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Heterochromatin protein 1 distribution during development and during the cell cycle in *Drosophila* embryos

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SUMMARY

Heterochromatin protein 1 (HP1) was initially discovered as a protein that is associated with the heterochromatin at the chromocenter of polytene chromosomes in *Drosophila* larval salivary glands. In this paper we investigate the localization of heterochromatin protein 1 in the diploid nuclei of *Drosophila* embryos. We focus on its association with the interphase heterochromatin in fixed embryos before and during cycle 14, the developmental time at which heterochromatin becomes most conspicuous, and also follow its localization during mitosis. The GAGA transcription factor was recently shown to be localized at sequences within α-heterochromatin in pre-cycle 14 embryos, and an antibody against this protein serves as a convenient marker for these sequences.

We find an enrichment of heterochromatin protein 1 in the intensely DAPI-staining regions near the apical surface of nuclear cycle 10 embryos. At this stage GAGA factor is localized into punctate structures in this same region. This enrichment for HP1 is markedly increased during nuclear cycle 14.

Surprisingly, whereas GAGA factor retains its association with the heterochromatin throughout the cell cycle, a significant fraction of HP1 is dispersed throughout the spindle around the segregating chromosomes during mitosis. This dispersed pool of heterochromatin protein 1 was observed during mitosis in both early and late *Drosophila* embryos and in an analysis of a bacterially produced 6× histidine-heterochromatin protein 1 fusion protein injected into living *Drosophila* embryos. When *Drosophila* tissue culture cells were prepared by a method which removes soluble protein and avoids fixation of the mitotic chromosomes, an enrichment for heterochromatin protein 1 in the heterochromatin of the chromosomes was discovered also.

Key words: heterochromatin protein 1, cell cycle, *Drosophila*, embryo, polytene chromosome, salivary gland

INTRODUCTION

The centromeres of virtually all higher eukaryotes are organized into a highly compact type of chromatin, resembling a level of organization in the metaphase chromosome, throughout the cell cycle (Heitz, 1928; Rattner and Lin, 1988). This chromatin (termed heterochromatin) differs in this respect from the bulk of the chromatin (euchromatin), which cycles between condensed and decondensed states. In the interphase nucleus, the heterochromatin is characteristically localized at the periphery of the nucleus and surrounding the nucleolus (Bouteille et al., 1983; Hancock and Boulikas, 1982; Franke et al., 1981; Brown, 1966).

The early embryonic development in *Drosophila* (Edgar et al., 1986), amphibians (Gurdon and Woodland, 1968; Newport and Kirschner, 1982), birds (Olszanska et al., 1984), and mammals (Bolton et al., 1984) is characterized by a period of rapid cell/nuclear cycles, little or no transcription, and a homogeneous appearance of the chromatin. Each of these embryos goes through an analogous transition period that involves a slowing of the cell cycle, the onset of transcription of most genes, and an increased heterogeneity in chromatin appearance. This transition takes place during nuclear cycles 10 to 14 in *Drosophila* (Edgar and Schubiger, 1986). The change in appearance of the chromatin during this transition might be partially attributed to histone H1 replacing the HMG-D protein (Ner and Travers, 1994). Associated with this change in chromatin organization is the appearance of a conspicuous chromocenter, at which the centromeric regions of each of the diploid chromosomes are clustered on the apical side of the nuclear envelope (Rabinowitz, 1941; Mahowald, 1968).

The distinct properties of heterochromatin are thought to be due to a specialized nucleoprotein organization in these regions. While euchromatin is enriched with unique coding DNA sequences, heterochromatin consists largely of repetitive DNA sequences (Miklos and Cotsell, 1990). Recent evidence suggests that many different repeated DNA sequences can induce heterochromatin formation presumably through their pairing to form specific topological structures (Dorer and Henikoff, 1994). Proteins that are associated with heterochromatin-specific DNA sequences have been found in both *Drosophila* and mammals. One of these, heterochromatin protein 1 (HP1), is present in *Drosophila* (James and Elgin, 1986), mice, and humans (Singh et al., 1991; Saunders et al.,

1993). This protein was first identified in *Drosophila* by virtue of its localization at the chromocenter of polytene chromosomes from larval salivary glands (James and Elgin, 1986). In addition, in *Drosophila melanogaster*, the GAGA transcription factor (Biggin and Tijan, 1988) is associated with the centromeres of mitotic chromosomes. Its localization is strikingly similar to the locations of two highly repeated satellite sequences at the centromeres (Raff et al., 1994; Lohe et al., 1993). These two satellites contain repeats of sequences to which GAGA factor has been shown to bind in vitro (Tsukiyama et al., 1994), and it is likely that this protein directly recognizes this DNA at the centromeres.

The mouse homologue of HP1 was recently shown to be located at the centromeres of mitotic chromosomes from mouse L cells (Wreggett et al., 1994) Whether HP1 is also located at the centromeres of *Drosophila* mitotic chromosomes has been unknown. Polytene chromosomes are derived from specialized cells that undergo multiple rounds of replication without chromosome segregation, and thus they are very different from the chromosomes of mitotically active cells. In particular, each region of the polytene chromosome has been replicated to different extents (Ashburner, 1980). The chromocenter consists of both α- and β-types of heterochromatin (Heitz, 1928). The α-heterochromatin, which is composed almost entirely of highly repetitive satellite DNA sequences (Miklos and Cotsell, 1990), is severely underreplicated in polytene chromosomes and therefore virtually invisible (Gall, 1973). The β-heterochromatin is composed of both middle repetitive elements and unique coding sequences, and it is replicated to almost the same level as euchromatin (Miklos and Cotsell, 1990). HP1 is distributed throughout the β-heterochromatin in the chromocenter (James et al., 1989). Whether it is also present in α-heterochromatin could not be determined because of the relative underrepresentation of these sequences in the chromocenter. Because the satellite DNA, of which α-heterochromatin is composed, constitutes up to 20% of the diploid genome, it is visible as large blocks on mitotic chromosomes. Therefore, if HP1 is also present in α-heterochromatin, it should be visible on *Drosophila* mitotic chromosomes.

In this paper we determine the intracellular locations of HP1 before and after the developmental changes that occur between cycles 10 and 14 in *Drosophila* embryos and investigate its presence on mitotic chromosomes in embryos and in tissue culture cells. As a marker for the centromere throughout the cell cycle, we have used the location of GAGA factor. Our results are consistent with HP1 being localized both in the heterochromatin of mitotic chromosomes and dispersed around the segregating chromosomes.

MATERIALS AND METHODS

Immunostaining

Embryos were fixed for five minutes in 37% formaldehyde followed by one hour in methanol/EGTA according to the method of Kellogg et al. (1988). Embryos were also fixed with methanol/EGTA only (Mitchision and Sedat, 1983) and with a mixture of 62% ethanol, 8% acetic acid, and 5% formaldehyde equilibrated in heptane (Wieschaus and Nusslein-Volhard, 1986). The fixed embryos were immunostained with a mouse monoclonal antibody against HP1 (1:200 dilution) kindly provided by S. C. R. Elgin (James and Elgin, 1986) and a mouse monoclonal antibody against histones (Chemicon, Catalog # MAB052) (1:10 dilution) that was directly labeled with *N*-hydroxysulfosuccinimide rhodamine using the procedure of Blakeslee and Baines (1976). Fixed embryos were also stained with an antibody prepared against GAGA factor in rabbits, which had been affinity purified against a bacterially produced fusion protein between glutathione-S-transferase and GAGA factor (1:500 dilution). Anti-HP1 immunostaining was detected with a fluorescein-labeled anti-mouse IgG secondary antibody (Cappel Laboratories) and anti-GAGA factor immunostaining was detected with a rhodamine-labeled anti-rabbit IgG secondary antibody (Cappel Laboratories) (1:500 dilution). Both secondary antibodies were preabsorbed to *Drosophila* embryos to reduce non-specific staining to minimal levels. Embryos co-stained with anti-HP1 and anti-GAGA factor antibodies were also stained with the DNA-specific stain, 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) to allow cell cycle and developmental staging. Embryos were also stained with each antibody alone to insure that a given staining pattern in a co-immunostaining experiment was specific for a given antibody and not the result of bleed-through from the second antibody. Also, the unknown in all double-label experiments (HP1) was detected with the fluorescein-label, the chromophore that is most likely to bleed into the second channel.

