Three euchromatic DNA sequences under-replicated in polytene chromosomes of *Drosophila* are localized in constrictions and ectopic fibers

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Abstract. We examined three regions of under-represented euchromatic DNA sequences (histone, *Ubx*, and 11A), for their possible correlation with euchromatic constrictions in polytene chromosomes of *Drosophila melanogaster*. Cloned sequences were hybridized to filters and to chromosomes prepared for light microscopy. Under-represented sequences hybridized to DNA within constrictions and in ectopic fibers. In contrast, adjacent sequences that were fully endoreplicated in the *Ubx* and 11A regions in polytene cells hybridized to sites just adjacent to their respective constrictions. For one region (*Ubx*), sequences under-represented in salivary gland cells were fully endoreplicated in fat body cells. For this particular region, the morphology of the polytene chromosomes differs between these two cell types in that the specific constriction is absent at this region in fat body polytene chromosomes, thus strengthening the correlation between under-representation and chromosome constrictions. Although all three sequences are in regions that have been classified by others as "intercalary heterochromatin," we detect no common functional or sequence organizational feature for these examples of under-represented DNA. We suggest that the lower efficiencies of the replication origins, or special regions of termination at these sites, are the primary cause of the under-replication, and that this under-replication is sufficient to confer the properties of intercalary heterochromatin.

Introduction

The replication of euchromatic DNA in polytene chromosomes of *Drosophila* is generally uniform, as inferred from the renaturation kinetics of diploid and polytene DNA (Dickson et al. 1971; Laird et al. 1973), and from quantitative Southern hybridization of cloned DNA sequences (Lifschytz 1983; Spierer and Spierer 1984). Satellite DNA sequences, located predominantly in the centromeric heterochromatin, and ribosomal DNA, also located in heterochromatin, are exceptions in that they are under-represented in salivary gland polytene chromosomes relative to the abundance of these sequences in mitotic chromosomes. The under-representation of these sequences is generally assumed to be due to under-replication (Rudkin 1969; Gall et al. 1971; Dickson et al. 1971). There are a few regions in the euchromatic arms of polytene chromosomes that are

distinctively wide or narrow, and these have been suggested to represent regions where DNA replication is somewhat advanced or retarded, respectively, relative to most euchromatic sequences (Laird 1980). Proportionally over-represented DNA sequences, which would include DNA puffs such as are observed in *Rhynchosciara* polytene chromosomes (Breuer and Pavan 1955; Glover et al. 1982) have not yet been reported for *Drosophila melanogaster*. Two examples of under-represented (and presumably under-replicated) DNA sequences in the euchromatic arms have, however, been noted (Lifschytz 1983; Spierer and Spierer 1984). Because both examples were reported to occur at or near constrictions, we examined further the relationship between replication levels and chromosome morphology. Specific cloned DNA sequences, located in or near prominent constrictions in salivary gland chromosomes, were tested for under-replication by quantitative Southern hybridization. The ^{32}P-labeled probes were hybridized to filter-bound DNAs prepared from larval imaginal discs, which are composed of diploid cells continuing to divide (we shall refer to these cells as mitotic cells and DNA extracted from them as mitotic DNA), from salivary glands, which have chromosomes of a high degree of polyteny, and from larval fat bodies, which have chromosomes of about fourfold lower polyteny than salivary gland chromosomes (Laird et al. 1980).

The three constrictions we examined, (11A, 39D, and 89E) have been described as regions of "intercalary heterochromatin." This term was first used by Kaufmann (1939) to describe regions that are located in the euchromatic arms of polytene chromosomes, but are like the centromeric heterochromatin in sensitivity to X-ray induced breaks in meiotic cells and in a tendency to associate with each other or with the chromocenter (ectopic pairing) in polytene cells. Since then, the proposed list of characteristics of intercalary heterochromatin has been expanded to include apparent structural weakness, late replication, and the presence of repeated sequences (see Zhimulev et al. 1982, for discussion). We relate our results to the definition and properties of intercalary heterochromatin, and we suggest that under-replication itself may account for many of these properties.

Materials and methods

Preparation of DNA. An Oregon R *D. melanogaster* stock obtained from J. Fristrom was raised on standard cornmeal medium at 18° C in uncrowded conditions. Mass isolated

