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Distinct Cytoplasmic and Nuclear Fractions of *Drosophila* Heterochromatin Protein 1: Their Phosphorylation Levels and Associations with Origin Recognition Complex Proteins

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Abstract. The distinct structural properties of heterochromatin accommodate a diverse group of vital chromosome functions, yet we have only rudimentary molecular details of its structure. A powerful tool in the analyses of its structure in *Drosophila* has been a group of mutations that reverse the repressive effect of heterochromatin on the expression of a gene placed next to it ectopically. Several genes from this group are known to encode proteins enriched in heterochromatin. The best characterized of these is the heterochromatin-associated protein, HP1. HP1 has no known DNA-binding activity, hence its incorporation into heterochromatin is likely to be dependent upon other proteins. To examine HP1 interacting proteins, we isolated three distinct oligomeric species of HP1 from the cytoplasm of early *Drosophila* embryos and analyzed their compositions. The two larger oligomers share two properties with the fraction of HP1 that is most tightly associated with the chromatin of interphase nuclei: an underphosphorylated HP1 isoform profile and an association with subunits of the origin recognition complex (ORC). We also found that HP1 localization into heterochromatin is disrupted in mutants for the ORC2 subunit. These findings support a role for the ORC-containing oligomers in localizing HP1 into *Drosophila* heterochromatin that is strikingly similar to the role of ORC in recruiting the Sir1 protein to silencing nucleation sites in *Saccharomyces cerevisiae*.

Key words: HP1 • ORC • SIR1 • heterochromatin assembly • silencing

In 1928, Heitz defined the term heterochromatin to describe regions of the chromosomes that, unlike the more typical euchromatin, do not undergo cyclical changes in condensation during the cell cycle. The functional significance of this distinct chromatin, most typically found at the centromeres and telomeres, has only begun to be fully recognized. In recent years, it has been demonstrated to function in a diverse group of processes, ranging from gene regulation (Weiler and Wakimoto, 1995), sister chromatid adhesion (Allshire et al., 1995), and meiotic homologue pairing (Dernburg et al., 1996*b*; Karpen et al., 1996) to providing focal points for mitotic chromosome condensation and organizing nuclear architecture (Hochstrasser et al., 1986; Kellum and Alberts, 1995; Bhat et al., 1996; Csink and Henikoff, 1996; Dernburg et al., 1996*a*; Gotta et al., 1996; Török et al., 1997).

Euchromatic genes in *Drosophila* are subjected to mosaic repression when juxtaposed to heterochromatin by a chromosome rearrangement (for review see Spofford, 1976). This so-called variegated position effect (PEV)[1] is formally analogous to the regulation of the mating type genes in budding yeast. These genes are maintained in a repressed state at a pair of silent loci and only become transcriptionally competent when moved to an active mating type locus (for review see Laurenson and Rine, 1992). In both PEV and yeast silencing, a gene that is fully capable of producing a functional product is believed to be inactivated by a heritably stable form of repressed chroma-

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1. *Abbreviations used in this paper*: ARS, autonomous replicating sequence; HP1, heterochromatin protein 1; ORC, origin recognition complex; PEV, variegated position effect; Sir, silencing information regulator.

tin. Transgenes inserted into *Drosophila* heterochromatin are packaged into an unusually highly ordered array of nucleosomes, and their promoters are resistant to endonuclease digestion (Wallrath and Elgin, 1995). The chromatin of the silent loci is similarly resistant to endonuclease digestion and to enzymes for DNA repair and methylation (Terleth et al., 1989; Singh and Klar, 1992; Loo and Rine, 1994).

Genetic experiments in both yeast and *Drosophila* have identified proteins that function in establishing or maintaining the silenced chromatin. In budding yeast, a set of silencing information regulator (Sir) proteins has been identified through mutations that interfere with silencing (Laurenson and Rine, 1992). Biochemical studies have provided evidence for interactions between these proteins, histones, and other chromatin proteins to form the silent state (Kurtz and Shore, 1991; Raff et al., 1994; Granok et al., 1995). Genetic screens for mutations that modify position effect variegation have been used in *Drosophila* to identify proteins that affect heterochromatin formation (for review see Grigliatti, 1991). The dose dependence of a large number of PEV modifiers has led to speculations that *Drosophila* heterochromatin may be similarly composed of a network of interacting proteins (Locke et al., 1988). Heterochromatin protein 1 (HP1) was the first product of a PEV modifier gene shown to have a heterochromatic localization (James and Elgin, 1986; James et al., 1989; Eissenberg, 1990). Because it apparently lacks DNA-binding activity, its localization into heterochromatin is thought to require other proteins.

The Sir proteins also have no known DNA-binding activity. The Sir1 protein is the first of these to be localized to silencing nucleation sites through the DNA-binding activity of the origin recognition complex (ORC) (Pillus and Rine, 1989; Chien et al., 1993; Triolo and Sternglanz, 1996; Fox et al., 1997). It then participates in the recruitment of the remaining Sir proteins to the site. The ORC multi-protein complex binds and initiates DNA replication from autonomous replicating sequence (ARS) elements distributed throughout the yeast genome (Bell and Stillman, 1992). It also recruits the Sir1 protein to one of these elements within the silencing nucleation sites. Moreover, mutants for the yeast ORC2 subunit display a silencing defect (Bell et al., 1993; Fox et al., 1995), and this defect can be complemented by the wild-type *Drosophila* ORC2 gene (Ehrenhofer-Murray et al., 1995). This finding suggests that the silencing function of ORC is conserved in *Drosophila*. More recently, the *Drosophila* ORC2 subunit was found to be enriched in centric heterochromatin and specific subunits of the ORC complex (1, 3, and 4) were shown to physically interact with HP1 (Pak et al., 1997). These findings pointed to a role for ORC in recruiting HP1 into *Drosophila* heterochromatin.

We have undertaken a biochemical analysis of HP1-interacting proteins to identify other protein components of *Drosophila* heterochromatin and to determine how HP1 is localized into it. Multiple oligomeric species of HP1 were identified in the maternally loaded cytoplasm of early embryos and compared with distinct nuclear fractions of the protein. The most tightly bound nuclear fraction was found to resemble the two largest cytoplasmic oligomers. Like the HP1 in these cytoplasmic species, this nuclear fraction of the protein was underphosphorylated and associated with *Drosophila* ORC proteins. We also found the localization of HP1 into heterochromatin to be perturbed in mutants for the ORC2 subunit. This result suggests a role for the ORC-containing cytoplasmic oligomers in localizing these HP1 phosphoisoforms into heterochromatin. Another nuclear fraction of the protein, which is more highly phosphorylated and is not ORC associated, was found to increase in abundance during the developmental stage when heterochromatin first becomes distinct as a cytological entity. This suggests an important role for this HP1 fraction also in heterochromatin formation.

Materials and Methods

Separation of HP1 Oligomers

A 0–2 h collection of *Drosophila* embryos was homogenized in buffer A (50 mM Hepes, pH 7.6, 10% glycerol [wt/vol], 1 mM Na-metabisulfite, 100 mM PMSF, 200 mM benzamidine, and a 1:100 dilution of protease inhibitor cocktail [1.6 mg/ml benzamidine and 1.0 mg/ml of each of the following: phenanthroline, aprotinin, leupeptin, and pepstatin]) plus KI (kinase inhibitors: 25 mM KCl with 25 mM NaFl and 80 mM β-glyceraldehyde) (1 g dechorionated embryos/1 ml buffer A plus KI). The extract was centrifuged at 10,000 *g* for 15 min to remove nuclei and cell debris and clarified by centrifugation at 100,000 *g* for 1 h. The protein concentration of the clarified extract was typically 60–80 mg/ml.

The extract (100 μl) was applied to a Superose 6 10/30 gel filtration column equilibrated with buffer A/.02 (2% glycerol) plus KI. 0.5-ml fractions were collected, precipitated with TCA, resuspended in SDS loading buffer, and then separated by SDS-PAGE. The fractionation of HP1 was monitored by immunoblot analyses with a polyclonal antibody prepared against a 6× his-HP1 fusion protein expressed in bacteria. Bio-Rad Laboratories (Hercules, CA) gel filtration standards (151–1901) were used for molecular weight calibration.

