Islands of Complex DNA are Widespread in Drosophila Centric Heterochromatin

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ABSTRACT

Heterochromatin is a ubiquitous yet poorly understood component of multicellular eukaryotic genomes. Major gaps exist in our knowledge of the nature and overall organization of DNA sequences present in heterochromatin. We have investigated the molecular structure of the 1 Mb of centric heterochromatin in the Drosophila minichromosome *Dp1187*. A genetic screen of irradiated minichromosomes yielded rearranged derivatives of *Dp1187* whose structures were determined by pulsed-field Southern analysis and PCR. Three *Dp1187* deletion derivatives and an inversion had one breakpoint in the euchromatin and one in the heterochromatin, providing direct molecular access to previously inaccessible parts of the heterochromatin. End-probed pulsed-field restriction mapping revealed the presence of at least three "islands" of complex DNA, *Tahiti*, *Moorea*, and *Bora Bora*, constituting approximately one half of the *Dp1187* heterochromatin. Pulsed-field Southern analysis demonstrated that Drosophila heterochromatin in general is composed of alternating blocks of complex DNA and simple satellite DNA. Cloning and sequencing of a small part of one island, *Tahiti*, demonstrated the presence of a retroposon. The implications of these findings to heterochromatin structure and function are discussed.

THE heterochromatic regions of multicellular eukaryotic genomes have puzzled chromosome biologists for over a century. Heterochromatin was initially differentiated from euchromatin by cytological criteria; the heterochromatin remained condensed throughout the cell cycle and exhibited unusual staining properties (HEITZ 1928; WHITE 1973). Heterochromatin is further distinguished from euchromatin by its paucity of genes, replication late in S phase, and high content of repetitive sequences (JOHN 1988). By these criteria, cytologists determined that most multicellular eukaryotic chromosomes contain both heterochromatin and euchromatin, the former usually confined to centromeric and telomeric regions. Heterochromatin constitutes a significant portion of individual chromosomes and of whole genomes; 15% of the human and 30% of the Drosophila genomes are heterochromatic (JOHN 1988). Some chromosomes, such as the *Y* chromosomes of many species, appear to be entirely heterochromatic (WHITE 1973). Despite the ubiquitous distribution of heterochromatin among multicellular eukaryotes, our understanding of its detailed molecular structure and its role in chromosomal, cellular, developmental and evolutionary processes is minimal.

The repetitive nature of heterochromatic DNA, and its ability to inhibit euchromatic gene function, has led some to suggest that heterochromatin is merely "junk" DNA having no utility to the cell. However, the view of heterochromatin as "inert" is based on a lack of knowledge and incomplete structural and functional analyses. Many essential functions reside in heterochromatic regions, including the highly active ribosomal RNA genes. In Drosophila, genes required for viability (*e.g.*, lethal mutable genes) and fertility (*e.g.*, the *Y*-linked male fertility factors) reside in heterochromatin (GATTI and PIMPINELLI 1992). Most importantly, heterochromatic DNA is essential for normal chromosome inheritance. In widely divergent multicellular eukaryotes, centromeres and other inheritance elements are positioned deep in centric heterochromatin (WHITE 1973; MURPHY and KARPEN 1995a,b), and heterochromatic sequences appear to be necessary for sister chromatid cohesion of mitotic and meiotic chromosomes (LICA *et al.* 1986; MIYAZAKI and ORR-WEAVER 1994). A number of other inheritance functions, defined in greatest detail in Drosophila, also require heterochromatic sequences. The disjunction of achiasmate chromosomes in female meiosis relies on centric heterochromatin (HAWLEY and THEURKAUF 1993; LE and KARPEN, unpublished data), and the meiotic pairing of the *X* and *Y* chromosomes in males requires the intergenic spacer of the ribosomal genes (MCKEE and KARPEN 1990; MCKEE *et al.* 1992). Heterochromatic sequences play critical roles in meiotic drive systems (Segregation Distortion and *X-Y* drive), characterized by the preferential, non-Mendelian recovery of one chromosome over its homologue (LYTTLE 1991).

A major impediment to understanding the biological

This article is dedicated to the memory of Ronald Christman, who died too young to make his mark in science.

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functions encoded by heterochromatin is a lack of knowledge of its structure. Analyses of the molecular composition and organization of heterochromatic regions present special problems not usually encountered in the study of euchromatin. Heterochromatin contains large amounts of repeated sequences, including highly repeated satellite DNAs, as well as middle-repetitive sequences, such as transposable elements and tandemly repeated ribosomal RNA genes. HEITZ (1934) used cytological criteria to separate centric heterochromatin into "α" and "β" components. α heterochromatin contains highly repeated satellite DNA and is severely underrepresented in Dipteran polytene chromosomes (GALL *et al.* 1971). β heterochromatin can be thought of as the "buffer" between euchromatin and α heterochromatin (MIKLOS and COTSELL 1990), contains middle repetitive transposon-like sequences and some single copy genes (*e.g.*, the *light* gene) (DEVLIN *et al.* 1990a), and is not severely underrepresented in polytene nuclei (YAMAMOTO *et al.* 1990). The structure and function of a few single copy β-heterochromatic genes [*e.g.*, *light* (DEVLIN *et al.* 1990a,b) and *suppressor of forked* (MITCHELSON *et al.* 1993)] have been investigated, but intensive molecular analysis of the more difficult centric heterochromatic regions has only recently been attempted. *In situ* hybridization to mitotic chromosomes and cytogenetic banding patterns have revealed the gross distribution of repeated DNAs within centric heterochromatin in a variety of organisms, including Drosophila (BONACCORSI and LOHE 1991; LOHE *et al.* 1993) and humans (DUNHAM *et al.* 1992; GRADY *et al.* 1992). However, the presence of these repeated DNAs has made finer structural analyses of heterochromatin, using standard molecular methods, extremely difficult. Simple cloning of heterochromatic sequences is hampered by instability of repeated sequences in host organisms such as *Escherishia coli* (BRUTLAG *et al.* 1977b; LOHE and BRUTLAG 1986) and *Saccharomyces cerevisiae* (FOOTE *et al.* 1992; COOPER *et al.* 1993), making conclusions about the overall structures of genomic copies dubious. Nevertheless, important information about the nucleotide composition of heterochromatin has been obtained from sequencing small clones of satellite DNA (LOHE and BRUTLAG 1986; JABS and PERSICO 1987; TYLER-SMITH 1987; ABAD *et al.* 1992).

There is a large gap between the physical information generated by cytogenetic studies and the details of satellite DNA sequences. The resolution afforded by cytological methods is poor, making it difficult to assess the molecular organization and sequence composition of heterochromatin below the megabase level (*e.g.*, the size and complexity of simple sequence arrays). Conversely, the limited nucleotide sequences of cloned heterochromatic DNAs have revealed little about the higher order organization and overall sequence composition of heterochromatin. Although pulsed-field electrophoresis provides the resolution necessary to bridge this information gap, the dispersion of repetitive DNAs throughout the genome makes it difficult to use them as probes to restriction map specific regions of heterochromatin. Chromosome-specific satellite DNAs have been used to restriction map repeated domains in mammals (WILLARD *et al.* 1986; JABS *et al.* 1989; WEVRICK and WILLARD 1989; MAHTANI and WILLARD 1990; ARN *et al.* 1991; WEVRICK *et al.* 1992), but the completeness of these maps has been limited by the lack of direct molecular access deep within individual satellite blocks. A number of important questions about heterochromatin structure remain unanswered. Are nonsatellite, or even single copy, sequences present in "deep" heterochromatin? How big are the tandem arrays of satellite DNA, and how are they organized with respect to other heterochromatic sequences?

We have investigated the molecular structure of the 1 Mb of centric heterochromatin present in the Drosophila minichromosome *Dp(1;f)1187* (*Dp1187*). This minichromosome contains the normal components and functions associated with multicellular eukaryotic chromosomes. *Dp1187* contains heterochromatin and euchromatin and is transmitted with high fidelity through meiosis and mitosis. However, its relatively small size of 1.3 Mb (1/30th the size of the normal *X* chromosome) makes it amenable to molecular analysis. Furthermore, *Dp1187* can be manipulated genetically because it carries scorable genetic markers and its presence is not essential to the viability of the organism. Limited restriction mapping previously suggested that a cluster of restriction sites, termed an "island of complex DNA," was present within 50–100 kb of the *Dp1187* euchromatin–heterochromatin junction (KARPEN and SPRADLING 1990, 1992). Here, we report that irradiation mutagenesis and pulsed-field restriction mapping reveals significant amounts of substructure deep within *Dp1187* centric heterochromatin. We also demonstrate that Drosophila heterochromatin in general is organized as alternating blocks of complex islands and satellite DNA, each hundreds of kilobases in length. The composition of the islands was investigated by cloning and sequencing a small piece of one such island, designated *Tahiti*, which revealed the presence of a *Doc* retroposon. Our results demonstrate that centric heterochromatin can be dissected in a directed fashion with molecular-genetic methods. The significance of these results to the problem of heterochromatin evolution and function is discussed.

MATERIALS AND METHODS

Drosophila stocks and culture: The *Dp 8-23* chromosome is a *Dp1187* derivative that contains *rosy*$^+$ *P* element (*PZ*) insertions at −246 kb and −185 (Figures 1 and 2A). This derivative of *Dp1187* was generated by interchromosomal transposition of one *PZ* element into −246 (KARPEN and SPRADLING 1992), followed by local transposition into the −185 site (TOWER *et al.* 1993). *Dp 8-23* and all derivatives reported here were kept

in a y ;ry^{506} background. All crosses were performed at 25° on standard Drosophila media, unless noted otherwise. See LINDSLEY and ZIMM (1992) for a description of all other stocks and mutations.

Irradiation mutagenesis of *Dp1187*: For each round of mutagenesis, ~1800 y ; ry^{506} ; *Dp 8-23*, y^+ry^+ males were placed in a cylindrical plexiglass chamber and then gamma irradiated with 4000 rads from a Cobalt-60 source. Forty-five to 50 males were immediately mated to 50 y ; ry^{506} virgin females (Figure 1) in individual bottles. Males were removed after 3–5 days, and female parents were transferred to new bottles twice. F1 progeny were scored for altered y^+ or ry^+ phenotypes, including y−ry+, y+ry−, y−ry±, y±ry− or y±ry± (see Table 1 legend). Males or females expressing these phenotypes were individually outcrossed in vials to three y ; ry^{506} virgin females or males, respectively. If the altered phenotype was heritable, a y ; ry^{506} ; *Dp* stock was established. The stock and derivative were named with a gamma (γ) followed by the parental bottle number. Genetic linkage analyses were carried out as described previously (KARPEN and SPRADLING 1992), by screening for segregation of the appropriate marker (y^+ or ry^+) from a second (SM5, *Cy*) or third (TM3, *Sb* ry^{RK}) chromosome balancer, a fourth chromosome dominant marker (ci^D), or by observing sex-limited transmission. Animals with an extra *Y* chromosome (*XXY* and *XYY*; Table 1) were produced by crossing *Dp*-bearing males to $\hat{XY}$, y / $\hat{XY}$, y ; ry^{506} virgin females ($\hat{XY} = Y^S X Y^L$).

PCR assay for the presence of the sc^8 breakpoint: One-tenth of the DNA prepared from a single fly (GLOOR *et al.* 1991) of the appropriate phenotype was used in each PCR reaction. Four primers were used, two that flank the sc^8 breakpoint (euchromatic side GK31 = 5′-CTCAGTGATTAC-GAAATC-3′, heterochromatic side RLG2 = ATCAGACCA-CCAAGACACCA; sequences generously provided by Dr. ROBERT GLASER), plus positive control primers that anneal to the *apterous* locus (JT1 = TAATGGCAAAGTAGGACTT and JT5 = GATACCGCCTCATCCAGTAT; sequences generously provided by Dr. JOHN THOMAS). The PCR reactions used standard buffer conditions [10 mM Tris, pH 8.0, 1 mM $MgCl_2$, 2.5 units Taq Polymerase (Stratagene, San Diego, CA), 200 μg/ml of each primer]. Components were mixed on ice and then cycling reactions were carried out with a 94° incubation for 4 min, followed by 35 cycles of 94° 1 min, 55° 1.5 min, and 72 ° for 2 min, in a Perkin Elmer 4800 Thermal Cycler. The presence of the sc^8 breakpoint was indicated by a 280-bp band after electrophoresis through 3% NuSieveGTG agarose (FMC, Rockland, ME); the production of a 550-bp band from the *apterous* locus was used as a positive control demonstrating that the PCR reactions worked.

Neuroblast squashes: Cytological evidence for the presence of a small free duplication or minichromosome was obtained by analyzing metaphase squashes from third instar larval brain neuroblasts. Standard squash methods were used (GATTI *et al.* 1994), and chromosomes were stained with DAPI (1 μg/ml in phosphate-buffered saline + 0.1% Triton X-100). For each line, 16 well-separated metaphases were scored for the presence of the fourth chromosome and a minichromosome (four metaphases from four different brains). Squashes only were included in the analysis when they displayed at least one visible fourth chromosome.

