"RFLP subtraction": A method for making libraries of polymorphic markers

(biotin–avidin affinity purification/mouse/polymerase chain reaction/DNA)

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ABSTRACT We developed a method, "RFLP subtraction," that isolates large numbers of unique sequence restriction fragment length polymorphisms (RFLPs) in a single experiment. The technique purifies small restriction fragments from one genome containing sequences that reside on large fragments in a related genome. We first isolate samples containing the small restriction fragments from two polymorphic strains. Subtractive hybridization then removes the fragments that are present in both samples. The remaining sequences are RFLPs: they occur on small fragments in one strain but not in the other. Here we use RFLP subtraction to make a library of hundreds of unique sequence RFLPs from two inbred mouse strains. We analyze and map a subset of the RFLPs and show that the genetic linkage of these markers can be rapidly determined by an efficient dot blot mapping technique. Several other potential applications of RFLP subtraction, including isolating region specific markers, are discussed.

Genetic markers corresponding to DNA polymorphisms have fueled the recent dramatic progress in genome mapping, gene isolation, and DNA diagnostics (1, 2). High-resolution genetic maps of polymorphic markers are being constructed for human, mouse, crop plants, and many other organisms of interest to biologists. These maps are indispensable for positional cloning of genes defined by mutation, such as those that cause inherited disease in humans or resistance to pathogens in crop plants. The current revolution in forensic, medical, and agricultural DNA diagnostic technologies is based on parallel detection of numerous polymorphisms that provide a DNA fingerprint.

A number of powerful new gene isolation and genome mapping methods could be built around a method that simultaneously isolates large numbers of unique sequence polymorphic markers. As yet, no such method has been developed. Polymorphic markers can be isolated serially by screening Southern blots with genomic probes, by amplifying genomic DNA with short oligonucleotide primers (3, 4), or by digesting amplified sequences with a panel of restriction enzymes (5). Two methods have recently been developed to clone polymorphic markers en masse. Repetitive polymorphic sequences can be cloned in large numbers by screening libraries with simple sequence repeats (6–8). Nonrepetitive restriction fragment length polymorphisms (RFLPs) have recently been isolated using a competitive hybridization method that purified 20 human RFLPs in one experiment (9).

Here we describe RFLP subtraction, which efficiently isolates many unique sequence RFLPs using subtractive hybridization (9–17). RFLP subtraction purifies sequences that occur on restriction fragments of a particular size in one strain but that are not represented in the same size class of fragments in a related strain. By applying RFLP subtraction

to genomic DNA from two inbred mouse strains we construct a library containing hundreds of unique sequence markers. We also demonstrate an efficient method for mapping the products of RFLP subtraction using dot blot hybridization.

MATERIALS AND METHODS

DNA. DNAs from mouse strains C57BL/6J, A/J, CBA/N (18), and BALB/K (19) were generously provided by J. Press (Brandeis University). DNAs from strains A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, and DBA/2J (18) and the AXB and BXA set of recombinant inbred strains (20) were purchased from The Jackson Laboratory. Oligonucleotides AGCACTCTCCAGCCTCTCACCGCA (OL24), GACACT-CTCGAGACATCACCGTCC (OL25), (biotin-dX)GACAC-TCTCGAGACATCACCGTCC (OL25B), GTTGGTT-TAAGGCGCAAG (OL30), (biotin-dX)AA(biotin-dT)TCT-TGCGCCTTAAACCAAC (OL31DB), GAC CTCGAGA-CATCACCGTCCA (OL38), AGCTTGGA-CGGTGATGTC-TCGAGAGTG (OL39), AATTCTTGCGCCTTAAAC-CAACA (OL40), AGCTTGTTGGTTTAAGGCGCAAGAA (OL41), and AGCTTGCGGTGAGAGG (OL42) were purchased from the Midland Certified Reagent (Midland, TX) and purified by polyacrylamide gel electrophoresis. Adaptors AD9, AD10, and AD11 are equimolar mixtures of oligonucleotides OL38 and phosphorylated OL39, OL40 and phosphorylated OL41, OL24 and OL42, respectively.

