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ISOLATION OF 2 NOVEL RFLP MARKERS AND THEIR LOCALIZATION AT 2q35 BY MICRODISSECTION AND SUBSEQUENT ENZYMATIC AMPLIFICATION

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Summary We previously constructed a chromosome 2q-specific genomic library and isolated a number of microclones. In the present study, we first analyzed with Southern hybridization whether any of the microclones represent restriction fragment length polymorphisms (RFLPs), and then tried to map RFLP markers physically, using the recently developed chromosome microdissection/enzymatic amplification method. Of 13 clones analyzed, two were RFLP markers; a clone, pM2C83, showed a four-allele *Msp*I RFLP, and the other, pM2C8, a two-allele *Rsa*I RFLP. In order to assign the two polymorphic markers, two chromosomal segments, 2q32-q35 and 2q35-qter, on the chromosome 2 from a karyotypically normal person were microdissected, and the DNA from each segment was amplified with the polymerase chain reaction (PCR) using marker sequence-specific primers. With this method, both of the clones were assigned to 2q35. These two RFLP markers must be useful for linkage analysis of genetic diseases whose loci are at around 2q35.
Key Words Novel RFLP markers, microdissection, polymerase chain reaction (PCR), mapping, 2q35

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INTRODUCTION

The chromosome microdissection technique has been developed as a convenient tool to isolate microclones from a defined chromosomal region of interest (Lüdecke *et al.*, 1989, 1990; Senger *et al.*, 1990; Johnson, 1990; Jinno and Niikawa, 1990; Kao and Yu, 1991; Djabali *et al.*, 1991). We previously constructed a human chromosome 2q-specific genomic library by means of microdissection, and isolated many single-copy DNA microclones with a size ranging 200–700 bp (Hirota *et al.*, 1992). These microclones are available for screening of corresponding larger genomic clones and/or cDNAs from phage, cosmid or yeast artificial chromosome (YAC) libraries. In addition, the microclones may represent restriction fragment length polymorphisms (RFLPs) (Lüdecke *et al.*, 1990; Hirota *et al.*, 1992). However, when using them directly in linkage analysis, narrowed assignments are an *a priori* requisite. For this purpose, chromosome fluorescence *in situ* hybridization (FISH) technique has generally been adopted (Hirota *et al.*, 1992), but FISH first needs the isolation of corresponding phage or cosmid clones with a minimal size of almost 1 kb, to be used as probes (Garson *et al.*, 1987). An alternative mapping procedure, a chromosome microdissection and subsequent polymerase chain reaction (CM-PCR) method, has recently been developed by Han *et al.* (1991) and by Spielvogel *et al.* (1992), which involved PCR of the DNA from a defined microdissected chromosomal region using a set of clone-sequence-specific primers. This method provided rapid mapping of DNA fragments with a size as small as microclones.

The present study deals with the isolation of novel RFLP markers from the previously constructed 2q-specific microclone library (Hirota *et al.*, 1992) and with mapping of the markers by the CM-PCR method.

MATERIALS AND METHODS

DNA microclones analyzed. We previously constructed a pUC19 2q-specific genomic library and isolated microclones from the library (Hirota *et al.*, 1992). These microclones were used in the present study. They included pM2C1, pM-2C4, pM2C7, pM2C8, pM2C9, pM2C12, pM2C28, pM2C58, pM2C63, pM2C64, pM2C70, pM2C71, and pM2C83, and all were single-copy sequences. Although they had been confirmed to be derived from human chromosome 2 by the hybrid cell panel analysis, all but one clone (pM2C4) had not precisely been mapped. The pM2C4 already assigned to 2q33.3 (Hirota *et al.*, 1992) was used as a positive control to evaluate the present mapping strategy.

Southern hybridization. The microclones were initially tested for RFLPs by Southern hybridization to DNAs that were isolated from six unrelated Japanese individuals and digested with each of six different restriction enzymes (*Msp*I, *Taq*I,

*Rsa*I, *Bgl*II, *Pst*I, and *Pvu*II). The enzyme digested DNA (6–10 μg) was separated by electrophoresis on 0.8% agarose gels and transferred to nylon membranes. Each microclone DNA was labeled with [^{32}P]dCTP using the multiprime DNA labeling system (Amersham International Plc., U.K.), and used as a probe. Hybridization was performed at 65°C for 12–24 hr in a mixture containing 0.125 M Na_2HPO_4 (pH 7.2)/0.25 M NaCl/7% SDS/1 mM EDTA/10% PEG 6000, and post-hybridization washing at room temperature for 15 min twice in 2×SSC and 0.1% SDS, and then for 30 min at 65°C twice in 0.5×SSC and 0.1% SDS. Hybridization signals were detected by autoradiography.

