipitation experiments with various peptides from HP2 have identified a single HP1 binding domain. Conserved domains in HP2, including those within the HP1 binding region, have been identified by recovering and sequencing *Su(var)2-HP2* from *D. willistoni* and *D. virilis*, as well as examining available sequence data from *D. pseudoobscura*. A PxVxL motif, shown to be an HP1 binding domain in many HP1-interacting proteins, is observed but is not well conserved in location and sequence, and does not mediate HP2 binding to HP1. The sole HP1 binding domain is composed of two conserved regions of 12 and 16 amino acids separated by 19 amino acids. Site-directed mutagenesis within the two conserved regions has shown that the 16 amino acid domain is critical for HP1 binding. This constitutes a novel domain for HP1 interaction, providing a critical link for heterochromatin formation in *Drosophila*.

Regions of every euchromatic genome, prominently the centromeres and the telomeres, are packaged as constitutive heterochromatin. These regions contain relatively few genes, and are made up primarily of repetitious sequences, such as satellite DNA and transposable elements. Heterochromatic domains are characterized by a condensed appearance of the interphase chromatin, a low level of meiotic recombination, and late replication in S phase. In *Drosophila*, a normally euchromatic gene that is mislocalized to heterochromatin by transposition or rearrangement will be silenced in some cells but not others, resulting in a variegating phenotype. This phenomenon is known as PEV¹, or position effect variegation (1). Mutations that suppress PEV (resulting in a loss of silencing) have identified many genes whose products are essential for heterochromatin formation (2,3).

Heterochromatin Protein 1 (HP1), one of the best-characterized nonhistone chromosomal proteins, has been implicated in both heterochromatin formation and gene silencing. HP1 is conserved from yeast (*Schizosaccharomyces pombe*) to humans and is consistently associated with pericentric heterochromatin (4). HP1 has been found to be part of multiprotein complexes that are necessary for the induction of heterochromatin formation. Proteins in the HP1 family contain an amino-terminal chromodomain and a carboxy-terminal chromoshadow domain separated by a linker region of variable length. The chromodomain has been found to bind to histone H3 methylated at lysine 9 (H3-mK9) (5,6); the chromoshadow domain is a protein-

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¹The abbreviations used are: PEV, position effect variegation; HP2, Heterochromatin Protein 2; HP1, Heterochromatin Protein 1; *Su* (var), Suppressor of position effect variegation; H3-mK9; histone H3 methylated on lysine 9; HP2-L, the larger isoform of HP2; HP2-S, the smaller isoform of HP2; TP, test peptide; KLH, keyhole limpet hemocyanin;

protein interaction domain that forms a homodimer (7) and binds to many other proteins, including SU(VAR)3-9, a histone H3-K9 methyltransferase (8-10) and SU(VAR)3-7, a zinc finger protein (11). Missense mutations that weaken HP1 interactions with H3-mK9, as well as truncations that delete the chromoshadow domain, show Su(var) activity (5,6,12).

In an attempt to identify additional proteins that interact with HP1 and may contribute to the formation of heterochromatin and large-scale condensation of the genome, a yeast two-hybrid screen was carried out using HP1 as bait; this identified Heterochromatin Protein 2 (HP2) (13). Genetic analysis showed that mutations in HP2 can be dominant suppressors of PEV. HP2 co-precipitates with HP1 from a *D. melanogaster* embryo extract and colocalizes with HP1 on *Drosophila* polytene chromosomes at the chromocenter, telomeres, and along the fourth chromosome. HP2 has two isoforms, HP2-L and HP2-S, which are approximately 365 and 176 kD respectively. HP2-S is made by an alternative splicing event whereby the fifth and sixth exons of HP2-L are spliced out. Both proteins have few recognizable structural motifs (13). See Figure 2 for a map of the intron/exon structure of HP2.

The original recovery of HP2 indicated an HP1 binding domain within the C-terminal exons, 8 and 9. A PxVxL motif located in the sixth exon led us to wonder whether other HP1 binding sites exist in HP2, allowing HP1 to bind to HP2 in multiple regions and to extend the reach of heterochromatin. Phage display experiments indicate that a PxVxL motif serves as an HP1 binding domain in many HP1-interacting proteins (14). In addition, the sixth exon of HP2 resembles the HP1 binding domain in ATRX, being rich in serine and charged amino acids (13). ATRX is a transcriptional regulator that localizes to the pericentric heterochromatin and the short arms of acrocentric chromosomes (15). The HP1 binding domain is unstructured in ATRX, suggesting that ATRX and HP1 interact with one another by an unstructured charge patch (16). This could also be the case for HP2 and HP1.

