Characterization and origin of extrachromosomal DNA granules in
Sarcophaga bullata

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Summary

We have used endonuclease treatment in situ, followed by Giemsa or ethidium bromide staining, for mapping repetitive sequences on the chromosomes of the flesh fly Sarcophaga bullata and thus for studying extrachromosomal DNA granules in this species. All three restriction enzymes employed (Haelll, AluI and HindIII) show the same cytological effects, except for a single interstitial band. In both polytene and mitotic chromosomes, chromatin resistant to these endonucleases presumably includes at least three previously unrecognized buoyant density satellites (1·663, 1·670 and 1·692 g ml⁻¹ in neutral CsCl), and is predominantly localized in the pericentric regions of all five autosomes. Mitotic treated chromosomes show that the entire rod-shaped X chromosome, but no part of the dot-like Y chromosome, consists of endonuclease-resistant chromatin. The most unusual heterochromatic component of polytene nuclei in this species, the 'extrachromosomal DNA granules', are also entirely resistant to digestion with endonucleases. We think that these DNA granules represent dispersed X chromatin and not, as previously assumed, extruded autosomal heterochromatin.

Key words: extrachromosomal DNA, heterochromatin, highly repetitive DNAs, restriction endonucleases.

Introduction

Very little is known about Sarcophaga bullata heterochromatin, including its most unusual component, the 'extrachromosomal DNA granules', which are found in the nuclei of polytene foot-pad cells during pupal development (Whitten, 1965). Samols & Swift (1979a, b) have found that quinacrine-bright chromatin includes a pericentric region in each of the five autosomes pairs as well as both X and Y chromosomes, in dividing gonial cells. In giant foot-pad cells, pericentric heterochromatin of three autosomes as well as DNA granules fluoresce intensely after quinacrine staining. Samols & Swift (1979a, b) have also reported that the two quinacrine-bright blocks in chromosome C and E share a specific DNA fraction with extrachromosomal granules. On the other hand, S. bullata genome was previously thought to be distinguished by the absence of satellite DNAs or any repetitive sequences in sex chromosomes. On this basis, Samols & Swift (1979b) conclude that extrachromosomal DNA granules “result from an extrusion process which takes place early during the polytenization of these cells”.

To investigate these problems, we used HaeIII, AluI and HindIII restriction endonucleases to digest both purified DNA and fixed cytological preparations of S. bullata.

Digestion of fixed chromosomes with a number of restriction enzymes, followed by staining with Giemsa or DNA-specific dyes, produces specific banding patterns (Lima de Faria et al. 1980; Mezzanotte et al. 1983a,b; Miller et al. 1983) similar or identical to those induced by conventional chemical treatments.
(Sumner, 1982). Even though the details of the mechanism remain to be elucidated, it seems that endonuclease-induced banding is due to differential DNA extraction (Bianchi et al. 1985; Mezzanotte et al. 1985). Chromosomal sites that remain intensely stained after DNA digestion are known to include clusters of repetitive nucleotide sequences lacking appropriate and sufficiently numerous targets for enzyme cleavage. Highly repetitive (satellite) DNA sequences, but also some of the moderately repetitive nucleotide sequences (e.g. the ribosomal genes), have been detected in chromosomes digested with different restriction enzymes (Mezzanotte et al. 1983b; Miller et al. 1983; Kaelbling et al. 1984; Bianchi et al. 1985; Mezzanotte, 1986). Digestion of cytological preparations with certain restriction enzymes can thus rapidly provide comprehensive and reliable information about the chromosomal organization of clustered repeated nucleotide sequences.

Our results indicate that it is possible to identify typical buoyant density satellites in Sarcophaga DNA and also to recover such satellites after digestion with HaeIII, AluI or HindIII. The same restriction enzymes are incapable of cleaving specific areas of autosome, as well as the entire X chromosome and the extrachromosomal DNA granules. On the basis of these findings, we offer a new interpretation of the origin of DNA granules.

