Molecular and Cytogenetic Analysis of the Heterochromatin-Euchromatin Junction Region of the *Drosophila melanogaster X* Chromosome Using Cloned DNA Sequences

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ABSTRACT

We have used three cloned DNA sequences consisting of (1) part of the *suppressor of forked* transcription unit, (2) a cloned 359-bp satellite, and (3), a type I ribosomal insertion, to examine the structure of the base of the *X* chromosome of *Drosophila melanogaster* where different chromatin types are found in juxtaposition. A DNA probe from the *suppressor of forked* locus hybridizes exclusively to the very proximal polytenized part of division 20, which forms part of the β-heterochromatin of the chromocenter. The cloned 359-bp satellite sequence, which derives from the proximal mitotic heterochromatin between the centromere and the ribosomal genes, hybridizes to the under replicated α-heterochromatin of the chromocenter. The type I insertion sequence, which has major locations in the ribosomal genes and in the distal mitotic heterochromatin of the *X* chromosome, hybridizes as expected to the nucleolus but does not hybridize to the β-heterochromatic division 20 of the polytene *X* chromosome. Our molecular data reveal that the *suppressor of forked* locus, which on cytogenetic grounds is the most proximal ordinary gene on the *X* chromosome, is very close to the junction of the polytenized and non-polytenized region of the *X* chromosome. The data have implications for the structure of β-heterochromatin–α-heterochromatin junction zones in both mitotic and polytene chromosomes, and are discussed with reference to models of chromosome structure.

THE structural changes that ensue when a dipteran mitotic chromosome is converted to a polytene one have been the subject of considerable research (HEITZ 1928, 1929, 1933, 1934; FUJII 1936, 1942; COOPER 1959; RUDKIN 1969; WOLF 1970; GALL 1973; LIFSCHYTZ 1983; ASHBURNER 1980; SPIERER and SPIERER 1984; DICKSON, BOYD and LAIRD 1971; LAIRD *et al.* 1974, LAIRD 1980; HAMMOND and LAIRD 1985; MIKLOS and COTSELL 1990). It is generally accepted that most of the euchromatic portion of a mitotic chromosome of *Drosophila melanogaster* goes through ten rounds of replication to form the familiar banded polytene structures seen for example in the nuclei of larval salivary glands (BRIDGES 1938; LEFEVRE 1976). The constitutively heterochromatic regions of mitotic chromosomes on the other hand replicate little, or not at all in polytene chromosomes (GALL, COHEN and POLAN 1971; GALL 1973; LAKHOTIA and JACOB 1974; ENDOW and GALL 1975; HOLMQUIST 1975; DICKSON, BOYD and LAIRD 1971; LAIRD *et al.* 1974; HAMMOND and LAIRD 1985).

We dedicate this paper to our colleague GEORGE LEFEVRE. His untimely death has deprived the scientific community of a brilliant Drosophila cytogeneticist. In his quiet gentlemanly manner he labored long and unsung to provide those precious hard won foundations on which so many biologists can now build further.

The cytogenetic characteristics of the junction zones between mitotic heterochromatin and mitotic euchromatin have remained controversial (GALL 1973; SCHALET and LEFEVRE 1973, 1976; LIFSCHYTZ 1978) and the molecular properties of such zones are almost totally unknown in any eukaryote. The most studied junction region has been that of the *D. melanogaster X* chromosome. This chromosome contains about 40 megabases of DNA and is partitioned into two cytologically distinct halves in diploid cells. The distal euchromatic half of the mitotic *X* chromosome contains approximately 1000 lethal genetic complementation groups. Its proximal mitotic half, termed constitutive heterochromatin, consists largely of repetitive DNA sequences (Figure 1a) (reviewed in BRUTLAG 1980; MIKLOS 1985; JOHN and MIKLOS 1979, 1988). This heterochromatic half also contains the 18S and 28S ribosomal genes and some type I insertion sequences (KIDD and GLOVER 1980; HILLIKER, APPELS and SCHALET 1980; HILLIKER and APPELS 1982).

The polytene *X* chromosome of larval salivary gland

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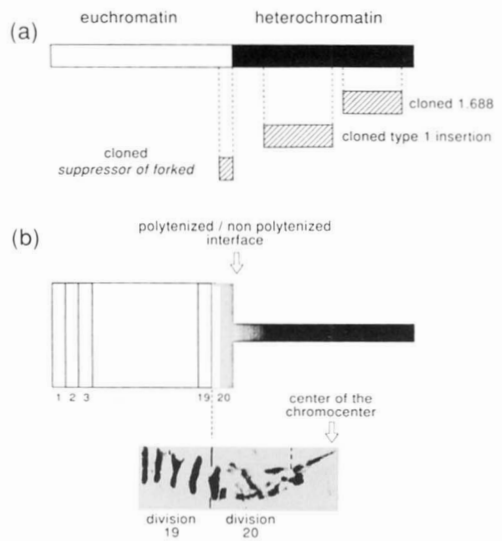

FIGURE 1.—Characteristics of mitotic and polytene chromosomes: (a) cytological domains (euchromatin, white; heterochromatin, black) and locations of some cloned DNA sequences (stippled) determined by *in situ* hybridizations (HILLIKER and APPELS 1982); (b) characteristics of the polytenized/nonpolytenized chromosomal regions: cytological appearance of the well banded polytene division 19, the poorly banded β-heterochromatic division 20, and the thin attenuated chromocentral strands joining the base of the chromosome to the center of the chromocenter (LEFEVRE 1976).

nuclei is seen in its banded euchromatic state for 19 of its polytene divisions, whereas division 20 has a poorly banded appearance (Figure 1b) (BRIDGES 1938; LEFEVRE, 1976). Division 20 can form part of the chromocenter and is referred to as being β-heterochromatic, a term introduced by HEITZ (1934). In describing the polytene chromosomes of dipteran larvae, HEITZ (1934) distinguished between the compact deeply staining chromocentral region, which he termed α-heterochromatin, and the granular and faintly staining loosely compacted chromocentral region which he termed β-heterochromatin. He suggested that the α-heterochromatin corresponded to the bulk of the constitutive heterochromatin seen in mitotic chromosomes.

The use of the word "heterochromatin," a cytological description, progressively became less exact as it accumulated a host of structural and functional properties (reviewed in COOPER 1959; HILLIKER, APPELS and SCHALET 1980; MIKLOS 1985; JOHN and MIKLOS 1979, 1988; MIKLOS and COTSELL 1990). The relationship between constitutive heterochromatin in mitotic chromosomes, and α- and β-heterochromatin in polytene chromosomes is still incompletely understood. There remains a blurred image of whether all heterochromatin is inert, whether it contains ordinary genes, or whether the effects that are ascribed to it are due to an unusual molecular architecture or to the activity of peculiar genes sequestered within it. In particular, the β-heterochromatin of the polytene *X* chromosome is commonly thought to be part of the mitotic heterochromatin.

