INTRODUCTION

Chromosomes are biological information storage and retrieval systems whose structure is intimately entwined with function. To accommodate replication and transcription, the chromosomal DNA is spread over a large volume and exposed to proteins in the nucleoplasm. Then, during the short period of mitotic prophase, the chromosomes can condense more than 250 times and separate cleanly from each other (Earnshaw, 1988). One of the open questions about chromosome structure is how active interphase chromatin is organized to facilitate both the requirements of interphase functions and the orderly condensation that occurs in mitosis.

One attractive idea, for which there is a great deal of evidence, is that the chromosome is organized into large domains, or loops, anchored to the nucleoskeleton or ‘nuclear matrix’. The loops are composed of the 10 and 30 nm chromatin fibers, and are thought to be of the order of 50-200 kb in length (reviewed by Wolff, 1992). There are at least two loop-type models for chromosome organization (Bodnar, 1988); a significant difference between them is whether chromosomes are ordered through stable intra-chromosomal associations derived from metaphase loop structures (Boy de la Tour and Laemmli, 1988; Gasser and Laemmli, 1987; Laemmli et al., 1992; Mirkovitch et al., 1984; Saitoh and Laemmli, 1994), or whether the loops result from dynamic, functional interactions with nucleoskeletal components (Gerdes et al., 1994; Hozák et al., 1993; Jackson and Cook, 1993). Up till now, in situ hybridization techniques have failed to reveal loops, or any other substructure, in intact interphase nuclei (Trask et al., 1989; Lawrence et al., 1990). As a result, more indirect methods, using exploded nuclear ‘halos’ (Mirkovitch et al., 1984; Gerdes et al., 1994) and biochemical probes (see, for example, Jackson et al., 1990) are usually used. As an alternative, we have developed a procedure that combines the techniques of in situ hybridization and high-resolution image analysis with the unique properties of Drosophila blastoderm embryos to determine the location of sequences in intact diploid nuclei with unprecedented spatial resolution.

We chose to examine the Notch gene, whose protein product is a transmembrane glycoprotein required throughout development in a variety of tissues (Fortini and Artavanis-Tsakonis, 1993) and which is expressed uniformly in all somatic cells of the cycle 14 embryo (Hartley et al., 1987). Notch is located near the telomere on the X chromosome, allowing us to observe a single copy in males or two copies in females (some of which are paired) (Hiraoka et al., 1993). The transcribed portion of the Notch gene is about 40 kb (see Fig. 1), large enough to resolve using in situ hybridization methods, but small enough to be contained in a relatively compact domain in the nucleus. The gene has been cloned and large portions sequenced, providing us with accurate information about the function of and distance between our hybridization probes. Furthermore, the fine structure of the Notch gene in salivary gland polytene chromosomes has been previously determined (Rykowski et al., 1988). Since Notch is not expressed in salivary glands, we can compare the geometries of the gene in
the active state in the embryo and of the same gene in the inactive state.

In our model system of choice, the nuclei of whole-mount late syncytial blastoderm *Drosophila* embryos, 1000–4000 morphologically identical nuclei lie in a sheet just beneath the embryo surface (see Foe et al., 1993, for review). Moreover, chromosomes are highly ordered within embryonic nuclei: the centromeres and telomeres are located on opposite ends of the the nucleus with centromeres toward the surface (Rabl orientation) (Foe and Alberts, 1985). This means that a particular gene locus will lie at the same distance below the embryo surface in all the nuclei. As importantly, the mitotic cycles of embryonic nuclei are almost completely synchronous from the second through the 13th nuclear division. The synchrony controls for variability caused by cell cycle-dependent differences in gene structure, while the regularity and large numbers of nuclei allow us to use statistical methods to accurately determine the relative positions of hybridization signals imaged using different fluorescence filter sets. We reasoned that, by examining *Notch* simultaneously in a large number of nominally identical cells, we could determine directly the 3-dimensional structure of a gene as it is expressed, thereby inferring some properties of the chromatin in which it is contained.

Our results demonstrate that probes that should colocalize do so to within about 100 nm. Using this technique, we find that hybridization signals from the 5′ and 3′ ends of the *Notch* gene do not colocalize. Rather, the distance between the two probes we used varies and represents a range of condensation levels from a compact 30 nm fiber to an open ‘heads on a string’ arrangement. The extent of elongation is at least as much as would be predicted, based on results from studies with polytene chromosomes (Lamb and Daneholt, 1979). We propose that gene elongation can be explained by a combination of decondensation caused by gene transcription, DNA replication and nuclear elongation, which take place at the same time.