Schneider tissue culture cells were cultivated in D-22 insect medium supplemented with 10% fetal calf serum on glass coverslips. The cells were fixed for five minutes in 3.0% formaldehyde, 0.1% glutaraldehyde in PBS (150 mM NaCl and 50 mM sodium phosphate, pH 7), followed by several five minute washes in PBS plus 0.1% Triton X-100. The fixed cells were then stained with a 1:200 dilution of the monoclonal supernatant against HP1 and a 1:500 dilution of the affinity purified rabbit polyclonal antibody against GAGA factor. Immunostaining was detected with a fluorescein-labeled anti-mouse IgG and a rhodamine-labeled anti-rabbit IgG (1:500 dilution) (Cappel Laboratories). Unfixed mitotic chromosomes were also prepared from Schneider tissue culture cells by the method of Wreggett et al. (1994) and stained with anti-HP1 antibodies.

Immunostaining was visualized by laser scanning confocal microscopy using a Nikon optiphot fluorescence microscope equipped with the Bio-Rad MRC-600 laser scanning confocal attachment. All images were collected using a Nikon 60× Plan Apo lens with a numerical aperture of 1.4 and a two or three-fold zoom. The MRC-600 Confocal Microscope Operating Software (CoMOS) Version 6.03 (Bio-Rad Microscience Ltd, 1992-92) was used in processing the images. A Sony color video printer UP5100 was used to print color photographs of the confocal images.

Fluorescence intensity quantitation

The histogram program of the CoMOS operating software was used to quantitate fluorescence intensity of the HP1 immunofluorescence signal. For Table 1, the intensity level was determined for four nuclei at the surface of the embryo and four nuclei at the nuclear midline of each of ten embryos during cycle 10 and during cycle 14. The nuclear midline was determined to be the point at which the nuclear diameter was greatest; the apical surface intensity was taken in the plane above the midline at which the intensity of the signal was greatest (usually in a plane where the diameter was ~1/2 the diameter at the midline). The apical surface/nuclear midline (A/NM) ratio was determined for each embryo from the average intensities in the four nuclei at the surface and in the four nuclei at the nuclear midline. The A/NM ratio for the anti-histone immunofluorescence signal was similarly obtained. These intensity values were compared only within a single embryo in order to provide an internal control for the fluctuations in overall intensities between embryos. The *t*-test was applied to determine that the ratio of the intensity of the HP1 signal is significantly different from that for the histone signal and that this ratio is significantly different in cycle 10 and cycle 14 embryos within a 99.995% confidence limit.

Quantitation of HP1 levels by western blot analysis

HP1 levels in the soluble and pellet fractions from cycle 14 interphase nuclei were determined by western blot analysis using the mouse monoclonal antibody against HP1. Nuclei were prepared by the method of Udvardy et al. (1985) from a 0-1 hour collection of embryos that was aged for 3 hours. The nuclei were resuspended in a buffer of 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethyl sulfony fluoride, 2 mM benzamidine HCl, and a 1:100 dilution of a protease inhibitor cocktail (Kellogg et al., 1989). The salt concentration of aliquots of the nuclear suspension was then adjusted to 150 mM KCl, 500 mM KCl, and 1.5 M $MgCl_2$ and centrifuged for 15 minutes in a TL-100 Beckman centrifuge at 16,000 ***g***. The pellet fraction was resuspended in a volume equal to that of the supernatant fraction and equal volumes were electrophoresed in an SDS-polyacrylamide gel. The protein bands were transferred to nitrocellulose for western blot analysis using the mouse monoclonal antibody against HP1 (kind gift from S. C. R. Elgin (James and Elgin, 1986) as probe.

Live analysis of HP1 localization

A cDNA encoding HP1 was obtained by polymerase chain reaction from the Nick Brown cDNA library (Brown and Kafatos, 1988). Using the published sequence for the gene encoding HP1 (Eissenberg et al., 1990), we synthesized an oligonucleotide complementary to the 5′ end of the gene (GCGCGCGGATCCAAAATCGACAACCCTGAGAG) and an oligonucleotide that is complementary to the 3′ end of the gene (CGCGCGAAGCTTCAGGATAGGCGCTCTTCGTA). The PCR product was cloned into the *Bam*HI/*Hin*dIII sites of the pQE-9 vector containing a 6× histidine tag. The 6× histidine-HP1 fusion protein was purified on Ni-NTA resin (QIAGEN, inc.) (Hochuli, 1990) followed by gel filtration on a Pharmacia FPLC Superose 12 column to separate monomeric protein from aggregates of the protein. Monomeric 6× histidine-HP1 was labeled with *N*-hydroxysulfosuccinimide fluorescein according to the method of Hyman et al., 1991. The labeled fusion protein was injected into living pre-cycle 10 embryos along with rhodamine-labeled histones H2A and B (Minden et al., 1989). The dynamics of HP1 and histone distribution were observed in the living embryo by confocal microscopy using a Nikon optiphot fluorescence microscope equipped with the Bio-Rad MRC-600 laser scanning confocal attachment and the Sony LVR-5000A laser video disc recorder (Sullivan et al., 1993). The images were collected using a Ziess 25× Plan-Neofluar lens with a numerical aperture of 0.8.

RESULTS

HP1 localization during early *Drosophila* development

To determine the localization of HP1 before and during cycle 14, embryos at various stages of development were fixed and co-stained with a fluorescein-labeled antibody against HP1 and a rhodamine-labeled anti-histone antibody. For comparison, embryos were similarly co-stained with antibodies against HP1 and GAGA transcription factor, the latter serving as a marker for the α-heterochromatin in the centromeres. Fluorescence confocal microscopy was used to detect the immunostaining.

