Chromosome microdissection and cloning in human genome and genetic disease analysis

(microcloning/universal amplification/linker adaptor/polymerase chain reaction)

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ABSTRACT A procedure has been described for microdissection and microcloning of human chromosomal DNA sequences in which universal amplification of the dissected fragments by Mbo I linker adaptor and polymerase chain reaction is used. A very large library comprising 700,000 recombinant plasmid microclones from 30 dissected chromosomes of human chromosome 21 was constructed. Colony hybridization showed that 42% of the clones contained repetitive sequences and 58% contained single or low-copy sequences. The insert sizes generated by complete Mbo I cleavage ranged from 50 to 1100 base pairs with a mean of 416 base pairs. Southern blot analysis of microclones from the library confirmed their human origin and chromosome 21 specificity. Some of these clones have also been regionally mapped to specific sites of chromosome 21 by using a regional mapping panel of cell hybrids. This chromosome microtechnology can generate large numbers of microclones with unique sequences from defined chromosomal regions and can be used for processes such as (i) isolating corresponding yeast artificial chromosome clones with large inserts, (ii) screening various cDNA libraries for isolating expressed sequences, and (iii) constructing region-specific libraries of the entire human genome. The studies described here demonstrate the power of this technology for high-resolution genome analysis and explicate their use in an efficient search for disease-associated genes localized to specific chromosomal regions.

Molecular analysis and fine structure mapping of the human genome require isolation of large numbers of DNA probes from defined regions of the chromosomes (1, 2). In addition, the effective use of genetic linkage analysis with polymorphic DNA probes from the human genome has localized more and more inherited diseases of unknown etiology, as well as specific forms of cancer, to refined regions of various chromosomes (3). Thus, the next major task is to isolate the genes underlying these diseases for better understanding, prevention, diagnosis, and treatment of the disease.

A direct approach to this objective, as proposed by Edstrom and his colleagues (4), is to use the chromosomal microdissection technique to remove physically the chromosome region of interest and to clone the minute quantities of the dissected chromosomal DNA by a microcloning procedure. This approach has been successfully applied first with the Drosophila polytene chromosome (4) and later with the mammalian metaphase chromosome (5).

Recently, a modification of this method was described by Ludecke et al. (6) in which significantly increased cloning efficiency was achieved by ligation of the dissected chromosomal DNA to a vector, followed by polymerase chain reaction (PCR) to amplify the dissected sequences. The PCR products were cloned into the plasmid vector pUC13. Using this method, these investigators obtained between 5000 and 20,000 clones from each of the dissected chromosome regions (6–9). The advantages of this method over the original microcloning procedure include (i) banded and stained chromosome preparations can be used, (ii) fewer dissected chromosome fragments are needed, and (iii) large numbers of microclones can be obtained.

Another method of PCR microcloning was described by Saunders et al. (10) and by Johnson (11) for a universal amplification of microdissected Drosophila polytene chromosome fragments. In this method, a polytene chromosome band was dissected, cleaved with either Sau3A or Mbo I, and ligated to a linker adaptor. The linker adaptor was constructed from oligonucleotides of 24-mer and 20-mer annealed to yield a 5' protruding Sau3A or Mbo I sequence. The 20-mer of the linker adaptor was used as a primer for PCR. The amplified products were digested again with Sau3A or Mbo I and cloned into the compatible pBluescript vector. Large numbers of clones were obtained by this method. More importantly, Saunders et al. (10) also compared the PCR microcloning method with the conventional microcloning method and showed that the PCR method generated probes representing >90% of the dissected genomic region, whereas the probes from the conventional method covered only 3–4% of the same dissected region. Moreover, the PCR microcloning by the linker-adaptor method appears to offer additional useful features over the PCR microcloning method of Ludecke et al. (6): (i) several different linker sequences and restriction enzymes can be used, and (ii) a more efficient sticky-end ligation, instead of blunt-end ligation, is used.

We have been interested in this chromosome microtechnology and have developed competence in microdissection of human chromosomes and the conventional microcloning technique, as described (1, 2). Because the PCR microcloning method offers significant advantages over conventional microcloning, we have adopted the Mbo I linker-adaptor technique into our microcloning procedure to amplify microdissected human chromosome sequences for cloning into the plasmid vector pUC19. Here we report the construction of a very large library comprising 700,000 recombinant microclones from 30 dissected chromosomes of human chromosome 21. Characterization of microclones from the library confirmed their human origin and chromosome 21 specificity. Some of the clones have also been assigned to specific regions of chromosome 21 by a hybrid mapping panel. The chromosome technology described here should offer great potential in providing large numbers of cloned sequences from a defined region of a chromosome for fine-structure physical mapping of the genome, and for an efficient search for disease genes of unknown etiology, but localized to refined regions of the chromosome.