**MATERIALS AND METHODS**

**Embryo preparation**

Oregon-R embryos are collected from population cages and aged to yield cycle 10-14 blastoderm embryos. Embryos are fixed, hybridized and mounted as previously described (Gunawardena and Rykowski, 1994; Rykowski, 1991). Briefly, Oregon R embryos are collected from a population cage for one hour and aged for 30 minutes to obtain embryos in cycle 14. The embryos are mass dechorionated in 50% bleach, 50% Buffer A (15 mM PIPES, pH 6.8, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, pH 7, 2 mM EDTA, pH 7, 0.5 mM spermidine, 0.2 mM spermine, 0.1% 2-mercaptoethanol), fixed in a two-phase medium of 4% formaldehyde in Buffer A and heptane in equal volumes. The embryos are devitellinized by incubation in 50% methanol/50% heptane (v/v) at −70°C followed by a temperature shock at 37°C for 30 seconds. The embryos are removed from the methanol phase and stepped into Buffer A. The embryos are used immediately for hybridization.

The fixation procedure results in preparations that retain excellent antigenicity and structural integrity (Mitchison and Sedat, 1983). Comparisons between live embryos and embryos fixed using this procedure have shown no apparent difference in chromosome structure resulting from preservation techniques (Hiraoka et al., 1989, 1990). Before hybridization, every embryo preparation is evaluated according to the criteria listed by Gunawardena and Rykowski (1994), to assure that the chromatin morphology is well preserved.

**In situ hybridization to DNA and RNA**

Probes are prepared by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer-Mannheim) to generate single-strand lengths averaging 75-150 bases. Embryos are prepared for hybridization by incubation with RNase A (1 mg/ml, Boehringer-Mannheim) in PBT (0.13 M NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, 0.1% Tween-20 (Surfact-Amp 20™, Pierce, Rockford, IL) for 10 minutes. The embryos are stepped into hybridization buffer (50% distilled formamide (Omnisolve, EM Science), 100 µg/ml herring testes DNA, 0.1% Surfact-Amp 20™, 4× SSC) and preincubated at 37°C for 1 hour. The embryos are heated to 70°C to denature the chromosomes, and then incubated in fresh hybridization buffer containing 10 µg/ml of each probe DNA which had been previously denatured. After hybridization at 37°C for 24 to 36 hours, the embryos are stepped in PBT. The embryos are incubated with a combination of FITC-labeled anti-digoxigenin and Texas Red-labelled avidin or rhodamine-labelled anti-digoxigenin (all from Boehringer-Mannheim). After rinsing, the embryos are counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.1 µg/ml) in Buffer A. The embryos are mounted in Buffer A (made at pH 7.2) and observed. Zero-distance data sets were made by hybridizing labelled DNA from a single location (P9.9 or H8.8, respectively), while the 29 kb data set was made by hybridizing both the P9.9 and the H8.8 probes.

Hybridization to RNA is done according to published procedures (Tautz and Pfeifle, 1989), using probes (H7.8 and H8.8) and embryos are prepared as above. Since the *Notch* gene is transcribed in cycle 14 (Hartley et al., 1987, and see below), it is important to distinguish hybridization to chromosomal DNA from hybridization to mRNA transcripts. When hybridizing to chromosomal DNA, the signals we observe are resistant to RNase treatment and dependent on denaturation of embryonic chromosomes. Hybridization signals are seen in early cycles and in mitosis, when transcripts are likely to abort (Shermoen and O’Farrell, 1991). Germ cells apparently do not transcribe the *Notch* gene (S. Gunawardena, unpublished), yet we have observed hybridization in germ cells. On the other hand, when hybridizing to RNA, we find that the signal is sensitive to RNase, does not require denaturation and disappears at mitosis (S. Gunawardena, unpublished). Thus we conclude that we can distinguish between DNA and RNA hybridization.

**Image acquisition**

Image data are recorded in three colors and three dimensions using a computational optical microscope to record separately the positions of the 5′ and 3′ hybridization probes and the nuclei as revealed by DAPI. The images are subsequently processed (see below) and analyzed to determine the positions of the hybridization signals in
Each set, the alignment of the FITC and Texas Red or rhodamine data sets, and the relative positions of the hybridization signals.

