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An actin-related protein in *Drosophila* colocalizes with heterochromatin protein 1 in pericentric heterochromatin

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SUMMARY

The actin-related proteins have been identified by virtue of their sequence similarity to actin. While their structures are thought to be closely homologous to actin, they exhibit a far greater range of functional diversity. We have localized the *Drosophila* actin-related protein, Arp4, to the nucleus. It is most abundant during embryogenesis but is expressed at all developmental stages. Within the nucleus Arp4 is primarily localized to the centric heterochromatin. Polytene chromosome spreads indicate it is also present at much lower levels in numerous euchromatic bands. The only other protein in *Drosophila* reported to be primarily localized to centric heterochromatin in polytene nuclei is heterochromatin protein 1 (HP1), which genetic evidence has linked to heterochromatin-mediated gene silencing and alterations in chromatin structure. The relationship between Arp4 and heterochromatin protein 1 (HP1) was investigated by labeling embryos and larval tissues with antibodies to Arp4 and HP1. Arp4 and HP1 exhibit almost superimposable heterochromatin localization patterns, remain associated with the heterochromatin throughout prepupal development, and exhibit similar changes in localization during the cell cycle. Polytene chromosome spreads indicate that the set of euchromatic bands labeled by each antibody overlap but are not identical. Arp4 and HP1 in parallel undergo several shifts in their nuclear localization patterns during embryogenesis, shifts that correlate with developmental changes in nuclear functions. The significance of their colocalization was further tested by examining nuclei that express mutant forms of HP1. In these nuclei the localization patterns of HP1 and Arp4 are altered in parallel fashion. The morphological, developmental and genetic data suggest that, like HP1, Arp4 may have a role in heterochromatin functions.

Key words: Chromatin, Actin-related protein, *Drosophila*, Heterochromatin-protein 1, Position effect variegation

INTRODUCTION

The actin-related proteins (arps) are one branch of an ancient and divergent group of protein families, the actin superfamily (Frankel and Mooseker, 1996). Among the other branches of the superfamily are the hsp/hsc70s, divergent forms of hsp/hsc70, sugar kinases and a variety of prokaryotic ATP-binding proteins. Their one common feature is the actin fold (Kabsch and Holmes, 1994), a tertiary structure centered around an ATP/ADP binding pocket. The actin fold undergoes major conformational shifts in response to the hydrolysis state of the adenine nucleotide, and such shifts are thought to be central to the functions of actin superfamily members. The arps are grouped into several classes that are highly conserved in eukaryotes. Each class is distinguished by its degree of similarity to actin. For example, the class 1 arps are approximately 55% identical to actins, while the class 3 arps are approximately 40% identical. *Drosophila* Arp4 is among the most divergent of the arps; it is only 28% identical to actin (Frankel et al., 1994). The arps exhibit functional as well as structural diversity. Arp1 is localized to microtubules and kinetochores, Arp2 and Arp3 are localized to the actin filament cortex underlying the plasma membrane (reviewed by Frankel and Mooseker, 1996; Mullins et al., 1996, 1997), and Arp5 in *Drosophila* is only expressed in testis (Fyrberg et al., 1994). An arp in *Saccharomyces cerevisiae*, act3p, has been localized to the nucleus (Weber et al., 1995; Jiang and Stillman, 1996) where it plays a role in transcriptional regulation and chromatin structure (Jiang and Stillman, 1996). We have been characterizing the functions of Arp4. It is also localized to the nucleus, where it is primarily associated with heterochromatin.

Centric heterochromatin is the highly condensed and largely transcriptionally inactive region flanking the centromere. In *Drosophila* a substantial part of each chromosome consists of this type heterochromatin, making it a major component of overall chromosome structure (Weiler and Wakimoto, 1995). Centric heterochromatin is thought to play a role in sister chromatid adhesion during mitosis and chromosome pairing

during meiosis (Murphy and Karpen, 1995). Heterochromatin also has the property of silencing the expression of genes that become juxtaposed to the heterochromatin by a chromosome rearrangement, with one break point located within the heterochromatin. In *Drosophila* several dozen genetic loci have been identified that modify silencing activity (Reuter and Spierer, 1992). One of these loci encodes HP1, a protein that has been localized to the heterochromatin throughout prepupal development (Elgin, 1996). Mutations in HP1 have also been correlated with abnormalities in chromosome segregation during *Drosophila* embryogenesis (Kellum and Alberts, 1995), implicating the protein in most of the known functions of heterochromatin. Our data indicate that Arp4 colocalizes with HP1 in the centric heterochromatin throughout prepupal development and that these two proteins undergo a series of changes in nuclear distribution that coincide with major shifts in nuclear functions. When nuclei are examined from flies that express mutant forms of HP1, there is an altered HP1 distribution and parallel alterations in the distribution of Arp4. These observations suggest a functional connection between the two proteins.

MATERIALS AND METHODS

Fly strains

All wild-type tissues were obtained from Canton S flies. The chimeric HP1 transgene is located on the second chromosome and is balanced with *Cyo*; this strain was provided by Dr Joel Eissenberg (St Louis University, St Louis, MO). The X chromosome deficiencies, kindly provided by J. Eeken (University of Leiden, Leiden, Netherlands), span regions 13C-14A, 13B-14B and 13C-14B and were carried as heterozygotes balanced with either *In(1)sc^{8}In(1)Δ49* marked with *Sxl* (provided by A. Schalet, Yale University) or *FM7c* marked with β-galactosidase under the *fushi tarazu* promoter (provided by S. Panzer, Yale University). The*Su(var)2-5^{02}* line was balanced with *Cyo*, in a *y^{-} w^{-}* background.

Fusion proteins and antibody generation

The Arp4 antibodies used in this study were generated against amino acids 359-398 in the coding sequence (Frankel et al., 1994). This region was subcloned into the pGEX vector (Amrad Corporation, Melbourne, Australia) and the glutathione S-transferase (GST) fusion proteins were injected into rats. Bacteria harboring the pGEX plasmids were induced with IPTG at an OD_{550} of 0.6-0.7, harvested after an additional 1.5 hours of growth, washed with a buffer that contained 15 mM Tris-HCl, pH 8.0, 1 mM DTT and 0.02 mM $CaCl_2$, resuspended in the same buffer (supplemented with the protease inhibitors aprotinin, leupeptin, chymostatin and pepstatin, all from Sigma Chemical Co., St Louis, MO), and lysed by two passages through a French pressure cell at 1,000 psi. The lysate was centrifuged at greater than 30,000 ***g***-hours, the supernatant collected, and Tris, NaCl and Triton X-100 were added to concentrations of 50 mM, 150 mM and 0.5%, respectively. This fraction was adsorbed to a glutathione affinity column (Sigma Chemical Co., St Louis, MO) and fusion protein was eluted with free glutathione. The eluted fractions were approximately 95% pure, as assessed by SDS-PAGE.

To rapidly purify sera from animals inoculated with GST fusion protein, amino acids 359-398 in the Arp4 sequence were subcloned into the pMal vector (New England Biolabs, Beverly, MA). The maltose-binding-protein (MBP) fusion protein was purified as described above for the GST fusion protein, except that the final step utilized an amylose affinity column (New England Biolabs, following the manufacturer's instructions). The purified MBP fusion protein was coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ, following the manufacturer's instructions).

The HP1 antibody is mouse monoclonal C1A9, which has been previously described (James and Elgin, 1986).

Purification of antibodies

Rat antisera reactive to the GST fusion protein were purified by two methods. The first method involved adsorption and elution from preparative western blots. Small batches of antisera were first adsorbed 4-6 times against nitrocellulose that contained GST with no added fusion sequence. When all anti-GST reactivity had been removed, the antiserum was adsorbed to nitrocellulose that contained the immunogen. The bound antibody was then eluted with acidic glycine, in a manner analogous to the elution of antibody from an affinity resin (Olmstead, 1981). Effective dilutions were determined empirically since the antibody concentration could not be measured directly. The second method involved adsorption and elution from an MBP fusion protein affinity column (Frankel et al., 1990). Antibodies purified by either method had identical specificities and yielded identical results. All of the data shown in this paper were obtained with antibody purified by the second method.

Several antibody controls were performed: pre-immune sera, secondary antibody only, a primary antibody (R26.4 rat monoclonal) directed against an irrelevant antigen (the rat form of the tight junction protein ZO-1; Stevenson et al., 1986), and anti-GST antibodies purified from the same sera used to purify the Arp4 antibody.

Transient overexpression of Arp4 in S2 cells

The full-length coding sequence was subcloned into the pRmHa-1 vector, which expresses the protein under metallothionein control. The procedure for transfection and induction is as described by Diederich et al. (1994), except that the induction was for 5 hours. Longer inductions resulted in widespread cell death.

Developmental western blot

Timed embryo collections were dechorionated and frozen at −70°C. S2 cells were collected, washed in PBS and the cell pellets stored at −70°C, as were the other tissue samples. In preparation for SDS-PAGE analysis, all tissue samples were thawed on ice and added to SDS buffer (the final concentrations were 2.3% SDS, 12% glycerol, 73 mM Tris-HCl, pH 6.7, and 0.5 mM Pefabloc; Boehringer Mannheim, Indianapolis, IN). The samples were rapidly homogenized and boiled. Aliquots were then saved for protein assays while the rest of the sample was prepared for SDS-PAGE analysis by adding: EDTA and EGTA to 10 mM, β-mercaptoethanol to 2% and bromophenol blue. The final gel samples contained 2% SDS and were stored at −70°C. Protein was determined by the BCA assay (Pierce Chemical Co, Rockford, IL). For each sample, 20 μg of total protein were loaded per lane of a 5-16% acrylamide minigel.