Preparation of Tracer and Driver. BALB/K and C57BL/6J DNAs were digested with HindIII and ligated to adaptors AD9 and AD10, respectively. DNA (60–80 ng) was electrophoresed on a 1% low-melting agarose gel (FMC). BALB/K fragments (250–1000 bp; tracer) and C57BL/6J fragments (120–1200 bp; driver) were purified from melted gel slabs by phenol extraction, precipitated with ethanol, and resuspended in 50 µl of TE (10 mM Tris·HCl/1 mM EDTA, pH 8.0). The tracer DNA (1 µl) was amplified in a 100-µl PCR reaction mixture for 25 cycles using OL25 as a primer, purified on a Sephacryl S-300HR (Pharmacia) spin-column, ethanol precipitated, and dissolved in water. Driver DNA (1 µl) was amplified in a 25-µl PCR reaction mixture for 20 cycles using biotinylated primer OL31DB. The PCR products were reamplified in six 200-µl PCR reaction mixtures for 30 cycles. After chloroform extraction, Sephacryl S-300HR spin-column chromatography, and ethanol precipitation, the sample (125 µg) was dissolved in water. PCR mixtures contained reaction buffer (Boehringer Mannheim), 200 µM (each) dNTP, 1 µM oligonucleotide primer, and 0.1 unit of Taq polymerase per µl (Boehringer Mannheim). Except where noted, the PCR regime was 20–30 cycles (30 sec at 94°C, 30 sec at 55°C, and 3 min at 72°C) followed by 10 min at 72°C.

Subtractive Hybridization. We combined 10 µg of driver, 100 ng of tracer, 20 µg of yeast tRNA, 5 µg of OL30, and 2 µg of OL25. The oligonucleotides were included to block annealing of the self-complementary ends of the driver and

Abbreviation: RFLP, restriction fragment length polymorphism.
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the tracer. The sample was lyophilized in a SpeedVac evaporator (Savant) and dissolved in 3.2 μl of 25 mM Na-EPPS (*N*-[2-hydroxyethyl]piperazine-*N'*-[3-propanesulfonic acid]; Sigma)/2.5 mM EDTA, pH 8.0 (at 20°C), overlaid with mineral oil, and denatured for 3 min at 100°C (14). After adding 0.8 μl of 5 M NaCl, the sample was incubated for 16–24 hr at 65°C. Following hybridization, the oil was removed, the sample volume was brought to 100 μl with 10 mM Tris·HCl, pH 8/1 mM EDTA/0.5 M NaCl (NTE), and Fluoricon avidin-polystyrene assay particles (100 μl; Idexx Laboratories, Westbrook, ME) were added (14, 21). The samples were incubated for 10 min at room temperature and spun in Spin-X filter units (Costar) to remove the beads. After washing the beads with NTE (100 μl), the filtrate containing the subtraction products was extracted with phenol/chloroform (1:1). Yeast tRNA (10 μg) was added and the DNA was ethanol precipitated and dissolved in water (50 μl). An aliquot (5 μl) was saved for further analysis. The rest of the sample was combined with 10 μg of driver, 5 μg of OL30, and 2 μg of OL25, and the next round of hybridization was set up as described above. A total of three rounds of hybridization was performed. Aliquots obtained after each round of subtraction (1 μl) were amplified in 50-μl reaction mixtures for 20 cycles using OL25 as a primer. We found that short sequences (<500 bp) are overrepresented in the amplified mixture. To achieve a more even distribution of fragment sizes, the amplified subtraction products were denatured and passed successively over two spin-columns packed with Sephacryl S-400 (Pharmacia).

Removing Poorly Hybridizing Fragments. The subtraction products (0.1 μl of 50 μl) were amplified in a 50-μl PCR reaction mixture for 20 cycles using biotinylated primer OL25B. To inactivate *Taq* polymerase, SDS was added to a final concentration of 0.1%, and an aliquot (25 μl) was denatured for 10 min at 99°C and allowed to reanneal for 2 hr at 72°C. DNA was applied to a Sephacryl S-300HR spin-column. The eluate was extracted with phenol/chloroform (1:1) and ethanol precipitated. DNA was further digested with 30 units of *Hind*III, ligated to nonphosphorylated adaptor AD11, treated with 50 μl of avidin beads as described above, ethanol precipitated, and dissolved in 25 μl of water. After mixing a portion of the sample (5 μl) with 10 μg of driver, 20 μg of yeast tRNA, and 5 μg of OL30, subtraction was performed as above. DNA (1 μl of 50 μl total) was incubated in PCR reaction mixture (Boehringer Mannheim) without *Taq* polymerase for 5 min at 72°C, the enzyme was added, the 3' ends of the adapter were filled in at 72°C for 5 min, and then the sample was amplified for 30 cycles (30 sec at 95°C and 3 min at 72°C) followed by extension for 10 min at 72°C (16).