To accumulate and analyze images, a highly sensitive optical system is used. The optical microscope system we use is a fluorescence wide-field, optical microscope (IMT-2, Olympus), connected to a scientific grade charged-coupled device (CCD) camera (Photometrics) cooled to −42°C to reduce thermal noise. The camera system has a 12 bit read-out and its response is linear over its entire range. The microscope includes highly selective excitation and emission filters (Omega Optical), multichroic mirrors (ChromaTechnologies), high numerical aperture lenses, high tolerance shutters (Uniblitz) and computerized stepping motor (Compumotor) (Agard et al., 1989; Rykowski, 1991). Using this method, three fluorophores can be visualized independently.

The embryo is roughly 0.42 mm in length and 0.15 mm in diameter (Sonnenblick, 1950) with about 100 nuclei per field (an area of 3490 µm²; see Foe and Alberts, 1983) lying just under the surface. During the first 55 minutes of nuclear division cycle 14, the nuclei change from a spherical to prolate elliptical shape, elongating from 5 to 10 µm in depth (Foe and Alberts, 1983). The Notch gene, which is near the telomere of the X chromosome, lies near the base of the nucleus as diagrammed in Fig. 2. To sample the same region of each embryo, the membranes in the embryos used to generate zero-distance data sets 1 and 2 show hybridization signal location

Three-dimensional data sets are processed to correct hot pixels using a nearest-neighbor average. Data stacks are normalized to correct for photobleaching and lamp flicker. The data sets are enhanced using both low-pass and high-pass Gaussian Fourier filters (sigma values=0.0433 and 2.38 µm⁻¹). Hybridization signals are marked interactively using PRIISM (Agard et al., 1989; Chen et al., 1990). After numbering the nuclei, the program is used to mark the position of the hybridization signals in each data set separately, generating a list of the X, Y and Z coordinates for each spot in both colors. The list of data points generated in this way is passed to a Mathematica notebook and an Excel worksheet, whose purposes are to verify the pairing of green and red signals, correct for alignment using a GRG2 algorithm (see below), and determine the distribution of inter-probe distances.

Several criteria are used when marking the hybridization points to minimize error and to objectively distinguish hybridization signals from background. Signals are bright enough to be seen in more than one optical section, while background spots are usually smaller and more dim. Thus, we mark only those signals that can be seen in several sections. When oblong signals are evident the mark is placed at the center of the object. Signals in incomplete nuclei at the edge of the image are ignored. In ambiguous cases the nuclei are rejected. We use preparations that show hybridization signals in both colors in over 50% of the nuclei in the optical field.

Alignment of data sets

Before we calculate the distance between two signals, we correct the data sets for translations caused by differences in alignment between the motorized fluorescence filters. In 2-dimensional samples, we use multi-colored fluorescent beads as fiducial markers. This method is inappropriate with 3-dimensional samples. Instead, we use an algorithm based on the Generalized Reduced Gradient Method (GRG2; Abadie and Carpenter, 1969) that determines an offset by minimizing the sum of the distances between all pairs of spots. The displacements calculated in this way are used to correct the X and Y coordinates of one data set to bring it into alignment with the other. We find that for the data sets analyzed here, the displacements range from 0.1 to 0.3 µm, and average about 0.2, which is comparable to offsets determined using fiducial marks in 2-dimensional data sets (R. Madacz and M. C. Rykowski, unpublished).

Because the chromosomes are oriented asymmetrically in the Z dimension, we cannot use the same assumptions for corrections of displacements in Z. On the other hand, displacement of the data sets in Z results from chromatic aberration rather than filter alignment. The distribution of the Z components for zero-distance data sets 1 and 2 shows that the Z distance distribution is symmetric, and it is centered at roughly zero (0.00008 µm). The lack of displacement on the Z dimension between the red and green data sets is expected for an optical system that is corrected for chromatic aberrations. For this reason, we corrected data sets for X and Y, but not Z misalignment using the minimization algorithm.

Determination of embryo age

We determine the age of the embryos by measuring the length of the invaginating membrane in fixed material after observing the hybridization signal (Schejter and Wieschaus, 1993). During nuclear cycle 14, membranes invaginate from the embryo surface, moving into the embryo interior between the nuclei. Eventually, the membranes fuse to enclose the cytoplasm around each nucleus. The moving front of membrane can be seen in phase-contrast micrographs of living embryos, and the extent of membrane movement can be determined.

The membranes in the embryos used to generate zero-distance data sets 1 and 2 and the 29 kb data set were invaginated to 12.3, 10.3 and 9.4 µm, respectively; we estimate their ages to be 38, 35 and 34±1 minutes into cycle 14.

