Letters to the Editor

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The −75A→C Substitution in the 5′ UTR of the Wilson Disease Gene Is a Sequence Polymorphism in the Mediterranean Population

To the Editor:

In their haplotype and mutation analysis of Wilson disease (WD) in Japanese patients, Nanji et al. (1997) report an A→C substitution at position −75 in the 5′ UTR of the WD gene, found in 1/42 WD chromosomes investigated. The authors considered this substitution to be a disease-causing mutation, and they postulated that the mutation adversely affects WD-gene expression, either by abolishing ribosome binding or by interfering negatively with transcriptional factor(s)–DNA binding. However, Nanji et al. (1997) did not report screening for the presence of the A→C mutation in normal chromosomes, to exclude the possibility that this mutation is a simple polymorphism.

Of 228 WD chromosomes analyzed in our study of WD in Mediterranean populations, we found the −75A→C substitution in 23 WD chromosomes that carry an unquestionable disease-causing mutation, as well as in 16 WD chromosomes in which the mutation has not yet been defined. The A→C substitution at position −75 was also detected in 15 (28%) of 54 normal chromosomes from the same Mediterranean population.

These data clearly indicate that −75A→C is a sequence polymorphism that most likely does not affect the function of the WD gene. We previously reported the same A→C substitution in the 5′ UTR (Figus et al. 1995); however, because of erroneous numbering of the nucleotide sequence in the sequence ladder, we incorrectly indicated its position as −74 instead of −75.

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Reply to Loudianos et al.

To the Editor:

We appreciate receiving additional information on the A→C substitution at position −75 in the 5′ UTR of the Wilson disease gene. When we reported this substitution (Nanji et al. 1997), we were careful to indicate that it was in the "putative promoter" region and that it *might* be associated with the disease. We pointed out that direct testing of the effect of the mutation on expression would be required to confirm the nature of the mutation. We described the results of the analysis of 21 normal chromosomes from a Japanese group, which is the same ethnic group as that of the patient. These normal-chromosome results were obtained from the analysis of the normal chromosomes in the heterozygous parents of the patients. None of the putative promoter mutations were identified in the normal sample. We did report, in table 3 of our previous study (Nanji et al. 1997), some alterations that we felt were definitely polymorphisms. Ap-

parently, the A→C substitution is more common in the normal Mediterranean population. We had missed this because of the error in the study by Figus et al. (1995), as is noted in the letter by Loudianos et al. (1998 [in this issue]). Promoter studies are currently in progress to determine the nature of the mutations reported by us.

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Reply to Burghes

To the Editor:

In his recent editorial entitled "When Is a Deletion Not a Deletion? When It Is Converted" Burghes (1997) correctly ascribes the cause of spinal muscular atrophy (SMA) to the loss or mutation of the telomeric copy of the *SMN* (survival motor neuron) gene. The reduction in SMN protein, as Burghes recognizes, most likely leads to motor-neuron death, by unknown mechanisms (Coovert et al. 1997; Lefebvre et al. 1997). He also outlines the probable role of centromeric copies of SMN in the modulation of disease severity (Campbell et al. 1997; Velasco et al. 1996; McAndrew et al. 1997). However, concerning a second SMA candidate gene, known as *NAIP* (neuronal apoptosis–inhibitory protein), Burghes states that "it appears likely that the deletion of *NAIP* marks the extent of the [genomic] deletion and that different forms of SMN_{cen} modify the SMA phenotype," thereby rejecting a role for *NAIP* in SMA pathogenesis.

On this final point we strongly disagree. During the past 2 years, our group, our collaborators, and other laboratories have shown that NAIP could be involved in SMA pathogenesis in several ways. First, in most populations the *NAIP* gene is deleted in the majority of type I SMA individuals. In some type I SMA populations, the deletion of SMN_{tel} extends to *NAIP* in >80% of affected chromosomes (Morrison 1996; Samilchuk et al. 1996; Velasco et al. 1996). Second, in the CNS, NAIP is expressed in at least eight distinct neuronal populations, including the motor neurons, all of which are affected in type I SMA (Towfighi et al. 1985; Murayama et al. 1991; Peress et al. 1986; Xu et al. 1997*b*). A number of NAIP-positive neuronal types (e.g., cholinergic neurons of the striatum), when subjected to ischemia, demonstrate both a significant increase in NAIP levels (Xu et al. 1997*a*) as well as a marked resistance to apoptotic death. Third, NAIP exerts an antiapoptotic effect in cultured cells (Liston et al. 1996) and affords hippocampal neuroprotection in vivo when overexpressed from a transgene (Xu et al. 1997*a*).

In view of these data, we find the assertion surprising that the *NAIP* gene serves merely as a marker of genomic-DNA deletion size. Clearly, formal proof of NAIP involvement in SMA pathogenesis must await further analysis (e.g., exacerbation of an SMA phenotype in SMN-deficient mice when expression of NAIP is compromised). However, we feel that it is likely that motor neurons from SMA individuals with deletions of both *NAIP* and SMN_{tel} are prone to apoptosis. As a result, the cells are less able to withstand the stress of SMN depletion and die earlier than they would otherwise, resulting in a more severe form of SMA.

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Reply to Mackenzie

To the Editor:

MacKenzie suggests that, in my editorial "When Is a Deletion Not a Deletion? When It Is Converted" (Burghes 1997), I have not ascribed sufficient significance to the role of the neuronal apoptosis inhibitory protein (NAIP) gene in spinal muscular atrophy (SMA). In particular, MacKenzie takes issue with the following statement: "Further work is required to clearly define the mechanism by which the converted alleles modify phenotype, and it is possible that deletion of adjacent genes, such as NAIP, could influence the exact severity of the phenotype. However, it appears most likely that the deletion of NAIP marks the extent of the deletion and that different forms of SMN^C modify the SMA phenotype" (Burghes 1997, p. 13).

