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PCR-mediated Cloning of *Hpa*II Tiny Fragments from Microdissected Human Chromosomes

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Vertebrate DNA contains a small fraction of unmethylated CpG-rich DNA sequences, many of which include the 5′ end of a gene. This fraction can be detected by its cleavage to tiny fragments with the methylation-sensitive restriction enzyme *Hpa*II. Thus, the isolation of *Hpa*II tiny fragments (HTFs) from a specific chromosome region may be a useful approach for making an inventory of the genes contained in it. Using microdissection, we have isolated DNA from human chromosome band 8q24.1. The DNA was digested with *Hpa*II, ligated to a *Cla*I-cut pUC plasmid, and amplified with *Taq* DNA polymerase and the standard M13/pUC forward and reverse sequencing primers. The amplification products were used to construct an HTF library, which is enriched for CpG-rich single-copy clones.

Positional cloning of disease loci in man, mouse, and other species requires the isolation of DNA from specific chromosome regions and the identification of candidate gene sequences. This approach has been employed successfully in chronic granulomatous disease,[1] Duchenne muscular dystrophy,[2] cystic fibrosis,[3] and several other diseases, but it can be tedious. Direct cloning of gene sequences from the region of interest may speed up such an undertaking. Region-specific probes can be isolated by microdissection of banded metaphase chromosomes and enzymatic DNA amplification,[4] but they are randomly distributed over the target region.[5–8] Unexpectedly, however, some microlibraries appear to be enriched for gene sequences.[9–11] It is possible that these libraries were constructed from gene-rich chromosome regions. Here we describe an approach aimed at the specific cloning of gene sequences from microdissected chromosome fragments.

In previous microcloning experiments, the chromosomal DNA was digested with the restriction enzyme *Rsa*I (recognition site GTAC), ligated to a *Sma*I-cut pUC plasmid, and amplified in a sequence-independent manner with DNA polymerase and the M13/pUC forward and reverse sequencing primers.[12] The modified procedure described here is based on the observation that many vertebrate genes have an unmethylated CpG-rich island at their 5′ end.[13] These islands can be detected by their cleavage to tiny fragments with the methylation-sensitive restriction enzyme *Hpa*II. Thus, the isolation of *Hpa*II tiny fragments (HTFs) from microdissected chromosome fragments should give direct access to gene sequences in the dissected chromosome region. Because *Hpa*II fragments have sticky ends, they cannot be ligated to the *Sma*I plasmid. Therefore, we have replaced the *Sma*I site by the recognition site for *Cla*I (ATCGAT), which generates sticky ends compatible with *Hpa*II (Fig. 1). Here we report on the use of this plasmid in the enzymatic amplification of *Hpa*II tiny fragments from total human DNA and human chromosome band 8q24.1.

MATERIALS AND METHODS

*Cla*I-vector construction and preparation

The pUC *Sma*I vector, which contains a single *Sma*I site flanked by *Eco*RI sites,[4] was digested with *Eco*RI, and the ends were filled in with dATP and dTTP using Klenow DNA polymerase. Unincorporated dNTPs and the released *Sma*I adaptor were removed by gel filtration through a Sephadex S-200 spun column (Pharmacia). The plasmid DNA was ligated to a synthetic *Cla*I linker (CATCGATG, New England Biolabs) and used to transform competent DH5α cells. One clone with the correct sequence was grown up and used for subsequent experiments. The plasmid was linearized with *Cla*I, purified by agarose gel electrophoresis, and isolated by the freeze-squeeze method.[14]

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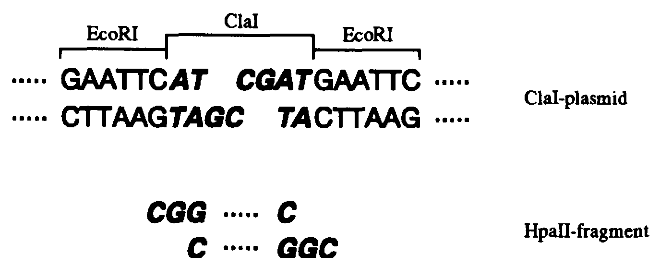

Figure 1 Universal enzymatic amplification of *Hpa*II fragments. *Hpa*II fragments are ligated to a *Cla*I-cut pUC plasmid containing a single *Cla*I site flanked by two *Eco*RI sites and are amplified with *Taq* DNA polymerase and the standard M13/pUC forward and reverse sequencing primers.