RESULTS

The task we have before us, to determine the fine structure of
chromatin in 3-dimensional nuclei, demands that the methods used are sufficient to detect small differences in the location of in situ hybridization signals. To assure that we can measure relatively short distances between chromosomal domains, we need to determine the resolution and accuracy of the in situ hybridization method. That is, we need to determine the reproducibility of signal localization, and establish the extent to which two differently labelled probes that should colocalize precisely will superimpose. These are non-trivial questions because the hybridization signal is likely to result from a combination of reporter molecules linked directly to the nucleic acid probe, as well as some accreted material perceived as background. The pattern of the secondary material is likely to be different for reporter molecules with different chemical properties, and may cause artifactual variations in apparent position of the signal. Variations in signal intensity might also arise from uneven distribution of the hybridizing DNA to its target. Moreover, optical artifacts resulting from refraction through the embryo may act to distort the images in a wavelength-dependent manner.

To control for these possible artifacts, we did two experiments resulting in what we call zero-distance data sets 1 and 2. In the first experiment, we labelled the H8.8 fragment (see Fig. 1 for probe location) with digoxigenin-11-dUTP and visualized the hybridization with both FITC- and rhodamine-labelled anti-digoxigenin. We reasoned that this situation would allow us the highest probability of complete overlap, as there is only one labelled DNA and the differently labelled antibodies are as similar as possible. We collected three 3-dimensional data sets (referred to collectively in the text as zero-distance data set 1) to observe the FITC (green) and rhodamine (red) hybridization signals, as well as the nuclear DNA stained with the DNA-specific dye, DAPI (blue), and analyzed them as described in Materials and Methods.

As expected, the red and green data sets overlapped very well, as shown for two different projections of an elongated hybridization signal (Fig. 3, top two rows). The first two panels in each row represent the rhodamine and fluorescein channels, respectively. Note that the patterns are very similar, even the backgrounds are nearly identical. The last panel shows a computational superimposition of the two signals, where green represents the fluorescein and red the rhodamine channels, respectively. Note that the signal appears a nearly uniform yellow color, indicating that the signals overlap almost exactly, with the same variations in intensity.

After marking the positions of the hybridization signals, the distribution of ‘inter-probe’ distances shown in Fig. 4A was obtained. The distribution shows a strong mode at zero distance, and a lower number of non-zero inter-probe distances. We made several measurements from the same data set and got the same distribution in each case, although individual values vary (data not shown). We conclude that the variation is the result of aliasing (i.e. fitting a continuous variation in intensity into discrete pixels) and observer error. To verify that the error is random, we replotted the data in two different ways to determine if the zero-inter-probe distances are non-randomly distributed. To visualize the 3-dimensional distribution of 5’ to 3’ orientation, we plotted the vectors representing the paths that connect the green signal to the red signal (Fig. 4B). The length of the vector represents the inter-probe distance, while the angle represents the orientation.

From this vector plot it is observed that the distribution in X, Y and Z is spherical, i.e. isotropic.

We also asked if the measured inter-probe distance depends on the location of the hybridization signals in the microscopic field. A systematic variation in inter-probe distance might result from optical aberrations introduced by the microscope or by the embryo itself. To determine if this is the case, we plotted the inter-probe distance as a function of the position of the nuclei in the microscopic field. The results are shown in
the field plot in Fig. 4C and demonstrate that there is no systematic relationship between the distance between signals and the position in the field. We conclude that the variation in apparent inter-signal distance results from random rather than systematic error. The sources of the error include slight variations between the positions of the brightest point in the signals, as well as observer error. The variation represents the accuracy of the method, about ±0.1 μm.

In the second zero-distance experiment, we determined the overlap between signals resulting from the same DNA probe labelled separately with different derivatives and visualized with different reporter molecules. In this way, we can assess the contribution of differential probe distribution to the target sequences, as well as differential reporter molecule background. We generated two hybridization probes from the same plasmid, P9.9, one labelled with digoxigenin, the other with biotin, and hybridized them simultaneously to embryonic chromosomes. The signals were visualized with FITC anti-digoxigenin and Texas Red-avidin and analyzed as above. The results of this analysis are shown in Fig. 4D-F. The distribution in Fig. 4D shows a strong mode at about 0.1 μm, with a distribution between 0 and 0.35 μm. Note that the mode of the distribution of inter-probe distances does not fall at zero, and only a minority of the signals colocalize precisely, even though the signals overlap substantially (Fig. 3, last two rows). We replotted the data as vector plot (Fig. 4E) and field plot (Fig. 4F) as described above and find that, as for the single-reporter system, the 3-dimensional distribution of inter-probe distances is isotropic and unrelated to the position of the nucleus in the microscopic field.

