Isolation of region-specific cosmids from chromosome 5 by hybridization with microdissection clones

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ABSTRACT

A method is described for the isolation of chromosome region specific cosmids. The 5q35 region of the long arm of human chromosome 5 was microdissected, digested with *Mbo*I, ligated to oligonucleotide adaptors, amplified by the polymerase chain reaction and cloned into a plasmid vector. Inserts which did not contain highly repetitive sequences were used to screen a chromosome 5 cosmid library by direct hybridization. There were 33 positive cosmid clones identified with 4 microclones. Individual cosmid clones were biotinylated and used as probes for fluorescence *in situ* hybridization to metaphase chromosomes. Of the 33 cosmids that were mapped, 29 localized to q35 and 4 to q34, demonstrating the specificity of the microdissection library and the cosmids.

INTRODUCTION

The technique of chromosome microdissection has been successfully used to produce DNA libraries that are specific to subregions of *Drosophila* and mammalian chromosomes (1,2). Microlibraries can be created from the dissection of as few as twenty human chromosome fragments using the polymerase chain reaction (PCR) to amplify DNA ligated to specific linker adaptors (3,4), or primed with oligonucleotides designed to amplify DNA sequences nonspecifically (5).

Clones generated by microdissection have been used as Southern hybridization probes to characterize specific disease loci such as the fragile X mutation on Xq27.3, Langer-Giedion syndrome on 8q, and the Down syndrome region on chromosome 21 (6–7). The next step in this type of approach is to isolate larger genomic clones such as cosmids or yeast artificial chromosomes (YACs,9), so as to fully saturate the entire chromosome region. Microdissection clones should represent excellent entry points into such larger insert libraries. In addition, this expansion from each entry point into a much larger contig should obviate some of the concerns that have been raised as to the sequence representation in microdissection libraries.

We have been interested in constructing a physical map of parts of the long arm of chromosome 5 and in obtaining large genomic clones from this region. Here we report the microdissection and

cloning of a library of short fragments from the chromosome region 5q35, and the use of clones from this library that were not highly repetitive to screen a chromosome 5 cosmid library. The specificity of the isolated cosmids and by implication the original microclones, for the 5q35 region was confirmed by fluorescence *in situ* hybridization (FISH) to metaphase chromosomes, demonstrating the utility and specificity of this method for rapidly obtaining large genomic clones and thus gaining entry into a specific chromosome region.

METHODS

Microdissection and PCR Amplification

The procedure used was a modification of a previously described method for the microdissection and amplification of *Drosophila melanogaster* polytene chromosomes (4). Metaphase spreads were prepared from human lymphoblastoid cell lines. Cells were pretreated with 5 µg/ml of ethidium bromide for 1 h prior to the addition of 0.1 µg/ml of colcemid (Gibco) for 30 min. After hypotonic treatment, the cells were fixed twice in 5:1 methanol/acetic acid and resuspended in 3:1 fix immediately before dropping onto coverslips. The coverslips were air dried for 5 min then baked at 90°C for 15 min before banding with trypsin-Wright's stain.

Chromosomes were microdissected using a Leitz micromanipulator and sterile borosilicate needles. Coverslips with metaphase spreads were inverted and mounted on a glass chamber. Microdissected fragments from 5q35 (20 in total) were transferred to approximately 1 nl droplet of buffered glycerol (4 parts 87% glycerol, 1 part phosphate buffered saline) on an adjacent silanized coverslip. After the dissection, the chamber was filled with paraffin oil (Photrex grade, Baker) saturated with M buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM NaCl). All the microchemistry solutions were dispensed with silanized glass micropipettes.

The droplet containing the chromosome fragments was fused with 1 nl of 0.1% sodium dodecyl sulfate (SDS) and 0.5 mg/ml proteinase K in M buffer and incubated at 37°C for 30 min. The droplet was extracted twice with 3 volumes of phenol equilibrated with M buffer, followed by spraying briefly with chloroform (4). After 10 min, 0.1 vol of *Mbo*I (8 units/µl, BRL) was fused with

the droplet using a microneedle. The glass chamber was incubated at 37°C for 2 hours in a moist petri dish. The chamber was returned to the microscope, and a 0.5 vol of ligase mix (0.25 M Tris-HCl, pH 7.5, 50mM MgCl₂, 5 mM ATP, 5mM dithiothreitol, 25% polyethylene glycol 8000 mixed 1:1 with T4 DNA ligase) and an equal volume of *Mbo*I linker adaptor (100 µg/ml in 10mM Tris-HCl, pH 7.5, 0.5 mM EDTA) were fused to the DNA-containing droplet. The two oligonucleotides used to prepare the linker adaptor were a 20-mer (3'-ACATG-ACGTGGTCGTTTAGG-5'), and a 24-mer (5'-GATCTGTA-CTGCACCAGCAAATCC-3'). The ligation was carried out at room temperature for 12 hours. The microdroplet was then taken up in 10 µl of distilled water and transferred to a 0.6 ml plastic tube.