Pulsed-field Southern analysis: *Preparation of high molecular weight DNA from embryos:* A modification of previously described methods (KARPEN and SPRADLING 1990, 1992) was used to prepare high molecular weight DNA from 12- to 15-hr collections of embryos. A detailed protocol is available upon request. Briefly, embryos were rinsed off apple juice/agar collection plates into weigh boats with 0.7% NaCl/0.4% Triton X-100, collected in a Nytex sieve, and rinsed twice with NaCl/Triton. Embryos were dechorionated in 50% bleach for 90 sec and then collected in a sieve, rinsed twice with NaCl/Triton, three times with 0.7% NaCl, and transferred to a microfuge tube (matched with plastic pestle, Kontes 749520-0000). The volume of embryos was estimated after gentle centrifugation (1 sec, 5000 rpm, Eppendorf Centrifuge 5415C); usually two 80 μl agarose "inserts" were prepared per 25 μl of embryos. After removal of the NaCl, 20 μl of homogenization buffer (0.1 M NaCl, 0.03 M Tris pH 8.0, 0.05 M EDTA, 0.5% Triton X-100, 7.7 mM β-mercaptoethanol) was added per insert. Embryos were homogenized with the plastic pestle and then centrifuged (3 sec 5 K) and rehomogenized twice. Cuticle and other contaminants were removed with a gentle centrifugation (2 sec 2 K), and the supernatant was gently transferred to a new tube with a cutoff pipette tip. Another 20 μl of homogenization buffer (per insert) was added to the homogenate, rehomogenized, and spun 2 sec 2K. The total volume of the combined supernatants was adjusted with homogenization buffer to produce the estimated number of inserts. An equal volume of 1.5% FMC InCert agarose (in 0.125 M EDTA pH 7.5, boiled 5 min in screw-cap microfuge tube and kept at ~50°) was added to the combined supernatants (prewarmed to 37° for 2 min) with a cutoff pipette tip, pipetted gently three times to mix, and then pipetted into molds. Once inserts gelled (≤10 min at 4°C), they were removed from the molds and incubated overnight at 50° in 2.5 ml per insert of NDS (0.5 M EDTA, 0.01 M Tris p H9.5, 1% Sarkosyl) plus 1 mg/ml Proteinase K (Merck).

Restriction digests: Agarose inserts were prepared and digested as described previously (KARPEN and SPRADLING 1990, 1992), except that two rinses in TE + phenylmethylsulphonyl fluoride were for 1 hr each, whereas the 4 TE only washes were reduced to 20 min each. For partial digests, one half inserts were preincubated with different enzyme concentrations (*e.g.*, 0, 1, 2, 5 and 20 units) for 2 hr at 4° and then digested at the appropriate temperature for 4 hr. Most of the enzymes were purchased from NEB (New England Biolabs, Beverly, MA) and some from Stratagene and Promega (Madison, WI).

Pulsed-field electrophoresis: Pulsed-field electrophoresis was performed in a CHEF apparatus (contour-clamped homogeneous electric fields, CHU *et al.* 1986) purchased from CBS Scientific (San Diego, CA). All gels were 100 ml of 1% HGT agarose (FMC), run in 1500 ml of 1/2 × TBE (SAMBROOK *et al.* 1989) at 180 volts, 12°, and 170 mA. The pulse conditions used for each gel are described in the Figure legends.

Blotting and hybridization: Pulsed-field gels were stained in 1 μg/ml ethidium bromide for 15 min, destained in water for 15 min, and then photographed with a Speedlight imaging system (Lighttools Research, San Diego, CA). After depurination for 12 min in 0.25 N HCl, a brief rinse in water, and two 15-min incubations in alkaline transfer buffer (1.5 M NaCl, 0.4 N NaOH), DNA was transferred by capillary action to Hybond N (Amersham) overnight. Blots were neutralized for 2–5 min each in 1× unblot (0.2 M Tris, pH 7.5, 2× SSPE) and then 5× SSPE crosslinked on the "auto crosslink" setting of a Stratalinker (Stratagene). Probes were prepared by random-hexamer labeling of gel purified fragments with ^{32}P-dCTP (Amersham, Arlington Heights, IL). Most hybridizations with heat denatured probes (10 min boil) were performed for 2 hr in a Techne HB2 oven at 65°, using QuickHyb buffer (Stratagene). Hybridizations with nick-translated 1.672 AATAT satellite probes (^{32}P-dATP) were performed overnight at 48°, in CHURCH and GILBERT hybridization buffer (CHURCH and GILBERT 1984). Signals were observed after exposure to film (Kodak XAR-5, 2 Dupont Intensifying screens) or analyzed with a Molecular Dynamics Phosphorimager.

Probes: Satellite DNA plasmids 1.672-38, 1.705-42 and

aDm23-24 (1.688) were generously provided by Dr. A. LOHE (LOHE and BRUTLAG 1986; LOHE *et al.* 1993). The probes described in Figure 2A (1–6) were all from subclones of phage lambda or cosmid genomic clones (see KARPEN and SPRADLING 1990, 1992; TOWER *et al.* 1993) in the vector pBSKS+ (Stratagene). The first part of the name (*e.g.*, sc101) refers to the parental phage or cosmid, the subsequent letters (*e.g.*, XH) refer to the restriction sites at the ends of the clone, and the number at the end refers to the size of the fragment (in kb). Probe 7 was a generous gift from Drs. P. ZHANG and A. SPRADLING and corresponds to plasmid-rescued sequences that flank a *PZ* insertion (ZHANG and SPRADLING 1993). Probe 8 was isolated from Car20, a *P*-element plasmid that contains a *rosy*$^+$ gene (RUBIN and SPRADLING 1983). The positions of the probes relative to the *Dp1187* map are as follows: 1 = 0 to −7.7, 2 = −28.6 to −30, 3 = −30 to −32.6, 4 = −40 to −51.5, 5 = −70 to −79, 6 = −120 to −122.5, 7 = −164 to −165. The rosy sequences constitute 7.2 kb of the 14.5 kb *PZ* elements inserted at −185 and −246 in *Dp 8-23* (KARPEN and SPRADLING 1992; TOWER *et al.* 1993).

Molecular weight determinations: Molecular weights were determined by comparison with the mobility of standards, including the 1-kb ladder (BRL, Gaithersburg, MD), *Hin*dIII digested lambda DNA, LMW marker (NEB), lambda concatamers (NEB), and *Saccharomyces cerevesiae* chromosomes (NEB). Labeled lambda and *S. cerevesiae* DNAs were included in the hybridizations to make the molecular weight determinations more accurate. Experimental fragment sizes were determined by exponential extrapolations between the migration distances of the nearest markers, using Excel for Macintosh 4.0 (Microsoft, Redmond, WA) and a program developed by K. MAGGERT and T. MURPHY.

Cloning of genomic DNA: Two hundred adult flies from inbred lines of *Dp 8-23* or *γ240* were homogenized and the DNA extracted by conventional methods (BENDER *et al.* 1983). After *Mbo*I digestion and conventional preparative electrophoresis [0.7% Seaplaque GTG agarose (FMC), 1/2× TAE (SAMBROOK *et al.* 1989)], molecular weight markers and one lane of each sample DNA were separated from the preparative gel, stained in ethidium bromide, photographed, blotted and hybridized with probe 5, as described above. The unstained preparative gel was lined up with the autoradiogram, and the region of the gel corresponding to the appropriate fragment size (1.2 kb for *Dp 8-23*, 1.5 kb for *γ240*) was excised. Gel slices were equilibrated in β-agarase buffer (NEB) and agarase treated (2 units/100 mg gel, 2 hr, 40°). Fragments were concentrated by ethanol precipitation and then ligated overnight to *Bam*HI-digested, CIAP-treated (calf-intestinal alkaline phosphatase, Boehringer Mannheim, Indianapolis, IN) pBSKS+ (Stratagene). After transformation into SURE cells (Stratagene) according to the instructions supplied by the manufacturer, colony lifts were produced by standard methods (SAMBROOK *et al.* 1989) and hybridized with probe 5 as described above. Positive colonies were picked and rescreened. Restriction digests of the plasmids and hybridization to pulsed-field blots of digested *Dp 8-23* and *γ240* were performed to confirm that the clones were derived from the −75 and +110 region, respectively.

DNA sequencing: The *Dp 8-23Mbo* 1.2 and *γ240Mbo* 1.5 clones were sequenced using the Sequenase 2.0 kit (United States Biochemicals). Sequencing of both inserts was initiated using the T3 and T7 primers that flank the pBSKS+ polylinker. These sequences were extended using primers (purchased from the Salk Institute Cancer Center Core Facility) derived from the initial sequences; sequencing by extension was continued until all regions of the clones were sequenced at least twice. The sequences were assembled and compared using Sequencher version 2.010 software (GeneCodes, Ann Arbor, MI), run on a Macintosh Quadra 950 computer.

RESULTS

A genetic screen for *Dp1187* minichromosome derivatives: Rearrangements that juxtapose heterochromatin with cloned, single-copy euchromatic regions are useful tools for mapping regions of repeated DNA. The origin of *Dp1187* is described in Figure 1A. The juxtaposition of euchromatin and heterochromatin in *Dp1187* (the *sc*^{8} breakpoint) provided an entry point for restriction mapping a limited portion of the heterochromatin (KARPEN and SPRADLING 1990, 1992). Here we describe a genetic screen for rearranged derivatives of *Dp1187* that provided access to uncharted portions of the 1 Mb of centric heterochromatin. Briefly, males carrying a genetically marked minichromosome derivative of *Dp1187* (*Dp 8-23*, Figure 1B) (TOWER *et al.* 1993) were irradiated and mated to females (Figure 1C). F1 progeny were scored for alterations in the expression of either the *yellow*$^+$ gene adjacent to the centric heterochromatin or the *rosy*$^+$ marker genes present in the subtelomeric domain (y$^-$, y±, ry$^-$ or ry±; ± refers to variable expression, see legend Table 1). Of the 483,000 irradiated chromosomes screened, 60 lines bred true for the altered phenotypes. These candidate derivatives were established in separate stocks, designated by "γ" (for gamma irradiation) followed by a number (that refers to the F1 bottle number).

Genetic linkage analyses and larval neuroblast squashes were used to determine whether the altered phenotypes were associated with a free duplication rather than a translocation (see MATERIALS AND METHODS). This analysis identified 16 translocations among the derivative lines, including seven in which the reciprocal parts of the translocations were recovered in separate stocks (see Table 1). Note that a few lines (*i.e.*, *γ845*, *γ308* ry$^+$ and *γ308* y±) were identified as translocations by neuroblast squashes but did not behave as translocations in the linkage analysis.

Altered *y*$^+$ or *ry*$^+$ expression could be caused by direct gene mutation or by rearrangements that induce position-effect variegation (PEV) (see KARPEN 1994 for review). Animals with an extra *Y* chromosome, a strong suppressor of heterochromatin-induced PEV, were scored for changes in phenotype. The results reported in Table 1 indicate that all null phenotypes (ry− or y−) were due to gene mutation or loss rather than severe PEV; in these lines, marker gene expression was absent even when an extra *Y* was present. In contrast, many of the variable phenotypes were suppressed (± to +) by the extra *Y* chromosome, indicating that the variable expression was due to PEV rather than gene mutation or chromosomal instability.

