

New polymorphic markers in the vicinity of the pearl locus on mouse Chromosome 13

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Abstract. We have used a *Mus domesticus*-*Mus spretus* congenic animal that was selected for retention of *Mus spretus* DNA around the pearl locus to create a highly polymorphic region suitable for screening new markers. Representation difference analysis (RDA) was performed with either DNA from the congenic animal or C57BL/6J as the driver for subtraction. Four clones were identified, characterized, and converted to PCR-based polymorphic markers. Three of the four markers equally subdivide a 10-cM interval containing the pearl locus, with the fourth located centromeric to it. These markers have been placed on the mouse genetic map by use of an interspecific backcross panel between *Mus domesticus* (C57BL/6J) and *Mus spretus* generated by The Jackson Laboratory.

Introduction

The pearl (pe) mutation is a recessive, hypopigmentation mutation that causes extensive alterations in the brain and visual system. The original pearl mutant, identified in the C3H strain, was localized to Chromosome (Chr) 13 by linkage to *As1* and *Lth1* (Elliot et al. 1985). This mutant allele was subsequently transferred into a congenic C57BL/6J strain, C57BL/6J Pin pe/pe (Avner et al. 1988). Though considered an anchor locus for a sparsely mapped region of mouse Chr 13, the pearl locus has only recently been integrated into mouse genetic maps that employ microsatellite-based markers (Justice and Stephenson 1993). We have endeavored to refine the position of pearl by the localization of linked markers, using the interspecific backcross panels made available by The Jackson Laboratory. Lawrence Pinto has bred a *Mus domesticus*/*Mus spretus* congenic animal in order to selectively introduce a limited region of *Mus spretus* genomic DNA into the *Mus domesticus* background, thereby creating a highly polymorphic region suitable for screening new markers. This congenic line has been previously employed for isolating markers linked to the pearl locus with *Mus spretus*-specific LINE-1 elements (Rikke et al. 1993). In this report, we present four markers isolated from a limited region of mouse Chr 13 that includes the pearl locus, using the representational difference analysis strategy developed by Lisitsyn and associates (1993). In addition to confirming the linkage of these markers with pearl, we have used them to integrate this region of Chr 13 into the current genetic map by mapping

them with one of the interspecific backcross panels recently developed by The Jackson Laboratory (Rowe et al. 1994).

Materials and methods

Fertile females from the cross of C57BL/6J Pin pe/pe and *Mus spretus* were backcrossed to C57BL/6J Pin pe/pe; progeny with normal phenotypes were selected. These animals were then backcrossed for a total of 12 generations in order to achieve a congenic line, B6/*spretus* pe^{+Pin}N12F6. From the calculations of Haldane, the estimated size of the retained *Mus spretus* DNA is 17 cM, with a <5% probability of an unlinked segment of *Mus spretus* DNA being present (Rikke et al. 1993).

Two sets of genomic subtractions using the representation difference analysis (RDA) method were performed (Lisitsyn et al. 1993). In one set, the genomic DNA from the inbred line, C57BL/6J, was used as a tester, and DNA from a homozygous B6/*spretus* pe^{+Pin}N12F6 was used as a driver. In the other subtraction, the roles of the two DNA samples were reversed. Two micrograms of each DNA were digested with *Bgl*III restriction endonuclease; RDA was then performed as described in the original procedure. The tester and driver DNAs were ligated to short double-stranded adaptor oligonucleotides to create amplicon DNAs as described in the original RDA method. The resulting DNA fragments from the two RDA experiments were each subcloned into a pUC118 plasmid. Probes for Southern blotting were prepared by random primer labeling of unique inserts excised from the plasmids. Equivalent amounts of tester and driver amplicon DNA were electrophoresed and blotted to nitrocellulose filters (Sambrook et al. 1989). Probes were hybridized to the filters at 68°C for 1 h in solutions containing 6× SSC, 5× Denhardt's solution, and 50 µg/ml of salmon sperm DNA. The filters were washed three times at 68°C in 1× Blot Wash (12 mM Na₂HPO₄/8 mM NaH₂PO₄/1.4 mM Na₄P₂O₇/0.5% SDS). The filters were then exposed to Kodak X-OMAT film. Only those probes that hybridized to the tester amplicon DNA but not to the driver amplicon DNA were further mapped and sequenced.