The current study utilizes coimmunoprecipitation in order to investigate potential HP2-HP1 interaction sites. We have also examined the evolutionary conservation of HP2 in four *Drosophila* species in order to identify domains of importance within the protein, including the HP1 binding domain. The species examined include *D. pseudoobscura*, *D. willistoni*, and *D. virilis*, which diverged from *D. melanogaster* 25-30, 30-40, and 40-60 million years ago, respectively. We find that neither the PxVxL domain, nor the domain similar to ATRX, nor any region outside of the originally identified HP1 binding region coprecipitates with HP1. We have identified a novel HP1 binding domain in the eighth exon that is conserved among the various species analyzed.

Experimental Procedures

Chromosome Staining

Squashing and immunofluorescent staining of polytene chromosomes from third instar larvae of *D. melanogaster*, *D. pseudoobscura*, and *D. virilis* was done as previously described (17). For HP1, the primary antibody is the mouse monoclonal antibody C1A9 described previously (18). For HP2, the primary antibody is a polyclonal rabbit HP2 antibody generated against a *D. melanogaster* cDNA product previously described (13). Secondary antibodies were labeled with Alexa Fluor 488 (green) and 594 (red).

Cross-species Westerns

Nuclei were isolated from 50 adult female flies of each species using a modified version of Protocol 1 from Wallrath *et al.* (19). Nuclei were lysed and DNA was sheared by resuspending the sample in load dye with a 22 gauge syringe. Samples were resolved on an 8% Tris-glycine polyacrylamide gel. The primary antibody used for western detection was a chicken anti-HP2

antibody generated against the exon 1 peptide MEDIEYLDEYKDZC conjugated to KLH, used at a dilution of 1:5000. The secondary antibody was horseradish peroxidase labeled goat anti-chicken IgY (Aves Lab) used at a dilution of 1:5000. Westerns were visualized using chemiluminescence.

Plasmid Construction

cDNA clones containing the short isoform of HP2 (RE12383=HP2-S, aa 1-276 and 1901-3257) and exon 1 through part of the sixth exon of the long isoform (LD29301, aa 1-1353) were obtained from the Berkeley Drosophila Genome Project. The LD29301 clone has a deletion of 2 amino acids which results in a premature stop codon. This sequence was corrected by replacement with a fragment of cDNA from LD30345 (also obtained from the Berkeley Drosophila Genome Project). Constructs were made from these two cDNAs by PCR amplification using primers with restriction sites on the ends, and then digesting the DNA and ligating it into the *Sac* I and *Not* I sites or the *Eco*R I and *Not* I sites, respectively, of pET28a. All of the smaller constructs created for the coimmunoprecipitation experiments were similarly generated by creating PCR products using RE12383, the modified LD29301, or one of the larger constructs made in a previous experiment as the template. The products were placed in either the *Sac* I and *Not* I or the *Eco*R I and *Not* I sites of pET28a. The pET41a vector, which incorporates a GST tag into the protein, was used when the transcription/translation products desired were so small that they were likely to be degraded in the rabbit reticulocyte lysate system. HP2 2188-2263, HP2 2188-2347, and HP2 2188-2418 are proteins made from PCR products containing sequence upstream of pET28a's T7 promoter through the HP2 coding region. Site directed mutagenesis was carried out and all products were confirmed by sequencing.

