

Overlapping Domains of the Heterochromatin-Associated Protein HP1 Mediate Nuclear Localization and Heterochromatin Binding

James A. Powers and Joel C. Eissenberg

Edward A. Doisy Department of Biochemistry and Molecular Biology, Cell and Molecular Biology Program, St. Louis University School of Medicine, St. Louis, Missouri 63104

Abstract. The *Drosophila* protein HP1 is a 206 amino acid heterochromatin-associated nonhistone chromosomal protein. Based on the characterization of HP1 to date, there are three properties intrinsic to HP1: nuclear localization, heterochromatin binding, and gene silencing. In this work, we have concentrated on the identification of domains responsible for the nuclear localization and heterochromatin binding properties of HP1. We have expressed a series of β -galac-

tosidase/HP1 fusion proteins in *Drosophila* embryos and polytene tissue and have used β -galactosidase enzymatic activity to identify the subcellular localization of each fusion protein. We have identified two functional domains in HP1: a nuclear localization domain of amino acids 152–206 and a heterochromatin binding domain of amino acids 95–206. Both of these functional domains overlap an evolutionarily conserved COOH-terminal region.

HHP1 is a 206 amino acid protein of *Drosophila melanogaster* that is associated primarily with the heterochromatic regions of *Drosophila* interphase and polytene chromosomes (James and Elgin, 1986; James et al., 1989). It is an essential protein (Eissenberg et al., 1992) with dosage-dependent effects on heterochromatin-mediated gene silencing (Eissenberg et al., 1990, 1992). To begin to understand the role of HP1 in the formation and maintenance of heterochromatin, one approach is to identify specific functional domains of HP1. Based on the characterization of HP1 to date, there are three properties intrinsic to HP1: nuclear localization, heterochromatin binding, and gene silencing. In this work we have concentrated on the identification of domains responsible for the nuclear localization and heterochromatin binding properties of HP1. The domains of protein structure that account for these properties may be discrete and separable, or may be assembled from amino acid residues dispersed throughout the protein sequence.

Recently, two highly conserved regions, which represent possible candidates for functional domains, have been identified in HP1. Paro and Hogness (1991) reported a 37 amino acid region near the NH₂ terminal of HP1 has a 65% sequence identity with the *Drosophila* Polycomb protein. Because both HP1 and Polycomb protein are chromosomal proteins and both are involved in mediating gene repression, it is attractive to think this homology may be responsible for a common functional property. Paro and Hogness (1991) have designated this region of homology the "chromo domain." Several labs have used DNA probes based on the chromo domain coding sequences to identify genes encoding other proteins that share this sequence. Using this strategy, HP1 homologs have been identified in *Drosophila virilis*

(Clark and Elgin, 1993), mealybug (Epstein et al., 1992), mouse (Singh et al., 1991), and human (Singh et al., 1991; Saunders et al., 1993). Sequence comparisons consistently show two highly conserved regions among the predicted protein products of these genes. One is, of course, the chromo domain. The other is a 65 amino acid COOH-terminal region that has 52% sequence identity with the two mouse homologs and *Drosophila melanogaster* HP1 (Fig. 1). The chromo domain contains a short cluster of basic amino acids similar to reported nuclear localization sequences (Dingwall and Laskey, 1991) and represented the best candidate for the nuclear localization domain. Sequence analysis of HP1 revealed no other probable nuclear localization or DNA binding motifs.

To determine whether distinct, functional domains of HP1 exist which are responsible for the localization and binding functions, we have constructed *Escherichia coli* (*E. coli*) LacZ/HP1 fusion plasmids containing a series of HP1 polypeptides. Fusion proteins were expressed in *Drosophila* and assayed in diploid and polytene tissue. Here we report the identification of overlapping nuclear localization and heterochromatin binding functional domains in the COOH-terminal half of HP1.

Methods

Drosophila Stocks

Drosophila melanogaster Canton S, v^{36F}; ry⁵⁰⁶ and the balancer stocks v^{36F}; CyO/Sco; ry⁵⁰⁶ and v^{36F}; Sb/TM2,ry⁵⁰⁶ (obtained from Dr. L. Searles, University of North Carolina, Chapel Hill) were grown at room

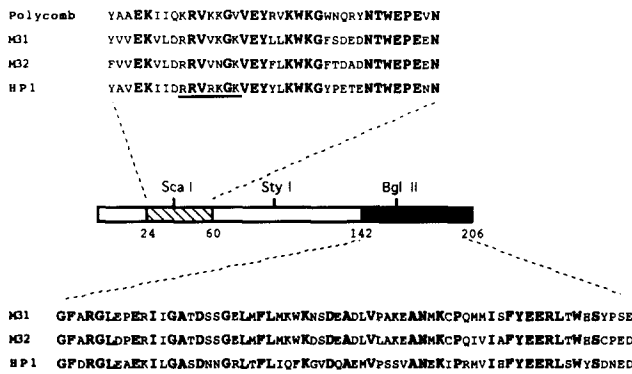


Figure 1. Two evolutionarily conserved regions encoded by the HPI cDNA. The center bar represents the 206 amino acids of HPI. Restriction enzyme sites used to make the β -galactosidase/HPI fusion constructs are shown above the bar and HPI amino acid numbers are shown below. The NH₂-terminal hatched region (detailed above the map) indicates the "chromo domain," a region of 37 amino acids that has 65% sequence identity with a similar region in the *Polycomb* gene product and a 62% identity with the mouse HPI homologues, M31 and M32. A cluster of basic amino acids, similar to identified nuclear localization motifs, is underlined. The COOH-terminal filled region (detailed below the map) indicates a region of 65 amino acids that has 52% sequence identity with the COOH-terminal region of the mouse homologues. Amino acids are shown in single letter code. Amino acids conserved among all proteins in the group are shown in bold type.

temperature in half pint plastic bottles or 23 ml plastic vials using a cornmeal based medium supplemented with dried bakers' yeast.

Plasmid Constructs

Many of the constructs used in this study were the result of a lengthy process of evolution. The fragments that make up each construct are identified with a brief description of the process involved. The complete details for the construction of each plasmid and the sequence of each are available upon request.

All constructs are cloned into the P-element transformation vectors pUCHsneo (Steller and Pirrotta, 1985) or pYCl.8 (Fridell and Searles, 1991). The *E. coli lacZ* gene under the *Drosophila Hsp70* heat shock promoter was obtained from the β -galactosidase expression vector p194.70ZT provided by D. J. T. Lis. p194.70ZT was modified by polymerase chain reaction mutagenesis using the oligonucleotides 5'-TCAGCTGAGCGCCGGTCGCTA-3' and 5'-CTTTTGTGACACCAGACCAACT-3' to remove the *lacZ EcoRI* site and stop codons. The 3' end of *lacZ* was amplified using these oligos; the amplification product was chloroform extracted and ethanol precipitated. The fragment was inserted into p194.70ZT prepared by removing the *EcoRI* fragment and filling the ends of the *EcoRI* site using T4 polymerase. The resulting plasmid codes for the entire β -galactosidase amino acid sequence.

