

Three-Dimensional Organization of *Drosophila melanogaster* Interphase Nuclei. II. Chromosome Spatial Organization and Gene Regulation

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Abstract. In the preceding article we compared the general organization of polytene chromosomes in four different *Drosophila melanogaster* cell types. Here we describe experiments aimed at testing for a potential role of three-dimensional chromosome folding and positioning in modulating gene expression and examining specific chromosome interactions with different nuclear structures. By charting the configurations of salivary gland chromosomes as the cells undergo functional changes, it is shown that loci are not repositioned within the nucleus when the pattern of transcription changes. Heterologous loci show no evidence of specific physical interactions with one another in

any of the cell types. However, a specific subset of chromosomal loci is attached to the nuclear envelope, and this subset is extremely similar in at least two tissues. In contrast, no specific interactions between any locus and the nucleolus are found, but the base of the X chromosome, containing the nucleolar organizer, is closely linked to this organelle. These results are used to evaluate models of gene regulation that involve the specific intranuclear positioning of gene sequences. Finally, data are presented on an unusual class of nuclear envelope structures, filled with large, electron-dense particles, that are usually associated with chromosomes.

EUKARYOTIC cells use a variety of mechanisms to ensure that genes are expressed in the cell types in which their products are required. The relative activity of a gene in different cell types can vary over at least eight orders of magnitude (31). It has been proposed that the specific positioning of genes within defined subnuclear compartments is a component of this remarkable range of control (12, 14, 30). In this paper we focus on the potential role of the three-dimensional arrangement of chromosomal loci in the tissue-specific regulation of gene expression.

Continued interest in this question has been generated by studies of the so-called nuclear matrix, an insoluble residue left after treatment of nuclei with high or low salt concentrations, detergent, and DNAase. Many claims for specific association of certain DNA sequences with this structure, particularly of transcribed and/or replicating sequences, have been made. However, the data are not yet consistent enough to make strong conclusions (reviewed in references 33, 36). Most significantly, no relationship between this *in vitro* fraction and a structure existing *in vivo* has been demonstrated.

The idea that certain DNA sequences can be segregated into specific subnuclear compartments as a means of gene regulation has received support from a study of DNAase-hypersensitive sites in isolated nuclei. There is an apparent concentration of such sites—thought to mark active loci—in the periphery of the nucleus (30). On the other hand, [³H]uridine pulse-labeling experiments on intact cells indi-

cate nascent transcripts are concentrated at euchromatin-heterochromatin boundaries, which are not necessarily at the nuclear surface (18), so the issue remains open.

Indirect analysis of interphase chromosome positions, such as the elegant experiments of Cremer et al. (16), has revealed that in many types of cells, chromosomes are maintained in relatively stable, polarized configurations within limited subnuclear territories. Unfortunately, it has not yet been possible to describe the dispositions of chromosomes beyond this gross structural level. A review of experiments suggesting nonrandom elements in interphase chromosome spatial organization can be found in Comings (15).

In the absence of direct data on the subject, a variety of hypotheses have been advanced that invoke a role for specific three-dimensional nuclear architectures in various aspects of gene regulation. In the present report, we test some of the predictions made by these models using a quantitative analysis of the three-dimensional folding of chromosomes in different polytene tissues of *Drosophila melanogaster* and in salivary gland cells subjected to functional perturbations. We show that chromosomes do not fold into determinate configurations, that specific chromosome-envelope interactions occur but are unlikely to be necessary for tissue-specific gene expression, that specificity in chromosome-nucleolus contacts is restricted to the nucleolar organizer, and that chromosome configurations are remarkably stable even during drastic shifts in the pattern of transcription. We also provide data on an unusual class of nuclear envelope structures that may be involved in nuclear-cytoplasmic exchange. In the

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Discussion section, the impact of these data on models of nuclear architecture will be considered.

Materials and Methods

Heat Shock and Ecdysone Treatment of Salivary Glands

Either of two physiological media were employed for dissections and incubations: the buffer of Shield and Sang (49) or Grace's insect medium (Gibco, Grand Island, NY) as modified by Ashburner (3). The latter led to slight nuclear shape changes but appeared otherwise satisfactory. Glands were mounted in buffer on a slide under a bridged coverslip and sealed with paraffin oil. A Zeiss Neofluar $\times 100/1.3$ NA objective lens (Carl Zeiss, Inc., Thornwood, NY) was used with bright field optics. To achieve optimal contrast, the field diaphragm was closed down as far as possible, the condenser aperture was fully open, and the condenser lens was brought just short of the Kohler condition, i.e., slightly further away from the sample. Immersion oil was put on the condenser lens. This technique was found to be superior to either phase-contrast or Nomarski differential interference-contrast methods.

In the first type of heat shock experiment, a temperature-controlled stage mount (52) was modified to allow the use of a high-resolution lens. Supports constructed from coverslips and slides were affixed to the mount and made to be slightly thicker than the slide/coverslip sandwich used to hold the glands. The mount was preheated to 37°C, and the slide was quickly taped to it. The assembly was taped to the stage of a Zeiss Universal microscope (Carl Zeiss, Inc.), slide side down, with the objective lens extending into the hole in the metal mount. 1–2 min is required between the taping of the slide and examination of the first nucleus. In some experiments nuclei were observed with a $\times 40/0.75$ NA lens. Photographs were taken at one or a few focal planes every 1–2 min for 20–35 min; between picture-taking times, the full depth of the nucleus was monitored.

In the second kind of experiment, glands were placed on the microscope, and two nuclei were selected for observation. Sets of bands visible in a selected focal plane in each were sketched. Pictures of 15–20 serial optical sections, spaced at one-notch intervals on the focus knob, were then taken. The slide was subsequently placed on a 37°C hot plate for 20 or 30 min and immediately placed back on the microscope stage. The original focal plane from each nucleus was identified from its sketch, and a parallel series of optical planes was photographed. In some experiments, another 1.5–2 h at room temperature was allowed to elapse, and the same focal planes were rephotographed. After treatment, glands were squashed in lactoacetoorcein to verify that the major heat shock loci were puffed. Other controls included treating glands identically except for the omission of the heat shock and testing glands for trypan blue exclusion after the experimental manipulations (a 0.4% dye solution was diffused into the space under the bridged coverslip; cells damaged by handling rapidly accumulated the dye and had pycnotic nuclei).

We checked the possibility that the heat shock loci were already clustered before the temperature shift. This was done by measuring the distances between all pairs of the nine heat shock loci (or the six major ones) in 17 unfixed nuclei (28) and finding the average (overall average = 13.9 ± 3.2 μm). This value was then compared to the same measurement for a set of nine (or six) control loci. To match the relative locations of the heat shock loci in the genome, the control loci were chosen by "projecting" the heat shock loci onto the other autosome (e.g., the 67B locus on 3L projects onto 27B on 2L). The control loci had a similar overall average separation (13.0 ± 2.6 μm) to the heat shock loci.

For the ecdysone treatments, Grace's insect medium with Ashburner's modifications (3) was used, and β -ecdysteroid (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 5 μM . Optical sections were collected in the same manner as above except that in one experiment, optical sectioning was done on a computer-controlled Zeiss Axiomat microscope (Carl Zeiss, Inc.) and images were stored on a magnetic disk. One lobe of the gland was squashed before each experiment to determine whether it had the PSI (intermoult) puffing pattern (4). After collecting data at 1–2-h intervals over an 8–12-h span, the gland was squashed to ascertain the final puffing stage reached. The heat shock puffing pattern was not observed. As controls, we analyzed puffing patterns of sets of PSI glands that were mounted on slides and exposed to ecdysone as above. The estimated stages, based on Ashburner's (4) data, were as follows: 4-h incubation (\sim PS6), 6-h (\sim PS8–9), 8-h (\sim PS10–11), and 10-h stages (\sim PS11–12). The kinetics appear similar to those described by Ashburner, but a few differ-

ences were noted, e.g., 74EF/75B remained slightly puffed at later stages and 78D was unpuffed early but puffed at 6–8 h. Cells were shown to be alive by trypan blue exclusion for at least 6.5 h after mounting in ecdysone.