**Materials and methods**

**DNA isolation**

Mid-pupae were removed from puparium cases, frozen in liquid nitrogen and pulverized with mortar and pestle. The powder was suspended in 0.15 M-NaCl, 0.1 M-EDTA (pH 8.0). After addition of sodium dodecyl sulphate (to 2%) and NaClO4 (to 1 M), the slurry was mixed by slow rotation (1 h at 25°C) with an equal volume of saturated phenol containing 0-1% 8-hydroxyquinoline. The aqueous phase, separated by centrifugation, was mixed with 2 vol. of ethanol and the precipitate was collected on a rod. The DNA was further purified by extraction with chloroform:isoamyl alcohol (24:1; v/v) and by digestion with pancreatic RNase A (Worthington). The DNA was finally dissolved in water.

**Endonuclease digestion of DNA**

The DNA (6-7 μg) was digested with 24 units of AluI or with 30 units of HaeIII or HindIII in a volume of 15 μl of 50 mM-NaCl, 10 mM-MgCl2, 1 mM-dithiothreitol, 10 mM-Tris·HCl (pH 7.5) at 37°C for 3, 9 and 27 h. All restriction endonucleases were obtained from Bethesda Research Laboratories. Digestion was stopped by the addition of 10 mM-EDTA. The NaCl concentration was adjusted to 1 M and the DNA was precipitated with 2 vol. of ethanol (5 min in a solid CO2-ethanol slurry). The precipitate was collected by centrifugation, air-dried and redissolved in 1 mM-EDTA, 10 mM-Tris·HCl (pH 8.0), by slow rotation for 1 day at 4°C. Control samples were treated identically, except that no endonuclease was added.

**Analytical CsCl pycnography**

Equilibrium centrifugation of DNA samples in neutral CsCl gradients was carried out in single-sector Kel-F centrifuges at 4200 revs min⁻¹ (25°C) in a Spinco model E ultracentrifuge according to Mandel et al. (1968). Ultraviolet absorption photographs were taken after 20 h and traced with a microdensitometer. Each sample included approximately 1 μg of bacteriophage SP82 DNA as a density standard (1.743 g ml⁻¹ as determined with reference to Escherichia coli DNA, 1.710 g ml⁻¹).

**Chromosome preparations**

Metaphase chromosomes from gonads and polytene chromosomes from foot-pad cells were prepared, respectively, 3 and 7 days after the white-puparium stage (pupae raised at 25°C). Except where indicated, only the larger foot-pad chromosomes of males were analysed. Tissues were fixed in methanol:acetic acid (3:1; v/v) for 5-10 min, transferred to 45% acetic acid for about 5 min and then squashed. After freezing and removing the coverslip, the preparations were immersed in 98% ethanol for 5-10 min and air-dried.

**Endonuclease digestion in situ**

Freshly prepared chromosome squashes were digested with HaeIII, AluI or HindIII in the same solution as used for the digestion of purified DNA. The enzymes (30-50 units in 100 μl of incubation buffer) were applied between slide and coverslip, and the preparations were incubated at 37°C in a moist chamber for 16 h. Digestion was stopped by rinsing the slides under running tap water and subsequently in 5 mM-EDTA (pH 7.5) for 5 min at 4°C. Preparations were stained for 5-10 min with 2.5% Giemsa in deionized water. Some preparations were subsequently destained in methanol:acetic acid (3:1; v/v) for 10 min, washed in tap water and stained with ethidium bromide (20 mg in 50 ml of: 7 mM-Na2HPO4, 2 mM-NaH2PO4, 180 mM-NaCl, 1 mM-Na2EDTA).

