competitor DNA were reduced from 100 ng and 200 μg to 10 ng and 20 μg, respectively. The composition of buffer B was 1.2 M sorbitol and 10 mM $KHPO_4$ at pH 7.5. After the second posthybridization wash in 40% formamide and 2× standard saline citrate (SSC), a 15-min wash at room temperature in 2× SSC and 0.1% Triton X-100 was done. The oligonucleotide probes were as follows: 5′-GCTT*GCCTTGTTGAATT*CTG-GTGAATT*GCCTGGTGTT*AATGAGGAAATT*GG-3′, 5′-GATGCCTT*AGTGATGGT*AGGCTTTGTTGT*-GGGCGCTCCGGT*CTCTTAGAT*A-3′, 5′-GGA-ACTT*GGACGACCTAGT*CGATTCCAATT*CCTT-GCCGT*AATTGAAACT*AT-3′, 5′-AT*GGTTCTA-TT*GGTTGGTGGACT*CATCGGCGGTGT*GACG-GGAGGAGTAAT*A-3′, 5′-AAGCT*TTGAAACTG-TT*CGTCTTTTTGT*GACTGGCATTT*GGCATGG-GAAAT*G-3′, and 5′-GT*CGAGAGCAAATCTAT*-GATAATTGGG*GACCTTGGGCT*TGGAGTGTAT*-GC-3′. The probes were directly labeled with a Cy3 fluorochrome at amino-modified thymidine residues indicated by the asterisks (*19*). To detect poly(A)$^+$ RNA, FISH was performed with T43 labeled with fluorescein isothiocyanate (*20*). In formamide-containing solutions, the concentration was reduced to 10% for poly(A)$^+$ RNA detection. Images were taken with an Olympus IX70 inverted epifluorescence microscope and Oncor (Gaithersburg, MD) imaging software, version 2.0.5.

24. Strain K5552, which encodes an epitope-tagged version of Ash1p (Ash1p-myc9), was grown to midlogarithmic phase, fixed, and processed for simultaneous FISH and immunofluorescence. After FISH, immunofluorescence was performed as described previously (*21*) with the following alterations. Antibody to myc was diluted 1:5 into a solution of 1× phosphate-buffered saline, 0.1% bovine serum albumin, 20 mM vanadyl ribonucleoside complex, and ribonuclease inhibitor (40 U/μl). The secondary antibody, goat antibody to mouse immunoglobulin G, conjugated to dichlorotrianzinyl amino fluorescein (Jackson Laboratories), was diluted 1:50 into the same solution.
25. Plasmid C3431 is a derivative of YEplac195 (*17*) carrying a Sal I–Sac I ASH1 fragment.
26. Plasmid pHZ18-poly(A) containing the *ADHII* 3′-UTR has been described (*5*). Plasmid pXMRS25 was constructed from pHZ18 (*22*) by insertion of an *ASH1* fragment generated by the polymerase chain reaction (PCR). The PCR product contained the last five amino acid codons of *ASH1* and extended 250 nucleotides beyond the stop codon. The *ASH1* fragment was subcloned into the Sac I site of pHZ18 by the inclusion of a Sac I restriction site in the PCR primers. The primers for PCR were 5′-GGGC-CCGAGCTCGAGACAGTAGAGAATTGATACATG-3′ and 5′-GGGCCCGAGCTCATCAGGATGAC-CAATCTATTGCGC-3′. To verify that no mutations were introduced by PCR, the *ASH1* region of plasmid pXMRS25 was confirmed by DNA sequencing.
27. We thank M. Rosbash for initiating our collaboration and D. Amberg, S. Brown, A. Bretscher, B. Haarer, and P. Novick for providing yeast strains. Supported by NIH grant GM54887 (to R.H.S) and NIH–National Institute of Child Health and Human Development fellowship 7 F32 HD08088-02 (to R.M.L).

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TECHNICAL COMMENTS

Genetic Complexity and Parkinson's Disease

Mihael H. Polymeropoulos *et al.* describe the genetic linkage of a large Parkinson's disease (PD) pedigree to chromosome 4q21-q23 (*1*). In this study, which affirms a long hypothesized genetic component to the disease, linkage was detected in a single large family with the use of an autosomal dominant model with 99% penetrance of the disease trait. The clinical presentation in this family, however, may differ from typical idiopathic PD because of the apparent autosomal dominant transmisson, early onset, rapid course, and less frequent occurrence of tremor as a significant sign (*2*). Thus, it is unclear whether the putative PD locus identified by Polymeropoulos *et al.* (which they termed PD1) is responsible for the majority of familial idiopathic PD cases.

