Changing chromatin dynamics and nuclear organization during differentiation in *Drosophila* larval tissue

Rajika Thakar and Amy K. Csink*

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213, USA

*Author for correspondence (e-mail: csink@andrew.cmu.edu

Accepted 14 December 2004
Journal of Cell Science 118, 951-960 Published by The Company of Biologists 2005
doi:10.1242/jcs.01684

Summary

Global changes in gene expression and exit from the cell cycle underlie differentiation. Therefore, understanding chromatin behavior in differentiating nuclei and late G1 is key to understanding this developmental event. A nuclear event that has been shown to specifically occur in late G1 is the association of two heterochromatic blocks in *Drosophila*. The *brown*^{Dominant} (*bw*^{D}) chromosome of *Drosophila melanogaster* contains a large block of heterochromatin near the end of 2R. This distal block associates with centric heterochromatin (2Rh), but not until at least 5 hours into G1. We used the *bw*^{D} allele as a model for nuclear organization to determine whether its association with the heterochromatic compartment of the second chromosomes (2Rh) strictly requires differentiation or if this change is a stochastic event, its occurrence being proportional to time spent in G1/G0 phase of the cell cycle. Fluorescence in situ hybridization on eye imaginal discs showed increased association between the *bw* locus and *2*Rh in differentiated cells. Interestingly, an increase in the number of nuclei showing *bw*^{D}*-2*Rh association in the brains of developmentally delayed larvae that were compromised for differentiation was also observed. Live fluorescence imaging showed that the kinetics of chromatin movement remains unchanged in the developmentally arrested nuclei. These observations suggest that nuclear reorganization is not directly controlled by specific inductive signals during differentiation and that this nuclear reorganization can happen in a cell, regardless of differentiation state, that is arrested in the appropriate cell cycle stage. However, we did see changes that appear to be more directly correlated with differentiation. Dynamic imaging in eye imaginal discs showed that the movement of chromatin is more constrained in differentiated cells, implying that confinement of loci to a smaller nuclear space may help to maintain the changed organization and the transcription profile that accompanies differentiation.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/5/951/DC1

Key words: Nuclear organization, Chromatin dynamics, Differentiation, Heterochromatin

Introduction

The correlation between nuclear organization and gene expression has been extensively studied in the past few years and is now well established. It has long been known that the bulk of the transcriptionally quiescent heterochromatin localizes to the nuclear periphery, and studies on mammalian tissue culture cells find that chromosomes occupy discrete territories during interphase (Manuelidis, 1985; Trask et al., 1988). More recently, it has been shown that transcriptionally competent regions preferentially localize to the periphery of these chromosomal territories (Verschure et al., 1999). This organization is observed not only at the level of the chromosome, but also at the level of a single genetic locus in the total space of the nucleus (for a review, see Spector, 2003). For example, the immunoglobin heavy chain locus is positioned at the nuclear periphery when inactive, but more centrally disposed when transcriptionally active (Kosak et al., 2002). Similarly, in a mouse lymphoid cell line, association of genes with heterochromatin is coincident with the downregulation of the corresponding gene transcripts (Brown et al., 1997).

What influences this nuclear reorganization? One answer is that chromatin structure itself dictates the position of a chromosomal region: chromatin with intrinsic or induced heterochromatic properties is localized to a specific region of the nucleus while chromatin in a euchromatic state is localized elsewhere. An example of this was found in *Drosophila* where the *brown*^{Dominant} (*bw*^{D}) allele contains a 1.6 Mb insertion of heterochromatin into the distal euchromatin of the right arm of chromosome 2 (2R) (Platero et al., 1998). Flies heterozygous for *bw*^{D} exhibit a variegated eye phenotype, showing that the wild-type gene on the homologous chromosome is also inactivated. Fluorescent in situ hybridization (FISH) on central nervous system (CNS) cells dissected from third instar larvae heterozygous for *bw*^{D} showed that the *bw* locus associates with centric heterochromatin of the second chromosome (2Rh) (Csink and Henikoff, 1996; Dernburg et al., 1996). It is thought that somatic pairing of the homologous interphase chromosomes (a phenomenon commonly found in almost all nuclei of Dipteran insects) allows the *bw*^{D} allele to drag the wild-type

homolog closer to heterochromatin, resulting in silencing of the wild-type gene.

Nuclei in higher organisms are known to undergo extensive changes in organization as they progress through both the cell cycle and development (for a review, see Francastel et al., 2000). Thus, it is not surprising that positioning of the bw^D heterochromatic insertion varies over the cell cycle. The association between bw^D and 2Rh is not apparent until at least 5 hours into G1. If the length of G1 in a specific cell type is sufficient for bw^D-2Rh association to form, the association is broken at the beginning of the subsequent S phase. No association is formed during G2 (Csink and Henikoff, 1998). The first 13 embryonic nuclear cycles contain only S and M phases. Consistent with this, the bw^D locus is not associated with 2Rh in nuclei from cycle 13 embryos (Dernburg et al., 1996). These studies suggest that silencing caused by interaction with heterochromatin or positioning in a heterochromatic compartment may not be possible until well into the G1 phase of the cell cycle. Studies in mammalian cells have also found that association of some silenced loci with pericentric heterochromatin occurs during G1 (Brown et al., 1999).

The larval CNS consists of a heterogeneous population of cells that are rapidly cycling, differentiating or are terminally differentiated. The changes in nuclear organization seen in differentiated cells may either be regulated by a specific event during differentiation or could merely be a consequence of increased time spent in the G1/G0 phase of the cell cycle. Even though heterochromatic associations occur within a population of cycling cells, the possibility that the association between bw^D and 2Rh is regulated by a specific event during differentiation was not ruled out (Csink and Henikoff, 1998). In this study, we have tested whether the change in nuclear organization of bw^D is correlated with specific events during differentiation.

We found that the number of cells that show association between bw^D and 2Rh is higher in differentiating cells as opposed to undifferentiated cells of the eye imaginal discs. However, we also observed that in larval CNS of developmentally arrested larvae, the number of nuclei where the bw^D locus is close to 2Rh increased. These data are more consistent with the interpretation that the length of G1 is responsible for increased association, rather than specific signal during differentiation. Of course, as terminal differentiation is often accompanied by cell cycle exit, increased association will be an indirect consequence of developmental progression. Interestingly, when we observed chromatin dynamics using time-lapse fluorescence microscopy we found that chromatin was more constrained in differentiated cells. These findings suggest that change in nuclear organization, although not established by a differentiation specific event, may be maintained by a differentiation controlled change in overall chromatin dynamics.

Materials and Methods

Fly lines and culture

All fly lines were maintained at 25°C on cornmeal molasses medium, except when reared for egg laying, which was done on grape-agar plates (Ashburner, 1989). Flies containing the *lac*O repeats inserted at position 1F on the X chromosome were obtained from A. Belmont (University of Illinois, Urbana, IL) (Vazquez et al., 2001) and were used to obtain P-element replacements of $p\{PZ\}\ Dcp\text{-}1^{8859}$, which is located 15 kb from the *bw* locus (Csink et al., 2002). The position and the integrity of *lac*O repeats were checked by polytene FISH on salivary glands from third instar larvae and by Southern blot analysis of DNA from adult flies. Fly line w^{1118}; $P\{w^{mc}, Ubq\text{-}mRFP\text{-}lacI\text{-}NLS\}$ was generated by P-element mediated germline transformation (Spradling, 1986). Other lines were obtained from the Bloomington Stock Center (flystocks.bio.indiana.edu) or were generated in our lab.

Yeast strains

A diploid yeast strain heterozygous for *erg2Δ* was obtained from the *Saccharomyces* genome deletion project (SGDP), strain record number 20788, MAT*A/α his3Δ1/his3Δ1 leu2Δ/leu2Δ met15Δ/MET15 lys2Δ/LYS2 ura3Δ/ura3Δ erg2Δ :: KANMX4/ERG2*. The haploid *erg2Δ* delete was obtained by random sporulation. PCR was done using primers as suggested by SGDP to confirm the deletion. Yeast used as an ERG2 (WT) control was a haploid strain, *MAT α his3 Δ200ura3-52 leu2 lys2*. Both the *erg2Δ* and the ERG2 control yeasts were in a S288C background.