**C-bandung**

This technique is considered capable of detecting regions of constitutive heterochromatin in fixed chromosomes (Arrighi & Hsu, 1971). A modification of the procedure employed by Bedo (1975) was used. Slides were incubated for at least 24 h in isopropanol at 4°C and treated with 1 M-HCl for 5 min at about 21°C, with a saturated solution of Ba(OH)2 for 5 min at 55°C, and finally with 2 x SSC (1 x SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate) for 3 h at 65°C. After each step, slides were thoroughly washed in tap water. Finally, chromosomes were stained with 5% Giemsa in deionized water for 30 min.

**Light-induced banding**

This technique is considered capable of detecting chromosomal areas containing A + T-rich DNA sequences (Mezzanotte et al. 1982). Squash preparations were immersed in a Methylene Blue solution (0-1% in tap water) and exposed to light for 16 h, essentially as described by Mezzanotte et al. (1982). The chromosomes were destained in 70% ethanol for 5 min, stained with 0.1% Acridine Orange in 2 x SSC for
3–5 min and washed in 2× SSC for at least 15 min before they were observed and photographed under fluorescence microscopy.

Results

Satellite DNAs

Mid-pupal DNA of *S. bullata* includes at least three buoyant density satellites (Fig. 1). Two light fractions (1·663 and 1·670 g ml⁻¹ representing, respectively, 0·5 and 2% of the total DNA) are completely separated from the bulk of the material in neutral CsCl gradients (Fig. 1a). The third satellite (3·5% of the total DNA) has only a slightly higher buoyant density (0·001 g ml⁻¹) than main-band DNA. This cryptic fraction was recognized only after extensive endonuclease digestion (Fig. 1c,d). The DNA preparation used in most of our experiments had a main-band buoyant density of 1·691 g ml⁻¹; estimates from two other DNA preparations ranged between 1·692 and 1·693 g ml⁻¹, somewhat closer to previously published values (1·693–1·694 g ml⁻¹, see Laird & McCarthy, 1969; Samols & Swift, 1979b).

The DNA used for endonuclease digestion was minimally purified to ensure that we started with high molecular weight material. Such preparations included a rapidly banding polysaccharide, which served as a convenient carrier for DNA recovery after enzyme digestion as well as a useful buoyant density marker (1·676 g ml⁻¹). The polysaccharide can be removed with alpha-amylase and does not conceal any satellite DNA (Fig. 1a).

All three satellite DNAs are completely resistant to either *Hae*III, *Alu*I or *Hind*III restriction endonuclease. Even after extensive digestion with *Hae*III or *Alu*I, the original amount of the l·670 g ml⁻¹ satellite (Fig. 1b) is fully recovered (Fig. 1c,d). The same is true for the l·663 g ml⁻¹ satellite, which is not resolved in the tracings (Fig. 1b,e). After 9 h incubation with *Hae*III or with *Alu*I, the cryptic satellite is clearly recognizable among the widely dispersed main-band DNA fragments, whereas digestion with *Hind*III for the same time is too short to reveal this satellite component (Fig. 1e). If *Hind*III digestion is extended to 27 h, the cryptic satellite DNA is clearly resolved.

Because of their low buoyant density (in the case of the two light satellites) and because of their resistance to endonuclease digestion, it is possible to postulate that the satellite DNAs represent simple, highly repetitive nucleotide sequences of nuclear origin.

Polytene chromosomes

Regardless of whether Giemsa or ethidium bromide was used, endonuclease-induced patterns of chromosome staining were identical. For brevity, we will refer only to Giemsa staining.

Fig. 2 shows the location of nuclease-resistant sites and sites of C-banding. Each of the five polytene chromosomes includes one special constricted site (presumably the centromere) within some compact amorphous material, the α′-heterochromatin. Here any two or three chromosomes may fuse and form partial chromocentres. The α′-heterochromatin of each of the five separate chromosomes is specifically resistant to digestion with either *Hae*III, *Alu*I or *Hind*III. An additional 14 bands are equally protected from digestion with any of the three nucleases. All are heavy bands adjacent to α′-heterochromatin. One of these bands (open circle in Fig. 2) may be assigned to the α′-heterochromatin, due to its resistance to C-banding (see below). The pericentric regions including the remaining 13 bands will be called β-heterochromatin.

