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Identification of Polymorphisms and Sequence Variants in the Human Homologue of the Mouse Natural Resistance–Associated Macrophage Protein Gene

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Summary

The most common mycobacterial disease in humans is tuberculosis, and there is evidence for genetic factors in susceptibility to tuberculosis. In the mouse, the *Bcg* gene controls macrophage priming for activation and is a major gene for susceptibility to infection with mycobacteria. A candidate gene for *Bcg* was identified by positional cloning and was designated "natural resistance-associated macrophage protein gene" (*Nramp1*), and the human homologue (*NRAMP1*) has recently been cloned. Here we report on (1) the physical mapping of *NRAMP1* close to *VIL* in chromosome region 2q35 by PCR analysis of somatic cell hybrids and YAC cloning and (2) the identification of nine sequence variants in *NRAMP1*. Of the four variants in the coding region, there were two missense mutations and two silent substitutions. The missense mutations were a conservative alanine-to-valine substitution at codon 318 in exon 9 and an aspartic acid–to-asparagine substitution at codon 543 in the predicted cytoplasmic tail of the NRAMP1 protein. A microsatellite was located in the immediate 5′ region of the gene, three variants were in introns, and one variant was located in the 3′ UTR. The allele frequencies of each of the nine variants were determined in DNA samples of 60 Caucasians and 20 Asians. In addition, we have physically linked two highly polymorphic microsatellite markers, *D2S104* and *D2S173*, to *NRAMP1* on a 1.5-Mb YAC contig. These molecular markers will be useful to assess the role of *NRAMP1* in susceptibility to tuberculosis and other macrophage-mediated diseases.

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Introduction

Tuberculosis is a major public health concern worldwide. About one-third of the world's population is infected with *Mycobacterium tuberculosis*, and 30 million people are expected to die in the next decade from tuberculosis (World Health Organization 1994). While environmental exposure to mycobacteria is necessary for the development of tuberculosis, intrinsic host factors are also important in determining the outcome of an infection with *M. tuberculosis*. For example, human twin studies show significantly greater concordance of disease among MZ twins compared with DZ twins of the same sex (Kallmann and Reisner 1943; Vogel and Motulsky 1986, pp. 213–214).

Genetic studies in the mouse have demonstrated that innate susceptibility to *M. bovis* (BCG), *M. lepraemurium*, *M. intracellulare*, and *M. avium*, as well as two nonmycobacterial species, *Salmonella typhimurium* and *Leishmania donovani*, is under the control of a single gene located in the proximal region of mouse chromosome 1 (Schurr et al. 1990*a*). This gene has been alternatively designated "*Lsh*," "*Ity*," or "*Bcg*" (Blackwell 1989). Susceptibility to infection is recessive to resistance. Resistant mice restrict the growth of the above-mentioned infectious agents in their reticuloendothelial organs, while rapid uncontrolled proliferation of injected pathogens occurs in susceptible mice. Analysis of the resistant and susceptible mice has implicated the mature tissue macrophage in resistance to infection and has led to the proposal that the *Bcg/Lsh/Ity* gene regulates macrophage priming for activation (Buschman et al. 1989; Blackwell et al. 1991).

A candidate gene for *Bcg*, originally designated "*Nramp*" (which we refer to as "*Nramp1*"), was isolated by positional cloning and was shown to be expressed in the macrophage (Vidal et al. 1993). Sequencing of the *Nramp1* gene from 27 inbred strains of mice showed that susceptibility to mycobacterial infection was associated with a nonconservative substitution of glycine to aspartic acid in the second putative transmem-

brane domain (Malo et al. 1994). A human homologue of the mouse gene has recently been cloned, and the exon-intron organization has been determined for 16 exons (Cellier et al. 1994). In the present communication we report on the localization of the human *NRAMP1* gene (originally designated "*NRAMP*") and on the identification of nine sequence variants. These variants should be useful for the genetic analysis of susceptibility to tuberculosis and other nonparasitic diseases of the macrophage in humans.