Subcloning of mRFP-LacI-NLS fusion protein expressed under the ubiquitin promoter

The ubiquitin promoter was obtained as a 1980bp *Bam*HI/*Kpn*I fragment from plasmid pCa4UbnlsGFPh27 (Davis et al., 1995) kindly donated by P. O'Farrell (University of California, San Francisco). LacI-NLS and 3′UTR were obtained as a *Bam*HI/*Cla*I fragment of plasmid p3′SS (Belmont et al., 1999) provided by A. Belmont (University of Illinois, Urbana, IL). Using mRFP1 in plasmid pRSETB (Campbell et al., 2002) obtained from R. Tsien (University of California, San Diego) as a template, the sequence for mRFP was PCR amplified with the forward primer 5′-CCATCGATATGGCCT-CCTCCG-3′ and reverse primer 5′-TCTTAGGATCCGGCGCCGGT-3′. *Cla*I and *Bam*HI restriction sites in the primers enabled subcloning with the ubiquitin promoter and *lac*I sequences in frame. All the above fragments except mRFP were first individually subcloned into pBluescript II KS(±) and were later put together in frame using appropriate combinations of restriction enzymes in pBluescript II KS(±). The construct was finally subcloned into the *KpnI/Sac*II site of pCasPer4 to obtain pCas4{Ubq-mRFP-LacI-NLS}. Expression and nuclear localization of the fusion protein were tested by transfecting SL2 cells before P-element transformation. The construct along with pP25.7wc Δ2-3 were micro-injected into w^{1118} embryos to obtain germline transformants as described previously (Spradling, 1986).

Microscopy

All microscopy on nuclei was carried out using a Deltavision (Applied Precision) system. The images were gathered with a cooled CCD camera using either a 60×/1.4 na UPlanApo (FISH and BrdU experiments) or 100×/1.35na PlanApo (live imaging experiments) objectives (Olympus). In the FISH experiments, images were gathered as 3D stacks, deconvolved using the Softworx software package (Applied Precision) and projected into 2D for measurements.

Fluorescence in situ hybridization on eye imaginal discs

Fluorescence in situ hybridization was done on eye imaginal discs from late third instar female larvae. Larvae heterozygous for bw^D were derived from a cross between $P\{w^{+mW.hs}=GawB\}elav[C155]$, w^-; $P\{w^{+mC}=UAS\text{-}syt.eGFP\}$ males and w^{1118}; bw^D female flies and compared to $P\{w^{+mW.hs}=GawB\}elav[C155]$, w^-; $P\{w^{+mC}=UAS\text{-}syt.eGFP\}$, bw^+ larvae. The eye-antennal imaginal discs were dissected from larvae along with the mouth hooks in nuclear structure preservation buffer (NSPB) (80 mM KCl, 20 mM NaCl, 15 mM PIPES

pH 7.0, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM DTT) supplemented with 0.1% deoxy-cholate and 0.1% triton-X-100. The discs were teased with a sharp tungsten needle to remove the peripodial membrane, subjected to hypotonic treatment in 1.0% sodium citrate for 5 minutes and dissected at the morphogenetic furrow in NSPB buffer under an epifluorescence dissecting microscope. The expression pattern of GFP was used to demarcate the furrow. After separating the posterior end of the eye discs, the anterior end was dissected away from the antennal disc and mouth-hooks. The separated anterior and posterior ends of the eye imaginal discs were incubated in 0.3 mg/ml solution of collagenase (Sigma) for 5 minutes at room temperature. The 2 mg/ml stock solution of collagenase was prepared in divalent cation free buffer (Ashburner, 1989). The discs were fixed in methanol(11): acetic acid(11): water(1) (MAW) for 30 seconds, and transferred to a drop of 45% acetic acid on a slide for a minute as described previously (Csink and Henikoff, 1996), except that the slide was pre-coated with 5-10 µl of 1 mg/ml stock solution of poly-lysine (Sigma) prepared in 50 mM sodium borate buffer pH 8.5. The tissue was further teased apart using a tungsten wire and squashed under a sialized coverslip. FISH was as described in (Csink and Henikoff, 1998). Nuclei were counterstained with DAPI (Molecular Probes). The two dimensional distance between the signals for the two probes was measured and corrected for by the nuclear radius (as determined by the nuclear area assuming a perfect circle). To prevent bias the images were coded and scored blind.

Developmental arrest of larvae and FISH on CNS

w^{1118}; bw^{D} and w^{1118} larvae were used. In all, 70-80 females were allowed to lay eggs on a grape agar plate for 2 hours. The embryos were collected, washed in sterile dH_2O, 70% ethanol and dechorionated by washing in 2.5% NaOCl (bleach) in 50% ethanol for 3 minutes and rinsed in Chang and Gehrings's solution (Ashburner, 1989). Bleach and ethanol prevent wild-type yeast contamination. Embryos were transferred to a sterile conical flask containing 5 ml of 0.5% dextrose in 2% water-agar gel and plugged with sterile rayon.

The agar was overlaid with either *erg2Δ* yeast *or WT* yeast. The yeast strains were grown as overnight cultures of 500 ml; care was taken to wash off the media in sterile distilled water. The pellet was suspended in twice the volume in mls of the wet weight of the pellet in dH_2O. For each overlay approximately 0.5 ml of the yeast paste was usually added as adapted from (Bos et al., 1976). More yeast was added 48 hours, 72 hours and 120 hours AED. The embryos were reared in these flasks at 25°C. They reached the third instar larval stage at around 96-100 hours AED. CNS from female larvae fed *WT* yeast as well as *erg2Δ* yeast were dissected at 110-115 hours AED. A second round of dissections was done for developmentally arrested larvae that had been fed *erg2Δ* yeast at 130-135 hours AED. By this timepoint all larvae reared on WT yeast had pupated. The protocol followed for dissection and FISH on CNS was as described previously (Csink and Henikoff, 1998). The images were acquired and analyzed as described above.

Measuring mitotic index

w^{1118} larvae were reared on either *erg2Δ* yeast or *WT* yeast and dissected at 110-115 hours AED (day 1) and 130-135 hours AED (day 2) (developmentally arrested larvae only). CNS were dissected and prepared as described previously (Ashburner, 1989). To prevent bias the slides were coded before imaging and each field was randomly chosen with the nuclei slightly out of focus before imaging, so that perception of mitotic figures would not influence field selection.

Determination of cell cycle phase by quantitating DAPI intensity

w^{1118}; bw^{D}, larvae were reared on *erg2Δ* yeast *or WT* yeast as described above. Larvae were used at 113 hours AED (reared on *erg2Δ* yeast *or WT* yeast) as well as 137 hours AED (reared on *erg2Δ* yeast). They were fed bromodeoxy-uridine (BrdU) laced food for 3 hours, prepared in the following manner: 0.3 g of dry agar was placed in a 35 mm×10 mm style cell culture dish (Corning). The agar was soaked with 1.6 ml of 1 mg/ml solution of BrdU stock solution (20 mg/ml in 40% ethanol). The stock solution of BrdU was diluted to 1 mg/ml in 0.5% dextrose and bromophenol blue tracker at 0.3 mg/ml. The larvae obtained from the conical flasks were fed the BrdU laced blue food for 3 hours at 25°C in a humid chamber, after which only those with blue food in the gut were selected. The larvae were washed and CNS dissected. The tissue and slides were processed as described (Csink, 2004).

Fields for imaging were chosen while looking only at the DAPI channel and were acquired at a single plane of focus. Quantitation of DAPI intensity was performed using Softworx software (Applied Precision). The total DAPI intensity per unit area was calculated for each nucleus and the background intensity was subtracted. The median nuclear intensity for each field was used as the normalizing factor. On the basis of the distribution of the ratios in a histogram, and the corresponding BrdU label, the ratios obtained were designated into three classes: Aneuploids (A) with ratio <0.5 and >2.6, G1 if the ratio fell between 0.5-1.20 and S/G2 if the ratio was between 1.2-2.6. Statistical significance of the distribution of cell cycle for calculating the P-value was computed using the G-test (Sokal and Rohlf, 1981).