SCHALET and LEFEVRE (1973, 1976) and LEFEVRE (1981) helped to clarify such issues at the cytogenetic level by examining the genic content of the heterochromatin-euchromatin junction region of the *D. melanogaster* mitotic *X* chromosome and comparing it to polytene divisions 19 and 20. From their cytogenetic analyses they concluded that; (1) most of division 20 is populated with ordinary sex-linked genes and their density in subdivisions 20A-D is no different from divisions 1 through 19, (2) the heterochromatin-euchromatin junction of the mitotic *X* chromosome is seen in the polytene *X* chromosome at or after subdivision 20D, and (3) the poorly banded, (or β-heterochromatic) 20A-D region in polytene chromosomes does not correspond to the proximal mitotic heterochromatin.

Following the detailed genetic analyses of LIFSCHYTZ and FALK (1968, 1969); LIFSCHYTZ (1971, 1975) and LIFSCHYTZ and YAKOBOVITZ (1978), it was suggested by LIFSCHYTZ (1978) that there is no single heterochromatin-euchromatin discontinuity, and that the base of the *X* chromosome is composed of heterochromatic segments of different lengths alternating with euchromatic segments. He suggested that "heterochromatic segments with various breakability potentials flank small groups of genes (or every gene) in sections 19 and 20."

Molecular cloning techniques now enable a number of previous uncertainties to be reexamined. First, subdivisions 19E, 19F and division 20, have been microdissected and microcloned (MIKLOS *et al.* 1988), and the gross molecular architecture of this area has begun to be determined by chromosomal walking within these two divisions (MIKLOS *et al.* 1984; DAVIES, PIRROTTA and MIKLOS 1987; DE COUET *et al.* 1987; YAMAMOTO *et al.*, 1987; HEALY, RUSSELL and MIKLOS 1988). Second, germ line clone analysis of the 26 contiguous genetic complementation groups in divisions 19 and 20 (PERRIMON, SMOUSE and MIKLOS 1989) has allowed a detailed genetic comparison to be made between the types of genes in euchromatic versus β-heterochromatic regions. Third, the most proximal ordinary gene at the base of the *X* chromosome, the *suppressor of forked* locus, *su(f)* (LINDSLEY and GRELL 1968; SCHALET and LEFEVRE 1973, 1976), has been cloned by transposon tagging using a *P*-element insertion mutation (A. MITCHELSON and K. O'HARE, manuscript in preparation).

By using a DNA sequence from the *suppressor of forked* transcription unit we have determined its cytological location in polytene chromosomes. Furthermore, we have probed polytene chromosomes with the cloned 359-bp repetitive DNA sequence (BRUTLAG 1980), since this is a marker for the proximal

mitotic heterochromatin between the centromere and the ribosomal genes (HILLIKER and APPELS 1982). We have used a cloned type I insertion probe (KIDD and GLOVER 1980), which is a member of a repetitive DNA family, known to be located both within the ribosomal genes as well as within the distal mitotic heterochromatin of the *X* chromosome (HILLIKER and APPELS 1982). In larval neuroblasts we have also cytologically examined the mitotic morphology of a particular deleted *X* chromosome which is known to be deficient for the *suppressor of forked* locus (*deficiency DCB1-35b*).

Our molecular data reveal that the *suppressor of forked* locus is very close to the border between the polytenized and nonpolytenized regions of the *D. melanogaster X* chromosome. The data are in total agreement with those of SCHALET and LEFEVRE (1973, 1976) who positioned this locus exclusively on cytogenetic grounds.

MATERIALS AND METHODS

Polytene chromosome squashes and biotinylated probe syntheses: Salivary gland chromosome squashes were made in 45% (vol/vol) acetic acid and were dehydrated in 98% (vol/vol) ethanol for 2–4 h. The preparations were air dried at room temperature and placed in 2 × SSC at 70° for 30 min and then treated with 0.01 N NaOH for 3 min (GREEN, YAMAMOTO and MIKLOS 1987).

Biotinylated probes were synthesized by standard nick translation methods using biotin-11-dUTP from Bethesda Research Laboratories (MIKLOS *et al.* 1988). The biotinylated probes were used at a concentration of 1 μg/400 μl of hybridization solution. DNA hybridizations were performed at 37° in a hybridization mix containing 50% (vol/vol) formamide; 4 × SSC, 1 × Denhardt's solution, 10% (wt/vol) dextran sulfate and sheared calf thymus DNA at 250 μg/ml. The slides were twice washed in 2 × SSC at 60° followed by a wash at room temperature in 1 × SSC. The washed slides were then treated with the BRL DNA detection system using alkaline phosphatase to visualize the hybridization sites. The fidelity and sensitivity of the *in situ* hybridizations was also checked using cloned probes from the *white* locus as controls, as well as cloned probes from various loci at the base of the *X* chromosome.

DNA probes: The *suppressor of forked* probe was a DNA fragment from the sequences flanking the *P* element insertion in the mutation *suppressor of forked*^{MS252} (A. MITCHELSON and K. O'HARE, in preparation). This *Bam*HI-*Xho*I fragment contains part of the *suppressor of forked* transcription unit (Figure 2). In Southern blots of wild-type (Canton S) DNA, this single copy probe detects a 2.1-kb *Hin*dIII fragment (data not shown). As a control for an *X*-linked single copy DNA probe we used a subclone of coordinates +6.8 to +9.0 of the *white* locus (LEVIS, BINGHAM and RUBIN 1982). This plasmid, pm11.3, detects a 5.6-kb *Hin*dIII fragment in Southern blots of Canton S DNA. The type I insertion sequence probe was the plasmid pC2 (KIDD and GLOVER 1980) and the 359-bp satellite probe was the plasmid pDm23 (CARLSON and BRUTLAG 1977).

DNA preparation and Southern blot hybridization conditions: Larval salivary glands and imaginal discs were hand dissected from third instar Canton-S larvae in 0.7% NaCl. The tissue was ground with a pestle in an Eppendorf microfuge tube in lysis buffer (30 mM Tris-HCl (pH 8.0), 100 mM EDTA). Proteinase K was added to 0.1 mg/ml and Sarkosyl to 1%. After incubation at 42° for 2 h, the mixture was deproteinized by extraction with phenol, phenol/chloroform (1:1) and chloroform. The DNA was precipitated with ethanol, recovered by centrifugation and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The DNA blot, synthesis of probe, hybridization and washing were by standard procedures (SAMBROOK, FRITSCH and MANIATIS 1989). Size markers were *Bst*EII fragments of lambda DNA.

