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GENOME METHODS

Microsatellite Hybrid Capture Technique for Simultaneous Isolation of Various STR Markers

Michal Prochazka[1]

Clinical Diabetes and Nutrition Section, Phoenix Epidemiology and Clinical Research Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, Arizona 85016

The microsatellite hybrid capture technique was designed to enrich simultaneously for various microsatellite repeats from a genomic clone. It is illustrated in this report that different repeat motifs, including polymorphic ones, can be efficiently isolated in a single experiment. In principle, this technique can be applied to any type of genomic clones to facilitate the isolation of informative markers for fine mapping of subchromosomal regions of interest, and for linkage and association studies of candidate genes for which no polymorphic markers are available yet.

Genetic studies of complex phenotypes, including heritable diseases, typically require genome-wide scans of highly informative DNA markers as the initial step in positional cloning of the underlying genes. Rapid advances in systematic mapping approaches were made possible by the recent development of high resolution maps of polymorphic short tandem repeat markers (STRs, microsatellites) covering the entire genome (Buetow et al. 1994; Gyapay et al. 1994). Such markers are typically generated from total, or chromosome-specific genomic DNA libraries, but fine mapping of discrete chromosomal segments and linkage or association analyses of specific candidate genes often require the isolation of novel polymorphic STRs from selected genomic clones covering the region of interest.

We are studying the genetics of non-insulin-dependent diabetes mellitus (NIDDM) in the Pima Indians, a Native American population with the highest reported prevalence of the disease in the world (Knowler et al. 1978). Because multiple genetic factors may contribute to NIDDM susceptibility in this population (Bogardus and Lillioja 1992), our strategies include a systematic genome-wide scan, as well as analyses of selected candidate genes using appropriate STRs. Because some of the candidate genes that we are examining were cloned and mapped just recently, no specific markers are available yet. Numerous methods were designed to generate novel markers from total genomic DNA or from individual genomic clones (Brenig and Brem 1991; Ostrander et al. 1992; Pandolfo 1992; Lyall et al. 1993; Armour et al. 1994; deSouza et al. 1994; Kandpal et al. 1994; Robic et al. 1994; Rowe et al. 1994; Brown et al. 1995; Chen et al. 1995), with an emphasis on $(CA)_n/(GT)_n$ repeats which are the most abundant class of polymorphic STRs in human (Beckmann and Weber 1992). Although we have previously isolated polymorphic $(CA)_n/(GT)_n$ repeats for some candidate genes (Mochizuki and Prochazka 1994; Prochazka et al. 1995), selective screening of genomic clones for only one motif was a limiting factor in the development of informative markers for our studies. To facilitate identification of new STRs, we designed the "microsatellite hybrid capture" technique, which utilizes a mixture of biotin-tagged probes corresponding to various repeat motifs and allows for simultaneous isolation of different STRs from the same genomic clone in a single experiment.

RESULTS AND DISCUSSION

The principle of the microsatellite hybrid capture technique is illustrated in Figure 1. The method was tested with two different P1 clones, designated here as P1-2855 and P1-CC1, which represent two unlinked candidate genes that could contribute to NIDDM susceptibility. The enrichment was monitored in each experiment by agarose gel electrophoresis of aliquots of the PCR-

[1]E-MAIL hzr@cu.nih.gov; FAX (602) 263-1647.

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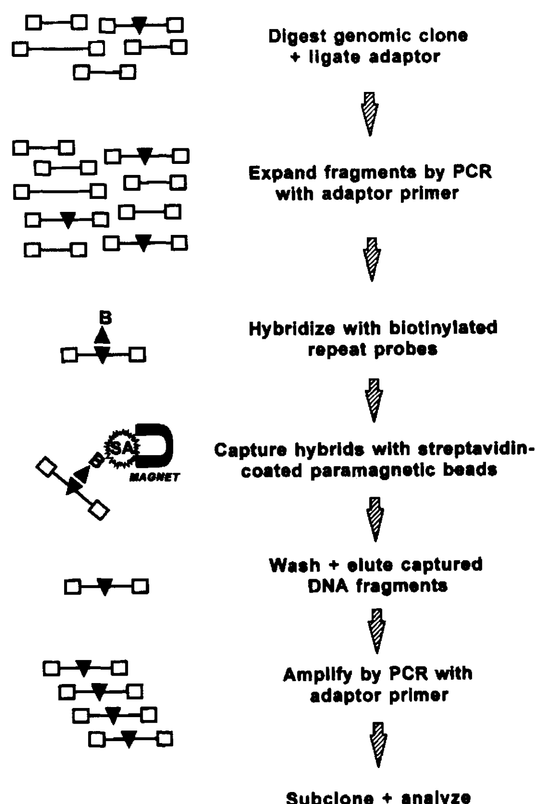