To remove the 5' untranslated sequences of the HPI cDNA, the *BglII* cDNA fragment was inserted into the *BamHI* site of pUC 13.10 μ g of the resulting plasmid was linearized with *EcoRI* followed by digestion with 32 units of *Bal31* (US Biochemical Corp., Cleveland, OH) for 140 seconds at room temperature in a total volume of 80 μ l. *SalI* linkers were ligated to the digested DNA. This population of plasmids was used to transform competent *E. coli* TGI cells. DNA was prepared from transformed colonies and was screened for deletions in the desired size range by *SalI* restriction enzyme digestion. DNA from selected colonies was sequenced and a clone was selected that removed all upstream sequences including the adenosine of the HPI ATG start site codon. This placed the added *SalI* site in frame with the polylinker *SalI* site at the 3' end of the *lacZ* gene. All fusion constructs that contain the NH₂-terminal coding sequence of HPI were made using this deletion, and therefore, start with the second HPI codon.

pG β -gal (plasmid using the pUCHsneo P-element vector and G418 selection that contains the β -galactosidase gene) contains the *XhoI/BglIII* fragment of the modified p194.70ZT plasmid that includes the *Hsp70* promoter,

the *lacZ* gene followed by the *EcoRI* to *SalI* fragment of the pUC19 polylinker and the *Hsp70* 3' termination sequence was inserted into the *SalI/BamHI* sites of pUCHsneo. The polylinker between the *lacZ* sequence and the *Hsp70* termination sequence codes for the following amino acids: GLU, PHE, GLU, LEU, GLY, SER, ARG, GLY, SER, SER, ARG, VAL, and ASP.

pG β /41 (plasmid using the pUCHsneo P-element vector and G418 selection that contains the β -galactosidase/amino acids 2-41 of HPI fusion) contains the *SalI/ScaI* fragment of the *Bal31* digested HPI cDNA inserted into *pG β -gal* at the *SalI* site and an *EcoRV* site from a synthesized oligonucleotide linker added to the polylinker. NH₂-terminal linker amino acids in this construct are: GLU, PHE, GLU, LEU, GLY, SER, ARG, GLY, SER, SER, ARG, VAL, ASP, and LEU. Non-HPI amino acids at COOH-terminal end are: PRO, ARG, and VAL.

pG β /95 contains the *SalI/StyI* fragment of the *Bal31* digested HPI cDNA inserted into *pG β -gal* at the *SalI* site and a *StyI* site from a synthesized oligonucleotide linker added to the polylinker. NH₂-terminal linker amino acid sequence is the same as *pG β /41*. Non-HPI amino acids at the COOH-terminal end are: GLY, LEU, GLU, SER, THR, LYS, ALA, and LYS.

pG β /153 contains the *SalI/BglIII* fragment of the *Bal31* digested HPI cDNA inserted into the *SalI* site of the *pG β -gal*. NH₂-terminal linker amino acid sequence is the same as *pG β /41*. There are no additional COOH-terminal amino acids.

pG β /206 contains the *BglIII/EcoRI* fragment of an HPI cDNA clone that encodes the COOH-terminal sequences of HPI and the 3' untranslated termination sequences of HPI. This was used to replace the *BglIII/EcoRI* fragment of *pG β /153*. The NH₂-terminal linker amino acid sequence is unchanged.

pG β /41-206 contains *SalI/ScaI* fragment of HPI removed from *pG β /206*. NH₂-terminal linker amino acids in this construct are: GLU, PHE, GLU, LEU, GLY, SER, ARG, GLY, SER, SER, and ARG.

pG β /41-153 contains the *SalI/ScaI* fragment of HPI removed from *pG β /153*. NH₂-terminal linker amino acid sequence is the same as *pG β /41-206*. There are no additional COOH-terminal amino acids.

pG β /41-95 contains the *SalI/ScaI* fragment of HPI removed from *pG β /95*. NH₂-terminal linker amino acid sequence is the same as *pG β /41-206*. Non-HPI amino acids at COOH-terminal end are: GLY, LEU, GLU, SER, THR, LYS, ALA, and LYS.

pG β /95-206 contains the *SalI/StyI* fragment of HPI removed from *pG β /206*. NH₂-terminal linker amino acids in this construct are: GLU, PHE, GLU, LEU, GLY, SER, ARG, GLY, SER, and SER.

pG β /93-153 contains the *SalI/StyI* fragment of HPI removed from *pG β /153*. NH₂-terminal linker amino acid sequence is the same as *pG β /95-206*. There are no additional COOH-terminal amino acids.

pG β /152-206 contains the *SalI/BglIII* fragment of HPI removed from *pG β /206*. NH₂-terminal linker amino acids in this construct are: GLU, PHE, GLU, LEU, GLY, SER, ARG, GLY, SER, SER, ARG, VAL, and GLU.

pV β /152-206 (P-element vector pYCl.8 with *vermillion* selection): *pG β /152-206* was digested to completion with *HindIII* and then partially digested with *EcoRI* to remove the entire *lacZ*/HPI fusion including the *Hsp70* promoter and HPI termination sequences. This fragment was inserted into the Bluescript-KS vector (Stratagene Cloning Systems, La Jolla, CA) at the *HindIII/EcoRI* sites. This fragment was then excised with *NotI/SalI* and inserted into pYCl.8 at the *NotI/SalI* sites.

pV β /95-206, *pV β /41-206*, *pV β /206*, *pV β /93-153*, *pV β /153*, were all made by replacing the *KpnI* fragment of *pV β /152-206* with the *KpnI* fragment of the appropriate *pG β* plasmid.

All plasmid DNA used for injection was prepared by banding once in a CsCl gradient using standard protocols.

Transformation

The protocol used for somatic transformation was essentially as described by Martin et al. (1986) and Shore and Guild (1987). Each plasmid at 1 mg/ml in injection buffer was injected into 10-30-min old Canton S embryos. To assay embryonic expression, embryos were injected at the posterior end and allowed to age overnight at room temperature. To assay expression in polytene tissue, embryos were injected at the anterior end. Hatched larvae were transferred to food vials and were grown at room temperature to the third instar larval stage.

The protocol used for germ line transformation was essentially that described by Spradling (1986). Fusion plasmids at 500 μ g/ml plus the helper plasmid *p π 25.7wc* (Karess and Rubin, 1984) at 250 μ g/ml in injection buffer were injected into the posterior end of 10-30-min old embryos. Hatched larvae were transferred to food vials and were grown to adulthood.

Fusion constructs cloned in the P-element vector pUCHsneo were injected into Canton S embryos. Injected G₀ adults were pair mated to Canton S flies. F₁ embryos were transferred to vials containing Instant *Drosophila* Medium (Carolina Biological Supply Co., Burlington, NC) supplemented with 1 µg/ml G418 (Gibco Laboratories, Grand Island, NY), 0.005% propionic acid (Sigma Chemical Co., St. Louis, MO), and 4.4 mM methylparaben (Sigma Chemical Co.) and heat shocked every other day for 30 min at 37°C. Surviving transformed F₁ adults were pair mated to Canton S flies and maintained on G418 vials and heat shocked every other day until stable lines were obtained. Transformed lines were obtained for the fusion constructs pGβ/41, pGβ/95, pGβ/153, pGβ/41-95, and pGβ/41-153.

After several attempts, no transformants could be obtained for any of the constructs that contained HPI amino acids 152–206. It may be that β-galactosidase fusion proteins expressing this peptide are detrimental to the flies. This would present a problem due to the requirement to continually express the fusion protein during the selection for transformants. The pUCHsneo vector includes the gene for neomycin resistance, which confers resistance to the aminoglycoside G418, also under the control of a heat shock promoter. Therefore, both the β-galactosidase/HPI fusion and the neomycin resistance gene are induced by heat shock and necessarily expressed during the selection process. To avoid this problem, the remaining β-galactosidase/HPI fusions were cloned into the *vermillion* P-element vector which expresses the *vermillion* gene under its own promoter (Fridell and Searles, 1991). This gene complements the eye color mutation in *vermillion* mutant flies, allowing for the selection of transformants without the expression of the HPI fusions.