Material and Methods

Somatic Cell Hybrids

The National Institute of General Medical Sciences (NIGMS) mapping panel 2 (Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ), which is made up of 24 hybrid cell lines that contain individual chromosomes as their only human complement (except for hybrid cell lines 07299 and 10478, which contain human chromosomes 1 and X, and 4 and 20, respectively), was used for the chromosomal assignment of *NRAMP1*. The somatic cell hybrids were grown according to the conditions specified by the supplier. After one passage of cells, DNA was extracted according to standard protocols using proteinase K treatment and phenol/chloroform extraction (Sambrook et al. 1989). Primers 2F (5′-CCCTCCCTTAATGAAGGATC-3′) and 4R (5′-CCACCACTCCCCTATGAGGTG-3′), which hybridize to the 5′ and 3′ exon-intron boundaries of exons 5 and 6 of *NRAMP1*, were used to amplify DNA from the hybrid panel.

Construction of a YAC Contig in the Vicinity of NRAMP1

We previously identified close to 2,000 chromosome 2–specific YAC clones from the CEPH Mark I YAC library by the interspersed repetitive sequence (IRS)–PCR approach (Liu et al., in press). These YACs were subsequently used for sequence-tagged site (STS) screening with a set of expressed sequence tags and microsatellite markers including primers specific for *NRAMP1* and *VIL* (Rousseau-Merck et al. 1988; Lu-Kuo et al. 1993) and *D2S104* and *D2S173* (Spurr et al. 1994). The primer sequences for *VIL* are Vil-3 (5′-TACAGTGAGGACCCATGTGC-3′) and Vil-4 (5′-TCTGTTAAGGGTTTCGGCTC-3′). YAC clones positive for *NRAMP1* and *VIL* were used for chromosome walking in order to construct a YAC contig. The protocols for preparing YAC DNA, amplifying YAC DNA with IRS-PCR primers, obtaining walking probes, and screening of the CEPH Mark I YAC library are described elsewhere (Liu et al., in press). The Baylor College YAC library was screened by Dr. G. Chinault with the Vil-3/4 primers. The mega YACs, 829_e_12 and 872_h_8, were isolated from a copy of the CEPH YAC libraries available in our laboratory.

Human Genomic DNA Samples

DNA samples were obtained from Epstein-Barr virus (EBV)–transformed cell lines of nine Native Canadians who are members of an extended multiplex tuberculosis family (Mah and Fanning 1991), from EBV-transformed cell lines of 27 individuals from 12 tuberculosis families from Hong Kong, and from white blood cells of 60 unrelated Caucasians and 20 unrelated Asians. High-molecular-weight genomic DNA was extracted from EBV-transformed cells and white blood cells, according to standard protocols (Sambrook et al. 1989), was quantified by standard UV spectroscopy, and was stored at 4°C in Tris-EDTA (pH 8.0).

Molecular Cloning

Subcloning of restriction fragments containing the 5′ end of the *NRAMP1* gene was done according to standard procedures by digestion of cosmid DNA with restriction enzymes and size separation of restriction products on agarose gels. A 1.6-kb *Pst*I fragment overlapping the 5′ region of the *NRAMP1* gene was identified by Southern analysis using a partial cDNA clone as a probe. The 1.6-kb fragment was extracted from agarose by using the Qiagen DNA purification kit and was subcloned in pBluescript KS+; fragment ends were sequenced by the dideoxy-chain termination method (Sanger et al. 1977) using the Sequenase kit (U.S. Biochemical) and T3- or T7-specific sequencing primers. Sequence analysis revealed the presence of a microsatellite ~150 bp into the 5′ region of the fragment. A 5′ PCR primer (5′-GACATGAAGACTCGCATTAG-3′) was designed and, together with the 3′ primer (5′-TCAAGTCTCCACCAGCCTAGT-3′), was used to test for microsatellite length variation. To allow visualization of the length polymorphism, amplification was done with a ^{33}P-labeled 5′ primer. The amplification products were digested with *Nco*I, and end-labeled fragments were sized on standard sequencing gels. The ligation and transformation of recombinant vectors into DH5α, as well as the selection and growth of recombinant bacteria, were done according to standard protocols (Sambrook et al. 1989). Plasmid DNA was isolated using the QIA prep-spin plasmid kit (Qiagen) and was used for dideoxy sequencing of the inserts. T-vector was prepared according to the protocol described by Marchuk et al. (1991), with minor modifications.