Abbreviations: PCR, polymerase chain reaction; YAC, yeast artificial chromosome.
MATERIALS AND METHODS

Cell Lines and Chromosome Preparations. For microdissection, we used one of our human−CHO-K1 cell hybrids, 38-14, which contains a single human chromosome 21 plus a human chromosome 9 (with a partial short arm). The human chromosome 21 in the hybrid can be distinguished from all other chromosomes even in unstained preparations. We have previously used this hybrid for microdissection of unstained preparations for conventional microcloning (2). Mitotic cells were harvested and fixed in 100% methanol instead of acetic acid/methanol (1:3) to avoid possible acid damage to the chromosomal DNA as described (2).

Chromosome Microdissection. We dissected the entire human chromosome 21 as the cloning material. In each microdissection experiment, 30 chromosomes were dissected with a de Fonbrune micromanipulator and the Leitz Laborlux D-FS high focal length microscope as described (2). Glass micro-needles with fine tips were constructed with a de Fonbrune microforge (12). The dissected chromosomes were assembled in a microdrop of proteinase K (stock solution, 0.5 mg of proteinase K per ml in 10 mM Tris-HCl, pH 7.5/10 mM NaCl/0.1% SDS), which was placed on a glass slide and covered with a larger drop of buffer-saturated paraffin oil (Merck 7162). No oil chamber was used.

Preparation of Microdissected Chromosome DNA. After dissection, a tiny drop of 10× fresh proteinase K was added to the original microdrop. The slide was incubated at 50°C for 2–3 hr to completely digest the protein of the chromosomes. The microdrop was then extracted three times with buffer-saturated phenol (ultr vape; BRL) and once with chloroform as described (12).

Preparation of Mbo I Linker Adaptor. The Mbo I linker adaptor was prepared by annealing two oligonucleotides of the following sequences as described by Johnson (11): a 24-mer including 5′-GAT CTG TAC TGC ACC AGC AAA TCC-3′ of which the 5′ end was phosphorylated, and a 20-mer including 5′-AGC AAA TCC-3′ with a dephosphorylated 5′ end (prepared by Research Genetics, Huntsville, AL). While the phosphorylated 5′ overhanging Mbo I end of the adaptor will ligate to the Mbo I-digested chromosomal DNA, the dephosphorylated 5′ termini at the blunt end of the adaptor will prevent them from self-ligation to form long repeating units, which would interfere with amplification. The two oligonucleotides were annealed at 60°C for 1 hr with a concentration of 100 μg/ml.

Ligation of Chromosomal DNA to Linker Adaptor. The phenol-extracted chromosomal DNA was digested at 37°C for 2 hr with 1/5th vol of Mbo I (BRL) from a stock of 10 units/μl. The enzyme was inactivated at 75°C for 20–30 min. The digested DNA was ligated to the adaptor by adding 1/2 vol of ligation reaction mixture containing 0.25 M Tris-HCl (pH 7.6), 50 mM MgCl₂, 5 mM ATP, 5 mM dithiothreitol, 25% polyethylene glycol 8000, and an equal volume of T4 DNA ligase (8 units/μl; BRL) mixed with another 1/2 vol of adaptor (100 μg/ml). The ligation mixture was incubated at 12°C for 16 hr.

Universal Amplification of Chromosomal DNA by Linker Adaptor. After ligation, the microdrop was transferred to an Eppendorf tube for amplification (13). The following PCR conditions were used: one cycle at 94°C for 5 min, at 45°C for 2 min, at 72°C for 3 min; 30 cycles at 94°C for 1.5 min, at 45°C for 2 min, at 72°C for 3 min; extended for 72°C for 10 min. The 20-mer of the adaptor was used as a primer. PCR was carried out in an automated thermal cycler (MicroCycler; Eppendorf).

Cloning into pUC19. After PCR, the amplified products were purified with GeneClean II (Bio 101, La Jolla, CA), digested with Mbo I, separated on agarose gel to remove adaptor molecules, purified again with GeneClean, ligated to dephosphorylated pUC19 vector (Boehringer Mannheim), and used in transformation with the highly efficient DH5α competent cells (BRL).