Structures of *Dp1187* γ derivatives: Derivatives lacking *y*$^+$ or *ry*$^+$ expression presumably contained muta-

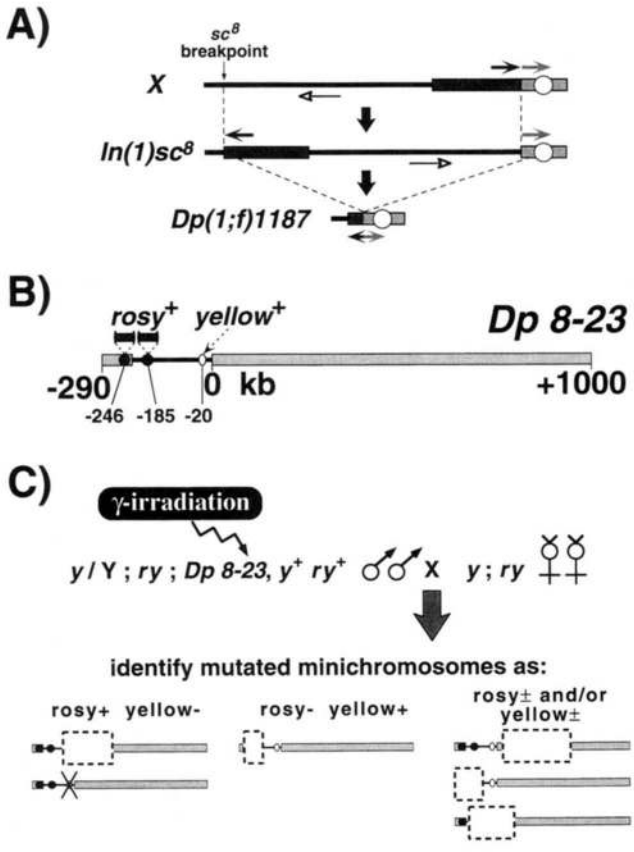

FIGURE 1.—Irradiation mutagenesis of *Dp1187*. (A) *Dp1187* origin and gross structure. *Dp(1;f)1187* was isolated by KRIVSHENKO and COOPER (LINDSLEY and ZIMM 1992) after irradiation-induced deletion of the *X* chromosome inversion $In(1)sc^8$ (see KARPEN and SPRADLING 1990 for a detailed description of the origin of *Dp1187*). The euchromatin (black line) retained in *Dp1187* is from the tip of the *X*, whereas the centric heterochromatin (gray box) is likely derived from the base of the *X*. Open circle marks the location of the centromere. The dark and light gray arrows show the orientation and gross positions of *Dp1187* heterochromatin regions, relative to the *X*. (B) Structure of *Dp 8-23*. Sequences to the left of the euchromatin/heterochromatin boundary ("sc^8 breakpoint," position 0 kb; see A) include *X*-tip euchromatin (black line) and the subtelomeric heterochromatin (gray box) (KARPEN and SPRADLING 1992); the 1 Mb of centric heterochromatin is shown as a gray box (0 to +1000 kb). Two $rosy^+$ (ry^+) marker genes (dark gray circles), introduced by *P*-element transposition, are present in the *Dp1187* derivative *Dp 8-23* (KARPEN and SPRADLING 1992; TOWER *et al.* 1993), in addition to the $yellow^+$ (y^+) gene (open oval) normally present in the *X* and *Dp1187* euchromatin. The kilobase coordinates correspond to those determined for the parental *Dp1187* (KARPEN and SPRADLING 1990, 1992). The actual size of *Dp 8-23* is 1320 kb, because each *PZ* insertion adds 14.5 kb to the 1290 kb length (see TOWER *et al.* 1993 for *PZ* structure and orientations). (C) The screen used to isolate *Dp 8-23* derivatives. Males were irradiated and crossed, and the F1 progeny screened for phenotypic changes in either the y^+ or ry^+ marker genes, as described in MATERIALS AND METHODS. Examples of possible types of internally altered derivatives, and their phenotypes, are shown below. ± indicates a variegating phenotype (mosaicism or variegation for *y*, intermediate expression for *ry*). Not shown are translocations with other chromosomes, and inversions.

tions or breakpoints (deletion, translocation or inversion) within the euchromatin of *Dp 8-23*, because the marker genes were located in this region of the chromosome. Two types of molecular analyses, PCR and pulsed-field Southern analysis, were used to elucidate the gross structures of the derivatives, and to identify *Dp γ* derivatives that also contained heterochromatic breakpoints.

A euchromatic primer and a primer corresponding to the 1.688 (359 bp) satellite were used in a PCR assay (see MATERIALS AND METHODS) to test for the presence of the original sc^8 breakpoint (the euchromatin/centric heterochromatin boundary, position 0 in Figure 1B). The results are summarized in Table 1. Three $y^- ry^+ Dp$ derivatives, *γ240*, *γ840*, and *γ1230*, failed to produce the appropriate PCR band, suggesting that these minichromosomes contained deletions that included both euchromatin and centric heterochromatin.

The gross molecular structures of all free duplication derivatives with altered y^+ or ry^+ phenotypes were determined by pulsed-field gel electrophoresis (PFGE) and Southern hybridization analyses. High molecular weight DNA was prepared from embryos in agarose inserts, and pulsed-field Southern blots were hybridized with the single-copy probes described in Figure 2A (see MATERIALS AND METHODS). Overall minichromosome size was determined by comparing the mobilities of the undigested free duplications to that of the 1.32 Mb parental *Dp 8-23*; representatives of all classes of derivatives are shown in Figure 2, B and C. The putative deletion derivatives identified by PCR analysis, *γ240*, *γ840* and *γ1230*, displayed bands substantially smaller than 1.3 Mb (1115, 1020 and 620 kb, respectively), after ethidium bromide staining (Figure 2B) or Southern hybridization (Figure 2C). Other derivatives, such as *γ1088*, *γ1111*, *γ1407*, and *γ878* displayed bands of 1245, 1130, 1077 and 1072 kb respectively; the presence of the sc^8 breakpoint in these derivatives (PCR, Table 1) suggested deletion of only euchromatin. Some derivatives, such as *γ238* and *γ158*, appeared very similar in size to the parental *Dp 8-23*. Finally, translocations, such as *γ1026*, exhibited substantially larger uncut chromosomes, beyond the separation range of these gels (≥1600 kb).

Pulsed-field Southern blots of uncut DNA were hybridized with the seven probes indicated in Figure 2A to determine which regions of *Dp1187* euchromatin were still present. An example of such an analysis performed on *γ1230* is shown in Figure 2D. *γ1230* retained sequences homologous to probes 6 and 8 (also 7, not shown), but lacked 1, 4 and 5 (also 2 and 3, not shown). Thus, the *γ1230* euchromatic breakpoint could be placed grossly between −75 and −122. Similar studies were performed on the other *Dp γ* derivatives (data not shown).

More precise localization of the euchromatic breakpoints, and of the structures of the derivatives, was accomplished by pulsed-field restriction analysis (see MA-

TABLE 1

Summary of phenotypic and structural analyses of *Dp8-23γ* irradiation derivatives

Line no.	Phenotype[a] *X/X* or *X/Y*	Phenotype[a] *X/XY* or *XY/Y*	NB[b]	PCR[c]	Notes[d]
			Deletions, inversions and insertions		
γ66	y±ry±	y+ry+	+	+	35 kb euchromatic deletion
γ238	y±−ry±	y±ry+	+	+	830 kb inversion, y±− partially *Y* suppressible
γ240	y−ry+	y−ry+	+	−	185 kb deletion
γ754	y±ry±	y+ry+	−	+	150 kb euchromatic deletion
γ840	y−ry±	y−ry+	+	−	300 kb deletion
γ878	y±ry−	y±+ry−	+	+	terminal deficiency, y± partially *Y* suppressible
γ1000	y±ry−	y±+ry−	+	+	terminal deficiency, y± partially *Y* suppressible
γ1088	y−ry+	y−ry+	+	+	75 kb euchromatic deletion
γ1111	y±ry±	y±+ry+	+	+	190 kb euchromatic deletion
γ1144	y*ry+	y*ry+	+	+	100 kb insertion, breakpoint between +50 and +1000, unusual *y* phenotype (dark brown cuticle)
γ1230	y−ry±	y−ry+	+[e]	−	700 kb deletion
γ1257	y±ry+	y+ry+	+	+	5 kb euchromatic deletion
γ1407	y±ry−	y±+ry−	+	+	terminal deficiency, *y*± partially *Y* suppressible
			Translocations		
γ121	y−ry+	y−ry+	−	+	T(Dp;2) and T(2;Dp), breakpoint at *y*
γ127	y−ry+	y−ry+	−	+	T(Dp;2) and T(2;Dp), breakpoint at *y*
γ162	y−ry+	y−ry+	−	+	T(Dp;2) and T(2;Dp) breakpoint at *y*
γ308	y−ry+	y−ry+	+[f]	−	T(Dp;X), breakpoint between −80 and −100
γ308	y±ry−	y+ry−	−	+	T(X;Dp), breakpoint between −80 and −100
γ379	y−ry+	y−ry+	+	−	T(Dp;Y), breakpoint between −100 and −160
γ379	y±ry−	y±+ry−	−	+	T(Y;Dp), breakpoint between −100 and −160; extra *Y* suppressed y± in abdomen but not thorax
γ817	y±ry+	y+ry+	−	+	T(Dp;3) and T(3;Dp), breakpoint between −40 and −100
γ849	y±y+	y+ry+	−	+	T(Dp;3) and T(3;Dp), breakpoint between −40 and −185
γ876	y−ry+	y−ry+	−	−	T(Dp;Y); breakpoint between −70 and −50
γ876	y±ry−	y+ry−	−	+	T(Y; Dp); breakpoint between −70 and −50
γ894	y±ry+	y+ry+	−	+	T(Dp;3) and T(3;Dp), breakpoint between −40 and −70
γ952	y−ry+	y−ry+	−	−	T(Dp;Y), breakpoint between −100 and −185
γ952	y±ry−	y±+ry−	−	+	T(Y;Dp), breakpoint between −100 and −185
γ1026	y±−ry+	y±−ry+	−	+	T(Dp;3) and T(3;Dp), breakpoint at *y*
γ1067	y±ry+	y+ry+		+	T(Dp;3) and T(3;Dp), breakpoint between −50 and −100
γ1227	y−ry+	y−ry+	+	−	T(Dp;Y), breakpoint between −70 and −185
γ1227	y±ry−	y+ry−	ND[g]	+	T(Y;Dp), breakpoint betwen −70 and −185
γ1235	y±ry−	y+ry−	ND	+	T(Y;Dp), breakpoint between −100 and −185
γ1235	y−ry+	y−ry+	−	−	T(Dp;Y), breakpoint between −100 and −185
γ1337	y−ry+	y−ry+	−	+	T(Dp;2) and T(2;Dp), breakpoint at *y*
γ1395	y−ry+	y−ry+	ND	−	T(Dp;Y), breakpoint between −100 and −185
γ1395	y±ry−	y±ry−	−	+	T(Y;Dp), breakpoint between −100 and −185
			No visible structural alterations[h]		
γ12	y*ry+	y*ry+	+	+	Unusual *y* phenotype, *y* wings, but rest of body appears normal
γ105	y±ry±	y±ry+	ND	+	Variegates for *y* and *ry*
γ140	y−ry+	y−ry+	+	+	
γ158	y−ry+	y−ry+	+	+	
γ182	y−ry+	y−ry+	+	+	
γ199	y*ry+	y*ry+	+	+	Unusual y phenotype, not *Y* suppressible
γ300	y±ry+	y+ry+	+	+	Variegates for *y*
γ359	y−ry+	y−ry+	+	+	
γ529	y−ry+	y−ry+	+	+	
γ648	y−ry+	y−ry+	+	+	
γ725	y±ry±	y±+ry+	ND	+	Variegates for *y*, y± partially *Y* suppressible
γ737	y−ry+	y−ry+	+	+	
γ742	y−ry+	y−ry+	+	+	
γ791	y−ry+	y−ry+	+	+	
γ792	y+ry±	y+ry+	+	+	Mutation in one of the *ry*+ genes?

TABLE 1

Continued

Line no.	Phenotype[a] *X/X* or *X/Y*	Phenotype[a] *X/XY* or *XY/Y*	NB[b]	PCR[c]	Notes[d]
			No visible structural alterations[h] (cont.)		
γ819	y±ry+	y+ry+	+	+	Variegates for *y*
γ862	y±ry+	y±+ry+	+	+	Variegates for *y*, y± partially *Y* suppressible
γ863	y−ry+	y−ry+	+	+	
γ869	y−ry+	y−ry+	+	+	
γ872	y−ry+	y−ry+	+	+	
γ875	y−ry+	y−ry+	ND	+	
γ888	y−ry+	y−ry+	+	+	
γ890	y−ry+	y−ry+	+	+	
γ910	y−ry+	y−ry+	ND	+	
γ918	y−ry+	y−ry+	ND	+	
γ919	y−ry+	y−ry+	ND	+	
γ921	y−ry+	y−ry+	ND	+	
γ1028	y*ry+	y*ry+	+	+	Unusual *y* phenotype; bristles on thorax nearly normal, abdomen cuticle chocolate brown in females, significantly darker in males
γ1244	y−ry+	y−ry+	+	+	
			Miscellaneous		
γ845	y−ry+	y−ry+	+		Complex rearrangement; T + inversion, T + insertion, or insertion + inversion

[a] + is wild-type, − is mutant, ± is a variegating phenotype (mosaicism or variegation for *y*, intermediate expression for *ry*); + > ±+ > ± > ±− > −. * indicates an unusual *y* phenotype (see Notes).

[b] Presence (+) or absence (−) of free duplication (minichromosome) in DAPI-stained larval neuroblast squashes (see MATERIALS AND METHODS).

[c] Presence (+) or absence (−) of the *sc*[8] breakpoint (position 0, Figure 1) as determined by PCR analysis (see MATERIALS AND METHODS).

[d] Additional information about derivatives; the breakpoint positions not described here are shown in Figure 4. T, translocation

[e] Minichromosome showed half the size and DAPI fluorescence of *Dp1187*.

[f] Minichromosome was approximately four times larger than *Dp1187*, similar in size and fluorescence intensity to the 4th chromosome.

[g] ND, not determined

[h] *y* or *ry* mutations with a structural change below the 5 kb resolution of these pulsed-field methods, including single- or multiple-base mutations.

