Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8448–8451, September 1992
Genetics

Linkage mapping by simultaneous screening of multiple polymorphic loci using *Alu* oligonucleotide-directed PCR

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Communicated by Louis M. Kunkel, June 10, 1992 (received for review March 16, 1992)

ABSTRACT **We present the use of our recently described multiple-loci polymorphic DNA markers ("alumorphs") for linkage mapping of the human genome. By using the polymerase chain reaction (PCR) with an *Alu*-specific primer we could reveal, in a single experiment, up to 20 genomic polymorphisms seen as the presence or absence of amplified DNA fragments originating from genomic segments flanked by *Alu* repeats. Using this approach we examined genomic DNA samples from two families with a history of pseudovitamin D-deficiency rickets (PDDR), an autosomal recessive disorder. An indication of linkage with the PDDR phenotype was found for one of the polymorphic bands, denoted 30A. A significant linkage [logarithm-of-odds (lod) score > 3.0] was obtained between this polymorphism and a number of chromosome 12q markers tightly linked to PDDR. The 30A band specifically hybridized to DNA digests from hybrid cell lines carrying a human chromosome 12, thus independently assigning the 30A marker to this chromosome. Since *Alu* elements are ubiquitous in human DNA, the use of alternative *Alu*-specific primers, which reveal different sets of *Alu*-flanked loci, should provide an efficient and rapid approach to human genetic mapping.**

Finding the map position of a genetic trait by linkage analysis requires random sampling of the genome with polymorphic markers. Compared with genomic screening using single-copy DNA markers (1), simultaneous analysis of DNA sequence variation at multiple loci (2, 3) is efficient and greatly increases the scope of mapping studies. Recently, a new approach based on PCR amplification with *Alu*-specific primers was used to reveal multiple-loci DNA markers, named alumorphs (2).

With more than half a million *Alu* elements (4) distributed randomly in the human genome, every region of several kilobase pairs is expected to contain a single-copy DNA segment flanked by these repeats (5). The intervening DNA fragment can be amplified by PCR with a single *Alu*-specific oligonucleotide primer when two flanking *Alu* elements are in the opposite orientation. Simultaneous amplification from many genomic loci results in multiple constant or variant bands (2). The former are present in all individuals examined, whereas the latter, due to allelic polymorphism, serve as potential DNA markers in linkage analysis. An allelic polymorphism is detected as the presence or absence of the amplified DNA product of a given length and may result from either (*i*) a recent insertion/deletion of one of the flanking repeats (6, 7) or (*ii*) length variability in the intervening sequence (8, 9). Segregation analysis of amplified DNA fragments representing multiple, randomly distributed polymorphic loci should lead to the rapid identification of a DNA marker linked to a genetic trait under investigation.

Using this approach, we analyzed genomic DNA samples from two families with a history of pseudovitamin D-deficiency rickets (PDDR; McKusick no. 26470; ref. 10). PDDR is an autosomal recessive disorder characterized by hypocalcemia, secondary hyperparathyroidism, and early onset of rickets resembling the clinical picture of common vitamin D deficiency (11); it is caused by impaired activity of the renal 25-hydroxyvitamin D 1α-hydroxylase, the enzyme producing an active form of vitamin D (12). The PDDR locus has been mapped to chromosome 12 by linkage analysis (13), prompting us to use this disease as a model system for alumorph mapping. Here we present a linkage between an alumorph marker and a number of loci from the PDDR region on chromosome 12. Physical localization of the disease-linked alumorph to chromosome 12 by hybridization with somatic cell hybrids illustrates one of the ways to integrate alumorphs within the existing genomic map.

MATERIALS AND METHODS

Genomic Samples. Clinical diagnosis of PDDR, DNA isolation, and analysis of restriction fragment length polymorphism by use of chromosome 12q markers (see Table 1) have been described (13).