Measurement and imaging of chromatin movement

To study the dynamics of chromosomal movement w^{-}, y^{-} *P{w^mc, lacO} ; P{w^mc, lacO} Dcp-1/CyO* females were crossed with w^{1118}, *P{w^mc, Ubq-mRFP-lacI-NLS}* males to obtain y^{-} w^{-} *P{w^mc, lacO}/w^1118, P{w^mc, Ubq-mRFP-lacI-NLS}; P{w^mc, lacO} Dcp-1/+* for imaging. Eye-antennal discs were dissected from third-instar female larvae obtained from the cross described above. The larvae were washed in 1× Ringers and the dissections were carried out in MM3 media (Ashburner, 1989) supplemented with 2.5% FBS. The discs were transferred to a poly-lysine-coated 40 mm coverslip and imaging was done in an FCS2 chamber (Bioptech) at 21° to 24°C, allowing us to maintain the primary culture during microscopy (Li and Meinertzhagen, 1995). Images were taken every 5 seconds at a single plane of focus. The length of the movies varied from of 140-500 seconds. For image analysis, polygons were drawn around the nucleus as well as the dots representing the *lac*O repeats on the X chromosome and near the *bw* locus on the 2nd chromosome, using the Softworx software. The distance between the center of mass of the two dots and the nuclear radius was computed using a Java program developed in lab. The mean step size (Table 2) was computed for Δt=10 seconds. The mean square change in distance $\langle \Delta d^2 \rangle$ for all possible values of Δt was computed using a macro on Excel and statistical calculations were performed on StatView (version 4.5). For increasing values of Δt the number of data points used to calculate $\langle \Delta d^2 \rangle$ decreases. This adds to noise in the data observed at later time points, confirming earlier findings that the approximation $\langle \Delta d^2 \rangle$ is reliable only for first 25-50% of the collection period for each nucleus (Heun et al., 2001; Vazquez et al., 2001). Hence to evaluate chromatin motion we first computed $\langle \Delta d^2 \rangle$ values for individual nuclei using the entire range of data. Since the duration of movies varied from 140 to 500 seconds, values corresponding to the first 50% of the length of movie (70-250 seconds) for each individual nucleus were used to evaluate chromatin motion as the average $\langle \Delta d^2 \rangle$. The values obtained until Δt=120 seconds are represented here. The diffusion coefficient (D) can be computed from the slope of the graph, based on the relation $\langle \Delta d^2 \rangle = 4D \langle \Delta t \rangle$ (Qian et al., 1991; Vazquez et al., 2001). Since chromatin motion is constrained, the change in distance over time reaches a standard value. The slope was hence calculated by taking only the linear portion of the graph into account at Δt=5-10 seconds for both differentiated as well as undifferentiated cells. For

undifferentiated cells at Δt=5 seconds data was representative of 814 data points, from 19 nuclei in 7 eye discs. The value of average $<\Delta d^2>$ at Δt=120 was calculated from 18 nuclei. In the case of differentiated cells, at Δt=5 seconds the data was obtained from 668 data points, 21 nuclei from 9 eye discs. The number of nuclei used to compute average $<\Delta d^2>$ at Δt=120 was 18.

Comparison of chromosomal movements in CNS of larvae reared on wild-type yeast as compared with *erg2Δ* was done in a similar manner. Larvae were dissected at 112-120 hours AED and 135-140 hours AED. Images were acquired in a single plane of focus every 2 seconds for a total of 200-400 seconds. As a control, movies were also acquired of tissue fixed in 2% paraformaldehyde (Sigma) with 0.1% Triton-X in 1× PBS for 5 minutes at room temperature. Diffusion coefficient was calculated using the average $<\Delta d^2>$ values at Δt 6-10 seconds in the interval corresponding to the linear portion of the graphs for live cells and on the slope of total data set for fixed cells. For larvae reared on wild-type yeast the plot at Δt=2 as well as Δt=120 seconds is representative of data obtained from 12 nuclei in the CNS dissected from 3 larvae, the number of data points used to compute the average $<\Delta d^2>$ value at Δt=2 seconds was 1685. For larvae reared on *erg2Δ* yeast on day 1 the number of nuclei at Δt=2 seconds was 10, dissected from 3 larvae. The number of data points used to compute the average $<\Delta d^2>$ value at Δt=2 seconds was 1746. The value of average $<\Delta d^2>$ at Δt=120 seconds was computed from 9 nuclei. For the developmentally arrested larvae on day 2, the numbers at Δt=2 and Δt=120 seconds were 13, 4, and 2221, respectively. Data for fixed cells at Δt=2 seconds was obtained from 10 nuclei dissected from 3 larvae, at Δt=120 seconds the number of nuclei was 4. The number of data points used to compute the average $<\Delta d^2>$ value at Δt=2 seconds was 1685.

Results

Differentiation and heterochromatic association of bw^D in eye imaginal discs

Association of bw^D with centric heterochromatin is assayed by measuring the distance between a probe specific for the 59E region near *bw* and a probe that recognized AACAC, a satellite sequence found only in a small subset of the heterochromatin of 2R. If the association between bw^D-2Rh is regulated by a specific event during differentiation, we would expect to see a larger proportion of cells with a smaller distance between the probes in differentiated cells when compared with undifferentiated cells. Additionally, if heterochromatic association strictly requires differentiation, we predict that the distribution of the interprobe distances in the undifferentiated cells would be the same in bw^D and bw^+ nuclei. To investigate this, we examined the nuclei in larval eye imaginal discs which contain cells spatially separated by differentiation state. During the mid third instar larval stage, owing to the shortening of cells in the disc center, a dorsal-ventral furrow formed in the eye disc. This morphogenetic furrow progressed across the disc in a posterior to anterior direction. Cells posterior to the furrow underwent differentiation, whereas those anterior to the furrow were synchronously dividing but unpatterned (Fig. 1A). Therefore, we used this tissue to test for a correlation between differentiation and change in nuclear location of the *bw* locus.

To aid in the separation of the two cell types, we used a fly-line that expresses GFP only in the differentiated cells posterior to the morphogenetic furrow. We dissected eye imaginal discs from crawling third instar larvae at the morphogenetic furrow, separating the differentiating cells from undifferentiated cells (Fig. 1A). The dissected tissues were subject to FISH on separate slides. No significant difference in the distance between *bw*-2Rh (corrected by the radius) in differentiated and undifferentiated cells from bw^+ larvae was found (Fig. 1B). However, in bw^D heterozygotes the distance between *bw*-2Rh significantly decreased in differentiated cells (Fig. 1B,C) when compared with undifferentiated cells. Interestingly, we also found a statistically significant decrease in the *bw*-2Rh distance in the undifferentiated cells from bw^D heterozygotes when compared with bw^+. Association between *bw*-2Rh in undifferentiated bw^D/bw^+ nuclei shows that differentiation is not strictly required for association. This observation suggests that increased association in differentiated cells may simply be a stochastic event and the increased association in the differentiated cells is due to their spending more time in G1. The experiments described below were performed to further test this possibility.

Heterochromatic associations in developmentally arrested larvae

To test if the repositioning of the bw^D heterochromatic block is specific to differentiation, we measured the association between bw^D and 2Rh in developmentally arrested larvae. If differentiation promotes association between bw^D and 2Rh, then developmental arrest, which prevents differentiation, should decrease association. Developmental arrest was accomplished by starving the larvae of the hormone ecdysone. Ecdysone is essential for morphogenesis in insects and regulates many aspects of cellular differentiation (Thummel, 1995). In *Drosophila melanogaster*, a small amount of ecdysone precursor is provided maternally, but later morphogenesis requires that the precursor be provided in the diet. This precursor, in turn, is the product of the *erg2* pathway in *Saccharomyces cerevisiae*, the primary food of *D. melanogaster*. When larvae are restricted to $erg2^-$ yeast, their development stalls in the third instar stage and they fail to pupate (Fig. 2A) (Bos et al., 1976). Larvae reared on the mutant yeast developed at the same rate as those fed wild-type yeast until mid third instar stage, 110-115 hours after egg deposition (AED) (referred to as 'day 1'). At around 118-120 hours AED larvae fed wild-type yeast started to pupate, whereas those fed mutant yeast remained in the third instar stage (Fig. 2A). Even at 144 hours AED, larvae fed mutant yeast failed to pupate, did not grow in size and would remain in the third instar stage till they begin to die about 5 days later. Larvae fed wild-type yeast stopped eating during the late third instar stage. By contrast, the developmentally arrested larvae continued to eat at least until 140 hours AED (referred to as 'day 2'). By feeding them wild-type yeast, we could rescue the developmentally arrested larvae at 120 or 135 hours AED; however, the frequency of rescue was slightly lower when rescued at 135 hours AED. All the larvae that successfully pupated, eclosed as fertile flies that appeared normal.