Coimmunoprecipitation Experiments

The HP2 constructs or PCR generated products were transcribed and translated using Promega's Quick Coupled Transcription/Translation System according to the manufacturer's protocol. Transcription/translation reactions were generally carried out for 60-90 minutes at 30° C as described in the protocol, but in some instances an incubation of 2 hrs at 25° was needed to obtain protein product. Products were ³⁵S-Met labeled except in some instances where HP1 was kept nonradioactive due to similar size as an HP2 product. Immunoprecipitations with an HP2 peptide known from previous experiments to bind to HP1 were done to verify the production of the nonradioactive product. The radioactive products were resolved by SDS-PAGE and detected by autoradiography to confirm that protein products of the correct size had been produced. Once the products were generated, 10-20 µl of the approximately 50 µl transcription/translation reaction was added to 10 µl of protease inhibitor cocktail and 1-10 µl of WA191 rabbit antibody (specific for HP1 and generated against the peptide CYAVEKIIDRRVRKGKVEYYLKWKG from the chromodomain) where appropriate, and brought up to 400 µl with binding buffer (20 mM Tris pH 8, 100-200 mM NaCl, 0.5% NP40, 0.5 mM PMSF). Higher salt was needed in some instances to eliminate nonspecific binding. The binding reaction went from 2 hrs. to overnight at 4° C while gently rocking. 0.005 g of Protein A Sepharose CL-4B was washed with binding buffer, and the binding reaction was added to the beads to incubate from 2 hrs. to overnight at 4° C. The beads were then collected by centrifugation and washed with binding buffer. The beads were resuspended in sample buffer, boiled, and released protein loaded on to either 4-20% gradient gels, or 15% or 18% polyacrylamide gels, depending on the desired separation. The gels were then fixed, treated with a fluorographic agent, dried down, and exposed to film.

Recovery, Sequencing, and Cross-species Comparison of HP2 Homologues

To obtain the genomic sequence of the *D. pseudoobscura* HP2 homologue, BLASTALL (20) was utilized to find sequence with high similarity to the *Su(var)2-HP2* gene, using tblastn and default parameters. Looking at an alignment between the two species, it appeared that one of the most highly conserved regions was in exons 2 and 3. We used this information to design a probe to screen genomic libraries from *D. willistoni* and *D. virilis* described in Bergman *et al.* (21), now available spotted on a single filter from BACPAC (<http://bacpac.chori.org>). This screen identified six fosmid clones from *D. willistoni* and four from *D. virilis* that were positive for HP2. Positive clones recovered from the *D. willistoni* library were 11I16, 5L17, 13B13, 33M24, 41H8, and 43F23; clones recovered from the *D. virilis* library were 35C22, 38N18, 42P13, and 46G10. One fosmid clone from each species was sequenced, 11I16 from *D. willistoni* and 42P13 from *D. virilis*. Subcloned libraries were created using mechanical shearing and approximately 400 clones were sequenced. Resulting reads were assembled using Phred, Phrap, and Consed (22-24) and regions of low quality were resequenced as necessary to obtain full-length sequence with Q scores above 30. In each case a 15 kb region including the gene for HP2 was analyzed by GENSCAN (25) (<http://genes.mit.edu/GENSCAN.html>) to approximate the coding sequence. Intron-exon boundaries were examined by eye and the final protein sequence was translated by Translate (<http://us.expasy.org/tools/dna.html>). The protein sequences from all four species (*D. melanogaster*, *D. pseudoobscura*, *D. willistoni*, and *D. virilis*) were used with multiple protein domain algorithms, including Pfam (26) (<http://pfam.wustl.edu>), ELM (27) (<http://elm.eu.org>) and SMART (28) (<http://smart.embl-heidelberg.de>). Multiple sequence alignment was performed with Clustalw (29) (<http://www.ebi.ac.uk/clustalw/>), MEME, (30) and Block Maker (31) (<http://blocks.fhrc.org>). Block Maker output was then used in LAMA (32) to search for similar domains in the public database. Block Maker and MEME output were used in MAST (33) searches to look for clusters of similar domains in the public database.

Results

The Heterochromatin Association of HP2 Is Conserved in *D. pseudoobscura*, *D. willistoni*, and *D. virilis*, As Are The Two Protein Isoforms

In order to determine whether HP2 is conserved in other *Drosophila* species and has the same distribution pattern, and hence implied function, polytene chromosomes from third instar larvae were stained with an HP2 antibody generated from a peptide in the C-terminal region of *D. melanogaster* HP2. The top panels of Figure 1A show a *D. virilis* squash by phase, and stained with antibodies specific for HP1 or HP2. HP1 and HP2 co-localize to the pericentric heterochromatin and to three prominent sites in the long chromosome arms. Neither localizes to the dot chromosome (arrowhead) which appears to be largely euchromatic in this species (18). *D. melanogaster* and *D. pseudoobscura* also exhibit colocalization of HP1 and HP2, indicating prominent association of HP2 with pericentric heterochromatin and the dot chromosome (arrowhead), shown in the lower panels. In these species, the dot chromosome is associated with HP1 and appears largely heterochromatic. Thus, HP2 has a similar distribution pattern as HP1 in different *Drosophila* species, implying a similar function. A western blot of proteins isolated from adult nuclei from *D. melanogaster*, *D. pseudoobscura*, *D. virilis*, and *D. willistoni*, shows that the four species all contain two protein isoforms of HP2 at similar apparent molecular weights (Figure. 1B). The predicted molecular weight of the smaller isoform is 176 kD, yet the apparent molecular weight is around 250 kD. This is most likely due to the high percentage of charged amino acids within the protein.