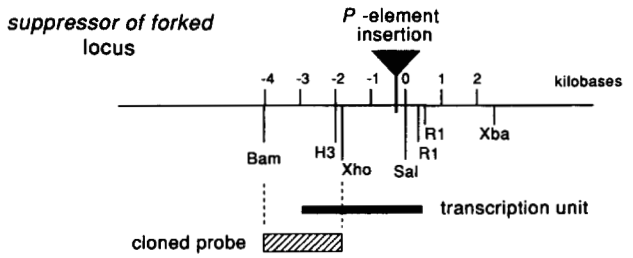

FIGURE 2.—DNA map of the *suppressor of forked* [*su(f)*] region. The approximate extent of the *su(f)* transcript is shown and the *Bam*HI-*Xho*I restriction fragment used for *in situ* hybridizations and DNA blotting is as indicated (A. MITCHELSON and K. O'HARE, unpublished results).

Mitotic chromosome preparations: These were performed essentially according to previous methods (MATSUDA, IMAI and TOBARI 1983). Third instar larval brains were dissected in 0.7% NaCl and transferred to 1.0% sodium citrate, 0.005% colchicine for 5 to 7 min. The tissue was then fixed in ethanol/glacial acetic acid/distilled water (3/3/4, vol/vol/vol) and minced with forceps. The slide was briefly washed in ethanol/glacial acetic acid 1:1 (vol/vol). One further drop of glacial acetic acid was added and the slide was allowed to air dry at room temperature. The chromosomes were stained with Giemsa (Merck) in 0.01 M sodium phosphate buffer (pH 6.8).

RESULTS

The *suppressor of forked* locus was cloned using a *P* element-mediated mutagenesis screen which yielded a *suppressor of forked* mutation denoted MS252 (A. MITCHELSON and K. O'HARE, in preparation). The relevant genomic region was recovered using *P* element DNA as a probe to screen a lambda bacteriophage library. Furthermore, a large DNA fragment which includes the entire *suppressor of forked* transcription unit has been reintroduced into the *D. melanogaster* genome by *P* element-mediated germ line transformation and has been found to rescue the *suppressor of forked* mutant phenotype (M. SIMONELIG and K. O'HARE, unpublished data). The mutant identification and molecular cloning of the *suppressor of forked* locus itself is the subject of a separate communication (A. MITCHELSON and K. O'HARE, manuscript in preparation). The molecular orientation of the 50-kb chromosomal walk within which the *suppressor of forked* transcript is found, has been determined relative to the centromere using chromosomal rearrangement breakpoints (K. O'HARE, L. E. KELLY and G. L. G. MIKLOS, unpublished results). The only major issue

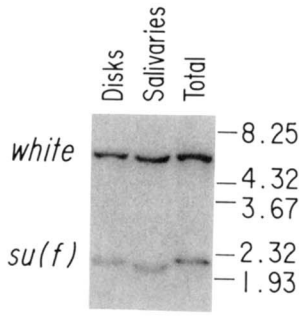

FIGURE 3.—Southern blotting experiments involving genomic DNAs from imaginal disks, larval salivary glands and whole adult flies. The two probes used in the hybridization are single copy DNA sequences from the *white* and *suppressor of forked* loci. The sizes of the marker fragments are in kilobases.

germane to the present work is that we have used a *bona fide suppressor of forked* probe for our *in situ* and Southern hybridization analyses (Figure 2).

Southern blotting experiments–Larval salivary gland DNA *vs.* imaginal disk DNA: On cytogenetic grounds (SCHALET and LEFEVRE 1973, 1976) positioned the *suppressor of forked* locus to the very proximal β-heterochromatic region of division 20 where the exact level of polytenization is unknown. We therefore firstly hybridized the *suppressor of forked* probe (the *Bam*HI-*Xho*I fragment shown in Figure 2) to restriction endonuclease cleaved genomic DNA from imaginal disks (which are largely diploid) as well as to DNA from larval salivary glands, (the replicative status of which is not known for the area under study). We also simultaneously used as a control, a single copy DNA probe from the *white* locus in euchromatic division 3.

The results of this experiment are shown in Figure 3. Hybridization is found to a 5.6-kb genomic fragment due to the *white* probe, and to a 2.1-kb fragment due to the *suppressor of forked* probe. Most importantly, it can be seen that the relative intensities of the 2.1-kb fragments are very similar for DNA derived either from diploid or from polytene sources. We have quantitated the amount of hybridizations to the *suppressor of forked* fragment in each lane relative to the amount of hybridization to the *white* fragment in the same lane. We find that the relative hybridizations of the *suppressor of forked* fragments in imaginal disks versus salivary glands are in the ratio of 10:8. Hence in the polytene condition, the *suppressor of forked* locus is about 80 per cent replicated within the sensitivity of our densitometric measurements. If the *suppressor of forked* locus were to be as underreplicated in polytene cells as the satellite DNA sequences in α-heterochromatin, then the signal for the 2.1-kb fragment in the "polytene" lane of this figure ought to be at least 100-fold reduced in its intensity relative to the "diploid" lane. Such is not the case, and it is clear that the *suppressor of forked* locus undergoes significant replication in larval salivary gland cells within the limits of these hybridization experiments. Furthermore, the hybridization intensity of the single 2.1-kb fragment from imaginal disk DNA indicates that it is likely to be single copy, or at least at low copy number within the genome.

In situ hybridizations: 1. The *suppressor of forked* probe: we utilized the same probe as above for *in situ* hybridizations to polytene chromosomes of larval salivary gland cells and the cytological results are seen in Figure 4. First, there is an intense staining reaction in the very proximal region of the *X* chromosome (Figure 4A) and nowhere else in the genome (data not shown). This signal is as strong as that achieved by other single copy containing cloned DNA inserts that we have routinely used as controls from elsewhere in the euchromatin of the *X* chromosome. The relative strengths of such *in situ* signals, albeit estimated by eye, are nevertheless concordant with the Southern blotting and hybridization data which we have just described, in revealing that the *suppressor of forked* locus is not significantly underreplicated in larval salivary gland nuclei.