We compared heterochromatic association in the CNS dissected from bw^+ and bw^D larvae 110-115 hours AED (day 1) that had been fed *erg2Δ* yeast and in CNS from similarly aged bw^+ and bw^D larvae fed wild-type yeast. We also compared CNS derived from developmentally arrested larvae reared on *erg2Δ* yeast that had stalled at this stage for about 24 hours, at 135-140 hours AED (day 2). At this time all larvae that had been fed wild-type yeast had pupated, thus no corresponding control of larvae fed wild-type yeast was

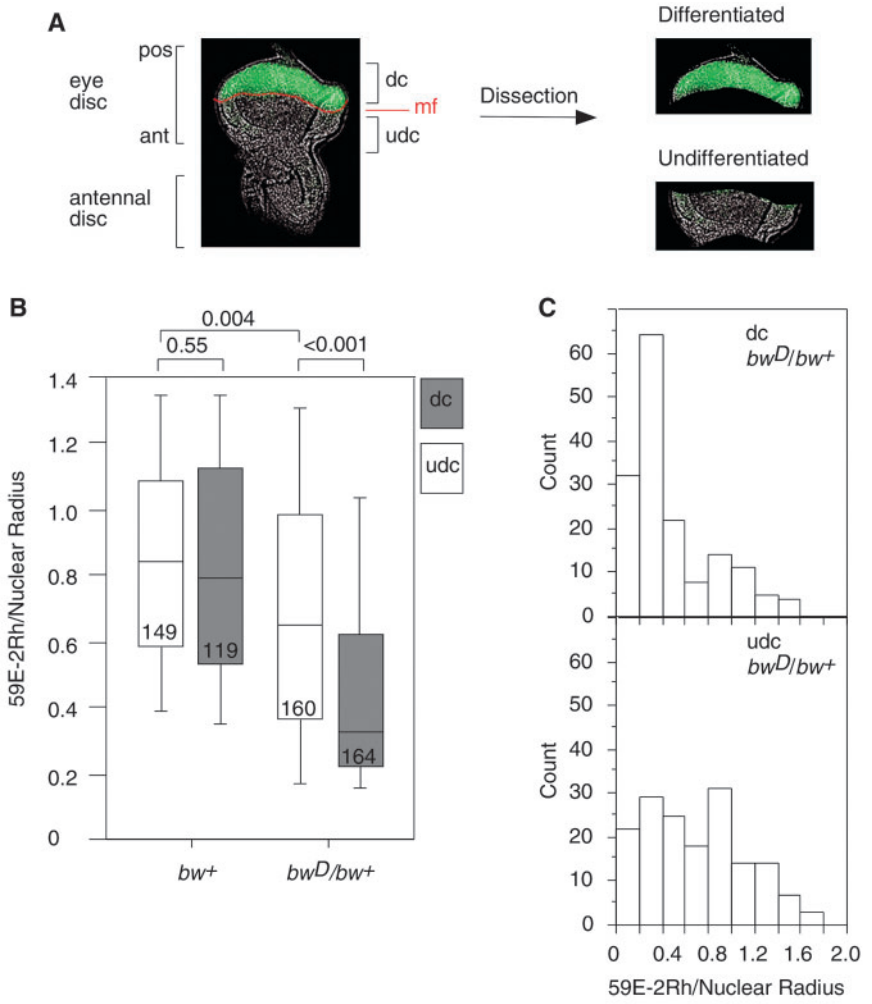

Fig. 1. Heterochromatic association in eye imaginal discs. (A) Separation of differentiated and undifferentiated cells. Image of an eye-antennal disc dissected from late third instar larvae. GFP is expressed in cells undergoing differentiation (dc) that are present posterior (pos) to the morphogenetic furrow (mf). No GFP expression is seen in the undifferentiated cells (udc) present anterior (ant) to the furrow. Dissection at the mf (demarcated by the red line) allows the separation of cells on the basis of their differentiation state. (B) To quantitate the association between bw^D and 2Rh, FISH was done on nuclei obtained from differentiating and undifferentiated cells that were either bw^+ or heterozygous for bw^D. The distance between the two probes was measured and divided by the radius of the nucleus stained with DAPI. The distribution of the data is represented in box plots. Box plots are calibrated representations of histograms wherein each horizontal line represents the 10th, 25th, 50th (median), 75th and 90th percentiles. The numbers inside the box plots represent the total number of nuclei studied. *P* values to determine statistical significance of the difference in the data were determined using the Mann-Whitney test for statistical significance and are depicted above the brackets. (C) Histograms showing the distribution of the distances between 59E and 2Rh in eye imaginal disc nuclei from bw^D/bw^+ larvae. In differentiated cells distribution is skewed towards the lower end.

possible. We chose to use CNS rather than eye imaginal discs, because CNS dissection and preparation was easier and the GFP expression in the fly line used in our earlier analysis varied greatly among larvae that had been fed the mutant yeast.

Contrary to our expectations, we found that in bw^D nuclei from larvae 130-140 hours AED (day 2) that had been fed *erg2Δ* yeast, there was a significant decrease in the distance between *bw*-2Rh (Fig. 2B,C). On day 1 the apparent association was not significantly different in nuclei that were fed either the wild-type yeast or the *erg2Δ* yeast. In the bw^+ nuclei, there was a slight difference in the distribution of the distance between *bw*-2Rh in larvae reared on *erg2Δ* or wild-type yeast at day 1, which seemed to be lost in the day 2 larvae. Taken together, these data, along with our observation in eye imaginal discs, argue against a causative role for a differentiation specific signal in regulating the changes in nuclear organization with respect to bw^D.

Cell cycle profile of developmentally arrested larvae

To follow up on our data from the developmentally arrested larvae, we examined the cell cycle profile of the larvae reared on *erg2Δ* yeast. We first compared the mitotic index in the CNS of larvae from the three different classes in Table 1. The difference in mitotic index was not statistically significant, indicating that feeding the larva *erg2Δ* yeast does not cause a cell cycle arrest during mitosis.

We next determined the proportion of cells in the G1 and S/G2 phase of the cell cycle by quantifying the DNA content in cells based on relative DAPI intensity. Larvae reared on the wild-type yeast were fed BrdU at 113 hours AED (day 1); those reared on *erg2Δ* yeast were fed BrdU at 113 hours (day 1) or at 137 hours (day 2) AED. In each case BrdU-laced food was fed for 3 hours, following which the CNS was dissected. Using the DAPI intensity and the presence or absence of BrdU labeling, we were able to determine the proportion of nuclei in G1 or S/G2 phases of the cell cycle (Csink and Henikoff, 1998). In addition, we were also able to determine if there was a change in the proportion of cells entering the S phase in larvae reared on *erg2Δ* yeast. In larvae fed *erg2Δ* yeast the proportion of cells in G1 was higher than that observed in larvae reared on wild-type yeast ($P<0.01$) (Table 1). In larvae reared on *erg2Δ* yeast, the proportion of cells in G1 was similar for day 1 as well as day 2. However, there was a significant decrease in the percentage of cells that were labeled with BrdU in the developmentally arrested larvae on day 2 as compared with day 1 ($P<0.001$). These results are consistent with an overall retardation of the cell cycle, a slight lengthening of G1 and/or a partial block of the G1-S transition.