Fusion constructs cloned in the P-element vector pYCl.8 were injected into v^{36F}; r^{y506} embryos. Injected G₀ adults were pair mated to v^{36F}; r^{y506} flies. F₁ adults were screened based on the rescue of the *vermillion* eye color mutation. Transformed F₁ adults were pair mated to v^{36F}; r^{y506} mates and successive generations were selected based on eye color until stable lines were obtained. Transformed lines were obtained using the *vermillion* vector for the fusions pVβ/93-153, pVβ/153, pVβ/206, pVβ/95-206, and pVβ/152-206. To date we have not obtained a transformed line for the fusion containing the HPI peptide 41-206 after many attempts.

To establish stable transformed lines, transformed flies were crossed to both the second chromosome (v^{36F}; CyO/Sc^o; r^{y506}) and third chromosome (v^{36F}; Sb/TM2, r^{y506}) balancer stocks to determine which chromosome carries the P-element insert. Transformed flies heterozygous for the proper balancer were pair-mated to establish balanced transformed stocks. If the insert was nonlethal, homozygous stocks were established. Homozygous stocks for transformants carrying an insert on the X-chromosome were established by multiple rounds of selection.

Immunodetection

The HPI antibody is a polyclonal rabbit serum directed against a synthetic peptide based on HPI amino acids 25–47 (a gift of R. Clark and S. C. R. Elgin, Washington University, St. Louis, MO).

For western blot analysis of fusion protein, 1-d old injected embryos were heat shocked at 37°C for 30 min and allowed to recover for 30 min. For each construct, 10 embryos were homogenized on ice in 50 µl of buffer (Laemmli, 1970) containing 5 µl of a proteinase inhibitor cocktail (10 µM benzamide-HCl, 1.0 mM PMSF, 1 µg/ml phenanthroline, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A) in a microfuge tube, incubated 2 min in a boiling-water bath and microfuged 30 s at 10,000 rpm to remove insoluble material. Proteins were then separated on 5%/10% discontinuous SDS-polyacrylamide gels (Laemmli, 1970) at 200 V for 45 min using the Bio-Rad Mini-Protein II system (Bio-Rad Laboratories, Richmond, CA). Proteins were electrophoretically transferred to nitrocellulose membrane in 25 mM Tris base, 192 mM glycine (free base), 20% methanol (w/v), pH 8.3 (Towbin et al., 1979). After transfer, the membrane was blocked with 1% BSA (fraction V; Sigma Chemical Co.) in TBST (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature. The blocking solution was replaced with either anti-HPI antibody at 1:10,000 or anti-β-gal antibody (5 Prime-3 Prime) at 1:5,000 diluted in TBST and incubated for 30 min at room temperature. The blot was washed three times for 5 min in TBST and incubated in a 1:7,500 dilution of anti-rabbit IgG-alkaline phosphatase conjugated secondary antibody (Promega Corp., Madison, WI) in TBST for 30 min at room temperature. The blot was again washed three times in TBST. Bands were detected using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega Corp.) as described (Harlow and Lane, 1988).

For immunohistochemical localization of HPI in intact salivary glands, salivary glands from Canton S third instar larvae were dissected and in-

cubated for 20 min at room temperature in Cohen and Gotchel (1971) gland medium containing 0.5% Triton X-100. Glands were then transferred to 2% buffered formaldehyde solution (2% formaldehyde, 2% Triton X-100, 10 mM sodium phosphate, pH 7, 2 mM KCl, 0.1 M NaCl) for 25 min at room temperature followed by a 10-min incubation in 45% acetic acid. Staining was done using the Vectastain Elite HRP kit (Vector Labs, Inc., Burlingame, CA). The glands were washed three times for 5 min in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated 60 min in HPI antiserum diluted 1:10,000 in TBSTB (TBS, 10% goat serum, 0.05% Tween-20). Glands were washed in TBSTB and incubated 30 min in a 1:200 dilution of biotinylated anti-rabbit IgG antibody (Vector Labs, Inc.) in TBSTB. Glands were washed once in TBSTB and twice in TBS and incubated in a 1% avidin DH: biotinylated HRP H complex for 30 min. Glands were washed in TBS and stained in 0.005% diaminobenzidine tetrachloride, 0.025% H₂O₂, 50 mM Tris-HCl, pH 7.2. Glands were mounted in 95% glycerol/PBS (10 mM sodium phosphate, pH 7.2, 150 mM NaCl).

The immunofluorescence localization of the β-galactosidase/HPI fusion proteins on polytene chromosomes was done as described in James et al. (1989) with the following modifications. Third instar larvae were heat shocked at 37°C for 30 min followed by a 30-min recovery. Salivary glands were dissected and incubated in gland medium for 8–10 min. Chromosomes were stained by incubating in anti-β-galactosidase antibody (Promega Corp.) at 1:5,000 and, for double labeling experiments, anti-HPI antibody at 1:1,000 diluted in TBSTB for 30 min followed by a 30 min incubation with the appropriate secondary antibodies. For the β-galactosidase antibody (TRITC) conjugated goat anti-mouse IgG (Sigma Chemical Co.) was used at 1:250 in TBSTB. For the HPI antibody, FITC conjugated goat anti-rabbit IgG (Sigma Chemical Co.) was used at 1:250 in TBSTB.

β-galactosidase Activity Staining

For staining of embryos, 1-d old injected embryos were heat shocked at 37°C for 30 min and allowed to recover for 30 min. Embryos were manually dechorionated and fixed in formaldehyde-saturated heptane for 2 min. Heptane was drawn off and replaced with PBS. Embryos were transferred to double stick tape, covered with a drop of PBS and manually devitellinized with a dissection needle. Devitellinized embryos were stained in a 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assay buffer (Simon et al., 1985). Stained embryos were mounted in 95% glycerol/PBS.

For staining of polytene tissue, transformed third instar larvae were heat shocked at 37°C and allowed to recover as described in Fig. 5. Transformed larvae grown on G418 selection food were necessarily heat shocked every other day. The transformants selected on the basis of eye color (*vermillion* constructs) were not heat shocked until it was necessary for staining. In an effort to obtain similar levels of staining with both groups of constructs, larvae transformed with the *vermillion* constructs were heat shocked multiple times over a few days and/or allowed to recover for longer periods of time (1–3 d). These variations in treatment improved the intensity of the stain but did not significantly alter the staining pattern. Tissue was dissected in 0.2% X-gal assay buffer and incubated at room temperature until the desired staining was obtained. Stained tissue was mounted in 95% glycerol/PBS.

Results

Determination of the HPI Nuclear Localization Domain

To determine whether a distinct portion of HPI can confer nuclear localization, a series of chimeric *lacZ*/HPI gene fusions were made. These include sequences of HPI cDNA fused to the COOH-terminus of *lacZ* under the control of the *Hsp70* heat shock promoter in a P-element transformation vector. HPI cDNA was divided into four regions, each coding for peptides of ~ one fourth of the protein sequence, using available restriction sites. Constructs include a series of COOH-terminal peptide deletions, a series of NH₂-terminal peptide deletions, and a series of internal peptides (Fig. 2 a). The proper reading frame of each construct was verified by sequencing (data not shown) and the expression of the expected β-galactosidase/HPI fusions was verified by Western blot analysis.