Single-Strand Conformation Analysis (SSCA)

The PCR reaction mix used for all SSCA PCR reactions contained 200 ng of genomic DNA, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, 0.07 mM dATP, 16 pmol of each primer, 1 mM $MgCl_2$, 10 mM Tris-

HCl (pH 8.0), 0.05% Tween-20, 0.05% NP40, 0.2 units of *Taq* DNA polymerase (Perkin Elmer Cetus), and 8 μCi of ^{35}S-dATP in a total reaction volume of 15 μl. The conditions for amplification were a single denaturation step of 3 min at 94°C, followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C–63°C primer-specific annealing temperature), and extension (1 min at 72°C), and a final extension step of 7 min at 72°C. The primers used for amplification of *NRAMP1* gene segments for which sequence variants were identified are listed in table 1. All SSCA variants were detected on 6% polyacrylamide/5% glycerol sequencing gels run at room temperature at a constant current of 28 mA for 5 h.

DNA Sequencing

PCR amplifications were carried out in 20-μl reaction volumes containing 100 ng of genomic DNA, 50 mM Tris-HCl (pH 8.3), 1.8 mM $MgCl_2$, 0.05% Tween-20, 0.05% NP40, 187.5 μM of each dNTP (Promega), 0.4 μM of each primer, and 1 unit of *Taq* polymerase (Perkin Elmer Cetus). Parameters for thermocycling were as follows: incubation for 4 min at 94°C, followed by 33 cycles of 1 min at 94°C, 30 s at 59°C, 1 min at 58°C, 1 min 40 s at 72°C, and a final extension step of 10 min at 72°C. For direct sequencing, the genomic PCR-amplification products were diluted 1:15 in H_2O without further purification and were subjected to thermal cycle sequencing using the Hot Tub DNA Sequencing System (Amersham). For clone sequencing, 1–2 μg of purified plasmid DNA were used for dideoxy sequencing using the Sequenase kit (U.S. Biochemical) with T3- or T7-specific sequencing primers. For each ligation, at least four clones were sequenced in both sense and antisense orientation.

Southern Analysis

Five micrograms of genomic DNA or 1 μg of cosmid DNA was digested with restriction enzymes, with conditions and quantities recommended by the supplier (New England BioLabs). The digested DNA samples were size separated on agarose gels, were transferred onto Hybond nylon membranes (Amersham), and hybridized with probes labeled to high specific activity (5×10^8 cpm/μg DNA) with α-^{32}P-dATP (specific activity >3,000 Ci/mmol; Amersham [Feinberg and Vogelstein 1983, pp. 213–214]) under high-stringency conditions as described elsewhere (Schurr et al. 1989). All *NRAMP1*-derived probes were preannealed with 400 μg of human placental DNA in 1 ml of hybridization medium for 1 h at 65°C.

Restriction-Site Analysis

Analysis of the restriction-endonuclease recognition sites in DNA sequences was carried out with the MacVector software package, version 4.1 (International Biotechnologies). Restriction-endonuclease digestions were done by using 5–10 U of enzyme per PCR reaction, under conditions recommended by the supplier (New England BioLabs). Restriction-enzyme digestion products were resolved by electrophoresis on 12% polyacrylamide gels stained with ethidium bromide; a 100-bp ladder was used as a size marker (Pharmacia).

Results

Physical Mapping of NRAMP1

The chromosomal localization of *NRAMP1* was determined by PCR amplification of genomic DNA extracted from the monochromosomal somatic cell hybrids of NIGMS panel 2 by using primers 2F and 4R. As shown in figure 1, the predicted PCR product of ~700 bp was amplified in human genomic DNA and in DNA obtained from hybrid 10826B, which contains human chromosome 2 as its only human chromosome. The fact that only one clearly identifiable band was amplified in human genomic DNA and in hybrid 10826B demonstrated the *NRAMP1* specificity of the primers and showed that *NRAMP1* is located on chromosome 2.