imaginal discs from late third instar larvae of this stock were a gift of Dr. Fristrom. Salivary glands and fat bodies were dissected from late third instar larvae. DNA was isolated using a modification of the method described by Bender et al. (1983b). Over a period of 1 h, salivary glands or larval fat bodies were dissected free of other tissue in *Drosophila* Ringer's solution (Ephrussi and Beadle 1936) and transferred to 0.1 ml of lysis buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris pH 9.1, 0.05 M EDTA, 0.5% SDS, 100 µg/ml proteinase K) at room temperature. Approximately 50 pairs of salivary glands or fat bodies from 25 larvae were collected, the tissue was disrupted by passage four times through a 23 g needle, and the mixture was incubated at 37° C for 1 h. It was then extracted with phenol, equal volumes of phenol and chloroform, and with chloroform only. The nucleic acids were precipitated by the addition of one-tenth volume of 3 M sodium acetate and three volumes of absolute ethanol. RNA was removed by dissolving the precipitate in 0.1 ml TE (10 mM Tris, 1 mM EDTA, pH 8), adding boiled RNase A to 50 µg/ml, and digesting for 30 min at 37° C. The organic extractions and ethanol precipitation were then repeated. DNA was stored in TE at 4° C. Imaginal disc DNA was extracted by the same method after combining equal volumes of lysis buffer and discs that had been stored in glycerol at −70° C. Purified DNA was digested overnight with EcoRI in the following buffer: 100 mM Tris pH 7.5, 50 mM NaCl, 10 mM MgCl₂. A small amount of each digested DNA was run on a minigel to determine the extent of digestion and to verify that the amounts of DNA run in parallel lanes of the gel would be comparable. EcoRI restriction fragments were separated on a 0.7% agarose gel run at 21–25 V for 18–20 h with approximately 1 µg of DNA per lane, and with 0.5 µg/ml of ethidium bromide included in the gel and the running buffer. The gel was photographed, depurinated, denatured and neutralized and the fragments were transferred to nitrocellulose by the method of Southern (1975). The gel was restained and rephotographed to verify that the transfer of fragments had been complete, and the filter was baked at 80° C for 1–2 h under vacuum.

Cloned DNAs. The histone clone used was aDm-3000-1, a single 4.8 kb histone repeat unit from cDm 500 (Lifton et al. 1978) cloned in pBR322 (D. Hogness, personal communication). Two cloned DNAs from the 89E region, p3139, 8 kb containing the 3′ exon and homeobox of the *Ubx* gene, and p3206, 2.6 kb containing the 5′ exon of the same gene, were subcloned from cosmids by M. Akam and obtained from R. Garber. Clones 0371, 348, and 133 were included in a series of overlapping clones from the 11A region obtained from T. Goralski and A. Mahowald.

Labeled probes. The above cloned DNAs, or, in some cases, restriction fragments isolated from them, were nick translated by standard methods (Maniatis et al. 1982). For hybridization to filters, the probes were labeled with ³²P-ATP; for in situ hybridization to squashed polytene chromosomes prepared for light microscopy, ³H-ATP and ³H-CTP were used.

Hybridization of probes to filters. Filters carrying DNA were pre-hybridized in sealed boilable food bags for 2–4 h at 65° C in the following buffer: 0.75 M NaCl, 0.25 M Tris pH 8, 0.005 M EDTA, 2 × Denhardt's solution, 0.1% SDS,

100 µg/ml sheared denatured salmon sperm DNA. The pre-hybridization buffer was removed from the bag and replaced by hybridization solution, which consisted of the same buffer containing 1 × Denhardt's solution, 10% dextran sulfate, and labeled probe that had been denatured by heating to 95°–100° C for 10 min. The bag was resealed and incubated at 68° C with gentle agitation for at least 18 h. After hybridization the filter was washed in 2 × SSC, 0.1% SDS (three washes, 5 min each) at room temperature, and in 0.1 × SSC, 0.1% SDS (two washes, 30 min each) at 65° C (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate.) The filter was dried briefly by blotting at room temperature or at 37° C, wrapped in Saran wrap, and exposed to Kodak X-AR5 autoradiographic film with two Cronex intensifying screens. In most cases the film was pre-flashed (Laskey and Mills 1977), and several exposures were made to obtain at least one in the linear range of the film. Autoradiographs were developed in Kodak D-19 developer and analyzed on a Helena Quick-Scan Jr. densitometer. All clones used as probes were hybridized to at least three filters, except for clone 348, which was hybridized to a single filter; the range of values obtained for under-representation of specific sequences is described below (see Results). In early experiments comparing cloned DNAs from several different chromosomal regions (results not shown), we found that the p3139 probe from the 89E region appeared to be replicated to the same relative extent in imaginal discs and in salivary glands. This probe bound to three fragments in the size range of other fragments to be examined, and we adopted it as the standard probe for normalizing DNA amounts. Filters were hybridized with the standard, analyzed by autoradiography, then hybridized with other probes. The densities of the hybridization bands from the standard sequence were compared and the ratio of their values in the imaginal disc and polytene DNA lanes was used to normalize for DNA amount in comparing the other probes. The first probe was not washed from the filter before the second hybridization unless the fragments detected by the two probes were so similar in size that they could not be easily distinguished. When it was necessary, hybridized probe was removed from filters by a 2 h wash at 65° C in the following solution: 2.5 mM Tris pH 8, 0.1 mM EDTA, 0.025% sodium pyrophosphate, 0.05 × Denhardt's solution (Thomas 1980). The washed filters were dried and exposed to film for a period of 1–3 days to verify that most of the hybridized probe had been removed.

