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Dodeca satellite: A conserved G+C-rich satellite from the centromeric heterochromatin of *Drosophila melanogaster*

(centromeric satellite/*Drosophila* yeast artificial chromosome library/*in situ* hybridization)

JOSÉ P. ABAD*, MAR CARMENA*, SIGRID BAARS*†, ROBERT D. C. SAUNDERS‡, DAVID M. GLOVER‡, PALOMA LUDEÑA§, CARLOS SENTIS§, CHRIS TYLER-SMITH¶, AND ALFREDO VILLASANTE*†‖

†Centro Nacional de Biotecnologia (CSIC), *Centro de Biología Molecular (CSIC-UAM), and §Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049-Madrid, Spain; ‡Cancer Research Campaign Cell Cycle Genetics Group, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, United Kingdom; and ¶Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom

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ABSTRACT **To identify sequences from the centromeric region, we have constructed a *Drosophila melanogaster* yeast artificial chromosome (YAC) library and screened it with purified DNA from the minichromosome Dp(1;f)1187 derived from the X chromosome. We describe the structure of one clone isolated in this way. This YAC is structurally unstable and contains tandemly repeated G+C-rich 11-mer and 12-mer units, which we call dodeca satellite. Most of this satellite is located near the centromere of an autosome. Cross-hybridizing sequences are found in the genomes of organisms as distant as *Arabidopsis thaliana* and *Homo sapiens*.**

The genomes of higher eukaryotes contain large amounts of simple and complex tandemly repeated DNA sequences, classically termed satellite DNA (for review, see ref. 1). It has been known since 1970 that satellite DNAs are located primarily in centromeric heterochromatin (2). However, their role in possible functions of this region of the chromosome, such as chromosome pairing and chromosome segregation, remains largely unknown. Studies on the organization of centromeric heterochromatin have been impeded by the structural properties of satellite DNA. Satellite DNA fragments can be difficult to clone in bacterial vectors (3) and are therefore underrepresented or absent from *Escherichia coli* libraries. In addition, their very large size and repetitive nature make it difficult to construct complete physical maps of these regions, even by pulsed-field gel electrophoresis (PFG).

Recently, the development of yeast artificial chromosomes (YACs) as vectors for the propagation of exogenous DNA in *Saccharomyces cerevisiae* (4) has opened up additional possibilities. Libraries have been constructed containing large fragments of DNA from organisms such as humans and *Drosophila* (5, 6). However, YAC clones containing human DNA showed structural instability for all tandemly repeated sequences examined (7). Little is known about the structural stability of *Drosophila* satellite sequences in YACs, but, even if they are also unstable, it may at least be possible to use such clones as a source of probes from the centromeric sequences.

The chromosomes of *Drosophila melanogaster* offer a good system for analysis of the centromeric heterochromatin of higher eukaryotes. Like many eukaryotes, they have a hemispherical kinetochore with bundles of microtubules attached, and their centromeric heterochromatin makes up a high proportion of each chromosome. In addition, it is possible to generate a panel of chromosomes with centromeric rearrangements by classical genetics, and many mutants in mitosis and meiosis are available.

A total of 11 simple repeated sequences have been cloned from gradient-purified satellite DNA of *D. melanogaster* and the nucleotide sequence of each repeat conforms to a formula $(RRN)_m(RN)_n$, where R is A or G and N is any nucleotide (8). In this study, we have developed an approach to the cloning of centromeric heterochromatin sequences from *D. melanogaster*. Thus, we have discovered a type of tandemly repeated DNA sequence that is located in the centromeric region of some *D. melanogaster* chromosomes and that cross-hybridizes with DNA from other species including *Arabidopsis* and humans.**

MATERIALS AND METHODS

***Drosophila* Stocks and Mammalian Cell Lines.** The *Dp(1;f)-1187* stock, XYS·YL, Df(1)259, y⁻w/C(1)RA, l(1) J1In(1)sc^8/Dp(1;f)1187, was kindly provided by A. Spradling (Carnegie Institute of Washington). Oregon R was used as a wild-type stock. The mammalian cell lines used were MTL and PEN (human lymphoblastoid male and female cells), 3E7 (mouse Y hybrid), 853 (hamster Y hybrid), Q998-8 (hamster Y hybrid; a gift from Carol Jones). The 3E7 and 853 revertants had lost the human Y chromosomes. The Q998-8 derivative had lost the Y long arm but retained the Y short arm.