Sucrose density gradient centrifugation was performed using a 5–20% (wt/vol) sucrose gradient in buffer A plus KI. The gradients were centrifuged at 50,000 rpm in a TLS55 rotor for 7 h, and 0.1 ml fractions were collected and analyzed as described above. A combination of Bio-Rad Laboratories (151-1901) and Pharmacia Biotechnology Inc. (17-0445-01; Piscataway, NJ) molecular weight standards were used for calibration.

Nuclear Salt Extractions

A 0–1 h collection of *Drosophila* embryos was aged for 2.3 h to obtain a homogeneous population of embryos in interphase of cycle 14 or for 1.5 h to obtain a population of cycle 12–13 embryos (70–80% in interphase). Nuclei were prepared from these embryos as previously described (Kellum et al., 1995) and sequentially extracted with nuclear extraction buffer (60 mM Hepes, pH 7.6, protease inhibitor cocktail [1:100] and 1% Tween-20) with an increasing concentration of KCl (60 mM, 0.5 M, and finally 1 M KCl). A constant volume was maintained for each extraction step. An equivalent volume of the pellet and supernatant fractions from each extraction was subjected to SDS-PAGE and immunoblotting was used to quantitate the level of HP1 in each fraction.

HP1 Phosphoisoform Analyses

Two-dimensional gel electrophoresis was performed as described by Eissenberg et al. (1994). The gel filtration fractions containing each oligomeric species of HP1 were pooled and concentrated 10-fold by Centricon-20 ultrafiltration. An equivalent amount of protein from each salt-extracted fraction was similarly separated by two-dimensional gel electrophoresis. HP1 isoforms were visualized by immunoblotting.

Immunoaffinity Chromatography

For use in the immunoaffinity chromatography, a rabbit polyclonal antibody was prepared against an NH_2-terminal peptide of HP1 (CIDNPESSAKVSDAEEEE) coupled to keyhole limpet hemocyanin (Harlow and Lane, 1988). The antibodies were affinity purified over a column of the 6× his-HP1 fusion protein coupled to Affigel-10 as described (Harlow and

Lane, 1988). Low affinity antibodies were eluted first with 1.5 M $MgCl_2$, and then high affinity antibodies were eluted with 100 mM glycine, pH 2.0. The immunoaffinity resin was constructed by covalently linking ~3 mg of high affinity antibodies to 1 ml protein A–agarose (Sigma Chemical Co.) with dimethlypimelidate (Harlow and Lane, 1988). A control column was similarly constructed by coupling 3 mg of rabbit IgG (G0261; Sigma Chemical Co.) to 1 ml of the same column matrix.

The fractions from a Sephacryl S-300 26/60 column containing each HP1 oligomer were pooled and equivalent amounts were incubated for 1 h at 4°C with the anti-HP1 immunoaffinity resin and with the control IgG column resin, in parallel. Each batch of resin was washed by rotation with 30 column volumes of buffer A plus 100 mM KCl (wash buffer) (three batch washes with 10 column volumes, 15 min each, at 4°C). Each batch of washed resin was then poured into a column and washed with another 15 column volumes of wash buffer. Proteins were then sequentially eluted from the anti-HP1 columns with the following buffers: Buffer A plus 0.5 M KCl, Buffer A plus 1.0 M KCl, 100 mM glycine, pH 2.0, plus 10% glycerol. All protein bound to the control IgG column was eluted in a single step with 100 mM glycine, pH 2.0, plus 10% glycerol. Immunoblot analysis was performed on the following samples (lanes): (*1*) unfractionated cytoplasmic extract (~200 μg, 1%); (*2*) pool of gel filtration fractions containing each oligomer before it had been applied to anti-HP1 resin (2% total); (*3*) pool of gel filtration fractions after incubation with anti-HP1 resin (2% total); (*4–6*) TCA-precipitate of each eluted fraction from anti-HP1 resin (in entirety); and (*7*) TCA precipitate of protein eluted from control IgG resin. Antibodies prepared against recombinant forms of DmORC2, DmORC6 (Pak et al., 1997), and HP1 that had been expressed in baculovirus or bacteria were used for immunoblot analyses. The immunoblot signals were visualized by enhanced chemiluminescence from an HRP-conjugated anti–rabbit IgG secondary antibody (NA934; Amersham Corp., Arlington Heights, IL).

For immunoaffinity purification of HP1 and associated proteins from the individual salt-extracted nuclear fractions, interphase nuclei were prepared from cycle 14 embryos (2.3–3.3 h after oviposition) and sequentially extracted with 60 mM KCl, 0.5 M KCl, and 1.0 M KCl as described above. Before immunoaffinity purification, the high salt fractions were adjusted to 60 mM KCl by dialysis against a series of buffers with progressively lower KCl concentration. HP1 and associated proteins were then immunoaffinity purified from each fraction using the protocol described above for the immunoaffinity purifications of the cytoplasmic oligomers. Protein was sequentially eluted from the anti-HP1 column with buffer A plus 0.5 M KCl followed by 100 mM glycine, pH 2.0. Protein bound to the control IgG column was eluted in a single step with 100 mM glycine, pH 2.0. Immunoblot analysis was performed on a TCA precipitate of each eluted fraction and a portion (2%) of each salt-extracted fraction before it had been applied to the anti-HP1 resin.

Immunostaining of ORC2 Mutant Chromosomes

For HP1 immunostaining of mitotic chromosomes, brains were dissected from third instar larvae in physiological saline solution (0.7% NaCl). For HP1 immunostaining, the brain tissue was placed in hypotonic solution (0.5% sodium citrate) for 2 min, and fixed in a drop of (5:1:0.5, methanol/acetic acid/distilled water) for 2 min. For GAGA immunostaining, the brain tissue was placed in 0.5% sodium citrate for 10 min, and then fixed in a drop of (5:2:3, methanol/acetic acid/distilled water) for 2 min. The tissue was gently homogenized in the fixative by drawing it through a syringe needle several times before squashing it under a coverslip. The slides were then placed in liquid nitrogen before removing the coverslip, immersed in PBS plus 1% Triton-X for 10 min, and then in a blocking solution of PBS with 1% nonfat dried milk for 30 min. For HP1 staining, a 1:20 dilution of the C1A9 monoclonal was used, whereas for GAGA staining a 1:100 dilution of an affinity-purified polyclonal antibody against GAGA factor was used. The slides were incubated overnight at 4°C with the primary antibody. The slides were then washed (three times for 15 min each) with PBS and incubated for 30 min at room temperature with secondary antibody. FITC-conjugated sheep anti–mouse IgG was used for HP1 immunostaining and FITC-conjugated donkey anti–rabbit IgG was used for GAGA immunostaining. The slides were washed (three times for 15 min each), stained with DAPI (4,6-diamidino-2-phenilindole) at 0.01 mg/ml, and then mounted in antifading medium.

Squashes of polytene chromosomes and HP1 immunostaining were performed as described by James et al. (1989). Chromosome preparations were analyzed using a computer-controlled Axioplan epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a cooled CCD camera (Photometrics, Tucson, AZ). The fluorescence intensity quantitation was performed using NIH Image software. Grayscale measurements (scale of 0–255 units/pixel) were obtained for the HP1 immunofluorescence and DAPI-staining signals in four separate regions (2,500 square pixels) at the chromocenter of 10 different chromosome spreads from wild-type and $k43^1$ homozygous mutant larvae. The gray-scale measurements were subtracted from 255 to obtain intensity measurements, and the ratio of the HP1 signal/DAPI signal for each chromosome spread was determined.

Results

HP1 Fractionates as Multiple Oligomeric Species from a Cytosolic Extract from Early Drosophila Embryos

Early *Drosophila* embryogenesis consists of a rapid series of nuclear divisions within a syncytial cytoplasm. The mitotic divisions occur before the onset of transcription in the embryo and rely upon a pool of maternally produced proteins loaded into the egg during oogenesis. To accomodate the demands of the rapid cell cycle events, the maternal proteins are often pre-assembled into intermediate multi-protein complexes (Kellogg et al., 1992; Zheng et al., 1995; Hirano et al., 1997). We hoped to make use of this feature of the early *Drosophila* embryo to purify a native multi-protein complex containing the heterochromatin-associated protein, HP1. Another feature of the early *Drosophila* embryo can also be exploited in biochemical studies of heterochromatin formation. Because heterochromatin does not become distinct as a cytological entity until the fourteenth nuclear division (Spofford, 1976), it is possible to monitor the process of HP1 assembly into heterochromatin by analyzing changes in the cytoplasmic and nuclear fractions of HP1 during this developmental transition.