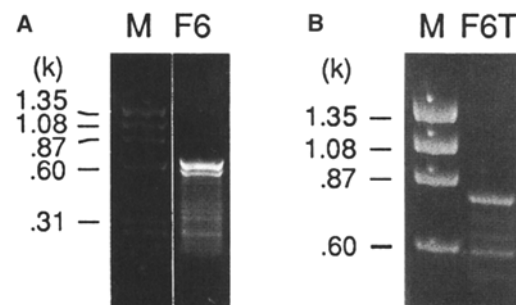


Fig. 1. Genomic DNA fragments isolated from RDA. (A) C57BL/6J Pin pe/pe DNA as the tester. Lane F6 shows the complexity and sizes of the isolated DNA fragments by RDA. (B) B6/*spretus* pe^{+Pin}N12F6 DNA as the tester. Lane F6T shows the isolated DNA fragments by RDA. In addition to the two major bands, at least four faint bands are also present. M identifies the lanes containing Φ X174 *Hae*III DNA fragments used as size markers.

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The nucleotide sequence data for clones reported in this paper have been submitted to GenBank and have been assigned the following accession numbers: U25821 (*D13Gor2*), U25824 (*D13Gor3*), U25825 (*D13Gor4*), and U25827 (*D13Gor1*).

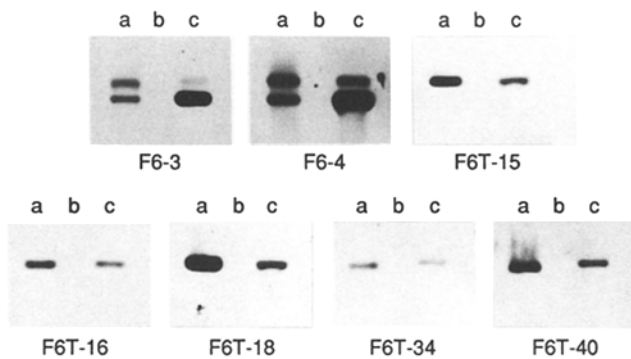


Fig. 2. Southern blots of the tester and driver amplicon DNA with RDA probes. Fragments F6-3, F6-4, F6T-15, F6T-16, F6T-18, F6T-34, and F6T-40 were used as Southern blotting probes, respectively. Lane **a** shows the positive control for each probe. Lane **b** contains the driver amplicon DNA that does not hybridize to the probe. Lane **c** illustrates the tester amplicon DNA that hybridizes to the probe. The identical sizes of the control bands and those seen in the tester amplicon DNA confirm that the cloned probes are representative of the original, amplified DNA. The double bands observed with the hybridizations with F6-3 and F6-4 probes may be the result of an internal restriction endonuclease cleavage site or an artifact from the gel electrophoresis and blotting. The presence of the additional bands does not affect the demonstration that these two cloned probes are not present in the driver amplicon DNA.

In order to confirm the localization of the positive probes within the *Mus spretus* region flanking the pearl locus in the B6/*spretus* $pe^{+Pim}N12F6$ mouse, the probes were hybridized to Southern blots consisting of either *EcoRI*, *PstI*, and *BamHI* restriction endonuclease digests or *BamHI*, *BglII*, *HindIII* restriction endonuclease digests of genomic DNAs from C57BL/6J, B6/*spretus* $pe^{+Pim}N12F6$, *Mus spretus*, and C3H mice. The DNA fragments were electrophoresed on 1% agarose gels and were transferred to either Zetabind (Cuno, Meridan, Conn.) or Hybond N+ (Amersham, Arlington Heights, Ill.) membranes. The nylon filters were baked for 2 h at 80°C to immobilize the DNA.