During interphase of cycle 14 in *Drosophila*, the centromeric heterochromatin is concentrated in the region of the nucleus located near the apical surface of the embryo (Foe and Alberts, 1983; Hiraoka et al., 1990). Both HP1 and GAGA factor were found to be similarly concentrated in this region (Fig. 1). The two proteins appear to be co-localized at many regions of the heterochromatin, but their localization patterns are not the same (Fig. 1B). Much of the GAGA factor protein is limited to a subset of HP1 staining sites (*yellow* areas in Fig. 1B), but occasionally it is found where HP1 is not (*red* areas

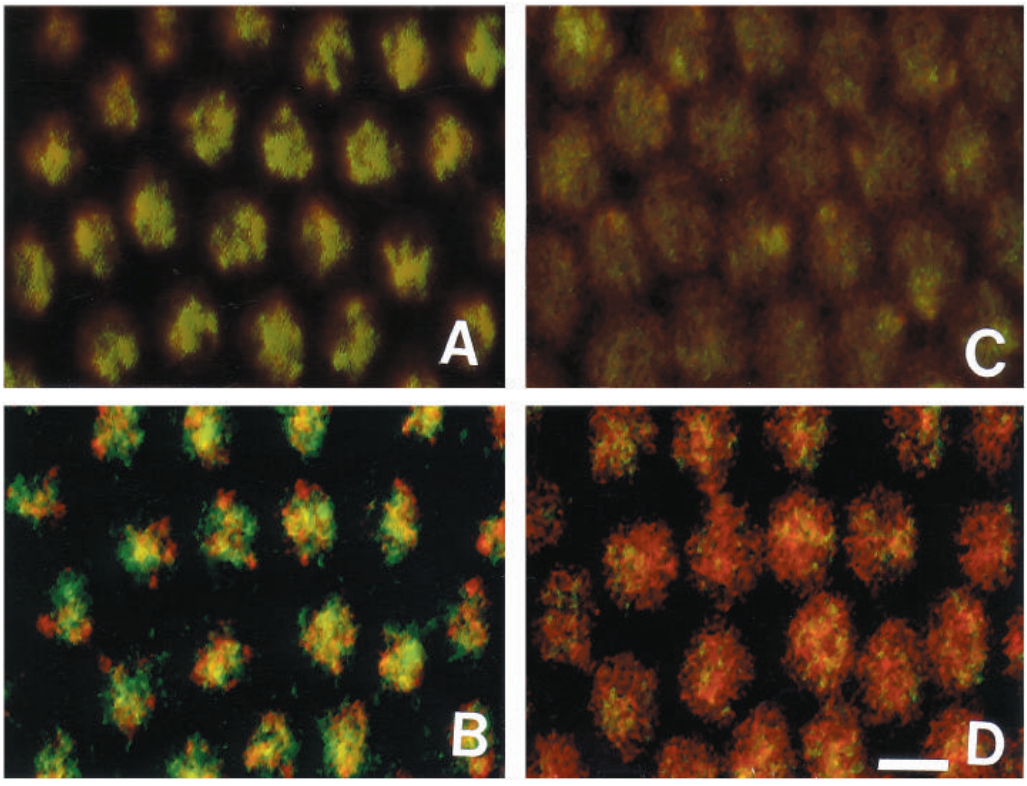


Fig. 1. HP1, GAGA factor, and histone localization in a cycle 14 embryo. (A) Fluorescein-labeled HP1 (*green*) and rhodamine-labeled histone (*red*) at the apical surface; (B) fluorescein-labeled HP1 (*green*) and rhodamine-labeled GAGA factor (*red*) at the apical surface; (C) fluorescein-labeled HP1 (*green*) and rhodamine-labeled histone (*red*) through the nuclear midline; (D) fluorescein-labeled HP1 (*green*) and rhodamine-labeled GAGA factor (*red*) through the nuclear midline. (Regions of overlap are in *yellow*.) Bar, 10 μm.

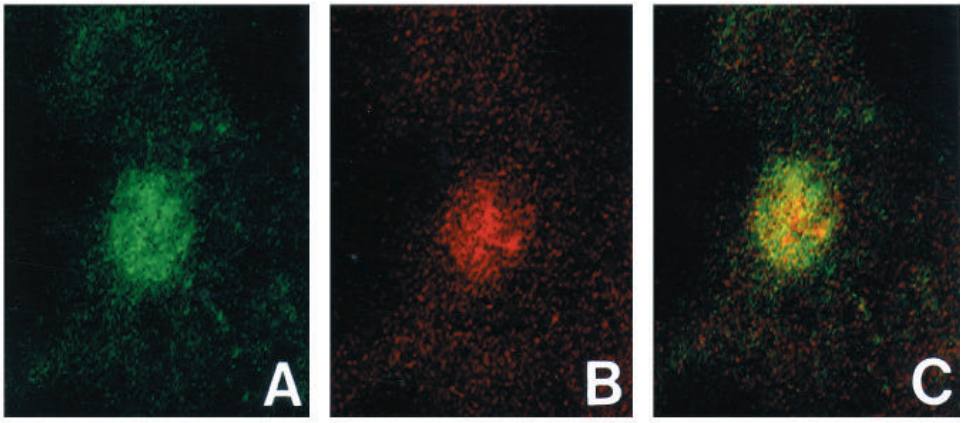


Fig. 2. HP1 and GAGA factor localization in a cycle 5 embryo. (A) Fluorescein-labeled HP1 (*green*); (B) rhodamine-labeled GAGA factor (*red*); (C) merged image of HP1 (*green*) and GAGA factor (*red*). (Regions of overlap are in *yellow*.) Bar, 10 μm.

Table 1. Fluorescence intensity of HP1 signal at the apical surface and at the nuclear midline in nuclear cycle 10 and nuclear cycle 14 embryos

Fluorescence intensity of HP1 signal in nuclear cycle 10 embryo			Fluorescence intensity of HP1 signal in nuclear cycle 14 embryo		
At apical surface(A)	At nuclear midline(NM)	Ratio A/NM	At apical surface(A)	At nuclear midline(NM)	Ratio A/NM
1) 168	124.0	1.36	1) 207	59.6	3.48
2) 138	69.7	1.99	2) 181	43.9	4.13
3) 111	57.3	1.95	3) 190	51.8	3.68
4) 125	74.5	1.70	4) 206	47.1	4.37
5) 147	70.4	2.09	5) 187	37.9	4.94
6) 99	57.5	1.73	6) 184	63.2	2.92
7) 110	76.0	1.46	7) 185	64.2	2.89
8) 142	83.8	1.70	8) 195	54.9	3.56
9) 134	71.2	1.89	9) 194	61.1	3.18
10) 120	74.3	1.62	10) 180	51.5	3.49
		Average=1.75 s.d.=0.05			Average=3.82 s.d.=0.55

The A/NM ratio for the anti-histone immunofluorescence signal was similarly determined from the intensity of the signal in four nuclei at the apical surface and four nuclei at the nuclear midline for each of five embryos. The average A/NM ratio during nuclear cycle 10 was 1.20±0.21 and during nuclear cycle 14 was 1.71±0.13.