HP1 Binding Is Limited To The C-terminal Domain of HP2

Although an HP1 interaction domain was identified in the C-terminal portion of HP2 by a yeast two-hybrid screen (13), this does not preclude the possibility that other HP1 interaction

domains exist in HP2. The polypeptides used to test different domains of HP2 for binding with HP1 are shown in Figure 2. Their ability to bind to HP1, as indicated by co-precipitation, is indicated by a plus or minus sign. Sequences within the sixth exon of HP2 were given special attention for two reasons. First, aa region 1328-1900 was tested due to the presence of a PxVxL motif (aa 1665-1669, PSVSL in HP2), which has been determined to be an HP1 binding domain in HP1-interacting proteins such as TIF1-beta and the p150 subunit of CAF-1 (14). Second, exon 6 has an amino acid composition similar to that of the HP1-binding domain of ATRX, a transcriptional regulator with Swi/Snf activity. We therefore investigated the possibility of HP1 binding at these sites, as well as other regions of the protein outside of the C-terminal domain of HP2.

However, *in vitro* coimmunoprecipitation experiments demonstrate an HP1 binding activity only in the C-terminal domain (as summarized in Figure. 2). Following *in vitro* expression, co-immunoprecipitation of ³⁵S-Met labeled HP1 and HP2 polypeptides was performed and the coprecipitated products were displayed using SDS-PAGE. Figure 3, lane 1 shows the HP2-S protein product, which contains the C-terminal domain previously shown to bind to HP1, but does not include exons five and six. As can be seen in lane 5 of Figure 3, the HP2-S protein binds to HP1 in this co-precipitation assay. Lanes 2 and 3 demonstrate that the results are not due to nonspecific binding. Lane 4 shows a mixture of HP1 and the HP2 TP (Test Peptide) for size reference. Parallel samples for the protein product which contains amino acids 1-1353, including the highly conserved N-terminal domain, are shown in lanes 6-10 of Figure 3. This HP2 polypeptide does not bind to HP1 as can be seen in lane 10, nor does the protein product of the region of the sixth exon which contains the PxVxL HP1 binding motif encompassing amino acids 1328-1900, as can be seen in lanes 11-15. These two peptides also contain the ATRX-like region described previously. Thus HP1 appears to bind only within the C-terminal domain of HP2.

A 99 Amino Acid Region Is Both Necessary and Sufficient For HP1 Binding to HP2

Once it had been determined that an HP1 binding domain resides only in the C-terminal portion of HP2, clones were made to narrow down the region responsible for HP1 binding. A clone encompassing this region, amino acids 2188-3257, produced a protein product that binds to HP1 as can be seen in lane 10 of Figure 4. Lane 9 shows the TP and HP1 for size reference. Clones were made that encompassed amino acids 2647-3027, 3006-3257 and 2647-3257. None of these clones produced protein products that bind to HP1. Experimental results for the clone containing amino acids 2647-3257 (which encompasses the two smaller clones) are shown in Figure 4, lanes 1-5. Lane 1 shows the ³⁵S-Met labeled HP2 TP; it can be seen in lane 5 that while HP1 is precipitated by the HP1 antibody, the HP2 protein product is not. A deletion series within the region encompassing amino acids 2188-3257 was done to narrow down the region for HP1 binding. The following clones were made in pET28a: amino acids 2188-3027, 2188-2665, and 2188-2512. All of the produced protein products bind to HP1. Figure 4 lane 15, shows the positive results obtained with the TP 2188-2512. Thus, we continued to do a C-terminal deletion series on this product. Peptides containing amino acids 2188-2263, 2188-2347 and 2188-2418 did not bind to HP1. As can be seen in Figure 4, lanes 21-25, a region of 99 amino acids (2414-2512) is sufficient for HP1 binding; negative results obtained with TP 2188-2418, lanes 16-20, and 2647-3257, lanes 1-5, indicate that this region is necessary for HP1 binding of the C-terminal domain.

