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Chromosomal Distribution of Rapidly Reannealing DNA in *Drosophila melanogaster*

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Abstract. Cytological hybridization has been used to localize fractions of rapidly reannealing DNA in salivary chromosomes of *Drosophila melanogaster.* Complementary RNA of high specific activity was transcribed from hydroxy-apatite-fractionated rapidly reannealing sequences and from selected buoyant-density fractions of total DNA. It was then hybridized to chromosome squashes after denaturation of DNA in NaOH. Highly "repeated" DNA sequences were detected over much of the chromosome, but were concentrated in chromocentric heterochromatin. A family of sequences with a low percentage of guanosine plus cytidine was highly concentrated in a particular region within the chromo-center. One "euchromatic" region near the tip of chromosome arm 3L also exhibited a concentration of repeated sequences.

The technique of nucleic acid hybridization on denatured cytological preparations has been used to localize ribosomal RNA cistrons in several organisms.[1–] In these experiments, labeled ribosomal RNA of high specific activity, or complementary RNA of very high specific activity (transcribed from ribosomal DNA sequences) was hybridized to alkali- or heat-denatured DNA in cytological preparations. Mouse satellite DNA was shown by this technique to be concentrated around the centromere in mouse metaphase chromosomes.[4,5]

The mouse density satellite DNA (a sequence 350 nucleotides in length repeated 10^6 times per haploid genome[6]) is an extreme example of the rapidly reannealing DNA existing in the genomes of a great number of eukaryotes.[7] This fraction of DNA, comprising 10–20% of the total in most organisms, consists of more or less similar (that is, cross-reacting under renaturation conditions) sequences present in large numbers per haploid genome. The roles that these rapidly reannealing sequences play is not yet clear. It has been suggested[8] that the 10^5–10^6 copy sequences in rodent DNA may function in chromosome organization or the regulation of some aspects of chromosome metabolism.

In the experiments to be reported, the method of cytological hybridization described by Pardue *et al.*[3] has been used in studying the chromosomal distribution of fractions of rapidly reannealing sequences in *Drosophila melanogaster,* which comprise about 13% of the total DNA. In a separate report[9] we described the hybridization of hydroxyapatite-isolated, rapidly reannealing DNA to CsCl gradient fractions of total DNA, in which a group of sequences low in G+C content was detected in addition to sequences with a base composition like the

bulk of the DNA. This paper presents the results of hybridization of complementary RNA, synthesized *in vitro* using total rapidly reannealing DNA and DNA from selected CsCl gradient fractions, to squashes of *D. melanogaster* polytene chromosomes.

Methods. Procedures used in the preparation of DNA from *Drosophila melanogaster* adults, the isolation of rapidly reannealing sequences by chromatography on hydroxyapatite, and preparative CsCl gradient ultracentrifugation have been described elsewhere.[9] Cytological hybrids were prepared as described.[3]

Slide preparation: Salivary glands were removed from late third instar larvae of *D. melanogaster* Oregon R, in Beadle-Ephrussi[10] solution, placed in a drop of 45% acetic acid on a gelatin subbed slide for a few minutes, then squashed under a coverslip. The coverslip was removed after freezing on dry ice and the slide was placed in 95% ethanol for 5–10 min, then air dried. Slides were prepared for hybridization as follows. Dried slides were placed in 0.2 N HCl at room temperature for 30 min and washed three times in 2 × SSC (standard saline citrate: 0.15 M NaCl–0.015 M sodium citrate). Endogenous RNA was then removed with 100 μg/ml of pancreatic RNase (Calbiochem) in 2 × SSC at room temperature for 2.5 hr. The slides were washed three times in 2 × SSC, dehydrated through ethanol, and dried. To denature the DNA, slides were placed in 0.07 N NaOH at room temperature for 2 min, dehydrated, and dried, and were then used for hybridization as described below.