Cloning and Analyzing the RFLP Subtraction Products. The amplified subtraction products were cut with *Hind*III and ligated to *Hind*III-digested dephosphorylated pBluescript KS(+) plasmid (Stratagene). After transforming *Escherichia coli* with the ligation products, single colonies were boiled and the inserts were amplified using M13 (−20) and M13 (reverse) primers (New England Biolabs). Markers were mapped using labeled inserts (22) to probe Southern blots containing *Hind*III-digested DNA (7–10 μg) from 31 strains from the AXB and BXA recombinant inbred set (20, 23, 24). Linkage analysis was performed using the MAP MANAGER (version 2.4) computer program (25) and the data base of strain distribution patterns (update of August 1993) kindly provided by K. Manly (Roswell Park Cancer Institute). The chromosomal locations of the markers were found using the program's "Find best location" subroutine. For probing colony lifts, vector sequences were removed from the amplified inserts by digestion with *Hind*III and gel purification.

Dot Blot Mapping. Short *Hind*III fragments from 6 mouse inbred strains and 31 strains from the AXB and BXA recombinant inbred set were gel-purified and amplified as described

above for preparation of the tracer DNA. PCR products were passed through Sephacryl S-300HR spin-columns, extracted with phenol/chloroform (1:1), and ethanol precipitated. DNA (1–2 μg each) was dot blotted and probed with amplified inserts from the RFLP library (26).

RESULTS

Strategy. RFLP subtraction is derived from genomic subtraction, a method that purifies DNA corresponding to deletion mutations (14, 16, 27, 28). Both methods purify fragments that are present in one population (the tracer) but absent in another (the driver). Purification is achieved by
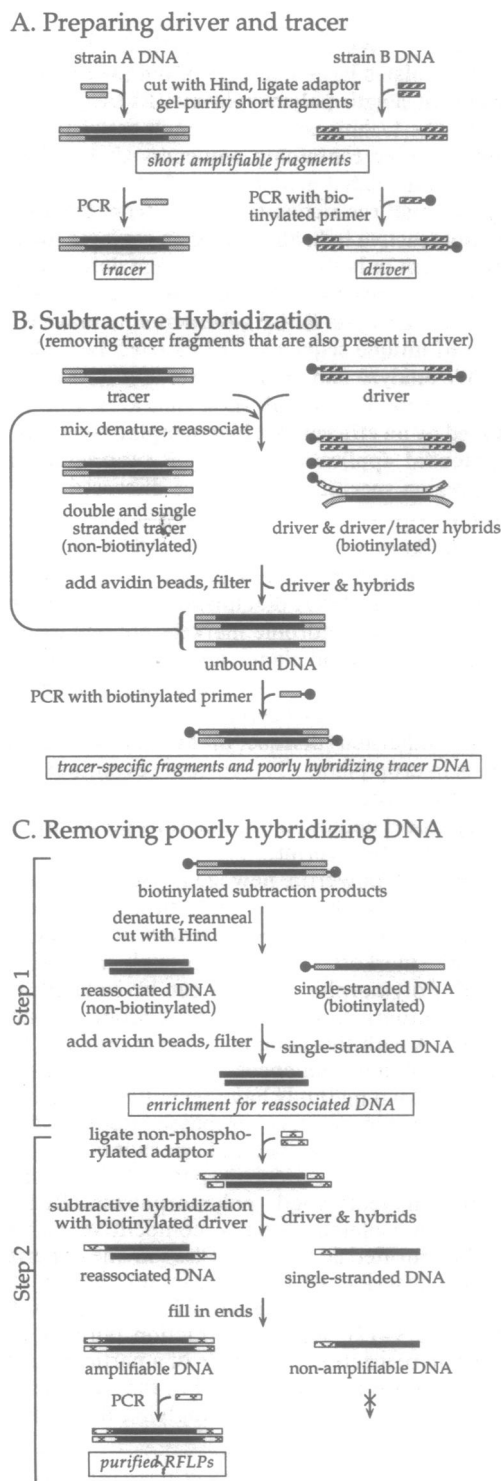

FIG. 1.   RFLP subtraction method.