Characterization of Microclones. After transformation, ampicillin-resistant white colonies were individually isolated for initial characterization. For determining the unique or repetitive sequence nature of the insert, labeled total human DNA was hybridized to filters containing recombinant microclones, as described (14). The insert size contained in the microclones was estimated by PCR in which specific primers flanking the cloning sites of pUC19 were used to amplify the insert sequence in each clone. Two sequences of 20-mer each were used as the primer: 5′-ACA GGA AAC AGC TAT GAC CA-3′ and 5′-CGT TGT AAA ACG ACG GCC AG-3′. The bacterial colonies containing recombinant pUC19 were used directly in PCR and the amplified sequences were separated on a gel containing 2% NuSieve and 1% agarose.

Identification of Microclones Derived from Dissected Human Chromosome 21. The PCR amplified products from each clone were used to prepare labeled probes for Southern hybridization to DNAs from human, cell hybrid 38-14 containing human chromosome 21, and the standard CHO. The PCR products were purified by GeneClean and labeled to high specific activities with [32P]dCTP by the primer-extension procedure (15) using both the random hexamers and the two specific primer sequences flanking the inserts. After separation on gel, the DNA was transferred to a Hybond-N+ nylon membrane (Amersham) for 2–3 hr by the alkaline-blotting procedure (transfer buffer, 0.4 M NaOH), according to the manufacturer's protocol. The filters were prehybridized at 65°C for 2 hr before the probes were added at a concentration of 1–3 × 10⁶ cpm/ml. Hybridization was carried out at 65°C for 16–20 hr. Stringent washing was used, including a final wash at 65°C for 10–20 min. The x-ray film was exposed for 2–4 days.

Regional Mapping of Microclones on Chromosome 21. A chromosome 21 regional mapping panel was kindly provided by David Patterson (Eleanor Roosevelt Institute). Only a partial panel including four hybrid lines was used: 153E7b (containing normal long arm), 21q+ (containing 21pter-q22.2), GA9-3 (containing 21q21.1–qter), and 1881c-13b (containing 21q21.3–qter). Detailed karyotypic analysis of these hybrids has been described (16).

Precautions in Minimizing Extraneous DNA in Universal Amplification. It is absolutely essential to enforce strict precautions to prevent propagation of contaminant DNA from extraneous sources into the highly sensitive process of universal amplification (17–19). No glassware washed in a common facility was used; disposable plasticware was used instead. Autoclaved distilled water, buffers, and reagents were used. For materials that are not autoclavable, exposure to UV (254 nm) will render the extraneous DNA not amplifiable (18). All buffers and reagents were prepared in an air-clean hood (19) and divided into small aliquots to be used only once and discarded. Similarly, all glass micropipettes constructed for microcloning were used only once and discarded. Only "safe" buffer was used to saturate paraffin oil and phenol. A control microdrop without insert DNA was always included in each PCR microcloning experiment; after PCR, if the control sample contained any trace of PCR products, the entire experiment was discarded. With these precautions strictly enforced, "mysterious" recombinant clones of unknown origin have been effectively excluded.

RESULTS

Microdissection of Human Chromosome 21. Chromosome preparations from hybrid 38-14 were used. The de Fonbrune micromanipulator is highly efficient for use in dissecting human chromosomes. The entire chromosome 21 was dis-
sected. The same dissection technique can be used to dissect a fragment from this chromosome, as shown in Fig. 1. It was convenient to dissect a fragment of 10–15 megabases from the middle part of the chromosome and a smaller fragment of 3–5 megabases from the ends. Thirty chromosomes were dissected and stored in proteinase K. Another microdrop containing only proteinase K was treated in the same fashion as the sample microdrop throughout the experiment to serve as a control.

**Universal Amplification of Microdissected Chromosome 21 Using Mbo I Linker Adaptor and PCR.** The sample microdrop containing the collected 30 pieces of human chromosome 21 and a control microdrop were treated with fresh proteinase K, extracted with phenol and chloroform, digested with Mbo I, ligated to Mbo I linker adaptor, and subjected to PCR. A sample of 15 μl of the 100-μl PCR products was used in gel electrophoresis to examine the amplified fragments. In parallel experiments, a series of control samples that received no chromosome 21 DNA were treated with different degrees of precautionary stringency designed to eliminate extraneous DNA. As shown in Fig. 2, the control sample in lane 4, which showed no detectable amplification of extraneous DNA, resulted only after strictly enforcing all precautions. In our cloning experiments, we only used those with control samples like that shown in lane 4 and discarded all other experiments that displayed even trace amounts of PCR products in control samples.

**Cloning Amplified Chromosome 21 Sequences into pUC19.** After PCR, the products were digested with Mbo I and ligated to pUC19 for transformation; 700,000 recombinant microclones were obtained. The clones from this library were characterized in detail.