Synthesis of complementary RNA: RNA polymerase used for the production of ^{3}H-labeled complementary RNA of high specific activity was prepared following exactly the procedure detailed by Burgess.[11] Enzyme activity was determined as described, using [^{3}H]ATP at 5 mCi/mmol. Activity-containing fractions from the DEAE-cellulose chromatography step (fraction 4) were pooled, precipitated with ammonium sulfate, dissolved in the storage buffer, and used without further purification. Assays for *Drosophila* DNA template activity were performed essentially as described by Burgess in reaction mixtures of the following composition: 20 units of enzyme per ml, 0.15 mM each of UTP, CTP, GTP, and [^{3}H]ATP (5 mCi/mmol), 0.15 M KCl, 0.04 M Tris (pH 7.9), 4.6 mM $MgCl_2$, 2 mM $MnCl_2$, 0.07 mM EDTA, 0.1 mM dithiothreitol, 0.4 mM potassium phosphate, pH 7.5 (to inhibit polynucleotide phosphorylase activity) plus 0.5 mg/ml bovine serum albumin. Incubation was at 37°C. DNA samples to be used in the preparation of complementary RNA were dissolved in 0.1 M Tris, pH 7.9, 0.5 mM EDTA.

The reaction mixture (0.25 ml) for the production of complementary RNA with high specific activity for hybridization was as above, except that the following amounts per 0.25 ml were used: 5 units of enzyme; 5–10 μg of DNA; GTP, 60 nmol; [^{3}H]ATP (21.7 Ci/mmol), [^{3}H]CTP (20.4 Ci/mmol), and [^{3}H]UTP (17.1 Ci/mmol), all from Schwarz BioResearch, 100 μCi each;[3] bovine serum albumin was excluded. The reaction was carried out for 60 min at 37°C. 20 μg of DNase I (Worthington, DPFF) in 0.75 ml of 0.04 M Tris, pH 7.9, was added and the solution was kept at room temperature for 30 min. Unlabeled *E. coli* ribosomal RNA (125 μg) was added as carrier, followed by sodium dodecyl sulfate to 1% and two volumes of water-saturated phenol. The mixture was shaken periodically for several hours at 4°C, then centrifuged, and the phenol phase was reextracted briefly with 0.25 ml of water. The aqueous phases were pooled, heated to 85°C for 3 min, then passed into a 0.9 × 9 cm Sephadex G-25 column. The column was eluted with deionized water and 1-ml fractions were collected. Aliquots of 10 μl were taken from each fraction, the RNA was precipitated with cold 5% trichloroacetic acid (TCA) in the presence of 100 μg of yeast soluble RNA, loaded onto Millipore filters, and counted in toluene-based scintillant. Fractions with high TCA-insoluble counts were pooled, passed through a 0.45 μm Millipore filter (less than 10% of the counts were retained), and made to 6 × SSC. Activities of the preparations were determined by applying aliquots as spots to filters and are given in the legends to Figs. 2, 4, and 5.

Cytological hybridization: Aliquots (100–200 μl) of the complementary RNA solutions were placed on slides, sometimes along with 125 μg of unlabeled *D. melanogaster*

ribosomal RNA for competition studies. Each slide received approximately 0.1 μg of complementary RNA. The slides were incubated in 100-mm plastic Petri dishes at 64°C for 18 hr. The atmosphere was kept humid by saturating filter paper in the plates with 6 × SSC, the slides being supported by rubber washers from centrifuge tube caps. After the incubation, the slides were washed three times in 6 × SSC, incubated in pancreatic RNase at 25 μg/ml in 2 × SSC for 1 hr at 37°C, then washed in 6 × SSC and dried after ethanol dehydration. Slides were then dipped in Kodak NTB-2 emulsion diluted 1:1 with distilled water, dried, and stored at 4°C for lengths of time specified in the figure legends. Autoradiographs were developed in D-19 for 2 min. After fixing and washing, slides were stained with Giemsa's solution.

Results. Production of complementary RNA: *E. coli* RNA polymerase, containing the sigma factor, was prepared as far as fraction 4 according to Burgess,[11] and was stored in buffer in 50% glycerol at −20°C and 100 units/ml. Preliminary tests showed that the enzyme transcribed *Drosophila* DNA 70–80% as efficiently as calf thymus DNA, the standard used for the enzyme assay.

In determining the kinetics of the polymerase reaction on DNA used in the preparation of high-specific-activity complementary RNA, 10-μl aliquots were removed from one of the reaction mixtures at specified times and incorporation was measured. Fig. 1 illustrates the reaction curve, from which it can be seen that the rate reached a plateau at about 30 min, and that after 1 hr, net degradation began to occur, although at a low rate. The total reaction mixture usually produced 0.5–1 μg of RNA in 1 hr (calculated from the specific activities of the substrates). The sedimentation properties of the RNA product have been estimated in isokinetic sucrose gradients, where the bulk sediments at 3–4 S.