The filters were initially prehybridized overnight at 42°C in 20 ml Hyb-N solution (10% dextran sulfate/40% formamide/4× SSC/20 mM Tris, pH 7.4/1× Denhardt's solution) containing 100 µl salmon sperm DNA (10 mg/ml) and 10 µl mouse Cot-1 DNA (10 mg/ml; Davis et al. 1986). In subsequent hybridizations, the filters were prehybridized a minimum of 1 h as above, without the mouse Cot-1. Probes were generated by random-prime labeling with ^{32}P -dCTP and Pharmacia's Oligolabelling Kit (Piscataway, NJ). The hybridization of the filters with the radioactively labeled probes was done overnight at 42°C. The filters were subsequently washed twice for 15 min in 2× SSC/0.1% SDS at room temperature and washed again for 30 min in 0.1× SSC/0.1% SDS at 60°C. The washed filters were exposed to Kodak X-OMAT film 1–4 days in the presence of an intensifying screen. The genomic DNA clones were sequenced by the dideoxynucleotide chain termination method (the Double-Stranded Cycling Kit from Epicenter Technologies, Madison, Wis.) with oligonucleotide primers complementary to the plasmid DNA flanking the insertion site (Sanger et al. 1977; Biggin et al. 1983).

The small genomic clones were initially tested by being used as hybridization probes to detect restriction fragment length variations on Southern blots. Those genomic clones that were within the *Mus spretus* or C3H regions were sequenced, and oligonucleotide primers were made for PCR-based detection. Nucleotide variations within the PCR markers were detected either by secondary restriction endonuclease digests, size differences of the products on a sequencing gel, or by a positive-null allele detection assay. Polymerase chain amplification was performed by an initial denaturation step of 94°C for 2 min followed by 30–40 cycles of 94°C 20 s; 65°C 20 s; and 72°C 30 s. The cycles were terminated with a final incubation at 72°C for 7 min (Dietrich et al. 1992). C57BL/6J, B6/*spretus* $pe^{+Pim}N12F6$, *Mus spretus*, C3H, or pearl DNA was used as templates in individual reactions. The PCR-derived DNA products were run on 6% sequencing gels to detect nucleotide variations. Once a variation was detected, these primers were used in reactions with the 96-member (C57BL/6 × *Mus spretus*) F_1 × *Mus spretus* backcross panel from The Jackson Laboratory (Bar Harbor, Maine). The genotyping data were provided to Lucy Rowe and Mary Barter at The Jackson Laboratory, who calculated the map positions and distances with respect to the other markers placed on this