S2 cell fractionation

S2 cells were grown as described by Fehon et al. (1990). A 1 ml sample of packed S2 cells was collected, washed in PBS and stored at −70°C. The pellets were thawed on ice in lysis buffer: 75 mM KCl, 20 mM imidazole, pH 7.2, 1 mM EGTA, 2.5 mM $MgCl_2$, 0.02% azide and protease inhibitors (aprotinin, leupeptin, chymostatin, pepstatin and Pefabloc). The cell suspension was homogenized on ice at a final volume of 5 ml, using a tight-fitting Dounce. Lysis was monitored by phase contrast microscopy. The lysate was sedimented at 1,100 ***g*** for 10 minutes. The supernatant was then recentrifuged; the clearance of all nuclei was confirmed by phase contrast microscopy. The first pellet was washed twice with lysis buffer and resuspended to 1.5 ml in the same buffer except that imidazole was 10 mM and $MgCl_2$ was 4 mM (crude nuclei). The resuspended pellet was added to a 60% (w/w) sucrose solution in the same buffer, for a final concentration of 45%. An equal volume of a 40% sucrose solution was layered over the 45%

solution, and centrifuged for 200,000 ***g***-hours. The nuclei were in the pellet, which was washed two times with lysis buffer. The final nuclear pellet was resuspended in lysis buffer and samples were checked by phase contrast and DAPI (4,6-diamidine-2-phenylindole) fluorescence, to determine whether the nuclei were intact and whether there were any contaminating organelles. The rest of the sample was either frozen at −70°C for gel analysis or fixed with 2% freshly dissolved EM-grade formaldehyde in the same buffer (30 minutes at 4°C). Fixed nuclei were washed twice with lysis buffer.

In preparation for SDS-PAGE, S2 cell fractions were homogenized in SDS buffer as described above for the developmentally staged tissue samples. Protein concentrations were determined by the BCA assay. For each fraction, 15 μg of total protein were loaded per lane of a minigel.

S2 cells that were not stored at −70°C were also fractionated, with similar results. Embryos were fractionated as described by Ashburner (1989), to obtain a crude nuclear pellet and a post-nuclear supernatant.

Tissue fixation for immunolocalization

Canton S and deficiency embryos (from all balanced strains) were examined by immunofluorescence. Embryos were collected and fixed in a manner that avoided anoxic conditions, which can induce chromosome condensation (Foe and Alberts, 1985; Foe et al., 1993). Collection, dechorionation (in 50% bleach) and washing (saline with 0.05% Triton as surfactant) were performed on an open steel mesh. Embryos were transferred to a glass tube, allowed to settle and all liquid overlaying the embryos was removed. The embryos were then spread along the sides of the tube, with a minimum amount of moisture to prevent drying, and allowed to recover from any potential anoxia for 16-18 minutes (Foe and Alberts, 1985). The fixative was 50% heptane and 50% PBS containing 4% freshly dissolved EM-grade formaldehyde and one of three buffers: no additions, 25 mM EGTA, or 25 mM EGTA and 4 mM $MgCl_2$. Fixation was for 30 minutes at room temperature with shaking. The embryos were devitellinized with methanol and stored at −20°C in methanol.

In order to optimize for the fixation of nuclei, the three buffers mentioned above were compared. It has been shown that cytoskeletal structures are well preserved in embryos fixed in PBS with EGTA (Therkauf, 1992) or PBS with EGTA and Mg^{2+} (Karr and Alberts, 1986). Embryos fixed in PBS-EGTA-Mg^{2+} had the strongest Arp4 antibody staining of nuclei. Qualitatively similar but less intense staining was obtained with PBS-EGTA. Nuclei were poorly stained after fixation in PBS with no additives.

Imaginal discs and other third instar larval tissues were hand dissected on ice in complete S2 cell tissue culture medium (see above) and stored on ice in tissue culture medium until fixation. Prior to fixation the discs were briefly washed with ice-cold PBS containing 25 mM EGTA and 4 mM Mg^{2+}. Ice cold fixative (2% freshly dissolved formaldehyde in PBS containing 25 mM EGTA and 4 mM Mg^{2+}) was added, and fixation was for 30 minutes in a room temperature water bath. For the HP1 chimera, larvae were maintained at 22°C and expression of the transgene was induced for 30 minutes at 37°C followed by one hour of recovery at room temperature.

Immunoblots and immunocytochemistry

SDS-PAGE and immunoblots were performed as described by Carboni et al. (1988). Nitrocellulose blots were blocked with 5% nonfat dried milk in TBS. The same blocking reagent was used for the dilution of primary and secondary antibodies. Other procedures are as described by Frankel et al. (1990) except that the secondary antibody was conjugated to alkaline phosphatase and the color reaction employed nitro tetrazolium blue (Sigma Chemical Company, St Louis, MO). Immobilon blots were treated as recommended by Boehringer Mannheim (Indianapolis, IN) for chemiluminescent detection with the exception that 1% blocking reagent (provided by the manufacturer) was used during the primary and secondary antibody incubations. Arp4 antibody purified by affinity chromatography was used at 2 μg/ml. Multiple exposures of chemiluminscent blots were quantitated with a Visage 2000 imaging system (Bio Image, Ann Arbor, MI).

Whole mount immunocytochemistry of embryos was performed as described by Fehon et al. (1991), except that the solution used for blocking and antibody dilution contained PBS, 0.1% Triton X-100, 3% normal goat serum, 10 mM EGTA, 0.02% azide and 4 mM Mg^{2+}. All incubations were at 4°C. Blocking was for 4-6 hours, while primary and secondary antibody incubations were performed overnight. Wash buffer consisted of PBS that contained 10 mM EGTA and 0.1% Triton. The final samples were mounted in Citifluor (Citifluor Corporation, London, England). Whole mount immunocytochemistry of third instar larval tissues was performed as described for embryos, except that tissues were blocked for longer periods of time. Arp4 antibody purified by affinity chromatography was used at a dilution of 2.5 μg/ml; the HP1 monoclonal was diluted 1/250 from an ascites fluid stock. All secondary antibodies were the double-labeling grade from Jackson ImmunoResearch Laboratories conjugated to either fluorescein, CY3 or CY5 (West Grove, PA); stock solutions prepared according to the manufacturer's directions were diluted 1/250.

When embryos were double-stained for DNA and antibody, 125 μg/ml of RNAse A was included in the secondary antibody incubation and Mg^{2+} was omitted from the buffer. Embryos were then washed twice in PBS-EGTA-Triton, incubated with 10 μg/ml of propidium iodide in PBS-EGTA-Triton for 20 minutes (to visualize DNA), then washed four more times before adding mounting medium.

S2 cell nuclei were blocked and incubated with primary antibody as above except that only two washes in PBS-Triton were performed before and after the secondary antibody incubation. Due to the pronounced tendency of nuclei to aggregate, Mg^{2+} was omitted from all buffers after fixation, and the nuclei were resuspended with a 27 gauge needle prior to the final wash.

Intact S2 cells were fixed and stained as described by Fehon et al. (1990) except that primary antibody incubations were overnight at 4°C and the solution for blocking and dilution of antibodies was as described above for embryo staining.

Stained tissues were examined on a Bio-Rad 600 confocal microscope as described by Xu et al. (1992), with the following modifications. To ensure spectral discrimination of the collected signal, each fluorochrome was excited consecutively, not simultaneously. Samples were illuminated with only one laser emission band, such that no signal was detected if the sample was excited with the fluorescein laser emission band and viewed with the CY3 filter, and vice versa.

Staining of polytene chromosome squashes

Third instar salivary glands were collected, fixed and squashed according to a protocol supplied by D. Bettler (Emory University, Atlanta, GA), modified by G. Doughty. Glands were dissected in PBS and fixed for 5 seconds in 3.7% formaldehyde, PBS, 0.01% Triton X-100 followed by 2 minutes in 3.7% formaldehyde, 50% acetic acid, 16% lactic acid. The glands were squashed and spread in the latter solution and frozen on dry ice as described by Urness and Thummel (1990). Slides were stored in 67% glycerol in PBS at −20°C. The solution for blocking and for the dilution of antibodies was PBS with 0.2% Triton, 1% normal goat serum and 0.04% azide. The Arp4 antibody was used at a concentration of 5 μg/ml and the HP1 monoclonal ascites stock was diluted 1/1,000; the primary antibody control was affinity-purified rat anti-GST used at 5 μg/ml. The incubations were as follows: slides were washed twice with PBS, blocked for 30 minutes at room temperature, briefly drained and primary antibody solution was added. Coverslips were placed over the slides followed by incubation in a humid chamber at 4°C for 14-18 hours. After two PBS washes they were again blocked for 30 minutes at room temperature and incubated in secondary antibody for 5-6 hours at 4°C. The slides were washed in PBS, stained with Hoechst 33258 (to visualize DNA), washed and mounted. DNA stain was examined using a Leitz orthoplan microscope. Antibody staining was examined using a confocal microscope, as described above.