***Alu*-Specific Primers.** In the analysis presented in this paper we used the dodecanucleotide R12A/267 (5′-AGCGAGACTCCG-3′). This primer name starts with a letter R, which denotes a primer identical to the *Alu* coding strand, thus directing amplification downstream (to the right) of a genomic *Alu* template; "L" primers, complementary to the coding strand, would direct DNA synthesis upstream of an *Alu* element. The two following digits define the length of the oligonucleotide (12 for dodecanucleotide), whereas the letter A indicates that the sequence is identical to the human *Alu* general consensus (14, 15). Finally, the number 267 indicates that our dodecanucleotide spans positions 267–278 of the *Alu* consensus. In addition to R12A/267 we also used the following primers: L12A/8 (5′-TGAGCCACCGCG-3′), L12A/57 (5′-TCAAGTGATCCG-3′), and R12B/267, which differs from R12A/267 by G → A substitution at the 3′-terminal nucleotide. Oligonucleotides were synthesized using a Gene Assembler (Pharmacia) and 5′-end-labeled using [γ-^{32}P]ATP (6000 Ci/mmol, NEN; 1 Ci = 37 GBq) and T4 polynucleotide kinase (Pharmacia).

DNA Amplification by PCR. The PCR was carried out in 10 mM Tris·HCl, pH 9.0/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin/0.01% Triton X-100/2% formamide/200 μM dNTPs/1 μM primer (a mixture of 1:4 or less of the labeled and the nonradioactive primer) with 25 ng of genomic DNA

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Abbreviations: PDDR, pseudovitamin D-deficiency rickets; PIC, polymorphism information content; lod, logarithm of odds.

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per reaction (20 μl). After an initial denaturation of 7 min at 94°C and the addition of 1 unit of *Taq* DNA polymerase at 4°C, 27 cycles of amplification (each consisting of 30 sec at 94°C, 45 sec at 50°C, and 120 sec at 72°C) were followed by a final 7-min extension at 72°C. The PCR was performed in a DNA thermal cycler (Perkin/Elmer–Cetus).

Polyacrylamide Gel Electrophoresis. Amplified DNA fragments were mixed with 0.2 volume of loading buffer (40% sucrose/0.05% bromophenol blue/0.05% xylene cyanol). About one-fifth of the PCR products was loaded onto a 6% polyacrylamide gel [acrylamide: *N,N'*-methylenebisacrylamide weight ratio of 29:1 in 1× TBE buffer (90 mM Tris borate, pH 8.3/2 mM EDTA)] cast in a Sequi-Gen 0.04 × 38 × 50-cm sequencing cell (Bio-Rad). Electrophoresis was carried out in 1× TBE buffer at room temperature at 14 V/cm for 17 hr. Gels were dried and exposed at −80°C with an intensifying screen for several hours (weak exposure) to 2 days (strong exposure).

Linkage Analysis. Linkage analysis was performed using the LIPED program of Ott (16), PC version of October 1986 (kindly provided by James A. Trofatter). Alumorphs (2) are dominant DNA markers similar to "randomly amplified DNA polymorphisms" (RAPDs) (17). In the linkage analysis we consider the presence or absence of an amplified band of a given length. The absence of an amplified band indicates a homozygote for the null (i.e., recessive) allele; its presence indicates either a homozygote for the plus (dominant) allele, or a +/0 heterozygote. For biallelic dominant markers the polymorphism information content (PIC), calculated assuming mapping of a rare dominant trait (1), corresponds to PIC $= p_0^2(1 - p_0^2)$, where p_0 denotes the null allele frequency. Thus, while in codominant markers such as restriction fragment length polymorphisms (1) the maximum PIC value occurs when allelic frequencies are equal (e.g., the maximum PIC of 0.375 in the case of two alleles), in biallelic, dominant alumorphs the maximum PIC of 0.25 is reached at $p_0 = (0.5)^{1/2}$ (i.e., about 0.7). The power of pedigrees used in this investigation for PDDR mapping was assessed using the program SLINK (18, 19) from the LINKAGE package (kindly provided by Jurg Ott). The maximum logarithm-of-odds (lod) score of 4.9 was obtained for both dominant and codominant biallelic markers. The corresponding expected lod values were 0.85 and 1.7, respectively, at a recombination fraction (Θ) = 0.0, assuming equal allele frequencies.

Chromosomal Localization of the PDDR-Linked Marker. The radioactive band representing the 30A marker was excised from the dried gel, eluted by incubation in 1 mM EDTA/0.5 M ammonium acetate/0.1% SDS, pH 8.0, for 18 hr at 37°C, and ethanol-precipitated. The eluted DNA fragment served as a template for a second PCR amplification (conditions as described above, except that no genomic DNA was present and the nonlabeled primer R12A/267 was used). The product was digested with a variety of restriction enzymes (BRL), Southern blotted, and hybridized (*i*) with itself and (*ii*) with total human DNA, to identify subfragments containing unique segments. A 1.8-kilobase-pair (kb) *Msp* I subfragment of the 30A band which did not hybridize to total human DNA was chosen as a single-copy probe for subsequent hybridizations. The panel of genomic DNAs from 18 human–hamster somatic hybrid cell lines was purchased from Bios (New Haven, CT; BIOSMAP somatic cell hybrid Southern blot DNAs, 1991, panel no. 1, lot no. NL001). DNA samples digested with restriction enzymes were resolved in 1% agarose gels (5 μg of DNA per lane). Southern transfer, probe labeling, and hybridization were performed as described (13).

