Microdissection and microcloning of the mouse X chromosome

(mdx/genetic mapping)

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ABSTRACT A wild mouse (CD) karyotype in which all the chromosomes bar the X, 19, and Y, are fused as metacentrics has been used for the microdissection and microcloning of a specific mouse X chromosome region. Dissection of a proximal region of the X chromosome encompassing the genetic loci Hprt to Tfm and including mdx has yielded 650 clones. A number of the recovered clones containing sizable inserts have been confirmed as X chromosome specific. This X chromosome bank of clones provides a start point for the isolation of the mdx locus. It is now clear that microdissection and microcloning can be applied to all mouse chromosomes, including the X chromosome, yielding premapped banks of clones that will greatly aid in the isolation and characterization of important genetic loci.

The mouse X chromosome has become a paradigm for the study of a variety of biological phenomena of molecular interest: X chromosome inactivation (1, 2), sex determination (3–6), and the comparative evolution of mammalian chromosomes (7, 8). Yet, very little is known of the detailed molecular structure or long-range organization of this or other mammalian chromosomes. In addition, the mouse X chromosome contains a number of genetic loci of exceptional interest; e.g., some, such as mdx [X-linked muscular dystrophy (9)], putative homologues to human X-linked genes of medical interest, e.g., Duchenne muscular dystrophy (10). To begin the molecular mapping of the mouse X chromosome and isolation of genetic loci of primary interest, we have used microdissection and microcloning (11, 12) to isolate DNA sequences from a specific X chromosome region. Genomic clones recovered from the microdissection and microcloning of a proximal region of the mouse X chromosome originate from the mouse X chromosome and provide a bank of clones highly specific to this chromosome.

METHODS

Preparation of Metaphase Spreads. Lymphocytes were collected from male and female CD mice by suborbital bleeding (16). Lymphocyte division was stimulated by concanavalin A treatment for 2–3 days and, subsequently, lymphocytes were arrested in metaphase by Colcemid. After treatment with KCl for 12 min, cells were fixed with methanol/acidic acid (3:1) and spread by air-drying. Metaphase spreads were stained with 1% Giemsa for photography.

Microdissection and Microcloning. Unstained metaphase spreads of the CD strain were prepared on coverslips according to the methods described above. Coverslips were inverted over an oil chamber and chromosomes were dissected by using glass microneedles with the aid of a de Fonbrunne micromanipulator as described (11, 12). All manipulations were observed under a phase-contrast microscope with a long working distance. Specially fine microneedles were drawn on a de Fonbrunne microforge and were used for the microdissection of the proximal region of X chromosomes well separated in the metaphase plate. After dissection, DNA was prepared and cloned essentially according to published micromethods (11, 12) but with some modifications. Briefly, fragments were pooled in a drop of protease K/0.5% NaDodSO4 residing on a separate coverslip in the oil chamber. After dissection was completed, the oil chamber was incubated at 37°C for 2 hr. After incubation, the nanoliter drop was extracted several times with phenol. After chloroform extraction to remove residual phenol the nanoliter drop was transferred to a fresh oil chamber and a nanoliter drop of restriction enzyme (EcoRI, 50 units/ml) was added and digestion proceeded by incubation at 37°C for 2 hr. Phenol extraction to inactivate enzyme and chloroform treatment were repeated with subsequent transference of the microdrop to a fresh oil chamber. A nanoliter microdrop (~400 pg) of EcoRI-digested vector, λgt10, was added, followed by a nanoliter microdrop (0.4 unit) of ligase. Ligation proceeded overnight at 4°C. After ligation, the microdrop was taken up in several microliters of buffer for in vitro packaging. Recombinants were selected by plating on an Escherichia coli C600hflA− host (14, 15). Individual plaques were picked and plate stocks were prepared by propagation on SM32 (16) using standard procedures.

Analysis of Microclones. A plate lysate method was used to prepare DNA from individual clones. Insert size of clones was determined by EcoRI or HindIII digestion and agarose gel electrophoresis. Two HindIII sites flank the RI insertion site of λgt10, and HindIII digestion provides a rapid and easy method for indirectly determining insert sizes of smaller clones without using large amounts of prepared DNA by observing the change in migration of a λgt10 HindIII 6.7-kilobase (kb) band. Gels were blotted onto nitrocellulose and hybridized to nick-translated total mouse DNA (108 dpm/μg) to identify clones containing repeat sequences (17). Prehybridization and hybridization conditions are as described below.