Figure 1 Principle of the microsatellite hybrid capture technique. (□) UNI-Amp adaptor; (▼, ▲) repeat motifs; (B) biotin label; (SA) streptavidin-coated paramagnetic beads.

expanded starting pools of DNA fragments, which were compared with PCR products amplified after the selection step (see Methods). Typically, a change in the pattern from a ladder of DNA bands (size range 0.25–1.5 kb) in the starting material to a few prominent bands in the captured samples was observed (not shown).

In the first set of experiments performed with P1-2855, approximately one-third of the colonies showed a strong signal upon hybridization with ^{32}P-labeled repeat probes. Nine positive clones with different insert sizes [determined by direct PCR on lysed transformed cells (Güssow and Clackson 1989)] were sequenced, and three separate STRs were identified as shown in Table 1. Based on this efficient enrichment for clones containing repeats, subsequent hybrid capture experiments with P1-CC1 were simplified by omitting the colony hybridization screening step. After the subcloning of the PCR-amplified capture genomic fragments, nine clones differing by their insert sizes were directly sequenced, and five of them contained STR motifs representing three separate microsatellites (Table 1).

Based on the available sequences of the inserts, it was possible to design unique PCR primers flanking four of the identified STRs (two from each P1 clone; Table 1). The chromosomal localization of these repeats was verified by PCR analysis of DNA from appropriate somatic cell hybrids, and the physical location of STRs from P1-2855 was further confirmed by PCR with ad-

Table 1. Summary of STRs Isolated by the Microsatellite Capture Technique

P1 clone	Repeat motif	Number of alleles	Heterozygosity
2855	$[(GAA/GAAA/GAAAA)^{a}\text{-}(GGAA)_{10}(GGCA)_{7}]$	7	0.79
	$(CA)_{8}$	1	0
	$(GA)_{36}$	ND[b]	ND[b]
CC1	$(AAC)_{11}$	3[c]	0.38
	$(AAAAG)_{6}$	1	0
	$(TC)_{18}(T)_{3}CTCC(CT)_{6}T(TC)_{3}(TA)_{7}(CA)_{5}$	ND[b]	ND[b]

[a]Imperfect repeat motif spanning 77 bp.
[b]Not determined because inserts did not contain suitable flanking sequence to design unique primers for PCR with genomic DNA.
[c]A fourth allele was observed in a DNA sample from a Caucasian control subject.

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ditional P1 and yeast artificial chromosome (YAC) clones carrying the same candidate gene (no other genomic clones were available for the locus represented in P1-CC1). The informativeness (heterozygosity) of these STRs was tested on genomic DNA samples from 13–24 Pima Indians, and one polymorphic marker was identified for each locus (Table 1).

Over the last few years, numerous techniques have been developed to facilitate isolation of polymorphic microsatellite markers (Brenig and Brem 1991; Ostrander et al. 1992; Pandolfo 1992; Lyall et al. 1993; Armour et al. 1994; deSouza et al. 1994; Kandpal et al. 1994; Robic et al. 1994; Rowe et al. 1994; Brown et al. 1995; Chen et al. 1995). Although some of the previously published strategies for isolation of STRs from total genomic DNA overlap with the approaches used in the microsatellite hybrid capture described here [biotin/streptavidin-based selection of a repeat motif (Kandpal et al. 1994; Brown et al. 1995) or simultaneous isolation of various STRs in the same reaction (Armour et al. 1994)], this technique offers several improvements.

The microsatellite hybrid capture allows for an efficient simultaneous enrichment of DNA fragments containing various STR motifs (di-, tri-, tetra-, pentanucleotides) from a single genomic clone in the same experiment, thus increasing the probability of rapidly finding polymorphic markers for a specific locus. The selectivity can be easily modified by changing the number and specificity of the biotin-tagged repeat probes and by altering the stringency conditions. Further advantages include a very short time (typically <4 hr) required to complete the enrichment and capture of the PCR-expanded fragments. Moreover, additional manipulation of the selected fragments by restriction endonuclease digestion for their subcloning is eliminated by taking advantage of a direct ligation of the PCR-amplified products using the TA cloning kit. The enrichment for repeat-containing fragments could be further increased by subjecting the eluted DNA to a second round of selection. As demonstrated here, however, the enrichment achieved by a single round of hybrid capture is sufficient to identify STR-containing constructs simply by direct sequencing of just a small number of clones from the captured sublibrary. This option of avoiding the use of radioactive probes represents further advantage over other methods, which typically require hybridization screening of the clones with ^{32}P-labeled repeat oligonucleotides.