In the mouse, *Nramp1* is closely linked to *Vil* (Malo et al. 1993). To estimate the physical distance between the human homologues of these genes, we carried out STS screening of a previously generated chromosome 2 CEPH Mark I YAC sublibrary, with a set of STS markers including primers specific for *NRAMP1, VIL,* and 50 microsatellite markers. Three YAC clones, 2A3A03 (96_a_3), 3G1H06 (211_h_6), and 4G6F03 (330_f_3), were identified that carried both *NRAMP1* and *VIL* (the CEPH YAC designations are given in parentheses). STS screening of the Baylor College YAC library, with primers specific for *VIL,* identified a YAC clone, B2, with an insert size of 200 kb. PCR analysis with primers specific for *NRAMP1* revealed that B2 also contained the *NRAMP1* sequence, suggesting a maximum distance of 200 kb separating these two loci. Thus, *NRAMP1* can be assigned to chromosome region 2q35, since *VIL* had previously been assigned to this chromosomal region (Rousseau-Merck et al. 1988; Lu-Kuo et al. 1993). A YAC contig of the region encompassing *NRAMP1* and *VIL* was constructed by chromosome walking in the CEPH Mark I YAC library by using IRS-PCR probes derived from YAC clones that included *NRAMP1* and *VIL* (fig. 2). Among the YAC clones identified by chromosome walking, clone 4L3E04 (312_e_4) tested positive for microsatellite markers *D2S104* and *D2S173.* We then searched Généthon's GenomeView database via the Internet (URL is http://www.genethon.fr) for *D2S173* and *D2S104,* and we identified a 210-kb YAC clone (829_e_12) that was positive for both markers. In

Table 1

NRAMP1 Polymorphisms and Sequence Variants

Name	Nucleotide/Amino Acid Change	Primers, 5′ to 3′ (PRC Product Size)	Polymorphic Enzyme	Allele[a]	Allele Frequency: Caucasian (n = 120)	Allele Frequency: Asian (n = 40)
$(GT)_n$	Microsatellite, 5′ to exon 1	GAC ATG AAG ACT CGC ATT AG TCA AGT CTC CAC CAG CCT AGT (~800 bp)	*Nco*I	Allele 1 = 286 bp Allele 2 = 288 bp Allele 3 = 290 bp	.73 .25 .02	.85 .10 .05
274C/T	C or T at nucleotide 274: TTC or TTT (Phe) at codon 66 in exon 3	TGC CAC CAT CCC TAT ACC CAG TCT CGA AAG TGT CCC ACT CAG (216 bp)	*Mnl*I	Allele 1 (T) = 167, 37, and 12 bp Allele 2 (C) = 102, 65, 37, and 12 bp	.27 .73	.12 .88
469+14G/C	G or C at nucleotide + 14 of intron 4	TCT CTG GCT GAA GGC TCT CC TGT GCT ATC AGT TGA GCC TC (624 bp)	*Apa*I	Allele 1 (G) = 624 bp Allele 2 (C) = 455 and 169 bp	.73 .27	.92 .08
577−18G/A	G or A at nucleotide −18 of intron 5	CTG GAC CAG GCT GGG CTG AC CCA CCA CTC CCC TAT GAG GTG (146 bp)	*Msp*I	Allele 1 (A) = 146 bp Allele 2 (G) = 125 and 21 bp	0 1.0	.02 .98
823C/T	C or T at nucleotide 823; GGC or GGT (Gly) at codon 249 in exon 8	CTT GTC CTG ACC AGG CTC CT CAT GGC TCC GAC TGA GTG AG (234 bp)	*Nar*I	Allele 1 (T) = 234 bp Allele 2 (C) = 135 and 99 bp	.02 .98	.15 .85
A318V	C or T at nucleotide 1029; GCG (Ala) or GTG (Val) at codon 318 in exon 9	TCC TTG ATC TTC GTA GTC TC GGC TTA CAG GAC ATG AGT AC (232 bp)	*Bso*FI	Allele 1 (Val) = 232 bp Allele 2 (Ala) = 171 and 61 bp	0 1.0	0 1.0
1465-85G/A	G or A at nucleotide −85 of intron 13	GCA AGT TGA GGA GCC AAG AC ACC TGC ATC AAC TCC TCT TC (241 bp)	*Bsr*I	Allele 1 (A) = 142, 75, and 24 bp Allele 2 (G) = 102, 75, 40, and 24 bp	.38 .62	.35 .65
D543N	G or A at nucleotide 1703; GAC (Asp) or AAC (Asn) at codon 543 in exon 15	GCA TCT CCC CAA TTC ATG GT AAC TGT CCC ACT CTA TCC TG (240 or 244 bp)	*Ava*II	Allele 1 (Asn) = 201 and 39 bp Allele 2 (Asp) = 126, 79, and 39 bp	.01 .99	.18 .82
1729+55del4	Deletion of TGTG in the 3′ UTR (55 nt 3′ to the last codon in exon 15)	Same as for D543N	*Fok*I	Allele 1 (−TGTG) = 240 bp Allele 2 (+TGTG) = 211 and 33 bp	.01 .99	.18 .82

[a] All alleles show Mendelian segregation (data not shown).