As part of an ongoing multicenter study of the genetics of idiopathic PD, we have ascertained 94 Caucasian families (a total of 213 affected relatives sampled: 108 affected sibpairs and 31 affected relative pairs) with at least two individuals in each family meeting clinical criteria for idiopathic PD (*3*). We have identified approximately 200 multiplex idiopathic PD families to ascertain for a genomic screen. The 94 families discussed here were those completely ascertained, with DNA sampled, at the time of the analysis. Linkage analysis of chromosome 4q21-q23 markers in these idiopathic PD families did not reveal evidence for linkage of an autosomal dominant, highly penetrant gene, as was described by Polymeropoulos *et al.* (*1*, *4*). We determined two-point log odds (lod) scores, with the use of the model of Polymeropoulos *et al.* as well as a low penetrance "affecteds-only" autosomal dominant model. These lod scores were strongly negative for markers *D4S2361*, *D4S2409*, *D4S2380*, *D4S1647*, and *D4S2623*. Multipoint analysis of the genetic map *D4S2361-17cM-D4S1647-10.5cM-D4S2623* supported these findings for both models, excluding the entire candidate region. We found no evidence for heterogeneity of either the two-point ($P > 0.20$) or multipoint (ln likelihood = 1) lod scores (*5*). Because the power of the parametric lod score method suffers when the genetic model is misspecified, we also used nonparametric analyses of affected relative pairs (*6*). As with the parametric lod score analysis, we found no significant evidence for linkage using either two-point or multipoint analysis; in this data set, the multipoint location scores (MLS) exclude the entire 27.5 cM region for recurrence risks to siblings as low as 2.5 (Fig. 1). Because the pedigree analyzed by Polymeropoulos *et al.* contained many younger onset cases (mean age at onset of the disease was 46), we repeated our analysis in the 22 families with at least one affected individual with an onset earlier than age 45; the analysis in the subset supported the results from the full sample (*7*).

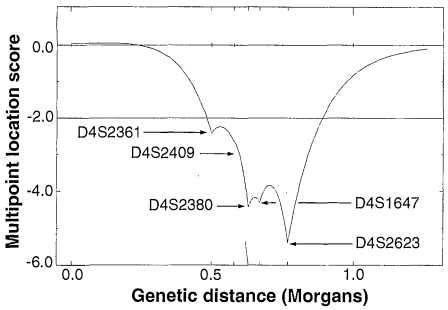

Fig. 1. Multipoint exclusion map for chromosome 4q21-q23 markers. The multipoint lod scores (MLS) within the region are all less than −2.0 at $\lambda_s = 2.5$, excluding the entire candidate region identified by Polymeropoulos *et al.* (*1*). Arrows indicate chromosome markers.

The absence of linkage to chromosome 4q21-q23 in our dataset indicates that there is genetic heterogeneity in PD. It is possible that the region identified by Polymeropoulos *et al.* harbors a disease locus responsible only for a rare autosomal dominant form of PD. Such a situation would be analogous to the genetics of Alzheimer's disease (AD), where mutations (in the amyloid precursor protein and the presenilin 1 and presenilin 2 genes) that cause autosomal dominant AD are responsible for less than 2% of all cases (*8*). Therefore, although the report by Polymeropoulos *et al.* is a first step in unraveling the genetic etiology of PD, other independent genetic effects likely remain to be discovered.

William K. Scott, Jeffrey M. Stajich, Larry H. Yamaoka, Marcy C. Speer, Jeffery M. Vance, Allen D. Roses, Margaret A. Pericak-Vance, and the ***Deane Laboratory Parkinson Disease Research Group*** (*9*), *Department of Medicine, Duke University Medical Center, Durham, NC, 27710, USA; E-mail: mpv@locus.mc.duke.edu*

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3. The families enrolled in this study were ascertained in the following manner. Each of the principal investigators of the 12 study sites identified idiopathic PD patients with one or more first-degree relatives with PD. All 94 families included in the analysis were responsive to levodopa. Specifically excluded were patients with a history of encephalitis, neuroleptic therapy within the year before diagnosis, evidence of

normal pressure hydrocephalus, or a clinical course with atypical features, suggesting secondary Parkinsonism. All first-degree relatives of these patients who consented to participate in this study were subsequently examined and queried regarding the above exclusion criteria and atypical clinical features. Each of these individuals was assigned a status based on their history and the number of the following clinical signs that were present: resting tremor, bradykinesia, and rigidity. Individuals were coded as "affected" if their examination demonstrated at least two of the signs and had no other etiologies for parkinsonism or atypical clinical features, "unclear" if they had only one sign but may have had a history of atypical clinical features, and "at risk" if they had no signs. Mean age at onset of PD symptoms in affected individuals was 61.4 years (SD, 13.1 years). Mean age at examination in affected individuals was 71.5 years (SD, 10.2 years) and in unclear or at risk individuals was 68.4 years (SD, 14.5 years).