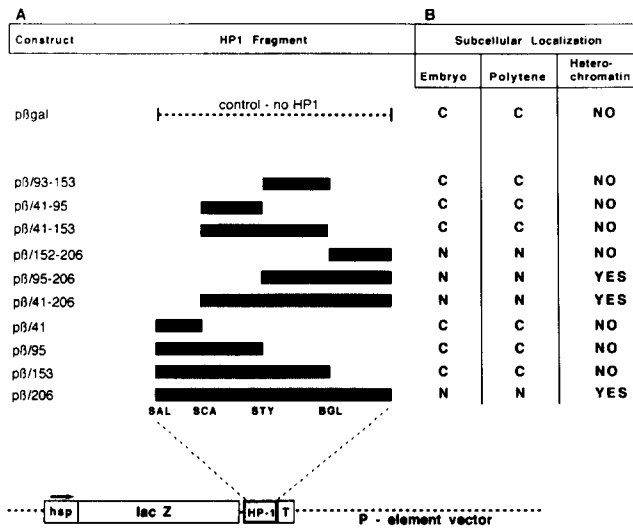


Figure 2. The COOH-terminal half of HP1 contains separable nuclear localization and heterochromatin binding domains. *A* diagrams the series of β -galactosidase/HP1 fusions. Construct name is listed at left and indicates the HP1 amino acids included in the fusion. HP1 cDNA fragments are fused in frame downstream of the *E. coli lacZ* gene under the heat shock promoter. Constructs that include the COOH-terminus of HP1 include the HP1 stop codons and 3' untranslated sequences. The remaining constructs contain the *Drosophila Hsp70* stop codons and 3' untranslated sequences. *B* summarizes the results of the subcellular localization studies. Each fusion construct was injected into pre-blastoderm embryos. For embryonic localization, 24-h old embryos were heat-shocked, allowed to recover for 30 min., then fixed and stained for β -galactosidase activity using X-gal. Results are summarized in the right-hand column: all fusions containing the COOH-terminal 55 amino acids of HP1 directed nuclear localization (N) of β -galactosidase, while in those lacking this domain, β -galactosidase localization was cytoplasmic (C). For localization in polytene cells, third instar larvae containing the fusions were heat-shocked and allowed to recover. Polytene tissue was dissected and stained for β -galactosidase activity using X-gal. Nuclear localization data are summarized in the center column and confirm the embryo data. Heterochromatin localization data are summarized in the right column: all fusions containing the COOH-terminal 112 amino acids directed heterochromatic localization of β -galactosidase.

Each construct was transiently expressed in *Drosophila* embryos after somatic transformation. Embryos were heat shocked briefly to induce fusion protein synthesis. Homogenates were then run on duplicate SDS-polyacrylamide gels and blotted. One blot was probed with β -galactosidase antibody (Fig. 3 *a*) to detect each of the fusion proteins. The mobility shift in each lane corresponds to the size of the HP1 peptide. The duplicate blot was then probed with a polyclonal anti-HP1 serum (Fig. 3 *b*) to verify the presence of HP1 peptides. The antibody was raised against a synthetic peptide based on the chromo domain sequence of HP1 (Clark and Elgin, unpublished observations). As expected, the HP1 antibody detected wild-type HP1 in each lane and all of the β -galactosidase fusion proteins that contain at least the first 40 amino acids of HP1. The wild-type HP1 band indicates approximately equal loading in each lane. The variation of the intensity of the fusion bands is probably due to differences in the amount of injected DNA or to differences in the effectiveness of heat shock induction.

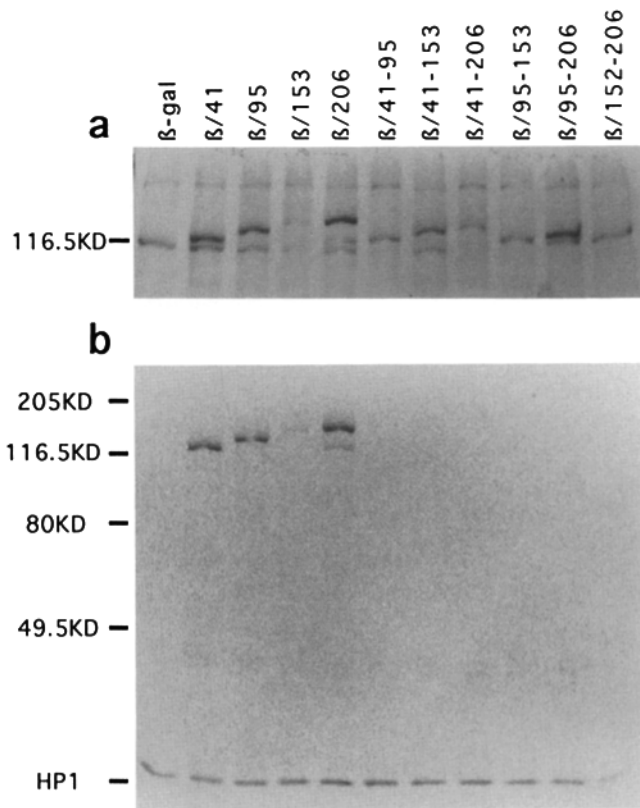


Figure 3. Embryonic expression of the β -galactosidase/HP1 fusion proteins. For each fusion construct, total protein was prepared from 10 heat-shocked embryos, and separated on duplicate SDS-PAGE gels and transferred to nitrocellulose. Blots were probed with primary antibody (*a*: anti- β -galactosidase, *b*: anti-HP1) and fusion proteins were detected with alkaline phosphatase conjugated secondary antibody. Note that the HP1 antibody is specific to an epitope encoded in the first 40 amino acids of HP1; only those fusions containing this peptide are detected. Wild type HP1 is detected at the bottom of *b*.

Subcellular localization of the fusion proteins was assayed in somatically transformed embryos using X-gal to stain for β -galactosidase enzymatic activity. Each construct was injected into ~ 100 embryos which were aged overnight, heat shocked to induce fusion expression, fixed, and stained for β -galactosidase enzymatic activity. In each embryo, a subset of cells showed varying levels of fusion protein expression (data not shown). Nuclear staining was present in all embryos that expressed a COOH-terminal HP1 peptide which includes amino acids 152–206 and was absent in embryos that did not express this peptide (summarized in Fig. 2 *b*). This identifies the COOH-terminal 54 amino acids of HP1 as necessary and sufficient for nuclear localization. This peptide does not contain a "consensus" nuclear localization sequence (Dingwall and Laskey, 1991). The chromo domain peptide has no nuclear targeting activity in this assay.

