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A Major Locus for Autosomal Recessive Retinitis Pigmentosa on 6q, Determined by Homozygosity Mapping of Chromosomal Regions That Contain Gamma-Aminobutyric Acid–Receptor Clusters

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Summary

Retinitis pigmentosa (RP) is the most common inherited retinal dystrophy, with extensive allelic and nonallelic genetic heterogeneity. Autosomal recessive RP (arRP) is the most common form of RP worldwide, with at least nine loci known and accountable for ~10%–15% of all cases. Gamma-aminobutyric acid (GABA) is the major inhibitory transmitter in the CNS. Different GABA receptors are expressed in all retinal layers, and inhibition mediated by GABA receptors in the human retina could be related to RP. We have selected chromosomal regions containing genes that encode the different subunits of the GABA receptors, for homozygosity mapping in inbred families affected by arRP. We identify a new locus for arRP, on chromosome 6, between markers *D6S257* and *D6S1644*. Our data suggest that 10%–20% of Spanish families affected by typical arRP could have linkage to this new locus. This region contains subunits *GABRR1* and *GABRR2* of the GABA-C receptor, which is the effector of lateral inhibition at the retina.

Introduction

Retinitis pigmentosa (MIM 268000) is the most common retinal dystrophy. The classic findings are nyctalopia, visual-field constriction, "bone spicula" pigmentation, attenuated retinal vessels, waxy pallor of the optic disk, and no results detectable by electroretinogram (Jiménez-Sierra et al. 1989).

RP presents important locus and allelic heterogeneity, with different modes of inheritance: autosomal dominant and recessive, X-linked, and digenic. Whether classic linkage analysis or the molecular study of candidate genes is used, the number of loci related to this group of disease genes continues to grow (Dryja and Li 1995).

Autosomal recessive RP (arRP) is the most common form of RP worldwide. In Spain, 47% of cases of the disease present autosomal recessive inheritance (C. Ayuso, personal communication). To date, nine loci related to arRP have been identified (Rosenfeld et al. 1992; McLaughlin et al. 1993; Knowles et al. 1994; van Soest et al. 1994; Dryja et al. 1995; Huang et al. 1995; Gu et al. 1997; Martínez-Mir et al. 1997; Maw et al. 1997 [MIM numbers for the loci identified by these studies are 180380, 180072, 600132, 600105, 123825, 180071, 180069, 601718, and 180090, respectively]). These loci account for only 10%–15% of all cases of arRP (Dryja et al. 1995).

The genes that form the biochemical machinery involved in photoreception have been, until recently, the mainstay of studies concerning the etiology of arRP (Dryja 1990). Toxicity mediated by excitatory neurotransmitters such as glutamate or aspartate is, however, a well-established concept in other neurological and neurodegenerative disorders (Lipton and Rosenberg 1994). We must remember that the rod is the first neuron of the visual pathway, establishing a glutamanergic synapsis with a rod bipolar cell, at the spherule, in the outer plexiform layer (Rapaport 1989). This synapsis is simultaneously modulated by different inhibitory efferents of horizontal cell axons and interplexiform cells (Kolb 1974). These cells contain gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the CNS (Sivilotti and Nistri 1991). GABA activates specific receptors, which are classified into three subtypes—GABAa, GABAb, and GABAc—according to the mechanism of action and pharmacological properties (Bormann and Feigenspan 1995). It is believed that the GABAergic efferents that reach the outer plexiform layer of the retina act as feedback of the visual pathway and seem to be the effectors of lateral inhibition (Kolb 1994).

We propose for RP a pathogenic mechanism that is consistent with the Lisman and Fain's (1995) hypothesis of the equivalence of light. In our model, the lack of

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targets (GABAergic receptors), at the spherule, for GABA provokes an abnormal release of glutamate from the synaptic vesicles of the rod, precipitating a cytotoxic effect on the adjacent tissue. This cytotoxicity is analogous to the degeneration that ensues when a dying excitatory neuron releases its neurotransmitter contents into surrounding tissue (Tanaka et al. 1997).