Genomic DNA Preparations and PFG. High molecular weight genomic DNA was prepared in two different ways: in solution or in agarose plugs. For *Drosophila* DNA preparations in agarose plugs, population cages were set up to collect 0- to 12-hr-old embryos. After dechorionation, embryos were disrupted in Ringer's solution supplemented with 50 mM EDTA. For analysis of large DNA fragments, three different PFG apparatuses were used: Pulsaphor system (Pharmacia), CHEF-DRII (Bio-Rad), and a "waltzer" rotational gel electrophoresis apparatus. In the first two systems, 1% agarose (Sigma type I) gels were run in 0.5× TBE (1× TBE is 90 mM Tris·borate/2 mM EDTA) while in the waltzer apparatus, 1.5% agarose gels were run in 0.5× TAE (1× TAE is 40 mM Tris·acetate/2 mM EDTA).

Construction of *Drosophila* YAC Library. The cloning vector pYAC-white was constructed by insertion of a white minigene driven by a heat shock protein 70 promoter into the unique *Sal* I site of pYAC-4 (4). This marker gene was obtained from plasmid pW8 (9) as a 4-kilobase (kb) *Sph* I/*Spe* I DNA fragment. The high molecular weight DNA was made from the *Dp(1;f)1187* stock. An agarose plug was partially digested with *Eco*RI. The digested DNA was ligated to

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Abbreviations: YAC, yeast artificial chromosome; PFG, pulsed-field gel electrophoresis.

‖To whom reprint requests should be addressed.

**The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M86306–M86309).

*Eco*RI/*Bam*HI digested and dephosphorylated pYAC-white vector and was then used to transform yeast strain AB1380 as described by Anand *et al.* (10).

DNA Probes, Hybridization, and Sequencing. The 4.4RR probe is a 4.4-kb *Eco*RI restriction fragment obtained from λsc 94 (11). DNA probes were purified by agarose gel electrophoresis and labeled by the random-primer procedure (12). Filter hybridization conditions were essentially as described by Church and Gilbert (13). Low-stringency hybridizations were performed at 55°C in the same conditions or in 10% dextran sulfate/0.5 M sodium phosphate/1% SDS/5× Denhardt's solution, and washed in 2× standard saline citrate/0.1% SDS at the same temperature or at 65°C. Sequencing was performed by the method of Sanger *et al.* (14) with Sequenase 2.0 and the dGTP analogue 7-deaza-dGTP.

***In Situ* Hybridization to Metaphase Chromosomes.** In the *in situ* hybridization to *Drosophila* mitotic chromosomes, the biotinylated probes were detected by an avidin-fluorescein conjugate. A full description of the method will be reported elsewhere (unpublished data). For *in situ* hybridization to human chromosomes, standard chromosome spreads from lymphocyte cultures were hybridized *in situ* under low-stringency conditions that allow hybridization between molecules sharing 60–65% homology. Hybridizations were performed essentially as described by Schwarzacher-Robinson *et al.* (15). The biotinylated probe was detected with streptavidin and biotinylated alkaline phosphatase.

RESULTS AND DISCUSSION

Strategy for Identification of Centromeric Heterochromatin Sequences. In 1953 Krivshenko and Cooper generated an extensive series of free X chromosome duplications by inducing large interstitial deletions within the inverted X chromosome *In(1)sc*^{8}. *Dp(1;f)1187* is the smallest with a size of <30% of the metaphase length of chromosome 4 (cited in ref. 16; see Fig. 1*A*). This free duplication is a key element in our cloning of centromeric region sequences. Preliminary characterization shows the size of this minichromosome to be ≈1.3 megabases (Mb), which includes ≈1 Mb of heterochromatin (ref. 17 and this paper). Thus, this minichromosome can be seen as a DNA molecule that is highly enriched in centromeric sequences and that can be used as a hybridization probe for identification of centromeric region clones. To isolate the DNA of this minichromosome we have used preparative PFG of intact DNA from embryos. This has been possible because we were able to detect the minichromosome by ethidium bromide staining of the gel (Fig. 1 *B* and *C*).