Nuclear Localization of β -gal/HP1 Fusions in Polytene Tissue

To verify the subcellular localization data from embryos, the experiments were repeated using third instar polytene tissue. The large size of polytene cells makes identification of cellu-

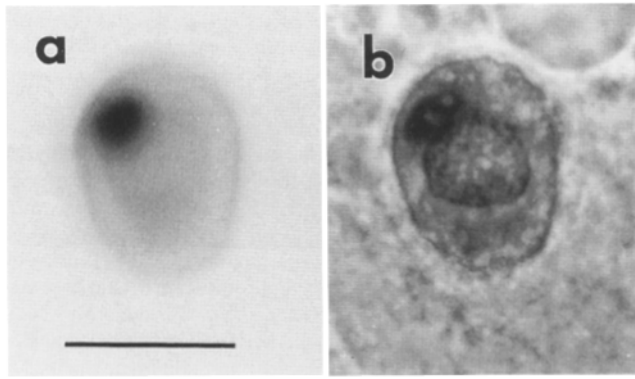


Figure 4. Polytene expression of the β -galactosidase/full-length HP1 control fusion protein. Fusion protein localization based on β -galactosidase activity detected with X-gal staining. (a) Bright field microscopy of a fat-body nucleus from a transgenic line showing expression of pV β /206 after 30-min heat shock and 30-min recovery. Bar, 20 μ m. (b) Same nucleus as in a using phase contrast microscopy to show position of the nucleolus.

lar structures easier and thus, allows subnuclear localization of the fusion proteins. Where possible, these experiments were done using transgenic lines containing the β -galactosidase/HP1 fusions introduced by P-element mediated transformation. We have not been able to establish a transgenic line for the fusion construct pV β /41–206. For this construct somatically transformed larvae were used. For the constructs pV β /206, pV β /95–206, and pV β /151–206 β -galactosidase staining was assayed in both somatically transformed and transgenic tissue with identical results.

To induce the expression of the fusion proteins, third instar larvae were heat shocked for 30 min and allowed a period of recovery to accumulate fusion protein before staining. Subcellular localization of the β -galactosidase/HP1 fusions was detected by staining for β -galactosidase activity in intact polytene tissue. The results in polytene tissue are completely consistent with the embryo data. The HP1 COOH-terminal peptide of amino acids 152–206 is necessary and sufficient for the localization of the β -galactosidase/HP1 fusion to the nucleus (Fig. 2 b and Fig. 5) in both diploid embryonic cells and in polytene third instar larval cells.

Heterochromatin Localization of β -gal/HP1 Fusions in Polytene Tissue

In the previous experiment, two distinct nuclear staining patterns were evident in the cells showing nuclear localization of the fusion constructs (Fig. 5, d, h–j). The first pattern, a distinct spot of stain inside the nucleus was first seen in the cells expressing the β -galactosidase/full-length HP1 fusion protein; pV β /206 (Fig. 4, Fig. 5 h). A spot of stain adjacent to the nucleolus (shown in Fig. 4), often appearing to be wrapped around the nucleolus, was consistently seen in these cells. This pattern is consistent with the location of the heterochromatic chromocenter and with the immunofluorescence staining pattern reported by James et al. (1989) for wild-type HP1 in intact polytene tissue. This staining pattern is also seen in cells expressing pV β /41–206 or pV β /95–206 (Fig. 5 i and j) which contain the HP1 nuclear localization domain and additional NH₂-terminal amino acids. To

confirm that this pattern is identical to the heterochromatin specific staining pattern of wild-type HP1, third instar salivary glands from wild-type flies were immunohistochemically stained with anti-HP1 primary antibody and HRP conjugated secondary antibody (Fig. 5 k). The staining pattern is identical to the pattern seen in the polytene cells expressing the fusions pV β /206, pV β /41–206, and pV β /95–206 (Fig. 4, Fig. 5, h–j). There are often one or two additional spots of stain, away from the nucleolus, seen in the salivary gland nuclei of both the control tissue and the tissue expressing the fusion constructs pV β /206, pV β /41–206, and pV β /95–206 (for example, compare k with h and i in Fig. 5).

In contrast to this pattern of distinct, localized spots of stain inside the nucleus is the second pattern of nuclear staining seen in cells expressing pV β /151–206, the construct containing the HP1 nuclear localization domain (Fig. 5 d). A generalized staining of the entire nucleus was seen in these cells. The examination of frozen sections of these cells confirmed a generalized staining pattern of the entire nucleus with no localized spots of stain evident (data not shown).

To verify that the specific spot of stain adjacent to the nucleus does indeed represent heterochromatin localization, β -galactosidase/HP1 fusion proteins were visualized on polytene chromosomes by immunofluorescence staining using an anti- β -galactosidase antibody. Chromosomes from cells expressing pV β /206 (data not shown) or pV β /95–206 (Fig. 6) show specific staining of the chromocenter, confirming the localization of the fusion proteins to heterochromatin. No staining was seen on chromosomes from cells expressing pV β /151–206 (data not shown). To verify that the heterochromatin localization seen on chromosomes from cells expressing pV β /95–206 represents the wild-type pattern of HP1 localization, these chromosomes were double stained using antibodies to both β -galactosidase and HP1. Both antibodies result in identical patterns of staining both at the chromocenter and at a few specific bands on the chromosome arms (Fig. 6, compare b and c).

These results identify an HP1 heterochromatin localization domain that is physically distinguishable from, but overlaps with, the nuclear localization domain. The fusion protein containing amino acids 152–206 of HP1 directs the nuclear localization of β -galactosidase but is insufficient for heterochromatin targeting. In contrast, the fusion protein which includes amino acids 95–206 of HP1 directs both the nuclear and the heterochromatic localization of β -galactosidase activity, indicating that the additional amino acid sequence required for heterochromatin localization is included in the interval 95–151. The peptide composed of amino acids 95–206 represents the COOH-terminal half of HP1. This peptide contains the entire COOH-terminal evolutionarily conserved region of HP1 but excludes the chromo domain. No DNA binding motifs are apparent in the HP1 sequence.

Discussion

We have identified overlapping HP1 nuclear localization and heterochromatin binding domains using a series of β -galactosidase/HP1 fusion proteins. Because flies homozygous for HP1 mutations are not viable, it was necessary to use a marker protein to identify the location of HP1 peptides on a background of wild-type HP1. We used the cytoplasmic protein β -galactosidase from *E. coli* as a marker protein be-