It is my opinion that this is a fair reflection of our current knowledge of the situation and that, at present, there is not adequate evidence to implicate NAIP as a major SMA-modifying gene. The first and foremost argument against involvement of NAIP comes from genetic studies. MacKenzie indicates that, in some type I SMA populations, the rate of NAIP deletion approaches 80%. However, in most cases in which a noninbred population has been studied, the rate of NAIP deletion in type I SMAs is 45%–50% (Cobben et al. 1995; Hahnen et al. 1995; Roy et al. 1995; Thompson et al. 1995; Velasaco et al. 1996; DiDonato et al 1997*b*). MacKenzie states that "we feel it likely that motor neurons from SMA individuals with deletions of both NAIP and SMN_{tel} are prone to apoptosis. As a result, the cells are less able to withstand the stress of SMN depletion and die earlier than they would otherwise, resulting in a more severe form of SMA." This would predict that the disease in those patients without a deletion of the NAIP gene would be mild, whereas the disease in those patients with a NAIP gene deletion would be severe. Patients with SMN^T intragenic mutations that still retain the NAIP gene would also be predicted to have a mild form of the disease. So a critical question is, Do the type I SMA cases without NAIP deletions show a clinical progression different from that seen in patients with an NAIP deletion? There is no clear difference between these two populations. In addition, type II/III SMA cases can have deletions of NAIP, as can carriers with no clinical phenotype (Cobben et al. 1995; Hahnen et al. 1995; Roy et al. 1995; Thompson et al. 1995; Rodrigues et al. 1996; Campbell et al. 1997; DiDonato et al. 1997*b*). Therefore, it does not always seem to be the case that motor neurons lacking NAIP are more sensitive to the loss of SMN^T. It could be argued that the type I SMA cases with an intact NAIP gene have another mutation, which is not detectable by current assays—and that they therefore are phenotypically equivalent—whereas type II/III SMA patients with a deletion of NAIP somehow make the NAIP protein in the motor neurons. This indicates two critical studies that are needed to substantiate NAIP as a major modifier of SMA. First, there must be detection of intragenic NAIP mutations in the type I SMA patients who have an intact NAIP gene. Second, there must be studies at the protein level that show reduction of the NAIP protein in motor neurons of type I SMA patients who do not have a deletion of the NAIP gene.

Apart from deletions of SMN^T, type I SMA patients with intragenic mutations of SMN^T and no detectable alteration in the NAIP gene have been identified (Burghes 1997, table 1). These patients have a clinical phenotype indistinguishable from those type I SMA cases who have NAIP deletions. If NAIP were a major modifier of SMA, it would be difficult to explain type I SMA cases who had an intact NAIP gene, since NAIP would be expected to exert its protective effect and to modulate the phenotype.

The identification of the NAIP gene in the SMA region gave an appealing candidate gene, since it indicated that apoptosis could have a key role in SMA. However, at the current time, in my view, it is not clear what role apoptosis plays in SMA. Does the motor-neuron cell activate the apoptotic pathway because the cell lacks a critical element or is SMA due to an actual defect in an apoptotic pathway? Mice do not have two SMN genes (SMN^C and SMN^T) on a chromosome; rather, they have one SMN gene on a chromosome (DiDonato et al. 1997*a;* Violet et al. 1997). Mice that lack this SMN gene die early in embryogenesis (Schrank et al. 1997), and the cells show definite apoptotic changes. This clearly highlights the importance of the SMN^C gene in human SMA patients, but it does not explain why the loss of SMN^T causes SMA. Indeed, the SMN protein has recently be shown to be important for snRNP biogenesis, presumably a critical function in a cell (Liu et al. 1997). Interestingly, snRNPs appear to be particularly enriched in motor neurons. So is SMA caused by disruption of snRNP formation in neurons or by disruption of apoptotic pathways? What is the role of SMN^C in motor neurons? These questions remain to be resolved.

I should also note that phenotypic modification in SMA is not entirely resolved by the conversion model that I have presented elsewhere (Burghes 1997). In particular, there are rare type II/III SMA families in which two sibs inherit the identical 5q13 haplotypes (Burghes et al. 1994; Cobben et al. 1995; Hahnen et al. 1995; Wang et al. 1996; DiDonato et al. 1997*b*); both sibs have no detectable SMN^T gene, but they show remarkably discordant phenotypes. This is not fully explainable by the conversion model and could indicate that genes outside the 5q13 region act as phenotypic modifiers. If alteration of apoptotic death is implicated in SMA, then genes such as bcl2 become candidates as phenotypic modifiers. However, it is equally likely that phenotypic modifiers of SMA effect the level of SMN expression in motor neurons by up-regulating the SMN^C gene to increase the level of SMN protein. If this is the case, an intriguing possibility is that up-regulation of the SMN^C gene serves as a therapeutic intervention in SMA. In conclusion, one component that modifies phenotype in SMA is the type of mutation in SMN^T; but, as yet, it is not clear what other factors can modify phenotype.

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Evidence for Paleolithic and Neolithic Gene Flow in Europe

To the Editor:

In recent Letters to the Editor, Cavalli-Sforza and Minch (1997) and Richards et al. (1997) discuss the relative contributions of the first Paleolithic colonizers of Europe, and of later Neolithic immigrants, to the gene pool of current Europeans. Using the method of median networks (Bandelt et al. 1995), Richards et al. (1996) demonstrated that most mitochondrial lineages coalesce at ancestors who presumably lived in the Paleolithic period, which, in Europe, means >10,000 years ago. Through an analysis of the geographic distribution of these lineages, they reached the conclusion that most mitochondrial alleles spread in Europe prior to the Neolithic period. Two implications of this finding were that (1) farming was essentially a local development, the spread of which was not accompanied by extensive gene flow, and (2) the gradients of allele frequencies described in many studies (starting with Menozzi et al. [1978] and reviewed in Cavalli-Sforza et al. [1994]) were not due to a Neolithic demic diffusion from the Near East (Ammerman and Cavalli-Sforza 1984), as is generally believed. Richards et al. (1996) interpreted the results of a simulation study of various population-expansion mechanisms (Barbujani et al. 1995) as supporting a Paleolithic origin of these clines.