Quantitative Analysis of Models

Chromosome–nuclear surface contact frequency plots are described in Hochstrasser et al. (28). The maximum distance from the surface used as a cutoff to generate each plot is given in Figs. 4 and 5. 2×2 contingency tests for the association of high-frequency peaks in each tissue (selected as those frequencies with a random probability of less than 0.05 as determined by Monte Carlo calculations) with sites of intercalary heterochromatin and with the peaks in other tissues were described previously (28). As before, the chromosome arms were divided into three-letter subdivisions (e.g., 10A–10C) for the contingency tests. To test if the overlap of contact peaks between tissues was truly due to specific local properties, we compared peak positions between nonhomologous arms in two tissues (prothoracic X vs. salivary 2L, etc.); peak positions were not correlated ($\chi^2 = 0.897$, $P > 0.3$). Chromosome intradistance plots and the methods employed to analyze them were detailed previously (28, 40). Distance to the nucleolar surface in prothoracic glands (27) was measured in a manner analogous to distance to the nuclear surface. A 2.25- μm cutoff was used for the contact frequency plots as this is near the largest average chromosome width in any nucleus.

Results

Chromosome Positions Are Not Dynamically Coupled to Gene Expression

It is possible that gene spatial arrangements are dynamic and undergo specific changes as the pattern of transcription changes. If they occur asynchronously within a tissue, such changes could account for the finding that chromosome configurations have only limited similarities between cells of the salivary gland (28). Different cells within the same gland are known to differ in the timing of glue granule formation (8) and in steady-state levels of RNA (47).

The hypothesis of dynamic gene positioning was tested by examining salivary gland chromosome dispositions before, during, and after heat shock. Raising the temperature of salivary glands from 25 to 37°C, *in vivo* or *in vitro*, leads to a dramatic change in the pattern of transcription; most genes previously active cease being transcribed while a specific new set of genes is activated (2). Rather than attempt to construct an entire new set of chromosome models from heat-shocked glands and compare them with our previous data, we examined the same nuclei in both states. This could be done with bright-field optical microscopy.

The experiment was performed in two ways. In the first, third instar salivary glands were mounted in physiological medium on a temperature-controlled stage heated to 37°C. This allowed nuclei to be monitored continuously for changes in chromosome positions. In the second, optical sections were first collected from several nuclei in a gland, the gland was then subjected to a 20–30-min heat shock, and a new set of optical sections were collected from the same nuclei. A total of 10 nuclei from five glands were studied in the latter set of experiments, about two dozen in the former. Fig. 1 shows corresponding optical sections from nuclei before and after heat shock. In Fig. 1 *g* an optical plane similar to those in Fig. 1 *c* and *f* has also been photographed 1.5 h (at room temperature) after heat shock. There are no prominent movements of chromosome segments in any of the nuclei from any of the experiments. Movements of ~ 1 μm or less may occur and are observed on rare occasions. It should be noted that before heat shock, the heat shock loci

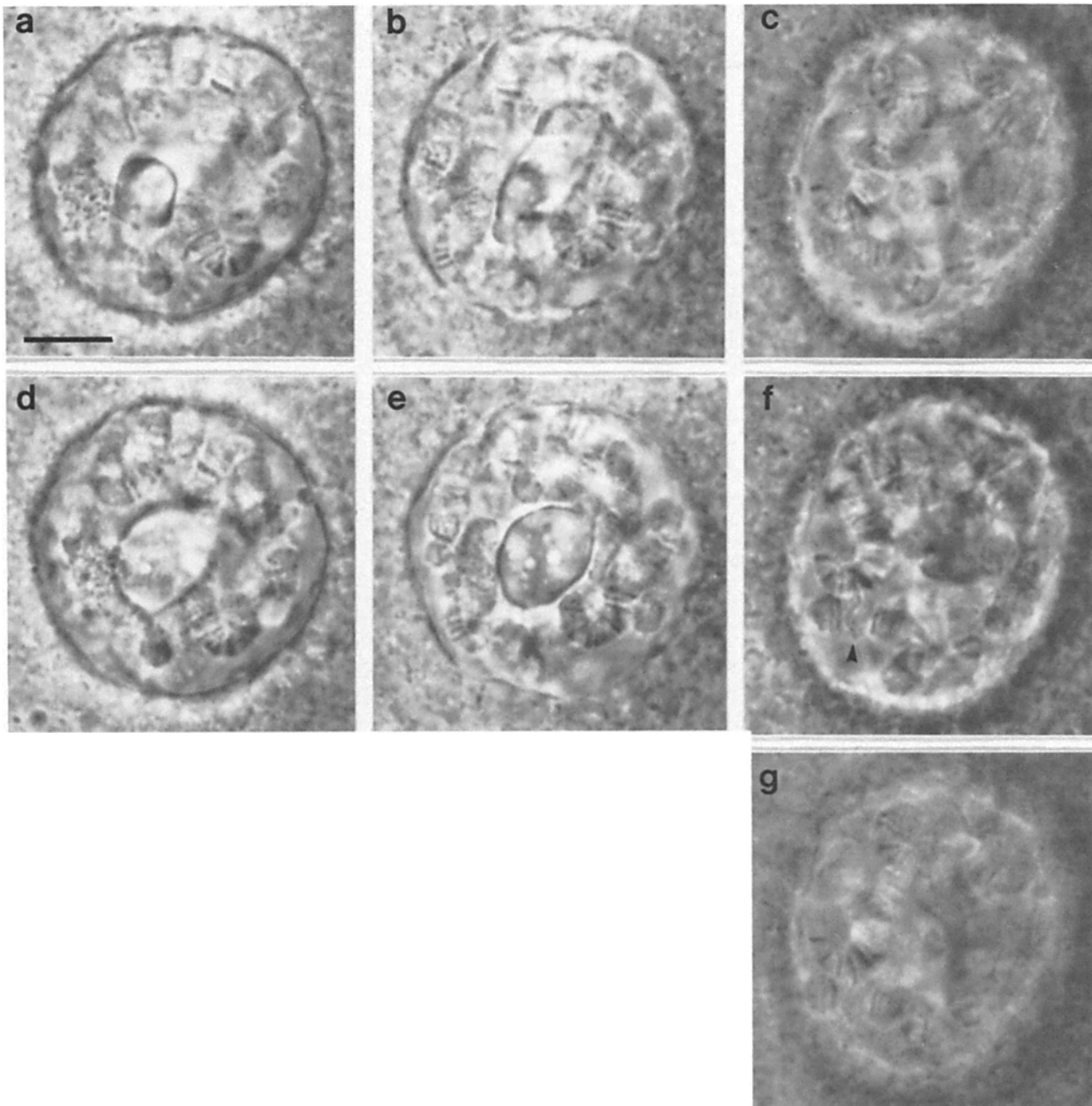


Figure 1. Effect of heat shock on chromosome positions. (a–c) Optical planes of nuclei in living salivary glands photographed before heat shock. (a and b) Different planes within the same nucleus. (d–f) Corresponding sections from the same nuclei shown in the first row but after a 30- (d and e) or 20-min (f) heat shock at 37°C. The similarity of chromosome positions is evident as is the change in nucleolar shape. (g) A section of approximately the same optical plane shown in c and f after the gland has been at room temperature for 1.5 h after the heat shock. A reversible change in optical clarity can be discerned. The arrowhead points to the “ballet skirt” in subdivision 68C of 3L; the 67B heat-shock locus is nearby (not visible in section). Bar, 10 μ m.

are neither preferentially localized in the nuclear periphery (28) nor closely clustered within any subnuclear domain (see Materials and Methods).

Two other observations from these experiments should be pointed out. First, there is a rather dramatic change in nucleolar shape and/or position after heat shock (Fig. 1). It tends to go from an irregular, asymmetric structure to one that is more spherical and simpler in outline. Changes in nucleolar morphology during heat shock have previously been noted in diploid cells and may be related to the block

in ribosome processing and assembly during such a stress (45). The effect is at least partially reversible; 1.5–2 h after returning to room temperature, the nucleolus is again more irregularly shaped. Second, the optical properties of the cell are often altered by heat shock. Immediately after treatment, the nucleus is optically much clearer and the nuclear boundary much more distinct. This may be due to the known cytoskeletal rearrangements that accompany heat shock, e.g., collapse of the intermediate filament network around the nucleus (10). This optical effect is also reversible (Fig. 1). It is

interesting that despite these cytological changes around and amidst the chromosomes, their configurations remain unperturbed.