Chromosome microdissection, and PCR amplification of the chromosomal DNA. For the purpose of direct mapping of the microclones which would represent RFLPs, we adopted the CM-PCR mapping method developed by Han *et al.* (1991) and by Spielvogel *et al.* (1992). Detailed microdissection procedures themselves were the same as described previously (Jinno and Niikawa, 1990; Hirota *et al.*, 1992; Deng *et al.*, 1992). In short, metaphase chromosomes were freshly prepared with the short-fixation method from an EBV-transformed lymphoblastoid cell-line of a karyotypically normal male. A chromosomal region, 2q33-qter, which was corresponding to the derivation of our microclones, was divided into two parts, regions A (2q32-q35) and B (2q35-qter) (Fig. 1, A and B). Each of the regions was microdissected under an inverted microscope with a fine glass needle by handling a micromanipulator. Since these chromosomal regions could not accurately been cut-out, a small part of the dissected fragments might contain common DNAs, especially at around 2q35. Five chromosomal pieces from each region were collected, and incubated with a proteinase solution, containing 0.5 mg/ml proteinase K/0.1% SDS/10 mM Tris-HCl (pH 7.5)/10 mM sodium chloride, at 37°C for 4 hr. Chromosomal DNA was extracted with phenol/chloroform.

In order to design primer DNAs for the PCR amplification of the microdissected chromosomal DNA, microclones showing RFLPs and the control clone (pM2C4) were partially sequenced by the dideoxy-chain termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (Sequenase, Biochemical Corporation, Cleveland, Ohio, USA; Catalogue No. 70700). Based on their sequences, clone-specific oligonucleotide primer pairs were synthesized (Table 1). All the agents used but the template DNAs were pre-treated with psoralen-UV (Jinno *et al.*, 1990) to avoid amplification of possible contaminated DNAs.

The DNA from each microdissected chromosomal region was transferred into 50 μl of a PCR reaction mixture (50 mM KCl/20 mM Tris-HCl, pH 8.5/1.5 mM $MgCl_2$/0.1% Tween 20/0.2 mM each of dNTP and 100 ng each of primers). After adding 2 U of Taq polymerase to the mixture, the DNA was PCR-amplified in an automated temperature controller (Program Temp Control System PC-7000, ASTEC Co., Tokyo). After the first 20 PCR cycles (95°C for 2 min, 55°C for 2 min, and 72°C for 2 min), another 1 U of Taq polymerase was added, and additional 20 cycles were performed. At the end of the 40th cycle, 10 μl reaction mixture was

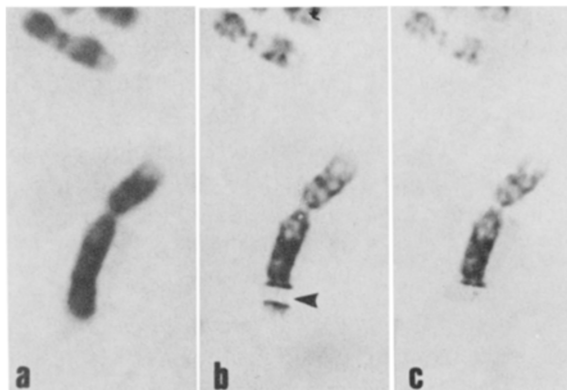

Fig. 1A. A chromosome 2 before microdissection (a), and after dissection of region A (2q32-q35) (b), and region B (2q35-qter) (c).

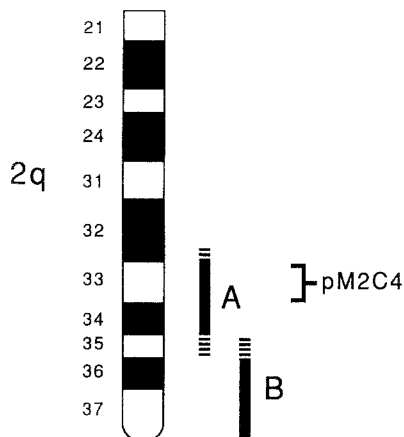

Fig. 1B. Localization of dissected regions A and B, and the pM2C4 on chromosome 2. Solid and dotted bars indicate confidently dissected regions and possible overlapping regions, respectively.