To explore this model further, we used homozygosity mapping (Lander and Botstein 1987) to analyze consanguineous families affected by arRP. For candidate chromosomal regions, we selected loci that contain genes encoding the different subunits of the GABA receptors. The aim of this study was to determine whether inhibition mediated by GABA receptors in the retina could be related to RP. The genes that encode the different subunits of these receptors are usually grouped into clusters throughout the genome: *GABRR1* and *GABRR2,* on 6q (Cutting et al. 1992); *GABRA5*, *GABRB3,* and *GABRG3,* on 15q; *GABRA2, GABRB1, GABRA4,* and *GABRG1,* on 4p; and *GABRA1, GABRA6, GABRB2,* and *GABRG2,* on 5q (Schantz Wilcox et al. 1992).

The location of these genes in the human transcript map (Schuler et al. 1996), together with the density of the genetic map available at present (Dib et al. 1996), permits both the selection of highly polymorphic short tandem-repeat (STR) markers linked to these clusters and the homozygosity analysis of consanguineous families affected by arRP.

With this strategy, we obtained linkage to the 6q cluster in a subgroup of families affected by arRP. This region contains the rho1 and rho2 subunits of the GABAc receptor (Cutting et al. 1991, 1992). Both subunits are expressed in the retina and form part of the GABAc receptor that is expected to mediate the lateral inhibition of the light responses in the vertebrate retina (Bormann and Feigenspan 1995). The physiological roles of GABAc receptors on horizontal cells have yet to be determined, although GABAc receptors may mediate regenerative responses in horizontal cells, since many horizontal cells that are GABAergic are also sensitive to GABA (Lukasiewicz 1996). In addition, excitatory synaptic transmission between bipolar cells and ganglion cells could be modulated by GABAc-receptor activation (Lukasiewicz 1996).

Subjects and Methods

Subjects

We selected 17 unrelated families or index patients. All of the index patients were the offspring of consanguineous marriages (table 1) and had typical RP symptoms, characteristic fundus examination, and nothing detectable by scotopic electroretinogram. Autosomal recessive inheritance was confirmed by the existence of two or more affected members of one family and/or the presence of first- or second-degree consanguinity among the parents, as were a complete lack of signs and symptoms in the parents of the patients.

Once we obtained evidence of linkage to the selected candidate region, 12 nuclear and noninbred families affected by arRP (with two or more affected members and the same clinical and electroretinographic criteria) were added to the study, to allow us to perform a linkage analysis with markers of the critical region (table 1).

Selection and Analysis of Markers of the Candidate Region in Families Affected by arRP

According to the Human Transcript Map (Schuler et al. 1996), the *GABRR1* gene is located on the long arm of chromosome 6, in a 5-cM region between *D6S445* and *D6S1644*. The gene *GABRR2* has been located, on the same map, between *D6S1601* and *D6S1570*. This location overlaps partially with that assigned to gene *GABRR1*. The minimum candidate region that contains both genes is of 7.2 cM and is found between *D6S445* and *D6S1570*.

To perform the screening of this region in inbred patients, we selected five highly polymorphic markers in the candidate region. The initial analysis was performed with the following map: *D6S445* (.70)–0.6 cM–*D6S1609* (.81)–0.8 cM–*D6S1627* (.80)–3.9 cM–*D6S1613* (.91)–1.9 cM–*D6S1570* (.78) (the heterozygosity of each marker is shown in parentheses, and the distance between two contiguous markers on the map is also shown). Information concerning the markers used was extracted from the Généthon public database and also from Genome Database (GDB).

Individuals homozygous for three or more contiguous markers were reevaluated, along with the rest of the available family, in order to allow us to perform a linkage analysis and to calculate the multipoint LOD scores, using the fixed map described above and running Mapmaker/Homoz software (Kruglyak et al. 1995). The allelic frequencies of the markers used in this analysis were obtained from GDB, except for markers *D6S1613* and *D6S1627,* for which the data derived from the families studied was used. Since the allelic frequency critically affects the multipoint analysis (Gschwend et al. 1996), all the allelic frequencies <.1 were overestimated up to a frequency of .1. For this reason, the multipoint LOD-score values obtained by Mapmaker/Homoz are conservative.