TERIALS AND METHODS). As shown in Figure 3A, the structures of all regions of *Dp 8-23* can be analyzed with *Not*I, *Sfi*I and *Xho*I digests, after hybridization with the appropriate probes. An example of such an analysis of *γ1230* is shown in Figure 3B. Probe 1 hybridizes to an 1100 kb *Not*I fragment in *Dp 8-23* that includes the entire 1 Mb of centric heterochromatin (Figure 3B, left). This band is absent in *γ1230* because probe 1 is deleted in this derivative. Probe 6 is retained in *γ1230* (see above and Figure 2D); the 70 kb *Dp 8-23Not*I fragment that hybridizes with probe 6 is replaced with a 490 kb fragment in *γ1230* (Figure 3B, right *Not*I panel). The *Sfi*I digest probed with *rosy* sequences (probe 8) demonstrates that the region to the left of −185 is the same in *Dp 8-23* and *γ1230* (the 70 and 54 kb bands; Figure 3B). In contrast, the 155 kb *Sfi*I fragment that spans the −40 to −185 kb region of *Dp 8-23* (plus 10 kb present in the −185 *PZ* element) was absent in *γ1230* and is replaced by a 495 kb fragment. The first *Sfi*I site to the left of the *γ1230* breakpoint is 10 kb into the *PZ* element inserted at −185 (KARPEN and SPRADLING 1992), since the *Sfi*I site at −40 was deleted in this derivative. Thus, the size of the deletion in *γ1230* was calculated by subtracting 495 from 1195 kb rather than 1040 kb (the fragment size observed in *Dp 8-23*). These digests confirm that *γ1230* has a 700 kb deletion, consistent with the estimates from uncut DNA (Figure 2, B–D). Similar analysis of the *Not*I results led to the same conclusion. The *γ1230* euchromatic breakpoint was placed precisely at −121 by hybridizing pulsed-field Southern blots with a collection of subclones from the −70 to −122 region. Probes to the left of −121 were retained in *γ1230*, whereas those to the right of −121 were absent. Finally, the heterochromatic breakpoint in *γ1230* could be placed at +580 (Figure 4) by combining information on the position of the breakpoint (−121) with the size of the deletion (700 kb).

γ238 was a special case, initially noteworthy because of its unusual y^+ expression. Animals that carried *γ238* (identified by ry^+ phenotype) were nearly y^-, with only

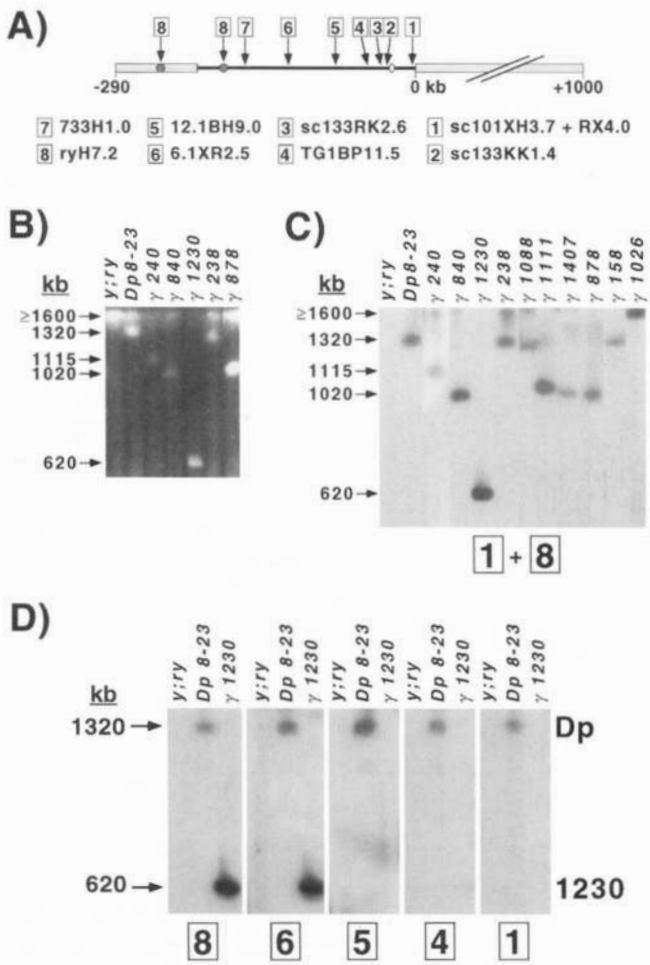

FIGURE 2.—Determination of the structures of *Dp1187* γ derivatives: analysis of uncut chromosomes. (A) Cloned euchromatic DNAs used as probes in the structural analyses of *Dp 8-23* γ derivatives. See MATERIALS AND METHODS for a description of the clones and their exact position within the *Dp1187* map. (B) Ethidium bromide stained pulsed-field gel. Electrophoresis of uncut high molecular weight DNA demonstrates the presence of different size minichromosomes. See MATERIALS AND METHODS for methods of DNA preparation and electrophoresis. Genomic pieces ≥1600 kb are not resolved under these pulsing conditions (40- to 100-sec pulses, 2-sec ramp, 28 hr) and accumulate in the wells (not shown) or the "compression zone" marked as ≥1600 kb). High molecular weight DNA from the *y*; *ry* background strain only shows staining in the ≥1600 kb region, whereas *y*; *ry* strains containing *Dp 8-23* or any of the γ derivatives shown above produces visible bands of 1320 kb or less. (C) Southern hybridization of uncut DNA from *Dp* γ derivatives. Probes 1 and 8 were used to visualize the sizes of the derivatives shown above, because not all derivatives could be seen after ethidium staining. All derivatives except *γ238*, *γ158* and *γ1026* are smaller than *Dp 8-23*; *γ238* and *γ158* are the same size, whereas *γ1026* is larger and remained in the compression zone. Pulse conditions were 50–110-sec pulses, 2-sec ramp, 28 hr. (D) Gross positioning of deletion breakpoints within the euchromatin. *γ1230* is used as an example of the method used for all the *Dp* γ derivatives. The same blot was hybridized sequentially with different probes from the *Dp1187* euchromatin. Probes 1, 4, 5, 6 and 8 hybridized to *Dp 8-23*, whereas only 6 and 8 were present in *γ1230*. This locates the euchromatic break between the left end of probe 5 and the right end of probe 6, between −80 and −120. Pulse conditions were the same as in C).

an occasional y^+ bristle, and a few y^+ spots on the posterior dorsal abdominal cuticle. This $y\pm$ phenotype was only partially suppressed by the presence of an extra *Y* chromosome (Table 1), suggesting that the alteration caused a strong position effect. The pulsed-field analyses of uncut DNA indicated that the overall size of *γ238* was not different from *Dp 8-23* (Figure 2, B and C), despite the dramatic change in phenotype. Similarly, all of the *Sfi*I (Figure 3C) and *Not*I (data not shown) fragments were the same in *γ238* and *Dp 8-23*. However, *Xho*I digestion revealed that a structural change had occurred between −40 and +50, changing the *Dp 8-23* 110 kb band into two bands of 280 and 12 kb (Figure 3C). These data suggested that *γ238* contained an inversion rather than a deletion, insertion or translocation. Hybridization with a series of probes from the 0 to −40 region placed the euchromatic breakpoint at −30 kb. The position of the heterochromatic breakpoint (+800, 200 kb from the end of the chromosome) was determined from the size of the larger *Xho*I fragment (200 kb = 280 kb *Xho*I fragment − 30 kb of euchromatin − 50 kb between the sc^8 breakpoint and the *Xho*I site at −50). The *γ238* structure shown in Figure 4 was further supported by the detailed restriction mapping described below. The *γ238* structure provides a likely explanation for the strong PEV phenotype—in this derivative only 30 kb of euchromatin, including the y^+ gene, lies between two large blocks of heterochromatin.

Pulsed-field restriction analysis was performed on all *Dp* γ derivatives in the same manner as described for *γ1230* and *γ238*. The structures determined by combining results from the molecular, genetic and cytological analyses are displayed in Figure 4. Three deletions were recovered with one euchromatic and one heterochromatic breakpoint: *γ240*, *γ840*, and *γ1230* contained minichromosomes of 1135, 1020 and 620 kb, respectively. As described above, *γ238* is an inversion with one euchromatic and one heterochromatic breakpoint. Purely euchromatic deletions were recovered, both interstitial (*γ66*, *γ1088*, *γ754*, and *γ1111*) and terminal (*γ878*, *γ1000*, and *γ1407*). Most of these large euchromatic deletions were recovered as lines with variegated *yellow* phenotypes ($y\pm$; Table 1). As described previously (TOWER *et al.* 1993; ZHANG and SPRADLING 1993), terminal and internal deletions within *Dp1187* euchromatin increase y^+ PEV, even when the deletions are located over 100 kb from the y^+ gene. Nearly half (29) of the derivatives had changes too small to be detected with these pulsed-field analyses (<5 kb). *γ158* is an example of this large class of derivatives (Figure 4 and Table 1). Finally, the fact that only one derivative (*γ845*, Table 1) had a structure that could not be determined demonstrates the benefits of using a molecularly defined chromosome for irradiation mutagenesis (see DISCUSSION).

Restriction mapping *Dp1187* heterochromatin reveals

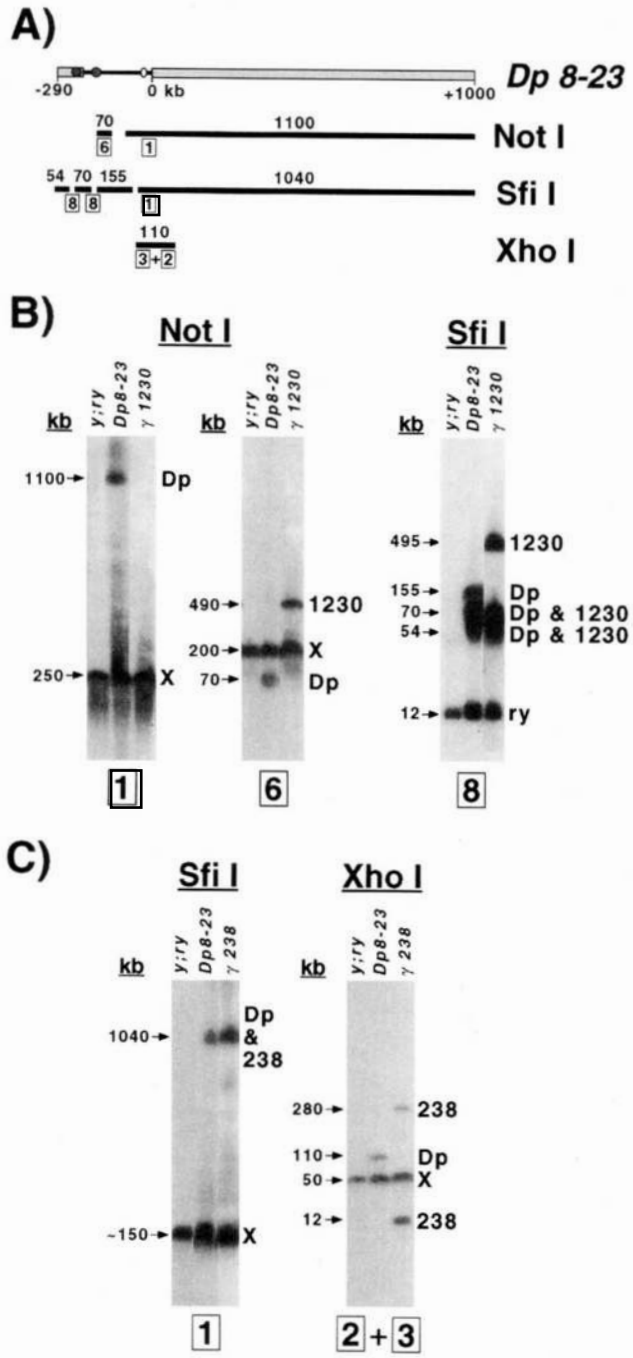

FIGURE 3.—Determination of the structures of *Dp1187* γ derivatives: analysis of digested DNA. (A) Sizes and positions of *Not*I, *Sfi*I and *Xho*I fragments used in the detailed analysis of *Dp 8-23* γ derivatives. Sizes (in kb) are shown above each fragment, whereas the appropriate probes (see Figure 2A) are shown boxed below. (B) Pulsed-field Southern analysis of *Not*I and *Sfi*I digests revealed the structure of *γ1230*. Sizes in kb are shown to the left of each blot, the source of each fragment (*X*, *Dp 8-23* or *γ1230*) to the right. The *Not*I digests probed with 1 (or 6 in the case of *γ1230*) reveal the structure of the centric heterochromatin, whereas the *Sfi*I digest probed with 8 (*rosy*$^+$) reveals the structure of the euchromatin and subtelomeric heterochromatin to the left of −40. The pulse conditions were 35–95-sec pulses, 2-sec ramp, 28 hr for *Not*I probe 1 and 35–105-sec pulses, 2-sec ramp, 22 hr for *Not*I probe 6 and *Sfi*I. (C) *Sfi*I and *Xho*I digests used to analyze the structure of *γ238*. The *Sfi*I digests probed with 1 demonstrate that the region to the right of −40 is the same in *Dp 8-23* and *γ238*, but the *Xho*I digests reveal that there is an alteration between −40 and +50. The normal size of the *Sfi*I fragment and the two new bands in *γ238* suggest the presence of an inversion, with a euchromatic breakpoint within probes 2 and 3. Pulse conditions for the *Sfi*I gel was 40–100-sec pulses, 2-sec ramp, 26 hr and for the *Xho*I digests 5–65-sec pulses, 2-sec ramp, 18 hr. See previous figures for a description of other symbols.

the presence of three islands of complex DNA: The isolation of *Dp1187* derivatives allowed us to investigate the detailed molecular structure of previously inaccessible parts of the heterochromatin. *Dp1187* deletion derivatives were restriction mapped by pulsed-field Southern analysis, using complete and partial digests with enzymes that cut frequently in euchromatin. Three derivatives with heterochromatic breakpoints (2 deletions, *γ240* and *γ1230*; one inversion, *γ238*) provided direct molecular access to regions beyond the +55/+90 island identified previously (KARPEN and SPRADLING 1990, 1992). We probed with euchromatic single-copy sequences adjacent to the new breakpoints in these derivatives, an approach used previously to map near the *Dp1187* euchromatin/heterochromatin junction (KARPEN and SPRADLING 1990).