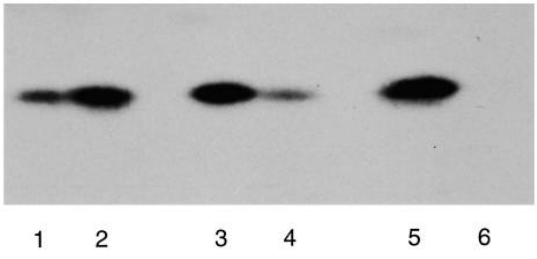


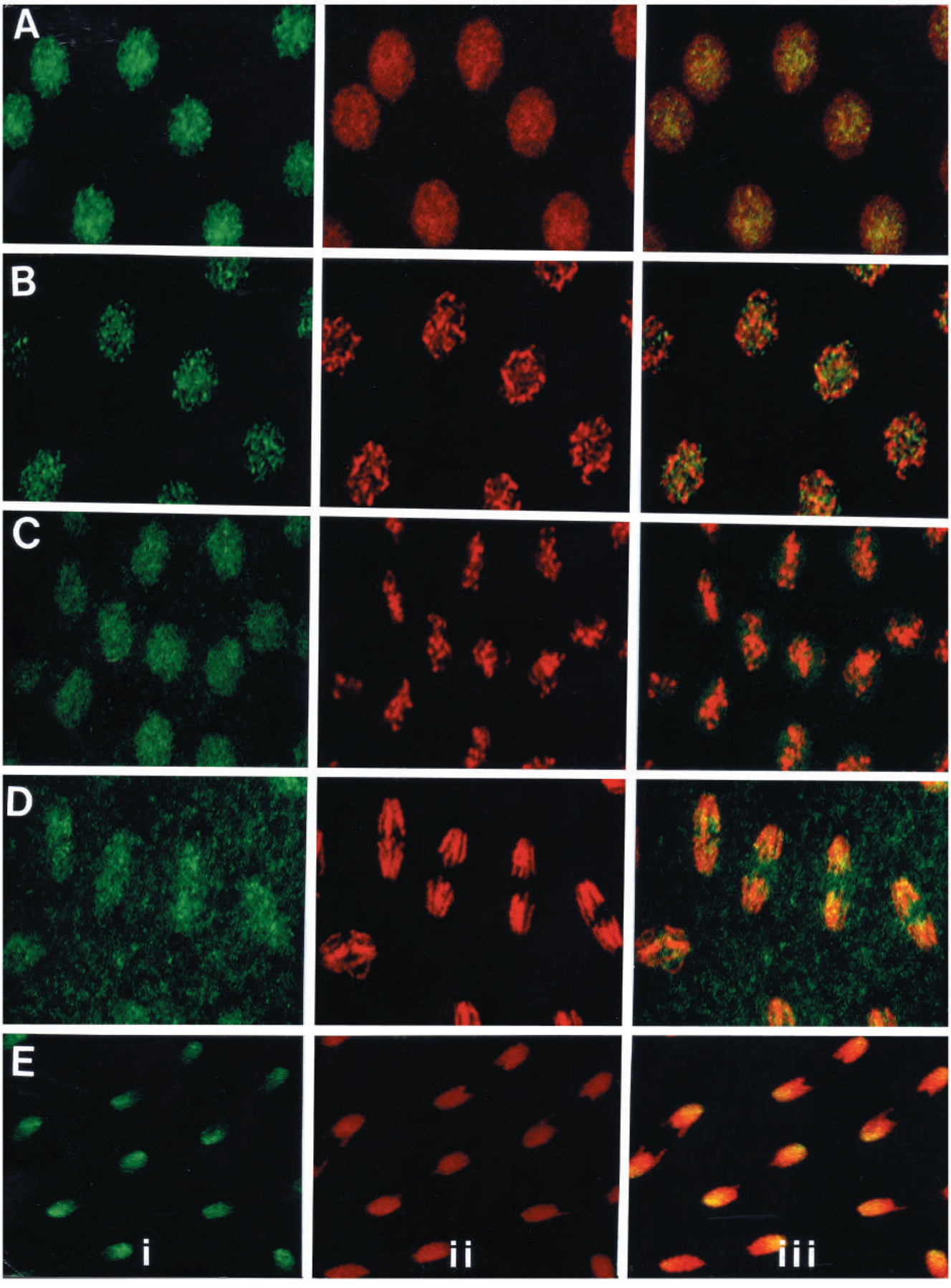
Fig. 3. Determination of HP1 levels in the soluble and pellet fractions from salt-extracted cycle 14 interphase nuclei. Samples were electrophoresed through a 10% SDS-polyacrylamide gel (Laemmli, 1970) and transferred to nitrocellulose for western blot analysis with anti-HP1 antibody. Lane 1, 5 μl of the soluble fraction and lane 2, 5 μl of the pellet fraction from nuclei extracted with 60 mM KCl; lane 3, 5 μl of the soluble fraction and lane 4, 5 μl of the pellet fraction from nuclei extracted with 0.5 M KCl; lane 5, 5μl of the soluble fraction and lane 6, 5 μl of the pellet fraction from nuclei extracted with 1.5 M $MgCl_2$.

in Fig. 1B). Reduced amounts of both proteins are present deeper in the nuclei. The interior localization of each protein appears to coincide with chromatin (see Fig. 1C). However, the GAGA protein is more widely distributed than HP1 throughout the chromatin in this region of the nucleus (*red* areas in Fig. 1D). The localization of GAGA factor deeper in the nucleus at this stage presumably reflects its transcriptional role in euchromatin. The HP1 present deeper in the nucleus (Fig. 1D) may correspond to the sites along the euchromatic arms of polytene chromosomes where the protein is found (James et al., 1989). Alternatively, some of the centromeric heterochromatin may extend into this region of the nucleus. Similar observations have been made with in situ hybridizations using DNA probes to heterochromatin sequences (A. Dernberg, personal communication).

Large maternal stores of HP1 and GAGA factor are present in the cytoplasm of the early embryo, but as early as nuclear cycle 3 it was possible to detect the presence of both proteins inside nuclei above this background. A cycle 5 nucleus is shown in Fig. 2. The chromatin in embryos at these early stages is relatively homogeneous in appearance, but some regions that have a slightly more compact appearance are revealed by immunostaining the embryos with anti-histone antibodies (data not shown). While both HP1 and GAGA factor are present throughout the nuclei, they are concentrated in these more compact regions. The GAGA factor staining (Fig. 2B) has a more punctate appearance than that of HP1, which looks more fibrous (Fig. 2A). As was observed in nuclear cycle 14 embryos, HP1 and GAGA factor sites of staining do not coincide exactly (Fig. 2C).

HP1 is present throughout the nuclei both before and after cycle 14; however, it is most concentrated at the apical side of each nucleus where the centromeric heterochromatin is located (Foe and Alberts, 1983; Hiraoka et al., 1990). In order to quantitate this enrichment, HP1 levels were determined from the intensity of its immunofluorescence signal. In each of ten embryos, the intensity of this signal was determined for four