Once linkage was established, 12 nuclear noninbred families affected by arRP underwent linkage analysis, with markers *D6S402, D6S1627,* and *D6S1613*. Our objective was to identify new families linked to this region (extension analysis). Those families that were either not informative or semi-informative in relation to

Table 1

Analysis of Linkage to the GABA-C Cluster at 6q in 29 Spanish Families Affected by arRP

arRP Family[a]	Consanguinity[b]	No. of Affected Sibs/ No. of Healthy Sibs	No. of Parents Available for Genotyping	Status of GABA-C Cluster Marker[c]
Inbred:				
RP5	1st	3/1	2	Homozygosity
RP23	2d	2/1	2	Heterozygosity
RP29	2d	2/0	2	Heterozygosity
RP65	1st	1/0	0	Heterozygosity
RP66	1st	2/0	1	Heterozygosity
RP77	1st	1/0	0	Heterozygosity
RP92	1st	1/0	0	Heterozygosity
RP95	2d	1/3	2	Heterozygosity
RP99	1st	2/1	1	Heterozygosity
RP108	2d	1/2	1	Heterozygosity
RP167	2d	1/0	0	Homozygosity
RP190	1st	2/3	1	Heterozygosity
RP193	1st	1/2	2	Heterozygosity
RP206	1st	1/0	2	Heterozygosity
RP216	1st	1/3	2	Heterozygosity
RP217	2d	1/2	2	Heterozygosity
RP260	2d	1/0	0	Heterozygosity
Noninbred:				
RP1	NA	2/3	1	(−∞/−∞)
RP19	NA	3/3	2	(−∞/−∞)
RP31	NA	3/2	2	(−∞/.73)
RP42	NA	2/3	2	(−∞/−∞)
RP73	NA	3/0	1	(.89/.37)
RP76	NA	3/1	2	(−∞/−∞)
RP116	NA	2/1	1	(−∞/.06)
RP156	NA	2/1	2	(−∞/−∞)
RP176	NA	2/2	1	(−∞/−∞)
RP178	NA	2/1	0	(.73/−∞)
RP203	NA	2/0	2	(−∞/−∞)
RP214	NA	2/7	1	(1.48/1.48)

[a] Underlining denotes families with positive linkage to the new locus (see fig. 1).

[b] NA = not applicable.

[c] The markers used for inbred families were *D6S445, D6S1609, D6S1627, D6S1613* and *D6S1570;* and the markers used for noninbred families were *D6S402, D6S1627* and *D6S1613*. The heterozygosity in inbred families is considered to be a criterion for exclusion of a locus (Lander and Botstein 1987). Data in parentheses for the noninbred families are two-point LOD scores at $\theta = .0$ for *D6S402* and *D6S1627*, respectively.

D6S402, D6S1627, or *D6S1613* were analyzed with flanking markers. For extension analysis, the LOD scores were calculated by use of the MLINK program from the LINKAGE package. Those families with positive LOD scores were reevaluated with 17 markers from the pericentromeric region of chromosome 6 (see below). The 29 families affected by arRP that were available underwent a homogeneity test with program HOMOG v3.35, with use of the results of the two-point LOD score obtained for markers *D6S402* and *D6S1627*.

Determination of the Critical Region and Multipoint LOD Scores

All the recessive inbred and noninbred families (RP5, RP73, RP167, and RP214) that presented linkage with the markers of the critical region were reevaluated, increasing the marker density of this interval, in order to allow us to perform a multipoint analysis of the region and to determine the minimum region that contains the new gene. For this study, we selected 12 new markers located in this chromosomal region (see fig. 1). The determination of the multipoint LOD score was performed with the Mapmaker/Homoz program (Kruglyak et al. 1995), by application of the information found in families RP5, RP73, RP167, and RP214. The allelic frequencies and thresholds of the markers used in this analysis were selected as described above. The estimated frequency of the mutated allele was .00125.