RESULTS AND DISCUSSION

Fig. 1*a* illustrates an analysis of a three-generation family affected with PDDR. Genomic DNA samples from the family members were amplified by PCR using a single ^{32}P-labeled *Alu*-specific dodecanucleotide primer, R12A/267, which directs DNA amplification of DNA segments flanked by *Alu* repeats in a tail-to-tail orientation (2, 14, 15). More than 40 bands of unequal intensity were resolved by electrophoresis

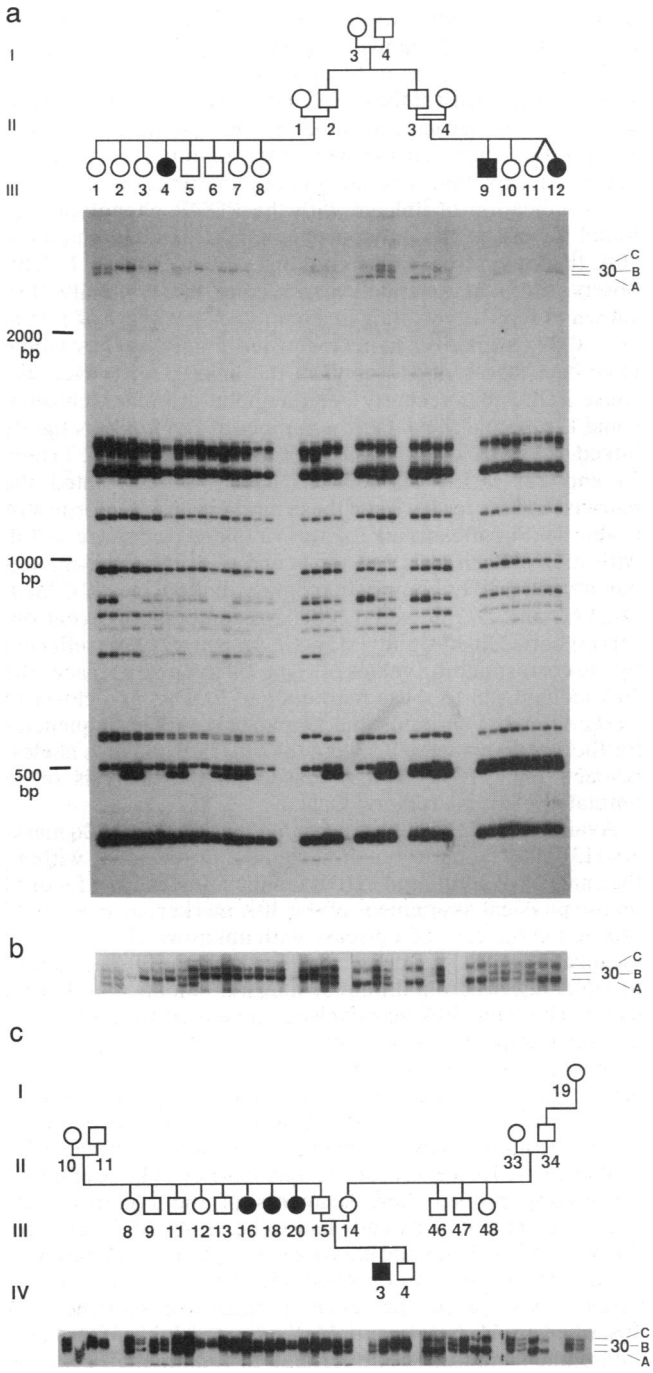

FIG. 1. Analysis of genomic polymorphisms involving *Alu* sequences (alumorphs) in two PDDR families (affected individuals are indicated by filled symbols). (*a*) The ^{32}P-labeled products of PCR amplification using an *Alu*-specific primer were analyzed by electrophoresis in a nondenaturing 6% polyacrylamide gel. Each individual from the pedigree shown on the top of the autoradiogram was analyzed in duplicate by two independent PCR amplifications, shown in two adjacent lanes on the gel. Molecular size marker [100-base-pair (bp) ladder; Pharmacia] is indicated at left. The lowest of the 30A–C cluster of three polymorphic bands indicated at the right corresponds to the 30A marker of interest. (*b*) Overexposed section of a gel containing the cluster of polymorphic bands 30A–C. (*c*) Segregation of polymorphic fragments 30A, 30B, and 30C in a second PDDR family (see ref. 13).