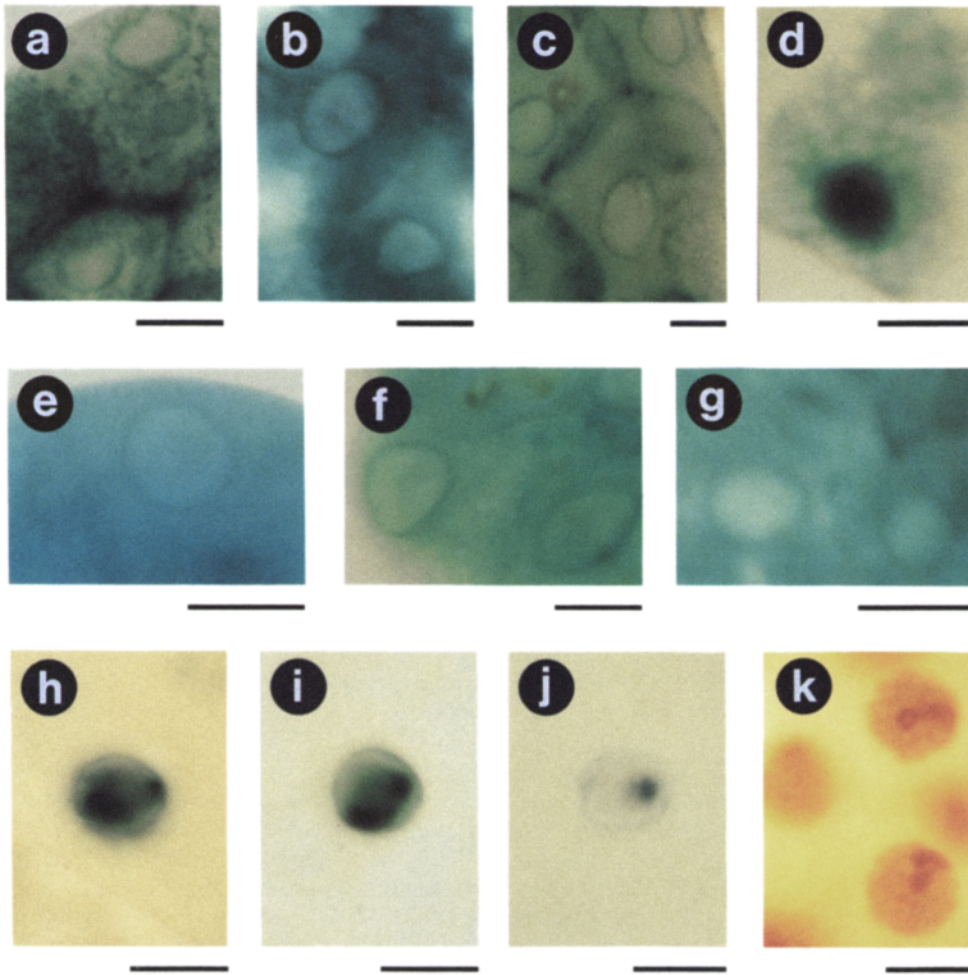


Figure 5. Intracellular localization of β -galactosidase/HP1 fusion proteins in polytene tissue. Fusion protein localization in *a-j* based on β -galactosidase activity detected with X-gal staining. *k* is the HP1 wild-type control detected by anti-HP1 antibody staining. (*a*) β -galactosidase fused to HP1 amino acids 2–41 remains cytoplasmic. Transgenic line, salivary gland, 30-min heat shock followed by 30-min recovery. Apparent blue stain in the nucleus is due to perinuclear staining. (*b*) β -galactosidase fused to HP1 amino acids 41–95 remains cytoplasmic. Transgenic line, salivary gland, 30-min heat shock followed by 60-min recovery. (*c*) β -galactosidase fused to HP1 amino acids 93–153 remains cytoplasmic. Somatic transformation, salivary gland, 30-min heat shock followed by 30 min recovery. Result is the same using transgenic line. (*d*) β -galactosidase fused to HP1 amino acids 152–206 concentrates in the nucleus but does not decorate the chromocenter. Transgenic line, salivary gland, 30-min heat shock followed by 20-h recovery. (*e*) β -galactosidase fused to HP1 amino acids 2–95 (containing the entire chromo domain) remains cytoplasmic. Transgenic line, salivary gland, 30-min heat shock followed by 30-min recovery.

(*f*) β -galactosidase fused to HP1 amino acids 41–153 remains cytoplasmic. Transgenic line, salivary gland, 30-min heat shock followed by 60-min recovery. (*g*) β -galactosidase fused to HP1 amino acids 2–153 remains cytoplasmic. Transgenic line, salivary gland, 30-min heat shock followed by 60-min recovery. (*h*) β -galactosidase fused to full length HP1 (amino acids 2–206) decorates the chromocenter. Pattern mimics that seen in anti-HP1 stained wild-type salivary glands (in *k*). Somatic transformation, fat body, 30-min heat shock followed by 24-h recovery. Result is the same using transgenic line. (*i*) β -galactosidase fused to HP1 amino acids 41–206 decorates the chromocenter. Somatic transformation, fat body, 30-min heat shock followed by 30-min recovery. (*j*) β -galactosidase fused to HP1 amino acids 95–206 decorates the chromocenter. Transgenic line, fat body, 30-min heat shocks on three consecutive days, followed by 72-h recovery. (*k*) Immunolocalization of HP1 protein in intact wild-type salivary glands. Detection is with HRP- labeled secondary antibody. Bar in each panel, 20 μ m.

cause it has been used successfully in a variety of organisms as a marker in localization studies (Burglin and DeRobertis, 1987; Nelson and Silver, 1989; Picard and Yamamoto, 1987), and because of the availability of a simple enzymatic assay. While it has been reported that β -galactosidase is not completely excluded from the nucleus (Kalderon et al., 1984; Moreland et al., 1987), we have not detected significant nuclear localization of β -galactosidase enzyme activity in control tissues expressing nonfused β -galactosidase or in any of our fusions that do not contain the nuclear localization sequence. This may be due in part to the requirement for β -galactosidase enzymatic activity in our experiments. β -galactosidase activity requires the formation of a tetramer of 116 kD monomers; therefore β -galactosidase monomers or fragments would not be detected in the nucleus by our activity stain.

The nuclear localization domain of HP1, identified in both embryos and third instar larvae polytene tissue, is present in

the COOH-terminal peptide of amino acids 152–206. This interval includes the most highly conserved region among the identified HP1 proteins. This region does not contain any of the short regions of basic amino acids that make up the typical nuclear localization sequence (reviewed in Dingwall and Laskey, 1991; and Garcia-Bustos et al., 1991). The only obvious cluster of basic amino acids present in HP1 is located in the chromo domain (Fig. 1), but the chromo domain is dispensable for the nuclear localization of the β -gal fusions presented here, and appears to have no nuclear targeting activity in the absence of the COOH-terminal domain. While we can not rule out the possibility that the chromo domain sequences may be in an unfavorable conformation in these fusions and may act as a second nuclear localization signal in wild-type HP1, this seems unlikely. None of the several fusion constructs that contain the chromo domain, presumably in differing conformational contexts, could direct detectable nuclear localization of β -galactosidase, while all of

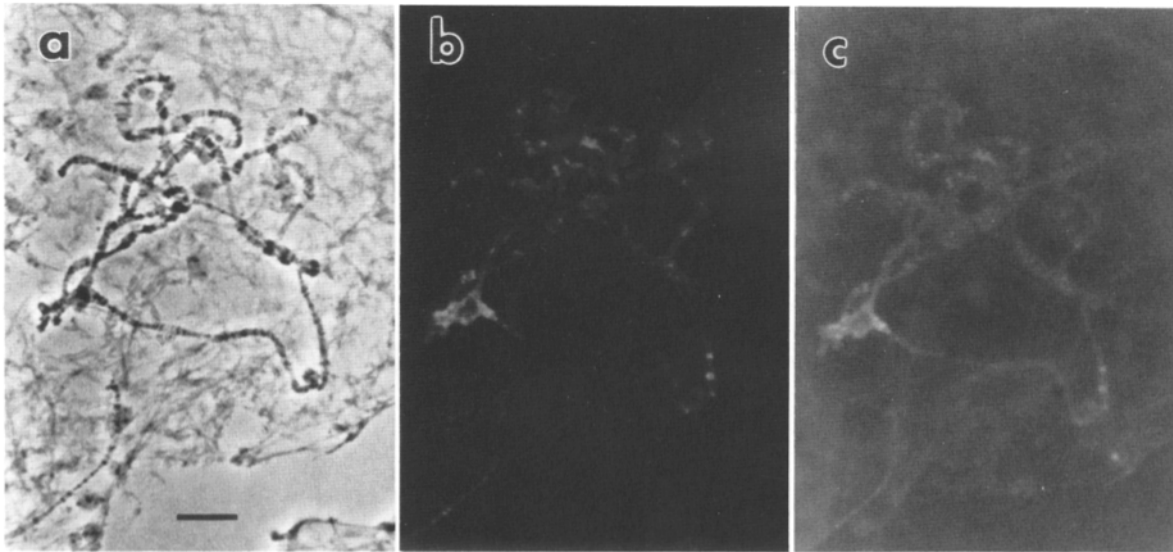


Figure 6. Double immunofluorescence staining of polytene chromosomes from a transgenic line expressing pV β /95–206. Third instar larvae were heat shocked 30 min followed by a 30-min recovery. (a) Phase contrast. Bar, 20 μ m. (b) Fluorescence microscopy, using an anti- β -galactosidase antibody to detect the fusion protein, shows staining of the chromocenter and several additional bands on the arms. (c) Fluorescence microscopy of the same chromosome using the anti-HP1 antibody to detect endogenous HP1 gives a pattern of staining identical to the distribution of β -galactosidase fusion protein seen in b.