4. Microsatellite markers spanning the region defined by Polymeropoulos *et al.* (*1*) were selected for the analysis; the resulting genetic map was: *D4S2361-7.7cM-D4S2409-5.3cM-D4S2380-4cM-D4S1647-10.5cM-D4S2623* (Cooperative Human Linkage Center, database is online at www.chlc.org). Two-point and multipoint lod scores were calculated with the use of the VITESSE software package [J. R. O'Connell and D. E. Weeks, *Nature Genet.* **11**, 402 (1995)], we assumed, as did Polymeropoulos *et al.*, autosomal dominant inheritance, a disease allele frequency of 0.001, and 99% penetrance. A low penetrance, "affecteds only" analysis was also performed. Allele frequencies were estimated from 75 unrelated Caucasian controls.
5. Heterogeneity analysis of two-point and multipoint lod scores was performed using the admixture test, implemented in the HOMOG software package [J. Ott, *Analysis of Human Genetic Linkage*, revised ed. (Johns Hopkins Press, Baltimore, MD, 1991)]. The asymptotic chi-square test is not valid for multipoint lod scores; therefore, a log likelihood comparison is used to assess heterogeneity.
6. Two-point affected-relative-pair analysis was performed using the SimIBD software package [S. Davis, M. Schroeder, L. R. Goldin, D. E. Weeks, *Am. J. Hum. Genet.* **58**, 867 (1996)]. Multipoint affected sibpair exclusion mapping was performed with the use of ASPEX [available from N. Risch, Stanford University, and based on N. Risch, *Am. J. Hum. Genet.* **46**, 229 (1990)] with the use of all five microsatellite markers. Estimates of the recurrence risk to siblings (λ_s) range from 3 to 150, with the majority of studies supporting a λ_s between 10 and 20 [R. C. Duvoisin, *Adv. Neurol.* **60**, 306 (1993); K. Kondo and K. Watanabe, *ibid.*, p. 346]. Exclusion mapping with the recurrence risk set at the low end of this range determined that the region can be excluded for $\lambda_s \geq 2.5$.
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9. The Deane Laboratory Parkinson's Disease Research Group is a multicenter study of the genetics of idiopathic Parkinson Disease coordinated by the Center for Human Genetics, Duke University Medical Center. The collaborating sites and principal investigators are: University of Minnesota, M. Nance; Ohio State University, J. Hubble; University of Kansas Medical Center, W. Koller; University of Pennsylvania Graduate Neurological Center, M. B. Stern and A. Colcher; Emory University School of Medicine, R. L. Watts; Rush Presbyterian–St. Luke's Hospital, C. Goetz and E. Pappert; Carolina Neurological Clinic, F. H. Allen Jr.; Baylor College of Medicine, J. Jankovic and W. Ondo; Marshfield Clinic, B. C. Hiner; University of California, San Francisco, M. Aminoff and G. Dowling; University of California, Los Angeles, G. W. Small.
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Polymeropoulos *et al.* present results of a genome-wide screen for genetic linkage in a large family with autosomal-dominantly inherited L-Dopa-responsive parkinsonism with Lewy-body pathology (*1*). They convincingly demonstrate linkage with polymorphic markers on chromosome 4q21-4q23, with a maximum two-point lod score of 6.00 for marker *D4S2380*. The locus was termed PD1. The role of the PD1 locus in other families with inherited parkinsonism and in sporadic PD remains to be investigated.

We have examined polymorphic markers closely linked to PD1 in 13 multigenerational families with inherited parkinsonism (Table 1). Affected members in all families exhibited at least two of the three cardinal clinical signs of PD (akinesia, rigidity, and resting tremor), as well as asymmetry at onset and a marked improvement on L-Dopa treatment. Rigorous exclusion criteria were applied (supranuclear ophthalmoplegia, cerebellar or pyramidal signs, and severe autonomic or postural disturbance within 2 years of onset). The wide range of age at onset and spectrum of clinical features, including the presence of dementia in addition to parkinsonism in some affected individuals, was similar to that observed in the family studied by Polymeropoulos *et al.* (*1*). No additional neurologic deficit was observed except for amyotrophy in one affected of family A. Multipoint analysis with eight polymorphic markers spanning the region from GATA *10G07* to *D4S2623* excluded the entire 17 cM region likely to contain PD1 in five of the families (families A, B, C, D, and IT-1). In one additional family (G), the major portion of the critical region was also excluded, with lod scores between −1.9 and −2 for the remainder of

Table 1. Demographic and clinical characteristics in 13 families with inherited parkinsonism.