Pairwise Comparison of The Su(var)2-HP2 Gene In *D. melanogaster*, *D. pseudoobscura*, *D. willistoni* and *D. virilis* Identifies Conserved Blocks

Upon release of the *D. pseudoobscura* genome, a tblastn search (20) was performed using the sequence of HP2 from *D. melanogaster*. The results suggested that a homologue exists with similar length, amino acid composition, and intron-exon structure (see Table 1 and Figure 5).

Su(var)2-HP2 was identified in *D. virilis* and *D. willistoni* as described above. In both cases, HP2 appears to have an amino acid composition and intron-exon boundaries similar to that in *D. melanogaster*. The length of the predicted larger isoform varies a great deal between the species; *D. pseudoobscura* has the longest version of the protein, 3366 amino acids long. *D. willistoni* HP2 has 2860 amino acids and *D. virilis* HP2 has 3078 amino acids. The predicted range of molecular weights is 316-362 kD. Most of the difference in length is due to the varying length of the sixth exon. Comparison of the predicted smaller isoforms shows that they are much closer in size, ranging from 1562-1633 amino acids in length and 171-176 kD in predicted molecular weight (see Figure 1B). Calculations of isoelectric point also suggest that the smaller isoforms are more similar among species than the larger isoforms. The pI range for the larger isoform is 5.72-7.63, while for the smaller isoform the range is 9.09-9.79.

Each predicted protein sequence was analyzed using several protein motif-predicting programs. *D. melanogaster* HP2 has two putative AT-hooks at amino acid positions 486 and 519. Protein sequences from all four species appear to have an AT-hook at a position corresponding to the second AT-hook, but not to the first. In addition to the conserved AT-hook, various protein motif-predicting programs detected 2-3 more AT-hooks in *D. pseudoobscura* and 1 in *D. willistoni* HP2. The location of these additional AT-hooks was not conserved among the species.

Based on the amino acid composition and size of the protein in each species, it is predicted that a PxVxL motif would be found in the protein. This domain was not found within the Block Maker (31) results, suggesting that it is not highly conserved between the species. A subsequent BLAST (20) search showed that a PxVxL motif does exist in each species, although the specific sequences and locations are not well conserved (*D. melanogaster*, aa 1665, PSVSL; *D. pseudoobscura*, aa 2617, PAVAL; *D. willistoni*, aa 1642, PDVSL; *D. virilis*, aa 1255, PPVLL). Some motif finders identified other possible domains with low E-values, but none were consistent within the four species studied.

Clustalw (29) and Block Maker(31) were used for multiple sequence alignment of the protein sequences. Multiple sequence alignment of the larger isoform shows that there are no significant blocks of homology within the sixth exon. Figure 5 presents a graphical output showing the most highly conserved blocks over the length of the smaller isoform. Conserved blocks were identified throughout the protein, particularly in the N-terminal exons 1-5. Two conserved blocks, labeled G and H, are present in the HP1 binding region detected by immunoprecipitation (Figure 4). Their sequences are shown in Figure 5. A tblastn search against the nr database shows no significant homology of these two blocks with other known DNA sequences. Other blocks highly conserved within HP2 show no significant results in tblastn searches. The conserved G and H blocks may play a critical role in HP1 binding.

Block H Is Necessary For HP1 Binding

The 99 aa peptide which is able to bind to HP1 contains two conserved domains, Block G (aa 2433-2444) and Block H (aa 2464-2479), separated by 19 amino acids in *D. melanogaster* (see Figure 5). These domains were examined by site-directed mutagenesis. In each construct, mutations were made in one domain and not the other. In Block G, AAFPVKITAASS, three mutations were introduced to generate the sequence AADPEKETASS, changing hydrophobic amino acids to charged amino acids. Block H was unchanged in this construct. Similarly, to test Block H, RKLCKVKNRRPYNKWL, the sequence was changed to RKLCEKINERPYNKEL. Block G was unchanged in this construct. As can be seen in Figure 6, mutations within Block G do not result in loss of HP2 polypeptide binding to HP1 (lane 5) while mutations within Block H eliminate binding to HP1 (lane 10). Thus, Block H is critical for HP1 binding.