**Determination of Microclones with Unique Sequence Inserts.** Analysis of 600 clones for unique or repetitive sequences revealed that 22% hybridized either strongly (highly repetitive) or less strongly (moderately repetitive), 20% hybridized weakly (slightly repetitive), and 58% showed no detectable hybridization (containing unique or very low copy sequences). Fig. 3 shows a typical colony hybridization of these microclones.

**Determination of Insert Size Contained in the Microclones.** We analyzed 60 clones for their insert size. The estimated sizes in these inserts ranged from 50 to 1100 base pairs (bp), with a mean length of 416 bp. All these 60 inserts have apparently different sizes. A gel showing the amplified inserts is shown in Fig. 4. Thus, the insert sizes contained in our microclones are generally larger than those obtained by the method of Horsthemske and co-workers (6–9), which averaged between 150 and 217 bp.

**Demonstration of Human Origin and Chromosome 21 Specificity of the Microclones.** Microclones were analyzed by Southern blot analysis to determine whether they were of human origin and chromosome 21 specific. Of 22 microclones analyzed, at least 19 were shown to be definitely human and chromosome 21 specific and 1 was from CHO. Typical hybridization results are presented in Fig. 5 for three of these microclones. All 19 clones hybridized to both human and hybrid 38-14 but not to CHO. The banding patterns in human and hybrid DNA are identical for each clone. Because the inserts were generated by Mbo I cleavage of the chromosomal DNA, the hybridizing genomic fragments cleaved with either HindIII or EcoRI had different sizes from the inserts. Thus, clone 21E-181 containing an insert of 660 bp hybridized to a HindIII genomic fragment of 11.7 kilobases (kb) (Fig. 5A). Clone 21E-199 containing an insert of 380 bp hybridized to a HindIII fragment of 13.2 kb (Fig. 5B). Clone 21E-56 containing an insert of 280 bp hybridized to a HindIII fragment of 14.1 kb (Fig. 5C) and to an EcoRI fragment of 1.4 kb (Fig. 5D).

No weak bands were detected in CHO, presumably because we used stringent hybridization and washing conditions in these experiments. However, we cannot rule out the possibility that homologous sequences may exist in CHO if less stringent conditions were used. For the 19 clones that

**Fig. 1.** Microdissection of the proximal long arm of human chromosome 21, including 21q11-q21. Unstained metaphase chromosomes were prepared from cell hybrid 38-14, containing an identifiable chromosome 21.

**Fig. 2.** A gel showing decreasing amounts of PCR products from contaminated DNA by increasing the stringency of control to eliminate extraneous DNA during microcloning. Lanes: 1, dissected chromosomal DNA sample; 2–4, controls without adding DNA. Lane 4 received the most stringent control and showed no detectable PCR products.

**Fig. 3.** Colony hybridization using grid filters containing 200 microclones per filter hybridized to labeled total human DNA.

**Fig. 4.** A 3% gel (2% NuSieve/1% agarose) showing amplified insert sequences from microclones. The actual insert size in each clone is calculated by subtracting 106 nucleotides from the amplified band, of which 40 nucleotides are the two primer sequences and 68 nucleotides are the pUC19 multiple cloning sites. Lanes: 1, 123-bp ladder marker; 2–13, inserts with, respectively, 240, 500, 390, 560, 370, 1100, 230, 140, 170, 510, 820, and 80 bp.
increased PCR products, we could expect that the clones in the library are largely from different sequences and cover a very large portion of the chromosome. This prediction can be tested by using samples of the microclones to screen yeast artificial chromosome (YAC) libraries for isolating corresponding YAC clones to analyze their genomic representation and coverage of the chromosome.

Because of the common occurrence of extraneous DNA unintentionally introduced into the samples during microdissection and microcloning, we have exercised highly stringent control to eliminate such contamination. This precaution is essential to ensure the absence of mysterious microclones in the library and to minimize the efforts involved in the characterization of individual clones for their human origin and chromosome specificity.

The small insert sizes, averaging 300–400 bp, in the microclones may present an advantage in cloning genomic regions containing inverted repetitive sequences, which can form palindromic structures and often render the clones unstable (20). A pair of inverted repeats several hundred base pairs long each would appear too large to be included in most of the microclones, thus potentially reducing unclonable regions in the human genome.