Fig. 3 shows Giemsa staining patterns of chromosome C before (A) and after (B–D) enzyme digestion. Untreated chromosomes appear to be evenly and intensely stained except for large puffs and sufficiently stretched interbands. However, in squashes treated with *Hae*III, *Alu*I or *Hind*III, only the α-heterochromatin and four adjacent bands are still as intensely stained as in control preparations. The dark nuclease-resistant bands are not only separated by lighter

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**Fig. 1.** Tracings of ultraviolet absorption photographs of *Sarcophaga* DNA in neutral CsCl equilibrium density gradients. Except for controls (a,b), DNA samples were digested with restriction endonucleases *Hae*III (c), *Alu*I (d) or *Hind*III (e) for 9 h as described in Materials and methods. One of the control DNAs (a) was more extensively purified, including digestion of the polysaccharide (1·676 g ml⁻¹) with alpha-amylase; the DNA concentration in A was also doubled to reveal the minor 1·663 g ml⁻¹ satellite in the tracing.
interbands, but also by a few faintly stained bands. Nuclease-resistant bands adjacent to the nucleolar region in chromosome A are also not arranged contiguously.

We found only a single interstitial site, the H-band, that is differentially resistant to endonuclease digestion. The H-band is distinguished from pericentric heterochromatin in that it resists digestion with HaeIII (Fig. 4A), but not AluI (Fig. 4B) or HindIII. The H-band is also distinguished from pericentric heterochromatin by its sensitivity to photo-oxidation. In fact, Acridine Orange differentially stains all the nuclease-resistant pericentric areas, but not the H-band, after photo-oxidation (Fig. 4D).

C-bands in polytene foot-pad chromosomes appear to be restricted to the five blocks of α-heterochromatin plus four additional sites: one band immediately adjacent to the α-heterochromatin in C, (Fig. 2, open circle; Fig. 3), the H-band, one puff and some intranucleolar chromatin (Figs 2, 4C: H, P, N). In some preparations, the response to C-banding was more graded than in others (cf. Bedo, 1975), but in all cases the nine sites just mentioned were consistently the most stained.

Staining of nucleolar chromatin after C-banding has been observed in other polytene cells (Bedo, 1975), but the distinctive staining of a single puff is curious though not unexpected. Prior observations (Bultmann, unpublished) have shown that other staining properties and ultrastructural characteristics of this particular puff (P) are unusually similar to those observed in the nucleolus. This small puff is present throughout the lifetime of well-developed foot-pad chromosomes and seems to be nearly invariable in size.

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Fig. 2. Heterochromatic sites in polytene chromosomes of S. bullata. This orcein-squash preparation of male dorsal foot-pad cells was made 9 days after the white-puparium stage. By this time the polytene chromosomes are fully developed and their DNA content has increased to 1024 or 2048 times the haploid level of 0.6 pg (Samols & Swift, 1979a). Also at this stage puffing activities are minimal and the banding pattern is therefore least distorted by large puffs (counterstained with light green-orange G). The preparation is extraordinary in that none of the five polytene chromosomes is broken. The most frequent weak points are marked by filled (breakage in 90% of the squashes) and open triangles (~50% breakage). Short (S) and long (L) chromosome arms are identified according to Whitten (1965). Also indicated are three clusters of DNA granules (G), the chromosomal sites resistant to digestion with HaeIII, AluI or HindIII (heavy bars) or only HaeIII (H), and the sites of C-banding (filled and open circles, also the H-band, a small puff (P), and some intranucleolar chromatin (N)). Bar, 10 μm.

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Fig. 3. Endonuclease digestion and C-banding of chromosome arm C,. Giemsa staining after the following treatments: A, control (mock-digestion, 16 h); B, HaeIII (50 units, 16 h); C, AluI (30 units, 16 h); D, HindIII (30 units, 16 h plus 60 units, 16 h); E, C-banding. In C and E, fusion of the α-heterochromatin of C, and A,. In B, one of the β-heterochromatin bands is associated with a chain of DNA granules. Bar, 10 μm.