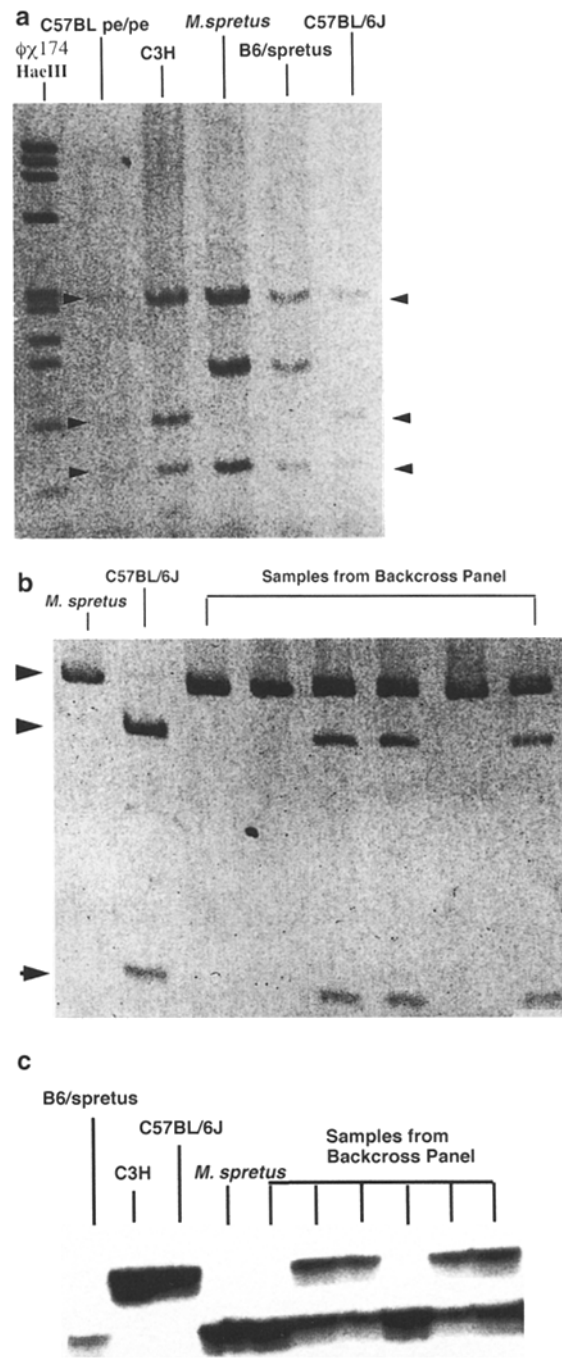


Fig. 3. Illustration of the PCR-based variations observed for F6-3, F6-4, and F6T-15. PCR reactions were performed using genomic DNA from various mouse lines and strains and/or from members of the interspecific backcross panel as templates. In each case, note that the banding pattern for the congenic mouse B6/*spretus* $pe^{+Pim}N12F6$ (noted as B6/*spretus* in the figure) matches that of the *Mus spretus*, therefore placing it within the interval spanning the pearl locus. **a.** F6-3. Thirty-microliter PCR reactions were performed, a 5-µl aliquot of which was digested in a 10-µl reaction with *HaeIII*; the entire digestion was then electrophoresed through a 6% polyacrylamide minigel. Fragment sizes for C57BL/6J, C3H, and C57BL/6J pe/pe are 271, 114, 78, and 52 bp. Note the loss of a site in *Mus spretus* and B6/*spretus* $pe^{+Pim}N12F6$, producing fragments of 271, 166, and 78 bp. Bands were identified through ethidium bromide staining. **b.** F6T-15. Methods are as described in "a" except that PCR products were cut with *AvaII*. Note that backcross panel members are easily typed as either homozygous *Mus spretus* or heterozygous C57BL/6J and *Mus spretus*. The upper arrow indicates the uncut 435-bp fragment; the lower arrows identify the 344-bp and 91-bp fragments generated by the *AvaII* site. **c.** F6-4. PCR reactions were performed with the reverse primer end-labeled with ^{32}P -ATP; products were run on a 6% sequencing gel. Bands were observed through autoradiography.

Table 1. Summary of primer sequences, detection methods, and allele assignments.

Clone	Name	Primer sequence	Detection method	Product Size	Assignment in B6/ <i>spretus</i> congenic	Comments
F6-1		none	SOUTHERN	not applicable	non- <i>Mus spretus</i>	
F6-2		none	SOUTHERN	not applicable	non- <i>Mus spretus</i>	
F6-3	<i>D13Gor2</i>	F: ttgtgtgtcccagtgagag R: ccagagctcattagccaac	RFLV/PCR	515 bp	<i>Mus spretus</i>	<i>Hae</i> III site lost in <i>Mus spretus</i> allele
F6-4	<i>D13Gor3</i>	F: tgaatgcccaaaaggaaga R: agggaaagactgccctgg	PCR	302 bp	<i>Mus spretus</i>	<i>Mus spretus</i> allele 4 bp smaller than C57BL/6J allele
F6T-15	<i>D13Gor4</i>	F: ttcagcaggatgagaagtggg R: ttgccagccaggatcttc	RFLV/PCR	436 bp	<i>Mus spretus</i>	<i>Ava</i> II site lost in <i>Mus spretus</i> allele
F6T-16		none	SOUTHERN	not applicable	non- <i>Mus spretus</i>	
F6T-40	<i>D13Gor1</i>	F: ctagttagtgaaccaaggctgac R: taatccagctctgagtggcacc	PCR	469 bp	C3H	Null <i>Mus spretus</i> allele

backcross panel. In instances in which a locus was not genotyped for a particular animal, analysis was made by inference; that is, if both of the typings adjacent to a missing locus were the same, then the missing locus was assumed to match, rather than hypothesizing a double recombination within that interval.