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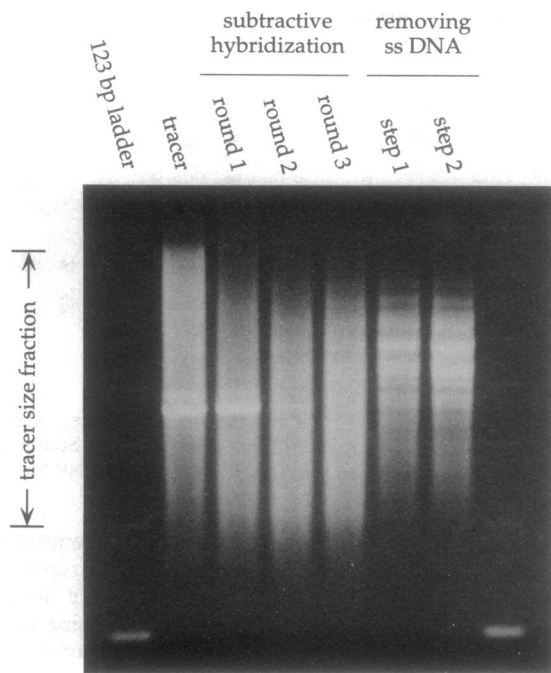*Proc. Natl. Acad. Sci. USA 91 (1994)*   6115

FIG. 2. Electrophoretic analysis of the products of RFLP subtraction on 2% agarose gels stained with ethidium bromide. From left to right: sized, amplified tracer DNA; the amplified products of subtractive hybridization (rounds one to three); the amplified products after removing poorly hybridizing sequences.

removing all of the fragments in the tracer DNA that have counterparts in the driver DNA using subtractive hybridization. In RFLP subtraction, the tracer is a size fraction of digested DNA from one strain and the driver is a similar size fraction from a polymorphic strain. The products obtained after removing the common sequences are RFLPs; they are sized tracer fragments whose driver counterparts are not found in the same size fraction.

There are three steps in RFLP subtraction: preparation of driver and tracer (Fig. 1*A*), subtractive hybridization (Fig. 1*B*), and removal of nonhybridizing sequences from the tracer (Fig. 1*C*). To prepare the driver and tracer DNA (Fig. 1*A*), we cut the genomic DNA from two related strains with *Hin*dIII and cap the ends of the fragments from each strain with a different oligonucleotide adaptor. The low molecular weight fragments are then purified from a slice of an agarose gel and amplified using one of the adapter strands as a PCR primer. We use a biotinylated primer to amplify the driver so that driver DNA can be removed following the subtractive hybridizations by binding to avidin-coated beads.

We perform three rounds of subtractive hybridization to remove tracer sequences that also occur in the driver (Fig. 1*B*). A small amount of tracer is mixed with an excess of biotinylated driver, and the mixture is denatured and allowed to reanneal. Most tracer sequences will hybridize to complementary biotinylated driver strands. Some tracer sequences, however, are not represented in the driver because they reside on large *Hin*dIII fragments (i.e., they are RFLPs) or are missing from the driver genome. These fragments will have no complementary biotinylated strands with which to anneal. The biotinylated driver DNA, and any tracer that has annealed to it, is then removed using avidin-coated polystyrene beads (14, 21). We find that 97% of denatured biotinylated driver DNA is reproducibly removed by this method. The unbound fraction is then subjected to two more rounds of subtractive hybridization and tracer DNA remaining after the third round is amplified.

Material obtained at this stage of subtraction is enriched for tracer-specific fragments as well as for fragments that reassociate poorly under the hybridization conditions we used. Tracer fragments that fail to reassociate (for example, those with extensive secondary structure) cannot be removed by biotinylated driver. It is essential to remove the poorly hybridizing sequences, which may represent a large fraction of the product at this stage.