When the *X* chromosome is stretched and undergoes distortion, the *suppressor of forked* area appears elongated, but a clear unhybridized stretch of chromosome is seen between the *suppressor of forked* signal and the middle of the chromocenter (arrowed in Figure 4B). Note that the base of the *X* chromosome has not broken away from the middle of the chromocenter, but is still attached to it via a slender chromosomal thread. Furthermore, the fact that the *suppressor of forked* signal in this preparation appears to extend throughout division 20 is a peculiarity of this region. SCHALET and LEFEVRE (1973) found, for example, that 20B-D is often oriented at right angles to division 19. It may either be that ectopic pairing of the very proximal *suppressor of forked* region with other areas in the distal part of division 20 gives rise to the observed *in situ* result, or else that the *suppressor of forked* region has been unusually distorted during cytological preparation.

It is clear from Figure 4A that the *suppressor of forked* probe is hybridizing to the proximal portion of division 20, an area denoted by SCHALET and LEFEVRE (1973, 1976) and LEFEVRE (1976) as region 20EF. This very proximal polytenized area is then connected to the middle of the chromocenter by a thin strand which does not become labelled with the *suppressor of forked* probe (Figure 4B).

2. The satellite probe: using a probe made from the cloned 359 bp satellite DNA sequence, which is found only in the proximal mitotic *X* heterochromatin (Figure 1a) (HILLIKER and APPELS 1982), the hybridization signal from this particular highly repetitive DNA is now seen as a small dot in the middle of the

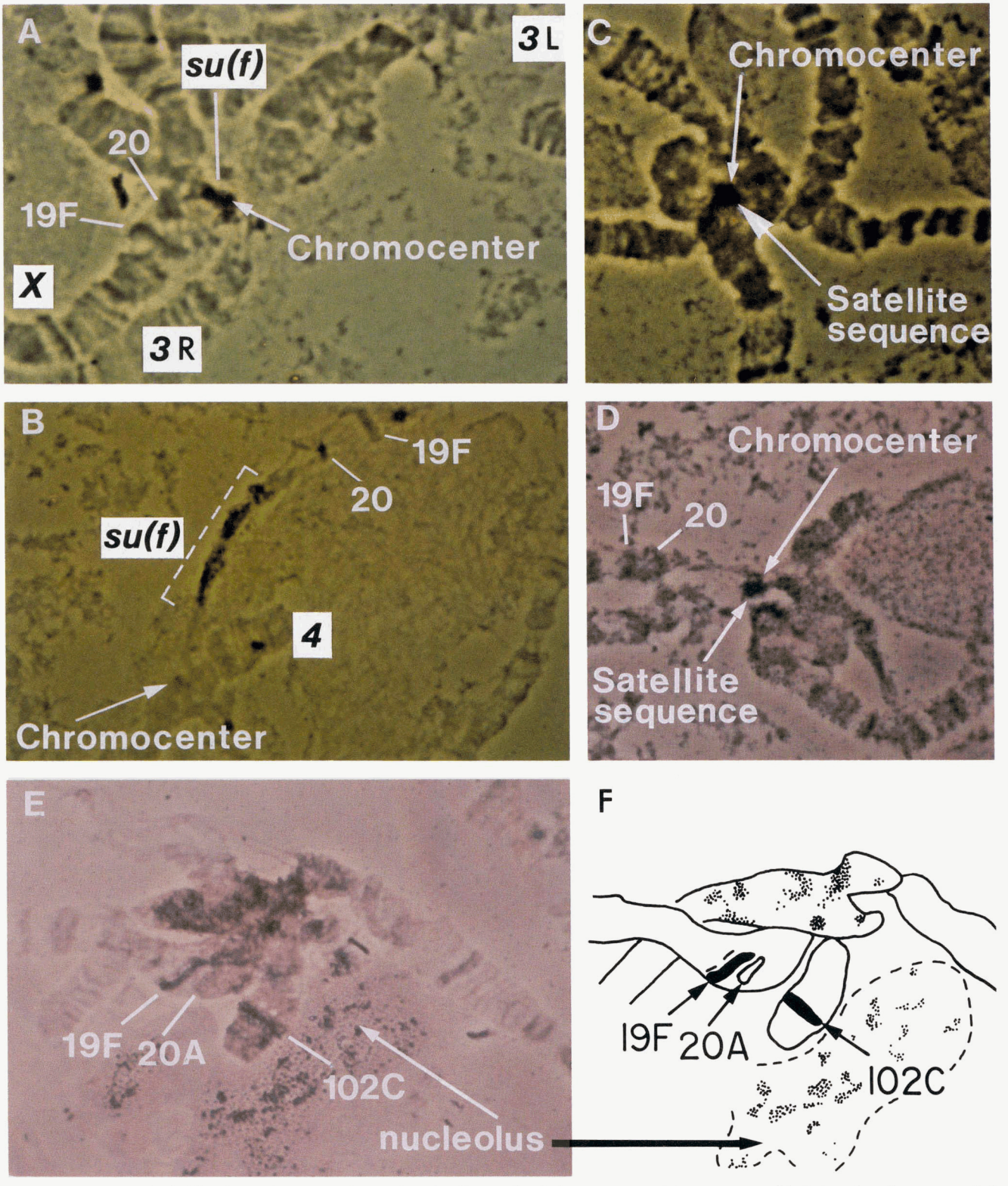

FIGURE 4.—*In situ* hybridizations of DNA probes to larval salivary gland chromosomes; (A) *suppressor of forked* probe; the center of the chromocenter is arrowed as are the relevant landmarks at the base of the *X* chromosome. (B) *suppressor of forked* probe; division 20 is highly stretched and the center of the chromocenter is arrowed. Note the thin strand, free of signal, which connects the positively hybridizing part of division 20 to the center of the chromocenter. (C) The 359-bp satellite DNA probe. (D) The 359-bp satellite DNA probe; division 20 is highly stretched. The signal is found only over the α-heterochromatin which constitutes the middle of the chromocenter. (E) the type I insertion probe; signals are found over the nucleolus, subdivision 102C on chromosome *4*, at the boundary of subdivision 19E/19F and throughout the β-heterochromatic areas of some chromosome arms. (F) the type I insertion probe; explanatory drawing. No significant hybridization signals are found in division 20, subdivision 102C is strongly labelled as is the 19E/19F boundary.

chromocenter (Figure 4C). The signal does not intrude at all into the polytenized portion of division 20. When the base of the *X* chromosome is stretched, the hybridization signal from this cloned satellite sequence is exclusively restricted to the middle of the chromocenter (the so called α-heterochromatin, Figure 4D) and very occasionally to the proximal part of the thin attenuated strand seen in this figure.