Analogous experiments were done by incubating glands with moulting hormone, β -ecdysone, and charting chromosome dispositions. This treatment closely simulates the sequence of puffing changes seen *in vivo* (3). Six nuclei from two glands have been examined over 8–12-h periods at 1- or 2-h intervals. These experiments are complicated by various cytological changes that occur over these long times, including changes in nuclear shape and/or orientation, changes in nucleolar shape, swelling of the lumen of the gland, and pronounced changes in optical clarity presumably due to accumulation of cytoplasmic glue granules. Hence, only a half dozen or so chromosome segments could be reliably identified at most or all the time points. All of these appeared unchanged in position or orientation (data not shown). So to a first approximation, this result agrees with the heat shock experiments and with *in vivo* controls done over a more limited timespan (28).

Nonspecific Chromosome Configurations in All Cell Types

The manner in which the genome is packaged in the interphase nucleus clearly has many tissue-specific aspects in both diploid (15) and polytene cells (27). For example, tissue-specific intranuclear locations and associations of centric heterochromatin typify nuclei in many cell types. To what extent this tissue-specificity involves the precise relative positioning of genes has been a matter of intense speculation (7, 12, 14, 39). With chromosome models from a large set of nuclei in different tissues, these speculations can now be experimentally tested.

Specific gene–gene structural interactions were not found in salivary glands (28). However, we did not know if this was generally true of interphase nuclei. We have now extended our analysis to nuclei from different tissues and with lower levels of polyteny. Also, the problem of cell type heterogeneity, which could not be rigorously ruled out for salivary gland cells, should be avoided in the “large cells” (46) of the middle midgut. Almost all the cells examined were right at the Ia/Ib midgut bend (27). Morphologically, these cells are obviously distinct from the cells around them; moreover, histochemical and ultrastructural studies, among others, clearly show that this cell type is segregated from neighboring midgut cell types (46).

Chromosome configurations have been analyzed in three new tissues: the prothoracic gland, the large cells of the middle midgut, and the hindgut (26, 27). Prothoracic gland nuclei, unlike nuclei from the gut tissues, are very similar in general chromosome organization to salivary gland nuclei. We have emphasized the use of intradistance plots to compare chromosome folding in different nuclei (28, 40). In these plots, the distance between every pair of loci on a chromosome model is measured and then plotted as an intensity value on a two-dimensional grid in which each axis represents cytological position along the chromosome. The pattern of intensities will be a mapped representation of the three-dimensional folding of the chromosome. Plots generated for a particular chromosome arm from every nucleus can be compared in several ways to look for similarities in chromosome folding.

Fig. 2 displays “rank order” intradistance plots for chromosome arm 2L from both prothoracic gland and midgut nuclei. What are ranked in ascending order in such plots are the intensity values (intradistances) at each point in the original set of plots (28, 40). To illustrate how one reads these plots, consider the point in Fig. 2 *a* marked by an arrowhead. This point represents the pair of cytological loci 25C and 27C. Its intensity value (shade of gray) in this rank = 5 plot represents a 3- μ m distance. Thus, 25C and 27C are within 3 μ m of each other (in the model) in at least 5 of the 11 nuclei examined.

From Fig. 2 *a* it is evident that very few loci show a common set of close physical interactions even in a small subset (less than half) of the prothoracic gland nuclei, and these common contacts are confined to the region along the diagonal of the plot, i.e., to loci that are necessarily close together by virtue of their cytogenetic position. Prothoracic gland chromosomes in these nuclei have an average diameter of 1.6 μ m, and the average length between each cytological division (e.g., 21A to 22A) is \sim 4 μ m (preceding paper). Thus, to continue with the example above, the 25C and 27C loci would be \sim 8 μ m apart if the chromosome ran perfectly straight through this region; to find that, in a limited number of nuclei, these two sites are within 3 μ m of each other is not surprising. Much of the chromosome in this tissue is tightly coiled; the off-diagonal features in Fig. 2 *a* could simply indicate that the bending of the chromosome needed to achieve this compaction sometimes occurs in similar segments in several nuclei. That the plot is primarily an indication of relatively nonspecific chromosome bending rather than specific locus–locus interactions is supported by the control shown in Fig. 2 *b*. To make this plot, the 11 original plots have had their intensity values shifted by different random distances along the diagonal; the resulting rank = 5 plot (Fig. 2 *b*) therefore reflects only the random overlap of intensity values in the original plots (see reference 28 for details). The randomized plot looks very similar to the original rank-order plot, having features as far off the diagonal as in the latter case.

The midgut rank = 5 plot in Fig. 2 *c* reveals the same lack of folding specificity. Because chromosomes are much straighter in this tissue, the intensity overlaps in the rank-order plots due to nonspecific bending drop off even more rapidly as one goes up in rank; darker values (closer appositions) rapidly shrink toward the diagonal. Fig. 2 *c* indicates that no two loci separated by more than approximately two cytological divisions on the chromosome are within 5 μ m of each other in even 20% (5 of 25) of the nuclei. The similarity of the randomized midgut plot (Fig. 2 *d*) to Fig. 2 *c* further suggests a lack of folding specificity. The absence of regularities in midgut chromosome configurations can be directly verified from the models. Fig. 3 shows stereopair models of 2L from four nuclei. Configurations range from an uncoiled U-shape to a tight ball of loops; local similarities are also wanting. Hence, it is unlikely that we are missing examples of specific three-dimensional configurations in the process of abstracting the information into intradistance plots. Hindgut chromosome models were also directly compared and found to lack consistent similarities. Other ways of comparing plots, e.g., by direct examination of individual intradistance plots from all the nuclei, confirm these conclusions. We have also failed to find specific interactions between loci on differ-

