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Isolation and Characterization of DNA Probes from a Flow-Sorted Human Chromosome 8 Library That Detect Restriction Fragment Length Polymorphism (RFLP)

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SUMMARY

We have used a recombinant DNA library constructed from flow-sorted human chromosome 8 as a source of single-copy human probes. These probes have been screened for restriction fragment length polymorphism (RFLP) by hybridization to Southern transfers of genomic DNA from five unrelated individuals. We have detected six RFLPs distributed among four probes after screening 741 base pairs for restriction site variation. These RFLPs all behave as codominant Mendelian alleles. Two of the probes detect rare variants, while the other two detect RFLPs with PIC values of .36 and .16. Informative probes will be useful for the construction of a linkage map for chromosome 8 and for the localization of mutant alleles to this chromosome.

INTRODUCTION

Currently, substantial interest and effort is being directed toward locating genes whose mutant alleles are responsible for inherited disease, using, primarily, arbitrary random DNA sequences that detect RFLP [1]. Locations have already been established for Duchenne muscular dystrophy [2], Huntington disease [3], and cystic fibrosis [4–6].

As the human linkage map becomes more complete, it will become possible to locate such deleterious genes using a strategy based upon a small set of suitably spaced, informative, test markers for each chromosome. The availabil-

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ity of 22 autosomal linkage maps will allow definitive exclusion of autosomal mutant alleles from 21 chromosomes as well as the assignment to one chromosome. Furthermore, map construction itself, when based upon the use of chromosome-specific probes, is more efficient [7]. The availability of recombinant human DNA libraries for specific human chromosomes, through the National Laboratory Gene Library Project, will facilitate this chromosome-specific approach.

We have chosen to investigate human chromosome 8 for a number of reasons. First, there are few genes assigned to this chromosome [8]. The only suitable polymorphisms for linkage analysis are RFLPs, detected by cloned genes or random probes. This chromosome participates in translocations and the formation of minute chromosomes that confer a cancerous cellular phenotype. The *myc* oncogene is often, but not always, involved in these chromosomal aberrations. Thus, probes derived from this chromosome may be of value for investigating mechanisms of tumorigenesis and developing treatment strategies.

Here, we describe the isolation of single-copy human DNA sequences from a flow-sorted human chromosome 8 library and the characterization of several probes that detect RFLP.

MATERIALS AND METHODS

Preparation of DNA Probes

The chromosome-specific gene library used in this work was constructed at the Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, California, under the auspices of the National Laboratory Gene Library Project, which is sponsored by the U.S. Department of Energy. This library, designated LL08NS02, was constructed by flow sorting human chromosome 8 from a CHO-human lymphocyte somatic cell hybrid, UV20HL21-27, that retained human chromosomes 4, 8, and 21. DNA isolated from the flow-sorted chromosomes was digested to completion with *Hin*dIII and cloned into the insertion vector Charon 21A. The resulting library has a complexity of 7.4×10^5 (20 genome equivalents).

We received a 1 ml aliquot of this library that titered at 1.7×10^8. This library was amplified by growth on the *supF* strains of *E. coli* DP50SupF and LE392. For each strain, 20 plates of NZY agar were overlaid with soft NZY top agar (7 gm/l agar at 42°C) mixed with bacterial plating cells infected with 8.5×10^4 phage. After overnight incubation at 37°C in a humid atmosphere, the plates were cooled and overlaid with 5 ml of 10 mM Tris, pH 7.5, 10 mM $MgCl_2$. The overlay solution was collected the following day and $CHCl_3$ added to lyse remaining bacteria. Phage were precipitated by adding NaCl to 1 M and 10 gm of polyethylene glycol 6,000 per 100 ml of supernatant. After 1 hr at 4°C, the phage were collected by centrifugation for 10 min at 11,000 *g*. The pellet was taken up in a small volume of 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, and debris removed by centrifugation. An equilibrium gradient was set up by adding 0.75 gm/ml CsCl and centrifuging for 16 hrs at 35,000 rpm in a Beckman SW50 rotor. Phage bands were detected by light scattering and DNA prepared by formamide extraction and ethanol precipitation. Phage DNA was digested with *Hin*dIII and the pooled inserts separated from vector lambda arms by agarose gel electrophoresis. Inserts isolated by electroelution were recloned into pUC13. After transformation into *E. coli* JM83, recombinants

were isolated by selecting white colonies from plates containing ampicillin and x-gal (5-bromo, 4-chloro, 3-indolyl-B-galactoside).

Preparation and Hybridization of Dot Blots

Aliquots of plasmid DNA were prepared from these isolates by extraction from 1 ml overnight cultures and spotted onto nitrocellulose filters. These filters were hybridized with total human DNA labeled with ^{32}P by nick-translation to a specific activity of at least 10^7 cpm per μg DNA. Some dots were hybridized with similarly labeled Syrian hamster DNA.