3. The type I insertion sequence: The type I insertion sequence (KIDD and GLOVER 1980) is known to occur in the ribosomal gene array in the middle of the mitotic heterochromatin of the *X* chromosome, as well as in the distal mitotic heterochromatin (HILLIKER and APPELS 1982). It is also known to have a prominent location on chromosome *4* (PEACOCK *et al.* 1981). In polytene nuclei it is known to hybridize to the nucleolus, to subdivision 102C and to the bases of at least two autosomal arms (PEACOCK *et al.* 1981).

When we hybridize a type I insertion probe to polytene chromosomes, we find that it goes to the published locations: (1) the nucleolus (Figure 4E), (2) subdivision 102C on chromosome *4* (Figure 4E), (3) extensively throughout parts of the β-heterochromatin of some autosomes (Figure 4E) and, (4) the boundary between subdivisions 19E and 19F (Figure 4E) where we have previously found a small cluster of type I-like sequences near the *uncoordinated* locus (MIKLOS *et al.* 1984; 1988; HEALY, RUSSELL and MIKLOS 1988).

Our *in situ* results are shown in Figure 4E and its associated explanatory drawing Figure 4F. Division 20 is free of signal, whereas the 19E/19F boundary, which contains a cluster of type I-like insertion sequences is positive (arrowed). Subdivision 102C on chromosome *4* is also clearly labelled. Parts of the β-heterochromatic regions of at least two autosomal arms are also positive as has been noted previously (PEACOCK *et al.* 1981; their Figure 4).

These *in situ* results, in which the type I insertion sequence does not hybridize to division 20, but does hybridize to the 19E/19F boundary, are in excellent agreement with the predictions from the microdissection and microcloning data reported earlier (MIKLOS *et al.* 1988). In that study, the polytenized base of the *X* chromosome was serially microdissected to yield four mini-libraries; libraries 1 and 2 emanated from division 20, library 3 was from subdivision 20A and the proximal part of 19F, and library 4 came from 19E and the distal part of 19F (MIKLOS *et al.* 1988; G. L. G. MIKLOS and J. A. DAVIES, unpublished data).

It is also known from previous cytogenetic and molecular studies that the 19E/19F boundary contains a cluster of DNA sequences which strongly cross hybridize to a type I probe (MIKLOS *et al.* 1984; HEALY, RUSSELL and MIKLOS 1988). When our 4 microdissection libraries containing a total of 1200 clones were challenged with the type I insertion sequence, 11 strongly hybridizing clones were detected in library 4, none in library 3, one in library 2 and none in library 1. These findings are thus in agreement with our *in situ* data for the base of the *X* chromosome. Type I sequences are not found to any significant extent in the polytenized part of division 20, nor in libraries 1, 2 and 3 which were made from division 20. However, they conveniently provide a hybridization control at the 19E/19F boundary, and as expected, are found at the highest frequency in library 4 which was made from these subdivisions.

In summary, the *suppressor of forked* probe hybridizes to the most proximal polytenized part of the *X* chromosome. The satellite probe, which occupies nearly all the proximal mitotic heterochromatin proximal to the ribosomal genes (Figure 1a) labels the middle of the chromocenter (the α-heterochromatin). The type I insertion probe, which marks the ribosomal genes and some of the distal mitotic heterochromatin of the *X* chromosome, is not found in the polytenized portion of division 20 (the β-heterochromatin).

Mitotic cytology: In order to examine further the position of the actual heterochromatin-euchromatin border of mitotic chromosomes, we used a particular deficiency *DCB1-35b*, which deletes DNA from band 19E8 through the *suppressor of forked* locus inclusively (SCHALET and LEFEVRE 1976). This deletion removes all of subdivision 19F and division 20. If it were to encroach significantly into the mitotic heterochromatin, we may be able to detect this by analyzing mitotic chromosomes in neuroblast nuclei.

It is already known from the work of SCHALET and LEFEVRE (1973) that the *suppressor of forked* locus is unlikely to be in the very distal mitotic heterochromatin of the *X* chromosome. These authors examined two particular chromosomal rearrangements, termed y^{x5} and y^{x15} in both polytene and mitotic configurations and concluded that the *suppressor of forked* location was likely to be euchromatic. However these cytological descriptions were called into question by LIFSCHYTZ (1978) who pointed out that the cytological resolution achieved by these authors on these two rearrangements was insufficient to warrant the above conclusions. In order to reexamine this question we used the *DCB1-35b* deficiency chromosome and significantly improved the cytological resolution in mitotic chromosomes relative to that of SCHALET and LEFEVRE (1973).

We constructed *DCB1-35b/yw* heterozygous individuals and examined the mitotic heterochromatins of the two *X* chromosomes in the same neuroblast cell. The proximal mitotic heterochromatin of an *X* chromosome is that between the centromere and the constriction due to the nucleolus organizer. It consists of the so called blocks A and B of COOPER (1959). The