in a nondenaturing polyacrylamide gel and visualized by autoradiography, as shown in Fig. 1*a* by an intermediate film exposure. Because of the complexity of this PCR, each DNA sample was amplified in duplicate to avoid artifacts and to assure reproducibility. The duplicates were run side by side so that each individual was represented by two adjacent lanes on the gel. About 20 bands were polymorphic and segregated as Mendelian markers in this and other pedigrees (data not shown). A majority of these polymorphisms were in repulsion with one another as indicated by the pairwise lod score analysis (not shown) using the LIPED program (16), consistent with their expected random genomic distribution (2, 5).

An indication of linkage with the PDDR phenotype was found for one of the polymorphic bands, denoted alumorph 30A, the lowest from a cluster of three (30A–C in Fig. 1). This observation was extended to a second PDDR family (13), shown in Fig. 1*c*, resulting in a combined lod score of 1.55 at $\Theta = 0.0$. Ordinarily, more extensive family studies would have been necessary to confirm the linkage. However, because PDDR has recently been mapped to human chromosome 12q14, and the data on a number of 12q markers tightly linked to PDDR were available for the families in Fig. 1 (refs. 13 and 22; M.L., unpublished data), we calculated the pairwise lod scores between these markers and alumorph 30A (Table 1). Significant linkage was obtained (lod score > 3.0) with the collagen *COL2A1* locus and with the three-marker haplotype, 3MH, comprising tightly linked loci: *D12S14*, *D12S17*, and *D12S6* (ref. 12). The recombination event observed between 30A and 3MH, *ELA1*, and *D12S4* (reflected by the corresponding values of $\Theta_{max} > 0$ in Table 1) places the 30A locus distal to these markers and PDDR, and closer to *COL2A1* on the chromosome 12 map (13). Allele frequencies for the 30A locus were 0.53 and 0.47 for null and plus alleles, respectively (PIC = 0.203), based on the analysis of 35 unrelated Caucasian individuals.

Availability of the data concerning PDDR-linked 12q markers (13) allowed us to obtain a significant lod score without the analysis of additional PDDR families. Instead, we focused on the physical assignment of the 30A marker, as one would proceed in the case of a disease with unknown chromosomal localization. This was done by hybridizing the 30A alumorph to DNA digests from a human–hamster somatic cell hybrid panel. The band 30A was excised and eluted from the dried autoradiographed polyacrylamide gel and the corresponding fragment was reamplified by PCR using the same R12A/267 primer that was used to reveal the alumorph pattern shown in Fig. 1*a*. This was the simplest and most direct way to obtain the marker DNA fragment in an amount sufficient for further tests. However, for the 30A marker to be useful as a single-copy probe, short segments of the repetitive *Alu* sequence present at its ends had to be removed. Therefore, the reamplified 30A band was digested with a combination of restriction enzymes and probed with ^{32}P-labeled human genomic DNA for the presence of repetitive sequences as described in *Materials and Methods*. A 1.8-kb *Msp* I 30A subfragment that did not hybridize to total human genomic DNA was chosen as the single-copy probe for subsequent Southern hybridizations. The 1.8-kb *Msp* I 30A probe revealed a single band of the same size in human DNA digested with *Msp* I and single bands of different lengths when other enzymes were used (Fig. 2*a*). Consequently, this probe was used in hybridizations with human–hamster chromosome panel DNAs digested with *Msp* I (Fig. 2*b*). Specific hybridization to DNA digests from hybrid cell lines carrying human chromosome 12 provided an independent assignment of the alumorph 30A and its linked loci to human chromosome 12.