Chromosomal distribution of rapidly reannealing sequences: In a separate report[9] we suggested that the rapidly reannealing fraction of *D. melanogaster* DNA is composed of families of similar sequences having from very few copies to about 200 copies each, with an average of about 40. It is evident, however, that with relatively short periods of autoradiographic exposure in the experiments reported here, only hybridization to the larger family sequences will be detected, since in both the transcription and the hybridization these sequences will be favored with respect to the species of complementary RNA (see also ref. 12). To a degree, then, the detection of families of diminishing size will be an inverse function of the length of exposure. It will be a direct function of the extent to which the sequences are clustered.

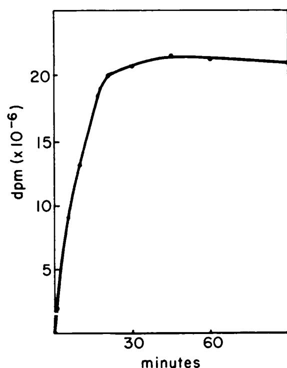

FIG. 1. Incorporation of [^{3}H]ribonucleotides into complementary RNA. The template was from a region I DNA preparation (see Fig. 3). Activities are expressed per 0.25 ml reaction mixture. The liquid scintillation spectrometer counted at an efficiency of about 30%.

For the experiments in this section, RNA was transcribed from the 13% rapidly reannealing DNA in *D. melanogaster*, as isolated on hydroxyapatite from the bulk of the DNA, then hybridized to late third instar larval salivary chromosome squashes. After a short exposure (3 days), the chromocenter was distinctly labeled, with grains also appearing elsewhere on the chromosomes (Fig. 2*a*). In favor-

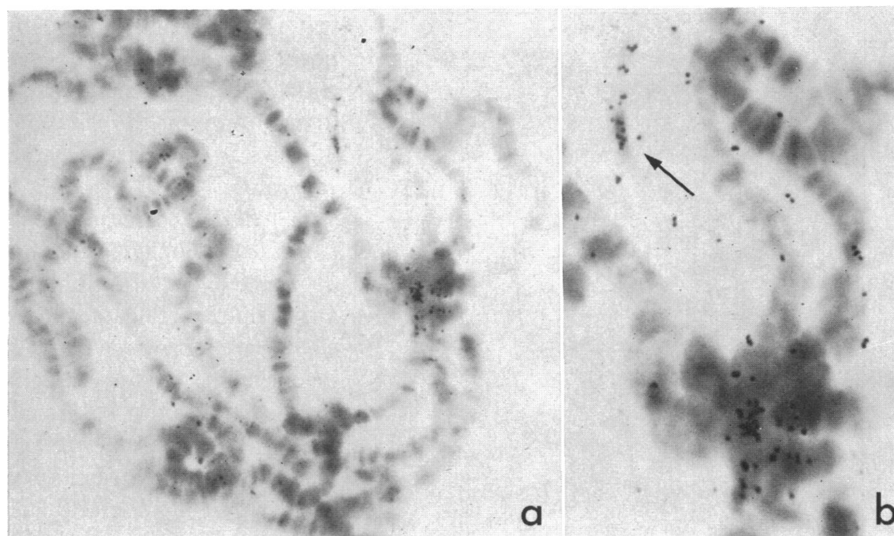

FIG. 2. (*a*) Complementary RNA transcribed from hydroxyapatite-fractionated rapidly reannealing *D. melanogaster* DNA was hybridized to alkali-denatured DNA in salivary chromosome squashes as described in *Methods*. The RNA preparation had a specific activity of 1.5×10^6 cpm/ml. Autoradiographic exposure was for 3 days. ×590.

(*b*). Higher magnification of (*a*). Note labeling over intranucleolar DNA (arrow) and the distribution of grains over the chromocenter. ×1450.

able squashes, several grains could be seen clearly localized to the intranucleolar DNA (nucleolar organizer), where the cistrons for ribosomal RNA are reiterated about 130 times per haploid genome[13] (Fig. 2*b*).