The PCR amplification was carried out in the following 100 µl reaction mixture: 50 mM KCl, 10mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, 200 µM of each of deoxynucleotide triphosphate, 2 µM of the 20-mer linker, and 2.5 units of Taq polymerase (Cetus). The DNA was amplified for 35 cycles (1 min at 94°C, 2 min at 55°C, and 3 min at 72°C) using a Perkin-Elmer-Cetus thermal cycler. A 10 µl aliquot from the first reaction was subjected to a second round of 35 cycles of amplification. The reaction mixture was spun through a Sephadex G-50 column, gel purified, and ligated into dephosphorylated BamHI digested Bluescript I (SK-) (Stratagene). After the ligation, the mixture was transformed into XL-1 blue competent cells (Stratagene).

Southern Hybridization

Inserts from the microdissection clones were isolated by PCR using T3 (ATTAACCCTCACTAAAG), and T7 (AATACGACTCACTATAG) primers specific to the vector sites flanking the inserts. The inserts were amplified for 30 cycles (1 min at 94°C,

1 min at 37°C, and 1 min at 72°C). An aliquot of the amplification mixture was electrophoresed in a 1.5% agarose gel, alkaline transferred to a nylon membrane (10), and hybridized with ³²P-dCTP labeled total human DNA using standard methods (11,12). Clones that did not hybridize to total human DNA were used to screen the chromosome 5 cosmid library.

Screening of the Cosmid Library

The human chromosome 5 cosmid library was constructed and provided by Larry Deaven (Life Sciences Division, Los Alamos National Laboratory). The cosmids were supplied as arrayed clones in 96-well plates. The library was plated in duplicate onto Immobilon-N (Millipore) pre-cut 6.5×11.5 cm membranes, placed on ampicillin (100 µg/ml) LB-agar plates, and grown overnight at 37°C. Cosmid clones on the membranes were processed using standard methods (10), UV-crosslinked (Stratalinker, Stratagene), and washed with a solution containing 1.5 M NaCl, 100 mM Tris-HCl pH 8.0, 1 mM EDTA, and 0.1% SDS at 65°C for 2 hours. After the removal of the bacterial debris, membranes were pre-hybridized in 5×SSPE, 5×Denhardt's, 100 µg/ml of denatured sonicated salmon sperm DNA, and 0.1% SDS for 2 hours. Hybridization was carried out for 16 hours at 65°C with ³²P-CTP labeled probes. The post-hybridization washes were in 2×SSC, 0.1% SDS at room temperature for 15 min, and 0.2×SSC, 0.1% SDS at 65°C for 30 min×2. Autoradiography was carried out overnight. Clones that were positive in duplicate were isolated and rescreened.

In Situ Hybridization

Microdissection clones were directly localized using ³H labelling as previously described (13). The hybridization of biotin labeled cosmids identified by the microdissected clones was performed using standard methods (14), with some modifications. Briefly, 10 µl of hybridization mixture containing 50% formamide, 10% dextran sulfate, 2×SSC, 1 µg of sonicated salmon sperm DNA, 0.5 µg of Cot1 DNA (Gibco), and 0.1 µg of biotinylated probe was denatured by heating to 70°C for 10 min. The probe mixture was cooled on ice for 2 min, incubated at 37°C for 15 min and applied to denatured metaphase spreads. Hybridization was

Figure 1. Microdissection of human chromosome 5. (A), Normal G-banding pattern of chromosome 5. The arrows indicate the microdissected region. (B), Banded metaphase with two chromosomes 5. The arrow head indicates the 5q35 region on the chromosome not dissected, and the arrow shows the other chromosome after the dissection of 5q35.

Figure 2. PCR amplified microdissection products before and after cloning into the Bluescript vector. (A), Ethidium bromide stained 1.5% agarose gel after electrophoresis of one-fifth of the PCR product (70 cycles). Lanes 1, λ-HindIII digest; lane 2, ΦX174-HindIII digest; lane 3, *Mbo*I linker adaptor without template DNA; lane 4, amplification of the 5q35 dissected DNA. (B), size range of vector inserts amplified with T3 and T7 primers. The first lanes on the left are ΦX174-HaeIII digests.

carried out at 37°C for 16 hours. Slides were washed for 3×2 min in 50% formamide, 2×SSC at 45°C and once in 2×SSC at room temperature. Detection of biotinylated probes was performed with 5 µg/ml of fluoresceinated avidin (Vector). The signals were amplified by incubating with biotinylated goat antiavidin (Vector), followed by fluoresceinated avidin. The chromosomes were counterstained with propidium iodide and DAPI.