The results of the analysis of the two-reporter system are substantially different from those of the single reporter case. If the errors contributing to the distribution were random, we would expect the mode to be zero with a broad distribution, as shown for the case in which a single reporter system was used. It appears from these results that two probes that are very close may appear artificially separated when they are labelled with different reporter systems. This result means that there is a lower limit to our ability to accurately determine the relative positions of two signals. We are currently investigating the basis for this effect. In the meantime, we will assume that the signal is likely to be accurate to no more than ±0.1 μm, with a minimum resolution of 0.1 μm.

With the limits of resolution established, we next sought to determine whether it is possible to resolve the positions of different portions of the Notch gene in interphase nuclei using the two-color in situ hybridization method. Using the methods...
outlined above, we hybridized two probes, one from the 5′ and one from the 3′ end of the Notch gene, separated by a center-to-center distance of 29.2 kb. As before, three 3-dimensional data sets (called collectively the 29 kb data set) were collected from a female embryo, a total of 98 nuclei containing 179 signal pairs. We examined a female data set because the larger number of hybridization signals provided a smoother distribution function. The distribution of inter-probe distances for each probe pair was determined and the results are plotted in Fig. 5A. For comparison, note that these distributions and the ones shown in Fig. 4 from the zero-distance data sets are normalized to compensate for the different number of chromosomes in each data set. The distribution of inter-probe distances is considerably more broad, and has a higher modal value than for either zero-distance data set. Clearly this distribution is significantly different from the control data sets. Likewise, inspection of a few of the signal pairs from this data set (Fig. 6) shows obvious differences in location and signal shape compared to those from either zero-distance data set.

The vector plot in Fig. 5B demonstrates that the 3-dimensional distribution of inter-probe locations is fairly uniform, as with the zero-distance data sets. However, in examining the distributions of the X, Y and Z components of the inter-probe distances, we find that while the distributions in X and Y are more broad than in the zero-distance cases, the distribution in Z broadens considerably more. This gives the vector plot a more football-shaped profile rather than a spherical one. Since the optical Z axis and nuclear axes are aligned, this increasing orientation of the gene in the optical Z axis represents an elongation along the nuclear axis.

We next asked if there is a preferential direction for the Notch gene along the embryo axis. We reasoned that if the gene is tethered to an immovable structure in the nucleus, the gene may be oriented preferentially. We determined the number of nuclei showing a 5′ to 3′, 3′ to 5′ or no orientation in Z. Results are shown in Table 1. As should be clear from Fig. 5B, there is no absolute 5′ to 3′ orientation, but there is a slight preference for a 5′ to 3′ orientation along the positive direction. That is, the 5′ (centromere-distal) end of the gene lies below that of the 3′ (centromere-proximal) end, consistent with the Rabl orientation of the chromosome. Since the orientation preference is slight and since the orientation has a significant component in the X-Y plane, we assume that the chromosome is gently coiled from the centromere to the telomere. These data must be confirmed by observing more data sets from embryos at different ages, but they may suggest that the chromosome is tethered to a structure so that, as the gene expands, it does so in a preferred direction.

We examined the orientation of homologous genes in the same nucleus to determine whether the orientation of the gene is likely to be the same as its pair, or whether it will have a random orientation. The results are summarized in Table 2. We find that, for those nuclei in which two individual hybridization signals can be distinguished, the orientation of the genes on the two homologs is more likely to be the same than that predicted on the basis of an assumption of independent distribution of gene orientations. We conclude that the orientation of the gene is dependent on local forces and may be determined in part by tethering of the gene to nuclear integument in a reproducible way.

![Image](322x491 to 550x719)

**Fig. 6.** A montage of rotated and projected images showing hybridization signals using probes H7.8 (5′) and H8.8 (3′) to a cycle 14 female embryo. The probes were labelled with biotin-16-dUTP and digoxigenin-11-dUTP, and visualized using avidin/Texas Red and anti-digoxigenin fluorescein, respectively. The images are rotated 90° so that the Z axis of the embryo runs top to bottom in the images. The three rows illustrate separate hybridization signals with the Texas Red on the left, the fluorescein in the center and the overlayed signals on the right. Notice the separation of the two probes with only a small overlap between the two (in yellow). Bar, 1 µm.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Orientation (% observed/% expected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 kb</td>
<td>8.6/1.4 0/6 7.4/15 7.4/31 55/40 21/6.2</td>
</tr>
<tr>
<td>Unpaired loci</td>
<td>0 4 0 2 2 2</td>
</tr>
<tr>
<td>Paired loci</td>
<td>6 70 24</td>
</tr>
</tbody>
</table>

The expected values were calculated on the assumption that the unpaired homologs would orient independently, based on the overall frequencies of orientations. ‘0’ refers to genes whose 5′ and 3′ ends are in the same plane; ‘+’ refers to genes oriented 5′ to 3′; and ‘−’ refers to genes oriented 3′ to 5′ relative to the nuclear axis.