those that contain the COOH-terminal domain showed obvious nuclear staining. In studies on the effects of context on nuclear localization sequences, only completely buried sequences were non-functional (Garcia-Bustos et al., 1991).

In the absence of a classical nuclear localization signal, it is possible that HP1 simply enters the nucleus by passive diffusion. However, while passive diffusion of small molecules (<60 kD) into the nucleus can be demonstrated experimentally, it is unclear whether this mechanism generally operates for small nuclear proteins in vivo (Silver, 1991). Histone 2B (15 kD) and several other small nuclear proteins contain functional nuclear localization sequences (reviewed in Garcia-Bustos et al., 1991), and histone H1 enters the nucleus in an energy and temperature dependent manner (Breeuwer and Goldfarb, 1990), suggesting that small proteins utilize an active nuclear translocation mechanism. HP1 may be transported into the nucleus piggyback style in association with another heterochromatin protein that contains a canonical nuclear localization signal. Moreland et al. (1987) have reported that yeast histone 2B contains a protein binding domain for histone 2A that is sufficient for the nuclear localization of a histone 2B/ β -galactosidase fusion protein and Booher et al. (1989) have shown that the formation of a CDC2-CDC13 complex is necessary for the localization of CDC2 to the nucleus.

In our effort to map a heterochromatin targeting activity, we were restricted to using only those β -galactosidase/HP1 fusions that contained the nuclear localization domain. Nevertheless, we could identify a domain sufficient for heterochromatic localization of the β -galactosidase fusions. The staining pattern seen in all the polytene cells expressing a fusion that contains the HP1 peptide of amino acids 95–206 is changed from a generalized nuclear staining with β -galactosidase/152–206 to a pattern of a distinct spot of stain adjacent to the nucleolus with one or more additional spots sometimes seen away from the nucleolus. This pattern is

consistent with the location of the chromocenter and is identical to the pattern seen in wild-type salivary glands stained using an antibody to HP1 (Fig. 5 k). The localization of fusion protein to the chromocenter was confirmed by immunofluorescence localization using polytene chromosome squashes, and in the case of the pV β /95–206 fusion, was identical to the localization of endogenous HP1 (Fig. 6). These data identify a functional domain that requires at least a portion of the amino acid 95–151 peptide in addition to the nuclear localization domain to specify specific heterochromatin localization. A heterochromatin targeting domain has also been identified in the NH₂-terminus of the human centromere-associated protein CENP-B (Pluta et al., 1992). This domain contains a helix-loop-helix motif and has been shown to bind to specific heterochromatin sequences. A comparison of the amino acid sequences of the heterochromatin targeting domains in the two proteins reveals no significant sequence homologies. The heterochromatin localization of HP1 is most likely mediated by protein-protein interactions rather than HP1 binding directly to DNA. No DNA binding motifs have been identified in the HP1 sequence, and it has been reported that HP1 does not bind directly to DNA (Singh et al., 1991). It seems likely that another heterochromatin structural protein binds to the HP1 heterochromatin localization domain and directly or indirectly mediates the binding of the protein complex to DNA.

We have often seen, in both the HP1 wild-type controls and in polytene cells expressing the β -galactosidase/HP1 fusions containing the heterochromatin localization domain, more than a single spot of stain at the chromocenter. James et al. (1989) reported that the staining pattern of HP1 on polytene chromosomes consistently includes regions in addition to the chromocenter, most notably the fourth chromosome and cytological region 31 on chromosome 2. This is one possible explanation for the additional spots we have seen in the intact

salivary glands. The appearance of these additional spots is also similar to the staining pattern seen by Saunders et al. (1991) in human cell lines using an antibody to a human HP1 homologue (Saunders et al., 1993). They report the existence of a subnuclear structural domain associated with chromatin that they call the Polymorphic Interphase Karyosomal Association). The function of these domains, and any possible role that HP1 might play in them is unknown at present.

The *Drosophila* protein HP1 was originally described as a tightly bound nonhistone chromosomal protein which is primarily associated with the pericentric heterochromatin (James and Elgin, 1986; James et al., 1989). The association of HP1 with heterochromatin appears to have functional significance. Normally euchromatic genes which become relocated to a heterochromatic site due to a chromosome rearrangement are frequently subject to mosaic repression at their new position. This phenomenon is termed heterochromatic position-effect variegation, and the silencing associated with position-effect variegation is believed to be a consequence of spreading of nearby heterochromatin into the euchromatin (Spofford, 1976; Eissenberg, 1989; Grigliatti, 1991). Because the degree of silencing in variegating rearrangements is sensitive to the dosage of HP1 (Eissenberg et al., 1992), it is believed that HP1 is a structural subunit of a protein complex capable of spreading in an assembly-driven process along the chromosome (Locke et al., 1988) and interfering with normal euchromatic gene function. The nature of this complex and the mechanism by which HP1 participates in its formation and propagation is not known.

The high degree of evolutionary conservation associated with NH₂-terminal and COOH-terminal motifs in the amino acid sequence of HP1 suggests a functional constraint acting in these regions. What these functions might be is not known. Our results show that a nuclear targeting signal resides within the COOH-terminal conserved sequence, but because the conserved sequences between amino acids 142 and 150 are not required for nuclear targeting, this activity alone would not account for the extent of conservation. The peptide domain sufficient for heterochromatin targeting (amino acids 95–206) encompasses the entire conserved region, and therefore could account for the functional constraint on evolutionary divergence in this region; further mutagenesis studies are underway to delineate the functions of conserved residues. It is interesting to note that the HP1 mutant allele *Su(var)2-5⁰⁴* encodes a truncated HP1 protein missing amino acids 169–206, and therefore, most of the nuclear targeting domain (Eissenberg et al., 1992).

Our experiments did not allow us to assign any functions specifically to the NH₂-terminal conserved region of HP1. In our assays, this region is dispensable for both nuclear targeting and heterochromatin binding. Imbedded in this region is a motif that shows high sequence homology to a 37 amino acid region in the *Polycomb* gene product (the chromo domain). While *Polycomb* protein, like HP1, is a chromosomal protein, it is localized exclusively to euchromatic sites on polytene chromosomes (Zink and Paro, 1989). Thus, it is perhaps not surprising that the chromo domain has no heterochromatin targeting activity in our assay. Because both *Polycomb* protein and HP1 play a role in mitotically heritable gene silencing, *Polycomb* protein at homeotic loci and HP1 at heterochromatic chromosome breakpoints, Paro and Hog-

ness have drawn the reasonable inference that these proteins “. . . use analogous mechanisms at the levels of higher order chromatin structure for the stable transmission of a determined state” (Paro and Hogness, 1991). Our results are consistent with the possibility that the NH₂-terminal conserved region, including the chromo domain, may be involved in higher order chromatin structure formation, perhaps through protein-protein contacts. Further studies are underway to identify functional properties that map to the chromo domain. These studies, in conjunction with studies to identify the other proteins that interact with the functional domains of HP1, should allow us to better understand the process of heterochromatin formation and its role in gene inactivation.