DNA Genotyping

The extraction of high-molecular-weight genomic DNA from peripheral blood of the patients and related

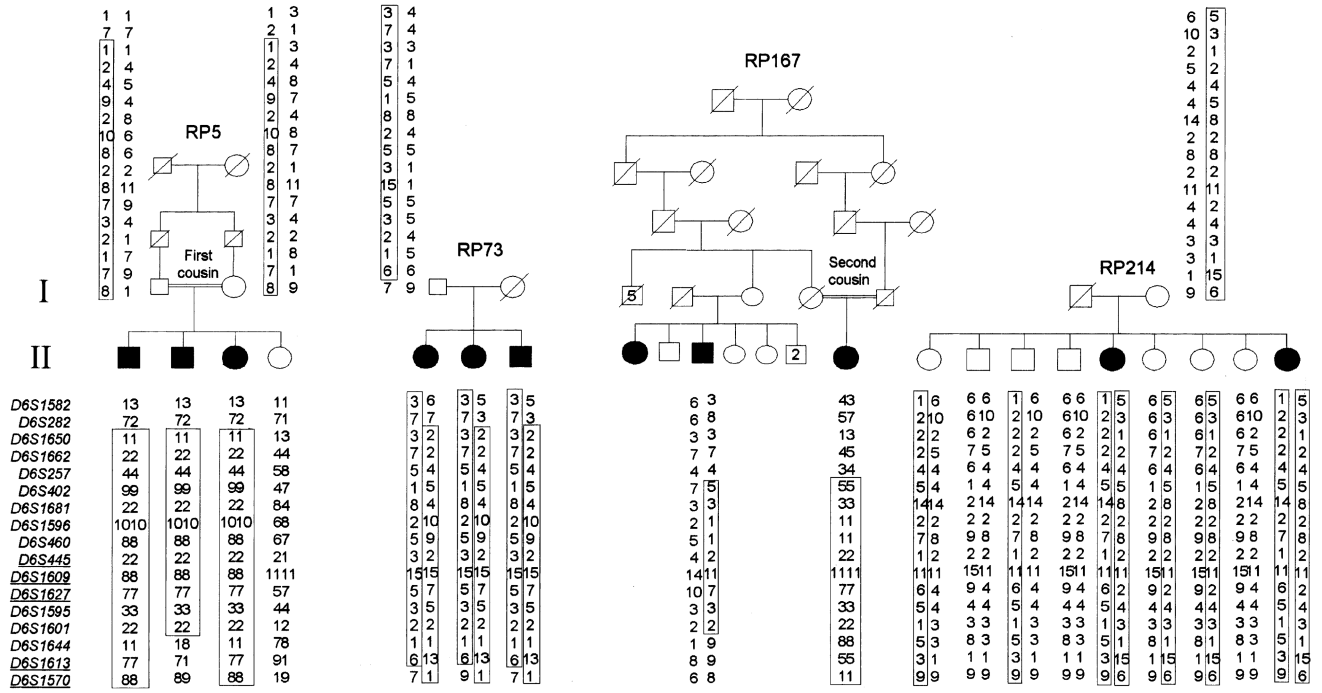

Figure 1 Haplotypes of families that present linkage to the new locus of arRP on 6q. The underlined markers correspond to those used in the initial screening of the region (see Subjects and Methods section).

family members was performed according to standard procedures (Dracapoli et al. 1994). The polymorphic markers (i.e., STRs) were genotyped individually by PCR. The primer pairs of the selected markers were generated in an oligonucleotide synthesizer (Oligo 1000 DNA synthesizer; Beckman). One of the oligonucleotides from each pair was 5′ labeled during synthesis with Cy5-amidyte fluorochrome (Pharmacia Biotech). All the PCR reactions were performed with 100 ng of genomic DNA, in a final volume of 10 μl. Each reaction contained 200 μM of each dNTP (Boehringer Mannheim), 10 pmol of each primer, 1 × PCR buffer Mg^{++} (Boehringer Mannheim), and 1 unit of *Taq* DNA polymerase (Boehringer Mannheim). The PCR was performed with an initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°–60°C (depending on the primer pair), and 1 min at 72°C; and the final extension was 7 min at 72°C. A total of 1–3 μl of each product of PCR was mixed (v/v) with loading buffer (100% deionized formamide and 10 mg of dextran blue/ml). The samples were denatured for 3 min at 95°C and were loaded onto a 0.5-mm 6% polyacrylamide gel with 7 M urea and 0.6 × Tris-borate EDTA. Electrophoresis was performed in the Alf-Express automatic sequencer (Pharmacia Biotech), at 250 V and 50°C. The sizes of the alleles obtained were determined by the Fragment Manager program (Pharmacia Biotech), by use of a standard-size fluorescent marker (Cy5 syzer, 50–500 bp; Pharmacia Biotech).