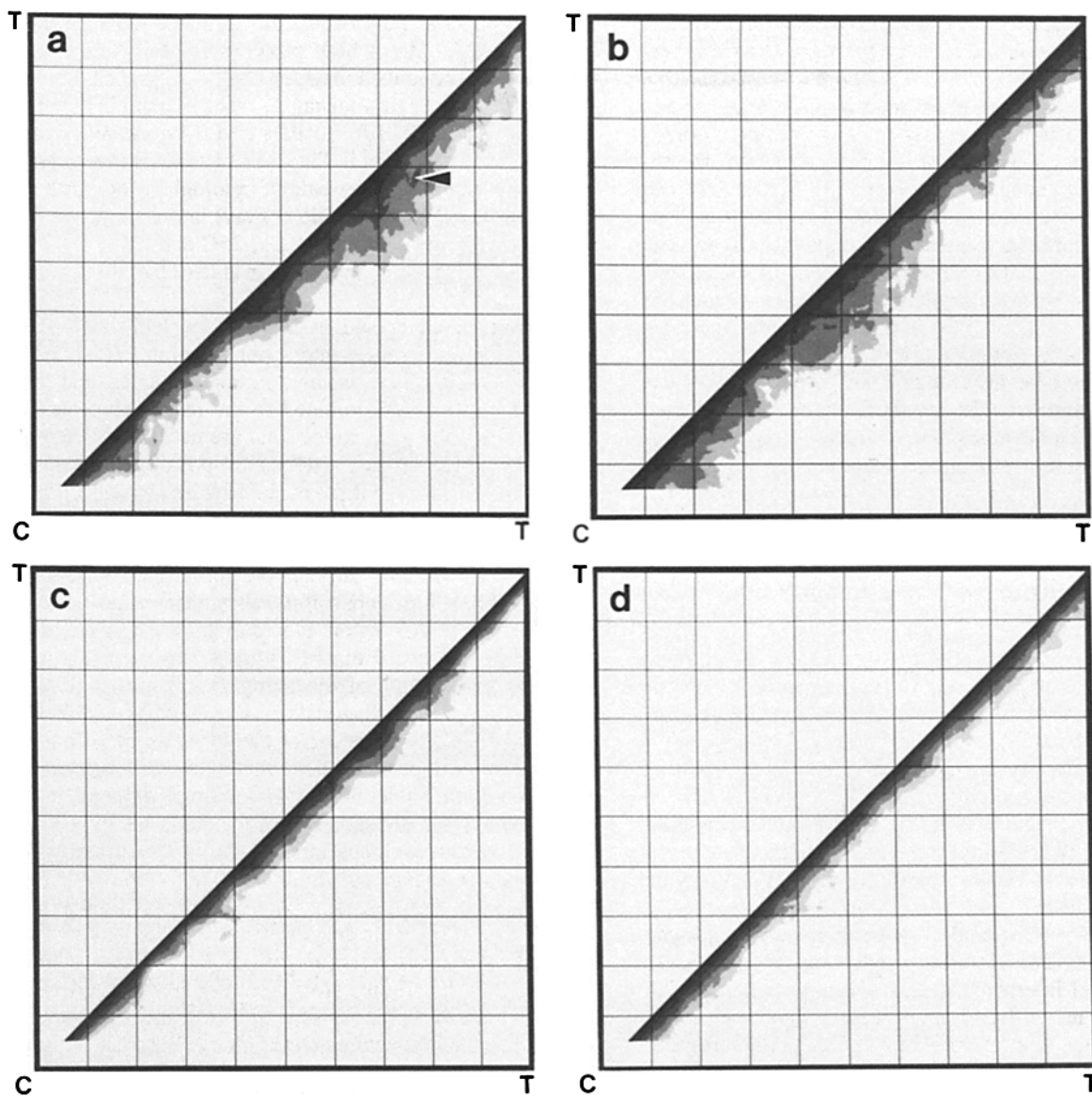


Figure 2. Three-dimensional folding of prothoracic gland and midgut chromosomes. (a) A rank = 5 rank-order intradistance plot for prothoracic gland chromosome 2L. The plot was generated from the original set of 11 2L intradistance plots. Distances between loci are represented by contoured intensity values; the darkest regions mark pairs of loci closest together in space. Distances are rank ordered at each pixel from the smallest (rank = 1) to the largest (rank = 11). Contour steps are separated by 1 μm , and distances $>5 \mu\text{m}$ are set to white. Cytological position is plotted along both axes with grid lines separated by two cytological divisions. T, telomere (21A); C, chromocentromere endpoint (40F, but cytology only followed to 40A). For further explanation, see text. The arrowhead marks an area representing the interaction of 25C and 27C, which are within 3 μm of each other in at least 5 out of 11 nuclei. (b) A rank = 5 rank-order intradistance plot generated from the same set of 11 prothoracic gland 2L intradistance plots except that the intradistance values were randomly displaced with respect to the cytogenetic sequence by a different amount in each (see text). Values are plotted as in a. (c) A rank = 5 rank-order intradistance plot for middle midgut chromosome 2L. The plot was made from the original set of 25 2L intradistance plots. (d) The analogous rank = 5 plot generated from the 25 midgut 2L intradistance plots that had been randomly displaced relative to one another.

ent chromosome arms using interdistance plots, although the general relative arrangement of chromosome arms can be determined this way. A specific tendency for loci to be positioned far from each other, beyond the general effect of the Rabl orientation found in some of the cell types, is not evident either. In summary, in no tissue have we found evidence for specific chromosome folding or specific interactions between a particular pair or small groups of loci.

Specific Chromosome–Nuclear Envelope Contacts in Different Tissues

Although chromosomes do not have determinate three-dimensional configurations, they may nevertheless form specific contacts with the nuclear envelope. This was shown to be the case in salivary glands (28). It is not known, however, what functional significance these specific surface contacts may have. One possibility is that specific genetic loci are

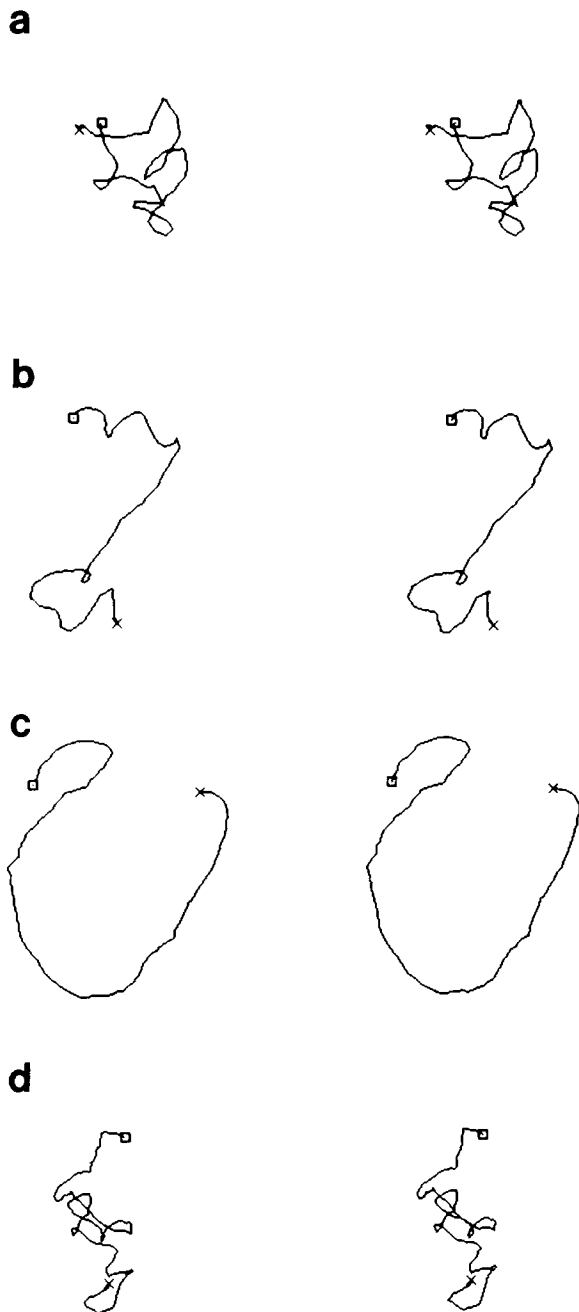


Figure 3. Chromosome arm 2L in four different middle midgut nuclei. The wide range of configurations is evident.

situated on the envelope in different cell types as a means of modulating their tissue-specific expression (12, 30). A comparison of high-frequency surface contacts in different tissues allows such an hypothesis to be experimentally tested.

Surface contact frequencies for the five major arms in both midgut and prothoracic gland nuclei are shown in Fig. 4. Peaks in these plots represent chromosomal loci that frequently contact the nuclear surface. Because peaks and valleys are expected even with random juxtapositions, a selection criterion must be used to choose those peaks that are high enough to make it relatively unlikely that they are due to random contacts. The Monte Carlo method for determining random probabilities was described previously (28). As