Collectively, these results suggest that increased association

between bw^D and 2Rh in the developmentally arrested larvae at 135 hours AED is because of an increase in the length of G1. In the day 1, *erg2Δ* fed larvae, although the proportion of cells in G1 was higher than that observed in larvae undergoing normal development, we did not observe a similar increase in association between bw^D and 2Rh. Since in these larvae the proportion of cells entering the S phase was only slightly different from those reared on wild-type yeast, we hypothesize that on day 1 more of the cells are in the earlier part of G1 where we do not expect to see association. On day 2 the cells would have been in G1 for a long enough period of time for a larger number of associations to form.

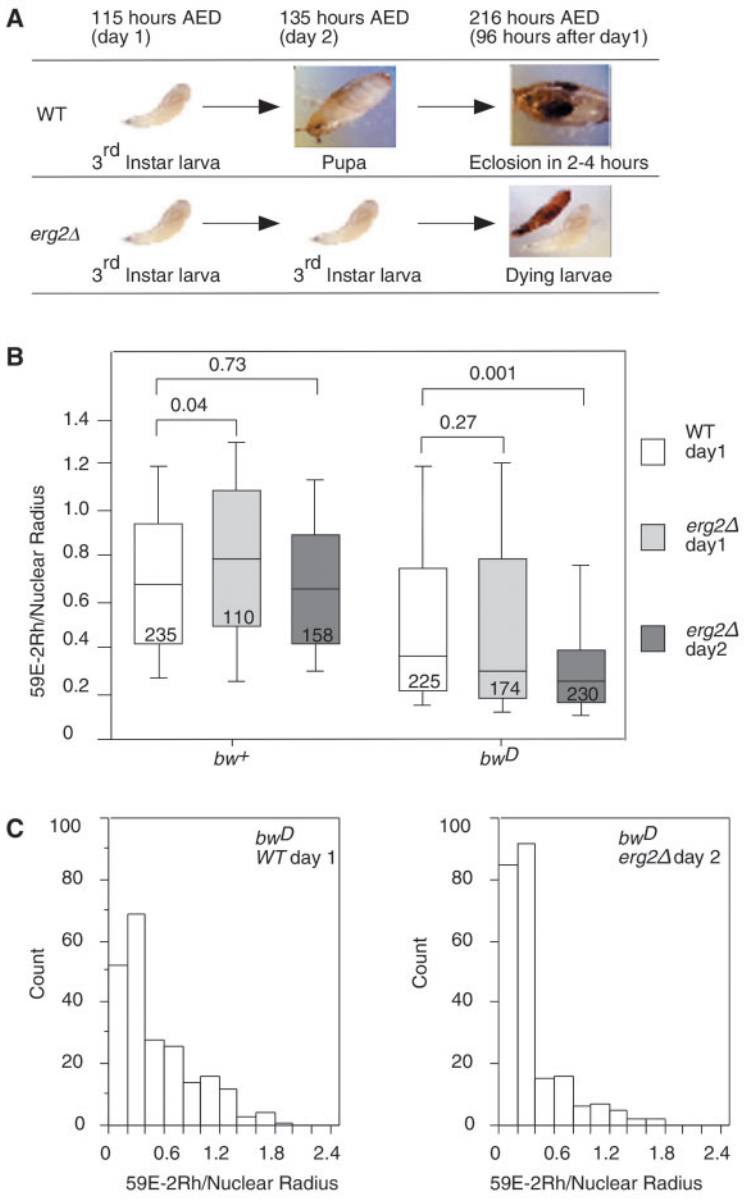

Fig. 2. Heterochromatic association in CNS nuclei from developmentally arrested larvae. (A) The effect of feeding larvae *erg2Δ* yeast. Larvae were reared on wild-type yeast (top) or *erg2Δ* yeast (bottom). Irrespective of the yeast fed, the larvae enter the mid-third instar larval stage and appear to undergo normal development until 115 hours AED. At 135 hours AED the larvae fed *erg2Δ* yeast remain stalled in the third instar stage and eventually die after a few more days. By this time, larvae fed wild-type yeast have pupated (they usually pupate at 118-125 hours AED) and are ready to eclose 216 hours AED. (B) Box plots (as in Fig. 1) show data from FISH on CNS from bw^+ and bw^D larvae. Larvae fed *erg2Δ* yeast were dissected at 110-115 hours AED (day 1) or 130-135 hours AED (day 2). Larvae fed wild-type (WT) yeast were dissected at 110-115 hours AED (day 1). *P* values (Mann-Whitney) are shown above brackets for the respective sets. (C) Histograms showing the distribution of the distances between 59E and 2Rh in bw^D larvae reared on WT yeast on day 1 and *erg2Δ* yeast on day 2.

Dynamics of chromosomal movements in primary cultures of eye imaginal discs

On the basis of our studies in fixed tissue, we were interested in learning if the difference in nuclear organization of bw^D arose due to a disparity in the dynamics of chromosomal movement in differentiated versus undifferentiated cells. Changes in chromatin dynamics during differentiation could play a major role in the changing patterns of gene expression. With advances in microscopy and the development of the *lac* operator (*lac*O) and *Lac*I-GFP fusion technique, the dynamics of movement of chromosomal regions can now be studied in live tissue (Belmont et al., 1999). Crosses were performed to produce flies that contained two copies of the *lac*O repeats, one marking the X chromosome and the other the *bw* locus on the second chromosome (Fig. 3A) and expressed a mRFP-LacI fusion protein. Nuclei from these larvae contained two bright dots. The unbound, but nuclear localized, mRFP-LacI was less bright than the spots and was used to demarcate the interphase nucleus (Fig. 3B, Figs S1, S2 and S3 in Supplementary material).

We compared the dynamics of chromosomal movement in differentiated cells posterior to the morphogenetic furrow to undifferentiated cells anterior to the furrow in primary cultures of eye imaginal discs obtained from third instar larvae. Differentiated cells were easy to recognize as they were part of the ommatidial clusters in the eye disc (Fig. 3B). Images were taken at a time interval of 5 seconds at a single plane of focus (for movies of nuclei from anterior and posterior cells, see Figs S1 and S2 in Supplementary material). To deduce the dynamics of movement, the distance between the two dots (d) was computed over time. A measurement of distance between the center of mass of two loci eliminates the need for correction for apparent motion that may arise due to nuclear rotation and translation (Vazquez et al., 2001). To determine the characteristics of chromatin movement, the mean square change in distance $\langle \Delta d^2 \rangle$ which is the average of Δd^2 values over all possible combinations of timepoints separated by Δt (Qian et al., 1991; Vazquez et al., 2001) was computed for each nucleus (Fig. 3C).

The diffusion coefficient (D) for undifferentiated cells was calculated at Δt=5-10 to be 3.3×10^{-4} μm^2/seconds and was comparable to that for differentiated cells 2.1×10^{-4} μm^2/seconds (Fig. 3C and Table 2). An unpaired *t*-test comparing the average $\langle \Delta d^2 \rangle$ values in differentiated versus undifferentiated cells for Δt values less than 25 seconds found no significant difference. However, for Δt values greater than 25 seconds the *t*-test

indicated that the values were significantly different. The average value of step size (Δd) for both differentiated and undifferentiated cells at a time interval of 10 seconds was found to be equivalent (Table 2). Although the rate of chromatin diffusion was found to be similar in both cell types, the plot for differentiated cells reached a plateau as early as Δt=50 seconds. The plateau height on the $<\Delta d^2>$ graph is referred to as the radius of confinement and is the size of the region through which the locus is free to diffuse. A similar plateau was not seen in undifferentiated nuclei during the time period studied (Fig. 3C), suggesting that chromatin motion is much more constrained in differentiated cells. This is exemplified by the smaller radius of confinement for the tagged chromosomes in differentiated cells, calculated from the graph in Fig. 3C and shown in Table 2. A comparison of the nuclear radius of undifferentiated and differentiated cells shown in Table 2 suggests that the decrease in the radius of confinement in differentiated cells may be due to a general nuclear contraction and/or chromatin compaction that accompanies differentiation.