X Chromosome Localization of Microclones. Mouse inbred line DNAs and hybrid cell DNAs were digested to completion with Taq I and were fractionated on 0.8% agarose gels and transferred to Zetaprobe membranes. Filters were initially hybridized overnight in 3× NaCl/Cit/10× Denhardt’s solution at 65°C followed by a second hybridization for 6 hr in 3× NaCl/Cit/10× Denhardt’s solution/denatured salmon sperm DNA (100 μg/ml)/poly(A) (10 μg/ml)/0.1% NaDodSO4 at 65°C (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate; 1× Denhardt’s solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). Microclone inserts were released from recombinants by EcoRI digestion and were separated on 1.4% low melting point agarose gels. Insert bands were cut out and water was added to 3 ml per g of gel and the gel was dissolved by heating in a boiling water bath for 7 min. Insert DNA was labeled by oligo-priming without

Abbreviation: kb, kilobase(s).

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further extraction according to a published method (18). Hybridization was carried out for 16 hr in 3 × NaCl/Cit/1 × Denhardt's solution/10% dextran sulfate/denatured salmon sperm DNA (100 µg/ml)/poly(A) (10 µg/ml)/0.1% NaDodSO4 at 65°C and a probe concentration of 1–5 × 10^6 dpml. After hybridization, filters were washed several times with 1× NaCl/Cit/0.1% NaDodSO4 at 65°C and exposed on Kodak XAR-5 film with intensification for 1–7 days.

RESULTS

Microdissection of the Mouse X Chromosome. Microdissection and microcloning involves the physical dissection of chromosomes from unstained metaphase spreads and their cloning by using specialized microprocedures. To unequivocally identify the mouse X chromosome within an unstained metaphase spread, we have used a wild strain of mouse (Mus musculus), CD (19, 20), in which all chromosomes apart from the 19, X, and Y, are fused as metacentrics (Fig. 1). The 19, X, and Y chromosomes are the only acrocentrics and are readily distinguishable; the X chromosome is clearly distinguishable from the 19 and Y on account of its much greater size (size ratio, 2.3:1). Arbitrarily, for the purposes of dissection, we have divided the mouse X chromosome into four equally sized regions: centromeric, proximal, distal, and telomeric (Fig. 2). As there is no direct correspondence between the genetic and physical maps of the mouse X chromosome, the exact genetic region dissected in any case is unclear. In Fig. 2, a genetic map is shown and its relationship to the physical map is indicated in order to demonstrate those genetic regions that are likely to be included in any regional dissection. As there is necessarily some variation in the region dissected, it is expected that some sequences from the centromeric and distal regions will be collected. Thus, the genetic region encompassed by a proximal dissection lies within the limits of the genetic loci Hprt and Tjf and includes the mdx locus (9).

Microcloning of Dissected X Chromosome Fragments. Dissected proximal X chromosome fragments (Fig. 2) from CD karyotypes were pooled in a proteinase K/NaDodSO4 nanoliter microdrop to release DNA. After phenol extraction, DNA was digested with EcoRI, phenol extracted again, and then ligated into the insertion vector λgt10, all using specialized microprocedures. After in vitro packaging, phage were plated on an E. coli hflA mutant host to selectively recover recombinant clones (14).

Analysis of X Chromosome Microclones. From 100 dissected proximal fragments (~2 pg of DNA), a total of 650 cl− phage clones were recovered (Table 1). Plating of a small proportion of the packaged microcloning on a nonselective strain [SM 32(22)] indicated that, in total, ~90,000 phage (recombinant and nonrecombinant) were recovered from the microcloning and subsequent in vitro packaging of microdissected proximal X chromosome DNA. Control ligations of the EcoRI-digested λgt10 vector alone demonstrated that only 1 in 10^9 phage recovered successfully grew on an E. coli hflA mutant host, indicating that a high proportion (>85%) of the 650 recovered cl− clones are true recombinants. A large number of the microclones were miniprepped and the insert size was estimated by restriction enzyme digestion. Some microclones (28 of 72) had very small (<0.1 kb) or undetectable inserts. Insert size of the remaining clones varied from 0.2 to 4 kb, with an average insert size of 0.4 kb. The miniprepped and digested clones were blotted and hybridized to total mouse genomic DNA to eliminate those clones containing repeat sequence DNA; 13% (9 of 72) of the clones hybridized strongly, including one microclone with a very small insert (<0.1 kb). The average insert size of sizable clones containing repeat sequences was 0.6 kb. The small average insert size has probably contributed to the relatively low number of clones containing significant repeat sequences; those clones containing repeat sequences are of higher average insert size. The overall small insert size of recovered genomic clones may be due to preferential acid hydrolysis of large EcoRI fragments during fixation of metaphase spreads.