**Table 2. 5′-3′ Orientation of the Notch genes in homologous chromosomes**

<table>
<thead>
<tr>
<th>Data set</th>
<th>Time into cell cycle (min)</th>
<th>Oriented 5′ to 3′ (%)</th>
<th>No orientation (%)</th>
<th>Oriented 3′ to 5′ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 kb</td>
<td>34</td>
<td>64</td>
<td>12</td>
<td>25</td>
</tr>
</tbody>
</table>

The expected values were calculated on the assumption that the unpaired homologs would orient independently, based on the overall frequencies of orientations. ‘0′ refers to genes whose 5′ and 3′ ends are in the same plane; ‘+’ refers to genes oriented 5′ to 3′; and ‘−’ refers to genes oriented 3′ to 5′ relative to the nuclear axis.
be unambiguous. Further, if the paired signals (9% of the total signals) are omitted from the analysis, the distribution of inter-probe distances is the same (data not shown). We conclude that any additional error introduced by measuring inter-probe distance in paired loci is insignificant.

We next sought an explanation for the rather broad distribution of inter-probe distances in the 29 kb data set. Some of the variation likely results from the limited resolution of the technique, but not all of it can be explained in this way. From the observation of chromosomal puffing in polytene nuclei, it is reasonable to suggest that transcriptional decondensation accounts, at least in part, for the increase in intragenic distances. It has previously been shown that transcription aborts during mitosis and is reinitiated at the start of interphase (Shermoen and O’Farrell, 1991). We might assume that initiation is simultaneous in all cells of the blastoderm, and that, assuming that the extension we see is the result of transcription, all genes should be elongated to the same extent. On the other hand, if initiation is not immediate and simultaneous in all nuclei, we might expect to see a broad distribution of condensation levels, even in a nominally synchronous population of nuclei. We decided to test directly whether the extent of transcription is the same in all cells by hybridizing in situ to nascent mRNA using the two color system.

![Fig. 7. A montage showing the same single sections of hybridization using H7.8 (5’ and H8.8 (3’) probes to the Notch transcript in a cycle 14 female embryo. The 5’ probe (Fig. 7A) was labelled with digoxigenin-11-dUTP and visualized with anti-digoxigenin fluorescein. The 3’ probe (Fig. 7B) was labelled with biotin-16-dUTP and visualized with avidin/Texas Red. Notice that the 5’ probe is evident in almost all of the nuclei but the 3’ probe is seen only in a few nuclei. Bar, 5 µm.](image)

![Fig. 8. Projection image of a single nucleus showing hybridization of H7.8 (5’) and H8.8 (3’) probes to the Notch transcript in a cycle 14 female embryo. The 5’ probe was labelled with digoxigenin-11-dUTP and the hybridization signal visualized using anti-digoxigenin fluorescein. The 3’ probe was labelled with biotin-16-dUTP and visualized with avidin/Texas Red. The embryo was also stained with DAPI. Notice that the 3’ probe (in red) is evident only on one homolog in a nucleus containing unpaired 5’ probe signals (in green); the extent of the nucleus is shown in blue. Bar, 1 µm.](image)
We produced two probes, H7.8 and H8.8, labelled in digoxigenin and biotin, respectively. H7.8 hybridizes exclusively to the large first intron, while H8.8 hybridizes to 3′ sequences, primarily exon (Fig. 1). The two probes were hybridized to RNA using established techniques (Tautz and Pfeifle, 1989) and visualized as above. We found that the 5′ probe hybridized in all data sets in which the 3′ probe did, but not the reverse, consistent with the hypothesis that Notch gene transcription begins fairly synchronously at the beginning of interphase (data not shown). We looked for embryos in which the extent of hybridization to the 3′ probe varied from one nucleus to the other, consistent with the hypothesis that either the time of initiation of transcription or the transcription rate is variable. Such a data set, from a female, is shown in Fig. 7. Note that the 5′ hybridization signals (Fig. 7A) are both brighter and more numerous than the 3′ signals (Fig. 7B). In several nuclei, two 5′ signals are apparent, but only one 3′ signal. A particularly striking example of such a nucleus is shown in Fig. 8. The 5′ signals are elongated in the image plane. Near one end of one 5′ signal is a definite region of hybridization to the 3′ probe, while the corresponding signal on the other homolog is absent. We conclude that while the Notch gene generally begins transcription at the beginning of interphase, there is some variability in either the initiation or the elongation rate. We further suggest that this variability may contribute to the broad distribution of inter-probe distances that we observed in the female data set.