The mapping strategy is illustrated in Figure 5, using *γ1230* and *Hin*dIII as an example. High molecular weight DNA was cut with *Hin*dIII, and blots from pulsed-field gels were probed with a single-copy fragment that lies between the last euchromatic cut site and the heterochromatin (probe 6). Due to a polymorphism in the euchromatin that included probe 6, the *X* chromosome produced a band of 92 kb, whereas an additional band of 58 kb was present in *Dp 8-23* DNA (Figure 5A). Note that all *Dp*-DNAs also contain *X*-specific fragments, because the minichromosome exists in addition to the normal chromosome complement. In *γ1230*, the 58 kb *Dp 8-23*-specific band was replaced by a unique 220 kb *Hin*dIII fragment. To determine the position of the first heterochromatic site for this enzyme, the position of the first euchromatic site to the left of the breakpoint had to be determined. The euchromatic *Hin*dIII site was placed 46 kb to the left of the −120 *Not*I site in *Dp 8-23* (at −166, Figure 5B) based on the results of *Not*I-*Hin*dIII double digests (data not shown). The 220 kb *γ1230Hin*dIII fragment was "anchored" in this manner to the −166 *Hin*dIII site, placing the first heterochromatic site 175 kb (220 minus 45) to the right of +580, at +755 (Figure 5B).

This "end-probed" mapping strategy was applied to *Dp 8-23*, *γ238*, *γ240* and *γ1230*, using the probes and enzymes presented in Figure 6A. *γ840* could not be used for this analysis because euchromatic clones from the vicinity of the breakpoint had not been isolated. Five separate but overlapping regions of *Dp1187* were mapped in this manner. Note that this strategy only

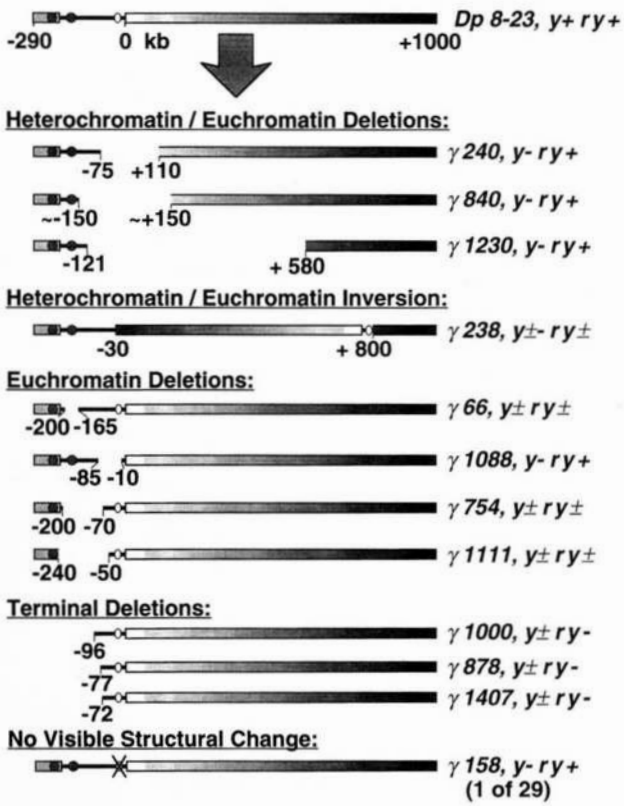

FIGURE 4.—Molecular structures of *Dp1187* γ derivatives. The structures and phenotypes of the *Dp* γ derivatives are shown in comparison to the parental *Dp 8-23*. The positions of the breakpoints are indicated below each drawing. The euchromatic breakpoint for γ*840* could only be grossly placed between −130 and −170 (−150 represents the average), because probes from this region were not available. The heterochromatic breakpoint for this 300 kb deletion was thus placed at +150, representing a range from +130 to +170. X indicates a change in the y^+ phenotype with no visible structural alteration. The centric heterochromatin is depicted with a shading gradient to highlight the structure of the γ*238* inversion. All other symbols are as described in previous figures.

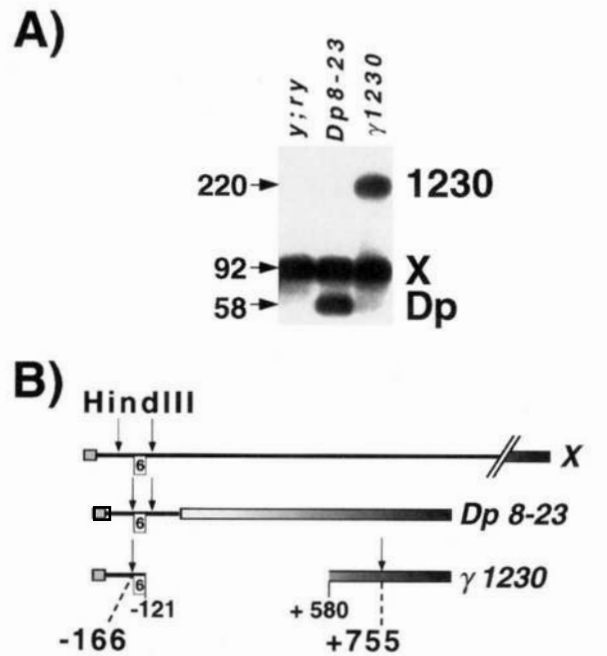

FIGURE 5.—Strategy for restriction mapping heterochromatin with *Dp1187* γ derivatives. (A) Example of Southern blot used to map a heterochromatic *Hin*dIII site with γ*1230*. Digests of DNA from the three strains were separated by pulsed-field electrophoresis (10–50-sec pulses, 1-sec ramp, 24 hr), blotted and hybridized with probe 6 as described in MATERIALS AND METHODS. Note that the 58 kb *Dp 8-23*-specific band is replaced by a 220 kb fragment in γ*1230*; all genotypes share the 92 kb *X*-specific band. (B) Positioning the heterochromatic *Hin*dIII site. The maps for the *X*, *Dp 8-23* and γ*1230* chromosomes are shown in the vicinity of probe 6. The +755 site was determined by adding the size of the heterochromatic portion of the γ*1230* fragment [175 kb = 220 − 45 (the size of euchromatic portion between the breakpoint and the "anchored" site (166 − 121 = 45 kb))] to the heterochromatic breakpoint (+580). The position of the anchored euchromatic site was determined from double digests of *Dp 8-23*. The site positions responsible for the *X* and *Dp Hin*dIII polymorphism are also shown.

allows the first heterochromatic site to be mapped when complete digests were performed. In some cases, partial digestions were used to map beyond the first site across the breakpoint (sites determined from partial digests are marked as *, Figure 6A). The *Dp1187* heterochromatin map shown in Figure 6B was generated by combining the maps from the individual derivatives. Many of the enzymes mapped to the same position in the different derivatives (circled sites in Figure 6A), supporting the validity of joining the maps in this manner. Examples of coincident sites include *Spe*I (+195), *Mlu*I (+289) and *Hpa*I (+401) in *Dp 8-23* and γ*240*, and *Hpa*I (+611) and *Sal*I (+733, +795) in γ*1230* and γ*238* (Figure 6A). Minor variations between the locations of sites in different derivatives (<5% of fragment lengths) were not considered significant. The amount of agreement between the separate maps is remarkably good, given the sizes of the fragments (especially for partial digests) and the resolution of these pulsed-field analyses. Significantly, there were no sites that contradicted the presence or absence of mappable sites in another derivative. For the combined map (Figure 6B), discrepancies in the positions determined from the individual maps (Figure 6A) were resolved by consensus, or by using the most accurately mapped site (smallest fragments and best resolved gels). The overlap between the maps also confirms that the derivatives were produced by internal deletion or inversion, rather than translocation of pieces from elsewhere in the genome.

The individual and combined restriction maps indicate that this 1 Mb of centric heterochromatin contains a surprising amount of substructure. Most notably, there are at least three regions of clustered restriction sites (black blocks, Figure 6B), separated by blocks of DNA with few sites (gray blocks, Figure 6B). Regions rich in highly repeated, simple sequence satellite DNA are expected to lack restriction sites, whereas clustering of 6 bp recognition sites should only occur when mid-

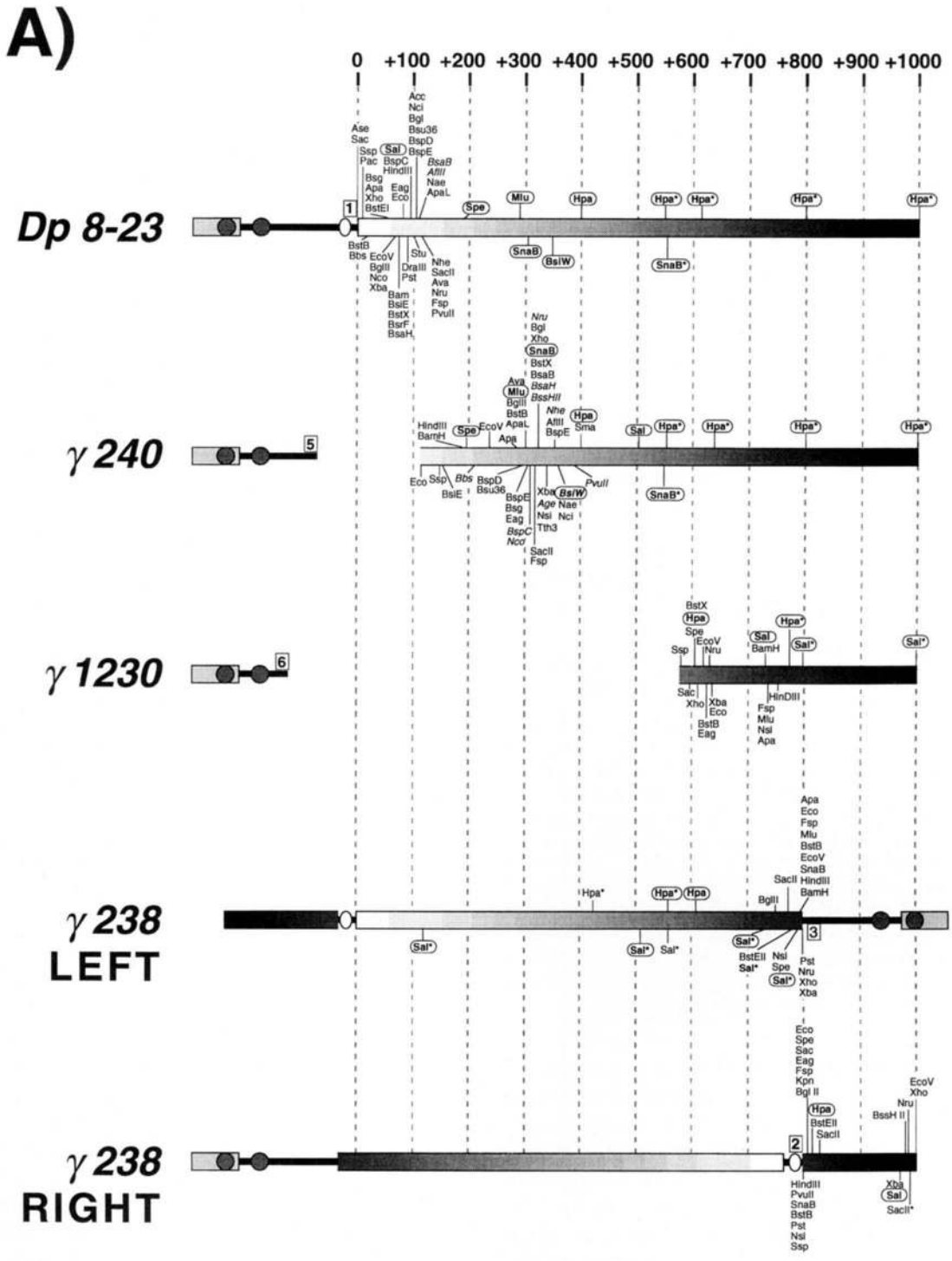

FIGURE 6.—Restriction maps of heterochromatin in *Dp1187* γ derivatives. (A) Restriction maps of individual derivatives. The separate maps generated from each of these derivatives are shown, with products of partial digests designated by an asterisk (*). Sites shown in italics were determined from fragment size and were not anchored in the euchromatin. Circled restriction sites coincide with sites mapped in at least one other derivative. Probes used for each map are shown in boxes. *γ238* is shown in two parts, corresponding to the left and right halves of the inversion, so the alignment of all five regions could be displayed. (B) Combined restriction map of *Dp1187* heterochromatin. The euchromatic sites for the enzymes that recognize 6 bp sites have been omitted for clarity. Black boxes are the islands of complex DNA *Tahiti, Moorea,* and *Bora Bora.* Grey blocks in center of *Bora Bora* indicate that this could consist of one large island, or two separate islands. 1.688 (= 359 bp) and 1.672 (= AATAT) show the approximate locations of these satellites (see text and Figure 7A); the exact distribution of the satellites is not known at this time (black vertical bars). The restriction map is complete for *Asc* I, *Not* I and *Sfi*I; it is likely complete for *Hpa*I and *Sal*I, but only in the heterochromatin. The map may be complete for other enzymes, but this cannot be determined from the method used (end-probing and complete digests). All other symbols are described in previous Figures.

