Cloning and characterization of a dispersed, multicopy, X chromosome sequence in Drosophila melanogaster

(middle repetitive DNA/X chromosome structure/DNA sequence homology)

GAIL L. WARING AND JANE C. POLLACK

Biology Department, Marquette University, Milwaukee, WI 53233

Communicated by Joseph G. Gall, December 24, 1986 (received for review May 12, 1986)

ABSTRACT We have isolated and characterized a dispersed middle repetitive DNA sequence from Drosophila melanogaster that is concentrated on the euchromatic portion of the X chromosome. In situ hybridization of the repeat unit to salivary gland chromosomes shows the sequence is distributed among approximately 10 major and 20 minor X chromosomal sites. Based on DNA sequence analysis of homologous sequences from three different cytogenetic regions, the 372-base-pair repeat unit appears to be (A+T)-rich and noncoding and shows strong sequence conservation among units from different chromosomal regions. The nature and distribution of this sequence are suggestive of the hypothetical X chromosome DNA sequences thought to be involved in the primary establishment of sex determination and dosage compensation in Drosophila.

The Drosophila genome consists of three kinetic components, highly repetitive, middle repetitive, and unique DNA (1). The middle repetitive fraction constitutes ≈17% of the total genomic DNA and includes the tandemly repeated rRNA, 5S RNA, and histone genes. Most of the middle repetitive DNA in Drosophila melanogaster, however, is not tandemly repeated but rather is dispersed over the entire genome (2). It is composed of at least 50–100 different sequence families, each of which generally consists of 10–100 closely related sequences (2). The number of sequences within a family seems to be conserved in different strains of the species, but their chromosomal positions are highly variable. In marked contrast, in this paper we describe a dispersed middle repetitive family with repeats at chromosomal positions that are conserved between strains. More importantly, the repeat units are concentrated in the euchromatic portion of the X chromosome. A functional link between family members is suggested by the extensive sequence homology found between repeats from different chromosomal regions. The intriguing possibility that these sequences may play a role in the primary establishment of sex determination and dosage compensation in Drosophila is considered.

MATERIALS AND METHODS

Preparation of Nucleic Acids. The Maniatis λ bacteriophage library of D. melanogaster genomic DNA (3) was screened by in situ plaque hybridization (4) under low stringency hybridization [4× SET (0.6 M NaCl/8 mM EDTA/0.12 M Tris-HCl, pH 8), 43% formamide, 37°C] and wash conditions (0.3 M NaCl/30 mM sodium citrate, pH 7, 37°C) with the SP6-labeled single-stranded RNA probe described in the text. Phage from single plaques were purified from liquid cultures by centrifugation in CsCl block gradients (5); phage DNA was purified with NaDodSO4, proteinase K, and phenol. For smaller quantities, DNA was prepared from phage lysates with RNase, NaDodSO4, and phenol.

Subclones were generated by inserting gel-purified restriction fragments into the appropriate sites of the pGem-1 and pGem-2 transcription vectors (Promega Biotec, Madison, WI). Recombinant plasmids were used to transfet Escherichia coli HB101. To confirm their identity, alkaline minipreparations of plasmid subclones (6) were digested with restriction endonucleases. The resultant fragments were separated on agarose gels, transferred to nitrocellulose paper, and probed with RNA transcripts prepared from p112-23R DNA or its derivatives (see Fig. 2). Large-scale plasmid preparations were made by modification of the method outlined by Promega Biotec.

Fly or embryonic DNA was prepared by homogenizing 300–500 flies or 1 ml of embryos in 5 ml of buffer (0.1 M NaCl/30 mM Tris-HCl, pH 8.0/10 mM EDTA/7.7 mM 2-mercaptoethanol) containing 0.5% Triton X-100. Following low-speed centrifugation (3000 rpm, 15 min) the pellet was resuspended in extraction buffer (30 mM Tris-HCl, pH 8.0/100 mM EDTA, 0.5 mg of proteinase K per ml) made 1% in sarkosyl. After digestion overnight at 37°C, DNA was purified by CsCl gradient centrifugation. Small quantities of embryonic and salivary gland DNA were prepared from sonicated tissues treated with RNase, proteinase K, and phenol/chloroform.