DISCUSSION

We have demonstrated that a two-color hybridization technique can reveal substructure in an expressed gene in Drosophila embryonic nuclei. We find that the Notch gene is in an open conformation as it is expressed during nuclear cycle 14. We also find that the gene is aligned along the Z axis, parallel to the nuclear axis; the 5′ to 3′ orientation (in which the 3′ end is uppermost in the nucleus) is preferred, though not strongly. We find that the number of chromosomes in an open conformation is broadly distributed. Hybridization to nascent mRNA suggests that some of this variability may result from variations in transcriptional initiation or elongation. Furthermore, in preliminary experiments (Gunawardena and Rykowski, unpublished), we have found an age-dependent variation in the mode of the inter-probe distance distribution, although our data are still insufficient to determine the exact timing of decondensation.

The first question to consider is whether the locations of the hybridization signals reflect the positions in the live embryo of the chromosomal sequences homologous to the probe DNA. That is: how well preserved are the embryos during fixation and hybridization? The fixation procedures have been developed to yield nuclei that closely resemble those in live embryos. The embryo preparations conform to the stringent requirements described in Materials and Methods, and on that basis we conclude that the embryos are well preserved. We conclude that, as far as possible, we have eliminated the possibility that the results we present are artifactual.

An alternative way to evaluate this in situ hybridization technique is to ask whether the results we find are consistent with our expectations based on results obtained using independent techniques. As discussed below, the observed inter-probe distances are in the range predicted by observations of expressed genes in polytene chromosomes (Lamb and Daneholt, 1979), but are quite different from those seen in mammalian cells (Lawrence et al., 1990; Trask et al., 1989). We discuss a possible explanation for the differences between our data and those obtained from mammalian cells that depends on the unique properties of the blastoderm embryo of Drosophila.

Previous results on the structure of the Notch gene in polytene chromosomes (where the gene is silent) showed that the expected condensation level for chromatin containing the Notch gene is 100-140 bp/nm (Rykowski et al., 1988). This would correspond to an inter-probe distance of 0.21-0.29 µm in the present experiments, assuming that the gene is in a linear form rather than a loop. If the gene were condensed at the level of interband (<20 bp/nm), the most decondensed portion of the polytene chromosome, we would expect an inter-probe distance in these experiments of at least 1.5 µm. Naked DNA (about 3.5 bp/nm) would extend 8.3 µm. The 29 kb data set showed a large number of decondensed chromosomes, about 90% of the nuclei showed an inter-probe distance between 0.1 and 1.5 µm, indicating a level of condensation between that expected for an inactive gene and that expected for 10 nm nucleosomal fiber. Thus, as expected, the chromatin structure of the active Notch gene in diploid nuclei is more open than that of the inactive gene in polytene nuclei. However, it should be mentioned that in other data sets, notably those from mitotic embryos, the inter-probe distance is less than seen in the 29 kb data set (Gunawardena and Rykowski, unpublished).

The results obtained here lead to a different conclusion than that reached for mammalian nuclei, in which no substructural differences between expressed and silent genes could be seen. We consider several possibilities. The first, and perhaps most important, is that the conclusions drawn previously rested on the similarity in the shape and size of the hybridization signal for expressed and non-expressed genes, rather than on the two-color technique employed here. It is clear that the combined signals from the 5′ and 3′ probes overlap substantially, and, if they were to be labelled in the same color, would likely appear globular. It is only by observing the relative locations of a large number of doubly labelled chromosomes that the extension of the gene is apparent.

It is also possible that functional and geometrical differences between mammalian and Drosophila nuclei are responsible for the apparently greater gene extension in Drosophila versus mammalian diploid genes. Among these are the localization of replication and transcription, and morphogenetic movements of the nucleus during blastoderm. Each of these factors will be considered in turn below.