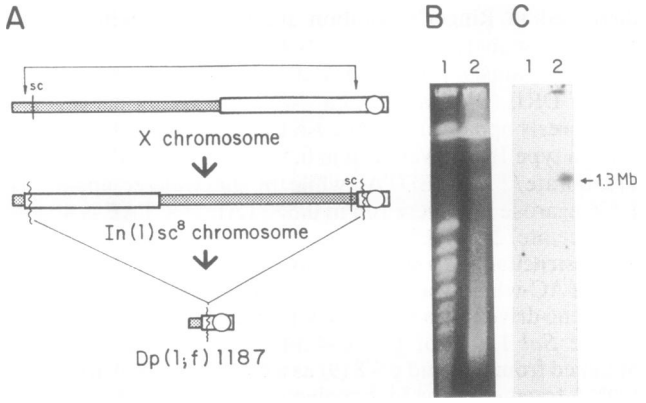

FIG. 1. Origin and size of *Dp(1;f)1187* minichromosome. (*A*) *Dp(1;f)1187* is the product of a deletion within the inverted X chromosome *In(1)sc*^{8}. Heterochromatin is shown as an open box and euchromatin is shown as a stippled box (the scheme is not drawn to scale). (*B*) Ethidium bromide staining of a pulsed-field gel. (*C*) Filter transfer hybridized with 4.4RR. Lanes: 1, yeast chromosomes from strain YP148; 2, DNA from *Dp(1;f)1187* stock. The gel was run in a waltzer apparatus for 43 hr with a 200-sec pulse time at 90 V.

In parallel, we have prepared a YAC library of *Drosophila* DNA partially digested with *Eco*RI. The library was constructed by using a modified YAC vector, pYAC-white, that contains a *Drosophila* visible marker. The DNA source for the library was the strain carrying *Dp(1;f)1187*. The library contains 2250 clones with an average size of 125 kb, ≈1.8 times coverage of the *Drosophila* genome. Screening this library with DNA from the minichromosome as a colony hybridization probe revealed 27 positive clones. DNA minipreparations of these clones were run on pulsed-field gels, transferred to nylon membranes, and hybridized again with minichromosome DNA. As a result of this second screening, clone yW6E2 gave one of the stronger hybridization signals.

Molecular Characterization of yW6E2. Analysis of the undigested YAC clone would be expected to show a single band. However, yW6E2 shows a major band of 60 kb and a minor band of 100 kb, and these are reduced in size by ≈15 kb (the size of the YAC vector arms) when digested by *Eco*RI. To investigate whether these two bands correspond to two unrelated clones or reflect instability of the sequences

C C C G T A C T C G G T

FIG. 2. Dodeca satellite sequence. The sequences of the dodeca satellite inserts in pBK6E201, pBK6E209, pBK6E215, and pBK6E218 are displayed to emphasize the 11- and 12-bp repeats.

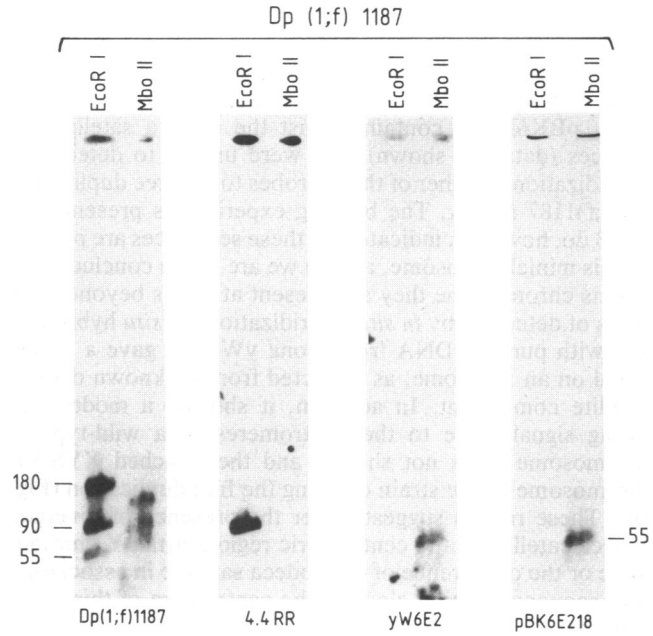

FIG. 3. Localization of dodeca satellite sequences in the *Dp(1;f)1187* minichromosome. *Dp(1;f)1187* DNA was digested with *Eco*RI or *Mbo* II and analyzed by PFG in a gel run in a Pulsaphor apparatus for 15 hr with a 60-sec pulse time, followed by 10 hr with a 90-sec pulse time at 200 V. A filter transfer was sequentially hybridized with the indicated probes.

originally cloned, the yW6E2 culture was streaked out and single colonies were picked for analysis. After separation by PFG and hybridization with a DNA fragment subcloned from the YAC yW6E2 (pBK6E218; see below), many bands are seen in each sample showing that these YACs contain common sequences and are very unstable (data not shown). This instability is similar to that found in human tandemly repeated DNA sequences cloned in YAC vectors (7).