The use of alumorph screening combined with linkage analysis and chromosome panel hybridization appears to be very effective in mapping autosomal recessive disorders and can be generalized to mapping any Mendelian trait. Despite the intrinsic lower informativity of alumorphs compared with codominant markers, one gains the information due to a possibility of the simultaneous analysis of a large number of loci (see also below). Using three different *Alu*-specific primers, we were able to score >50 distinct alumorphic bands in the family shown in Fig. 1*a*. Most of the polymorphisms seem to result from the presence or absence of one of the flanking *Alu* elements and hence consist of two alleles. Some polymorphic bands (such as those at about 500 bp or 540 bp in Fig. 1*a*) appear to segregate as allelic length variants of a single genomic locus differing by only a few nucleotides. This increases the informativity of alumorphs by supplementing the system with codominant oligo- or multiallelic polymorphisms.

In future mapping projects, additional *Alu*-specific primers targeting different sets of genomic loci will have to be used in order to find linkage to genetic traits of interest. A great variety of such primers can be designed based on the analysis of sequence diversity among *Alu* elements, their evolutionary past, and their mechanism of dispersion (refs. 14, 15, and 20 and references therein). Experimental conditions can be manipulated as well. For example, in comparison to our initial use of a 23-mer oligonucleotide primer (R23A/101) (2), the signal-to-noise ratio can be significantly improved by using primers as short as dodecanucleotides, by adding 1–3% formamide to the reaction mixture, and by using primers labeled at the 5′ terminus prior to PCR rather than incorporating radioactive nucleotide during DNA amplification. We have also observed that the primer R12B/267, which ends with a CpA dinucleotide (see *Materials and Methods*), generates a different and less polymorphic pattern of amplified DNA fragments (not shown) than the consensus-derived R12A/267 primer, which terminates in CpG. Although additional experiments are needed to confirm whether all primers containing intact CpG dinucleotides [thus presumably targeting younger *Alu* elements (14, 20)] will reveal more polymorphisms, it appears that a single substitution at the 3′ end of a primer is sufficient to change the amplification specificity. The "all or none" character of alumorphic markers makes them especially suitable for automation using fluorescence detection and commercial DNA sequencers. This approach can also be generalized to other interspersed repeats. In fact, even random oligodeoxynucleotides used as

Table 1. Pairwise lod scores between alumorphic fragment 30A and chromosome 12q markers

Chromosome 12q marker	Z_{max}	θ_{max}	Recombination fraction θ						
			0.00	0.01	0.05	0.10	0.20	0.30	0.40
COL2A1/Pvu II	3.22	0.00	3.22	3.21	2.89	2.55	1.87	1.17	0.46
*COL2A1/Hin*fI	2.38	0.00	2.39	2.38	2.20	1.99	1.54	1.01	0.42
ELA1/Taq I	1.04	0.11	$-\infty$	−0.62	0.92	1.03	0.93	0.65	0.26
D12S15/Taq I	1.57	0.00	1.57	1.57	1.42	1.27	0.94	0.58	0.21
D12S4/Taq I	2.85	0.07	$-\infty$	1.44	2.82	2.76	2.26	1.52	0.61
3MH*	3.37	0.05	$-\infty$	2.09	3.37	3.23	2.46	1.67	0.66
PDDR	1.55	0.00	1.55	1.54	1.36	1.17	0.79	0.31	0.13

*Three-marker haplotype based on *Msp* I polymorphisms in three tightly linked loci: *D12S14*, *D12S17*, and *D12S6*.