In situ hybridization. Squash preparations of salivary gland chromosomes were made according to the method of Atherton and Gall (1972), with the addition of a heat treatment (Bonner and Pardue 1976). RNase treatment, denaturation, and hybridization followed the method of Gall and Pardue (1971) with some modifications. In several experiments the RNase treatment was omitted. In most experiments, a 10 min treatment with acetic anhydride (5 ml/l 0.1 M Tris pH 8, followed by rinsing in 2 × SSC, dehydrating in ethanol, and air drying) was included before the denaturation step (Hayashi et al. 1978). The hybridization buffer used was 0.4 M NaCl, 50% formamide, 0.01 M Pipes pH 6.5. Nick-translated DNA was denatured by boiling for 10 min, then added to the hybridization buffer to give a concentration of approximately 25000 cpm/µl. Ten microliters of probe in buffer was placed on the slide over the chromosomes and covered with an 18 mm square cover slip. The

slide was placed in a moist chamber with hybridization buffer in the bottom of the chamber, or the edges of the cover slip were sealed with rubber cement. The hybridization reaction was allowed to proceed at 37° C for at least 18 h. Cover slips were removed in 2 × SSC at room temperature, and non-specifically bound DNA was removed by two additional rinses in 2 × SSC at 50° C. After the slides had been dehydrated and air dried, autoradiography was carried out according to the method of Gall and Pardue (1971).

Results

Three constrictions in salivary gland chromosomes contain under-represented sequences

One component of the hypothesis that polytene chromosome morphology is correlated with replication levels predicts that constrictions contain sequences that are delayed or halted in replication (Laird 1980). We tested this by measuring the amount of DNA, relative to mitotic DNA, of sequences located in three constrictions in salivary gland chromosomes. These constrictions are located at region 39 D on chromosome 2L, the location of the histone genes, region 89 E on chromosome 3R, the location of the bithorax complex, and region 11 A on the X chromosome.

Histone genes. Histone genes appear to be between one and two steps behind in replication in salivary gland chromosomes (Fig. 1). While the standard probe (p3139, see Materials and methods) shows bands of similar density in the imaginal disc and salivary gland lanes, the histone DNA band is fainter in the salivary gland lane. After normalizing for a small difference in DNA amount shown by the relative density of the standard probe in the different lanes, the amount of histone DNA detected in the salivary gland lane is 31% of the amount in the imaginal disc lane. For three other similar filters, the level of histone sequences in salivary gland DNA relative to imaginal disc DNA ranged from 18% to 43%. These results confirm those of Lifschytz (1983), who reported that histone sequences were 33% to 50% as abundant in salivary gland DNA as in pupal DNA. Hammond and Laird (1985), using quantitative in situ hybridization to cytological preparations of salivary gland chromosomes, have also reported under-representation of histone sequences in these chromosomes, finding them to be 18% as abundant as in imaginal disc cells.

Labeled probes prepared from histone sequences, including the clone that we used in this study, have been shown to hybridize in situ to the 39 DE region (Hammond 1984), which appears as a prominent constriction in 2L chromosome arms that are slightly stretched (Pardue et al. 1977; Lifschytz 1983). In some preparations, ectopic fibers extending from this region are labeled, while the chromosome regions to which they are ectopically attached remain unlabeled (Pardue et al. 1977, Fig. 8; Lifschytz 1983, Fig. 8).

Ubx sequences. Spierer and Spierer (1984) have mentioned unpublished data on under-representation of sequences at 89 E, which is the location of the bithorax complex and a prominent constriction in salivary gland chromosomes 3R. We obtained two cloned plasmids from this region: p3139, which includes the 3′ exon and homeobox of the Ubx gene, and p3206, which contains the 5′ exon of the
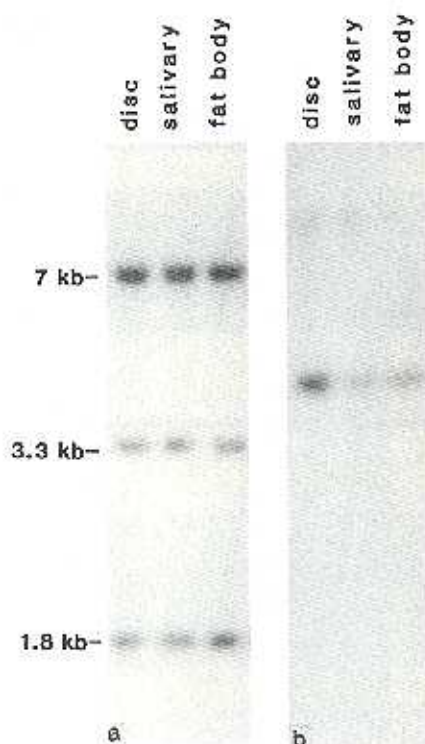

Fig. 1 a, b. Histone sequences are under-represented in salivary gland and fat body DNA. DNA from imaginal discs, salivary glands, and larval fat bodies was digested with EcoRI, separated by electrophoresis on a 0.7% agarose gel, and transferred to nitrocellulose filters. a Filter hybridized with the control probe (p3139, 3′ Ubx sequences); b The same filter hybridized with histone sequences. Densitometry measurements indicated slight differences in the amount of DNA in the control lanes (within 20%), and values for the binding of the experimental probes were normalized to account for these differences. After normalization for DNA amount, the histone sequences were calculated to be 31% as abundant in salivary gland DNA as in imaginal disc DNA, and 34% as abundant in fat body DNA as in imaginal disc DNA. These values take into account the small amount of hybridization to high molecular weight material seen as a faint band toward the top of the filter. This material ran at the same position as control undigested DNA (not shown), and varied in amount from preparation to preparation

same gene (R. Garber, personal communication). Our initial experiments indicated that the 3′ Ubx sequences are not under-represented in DNA from polytene chromosomes, and so we used this probe as our standard. The sequences at the 5′ end of this gene, however, were under-represented in salivary gland DNA (Fig. 2). Densitometry measurements, normalized for DNA amount, indicated that the 5′ Ubx sequences are 22% as abundant in salivary gland DNA as in imaginal disc DNA. For seven similar filters hybridized with the 5′ Ubx probe, this relative abundance ranged from 22% to 40%, which is approximately the same degree of under-representation that we detected for histone sequences in salivary gland DNA.