DNA of yW6E2 was purified by PFG and digested with several restriction enzymes to find the appropriate one to use in subcloning fragments of the YAC. Restriction enzyme *Spe* I generated multiple fragments between approximately 0.1 and 2 kb and was chosen to generate fragments for subcloning in pBluescript KS−. Several subclones were obtained and four of them—pBK6E201, pBK6E209, pBK6E215, and pBK6E218—were sequenced (Fig. 2). All the subclones are made up of a tandemly repeated sequence based on the pseudopalindromic 11- and 12-bp CCCGTACTGGT and CCCGTACTCGGT consensus sequences. We call this repetitive sequence dodeca satellite. These sequences show the following features: (*i*) they are the same except for the presence of a C in position 9 of the 12-mer, (*ii*) the 12-mers are usually found in groups of 3 or 4 monomers, (*iii*) the 11-mer following each of these groups is usually a variant in the consensus with a change in position 7 from a C to a T. These features suggest the existence of a less well defined higher-order structure. The search with the dodeca satellite consensus sequences in the GenBank and EMBL data bases did not detect any other similar repeated sequences. The dodeca satellite does not conform to the general formula $(RRN)_m(RN)_n$ for *Drosophila* satellites and represents a newly discovered class of G+C-rich satellite in *Drosophila*.

Organization of Dodeca Satellite DNA in the Genome. To localize dodeca satellite sequences in *Dp(1;f)1187*, this minichromosome was purified by PFG, digested with the restriction enzymes *Eco*RI and *Mbo* II, separated by PFG, blotted, and sequentially hybridized with different probes (Fig. 3). The hybridization of *Dp(1;f)1187* DNA to itself showed several large fragments (50–180 kb) for both enzymes. Since *Eco*RI and *Mbo* II digest on average every 4 and 0.7 kb, respectively, in euchromatin DNA, these fragments are likely to be derived from heterochromatic regions. Together they account for >300 kb of minichromosome heterochromatin. 4.4RR, a unique sequence immediately adjacent to the distal breakpoint of *In(1)sc*^{8}, hybridizes to a 90-kb *Eco*RI fragment; thus, this 90-kb fragment probably spans the boundary between euchromatin and heterochromatin of the minichromosome, in agreement with the data shown by Karpen and Spradling (17). Hybridization with yW6E2 DNA or its subclone pBK6E218 detected a 55-kb *Mbo* II fragment. No large *Eco*RI fragment was detected, although yW6E2 itself contains large *Eco*RI fragments. The lack of large hybridizing *Eco*RI fragments, together with the low signal shown by the *Mbo* II fragment indicates that clone yW6E2 did not come from the minichromosome. Thus, this clone was detected in the library because of cross-hybridization of the minichromosome to related sequences elsewhere in the genome.

To investigate the structure of these sequences, genomic DNA was digested with restriction enzymes and hybridized with the dodeca satellite (Fig. 4). *Bam*HI, *Eco*RI, and *Xho* I