Cavalli-Sforza and Minch (1997) argued that sequences of the mtDNA hypervariable region are not suitable for reconstructing evolutionary processes at this scale, because the high mutation rates at some sites cause an excess of random noise. In addition, a high female mobility might have blurred some previously existing geographic patterns. They suggested that a figure of ~25% might realistically represent the contribution of Neolithic immigrants to the gene pool of Europeans, because, in principal-component analyses of allele frequencies, a clinal component accounts for one quarter of the genetic variance (Menozzi et al. 1978; Piazza et al. 1995). If that were the case, there would be little overall disagreement; given the approximate nature of any such estimates, the figure (15%) proposed by Richards et al. (1997) may not differ significantly. We would like to suggest a third possibility—namely, that the available mitochondrial data do not contradict a much larger Neolithic contribution and that envisaging the current European gene pool as essentially a product of an Upper Paleolithic colonization may create more problems than it solves.

There are four traditional reasons to believe that there was a major Neolithic contribution to the European gene pool: (1) the continentwide gradients of allele frequencies; (2) their correlation with the archaeological record; (3) their overlapping with areas defined by linguistic criteria; and (4) their similarity to the gradients theoretically predicted under, or generated in simulation studies of, a model of demic diffusion. None of these pieces of evidence is proof, but in this field there is little that one can really prove. The point, at this stage, is to find the simplest explanation that accounts for most (or, possibly, for all) observed population characteristics. Of course, speaking of Paleolithic versus Neolithic processes is an oversimplification of phenomena that were certainly more complicated. However, such a highly schematic opposition is useful for the sake of clarity.

As for the gradients detected for roughly one third of the alleles studied in Europe (Sokal et al. 1989) (point 1), few doubt that they result from some form of population movement. Indeed, random genetic drift alone cannot generate nonrandom patterns on such a broad scale, and major selective effects on many independent loci appear unlikely (Ammerman and Cavalli-Sforza 1984; but see also Fix 1996). The problem is *when* those movements took place. As Richards et al. (1996, 1997) pointed out, the correlation with archaeological gradients (point 2) and, specifically, with the first evidence of farming activities (Sokal et al. 1991) now seems less cogent. Indeed, evidence is emerging that, not only in the Neolithic but also in the Paleolithic period, the main population movements occurred along a southeast-northwest axis (Richards et al. [1997] and references therein). If so, whatever the relative importance of the two temporal phases, both should have determined similar clines of gene frequencies. On the contrary, however, if it were shown that Paleolithic populations moved

largely in other directions, the longitudinal clines should still be taken as evidence for a major Neolithic contribution to the European gene pool.

What does not seem equally easy to reconcile with both views is the linguistic evidence (point 3). Renfrew (1987) proposed that the genes of Anatolian and Near Eastern populations, the technologies for farming and animal breeding, and the Indo-European languages were brought to Europe in the course of the same expansion, starting some 10,000 years ago. Renfrew (1991) also suggested that, in three other linguistically related areas (where Afro-Asiatic, Altaic, and Elamo-Dravidian languages are or were spoken), farming technologies could have spread at once with languages and with the people who spoke them. When a large-scale analysis of genetic data was performed, all four areas identified by Renfrew showed, for most genes, highly significant clinal patterns (with a lower statistical significance for the Afro-Asiatic–speaking area), some of which were not apparent if linguistic affiliations were disregarded. The geographic limits of those clines corresponded to major language barriers, and similar clines were not observed for language families that were not supposed to have spread during the Neolithic period (Barbujani and Pilastro 1993; Barbujani et al. 1994).

In synthesis, gene-frequency clines correspond to linguistic areas that, presumably, were established in the Neolithic period, not only in Europe, but in three other regions of Eurasia and North Africa, where clines were sought on the basis of linguistic evidence and were detected only when the different linguistic groups were analyzed separately. The main language families of Eurasia are unlikely to have spread in earlier time periods; several linguists are very reluctant to accept dates much earlier than 5,000 years ago (Coleman 1988). Therefore, the correlation between gene frequencies and languages seems difficult to explain if one assumes a limited genetic contribution of Neolithic farmers. One should imagine that, in four regions of Eurasia and North Africa, the routes of spread of four Neolithic language groups overlapped by chance with the routes followed by the first Paleolithic colonizers; this does not seem a parsimonious hypothesis.

Simulation results (point 4) also seem to be in better agreement with a comparatively recent dispersal of the Europeans' ancestors. Rendine et al. (1986) and Barbujani et al. (1995) showed that Neolithic population expansions that started in the Near East and were accompanied by little admixture do produce clines that are not observed under alternative models. Although the relative contributions of Paleolithic and Neolithic groups are difficult to calculate from the published results, in Rendine et al. (1986), population sizes were increased 3–25 times in the process, largely because of demographic growth among Neolithic farmers. As we understand it, this means that the Paleolithic contribution can be estimated to be between one third and a much lower value. The second study showed that simulated allele-frequency patterns significantly resemble the observed ones if the expanding population largely or entirely replaced any pre-existing settlers (Barbujani et al. 1995). Therefore, all simulation results obtained so far are certainly consistent with diffusion of Neolithic populations who were ancestral to most current Europeans. Conversely, the alternative view, which involves a series of founder effects during the initial colonization of Europe and which is conceivable in principle (Barbujani et al. 1995; Richards et al. 1996), has not yet been formally tested.