Preparation and Hybridization of Southern Transfers

Genomic human DNA was isolated from cultured lymphoblasts, obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey. These cell lines, which are identified by a GM prefix, were derived from a collection established by Dr. R. White and colleagues from Mormon pedigrees for linkage analysis. Restriction enzymes were purchased from BRL and New England Biolabs. Restriction digests were prepared by overnight incubation, using appropriate buffer and temperature conditions as suggested by the manufacturer. Generally, 4–5 μg aliquots of DNA were digested with 0.5–1 U of enzyme per μg. Agarose gels were prepared at 0.6%–0.8% concentration in 0.89 M Tris-borate, 0.89 M boric acid and electrophoresis carried out overnight at 25–35 V. The gels were treated with 0.25 M HCl for 15 min to partially depurinate DNA. They were then soaked 30–60 min in 1.5 M NaCl, 0.5 M NaOH to denature DNA and neutralized 30 min in 1 M ammonium acetate, 0.02 M NaOH. A 0.45 μm pore size Nytran (Schleicher and Schuell) membrane was wetted with the neutralization buffer and Southern transfer carried out for at least 3 hrs using as transfer buffer 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate). After transfer, the membranes were rinsed 5 min in 6× SSC and dried. They were then baked for 2 hrs at 80°C. The transfers were prehybridized at 40°C for at least 2 hrs in 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA (pH 7.4), 5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% PVP-40), 100 μg/ml sheared, denatured, salmon sperm DNA, and 0.5% SDS. This buffer was replaced with an identical solution containing cloned insert DNA labeled to a specific activity of 3–5 × 10^8 cpm/μg by nick-translation. For each genomic blot of about 15 × 10 cm, an 8 ml vol of hybridization solution containing 1–2 × 10^7 cpm was used. After overnight hybridization at 40°C, blots were washed in 1× SSC, 0.1% SDS at room temperature for 30 min, then for 30 min in 0.1× SSC, 0.1% SDS at 62°C. After blotting dry, the transfers were enclosed in plastic wrap and autoradiography was carried out for 1–4 days at −70°C using either Kodak XAR or XRP-1 X-ray film with Dupont Cronex Quanta III intensifying screens.

RESULTS

Isolation and Characterization of DNA Probes from the Chromosome 8 Library

Subcloning into plasmids. The LL08NS02 phage library was amplified on both DP50SupF and LE392, and 1–2 × 10^{12} phage were collected from plate lysates. These phage preparations were subjected to equilibrium CsCl gradient centrifugation. Both preparations gave two phage bands. An upper sharp band was estimated to account for 5%–10% of the total while the majority of phage lay in a broad lower band. Each preparation yielded about 100 μg of DNA (sum

of both bands). Agarose gel electrophoresis of *Hin*dIII digests showed that the lower band consisted of phage with *Hin*dIII inserts while DNA isolated from the upper band yielded phage arms only.

These inserts were electroeluted and recloned into the *Hin*dIII site of pUC13. After transformation, a total of 76 white colonies were picked from indicator plates and used for plasmid preparation.

Screening for human repetitive sequences. These plasmids were screened for repetitive DNA by hybridizing with ^{32}P-labeled human DNA. This probe hybridized to 29 isolates using dot blots and an additional nine isolates using Southern transfers. In addition, eight isolates lacked identifiable inserts, four contained very small inserts, two were lost, and three were identical to other isolates (this identity being confirmed by hybridization). These isolates were not further characterized.

Characterization of isolates that lack human repetitive DNA. The remaining 21 isolates were then screened with ^{32}P-labeled Syrian hamster DNA. This probe hybridized to dot blots of two isolates.

Southern transfers of human and hamster genomic *Hin*dIII-digested DNA were then probed with the 19 remaining isolates. Three probes hybridized to hamster DNA only, one to a single band while the others produced smears characteristic of a repetitive sequence. Eight probes hybridized to human DNA only, no hybridization was observed with seven, and one hybridized to both hamster and human DNA. The cross-hybridizing probe detected two fragments in hamster DNA, while the single human fragment detected was identical in size to the insert indicating human origin. Two of the eight probes hybridizing to human DNA alone detected multiple *Hin*dIII fragments, while the remaining six detected single fragments equal in size to the *Hin*dIII plasmid insert.

Thus, of the 52 isolates that gave definitive results, 38 (73%) contained repetitive human, five (10%) contained hamster, and nine (17%) contained low-copy human sequences.

