Method for systematic targeted isolation of homologous cDNA fragments in a multiplex format

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In this study, a two-step method for systematic multiplex cloning of homologous cDNAs from related species was developed. The first step, called MUCH (multiplex cloning of homologous genes), is cloning of partial but authentic cDNA fragments of homologous cDNAs by hybridization to arrayed cRNA probes of specified genes on a nylon membrane, followed by PCR amplification of the hybridized fragments. The second step is PCR-based screening of a library that contains longer cDNA inserts based on the sequences obtained in the first step. To evaluate this method, we tried to isolate mouse counterparts of 53 human large cDNAs by MUCH and could successfully isolate 32 mouse counterpart cDNAs from a single library. Complete sequencing of two mouse cDNAs isolated by PCR-based screening further demonstrated that this method enabled us to isolate multiple homologous cDNAs in parallel. We thus expect that this method could be applied to high-throughput cloning of homologous cDNAs in related species.

INTRODUCTION

As comparative genomic approaches are becoming popular in functional genomic analyses, a technique that facilitates the efficient parallel cloning of homologous cDNAs for multiple genes in related species is needed. Thus far, in the absence of partial cDNA sequence of the target gene, the most popular approach for isolation of homologous cDNA is colony hybridization, based on cross-hybridization of a probe derived from a gene of interest. However, since optimized cross-hybridization conditions vary from gene to gene, it is not easy to isolate homologous cDNA clones derived from multiple genes by colony screening based on cross-hybridization. To solve this problem, we devised a method for targeted cloning of homologous genes that enables us to isolate cDNA clones for multiple genes in parallel. In this method, we first obtain partial but authentic cDNA sequences by multiplex cloning of homologous genes (MUCH) and then screen a cDNA library using the obtained perfect cDNA sequence information for isolation of longer cDNAs. The results shown in this study demonstrate that this method

could be successfully applied to the isolation of mouse homologues of human long cDNAs, known as KIAA cDNAs (<http://www.kazusa.or.jp/huge>) (1).

MATERIALS AND METHODS

Two-Step Homologous cDNA Cloning Strategy

The cloning strategy that we developed in this study is shown schematically in Figure 1. Here, we used human KIAA (hKIAA) cRNAs (1) as probes to target mouse homologous cDNAs (mKIAA cDNAs).

Preparation of Mouse cDNA Fragments

Mouse brain mRNA was prepared from 6-week-old BALB/c male mice as previously described (2). Double-stranded cDNA was synthesized from 5 µg of poly(A)⁺ RNA using the SuperScript™ Plasmid System (Invitrogen, Carlsbad, CA, USA). Briefly, the first-strand cDNA was synthesized using SuperScript II RNaseH⁻ reverse transcriptase and T7-NotI-(dT)₁₆ primer (Figure

2A); a minor modification was that the reaction was performed at 37°C for 30 min and then at 42°C for 30 min. After SP6 adaptor (Figure 2B) ligation to both ends of double-stranded cDNA, the cDNA fragments were size-fractionated on 1% low-melting-temperature agarose gel, and cDNAs larger than 3 kb, our targeted mKIAA size range, were retrieved (2,3). Since these larger cDNA fragments were obtained in low quantities, cRNA amplification was carried out using a MEGAScript™ T7 kit (Ambion, Austin, TX, USA), and the cRNA was purified with an RNeasy® Mini kit (QIAGEN, Valencia, CA, USA) before being converted to double-stranded cDNA. Double-stranded cDNA was synthesized as described above using SP6 UP primer (Figure 2B) and 3.6 µg of purified cRNA. The resulting cDNA fragments were digested with *Hae*III, purified with a PCR purification kit (Invitrogen), and then A-tailed with dATP and *Taq* DNA polymerase (Takara, Kyoto, Japan) at their 3' ends by incubating at 70°C for 30 min. After purification, as described above, 20 ng of the cDNA fragments were ligated with 50 ng pT7Blue T-Vector (Novagen, Madison, WI, USA) using T4 DNA ligase (Invitrogen) by overnight incubation at 16°C. After phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation, the ligation mixture was suspended in water and the inserted *Hae*III-digested cDNAs were amplified from 1/20 (v/v) of the ligation mixture using the PCR with *Taq* DNA polymerase and pT7 S1 and pT7 AS1 primers (Figure 2C). The PCR thermal cycling was performed on an iCycler™ (BIO-RAD, Hercules, CA, USA) and conditions were as follows: initial denaturation step, 95°C for 1 min; amplification cycle (30 cycles), 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; final extension step, 72°C for 5 min. After removing small PCR products (<100 bp) by size fractionation on 1% low-melting-temperature agarose gel electrophoresis, the purified cDNA fragments were recovered and allowed to hybridize with probes on nylon membranes as described below.