Table 1. Sequences of three primer sets used in the present study.

Corresponding DNA to be amplified	Primer	Sequence (5′-3′)	Predicted size of amplified DNA	Annealing temperature
pM2C83	C83f	CAGCATTTCCTAGGAGTGAC	138 bp	55°C
	C83r	ACACTTCTGATTAGAGTCCC		
pM2C8	C8f	ACTCAGTCCTGCTCCAACTT	73 bp	55°C
	C8r	GCTCCAGAATGATTATCTGG		
pM2C4	C4f	AGGAAGCGTAGGGAGTAGAT	109 bp	55°C
	C4r	AGGATCTTGGTAAATGGTTACC		

subjected to electrophoresis on a 6% polyacrylamide gel and the PCR products were visualized by ethidium bromide staining.

RESULTS

Identification of RFLPs

Of the 13 microclones analyzed with Southern hybridization, two, pM2C83 and pM2C8, represented polymorphisms. The probe pM2C83 with a size of 550 bp identified a *Msp*I RFLP with four alleles (6.8 kb, 6.2+0.6 kb, 4.6 kb, and 4.0+0.6 kb) (Table 2 and Fig. 2a). A 0.6 kb fragment was observed, always accompanied with the 6.2 and the 4.0 kb fragments. These four alleles segregated in two families in an autosomal codominant fashion (Fig. 2a). The other probe pM2C8 with a size of 600 bp detected an *Rsa*I RFLP with 2 alleles, 2.9 and 1.1 kb (Fig. 2b). Allele frequencies at the two loci among 40 or 60 unrelated Japanese

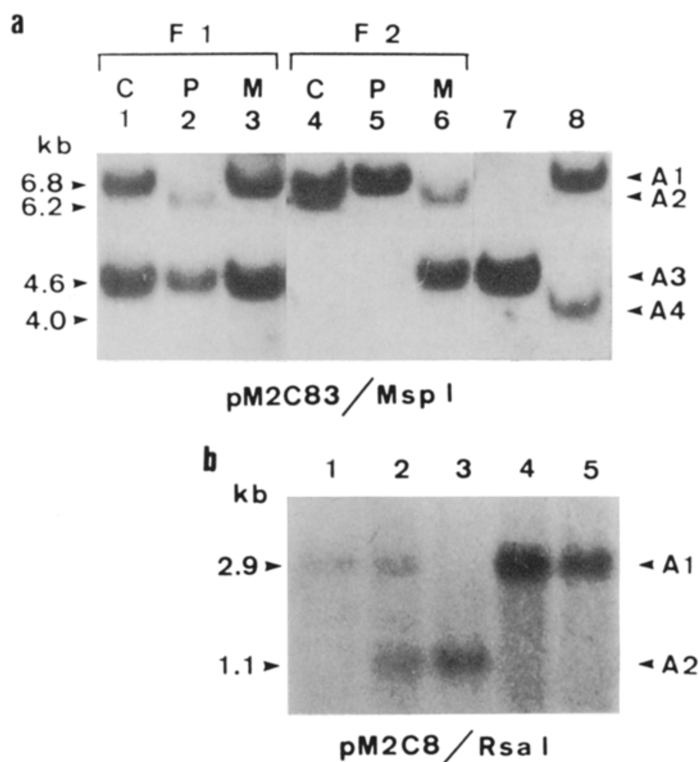

Fig. 2. RFLPs detected by two microclones, pM2C83 (a) and pM2C8 (b). a: Arrowheads on the right induicate 4 alleles which are detected in 8 individals (lanes 1–8) and transmitted from father (P) or mother (M) to a child (C) of family 1 (F1) and/or family 2 (F2). b: Two alleles are shown by arrowheads on the right.

Table 2. Allele frequencies of pM2C83 and pM2C8 polymorphisms in the Japanese.