Neither Block G Nor Block H Alone Are Sufficient For HP1 binding

We wished to determine whether Block G or Block H alone could bind to HP2 or if a larger fragment containing Block G, Block H, and the intervening sequence were needed for binding. Thus, PCR products were used to generate clones that encoded Block G or Block H fused to a GST tag in pET41a. A clone was also made including both domains and the 19 amino acids in between them. The 99 aa region of HP2 that was previously found to be both necessary and sufficient for HP1 binding (amino acids 2414-2512) was also cloned into this vector as a positive control. The additional size provided by the GST tag prevents ubiquitin-dependent proteolytic degradation in the rabbit reticulocyte lysates used in the transcription/translation system. HP1 was expressed from the pET28a vector, to avoid the possibility of generating dimers of HP1 and the TP via the GST tag (34). Immunoprecipitations were performed as described above, except that due to the similarity in size of the HP2 protein products with HP1, nonradioactive HP1 was used in tests with the GST-Block H and the GST-Block G products. Successful production of HP1 was monitored by precipitation of the protein product of HP2 that is necessary and sufficient for HP1 binding. GST-Block H and GST-Block G alone are not sufficient for binding to HP1, as can be seen in lanes 5 and 10 of Figure 7. A construct containing both domains linked by the 19 amino acids does bind to HP1, as can be seen in Figure 7, lane 15. Coupled with the prior results, this indicates that while Block H is critical for HP1 recognition, the 19 amino acid linker and possibly Block G lend additional amino acids most likely needed for stabilization.

Discussion

In this study, we have performed coimmunoprecipitation assays along with a comparative analysis of *Su(var)2-HP2* and the predicted protein in different *Drosophila* species to discover a novel HP1 binding domain. The presence of HP2 homologues, and evidence in support of conservation of function in other *Drosophila* species, is provided by immunostaining of polytene chromosomes from *D. pseudoobscura* and *D. virilis* with an HP2-specific antibody generated from a *D. melanogaster* HP2 peptide, as well as by Western analysis which shows that the two protein isoforms are conserved among the four species studied. Previous work has shown that HP2 localizes to the pericentric heterochromatin, the telomeres, and to the small fourth chromosome as well as to a few euchromatic sites within the long chromosome arms in *D. melanogaster*. We observe the same pattern in *D. pseudoobscura*; in *D. virilis* the dot chromosome is not stained with either antibody, as previously reported using antibodies specific for HP1 (18). Congruence of the HP1 and HP2 distribution patterns in the different species as well as conservation of the two protein isoforms of HP2 leads us to conclude that an HP2 product is present in the four species studied and plays a similar role as in *D. melanogaster*.

The only binding partner known for HP2 at present is HP1, as originally determined by a yeast two-hybrid assay mentioned previously. We sought to determine the HP1 binding domain in HP2. Work by Shaffer *et al.* (13) had indicated that HP1 binds to a site in the C-terminal domain of HP2. A C-terminal polypeptide of HP2 was pulled out of a yeast two-hybrid screen using HP1 as bait. This polypeptide had a rearranged sequence, as previously described. Using HP2-S (RE12383) obtained from the Berkeley *Drosophila* Genome project we were able to confirm that a clone with the correct sequence in the C-terminal region makes a polypeptide that binds effectively to HP1. We were also interested in determining whether HP1 might bind to multiple domains of HP2. Given its large size, HP2 might provide a scaffold for multiple interactions with HP1. We therefore performed coimmunoprecipitation assays as done with HP2-S to see if HP1 could co-precipitate various HP2 Test Peptides (TPs) in the presence of HP1 antibodies. However, only a single HP1 binding site was detected, present in the eighth exon. HP2 polypeptides containing PxVxL, reported as an HP1 binding motif in numerous HP1 binding

proteins, and an ATRX-like region, do not co-precipitate with HP1. Recent work has indicated that the HP1-binding domain in ATRX is a variant PxVxL motif that is manifested as LxVxL and is not simply a region of charged amino acids serving as an HP1 binding domain as had been speculated (35). While the PxVxL motif occurs, it is not well conserved in sequence and location among the HP2 proteins of the *Drosophila* species examined in this study. Given the inability of this domain to bind to HP1 in coimmunoprecipitation assays and the lack of specific conservation, this domain does not appear to be important for HP1 binding to HP2. The PxVxL peptide is found in only a subset of HP1 interacting proteins, including TIF1-beta and the p150 subunit of CAF-1 (14). This peptide is not found in several other proteins known to interact with HP1, including the actin-related protein ARP4 (36), inner centromere protein INCENP (37), origin recognition complex (ORC) proteins (38), or SU(VAR)3-9 (8). These proteins most likely bind to HP1 in a different manner than proteins that contain the consensus pentamer, and may bind to HP1 in a manner similar to HP2. The consensus pentamer has been found to bind across the interface of the HP1 chromoshadow domain dimer, inserted in a β -sheet between strands from each monomer (39). It is not known how proteins without the PxVxL motif bind to HP1. None of the non-PxVxL-containing proteins mentioned above appear to have any domains similar to the HP1 binding site in HP2, or the HP1 binding site (where known) in other non-PxVxL proteins. Crystallization or NMR studies of these HP1 binding proteins with HP1 may shed light on another method of HP1 binding.