Preparation of hKIAA cRNA-Immobilized Nylon Membrane

cRNA of each hKIAA cDNA was

synthesized from the *Not*I-linearized cDNA plasmids using a MEGascript T7 kit, followed by purification of cRNAs with an RNeasy Mini kit. After heat denaturation, 1 μ L of each of the cRNAs (100 ng/ μ L) was manually arrayed onto a 4.3 $\times$ 2.8 cm PALL Bio-dyne® A membrane (PALL, East Hills, NY, USA). The membranes were baked for 2 h in a Vacubaker AB-1890 (ATTO, Tokyo, Japan) and UV-irradiated in a Stratalinker® 2400 UV crosslinker (Stratagene, La Jolla, CA, USA).

Enrichment of mKIAA cDNA Fragments by Hybridization to the hKIAA cRNA Probe

Hybridization of mouse cDNA fragments to the hKIAA cRNAs immobilized on the nylon membrane was carried out in a hybridization vial (Mighty vial No. 2; Maruemu, Osaka, Japan) in a Hybrid Midi Dual Hybridization Oven (Thermo Electron, Beverly, MA, USA). After prehybridization with 50 μ g heat-denatured mouse Cot-1 DNA® (Invitrogen) for 1 h at 60°C in 500 μ L PerfectHyb™ buffer (TOYOBO, Osaka, Japan), the cRNA-spotted nylon membrane was subjected to hybridization in 250 μ L PerfectHyb buffer containing 200 ng heat-denatured PCR-amplified

Figure 1. Isolation of homologous cDNAs. The first step, multiplex cloning of homologous genes (MUCH), was developed to obtain partial but authentic sequences of targeted genes. The determined sequences were used for primer design to select clones by PCR-based screening in the second step. To perform two-step PCR-based screening, duplicate sets of culture plates were prepared from the master plates. One plate was used for 24 pools of the same column cultures and the other was used for 16 pools of the same row cultures. Plasmid DNAs were purified from these pooled cultures using an MFX-9600 Magnia® Nucleic Acid Purification System (TOYOBO). For preparation of the plate-pooled plasmid DNAs, an equal amount of 16 row-pooled plasmid DNAs were mixed. In the first round of screening, these 24 plate-pooled plasmid DNAs were used as templates for PCR and the plate containing a target clone was identified by gel electrophoresis. In the second round of screening, 24 column-pooled plasmid DNAs and 16 row-pooled plasmid DNAs of the positive plate were used as templates for PCR and then the location of the well containing the positive clone was determined, as in the first round of screening. Each PCR was performed in 10 μ L of reaction mixture containing the mKIAA specific primers, *Taq* DNA polymerase and anti-*Taq* High, with a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

mouse cDNA fragments, 50 µg mouse Cot-1 DNA, and 2 µg polyadenylic acid. After overnight hybridization at 60°C, the membrane was successively washed in 2× standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min, at 60°C for 15 min, and finally twice in 0.1× SSC/0.1% SDS at 60°C for 20 min. After being briefly rinsed with water, each cRNA-spotted area of the nylon membrane was cut out using a disposable biopsy punch (3-mm diameter; BP-30K, Kai Industry, Gifu, Japan). The excised membranes were transferred separately into 0.5-mL tubes, or to wells in a 96-well plate, and then washed with 100 µL of water at 65°C for 10 min. Finally, the hybridized mouse cDNA fragments were eluted from the excised membrane in 100 µL of water by incubation at 95°C for 5 min. Ten microliters of each eluate were subjected to hot-start PCR on an iCycler using *Taq* DNA polymerase, anti-*Taq* High (TOYOBO) and pT7 S1 and pT7 AS1 primers in 50 µL under the same conditions described for preparation of mouse cDNA fragments. After the amplification, 5 µL of each PCR mixture were electrophoresed on 3% NuSieve® 3:1 agarose gel (Cambrex, East Rutherford, NJ, USA), and the amount of each PCR product was estimated by comparison with fluorescent staining intensities of known amounts of DNA using ImageQuant™ software (version 5.0) on a FluoroImager 595 (Amersham Biosciences, Piscataway,