In principle, the microsatellite hybrid capture technique can be applied to any type of genomic clones [including P1s, bacterial artificial chromosomes (BACs), cosmids, λ phages, and gel-purified YACs], and it should facilitate isolation of new markers in genomic areas of interest in humans, as well as in other organisms.

METHODS

P1 clones were isolated at Genome Systems, Inc. (St. Louis, MO), and Centre d'Etude du Polymorphisme Humain (CEPH) YAC clones were obtained from Research Genetics, Inc. (Huntsville, AL). Genomic DNA from appropriate somatic cell hybrids was obtained from the National Institute of General Medical Sciences repository (Coriell Institute, Camden, NJ). Heterozyosity of markers was tested as described (Thompson et al. 1995), using genomic DNA samples from 13–24 Pima Indians who were not first degree relatives.

For the hybrid capture procedure, purified P1 DNA (10 ng) was digested separately with 4-base cutting restriction endonucleases that generate blunt-ended fragments (*Alu*I, *Hae*III, *Rsa*I), and the samples were desalted with the QIAquick nucleotide removal kit (Qiagen Inc., Chatsworth, CA). Approximately 300 pg was ligated with 2 pmoles of UNI-Amp adaptor using the UNI-Amp kit (Clontech Laboratories, Palo Alto, CA) in a 10-μl volume at 16°C for 20–24 hr, and 1 μl was subsequently used in PCR to expand the modified fragments. PCR was performed in the GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT) in a 25-μl volume containing 1× PCR buffer wih 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.625 units of AmpliTaq (all reagents from Perkin-Elmer), and 10 pmoles of the UNI-Amp primer (Clontech Laboratories). To avoid amplification of nonspecific products, TaqStart antibody (Clontech Laboratories) was routinely included in all PCR reactions as recommended by the manufacturer. After an initial denaturation at 96°C for 3 min, the samples were subjected to 30 PCR cycles (96°C for 20 sec, 60°C for 30 sec, 72°C for 3 min with 10 sec added to the extension time in each cycle), followed by a final elongation at 72°C for 5 min.

An aliquot containing ~30 ng of the PCR product (estimated from an ethidium bromide-stained minigel) was mixed with a pool of 5′-biotinylated repeat probes (1.5 pmole of each motif) in 6× SSC/0.1% SDS, denatured at 95°C for 5 min, and hybridized at 60°C for 1 hr with intermittent mixing. The 5′-biotinylated oligonucleotides included $(CA)_{12}$, $(CT)_{12}$, $(AAN)_9$, $(AAAN)_7$, (N = C, G, or T), $(CGG)_6$, and $(CAG)_6$ (synthesized at Research Genetics), which represent the most common STR motifs in the human genome (Beckmann and Weber 1992). After hybridization, 200 μg of streptavidin-coated paramagnetic beads (Dynal Inc., Lake Success, NY) equilibrated in 6× SSC was added, and the suspension was mixed continuously for 15 min to capture the biotin-containing hybrids. The beads were concentrated in the Dynal MPC-M magnetic stand, washed in 6× SSC/0.1% SDS (twice for 15 min at room temperature, and three times at 60°C), followed by two washes in 6× SSC at room temperature.

The captured DNA strands were eluted with 100 μl of fresh 0.1 N NaOH at 60° for 5 min, and after the addition

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of an equal volume of 1 M Tris (pH 7.5), the samples were desalted and concentrated in an Ultrafree-MC filter unit (30,000; Millipore, Bedford, MA) to a final volume of 10 μl. One-tenth of the purified single-stranded DNA was amplified with the UNI-Amp primer as described above, and the PCR products were subcloned directly into the pCRII vector using the TA cloning kit (Invitrogen Corporation, San Diego, CA). In experiments with P1-2855, transformed colonies were subsequently screened by standard hybridization (Sambrook et al. 1989) with a mixture of 5′ ^{32}P-labeled oligonucleotides corresponding to the sequences of the biotinylated capture probes. The inserts were sequenced with the PRISM Sequenase Terminator double-stranded DNA sequencing kit on the ABI automated sequencer model 373A (Perkin-Elmer).

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