We wished to determine if HP1 might be complexed with other proteins in this maternal cytoplasm. Therefore, we prepared an early embryo cytoplasmic extract from it and fractionated it by two different sizing methods. Using immunoblot analysis to monitor HP1 fractionation, we found it to migrate as three distinct oligomeric species during gel filtration chromatography (Fig. 1 *A*) and sucrose density sedimentation (Fig. 1 *B*). Each oligomer in the gel filtration experiment behaved as a species larger than the 23-kD size predicted from the amino acid sequence of HP1. The predominant species had an apparent molecular weight of 80 kD, whereas ~10% of the protein migrated as larger oligomers of 450 and 900 kD. The relative proportion of large to medium oligomer varied from preparation to preparation, suggesting that they may be interrelated. When the gel filtration data were combined with the sedimentation values determined for each species (2.6 S, 11.5 S, and 17.5 S, Fig. 1 *B*), we calculated true molecular weights of 39, 290, and 720 kD for them.

HP1 Oligomers Contain Different HP1 Phosphoisoforms

Eissenberg et al. (1994) showed that HP1 can be separated into eight distinct isoforms with varying degrees of phosphorylation, primarily at serine and threonine residues. They also found a correlation between HP1 phosphorylation and its assembly into cytologically distinct heterochromatin during cycle 14. To determine if HP1 phosphorylation might play a role in its oligomerization in the

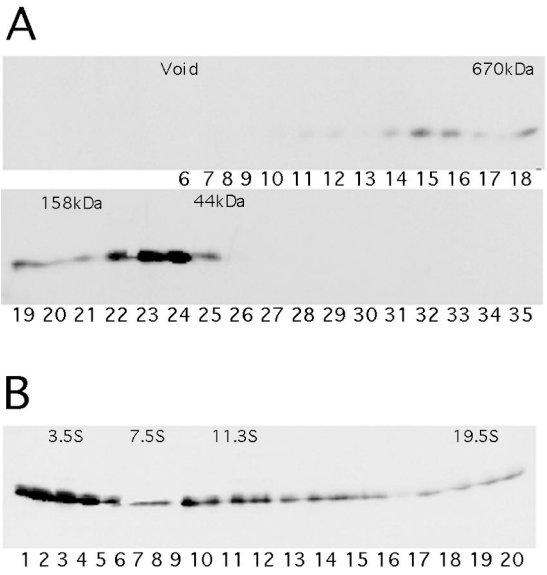

Figure 1. HP1 fractionates as multiple oligomeric species from a cytosolic extract from early *Drosophila* embryos. Immunoblot analyses of (*A*) fractions from a Superose 6 gel filtration column (HP1 oligomers: large, fractions 13–15; medium, fractions 18–20; small, fractions 22–24) and (*B*) fractions from sucrose density sedimentation (HP1 oligomers: large, fractions 17–20; medium, fractions 8–14; small, fractions 1–5). The peak fractions for molecular weight standards: (*A*) thyroglobulin, 670 kD; gamma globulin, 158 kD; ovalbumin, 44 kD; and (*B*) thyroglobulin, 19.5 S; catalase, 11.2 S; aldolase, 7.3 S; and ovalbumin, 3.5 S are indicated.

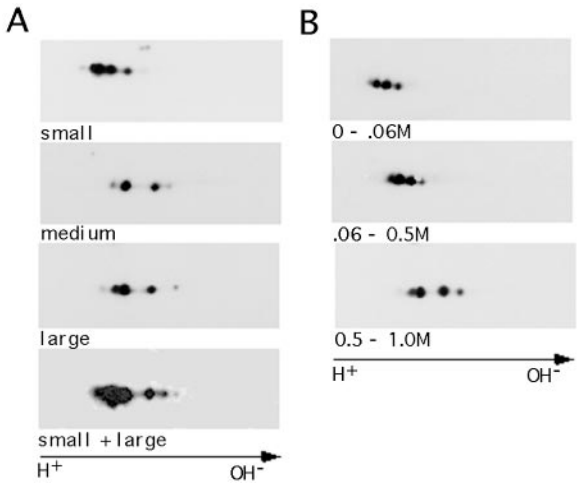

Figure 2. The cytoplasmic oligomers of HP1 and differentially salt-extracted nuclear fractions contain different HP1 phosphoisoforms. Immunoblot analyses of phosphoisoforms in (*A*) small, medium, and large HP1 oligomers, and a mixture of small and large oligomers. (*B*) Immunoblot analyses of HP1 in salt-extracted fractions (*0–0.06 M*, *0.06–0.5 M*, and *0.5–1.0 M* KCl) from cycle 14 interphase nuclei.

cytoplasm we examined the phosphorylation state of HP1 in each oligomer. The gel filtration fractions containing each oligomer were pooled and concentrated before subjecting them to two dimensional electrophoresis. Immunoblotting was then used to visualize the HP1 isoforms in each pooled fraction (Fig. 2 *A*). The isoform profiles of the large- and medium-size oligomers were very similar, but they were distinctly different from that of the small oligomer. Whereas the small oligomer was enriched with highly phosphorylated isoforms (Fig. 2 *A*, *small*), the large (Fig. 2 *A*, *large*), and medium (Fig. 2 *A*, *medium*) species contained an enrichment of underphosphorylated isoforms. When a mixture of the large and small oligomer fractions was subjected to similar analyses, the full spectrum of six to eight phosphoisoforms was obtained (Fig. 2 *A*, *small* and *large*). This experiment confirmed that the profiles of the small and large oligomers were distinct and allowed us to determine the alignment between them. We found the two most basic spots of the small oligomer profile to overlap with the two most acidic spots of the large oligomer profile; the most acidic of the eight spots were uniquely found in the small oligomer and the most basic spots were unique to the large.

Relationship of Cytoplasmic Oligomers to Nuclear Fraction of HP1

We wished to determine what relevance the cytoplasmic oligomers might have to the process by which HP1 is assembled into nuclei. Because a correlation has been found between HP1 phosphorylation and its assembly into heterochromatin (Eissenberg et al., 1994), we decided to compare the phosphoisoform profiles of the cytoplasmic oligomers to that of the nuclear fraction of HP1. Three distinct subpopulations of HP1 can be differentially salt extracted from cycle 14 interphase nuclei (Kellum et al., 1995), the developmental stage at which heterochromatin first becomes a distinct cytological entity (Spofford, 1976). The subpopulations were fractionated by sequentially extracting a preparation of interphase nuclei from cycle 14 embryos with increasing concentrations of potassium chloride. The proteins from sequential extractions (0–60 mM KCl; 60 mM–0.5 M KCl; and 0.5–1 M KCl) were then separated by two-dimensional electrophoresis and immunoblotting was used to visualize HP1 phosphoisoforms. Each fraction was found to contain distinct HP1 phosphoisoforms (Fig. 2 *B*). The most salt-resistant fraction of the protein (Fig. 2 *B*, *0.5–1.0 M*) had a distinctly more basic profile than the fraction that could be extracted with low salt (Fig. 2 *B*, *0–0.06 M*). Interestingly, the isoform profile of the high salt fraction closely resembled those of the two large cytoplasmic oligomers (Fig. 2 *A*, *medium* and *large*). In contrast, the fraction of the protein that could be extracted from nuclei with low salt (0–60 mM) had an isoform profile more closely resembling that of the small oligomer. The majority of HP1 in these nuclei was removed in the medium salt extraction step, and this fraction had an isoform profile that was intermediate to those of the high and low salt-extracted fractions (Fig. 2 *B*, *0.06–0.5 M*).