Can one say that allele frequencies support a Neolithic model, whereas DNA data support a Paleolithic model? We do not think so. The main finding of the study by Richards et al. (1996) is that most mutations that characterize the mitochondrial haplotypes of present-day Europeans occurred in the Paleolithic period. We do not question that conclusion, but we do not think that the age of a group of haplotypes can be mechanically equated to the age of the population from which they came, especially if these haplotypes are also found elsewhere. Certain alleles of the HLA-DRB1 locus arose millions of years ago and are shared by our species and others (see Ayala 1995). Their presence in European populations does not imply that Europe was colonized prior to the separation between humans and chimps. Similarly, suppose that some Europeans colonize Mars next year: If they successfully establish a population, the common mitochondrial ancestor of their descendants will be Paleolithic. But it would not be wise for a population geneticist of the future to infer from that a Paleolithic colonization of Mars.

The average coalescence time of two sequences sampled from two diverging populations is, in general, older, or much older, than the split of the groups. Unless a group colonizing a new territory passes through a strong and long-lasting bottleneck, part of its initial diversity will be maintained (Nei et al. 1975; Tajima 1983). Therefore, the coalescence times inferred from samples of its descendants will be close to the coalescence times of the population of origin, and these times will consistently overestimate the age of the derived populations. Clearly, inferences from population history must be based on measures of genetic diversity *between populations*, not *between molecules*. Among the appropriate statistics is Nei's d_{AB} distance—the average sequence difference between the haplotypes of two different samples, diminished by the average pairwise difference within samples (Nei 1987). In populations at equilibrium, this distance is linearly related, through the mutation rate, to the divergence time between pairs of populations. If one calculates d_{AB} from the mitochondrial data of Rich-

ards et al. (1996) and uses the divergence rate proposed in that article (1/10,500 years), Middle Easterners appear to be separated from all other European samples by <4,000 years. Although recent gene flow has probably reduced the levels of population differentiation, these figures do not point to population splits that predate the Neolithic period in Europe.

A link between molecules and populations can be established. However, to do that, one should focus on haplogroups that are geographically restricted to the area of interest—Europe, in our case—a point that Richards et al. (1996) did not thoroughly consider. Among the haplogroups recognized by Richards et al., three have not yet been observed outside Europe; they coalesce, respectively, at $6,000 \pm 2,000$ (haplogroup 2A-C), $12,500 \pm 6,000$ (haplogroup 2A-W), and $17,400 \pm 2,000$ (haplogroup 4) years ago. Once again, these dates are not in conflict with a mostly Neolithic origin of the European populations.

When considered at the population level, other DNA polymorphisms suggest a recent separation of European groups. In a study of seven DNA polymorphisms, we used a measure of microsatellite diversity (Goldstein et al. 1995) to estimate times since population divergence in Europe. Geographic clines were broad and significant, and none of the comparisons between European and Near Eastern populations led to estimates $\geqslant$10,000 years, with the single, predictable exception of Saami (Lapps) (Chikhi et al., in press). We are more than ready to accept that future analyses of larger numbers of loci may somewhat modify this conclusion. However, at present, all the molecular data analyzed at a continental scale appear fully consistent with a major Neolithic phenomenon that left its marks on present levels of population diversity. The European distribution of two Y-chromosome polymorphisms is in nice agreement with this conclusion (Semino et al. 1996). It is quite possible that the gene pools of certain isolated groups, Saami (Lahermo et al. 1996), Ladins (Stenico et al. 1998), and Basques (Calafell and Bertranpetit 1994) among them, contain a greater proportion of alleles derived from the first, Paleolithic colonizers. But this seems the exception, not the rule.

In synthesis, we believe that any model that suggests a largely Paleolithic origin of the European gene pool should incorporate an explanation of (1) the correlation between genetic data and linguistic patterns that are highly unlikely to have been established prior to the Neolithic period; (2) the simulation results showing that clines are generated by population processes supposed to have occurred in the European Neolithic period; and (3) the admittedly limited, but internally consistent, evidence from studies of microsatellite diversity. On the contrary, all these findings, as well as the distribution of mitochondrial diversity (to the clarification of which Richards et al. have contributed), can easily fit within a model of extensive demographic replacement associated with the dispersal of Near Eastern farming populations.

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Reply to Barbujani et al.

To the Editor:

We agree entirely with Barbujani et al. (1998 [in this issue]) that the age of a group of haplotypes cannot be mechanically equated to the age of the population from which they come and that such an uncritical equation would artificially elevate the estimated age of the population under study. However, our analysis (Richards et al. 1996, p. 194) focuses not simply on haplogroups, but on haplotypes within haplogroups. Such an analysis depends critically on the correct identification—by cross-population comparison of lineages—of all of the major founder haplotypes, which can then be used as a baseline from which to date the founder events associated with each cluster of haplotypes. This is exemplified in our paper by the identification of a number of distinct founder haplotypes in lineage group 2A, picked out on the basis of their presence as shared ancestral nodes in the European and Near Eastern phylogenies, which root deeply (during the Upper Paleolithic period) in the Near Eastern data but which have accumulated only a small amount of variation—equivalent to ~10,000 years or so—within Europe. This suggests, to us, expansion into Europe from the Near East during the Neolithic period. Of the various lineage clusters that we identified in Europe and the Near East, only group 2A showed this pattern; other clusters did not show evidence of recent founder events within Europe.