We apply two different procedures in succession to remove sequences that fail to hybridize efficiently. The DNA obtained after three rounds of subtractive hybridization is amplified with biotinylated primer, denatured, and renatured (Fig. 1*C*, step 1). Efficiently hybridizing sequences reassociate, while the nonhybridizing DNA remains single stranded. The DNA is then digested with *Hin*dIII to selectively remove the biotinylated ends from the desired reassociated product. Poorly hybridizing contaminants, in contrast, cannot be cut since they are single stranded and will therefore remain biotinylated. Avidin affinity chromatography now removes these nonreassociated (biotinylated) contaminants from the reassociated (nonbiotinylated) products.

To ensure complete removal of the nonhybridizing sequences, we include an additional step that selects for DNA that reassociates efficiently. In this step, based upon a previously published procedure (16), the subtraction products are first ligated to a nonphosphorylated adapter. Since both adapter strands lack a 5' phosphate group, only one of them actually forms a covalent bond with DNA, yielding fragments tailed with an oligonucleotide at the 5' ends (Fig. 1*C*, step 2). The DNA is then mixed with an excess of biotinylated driver and subtractive hybridization is performed as above. The product, composed of double-stranded and single-stranded DNA, is amplified; this time, however, only the double-stranded fragments can serve as templates in the PCR. In the first cycle of amplification *Taq* polymerase fills in oligonucleotide overhangs on the double-stranded fragments and the PCR proceeds as usual. In contrast, single-stranded DNA fragments cannot be amplified because they lack sequences complementary to the primer at the 3' end. The products of this step are the tracer sequences that cannot anneal to the driver but that efficiently self-anneal. We clone and analyze the amplified material obtained after this final purification step.

RFLP Subtraction of Mouse Genomic DNA. In reconstruction experiments we first showed that RFLP subtraction can purify a single λ phage restriction fragment present in the tracer at single copy level from the rest of the mouse genome (data not shown). We then applied RFLP subtraction to construct a library of RFLPs using sized DNA from strain BALB/K as tracer and sized DNA from strain C57BL/6J as driver. Fig. 2 shows the electrophoretic analysis of the amplified products after each round of subtraction. A complex pattern of bands emerged after the third round of subtraction and was clearly visible after the first of the two steps that remove the nonhybridizing sequences. The pattern of bands remains similar after the second step, indicating that the first step removed the bulk of the poorly hybridizing single-stranded products. After cloning the RFLP subtraction products we amplified the inserts from 26 randomly picked colonies. All 26 colonies contained inserts ranging in size from 250 to 700 bp. Two of the inserts (nos. 6 and 23) contained an internal *Hin*dIII site, probably as a result of ligating two fragments into one vector molecule. These clones were omitted from further analysis.

To determine if the subtraction products were indeed polymorphic, we hybridized 22 different inserts (2 of the 24 inserts were represented twice; see below) to Southern blots containing *Hin*dIII-digested genomic DNA from the strains that we had used to make the tracer (BALB/K) and the driver (C57BL/6J). All of the probes revealed polymorphisms be-
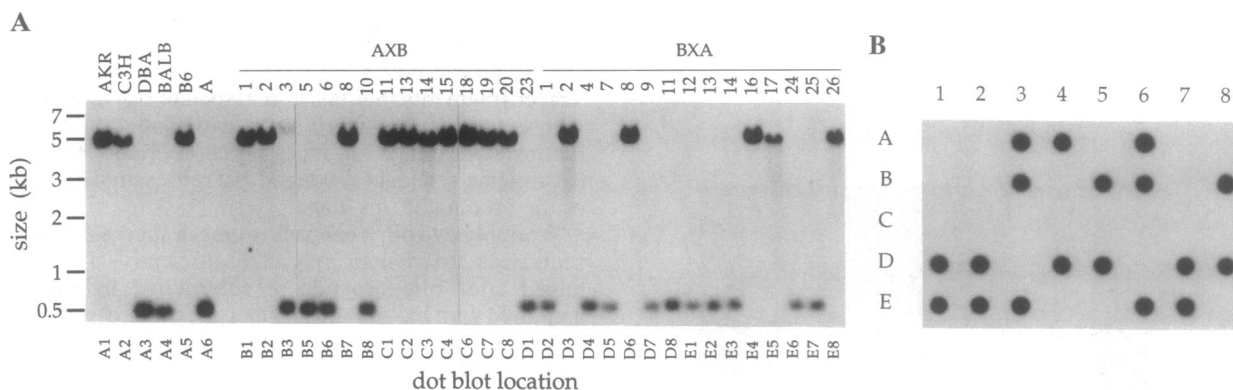
FIG. 3.   Hybridization of clone 17 to DNA from various mouse inbred strains and the AXB and BXA recombinant inbred set. The strain designations are B6 = C57BL/6J, BALB = BALB/cJ, DBA = DBA/2J, A = A/J, AKR = AKR/J, and C3H = C3H/HeJ. (*A*) Southern blot analysis. (*B*) Dot blot mapping. Wells A7, A8, and C5 were not loaded. DNA from strain AXB4 was analyzed in dot blot (B4) but not on the Southern blot.