Enzymatic Amplification of *Hpa*II Fragments from Total Human DNA

Human genomic DNA (5 μg) was digested with *Hpa*II, extracted with phenol and chloroform, and precipitated with ethanol. An aliquot of this DNA preparation (50 ng) was ligated to 450 ng of *Cla*I-digested *Cla*I plasmid DNA with 5 units of T4 DNA ligase in the presence of 15% polyethylene glycol 8000 (final volume 15 μl). Fifteen picograms of ligation products were treated with 1 unit of *Cla*I and amplified with *Taq* DNA polymerase (2 units) and 1 μM M13/pUC forward and reverse sequencing primers (30 cycles of 1 min at 94°C, 2 min at 45°C, and 3 min at 72°C in a Perkin-Elmer Cetus Thermal Cycler, final volume 100 μl). Half of the amplification products were treated with 20 units of *Eco*RI (final volume 100 μl) and purified on a Sephacryl S-300 spun column (Pharmacia). One-sixth of the column eluate was used for ligation to 20 ng of *Eco*RI-digested pT7T3-18U treated with phosphatase (Pharmacia, final volume 20 μl). One microliter of this ligation mixture was used to transform MAX efficiency competent DH5α cells (BRL). Approximately 400 colonies were obtained. Twelve clones were picked at random.

Microdissection and Library Construction

Microdissection of 8q24.1 was performed essentially as described previously.[15] Briefly, cultured amniotic fluid cells were harvested using the pipette method[16] (time of fixation 10–20 sec). Metaphase-stage cells were spread on clean coverslips and G-banded with trypsin and Giemsa (GTG). Chromosomes were dissected on an inverted microscope (magnification 1250x) with extended siliconized glass needles and a micromanipulator. Twenty chromosome fragments were pooled. Microreactions were performed as described previously,[12,15] except that the DNA was digested with *Hpa*II and ligated to the *Cla*I plasmid. Ligation products were treated with *Cla*I, amplified by PCR, and cloned into pT7T3-18U as described above. From 1 μl of ligation mixture, 19 colonies were obtained. Fifteen clones were picked at random.

Sequence Analysis

Recombinant plasmids were purified on Qiagen-20 tips. The *Cla*I plasmid and HTF clones from total human DNA were sequenced by the dideoxy chain-termination method using [α-^{35}S]dATP and Sequenase (USB). Microclones were sequenced on an Applied Biosystems 373A DNA Sequencer using fluorescence-tagged dideoxynucleotides and the cycle-sequencing procedure (Applied Biosystems). Sequence comparisons and data bank searches were performed with the Beckman Microgenie computer program.

Southern Blot Analysis

HTF clones from total human DNA were labeled by random oligonucleotide priming[17] and used to probe Southern blots containing *Eco*RI-, *Hin*dIII-, *Ban*II-, *Bam*HI-, and *Taq*I-digested human DNA. Microclones were labeled by PCR[18] and used to probe Southern blots containing *Bam*HI- and *Taq*I-digested DNA from man, hamster, and a chromosome 8-only hybrid cell line (10156B, Camden Cell Repository, NJ). Hybridizations were performed essentially as described previously,[19] except that 50% formamide was included in the incubation buffer and that hybridizations were performed at 42°C. The final wash was usually at 65°C in 150 mM sodium chloride, 15 mM sodium citrate, and 0.1% SDS.

RESULTS

Enzymatic Amplification of *Hpa*II Tiny Fragments from Total Human DNA

Total human DNA was digested with *Hpa*II and ligated to a *Cla*I-cut plasmid which contains a single *Cla*I site flanked by two *Eco*RI sites (Fig. 1). Polyethylene glycol (15%) was included in the ligation mixture to obtain long concatemers,[20] which are more efficiently primed in the PCR than circular molecules. A 10-fold excess of plasmid DNA over genomic DNA was used to assure that each *Hpa*II fragment was flanked by two plasmid molecules. After ligation, nonrecombinant *Eco*RI-*Cla*I-*Eco*RI linker sequences were recut with *Cla*I. Because *Cla*I sites are lost by ligation to *Hpa*II fragments and *Cla*I cuts infrequently in *Hpa*II tiny fragments, this step leaves most of the recombinant molecules intact.