We believe that a phylogeographic analysis such as this—which is indeed based on molecules rather than on populations—is capable of a much finer resolution than one based on distance statistics, such as that suggested by Barbujani et al. (1998). Moreover, the particular statistic used is misleading, as it is based on a model of populations of constant size at mutation-drift equilibrium, which is patently unsuitable for application to Europe and the Near East. However, an important weakness of our published analysis is the meager volume of comparative data from the Near East: essentially 42 individuals, mostly from the Arabian peninsula. In subsequent work, we are extending the analysis to a much larger sample from southwestern Asia, to improve our confidence that most founder haplotypes have been identified. We aim to report our conclusions in the near future.

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Reply to Hofmann et al.

To the Editor:

In the June issue of the *Journal*, Hofmann et al. (1997) commented on our study of Leber hereditary optic neuropathy (LHON; MIM 535000 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?535000]) multigeneration pedigrees (Mackey et al. 1996). Hofmann et al. made two main points in their letter, the first of which was that a mutation at nucleotide 15257 of the mitochondrial cytochrome *b* gene has a pathogenic role in LHON. This is a long-standing and unresolved controversy that epitomizes the pitfalls that beset the identification of pathogenic mtDNA mutations. Their second point, which is not an issue that we addressed, was that "so-called" secondary LHON mutations, in their terminology, play an etiologic role in other neurological disorders.

There is broad agreement that mtDNA mutations at nucleotides 3460, 11778, and 14484 are pathogenic LHON mutations, but there is disagreement over the pathogenic role of the 15257 mutation (Howell 1994, 1997*a*; Oostra et al. 1994). In our previous reports, including that by Mackey et al. (1996), the term "primary" was applied to the 3460, 11778, and 14484 LHON mutations. LHON is maternally inherited, but the penetrance is incomplete. The pathogenic mtDNA mutation is thus the predominant risk factor, but additional etiologic factors are required for manifestation of the optic neuropathy (Howell et al. 1997*a,* 1997*b*). In that sense, the 3460, 11778, and 14484 mtDNA mutations have a *primary* pathogenic role in LHON. Several additional mtDNA mutations have been identified that may have an etiologic or pathogenic role in LHON, including some that may augment or modify the phenotypic effects of the three known pathogenic mutations (see below). As a result of this uncertainty, the nomenclature has become commensurately more complicated. For example, Brown and Wallace (1994) list 16 mutations that have primary, secondary, or intermediate roles in LHON, although even this list is now incomplete (e.g., see Howell et al. 1998, and references therein).

The purpose of our previous study (Mackey et al. 1996) was to identify the pathogenic mtDNA mutations in LHON pedigrees. A total of 159 families (comprising ~12,000 maternal relatives) from Australia and northern Europe were analyzed, because these are countries where extensive genealogies are more easily obtained. We limited our study to large multigeneration LHON families, to avoid the ambiguities that arise with singleton cases of bilateral optic neuropathy in which maternal inheritance is lacking. The majority of sporadic cases of a LHON-like optic atrophy are not associated with the 3460, 11778, or 14484 LHON mutations (Chan et al. 1996). *None* of these 159 LHON families carried the 15257 mutation in the absence of one of the three previously established LHON mutations, although it was associated with one of the three mutations in six LHON families. This association has also been found in other LHON families (Howell et al. 1993; Oostra et al. 1994), as well as among those analyzed by Hofmann et al. (1997). The penetrance of pathogenic mutations is not increased in the LHON families whose mtDNAs also harbor the 15257 mutation (e.g., see Howell et al. 1993; Torroni et al. 1997). These negative results argue against a pathogenic role for the 15257 mutation, because LHON is a disorder whose penetrance is particularly dependent on the action of secondary etiologic factors (Howell 1997*a,* 1997*b*).

In contrast to our results, Obermaier-Kusser et al. (1994) reported a LHON family, with multiple affected family members that span multiple generations, that carries the 15257 mutation but not one of the three previously identified pathogenic LHON mutations. Hofmann et al. (1997) report a total of 55 optic neuropathy index cases, 3 of which are 15257 plus 11778 and 6 of which are 15257 plus 14484, but 5 of which are "15257 only." It is not clear whether these 15257-only cases are distant relatives of the LHON family described elsewhere (Obermaier-Kusser et al. 1994) or have affected maternal relatives. It is precisely because of the experimental and analytical difficulties inherent to singleton cases that we undertook our study of multigeneration families. More important, sequencing analysis has not been performed, either for the 15257-only LHON family or for the new 15257 cases, and the presence of a rare, unidentified pathogenic mutation cannot be ruled out.

The 15257 mutation has been detected at a low frequency (Brown et al. 1992; Kalman et al. 1995; and especially see Torroni et al. 1997) in normal control subjects, a result that argues against a pathogenic role. However, population surveys of normal controls should capture individuals who harbor a pathogenic LHON mutation but who, because of the incomplete penetrance, are not clinically affected. The 3460, 11778, and

14484 LHON mutations, which tend to produce a relatively high-penetrance form of LHON (Mackey 1994; Chan et al. 1996), have *not* yet been detected in such surveys. If the 15257 mutation produces an unusually low-penetrance form of LHON, this would explain both the absence of 15257-only multigeneration LHON families in our survey and the presence of the 15257 mutation in normal control populations. That explanation, however, does not fit the observation that the 15257 LHON family of Obermaier-Kusser et al. (1994) has a *high* penetrance.

Hofmann et al. (1997) also discuss haplogroup clustering and the 15257 mutation. Haplogroup clustering was first reported by Johns and Berman (1991), who showed that the frequency of what they termed *secondary* LHON mutations at nucleotides 4216 and 13708 were associated more often with 11778 LHON patients than with normal controls. It is now recognized that the 4216 and 13708 sequence changes define one of the major European haplogroups (designated "A" by Hofmann et al. [1996], "2A" by Richards et al. [1996], and "J" by Torroni et al. [1996]). Both the 11778 and 14484 mutations cluster, or are associated preferentially, with this haplogroup, and ~75% of 14484 LHON patients or pedigrees carry mitochondrial genomes that belong to this haplogroup (Johns 1994; Brown et al. 1997; Torroni et al. 1997). There is no satisfactory explanation for this clustering, although it has been proposed that one or more of the mtDNA sequence changes in this haplogroup markedly increases the penetrance of the 11778 and 14484 LHON mutations, particularly in the case of the latter (Brown et al. 1997; Torroni et al. 1997). The 15257 mutation, with rare exception, is also associated with this haplogroup (Howell et al. 1995; Brown et al. 1997; Torroni et al. 1997).