Results

In the initial screening of the candidate region on 6q, 2 of the 17 inbred patients affected by arRP (patients RP5.II-1 and RP167.II-8), although unrelated, showed homozygosity for all the markers selected from this region (see fig. 1 and table 1). The former of these two patients, RP5.II-1, is the offspring of a first-cousin marriage (consanguinity coefficient $F = \frac{1}{16}$). Both parents, two affected siblings, and a healthy child were all available for a linkage analysis. In the linkage analysis performed on family RP5, a recombination phenomenon can be observed between markers *D6S1601* and *D6S1644* in patient RP5.II-2. All the markers between *D6S1650* and *D6S1601* are homozygous by descent in the affected patients, whereas the healthy individual appears to be a noncarrier (see fig. 1).

Only two members of family RP167 were available for genotyping. As can be seen in figure 1, patient II-8 is the child of a second-cousin marriage ($F = \frac{1}{64}$). In this case, the region of homozygosity in RP167.II-8 includes all the markers between *D6S402* and *D6S1570,* which overlaps with the region of homozygosity identified for family RP5. Besides, the other available affected member

of the family (individual RP167. II-3, cousin of the patient) has inherited, between markers *D6S402* and *D6S1601,* the same chromosome region from their mother as has been inherited by the index patient (RP167. II-8).

It is worth pointing out that all the affected members of both inbred families (RP5 and RP167) share the same alleles at *D6S1627, D6S1595,* and *D6S1601* (7/7, 3/3 and 2/2, respectively), which could suggest linkage disequilibrium. All markers are found in the region that, according to the human transcript map (Schuler et al. 1996), contains the *GABRR1* gene.

The combined information from both families defines a minimum critical region of 16.1 cM between *D6S257* and *D6S1644,* situating the new gene for arRP on the long arm of chromosome 6.

The LOD scores in these families were calculated by use of a map with five fixed polymorphic markers, by use of Mapmaker/Homoz software (see Subjects and Methods section). We obtained multipoint LOD-score values of 2.16 at 0.5 cM, for family RP5, and 0.96 at 1 cM, for family RP167. The combined multipoint LOD score for both families is 3.1037 at 0.6 cM of the fixed map (near *D6S1629*).

Once linkage was established for the markers of our candidate region, we performed an extension analysis. We added to the study a total of 12 new, unrelated, and noninbred families affected by arRP. These 12 families were analyzed by use of markers linked to the critical region (table 1). Again, we obtained positive LOD scores for two new families (RP73, LOD score 1.18 at recombination fraction [θ] = .0, in *D6S1596*; and RP214, LOD score 1.48 at θ = .0, in *D6S1627*) (fig. 1). The homogeneity tests for markers *D6S402* and *D6S1627,* performed in 29 families affected by arRP, gave the following results: LOD score calculated under the assumption of locus heterogeneity (hLOD) 5.0151 at θ = .0 and α = .18, for *D6S402;* and hLOD 1.6748 at θ = .0 and α = .13, for *D6S1627.* Notwithstanding the small sample available, the value for *D6S402* is consistent with linkage. Also, in family RP73, the paternal chromosome inherited by the three affected siblings shares the same haplotype, for markers *D6S1595* and *D6S1601,* that is present in the two inbred families (RP5 and RP167) (see fig. 1).

With the combined information from families RP5, RP73, RP167, and RP214, we performed an additional multipoint analysis of the critical region, including 34 cM of the pericentromeric region of chromosome 6, using 17 polymorphic markers (mean heterozygosity .77). Using the Mapmaker/Homoz software, we obtained a maximum multipoint LOD score of 5.43 in 28 cM of the fixed map, close to *D6S1627* (fig. 2).