Labeling Nucleic Acids. Nick-translation of DNA was carried out as described by Maniatis et al. (6). For high-specific-activity RNA probes, DNA cloned into pGem transcription vectors was linearized and transcribed with SP6 or T7 RNA polymerase as described by the supplier (Promega Biotec). Transcripts were labeled by including 12 μM [32P]GTP in the transcription reaction mixture.

DNA Sequence Analysis. Drosophila DNA fragments cloned into pGem transcription vectors were sequenced according to the manufacturer's protocols (Promega Biotec). Briefly, linearized DNA was denatured, reannealed in the presence of primers, and sequenced with DNA polymerase I (Klenow fragment) by the dideoxy chain-termination method (7). Reaction mixtures were analyzed on 4% and 8% acrylamide gels containing 8 M urea.

In Situ Hybridization to Polytenic Chromosomes. Polytenic chromosome squashes were prepared as described by Bonner and Pardue (8). Hybridization with biotinylated probes was carried out according to Enzo Biochemicals (New York). DNA was nick-translated using Bio-11-dUTP (Bethesda Research Laboratories) and single-stranded DNA transcripts were synthesized using Bio-11-UTP (Bethesda Research Laboratories). Signal detection was achieved by using a streptavidin/biotinylated peroxidase complex (Enzo Biochemicals).

RESULTS

Isolation of Dispersed X Chromosome-Linked Sequences Homologous to 7F Region Sequences. In previous studies on
early eggshell gene expression in *Drosophila* we isolated three independent cDNA clones complementary to follicle cell RNAs synthesized during stage 10 of oogenesis. Genomic clones complementary to one of the cloned cDNAs hybridized *in situ* to region 7F of the X chromosome. 7F has been identified as a major chorion structural gene site (9). Unlike other overlapping genomic clones from this region, one genomic clone (112) hybridized to 8B in addition to 7F. Comparison of the restriction maps of the overlapping 7F region clones indicated that the 8B hybridization must be due to homologous sequences within either the 3.2- or 2.3-kilobase (kb) EcoRI restriction fragments just proximal to the chorion gene cluster (10). Accordingly, these RI fragments were subcloned into pGem-2 transcription vectors, nick-translated with biotinylated dUTP, and hybridized *in situ* to polytene chromosomes. The 3.2 subclone hybridized only to 7F, whereas the 2.3 subclone (p112-2.3R) gave signals at three sites: 7E, 7F, and 8B (data not shown).

The comparable intensities of the p112-2.3R hybridization signals at all three sites suggested extensive sequence homology within these regions. As a first step in determining the nature of these homologies we screened the Maniatis library for genomic clones containing sequences complementary to p112-2.3R. To increase the probability of recovering genomic clones from regions other than 7F, we used single-stranded RNA probes and low stringency hybridization and wash conditions. A 2.3-kb SP6 transcript made *in vitro* from p112-2.3R was used to screen ~50,000 plaques. Based on our prior experiences, the probe hybridized to about 20 times the number of plaques (125) expected for a single-copy sequence. Fifteen positive plaques were picked at random for further analysis. EcoRI restriction digests of the phage DNAs indicated that we had isolated nine new independent nonoverlapping clones. Southern blot analysis (11) showed that all nine phage contained sequences homologous to p112-2.3R (Fig. 1). Though several phages showed more than one homologous fragment, five of the phages shared a 400-base-pair (bp) hybridizable fragment.

To determine the cytogenetic location of the cloned sequences, the phage DNAs were nick-translated using biotinylated dUTP and hybridized *in situ* to salivary gland chromosomes. All of the phage gave a primary hybridization signal on the X chromosome (Fig. 2). In addition, several of the phage gave secondary signals at 7E and 8B. Since the primary hybridization signals were always on the X chromosome, these results strongly suggested that sequences homologous to p112-2.3R were dispersed but restricted to the X chromosome.

**Defining the Cloned Repeat Unit.** To more closely define the extent of the homologous region, double digests of p112-2.3R were electrophoresed, transferred to nitrocellulose, and hybridized with homologous sequences subcloned from three different cytogenetic regions (11A, 13B, 11EF). Sequences subcloned for probes included a 2.3-kb EcoRI fragment from X25, a 0.4-kb EcoRI fragment from X12 (pX-12R, Fig. 2), and a 3.5-kb PstI fragment from X30 (pX30P, Fig. 2) that included all of the 7F homologous sequences contained in this genomic clone. *Hae* III--EcoRI double digests of p112-2.3R yielded three *Drosophila* fragments: a 1200-bp *Hae* III fragment and two EcoRI--*Hae* III fragments, 600 and 500 bp in length (Fig. 2). Independent Southern blots of the 112-2.3R double digests hybridized with the subcloned probes from each of the three cytogenetic regions (11A, 13B, 11EF) showed that only the 600-bp EcoRI--*Hae* III fragment contained homologous sequences.