The Large HP1 Oligomers Contain Drosophila ORC Subunits

The similarity of the isoform profile of the most salt-resistant fraction of HP1 to those of the two large cytoplasmic oligomers suggested that they may be related and that the

HP1 in these oligomers may be targeted for localization into the salt-resistant nuclear sites. As possible intermediates in heterochromatin assembly, they might contain other proteins with which HP1 will be associated in heterochromatin. A number of observations prompted us to investigate whether the large HP1 oligomers contain ORC proteins. The *Drosophila* ORC2 subunit was recently found to have an enriched localization in heterochromatin, and three of the six ORC subunits (ORC1, 3, and 4) were shown to physically interact with HP1 (Pak et al., 1997). These observations, along with the known role for ORC in recruiting Sir1p to the silent loci in budding yeast (Bell et al., 1993; Chien et al., 1993; Fox et al., 1995; Triolo and Sternglanz, 1996) suggested a mechanism by which the large cytoplasmic oligomers might target HP1 into the salt-resistant nuclear sites.

To examine the possibility that the large cytoplasmic oligomers contain ORC subunits we first asked whether they co-fractionate with one of them. To this end, the fractions from the gel filtration experiment were probed with antibodies recognizing the DmORC2 subunit. We found that this subunit did, indeed, co-fractionate with the large HP1 oligomer (Fig. 3 *A*). To determine if ORC proteins were actually components of the large HP1 oligomers, we then immunoaffinity purified each oligomer from the gel filtration fractions. A polyclonal antibody was prepared against a peptide located next to the conserved chromodomain of HP1 for the immunoaffinity purification. In immunoblot analyses, this antibody recognized an HP1 fusion protein and a single 29-kD polypeptide from the unfractionated cytoplasmic extract (Fig. 3 *B*). The gel filtration fractions containing each oligomeric species were incubated with the antibody linked to protein A–agarose beads. As a control, an equivalent volume of each pool of gel filtration fractions was incubated with a similar preparation of a nonspecific IgG linked to agarose beads. After extensive washing (~50 column volumes), the proteins retained on the anti-HP1 column were eluted according to their binding affinities by progressively increasing the stringency of the eluting buffer. The column was first eluted with a buffer containing 0.5 M KCl, followed by one of 1 M KCl, and finally with a mild acid (100 mM glycine, pH 2.0) to disrupt the bond between the antibody and HP1. The protein retained on the control IgG resin was removed by a single elution with glycine, pH 2.0. The elutions from each column were TCA precipitated and subjected to SDS-PAGE analyses. (Fig. 3 *C*, lanes *4–7*). For quantitative comparisons, the gels also contained an aliquot of the unfractionated extract (Fig. 3 *C*, lane *1*) and the gel filtration fractions containing each HP1 oligomer before (lane *2*) and after (lane *3*) binding to the anti-HP1 column. We then used immunoblot analyses to test for the presence of HP1 and DmORC subunits 2 and 6 in the eluted fractions. Both ORC subunits were found to co-purify with HP1 from the gel filtration fractions containing the large HP1 oligomer and from those containing the medium oligomer, but not from the fractions containing the small oligomer (Fig. 3 *C*, lanes *4–6*). Neither ORC subunit nor HP1 was retained on the control IgG column (Fig. 3 *C*, lane *7*). By comparing the immunoblot signals for ORC2 and ORC6 in the gel filtration fractions before they were applied to the anti-HP1 column (Fig. 3 *C*, lane *2*) to their signals in the elution fractions (lane *6*), we estimated that ~6–10% of each ORC subunit was specifically retained on the anti-HP1 column during the purification of the large HP1 oligomer (~4% was tightly retained). By comparing the

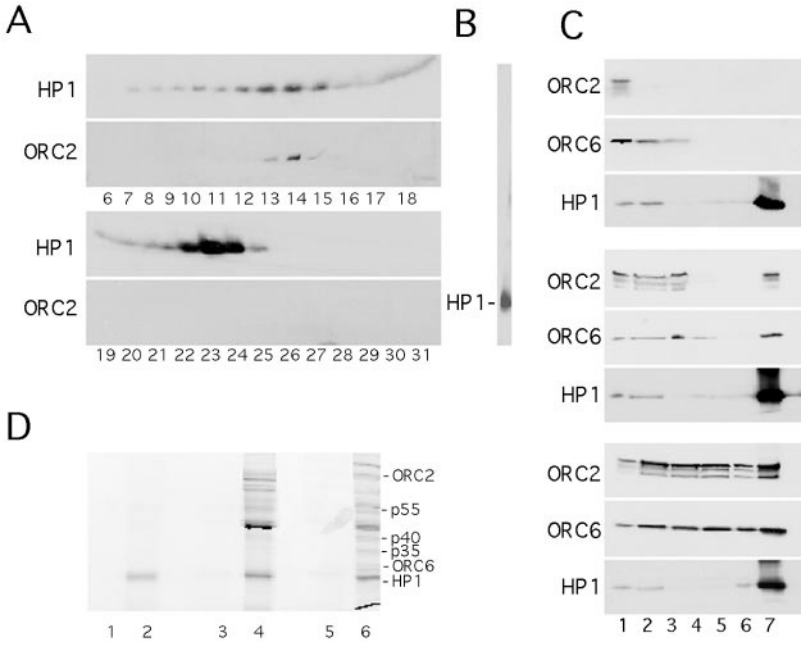

Figure 3. DmORC subunits coimmunoaffinity purify with large and medium HP1 oligomers. (*A*) Immunoblot analyses of fractions from Superose 6 gel filtration column with antibodies that recognize DmORC2 and HP1. (*B*) Immunoblot analyses of unfractionated cytoplasmic extract with antibody prepared against HP1 peptide for immunoaffinity purifications. (*C*) Immunoblot analyses of immunoaffinity-purified small, medium, and large HP1 oligomers with antibodies that recognize DmORC2, DmORC6, and HP1. Each gel is loaded as follows: lane *1*, total cytoplasmic extract, 200 μg; lane *2*, pooled gel filtration fractions applied to anti-HP1 resin, containing small, medium, and large oligomers, respectively, 2% total; lane *3*, flowthrough from each anti-HP1 column, 2% total; lane *4*, 0.5 M KCl elution; lane *5*, 1.0 M KCl elution; lane *6*, 100 mM glycine, pH 2.0 elution (~2 μg protein) from each anti-HP1 column; and lane *7*, total protein eluted from control IgG column. (*D*) Coomassie-stained profile of glycine eluate from control and anti-HP1 columns loaded with gel filtration fractions containing small (lanes *1* and *2*), medium (lanes *3* and *4*), and large (lanes *5* and *6*) oligomers, respectively. Polypeptides corresponding to immunoblot signals for DmORC2, DmORC6 (*ORC2* and *ORC6*), HP1, and three novel polypeptides (*p55*, *p40*, and *p35*) are indicated.

immunoblot signals for the two DmORC subunits in the unfractionated cytoplasmic extract (Fig. 3 *C*, lane *1*, ~200 μg total protein) to their signals in the glycine elution (lane *6*, ~2 μg total protein), we could also estimate that the purification of the large oligomer resulted in an ~1,000-fold enrichment of each ORC subunit. The DmORC subunits were more tightly retained on the anti-HP1 column during the purification of the medium oligomer; both proteins resisted removal by salt and required the weak acid of glycine to be eluted (Fig. 3 *C*, lanes *4–6*).