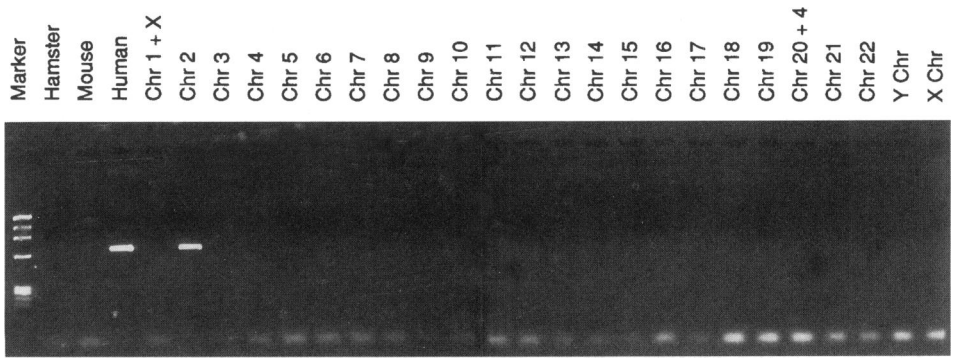

Figure 1 PCR analysis of monochromosomal somatic cell hybrids. Human, hamster, and mouse genomic DNAs were used as controls. *Hae*III-digested ΦX174 DNA was used as the size marker.

addition, we identified a mega YAC clone (872_h_8), from the Quickmap database (Cohen et al. 1993), that was positive for *D2S173*. DNA from this YAC clone was tested using primers specific for *VIL* and *NRAMP1*, and positive amplification was observed for both markers. Taken together, the results of these experiments demonstrated that *VIL*, *NRAMP1*, *D2S173*, and *D2S104* are physically linked on a chromosomal segment with an estimated size of 1.5 Mbp (fig. 2).

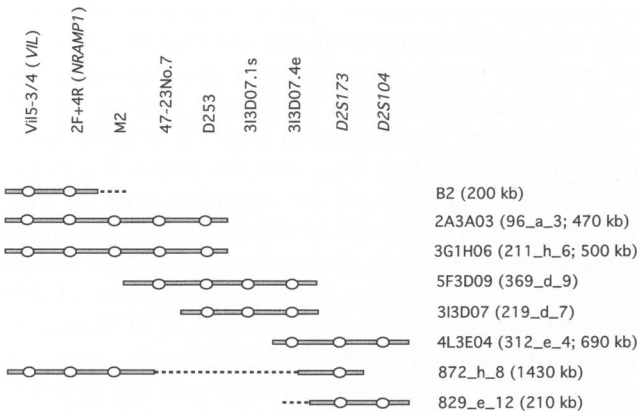

Figure 2 Genomic cloning of the region that includes *NRAMP1* and *VIL*. A likely order of markers and loci is given at the top of the figure. Only the YAC clones belonging to a minimum tiling path and five of the chromosome-walking probes (M2, 47-23No.7, D253, 3I3D07.1s, and 3I3D07.4e) are shown; however, the tiling path and the order of probes were derived from all the data. The order of probes has not been independently established, and other orders are likely. YACs, which are not drawn to scale, are represented as shaded bars; the names of YAC clones are given to the right of the bars. The CEPH YAC designations for CEPH Mark I YAC clones and the known sizes of YACs are indicated in parentheses. An open ellipse on a bar indicates that the YAC clone was identified by the corresponding probe. Dotted lines on three of the YACs (B2, 872_h_8, and 829_e_12) indicate that these YACs were not tested for the presence of the chromosome-walking probes. Probe M2 is an IRS walking probe derived from clone 872_h_8 and was used to screen the CEPH Mark I YAC library. The estimated size of the contig is 1.5 Mb.