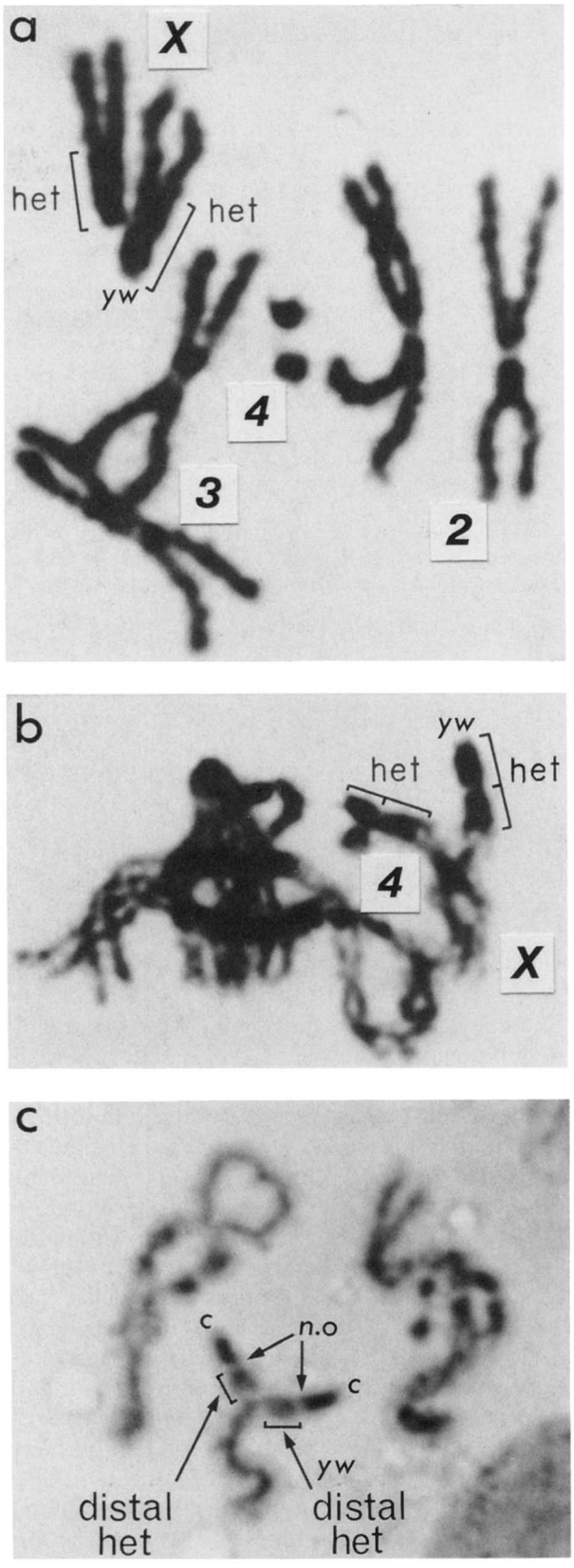

FIGURE 5.—Mitotic cytology of *DCB1-35b/yw* individuals. The *X* chromosomes and their associated euchromatic and heterochromatic regions are as indicated, as are autosomes *2, 3* and *4*; (a) late prophase; (b, c) early prophase. The proximal mitotic heterochromatin of the *yw* chromosome is longer than that of the *DCB1-35b* chromosome and the two chromosomes are thus distinguishable in the same cell (b). The constriction marking the nucleolus organizer (n.o) in the middle of the heterochromatin is as indicated. The distal heterochromatic regions of both chromosomes are shown in panel (c).

distal mitotic heterochromatin is that between the nucleous organizer and the euchromatin and it consists of the so called blocks C and D of COOPER (1959). We used this particular *yw* chromosome since it has more DNA in its proximal heterochromatic region between the centromere and the ribosomal genes than most chromosomes we have examined, and is thus readily distinguishable from its *DCB1-35b* homolog. By contrast, the size of the distal heterochromatin of the *yw* chromosome appears to be the same as that of the distal heterochromatin of other *X* chromosomes we have examined.

The complete karyotype of such heterozygous individuals is shown in Figure 5, a, b and c. In Figure 5b, it is clear that the *yw* chromosome is distinguished from its *DCB1-35b* homolog by the increased length of its proximal *X* heterochromatin. The constriction due to the nucleolus organizer between the proximal and distal parts of the *X* heterochromatin is seen in both *X* chromosomes. Examination of even earlier prophase stages in which the euchromatic regions of chromosomes are paired, but the heterochromatic ones are not (Figure 5c), reveals that the distal heterochromatic regions are largely intact in the *DCB1-35b* chromosome. The centromeres (c) and the nucleolus organizer (n.o) are as shown. This is the best resolution we have achieved using the light microscope and it is clear that most of the distal heterochromatin is present in the *DCB1-35b* chromosome.

We conclude that deficiency *DCB1-35b* does not extend significantly into the mitotic heterochromatin of the *X* chromosome. It is unlikely therefore that the *suppressor of forked* locus is located deep within the distal mitotic heterochromatin of the *X* chromosome.

DISCUSSION

Our results remove some of the uncertainties from interpretations of chromosome structure at the base of the *X* chromosome, and they are totally in agreement with the conclusions drawn by GALL, COHEN and POLAN (1971), and GALL (1973) concerning the basic rules of mitotic to polytene conversion. The data also bear out the postulates made five decades ago by HEITZ (1934) and the data of FUJII (1936, 1942) concerning the transition from mitotic to polytene chromosomes. Our molecular data also totally support the original conclusions of SCHALET and LEFEVRE (1973, 1976) based on cytogenetic analyses.

Mitotic, α- and β-heterochromatin in *D. virilis*: HEITZ (1928, 1929, 1933, 1934) clearly enunciated at the cytological level, the morphological concept of

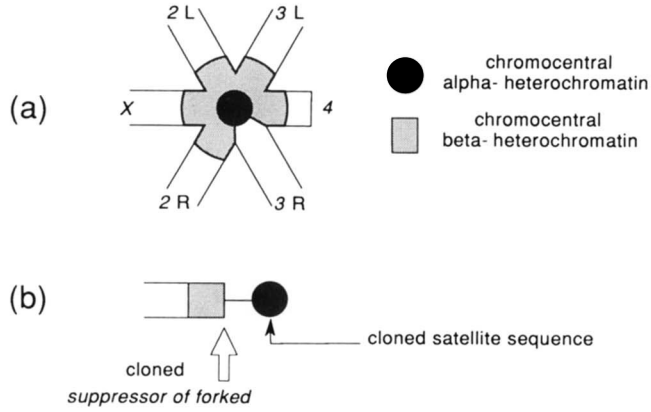

FIGURE 6.—Idealized representation of the chromatin structures at the bases of the various chromosome arms; (a) the three different chromatin types euchromatin, β-heterochromatin and α-heterochromatin are indicated. It should be noted that chromosome *4* is cytologically difficult to classify in terms of chromatin types. We have therefore only tentatively assigned a basal β-heterochromatic region to this chromosome. (b) hybridization sites in a stretched base of the *X* chromosome using the single copy and repetitive DNA probes.

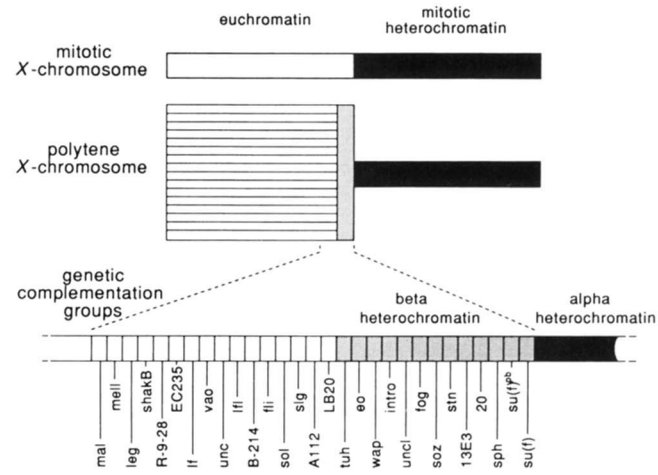

FIGURE 7.—Summary of the mitotic and polytene structures of the *X* chromosome and the positions of the genetic complementation groups (data from GALL 1973; SCHALET and LEFEVRE 1973, 1976; HILLIKER and APPELS 1982; MIKLOS *et al.* 1987, 1988; GREEN, YAMAMOTO and MIKLOS 1987; PERRIMON, SMOUSE and MIKLOS 1989; MIKLOS and COTSELL 1990).

heterochromatin. In particular, his definition on mitotic chromosomes concerned regions which retained a compact structure during most of the cell cycle. In polytene chromosomes, HEITZ (1934) suggested that there were two distinct chromocentral regions, a compact central α-heterochromatin, and a loosely compacted peripheral β-heterochromatin. In examining *Drosophila virilis*, he found that about half of each chromosome consisted of a large block of mitotic heterochromatin, but only some polytene chromosome arms had β-heterochromatin. He suggested therefore that the bulk of the mitotic heterochromatin corresponded to the α-heterochromatin seen in polytene nuclei.