	Family	Number of affected relatives	Affected relatives examined	Age at onset* (years)	L-Dopa response	Atypical features	Reference†
1	A	12	4	51 (35 to 60)	Positive	Amyotrophy and dementia in some	(*2, 3*)
2	B	8	4	62 (51 to 82)	Positive	Dementia in some	(*2, 3*)
3	C	11	4	60 (55 to 66)	Positive	None	(*2, 3*)
4	D	18	5	63 (48 to 78)	Positive	None	(*3, 4*)
5	G	11	4	56 (48 to 74)	Positive	Dementia in some	(*3*)
6	IT-1	5	4	54 (36 to 89)	Positive	None	(*5*)
7	K	4	4	45 (37 to 63)	Positive	None	–
8	FR-041	3	3	63 (60 to 65)	Positive	None	–
9	FR-722	8	5	59 (53 to 64)	Positive	None	(*6*)
10	FR-727	4	3	41 (31 to 52)	Positive	None	–
11	FR-755	4	3	38 (29 to 52)	Positive	None	–
12	UK-A	14	5	53 (42 to 70)	Positive	None	–
13	UK-B	5	5	37 (31 to 41)	Positive	None	(*7*)

*Mean and range. †Unpublished (–).

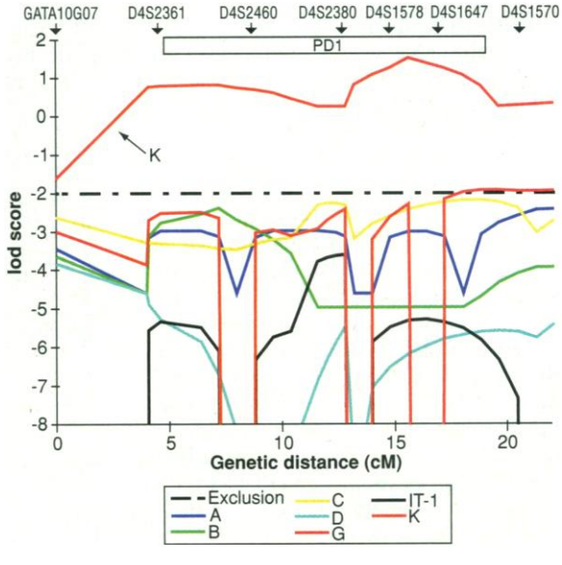

Fig. 1. Multipoint linkage analysis of the PD1-region on chromosome 4q21-q23 in seven families with familial parkinsonism. Polymorphic DNA fragments were amplified by PCR with the use of published primer sequences and a standard protocol. Multipoint analysis was performed using GENEHUNTER (*8*), and two-point analysis was done using VITESSE (*9*). An autosomal dominant model with an age-dependent penetrance was assumed. As was done by Polymeropoulos *et al.* (*1*), unaffected individuals were set to be unaffected only when they were older than the mean age of onset in the respective families; all other unaffected individuals were treated as unknown. Frequency of the disease allele was set to 0.001. Marker allele frequencies were set to be equal for all alleles. Estimating marker allele frequencies from founders in the pedigrees did not alter multipoint lod scores significantly.

the interval (Fig. 1). Data from one (previously unpublished) family of southern Bavarian origin showed positive lod scores with a maximum multipoint score of 1.5 (family K, Fig. 1). This lod score is close to the theoretical maximum in this relatively small family.

In six families (FR-041, FR-722, FR-727, FR-755, UK-A, and UK-B), only the two polymorphic markers most closely linked to PD1 (*D4S1647* and *D4S2380*) have been analysed. Obligate recombinations (no allele shared by all affecteds) were observed in five of these families either for each of the markers individually (three families), or for the haplotype of both markers (two families), again strongly arguing against linkage with the PD1 locus. In one family (FR-041), a positive pairwise lod score was obtained for *D4S2380* (0.29 at Theta = 0). Positive lod scores in families K and FR-041 may reflect true linkage, but they may also be a result of random fluctuations, because the relatively small size of these families precludes definite proof of linkage.