NJ, USA). For further enrichment, the hybridization-based capture of the amplified mKIAA cDNA fragments was carried out with pre-excised pieces of cRNA-spotted nylon membrane in either a 0.5-mL tube or a well in a 96-well plate. The hybridization for this second-round enrichment was performed in 100 µL PerfectHyb buffer containing 20–30 ng heat-denatured amplified mKIAA cDNA fragments, 1 µg polyadenylic acid, and 10 µg mouse Cot-1 DNA at 60°C for 3 h. The washing steps, the elution step, and PCR were performed exactly as described for the first-round enrichment, except that nested PCR primers (pT7 S2 and pT7 AS2; Figure 2C) were used for the PCR in the second-round enrichment.

Identification of mKIAA cDNA Fragments by Sequencing

As all of the PCR-amplified cDNAs should contain *Sal*I and *Bam*HI sites close to the end of each fragment (as seen Figure 2C), the second-round PCR products were double-digested with these endonucleases, purified using the PCR purification kit, and then ligated into *Sal*I-*Bam*HI digested pSPORT 1 vector (Invitrogen). The ligation products were introduced into DH5α™ competent cells (Invitrogen) by chemical transformation. Eight to thirty transformants were randomly picked and their insert fragments were amplified by direct colony PCR using M13 forward and T7 primers and

processed for sequencing analysis as previously described (3). The obtained cDNA sequences were subjected to a BLAST search against our in-house human cDNA database constructed from public DNA sequence databases by eliminating as many expressed sequence tags (ESTs) and genomic sequences as possible. Query cDNA fragments that showed the best homology to the probed hKIAA were selected as candidate mKIAA cDNAs.

PCR-Based Screening of cDNA Library

After obtaining authentic mouse cDNA sequences as described above, specific primers for each mKIAA cDNA were designed using OLIGO™ Primer Analysis Software (MBI, Cascade, CO, USA) and prepared for PCR-based screening of a cDNA library. Each randomly sampled cDNA clone selected from a size-fractionated mouse brain-derived cDNA library (9–11 kb plasmid size) (2) was separately cultured in a

Figure 2. Primer and adaptor sequences designed for construction of mouse *Hae*III cDNA library. (A) T7-*Not*I-(dT)₁₆ primer sequence. (B) SP6 adaptor sequence. This adaptor was prepared by self-annealing of the upper 29-mer oligonucleotide (also used as SP6 UP primer) and lower 27-mer oligonucleotide. In the latter, 5' cytidine-deoxyribose was phosphorylated, indicated as -P. (C) Primers designed in the region of the multiple cloning site in pT7Blue T-vector. Mouse *Hae*III cDNA fragments were ligated into the *Eco*RV T-cloning site.

Figure 3. Enrichment of mKIAA0302 and mKIAA0338 cDNA fragments by multiplex cloning of homologous genes (MUCH). The first- and second-round PCR products were electrophoresed on each lane. Though the first-round products gave only faint band(s), the second-round products showed strong bands of between 150 and 400 bp in length. The fragments smaller than 100 bp were background products amplified from noninserted pT7Blue vectors.