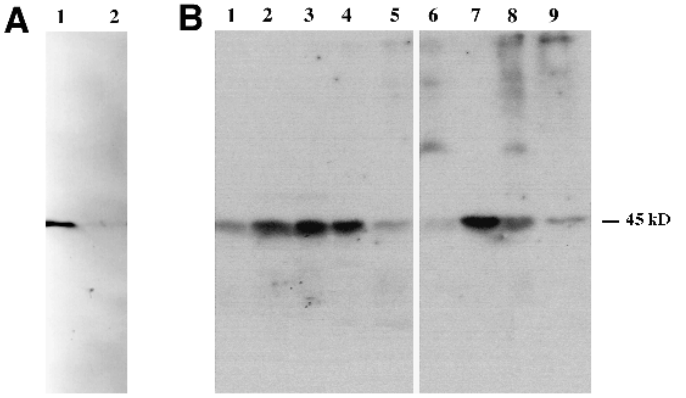


Fig. 1. Overexpression of Arp4 in S2 cells and developmental changes in endogenous Arp4 levels. Equal amounts of protein were loaded in each lane of the gels used for transfers; immunoblots were probed with affinity-purified antibody raised against *Drosophila* Arp4. The blot in A was visualized with alkaline phosphatase-conjugated secondary antibody, while the blot in B was visualized with HRP-conjugated secondary antibody and chemiluminescence. (A) Transient overexpression of the Arp4 coding sequence in S2 cells, a *Drosophila* tissue culture line derived from embryonic cells. Lane 1, transfected cells after induction; Lane 2, non-transfected cells. (B) Levels of Arp4 in developmentally staged tissue samples. Lane 1, 0-3 hour embryos; lane 2, 3-6 hour embryos; lane 3, 8-12 hour embryos; lane 4, 14-18 hour embryos; lane 5, first and second instar larvae; lane 6, male and female adults; lane 7, S2 cells; lane 8, ovaries; lane 9, imaginal disc-enriched tissue (approximately 50% discs and 50% salivary glands).

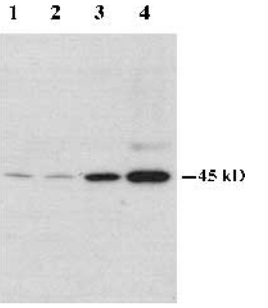


Fig. 2. Arp4 cofractionates with the nucleus. Nuclei were purified from S2 cells; equal amounts of protein were loaded in each lane of the gel used for transfer. The samples are as follows: lane 1, whole cell homogenate; lane 2, low speed supernatant, cleared of all nuclei; lane 3, low speed pellet, enriched for nuclei; lane 4, sucrose gradient-purified nuclei.

RESULTS

Generation of antibodies against Arp4

As a first step in characterizing the function of Arp4, antibodies were raised against a region of the Arp4 coding sequence which by alignment analysis had little similarity to corresponding regions in actins or other arps. The purified antibody only detected fusion proteins that contained the immunogen and exhibited no cross-reactivity against endogenous bacterial proteins (data not shown). The antibody was monospecific in immunoblots of *Drosophila* embryo proteins for an antigen that had the predicted molecular mass of the Arp4 gene product, 45 kDa (see below). In order to demonstrate that this antigen was the gene product, the full-length coding sequence was transiently overexpressed in S2 cells. The induced protein was immunoreactive and comigrated with the 45 kDa antigen present in uninduced S2 cells (Fig. 1A). Since the arps are not only related to actin but to the heat shock 70 proteins (Frankel and Mooseker, 1996), induction of Arp4 expression by heat shock was examined; expression in embryos was not affected by heat shock (data not shown).

Levels of Arp4 expression during development

We had previously shown that the message for Arp4 is maternally supplied and is most abundant in embryos (Frankel et al., 1994). In order to follow the developmental expression of the Arp4 protein, tissues were collected at different stages and analyzed by immunobloting (see Fig. 1B). Arp4 protein was most abundant in embryos and the S2 cell line (which is of embryonic lineage). While some protein was present in 0-3 hour embryos, the amount increased several-fold during embryogenesis. The 45 kDa protein was the only antigen detected in those tissues displaying the highest levels of the protein. In other tissues the antibody detected both the 45 kDa protein and variable amounts of higher molecular mass antigens (see Discussion).

Additional evidence for a maternally-derived pool of Arp4 protein was obtained from three fly strains carrying X chromosome deficiencies that span the region containing the Arp4 gene. Embryos hemizygous for each deficiency (males) die late in embryogenesis. Arp4 protein could be observed by immunofluorescence in mutant embryos until the point where they began to undergo degeneration (data not shown).

Arp4 is a nuclear protein

To determine the subcellular localization of Arp4, two types of experiments were performed, subcellular fractionation and indirect immunofluorescence. Both analyses indicated that Arp4 was present in the nucleus. S2 cells were initially separated into crude nuclear and non-nuclear fractions. The crude nuclei were then further purified on a discontinuous sucrose gradient, yielding a final fraction that was homogeneous by phase contrast microscopy and DAPI fluorescence (data not shown). Immunoblot analysis indicated there was a substantial enrichment for Arp4 in the nuclear fractions (Fig. 2). Similar results were obtained after the fractionation of *Drosophila* embryos (data not shown). The abundance of Arp4 was estimated by calibrating blots with known amounts of pure fusion protein. Arp4 was approximately 0.001% of the total protein present in purified S2 cell nuclei.

Purified S2 cell nuclei were fixed and examined by immunofluorescence (Fig. 3). All nuclei contained Arp4, in a pattern consisting of several spots of fluorescence and a more diffuse staining throughout the rest of the nucleoplasm. Staining was not detected along the nuclear membrane. A similar staining pattern was detected in the nuclei of intact fixed S2 cells (data not shown), post-gastrulation embryos and the diploid cells of the imaginal discs (see below). A more detailed description of the nuclear localization patterns of Arp4 is presented in the following sections. The same staining patterns were obtained using Arp4 antibodies purified by two methods (see Materials and Methods). Control antibodies gave a faint background of cytoplasmic stain and a complete absence of nuclear stain (for S2 nuclei see Fig. 3D, for embryos and other tissues data not shown). The controls included pre-immune serum, a primary antibody raised against an unrelated vertebrate protein (see Materials and Methods), anti-glutathione transferase purified from immune serum and secondary antibody alone.

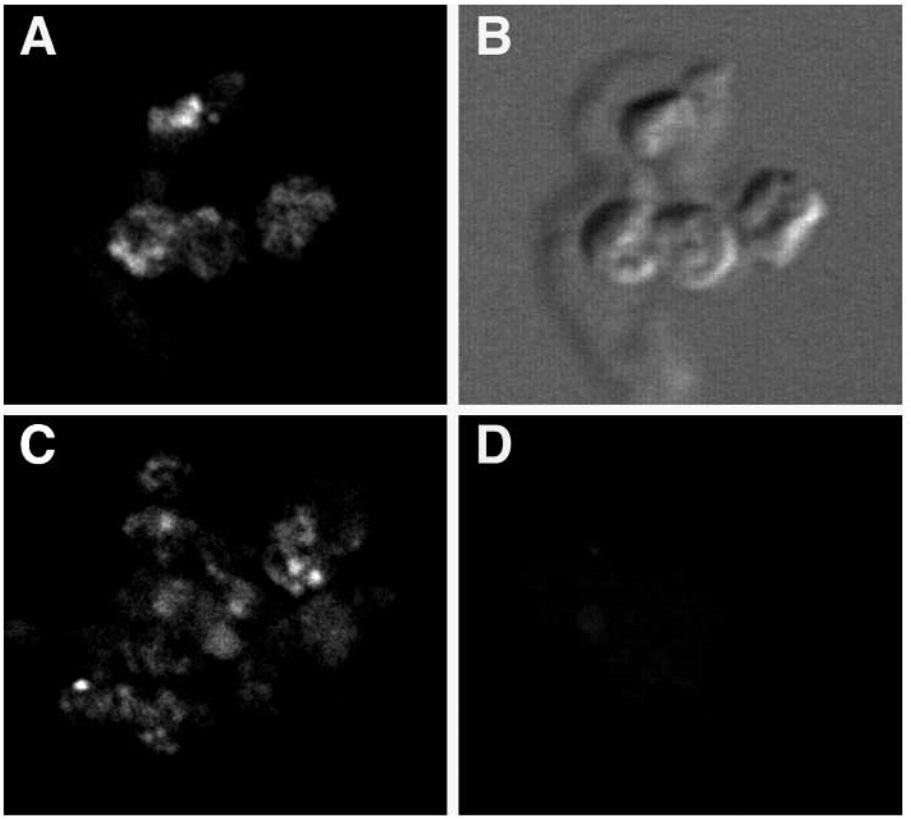


Fig. 3. Arp4 immunofluorescence in isolated S2 cell nuclei. Nuclei from the same fraction run on lane 4 of Fig. 2 were fixed and stained with Arp4 antibody (A and C) or control antibody (D). (A and B) Views of the same field visualized with Arp4 antibody (A) or Nomarski optics (B). The nuclei average 4-5 μm in diameter and are aggregated into clusters.

Arp4 colocalizes with the heterochromatin-associated protein HP1 during embryogenesis and in imaginal discs

