The relative positions of condensed chromosomes are maintained between divisions in the epidermis of *Calpodes ethlius*

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Summary

The larval epidermis of *Calpodes ethlius* (Lepidoptera, Hesperiidae) is a syncytium of doublets where sibling cells are twins that remain connected by residual midbodies between mitoses. Twins resemble one another more than their other neighbours in such structural features as the shape and number of nucleolar particles and the number of actin bundles. We have now found that they also resemble one another in the position of the condensed chromosomes that occur in female cells. Female lepidopteran cells contain one or more particles of condensed chromatin, depending on their ploidy. In the epidermis, nuclei with two condensed chromosomes are found in pairs and are separated by the same distances. However, clones of cells with multiple condensed chromosomes are not all alike, suggesting that chromosomes are repositioned at mitosis. Separation distances between chromosomes remain the same between but not through cell divisions, suggesting that determinants for nuclear structure are conserved through interphase and relaxed at mitosis. Although the condensed chromosomes of sibling nuclei resemble one another in their separation, they differ in their orientation, as would be expected if whole nuclei rotate in the plane of the epithelium.

Key words: chromosome position, nuclear structure, pattern pairing, condensed chromosomes.

Introduction

Two insect orders, Lepidoptera and Trichoptera, have heterogametic females with ZW sex chromosomes (Feltwell, 1982; Robinson, 1971). Female insect cells have a condensed chromosome presumed by Moore (1966) to be the Z chromosome. The nuclei of all cells of female *Calpodes ethlius* (Lepidoptera, Hesperiidae) contain such particles of condensed heterochromatin, which mark the position of the inactive chromosome within the nucleus (Locke, 1990). In epidermal cells they are roughly spherical, about 1.0 µm in diameter. Polyploid cells of other tissues contain single proportionately larger particles, reaching 10 µm in diameter in oenocytes (Locke, 1969). In larval epidermal cells polyploidy is reflected in the number rather than the size of the particles (Locke, 1988). The point of interest for the present study is that when there are two of these particles, there are also two in the nucleus of an adjacent epidermal cell.

In the 5th stage larval epidermis all divisions are lateral. Sibling cells retain their midbody between mitoses that may be several days apart, creating an epithelium of Siamese twin cells one cell thick. Twins resemble one another more than their other neighbours in such structural features as the shape and number of nucleolar particles (Locke and Leung, 1985) and the number of actin bundles (Delhanty et al., 1991). In this paper we describe how the distances separating condensed chromosomes in twinned cells remain the same between but not through cell divisions, suggesting that determinants for nuclear structure are conserved through interphase and relaxed at mitosis.

Materials and methods

Experimental animal

A colony of *Calpodes ethlius* (Lepidoptera, Hesperiidae) was kept in a greenhouse where the larvae were reared on Canna lily plants. The caterpillars were easily sexed because the ovaries and testes are visible through the transparent integument. The timing of larval development was standardized by rearing them in an incubator at 22°C on a 12:12 h, day:night, cycle. The 5th stadium (the time from the 4th - 5th ecdysis until the 5th - pupal ecdysis) took 8 days (Locke, 1970). Epidermal cells divide 36 h before ecdysis and again 90 h into the 5th stadium. Microscope observations were made on the epidermis of 5th stage female larvae in the period between divisions.

Preparation of the epidermis

Fifth stage caterpillars are up to 7.0 cm long and 1.5 cm wide. The integument (cuticle + the single layer of epidermal cells) was kept flat but with its natural proportions, by coating caterpillars with 5% aqueous gelatin, drying, and attaching them to a microscope slide with “krazy glue”. The glue adhered more firmly, but not permanently, to the cuticle after coating with gelatin. The glued larvae were then separated from the slide and fixed by influ-
tion with 5% formaldehyde. The glued integument was cut from the larva and fixation continued for 15 min. The integument was permeabilized for 10 min in 5% formaldehyde containing 1% Triton X-100. The glue mold, muscle and fat body were removed, leaving cuticle with epidermal cells preserved in one plane but otherwise in their natural positions.

Staining epidermal nuclei

Sheets of integument were incubated in 10 ml RNase (ICN Biochemicals) solution (1 mg RNase/ml 5×10⁻² M phosphate buffer, pH 7.4) at 34-37°C overnight, washed in distilled water, cut into 1 cm² segments and stained for 15 min in 1 ml of a solution containing 20 µg of propidium iodide. Excess stain was removed by three 5 min washes in distilled water. The integument was mounted in a 1:9 (v/v) phosphate buffer (pH 7.4):glycerol solution, with the epidermis facing a “0” coverslip.

