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ORIGINAL ARTICLE

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Homozygosity and linkage disequilibrium mapping of autosomal recessive distal myopathy (Nonaka distal myopathy)

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Abstract Autosomal recessive distal myopathy or Nonaka distal myopathy (NM) is characterized by its unique distribution of muscular weakness and wasting. The patients present with spared quadriceps muscles even in a late stage of the disease. The hamstring and tibialis anterior muscles are affected severely in early adulthood. We have localized the *NM* gene to the region between markers D9S319 and D9S276 on chromosome 9 by linkage analysis. To further refine the localization of the *NM* gene, we conducted homozygosity and linkage disequilibrium analysis for 14 patients from 11 NM families using 18 polymorphic markers. All of the patients from consanguineous NM families were found to be homozygous for six markers located within the region between markers D9S2178 and D9S1859. We also provided evidence for significant allelic associations between the *NM* region and five marker loci. Examination of the haplotype analysis identified a predominant ancestral haplotype comprising the associated alleles 199-160-154-109 (marker order: D9S2179-D9S2180-D9S2181-D9S1804), present in 60% of *NM* chromosomes and in 0% of parent chromosomes. On the basis of the data obtained in this study, the majority of *NM* chromosomes were derived from a single ancestral founder, and the *NM* gene is probably located within the 1.5-Mb region between markers D9S2178 and D9S1791.

Key words Quadriceps-sparing myopathy · Hereditary inclusion body myopathy · Distal myopathy with rimmed vacuole formation · Ancestral haplotype · Founder

Introduction

Autosomal recessive distal myopathy is a slowly progressive muscular disorder known as Nonaka distal myopathy (NM) in Japan (Nonaka et al. 1981). The profile of NM includes muscular weakness and wasting, which is prominent in the tibialis anterior and hamstring muscle in early adulthood. The spared quadriceps muscle even in late adulthood is a

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unique feature of this myopathy. The muscle biopsy specimen discloses a vacuolar myopathy without inflammation (Matsubara and Tanabe 1982; Nonaka and Engel 1985, 1998; Nonaka 1999; Mizusawa et al. 1987; Sunohara et al. 1989; Barohn et al. 1998; Illa 2000).

These clinical and pathological characteristics are strikingly similar to those of hereditary inclusion body myopathy (hIBM or quadriceps-sparing myopathy)(Argov and Yarom 1984). Askanas and Engel (1995) suspected that these myopathies were the same entity. Thus far, the molecular background of these myopathies remains elusive, although several research groups have performed genetic and linkage analysis on these myopathies as well as on other types of distal myopathies (Laing et al. 1995; Ahlberg et al. 1998a, b; Haravuori et al. 1998).

Murakami et al. (1995) and Askanas and Engel (1995, 1998) showed that muscle fibers of patients with NM and hIBM show accumulation of beta-Amyloid protein, as do the brains of patients with Alzheimer disease. Mutations in exons 16 and 17 of the beta-amyloid precursor protein (βAPP) gene on chromosome 21 were identified in patients with early onset familial Alzheimer disease (Mullan et al. 1992; Levy et al. 1990). Sivakumar et al. (1995) investigated the presence of similar mutations in exon 16 and exon 17 of βAPP in patients with hIBM by direct sequencing. However, no mutation was observed in either exon, and they speculated that accumulation of βAPP was an epiphenomenon unrelated to the pathogenesis of hIBM.

By applying linkage analysis, Mitrani-Rosenbaum et al. (1996) mapped the disease locus responsible for hIBM in Persian-Jewish families to chromosome 9p1-9q1. Extended work by Argov et al. (1997) demonstrated that the gene for hIBM with spared quadriceps was linked to chromosome 9p1-9q1 in families of Afghani Jews, Iraqi Jews, and non-Jews originating from India. By contrast, no linkage was shown between the disease locus and chromosome 9 in a French-Canadian hIBM family in which affected individuals showed white-matter involvement. The results suggested that hIBM encompasses several syndromes (Askanas and Engel 1995).

Ikeuchi et al. (1997) also used linkage analysis to localize the *NM* gene to a 23.3-cM interval defined by polymorphic markers D9S319 and D9S276 on chromosome 9. Interestingly, the candidate region for the *NM* and *hIBM* genes overlapped in a 15-cM interval between markers D9S165 and D9S276, indicating that both disorders might result from allelic mutation of the same gene (Askanas 1997).

In a previous study by Ikeuchi et al. (1997), no linkage disequilibrium was found between the *NM* region and marker loci. If linkage disequilibrium between the disease locus and marker loci can be detected, a powerful approach by which to narrow the critical region toward which positional cloning efforts should be directed will be available. Here we describe homozygosity and linkage disequilibrium mapping in order to greatly refine the assignment of the *NM* gene by using newly developed polymorphic markers, and we present the evidence for linkage disequilibrium between the *NM* region and several marker loci.