It is relatively simple and efficient to isolate large numbers of microclones containing unique sequences by colony hybridization. At least half of the clones in the library can be expected to contain single or low-copy sequences. Thus, thousands of microclones with unique sequences can be conveniently isolated and used in screening other human genomic libraries for larger inserts. In an initial attempt, we used four of our unique sequence microclones to screen the human YAC library from Washington University (21). This YAC library contains $\approx 56,000$ clones (5 times the human genome equivalent) with a mean insert size of 250 kb. After two-thirds of the library was screened, five positive YAC clones were identified with inserts ranging from 50 to 360 kb. These experiments demonstrate the feasibility of using microclones with very small inserts (several hundred base pairs) to isolate corresponding large genomic fragments of several kilobases in the YAC clones. The YAC clones isolated from a chromosomal region can be used to establish contiguous sequences to cover the entire dissected genomic region. The short inserts contained in the pUC19 microclones can be conveniently sequenced to identify unique sequences to serve as “sequence-tagged sites” as proposed by Olson et al. (22). Thus, the chromosome microtechnology described here fulfills our initial intention for gaining quick and direct access to a defined human chromosomal region to isolate large numbers of clones to facilitate genomic analysis and search for disease genes (1, 2).

The availability of large numbers of unique sequence probes from an important chromosomal region is also valuable in screening various cDNA libraries for isolating expressed sequences from the region. This approach is particularly appealing because (i) cDNA sequences have functional significance and are valuable materials for early phases of genomic sequencing, and (ii) cDNA clones isolated from relevant tissues can serve as candidate genes for an effective search for particular disease genes assigned to the region. We have already begun to use this approach to attempt to isolate as many cDNA clones as possible from human chromosome 21. Filters containing Agt11 phage plaques from a human liver cDNA library were screened with our chromosome 21 microclones identified to possess only unique sequences. Inserts from these microclones were independently amplified and the amplified inserts were pooled, 10 clones in a group, to prepare labeled probes. After screening 100,000 phage from the cDNA library probed with 100 unique sequence microclones, at least 4 positive cDNA clones were identified. Since the number of cloned genes on chromosome 21 is still

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**DISCUSSION**

The power of PCR in amplifying small amounts of microdissected chromosome sequences has greatly increased the efficiency of cloning these fragments into plasmid vectors. The extent of amplification (several orders of magnitude increase by 30 cycles of PCR) can be expected to compensate effectively for the loss of the dissected sequences due to the decreased cloning efficiency by several orders of magnitude after cleavage, dephosphorylation, insertion, and transformation imposed on plasmid vector cloning. For human chromosome 21 of $\approx 50$ megabases, complete digestion by Mbo I can generate 120,000–170,000 Mbo I fragments with an average length of 300–400 bp. We used 30 pieces of chromosome 21 and obtained 700,000 microclones with a mean size of 416 bp. Analysis of 60 microclones from this library showed that they all have apparently different insert sizes. If we assume that the decreased cloning efficiency of the dissected sequences can be effectively balanced out by the

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**Fig. 5.** Southern blot analysis showing that the microclones from the chromosome 21 library are of human origin and chromosome 21 specific. The microclones hybridized to DNAs from human and hybrid 38-14 (containing human chromosome 21 from which dissection was made), but not to CHO. Stringent hybridization and washing conditions were used in these experiments.

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limited, 16 genes cloned so far (3), a large collection of cDNA clones from chromosome 21 isolated by this approach should be extremely useful as candidate genes for the search of specific genes underlying Down syndrome and other neurological diseases on this chromosome.

The chromosome microtechnology described here can be used for molecular structure and sequence organization analyses of interesting cytogenetic landmarks—e.g., centromeres, telomeres, fragile sites, centric heterochromatin, acentric double minutes, centric minichromosomes, homogeneously staining regions, translocation and deletion breakpoints, and unstable chromosome regions.

This microtechnology is also useful in providing region-specific probes to fill large gaps that currently exist in the genetic linkage maps where DNA probes are sparse or nonexistent—e.g., the large gaps (>40 centimorgans) in 1q, 2p, 6p, 6q, 9p, 10p, 11q, 12q, 13q, 14q, 15q, 17q, 18q, etc. (23).

Microdissection can be directed to these regions and highly polymorphic microclones can be identified and used for this purpose.

The analysis of the human genome and the search for disease genes are both challenging and rewarding. These studies require development of many powerful molecular genetic approaches and methodologies. A better understanding of the human genome should provide insight into the structure and functional relationships of specific genes and DNA sequences in normal and diseased states. Successful isolation of genes underlying genetic diseases may lead to prevention, diagnosis, and therapy of many devastating diseases, including cancer. The chromosome microtechnology described here appears to offer an additional powerful methodology to achieve these goals.

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