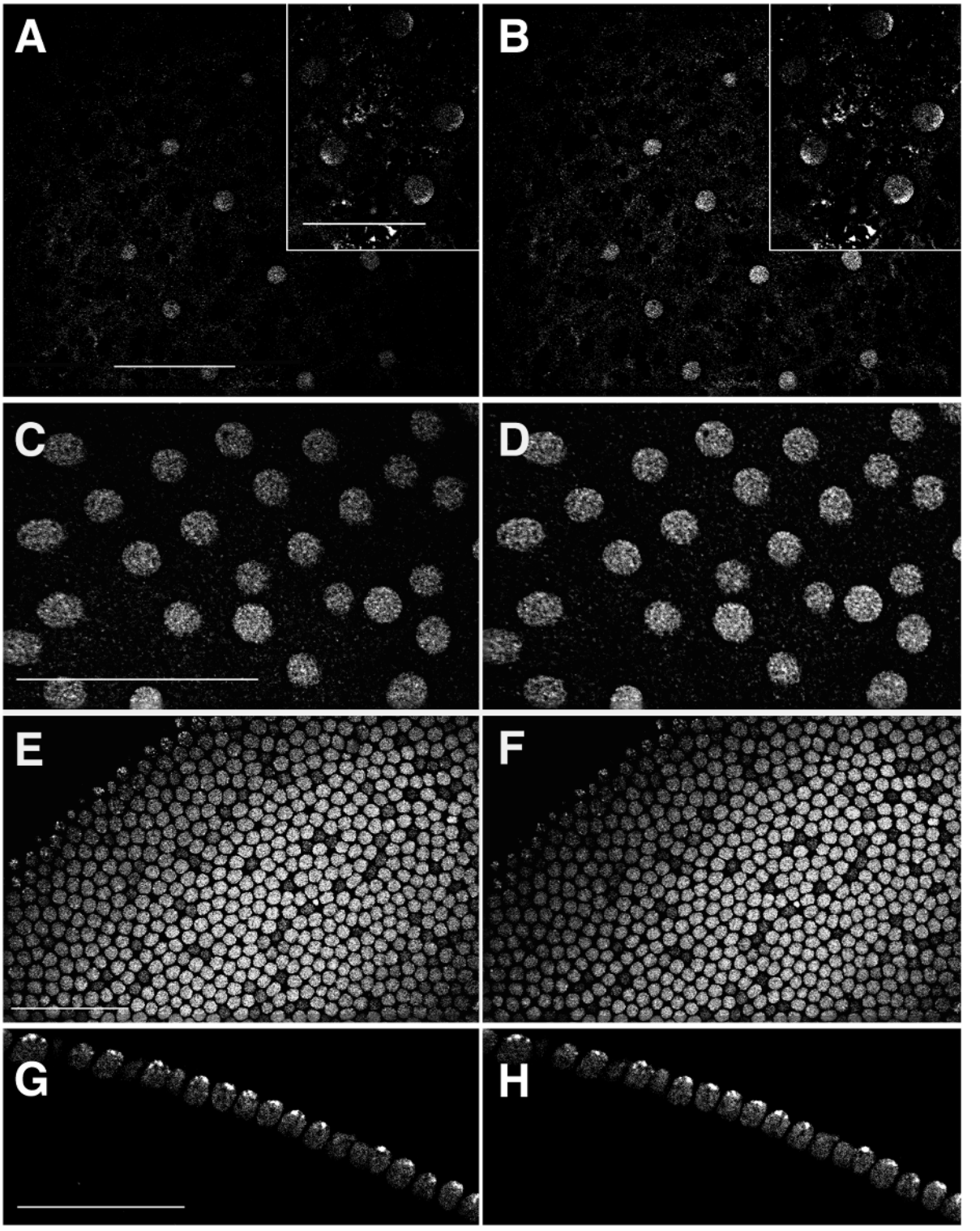
Preliminary studies indicated that Arp4 was localized in a pattern similar to that reported for HP1, a nuclear protein in *Drosophila* that is predominantly associated with heterochromatin (James and Elgin, 1986; James et al., 1989). Embryos were stained with both affinity-purified Arp4 antibody and a monoclonal antibody directed against HP1. The distribution patterns of the two proteins were temporally and spatially superimposible (Fig. 4).

Several aspects of early *Drosophila* embryogenesis make it informative for the examination of nuclear maturation and heterochromatin formation (reviewed by Foe et al., 1993). The first 13 nuclear divisions occur in a syncytium. In the pre-blastoderm embryo (nuclear cycles 1-8), the nuclei remain in the interior of the embryo and are transcriptionally inactive. This period coincides with the most rapid nuclear divisions (10 minutes per cycle), when there is no cytologically detectable heterochromatin. Given the late onset of replication in heterochromatin, it may be advantageous to avoid fully elaborated heterochromatin structures. The nuclear cycle slows after cycle 10, but consists only of S and M phases through nuclear cycle 14.

Arp4 and HP1 staining are first detected in the pre-blastoderm embryo. Fig. 4A and B show embryos after nuclear cycles 5 or 6 (the middle of stage 2 of *Drosophila* embryogenesis). Every nucleus exhibits punctate stain distributed throughout the nucleoplasm, with a crescent of higher intensity at the periphery of each nucleus (insets in Fig. 4A and B). By nuclear cycles 9-10 most nuclei have migrated to the egg cortex and the cell cycle begins to lengthen. Arrival at the cortex is marked by an increase in the level of Arp4 and HP1 stain, though the overall pattern remains unchanged. Many of the peripheral crescents appear to be oriented apically at this stage. Fig. 4C and D show embryos in interphase 10 (stage 3 of embryogenesis). By cycle 11 zygotic transcription is initiated and all of the cortical interphase nuclei have oriented their centromeres apically. Intense apical spots of Arp4 and HP1 appear at this stage, coincident with the first steps in heterochromatin condensation. This pattern is maintained until cellularization is completed (at the end of nuclear interphase 14). Fig. 4E and F show a stage 4 embryo, at interphase 12. The optical section is through the center of the nuclei, parallel to the embryo's surface. A cross-section through a similar embryo is shown in G and H. Cellularization is immediately followed by gastrulation. After gastrulation Arp4 and HP1 attain a nuclear pattern similar to that observed in non-embryonic cells. This consists of one or several intense foci, which are either perinucleolar or at the nuclear periphery, and less intense staining in the rest of the nucleus. Fig. 4I-L show embryos shortly after gastrulation, during early germ band expansion. All nuclei stained for both proteins throughout embryogenesis (data not shown).

It has previously been reported that HP1 becomes dispersed in the cytoplasm during mitosis (Kellum et al., 1995). We observed a dispersion of both Arp4 and HP1 in mitotic embryonic cells (Fig. 4). Groups of mitotic cells are shown in Fig. 4I-L; the identity of these mitotic domains was confirmed by double-staining embryos with the HP1 antibody and propidium iodide (data not shown).

All imaginal disc nuclei exhibited intense staining for Arp4 and HP1. The two proteins colocalized in a pattern similar to that observed in post-gastrulation embryos; however, only one



intense focus of stain was observed per nucleus, and it was almost always located at the nuclear periphery (Fig. 5A-D). In the eye disc a similar nuclear pattern was present on both sides of the morphogenetic furrow (Fig. 5E and F).

Arp4 is concentrated in heterochromatin

While Arp4 and HP1 were colocalized in a variety of polyploid larval tissues (such as fat bodies and the ring gland, data not shown), the most informative data were from the polytene cells of the salivary gland. Polytene chromosome spreads from Dipterans provide a unique opportunity to visualize chromosome structure, but the fixation and spreading required to obtain reproducible banding patterns is not always optimal for protein localization. We therefore examined the distribution of Arp4 and HP1 in intact salivary gland nuclei (Fig. 6). HP1 was used as a marker for the centric heterochromatin. The heterochromatin

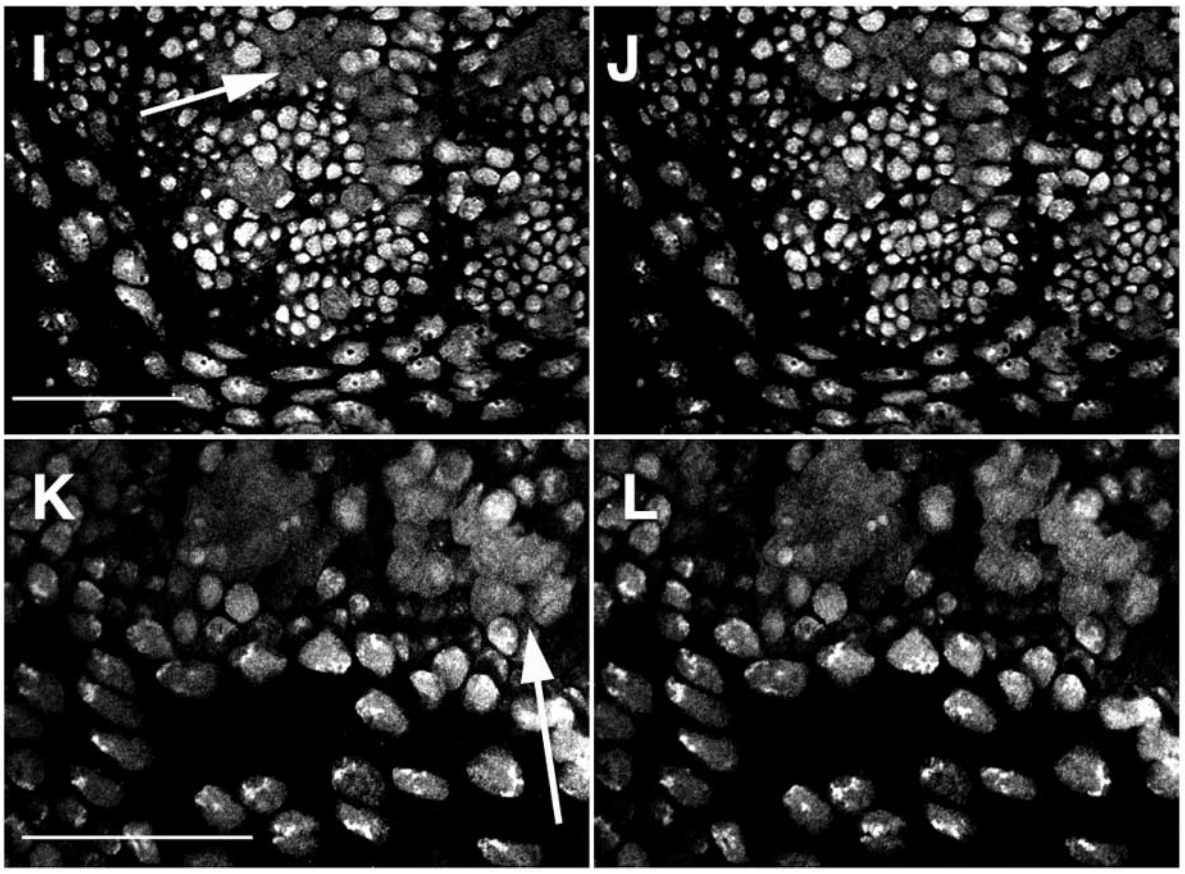


Fig. 4. Confocal images of *Drosophila* embryos stained for Arp4 and HP1. (A,C,E,G,I,K) Arp4 localization; (B,D,F,H,J,L) HP1 localization. Bars, 50 μm. (A-B) The middle of stage 2, after nuclear cycle 5 or 6. The insets are a different embryo from approximately the same stage. (C-D) Stage 3, after nuclear cycle 9. The optical section is through the center of the nuclei. (E-F) A stage 4 syncytial blastoderm, during the interphase of nuclear cycle 12. The optical section is at the surface of the embryo, at the center of most of the nuclei. (G-H) A stage 4 syncytial blastoderm, in optical cross-section. Most nuclei form a monolayer at the surface of the embryo. The interior of the embryo is at the lower left. The bright foci mark the apical portion of each nucleus. (I-J) A dorsal view of an embryo early in germ band expansion (after gastrulation). (K-L) An embryo at a similar stage to the one in I-J, but at a higher magnification. Arrows point to examples of mitotic cells in I and K.