Based on the co-distribution of HP1 and HP2 in the polytene chromosomes of all species examined, and the conservation of HP1, we expect the HP1 binding site to be well conserved among the different *Drosophila* species. Upon comparison of HP2 in the various species, we find that HP2-S is more conserved than HP2-L, although both protein isoforms persist throughout the evolution of *D. melanogaster*, *D. pseudoobscura*, *D. virilis*, and *D. willistoni*. There is approximately 50% similarity of HP2-S in the different species, including many small, highly conserved blocks (see Figure 5) that may serve as protein binding sites or regions of enzymatic function. Regions within the sixth exon and at the beginning of the eighth exon are highly variable between the species. Despite regions of sequence conservation, protein motif algorithms do not detect any recognizable sequence motifs in this protein that are conserved among the species other than the presence of putative AT-hooks. While HP2 from all *Drosophila* species examined have at least one AT-hook, these motifs are not in the same regions of the protein. Previous work indicates that the AT-hooks are insufficient to mediate binding to AT-rich satellite DNA². The lack of conserved, known motifs suggests that higher order protein folding may determine functional domains. Because HP2 is essential in *D. melanogaster*, we suspect that functional homologues exist in other organisms, but that the highly unstructured nature of HP2 allows considerable drift in sequence. As of yet, HP2 homologues have not been found in the sequenced genomes of the honey bee or mosquito. HP2 rabbit antibodies crossreact with a protein that binds to the pericentric heterochromatin in mealybug mitotic chromosomes, indicating a recognizable homologue³.

Once it was determined through coimmunoprecipitation experiments that HP1 binds only within one domain of HP2, we sought to better define the required domain.

Coimmunoprecipitation experiments ultimately identified a peptide of 99 amino acids that was both necessary and sufficient for binding to HP1. Through comparative sequence analysis it was found that within this region there are two conserved domains, Block G and Block H. Site directed mutagenesis of selected amino acids in these two domains indicates that Block H is essential for HP1 binding. A linker region of 19 amino acids bridges the two domains, although this length is variable among the different *Drosophila* species. Peptides made up of only Block G or Block H linked to GST are not able to bind to HP1. This is not surprising, given that a 12

²G. E. S. and S. C. R. E., unpublished observations

³Giorgio Pranter, University of Tuscia, personal communication

or 16 amino acid peptide may not be long enough to fold correctly for a binding pocket to be available. Thus, some part of the linker region or simply a bulkier fragment is important for HP1 to bind to HP2 in addition to Block H. Our findings based on coimmunoprecipitation and comparative sequence analysis have allowed us to identify a new HP1 binding domain that is likely to be critical for heterochromatin formation.

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FIGURE 1.

HP2 mimics the HP1 distribution pattern on polytene chromosomes in different *Drosophila* species and its protein splice variants are conserved. Immunofluorescent staining of polytene chromosomes and Western blots were done to confirm the presence of functional HP2 homologues in other *Drosophila* species. *A*, *D. virilis* squash viewed by phase contrast microscopy (top left panel). HP1 and HP2 have a coincident staining pattern, including prominent staining of the pericentric heterochromatin and three sites in the long chromosome arms (top middle and top right panels). The same experiment was done in *D. melanogaster* and *D. pseudoobscura*; the chromocenter is shown at higher magnification (lower panels). In these species, in addition to the pericentric heterochromatin, the dot chromosome is stained as well (arrowhead). *B*, Nuclei were isolated from adult flies from four species of *Drosophila* (M: molecular weight marker in kD, Dm: *D. melanogaster*, Dp: *D. pseudoobscura*, Dw: *D. willistoni*, Dv: *D. virilis*). Western blots confirm that all four species have two protein isoforms of similar size.

FIGURE 2.

A schematic diagram of the HP2 test peptides (TP) used in coimmunoprecipitation experiments. The exon structures of HP2-L and HP2-S are shown (top). The AT-hooks are located in exon 6 (black boxes) as is the PxVxL domain (*). The TPs with their amino acid boundaries are given. A (+) or (-) indicates the ability of the TP to bind to HP1 (right side).