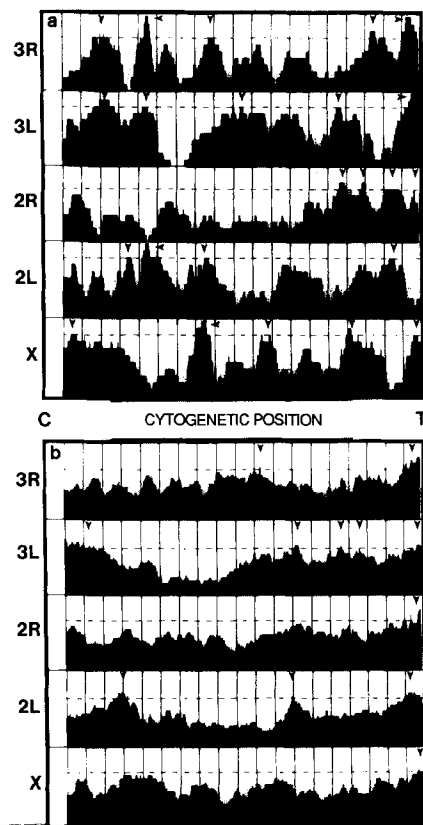


Figure 4. Nuclear surface contact frequencies for the five major chromosome arms. (a) Surface contact frequencies for chromosomes in 11 prothoracic gland cells. A locus was considered to contact the surface if it fell within $0.75 \mu\text{m}$ of the convex polygon in which the model was inscribed (40). This cutoff is slightly smaller than that used for midgut chromosomes (b) because these chromosomes are thinner, and with the smaller number of nuclei, the plots are more sensitive to random contacts. The abscissa represents cytological position, with each vertical division representing one cytological division (e.g., 1A-1F) in the respective arms (13). The ordinate scale runs from 0 to 11 (out of 11). T, telomere; C, chromocenter. The values in the left-most division in each plot have been set to 0 because the cytology is not followed into these areas. Cytology was noted on the models at 20 loci on each chromosome (telomeres and all A1 bands, e.g., 15A1); cytological points in between were placed by linear interpolation between the identified loci (40). The telomeres in the 2R and 3R plots fall one letter subdivision short of the right end. The arrowheads highlight regions where the contact frequency is at or above the $P < 0.05$ cutoff (indicated by the dashed lines). Note that broad peaks could represent the overlap of two or more attachments, e.g., there are three strong intercalary heterochromatin sites between 83D and 84D. (b) Surface contact frequencies for chromosomes in 25 middle midgut cells. All aspects of the plot are exactly as in a except that the ordinate scale is 0-25 (out of 25) and the plots were generated with a $1.0\text{-}\mu\text{m}$ cutoff distance. Cytology on the X chromosome in two of the three male nuclei could not be identified along the entire arm, so the plot of X chromosome contacts includes 23 chromosomes rather than 25 (ordinate scale is therefore 0-23).

before, we have chosen a cutoff of $P < 0.05$ (random probability of occurring this many or more times) for selecting frequency peaks that are likely to represent specific contacts. As a comparison with a large, new set of models from salivary gland nuclei has shown, this cutoff provides a relatively con-

servative estimate of the number of specific surface contacting loci (Mathog, D., and J. W. Sedat, manuscript submitted for publication).

As in salivary glands, different loci on prothoracic gland chromosomes vary markedly in the frequency with which they are found in the nuclear periphery (Fig. 4 *a*). At least 23 loci (*arrowheads*) are on the envelope at frequencies that are unlikely, by the above criterion, if contacts were only made randomly. Significantly, a very strong concordance between this set of loci and the high-frequency contacts in salivary glands has been found (Fig. 5). The relation was assayed with a 2×2 contingency test (Materials and Methods) and found to be highly significant ($\chi^2 = 35.6$, $P \ll 0.001$). These sites are also closely associated with intercalary heterochromatin loci (6, 54) by the same test ($\chi^2 = 23.3$, $P \ll 0.001$).

The similarity between the two tissues extends further. First, in both tissues the average frequency of surface contacts is lowest in chromosome arm 2R. Second, of the 15 high-frequency contacts identified in the salivary gland, 12 of them overlap sites found in the prothoracic gland. The exceptions are instructive. One of them is the single salivary gland peak that was not at an intercalary heterochromatin position (72B). The other two peaks not found in prothoracic glands are centered at 64C and 67D; in both cases, these loci fall just below the $P < 0.05$ cutoff and may thus represent sampling effects rather than real differences. Another possibility, for 64C at least, is that a nearby intercalary heterochromatin site interferes with its ability to reach the surface; 65C has a strong peak in the prothoracic gland data. The 19CE peak is actually found in salivary glands as well, although we chose not to discuss it because of its proximity to the surface contacting chromocenter. The chromocenter is usually not on the surface in prothoracic gland nuclei (27). Finally, of the 10 prothoracic gland high-frequency contacts not found in the original salivary gland data set, seven actually do form peaks in another collection of salivary gland nuclei (Mathog, D., and J. W. Sedat, manuscript submitted for publication), and eight (including all seven of the above) overlap intercalary heterochromatin loci (4D, 59C, 60F, 61A, 65C, 75C, 86D, and 89E). Thus, 20 of the 23 high-frequency contacts in prothoracic gland nuclei have corresponding frequency peaks in salivary gland nuclei, and 21 of 23 involve intercalary heterochromatin loci. The extent of variation between the two tissues is comparable to the variation seen between different samples of salivary gland nuclei. In conclusion, the specificity of peripheral localization of chromosomal loci appears to be indistinguishable between the two tissues and in both is associated with sites containing intercalary heterochromatin.

A very different picture emerges when chromosome–nuclear surface appositions are analyzed in middle midgut nuclei (Figs. 4 *b* and 5). The characteristic pattern of surface contacts seen in the previous tissues is no longer present. In fact, an apparent loss of specificity is seen. Only 12 loci show contact frequencies at or above the $P < 0.05$ cutoff, and five of these are at telomeres. The patterns of peaks are also flatter; this is reflected in a lower variance in peak height across each plot as compared to salivary and prothoracic gland plots. The very-high-frequency peaks that characterize the latter tissues, e.g., at 12EF on X and 35AC on 2L, are absent. Nonetheless, there remains a small overlap in the patterns

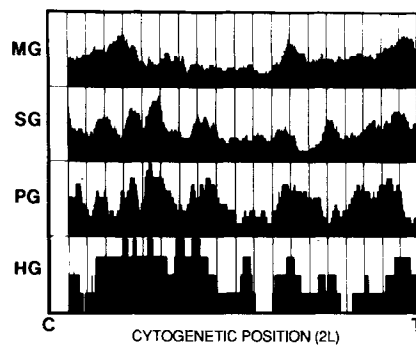


Figure 5. Comparison of nuclear surface contact frequencies for 2L in four tissues. Plots are made as in Fig. 4, and include two plots from that figure. (MG) Midgut (25 nuclei; cutoff distance of $1 \mu\text{m}$). (SG) Salivary gland (24 nuclei; cutoff distance of $1 \mu\text{m}$). Reprinted from Hochstrasser et al. (28). (PG) Prothoracic gland (11 nuclei; cutoff distance of $0.75 \mu\text{m}$). (HG) Hindgut (four nuclei; cutoff distance of $0.5 \mu\text{m}$).

between tissues. Midgut contacts are associated with both prothoracic ($\chi^2 = 7.95$, $P < 0.01$) and salivary gland ($\chi^2 = 8.97$, $P < 0.01$) frequency peaks; however, if the telomeric divisions are omitted from the comparison, the former association no longer achieves significance ($\chi^2 = 1.60$, $P < 0.2$). A correlation with intercalary heterochromatin, largely due to the telomeric contacts, is also observed ($\chi^2 = 12.1$, $P < 0.001$). Possible reasons for the dichotomy between surface contacts in midguts and the other tissues will be considered in the Discussion section.

With only four hindgut nuclei reconstructed, a complete analysis of surface apposition frequencies cannot be done, but a preliminary survey is suggestive. In Fig. 5, the surface-contact frequency plot for chromosome arm 2L is compared with the corresponding plots in the other three tissues. There is already some indication of the pattern of contacts seen in the prothoracic and salivary glands, especially in the 33–36 interval. Contingency tests, using only regions that are on the surface in all four hindgut nuclei, show a significant association with the peaks in all the other tissues as well as with intercalary heterochromatin (data not shown).

That certain chromosomal loci localized to the nuclear periphery was assumed to be due to their specific attachment to the nuclear envelope (28), but because the attachments cannot be directly visualized in light microscope images, this assumption was unproven. To study these presumptive envelope–chromosome interactions, an electron-microscopic analysis of thin-sectioned polytene cells was undertaken. Although it is easy to find chromosome segments abutting the envelope in such sections, this is not sufficient evidence for an actual attachment. However, we have found a number of examples of regions of the envelope that are pulled inward toward a chromosome (Fig. 6). These are either attached to long chromatin fibers (Fig. 6 *b* and *c*) or to a stretch of densely staining chromatin with many short fibers (Fig. 6 *a*). As with chromocentral attachment to the envelope (27), it is not possible to ascertain whether the fibers attach to the edges of nuclear pores or to the lamina, although our prejudice favors the latter. In addition, it may be noted from Fig. 6 *b* that there is no concentration of nuclear pores near the sites of chromosome attachment, pores appearing essentially close-packed throughout the envelope.