was often positioned at the periphery of the nucleus, though extensions could be observed in the interior. HP1 had a speckled pattern in the central mass of heterochromatin (the chromocenter), with a banded morphology in chromosome 4 (Fig. 6B) and in the proximal regions of the other chromosome arms (Fig. 6E). Arp4 was highly concentrated in the heterochromatin relative to the rest of the nucleus and its staining pattern there was identical to that of HP1 (Fig. 6A and C, D and F; also see Fig. 8E-F). Both proteins could also be seen in faint bands dispersed thoughout the rest of the nucleus (Fig. 6J-L). These bands were more apparent when images were acquired at an elevated gain setting that overexposes the heterochromatin staining (Figs 6G-I, 8A-D). The positions of these bands were consistent with a localization along the chromosome arms, which should be coiled within the nucleoplasm (Hochstrasser et al., 1986). Fig. 6G-L show different sections of the same nucleus, where G-I is a surface section and J-L is a more interior section.

Spread chromosomes show that the localization patterns of Arp4 and HP1 closely match in heterochromatin and overlap in euchromatin

Staining of chromosome spreads with antibodies to Arp4 and HP1 confirmed the presence of Arp4 and HP1 in numerous euchromatic bands (Fig. 7). The localization of Arp4 and HP1 in the chromosome spreads had all of the features seen in intact nuclei. The substructure of the heterochromatin was better preserved in intact nuclei, since the heterochromatin in the spreads was often stretched and distorted. However, enough of this substructure was preserved to indicate an almost complete superposition of Arp4 and HP1 in heterochromatin (Fig. 7A). The spreads also allowed the identification of chromosome 4, which has a high percentage of heterochromatin. Both Arp4 and HP1 were primarily localized to the heterochromatin with only minor distributions in the euchromatin. The chromosome spreads indicated that the set of euchromatic bands labeled by the Arp4 and HP1 antibodies overlap but are not coincident (Fig. 7B and C).

Colocalization of Arp4 and HP1 is maintained after genetic manipulation

The parallel developmental changes in the subnuclear localizations of Arp4 and HP1 suggest that the two proteins may be involved in similar functions and/or structures. In order to test for such a connection, colocalization was assessed in two strains

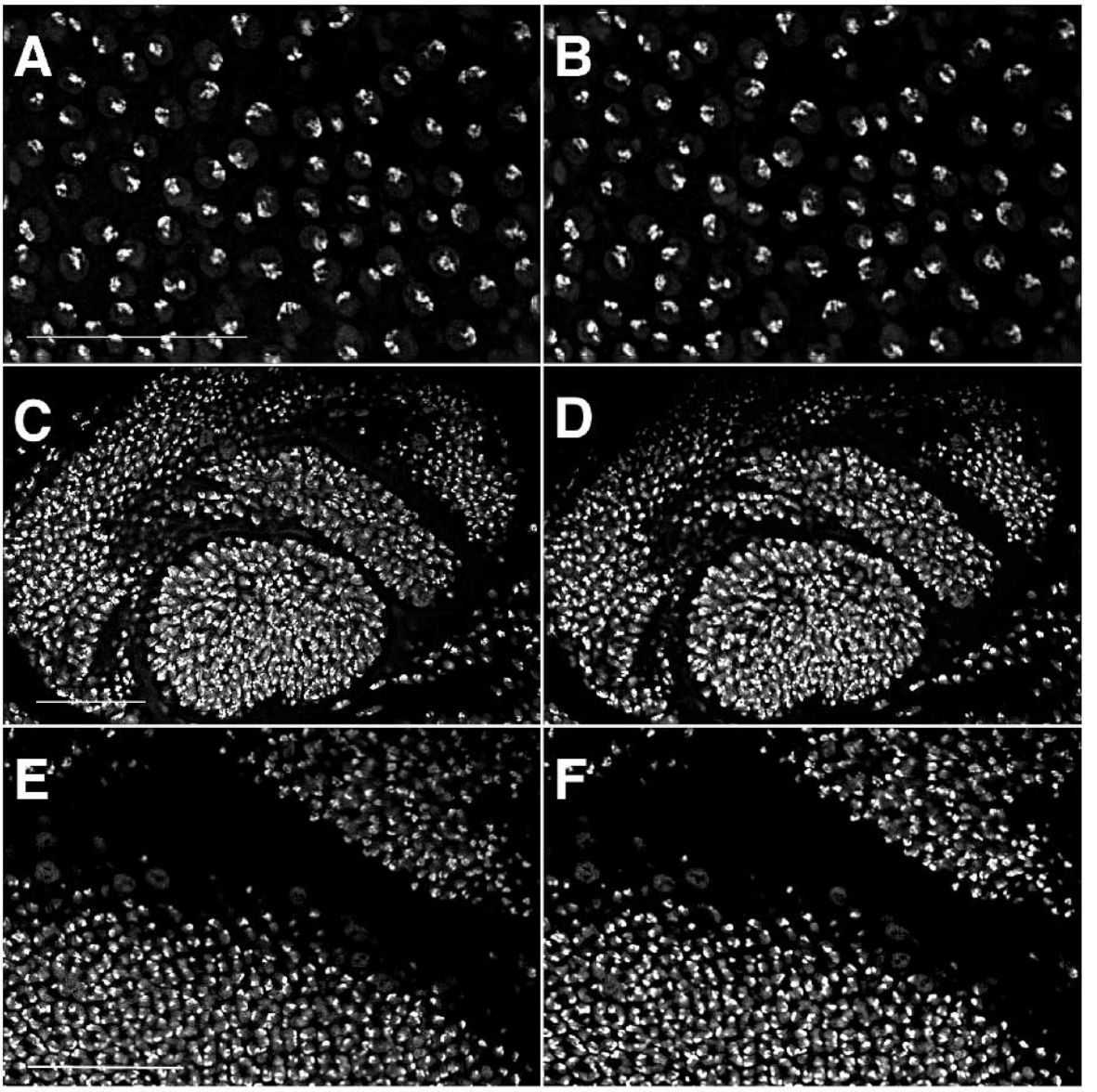


Fig. 5. Arp4 and HP1 colocalize in imaginal discs. (A,C,E) Arp4 localization; (B,D,F) HP1 localization. When tissue was stained with control antibodies there was no discernable signal (data not shown). Bars, 50 μm. (A-B) The outer layer of cells surrounding a leg disc, at a higher magnification. (C-D) Lower magnification view of a leg disc. (E-F) Higher magnification view of an eye disc. The morphogenetic furrow runs from the upper left to the lower right. Cells are organized into ommatidia behind (below in this image) the furrow but not before (above in this image) the furrow.

of *Drosophila* that express mutant forms of HP1. The first strain is heterozygous for a missense mutation in HP1 and exhibits a decrease in heterochromatin-mediated gene silencing (Platero et al., 1995). The second strain overexpresses a chimeric form of HP1 in a wild-type background (Platero et al., 1995). Previous studies using this strain indicated that both chimeric HP1 and the endogenous wild-type HP1 have altered nuclear localization patterns. Localization patterns were examined in intact polytene nuclei, for the following reasons. (a) It was not clear if mutant protein would remain associated with chromatin. If not, then it would be lost in preparations of spread chromosomes. (b) The three-dimensional structure of the centric heterochromatin was better preserved in intact nuclei, making any alterations in its morphology easier to detect.