The Ubx region is estimated to be 75 kb long and is located in the centromere proximal half of the bithorax complex (Bender et al. 1983a; Akam et al. 1984). To determine more precisely the location of the Ubx cloned sequences relative to the constriction, we hybridized ³H-labeled nick- translated probes to squashed salivary gland
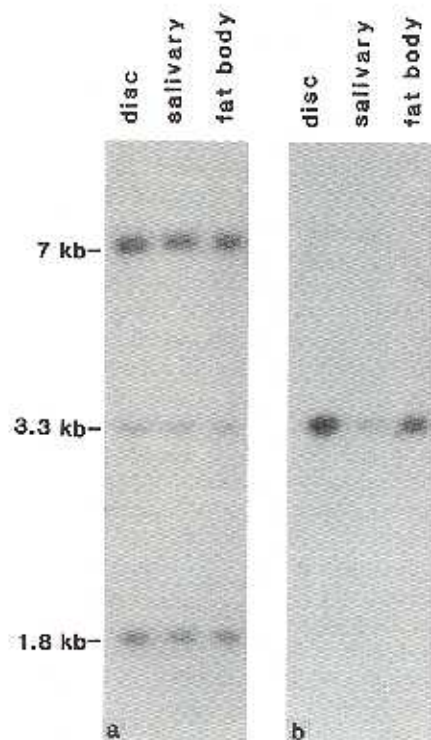
Fig. 2a, b. 5' *Ubx* sequences are under-represented in salivary gland, but not in fat body, DNA. Filters were prepared and hybridized as in Figure 1, but the filter was washed to remove the control probe before hybridization with the 5' *Ubx* probe. a Filter hybridized with control (3' *Ubx*) probe; b same filter hybridized with 5' *Ubx* probe. After normalization for slight differences in DNA amount, the 5' *Ubx* sequences were calculated to be 22% as abundant in salivary gland DNA as in imaginal disc DNA, but 89% as abundant in fat body DNA as in imaginal disc DNA

chromosomes. In general, the hybridization of each of the probes occurred at or near the 89 E constriction. In several sets of chromosomes, the right arm of chromosome *3* was slightly stretched in the 89 E region, which made the constriction more obvious. In these chromosomes, hybridization of the 3' *Ubx* probe was primarily to the region just proximal to the constriction, with few or no grains located over the constriction itself (Fig. 3a). In similarly stretched chromosomes, the 5' *Ubx* probe, which filter hybridization had shown to be under-represented relative to the 3' sequences, produced grains located primarily over the constriction (Fig. 3b). These results support the hypothesis that constricted regions are areas of DNA under-replication. In some preparations, ectopic fibers appeared to contain the region of hybridization; as in the case of the labeled fibers at the histone locus (above) and the 11 A region (below), the ectopic chromosomal region to which these labeled fibers attached was not labeled (Fig. 3c).

The 11 A region. If DNA under-representation at constricted regions is a general phenomenon, rather than a special property of the histone and bithorax regions, sequences localized to other constrictions should show similar under-representation in salivary gland DNA. A prominent constriction is located on the *X* chromosome at the 11 A region. We hybridized ^{32}P-labeled probes from the two ends and the middle of a 100 kb chromosome walk in this region to filters that had previously been probed with the DNA
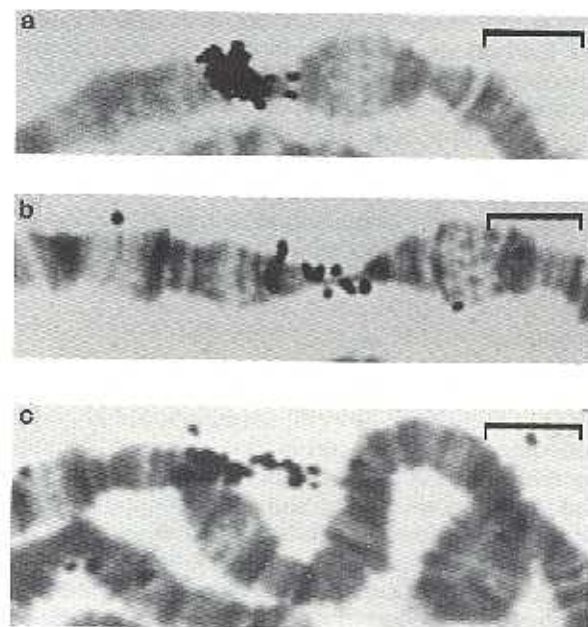

