PCR-mediated Cloning of Hpall Tiny Fragments from Microdissected Human Chromosomes

Bernhard Horsthemke, Uwe Claussen, Sonja Hesse, and Hermann-Josef Lüdecke

1Institut für Humangenetik, Universitätsklinikum Essen, W-4300 Essen 1, Germany, and 2Institut für Humangenetik, Universität Erlangen, Erlangen, Germany

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 Hpall. Thus, the isolation of Hpall 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 Hpall, ligated to a Clal-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 Rsal (recognition site GTAC), ligated to a SmaI-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 Hpall. Thus, the isolation of Hpall tiny fragments (HTFs) from microdissected chromosome fragments should give direct access to gene sequences in the dissected chromosome region. Because Hpall fragments have sticky ends, they cannot be ligated to the SmaI plasmid. Therefore, we have replaced the SmaI site by the recognition site for Clal (ATCGAT), which generates sticky ends compatible with Hpall (Fig. 1). Here we report on the use of this plasmid in the enzymatic amplification of Hpall tiny fragments from total human DNA and human chromosome band 8q24.1.

MATERIALS AND METHODS

Clal-vector construction and preparation

The pUC SmaI vector, which contains a single SmaI site flanked by EcoRI sites (4), was digested with EcoRI, and the ends were filled in with dATP and dTTP using Klenow DNA polymerase. Unincorporated dNTPs and the released SmaI adaptor were removed by gel filtration through a Sephadex S-200 spun column (Pharmacia). The plasmid DNA was ligated to a synthetic Clal 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 Clal, purified by agarose gel electrophoresis, and isolated by the freeze-squeeze method (14).

Enzymatic Amplification of Hpall Fragments from Total Human DNA

Human genomic DNA (5 µg) was digested with Hpall, extracted with phenol and chloroform, and precipitated with ethanol. An aliquot of this DNA preparation (50 ng) was ligated to 450 ng of ClaI-digested ClaI 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 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 EcoRI (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 EcoRI-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 cover slips and G-banded with trypsin and Giemsa (GTG). Chromosomes were dissected on an inverted microscope (magnification 1250×) 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 Hpall and ligated to the ClaI plasmid. Ligation products were treated with ClaI, 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 ClaI plasmid and HTF clones from the total human DNA were sequenced by the dideoxy chain-termination method using [α-32P]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 database 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 EcoRI-, HindIII-, BamHI-, and TagI-digested human DNA. Microclones were labeled by PCR (18) and used to probe Southern blots containing BamHI- and TagI-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 Hpall Tiny Fragments from Total Human DNA

Total human DNA was digested with Hpall and ligated to a ClaI-cut plasmid which contains a single ClaI site flanked by two EcoRI sites (Fig. 1). Polyeethylene 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 Hpall fragment was flanked by two plasmid molecules. After ligation, non-recombinant EcoRI-ClaI-EcoRI linker sequences were recut with ClaI. Because ClaI sites are lost by ligation to Hpall fragments and ClaI cuts infrequently in Hpall 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 EcoRI 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.
TABLE 1 Properties of HTF Clones from Total Human DNA

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

(ND) Not determined.

(mean 138). All clones contained half a Clai and half a HpaII 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 HpaII 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 Rsal microdissection library was already available. 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, the DNA (estimated amount 300 fg) was digested with HpaII and ligated to the Clai plasmid. The ligation products were treated with Clai 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 Clai and half a HpaII 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 HpaII, ligation of the DNA fragments to a Clai-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 HpaII fragments are very large and fail to be amplified by the PCR. Thus, our technique selects for HpaII tiny fragments. These fragments are derived from unmethylated CpG-rich DNA sequences, many of which include the 5’ end of a gene. The G + C content of the HpaII microclones (61%) is close to that of HTF islands. It is significantly higher.
than the G + C content of Rsal 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 HpaII microclones contain CpG dinucleotides, whereas 7 of 25 (28%) Rsal microclones analyzed do not contain any CpG at all (ref. 4 and unpublished results). These results prove that the HpaII clones are derived from CpG-rich islands.

Most of the HpaII clones are repeat-free. Only 1 of 11 clones obtained from total human DNA contained Alu-repeative DNA. As most of the Alu sequences contain HpaII 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. These results indicate that gene sequences can be recovered with our technique.

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

The HpaII 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.

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B Horsthemke, U Claussen, S Hesse, et al.

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