The protein profile of the glycine eluate from each immunoaffinity purification was also examined by Coomassie blue staining (Fig. 3 *D*). A prominent 29-kD polypeptide that aligned with the immunoblot signal for HP1 was recovered in the glycine elution from each purification (Fig. 3 *D*, lanes *2*, *4*, and *6*). This polypeptide was absent in the eluate from the control IgG column for each purification (Fig. 3 *D*, lanes *1*, *3*, and *5*). The immunoaffinity-purified small oligomer was largely devoid of other polypeptides (Fig. 3 *D*, lane *2*). On the basis of this result and its molecular weight, we anticipate that the small oligomer is a homodimer of HP1. In contrast, Coomassie blue staining of the immunoaffinity-purified large and medium oligomers revealed the presence of other proteins and both similarities and differences in their compositions (Fig. 3 *D*, lanes *4* and *6*). These results suggested that the medium complex may serve as an intermediate in the assembly of the large oligomer or it could result from the in vitro instability of the large oligomer. Both oligomers contained polypeptides in the molecular weight range of five out of six DmORC subunits. The bands corresponding to DmORC2 and DmORC6 were identified by aligning their immunoblot signals from one half of a nitrocellulose filter to which the proteins of each oligomer had been transferred with the Ponceau S–stained protein profile on the other half. Peptide sequence or immunoblot analyses will be required to determine the identities of the remaining polypeptides. The large oligomer differed from the medium one in containing a polypeptide in the molecular weight range of the DmORC1 subunit. Also, unlike the medium oligomer, it contained two polypeptides (p40, p35) with molecular weights distinct from those of any DmORC subunit. Another such polypeptide (p55) was present in both the large and medium oligomers.

Salt-resistant Fraction of Nuclear HP1 Is Associated with DmORC Subunits

The phosphoisoform data described above demonstrate a similarity between the HP1 in the large cytoplasmic oligomers and the salt-resistant fraction of the protein in interphase nuclei. We wished to determine if this nuclear fraction of HP1 was also similarly associated with DmORC subunits. Therefore, we immunoaffinity-purified HP1 and associated proteins from each nuclear fraction. Each salt-extracted fraction was first adjusted to a concentration of 60 mM to allow any protein–protein interactions disrupted during salt extraction to reform and to standardize the conditions for immunoaffinity purification of each fraction. Each fraction was then incubated with the anti-HP1 antibody linked to protein A–agarose. An equivalent volume of each fraction was also incubated with a non-immune IgG linked to protein A as a control. After extensive washing (>50 column volumes), the proteins retained on the anti-HP1 resin were sequentially eluted with 0.5 M KCl, and then 100 mM glycine, pH 2.0. The individual elutions from each purification were precipitated with TCA and subjected to SDS-PAGE. An aliquot of each salt-extracted fraction (1% of total) before it had been applied to the anti-HP1 resin was loaded on the same gel. Immunoblot analyses were used to test for the presence of HP1 and DmORC subunits 2 and 6 in the salt-extracted fractions and in the eluted fractions from each immunoaffinity purification. The immunoblot analyses determined that HP1 and both ORC subunits were present in all three salt-extracted fractions. Nevertheless, the two ORC proteins only co-purified with HP1 from the fraction extracted by high salt (0.5–1 M KCl) (Fig. 4 *A*). This result demonstrates another similarity between this nuclear fraction of HP1 and the large cytoplasmic oligomers.

Changes in HP1 Fractions Accompanying Heterochromatin Assembly

The results described above demonstrate several differences among the three differentially extracted populations of HP1 in interphase nuclei. Besides their different sensitivities to salt extraction, they also contain different levels of phosphorylation and are differentially associated with ORC proteins. The nuclei for these experiments were prepared from embryos at a developmental stage after heterochromatin had become cytologically distinct (cycle 14). We wished to determine if heterochromatin formation during cycle 14 is associated with a change in the relative abundance of the individual HP1 populations. The medium salt-extractable population (Fig. 4 *B*, *cyc14*, lane *2*) was the predominant HP1 fraction in these cycle 14 nuclei, whereas the low salt- (Fig. 4 *B*, *cyc14*, lane *1*) and high salt-extractable populations (Fig. 4 *B*, *cyc14*, lane *3*) each constituted minor fractions.

When we compared the relative abundance of each fraction in cycle 14 nuclei to that in pre-cycle 14 nuclei, we found that the medium salt-extractable fraction did not become the major fraction until cycle 14 (Fig. 4 *B*,

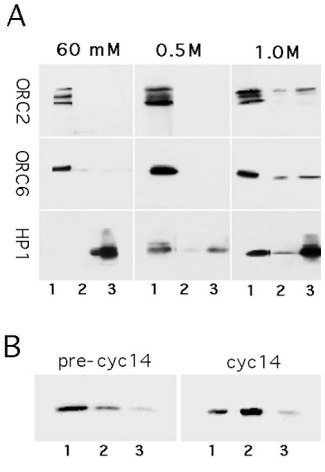

Figure 4. Distinct nuclear fractions of HP1 and their associations with DmORC proteins. (*A*) Immunoblot analyses of immunoaffinity-purified fractions from salt-extracted interphase nuclei (*60 mM*, *0.5 M*, and *1.0 M*) with antibodies that recognize HP1, DmORC2, and DmORC6. Each lane contains: (*1*) 4 μg (1%) of each salt-extracted nuclear fraction applied to anti-HP1 resin; (*2*) 0.5 M KCl elution; and (*3*) 100 mM glycine, pH 2.0, elution from anti-HP1 column. (*B*) Quantitation of HP1 in salt-extracted nuclear fractions: (*1*) 0–60 mM KCl; (*2*) 60 mM–0.5 M KCl; and (*3*) 0.5–1 M KCl from pre-cycle 14 and cycle 14 embryos.

pre-cyc14, lane *2*). Before this time, the least salt-resistant population of the protein was the major fraction (Fig. 4 *B*, *pre-cyc14*, lane *1*). During cycle 14, the low salt fraction decreased in abundance with a concomitant increase in the medium salt fraction (Fig. 4 *B*, lanes *1* and *2*). We also noted that a significant fraction of HP1 was present in the most salt-resistant form that is ORC associated before heterochromatin becomes fully formed during cycle 14 (Fig. 4 *B*, *pre-cyc14*, lane *3*).

Heterochromatic Localization of HP1 Is Affected in ORC2 Mutants

Since our studies showed a physical association of ORC proteins with HP1 in specific cytoplasmic and nuclear fractions, we wished to determine if the localization of HP1 into heterochromatin would be influenced by a mutation in one of the ORC subunits. Mutants for the gene encoding the DmORC2 subunit (*k43*) were recovered in a screen for larval lethal mutants with mitotic defects (Gatti and Baker, 1989). The *k43* mutant displayed a phenotype of diploid larval disks with an abnormally low mitotic index and irregularly condensed and fragmented chromosomes. The gene affected in this mutant was recently found to encode the DmORC2 subunit (Landis et al., 1997). To determine whether a mutation in the ORC2 subunit would affect the localization of HP1 into heterochromatin we immunostained chromosomes from homozygous *k43* mutant larvae. They were compared with the immunostaining in heterozygous sibling and wild-type larvae. For this study, the fixation conditions were optimized to preserve an enriched localization of HP1 in the heterochromatic regions of diploid mitotic chromosomes as well as interphase nuclei.

Squashes of diploid larval brain discs were prepared from individuals homozygous for $k43^{1}$ or $k43^{\gamma 4}$ and immunostained with the C1A9 mAb that recognizes HP1 (James and Elgin, 1986; James et al., 1989). Homozygous mutant larvae could be discriminated from their heterozygous siblings carrying a balancer chromosome with a dominant larval marker (TM6B). Comparisons of the immunostaining in homozygous individuals (n = 30), heterozygous individuals (n = 10), and wild-type controls (n = 10) revealed that the localization of HP1 into the heterochromatin of interphase nuclei (Fig. 5 *A*) and mitotic chromosomes (Fig. 5 *B*) is strongly perturbed in the ORC2 homozygous mutant larvae. In interphase nuclei, a punctate pattern of enriched HP1 staining was observed within the heterochromatic regions marked by intense DAPI-staining in the AT-rich satellite repeat DNA sequences (Fig. 5 *A*, *a*). HP1 staining was also highly enriched in the centric heterochromatin of mitotic chromosomes and