Fig. 3a–c. In situ hybridization of cloned probes to 89 E region. a The 3' *Ubx* probe hybridizes primarily to the region proximal to the constriction. b The 5' *Ubx* probe produces grains primarily over the constricted region. c Region of ectopic pairing labeled with 5' *Ubx* probe. The short ectopic fibers and 89 E are labeled; the ectopic region to which they attach is unlabeled. Bars represent 5 µm

standard. One of these clones contained a fragment that appeared to be under-represented in salivary gland DNA. The most proximal of the clones tested, 133, hybridized to several EcoR1 fragments, including a large fragment that ran close to the position of control undigested DNA. This hybridizing fragment appeared on all filters tested as a heavy diffuse band in the imaginal disc lane and in each case as a much lighter band in the salivary gland DNA lane (Fig. 4). For all three 11 A probes, the fragments ranging in size from about 2 to 7 kb were proportionally replicated in the imaginal disc and the salivary gland DNA. Other workers have reported that restriction fragment larger than 5 kb may be artifactually under-represented due to shearing (Spierer and Spierer 1984), and hybridization to high molecular weight fragments may not be reproducible (Lifschytz 1983). We do not think that the under-representation of the high molecular weight DNA is artifactual in this case, for we observed these bands on all the filters that we hybridized with this probe (four filters prepared with DNA from three separate DNA isolations and restriction digests). Because of background, diffuseness, and the great difference in density between the bands in the imaginal disc and the salivary gland DNA lanes, the relative density of the bands was difficult to measure on most of the autoradiographs. We estimate from comparing several exposures of the filter shown in Figure 4b, that sequences in this high molecular weight band are at most 25% as abundant in salivary gland DNA as in imaginal disc DNA. Sequences at the proximal end of the walk are repeated (T. Goralski, personal communication), and isolation and nick translation of a 5.3 kb EcoR1 fragment located at the centromere-proximal end of clone 133 indicated that sequences located in this fragment are responsible for the hybridization to
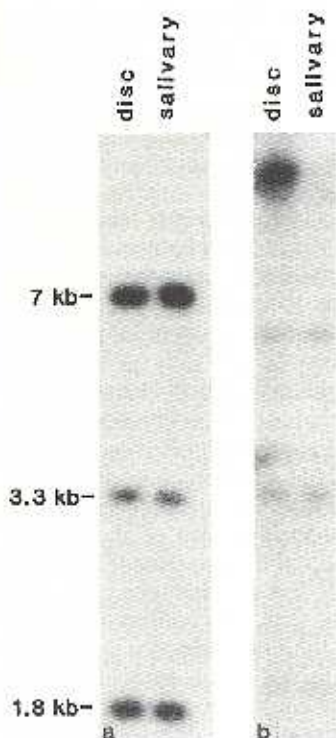
Fig. 4a, b. Sequences located at 11 A are under-represented in salivary gland DNA. The filter was prepared, hybridized and analyzed as in Figures 1 and 2. a Filter probed with control 3′ *Ubx* probe. b The same filter probed with clone 133, from the proximal end of the 11 A walk. Only the band of high molecular weight DNA (>23 kb) shows a substantial difference in density when the imaginal disc and salivary gland DNAs are compared. Densitometry measurements of this filter indicated that these sequences are at most 25% as abundant in salivary gland DNA as in imaginal disc DNA. Two other clones from the 11 A walk, 0371 from the distal end, and 348 from the middle, failed to show any substantial differences between imaginal disc and salivary gland DNA, when hybridized to similar filters

high molecular weight DNA on the filters (results not shown). The high molecular weight band in Figure 4 is five times as dense as expected for single-copy DNA, based on the density of the band at about 3.3 kb, and the relative sizes of the probe fragments responsible for the hybridization to the bands. We conclude from this that the sequences in the high molecular weight DNA are repeated approximately fivefold in mitotic DNA.