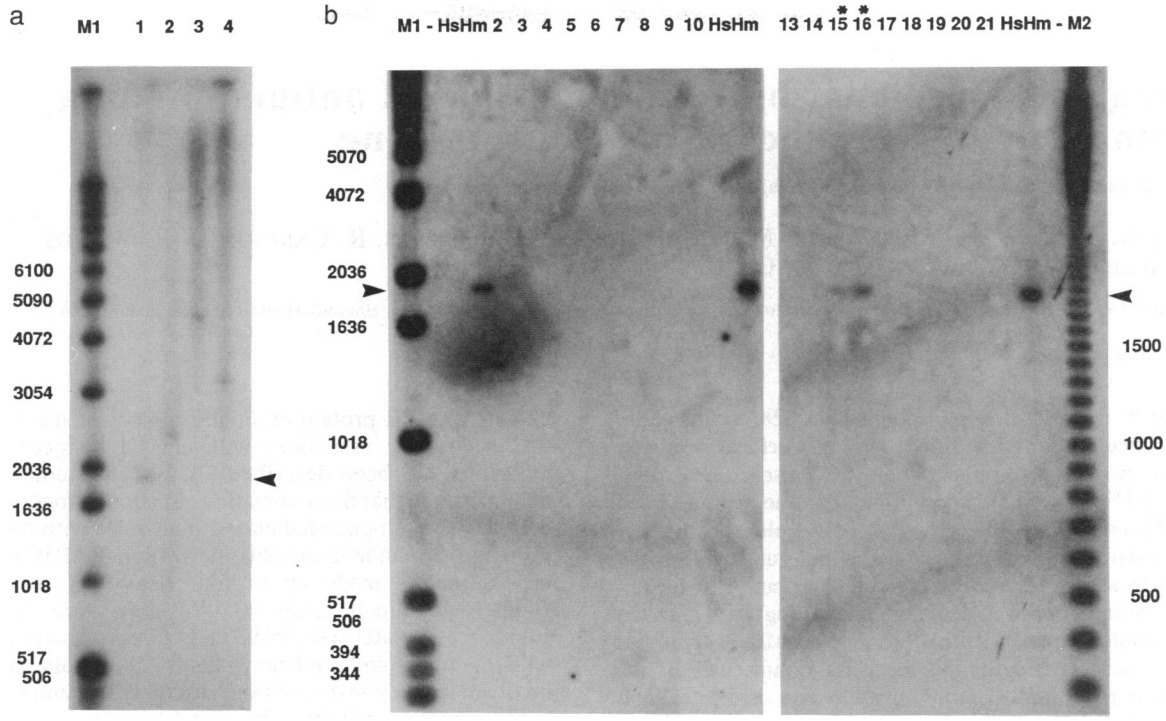

FIG. 2. Chromosomal assignment of the 30A marker by Southern hybridization to DNA from a human–hamster somatic cell hybrid panel. (*a*) Hybridization of a nonrepetitive segment of the 30A fragment to human genomic DNA digested with *Msp* I (lane 1), *Taq* I (lane 2), *Eco*RI (lane 3), or *Hin*dIII (lane 4). (*b*) Hybridization to *Msp* I-digested DNAs from a panel of human–hamster somatic cell hybrids (BIOSMAP somatic cell hybrid Southern blot DNAs, 1991, panel no. 1). Lanes 2–10 and 13–21 correspond to the Bios numbering (hybrid lines: 867, 854, 423, 860, 803, 909, 1006, 811, 967 and 734, 968, 683, 507, 750, 1099, 324, 940, 983, respectively). DNA samples in lanes 15 and 16, indicated by asterisks, originate from the hybrid lines (683 and 507, respectively) containing a human chromosome 12. The lower human chromosome 12 content in the hybrid line 683 explains the weaker hybridization in lane 15 as compared to lane 16. The 1.8-kb *Msp* I-specific band is indicated by arrowheads at the sides of the autoradiograms. Lanes Hm and Hs, human and hamster DNA digests, respectively. Lanes M1 and M2, the 1-kb (BRL) and 100-bp (Pharmacia) molecular size markers, respectively.

PCR primers reveal polymorphisms, so-called RAPDs (see *Materials and Methods*), which were successfully applied as dominant markers in mapping plant genomes (17). The use of primers complementary to human *Alu* repeats provides a means to increase the number of loci that can be scrutinized in a single experiment.

We have shown that the possibility of simultaneous typing of dispersed genomic loci by PCR accelerates the mapping process so that it can be successfully carried out by individual investigators. In contrast to "classical" markers, the chromosomal location of most alumorphs is unknown at present. We have shown that this is not an obstacle, since it is relatively easy to localize the polymorphic band linked to the trait of interest. The use of alumorphs in ongoing disease-mapping projects will assist in the rapid assembly of their linkage map and in the assignment of each alumorph to a chromosomal location. Once an alumorph-based genomic map is established, finding a chromosomal position through linkage to an alumorph will become straightforward. At that stage, this system of multiple-loci screening will also become very useful for the mapping of quantitative, multigenic traits where simultaneous screening of the entire genome with markers is of particular importance (21). Finally, tagging alumorphs with sequence-tagged sites would greatly facilitate their identification and integration within existing chromosomal and genetic maps of the human genome.

We are very grateful to Guy Cardinal for calculations using the SLINK program and to Grant Mitchell, Andrea Richter, and Louise Simard for their comments on the manuscript. This investigation was supported by the Fonds de Recherche en Santé du Quebec.

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