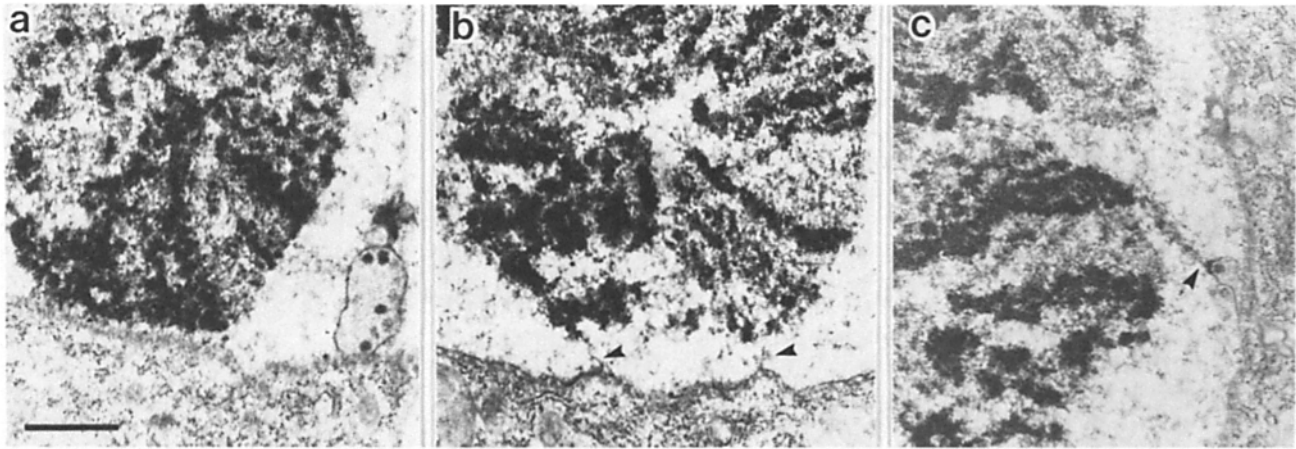


Figure 6. Salivary gland chromosome connections to the inner nuclear envelope. (a) Densely banded region of a chromosome sending many short fibers to the envelope; the envelope appears to be drawn up against the chromosome. The unusual structure next to the chromosome is described in the text. (b) A pair of salivary gland chromosome fibers (arrowheads) run to the envelope and appear to pull the attached regions inward. (c) A prominent ectopic fiber (arrowhead) attached to an infolded region of the inner envelope. Bar, 1 μm .

Only the Base of the X Chromosome Is Specifically Associated with the Nucleolus

Several reports claim specific interactions between certain chromosomal loci and the nucleolus in *Drosophila* salivary glands (1, 51). One locus in particular, 56EF, may be expected to associate with the nucleolus because it contains the genes coding for 5S ribosomal RNA. It was possible to trace out the nucleolar surface in prothoracic cells (27). Plots of the frequency of appositions between loci on chromosome X or 2R and the nucleolus are shown in Fig. 7. The number of close contacts between any particular locus (on any arm) and the nucleolar surface is low. 56EF is never within the 2.25- μm cutoff used to generate the plot. In 10 of 11 nuclei it is at least 5 μm away and in three nuclei it is over 10 μm away. The only euchromatic locus that appears with high frequency on the nucleolus is 19F/20A, at the proximal end of X (arrow). However, the nucleolar organizer is located in the heterochromatin at the base of X, and because the chromocenter is generally draped over the nucleolus (27), the 19F/20A peak may simply reflect this proximity. Small peaks at the base of several other chromosome arms support this contention.

That the base of the X chromosome is regularly associated with the nucleolus is not necessarily a trivial point. There are both cytological and biochemical data suggesting that

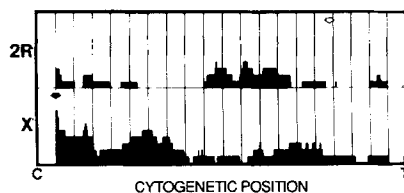


Figure 7. Nucleolar contact frequencies for X and 2R in prothoracic gland nuclei. Plots are generated and represented in the same way as nuclear surface contact frequency plots, e.g., Fig. 4. The cutoff distance (between the chromosome model and the nucleolar surface) used to make the plot was 2.25 μm . The solid arrow points to 19F/20A, which is on the nucleolar surface in 8 of 11 nuclei. The open arrow points to 56EF, the locus containing the 5S RNA genes, which is never within the 2.25- μm cutoff.

ribosomal DNA, which is largely located within the nucleolus (43), can be extrachromosomal in polytene cells (48, 55). In this state, it need not remain closely linked to the X chromosome. Interestingly, the euchromatic proximal end of X (around 20A) is often on the opposite side of the nucleolus from the rest of the chromocenter (see the model in Fig. 3 a of the preceding article), and DNA extending in toward the centrally located chromatin within the nucleolus is sometimes seen. It is as if the nucleolus, expanding from the nucleolar organizer, pushed the distal region of the X chromosome away from the rest of the chromocenter as it grew. Although not definitive, these observations suggest that in this tissue at least, nucleolar DNA is still continuous with the X chromosome. Finally, although no single locus associates with the nucleolus very often, it was noted that almost three-quarters of the X chromosome, on average, lies within a 5- μm shell around this organelle, whereas for the autosome arms, this average ranges between 34–56%.

Discussion

The experiments described here were directed toward testing for a possible role of three-dimensional chromosome folding in modulating gene expression and toward examining specific chromosomal interactions with different nuclear structures. Chromosomal loci are not repositioned within the nucleus when the pattern of transcription changes during heat shock or ecdysone treatment, and they are not specifically configured in any of the differentiated cell types studied. On the other hand, a specific subset of chromosomal loci is attached to the nuclear envelope, and this subset is very similar in two of the tissues and possibly a third. No high-frequency interactions between any euchromatic locus and the nucleolus were found except near the base of the X chromosome, which contains the nucleolar organizer.

These data will be used to evaluate some of the models invoking a role for the three-dimensional folding of the interphase genome in regulating gene expression. In what follows, we implicitly regard a polytene interphase cell as equivalent to a differentiated diploid cell. This is a reason-

able assumption. Polytene tissues are metabolically active and must synthesize many of the enzymes and molecules present in diploid cells. They are characterized by tissue and stage-specific patterns of gene expression (4). The same transcriptional machinery and DNA regulatory elements used in diploid cells are found in polytene cells as well (41). DNA in both classes of cells is organized into nucleosomes (25) and into chromatin structures with specific patterns of DNAase-hypersensitive sites (41). The occurrence of transitions between polytene and endopolyploid states and even from a polyploid back down to a diploid state (5) also argues against a fundamental difference in genome architecture between these kinds of cells. Finally, we have found many aspects of polytene chromosome packing that are seen in diploid cells as well (27, 28). Possible exceptions to this general similarity will be evaluated below.

Gene Regulation by Relative Gene Positioning

Some authors have proposed that particular gene sequences may be actively maneuvered into different functional compartments within the nucleus. Hutchison and Weintraub (30), for example, suggest that one step in activating a gene may be to tag it in a way that allows the gene to move into an active compartment. The experiments described here, using heat shock or ecdysone treatment to reset the pattern of transcription in salivary glands while the dispositions of chromosomes were followed, argue against repositioning of genes playing a part in gene regulation in a terminally differentiated cell. Chromosome positions are remarkably stable, even in the face of cytoskeletal rearrangements and changes in nucleolar shape and position (Fig. 1). These results also render highly improbable the idea that the wide range in chromosome configurations seen in salivary glands (28) was due to asynchronous functional states in the collection of cells examined.

A number of theories propose that genes are positioned within the interphase nucleus in a tissue-specific manner (14, 15). The most extreme models posit a specific three-dimensional genome structure in each differentiated cell type (12, 51). This view is succinctly stated by Steffenson: "Simply, gene placement is nuclear determination" (51). Other models are described in vaguer terms, but also invoke higher-order gene-gene interactions or superdomains (7, 19, 53) that are thought to "reflect strong selection for their economic and efficient action" (7).