The colocalization of Arp4 and HP1 was maintained in each mutant strain. Nuclei from heterozygotes carrying the missense mutation, *Su(var)2-5*^{02}, exhibited two major differences in localization compared to wild-type controls (Fig. 8). The majority of nuclei had diffuse Arp4 and HP1 stain in the interchromosomal spaces (Fig. 8G-J); such diffuse staining was only rarely detected in wild-type nuclei (Fig. 8A-D; in these nuclei the interchromosomal spaces are totally lacking stain). In addition, a minority of nuclei exhibited alterations in heterochromatin morphology (Fig. 8K and L) of a kind never seen in the wild-type (Fig. 8E and F). Instead of a tight chromocenter that was continuous with the proximal regions of the chromosome arms, there was a loose grouping of bands. These altered chromocenters were distinguished from the euchromatic bands by their higher stain

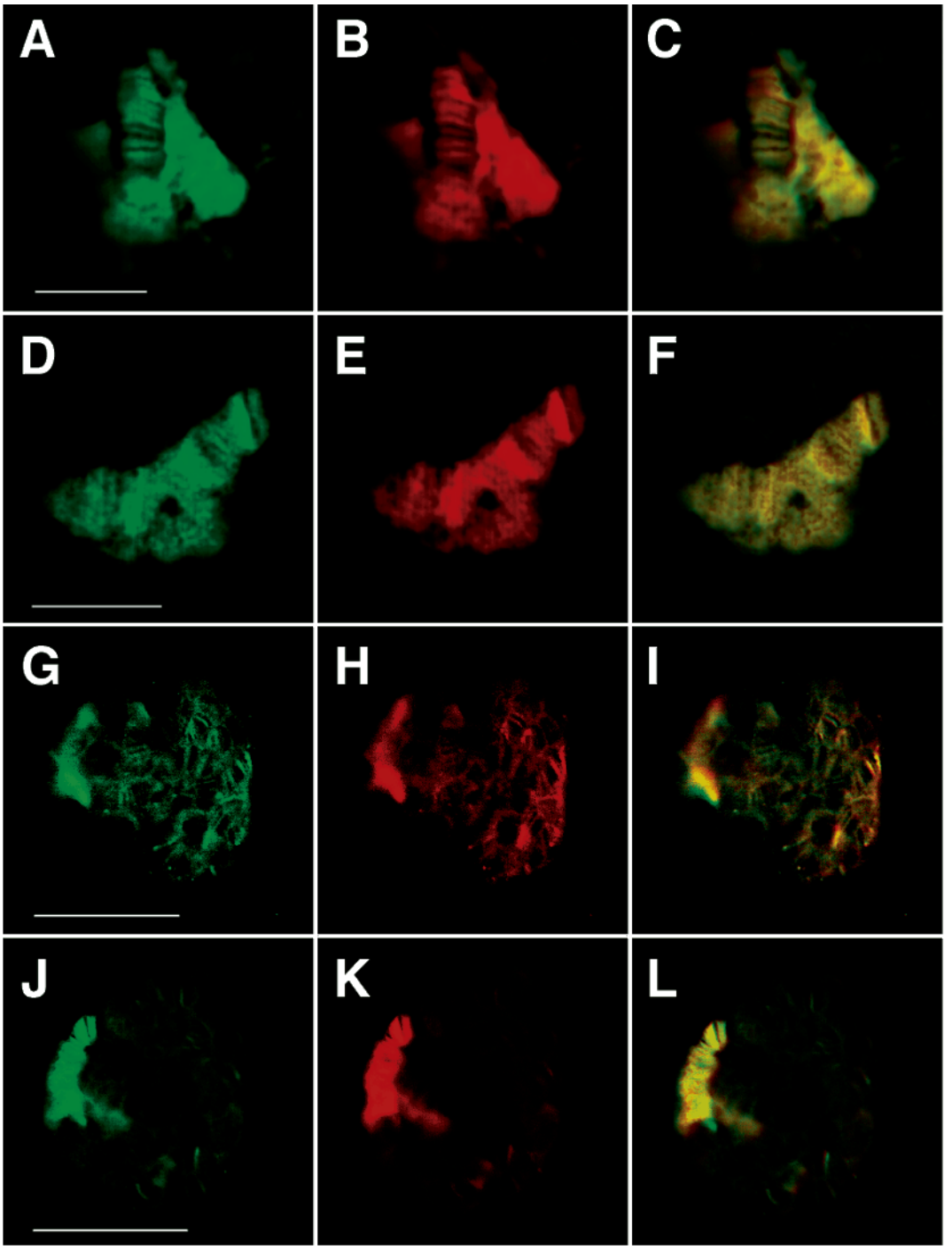


Fig. 6. Intact polytene nuclei stained for Arp4 and HP1 show that Arp4 is concentrated in the centric heterochromatin. Third instar salivary glands were fixed and stained as whole mounts. By Normarski optics the glands were intact and of normal morphology (data not shown). (A,D,G,J) Arp4 localization (green); (B,E,H,K) HP1 localization (red); (C,F,I,L) superpositions of the two staining patterns. When tissue was stained with control antibodies there was no discernable signal in either the green or red channels (data not shown). Bars: 10 μm (A-F); 25 μm (G-L). (A-C) An optical section showing chromosome 4. (D-F) An optical section through the centric heterochromatin, which is at the margin of the nucleus. (G-I) A surface view of a nucleus, imaged at a higher gain than the pictures in A-F to facilitate visualization of euchromatic bands. Bands are visible in an overall pattern that conforms to the packing expected for the chromosome arms. (J-L) A different optical section of the same nucleus shown in G-I, imaged at a lower gain than in G-I to facilitate the visualization of both heterochromatic and euchromatic stain. Part of the centric heterochromatin is in focus.

intensity. However, the heterochromatin/euchromatin differential in staining intensity was much less than in wild-type nuclei.

The chimeric form of HP1 was constructed by removing the HP1 chromo domain and substituting the chromo domain from Polycomb (Pc) (Platero et al., 1995). These domains are the only portions of HP1 and Pc that are similar. In the case of Pc, its chromo domain has been shown to be sufficient for appropriate targeting of the protein. Pc only localizes to euchromatic sites. The chimeric form of HP1 localizes to the sites normally occupied by Pc and recruits endogenous wild-type HP1 to the Pc sites. The chimeric HP1 is encoded by a transgene under heat shock control and is expressed as a β-galactosidase fusion protein; since the HP1 monoclonal antibody used for most of these studies does not detect the chimera, it was detected with β-galactosidase antibodies.

Larvae from the strain carrying the transgene were either maintained without heat shock, as controls, or were subjected to one round of heat shock prior to gland dissection. Intact salivary glands were labeled simultaneously with antibodies specific to Arp4, HP1 and chimeric HP1 (anti-β-galactosidase). Fig. 9 shows the localization patterns for the three antibodies. Since the control nuclei had no detectable β-galactosidase

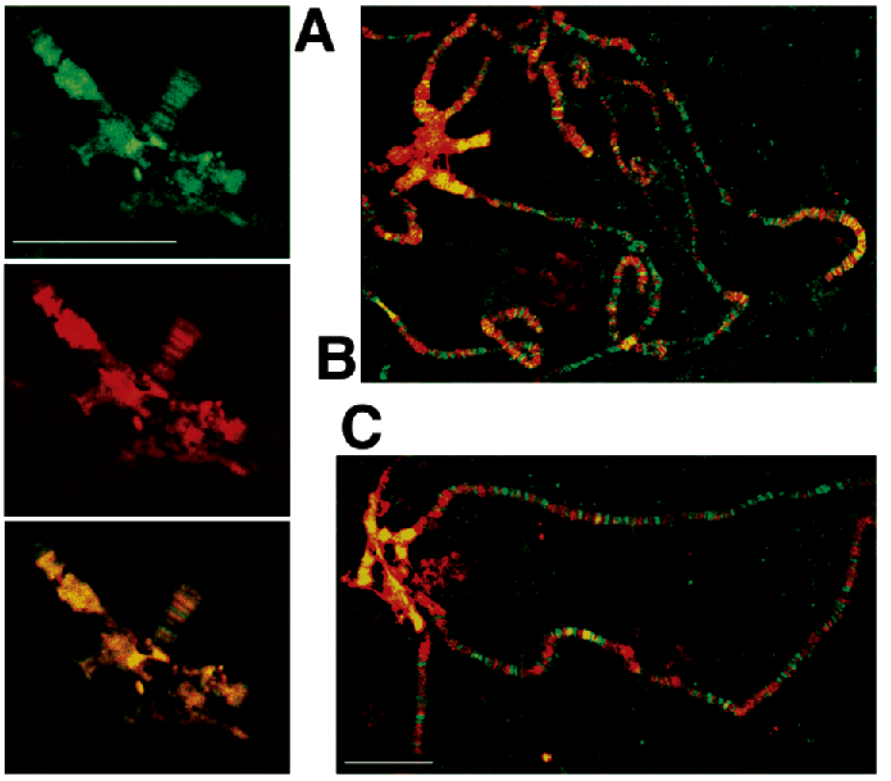


Fig. 7. Arp4 and HP1 colocalize in heterochromatin and are present in overlapping sets of euchromatic bands. Polytene chromosome spreads were stained for Arp4 (green) and HP1 (red). Bars, 25 μm. (A) A high magnification view of the centric heterochromatin. The banded structure is chromosome 4. (B-C) Views of two sets of chromosomes, photographed at a gain which maximizes the resolution of euchromatic bands but overexposes the heterochromatic pattern. Only the superposition of the two channels is shown. (B) This field has a high representation of chromosome ends; (C) this field shows the central regions of two euchromatic arms.

stain, only the Arp4 and HP1 patterns are shown. Without heat shock induction, Arp4 and HP1 precisely colocalize (as shown for wild-type nuclei above). With heat shock induction, HP1 is recruited to the same bands that label with the β-galactosidase antibody and represent Pc sites. Arp4 is also localized to these sites. In the presence of chimeric protein, the Arp4 and HP1 patterns remain qualitatively identical, but the relative band intensites differ from what is seen in the controls. Very little chimeric HP1 was detected in the heterochromatin.

DISCUSSION

Arp4 is a nuclear protein

Embryos and S2 cells were used to demonstrate the nuclear localization of Arp4 protein by two independent means, immunofluorescence and biochemical fractionation. The antibodies used in these studies were raised against a unique region of the Arp4 amino acid sequence. When the Arp4 coding sequence was overexpressed in bacteria and *Drosophila* tissue culture (S2) cells, only an antigen with the predicted molecular mass of 45 kDa was detected. Similarly, only a 45 kDa antigen was detected in embryos and S2 cells, which had the highest levels of the 45 kDa protein among all tissues and cells that were sampled. The reactivity of the Arp4 antibody was established by titration with pure Arp4 fusion protein; the antibody recognizes the 45 kDa protein with high affinity (data not shown). It was also determined that the endogenous 45 kDa antigen was present at low levels in its most abundant sources; a single S2 cell contains approximately 2,000 copies of the molecule. The antibody never detected an antigen migrating with the molecular mass of HP1 (James and Elgin, 1986) but did detect variable amounts of high molecular mass antigen in samples that contained post-mitotic tissues. The affinity and specificity properties of the antibody suggest that these antigens may be post-translationally modified forms of the Arp4 gene product.