Table 1. mKIAA cDNA Fragments Isolated by MUCH^a

mKIAA No.	Sequence Identity to hKIAA (%)	E Value	Hit Length (nt)	mKIAA No.	Sequence Identity to hKIAA (%)	E Value	Hit Length (nt)
0284	80.0	1.5×10^{-129}	261	1814	88.2	3.6×10^{-150}	228
0294	83.4	9.0×10^{-205}	344		89.5	1.5×10^{-101}	153
	83.0	4.0×10^{-143}	247		90.3	2.4×10^{-82}	124
0297	90.5	3.4×10^{-138}	200	1821	84.8	1.2×10^{-202}	329
	90.0	7.3×10^{-61}	102	1828	85.5	1.6×10^{-91}	162
0300	80.0	4.4×10^{-71}	140	1831	88.9	2.5×10^{-125}	189
0303	85.6	3.8×10^{-69}	118		88.2	5.5×10^{-122}	187
0308	93.8	1.4×10^{-55}	81		84.5	1.8×10^{-56}	139 ^c
0309	88.2	2.0×10^{-111}	181		87.5	7.5×10^{-36}	64
	85.6	2.2×10^{-92}	163	1833	83.1	6.8×10^{-108}	189
0310	87.4	3.5×10^{-144}	223		84.2	5.2×10^{-76}	133
0311	95.4	3.4×10^{-169}	219		90.7	4.2×10^{-59}	100
	90.6	6.0×10^{-143}	215	1836	76.3	1.0×10^{-107}	358
	95.8	2.9×10^{-127}	166		84.4	2.0×10^{-102}	199
0313	83.6	8.5×10^{-190}	318		85.3	1.2×10^{-53}	95
	83.8	1.0×10^{-141}	240	1837	91.1	6.8×10^{-201}	282
0319	85.2	4.3×10^{-127}	209		92.6	5.9×10^{-106}	149
0323	84.0	1.6×10^{-153}	263		87.2	6.8×10^{-77}	125
	87.6	1.7×10^{-69}	113	1843	87.1	2.3×10^{-104}	173
	77.2	1.6×10^{-17}	60		92.8	2.4×10^{-119}	166
0353	84.9	5.1×10^{-73}	126	1848	89.5	1.5×10^{-101}	153
0364	92.1	9.7×10^{-127}	178		87.2	3.4×10^{-42}	79
	91.1	7.5×10^{-67}	101		90.2	3.7×10^{-29}	51
0380	90.1	0	434	1849	76.1	4.9×10^{-126}	287
	89.4	2.7×10^{-208}	303		74.8	4.3×10^{-96}	223
1805	89.3	5.8×10^{-165}	243		88.4	4.2×10^{-59}	109
	93.0	2.2×10^{-101}	142	1854	74.1	1.6×10^{-17}	54 ^b
1808	86.1	3.2×10^{-216}	339	1855	90.7	2.3×10^{-141}	218
	84.8	3.0×10^{-139}	230		84.8	9.4×10^{-59}	132 ^c
	89.9	5.9×10^{-106}	158		87.5	1.1×10^{-57}	96
	89.2	2.3×10^{-104}	158	1856	77.8	4.8×10^{-92}	189
1811	87.5	1.4×10^{-179}	280	1862	82.4	3.0×10^{-43}	85
	93.7	7.4×10^{-129}	175				
	88.2	7.8×10^{-45}	76				

Mean value was 86.8% for sequence identity to hKIAA and 182 nucleotides (nt) for hit length. MUCH, multiplex cloning of homologous genes. E (expect) value is an estimate of statistical significance; the lower the E value, the more statistically significant the hit.

^aAfter mKIAA cDNAs were selected out by the BLAST search, each mKIAA was aligned to orthologous hKIAA by our in-house FASTA program.

^{b,c}All of the cDNA fragments were hit to coding sequences of the corresponding hKIAA genes except for those noted as "b" (hit to 5' untranslated region) and "c" (hit to 3' untranslated region).

single well of a 384-well plate to make 24 master plates of mKIAA clones (total 9216 clones). Using these master plates, PCR-based screening of the cDNA clones was performed, essentially as described by Chinault et al. (4), which is illustrated in the lower panel of Figure 1 and described in detail in its legend. The isolated cDNAs were characterized by entire sequencing using a shotgun strategy as described previously (3).

RESULTS AND DISCUSSION

Isolation of mKIAA0302 and mKIAA0338 cDNA Fragments by MUCH

The first step, termed MUCH (Figure 1), was carried out to determine the exact sequences of partial cDNA fragments of homologous genes. To improve the hybridization kinetics

and the PCR amplification efficiency in MUCH, the cDNAs to be screened were downsized by restriction enzymes with four-base recognition. Since the nucleotide sequence identity between homologous cDNAs usually varies along the molecule, the downsizing of cDNAs would also make it possible to apply stringent hybridization conditions in MUCH because highly conserved regions should be retrieved even under these conditions. This would allow us to carry out hybridization against multiple different probes under the constant conditions. To avoid artifactual amplification of cDNAs from immobilized probes by PCR, we used cRNA, in place of cDNA, as immobilized probe on the nylon membrane.