In addition to haplogroup J, there is a second haplogroup, which carries the mutation at nucleotide 4216 and a mutation at nucleotide 4917 (designated "B" by Hofmann et al. [1997], "2B" by Richards et al. [1996], and "T" by Torroni et al. [1996]). We (Howell et al. 1995), Hofmann et al. (1997), and Richards et al. (1996) agree that European haplogroups J and T are members of the same phylogenetic cluster (but, for the alternative view that the two haplogroups are less closely related, see Torroni et al. [1996, 1997]). It was initially concluded that there was also clustering of LHON mutations with the 4216+4917 combination of mutations (Johns and Berman 1991), but more-recent results do not support this conclusion (Torroni et al. 1997). Hofmann et al. (1997) report two LHON patients in whom the 15257 LHON mutation is associated with the mutation at nucleotide 4917. Furthermore, they state that *one* of these patients did not carry a LHON mutation at nucleotide 3460, 11778, or 14484. It is difficult to assess the ramifications of those results, but Torroni et al. (1997) have reported the 4917+15257 combination in a *normal* control. Furthermore, Torroni et al. (1997) have shown that the mitochondrial genomes from their LHON pedigrees cluster with the haplogroup J subbranch that does *not* carry the 15257 mutation (see their fig. 2), results that imply that the 15257 mutation does not increase LHON penetrance (see above).

The second major point raised by Hofmann et al. (1997, p. 1540) is that secondary LHON mutations are frequently associated with non-LHON neurological disorders. As evidence for this proposition, they cite recent studies that found an increased frequency of the 15257 and 13708 mutations, both in a cohort of patients with multiple sclerosis (MS) and visual impairment (Mayr-Wohlfart et al. 1996) and in a group of patients with varied neurodegenerative disorders (Rödel et al. 1996). However, even if the results of Kalman et al. (1995) and Mayr-Wohlfart et al. (1996) are pooled, there is no statistically significant association between the 15257 mutation and MS (data not shown). Second, there is no significant association between the 13708 mutation and MS in the pooled data from these two studies, nor when they are pooled with the much larger study by Kellar-Wood et al. (1994). It is also possible to use the pooled data of Kalman et al. (1995) and Mayr-Wohlfart et al. (1996) to show that there is no significant association between MS and haplotype J (data not shown). Rödel et al. (1996) have reported that 7 of 200 neurology patients from a southern German population harbor mtDNAs with the 13708+15257 array of secondary LHON mutations, although the frequency of this combination of sequence changes among normals from the same geographic region is not specified. Richards et al. (1996) have shown that haplogroup J (which they term "2A") has a heterogeneous geographic distribution throughout Europe and the Middle East, and the results of Rödel et al. (1996) thus require wider surveys of haplogroup J population samples.

Hofmann et al. (1997) have reported that the mtDNA from five of their eight patients with DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness) belong to the 4216+4917 haplogroup (haplogroup T of Torroni et al. [1996]), a frequency (63%) much higher than the frequency (9%) of this haplogroup among the controls in their study. In addition, there appears to be an increased frequency of MS patients with mtDNAs who belong to this haplogroup (Kalman et al. 1995; Mayr-Wohlfart et al. 1996). In these two studies, the pooled frequencies were 16/153 in MS patients and 7/174 in the controls ($P < .05$, as determined by a χ^2 test). Hofmann et al. thus conclude that the 4216, 4917, and 13708 sequences are not secondary LHON mutations but that, instead, they are mtDNA mutations that have an etiologic role (possibly secondary) in a variety of neurological disorders. We believe that such a con-

clusion is premature and that more-extensive screening of both patients and controls from geographically diverse European subpopulations is required. For example, mtDNA haplotype analysis of another set of 28 Caucasian DIDMOAD patients has not revealed haplotype clustering (J. Poulton and T. Barratt, unpublished data). Especially within small geographic areas, there may be, between *nuclear* genotypes and mitochondrial haplotypes, preferential associations that may be statistically significant but that do not signal an etiologic role for the mitochondrial genome in the disease process (for an analysis of the relative nuclear and mitochondrial genetic contributions from founders in isolated populations, see Heyer [1995]).

In summary, it is our view that the available data do not *yet* indicate that the 4216, 4917, 13708, and 15257 mutations (and any sequence changes associated with haplogroups J and T) are pathogenic, either in LHON or in other neurological abnormalities. We agree with Hofmann et al. (1997) that further investigation is warranted.

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Characterization of 10p Deletions Suggests Two Nonoverlapping Regions Contribute to the DiGeorge Syndrome Phenotype

To the Editor:

DiGeorge syndrome (DGS; MIM 188400 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?188400]) is a developmental-field defect characterized by abnormalities of structures derived from the pharyngeal arches and pouches (for a review, see Driscoll and Emanuel 1996). The vast majority of individuals with DGS have been found to have deletions of chromosomal region 22q11.2. However, a small number of patients have been shown to have deletions of chromosome 10p, with normal chromosome 22s (for a review, see Greenberg et al. 1988). We report here the location and extent of the deletion on chromosome 10, determined by means of a combination of heterozygosity tests and FISH analysis, in five DGS patients. Our results do not support the existence of a single, commonly deleted region on 10p in these five patients. Rather, they suggest that deletion of more than one region on chromosome 10p can be associated with the DGS phenotype.