Identification of Polymorphisms and Sequence Variants in the NRAMP1 *Gene*

We used several methods to search for mutations in the *NRAMP1* gene in genomic DNA of a panel of individuals that included 9 members of a Native Canadian kindred and 24 individuals from 11 Hong Kong families. Primers for 15 of the 16 exons were derived in the process of identifying the exon-intron organization of *NRAMP1* (Cellier et al. 1994). The majority of these primers were located within intronic sequences, to allow amplification of the exon-intron splice consensus sequences. The DNA samples were screened by SSCA of all *NRAMP1* exons except for exon 1. Direct sequencing revealed four SSCA variants that were due to mutations in the coding region and one that was in intronic sequences. Two variants were predicted to cause amino acid substitutions: A318V, an alanine-to-valine substitution at codon 318 in exon 9; and D543N, an aspartic acid–to-asparagine substitution at codon 543 in exon 15 (table 1). Two variants were silent nucleotide substitutions: 274C/T in codon 66 (phenylalanine) in exon 3 and 823C/T in codon 249 (glycine) in exon 8. The intronic variant, 577−18G/A, was a G-to-A nucleotide substitution near the 3′ end of intron 5. The mutations have been designated according to the nomenclature suggested by Beaudet and Tsui (1993); the nucleotides and codons were numbered according to the sequence of GenBank accession number L32185.

In the process of DNA sequencing to determine the sequence changes of the SSCA variants, we found two additional mutations: 1465−85G/A, a G-to-A nucleotide substitution near the 3′ end of intron 13; and 1729+55del4, a 4-bp TGTG deletion located 55 nt downstream of the last codon in exon 15. This 4-bp deletion creates a heteroduplex of the PCR products of individuals who are heterozygous for the insertion/deletion polymorphism. By Southern analysis, an *Apa*I RFLP was detected (allele 1 = 5 kb; and allele 2 = 4 kb + 1 kb) by using an *NRAMP1* cDNA probe. Sequential

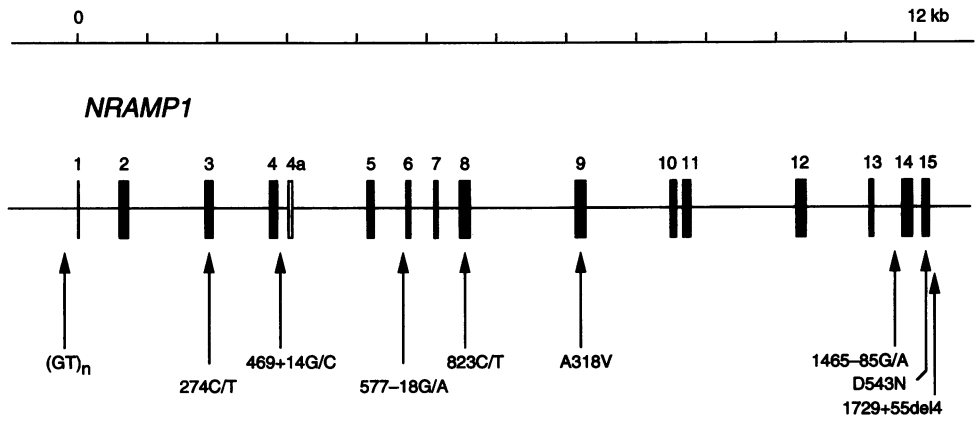

Figure 3 Location of polymorphisms and sequence variants in *NRAMP1*. The schematic diagram of the exon-intron organization was adapted from Cellier et al. (1994).

hybridization using *NRAMP1* exon probes to DNA from individuals heterozygous for the RFLP, along with direct sequencing, identified the polymorphism, designated "469+14G/C," as a G-to-C nucleotide substitution in intron 4. Finally, a microsatellite was identified on a 1.6-kb genomic *Pst*I fragment overlapping exons 1 and 2 of *NRAMP1* and was located ~800 bp 5′ to the *NRAMP1* start codon. The structure of the repeat was determined to be $(GT)_7AC(GT)_5AC(GT)_9$. We did not find any variants in exon 4a, which is an alternatively spliced exon (fig. 3) and codes for an inverted Alu Sx element (Jurka and Milosavljevic 1991), either by direct sequencing of 6 individuals or by SSCA of 33 individuals composing our screening panel and of 24 unrelated Caucasians.