RESULTS

The 5q35 region that we targeted for this study is shown in Figure 1A. Fragments were microdissected from twenty chromosomes similar to those in Figure 1B. The DNA was cut with *Mbo*I, ligated to linker-adaptors and amplified by PCR (Fig.2A). An aliquot of the PCR product was then cloned into the *Bam*HI site of a Bluescript plasmid yielding approximately 16,000 recombinant clones per microgram of insert DNA.

The inserts from 100 clones were amplified by PCR using T3 and T7 primers. The size range of the inserts was from 50 bp to 1400 bp with a mean of 435 bp (Fig.2B). Hybridization of total human DNA to the inserts revealed that 15% contained highly repetitive sequences. The depth of the microdissection library was assessed by hybridizing 10 microclones back to a panel of 100 random inserts. Only two of the ten inserts (MC-3 and MC-25) were represented more than once.

Inserts that did not hybridize with total human DNA were then used for *in situ* hybridization to determine if they localized to 5q35. One insert of 1400 bp was labeled with ³H and hybridized to normal human metaphases to directly determine whether it was indeed derived from the targeted region. There were 31 out of 228 (14%) grains on chromosome 5, and 17 (55%) of the 31 localized to q34-35.

To determine whether inserts from the microdissection library could be used to isolate large genomic clones, we hybridized 4 inserts that did not contain highly repetitive sequence elements to chromosome 5 cosmid filters. There were 33 cosmids identified and confirmed on secondary screening. Only 4 cosmids were identified in common with 2 probes. To determine the localization of the cosmids, clones were biotinylated and hybridized to metaphase chromosomes. Of the 33 cosmids used for *in situ*, 29 were localized to 5q35 and 4 to 5q34 (Fig.3). Two of the microdissection clones, MC-3 and MC-25, hybridized to 15 and 14 cosmids, respectively. The majority of these cosmids were mapped to q35, but 4 were localized to q34.

DISCUSSION

The microdissection and cloning of human chromosomes is an important method of generating DNA markers for specific regions of the genome. We have produced a microdissected library from 5q35 in an effort to generate specific DNA probes which could be used to isolate larger genomic fragments.

The characteristics of the 100 microclones which we analyzed are not appreciably different from the previously described chromosome 21 library which was amplified using an *Mbo*I linker-adaptor strategy (8). The number of clones with highly repetitive sequences was 15% in the 5q35 library compared to 22% for chromosome 21, and the mean insert lengths, 435 bp and 416 bp, were also very similar for 5q35 and 21, respectively. Another method that has been used to generate microlibraries involves digesting dissected chromosome fragments with *Rsa*I, ligating into *Sma*I-cut pUC, PCR amplification with M13/pUC primers, and cloning the PCR product into pUC13 (6,7). Although this strategy has produced representative and specific libraries from several regions of the genome, it has consistently produced mean insert sizes which are one-half that of the inserts generated from the *Mbo*I linker-adaptor method for reasons that remain unclear.

Two of the microclones from the 5q35 library hybridized to a large number of cosmids; the majority localized to q35, but 4 mapped to q34. These results suggest that the microclones contained low copy number repetitive sequences which are chromosome region specific. Certain classes of repetitive sequences like the variable number of tandem repeats (VNTRs) are preferentially located in the telomeric regions of chromosomes, including 5q35 (15,16). A preliminary screening of the cosmids described in this report with a 154 bp probe containing the consensus sequences from several VNTRs suggests that 11 of the 33 cosmids contain tandem repeats (DLS, unpublished).

The isolation of chromosome region specific cosmids or YACs using microdissection clones permits the confirmation of the specificity of the library while at the same time making large genomic fragments available for the generation of contig maps. This has the additional advantage of allowing immediate screening for cosmid clones without the obligatory DNA sequencing and oligonucleotide synthesis steps necessary in PCR screening schemes.

Microdissection clones from chromosome 21 have been used in an attempt to isolate chromosome specific cDNAs (8). It is doubtful that the representation of any microdissection library that has undergone 35 or more cycles of PCR will adequately

Figure 3. Demonstration of the localization of cosmids isolated with independent microclones to metaphase chromosomes by fluorescence *in situ* hybridization. (A,B,C), cosmids GL-A3, GL-A5 and GL-A8. The arrows indicate the hybridization of all three cosmids to 5q35.

reflect the complexity of the starting material. However, by moving from microdissection clones to cosmids and YACs, and then from these larger genomic regions to the cDNAs that they encode (17,18), it may be possible to isolate chromosome band-specific cDNAs throughout the genome.

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