We examined the extent of the chromosome-10p deletions in five patients. Phenotypic characterizations of three (GM6936, CH92-304, and CH95-199) of the five patients were reported elsewhere, by Greenberg et al. (1986), by Monaco et al. (1991) and Pignata et al. (1996), and by Lipson et al. (1996), respectively. Molecular characterizations of the deletions present in GM6936 and CH95-199 were also reported by Daw et al. (1996). Patient CH95-199 is designated as "P3" in Daw et al. (1996). The remaining two patients were referred to us after their deletions were detected by means of high-resolution cytogenetic analysis. All of the patients exhibit at least one of the classic features of DGS (cardiac defect, hypocalcemia, and/or immune defect). Clinical findings are summarized in table 1.

We first performed heterozygosity mapping by use of markers between loci D10S249 and D10S213. These sequence-tagged sites (STSs) correspond roughly to the cytogenetic location 10p15-10p12 (Chumakov et al. 1995). Three of the five patients had a single allele for the three most distal markers, D10S249, D10S591, and D10S189; this finding is consistent with a terminal deletion of 10p (table 2). The remaining two patients were heterozygous, at loci D10S249, D10S591, and D10S189, but had single alleles for a series of more centromeric markers (table 2); this finding is consistent with the presence of an interstitial deletion. As shown in table 2, there is no marker for which all patients have a single allele, which suggests that there is no common region-of-deletion overlap among these five patients.

To confirm the heterozygosity-mapping results, we performed FISH analysis on these five patients, using YACs from the region. All YACs were from the CEPH/Genethon megaYAC library, except 194G1, which was isolated from the smaller-insert CEPH library. FISH analysis confirmed and extended the results obtained from the heterozygosity tests: there is no common region of overlap among all five patients (fig. 1). Four of the five patients have a common region of deletion that includes the shortest region-of-deletion overlap (SRO) described in Daw et al. (1996). Two of these four patients, GM6936 and CH95-199 ("P3"), were included in Daw et al. (1996), and the other two do not narrow the SRO further. Patient CH92-092, although without a deletion for this SRO, does share an extensive region of deletion with the other two patients with terminal deletions. Further, it is possible that there is a small region-of-deletion overlap between CH92-092 and CH92-304. The endpoints of the deletions in these two patients could not be defined precisely because the YAC contig is not continuous from D10S1431 to D10S226. On the basis of the results presented here, it is not possible to attribute

Table 1

Clinical Features of Five Patients with 10p Deletions

Patient	Sex	Cardiac Defect	Hypocalcemia	Immune Defect	Cleft Palate	Facial Dysmorphia	Developmental Delay	Other[a]
CH92-092	F	−	+	+	−	−	+	Right renal hypoplasia, strabismus, high arched palate
CH92-304[b]	M	−	+	+	−	+	+	. . .
CH92-319	F	−	+	−	−	+	. . .[c]	Hearing loss, short neck, syndactyly (digits 3 and 4), long fingers
CH95-199[d,e]	F	+	−	+	+	+	+	GE reflux, finger contractures
GM6936[f]	F	−[g]	+	+	−	+	+	Ureteral reflux, recurrent UTI, short neck, mild pectus excavatum

NOTE.—Plus sign (+) = presence of the condition, and minus sign (−) = absence of the condition.
[a] GE = gastroesophageal; UTI = urinary tract infection.
[b] Reported by Monaco et al. (1991).
[c] Patient too young to be evaluated.
[d] Reported by Lipson et al. (1996).
[e] This patient is referred to as "P3" in Daw et al. (1996).
[f] Reported by Greenberg et al. (1986).
[g] Probable pulmonic branch stenosis.

the DGS phenotype to deletions of only one interval on chromosome 10p. Rather, these results suggest that there are at least two regions on chromosome 10p that, when deleted, can result in features seen in DGS.

After identification of two nonoverlapping regions of 10p loss, we screened cytogenetically normal DGS patients for submicroscopic deletions of 10p. These patients had been the subjects of molecular study and had been shown not to have deletions of 22q11.2. Samples for cytogenetic analysis were available from 11 patients who had the diagnosis of DGS and no detectable deletion of 22q11. These patients appear to have normal chromosome 10s by analysis with high-resolution G-banding. We performed FISH analysis on these 11 patients, using YACs from two deleted regions on chromosome 10p. YACs 916D6 and 959B9 map to the SRO defined by Daw et al. (1996), and YAC 944D12 maps to a second, more telomeric locus (see fig. 1). None of the patients we examined appeared to have deletions. However, because the YACs are large, it is possible that a small deletion within these regions would not have been detected. When a smaller deletion interval is defined, perhaps these patients can be reexamined.

In conclusion, we examined chromosome 10p deletions in five patients diagnosed with DGS. Our results indicate that there is no apparent common region of overlap between these five patients. Instead, these results suggest that, in addition to the SRO reported by Daw et al. (1996), there is a second, more telomeric region on chromosome 10p that, when deleted, can produce a

Table 2

Results of Heterozygosity Tests

	MARKER (POSITION)									
PATIENT	D10S249 (0 cM)	D10S591 (12.3 cM)	D10S189 (17.3 cM)	D10S1431	D10S226 (26.2 cM)	D10S465	D10S585 (28.9 cM)	D10S570 (32.1 cM)	D10S611	D10S213 (56.9 cM)
CH92-92	1	1	1	1	2	2	2	2	2	2
CH92-304	2	2	2	1	1	1	1	1	1	2
CH92-319	1	1	1	1	1	1	1	1	1	2
CH95-199	2	2	2	2	2	2	1	1	2	2
GM6936	1	1	1	1	1	1	1	2	2	2

NOTE.—Markers are named in order; the most distal markers are on the left. Map positions are given for those markers currently available from the Whitehead Institute/MIT Center for Genome Research. The numbers "1" and "2" indicate the number of alleles present at a particular locus. Markers that have been underlined are contained within the YACs shown in figure 1.