Microscopy

Epidermal cells were examined with a Bio-Rad MRC600 confocal laser-scanning microscope, using a Nikon Planapo ×60 oil immersion objective (NA = 1.4) on a Nikon Diaphot-TMD inverted microscope. The microscope was equipped with an argon ion laser and a filter, excitation wavelength = 514 nm.

Selection criteria for nuclei

Large flat fields of epidermal cells were scanned using conventional epi-fluorescence to find paired nuclei, each containing two condensed chromosome particles. Clusters with more than two nuclei having double particles were not used, since selection of pairs then became subjective.

Optical sectioning

Selected pairs of nuclei were optically sectioned (0.1 µm/section) from apical to basal surfaces at zoom 4, 6 or 8 (Fig. 1A). Image averaging of each section reduced background noise. A z-series consisted of 40-70 sections. An extended focus image was formed by stacking all the optical sections. The same field was later optically sectioned at a lower zoom (and at 0.2 or 0.3 µm/section) to show the relationship between pairs of nuclei and the surrounding cells.

3D measurement of particle separation

The x-y (or 2D) separation between particles was measured directly from the extended focus image. The z separation was measured by counting the number of sections between the maximal particle diameter and multiplying by the stepping motor increments (i.e. 6 sections × 0.1 µm per section = 0.6 µm). The possible error described by Leung and Jeun (1992) in determining the centre of spherical objects was insignificant because of the small size of the condensed chromosome (less than or equal to 1 µm in diameter). The 3D separation distance was calculated using the x-y and z distances in the Pythagorean theorem (Fig. 2B). Only the 3D results are presented because there was no significant difference between the 3D and 2D measurements (paired sample t-test, P>0.05), probably because the nuclei were flattened ovals rather than spheres.

Measurement of the angle formed by the line joining particles and the mitotic plane

Straight lines were drawn on an extended focus image to connect the double particles within each nucleus. The angles made by these lines with the estimated previous plane of division were measured (Fig. 3A).

Statistical analysis

Two separate one-way analysis of variance (ANOVA) tests were used to determine whether (1) the angle and (2) the condensed chromosome separation distances were significantly different between the 18 pairs of measurements in 18 twinned cells.

Results

Nuclei in female Calpodes contain condensed chromosomes

Fields of epidermal cells from female larvae stained for DNA with propidium iodide showed that all nuclei contained small dense particles. The particles were about 1 µm in diameter, roughly spherical and more brightly fluorescent than the rest of the nucleus, suggesting that their DNA is more densely packed than the surrounding heterochromatin (Fig. 1). The particles are presumed to be condensed chromosomes. Most epidermal nuclei were the same size and had single particles, but some larger cells had two or more. Nuclei with two (Fig. 1A,B) or three particles (Fig. 1C,D) always occurred in pairs. The pairs are presumed to be sibling cells because multiple particles never occurred singly and the pairs were always surrounded by cells with single particles.

The separation of paired condensed chromosomes

The positions of condensed chromosomes were determined in 18 pairs of nuclei. Nuclei were reconstructed in 3D from stacks of 0.1 µm thick optical sections. Vertical profiles showed that the nuclei were usually ovals compressed in the plane of the epithelium (Fig. 2A). The horizontal (x-y distances) and the vertical (z distances) were measured as in Fig. 2B. The real separation of the chromosomes, (x²+y²+z²)¹⁄₂, was then calculated. The separation between particles in unpaired nuclei varied from 1 to 14 µm (Fig. 2D), confirming that the condensed chromosomes do not have a constant position in the nucleus (Fig. 2C). However, the separation of double chromosomes was similar in pairs of nuclei (ANOVA, P<0.0005), from which we conclude that sibling cells resemble one another more than they resemble other cells nearby.