We thank Drs. S. C. R. Elgin and R. F. Clark for anti-HP1 serum, and Drs. J. Wallis, D. Coulter, J. Ryerse, and H. P. Zassenhaus for advice and encouragement. We hereby acknowledge that H. P. Zassenhaus was right about the use of a β -gal activity stain.

This work was supported by Biomedical Research Support Grant funds from St. Louis University, American Cancer Society Institutional Grant IN-124, and National Institutes of Health grant GM 40732.

Received for publication 27 July 1992 and in revised form 30 September 1992.

References

- Booher, R. N., C. A. Alfa, J. S. Hyams, and D. H. Beach. 1989. The fission yeast *cdc2/cdc13/suc1* protein kinase: regulation of catalytic activity and nuclear localization. *Cell*. 58:485–497.
- Breeuwer, M., and D. S. Goldfarb. 1990. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell*. 60:999–1008.
- Burglin, T. R., and E. M. De Robertis. 1987. The nuclear migration signal of *Xenopus laevis* nucleoplasmin. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2617–2625.
- Clark, R. F., and S. C. R. Elgin. 1993. Comparison of the heterochromatin protein I sequences of *Drosophila virilis* and *Drosophila melanogaster* show two highly conserved domains. *Nucleic Acids Res.* In press.
- Cohen, L. D., and B. V. Gotchel. 1971. Histones of polytene and non-polytene nuclei of *Drosophila melanogaster*. *J. Biol. Chem.* 246:1841–1848.
- Dingwall, C., and R. A. Laskey. 1991. Nuclear targeting sequences—a consensus? *Trends Biochem. Sci.* 16:478–481.
- Eissenberg, J. C. 1989. Position effect variegation in *Drosophila*: towards a genetics of chromatin assembly. *BioEssays*. 11:14–17.
- Eissenberg, J. C., T. C. James, D. M. Foster-Hartnett, T. Hartnett, V. Ngan, and S. C. R. Elgin. 1990. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* 87:9923–9927.
- Eissenberg, J. C., G. D. Morris, G. Reuter, and T. Hartnett. 1992. The heterochromatin-associated protein HP1 is an essential protein in *Drosophila* with dosage-dependent effects on position-effect variegation. *Genetics*. 131:345–352.
- Epstein, H., T. C. James, and P. B. Singh. 1992. Cloning and expression of *Drosophila* HP1 homologs from a mealybug, *Planococcus citri*. *J. Cell Sci.* 101:463–474.
- Fridell, Y.-W. C., and L. L. Searles. 1991. *Vermilion* as a small selectable marker gene for *Drosophila* transformation. *Nucl. Acids Res.* 19:5082.
- Garcia-Bustos, J., J. Heitman, and M. N. Hall. 1991. Nuclear protein localization. *Biochim. Biophys. Acta.* 1071:83–101.
- Grigliatti, T. A. 1991. Position-effect variegation: an assay for nonhistone chromosomal proteins and chromatin assembly and modifying factors. *Methods Cell Biol.* 35:587–627.
- Harlow, E., and D. Lane. 1988. *Antibodies, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 726 pp.
- James, T. C., and S. C. R. Elgin. 1986. Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila* and its gene. *Mol. Cell Biol.* 6:3862–3872.
- James, T. C., J. C. Eissenberg, C. Craig, V. Dietrich, A. Hobson, and S. C. R. Elgin. 1989. Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of *Drosophila*. *Eur. J. Cell Biol.* 50:170–180.
- Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear localization. *Cell*. 39:499–509.
- Karess, R. E., and G. M. Rubin. 1984. Analysis of P transposable element functions in *Drosophila*. *Cell*. 38:135–146.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of

- the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Locke, J., M. A. Kotarski, and K. D. Tartof. 1988. Dosage dependent modifiers of position effect variegation in *Drosophila* and a mass action model that explains their effect. *Genetics*. 120:181-198.
- Martin, P., A. Martin, A. Osmani, and W. Sofer. 1986. A transient expression assay for tissue-specific gene expression of alcohol dehydrogenase in *Drosophila*. *Dev. Biol.* 108:574-580.
- Moreland, R. B., G. L. Langevin, R. H. Singer, R. L. Garcea, and L. M. Hereford. 1987. Amino acid sequences that determine the nuclear localization of yeast histone 2B. *Mol. Cell. Biol.* 7:4048-4057.
- Nelson, M., and P. Silver. 1989. Context affects nuclear protein localization in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 9:384-389.
- Paro, R., and D. S. Hogness. 1991. The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 88:263-267.
- Picard, D., and K. R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3333-3340.
- Pluta, A. F., N. Saitoh, I. Goldberg, and W. C. Earnshaw. 1992. Identification of a subdomain of CENP-B that is necessary and sufficient for localization to the human centromere. *J. Cell. Biol.* 116:1081-1093.
- Saunders, W. S., C. A. Cooke, and W. C. Earnshaw. 1991. Compartmentalization within the nucleus: discovery of a novel subnuclear region. *J. Cell. Biol.* 115:919-931.
- Saunders, W. S., C. Chue, M. Goebel, C. Craig, R. F. Clark, J. A. Powers, J. C. Eissenberg, S. C. R. Elgin, N. F. Rothfield, and W. C. Earnshaw. 1993. Molecular cloning of a human homolog of *Drosophila* heterochromatin protein HP1 using anticentromere autoantibodies with *anti-chromo* specificity. *J. Cell. Sci.* In press.
- Shore, E. M., and G. M. Guild. 1987. Closely linked DNA elements control the expression of the *Sgs-5* glue protein gene in *Drosophila*. *Genes Dev.* 1:829-839.
- Silver, P. A. 1991. How proteins enter the nucleus. *Cell*. 64:489-497.
- Simon, J. A., C. A. Sutton, R. B. Lobell, R. L. Glaser, and J. T. Lis. 1985. Determinants of heat shock-induced chromosome puffing. *Cell*. 40:805-817.
- Singh, P. B., J. R. Miller, J. Pearce, R. Kothary, R. D. Burton, R. Paro, T. C. James, and S. J. Gaunt. 1991. A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res.* 19:789-794.
- Spofford, J. B. 1976. Position-effect variegation in *Drosophila*. In *The Genetics and Biology of Drosophila*, Vol. 1c. M. Ashburner, and E. Novitski, editors. Academic Press, New York. 955-1018.
- Spradling, A. C. 1986. P element-mediated transformation. In *Drosophila*, a practical approach. D. B. Roberts, editor. IRL Press, Oxford. 175-197.
- Steller, H., and V. Pirrotta. 1985. A transposable P vector that confers selectable G418 resistance to *Drosophila* larvae. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:167-171.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350-4354.
- Zink, B., and R. Paro. 1989. In vivo binding pattern of a trans-regulator of homeotic genes in *Drosophila melanogaster*. *Nature (Lond.)*. 337:468-471.