An aliquot of the ligation products corresponding to 1.5 pg of human DNA was amplified with *Taq* DNA polymerase and the M13/pUC forward and reverse sequencing primers. Thirty cycles of denaturation, annealing, and elongation were used. After *Eco*RI digestion and gel filtration, an aliquot of the amplification products was cloned into the plasmid vector pT7T3-18U. In one transformation experiment, approximately 400 clones were obtained. Based on this finding, we estimate that the whole library contains approximately 48,000 clones (yield 32 clones/fg DNA).

Twelve clones (t1–t12) were picked at random. Clone t1 grew poorly and could not be analyzed in detail. As estimated from agarose gel electrophoresis, it contains an insert of approximately 130 bp. Clones t2–t12 were sequenced and used to probe human genomic Southern blots. All clones were different (Table 1). The insert size ranged from 107 to 179 bp

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TABLE 1 Properties of HTF Clones from Total Human DNA

Clone	Insert size (bp)	C + G (%)	CpG/GpC	Number of bands on Southern blot
t1	130	ND	ND	ND
t2	121	53	12/5	1 (rDNA)
t3	141	50	5/8	smear (*Alu*)
t4	179	57	3/10	1
t5	121	65	10/12	1
t6	160	46	3/8	2
t7	168	52	4/14	1
t8	156	56	8/11	2
t9	130	52	5/9	1
t10	107	50	7/4	1
t11	125	41	2/5	1
t12	117	60	6/12	1

(ND) Not determined.

(mean 138). All clones contained half a *Cla*I and half a *Hpa*II site (5′-ATCGG... CCGAT-3′) at both ends (Fig. 1). The G + C content ranged from 41% to 65% (mean 54%). The CpG/GpC ratio ranged from 0.29 to 2.4 (mean 0.8).

Except for t2 and t3, significant homologies to other primate DNA sequences were not found. t2 contains a fragment from 18S ribosomal RNA gene (base pairs 1857–1917). t3 contains a fragment from an *Alu* repeat. In agreement with these findings, t2 detected one strongly hybridizing fragment on genomic Southern blots, while t3 gave a smear. Clones t4–t12 detected one or two restriction fragments.

Enzymatic Amplification of *Hpa*II Tiny Fragments from Microdissected Human Chromosome Band 8q24.1

Encouraged by the finding that most of the random HTF clones detected single-copy sequences and that a known gene (rRNA gene) had been recovered, we applied our amplification system to human chromosome band 8q24.1, for which an *Rsa*I microdissection library was already available.[4] The target region was excised from 20 metaphase chromosomes, which had been G-banded with trypsin-Giemsa (Fig. 2). Using the microreaction technique described previously,[15] the DNA (estimated amount 300 fg) was digested with *Hpa*II and ligated to the *Cla*I plasmid. The ligation products were treated with *Cla*I and amplified as described above. In one transformation, 19 clones were obtained. Based on this finding we estimate that the whole library contains 4500 clones (yield 15 clones/fg DNA).

Fifteen clones were picked at random and used to probe human genomic Southern blots. Eight clones did not give any signal, and seven clones detected single-copy sequences. Repetitive sequences were not detected. Sequence analysis of the seven single-copy clones revealed that two sequences (m1 and m3) were represented in two clones and one sequence (m2) was represented in three clones (Table 2). All three sequences contained half a *Cla*I and half a *Hpa*II site at both ends. The size of the sequences ranged from 58 to 95 bp (mean 71 bp). The G + C content ranged from 57% to 66% (mean 61%). The CpG/GpC ratio ranged from 0.38 to 1.33 (mean 0.9).

To determine whether m1–m3 are derived from chromosome 8, insert DNA was used to probe Southern blots containing DNA from man, hamster, and a human–hamster hybrid cell line containing chromosome 8 as the only human chromosome. All three clones gave positive signals with the hybrid cell line (Fig. 3). m2 detects a homologous sequence in hamster DNA.

DISCUSSION

The technique described in the present paper aims at the specific cloning of gene sequences from defined regions of vertebrate chromosomes. It is based on microdissection of banded metaphase chromosomes, digestion of the chromosomal DNA with the methylation-sensitive restriction enzyme *Hpa*II, ligation of the DNA fragments to a *Cla*I-cut pUC plasmid, and amplification of the ligation products with *Taq* DNA polymerase and the M13/pUC forward and reverse sequencing primers. Owing to the high degree of DNA methylation in vertebrate DNA, most of the *Hpa*II fragments are very large and fail to be amplified by the PCR. Thus, our technique selects for *Hpa*II tiny fragments. These fragments are derived from unmethylated CpG-rich DNA sequences, many of which include the 5′ end of a gene.[13]