Consistent with the presence of Arp4 in the nucleus, two potential nuclear localization signals (Dingwall and Laskey, 1991) are present in the coding sequence, at amino acids 34-41 (KAKSERRR) and 336-343 (PRLKRDLR). A potential nuclear localization signal is also present in a yeast arp (act3p) sequence (Weber et al., 1995) but not in the sequences of any actins or other arps studied to date. The localization of Arp4, like the localization of HP1 (Kellum et al., 1995), is cell cycle dependent. Both proteins become dispersed in the cytoplasm during mitosis. No cytoplasmic staining was detected in interphase cells. However, only approximately half of the Arp4 protein present in whole S2 cell lysates fractionated with the nucleus (Fig. 2; quantitation not shown). While some of the non-nuclear Arp4 may be due to the cytoplasmic component present in mitotic cells, it appears likely that most is due to leakage from nuclei during homogenization.

Arp4 colocalizes with HP1

Our results indicate that the distributions of Arp4 and HP1 are temporally and spatially similar. The two proteins are maternally supplied (for HP1 see Eissenberg et al., 1994) and show superimposible patterns within nuclei during embryogenesis. Colocalization was also observed in all of the other intact fixed tissues that were examined.

HP1 was initially characterized as a protein tightly associated with chromatin and primarily localized to heterochromatin (James and Elgin, 1986; James et al., 1989). The sequence of HP1 reveals no known DNA binding motifs, and several studies are consistent with a chromatin association mediated by protein-

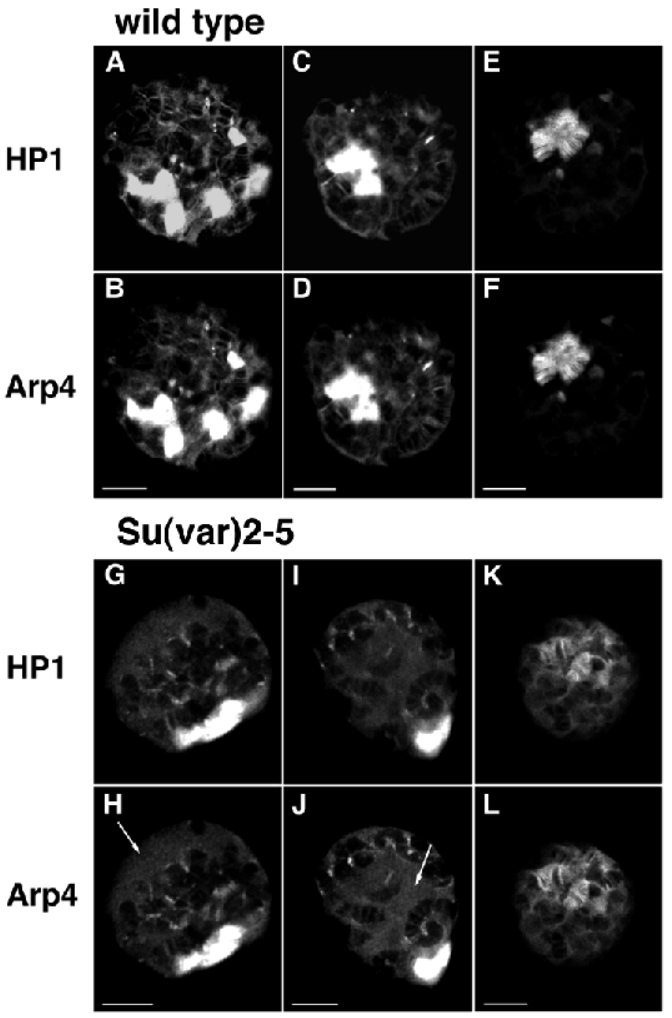


Fig. 8. Intact polytene nuclei from flies heterozygous for the HP1 mutation *Su(var)2-5^{02}* exhibit parallel alterations in the localization patterns for Arp4 and HP1. (B,D,F,H,J,L) Arp4 localization; (A,C,E,G,I,K) HP1 localization. Bars, 10 μm. (A-F) Three wild-type nuclei, double-labeled for HP1 and Arp4; see also Fig. 6. The images in A-D are taken at a gain that allows euchromatic bands to be visualized but overexposes the centric heterochromatin. Areas of nucleoplasm that are free of chromatin are also devoid of stain. E and F show part of the chromocenter and are taken at a gain that allows the visualization of heterochromatin but is underexposed for the euchromatic bands. (G-L) Three nuclei from *Su(var)2-5^{02}* heterozygotes. In G-J the interchromosomal nucleoplasm (arrows) contains diffuse immunoreactivity. These images are taken at a gain that allows euchromatic bands to be visualized but overexposes the heterochromatin. K and L show most of the chromocenter from a mutant nucleus. It contains less tightly packed bands than a wild-type chromocenter (see E and F) and stains less intensely relative to the euchromatic bands.

protein interactions (Powers and Eissenberg, 1993; Platero et al., 1995). Mutations at the *Su(var)205* locus, originally recovered as dominant suppressors of position effect variegation (PEV), were found to be alterations in the HP1 gene (Eissenberg et al., 1990, 1992). In PEV, epigenetic differences arise in the expression of a gene that are dependent upon chromosomal location and are often correlated with proximity to heterochromatin (Reuter and Spierer, 1992). Additional evidence has strengthened the connection between HP1, heterochromatin formation, and gene silencing (Eissenberg et al., 1992; Eissenberg and Hartnett, 1993): overexpression of HP1 from a heat shock promoter enhances PEV, as do duplications of the HP1 gene, while deletions of the HP1 gene act as haplo-abnormal suppressors of PEV. When reporter transgenes are inserted at a variety of chromosomal sites, genes inserted proximal to centric heterochromatin or in the fourth chromosome exhibit a PEV that is suppressed by HP1 mutations (Wallrath and Elgin, 1995).

Both intact polytene nuclei and spread polytene chromosomes from lysed salivary gland nuclei provide a high resolution image of nuclear structures. Therefore, while the euchromatic component of the Arp4/HP1 stain appeared diffuse in diploid nuclei, it could be resolved as discrete bands in the two polytene preparations. Both intact nuclei and spread chromosomes showed almost complete colocalization of Arp4 and HP1 in the heterochromatin. Chromosome spreads indicated that Arp4 and HP1 have overlapping but distinct euchromatic distribution patterns. Euchromatic sites visible in intact nuclei showed a complete colocalization of the Arp4 and HP1 bands. It is possible that some Arp4 and/or HP1 protein was lost during the chromosome spreading process, an interpretation consistent with the cell fractionation data (see above).

Developmental changes in Arp4/HP1 localization

The successive changes in the Arp4/HP1 localization pattern during early embryogenesis can be interpreted in terms of changes in heterochromatin condensation and nuclear maturation. The largely inactive nuclei of the pre-blastoderm embryo have a granular distribution of Arp4 and HP1 throughout the nucleoplasm, with a higher concentration in peripheral crescents. The crescents may represent the demarcation of a nuclear domain that will become an area for heterochromatin condensation. Embryonic nuclei undergo changes in chromatin structure concommitent with the onset of zygotic transcription (Ner and Travers, 1994). At this time, the intensity of staining for both proteins increases, followed by the appearance of foci that lie close to the positions of interphase centromeres. These foci may represent the initial stages of heterochromatin condensation. After cellularization, changes occur in the size and shape of the Arp4/HP1 foci which are consistent with the formation of a large chromocenter at this stage.

A very similar progression of HP1 localization patterns during embryogenesis has been observed by Kellum et al. (1995). In particular, HP1 was detected in preblastoderm nuclei before zygotic transcription is initiated, and it was observed to concentrate in a pericentromeric position after the onset of zygotic transcription. These investigators quantitated the relative apical and basal immunofluorescence obtained with HP1 and histone antibodies during cellularizaion. The apical/basal ratio for HP1 was more than twofold greater than the ratio for the histones, indicating that the apical concentration of HP1 was not simply due to the amount of DNA in the apical regions. This evidence is consistent with the preferential concentration of HP1 in the nascent heterochromatin of these nuclei.