The molecular data on *D. virilis* heterochromatin were provided by GALL, COHEN and POLAN (1971), GALL (1973), GALL and ATHERTON (1974) and HOLMQUIST (1975). These authors demonstrated that the major satellite DNAs which comprise over 40% of the genome in diploid tissues, and which are exclusively located in mitotic heterochromatin, are undetectable in buoyant density gradients of DNA from larval salivary glands. Furthermore, Satellite 1 hybridizes almost exclusively to the α-heterochromatin of polytene chromosomes and generalized "labeling of the β-heterochromatin is not seen even after long exposures." These authors concluded that mitotic heterochromatin in *D. virilis* either does not replicate or replicates very little during polytenization. Its residual amount is seen as the compact α-heterochromatin in the chromocenter of polytene chromosomes.

Mitotic, α- and β-heterochromatin in *D. melanogaster*: In *D. melanogaster* α-heterochromatin is not readily apparent in polytene chromosomes using the light microscope. Electron microscopic analyses, however, reveal two types of chromatin organization in the chromocenter (LAKHOTIA and JACOB 1974). First, there is a compact central block which is surrounded by a large diffuse granular area. EM autoradiography of the large granular chromocentral area shows it to be active in RNA synthesis at a level comparable to other euchromatic regions of the *X* chromosome, whereas the compact central block is completely inactive in RNA synthesis (LAKHOTIA and JACOB 1974). Clearly *D. melanogaster* has α- and β-heterochromatin, and their individual cytological characteristics reflect their transcriptional properties (MIKLOS and COTSELL 1990).

We believe that the summary in Figure 6 and 7 at present best approximates the cytogenetic and molecular organization of the base of the *X* chromosome. In a largely undistorted preparation, the mitotic heterochromatin of each *D. melanogaster* chromosome arm fuses to form the chromocentral α-heterochromatin (Figure 6a). The diffuse β-heterochromatin, on the other hand, is formed from chromosomal regions adjacent to the mitotic heterochromatin of each arm (except the right arm of chromosome *3*, which under normal conditions exhibits no β-heterochromatin). Chromosome *4* is cytologically ambiguous, and on the basis of some of its genetic behavior, as well as its molecular properties, gives evidence that it could be unusual in terms of its chromatin makeup (reviewed in MIKLOS and COTSELL 1990). At the cytological level, however, it exhibits no truly β-heterochromatic region (M. YAMAMOTO, unpublished results).

When the bases of the chromosome arms are stretched during some cytological preparations, the base of the *X* chromosome can appear as in Figure 6b. Thin strands join the α-heterochromatin to the poorly banded β-heterochromatin. The cloned repetitive sat-

ellite DNA sequence from the proximal mitotic heterochromatin of the *X* chromosome hybridizes to the chromocentral α-heterochromatin with occasionally some signal in the thin attenuated strand. The single copy cloned *suppressor of forked* DNA probe hybridizes exclusively to the very proximal polytenized part of the β-heterochromatin of the *X* chromosome. From both the Southern blotting and *in situ* hybridizations, it is clear that this *suppressor of forked* region is at least 80% polytenized. Thus the very proximal β-heterochromatic part of division 20 undergoes significant polytenization, even though it looks granular and is poorly banded. The situation may be analagous to that described by HENIKOFF (1981). He found that in a heterochromatic/euchromatic translocation adjacent to the heat shock genes in subdivision 87C, the region was nearly fully polytenized when a molecular probe was employed, but cytologically the area appeared diffuse and unbanded and its degree of polytenization was not readily apparent.

Alternatives to a single mitotic heterochromatin-euchromatin junction: LIFSCHYTZ (1978) originally suggested that a single discrete mitotic heterochromatin-euchromatin junction did not exist and that the base of the *X* chromosome is in fact composed of heterochromatic segments of different lengths which alternate with euchromatic segments. This hypothesis, however, is based on a data set of X-ray-induced exchanges, not on a cytological description of mitotic *X* chromosomes. LIFSCHYTZ (1978) found that there was a major hot spot for X-ray-induced breaks in subdivision 20A and argued that this was due to the presence of a large intercalary heterochromatic segment. His arguments are predicated on the assumption that susceptibility to X-ray-induced breakage is a characteristic of mitotic heterochromatin (wherever it is found along a chromosome) and hence the presence of mitotic heterochromatin can be inferred from regions which have high breakability potentials.

An alternative explanation for the distribution of X-ray-induced breaks is that some gene regions and their associated DNA landscapes are very large. It may well be, for example, that a long region of essentially single copy DNA may contain only a single lethal genetic complementation group. There is no reason why this should not be as much a hot spot for X-ray-induced breakage and reunion as an equally long region of repetitive DNA sequences. At the cytological level such a region could be euchromatic or heterochromatic. It may be significant that in mapping our single copy containing microclones obtained from microdissection of the base of the *X* chromosome (MIKLOS *et al.* 1988), we have found that subdivision 20A does contain a disproportionately large number of non cross hybridizing single copy containing microclones relative to subdivision 19F (G. L. G. MIKLOS and J. A. DAVIES, unpublished results). We already know from these data, as well as from chromosomal walks in this area, that the *extra-organs* region in subdivision 20A (Figure 7), which contains only a single lethal genetic complementation group, contains a very large amount of DNA relative to some of the single lethal genetic complementation groups in subdivision 19F. This may well account for subdivision 20A being a hot spot for X-ray-induced exchanges independently of its particular molecular makeup.