Discussion

The extensive nonallelic heterogeneity found in inherited disorders such as nonsyndromic deafness or RP is, no doubt, the greatest obstacle to finding the loci responsible for these diseases (Strachan and Read 1996). In arRP, ~80% of the pedigrees do not appear to be linked to any of the nine loci identified thus far (Dryja et al. 1995). In spite of this, the allele-sharing methods, such as homozygosity mapping, make possible, with relatively scarce samples, the identification of new loci.

With homozygosity mapping, three completely informative inbred individuals would be sufficient to achieve a LOD score >3 (Farrall 1993). The informativeness of families RP5 and RP167 is enough to obtain linkage. On the other hand, the data proceeding from haplotype and extension analysis suggest that this new locus could explain 10%–20% of the cases of arRP in Spain, since we have found linkage in 4/29 (13.7%) families affected by arRP, and since the values of the homogeneity tests for marker D6S402 are found to be within the range of linkage. We do not yet know whether these results can be confirmed in other populations. In this case, the experiment would have to be applied by other study groups to families with typical arRP.

This is the third gene identified, in relation to RP, on chromosome 6. According to the human transcription map, the new locus appears 14.4 cM below the *RDS/peripherin* gene (responsible for forms of autosomal dominant RP, digenic RP, and certain forms of macular degeneration [MIM 179605]) and 26 cM below *RP14* (an arRP locus located at 6p21) (Knowles et al. 1994). Another three loci exist that are related to eye diseases and that colocalize on 6q: two for maculopathies (MIM 136550 and 600110) on 6q11-6q16.2 (Small et al. 1992; Stone et al. 1994) and one for progressive bilateral chorioretinal atrophy (MIM 600790), which partially overlaps with the other two (Kelsell et al. 1995). More distal is the locus for oculodentodigital syndrome (MIM 164200), on 6q22-q24 (Gladwin et al. 1997). The seven loci on 6p21-6q24 that are related to eye diseases confirm the great richness of retinal-specific transcripts in this region of the genome (Small et al. 1992).

The results of our study show the power of the combination of the Human Transcript Map with the genetic map developed by Généthon. By selection of a group of genes specific for the retina and with only five markers (in the initial screening), linkage was obtained. We believe that the development of tissue-specific transcription maps will permit the design of screening strategies that are even more powerful.

The results of our study and others (Fukai et al. 1996; Pastural et al. 1997) support the approach of screening regions of the genome that contain candidate genes (the indirect candidate-gene approach), prior to a systematic