We have now examined the spatial organization of nuclei in four different tissues spanning three to four levels of ploidy. In none of them have we found any indication of specific chromosome configurations. This is especially clear in midgut nuclei, where a chromosome can range from a tight ball of coils to a horseshoe shape with no coils at all (Fig. 3). Additional, indirect evidence includes the variable association of midgut centromeric regions, including apparent chromosome breakage, and occasional interwound fold-back loops forming in nonsimilar chromosome segments (27). There are also no obvious subpopulations of cells within a tissue that show specific longer-range interactions (Fig. 2), although complex combinatorial schemes involving large numbers of loci in different but specific interactions with one another cannot be ruled out. It must be emphasized that the nuclei in this study have been subjected to a minimum of manipulation and were examined under conditions

as close to *in vivo* as possible. Moreover, the ability to superimpose the cytogenetic sequence of bands onto the chromosome models has made possible an unprecedented level of precision in making comparisons between nuclei. It therefore may be stated that a tissue-specific three-dimensional genome architecture is not a general feature of interphase nuclei.

In addition to the many similarities between diploid and polytene cells (see above), the fact that low polyteny chromosomes, as well as those in salivary glands, do not have specific configurations lends credence to an extrapolation of our conclusions to diploid cells. Strictly speaking, however, specific locus-locus interactions in diploid chromosomes cannot be unequivocally dismissed even though it is unclear why such interactions would be important in diploid cells and not polytene ones, because if anywhere, it is in very large nuclei that one might expect diffusion times of factors going between loci to become a significant limitation. It also is possible, in principle, that specific interactions are important early in development, but "relax" in terminally differentiated cells. Finally, the data in Figs. 2 and 3 do not address the organization of DNA at the level of bands or small stretches of bands. Euchromatin position effects, for instance, are now well known (50); our results indicate they are not the result of long range locus-locus interactions.

Gene Regulation by Gene Positioning in Subnuclear Compartments

Direct gene-gene interaction represents only one possible type of higher-order functional compartmentalization. Another potential compartment is the nuclear periphery (24, 30), where specific genes in each differentiated cell type may be "gated" via nuclear pores to the cytoplasm (12; also see reference 35). The localization of specific chromosomal loci to the nuclear periphery in salivary glands (28) could be taken as support for this hypothesis. However, the specificity of peripheral localization is almost identical to that found in the prothoracic gland (Figs. 4 and 5). Thus, the genes that are usually located on or very near the nuclear surface are essentially the same in the two cell types. Yet prothoracic and salivary glands have very different patterns of gene activity (27, 29). For example, of the 24 prominent puffs found on 3L, a minimum of 14 or 58% are active in only one of the two tissues (29). The fact that few of the contact loci are on the surface in 100% of the nuclei also suggests that a gene need not be on the surface to function properly (although the method of measurement may miss occasional contacts and attachments to envelope infoldings could be missed).

One could argue that the similar positions of high-frequency contacts in the two tissues (and possibly the hindgut as well) reflect the presence at these loci of "housekeeping genes" that are expressed in all tissues and must be localized at the envelope. It is unlikely that surface localization is essential for the correct regulation of such genes, however, given the reduced and/or altered specificity of envelope contacts in the middle midgut (Fig. 4). Many of the high-frequency contacts in the other tissues are only occasionally on the surface in this tissue, and considerably fewer loci regularly make contacts. The different contact pattern found in midgut cells may be related to the fact that these nuclei are subject to strong deformations that probably contribute to chromosome tangling and possibly breakage (27). These

deformations may sometimes shear away envelope attachments as well, thus partially washing out the specificity of contacts. Alternatively, the relative timing of envelope attachment and chromosome decondensation during early interphase may be different in these cells, presenting, for example, fewer potential binding sites to the surface, or the very straight midgut chromosomes may not bend frequently enough to allow the dispersed set of intercalary heterochromatin sites to regularly appose the surface.

It is interesting that the similarity between the high-frequency surface contacts in prothoracic and salivary glands does not extend to the chromocenter (preceding article). As with the chromocenter, the electron-microscopic data (Fig. 6) implicate direct attachment to the envelope of loci with characteristics of intercalary heterochromatin as the cause of specific peripheral localization. This is supported by the strong correlation of high-frequency envelope contacts with such loci. Thus, intercalary heterochromatin may not simply be a very small amount of the same sort of chromatin that makes up the chromocenter, but may well have distinct structural and functional properties.

The common feature of intercalary heterochromatin that is thought to lead to properties such as constriction in squashes and ectopic fibers is the presence of underreplicated, tandemly repeated DNA sequences (54). Perhaps this also accounts for its avid envelope binding, e.g., by pairing to repeated structural motifs in the polymeric lamina or by allowing the cooperative assembly of a chromatin structure with high envelope affinity. One perplexing aspect of the general association between intercalary heterochromatin loci and envelope binding is that a number of such loci (e.g., 3C, 11A) are in fact not in frequent contact with the surface. It will be interesting to learn what molecular properties might distinguish these exceptional cases. For example, certain nonhistone proteins are known to bind to a subset of intercalary heterochromatin loci (34), and such proteins may mediate envelope attachment. This can be tested by antibody staining of intact polytene or diploid nuclei.

Another potential functional compartment within the nucleus is the perinucleolar zone. Certain genes involved in nucleolar function, e.g., the 5S RNA genes, may localize to the nucleolus (51). However, we find no specific associations in prothoracic gland nuclei except in the vicinity of the nucleolar organizer (Fig. 7). This is actually consistent with *in situ* hybridizations done in *Xenopus* which show the same distribution of 5S DNA in both wild-type and anucleolate somatic cells (44). As mentioned in Results, the close link between the nucleolar organizer and the nucleolus may well indicate that nucleolar DNA is not extrachromosomal in this tissue as it has been suggested to be in salivary glands (48 and references therein).

A final nuclear compartment to consider is the hypothesized nuclear matrix (36). We have no evidence for or against such a structure and so cannot test whether genes are specifically positioned with respect to it except insofar as the nuclear lamina is considered a matrix component. A major protein in the matrix fraction is topoisomerase II; it was shown to localize along chromosomes in *Drosophila* salivary glands (9). Topoisomerase II is also known to bind to specific DNA sequences and to be associated with replicating DNA (42). These findings highlight a major point of contention about the internal matrix, namely, whether it is a coherent

structural entity *in vivo* or, instead, simply represents a set of chromosomal components of low solubility. Unfortunately, these alternatives cannot be distinguished by the available data.

Transvection Effects and Nuclear Structure

One unexplained genetic phenomenon often interpreted in terms of a specific three-dimensional genome architecture is the transvection effect. In this effect, the phenotype associated with a pair of alleles appears to depend on their ability to synapse or their physical proximity to each other. It has been documented in both diploid and polytene tissues (32, 38). For instance, in some genotypes, the white locus may behave in a synapsis-dependent fashion. The w^{DZL} allele of white, for example, when combined with a wild-type copy, yields yellow-eyed instead of red-eyed (wild-type) flies only if the two alleles can pair (11). Related to this is the interaction of the *zsk* gene with white. In a *z'* background, the color of the flies' eyes are yellow when the two wild-type copies of the white gene are paired and red when they are not (32).

Bingham and Zachar (11) favor an explanation of transvection in which the synapsed pair of alleles are positioned as a unit in a specific compartment within the nucleus. The w^{DZL} allele, when synapsed with the wild-type copy, is thought to force the latter into a nuclear position inappropriate for its expression. In *z'* flies, the correct positioning of the white genes is presumably antagonized as well, although only if they are paired. The evidence from our analysis weighs against this model, unless the hypothetical compartment to which transvection-sensitive genes are supposed to segregate is some as yet undefined structure(s) diffusely distributed within the nucleus. Transvection-sensitive genes, including *Sgs-4*, which is active in the salivary gland (38), are not specifically localized near the nuclear surface, the nucleolus, or one or a few other chromosomal loci. Transvection is therefore more likely to be due to a local effect on the paired loci.