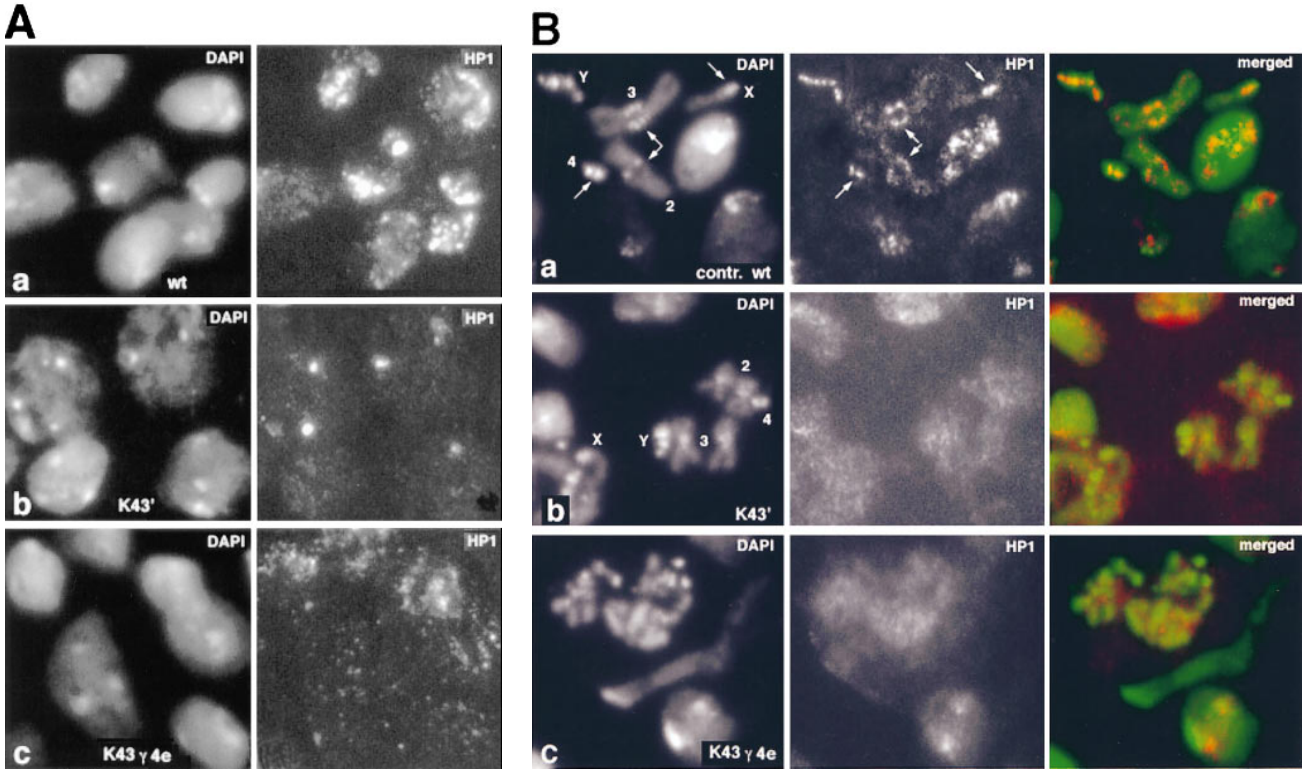

Figure 5. Heterochromatic localization of HP1 in diploid nuclei is perturbed in mutants for ORC2 gene. (*A*) Interphase nuclei from (*a*) wild-type or heterozygous *k43* larvae; (*b*) homozygous $k43^{1}$ larvae; and (*c*) homozygous $k43^{\gamma 4}$e larvae, stained with DAPI (*left panels*) and immunostained with antibodies that recognize HP1 (*right panels*). (*B*) Metaphase chromosomes from (*a*) wild-type or heterozygous *k43* larvae; (*b*) homozygous $k43^{1}$ larvae; and (*c*) homozygous $k43^{\gamma 4}$e larvae, stained with DAPI (*left panels*) and immunostained with antibodies that recognize HP1 (*center panels*); pseudo-color merged images (*right panels*), HP1-immunostaining (*red*) and DAPI-staining (*green*). Individual chromosomes are indicated (*2*, *3*, *4*, *X*, and *Y*).

along the heterochromatic Y chromosome from wild-type and heterozygous ORC2 mutant larvae (Fig. 5 *B*, *a*). The HP1 staining was more diffusely localized throughout the interphase nuclei from larvae that were homozygous for either allele of the ORC2 gene (Fig. 5 *A*, *b* and *c*). The defect in the localization of HP1 into interphase heterochromatin appeared to be more severe in the $k43^{\gamma 4}$ allele, which has a frameshift in the ORC2 protein coding region beginning at amino acid 41 (Fig. 5 *A*, *c*). In a few nuclei from $k43^{1}$ homozygous larvae, we observed an abnormal pattern of HP1 localized into a single bright spot (Fig. 5 *A*, *b*). The localization of HP1 into the heterochromatin of mitotic chromosomes was also perturbed in homozygous mutant larvae (Fig. 5 *B*, *b* and c). Instead of the punctate pattern of enriched HP1 staining observed in these regions of the chromosomes from wild-type larvae, the protein was diffusely localized throughout the nucleus and cytoplasm in the homozygous mutants.

We wished to determine if the perturbation in HP1 staining in the ORC2 mutant might reflect a disruption in the ORC-dependent replication of heterochromatic sequences rather than a role for ORC in recruiting HP1 into heterochromatin. To this end, we immunostained chromosomes from the ORC2 mutant larvae with antibodies that recognize a different heterochromatin-associated protein, GAGA factor. Unlike HP1, GAGA factor is thought to bind directly to satellite DNA sequences (AAGAG and AAGAGAG repeats) that are concentrated in heterochromatin (Raff et al., 1994). Thus, a perturbation in its localization might reflect a disruption in the replication of heterochromatic DNA. From comparisons of GAGA immunostaining on homozygous mutant ($n = 16$), heterozygous mutant ($n = 6$), and wild-type ($n = 6$) chromosomes, we concluded that the ORC2 mutation caused little perturbation in the localization of GAGA into heterochromatin (Fig. 6). Prominent sites of GAGA immunostaining were observed in the centric heterochromatin of the second chromosome and in regions distributed throughout the Y chromosome from wild-type larvae. A similar pattern of GAGA immunostaining was observed in the centric heterochromatin of chromosome 2 from homozygous *k43* mutant larvae, even in chromosomes that displayed an abnormal condensation phenotype (Fig. 6 *C*, *2R* and *2L*). In many nuclei from the homozygous mutant larvae, the GAGA staining on the Y chromosome was also unaffected (Fig. 6, *inset*). In some nuclei, however, we did observe a slight reduction in the area of the GAGA-staining regions along the Y chromosome (Fig. 6 *B*, *arrows*). This small effect on GAGA staining, nevertheless, was modest in comparison to the perturbation we observed in HP1 localization along this and other chromosomes.

Tower and co-workers (Landis et al., 1997) reported that the polytene tissues of all *k43* mutant alleles have normal size and levels of ploidy, indicating that the maternally