The G + C content of the *Hpa*II microclones (61%) is close to that of HTF islands.[13] It is significantly higher

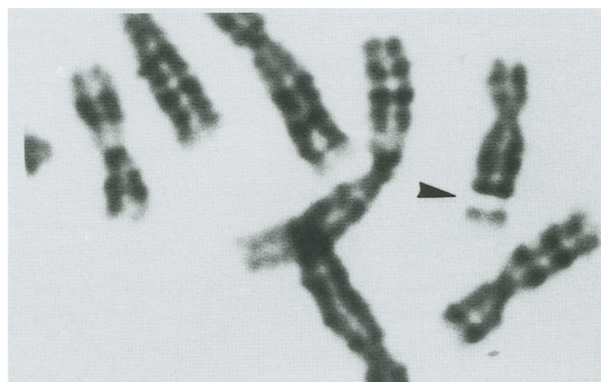

Figure 2 Microdissection of chromosome 8. Human metaphase chromosomes were prepared and dissected as described in Materials and Methods. Chromosome band 8q24.1 was targeted. The arrow indicates the dissected region.

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TABLE 2 Properties of HTF Clones from Microdissected DNA

Clone	Number of clones	Insert size (bp)	G + C (%)	CpG/GpC	Chromosome 8
m1	2	59	61	4/3	+
m2	3	58	66	7/7	+
m3	2	95	57	3/8	+

than the G + C content of *Rsa*I microclones derived from the same chromosome region (39%; ref. 4 and unpublished results) and the human genome in general. The same holds true for the CpG/GpC quotient (0.9% vs. 0.4%). All *Hpa*II microclones contain several CpG dinucleotides, whereas 7 of 25 (28%) *Rsa*I microclones analyzed do not contain any CpG at all (ref. 4 and unpublished results). These results prove that the *Hpa*II clones are derived from CpG-rich islands.

Most of the *Hpa*II clones are repeat-free. Only 1 of 11 clones obtained from total human DNA contained *Alu*-repetitive DNA. As most of the *Alu* sequences contain *Hpa*II sites, these findings suggest that *Alu* sequences are methylated or that they are selected against in our amplification and cloning procedure.

One HTF microclone sequence is conserved in hamster DNA and may contain gene or regulatory sequences. No significant homologies to other DNA sequences were found in a data bank search. One of the clones obtained from total human DNA contained part of the rRNA gene, which is present in multiple copies within the nucleolus organizer region of acrocentric chromosomes.[21,22] These results indicate that gene sequences can be recovered with our technique.

Compared to the 8q24.1 *Rsa*I microdissection library, the *Hpa*II microdissection library contains fewer clones and is much less complex. This result was expected, because the human genome contains fewer *Hpa*II tiny fragments than *Rsa*I fragments. The high redundancy of clones in the *Hpa*II library, however, makes the analysis more tedious. The high frequency of *Hpa*II microclones that do not hybridize to human DNA (8 of 15), however, is probably not related to the use of *Hpa*II, but to chromosome fixation-induced DNA damage.[12]

The *Hpa*II microclones will now be used to clone larger pieces of DNA and to identify gene sequences in 8q24.1. Deletion of a set of unknown genes in this region leads to the Langer–Giedion syndrome, which is characterized by craniofacial dysmorphism and skeletal abnormalities. It is one of our goals to identify these genes.

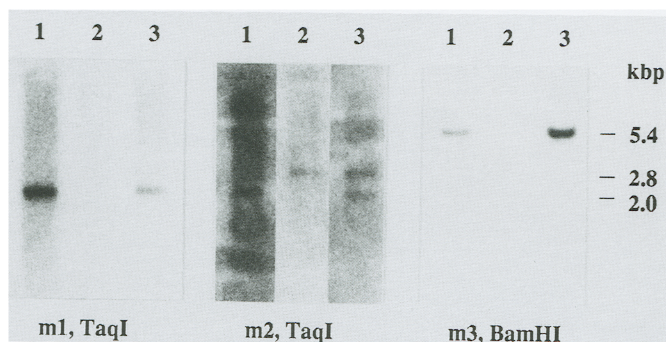

Figure 3 Chromosomal localization of HTF microclones. Microclones were labeled with ^{32}P and used to probe Southern blots containing total human DNA (lane *1*), total hamster DNA (lane *2*), and DNA from a chromosome 8-only hybrid (lane *3*). Fragment sizes were estimated with the help of *Hin*dIII-digested λ DNA and are given in kilobase pairs (kbp).

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