tween these two strains (Table 1). Of the 22 probes, 18 hybridized to unique short fragments (<1 kb) in BALB/K and to unique long fragments (1.2–20 kb) in C57BL/6J. Three probes detected short alleles in BALB/K but did not hybridize to C57BL/6J. One of the 22 probes hybridized to a low copy number repeated sequence that is present in both strains; only the tracer, however, contained a short allele. These data indicate that we have constructed a mouse RFLP library composed almost entirely of nonrepetitive markers.

To estimate the number of different RFLPs in the library, we probed replica filters containing about 5000 colonies each with the 24 amplified inserts (Table 1). Of 24 probes, 22 showed unique nonoverlapping hybridization patterns, while 2 pairs

Table 1.   Hybridization analysis of random markers from the RFLP library

Clone no.	Abundance in library*	Allele size, kb			Chrom. no.	lod score†
		B6 (driver)	BALB/K (tracer)	A		
1	22	11.3	0.4	12	Unlinked	
2	25	NH	0.7	0.7	8	9.0
3	23	3.5	0.5‡	0.5‡	17	9.3
4	6	10.5	0.6	0.6	13	4.8
5	7	5.9	0.6	5.9	ND	ND
7	3	7.5	0.6	0.6	19	3.2
8	3	7.2	0.4	0.4	7	4.5
9	7	>12	0.3	0.3	11	7.1
10	17	1.5	0.5	0.5	4	5.6
11	18	11	0.6	11	ND	ND
12	115	NH	0.5	0.5	14	9.0
13	20	Polymorphic repeat			12	8.4
14	205	3.8	0.5	0.5	10	5.8
16	64	NH	0.5	NH	ND	ND
17	165	5.1	0.5	0.5	6	7.1
19	4	7.8	0.5	8.6	17	2.5
20	18	1.5	0.6	0.6	ND	ND
21	48	3.2	0.4	3.2	ND	ND
22	1	1.2	0.7	0.7	1	5.8
24	6	2.6	0.8	2.6	ND	ND
25	127	6.7	0.6	6.7	ND	ND
26	74	1.6	0.5	0.5	14	9.0

The strain designations are B6 = C57BL/6J, DBA = DBA/2J, and A = A/J. NH, no hybridization detected; ND, not determined; Chrom., chromosome.
*Number of colonies in the library (of 5000 plated) that hybridize to markers.
†Logarithm of odds (lod) score for linkage to the most tightly linked marker in the data set.
‡Clone 3 also hybridizes weakly to 3.3-kb and 4.3-kb bands in these strains.

(nos. 11 and 15; nos. 14 and 18) hybridized to the same two sets of colonies. Thus, the 24 randomly picked clones represent 22 different RFLPs. We found that the clones were not represented equally in the library (Table 1). Assuming that the sample of 24 analyzed clones is representative of the entire library, we can roughly estimate the total number of distinct RFLPs as:

$$\left(\frac{5000}{24}\right) \sum_{i=1}^{24} m_i^{-1} \approx 600 \text{ RFLPs},$$

where m_i is the number of clones in the library that hybridize to the *i*th clone of our sample (Table 1).

We mapped 15 markers that detected polymorphic *Hin*dIII fragments in C57BL/6J and A/J mouse DNA (Table 1) by probing Southern blots containing *Hin*dIII-digested DNA from 31 members of the AXB and BXA recombinant inbred set (20). Fig. 3*A* shows a representative Southern blot that was probed with clone 17. Table 1 shows that 14 markers were assigned to particular mouse chromosomes with high level of confidence (logarithm of odds score 2.5–9). Including 8 commonly used inbred mouse strains in the Southern blot analysis (Fig. 3*A*) demonstrated that, as expected, the number of informative clones was greatest for the strains thought to be most distantly related (data not shown).