To verify that the recovered clones do indeed originate from the X chromosome, we have hybridized a number of low copy microclones carrying sizable inserts to hybrid cell lines—MAE-28 and MAE-32 (21)—containing the mouse X chromosome. MAE-28 contains Chinese hamster chromosomes plus mouse chromosomes X and 12, while MAE-32 contains Chinese hamster chromosomes plus mouse chromosomes X and 16. E-36, the parent cell line, contains only Chinese hamster chromosomes. DNAs from the inbred mouse lines, C57BL/10 and SWR, and from MAE-28, MAE-32, and E-36 were digested with Taq I, electrophoresed on agarose gels, blotted, and hybridized to a variety of X chromosome microclones chosen at random (Fig. 3). Five clones were tested and all were shown to be X chromosome specific. Hybridizing fragments observed in mouse DNAs, C57BL/10 and SWR, are also observed in MAE-28 and

FIG. 1. Chromosomes of the CD strain of mouse. Metaphase spreads from lymphocyte cells of male and female individuals of the wild CD strain (19, 20) of Mus musculus. The X chromosome is marked.
MAE-32 but not in E-36, confirming the location of the microclones on the X chromosome. In the case of two clones, 10 and 44, weakly hybridizing fragments common to E-36, MAE-28, and MAE-32 are seen, representing weakly homologous sequences in the Chinese hamster genome. In addition, the larger of the two X chromosome fragments detected by clone 44 shows a Taq I restriction fragment length polymorphism between the SWR and C57BL/10 inbred strains. Clone 44 also shows some background hybridization to all lanes, indicating that it still carries some repeat sequence DNA not detected in the initial screening. In clone 43, apart from the strongly hybridizing X chromosome fragment common to C57BL/10, SWR, MAE-28, and MAE-32 DNAs, a variety of weakly hybridizing fragments of variable intensity are observed in mouse genomic DNAs but not in MAE-28 and MAE-32 DNAs even after long exposure. These fragments may represent weakly homologous sequences to the X chromosome clone 43 with an autosomal location.

**DISCUSSION**

By using Robertsonian translocations to identify individual autosomes in unstained metaphase spreads, regional microdissection can provide banks of clones from individual autosomes (22). But, the X chromosome does not participate in Robertsonian fusions (20). However, by a process of exclusion, involving the majority of autosomes in Robertsonian fusions and leaving the X chromosome free, we have demonstrated here the microdissection and microcloning of an individual region of the mouse X chromosome. Thus, it is now possible to microdissect and microclone all mouse chromosomes.

Genomic clones originating from dissected proximal X chromosome fragments are X chromosome specific and provide a bank of clones premapped to the mouse X chromosome. While there must be some variation in the region dissected, the technique clearly provides a method for the rapid isolation of clones from a discrete physical region of the genome. Genetic experiments will enable us to define exactly the genetic limits of the microclones obtained, and at the same time, to define those clones tightly linked to interesting genetic loci.

Some 550 microclones, of which 50% may contain usable genomic inserts, have been obtained from the microdissec-

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**Table 1. Microdissection and microcloning of the proximal region of the mouse X chromosome**

<table>
<thead>
<tr>
<th>No. of chromosome fragments dissected</th>
<th>100</th>
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<tbody>
<tr>
<td>No. of clones recovered</td>
<td>650</td>
</tr>
<tr>
<td>Size range of inserts (72 clones analyzed)</td>
<td>&lt;0.1–4 kb</td>
</tr>
<tr>
<td>Average insert size (44 clones)</td>
<td>0.4 kb</td>
</tr>
<tr>
<td>% clones containing repeat sequences</td>
<td>13%</td>
</tr>
<tr>
<td>Average insert size of clones</td>
<td>0.6 kb</td>
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Average insert size was determined by using only microclones with sizable inserts, >0.1 kb (44 of 72 clones analyzed). Percentage clones containing repeat sequences was also determined by using only clones with sizable inserts, >0.1 kb (8 out of the 9 strongly hybridizing clones).
tion of a region of the mouse X chromosome, =25 cM long. Given a random distribution of proximal microclones along the chromosome region dissected, the clones may be able to differentiate very small genetic distances (=0.1 cM). The proximal bank of X chromosome microclones might therefore be expected to contain DNA sequences tightly linked to important genetic loci, such as mdx, that will lead to their eventual isolation and characterization. The provision of highly specific banks of microdissected clones from all mouse chromosomes should rapidly facilitate the future mapping of the entire mouse genome.

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