Results

The representational difference analysis technique was successful for both the forward (C57BL/6J DNA as tester) and reverse (congenic DNA as tester) attempts (Fig. 1). Seven (F6-3, 4; F6T-15, 16, 18, 34, and 40) of the original 11 probes hybridized appropriately with the tester amplicon DNA and did not hybridize with the driver amplicon DNA (Fig. 2). These inserts were then partially sequenced with plasmid-derived primers. On the basis of sequencing data, F6T-15 was identical to F6T-18, and F6T-16 was identical to F6T-34.

The genomic clone F6T-16 produced hybridization patterns with the genomic DNA of the congenic line, B6/*spretus* pe^{+Pin}N12F6, that were identical to those from the C57BL/6J parental DNA for three restriction enzymes (*Pst*I, *Hind*III, and *Eco*RI). The clone F6T-40 identified bands that did not correspond to either the pattern of C57BL/6J or of *Mus spretus*. Additional Southern blots employing *Bgl*II restriction endonuclease digestions indicated that F6T-16 and F6T-40 recognized C3H alleles in the B6/*spretus* pe^{+Pin}N12F6 animal. The region of B6/*spretus* pe^{+Pin}N12F6 that was replaced with *Mus spretus* DNA represents a subset of the C3H region flanking the pearl locus that was transferred into the C57BL/6J pe/pe congenic line (data not shown). The majority of the C3H-derived genome in B6/*spretus* pe^{+Pin}N12F6 lies centromeric to the *Mus spretus* region, while only a very small C3H-derived segment is present at the telomeric boundary.

The clones designated F6-3, F6-4, F6T-15 were found to be within the *Mus spretus* region of B6/*spretus* pe^{+Pin}N12F6 by restriction fragment length variations. Markers, F6-3, F6-4, F6T-15, and F6T-40 were sequenced, converted to PCR-based assays, and mapped on The Jackson Laboratory backcross panel. These genomic sequences have been registered in Genbank (F6-3: Accession #U25821; F6-4: Accession #U25824; F6T-15: Accession #U25825; F6T-40: Accession #U25827). Samples of the PCR variations are shown in Fig. 3.

Nucleotide variations with these PCR-based markers were detected either directly or by restriction fragment length differences (see Table 1). A size variation for F6-4 was detected directly, while F6-3 and F6T-15 PCR products were cleaved with *Hae*III or *Ava*II enzyme to detect variations, respectively. In the case of F6T-40, the primers produced a null allele for the *Mus spretus* DNA template. The (C57BL/6J × *Mus spretus*) F₁ × *Mus spretus* Jackson interspecific backcross panel was used for mapping F6T-40 and the other markers, in part because *Mus spretus* null alleles could be readily accommodated. The results of the backcross panel are summarized in Fig. 4. The incorporation of these RDA-derived markers onto the genetic map is shown in Fig. 5. The standardized, approved names for each of these markers is reflected on this map: *D13Gor1* = F6T-40; *D13Gor2* = F6-3; *D13Gor3* = F6-4; and *D13Gor4* = F6T-15. In a separate paper we have integrated this genetic map for the region of mouse Chr 13 with additional microsatellite markers and genes, thus providing for a realigned framework of this portion of the mouse genome.

Discussion

Representational difference analysis (RDA) is a powerful means of generating polymorphic markers to a select region of the genome.