Table 1. Comparison of cell cycle parameters of CNS cells from larvae reared on *erg2Δ* or wild-type (*WT*) yeast

Yeast fed, time dissected	Mitotic index*	% G1†	%BrdU label†
WT	1.16	69.85	9.97
erg2Δ day 1	1.36	73.14	8.39
erg2Δ day 2	0.99	72.20	4.48

*The mitotic index in the CNS of larvae reared on *WT* yeast dissected at 110-116 hours. AED was compared to larvae fed *erg2Δ* yeast dissected at 110-116 hours AED (day 1) and at 130-136 hours (day 2). For each set, data was collected from 12-15 larvae and 4-10 fields were studied per larva. All nuclei in the field were counted. The mitotic index was computed by calculating the percentage of cells undergoing mitosis. The difference in the mitotic index for each set was not found to be statistically significant as calculated by G-test for goodness of fit.

†In a separate experiment, larvae were fed BrdU laced food for 3 hours and immediately dissected. Quantitation of DNA content based on DAPI fluorescence intensity allowed us to categorize the cells in G1 or S/G2 phase of the cell cycle. Nine to ten larvae were used for each time point per yeast fed and at most five fields were scored per larva. As shown in the table, an increase in the proportion of cells in G1 phase was observed in larvae reared on *erg2Δ* yeast (*P* value <0.01 computed by G-test). A decrease in the proportion of cells labeled with BrdU was seen in larvae fed *erg2Δ* yeast at 136 hours AED (*P* value <0.001 computed by G-test) when compared to larvae dissected at 110-116 hours irrespective of the yeast fed.

Dynamics of chromosomal movement in developmentally arrested larvae

Our observations suggest that the association between bw^D and 2Rh is a factor of time spent in G1/G0 phases of the cell cycle since the previous mitosis. This, in turn, implies that the movement of *bw* towards 2Rh could either be a slow directed movement or a random walk event. In both these cases the probability of association would increase if more time is available for the event. If this is indeed the case, then the establishment of association between bw^D and 2*R*h observed in the developmentally arrested larvae should not arise due to a change in the rate of chromatin movement. We therefore compared chromatin movement in primary cultures of the CNS derived from larvae fed wild-type yeast to those reared on *erg2Δ* yeast (for movies of nuclei from CNS cells, see Fig. S3 in Supplementary material). We followed the movement

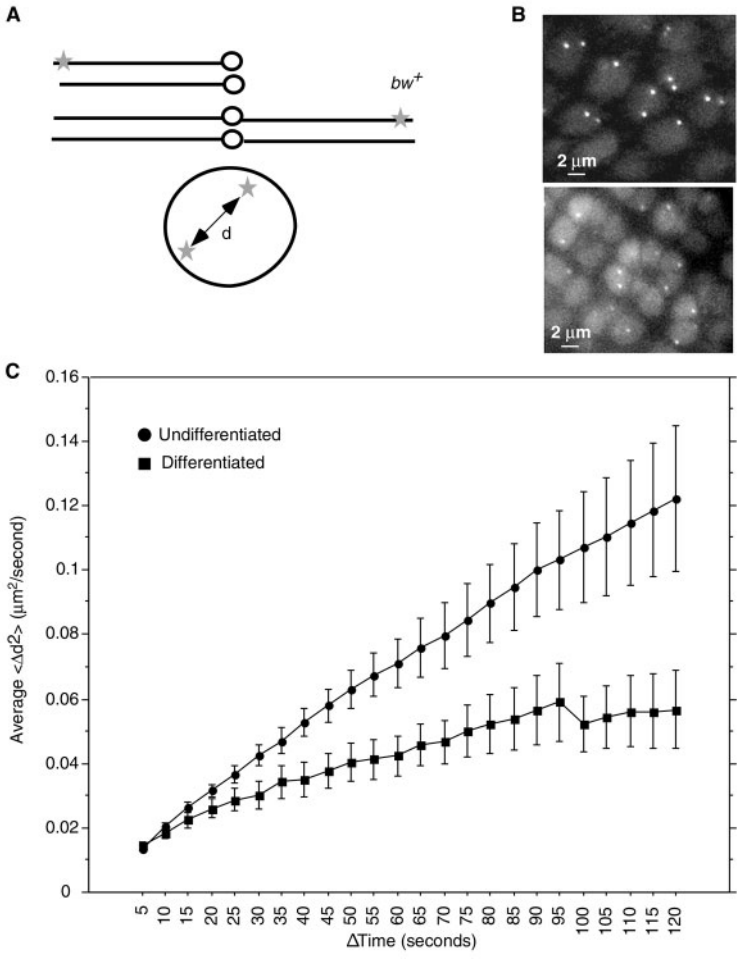

Fig. 3. The dynamics of chromosomal movement in differentiated and undifferentiated cells in primary cultures of eye imaginal discs. (A) Location of *lac*O repeats (stars) at 1F on the X and 59E on the 2nd chromosomes. The bottom circle is a schematic of these markers in interphase nuclei; d is the measure recorded at each time point. (B) Representative images from larvae containing the two above described *lac*O repeats and expressing mRFP-LacI fusion protein under an ubiquitin promoter. The top panel shows undifferentiated nuclei present anterior to the morphogenetic furrow and the bottom panel shows differentiated cells from ommatidial clusters present posterior to the furrow. The background fluorescence of mRFP demarcates the nucleus. The two bright dots are the two loci tagged with *lac*O repeats bound with mRFP-LacI fusion protein expressed under an ubiquitin promoter. (C) Plot of the average of mean square change in distance $<\Delta d^2>$ vs. the time interval (Δt). The distance between the center of masses of the two tagged loci was computed over time. For undifferentiated cells data was collected from 19 nuclei from 7 eye discs dissected from third instar larvae. In the case of differentiated cells, the data was obtained from 21 nuclei from 9 eye-discs. Error bars show ± 1 standard error of the mean. Comparison of the height of the graphs reveals that the two loci are confined to a smaller nuclear space in differentiated cells, suggesting that chromosomal movement is more constrained during differentiation.

Table 2. Measurement of nuclei from *Drosophila* larval tissue

	Mean step size (μm)	Standard deviation (μm)	Radius of confinement (μm)	Diffusion coefficient (μm^2/seconds)	Mean nuclear radius (μm)
Undifferentiated	0.11	0.089	>0.30	3.3×10^{-4}	2.98
Differentiated	0.10	0.086	0.22	2.1×10^{-4}	1.73
WT day 1	0.10	0.093	>0.29	3.7×10^{-4}	2.64
erg2Δ day 1	0.08	0.068	>0.26	1.9×10^{-4}	2.57
erg2Δ day 2	0.09	0.082	>0.28	2.7×10^{-4}	2.60
Fixed	0.04	0.036	0.06	1.0×10^{-7}	2.39

The mean step size was calculated at Δt=10 seconds. Since only the graphs for average $<\Delta d^2>$ vs. Δt for differentiated cells and fixed control reach a plateau (Fig. 3C and Fig. 4), the values for the radius of confinement for the other data sets were computed from the $<\Delta d^2>$ values at Δt=120 seconds. These values represent the lower limit of the radius of confinement.

of the *bw* locus with respect to the X chromosome, as was done in eye imaginal discs (Fig. 4). As theorized, the average $<\Delta d^2>$ among larvae reared on wild-type yeast when compared with those reared on *erg2Δ* yeast on either day 1 or day 2 was not statistically significant at any Δt (computed by unpaired *t*-test). The diffusion coefficients were also similar (Table 2). As a control we also imaged fixed nuclei and found that the plot for average $<\Delta d^2>$ did not change with increasing time intervals. The diffusion coefficient was found to be 1.0×10^{-7} μm^2/seconds, confirming that the change in average $<\Delta d^2>$ over time, observed in live tissue, was not an artifact of drift or jitter that may arise during microscopy. The average step size of chromosomal movements at a time interval of 10 seconds was also found to be similar irrespective of the yeast fed or the time of development (Table 2).

Discussion

A cell experiences vast changes in its transcription profile during differentiation. In addition, the nucleus can undergo changes in volume, shape, global chromatin condensation and distribution, and qualitative and quantitative changes in nuclear protein content (reviewed by Francastel et al., 2000). We show that in addition to these changes, there is a change in chromatin dynamics. Interestingly, it seems that, at least in the differentiating eye imaginal discs, this change is mostly due to a decreased area of confinement, not to a change in the rate of movement.