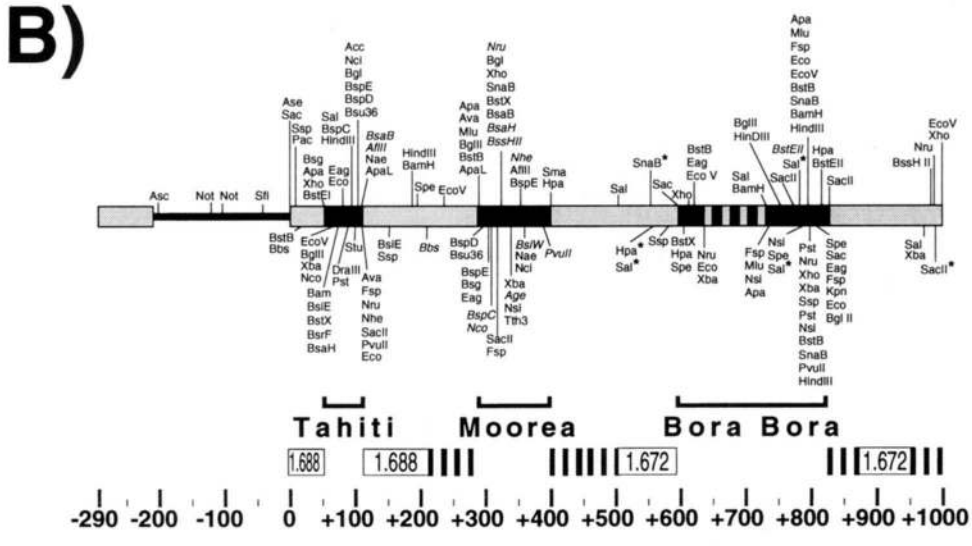

FIGURE 6.—*Continued*

dle-repetitive or single copy sequences are present (complex DNA). We called the regions of clustered restriction sites "islands of complex DNA," distinguished by the names *Tahiti*, *Moorea* and *Bora Bora*. The island *Tahiti* was partially mapped in previous studies (KARPEN and SPRADLING 1990, 1992), but the number of islands and their overall organization within centric heterochromatin could only be determined with the more extensive mapping presented here. *Tahiti* includes 60 kb of DNA (+50 to +110), and is separated from the 110 kb island *Moorea* (+290 to +400) by 180 kb. There are few restriction sites in the 200 kb region between *Moorea* and the 230 kb island *Bora Bora* (+600 to +830); however, the distribution of mapping entry points provided by the derivatives makes the map somewhat less reliable in this region (see DISCUSSION). The organization of restriction sites within *Bora Bora* suggests that it contains two separate regions with complex DNA of 40 and 100 kb (+600 to +640, +730 to +830), separated by 90 kb without restriction sites (small gray blocks within *Bora Bora*, Figure 6B); further mapping is necessary to determine whether this central region contains satellite DNA (see DISCUSSION). Finally, the right end of the chromosome contains a cluster of 6 bp recognition sites spread over a 30 kb region (+970 to +1000, Figure 6B). This region may contain the middle-repetitive sequences found in Drosophila subtelomeric heterochromatin, previously identified at the telomeres of *Dp1187* (KARPEN and SPRADLING 1992), chromosome *3* (LEVIS *et al.* 1993), and other chromosomes (VALGEIRSDOTTIR *et al.* 1990; BIESSMANN *et al.* 1993).

Finally, the region between *Tahiti* and *Moorea* includes the junction produced when *Dp1187* was generated from *In(1)sc*^{8} (tail-to-tail junction between dark and light gray arrows, Figure 1A). This junction lies between +195 and +236; the *Dp1187* and *In(1)sc*^{8} restriction maps are identical from 0 through the +195 *Spe*I site (Figure 6B) and diverge after +236 (the position of a *Hpa*I site found only in *In(1)sc*^{8}, data not shown). Therefore, *Tahiti* was present in the parental *In(1)sc*^{8}, and was not created during the production of *Dp1187*. Similarly, *Moorea* starts at +290, 54 kb from the furthest possible transition point at +236, and therefore is unlikely to have been fabricated during *Dp1187* production. However, the orientations and positions of *Tahiti* and *Moorea* are different in the normal *X*. *Tahiti* must be reversed with respect to *Moorea*, in comparison with their orientation in the normal *X*, and they likely come from somewhat different parts of the *X* heterochromatin (see Figure 1A).

Gross localization of satellite DNAs within *Dp1187*: We have grossly localized the 1.688 (359 bp) and 1.672 (AATAT) satellites within *Dp1187* heterochromatin, using these DNAs as probes in pulsed-field Southern analyses. Although these satellites are present at many sites within the Drosophila genome (LOHE *et al.* 1993), in this case they can be mapped to specific domains in *Dp1187* because PFGE separates intact minichromosomes from the rest of the genome. The presence of 1.688 sequences in uncut *Dp1187*, *γ240*, *γ840* and *γ238*, but not *γ1230* (Figure 7A), indicates that the majority of this satellite is present between 0 and +580, most likely in two separate blocks (Figure 6B). One block corresponds to the region between 0 and the start of *Tahiti*, previously shown to contain at least some 1.688 satellite by sequence and restriction analyses (GLASER *et*

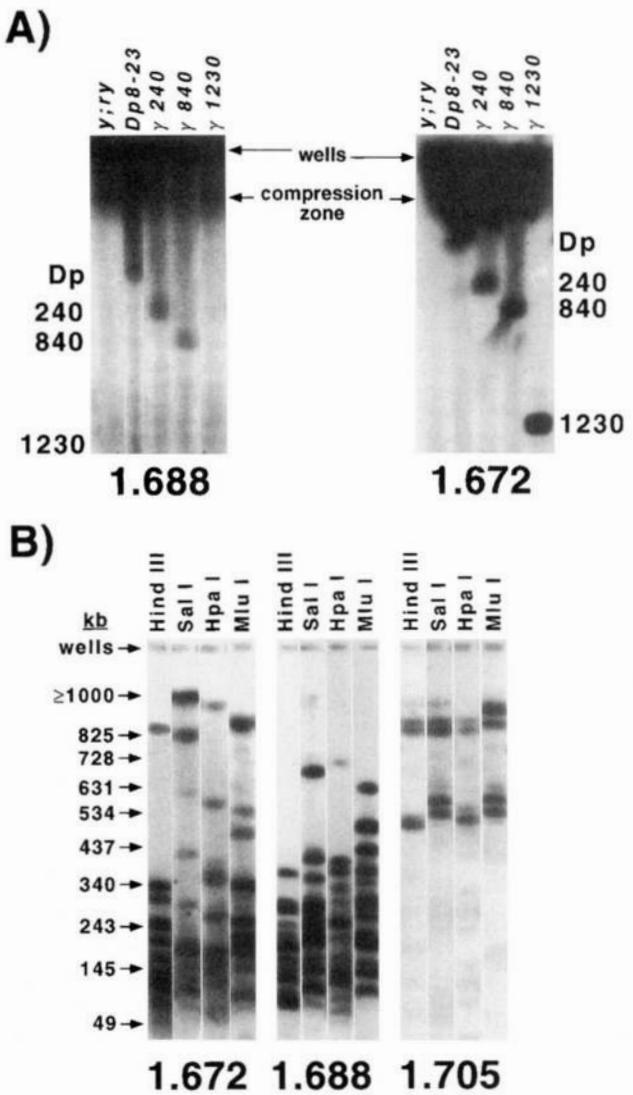

FIGURE 7.—Satellite DNA distribution in *Dp1187* and other heterochromatic regions. (A) Gross localization of satellites in *Dp1187* heterochromatin was accomplished by hybridization to pulsed-field Southern blots of uncut DNA from the derivatives shown above each lane (pulse conditions: 50–110-sec pulses, 2-sec ramp, 28 hr). 1.672 = AATAT, 1.688 = the 359 bp complex satellite. Note that *γ1230* is the only derivative that lacks the 1.688 and that *γ1230* has approximately as much 1.672 as the other derivatives after normalizing for the amount of minichromosome DNA (data not shown). (B) Analysis of satellite-containing regions in the whole Drosophila genome. A pulsed-field Southern blot of high molecular weight *y* ; *ry* DNA digested with the indicated enzymes was probed sequentially with the satellite DNAs indicated below (pulse conditions: 10–70-sec pulses, 2-sec ramp, 26 hr). Sizes in kb are shown to the left. 1.705 = AAGAG.

al. 1992). However, the presence of 1.688 in derivatives that lack this region and most or all of *Tahiti* (*γ240* and *γ840*) also placed 1.688 DNA to the right of +110. The absence of 1.688 in *γ1230* demonstrates that the last 420 kb of *Dp1187* heterochromatin lacks this satellite but does not rule out the presence of 1.688 in the region between the end of *Moorea* and +580.

Similar experiments indicate that all of the *Dp γ* derivatives contain the 1.672 repeat, including the severely deleted *γ1230* derivative (Figure 7A). In fact, *γ1230* retains most of the 1.672 repeat present in *Dp1187*, placing the majority of these AATAT repeats in the region between +580 and +1000 (signals were normalized for total DNA present in the uncut bands, determined by hybridization with a single copy probe; data not shown). The absence of restriction sites in the region from +820 to +970 (Figure 6B) for a large number of enzymes suggests that this region contains a block of 1.672. Restriction sites are also absent in the region from +580 to +600 (Figure 6, A, *γ1230* map, and B); in fact, a collection of 14 enzymes that recognize 4 bp sites failed to cut in this 20 kb region (data not shown). The presence of 1.672 satellite between +580 and +600 is suggested by two studies. First, only one enzyme, *Ssp*I, cuts in this 20 kb region (Figure 6), and it recognizes a variant of AATAT (AATATT recognition site). Second, combining a primer from the euchromatic side of *γ1230* and a 30-mer of ATATT (sequence anti parallel to AATAT) results in the PCR amplification of fragments > 350 bp only from *γ1230*-containing DNA (data not shown). Finally, there is not enough data to support or refute the possibility that the central portion of *Bora Bora* (+640 to +720, Figure 6B) contains 1.672 satellite DNA. Further studies using the restriction map of *Dp1187* heterochromatin should more precisely localize these satellite DNAs and help determine their overall organization.

Other regions of Drosophila heterochromatin are organized as alternating blocks of satellite and complex DNA: The organization of *Dp1187* centric heterochromatin into islands of complex DNA separated by blocks rich in satellite DNA could be specific to this minichromosome and *In(1)sc*^{8} (see above). Southern restriction analysis was performed on total genomic DNA, with satellite probes, to determine whether the island organization is conserved throughout Drosophila centric heterochromatin. High molecular weight embryo DNA was digested with *Hin*dIII, *Sal*I, *Hpa*I, or *Mlu*I, and fragments ranging from 10 to 1000 kb were separated on pulsed-field gels. Southern blots were hybridized sequentially with the simple satellite 1.672–38 (AATAT), the complex 359 bp satellite 1.688, and the simple sequence satellite 1.705–42 (AAGAG). The fragment-size distribution for 6 bp recognition sites should reflect the distance between complex sequences. If the satellite DNAs are organized as long blocks without regularly spaced complex islands, then fragments should have been predominantly >1000 kb, as is seen for the rare cutters *Not*I and *Sfi*I (data not shown). However, for all enzymes and both the 1.672 and 1.688 satellites, relatively low molecular weight fragments (50–500 kb) were predominant (Figure 7B). There were fewer fragments observed for the less prevalent 1.705 satellite, which were distributed in the 500–600 and 800–900 kb ranges. The fact that different enzymes produce sim-