With longer exposures the chromocenter became heavily labeled. More grains appeared over the chromosome arms, but with no discernible pattern. The presence of 625 μg/ml of unlabeled *D. melanogaster* ribosomal RNA during hybridization appears to have had no effect on the intensity of labeling over the chromocenter or chromosome arms, although it considerably reduced grains over the nucleolar organizer. Only light and inconsistent labeling over the telomeres has been detected in these preparations to date.

Distribution of selected %G+C fractions. Our previous report[9] demonstrated that within the main band of *D. melanogaster* DNA as centrifuged to equilibrium in CsCl, a disproportionate percentage of the rapidly reannealing DNA appears in a small region on the light side, indicating the presence of a more or less homogeneous population of sequences having a %G+C lower than the bulk of the DNA. A preparation of DNA enriched for these sequences was obtained by successive centrifugations in CsCl, as described in the legend to Fig. 3 (region II DNA). RNA transcribed from this DNA was hybridized to chromosome squashes. Similar preparations were made for the DNA in region I of Fig. 3. In both these cases, it is assumed that hybridization is occurring to large family sequences, as discussed above.

(*a*) *Region II.* After 4 days' exposure, labeling was light and confined to the chromocenter, as with the short exposures for the total rapidly reannealing

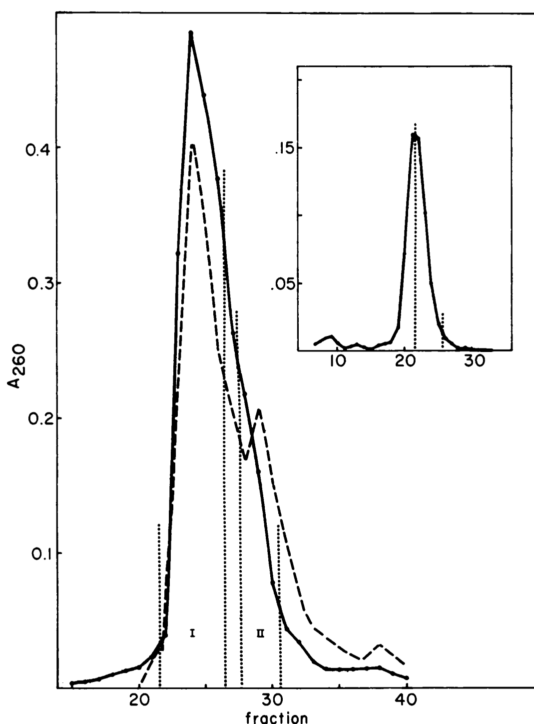

FIG. 3. Preparation of selected buoyant-density fractions of DNA. Total *D. melanogaster* DNA was centrifuged to equilibrium in CsCl as described elsewhere.[9] The broken-line profile illustrates the result of hybridizing hydroxyapatite-fractionated rapidly reannealing [^{3}H]DNA to CsCl gradient fractions, where the population of low G+C sequences is detectable. Fractions in region I were pooled, precipitated, and dissolved in buffer. Fractions in region II were recentrifuged in CsCl (*inset*), and DNA within the broken lines was concentrated by centrifugation (20 hr at 65,000 rpm) and dissolved in buffer for transcription.

fraction. With longer exposures (16–21 days), however, there was little change in the distribution of the label. It appears, in fact, that the grains were closely clustered in a region *within* the chromocenter (Fig. 4*a*). Some grains were dispersed over the chromosome arms, but labeling of the nucleolar organizer was light and inconsistent. This labeling pattern was more or less duplicated in all slides of this series, and indicates that the rapidly reannealing sequences in region II of the CsCl gradient profile are primarily confined to a portion of the chromocenter, in contrast with the distributions for the other complementary RNA preparations (compare with Figs. 2 and 5*b*).

(*b*) *Region I.* With a short exposure the chromocenter was heavily labeled, with light labeling over the arms. Later the chromosome arms were moderately labeled. Certain specific regions of the arms appeared heavily labeled, but the relatively poor preservation of the detail of banding pattern has made their identification difficult. A particular region near the tip of the left arm of chromosome 3 was labeled in nearly all nuclei in these slides (Fig. 5*a*–*c*). After long exposures, grains appeared over much of the chromosome (Fig. 5*d*). Again telomeres were only lightly labeled and little, if any, labeling occurred over the nucleolar organizer. The clustering of grains within the chromocenter was seen in these preparations, as in those with region II being used as the DNA template, but label was distributed over the rest of the chromocenter as well (as in the pattern for total rapidly reannealing DNA). No obvious Y-chromosome specific group of rapidly reannealing sequences was observed in any of these preparations. χ^2 tests indicated that grain distributions in Region I and the rapidly reannealing fraction were not significantly different ($0.50 > P > 0.20$), while they both differed significantly from the distribution for Region II ($0.01 > P$).