To establish optimum conditions for MUCH, we first carried out cloning of two mouse cDNAs homologous to hKIAA0302 (spectrin β III) and hKIAA0338 (type I brain 4.1/4.1N) because their mRNA levels in the brain were relatively high (5,6), thereby making them suitable controls. Figure 3 shows the gel image of PCR products after the first- and the second-round enrichment for mKIAA0302 and mKIAA0338 cDNAs under the hybridization conditions described in Materials and Methods. In both the mKI-

AA0302 and mKIAA0338 cDNAs, it was evident that there were more products after the second-round enrichment than after the first round. Results of Southern hybridization using rat KIAA0302 or KIAA0338 probes (5,6) suggested that most of fragments seen in Figure 3 were actually derived from mKIAA0302 or mKIAA0338 cDNAs, as expected (data not shown). After cloning the secondarily enriched cDNA

fragments, we analyzed the sequences of each set of 10 randomly isolated clones. The results showed that nine clones (six independent cDNA groups) and seven clones (four independent cDNA groups) contained cDNA fragments of the highest similarity to hKIAA0302 and to hKIAA0338, respectively. The nucleotide sequences of all of the isolated cDNA fragments (from 66 to 347 bp in length) corresponded to coding sequences (CDSs) of hKIAA0302 or hKIAA0338, with the sequence identities ranging from 81.4% to 93.1%. Thus, we considered that the conditions of hybridization, washing, elution, and amplification used were stringent enough for the isolation of homologous cDNA fragments.

Parallel Isolation of Multiple mKIAA cDNA Fragments by MUCH

Next, we applied the MUCH technique for the parallel isolation of multiple mKIAA cDNA fragments. In practice, we attempted to isolate 53 mKIAA cDNAs by either a tube reaction (21 mKIAA) or a 96-well plate reaction (32 mKIAA). The 96-well plate format was designed to test the feasibility of using MUCH for high-throughput cloning.

Table 1 summarizes the results of MUCH. Thirty-two mKIAA cDNAs were obtained and classified into 66 independent cDNA groups. The success rates in the tube format and the plate format were 71.4% (15/21) and 53.1% (17/32), respectively, and the overall success rate was 60.4% (32/53). The nucleotide sequence identities of these KIAA homologous cDNA fragments ranged from 74.1% to 95.8% with a mean value of 86.8% (the mean length was 182 bp). The observed mean value was very close to that reported as the mean value of the sequence identity between mouse and human orthologous genes by Makalowsky et al. (7). Among the 66 cDNA fragments thus isolated, 63 fragments originated from CDSs of their corresponding hKIAA genes while three fragments were from the 5' or 3' untranslated region (UTR). In addition, although we were not sure whether this method would enable the isolation of cDNAs that did not contain any conserved protein domains, the successful isolation of mKIAA0323, mKIAA1833, and

mKIAA1843 cDNA fragments clearly showed that the presence of conserved protein domains was not prerequisite.

PCR-Based Screening and Sequencing of the Isolated mKIAA cDNA Clones

Based on the obtained sequences of mKIAA cDNA fragments, we designed gene-specific PCR primers and screened out full-length cDNA clones from a previously constructed library (2). As a demonstration, we carried out isolation of cDNA clones for mKIAA1828 and mKIAA1831. We isolated one positive clone for each gene by PCR-based screening and then determined their insert sequences. The total nucleotide lengths of the isolated mKIAA1828 and mKIAA1831 cDNAs were 2921 and 2923 bp with CDS sizes of 587 and 481 amino acid residues, respectively. These isolated cDNA insert sizes were much smaller than expected because the cDNA plasmids were isolated as a plasmid dimer. These nucleotide sequences were deposited to the DNA Data Bank of Japan (DDBJ), GenBank®, and the European Molecular Biology (EMBL) databases under the accession numbers AB096262 for the mKIAA1828 cDNA and AB096263 for the mKIAA1831 cDNA. By referring to a human-mouse homology map (http://www.ensembl.org/Mus_musculus/syntenview/), it was confirmed that both the mKIAA1828 and mKIAA1831 cDNAs mapped to the chromosomal location predicted by the conserved synteny between human and mouse. Therefore, we concluded that our system worked efficiently in the isolation of orthologous genes.

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