We determined the chromosomal location of probes from the two ends and the middle of the 11 A walk by in situ hybridization to squashed salivary gland chromosomes. All three probes hybridized close to the constriction, but only the proximal probe (133), which contained sequences under-represented in salivary gland DNA, showed grains over the constriction in favorably stretched X chromosomes (Fig. 5). These grains do not extend the entire length of the constriction, but are located only in the distal half to two-thirds. In some preparations, ectopic fibers from the 11 A region showed labeling with the 133 probe. These labeled fibers were attached to an unlabeled chromosome region (Fig. 5e). Even with a 37 day exposure of the 133 probe (compared with the 11 day exposures shown in Fig. 5), and with a 50 day exposure of a probe made from the isolated 5.3 kb fragment, we detected no other site of
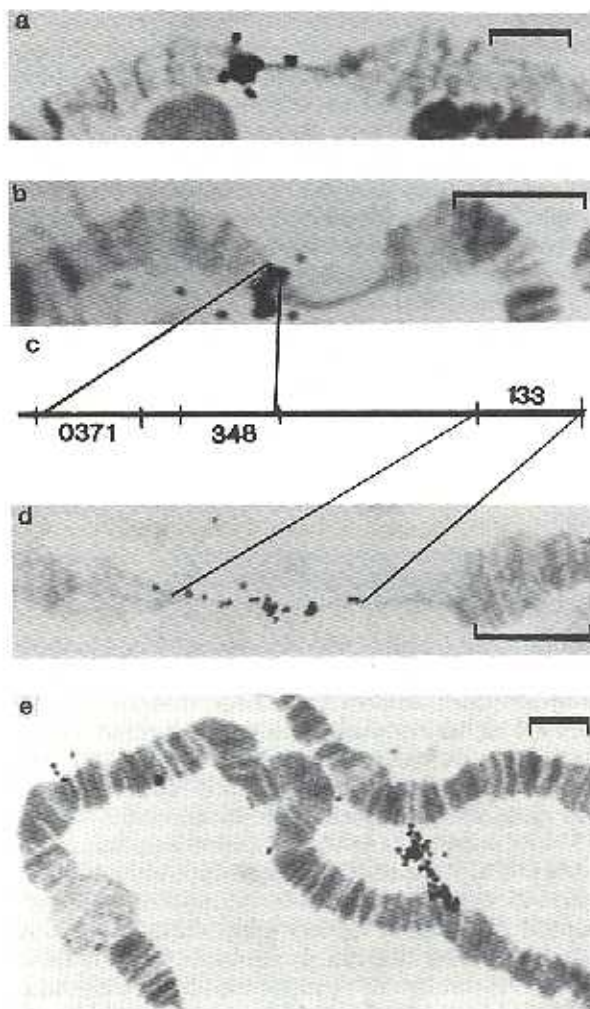

Fig. 5 a–e. In situ hybridization of cloned probes from the 11 A walk. a Clone 0371, the most distal clone, hybridizes to the region distal to the constriction. b Clone 348, from the middle of the walk, hybridizes to the region distal to the constriction. c Diagram showing the relative positions of the three clones used as probes. The region spans approximately 100 kb of DNA. d Clone 133, the most proximal clone, hybridizes to approximately two-thirds of the extended constriction in this preparation. e Ectopic fibers labeled with probe prepared from clone 133. While the ectopic fibers are heavily labeled, there is no labeling of the ectopic chromosomal region to which the fibers attach. Bars represent 5 µm

reproducible hybridization for these sequences in salivary gland chromosomes. We conclude therefore that sequences located in the 11 A constriction are under-replicated at this site.

Are sequences that are under-represented in salivary glands under-represented in other polytene tissues?

We isolated DNA from larval fat body and probed it with the histone and 5′ *Ubx* probes. Histone sequences appeared to be under-represented in fat body chromosomes. In Figure 1, the level of histone sequences detected in fat body DNA is 34% of that found in imaginal disc DNA, and two similar filters gave a range of 34% to 38%. This is similar to the degree of under-representation of histone sequences in salivary gland DNA, where the level detected was 18% to 43% of that found in imaginal disc DNA.

5′ *Ubx* sequences, in contrast to histone genes, and in contrast to their apparent under-representation in salivary gland chromosomes, were polytenized to the same extent as control sequences in fat body chromosomes. In Figure 2, the amount of 5′ *Ubx* DNA detected in fat body DNA is 89% of the imaginal disc value, and the values for two other filters hybridized with the 5′ *Ubx* probe are 100% and 103%. These filters were subsequently probed with the histone probe, and they showed under-representation of the histone sequences in both salivary gland and fat body DNA. Thus, one set of sequences (histone genes) that is under-represented in salivary glands is under-represented in at least one other polytene tissue, the fat body, but another sequence (5′ *Ubx*) that is under-represented in salivary glands is not under-represented in this second polytene tissue.

Discussion

Organization and transcription pattern of these under-represented sequences

We have examined specific DNA sequences located in three different constrictions in the euchromatic arms of salivary gland polytene chromosomes *X*, *2* and *3*. In each case the sequences are under-represented in salivary gland DNA. We assume that this under-representation is due to the failure of these sequences to undergo as many rounds of replication as most of the sequences in euchromatin. In the case of 5′ *Ubx* sequences, under-replication does not occur in fat body DNA. Richards (1980) analyzed fat body chromosomes in *D. melanogaster* and found most of the bands corresponded to bands in salivary gland chromosomes. A few regions remained difficult to correlate. One of these regions was 89 B through 89 E, where the fat body chromosomes appear to contain more bands and the region does not contain the constriction apparent in salivary gland chromosomes. Rather, there is in fat body chromosomes a constriction distal to 89 E. The constriction displacement strenghtens the correlation between under-replication and chromosome morphology. A stronger conclusion would however require in situ hybridization of the 5′ *Ubx* probe to fat body chromosomes.

Does the morphological difference between salivary gland and fat body chromosomes reflect a functional difference in the 5′ *Ubx* site in the two tissues? Akam (1983) used the 5′ *Ubx* probe to detect the presence of homologous transcripts by in situ hybridization to RNA in sections of third instar larvae. He found no hybridization above background in salivary gland tissue, indicating that this gene is not transcribed in salivary glands. In fat body tissue, a small percentage of the nuclei was labeled (<10%), suggesting that *Ubx* might be involved in transcription in fat body or fat body-associated cells at some time during the larval period.