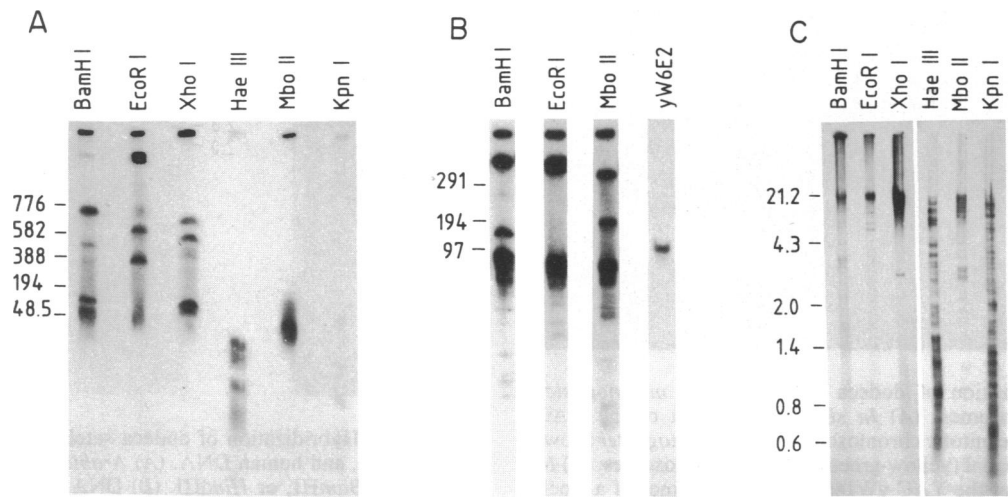

FIG. 4. Structure of dodeca satellite in the *Drosophila* genome. DNA from the *Drosophila Dp(1;f)1187* stock was digested by the enzymes indicated and the fragments were fractionated by conventional electrophoresis or by PFG under conditions resolving three different size ranges. The following running conditions were used: CHEF-DRII apparatus for 30 hr with a 100-sec pulse time at 150 V (*A*), CHEF-DRII apparatus for 24 hr with a 30-sec pulse time at 150 V (*B*), conventional 1% agarose gel electrophoresis (*C*). The insert DNA from pBK6E218 was used as the hybridization probe. yW6E2 is shown in *B* for comparison.

cleave the bulk of dodeca satellite into very large fragments, but each enzyme also yields distinctive bands between 1 and 20 kb (Fig. 4). The addition of the sizes of the bands produced by each enzyme indicates that, if each band is a single fragment composed entirely of the satellite, the dodeca satellite is distributed over ≈1 Mb. In contrast, *Hae* III and *Kpn* I produce mainly fragments that are <50 kb, while most *Mbo* II restriction fragments are ≈50 kb (Fig. 4). There is no genomic *Eco*RI restriction fragment similar in size to the largest insert detected in yW6E2 preparations (Fig. 4*B*). It is most likely that this insert comes from an even larger *Eco*RI fragment, which must be at least 350 kb. A less likely possibility is that yW6E2 is derived from a smaller genomic *Eco*RI fragment by recombination in yeast. To obtain clones of other members of this sequence family, we probed the YAC library using the insert of clone pBK6E218. Three additional positive clones—yW14G7, yW19B7, and yW21F8—were obtained.

To determine the chromosomal location of this new satellite, purified YACs were used for *in situ* hybridization to *Drosophila* metaphase chromosomes. Hybridization with yW6E2 shows that dodeca satellite sequences are present in the centromeric region of an autosome (Fig. 5*A*). The same result was obtained by using a subcloned fragment of this YAC, pBK6E218, containing just the dodeca satellite sequences (data not shown). We were unable to detect any hybridization of either of these probes to the free duplication Dp(1;f)1187 *in situ*. The blotting experiments presented in Fig. 3 do, however, indicate that these sequences are present on this minichromosome, and so we are led to conclude that on this chromosome they are present at levels beyond their limits of detection by *in situ* hybridization. *In situ* hybridization with purified DNA from clone yW14G7 gave a strong signal on an autosome, as expected from its known dodeca satellite component. In addition, it showed a moderately strong signal close to the centromeres of a wild-type Y chromosome (data not shown) and the attached XYS·YL chromosome of the strain carrying the free duplication (Fig. 5*B*). These results suggest either the presence of diverged dodeca satellite in the centromeric region of the Y chromosome or the occurrence of the dodeca satellite in association with sequences found close to the centromere of this chromosome.