To identify the basic repeat unit, sequences from three different regions (7F, 11EF, 13B) were subcloned into pGem transcription vectors, linearized, and sequenced from oppo-
site ends using the dideoxy chain-termination method. The homologous portion of p112-0.6RH was reduced to a 400-bp EcoRI–Alu I fragment (p112-0.4RA) by additional restriction enzyme analyses and Southern blotting experiments. Most of the homologous sequences in pX30P were reduced to 400-bp fragments by either EcoRI (pX30P-0.4R) or Hae III (pX30P-0.4H) cleavage (see Fig. 2). The DNA sequences of homologous subclones from 112, X30, and X12 are shown in Fig. 3. Following manual alignment of the three sequences, a 372-bp consensus sequence was derived. As is evident from Fig. 3, a high percentage of each sequence (87–90%) matches the consensus sequence. The DNA appears to be noncoding since at least 2, and as many as 10, termination codons are found in all six possible reading frames for all three sequences. The consensus sequence as a whole is (A+T)-rich (72%) and is marked by several stretches of at least 6 bp that are 100% A+T. Other than the 30-bp direct repeats indicated in Fig. 3 (73% homologous) there are no distinctive indirect repeats or regions of dyad symmetry. The sequences shown represent a nonrandom subset of repeats since all were generated by EcoRI digestion.

Our preliminary studies had shown that EcoRI and Hae III recognized the fundamental 372-repeat unit in pX30P. Following double digestions with EcoRI and Hae III, most of the homologous sequences were contained in 300-bp fragments. Taken together these data suggested a tandem arrangement of repeat units within pX30P similar to that depicted in Fig. 2. Sequence analysis of a Hae III subclone (pX-30-0.4H) (not shown) confirmed the proposed sequence overlap and verified that our consensus sequence represented an entire repeat unit.

Localization of the Repeat Unit on the X Chromosome. The results from our phage screen strongly suggest that sequences homologous to the 7F region were confined to the X chromosome. To confirm this finding the subcloned repeat unit p112-0.4RA was hybridized to Oregon R salivary gland chromosomes. Our previous studies had indicated that hybridization signals using double-stranded biotinylated DNA probes of <1 kb were difficult to detect. In an attempt to increase the sensitivity of detection we synthesized a 400-bp biotinylated SP6 RNA transcript in vitro. As shown in Fig. 4B, the RNA probe showed several sites with an intense hybridization signal and several with a clearly discernible, albeit weaker, signal (bars). Other than one minor site on the second chromosome, all signals were restricted to the euchromatic portion of the X chromosome. The distribution on the X chromosome was striking in that the proximal and distal parts were devoid of signals. All sites were in the midportion of the X chromosome between cytogenetic regions 4 and 15. To test whether this distribution was characteristic of another fly strain, the SP6 transcript was hybridized in situ to Canton S polytene chromosomes rather than Oregon R. The major sites of hybridization were consistent with the Oregon R results and again no signals were apparent in the most proximal and distal regions of the X chromosome.

Although our in situ hybridization results indicated otherwise, the possibility remained that some repeat units lie within the heterochromatic regions of the autosomes since centromeric heterochromatin is underreplicated in polytene nuclei. Fig. 5 shows two lines of evidence that suggest that most, if not all, of the repeat units are in euchromatic regions. When fly genomic DNA was digested with restriction enzymes that failed to cut within the repeat unit (Pst I, HindIII), 8–10 distinct bands hybridized with the 112-0.4RA probe (Fig. 5A). It is likely that these bands represent different genomic regions since the repeat units in all of the genomic clones shown in Fig. 2 were contained in single Pst I or HindIII restriction fragments (data not shown). The close correspondence between the number of major in situ hybridization sites (=10) and the number of bands in the genomic digests is consistent with clustering of most of the repeat units in the euchromatic portion of the X chromosome. The semiquantitative dot blot data shown in Fig. 5B also suggest that most of the repeat units lie in euchromatic regions. Diploid embryonic DNA and DNA from polytene salivary glands were dot blotted and hybridized to either the repeat unit (112-0.4RA) or a single-copy X chromosome sequence from region 3C (T. Burke and G.L.W., unpublished data). If a significant number of repeat units is located in centromeric heterochromatin, 112 sequences should be underrepresented in salivary gland DNA. The upper portion of Fig. 5B shows the relative amounts of 112 homologous sequences in the embryonic (E) and salivary gland (SG) samples, whereas the lower portion indicates the relative amounts of X chromo-