While our results argue against many of the exotic models outlined above, the finding of indeterminate chromosome configurations, coupled to their relatively static positions over both short and long developmental periods, is not without potential genetic consequences. These conditions could place important constraints on DNA transactions that depend on the juxtaposition of particular DNA sequences, e.g., recombination, certain DNA repair mechanisms (20), or some types of transcriptional control. A possible example of the latter is suggested by *in vitro* evidence that transcription factors can move between DNA molecules by a rapid direct transfer mechanism (21, 37). The assembly of a transcriptionally competent 5S RNA gene complex has been proposed to occur via a metastable intermediate that must exist for a certain minimum time; if there is a limiting amount of transcription factor, rapid DNA-mediated transfer of factors between sites could prevent this minimum time from being reached (37). Our data suggest that this mechanism could not operate effectively *in vivo* unless the multiple binding sites were clustered because transfer is unlikely to be sufficiently rapid over very long, statically arrayed chromosomes (21).

Clustering *in cis* may be selectively maintained for multicopy or otherwise related genes that are subject to this mode of regulation. At least two other situations where transfer of transcription factors between closely positioned DNA

molecules may be important can be suggested. The zeste-white interaction could be explained by supposing that in a z^1 background, a limiting amount of a positive transcription factor(s) for white is available and can transfer rapidly between synapsed gene copies, preventing a metastable transcription complex from existing on either copy long enough to be actively assembled. The paired chromatids in polytene nuclei may provide another example, with DNA-mediated transfer allowing the hundreds or thousands of gene copies to function as a tightly regulated unit even through many rounds of replication.

Appendix

The unusual envelope structures often seen near chromosome attachments (Fig. 6, *a* and *c*) were found in many regions, usually filled with large particles. Fig. 8 provides further examples. The “pods” appear to involve infoldings of the inner nuclear membrane only. The electron density and appearance of the material inside them is distinct from both nucleoplasm and cytoplasm. They are surrounded by a dense

layer similar to the nuclear lamina that is continuous with it, while examples of circular structures, that may be pore annuli, have been seen in grazing sections (Fig. 8 *f*). The pods often (always?) occur together with double-membrane “blebs” (23) into the cytoplasm and occasionally, are also associated with double-membrane invaginations. These features raise obvious questions about the structural plasticity of envelope components. Pods have also been seen in the midgut (Fig. 8 *d*). In reexamining the micrographs of blebs made by Gay (22, 23), we sometimes discerned material similar to what is described here; however, pod structures were not well preserved and were regarded as dense chromosomal fibers (23). Gay found that blebs only appeared when the cytoplasm was filling with glue granules, as in the glands in which we found pods.

The particles in the perinuclear pods are large, with apparent sizes from ~ 100 to >200 nm. Large numbers may fill a large pod. Their substructure suggests they involve aggregates of smaller particles (Fig. 8 *g*). What they are is not clear. We are testing the proposition that they are RNP with immunogold labeling. A potential parallel with the large

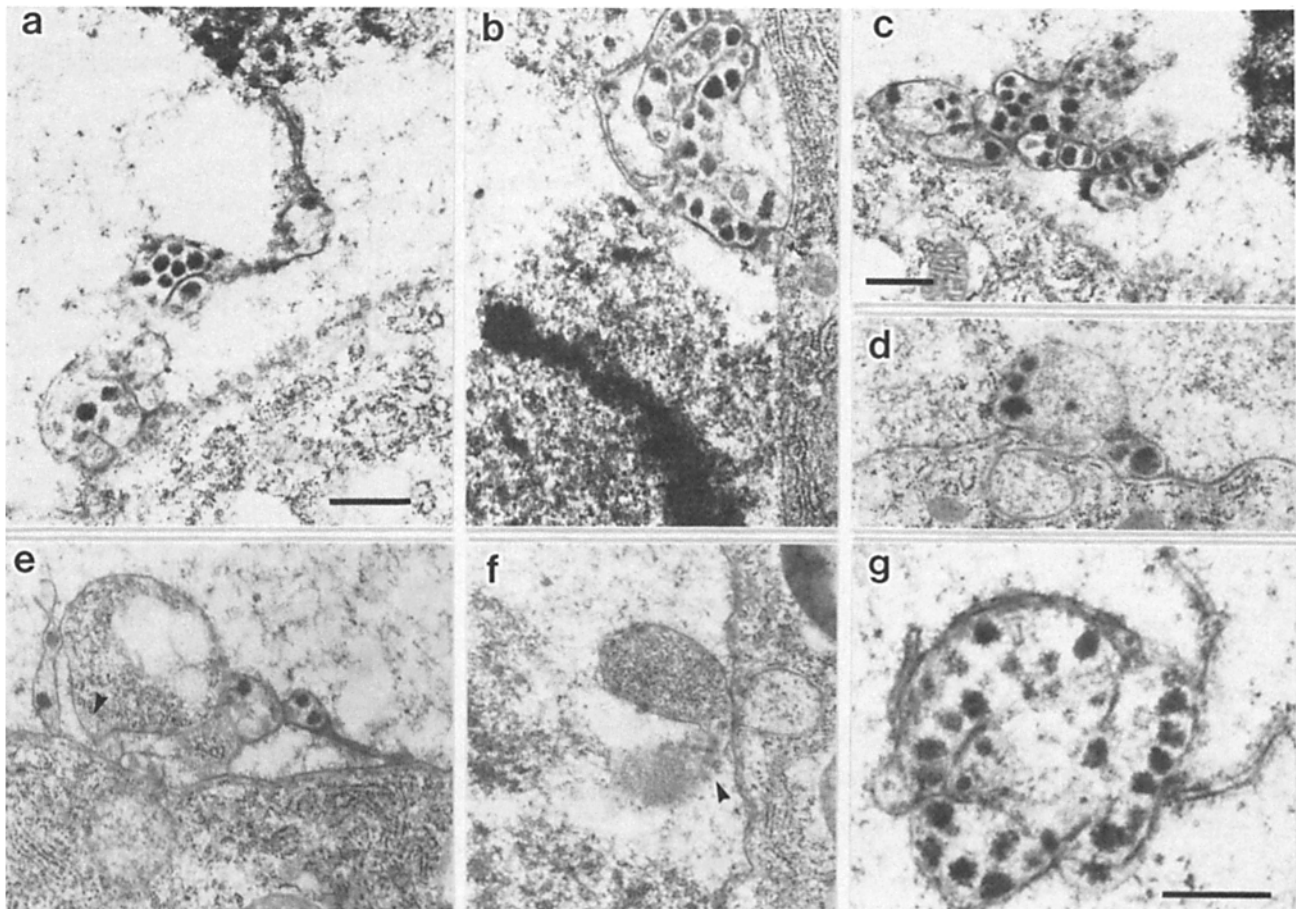


Figure 8. Podlike infoldings of the nuclear envelope. (*a–c*) Different multichambered pods in salivary gland nuclei, often extending a laminate arm to a chromosome. The large particles with variable electron densities are usually roughly circular in cross section but other forms, including a horseshoe shape, are also seen. (*d*) Pod associated with a double-membrane bleb into the cytoplasm of a middle midgut cell. (*e*) A complex envelope structure including a double-membrane invagination containing ribosomes and glue granules, a bleb, and particle-containing pods. The arrowhead points to a region where the outer envelope is separating from the inner one within the invagination. (*f*) A grazing section of a pod forming from a double-membrane invagination. An area with structures suggestive of pore annuli is indicated by arrowhead. *e* and *f* are from serial sections of salivary gland nuclei. (*g*) A higher-magnification view of a salivary gland pod structure. The dense particles appear to be made up of smaller particles. Bars, 0.5 μm .

RNP particles found at the 93D locus (17) may be pointed out. Their size is similar to those in the pods, and they also may be aggregates of smaller particles. The 93D particles accumulate to high levels during heat shock. Conceivably, accumulation of particles in both cases is due to a backup in RNP processing or transport. However, pods are unlikely to involve transcripts made only at the chromosome locus directly attached inasmuch as our data argue against stage or tissue-specific attachments of loci.

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