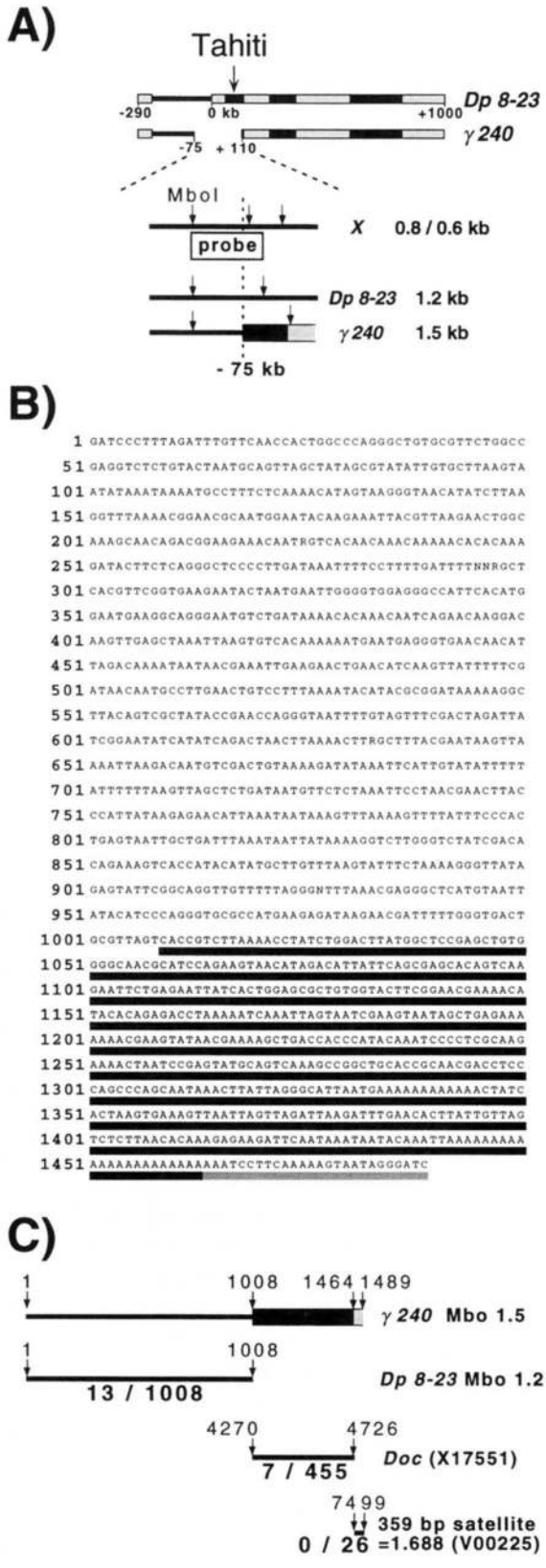

FIGURE 8.—The island *Tahiti* contains sequences homologous to the *Doc* retroposon. (A) Strategy for cloning a region of the island *Tahiti*. The overall structures of *Dp 8-23* and *γ240*, with an expansion of the −75 region, are shown. The positions of the *Mbo*I sites (gray lines) are indicated above each chromosome (*X*, *Dp 8-23* and *γ240*), and the sizes of the fragments seen after hybridization with the probe (a subclone of 5, see Figure 2) are shown to the right. All other symbols are described in previous figures. (B) Sequence of the *γ240Mbo* 1.5 kb fragment. The euchromatic portion of the sequence is indicated by a thin underline and was determined by comparison to the sequence of the *Dp 8-23Mbo* 1.2 fragment. The thick black underline represents the region homologous to the 3′ end of the *Doc* retroposon, and the thick gray underline indicates sequences homologous to the 1.688 satellite. (C) Extent of homology of the *γ240Mbo* 1.5 sequence to *Dp 8-23Mbo* 1.2, *Doc* and 1.688 satellite DNAs. The GenBank accession numbers for the *Doc* and 1.688 clones are shown in parentheses, and the nucleotide positions for the homologous regions are shown at the ends of each line. Above each line the number of mismatches plus gaps/total number of nucleotides are indicated.

ilar size fragments, for a particular satellite, is consistent with clustering of sites at the ends of satellite blocks. We conclude that the majority of the satellite-containing regions of the Drosophila genome are organized as alternating blocks of satellite and complex DNA, similar to the organization of the 1 Mb of *Dp1187* heterochromatin. In fact, the size distribution seen in Figure 7B suggests that the spacing of islands in the genome (predominantly 100–400 kb) is very similar to the separation of the *Dp1187* islands.

The complex island *Tahiti* contains sequences homologous to the *Doc* retroposon: We cloned a small portion of *Tahiti* to gain some knowledge of the sequence composition of the islands. The presence of cloned single copy sequences adjacent to heterochromatin in *Dp1187* deletion or inversion derivatives provides a method for isolating fragments that span the breakpoint and include euchromatin plus the desired region of heterochromatin. This approach was used previously to isolate a clone that crossed the sc^8 breakpoint in *Dp1187* (position 0, Figure 6B), demonstrating the presence of the 1.688 satellite (GLASER *et al.* 1992). Here we used the *γ240* derivative, and probe 5, to clone a 1.5 kb *Mbo*I fragment that included euchromatic sequences from the −75 region fused to heterochromatic DNA from +110, the right end of *Tahiti* (Figure 8A). A 1.2 kb *Mbo*I fragment from the parental *Dp 8-23* also was cloned to identify the euchromatic portion of the *γ240* clone. *Mbo*I polymorphisms allowed these minichromosomal DNAs to be separated from homologous fragments present on the *X* chromosome (0.8 + 0.6 kb, Figure 8A). Briefly (see MATERIALS AND METHODS for details), separate plasmid libraries were produced from size-selected *Mbo*I fragments present in *γ240* and *Dp 8-23* DNA and then plated and screened with probe 5. The sequences of the 1.2 kb *Dp 8-23* fragment and the 1.5 kb *γ240* fragments were compared (see MATERIALS AND METHODS) and shown to be highly homologous over the first 1012 bases (only 12 mismatches and 7 gaps, Figure 8C, most due to sequencing errors or ambiguities), this comparison identified the euchromatic portion of the *γ240* clone (no underline, Figure 8B).

The remaining 480 bases represents DNA from *Tahiti* (black and gray thick underline, Figure 8B). A database search revealed that two types of repeated DNA were contained in this region. Bases 1012–1468 (black bar, Figure 8B) contained a near-perfect match (9 mismatches in 457 bases, Figure 8C) to the 3′ end of the *Doc* retroposon (O'HARE *et al.* 1991), whereas the last 23 bases of the clone (gray bar, Figure 8B) were identical to a portion of the 1.688 satellite (Figure 8C). Thus, *Tahiti* contains at least a partial *Doc* retroposon. The position of the *γ240* heterochromatic breakpoint (+110) suggests that the *Doc* sequence may lie at the end of the island *Tahiti* and that the 1.688 satellite sequence represents the start of a block of satellite that separates *Tahiti* and *Moorea* (Figure 6B). It is unclear at this time whether the islands are composed entirely of middle-repetitive transposons or if single copy (genes?) and/or satellite DNA also are present.

DISCUSSION

The presence of repeated DNAs, especially highly repeated simple satellite, has made the molecular-genetic dissection of the heterochromatic regions of multicellular eukaryotic chromosomes extremely difficult. Consequently, the sequences responsible for many of the functions associated with heterochromatin, such as kinetochore formation and sister chromatid cohesion, remain obscure. We have focused on understanding the structure and function of one region of Drosophila heterochromatin, the 1 Mb of centric heterochromatin present in the minichromosome *Dp1187*. Here we described the generation and structural analyses of rearranged *Dp1187* derivatives. Three internal deletions and an inversion were recovered that provided molecular entry points into previously inaccessible portions of the *Dp1187* heterochromatin. End-probed pulsed-field restriction mapping revealed the presence of at least three islands of complex DNA, named *Tahiti*, *Moorea* and *Bora Bora*. These islands were separated by blocks deficient in 6 bp restriction enzyme recognition sites. This digestion pattern suggests that these interisland "seas" contain satellite sequences, whereas the complex islands must contain middle-repetitive and/or single-copy sequences. Southern hybridization analysis grossly localized two known Drosophila satellite DNAs within the *Dp1187* heterochromatin. Satellite probes hybridized to digests of total genomic DNA revealed that the organization seen in *Dp1187* is ubiquitous: Drosophila satellites are organized as relatively short (100–400 kb) blocks separated by complex DNA. Finally, cloning of a small portion of the island *Tahiti* demonstrated the presence of sequences homologous to the *Doc* retroposon (O'HARE *et al.* 1991), juxtaposed with satellite DNA. We conclude that there is a surprising and significant amount of substructure deep within Drosophila centric heterochromatin and that the presence of complex DNA within these regions will greatly facilitate future structural and functional studies. These results, in combination with previous studies (YOUNG and SCHWARTZ 1981; DEVLIN *et al.* 1990a; DIBARTOLOMEIS *et al.* 1992; GATTI and PIMPINELLI 1992; KARPEN and SPRADLING 1992; BIESSMANN *et al.* 1993; LEVIS *et al.* 1993; ZHANG and SPRADLING 1995), indicate that chromosomes in multicellular eukaryotes do not contain domains with distinct molecular components, as suggested by use of the cytological terms euchromatin, *α* heterochromatin and *β* heterochromatin. Rather, at the molecular level, chromosomes are a continuum of changing sequence composition, containing regions that differ in the relative amounts, and not the strict presence or absence, of highly repetitive, middle-repetitive and single-copy DNAs.

The utility of generating random rearrangements in a chromosome of known molecular structure: The pioneering studies of H.J. MULLER (MULLER 1930) introduced irradiation mutagenesis to the study of chromosome biology and gene function. Induced chromosome rearrangements have demonstrated the importance of chromosome position and interhomologue communication to gene expression (reviewed in TARTOF and HENIKOFF 1991; KARPEN 1994), increased our knowledge of chromosome functions (*e.g.*, meiotic disjunction) (GRELL 1976; HAWLEY *et al.* 1993) and facilitated gene cloning (BENDER *et al.* 1983). However, induced rearrangements have had limited use in understanding the molecular basis for heterochromatin structure and function. The limits of cytogenetic analysis in determining the structure and complexity of rearrangements is even more pronounced for heterochromatin, where the resolution of the banding patterns (GATTI and PIMPINELLI 1992) is far below that available for the euchromatin (SAURA *et al.* 1993). The overall structure and composition of rearranged chromosomes must be understood to identify reliably the structural components responsible for chromosomal functions, for example, the sequences involved in meiotic homologue pairing. Irradiation mutagenesis of a chromosome that is small enough to be analyzed in its entirety with PFGE, as described here for *Dp1187*, circumvents these problems. Although breaks were still induced at random, the structures of the derivatives could be confirmed with restriction mapping. Our studies also demonstrate that a large amount of structural information can be obtained from only a few breakpoints within a molecularly defined chromosome, demonstrating that random induction of breaks can be a useful tool. Finally, it is likely that more types of rearrangements were recovered in this analysis because the minichromosome is nonessential; the lethal-mutable genes present on *Dp1187* are also present on the *X*. Only rearrangements that eliminated chromosome transmission (*e.g.*, a centromere deletion) and deletions that removed both marker genes could not be recovered in this screen.

The structure of *Dp1187* heterochromatin: Here we demonstrate by molecular-genetic methods that Drosophila centric heterochromatin contains blocks or islands of complex DNA that alternate with stretches of highly repeated satellite sequences. Surprisingly, complex islands constitute nearly half of the 1 Mb of *Dp1187* centric heterochromatin. Although the methods and results described here provide insight into understanding the structure of this difficult region of the genome, there are many questions left unanswered.

From the digestion patterns, it is clear that the *Dp1187* islands must contain single-copy and/or middle-repetitive sequences. Cloning and sequencing demonstrated that one small region of *Tahiti* contains sequences homologous to the *Doc* retroposon. Previous *in situ* and blot hybridization analyses have suggested that transposable elements are abundant in Drosophila β-heterochromatin (YOUNG 1979; CAIZZI *et al.* 1993; CHARLESWORTH *et al.* 1994a) and in clones derived from the *Y* chromosome (HOCHSTENBACH *et al.* 1994). Recently, *in situ* hybridization to metaphase chromosomes was used to demonstrate that sequences with homology to known transposable elements are present in more proximal regions of the centric heterochromatin (PIMPINELLI *et al.* 1995). However, the resolution of *in situ* hybridization to metaphase chromosomes is low (approximately 500 kb to 1 Mb), and the extent of homology and functional conservation of heterochromatic transposons could not be addressed with these studies. The greater resolution of the *Dp1187* restriction map provides detailed information on the molecular organization of complex DNA in centric heterochromatin; conversely, the *in situ* hybridization studies (PIMPINELLI *et al.* 1995) strongly suggest that the identification of a *Doc* retroposon in *Tahiti* is representative, and that complex islands in general are likely to contain transposons or transposon fragments.

Given our limited information at this time, it is possible that the islands in "deep" centric heterochromatin contain a wide spectrum of complex DNAs, including incomplete and complete transposons, and even mutable genes. The molecular resolution of our pulsed-field mapping (in kb) also leaves open the possibility that the islands contain short stretches of satellite DNA mixed with the complex sequences. In fact, the *Tahiti* clone contained 1.688 satellite in addition to the *Doc* DNA. Interspersion of satellite and transposons has been found in randomly isolated genomic clones reported in the literature (CARLSON and BRUTLAG 1978; LOHE and BRUTLAG 1987; HOCHSTENBACH *et al.* 1993). One interesting cosmid clone contained two *Doc* elements flanking a 1.688 satellite monomer (SLOBODKIN and ALATORTSEV 1992). Despite the presence of similar sequences, this cosmid and the *Tahiti* clone are clearly distinct, since the *Doc* insertion sites within the 1.688 satellite are different. Furthermore, the chromosomal location of this cosmid, and other clones with satellite-transposon interspersion, is unclear. These clones could be derived from β heterochromatin or satellite blocks and not necessarily centric islands. Their origin could even be euchromatic, since 1.688-like sequences are present at a number of locations on the euchromatic *X* chromosome (DIBARTOLOMEIS *et al.* 1992). In contrast, the methods used here produced a heterochromatic clone (*γ240 Mbo* 1.5) whose genomic origin is known precisely. We do not believe that the *Tahiti* clone demonstrates interspersion of satellite and complex DNA within the island. Rather, the position of the *γ240* breakpoint at +110 strongly suggests that it is close to the right end of the island, and that the *γ240Mbo* 1.5 clone represents the transition point between the island and the 1.688 interisland satellite block. Cloning and sequencing of more extensive portions of *Tahiti, Moorea* and *Bora Bora* will be necessary to determine the types of sequences present in the islands, and their organization.