Dot Blot Mapping. The nature of the products of RFLP subtraction makes it feasible to genetically map them by a simple dot blot assay. The insert sequences in the RFLP library share a common feature: they are found on short *Hin*dIII fragments in the tracer DNA but not in the driver DNA. Thus, we can map the markers by hybridizing them to dot blots containing the short fragments of recombinant strains. In this assay a positive hybridization signal indicates that a strain inherited the tracer allele, while a negative signal indicates that it inherited the driver allele. To test this method we prepared and amplified the short *Hin*dIII fragments from 37 strains, applied the DNA to a filter, and probed the dot blot with labeled insert from clone 17 (Fig. 3*B*). The dot blot produced the same strain distribution pattern as the corresponding Southern blot (Fig. 3*A*), indicating the effectiveness of the dot blot mapping technique.

DISCUSSION

We have developed a method, RFLP subtraction, for constructing libraries of unique sequence RFLPs. The method isolates the small restriction fragments from one strain (the tracer) that have no small counterparts in digested DNA from another strain (the driver). By applying RFLP subtraction to two inbred mouse strains, C57BL/6J and BALB/K, we constructed a library containing hundreds of different mouse

RFLPs. Genetic analysis indicates that the cloned markers map to dispersed sites in the nuclear genome.

Three of the 22 markers that we analyzed did not hybridize to the driver DNA and therefore correspond to insertions or deletions. One of these clones (no. 12) shares an identical strain distribution pattern with another clone (no. 26) that does hybridize to both driver and tracer and also a previously mapped marker near the T-cell receptor α locus (29). We infer that clones 12 and 26 flank a rearrangement breakpoint, located close to the T-cell receptor α gene on chromosome 14.

RFLP subtraction is efficient with regard to time, starting materials, and yield of RFLPs. The theoretical yield of RFLPs depends on the level of polymorphism between the two strains and the fraction of HindIII fragments that are found in the tracer size fraction. Based on a polymorphism level between 0.1% and 0.4% (30), we expect 350–1250 RFLPs in the tracer size fraction. Our library of 600 RFLPs therefore contains a substantial fraction (50–100%) of the expected polymorphic markers. This compares favorably with a recently published method for cloning unique sequence RFLPs, representational difference analysis, which purified 20 RFLPs from humans, representing about 2% of the theoretical yield (9). Several factors may contribute to the high yields achieved in this study. Using a precise method of size selection precludes removal of desirable RFLPs by large fragments that may otherwise contaminate the driver. We minimize the tendency for some RFLPs to proliferate at the expense of others by limiting the number of amplification steps and by introducing self-annealing steps only at the end of our procedure when the desired sequences are at high enough concentrations to anneal completely.

Markers identified by RFLP subtraction can be rapidly mapped using a simple dot blot technique (Fig. 3B). Several important features of this method make it attractive for large-scale mapping projects. DNA for dot blotting is prepared by amplification of short restriction fragments; therefore <1 μg of genomic DNA from individual strains can provide an unlimited supply of material. Hundreds of strains can be scored in a single hybridization experiment and multiple hybridizations can be carried out simultaneously. This strategy can cut the costs of mapping genomes since no sequencing or primer synthesis is required. The dot blot mapping technique, being cost-effective, rapid, and amenable to automation, provides an attractive alternative to existing mapping strategies.

Isolating differences between genomes has many applications. RFLP subtraction provides a powerful and general method for cloning genes defined by mutation. To obtain markers surrounding a gene, the source of the driver could be a congenic strain (whose genetic background is identical to the tracer strain at all sites except near the locus of interest), a cell line (that differs from the tracer cell line at one chromosomal location only), or the phenotypically pooled progeny of a cross between the strain that is the source of the tracer and a polymorphic strain that has a different allele at the locus of interest (31, 32). Using RFLP subtraction to isolate DNA from regions that have lost heterozygosity in tumor cells could lead to the discovery of new tumor suppressor genes. Similarly, new instances of developmentally programed loss of heterozygosity (like the rearrangement of immunoglobulin loci) could be detected by applying RFLP subtraction to DNA from different tissues. Novel viral or microbial pathogens might be detected by using the method to isolate DNA unique to diseased tissue. Applying RFLP subtraction to related pathogens could generate useful markers for determining the identity of a strain causing an infection.

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