Can replication of the Notch gene during cycle 14 account for the elongation that we observe? By analogy with the replication timing of mammalian euchromatic genes (D’Andrea et al., 1983; Goldman et al., 1984), we might assume that Notch is replicated in the first part of S phase (which begins immediately in cycle 14), but we have not directly correlated gene elongation with replication time. Replication has been shown to be localized to a few hundred centers (Nakamura et al., 1986; Nakayasu and Berezney, 1989), sometimes called ‘factories’, distinct from those used for transcription, that are fixed to the nucleoskeleton (Hozák et al., 1993). Chromosomal
DNA is reeled through the factories (Hozák et al., 1993), which contain factors required for replication initiation and elongation, and post-replicative modification (reviewed by Spector, 1993). The placement of replication centers in Drosophila (Yamaguchi et al., 1992), about 1-2/µm³, would suggest that DNA from as far away as 0.5 µm might be recruited to a particular center. We would expect that elongation would occur in random directions in each nucleus, assuming that DNA associates with the nearest center, and that the centers are randomly placed. Thus, association with replication centers might account for the extent but not the direction of elongation.

Can transcriptional decondensation account for our observations? Results of others have shown that nuclear division disrupts transcription (Shermoon and O’Farrell, 1991), which resumes at the beginning of interphase. The transcription elongation rate has been estimated to be 1.1-1.4 kb/min (Shermoon and O’Farrell, 1991; Thummel et al., 1990). If we assume that Notch gene transcription restarts at the beginning of interphase and proceeds at the rates determined for other genes, the 40 kb Notch gene will take 28-36 minutes to be completely transcribed. The embryo from which the 29 kb data set was obtained was aged 34 minutes, based on the extent of invagination of the cytoplasmic membranes measured in phase contrast. By this time, the gene could have undergone one complete round of transcription in each nucleus, so most decondensation resulting from transcription should have occurred by this time. The fully transcribed gene is conceivably quite flexible, and might be allowed to move as a result.

Flexibility of the gene alone, however, is not enough to explain the decondensation of the gene; we still need to propose some motive force that acts to extend the gene. In polytene chromosomes, a buildup of transcription products appears to extend the chromatin (see below). However, some of that is probably caused by congestion in the large chromatin arrays which comprise polytene chromosomes; steric factors may be less important when chromosomes consist of single fibers. It is conceivable that genes become elongated when they are pulled into transcription centers in which splicing factors and other components are concentrated (Carter et al., 1993; Xing et al., 1993). However, transcription in diploid nuclei of Drosophila appears to be evenly distributed throughout the diploid nucleus (Zachar et al., 1993). Moreover, recruitment to centers fails to cause gene elongation in mammalian nuclei; we argue that recruitment to transcriptional centers is insufficient to explain gene elongation.

Because of the broad distribution and directionality of elongation, we suggest that the elongation of the Notch gene is due primarily to morphogenetic changes of the nucleus rather than the requirements of transcription or replication. During cycle 14, the nucleus doubles in length as it is enclosed in cell membranes. Since the distribution of chromatin remains fairly uniform over the nucleus during this time, fibers may be drawn along the nuclear axis in the flow of nucleoplasm. We propose the analogy of chromosomal puffing as seen in polytene chromosomes as a model for what we see in diploid embryonic nuclei. In the banded polytene chromosomes of dipterans, transcriptionally active genes often appear diffuse and more voluminous than inactive bands; that is, they are ‘puffed’. Electron microscopic examination of puffed regions shows that expansion results from the decondensation of the chromatids and from the accumulation of protein and RNA at the site of transcription (Lamb and Daneholt, 1979). Within the nucleus, puffs appear to swell at least as much in width as in length (Björkroth et al., 1988). There appear to be no structural impediments to elongation within the nucleus (Ericsson et al., 1989); the bulk of the adjacent chromosomal regions probably act to prevent elongation. Puffed regions can be greatly elongated when freed from the confines of the nucleus and pulled or squashed. In contrast, unpuffed bands retain much of their native appearance even after squashing. This suggests that decondensation during transcription allows greater extension than is possible for transcriptionally inactive regions and that elongation most likely results from external forces acting to extend the chromatin fiber.

A possible scenario is that, as the nuclear envelope expands, the chromosomes undergo a general decondensation relative to their telophase state. Genes that are decondensed further by transcription would move more freely than those still contained in 30 nm fibers. The effect may be more extreme for those genes lying, as Notch does, toward the telomere, as telomeres are known to cluster at the base of the nucleus. Extension of the gene by nuclear movement might be quite variable from nucleus to nucleus, explaining the wide distribution that we see in inter-probe distances. Moreover, the slight preference for the gene to orient with the 5′ end closer to the telomere may be a consequence of the Rabl orientation of the chromosome and independent of its interphase function.

The present work demonstrates the potential for exploration of the fine structure of the genome using high-resolution hybridization and imaging techniques. Using these techniques it should be possible to determine the relative contributions of each of the potential forces acting to decondense the chromosome, and to infer the kinds of attachments responsible for the chromosomal movements that we see.

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