In most cases we used end-probing of complete digests to map only the first restriction site adjacent to the breakpoint, leaving open the possibility that additional sites exist beyond the mapped site. For example, from the *Dp 8-23* mapping we could not determine whether there were other *Eco*RI sites within *Tahiti*, in addition to the +81 site. In fact, the *γ240Mbo* 1.5 clone contains an *Eco*RI site, placing at least one other site within *Tahiti*, at +109.5. The use of partial digests, the overlap between maps, and the distribution of the breakpoints give us confidence that the general structure is as described for most of the 1 Mb of *Dp1187* heterochromatin, especially the regions between 0 and +400 kb and +580 to +1000. However, some regions of *Dp1187* heterochromatin are incompletely mapped because of the distribution of heterochromatic breakpoints and the mapping method. In particular, the region between *Moorea* and *Bora Bora* clearly contains satellite DNA, but it could also contain another island. The results of partial digests suggest that if another island exists within this region, it could only be near +550 (Figure 6B).

The center of the island *Bora Bora* contains a 90 kb region that failed to digest with the enzymes used to map *γ1230* and *γ238*. This is not likely to be an artifact of the end-probing method. In the *γ1230* mapping, nine different enzymes (seven complete and two partial) failed to cut before and within this block yet cut farther away, between +730 and +800. It is possible that *Bora Bora* is really two islands, the first present between +600 and + 640 (to the *Xba*I site, Figure 6A) and the second from +730 to +830. Alternatively, the +640 to +730 region may contain complex DNA that fails to digest due to an unusual chromatin or secondary structure (GOTTSCHLING 1992; SINGH and KLAR 1992; WRIGHT *et al.* 1992) or base modification (GOMMERS-AMPT *et al.* 1993) that might persist through the relatively mild high molecular weight DNA preparation used here. We have observed that other regions of

Dp1187 heterochromatin digest incompletely with some enzymes, despite complete digestion of euchromatin in the same samples. An aberrant nucleosome pattern has been observed in the single-copy central core of the *S. pombe* centromere (POLIZZI and CLARKE 1991; UEKI *et al.* 1993; ALLSHIRE *et al.* 1994); perhaps this property is shared with Drosophila centromeres. It is important to note that although we have confirmed the presence of 1.672 AATAT satellite within the +580 to +600 region, no known satellite has been definitively associated with the +640 to +730 region.

Finally, the distribution of restriction sites at the right end of the *Dp1187* map (Figure 6B) suggests that heterochromatic telomeres may share structural features with euchromatic telomeres. The enzymes listed near the right end of the minichromosome may not correspond to sites, since the end of any chromosome acts as a universal "polylinker." However, the sites we have mapped in the right end of *Dp1187* are spread over a 30 kb region, from +970 to +1000. This region likely contains complex DNA. Further analyses will be necessary to determine whether the middle-repetitive components of subtelomeric regions seen in Drosophila (YOUNG *et al.* 1983; DANILEVSKAYA *et al.* 1992; KARPEN and SPRADLING 1992; LEVIS *et al.* 1993) and many other species (BROWN *et al.* 1990; DE LANGE *et al.* 1990; LOUIS and HABER 1992) are present in the *Dp1187* heterochromatic telomere. It will be interesting to determine whether the presence of adjacent satellite DNA, and the proximity to the centromere in the case of *Dp1187* and other acrocentric chromosomes, significantly affects the structural and functional evolution of heterochromatic telomeres.

The structure of satellite DNA blocks in Drosophila and other multicellular eukaryotes: *In situ* hybridization to metaphase chromosomes (BONACCORSI and LOHE 1991; LOHE *et al.* 1993) and density gradient centrifugation (BRUTLAG *et al.* 1977a; BRUTLAG *et al.* 1978) suggested that the satellite DNA present in Drosophila α or centric heterochromatin is organized into large (>1 Mb) blocks; however, the resolution of these methods are poor, leaving room for more structural complexity. We have shown here that complex islands alternate with blocks of satellite DNA in the centric heterochromatin of *Dp1187* and the Drosophila genome in general. However, even greater complexity in heterochromatin structure is hinted at by the identification of some restriction sites within the satellite blocks (*e.g.*, the block between *Tahiti* and *Moorea*, Figure 6B). These restriction sites could result from rare mutations within relatively "pure" arrays of satellite DNA (LOHE and BRUTLAG 1986). Alternatively, very short regions of complex DNA ("miniislands"), or perhaps even individual transposons, could be present within the satellite blocks. Determining the extent and precise organization of the satellites within these regions is the focus of ongoing detailed restriction mapping.

Published studies have described the gross organization of satellite DNAs in other multicellular organisms. Pulsed-field restriction analysis using chromosome-specific human alphoid satellite DNA probes (WILLARD *et al.* 1986) produced megabase-size fragments, suggesting a structure that differs from that reported here for Drosophila (JABS *et al.* 1989; WEVRICK and WILLARD 1989; MAHTANI and WILLARD 1990; ARN *et al.* 1991). However, recent analyses have shown that the centromeric regions of human chromosomes do contain complex DNAs, such as LINE elements (WEVRICK *et al.* 1992). It is also possible that complex DNAs are well represented in alphoid satellite arrays, but that they are not accessible to restriction enzymes, as suspected for regions of *Dp1187* heterochromatin. More precise molecular mapping, and direct testing for the presence of complex islands, will require single-copy entry points deep within the mammalian alphoid arrays. The genetic approaches for generating and characterizing rearranged derivatives, described here for a Drosophila minichromosome, would be difficult to apply to mouse or human systems. However, many rearranged chromosomes with heterochromatic breaks exist in nature and tissue culture cells, arise frequently in cancer (LARIZZA *et al.* 1989; DUTRILLAUX *et al.* 1990; HAAS 1990) and could be used to probe heterochromatin structure in humans and mice. In addition, mammalian minichromosomes (CARINE *et al.* 1989; HAAF *et al.* 1992) could be very useful in future studies of heterochromatin structure and function.

The evolution and function of complex islands: Do islands of complex DNA function in evolution or development? It is possible that the islands contain single-copy genes, such as those identified in β heterochromatin (DEVLIN *et al.* 1990a,b; EBERL *et al.* 1993). Genes located deep within centric heterochromatin would likely be regulated in an unusual manner, since the presence of extensive satellite DNA usually represses euchromatic gene function (PEV) (reviewed in KARPEN 1994). SANDLER and co-workers (PIMPINELLI *et al.* 1985) hypothesized that a heterochromatin location may be reserved for genes that are expressed at unusual times in development. One example is the preblastoderm stages in Drosophila, when the absence of visible heterochromatin (FOE and ALBERTS 1985; VLASSOVA *et al.* 1991) could permit the expression of heterochromatic genes. If low copy number transcription units are present in heterochromatin, perhaps these are genes that need to be repressed completely in most cells and stages (PIMPINELLI *et al.* 1985). Cloning and sequencing of the islands will be necessary to determine whether coding sequences are present and whether they represent specially regulated genes.

The islands also may contain sequences necessary for normal chromosome inheritance, such as kinetochore formation (BLOOM 1993), sister-chromatid cohesion (MIYAZAKI and ORR-WEAVER 1994), meiotic pairing

(HAWLEY *et al.* 1993; HAWLEY and THEURKAUF 1993), and replication. The transmission behavior of *Dp1187* derivatives indicates that the island *Bora Bora* is an essential component of the *Dp1187* centromere (MURPHY and KARPEN 1995a), but further investigations are necessary to determine whether islands in general play a role in inheritance. The *Dp1187* derivatives described here provide important tools for future functional analyses of the islands, and of heterochromatin in general.

The replication of heterochromatin poses a number of problems for multicellular eukaryotes, due to the prevalence of highly repeated sequences in these regions. It is likely that many satellite DNA repeat units lack the sequences necessary to initiate replication (origins). Extensive domains of these tandemly repeated DNAs would have to be replicated from origins in the flanking DNA, a function that could be provided by the complex islands. Thus, chromosome replication could provide selective pressure for the initial formation and accumulation of islands of complex DNA. In this model of heterochromatin evolution, the extent of satellite expansion and the maximum allowable interisland distance would be constrained by the requirement for complete genome replication during S phase. The replication of heterochromatin late in S phase, described for a number of organisms (GOLDMAN *et al.* 1984; TEN *et al.* 1990; TEN and COHEN 1993), may be a consequence of interisland distance being greater than the average replicon size for euchromatin (~100 kb in Drosophila) (BLUMENTHAL *et al.* 1974; MCKNIGHT and MILLER 1977), initiation late in S (MCCARROLL and FANGMAN 1988; FERGUSON *et al.* 1991; FERGUSON and FANGMAN 1992), or a reduced rate of elongation through long stretches of simple repeated DNA (FRY and LOEB 1994; KUNST and WARREN 1994; SHIBATA *et al.* 1994). Late replication suggests that heterochromatin is on the edge of acceptable replication timing. Indeed, the correlation between altered replication timing and fragile site formation (HANSEN *et al.* 1993) demonstrates the potential consequences of abnormally late replication. A similar shift in replication timing for all of the heterochromatin would have even more catastrophic consequences for the cell, given that it constitutes 30% of the Drosophila genome and 15% of the human genome. Conversely, the minimal interisland distance may be determined by different functional requirements, for example, the presence of centromeres within heterochromatin in widely divergent species (WHITE 1973) may be conserved because normal inheritance requires extensive blocks of satellite DNA. Thus, heterochromatin structure would continue to evolve by balancing the constraints imposed by replication (shortening interisland distance) with chromosome inheritance requirements and recombination mechanisms ("molecular drive") (DOVER 1993) that drive the expansion of satellite arrays. This evolutionary hypothesis is difficult to test directly, but support could be obtained by mapping origin distribution within heterochromatin, using clones of island DNA and two-dimensional gel electrophoresis (BREWER and FANGMAN 1987).

The presence of transposons, such as the *Doc* element in *Tahiti*, presents a number of interesting possibilities for complex island evolution and function. Transposon insertion within satellite DNA provides a plausible mechanism for initiating island formation. The presence of even a single element would facilitate island expansion by providing a target for subsequent insertions; the chromatin structure and/or sequence complexity of a transposon could make it much more receptive to subsequent insertions than the satellite DNA. Thus, the frequency of insertions within satellite regions need not be high for islands to form efficiently: the impact of stochastic, infrequent insertion events could be magnified by basic transposon biology. The recent demonstration of high frequency *P*-element insertion within heterochromatin (ZHANG and SPRADLING 1994) supports the idea that transposons can act to expand complex islands, though the ability of *P* elements to insert in satellite DNA is still unclear (COOK and KARPEN 1994). Transposons could become fixed within heterochromatin by mutational or transcriptional inactivation of transposition (reviewed in CHARLESWORTH *et al.* 1994b), coupled with selective pressure for retention. Both a *cis* role in chromosome function (*e.g.*, replication of heterochromatin, see above) and a *trans* role in the regulation of transposon behavior across the genome could drive the retention of transposons within heterochromatin. Alternatively, suppressed recombination in heterochromatic regions also could contribute to retention of active or inactive transposons (reviewed in CHARLESWORTH *et al.* 1994b). The extent of homology between the *Tahiti* clone and the 3′ end of a euchromatic *Doc* element (O'HARE *et al.* 1991) suggests that this heterochromatic element may be functional. Even partial elements, such as those seen in scrambled clusters of transposons (NURMINSKY *et al.* 1994), may play a significant role in regulating transposition in the germline and during development (SPRADLING *et al.* 1993; SPRADLING 1994). It will be necessary to determine first the precise structure of the complex islands, and the completeness of transposons such as the *Tahiti Doc* element, before specific models can be proposed and tested.

In conclusion, the isolation and characterization of *Dp1187* derivatives have proven extremely useful in analyzing the structure of a difficult region of eukaryotic genomes, the heterochromatin. The success of the approaches described here provides information, methodology and tools for further analyses of heterochromatin structure and function in Drosophila, and possibly other organisms. Combined with other current approaches to heterochromatin studies (ROSEMAN *et al.* 1993; DORER and HENIKOFF 1994; ZHANG and SPRADLING 1994), we expect that the mysterious aspects of

heterochromatin will soon begin to yield to direct analyses, leading to a greater understanding of the role(s) of heterochromatin in cell biology, genetics and evolution.

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