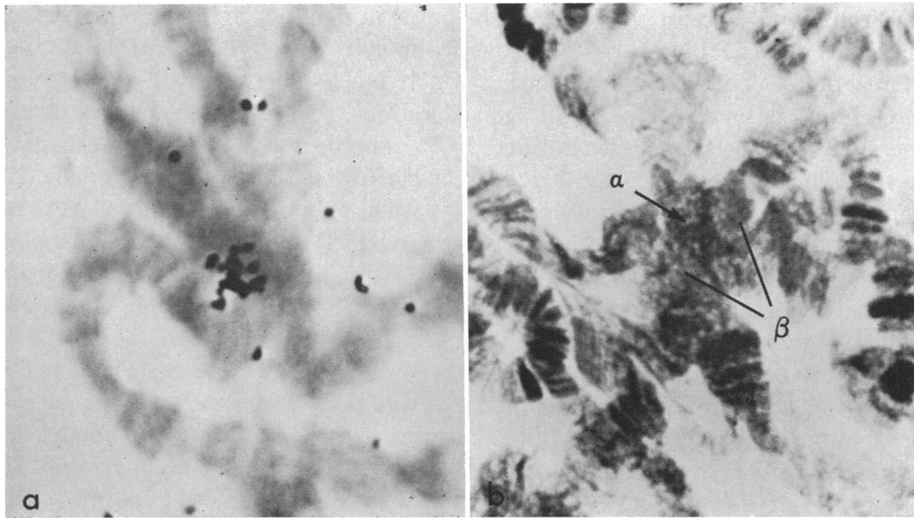

FIG. 4. (*a*) Hybridization of region II complementary RNA to the chromocenter. Relatively light labeling occurred over the chromosome arms, and grains were primarily clustered in a region within the chromocenter. The complementary RNA preparation had a specific activity of 3.2×10^6 cpm/ml. Exposure was for 8 days. ×2000.

(*b*). Feulgen preparation of a salivary chromosome squash illustrating the regions of α and β heterochromatin. ×2000.

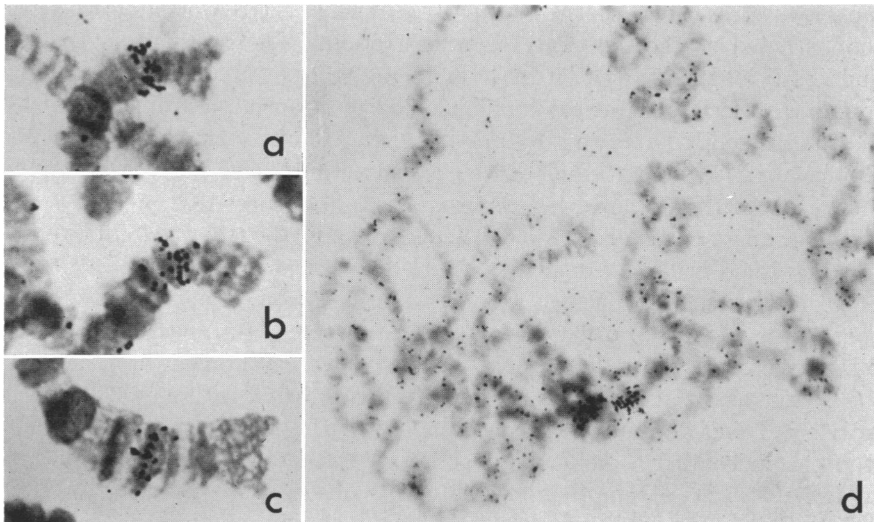

FIG. 5. (*a–c*) A region near the tip of the left arm of chromosome 3 is heavily labeled when complementary RNA from region I is hybridized to salivary chromosomes. Specific activity of the RNA was 1.7×10^6 cpm/ml. 21-day exposure. ×1600.