FIGURE 3.

HP1 binding is limited to the C-terminal domain of HP2. Coimmunoprecipitation experiments were carried out with HP1 (206 aa) and TPs from HP2. In the first experiment (lanes 1-5), HP2-S (*) was tested for its ability to bind to HP1 (arrowhead). The ^{35}S -Met labeled HP2-S product and a mixture of the HP2-S product plus ^{35}S -Met labeled HP1 are shown for size comparison (lanes 1 and 4). It can be seen that the TP is not precipitated in the absence of either the HP1 antibody and HP1 (lane 2), nor is the TP immunoprecipitated by the antibody in the absence of HP1 (lane 3). HP2-S and HP1 coprecipitate with an HP1 antibody (lane 5). Experiments using the TP encoding amino acids 1-1353 and 1328-1900 were done similarly (lanes 6-10 and 11-15). The N-terminal half of HP2 (exons 1-5 plus most of 6) does not immunoprecipitate in the presence of HP1 and HP1 antibodies (lane 10). This is also true for a peptide containing the PxVxL motif (lane 15).

FIGURE 4.

A 99 amino acid domain is both necessary and sufficient for HP1 binding. Two domains that span the eighth and ninth exons of HP2 were tested to see if the resulting peptide could bind to HP1. The TP (*) alone (lane 1), and mixed with HP1 (arrowhead) (lane 4) can be seen for size comparison. Neither the TP alone nor the TP plus antibody without HP1 results in immunoprecipitation of the TP by Protein A Sepharose beads (lanes 2 and 3). Results using the TPs indicated show that peptide 2647-3257 is insufficient for coprecipitation by HP1 (lane 5), but TP 2188-3257 is coprecipitated (lane 10). Only the N-terminal portion of this peptide (aa 2188-2512) is required for coprecipitation (lane 15), and of this region, peptide 2188-2418 does not bind to HP1 (lane 20). (In this case HP1 is not seen since it is nonradioactive.) The peptide shown in lane 21 (aa 2414-2512) was then deemed to be both necessary and sufficient to bind to HP1 (lane 25).

FIGURE 5.

Block Maker results for HP2-S. Block Maker was used to find regions of sequence similarity among the protein sequences of the smaller isoforms of HP2. Above is the graphical output for the MOTIF block making algorithm (*D. melanogaster*, Dm, *D. pseudoobscura*, Dp, *D. willistoni*, Dw, and *D. virilis*, Dv). Two conserved blocks from each species are boxed (G and H) and the sequences shown. A consensus sequence is given below (con). Conservation of hydrophobic amino acids is indicated by a (+). Where the amino acids are stacked, the amino acid on top occurs in half of the cases and the amino acid on bottom occurs in the other half of the cases. An X indicates that various amino acids occur in this position. Sites mutated in the *D. melanogaster* sequence in subsequent tests are underlined.

FIGURE 6.

Block H is necessary for HP1 binding. Coimmunoprecipitations were done as previously discussed with HP1 and HP2 TPs. In this experiment, either Block G or Block H was mutated by site directed mutagenesis in an HP2 clone encompassing amino acids 2414-2512. The ³⁵S-Met labeled HP2 TP (G) (*) is shown for size reference (lane 1). Controls as done previously are seen (lanes 2, 3, and 4) and it is shown that the mutated TP (G) can bind to HP1 (arrowhead) (lane 5). The TP (H) and HP1 are again seen for size reference (lanes 6 and 7). Controls were done as previously described (lanes 8, 9 and 10). Mutations within the Block H conserved domain abrogate binding to HP1 (lane 11).

FIGURE 7.

Block G, Block H, and the spacer region in between them, is sufficient for HP1 binding. Block G linked to GST can be seen in lane 1 for size comparison (*). In lane 5 it can be seen that the GST-Block G peptide cannot bind to HP1. Lanes 2, 3, and 4 are control lanes. HP1 is nonradioactive in this case due to the similarity in size to the TP. GST-Block H can be seen in lane 6 and its inability to bind to HP1 is shown in lane 10 and controls are seen in lanes 7, 8, and 9. The 47 amino acid TP (Block G-19 amino acids-Block H) linked to GST (*) can be seen for size comparison (lane 11). This peptide is immunoprecipitated with HP1 (arrowhead) (lane 15); the requisite controls are shown in lanes 12, 13, and 14.