Fig. 4. HP1 and histone distribution throughout the cell cycle. Fluorescein-labeled HP1 (*green*) and rhodamine-labeled histone (*red*) in nuclear cycle 10 embryos during: (A) interphase; (B) prophase; (C) metaphase; (D) anaphase; (E) telophase. The interphase and prophase images are a Z series of images taken in six different focal planes through the nuclei, while those for metaphase, anaphase, and telophase are taken in a single plane. Bar, 10 μm.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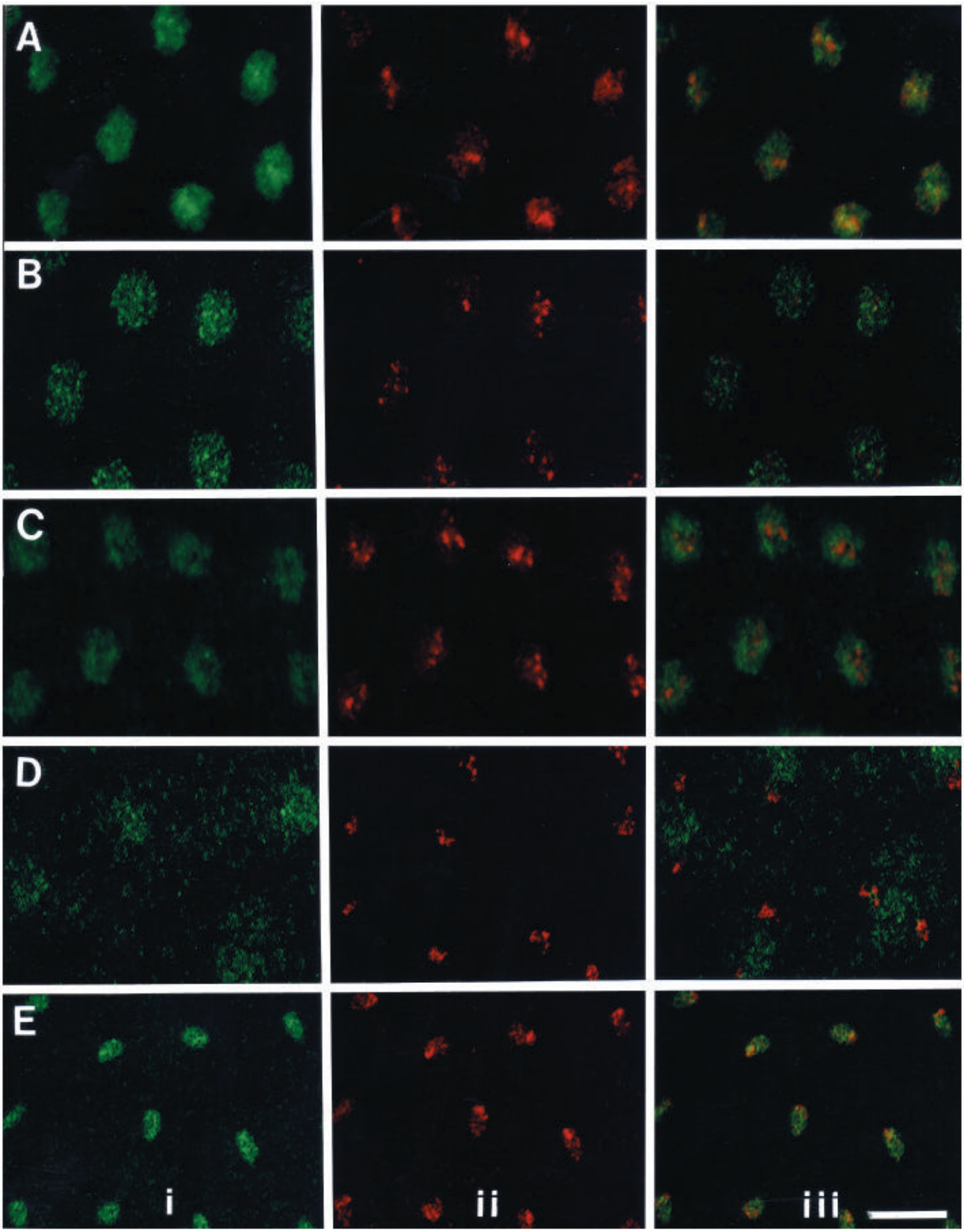
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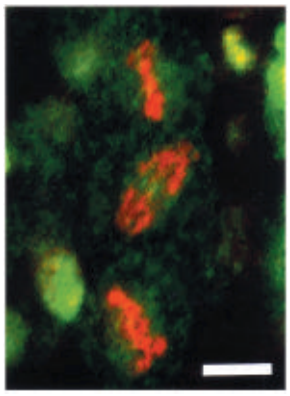

Fig. 6. HP1 and histone localization in a cycle 14 mitotic domain. Fluorescein-labeled HP1 (*green*) and rhodamine-labeled histone (*red*). Bar, 10 μm.

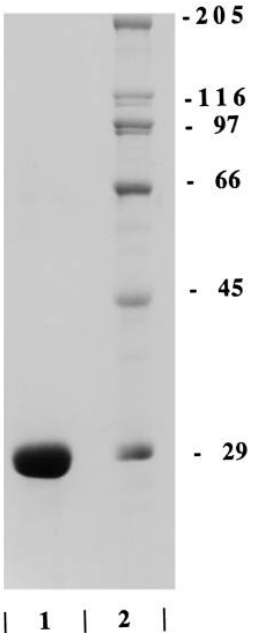

Fig. 7. HP1 fusion protein used for injection into living embryo. Samples were electrophoresed through a 10% SDS-polyacrylamide gel (Laemmli, 1970) and stained with Coomassie Blue. The 6× histidine-HP1 fusion protein is shown in lane 1 and molecular mass standards are shown in lane 2.

nuclei at the apical surface and for four nuclei at the nuclear midline (Table 1). The data was averaged for the nuclei at both locations in each embryo and used to calculate the ratio of the intensity at the apical surface relative to the intensity near the nuclear midline (apical/nuclear midline or A/NM ratio). As shown in Table 1, the average A/NM ratio for nuclear cycle 10 embryos was 1.8, while in nuclear cycle 14 embryos this average was 3.8. These numbers should be compared to an A/NM ratio for the intensity of the anti-histone immunofluorescence signal of 1.2 in cycle 10 embryos and 1.7 in cycle 14 embryos. Thus, HP1 is enriched in the chromatin at the apical end of the nucleus where centromeric heterochromatin is located: a small enrichment is seen during cycle 10, but a stronger asymmetry relative to histone appears by cycle 14.

The HP1 quantitation by fluorescence intensity is in agreement with quantitation of HP1 in the soluble and pellet fractions from interphase nuclei prepared from cycle 14 *Drosophila* embryos. When the nuclei were resuspended in 60 mM KCl there was a roughly three- to four-fold enrichment for HP1 in the pellet fraction as determined by western blot analysis (Fig. 3, lanes 1 and 2). A $MgCl_2$ concentration of 1.5 M was required in order for HP1 to be completely extracted from the pellet fraction (Fig. 3, lanes 5 and 6).

Cell cycle dynamics determined in fixed embryos

We were interested in determining whether HP1 is localized at the centromeres of the chromosomes in mitotically active diploid nuclei of *Drosophila* embryos. The localization of HP1 as the nuclei progress through the cell cycle was also of interest. Because the early nuclear divisions occur rapidly and synchronously in a syncytium (Foe and Alberts, 1983), cell cycle dynamics can be most easily observed in pre-cycle 14 embryos. In Fig. 4, the distributions of HP1 and histones are shown throughout nuclear cycle 10. For comparison, the localizations of HP1 and GAGA factor during the same nuclear cycle are shown in Fig. 5.

As can be seen in Fig. 5A-E, GAGA factor (*red*) remains associated with the centromeres through the entire cell cycle. During interphase, both HP1 (*green*) and GAGA factor are present throughout the nuclei, but are concentrated in a region at the apical surface. GAGA factor is concentrated into punctate structures, while HP1 staining appears more fibrous and diffuse (Fig. 5A). When the chromosomes detach from the nuclear envelope and condense during prophase, only some HP1 staining overlaps with the anti-histone staining that localizes the chromatin. HP1 (*green*) is also located between and around the chromatin fibers (*red*) (Fig. 4B). There is little overlap between anti-HP1 staining and anti-GAGA factor staining during this cell cycle stage (Fig. 5B). After the chromosomes are fully condensed on the metaphase plate, HP1 is mostly dispersed in an area that surrounds the condensed chromosomes (Fig. 4C). At this time, little of the HP1 staining overlaps with anti-GAGA factor staining (Fig. 5C). During anaphase, a significant fraction of HP1 is not concentrated on the chromosomes but is instead dispersed around them (Fig. 4D). By late anaphase HP1 fills the entire space occupied by the segregating chromosomes and spindle. At telophase, HP1 accumulates over the chromosomes and is enriched in the centromeric regions as the chromosomes begin to decondense and the nuclear envelope reforms (Fig. 4E). The HP1 staining now coincides partially with anti-GAGA factor staining where it remains until the following prophase (Fig. 5E).