Analysis of the sequence variants revealed that the nucleotide changes either created or destroyed a recognition site for restriction endonucleases (except for the microsatellite), which allowed us to develop a PCR assay for each of the variants (table 1). YAC clone 2A3A03 was used as a positive control for specificity of PCR assays of *NRAMP1* sequences. To determine if the sequence variants were polymorphic, we tested DNA samples from 60 Caucasians and 20 Asians. The D543N missense mutation in exon 15 and the 4-bp deletion, 1729+55del4, in the 3′ UTR appear to be in absolute linkage disequilibrium. The allele frequencies differed between the two ethnic groups, for four polymorphisms: 469+14G/C, 823C/T, D543N, and 1729+55del4 (Fisher exact test; $P < .05$). The 1465−85G/A polymorphism in intron 13 had the highest expected heterozygosity, in both the Caucasian and Asian samples (.47 and .46, respectively).

The observed allele counts for the nine variants are shown in table 2, for 22 unrelated members of 12 Hong Kong families and for 8 unrelated members of the Canadian kindred. We detected no significant association between tuberculosis disease status and the *NRAMP1* alleles in the small sample from either of these groups (Fisher exact test; $P > .05$).

Discussion

The evolutionary conservation of chromosomal segments between mouse and human is well documented, and comparative mapping has been employed successfully for the identification of a number of genes or disease loci (for reviews, see Nadeau et al. 1992; Liu et al. 1993; Searle et al. 1994). We have shown previously that a 35-cM chromosomal segment that includes *Bcg* on proximal mouse chromosome 1 is conserved in human chromosome region 2q33-qter (Schurr et al. 1990*b*). Among the conserved loci, *Vil* was the locus most closely linked to *Bcg*, and the physical distance between *Vil* and *Nramp1*, the candidate gene for *Bcg*, was determined to be ~50 kb (Malo et al. 1993). In the present study, we have physically mapped the human *NRAMP1* gene to within a maximum distance of 200 kb of *VIL*, in chromosome region 2q35, by PCR analysis of somatic cell hybrids and by YAC cloning. Both this chromosomal location of *NRAMP1* and the observed high sequence similarity with mouse *Nramp1* (Cellier et al. 1994) strongly argue against the possibility that human homologues of either the mouse chromosome 17 *Nramp*-related sequence (Dosik et al. 1994) or the mouse chromosome 15 *Nramp2* gene (Gruenheid et al., in press) were amplified in our experiments. A possible human homologue of *Nramp*-related sequence is expected to map to chromosome region 6q27 (Dosik et al. 1994), and human *NRAMP2* has been mapped, by in situ hybridization, to 12q13 (S. Vidal and P. Gros, personal communication). Furthermore, the estimated maximum distance between *VIL* and *NRAMP1* on chromosome 2q35 was similar to the observed physical distance between *Nramp1* and *Vil* on mouse chromosome 1. Our results, therefore, support the validity of mouse-human

Table 2

Distribution of Alleles of *NRAMP1* Polymorphisms and Sequence Variants in Unrelated Individuals from Tuberculosis Families

		Sequence Variant																	
		$(GT)_n$			274C/T		469+ 14G/C		577− 18G/A		823C/T		A318V		1465− 85G/A		D543N		1729+ 55del4	
Disease Status[a]	No. of Individuals	1	2	3	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Hong Kong families:																				
TB(+)	9	18	0	0	0	18	18	0	0	18	2	16	1	17	2	16	3	15	3	15
TB(−)	13	22	2	2	2	24	24	2	2	24	1	25	0	26	8	18	3	23	3	23
Canadian kindred:																				
TB(+)	3	4	2	0	2	4	4	2	0	6	1	5	0	6	3	3	1	5	1	5
TB(−)	5	8	2	0	1	9	9	1	0	10	2	8	0	10	3	7	3	7	3	7

[a] TB(+) = affected with tuberculosis; and TB(−) = not affected with tuberculosis.

comparative genome analysis on the level of physical maps.

We have identified nine sequence variants in *NRAMP1*. Seven of these variants are silent substitutions or are located in introns or UTRs of the gene. Only two of the sequence variants are predicted to cause amino acid substitutions: an alanine-to-valine substitution at codon 318 (A318V), which changes an amino acid located between the predicted fifth and sixth transmembrane domain, and an aspartic acid–to-asparagine substitution at codon 543 (D543N) in the predicted cytoplasmic carboxyl-terminal end of the protein. The alanine-to-valine substitution is a conservative substitution and is not expected to influence the function of the NRAMP1 protein. However, the substitution of a negatively charged aspartic acid by an uncharged asparagine residue could affect function of the protein. The homologous codon at the corresponding position in the mouse Nramp1 protein codes for a glutamic acid (Vidal et al. 1993), another amino acid with a negatively charged side chain. This provides support for the functional relevance of a negatively charged residue at codon 543. Additional knowledge of the function of NRAMP1 protein is required for inference of the effects of these missense mutations.