(*b*). Hybridization of region I complementary RNA to salivary chromosomes. Chromocenter and chromosome arms are generally labeled. Specific activity, 2.9×10^6 cpm/ml; exposure, 9 days. ×650.

Discussion. Several points should be borne in mind when interpreting the results of cytological hybridization experiments with a heterogeneous population of reactants. Some factors dictating which DNA sequences will be detected have been discussed above; other considerations, such as the specificity of the reaction, have been treated elsewhere.[1,3,14] The degree of specificity obtained in the hybridizations reported here cannot be fully ascertained, but some confidence is given by the localization of grains when region II DNA served as template, and by the reduction in labeling of the nucleolar organizer when unlabeled ribosomal RNA was included during hybridization and when RNA copied from selected regions of CsCl gradients was used (see below). In addition, labeling is minimal on slides in which the DNA was not deliberately denatured.

Labeling over the nucleolar organizer occurred only in those preparations for which the total rapidly reannealing DNA fraction served as template in the production of complementary RNA. From a consideration of the reported[15] base composition of the 38S ribosomal RNA precursor molecule in *Drosophila* (about 31% G+C) it is not surprising that the density gradient fractions taken for transcription contained few, if any, of the ribosomal RNA cistrons, since DNA of such low %G+C would band at a much lower density than the bulk of the DNA (42% G+C, ref. 9). It is unusual, however, that the precursor to ribosomal RNA in *Drosophila* contains only 31% G+C. In other organisms, including *Chironomus*[16] and *Rhynchosciara*,[3] the base composition of ribosomal RNA cistrons is consistently higher than the total DNA.[17-19]

In the preparations described here, grain clusters appeared first (and most intensely in longer exposures) over the chromocenters of the salivary chromosomes, regardless of whether total rapidly reannealing DNA or buoyant-density fractions acted as the template for transcription. These results suggest that sequences belonging to the larger families are concentrated there. As judged from label intensity, sequences complementary to transcripts from the full 13% rapidly reannealing DNA and from region I of the density gradient profile are to be found most frequently in a localized region within the chromocenter, and less so in the rest of the proximal heterochromatin. Sequences that are less "reiterated" or are less concentrated in particular regions (the two possibilities cannot be distinguished in these preparations) are distributed over much of the rest of the chromosomes. In contrast, RNA transcribed from region II DNA (some properties of which are discussed above; see also ref. 9) appears to bind predominantly to only a portion of the chromocenter. Whether these sequences are the same as, or adjacent to, a part of those detected with region I DNA as template is being determined. Nevertheless, of the rapidly reannealing DNA in the chromocenter, a particular fraction is considerably more concentrated than the rest. It is tempting to relate this observation (Fig. 4*a*) to Heitz' description[20] of α and β heterochromatin in the chromocenter (Fig. 4*b;* see also ref. 21), and to suggest that the α fraction (a rapidly reannealing population of low %G+C sequences) is associated with the centromere, and β (comprised in part of some other fractions of the rapidly reannealing DNA) is the remaining proximal heterochromatin of the chromosome arms and the Y chromosome. The possibility of equating two fractions of rapidly reannealing DNA with the two morphological fractions of heterochromatin is somewhat confused, however, by

Rudkin's suggestion[22] that a fraction of salivary chromosome heterochromatin continues to replicate while the rest does not during polytenization. Such a labeling pattern as we have obtained with region I complementary RNA would result if heterochromatin DNA were comprised of a large number of similar sequences, only some of which were concentrated (by replication) in the α region. The specificity of labeling we have found for region II DNA argues against this.

The results clearly indicate that large family sequences are found concentrated in the chromocentric heterochromatin, with a particular low G+C fraction localized around what may be the centromere. This observation agrees with the findings that in mouse,[4,5] salamander,[23] and *Rhynchosciara*,[24] highly repetitive DNA is localized around the centromere. In the mouse and *Rhynchosciara*, these sequences are found in a light buoyant-density satellite.[8,24]

The appearance of grains over the arms of the various chromosomes, with indications of some regions having concentrations of rapidly reannealing sequences (Fig. 5*a–c*), is potentially interesting and will be the subject of a more detailed investigation on the distribution and thermal stabilities of the hybrid duplexes. Studies on band duplications such as Bar [25] and pseudoallelism at several loci[26] suggest that several instances of multiple copies of identical and related sequences are to be found in *Drosophila* euchromatin.

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