A similar pattern of HP1 localization is also observed in older embryos after the formation of a conspicuous chromocenter. During cycle 14, a G_2 phase is acquired in the cell cycle (Edgar and O'Farrell, 1989), and nuclear divisions no longer occur synchronously but in discrete mitotic domains (Foe, 1989). These changes in cell cycle regulation do not appear to alter the dynamic distribution pattern of HP1 that was observed in early embryos (Fig. 6).

Several other protocols for fixing *Drosophila* embryos were also used, and all but one (fixation with methanol/EGTA only) yielded results similar to those described above. When embryos were fixed with methanol/EGTA only (Mitchison and Sedat, 1983), HP1-staining was not enriched in the heterochromatin of cycle 14 interphase nuclei. Using another

Fig. 5. HP1 and GAGA factor distribution throughout the cell cycle. Fluorescein-labeled HP1 (*green*) and rhodamine-labeled GAGA factor (*red*) in nuclear cycle 10 embryos during: (A) interphase; (B) prophase; (C) metaphase; (D) anaphase; (E) telophase. The interphase and prophase images are a Z series of images taken in six different focal planes through the nuclei, while those for metaphase, anaphase, and telophase are taken in a single plane. Bar, 10 μm.

commonly used protocol for fixing *Drosophila* embryos (Wieschaus and Nusslein-Volhard, 1986), HP1 immunostaining results were similar to those described above with a formaldehyde fixation, followed by methanol/EGTA. However, both GAGA factor staining and histone staining were perturbed in embryos fixed by this method. Finally, embryos were also fixed in formaldehyde and then devitellinized by hand rather than by incubating in methanol/EGTA. The results for HP1, GAGA factor and histone staining when using this fixation protocol were also similar to those described above.

While HP1 immunolocalization is coincident with chromatin during interphase, most of the HP1 does not appear to be directly associated with the chromosomes during mitosis. Some of the lack of coincidence between HP1 staining and the chromosomes (histone staining) during mitosis could be the result of the images being out of register. However, this is not likely, since the two images are generated from a single excitation beam, and their expected register was confirmed by using both rhodamine- and fluorescein-labeled secondary antibodies to detect HP1 in the same embryo (data not shown).

The localization pattern of HP1 has a fibrous appearance throughout the cell cycle. In the interphase heterochromatin this probably reflects its association with chromatin fibers. However, during mitosis the fibrous appearance may simply be the result of soluble protein filling the spaces between chromosomes and spindle microtubule fibers.

Cell cycle-regulated dynamics in living embryos

The failure to detect HP1 at the centromeres of mitotic chromosomes could be due to antibody inaccessibility in the highly compact heterochromatin structure in fixed chromosomes (Wreggett et al., 1994). It is also possible that the HP1-staining that is diffusely localized during mitosis is due to recognition of a cross-reacting antigen by the antibody against HP1. To circumvent these technical problems, we injected a fluorescent HP1, which had been produced in bacteria, into living embryos and observed its distribution throughout the cell cycle. The protein used was HP1 fused to a short tag of six histidines at its N-terminus. This protein was purified by nickel agarose chromatography (Hochuli, 1990) followed by gel filtration to separate the monomeric HP1 species from large aggregates of the protein (Fig. 7).

The purified HP1 fusion protein was labeled with fluorescein and coinjected with rhodamine-labeled histones H2A and H2B into pre-cycle 10 embryos. Using confocal microscopy, the localizations of the HP1 fusion protein and histones were then observed simultaneously in the living embryo under conditions that have been shown not to kill wild type embryos (Sullivan et al., 1993; R. Kellum, personal observations). The results are shown in Fig. 8. These localizations are similar to those obtained from immunostaining fixed specimens. Most notably, the diffuse pattern of localization surrounding the chromosomes during mitosis is observed in the live analysis (Fig. 8A-C). As with the immunostaining analyses, during

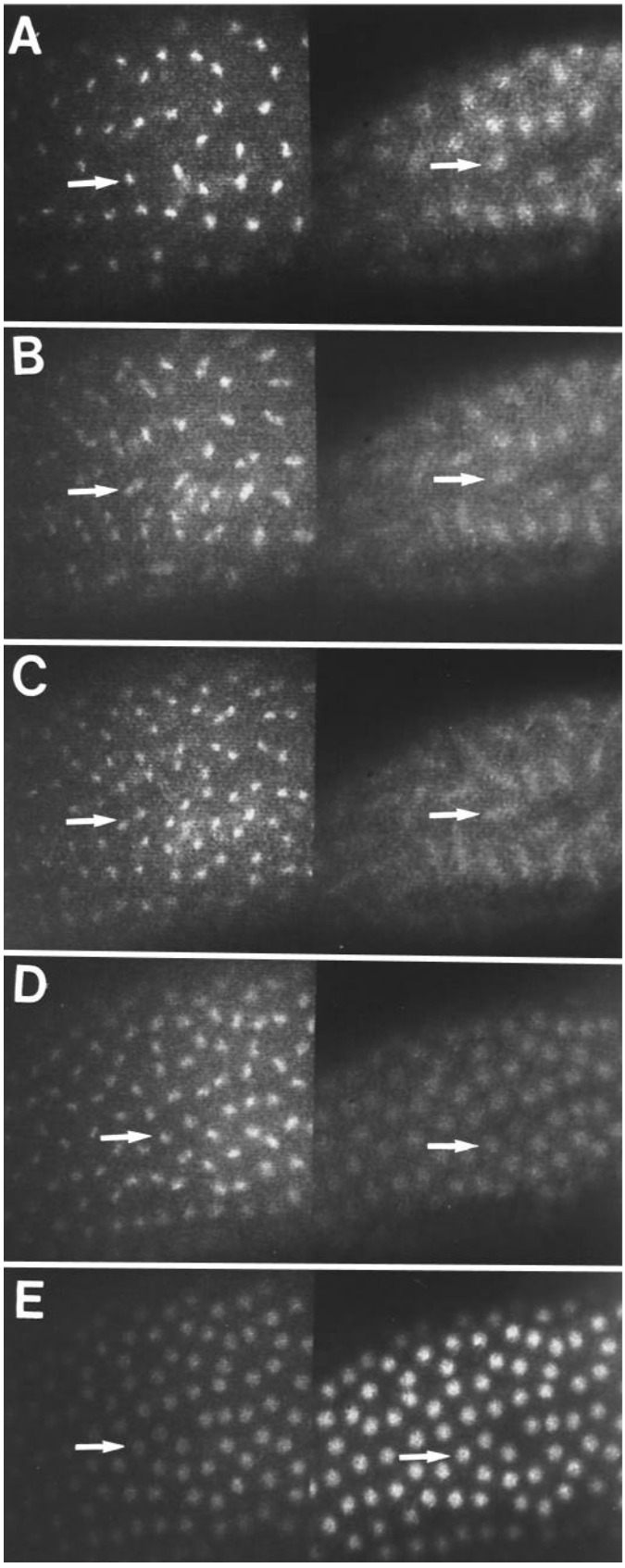

Fig. 8. Live analysis of HP1 fusion protein distribution. Successive micrographs of a single living embryo co-injected with rhodamine-labeled histones H2A and B (left panels) and fluorescein-labeled HP1 (right panels) during: (A) metaphase; (B) early anaphase; (C) late anaphase; (D) telophase of nuclear cycle 10; and (E) interphase of nuclear cycle 11. The images were collected at low magnification to insure that the nuclei remained in focus in their entirety throughout the cell cycle. Bar, 100 μm. Arrow, the same nucleus throughout.

anaphase we were unable to detect HP1 on the mitotic chromosomes above the background of the diffusely localized protein. During telophase the diffusely localized HP1 coalesces on or around the chromosomes and is segregated into the daughter nuclei (Fig. 8D).