Probe	Locus	Enzyme	Allele			Number of chromosome
			Symbol	Size (kb)	Frequency	
pMC83	2q35	*Msp*I	A1	6.8	0.48	120
			A2	6.2+0.6	0.13	
			A3	4.6	0.38	
			A4	4.0+0.6	<0.01	
pM2C8	2q35	*Rsa*I	A1	2.9	0.67	80
			A2	1.1	0.33	

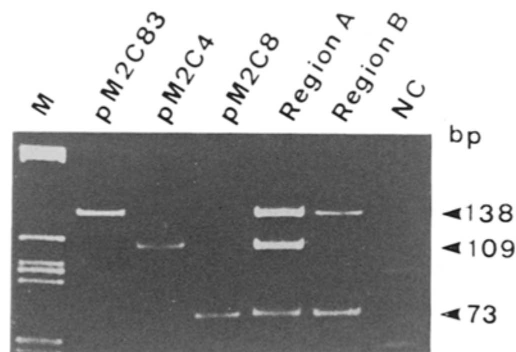

Fig. 3. Polyacrylamide gel electrophoresis of PCR products with combinations of primer sets. Lanes M and NC (b) are for size marker and negative control DNAs, respectively.

are shown in Table 2. The *Msp*I RFLP detected by pM2C83 was much informative, showing 58% heterozygosity among 60 Japanese individuals, although the 4.0+0.6 kb allele was very rare. The *Rsa*I detected by pM2C8 showed 40% heterozygosity in 40 Japanese.

Mapping of polymorphic microclones

The PCR on total human DNA using a primer pair, C83f and C83r, led to a single product with a predicted size of 138 bp (Fig. 3). Likewise, the PCR of total human DNA with the other two sets, C8f/C8r and C4f/C4r, amplified a predicted 73 bp fragment and a 109 bp fragment, each as a single band, respectively. These experiments confirmed the results of previous Southern blot analyses that all the three clones were single-copy sequences (Hirota *et al.*, 1992), and indicated that our primer pairs could detect the corresponding sequences from total genomic DNA.

Using a mixture of the three primer sets, the PCR of the DNA from each of the dissected chromosomal regions A and B amplified both the 138 and the 73 bp

fragments, in addition to the 109 bp fragment. Using the C4f/C4r set, only a 109 bp positive control fragment was amplified in the DNA from region A but not from region B (Fig. 3).

DISCUSSION

We isolated two novel RFLP markers, pM2C83 and pM2C8, from a 2q-specific pUC19-library which had been constructed by means of microdissection (Hirota *et al.*, 1992). Although these microclones had a size as small as 0.6 kb, they could directly be used as probes for the standard Southern hybridization.

Since the two markers had not accurately been assigned, we tried to map them by the CM-PCR mapping strategy, in which a 2q32-q35 region (region A) and a 2q35-qter region (region B) from a normal male were separately microdissected and the subsequent PCR was performed using clone-sequence-specific primers. When using the primer set for the control microclone (pM2C4) with known localization, only a DNA fragment with a predicted size was PCR-amplified at the predicted chromosomal region, whereas when using a mixture of three sets of primers each specific for each clone, PCR products for both pM2C83 and pM2C8 were observed at either region A or B. This might indicate that the two dissected chromosomal regions overlapped each other, and that both pM2C83 and pM2C8 are localized at an estimated overlapping site, 2q35.

In one of the original CM-PCR methods, Han *et al.* (1991) microdissected only one piece of a chromosomal segment and amplified the chromosomal DNA. To avoid possible contaminations or non-specific amplifications, they adopted the nested PCR. It has been pointed out that dissected chromosomal DNAs are often damaged during the microdissection procedures (Lüdecke *et al.*, 1990; Senger *et al.*, 1990; Hirota *et al.*, 1992), leading to the PCR amplification of some curious DNAs. In the present study, a combination of an employment of the psoralen-UV method, dissecting more numbers of chromosomal pieces and reduced PCR cycles could overcome these problems and provided rapid and efficient mapping of our microclones. The CM-PCR method is thus simple and highly specific, and useful for mapping of microclones. Although in general, the longer sequencing of microclones, the more appropriate primers for PCR are obtained, primers covering 200–300 bp of template DNA would actually work as shown in the present study. In addition, multiplex PCR was also available for simultaneous mapping of several DNA markers.

Each RFLP marker isolated and mapped in our study is highly informative, and may contribute to the construction of a linkage map involving a 2q35 region, to which the genes for Waardenburg syndrome type I, rhabdomyosarcoma, and for Ehlers-Danlos syndrome type X are mapped (Human Gene Mapping 11, 1991).

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