If division 20, or even just subdivision 20A is one in which the mitotic heterochromatin sputters out into euchromatin, producing small islands of mitotic heterochromatin between which a number of genes are sequestered, then we think that it needs to be demonstrated at the cytological level in mitotic chromosomes that this is indeed the case. It is not sufficient to infer the existence of a cytological chromatin type from X-ray-induced breakage probabilities. Owing to the limitations of the light microscope, the LIFSCHYTZ hypothesis is presently very difficult to disprove, based as it is on cytologically invisible "intercalary" heterochromatic blocks at the base of a mitotic chromosome. It is perhaps prudent to place it aside until either the molecular architecture of division 20 is better described, or the cytological resolution of the equivalent area in mitotic chromosomes is more finely analyzed.

Furthermore, the very existence of "intercalary" heterochromatin, which it should be remembered is based not on cytological appearance, but which has been inferred on the basis of properties such as susceptibility to X-ray-induced breakage and reunion, has been called into question. LAMB and LAIRD (1987) have shown that some "intercalary" heterochromatic regions are unlikely to be more than normal euchromatic sequences which have delayed DNA replication patterns.

We believe that it is more important to now know that the *suppressor of forked* locus is very close to the polytenized/nonpolytenized border of polytene chromosomes. Our *DCB1-35b* mitotic cytology, together with the data of SCHALET and LEFEVRE (1973, 1976) on the two rearranged chromosomes y^{x5} and y^{x15}, as well as our *suppressor of forked* molecular polytenization data, together indicate that at the cytological level the *suppressor of forked* locus is very close to the β-heterochromatin/α-heterochromatin junction of the *X* chromosome. Although there is still a possibility, because of the limitations of the light microscope, that the *suppressor of forked* locus lies within the very distal mitotic heterochromatin (part of COOPER's block D), this heterochromatic region would then have to undergo significant polytenization in order to conform to our data.

In addition, we have compared some of the clones obtained from the 50-kb *suppressor of forked* chromo-

somal walk (A. MITCHELSON, M. TUDOR and K. O'HARE, unpublished data) with clones from the microdissection of this region (MIKLOS *et al.* 1988). The chromosomal walk clones derive from a bacteriophage library made from diploid tissues, whereas the microdissection clones are from a single polytene *X* chromosome. Comparison of these two sets of clones has so far revealed no differences (K. O'HARE and G. L. G. MIKLOS, unpublished data). We therefore conclude that during the conversion from a mitotic to a polytene chromosome there are no gross DNA rearrangements (of the types found in immunoglobulin gene rejoining events for example) in this particular region.

While we still know very little about the molecular architecture of the β-heterochromatin of any chromosome, we do know that the β-heterochromatin of the *X* chromosome of *D. melanogaster* is unlikely to harbour significant amounts of the major satellite DNAs (MIKLOS *et al.* 1988). We have probed our microdissection libraries with the 9 different cloned satellite DNA sequences reported by LOHE and BRUTLAG (1986) and have not found significant cross hybridization to any of our 1200 clones. The caveat remains however, that none of these 9 simple sequence tracts may individually exist in lengths of less than 11 kb. If this were so, they could not have been cloned in the bacteriophage vector used in our microcloning experiments, as it only accepts inserts up to a maximum size of 11 kb (MIKLOS *et al.* 1988).

Division 20 does, however, contain a disproportionate amount of DNA sequences which cross hybridize to mobile elements when it is compared to subdivisions 19E and 19F (J. COTSELL, A. MITCHELSON, M. TUDOR, K. O'HARE and G. L. G. MIKLOS, unpublished results). It also contains other repetitive, nonsatellite DNA sequences (YOUNG *et al.* 1983; DONNELLY and KIEFER 1986, 1987; MIKLOS *et al.* 1988) some of which are overrepresented relative to subdivisions 19E and 19F. These may be previously undescribed active transposable elements, inactivated transposable elements, clustered-scrambled repeats (WENSINK, TABATA and PACHL 1979), or moderately repetitive DNA sequences of a more sedentary nature. It should also be noted that the β-heterochromatic regions of most chromosome arms, and chromosome *4* in particular, are deficient in long tracts of mono- and dinucleotide sequences such as $(C)_n$ and $(CA)_n$ (PARDUE *et al.* 1987; LOWENHAUPT, RICH and PARDUE 1989).

Why division 20, and the bases of the other polytene chromosome arms cytologically appear as β-heterochromatic is still not known. These β-heterochromatic regions bind special non-histone chromosomal proteins (JAMES and ELGIN 1986), and could conceivably also be unusual in terms of their replicative properties, as has been discussed for the general case of delayed but active replication forks (LAIRD, HAMMOND and LAMB 1987). This issue has been treated in detail elsewhere (MIKLOS and COTSELL 1990).

We believe that the description we have portrayed in Figure 7, is most consistent with all the available data and represents an amalgam of the data sets of GALL (1973); SCHALET and LEFEVRE (1973, 1976); LAIRD *et al.* (1974); LAKHOTIA and JACOB (1974), and our own. It is also seen from this figure that the β-heterochromatic division 20 contains a minimum of 13 genetic complementation groups from *tumorous head* to *suppressor of forked* (SCHALET and LEFEVRE, 1973, 1976; LEFEVRE 1981; LEFEVRE and WATKINS 1986; GREEN, YAMAMOTO and MIKLOS 1987; MIKLOS *et al.* 1987, 1988; PERRIMON, SMOUSE and MIKLOS 1989). The genetics and cell biological characteristics of these genes within β-heterochromatin is unremarkable when compared to the characteristics of their neighbors in the immediately adjacent euchromatic subdivisions 19E and 19F, namely, the *maroonlike* to *LB20* region (PERRIMON, SMOUSE and MIKLOS 1989). Thus on the criteria of gene density and gene types, the β-heterochromatin of the *X* chromosome and its adjacent euchromatin are functionally indistinguishable (MIKLOS and COTSELL 1990).

In summary, by using several cloned probes, we have molecularly confirmed and extended the conclusions of SCHALET and LEFEVRE (1973, 1976) which were based on careful cytogenetic analyses of polytene chromosomes. We would reiterate that the *suppressor of forked* locus is indeed the most proximal ordinary sex-linked gene and that it is located in subdivisions 20EF. The mitotic heterochromatin of the *X* chromosome is very probably proximal to this locus, although we are unable as yet to put a firm figure on its distance. Our molecular data, showing that the *suppressor of forked* locus is in a largely polytenized state and is close to the β-heterochromatin/α-heterochromatin junction, bears out the prophetic prediction of SCHALET and LEFEVRE (1973) that "the polytenized portion of section 20, which heretofore has traditionally been considered as heterochromatic corresponds, in fact, with the euchromatic portion of the mitotic *X* chromosome."

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