Notwithstanding the potential difficulties associated with live analysis of a eukaryotic protein that was produced as a fusion protein in bacteria, the agreement between the results of the live analysis of HP1 localization and the analyses of fixed specimens (using a number of different fixation protocols) strengthens the validity of the results from each type of analysis.

HP1 localization in *Drosophila* tissue culture cells

The mouse homologue of HP1 was recently shown to be located at the centromeres of metaphase chromosomes in mouse L cells (Wreggett et al., 1994). In order to more closely repeat the type of experiment performed by Singh and coworkers (Wreggett et al., 1994) with the mouse homologue, we decided to immunostain *Drosophila* tissue culture cells. Tissue culture cells and embryonic nuclei may differ in two properties that could potentially affect HP1 localization. First, prior to nuclear cycle 13 the *Drosophila* embryo is a syncytium of nuclei and the nuclear envelope is not completely broken down during mitosis (Hiraoka et al., 1990; Stafstrom and Staehelin, 1984); this might impede the release of soluble HP1 into the cytoplasm during mitosis such that any protein that is located on the chromosomes is obscured from view. Also, nuclei during these early nuclear divisions have not yet acquired G_1 and G_2 stages of the cell cycle (Edgar et al., 1986; Edgar and O'Farrell, 1989).

In formaldehyde/glutaraldehyde-fixed cells from both Schneider (Fig. 9, *arrow i*) and KC tissue culture cell lines (data not shown), HP1 (*green*) was found to be concentrated at the nuclear periphery during interphase, consistent with its localization in heterochromatin. GAGA factor (*red*), in contrast, is localized in a punctate pattern throughout the nucleus at this time (Fig. 9, *arrow i*). Consistent with the results in embryos, GAGA factor was localized at the centromeres of the chromosomes in tissue culture cells during mitosis (Fig. 9, *arrow a*). In contrast, HP1 was diffusely localized throughout the entire cell cytoplasm during mitosis, and we were unable to detect any specific staining on the chromosomes (Fig. 9, *arrow a*). This cytoplasmic staining contrasts with the nuclear

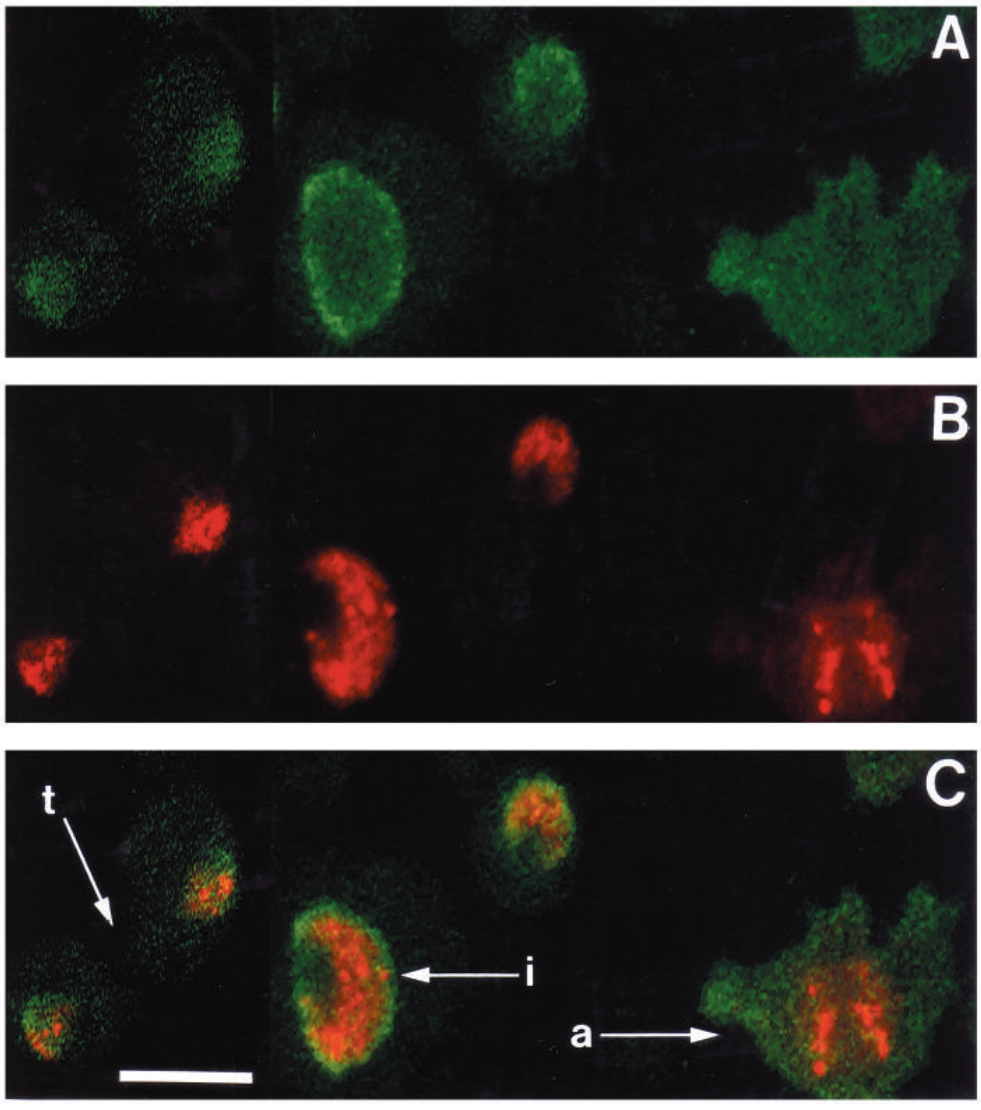

Fig. 9. HP1 and GAGA factor distribution in Schneider tissue culture cells. Fluorescein-labeled HP1 (*green*) and rhodamine-labeled GAGA factor (*red*) in Schneider tissue culture cells. Cells in various stages of the cell cycle are indicated by arrows and the letter: i, interphase; a, anaphase; t, telophase. Bar, 5 μm.