Screening for RFLP. The seven unique sequence human probes that detected single *Hin*dIII fragments were screened for RFLP. Digests were prepared from five unrelated individuals for each probe-enzyme combination screened. Thirteen different restriction enzymes were used, and 53 of the possible 78 probe-enzyme combinations were screened. A total of 89 restriction fragments were observed that included 741 base pairs (bp) in their restriction endonuclease recognition sites. We detected six RFLPs. Four probes detected polymorphism (table 1), with two of them detecting two different RFLPs.

Segregation of RFLPs in Families

The minor alleles detected by D8MGV2 and D8MGV5 are rare. Only one heterozygote was detected with each of these probes among unrelated individuals (table 2). Consequently, these estimates of minor allele frequency and PIC value are unreliable. A family segregating for D8MGV2 is shown in figure 1*A*.

Segregation of the *Taq*I RFLP detected by D8MGV3 is shown in figure 1*B*. The *Hin*dIII alleles appear to be in complete linkage disequilibrium with the

TABLE 1

PROBES DETECTING RFLP

PLASMID	*Hin*dIII INSERT		RESTRICTION ENDONUCLEASE	SIZE OF GENOMIC FRAGMENTS (kb)		
	Fragment	Size		A1	A2	Constant bands
pBS8.6	D8MGV2	3.4	*Hin*dIII	3.4	3.1	...
pBS8.60	D8MGV3	3.5	*Hin*dIII	3.5	6.8	...
			*Taq*I	3.5	3.2	1.7, 7.0
pBS8.9	D8MGV4	5.2	*Rsa*I	1.7	2.1	0.5, 0.68, 0.75, 2.5
			*Hin*fI	1.3	1.37	0.83, 0.95
pBS8.61	D8MGV5	6.5	*Eco*RV	3.8	4.7	20

NOTE: A1 and A2 refer to the major and minor alleles, respectively.

*Taq*I alleles in repulsion phase. The RFLPs detected by D8MGV4 also appear to show complete linkage disequilibrium in coupling phase. A family segregating for D8MGV4 is shown in figure 1*C*.

In addition, the log relative Mendelian likelihood [9], which determines the likelihood of Mendelian segregation compared to a random distribution of phenotypes, was calculated (table 2). Each pair of alleles also appears to be in Hardy-Weinberg equilibrium since no significant chi-square values were obtained.

DISCUSSION

Chromosome 8 is one of the less well-marked chromosomes in the human genome. A total of eight genes and no arbitrary DNA segments were assigned at Human Gene Mapping 8 (1985); four of these genes detect RFLP [8]. We have used a flow-sorted library as a source of chromosome 8 specific probes for developing additional markers for this chromosome.

The insertion vector Charon 21A was used for this library. Since we planned to maintain useful recombinants as plasmids, we chose to randomly subclone the phage inserts into plasmids prior to characterization. Of the 52 isolates that gave definitive results, five were of hamster origin and 47 were of human origin.

TABLE 2

CHARACTERIZATION OF DNA MARKERS

PROBE-ENZYME COMBINATION	MINOR ALLELE		PIC VALUE	LOG RELATIVE* MENDELIAN LIKELIHOOD
	Fraction	Frequency		
D8MGV2:*Hin*dIII	1/66	.015	.03	3.84 (13)
D8MGV3:*Hin*dIII	42/104	.40	.36	6.43 (84)†
D8MGV3:*Taq*I	30/76	...	...	2.83 (33)†
D8MGV4:*Rsa*I	9/86	.10	.16	2.08 (15)
D8MGV4:*Hin*fI	2/30	...	...	...
D8MGV5:*Eco*RV	1/28	.036	.07	...

* Figures in parentheses indicate the no. offspring typed.

† These data are from different families and are independent of each other.