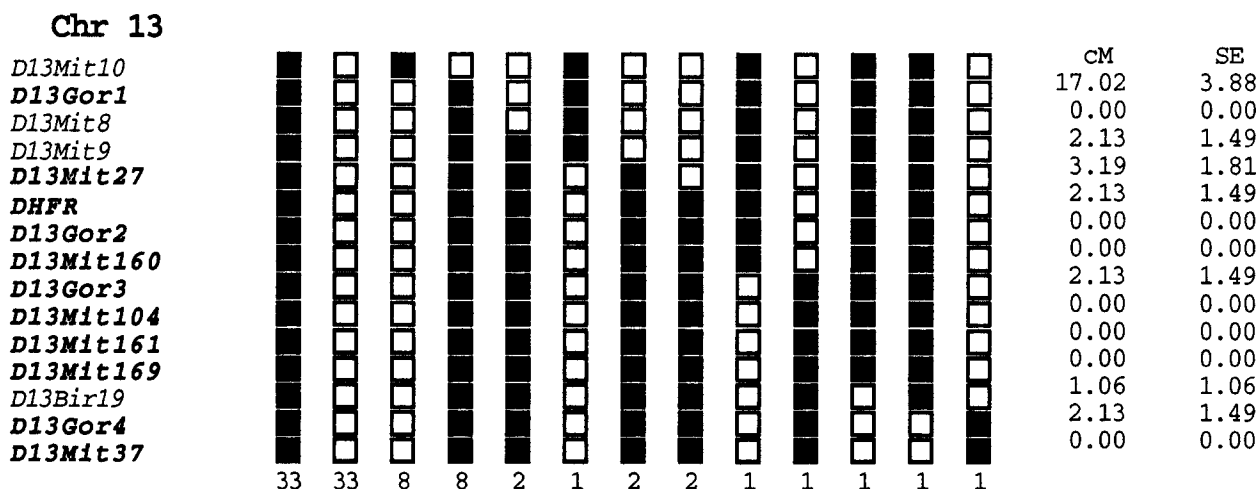


Fig. 4. Placement of RDA-derived clones on The Jackson Laboratory's Interspecific Backcross panel (C57BL/6J × *Mus spretus*) F₁ × *Mus spretus*. Data points in outline are inferred phenotypes (see text).

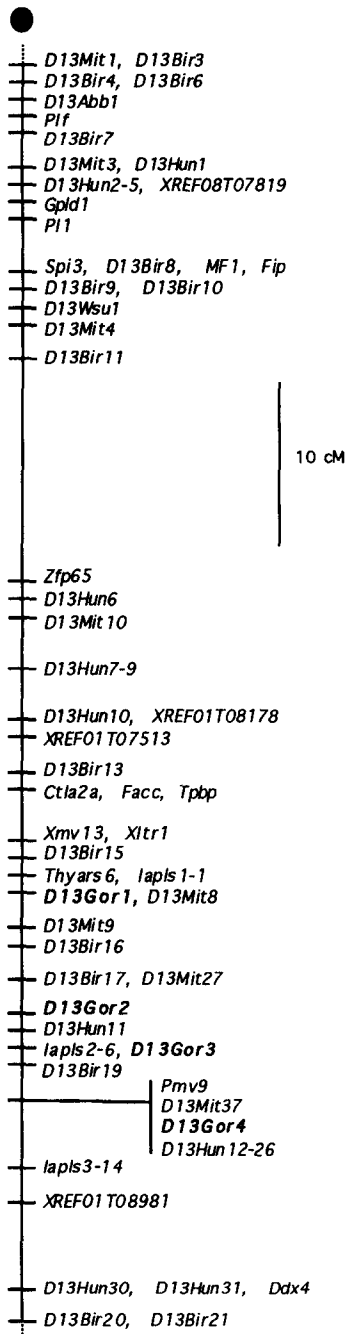


Fig. 5. Distal map of mouse Chr 13 as configured by Lucy Rowe and Mary Barter of The Jackson Laboratory, detailing the location of the RDA-derived markers with respect to other markers mapped on the The Jackson Laboratory interspecific backcross panel. Note that *D13Gor1* = F6T-40; *D13Gor2* = F6-3; *D13Gor3* = F6-4; and *D13Gor4* = F6T-15.

Markers can be identified between *Mus spretus* and *Mus domesticus* DNA as well as between *Mus domesticus* strains (C3H and C57BL/6J). The RDA method converts the genomic DNAs of the target and driver into restricted sets of PCR-amplified genomic fragments. For a limited group of restriction fragment length variations, one genomic source will contain an amplified genomic fragment, while the other PCR-generated DNA pool will lack representation of the corresponding genomic fragments that exceed the size constraints of routine PCR. The variations exploited by the RDA approach can be easily identified by Southern blots of genomic DNA. Though only a limited number of markers are generated with a single restriction enzyme digestion, the number of markers can be increased by using each DNA source as a separate driver and by using additional restriction endonucleases. The conversion of the RDA markers to PCR-based assays can be performed effectively by using additional restriction endonucleases to detect internal nucleotide variations, single-stranded conformational analysis, or a combination of these methods.

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