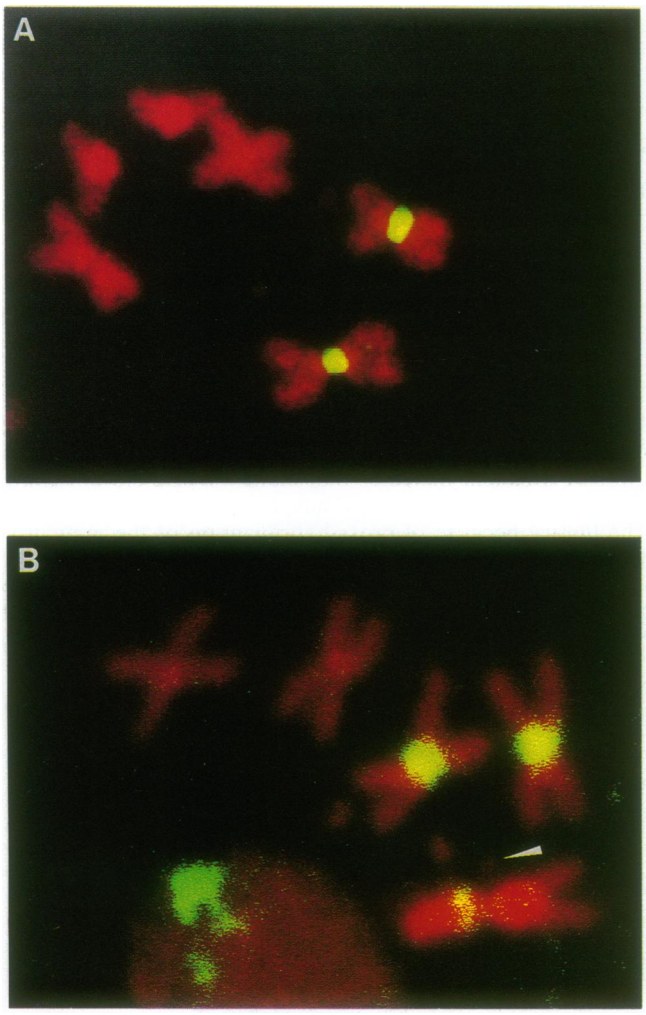

FIG. 5. Localization of dodeca satellite in *D. melanogaster* metaphase chromosomes. (*A*) *In situ* hybridization of the YAC yW6E2 to wild-type mitotic chromosomes of *D. melanogaster* showing hybridization signal (yellow-green) to a pair of autosomes. (*B*) *In situ* hybridization of the YAC yW14G7 to chromosomes of a stock carrying the free duplication *Dp(1;f)1187*. In addition to the strong hybridization to one autosome pair, a moderate signal is also seen on the attached XYS·YL chromosome. It is just possible to discern the free duplication (arrowhead), which does not show any hybridization signal. Hybridization signal is also seen to the interphase nucleus in the lower part of the figure.

Conservation of Dodeca Satellite. With few exceptions, the major satellite DNAs are labile during evolution. It is not generally expected that different species will share similar satellite DNA sequences. However, the $(TTAGGG)_n$ repeat is found in all vertebrate telomeric regions and in the pericentric regions of many vertebrate chromosomes (18). To determine whether sequences related to the dodeca satellite are present in other species, hybridizations were performed at lower levels of stringency. We have found cross-hybridization to DNA of five *Drosophila* species—*D. simu-*

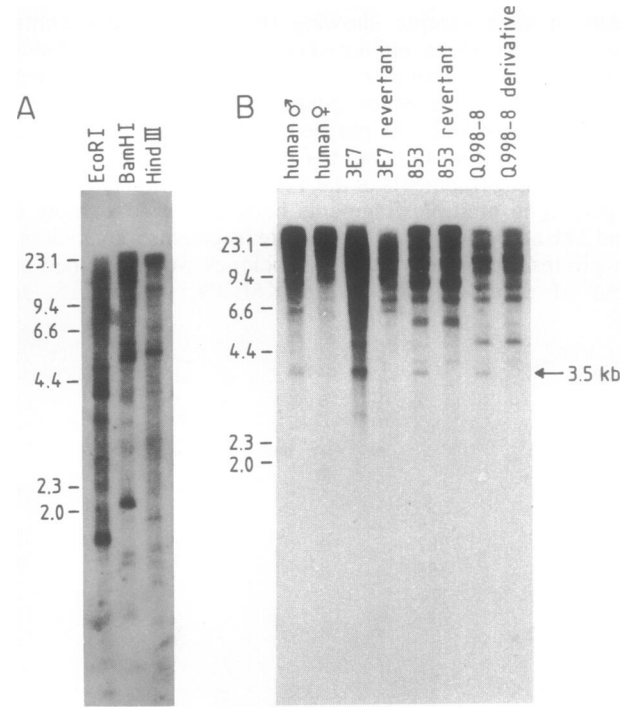

FIG. 6. Hybridization of dodeca satellite to *Arabidopsis*, hamster, mouse, and human DNA. (*A*) *Arabidopsis* DNA was digested by *Eco*RI, *Bam*HI, or *Hin*dIII. (*B*) DNA samples identified above each lane were digested with *Eco*RI. Human male, 3E7 (mouse Y hybrid), 853 (hamster Y hybrid), and Q998-8 (hamster Y hybrid) DNAs contain the long arm of the human Y chromosome. The other samples do not. DNAs were run on conventional agarose gels, transferred to filters, and hybridized with insert DNA from pBK6E218.