Thus, from the *Ubx* example, it could be argued that under-replication reflects transcriptional inactivity. In general, however, there is little evidence for a strong correlation between under-replication and lack of transcriptional activity in the sequences we have examined. Transcription in the 11 A and histone regions has not been investigated directly, to the best of our knowledge, but we expect the latter to be active, in contrast to the apparent inactivity of the *Ubx* region. Salivary gland DNA is complexed with

histone proteins (Cohen and Gotchel 1971; Holmgren et a 1985), and transcription of histone genes is coordinate with DNA synthesis in *Drosophila* as it is in other organism (Anderson and Lengyel 1980). Thus, there is no obviou correlation between transcriptional activity and under-repl cation of sequences in salivary gland chromosomes.

The three probes used hybridized in situ to a singl chromosomal site in each case, indicating that they do n contain dispersed repetitive sequences. The histone gene are known to be repeated about 100-fold and clustered the 39 DE locus (Lifton et al. 1978; Pardue et al. 1977 The 11 A probe appears to contain a repetitive elemen and since this probe hybridizes in situ only to the 11 region, we infer that 11 A contains a small cluster of r peated sequences. Under our hybridization conditions, th 5′ *Ubx* probe appeared not to contain repeated sequences compared with the histone and 11 A probes, a longer expo sure was required to obtain an adequate signal, even takin into account the specific activity and size of the probe Ananiev et al. (1978) have reported the presence of a repeti tive element, Dm225, at 89EF, but micrographs of in sit hybridization of Dm225 show that it hybridizes to man chromosomal sites, and when the 89 EF region is stretched the Dm225 probe hybridizes to the region adjacent to th constriction, rather than to the constriction itself (Ananie et al. 1978, Figs. 1, 3d). Because of these differences in th pattern of in situ hybridization, we infer that Dm225 se quences are not present in our probe, in the constriction or in ectopic fibers. Our three probes are obviously dissimi lar in structure, in that they do not all contain repeated sequences.

Temporal and tissue-specific pattern of DNA replication

We suspect that these under-represented sequences are de layed, rather than completely blocked, in replication. It ha been shown by quantitative in situ hybridization that two sequences under-represented in salivary glands (histone and ribosomal RNA genes) appear to participate fully ir the last two to three rounds of replication in these cells (Hammond and Laird 1985). Even a severely under-repre sented sequence, the 1.705 gm/cm^3 satellite, undergoes al most a complete doubling in abundance during the final endoreplicative steps. This implies that the inhibition of replication that leads to the under-representation of these sequences occurs fairly early in endoreplication, and that the inhibition can be relieved at a subsequent stage. For example, the under-representation of histone sequences in both salivary gland and fat body polytene DNA could be accounted for by the failure of these sequences to partici pate in two early rounds of replication, with a normal or nearly normal degree of DNA synthesis in subsequent rounds. The 5′ *Ubx* probe is about fourfold under-repre sented in salivary gland DNA. This indicates that these sequences have missed two rounds of DNA replication in these chromosomes. The fact that these sequences are not under-represented in the fat body implies that even if the sequences examined in this study possess some similar prop erty responsible for their under-replication, for example, unusual replication or termination sites, the sensitivity or efficiency of these sites must vary among the sequences and in different tissues.

We suggest that the under-representation is a conse quence of tissue-specific changes in the efficiencies of repli-

cation origins or termination sites in these polytene tissues. In order to determine which sequences are responsible for under-representation and the presence of constrictions in polytene chromosomes, sequences could be inserted by P-element transformation (Rubin and Spradling 1982) into ectopic chromosome sites and tested for under-representation and presence in novel constrictions at these sites. These experiments could also indicate whether the under-represented sequences we have detected are necessary and sufficient for induction of under-representation and constrictions at new chromosome sites, or if flanking sequences are required to produce these effects.

The sequences examined are located in intercalary heterochromatin

In addition to their under-representation and location in constrictions in salivary glands, the three sequences examined share the property of ectopic pairing with non-homologous chromosome sites (Pardue et al. 1977; Lifschytz 1983; Figs. 3c and 5e of this paper). This tendency to pair with other sites was one of the characteristics noted by Kaufmann (1939), who first used the term intercalary heterochromatin to describe sites that, although located in the euchromatic arms of polytene chromosomes, had some properties of the centromeric heterochromatin. Since that time, a number of studies have attempted to define intercalary heterochromatin in terms of some common function or structural organization (for discussion see Zhimulev et al. 1982; Bolshakov et al. 1985). Some of the proposed correlates with intercalary heterochromatin are weak points (constrictions or regions easily broken when salivary glands are prepared for light microscopy), late replication in polytene chromosomes, ectopic pairing, chromosome rearrangement breakpoints, and the presence of repeated sequences, (either clusters of genes repeated at the site, or moderately repeated, dispersed sequences).