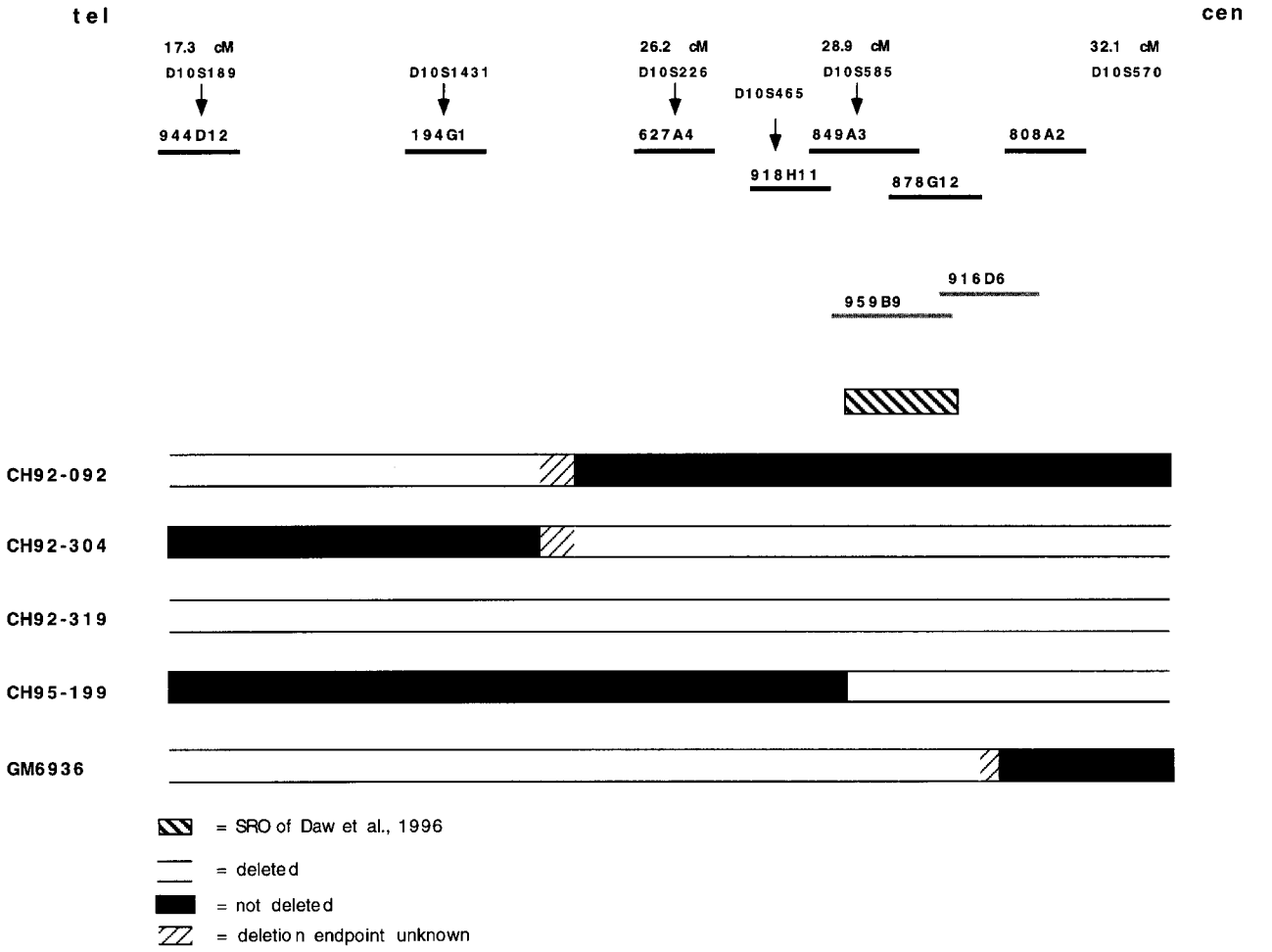

Figure 1 Diagram showing the regions-of-deletion overlap, as determined by the combination of heterozygosity tests and FISH analysis. At the top of the figure are YACs that were used for FISH analysis. Two of the three YACs used for the analysis of chromosomally normal patients, 959B9 and 916D6, are stippled, because the 10p-deletion patients were not tested with these two YACs. The STSs that are positive for a given YAC are shown. Map positions for several of the markers are also shown (taken from Hudson et al. [1995], with supplementary data from the Whitehead Institute/MIT Center for Genome Research, Human Genetic Mapping Project, data release 12 [July 1997]).

DGS phenotype. Alternatively, it is possible that either CH92-092 or CH95-199, the two patients who appear to have no region-of-deletion overlap, has a small internal deletion, rearrangement, or point mutation that maps within the region that is deleted in the other patients. Further characterization of these patients, as well as analysis of additional DGS patients with deletions on chromosome 10p, will enable us to distinguish between these possibilities. These findings also suggest that it is premature to screen for microdeletions of 10p in DGS patients in whom no 22q11 deletions have yet been identified.

One interesting feature of the patients examined here is that there is no obvious correlation between the phenotypic traits of the patients and the extent of the deletion. In particular, the patient (CH92-319) with the largest deletion exhibits one of the less severe phenotypes. Because of this observed variability, it is not possible to associate deletions in one region of the chromosome with a particular phenotype. The lack of a correlation between the size of a deletion and the phenotype is also observed in patients with deletions on chromosome 22, and may be a characteristic of haploinsufficiency disorders.

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