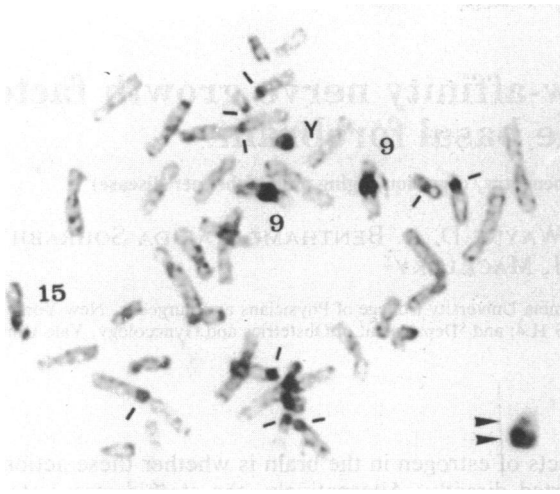

FIG. 7. Localization of dodeca satellite in human metaphase chromosomes. *In situ* hybridization was carried out with biotinylated insert DNA from pBK6E218. Markings point out the weaker signals observed over the pericentromeric regions of some chromosomes. (*Inset*) Enlargement of the Y chromosome to show the pericentromeric and nonpericentromeric hybridization signal (arrowheads).

lans, D. buzzatii, D. hydei, D. immigrans, and *D. virilis* (data not shown). Thus, dodeca satellite-like sequences have been detected in species separated for >60 million years. To extend our analysis, we have chosen one plant species (*Arabidopsis thaliana*) and three mammalian species (*Cricetulus griseus, Mus musculus, Homo sapiens*). As shown in Fig. 6, all these species contain sequences cross-hybridizing with the dodeca satellite.

The dodeca satellite-like sequences were localized in the human genome by *in situ* hybridization (Fig. 7). Labeling can be seen at the pericentromeric regions of several chromosomes. The main sites of labeling were in the pericentric regions of chromosomes 9 and 15 and on the long arm of the Y chromosome. A similar pattern of hybridization has been found with probes belonging to the 5-bp satellite 3 family (15). The relationship between *Drosophila* dodeca satellite and the human satellite 3 family is also shown in Fig. 6. Hybridization of the dodeca satellite probe to *Eco*RI digests of male and female human DNA produced a complex pattern, which included a 3.5-kb male-specific fragment (Fig. 6*B*, lanes 1 and 2). This fragment is likely to lie on the Y chromosome, and this possibility is confirmed by its presence in three independent "Y only" hybrids (lanes 3, 5, and 7) and its absence in revertants of two of them (lanes 4 and 6). It can be localized to Yq by its absence from the Q998-8 derivative, which has lost Yq sequences (lane 8). These cross-hybridizing sequences could represent a newly discovered human sequence family but are more likely to correspond to the satellite 3 family DYZ1, which consists of tandemly repeated 3.5-kb *Eco*RI fragments located on Yq. Weak homology between the dodeca satellite consensus sequence and the satellite 3 sequence data of Prosser *et al.* (19) can be found: CCCGTACTGGT and CAACCCGAAT(GGAAT)$_n$.

In conclusion, we have found a G+C-rich satellite in the centromeric region of *D. melanogaster* chromosomes, which has similarity to repeated sequences in widely different species. We can suggest two kinds of explanation for this similarity. Dodeca satellite and cross-hybridizing satellites might arise independently, for example by similar pathways of divergence from widespread sequence such as the telomeric TTAGGG (animals) or TTTAGGG (plants). Alternatively, dodeca satellite might be conserved, for example because of an important role in centromeric function. If these satellites have a function in segregation, they should be present at all centromeres in each species. However, they were only detected in a subset of chromosomes. Smaller amounts or diverged sequences could be present on other chromosomes. Testing these ideas will require further structural analysis and development of appropriate assays for centromere function.

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