Heterozygosities for the nine polymorphisms detected in the *NRAMP1* gene were low or moderate, with the highest expected value, .47, being seen for the 1465 −85G/A polymorphism in intron 13. Two microsatellite markers, *D2S104* and *D2S173*, were found to be closely linked to the *NRAMP1* region by physical mapping. The expected heterozygosities of *D2S104* and *D2S173* were .72 and .70, respectively (Spurr et al. 1994). The combination of these two highly informative microsatellite markers in the vicinity of the *NRAMP1* gene and the *NRAMP1* sequence variants should greatly facilitate the study of the role of the *NRAMP1* gene in disease susceptibility.

"The problem of the extent to which genetic factors enter into susceptibility to tuberculosis is one of the oldest in human genetics" (Neel and Shull 1954, p. 292). Infection by *Mycobacterium tuberculosis* results in a wide spectrum of phenotypic manifestations, ranging from skin-test sensitivity to purified protein derivative in individuals without detectable tubercle bacilli to fully developed pulmonary disease characterized by multibacillary granuloma that may cause death if the infection is left untreated (Lenzini et al. 1977). The clinical phenotype is almost certainly modulated by acquired immune responses. Among immunologically competent individuals, the quality and quantity of the specific antitubercle immune response depend on genetic factors, prior exposure to *M. tuberculosis* or other related environmental mycobacteria, and/or BCG vaccination (Dubos and Dubos 1987; Sifford and Bates 1991). Thus it appears from the complex etiology of tuberculosis that inadequate classification of tuberculosis patients with respect to clinical, vaccination, and exposure history will result in both etiologic heterogeneity and loss of power either to detect a major susceptibility gene or to confirm a candidate gene such as *NRAMP1*. We failed to detect a statistically significant association between *NRAMP1* variants and tuberculosis disease status in two samples of unrelated individuals. This could indicate that there is no predominant predisposing mutation in these families. However, our sample sizes were small, and both cases and controls were selected on the basis of being parents or grandparents of multiplex tuberculosis families. In addition, incomplete penetrance and sporadic cases of tuberculosis would further reduce the power to detect an association. A recessive susceptibility model with liability classes based on clinical status, age, exposure, and BCG vaccination (Boothroyd 1994) will be used in linkage analysis of *NRAMP1* and tuberculosis susceptibility. The *NRAMP1* variants described in the present report will also be useful for the genetic analysis of human

susceptibility to leprosy, typhoid fever, and Kala Azar, in analogy to susceptibility to intracellular parasites under *Bcg/Lsh/Ity* control in the mouse (for review, see Blackwell 1989; Blackwell et al. 1991; Shaw et al. 1993).

Numerous studies have established mouse *Nramp1* as an important regulatory element in the pathways of macrophage differentiation (Buschman et al. 1989; Blackwell et al. 1991). This suggests human *NRAMP1* as a candidate disease gene for a large number of genetic defects involving the macrophage, most notably autoimmune diseases (Ivanyi 1994). For example, genetic studies in NOD mice have mapped a diabetes susceptibility gene to the vicinity of *Bcg* (*Nramp1*) on proximal mouse chromosome 1 (Cornall et al. 1991), suggesting that human *NRAMP1* may act as a diabetes susceptibility gene. In addition, *NRAMP1* may play a role in the efficacy of BCG immunotherapy of patients with bladder cancer. Of the patients who suffered from superficial bladder cancer and were eligible for BCG immunotherapy, only 30%–50% responded to BCG immunotherapy (Morales et al. 1992). Failure of BCG treatment has been linked to an inefficient inflammatory response to instilled BCG (Torrence et al. 1988), which implies that *NRAMP1* may control the response to BCG immunotherapy. Thus, the study of *NRAMP1* expression and mutation analysis can be applied to a wide range of human diseases in addition to infectious diseases.

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