Fig. 4. Endonuclease digestion, C-banding and photo-oxidation of chromosome arm A,. Giemsa staining after 16 h digestion with 50 units HaeIII (A) or 30 units AluI (B), or after C-banding (C). Acridine Orange fluorescence (reverse contrast print) after photo-oxidation (D). The HaeIII-resistant band (H), a puff (P) and nucleolar material (N) are labelled as in Fig. 2. Bar, 10 μm.

DNA granules

In squashes of the very large dorsal foot-pad cells of males, the DNA granules are usually seen loosely attached to each other and to various chromosome sites (Fig. 2; also cf. Roberts et al. 1976), but many granules are widely scattered. Scattering and possible loss of DNA granules is avoided in the less polytene dorsal foot-pad cells of females (Fig. 5) or even smaller tarsal trichogen cells of males (Fig. 6). In both males and females, all DNA granules remain intensely stained following digestion with HaeIII, AluI or HindIII (Fig. 3B; Figs 5–6). DNA granules are also insensitive to photo-oxidation.

Metaphase chromosomes

The chromosome complement in mitotic and first meiotic gonial cells consists of five pairs of large autosomes and a pair of small sex chromosomes. The male is the heterogametic sex, carrying a rod-shaped X and a dot-like Y chromosome.

Each of the five autosomes contains AluI-resistant pericentric chromatin (Fig. 7), in amounts that are not fully represented in polytene chromosomes. Thus, on the basis of length measurements, we estimate that in gonial cells, individual autosomes contain at least 20–25% AluI-resistant material, whereas in polytene cells they contain no more than 2–8%. Overall, the amount of presumptive autosomal heterochromatin declines from about 35% to about 5% during polytenization.

AluI has no effect on the X chromosome, whereas the Y chromosome is completely digested (Fig. 7A,B).
**Discussion**

Our results indicate that at least three highly repetitive DNAs exist in *S. bullata* genome. Previous studies failed to recognize such satellites, possibly lost during DNA purification, and suggested that typical satellite DNAs may be replaced by less-repetitive nucleotide sequences in this species (Samols & Swift, 1979b). The fact that the three enzymes used do not attack any of the satellite DNAs permitted the detection of one highly repetitive DNA that is cryptic by standard equilibrium centrifugation analysis. Therefore, the cytologically detectable endonuclease-resistant *Sarcophaga* DNA fraction presumably includes at least the three buoyant density satellites that are resistant to either HaeIII, AluI or HindIII.

In metaphase chromosomes, AluI digestion shows a pattern similar, but not identical, to the C- or Q-banding pattern obtained by Samols & Swift (1979a,b). The most striking feature of AluI-treated chromosomes, as compared to C- or Q-banded chromosomes, is the possibility of differentiating the X from the Y chromosome. In fact, the former is completely resistant, while the latter is extensively attacked by AluI. The differential sensitivity of the two sex chromosomes to AluI provides a reliable means of identifying such chromosomes and may replace morphological criteria, which are unreliable due to the fact that the sex chromosomes in females are often shaped like the Y rather than the X in males (Fig. 7C,D).