Double chromosomes have different orientations in paired nuclei

Separation is a measure of the way that the condensed chromosomes are arranged within each nucleus. The direction

Fig. 1. Nuclei in epidermal cells from female Calpodes larvae contain particles of condensed chromatin. Propidium iodide staining of the epidermis for DNA shows most nuclei with single particles, but a few are polyploid and have two or more. Nuclei with more than one particle always occur in pairs. (A) Most cells have single particles, but one pair enlarged in (B) has two in each nucleus (l). (D) The pair of cells enlarged in (C) has 3 chromosomes (l). Pairs of nuclei are presumed to be in sibling cells because they are surrounded by fields of smaller nuclei all having single condensed chromosomes. Confocal summation of optical sections taken from cells about 4 days after the last mitosis. Bars, 5 µm.
of the separation is a measure of nuclear orientation in relation to structures outside the nucleus. If chromosome orientation is conserved in the whole nucleus between mitoses, as with the separation distances, then we might expect that the orientation of the double chromosome pairs would remain as mirror-images. With other nuclear components such as nucleolar particles, for example, orientation as well as separation is mirror-imaged in recently divided cells (Locke and Leung, 1985). On the other hand, if nuclear components can rotate, then the orientation of the heterochromatin might differ between pairs, even though their separations might remain constant. We therefore measured the angles subtended by the lines joining chromosomes and the plane between paired cells.

The angles subtended by the lines joining double particles and the plane separating paired cells were measured as in Fig. 3A. Angles measured in 18 nuclear pairs similar to Fig. 3B were found not to be mirror imaged (ANOVA, \(P>0.05\)). The orientations of sibling nuclei to one another are therefore not constant, even though they may be paired with respect to chromosome separation. We conclude that the components that conserve the separation distance
between chromosomes during interphase are free to change their orientation in the plane of the epithelium (Fig. 6, below).

**Pairs of chromosomes in clusters are not all separated by the same distance**

Double chromosomes usually occurred in pairs (as in Fig. 1A,B) but occasionally they were in fours (Fig. 4A) or even larger clusters (Fig. 4B). The low frequency of double chromosomes in pairs makes it unlikely that the clusters are chance associations of pairs. The clusters were all enclosed within fields of nuclei having single particles. Since the clusters were usually in fours, we presume that they are clones derived by division of pairs. Particles in these clusters of four did not all have the same separation. The separation was in two pairs as in Fig. 4A. Such double pairs were excluded from the calculations to show pairing, because it would have required the subjective selection of pairs from fours. Although the separations were clearly grouped in twos, each pair differed from the other pair, presumably because they were separated in lineage by an additional mitosis (Table 1). In larger clones (Fig. 4B), pairs could be matched subjectively but the separations extended over the whole range recorded in Fig. 2D. These observations suggest that whatever may determine the arrangement of chromosomes between mitoses, it is not conserved through mitoses.

**Table 1. Pairs of condensed female chromosomes in clones of four or more nuclei are not all separated by the same distances**

<table>
<thead>
<tr>
<th>Chromosome separation, 3D measurement (µm)</th>
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<td>Clone 1</td>
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</tr>
<tr>
<td>2.5</td>
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<td>4.4</td>
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<td>11.8</td>
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The separations of chromosomes in nuclei like those shown in Fig. 4 were measured. Although the nuclei could be subjectively grouped in pairs according to chromosome separation, they were not all alike.

**Discussion**

The arrangement of condensed chromosomes in sibling nuclei allows five conclusions: (1) after mitoses, sibling cells are alike in the position of condensed chromosomes. Patterns must therefore be replicated. (2) Chromosome position is conserved through interphase. Pattern is therefore not only replicated, but inherited. (3) Patterns do not survive through mitosis. The inheritance is transient. (4) There is no constant position of chromosomes in fields of epidermal cell nuclei. The repositioning of chromosomes during the cell cycle allows separation to vary, causing differences from cell to cell across the epithelium. (5) Nuclei may rotate in the plane of the epithelium, changing the orientation between nuclei but not the separation of chromosomes within nuclei. The conclusions are summarized in Figs 5 and 6.

**Pattern replication**

Since the early work of Heitz (1957), numerous studies have shown similarities between recently divided cells (Jordan et al., 1982; Locke and Leung, 1985; Rawlins and Shaw, 1990; Rawlins et al., 1991; Mathog and Sedat, 1989), giving evidence from a variety of cell types that similar spatial information determining the distribution of components is passed to daughter cells. In *Calpodes* epidermal nuclei, the inheritance of the spatial arrangement of condensed chromosomes can be recognized even several days after division, perhaps because of the conservation of midbodies.
The inheritance of chromosome position and its conservation through interphase

Similarities of condensed chromosome separation and nucleolar pattern persist in sibling nuclei for 5-6 days between mitoses, implying that particular nuclear structures are stable during interphase. The idea that chromosomes maintain their position during interphase agrees with studies on *Drosophila* embryo cells, where the relative positions of decondensation sites at the beginning of interphase are the same as early condensation sites at the next prophase (Hiraoka et al., 1989). Chromosomes in interphase nuclei are not like bowls of spaghetti, as they are depicted in even the most up-to-date textbooks (Alberts et al., 1989, pp. 762 and 767; Darnell et al., 1990, p. 155). Either as a result of their association with the nucleoskeleton or because of close packing, interphase chromosomes occupy discrete domains that are not intermixed with other chromosomes (Heslop-Harrison and Bennett, 1990).