| p112-0.4RA | ATGCAATACG | ATTGCT-ATT | TAAAAACCA | ACTCAGGAG | GATGCAATT | CCAATTTGG | GCCCATTTT |
| pX-12R | TTAATTTTTT | GCTATTAAAA | CGAAATAAA | TT-GTCAAA | AATACGGG | AAAGATTTT | TGCCAAAAT | NTAATATTT |
| pX-30P-0.4R | C* | C* | C* | C* | C* | C* | C* |

Fig. 3. Nucleotide sequence of the X chromosome repeat unit. The DNA sequences of homologous sequences from three different cytogenetic regions are shown (p112-0.4RA, pX-12R, pX-30P-0.4R). The top line represents the consensus sequence. Identical nucleotides are represented by asterisks; 1-bp gaps in the consensus sequence are represented by dashes. The underlined regions indicate direct repeats found within the consensus sequence that share 73% homology over a 30-bp region. (N = C, T, A, or G; P = A or G; K = T or G; M = C or G; L = A or T; J = A or C.)
some DNA. The comparable ratios of SG:E sequences with both probes indicate that most repeat units reside in euchromatic regions.

Repeat Unit Copy Number. To estimate the number of copies of the repeat unit per haploid genome a Southern blotting experiment was performed. Known quantities of DNA from adult females were digested with either EcoRI or Hae III, electrophoresed, transferred to nitrocellulose, and hybridized with p112-0.4RA. To estimate copy number, the strength of the hybridization signal was compared with that of known amounts of the pX-12R repeat unit (Fig. 2). The range of plasmid DNA used corresponded to the amounts of insert expected for 25–500 copies of the repeat unit per haploid genome. The existence of a small ladder of hybridizing fragments in the complete Hae III digest shown in Fig. 6, lane G (400, 800, 1200 bp), suggests that some of the repeat units are organized into tandem arrays in which the Hae III restriction site has been lost. The intensity of hybridization to higher molecular weight DNA in the EcoRI lane (lane F) suggests more limited conservation of EcoRI sites in the repeat units. Based on integration of the hybridization signals in the Hae III digest, we estimate there are 300–400 copies of the repeat unit per haploid genome. A similar copy number was estimated in a similar Southern blot experiment using pX30P-0.4R as probe.

**DISCUSSION**

In this paper we have reported the cloning and characterization of a family of dispersed repeated DNA sequences concentrated on the X chromosome in *D. melanogaster*. Approximately 300–400 copies of the repeat unit are distributed between cytogenetic regions 4 and 14A. The most striking feature of this repeated sequence family is its virtual confinement to the euchromatic portion of the X chromosome. Reports of other chromosome-specific sequences in eukaryotes are limited. Kunkel et al. (12, 13) have reported Y-specific repeated sequences on the human Y chromosome and Tone et al. (14) have reported 2 W-specific repetitive sequences in domestic fowl. The W chromosome in birds and the Y chromosome in mammals are largely heterochromatic in somatic cells. The sex chromosomal localization of these repetitive elements implies a possible role in heterochromatization. A repeated gene family localized primarily in the pericentric region of the human X chromosome has been reported by three groups (15–17). In *Drosophila*, Bkm-related sequences are localized on the X chromosome (18). Unlike

---

**Fig. 4.** Cytogenetic distribution of sequences homologous to p112-0.4RA. Biotinylated RNA was synthesized from linearized p112-0.4RA DNA with SP6 polymerase and hybridized to salivary gland chromosomes prepared from third-instar Oregon R larvae. (A) Peroxidase reaction signals superimposed upon the Giemsa-stained chromosome banding pattern. (B) Giemsa signals shown in A have been selectively reduced with a blue filter to facilitate visualization of the orange-brown peroxidase signals. Examples of some of the less intense hybridization signals are indicated by the bars. The arrows in B and C show the position of the most proximal X chromosome signal at 14A. (C) An independent spread of the region of intense hybridization shown between the arrowheads in B. The tip of the X chromosome is marked by the X in all three panels. The minor autosomal peroxidase signal in region 26A of the second chromosome can be seen in the upper right-hand corner of B.