One may imagine that the diffusion coefficient would be influenced by several factors, including the degree of compaction of the chromatin fiber, the anchoring of chromatin to nuclear structures and the rigidity or density of materials that may contribute to a nuclear matrix. Studies of Sedat and colleagues have found that the diffusion coefficient is different in various cell types in *Drosophila*. In spermatocytes derived from third instar larvae, the rate of chromatin diffusion in nuclei in mid G2 was found to be 1×10^{-3} μm^2/seconds, whereas for late G2 the value is 5×10^{-5} μm^2/seconds (Vazquez et al., 2001). The diffusion coefficient of chromatin in nuclei from early *Drosophila* embryos has been reported as 2×10^{-3} μm^2/seconds (Marshall et al., 1997). The calculated diffusion coefficients in our studies fall between these two extremes; they ranged from $1.9\text{-}3.7\times10^{-4}$ μm^2/seconds (Table 2) and are close to the value of 1.2×10^{-4} μm^2/seconds found for chromatin in mammalian tissue culture cells (Chubb et al., 2002). However, it is interesting to note that their seems to be little to no change

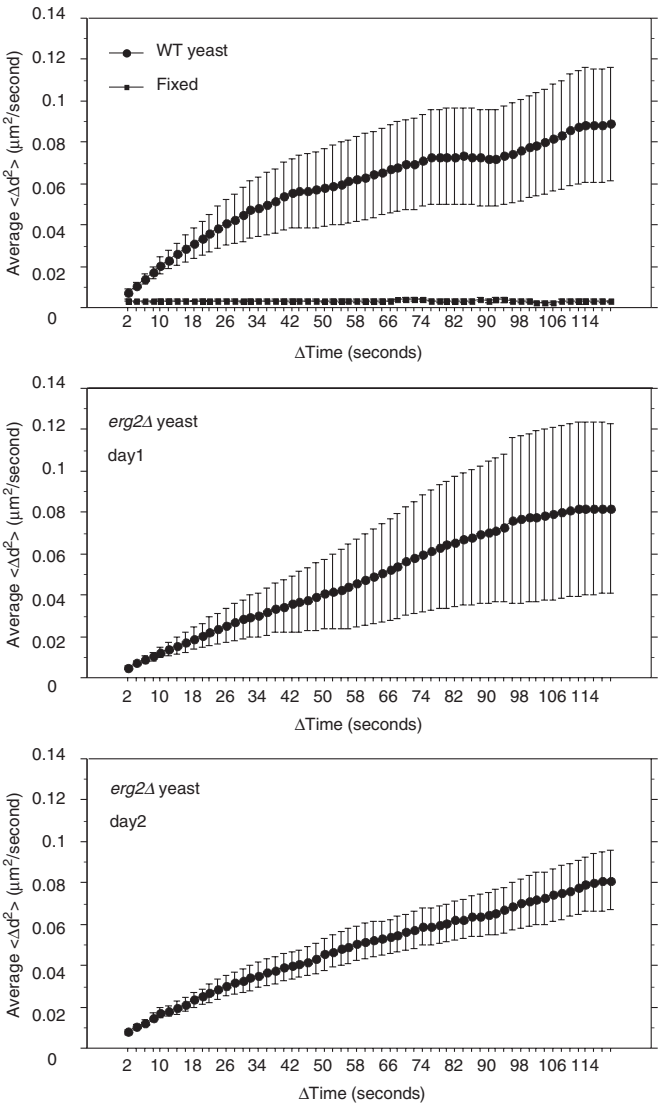

Fig. 4. Comparison of the dynamics of chromosomal movement among larvae reared on wild-type yeast and developmentally arrested larvae reared on *erg2Δ* yeast. No significant change in chromatin motion was observed in larvae reared on *erg2Δ* yeast when compared with those reared on wild-type yeast. For larvae reared on wild-type yeast the data is from 12 nuclei in the CNS dissected from 3 larvae. For larvae reared on *erg2Δ* on day 1 the number of nuclei was 10, dissected from 3 larvae and on day 2 the number of nuclei was 13 from 4 larvae. For fixed nuclei the numbers were 8 and 3, respectively. Error bars show ± 1 standard error of the mean.

in the diffusion coefficient over development in the system we have examined.

We have also shown that a specific rearrangement of interphase nuclear organization, the association of two blocks of constitutive heterochromatin, is correlated with differentiation. However, our results suggest that the heterochromatic association between bw^D and 2Rh is not a result of a discrete event during differentiation. Rather, association is a consequence of time spent, since the last mitosis, in either G1 or G0 phase of the cell cycle. The increased association between bw^D-2Rh is seen not only in cells undergoing differentiation but also in the developmentally arrested larvae with a lengthened G1 phase, as well as some undifferentiated cells of the eye imaginal disc. Association is also observed in cells that will re-enter S phase (Csink and Henikoff, 1998). These findings suggest that the association could be regulated by a factor that accumulates or is lost slowly from the nucleus. This gradual change would promote heterochromatin cohesion after mitosis, independent of differentiation. Indeed, some of the changes in nuclear organization seen during differentiation may be simply due to the fact that differentiation is accompanied by an extended G1 or exit from the cell cycle. We did see an increase in the confinement of a locus on differentiation. Therefore, it is possible that once a specific nuclear organization is established, its maintenance is assisted by a differentiation specific event that confines chromosomal movement.

What aspects of chromatin and nuclear structure could gradually change to promote association? Heterochromatin is known to be inherently sticky, and although this interaction is sequence independent (Sage and Csink, 2003), it is probably mediated by proteins that recognize the repetitive nature of heterochromatin (Dorer and Henikoff, 1997). The delayed association between bw^D and 2Rh and the fact that centromeric regions do not associate to form a chromocenter in most diploid tissues of *Drosophila* show that heterochromatin does not always self associate. These findings suggest that heterochromatic cohesion is regulated. Indeed, there are proteins that are known to associate with heterochromatin during mitosis, but gradually dissociate at a slow rate usually over a period of a few hours during interphase (Platero et al., 1998). Other proteins, like HP1 are removed from chromatin just before mitosis and redeposited as the cell exits mitosis (Kellum et al., 1995; Murzina et al., 1999). Moreover, the binding of HP1 to stable heterochromatic domains has been found to be highly dynamic (Cheutin et al., 2003). It has been speculated that this transient binding allows for competition among proteins to bind to heterochromatin and regulate its chromatin structure and cohesion.

There are several possible factors that participate in the alterations in nuclear structure during development and may contribute to the changes that increase chromatin confinement and promote bw^D association. The composition of nuclear lamina is known to change as a cell undergoes differentiation and progresses through the cell cycle. Lamins have been shown to directly and indirectly interact with chromatin or chromosomal binding proteins (reviewed by Goldman et al., 2002). Therefore, the increased confinement to a smaller nuclear space during differentiation that we see in the eye discs may be due to specific changes in the nuclear lamina or its associated proteins that in turn regulate their interaction with chromatin. HP1, a protein known to promote bw^D-2Rh associations (Csink and Henikoff, 1996), interacts with the nuclear envelope protein lamin B Receptor (LBR) (Ye and Worman, 1996). Changes in the phosphorylation profile of HP1 have been correlated with its cell cycle dependent assembly on chromatin, protein-protein interactions and heterochromatinization (reviewed by Kellum, 2003). Modification of HP1, during differentiation, may alter its interaction with the nuclear lamina as well as with chromatin. This in turn might help maintain the nuclear organization necessary to obtain the transcription profile accompanying differentiation.

Because the *bw* locus is present on the tip of the right arm of the second chromosome, its association with 2Rh in nuclei heterozygous for bw^D requires large-scale chromosomal movements. If the movement of bw^D towards 2Rh is a fast directional event we would expect to see triggered, saltatory chromatin reorganization in a short time window. So far, we have detected no such chromosomal movements. Instead, it appears that the movement of bw^D towards 2Rh is a slow directed movement or a random walk event where the probability of association would increase if more time was available for the event. This aspect of differentiation could then be viewed as being due to an extended G1, which would provide more time for establishment of final nuclear organization. Previous studies in *Drosophila* spermatocytes (Vazquez et al., 2001) and in *S. cerevisiae* (Heun et al., 2001) show that loci undergo both rapid, local saltatory movements as well as occasional long range movements (Gasser, 2002). The direction of these movements seems to be quite random. We postulate that bw^D randomly moves about its area of confinement within the nucleus in early G1. If it encounters 2Rh during early G1 it should be able to move away, but if it encounters 2Rh during late G1, due to the increase in the stickiness of heterochromatin, it can associate with 2Rh. Such association, and other later changes in nuclear organization, would then be stabilized by an overall increase in the confinement of chromatin.