The redetermination of chromosome separation distance at mitosis

Clones are not formed from similar cells, suggesting that chromosomes are repositioned between interphases. Nucleolar patterns also resemble one another in sibling pairs but not larger groups of nuclei (Locke and Leung, 1985). Release from the old nuclear skeleton and assembly on the mitotic spindle, followed by release from the spindle and recapture by the new nuclear skeleton, might be expected to introduce some randomness to the position of a chromosome. Relative chromosome position is reordered during the transition from prophase to metaphase in *Crepis capillaris*, where all chromosome pairs are associated with their homologues during prophase, but by metaphase the association only persists between nucleolar organizer chromosomes (Oud et al., 1989). A relationship between chromosomes and cytoplasmic microtubules precedes the metaphase rearrangement in mouse 3T3 fibroblasts. Prometaphase chromosome configuration translates interphase spatial order into order at the metaphase plate (Chaly and Brown, 1988). Once on the metaphase plate even quite large movements do not affect the relative positions of chromosomes. These relative positions might be expected to be transferred to the new interphase nuclear skeleton.

The variability of chromosome position in fields of nuclei

Because the interphase patterns shared by sibling cells do not survive through the next mitosis, genetic clones may consist of cells with differently patterned nuclei. Even

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**Fig. 4.** Pairs of double chromosomes that are in clones are not all separated by the same distance. (A) Nuclei with double chromosomes occasionally occur in fours that are presumed to be the daughters of sibling pairs. The chromosomes of one pair are separated by 3.3 and 4.0 µm, and the other pair by 5.2 and 6.1 µm. Bar, 10 µm. (B) Larger clones can be grouped subjectively into pairs with separations that may extend over the whole range. Bar, 25 µm. See Table 1.
clones of four cells are not all alike in the positions of their sex chromosomes. Our findings confirm observations showing that some chromosomes have different positions in nuclei of cells that are otherwise genetically and developmentally similar. For example, the separation of twin Barr bodies in tissue cultures of XXX human fibroblasts varies from cell to cell (Belmont et al., 1986), much like the distribution of condensed chromosomes in Calpodes. Although these examples show that chromosomes can differ in position in otherwise identical cells, other nuclear components may have tissue-specific positional determinants or domains that are similar in a general way from cell to cell. Three overlapping kinds of domain have been described in interphase nuclei: chromosomal, nucleolar and heterochromatin (Hilliker and Appels, 1989). For example, centromeres and telomeres lie at opposite poles in Drosophila blastoderm nuclei (Hiraoka et al., 1990); the nucleolar organizer regions on chromosomes 4 and 7 in Pisum sativum occupy special sites (Wolff and Quednau, 1988); human chromosomes are confined to domains in human-hamster hybrid cell lines (van Dekken et al., 1989); centromeres may be organizing centres for cell type-specific interphase chromosome patterns (Manuelidis and Borden, 1988). Even Barr bodies, which have variable positions, may have preferred domains near the nuclear envelope (Belmont et al., 1986). Variability in position does not imply that there is no determinant for that position, only that such a determinant may be differently placed in otherwise similar nuclei. Attention was drawn to this transient inheritance of patterns in otherwise variable sibling cells by calling it somatic inheritance (Locke, 1990).

Nuclear rotation with conservation of separation

The idea that nuclei may rotate in the plane of the surface agrees with observations on the orientation of the nuclear groove. The apical nuclear surface has a single deep fold, appearing in whole mounts as a clear bar with only half the thickness of the nucleus (Locke, 1985). The grooves have all orientations in the plane of the epithelium. Rotation of linked nuclear material in the plane of the surface but not in the vertical plane is the simplest explanation for the conservation of separation distance concurrent with changed chromosome orientation. Rotation in the plane at right angles to the surface is less probable because it would require nuclear components to bend, changing separation distances (Fig. 6). Nuclear rotation has been described in other cell types, for example in mouse neurones (DeBoni and Mintz, 1986).

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