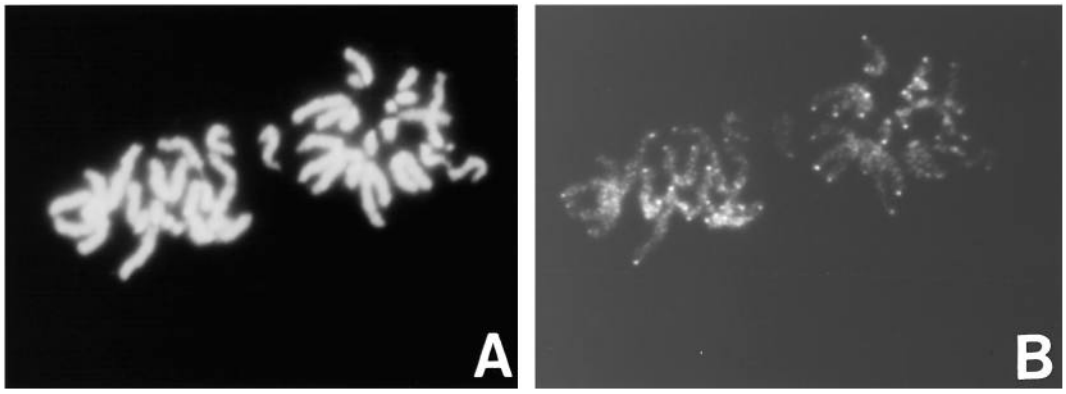

Fig. 10. HP1 localization on unfixed mitotic chromosomes from Schneider tissue culture cells. (A) DAPI staining; (B) anti-HP1 staining.

staining for HP1 observed in embryos (see Fig. 4D). This difference might be simply explained if the nuclear envelope is more completely broken down during mitosis in the tissue culture cells. As was observed in fixed embryos, HP1 seems to reassociate with the centromeric regions of the chromosomes during telophase, colocalizing with GAGA factor (Fig. 9, *arrow t*). Also, as was observed for the HP1 staining in embryos, some of the HP1 staining has a fibrous appearance throughout the cell cycle.

Wreggett et al. (1994) were only able to detect anti-HP1 immunostaining at the centromeres of metaphase chromosomes when the chromosomes were not fixed. When we stained unfixed tissue culture cells prepared by the methods used by these authors, we also were able to detect HP1 staining on the mitotic chromosomes (Fig. 10). Staining was seen throughout the chromosome arms in a somewhat banded pattern, with enrichment at the centromeres and the telomeres. Wreggett et al. (1994) also detected staining at sites in addition to the centromeres, including regions containing the amplified dihydrofolate reductase gene with a block of centromeric heterochromatin.

DISCUSSION

HP1 distribution during the cell cycle

We began this analysis of HP1 localization in embryos with the expectation that HP1 would be associated only with the centromeric heterochromatin and that it would maintain this association throughout the cell cycle. Instead, we found that while HP1 is enriched at the apical region of the nuclei where the heterochromatin is located during interphase, it is not exclusively localized within the heterochromatin of interphase nuclei (Fig. 1 and Table 1). We were also surprised to find that during metaphase and anaphase in both fixed and living embryos, HP1 was not localized exclusively on the chromosomes but was diffusely localized in an area surrounding the chromosomes (Figs 4,5,6 and 8). This diffusely localized protein apparently obscures from view any HP1 that is associated with the chromosomes. Using GAGA factor as a marker for a subfraction of the α-heterochromatin that is clearly visible at the centromeres during mitosis, we are unable to detect a marked enrichment for HP1 staining in these regions. In *Drosophila* tissue culture cells HP1 is dispersed throughout the cytoplasm during mitosis. Even though the protein that is dispersed during mitosis in these cells is not confined to the nuclear region, staining was still not observed on the mitotic chromosomes in these cells. However, when *Drosophila* tissue culture cells were prepared without fixation according to the method of Wreggett et al. (1994), we were able to detect an enrichment for HP1 at the centromeres and at the telomeres of unfixed mitotic chromosomes. This result may indicate that the anti-HP1 antibody is only accessible to the HP1 associated with the heterochromatin in mitotic chromosomes when the chromosomes are not fixed. It also demonstrates that the HP1 that was diffusely localized during mitosis probably prevented us from seeing this population of HP1 in the live analysis.

Unlike *Drosophila* HP1, the mouse homologue of HP1 may be localized exclusively on the chromosomes during mitosis (Wreggett et al., 1994). Why *Drosophila* HP1 behaves differently from its mouse homologue during mitosis is a puzzle. It is possible that a fraction of the mouse homologue is also dispersed during mitosis, but this population of protein is removed during the preparation of the unfixed chromosomes. During this preparation, cells are first incubated in a hypotonic solution, centrifuged onto glass coverslips and incubated in a detergent-containing buffer. The difference might also reflect a difference in the sequences of the two proteins. The sequences of the two proteins contain two regions of homology, one of approximately 65% identity over 37 amino acids at the N terminus and another of approximately 50% identity over 65 amino acids at the C terminus (Powers and Eissenberg, 1993). However, the approximately 80 amino acids lying between the two regions of homology have diverged.

What might be the function of the dispersed pool of HP1?

Presumably, only the HP1 which is associated with the chromosomes is functional. The possibility that the pool of protein that is dispersed during mitosis could have some role is made less likely by the observation that HP1 in tissue culture cells is not confined to the nuclear region as it is in embryos, but instead much of the protein is dispersed throughout the cell cytoplasm.

It is not clear whether this dispersed protein originates from a soluble pool of protein that exists throughout the cell cycle or reflects dissociation of a fraction of the protein from the heterochromatin during mitosis. In cycle 5 and cycle 10 embryos most of the GAGA factor is limited to specific regions of the nucleus, presumably to the AAGAG and AAGAGA repeats in α-heterochromatin (Lohe et al., 1993; Raff et al., 1994). HP1

is distributed diffusely throughout the nucleus with about a two-fold enrichment at the apical surface that is coincident with GAGA factor staining (Figs 2, 4 and 5). This enrichment increases to four-fold when the heterochromatin becomes most conspicuous during cycle 14 (Fig. 1). Salt extractions of interphase cycle 14 nuclei reveal a pool of HP1 that is soluble in 60 mM KCl, with the majority of the protein remaining in the pellet fraction until the concentration of salt is increased to 1.5 M $MgCl_2$. These results indicate the presence of a pool of soluble HP1 throughout the cell cycle, which constitutes a decreasing fraction of the total nuclear protein as embryonic development proceeds. The presence of a pool of HP1 surrounding the segregating chromosomes in mitotic cells even after a conspicuous chromocenter has formed (Fig. 6) suggests that some of the dispersed protein could occur as a result of cell cycle regulation of HP1 distribution.

The formation of a conspicuous chromocenter at cycle 14 probably reflects covalent modification of heterochromatin-associated proteins or the addition of new members to the heterochromatin complex. In this regard, Eissenberg et al. (1994) recently found multiple phosphorylation states of *Drosophila* HP1 and a correlation between the highly phosphorylated forms and this developmental stage.

The more static pattern of GAGA factor location during the cell cycle can be attributed to the fact that GAGA factor, which has been shown to bind AG/TC sequences in vitro (Tsukiyama et al., 1994), is likely to be directly bound to specific satellite DNA sequences in heterochromatin. In contrast, HP1 is not known to have any DNA binding activity (Singh et al., 1991), and therefore it may be located more peripherally in the heterochromatin structure, possibly recognizing specific topological structures formed by the pairing of DNA repeats (Dorer and Henikoff, 1994) or a specific histone isoform (such as histone H4 acetylated at lysine 12 which has a distribution on polytene chromosomes similar to that of HP1 (Turner et al., 1992)). Such a peripheral location would be consistent with a role for HP1 in the higher order organization of heterochromatin within the interphase nucleus.

In a wide variety of cell types, the centromeric heterochromatin is localized in a distinct domain on the nuclear envelope during interphase (Bouteille et al., 1983; Hancock and Boulikas, 1982; Franke et al., 1981; Brown, 1966). For example, during interphase of cycle 14 in *Drosophila*, this heterochromatin is organized into a conspicuous chromocenter that is associated with the nuclear envelope on the apical side of the nucleus. The dispersed pool of HP1 during mitosis, which becomes conspicuously enriched in the centromeric regions of the chromosomes during telophase, may reflect a role for it in reassembling the interphase nucleus after mitosis.

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