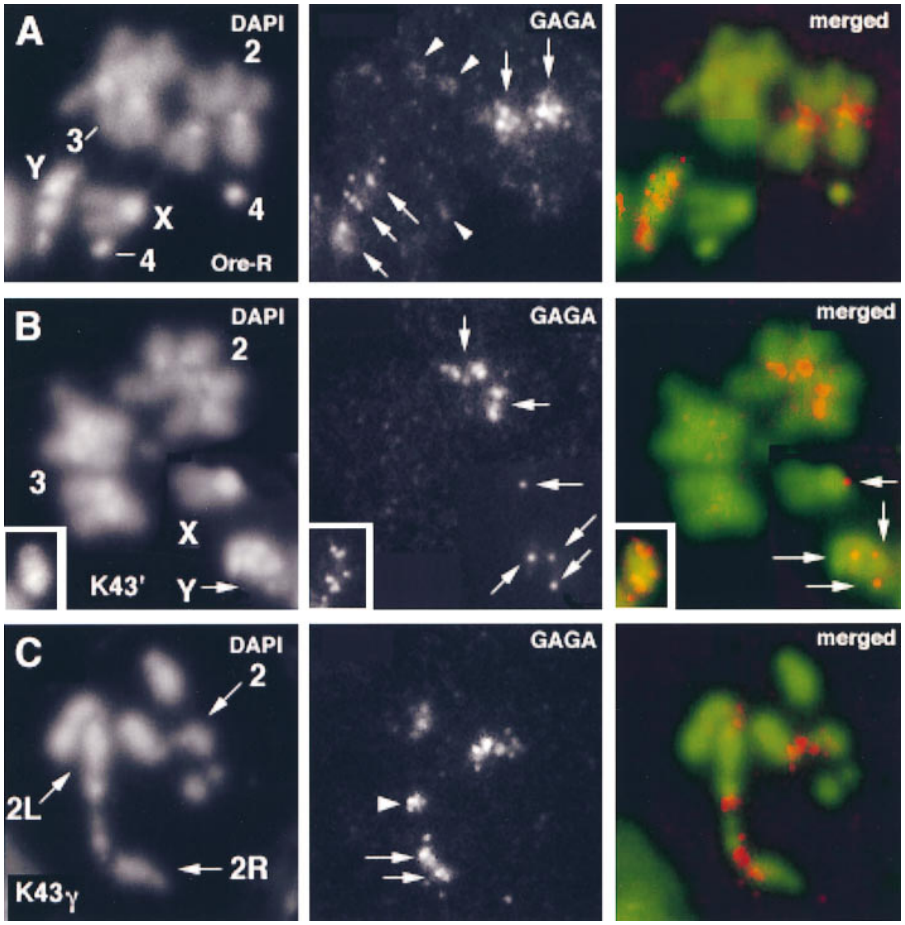

Figure 6. ORC mutations have minimal effect on GAGA localization into heterochromatin. Metaphase chromosomes from (*A*) wild-type or heterozygous *k43* larvae; (*B*) homozygous $k43^{1}$ larvae (representative Y chromosome exhibiting: normal [*inset*] and abnormal [*arrow*] GAGA localization); (*C*) homozygous $k43^{\gamma 4}$e larvae stained with DAPI (*left panels*) and immunostained with antibodies that recognize GAGA (*center panels*); pseudo-color merged images (*right panels*), GAGA-immunostaining (*red*) and DAPI-staining (*green*). Individual chromosomes are indicated (*2*, *3*, *4*, *X*, and *Y*).

contributed ORC2 protein is sufficient to support these tissues or that ORC has no role in DNA replication in polytene cells. Upon examination of the polytene chromosomes from homozygous *k43* mutant larvae, we also found no effect on the polytenization of chromosomes from the $k43^{1}$ mutant larvae (Fig. 7 *b*). We did, however, observe evidence of poor polytenization in the $k43^{\gamma 4}$ mutant (Fig. 7 *c*). The intensity of the HP1 immunofluorescence signal also appeared to be reduced at the chromocenter in both mutant alleles. We quantitated the HP1 signal relative to the signal for DAPI staining in $k43^{1}$ homozygotes, which lacked any apparent defect in polytenization. The DAPI-staining signal at the chromocenter was unaffected in this mutant allele in comparison to wild type. However, after the HP1 immunofluorescence signal was normalized against the DAPI signal, we found it to be reduced (16%) in the homozygous mutant (average ratio 1.46 ± 0.2, $n = 10$) in comparison to wild type (average ratio 1.74 ± 0.1, $n = 10$).

Discussion

HP1 is a highly conserved heterochromatin associated protein (James and Elgin, 1986; Singh et al., 1991; Saunders et al., 1993; Allshire et al., 1995), yet because it has no known DNA-binding activity, little is understood about how it is incorporated into the heterochromatin of any organism. To address this question, we have undertaken a biochemical analysis of distinct cytoplasmic and nuclear fractions of the protein in *Drosophila* embryos. We have immunoaffinity purified three oligomeric species of HP1 from the maternally loaded cytoplasm of the early embryo and found that the two larger oligomers specifically contain underphosphorylated isoforms of HP1 and *Drosophila* ORC subunits. ORC is required to initiate DNA replication from specific DNA-binding sequences (ARS) (Bell and Stillman, 1992). It also has a role in establishing silencing in budding yeast by recruiting the Sir1 protein to ARS

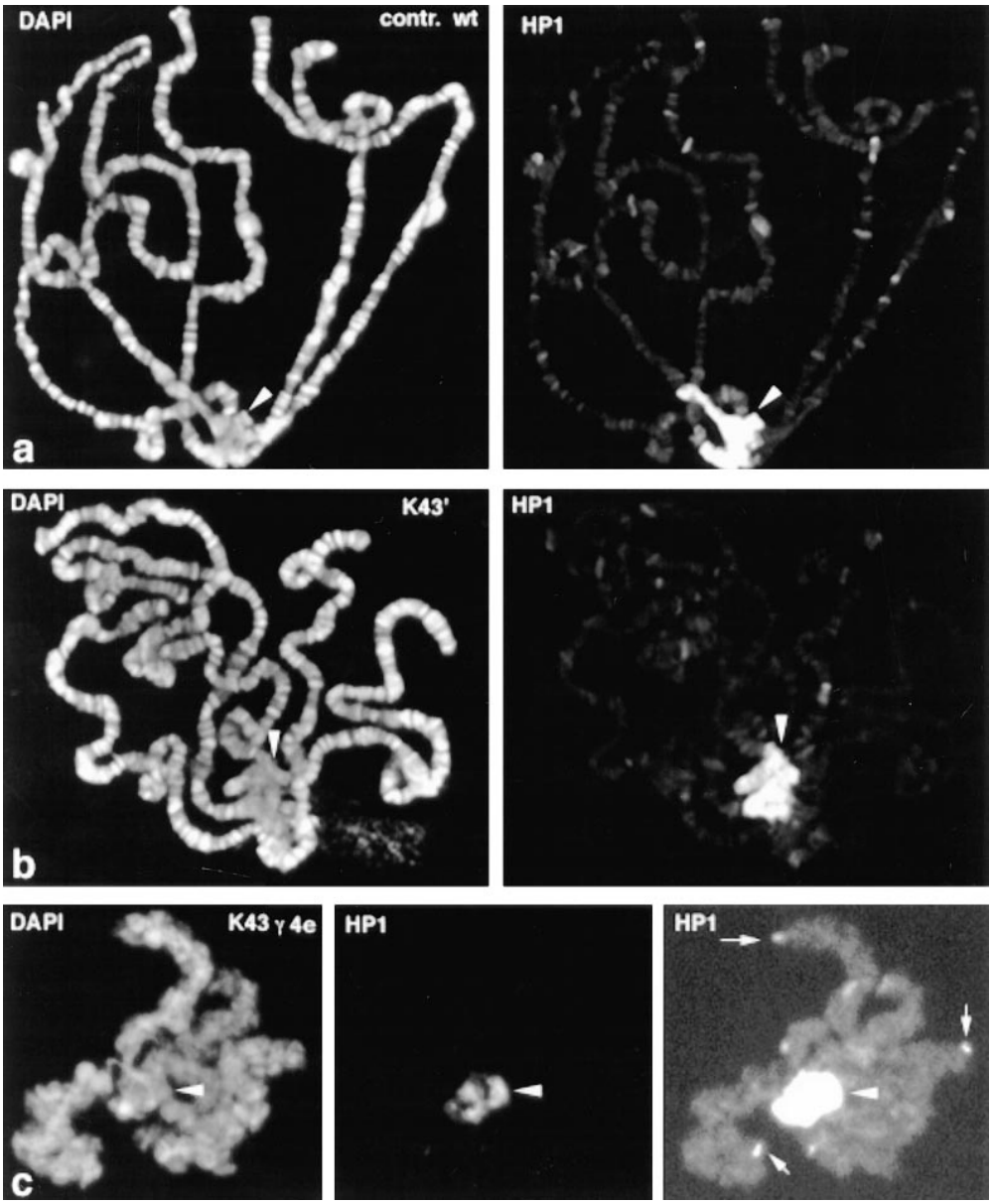

Figure 7. Polytenization of chromosomes and HP1 enrichment at the chromocenter of polytene chromosomes is affected by ORC2 mutations. HP1 immunolocalization (*right panel*) on DAPI-stained polytene chromosomes (*left panel*) from: (*A*) wild-type larvae (*arrowhead* indicates the chromocenter); (*B*) $k43^{1}$-homozygous mutant larvae; and (*C*) $k43^{\gamma 4}$e homozygous mutant larvae (enhanced exposure of HP1 immunostaining in *far right panel*, showing reduced HP1 signal at euchromatic sites of HP1 localization also).

elements located within the silencing nucleation sites (Bell et al., 1993; Chien et al., 1993; Fox et al., 1995, 1997; Triolo and Sternglanz, 1996). The cytoplasmic fraction of HP1 associated with the large oligomers resembles the most tightly bound nuclear fraction of the protein in phosphorylation state and a similar association with ORC subunits. We propose that ORC functions to target HP1 to these nuclear binding sites by a process that is analogous to the recruitment of the Sir1 protein to yeast silencing nucleation sites. Our finding that the heterochromatic localization of HP1 is perturbed in mutants for the ORC2 gene supports this model and indicates that this is the basis for the suppressor of variegation phenotype of ORC2 mutants (Pak et al., 1997).