**Fig. 5.** Analysis of DNA from diploid and polytene tissue. (A) Five micrograms of embryo DNA was digested with *Pst* I (P) or *Hind* III (H), electrophoresed in 0.8% agarose, transferred to nitrocellulose, and hybridized with 32P-labeled SP6 transcripts made from p112-0.4RA template DNA. (B) Serial dilutions of DNA from salivary gland tissue (SG) or two independent embryonic DNA preparations (E) were spotted onto nitrocellulose and hybridized with 32P-labeled SP6 transcripts made from either p112-0.4RA DNA (upper) or single-copy DNA subcloned from region 3C of the X chromosome (lower). Filters were hybridized at low stringency and washed in 0.3 M NaCl/30 mM sodium citrate, pH 7, at 37°C or 68°C for the 112 and 3C series, respectively. Five times more DNA (25–200 ng) was spotted in the 3C than the 112 series (5–40 ng).
the sequences reported in this paper, cloned Bkm DNA sequences consist of a simple tetranucleotide repeat, GATA, and are concentrated in the proximal region of the X chromosome (19).

Recent models on the primary establishment of sex determination and dosage compensation in Drosophila have postulated the existence of multiple copies of noncoding DNA sequences on the X chromosome (20, 21). In Drosophila sex and dosage compensation are determined by the ratio of the number of X chromosomes to the number of sets of autosomes (X/A ratio) (22). Genotypes with an X/A = 1.0 are female, those with ratios = 0.5 are male, and animals with intermediate ratios are sexual mosaics composed of male and female cells (reviewed in ref. 23). The X/A ratio is believed to act through an X-chromosome-linked control gene, Sxl, that exerts two functions (24, 25). High levels of Sxl expression dictate the female pathway of sexual differentiation as well as a low transcription rate for X-chromosome-linked genes. Low levels of Sxl activity result in the male pathway and a high X chromosome transcription rate. Based on genetic analyses of sex-determining genes, Chandra and colleagues (20) have formulated a model on how the state of activity of the Sxl locus can be related to the X/A ratio. There are three essential features of the model: (i) multiple sex-determining but noncoding sequences on the X chromosome provide high-affinity binding sites (σ) for a repressor (R) of Sxl function, (ii) the Sxl gene binds R with low affinity, and (iii) R is an autosomal gene product present in equal but limited amounts in female and male embryos. In females the (σ) sites on the two X chromosomes bind all R molecules, leaving Sxl accessible for transcription. In males, with half the number of (σ) sites there is a surplus of R molecules available for binding at Sxl, thereby preventing its transcription.

Indirect evidence for the multiplicity of sex-determining sequences on the X chromosome comes from early studies on triploid intersexes (26, 27). Triploid intersexes (X/A = 0.67) display a mosaic phenotype of male and female cells. Addition of X chromosome duplications (i.e., X/A → 1.0) led to feminization; deletions (X/A → 0.5) led to masculinization. The effect was proportional to the size of the duplication or deletion and was independent of chromosomal region. These results suggested a purely quantitative effect compatible with several sex-determining sequences scattered along the X chromosome. Recent observations on the effects of X chromosomal aneuploidy on the X transcription rate are also consistent with the hypothesis that elements involved in the primary establishment of dosage compensation are located throughout the X chromosome (28–30).

Although function is clearly speculative at this stage, the noncoding nature of our sequence (XDM), its concentration on the X chromosome, and its strong sequence conservation at dispersed chromosomal sites are all reminiscent of Chandra’s hypothetical sex-determining sequences. Steinmann-Zwicky and Nothiger have reported sequences localized in the distal region of the X chromosome with a strong feminizing effect (31) that challenges this traditional view of multiple female-determining factors distributed throughout the entire chromosome. However, unlike these localized sequences that appear to be necessary for proper Sxl activation, we suggest that our XDM sequences might be involved in assessing the X/A ratio.

We thank Ms. B. DeNoyer for help in the preparation of the manuscript and S. Hennen and R. Hawley for their helpful comments. This work was supported by National Science Foundation Grants PCM-8203171 and DCB-8502633 to G.L.W., who was the recipient of Research Career Development Award HD00524.