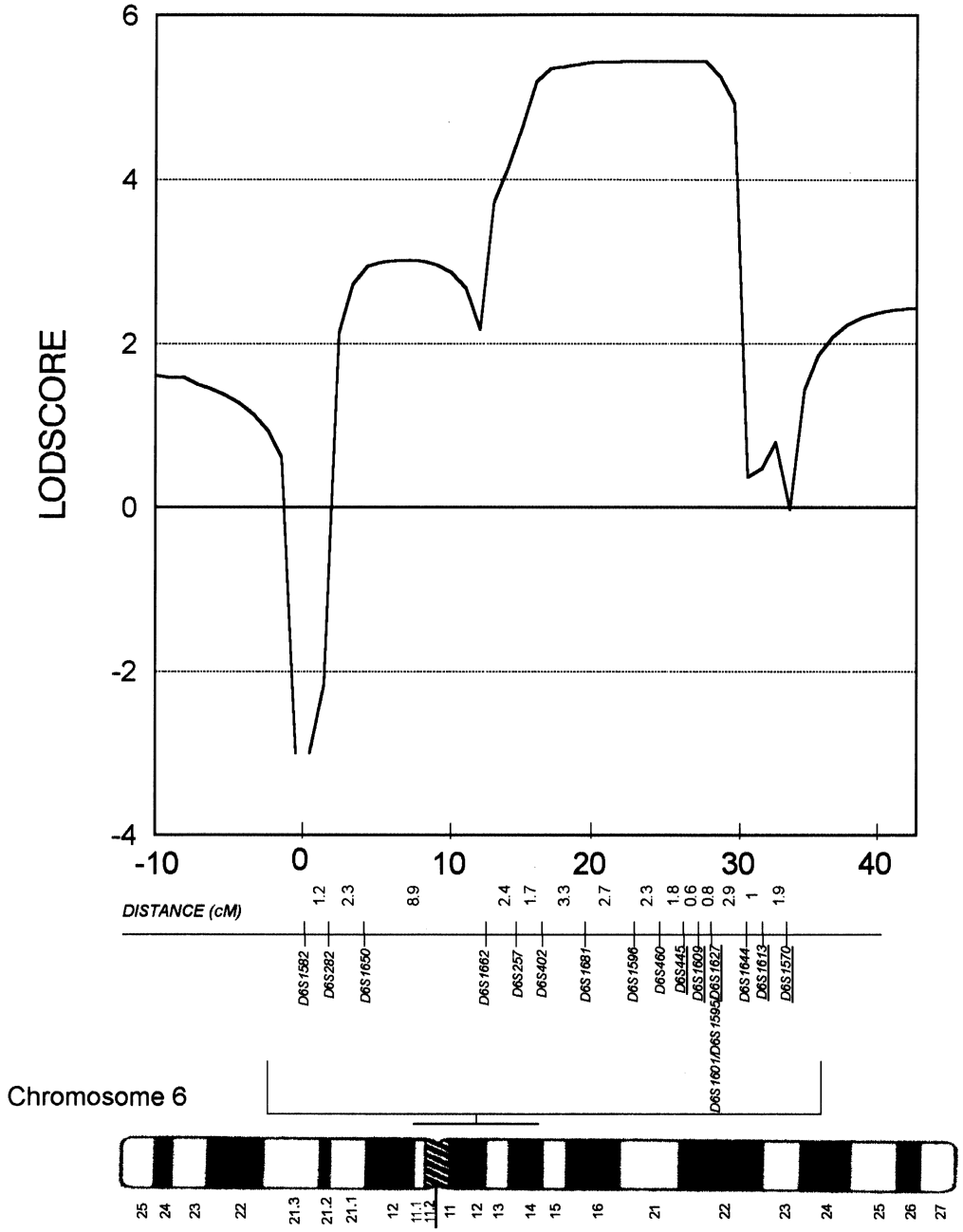

Figure 2 Multipoint analysis of the families showing linkage (RP5, RP73, RP167, and RP214) in a region of 34 cM between markers *D6S1582* and *D6S1570*. Sex-averaged genetic distances (in cM) between the markers are given (Dib et al. 1996). The centromere of chromosome 6 is located between *D6S257* and *D6S402*. The underlined markers correspond to those used in the initial screening of the region (see Subjects and Methods section).

search throughout the totality of the genome (genome-wide screening) (Inglehearn 1997), mainly because of the cost/benefit ratio of the projects. This would be the case for diseases with genetic heterogeneity, where a great number of nuclear families and a high density of markers (spaced every 5 cM) would be necessary in order to allow us to obtain linkage through genomewide screening (Gschwend et al. 1996).

The GABA receptors contained in this region are our first candidates for mutation analysis. This chromosomal

region was selected because it contained *GABRR1* and *GABRR2,* the genes of expression in the retina. If any member of this cluster—*GABRR1, GABRR2,* or any other subunit not yet characterized—finally presents mutations, there would be important implications for the future design of search strategies for RP genes, since GABA receptors are a superfamily of genes (DeLorey and Olsen 1992), and since other, related gene clusters could be involved with RP. Some are being studied by our group at present.

GABRR1 and *GABRR2* represent attractive candidate genes within the critical region that we have defined for arRP. However, we note that their involvement in the disease remains to be demonstrated and that a large number of expressed dequence tags also map to this interval. If further genetic and physiological studies confirm that GABAergic modulation is involved in retinal degeneration, other functions and genes at the level of the first synapsis would be RP candidates, aside from the phototransduction cascade and retinal vitamin-A metabolism (Dryja 1990; Gu et al. 1997; Maw et al. 1997).

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Généthon, http://www.genethon.fr
Genome Database, http://gdbwww.gdb.org
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/htbin-post/Omim (for macular degeneration, maculopathy, oculodentodigital syndrome, progressive bilateral chorioretinal atrophy, retinitis pigmentosa, and markers)

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