The capacity of heterochromatin to mediate gene silencing changes over the course of development (Lu et al., 1996). Silencing is most effective early in embryogenesis, at the cellular blastoderm stage, and in imaginal discs. Concentrated

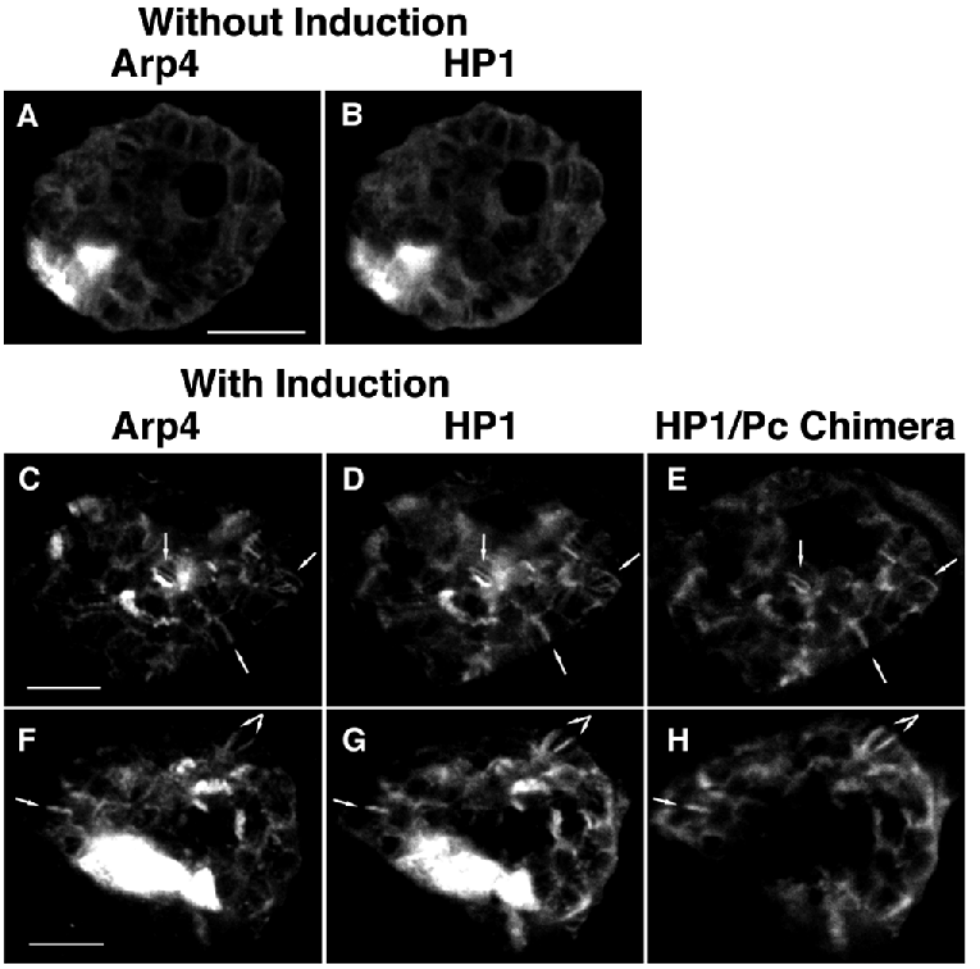


Fig. 9. Polytene nuclei from flies carrying a transgene that expresses an HP1/Polycomb chimera under heat shock control. (A,C,F) Arp4 localization; (B,D,G) HP1 localization; (E and H) localization of the HP1/Polycomb chimera (anti-β-galactosidase). Bars, 10 μm. (A-B) Nuclei that have not had expression of the chimera induced. No immunoreactivity was detected with the antibody against the chimera (data not shown). (C-H) Two nuclei from glands that had expression induced with one round of heat shock. The nuclei were triply labeled. Some bands stain for all three proteins. A subset of these bands is highlighted with arrows.

foci of Arp4/HP1 appear at the time that silencing is first detected. There is a decrease in the amount of silencing after gastrulation, coincident with a shift in the shape and size of the Arp4/HP1 foci. Our data indicate that Arp4 and HP1 maintain their colocalization before, during, and after major changes in heterochromatin function.

Colocalization of Arp4 and HP1 is maintained after genetic manipulation of HP1

Flies heterozygous for lethal HP1 mutations have decreased PEV, indicating a decreased amount of heterochromatin-mediated gene silencing. One of these mutations, *Su(var)2-5*^{02}, is the result of a missense mutation in the chromo domain. Overexpression of the mutant protein fails to complement other HP1 mutations in the PEV assay, indicating that the mutant protein is not functional with regard to gene silencing (Platero et al., 1995). Our data indicate that flies heterozygous for this mutation have parallel alterations in the localization of HP1 and Arp4. The alterations are consistent with a loss in the ability of the mutant HP1 protein to bind to chromatin, and with a shift of some Arp4 and HP1 from a bound to an unbound state. We detect an increased amount of HP1 and Arp4 in the interchromosomal nucleoplasm of mutant larvae. In some nuclei the chromocenter has a more loose, less compact morphology and a decreased staining intensity.

The colocalization of Arp4 and HP1 was further tested by examining nuclei from flies that overexpress a chimeric form of HP1. The chimera has been shown to localize to sites normally occupied by Polycomb (Pc) in addition to sites normally occupied by HP1 (Platero et al., 1995). Pc is a protein, exclusively associated with euchromatic regions, that helps mediate the repression of homeotic genes (Orlando and Paro, 1995). The chimera has also been shown to recruit endogenous HP1 to the Pc sites, resulting in an ectopic localization of HP1 within the euchromatin. In nuclei expressing the chimeric HP1, Arp4 remained colocalized with HP1 at all sites, both old and new.

Drosophila proteins implicated in the structure or function of heterochromatin

Many chromatin-associated proteins have been characterized in *Drosophila* and several have been localized to heterochromatin: HP1 (James and Elgin, 1986; James et al., 1989), GAGA factor (Raff et al., 1994; Kellum et al., 1995) and *modulo* (Garzino et al., 1992). The evidence for a functional role is strongest for HP1, the only *Drosophila* protein previously reported to be localized to the heterochromatin of polytene chromosomes. Arp4 is now identified as the second protein showing such a localization. GAGA factor has been localized to the centromeric region of embryonic metaphase chromosome spreads (Raff et al., 1994), and the staining pattern in intact fixed embryos indicates there is a centromeric

association during interphase (Kellum et al., 1995). However, GAGA factor and HP1 have different localization patterns during embryonic mitoses and exhibit only limited overlap during interphase. In polytene chromosomes GAGA factor localizes to many euchromatic bands but not to the heterochromatin (Raff et al., 1994). *modulo* is expressed in all nuclei of the embryo prior to cellularization; after cellularization its expression becomes restricted to cells of particular lineages (Garzino et al., 1992). The *modulo* pattern in blastoderm nuclei is perinucleolar and apical, consistent with a heterochromatic localization. We have presented evidence that Arp4 and HP1 colocalize in the heterochromatin during all stages of prepupal development, that at each stage of development this pattern is present in all interphase nuclei, and that Arp4 and HP1 show similar changes in localization during the cell cycle. In addition, the colocalization of the two proteins is maintained when genetic manipulations alter the properties of HP1 and cause shifts in its nuclear localization pattern.

A new type of chromatin-associated protein

The arps are part of the larger actin superfamily. A characteristic common to every branch of the superfamily is the ability to participate in a multiprotein complex, where the assembly of the complex is reversible and highly regulated (Frankel and Mooseker, 1996). Arp1 is part of the dynactin complex associated with microtubules. Arp2 and Arp3 are part of a multiprotein complex associated with the actin cytoskeleton. Future studies will determine if Arp4 is part of a multiprotein complex, and what role that complex may play in heterochromatin. While Arp4 has close family ties to a diverse set of non-nuclear cytoskeletal proteins, it has little in common with its nearest neighbors. We have compared the Arp4 amino acid sequence to the sequences of a variety of *Drosophila* proteins involved in transcriptional regulation, PEV and chromatin structure. Arp4 has no regions of homology with HP1 (Eissenberg et al., 1990), Polycomb (Paro and Hogness, 1991), GAGA factor (Soeller et al., 1993), *modulo* (Krejci et al., 1989), *trithorax* (Mazo et al., 1990), *Su(var)3-7* (Cleard et al., 1995), *Su(var)3-9* (Tschiersch et al., 1994), *Evar)3-93D* (Dorn et al., 1993) and several high mobility group proteins. In addition, there are no obvious DNA binding motifs or protein association domains, such as the chromo domain shared between HP1 and Polycomb (Paro and Hogness, 1991; Platero et al., 1995).

A role for Arp4 in chromatin function cannot be predicted on the basis of sequence analysis, but our morphological data point towards such a role. In *S. cerevisiae* genetic data have been obtained which implicate another arp in chromatin function. Act3p, called Arp4 in nomenclature proposed for the yeast arp family (Poch and Winsor, 1997), is also localized to the nucleus (Weber et al., 1995; Jiang and Stillman, 1996) and like *Drosophila* Arp4 is highly divergent from actin (Harata et al., 1994). The sequence similarity between Arp4 in *Drosophila* and yeast act3p is no higher than the similarity between *Drosophila* Arp4 and other classes of arp. Act3p has been linked to a new form of epigenetic 'variegation' in *S. cerevisiae* (Jiang and Stillman, 1996), where variegation describes the expression patterns of colonies derived from genetically identical cells. Using a comprehensive set of genetic assays, act3p-mediated variegation was shown to be distinct from the position-dependent variegation associated with yeast telomeres and mating type loci.

The colocalization of Arp4 with HP1 in the centric heterochromatin suggests a potential functional connection. This inference is strengthened by the tight spatial and temporal linkage in the localization patterns of each protein throughout development and after genetic manipulation. During development, changes in the Arp4 and HP1 localization patterns correlate with shifts in nuclear function (transcriptional competence, variegation competence and cell cycle regulation). In a mutant fly strain that is known to have an altered HP1 localization, Arp4 remained colocalized. In another fly strain carrying an HP1 mutation known to have an affect on gene silencing, we detected parallel changes in the localizations of both HP1 and Arp4. Genetic analysis of Arp4 will more fully reveal the role it plays in chromosome functions.

We thank Joel Eissenberg for providing the fly strain expressing the HP1 chimera, Glenn Doughty for his expert third instar larval salivary gland squashes, and Steve Warren for his support. Special thanks to Valerie Mermall, Lori Wallrath, Chris Shaffer, and Iain Dawson for helpful discussions and critical readings of the manuscript, to Lynn Cooley, Esther Verheyen, Karen Purcell, Robert Lake and Penny Post for critical reading of the manuscript, and to members of the Mooseker, Artavanis-Tsakonas and Elgin laboratories for advice and support. This work was supported by National Institutes of Health grant DK25387 to M. S. Mooseker, National Institutes of Health Research Service Award F32-GM14444 and Pathology Training grant T32-DK07556-18 to S. Frankel, grants from the Howard Hughes Medical Institute to S. Artavanis-Tsakonas and National Institutes of Health grant HD23844 to S. C. R. Elgin.

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