In polytene foot-pad chromosomes, nearly all endonuclease-resistant DNA is located in pericentric regions, which we have equated with α- and β-heterochromatin (Heitz, 1934). As in *Drosophila*, the α-heterochromatin in *Sarcophaga* is readily distinguished from euchromatin, both morphologically (irregular clumps of compact chromatin) and functionally (formation of chromocentres). Yet, in contrast to *Drosophila* but resembling other organisms such as *Nematocera* (Beermann, 1962), the *Sarcophaga* β-heterochromatin is not amorphous and consists of irregular bands and interbands. Nevertheless, we have been able to distinguish between β-heterochromatin and euchromatin, due to the different resistance to both endonuclease and photo-oxidation treatments found in the two cases. The α-heterochromatin was unaffected by nuclease digestion or photo-oxidation, like β-heterochromatin. The three major blocks of nuclease-resistant pericentric heterochromatin of polytene chromosomes A, C and E seems to be identical to the three major quinacrine-bright regions recognized by Samols & Swift (1979b).
The H-band is the only endonuclease-resistant site recognizable in polytene foot-pad chromosomes outside the pericentric heterochromatin. This band is also resistant to C-band treatment, but is not detectable after photo-oxidation. We can exclude the possibility that endonuclease resistance is due to the particular compactness of H-band, since this site is resistant to HaeIII, AluI or HindIII. We thus suggest that H-band contains DNA sequences different from those located in pericentric heterochromatin.

Quinacrine staining has been used to relate heterochromatin to DNA granules as an identical bright fluorescence that is present in both cases (Samols & Swift, 1979a,b). It was also shown that DNA granules do not include ribosomal DNA cistrons. Here we can add that DNA granules and pericentric heterochromatin are equally resistant to HaeIII, AluI or HindIII digestion. There is therefore no doubt that DNA granules are part of the Sarcophaga heterochromatin. The question remains, however, as to what kind of heterochromatin the DNA granules consist of. According to the extrusion hypothesis (Swift et al. 1978; Samols & Swift, 1979a,b), the DNA granules originate by a diminution process from pericentric heterochromatin of autosomes C and E. On the basis of the observations reported below, we propose the alternative view that DNA granules may be sex chromatin or, more specifically, X chromatin.

Structural features of pericentric autosomal heterochromatin such as the occurrence of constriction (Fig. 2E) are not necessarily related to the production of the DNA granules. In well-spread preparations, for instance, constrictions at the α-heterochromatin are present not only in chromosomes C and E, but also in the three other autosomes that are assumed not to be involved in chromatin extrusion. The weak points at which chromosomes break most frequently during squashing (Fig. 2: triangles) are also no more prominent near the presumptive sites of chromatin extrusion than near other pericentric heterochromatin. Additionally, AluI-resistant pericentric chromatin is under-represented in each of the five polytene chromosomes, not only in chromosomes C and E. This would imply that heterochromatin extrusion and heterochromatin replication are two interdependent mechanisms, which must be regulated in a highly coordinate and chromosome-specific manner. On the other hand, the in situ hybridization data of Samols & Swift (1979a,b) are not conclusive in establishing specific sequence homologies between DNA granules and pericentric heterochromatin of chromosomes C and E. For instance, it should be explained why the in situ hybridization with a cRNA prepared from a rapidly reassociating DNA fraction showed that "autoradiographic grains were seen over the heterochromatic areas of chromosomes C and E after a 10 day incubation", while the granules became positively labelled only if the hybridized slides were allowed to incubate for much longer periods" (i.e. 67 days, see Samols & Swift, 1979b). Lastly, the assumption that repetitive nucleotide sequences are absent in S. bullata sex chromosomes was never adequately tested by Samols & Swift (1979a,b). Our data indicate that the X chromosome contains one or more of the satellite DNA sequences, just like the DNA granules or any of the pericentric heterochromatic blocks. Endonuclease-resistant heterochromatin comprises not only the entire X chromosome, but also the pericentric areas of all five autosomes, thus excluding any peculiar base composition in chromosomes C and E.

The DNA granules appear to be stained equally in males and females after AluI treatment, despite the fact that this enzyme is capable of distinguishing between X and Y chromosomes in metaphase preparations (Fig. 7). On this basis, we conclude that the X chromosome may be co-replicated during polytenization, initially forming a cohesive chromocentric mass, which eventually dissociates into fairly discrete granules. On the contrary, the Y chromosome does not contribute to the DNA granules in foot-pad cells, but rather remains unreplicated, like the Y chromosome in Drosophila melanogaster salivary glands.

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