We conclude that mutations at the PD1 locus are probably a rare cause of autosomal-dominant parkinsonism. The role of the PD-1 gene in sporadic PD is still to be determined.

T. Gasser, *Department of Neurology, Klinikum Großhadern, 81377 Munich, Germany;* **B. Müller-Myhsok,** *Bernhard-Nocht-Institute for Tropical Medicine, Hamburg 20359, Germany;* **Z. K. Wszolek,** *Section of Neurology, University of Nebraska, Omaha, NE 68198–2045, USA;* **A. Dürr,** *INSERM U289, Paris 75013, France;* **J. R. Vaughan,** *Institute of Neurology, The National Hospital Queen Square, London WC1N 3BG, UK;* **V. Bonifati** and **G. Meco,** *Dipartimento di Scienze Neurologiche, Università "La Sapienza," Rome 00142, Italy;* **B. Bereznai,** *Department of Neurology, Klinikum Großhadern;* **R. Oehlmann,** *Bernhard-Nocht-Institute for Tropical Medicine;* **Y. Agid** and **A. Brice,** *INSERUM U289;* **N. Wood,** *Institute of Neurology, The National Hospital Queen Square,* and the *European Consortium on Genetic Susceptibility in Parkinson's Disease (GSPD) (10)*

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Response: Scott *et al.* and Gasser *et al.* are discussing genetic studies of families with PD that are designed to examine whether a locus that we previously reported (*1*) on chromosome 4q21-q23 is operating in their sample. The results of Scott *et al.* in 94 Caucasian families do not demonstrate linkage even when the 22 families with earlier onset are examined separately. Similarly, Gasser *et al.* exclude linkage in 13 multigenerational families with Parkinson's disease, with the exception of one family for which they achieved a maximum multipoint lod score of 1.5 for genetic markers in the 4q21-q23 region. Cumulatively, these comments suggest that the chromosome 4 locus will not account for the majority of familial Parkinson's disease and will be expected to operate only in a small percentage of families with the illness.

We have recently demonstrated that a mutation in the alpha synuclein gene is responsible for the phenotype in four families with early onset Parkinson's disease (*2*). Because the mutation was not detected in 50 individuals with sporadic PD, or in two other families with late onset of the illness, we concluded that mutations in the alpha synuclein gene will not account for the majority of the genetic factors of PD, but rather for a proportion of those families with an early onset autosomal dominant form of the illness. These results are in agreement with the observation of Scott *et al.* and Gasser *et al.*, and suggest that the understanding of genetic complexity of Parkinson's disease is just beginning to take shape.

Mihael H. Polymeropoulos
Laboratory of Genetic Disease Research, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA
E-mail:mhp@aeolus.nhgri.nih.gov

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Experiments in a Parkinson's Rat Model

Derek L. Choi-Lundberg *et al.* present evidence (*1*) that a replication-defective adenoviral (Ad) vector that encodes human glial cell line–derived neurotrophic factor (GDNF) protects dopaminergic neurons in substantia nigra (SN) in rats from progressive degeneration induced by the neurotoxin 6-hydroxydopamine (6-OHDA) that has been injected into the striatum. These results are important because of possible applications of Ad vector–mediated GDNF gene therapy in patients with Parkinson's disease. The experimental design used by Choi-Lundberg *et al.*, however, raises some concerns.

Choi-Lundberg *et al.* (*1*) injected 6-OHDA into the striatum of rats 7 days after labeling SN neurons with the retrograde fluorescent tracer fluorogold (FG). Thus, the neurotoxin acted mainly on SN neurons that were loaded with FG. Because of neuronal death and membrane disruption, the fluorescent tracer diffused in the extracellular space, from where it might have been incorporated by other cells. That such an uptake of tracer really occurred in the experiment by Choi-Lundberg *et al.* is demonstrated by figure 2, C through G, in their report, showing that microglia and other non-neuronal cells in the SN have been labeled with FG. Similar to non-neuronal cells, SN neurons that survived the neurotoxin might have incorporated the tracer through their cell membranes (*2*).

To conclude, the finding (*1*) of a reduced loss of FG-labeled neurons in the SN of GDNF-treated rats does not necessarily imply a neuroprotective action of GDNF. A control in which the injection of FG is made after the complete or nearly complete degeneration of the SN neurons would seem to be necessary to definitely support the conclusions made by Choi-Lundberg *et al.*

Roberto Pallini
Alessandro Consales
Liverana Lauretti
Eduardo Fernandez
Department of Neurosurgery, Catholic University School of Medicine, Rome 00168, Italy

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