We thank Roger Tsien, Patrick O'Farrell and Andrew Belmont for plasmids. We thank Andrew Belmont for Drosophila lines. Shoba Subramanian advised us on the preparation of the *erg2Δ* line. The SGDP provided yeast strains. Fred Lanni and Andrew Gove provided advice concerning the calculations in this paper. Andrew Gove wrote the Excel macro used to calculate $<\Delta d^2>$ and a program used to facilitate measurement of the distances between the loci. We thank Joy Sabl and Jon Minden for comments and insights on an earlier version of this paper. This work was supported by an American Cancer Society grant (RSG-00-073-04-DDC) to A. Csink.

References

Ashburner, M. (1989). *Drosophila: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Belmont, A. S., Li, G., Sudlow, G. and Robinett, C. (1999). Visualization of large-scale chromatin structure and dynamics using the lac operator/lac repressor reporter system. *Methods Cell Biol.* **58**, 203-222.

Bos, M., Burnet, B., Farrow, R. and Woods, R. A. (1976). Development of Drosophila on sterol mutants of the yeast Saccharomyces cerevisiae. *Genet. Res.* **28**, 163-176.

Brown, K. E., Guest, S. S., Smale, S. T., Hahm, K., Merkenschlager, M. and Fisher, A. G. (1997). Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* **91**, 845-854.

Brown, K. E., Baxter, J., Graf, D., Merkenschlager, M. and Fisher, A. G.

(1999). Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. *Mol. Cell* **3**, 207-217.

Campbell, R., Tour, O., Palmer, A., Steinbach, P., Baird, G., Zacharias, D. and Tsien, R. (2002). A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877-7882.

Cheutin, T., McNairn, A. J., Jenuwein, T., Gilbert, D. M., Singh, P. B. and Misteli, T. (2003). Maintenance of stable heterochromatin domains by dynamic HP1 binding. *Science* **299**, 721-725.

Chubb, J. R., Boyle, S., Perry, P. and Bickmore, W. A. (2002). Chromatin motion is constrained by association with nuclear compartments in human cells. *Curr. Biol.* **12**, 439-445.

Csink, A. K. (2004). Analysis of chromosomes of the larval CNS by FISH and BrdU labeling. *Methods Mol. Biol.* **247**, 343-352.

Csink, A. K. and Henikoff, S. (1996). Genetic modification of heterochromatic association and nuclear organization in Drosophila. *Nature* **381**, 529-531.

Csink, A. K. and Henikoff, S. (1998). Large-scale chromosomal movements during interphase progression in Drosophila. *J. Cell Biol.* **143**, 13-22.

Csink, A. K., Bounoutas, A., Griffith, M. L., Sabl, J. F. and Sage, B. T. (2002). Differential Gene Silencing by trans-heterochromatin in Drosophila melanogaster. *Genetics* **160**, 257-269.

Davis, I., Girdham, C. H. and O'Farrell, P. H. (1995). A nuclear GFP that marks nuclei in living Drosophila embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. *Dev. Biol.* **170**, 726-729.

Dernburg, A. F., Broman, K. W., Fung, J. C., Marshall, W. F., Philips, J., Agard, D. A. and Sedat, J. W. (1996). Perturbation of nuclear architecture by long-distance chromosome interactions. *Cell* **85**, 745-759.

Dorer, D. R. and Henikoff, S. (1997). Transgene repeat arrays interact with distant heterochromatin and cause silencing in cis and trans. *Genetics* **147**, 1181-1190.

Francastel, C., Schubeler, D., Martin, D. I. and Groudine, M. (2000). Nuclear compartmentalization and gene activity. *Nat. Rev. Mol. Cell Biol.* **1**, 137-143.

Gasser, S. M. (2002). Visualizing chromatin dynamics in interphase nuclei. *Science* **296**, 1412-1416.

Goldman, R. D., Gruenbaum, Y., Moir, R. D., Shumaker, D. K. and Spann, T. P. (2002). Nuclear lamins: building blocks of nuclear architecture. *Genes Dev.* **16**, 533-547.

Heun, P., Laroche, T., Shimada, K., Furrer, P. and Gasser, S. M. (2001). Chromosome dynamics in the yeast interphase nucleus. *Science* **294**, 2181-2186.

Kellum, R. (2003). HP1 complexes and heterochromatin assembly. *Curr. Top. Microbiol. Immunol.* **274**, 53-77.

Kellum, R., Raff, J. W. and Alberts, B. M. (1995). Heterochromatin protein 1 distribution during development and during the cell cycle in Drosophila embryos. *J. Cell Sci.* **108**, 1407-1418.

Kosak, S., Skok, J., Medina, K., Riblet, R., Le Beau, M., Fisher, A. and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science* **296**, 158-162.

Li, C. and Meinertzhagen, I. A. (1995). Conditions for the primary culture of eye imaginal discs from Drosophila melanogaster. *J. Neurobiol.* **28**, 363-380.

Manuelidis, L. (1985). Individual interphase chromosome domains revealed by in situ hybridization. *Hum. Genet.* **71**, 288-293.

Marshall, W. F., Straight, A., Marko, J. F., Swedlow, J., Dernburg, A., Belmont, A., Murray, A. W., Agard, D. A. and Sedat, J. W. (1997). Interphase chromosomes undergo constrained diffusional motion in living cells. *Curr. Biol.* **7**, 930-939.

Murzina, N., Verreault, A., Laue, E. and Stillman, B. (1999). Heterochromatin dynamics in mouse cells: interaction between chromatin assembly factor 1 and HP1 proteins [In Process Citation]. *Mol. Cell* **4**, 529-540.

Platero, J., Csink, A., Quintanilla, A. and Henikoff, S. (1998). Changes in chromosomal localization of heterochromatin binding proteins during the cell cycle in Drosophila. *J. Cell Biol.* **140**, 1297-1306.

Qian, H., Sheetz, M. P. and Elson, E. L. (1991). Single particle tracking. Analysis of diffusion and flow in two-dimensional systems. *Biophys. J.* **60**, 910-921.

Sage, B. T. and Csink, A. K. (2003). Heterochromatic self-association, a determinant of nuclear organization, does not require sequence homology in Drosophila. *Genetics* **165**, 1183-1193.

Sokal, R. R. and Rohlf, F. J. (1981). *Biometry*. New York: W. H. Freeman.

Spector, D. L. (2003). The dynamics of chromosome organization and gene regulation. *Annu. Rev. Biochem.* **72**, 573-608.

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila a practical approach* (ed. D. B. Roberts), pp. 175-198. Washington, D.C.: IRL Press.

Thummel, C. S. (1995). From embryogenesis to metamorphosis: the regulation and function of Drosophila nuclear receptor superfamily members. *Cell* **83**, 871-877.

Trask, B., van den Engh, G., Pinkel, D., Mullikin, J., Waldman, F., van Dekken, H. and Gray, J. (1988). Fluorescence in situ hybridization to interphase cell nuclei in suspension allows flow cytometric analysis of chromosome content and microscopic analysis of nuclear organization. *Hum. Genet.* **78**, 251-259.

Vazquez, J., Belmont, A. S. and Sedat, J. W. (2001). Multiple regimes of constrained chromosome motion are regulated in the interphase Drosophila nucleus. *Curr. Biol.* **11**, 1227-1239.

Verschure, P. J., van Der Kraan, I., Manders, E. M. and van Driel, R. (1999). Spatial relationship between transcription sites and chromosome territories. *J. Cell Biol.* **147**, 13-24.

Ye, Q. and Worman, H. J. (1996). Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to Drosophila HP1. *J. Biol. Chem.* **271**, 14653-14656.