The role of ORC in yeast silencing is genetically separable from its function in DNA replication. Specific alleles of the ORC2 and ORC5 genes can function in DNA replication but not in silencing, and vice versa (Fox et al., 1995; Bell et al., 1993; Dillin and Rine, 1997). Whether the heterochromatin assembly and DNA replication functions for ORC are also separable in *Drosophila* is not known. If they are, it is puzzling that the DNA-binding activity of ORC has been specifically co-opted for the recruitment of proteins into heterochromatin and that this function has been conserved even though the specific heterochromatin proteins involved have not been.

Possible Roles for Specific Nuclear Fractions of HP1

Our model predicts that the ORC-associated underphosphorylated HP1 isoforms in the early cytoplasm are targeted for ORC-binding sites in heterochromatin. When bound to these nuclear sites they may function to nucleate heterochromatin assembly. Consistent with such a specialized role for these phosphoisoforms, they constitute a minor fraction of the protein in interphase nuclei. At the silencing nucleation sites in yeast, the ORC-binding sequences are flanked by binding sites for the RAP-1 and ABF-1 proteins. These proteins act in conjunction with ORC and Sir1p to recruit the Sir3 and Sir4 proteins to the nucleation sites (Kurtz and Shore, 1991; Hecht et al., 1995; Moazed et al., 1997). The ORC-binding sites in *Drosophila* heterochromatin may be similarly flanked by binding sites for other DNA-binding proteins. These proteins may serve dual functions: one in specifying that the HP1-associated ORC binds only to ORC-binding sites in heterochromatin and another in recruiting other heterochromatin proteins to the region. Candidates for such proteins include a possible *Drosophila* homologue of RAP-1 (presented by Strausbaugh, L., M. Crayton, M. Sommer, and A. Baldo at the 39th Annual *Drosophila* Research Conference in Washington, DC, on March 25–29, 1998), GAGA factor (Kurtz and Shore, 1991; Raff et al., 1994; Granok et al., 1995), and the novel polypeptides that co-purified with the large cytoplasmic oligomer of HP1 (p55, p40, and p35). The ORC-binding sites in *Drosophila* heterochromatin are likely to be interspersed with a complex array of heterochromatic DNA sequences, with the secondary structure or repetitive nature of the sequences possibly playing an important role (Dorer and Henikoff, 1994; Le et al., 1995).

The function of the most abundant HP1 fraction that is more highly phosphorylated and can be extracted from nuclei by medium salt is a bigger puzzle. We observed a specific increase in the abundance of this fraction during cycle 14, the stage when heterochromatin first becomes a distinct cytological entity (Spofford, 1976). This result indicates that this fraction also plays a prominent role in heterochromatin assembly. Mutants for the ORC2 subunit exhibited a general disruption in the localization of HP1 throughout the heterochromatin, suggesting that ORC is required to localize not only the underphosphorylated isoforms with which it is physically associated, but also the more highly phosphorylated isoforms. These isoforms may be found in the heterochromatin that is extended from the ORC-associated underphosphorylated isoforms. This proposed function may be more analogous to that of the Sir3 protein in budding yeast. Among the Sir proteins, it has the unique ability to spread from the telomeres into flanking euchromatin when over-expressed (Renauld et al., 1993; Hecht et al., 1996). In view of its abundance, this fraction of HP1 may also be involved in many of the protein–protein interactions within heterochromatin or between the heterochromatin and other nuclear structures, for example, lamin B receptor (Ye and Worman, 1996), *Su(var)3-7* gene product (Cleard et al., 1997), and actin related protein 4 (Arp4) (Frankel et al., 1997).

Heterochromatin Assembly and Phosphorylation

Our results indicate a specific role for the underphosphorylated HP1 isoforms in heterochromatin assembly. On the surface, this appears to be at odds with the evidence that increased phosphorylation of HP1 accompanies its assembly into heterochromatin during cycle 14 (Eissenberg et al., 1994). Our studies differed from this previous study in that we examined phosphorylation levels of specific fractions of HP1 in interphase nuclei, whereas the previous study was conducted on the total pool of HP1 in embryos. Our finding that the highly phosphorylated medium salt-sensitive fraction increases in abundance during cycle 14 does indeed support a role for HP1 phosphorylation in heterochromatin assembly. That dephosphorylation of some heterochromatin component is also important for heterochromatin formation is demonstrated by the *Su(var)* phenotype for a gene that encodes a type 1 serine-threonine phosphatase (Baska et al., 1993). The nucleating function we propose for the minor underphosphorylated fraction of HP1 could partially account for this phosphatase requirement.

Pleiotropic Effects of ORC2 Mutation

The ORC protein complex was purified from yeast as an activity that binds to ARS elements in vitro and has been shown to be required to initiate DNA replication in vivo (Bell and Stillman, 1992; Bell et al., 1993; Fox et al., 1995). The earliest mutant to be recovered in an ORC subunit came from a *Drosophila* screen for larval lethal mutants displaying mitotic defects (Gatti and Baker, 1989). The *k43* mutant from this screen exhibited small or missing diploid imaginal disks and was only recently shown to encode the DmORC2 subunit (Landis et al., 1997). Landis et al. (1997) noted that the mitotic divisions in the early embryo were unaffected in each of multiple alleles of the *k43* gene, suggesting that the animals are able to survive

until late larval stages with the maternally loaded ORC2 protein. The mitotic defects then become apparent, as the maternal pool of protein becomes gradually depleted.

We examined the heterochromatic localization of HP1 in two different mutant alleles of the *k43* gene. The defect we observed for the localization of HP1 into the heterochromatin of diploid cells was apparently one of the earliest phenotypes to become visible as the maternal supply neared depletion. We did not observe a similar perturbation in the heterochromatic localization of GAGA factor in homozygous mutant larvae, indicating that the replication of heterochromatic binding sequences for GAGA factor was relatively unaffected by the ORC2 mutation. From these results, we conclude that the defect in HP1 localization reflects a role for ORC in recruiting HP1 into heterochromatin rather than an under-representation of heterochromatic DNA.

Landis et al. (1997) did not observe a replication defect in the polytene tissues of any mutant allele. This led them to speculate that either the maternal ORC2 protein is sufficient to support all ORC-related functions in polytene cells or it is not required for replication in these cells. We were able to see a less-pronounced effect of the ORC2 mutations on both the polytenization and the heterochromatic localization of HP1 in polytene tissues. Because immunolocalization experiments have shown that the ORC2 protein is present on polytene chromosomes (Pak et al., 1997), we conclude that ORC is probably also required for replication in polytene tissues. The defects in polytene cells may be less pronounced than in diploid cells, because the maternal pool of ORC2 protein is less effectively depleted in polytene cells undergoing multiple rounds of replication without intervening cell divisions.

The irregular condensation and fragmentation of chromosomes observed in *k43* mutants could be a consequence of incomplete DNA replication, or they could reflect a role for ORC in heterochromatin formation. Interestingly, a number of heterochromatin-associated proteins exhibit a similar chromosome condensation phenotype (Kellum and Alberts, 1995; Bhat et al., 1996; Török et al., 1997). The relationship between heterochromatin and mitotic chromosome condensation is not understood. However, this phenotype might reflect a role for heterochromatin, as the latest replicating portion of the nucleus (Lima de Faria and Jaworska, 1968; Newlon, 1988), in coordinating the completion of S phase with entry into mitosis. Such a role for heterochromatin might also account for the conserved linkage between heterochromatin assembly and ORC function.

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