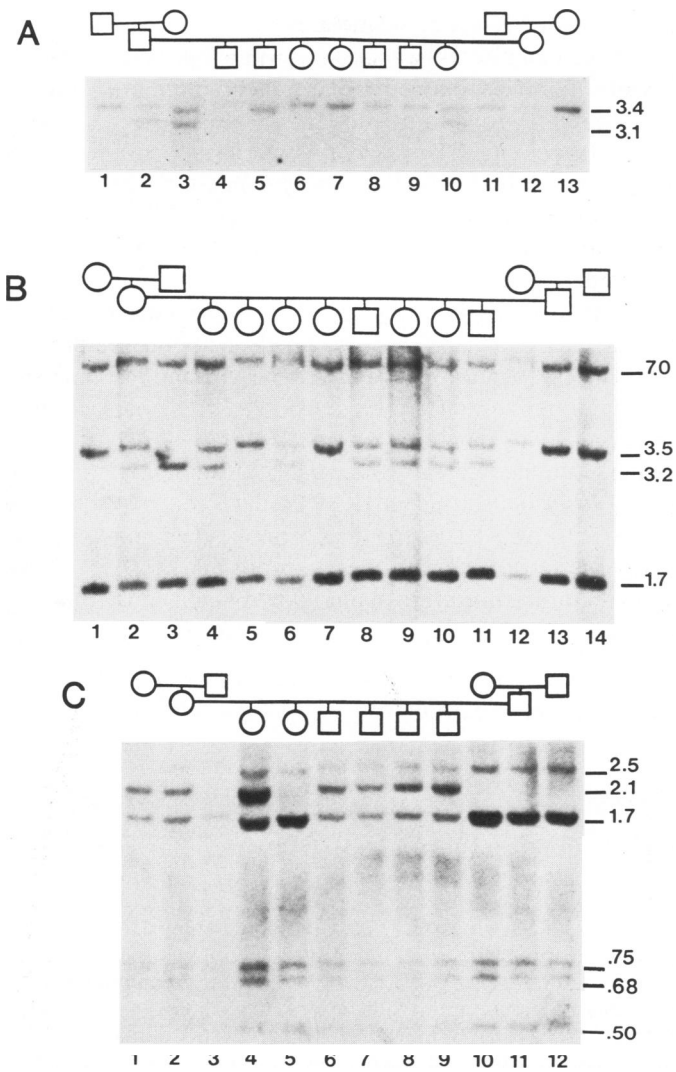

FIG. 1.—*A*, Segregation of D8MGV2 *Hin*dIII fragments in family 981 (Utah pedigree K-13291). *Lane 1*, I-1:GM7435; *2*, II-1:GM6995; *3*, I-2:GM7037; *4*, III-1:GM7018; *5*, III-2:GM7036; *6*, III-3:GM6981; *7*, III-4:GM6980; *8*, III-5:GM7047; *9*, III-6:GM7433; *10*, III-7:GM7058; *11*, I-3:GM6986; *12*, II-2:GM6997; *13*, I-4:GM7045. *Lanes 2–4* and *10* are heterozygotes, while the others are A1/A1 homozygotes. *B*, Segregation of D8MGV3 *Taq*I fragments in family 985 (Utah pedigree K-1341). *Lane 1*, I-1:GM6985; *2*, II-1:GM6991; *3*, I-2:GM6993; *4*, III-1:GM7343; *5*, III-2:GM7044; *6*, III-3:GM7012; *7*, III-4:GM7344; *8*, III-5:GM7021; *9*, III-6:GM7006; *10*, III-7:GM7010; *11*, III-8:GM7020; *12*, I-3:GM7055; *13*, II-2:GM7048; *14*, I-4:GM7034. *Lanes 2*, *4*, *6*, and *8–11* are heterozygotes, *lane 3* is an A2/A2 homozygote, while the others are A1/A1 homozygotes. *C*, Segregation of D8MGV4 *Rsa*I fragments in family 984 (Utah pedigree K-1340). *Lane 1*, I-1:GM7056; *2*, II-1:GM7019; *3*, I-2:GM7022; *4*, III-1:GM7062; *5*, III-2:GM7053; *6*, III-3:GM7008; *7*, III-4:GM7040; *8*, III-5:GM7342; *9*, III-6:GM7027; *10*, I-3:GM7000; *11*, II-2:GM7029; *12*, I-4:GM6994. *Lanes 1*, *2*, *4*, and *6–9* are heterozygotes, while the others are A1/A1 homozygotes. Fragment sizes are indicated *down the right-hand side of the figure*.

Thus, the admixture of hamster sequences in this library seems to be about 10%. Most of these isolates, four hamster and 38 human, contained repetitive sequences, while unique sequence isolates accounted for about 20% of the total. Two of the nine low-copy human isolates detected multiple human genomic *Hin*dIII fragments. These isolates were not screened for RFLP since the related sequences detected by these probes might be distributed throughout the genome. Although one of the isolates cross-hybridized with hamster DNA, the insert was identical in size with the human genomic *Hin*dIII fragment, indicating its human origin.

Screening human restriction digests with these unique sequence human probes yielded six RFLPs in 741 bp screened or about one per 125 bp. However, since two pairs of markers appear to be in complete linkage disequilibrium and two are rare variants, the yield of RFLPs that are useful for map construction is only two, or about one in 370 bp.

These markers, defined by arbitrary DNA fragments, the chromosome 8 marker that we described [10], and RFLPs detected by cloned genes [8] together provide six useful RFLPs for the chromosome 8 map. Construction of a 20 cM map is predicted to require over 30 more markers [7]. These additional markers can be readily acquired by using the flow-sorted chromosome 8 library as described here.

NOTE ADDED IN PROOF: The probes reported here have all been assigned to chromosome 8 with the exception of pBS8.60 (D8MGV3), which is located on chromosome 4.

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