The sequences that we have studied show some but not all of these features. As described above, all three sequences are located in constrictions and engage in ectopic pairing. They reside within regions of late replication in salivary gland chromosomes (Arcos-Teran 1972; Zhimulev et al. 1982). Chromosome rearrangement breakpoints, originating in meiotic cells, are frequent in the 11A region (Kaufmann 1939; Mukhina et al. 1981, cited by Zhimulev et al. 1982), but we do not yet know if these breakpoints occur precisely within the under-represented sequences we have studied. Chromosome rearrangement breaks are not a prominent feature of either the 39DE or 89E locus (Mukhina et al. 1981; Zhimulev et al. 1982). The histone and 11A sequences are repeated, but the 5' *Ubx* sequences are not. All three probes do, however, hybridize to constrictions and ectopic fibers and are therefore reasonably classifiable as intercalary heterochromatic DNA.

Does under-replication explain the properties of intercalary heterochromatin?

In this study and in previous studies of possible over- and under-replication of DNA sequences in the euchromatic arms of *Drosophila* salivary gland chromosomes (Lifschytz 1983; Spierer and Spierer 1984), sequences located in regions considered to be intercalary heterochromatin were found to be under-replicated. Is under-replication a common property of intercalary heterochromatin regions, or are there several different classes of intercalary heterochromatin, with different underlying bases of structural organization? One chromosomal region, 56F, the location of 5S genes, may be an example of a class different from the intercalary heterochromatin sites that we have found to be under-replicated. The 56F region displays many of the features of intercalary heterochromatin, including late replication and ectopic pairing, but does not show weak point behavior or major constrictions (Bolshakov et al. 1985), and the 5S genes are apparently not under-represented in salivary gland DNA (Szabo et al. 1977). It has not been demonstrated, however, that 5S DNA is included in the ectopic fibers at 56F, and this region contains a number of other kinds of sequences (Zhimulev et al. 1982), which might be under-replicated, responsible for heterochromatic properties, or both. We note that under-replication is only an extreme form of late replication (see below).

Not all features associated with intercalary heterochromatin are manifested together in regions generally described as intercalary heterochromatin regions (see above; Zhimulev et al. 1982; Bolshakov et al. 1985). Of the properties mentioned above, those that show the most frequent correlation, according to Bolshakov et al. (1985) are weak point behavior, ectopic pairing, late replication, and hybridization with cRNA (putatively repetitive sequences transcribed in *Drosophila* tissue culture cells). How might these features be associated with under-replication? (i) *Late replication* is considered to be a property of heterochromatin in general (Lima-de-Faria 1959; Brown 1966). It has been proposed that DNA synthesis in polytene chromosomes can be described as being under "relaxed" control: it is not required that all sequences replicate during one round of replication, before the next round can begin (Laird et al. 1987). Under the conditions of relaxed DNA synthesis in polytene chromosomes, the late replicating sequences, especially if they are relatively condensed, would be expected to be most likely to miss a round or two of endoreplication, resulting in under-representation. (ii) *Weak points* are expected for regions of under-replication, where failure of some strands to replicate results in a reduction of the number of chromatin strands at the region (Darlington and La Cour 1940). Such under-replicated sites would be weak points because of the greater force per chromatin strand, compared with the force per strand in fully replicated regions. (iii) *Ectopic fibers*: Ashburner (1980) has pointed out that regions of under-replication would contain multiple replication forks and has suggested that ectopic pairing fibers originate from branch migration of single strands at "frozen" replication forks in these under-replicated regions. Our study of three specific sequences confirms the predicted correlation of under-replication and ectopic fibers. In situ hybridization with the three probes has indicated that the sequences are located in ectopic fibers. There was no hybridization of the probes in those chromosomal sites to which the ectopic fibers were attached, suggesting that there was no major sequence homology between the regions. Cytological studies of ectopic pairing, however, indicate that ectopic pairing is not random, and that some sites pair frequently, implying a limited homology between paired sites (Slizynski 1945; Kaufmann and Iddles 1963). It is possible that limited homology exists between the sequences contained in our probes and the sites to which the ectopic fibers appear to attach, but it cannot be ruled out that other sequences in the ectopic fibers are responsible for the pairing behavior,

and the probe sequences are passively carried along. Thus, sequences contained in the cRNA that is reported to hybridize to intercalary regions (Gvozdev et al. 1980; Bolshakov et al. 1985), would also be candidates for a role in this pairing behavior. Alternatively, the observation that ectopic pairing occurs most frequently between sites located relatively close together on the same chromosome (Zhimulev et al. 1982) suggests that physical proximity of single-stranded DNA regions in the nucleus may be more important than homology for the frequency of ectopic pairing. There may be a single-strand binding protein, for example, sufficient to hold together two single-stranded DNA molecules, even in the absence of DNA sequence homology.

In conclusion, we have confirmed one component of the hypothesis that the morphology of polytene chromosomes is correlated with the local level of polyteny (Laird 1980). In addition, our results provide support for the conclusion that two of the major properties of intercalary heterochromatin – constrictions or weak points, and ectopic pairing fibers – are consequences of the third major property, late replication. For some sites of intercalary heterochromatin, for example, 11A, this late replication may also occur in meiotic cells, leading to increased frequency of meiotic chromosome rearrangements. Thus, many intercalary heterochromatin sequences are likely to be normal euchromatic sequences that have become, in some cells, late or delayed in their pattern of DNA replication. We retain the term intercalary heterochromatin for historical reasons, but it should be understood that many of these sequences may not be heterochromatic in any sense other than their pattern of DNA replication.

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