Subjects and methods

Patients and families

Linkage has been shown between the disease locus and chromosome 9 in 2 of 11 families (families 6 and 7) (Ikeuchi et al. 1997). The other 9 families who permitted analysis of DNA samples were not previously reported. Thus, 11 pedigrees with 14 NM patients and 25 unaffected family members (17 parents and 8 siblings) were included in this study. All of the families were Japanese, and consanguinity was present in 6 of 11 families. The clinical history of living members was ascertained through interviews, examinations, and medical records. The affection status of deceased members was determined from medical records or reports from other family members.

Clinical diagnosis of NM was established by at least one neurologist based on clinical course, family history, and neurological examinations, giving special attention to the presentation of the predominantly affected tibialis anterior muscle and the spared quadriceps muscle. Affection status was confirmed by the presence of the characteristic inclusion bodies in a muscle biopsy specimen. Unaffected individuals in this study had been evaluated by at least one neurologist.

One symptomatic patient (family 1-6) whose muscle biopsy specimen lacked typical inclusion bodies was considered to have NM on the basis of the presence of typical clinical symptoms and the fact that a clinically symptomatic sibling showed characteristic inclusion bodies in his muscle biopsy specimen. Except for this patient, the biopsied specimens of all affected subjects showed typical inclusion bodies.

Another symptomatic patient (family 4-4), in whom onset occurred at age 15, showed relatively rapid progression of muscle atrophy. This subject was included in this study because clinical and pathological studies confirmed that he had NM.

Genomic DNA preparation

Twenty milliliters of anticoagulated venous blood was collected from each participating living family member after informed consent had been obtained. Red blood cells were lysed by use of hypotonic osmotic shock for 1 min. Lymphocytes were harvested and then digested with sodium dodecyl sulfate and proteinase K overnight at 37°C. Subsequently, standard phenol/chloroform extraction followed by ethanol precipitation was used to prepare genomic DNA.

Development of new microsatellite markers

New polymorphic DNA markers including dinucleotide and tetranucleotide repeats were searched for in the human genome sequence database (http://www.ncbi.nlm.nih.gov/). After the primer sets were designed, variations in dinucleotide- and tetranucleotide-repeat numbers were examined

by polymerase chain reaction (PCR) amplification of the genomic DNA of 44 unrelated Japanese individuals. Highly polymorphic markers were then selected for homozygosity mapping and linkage disequilibrium analysis.

Genotyping

Eighteen polymorphic microsatellite markers, which map the region between markers D9S1678 and D9S273 and cover an estimated region of 15 cM on chromosome 9p1, were used to narrow the *NM* region. Nine primer sequences for the markers used have been published (Dib et al. 1996). The additional markers were newly generated in this study, and five important primer sequences are described in Table 1. Genotyping was performed by use of 6-FAM (Applied Biosystems, Tokyo, Japan) labeled primers and an ABI 310 Genetic Analyzer (Applied Biosystems) equipped with GeneScan software (Applied Biosystems).

PCR amplification was performed in a final volume of 12.5 μl containing of 10 ng of genomic DNA, 2.5 μmol of each primer, 0.75 units of Taq polymerase (Takara, Tokyo, Japan), 200 μmol each of deoxyribonucleotide triphosphate, and reaction buffer provided by the company (Takara). After denaturation at 95°C for 5 min, samples were subjected to 30 cycles of 30 s each at 95°C for denaturing, 30 s at the optimum annealing temperature, and 3 min at 72°C for extension, followed by a final extension at 72°C for 7 min.

After amplification, 0.5 μl of the reaction mixture was mixed with 10 μl of deionized formamide (Amresco, Solon, OH, USA) and 0.5 μl of the TAMRA-labeled molecular marker (GeneScan 500, Applied Biosystems). The mixed samples were denatured at 95°C for 10 min and immediately chilled on ice. The samples were then electrophoresed using an ABI 310 Genetic Analyzer. The amplified DNA fragments were analyzed by GeneScan software.

Ratio of homozygosity

To assess the statistical significance of associations, the percentage of homozygotes in patients versus that in unaffected relatives in consanguineous families was compared using standard χ-squared formulas, along with the Yates correction if the size of any of the expected classes was fewer than five samples.

Linkage disequilibrium analysis

Differences in the overall distribution of alleles on disease-bearing and normal chromosomes were tested by standard χ-squared formulas, along with the Yates correction if the size of any of the expected classes was fewer than five samples.

Allele counting prior to the linkage disequilibrium analysis was performed as follows:

In affected offspring of consanguineous marriages, if the marker genotypes were homozygous, only a single marker allele was counted in determining the marker allele frequency on the disease chromosomes. For individuals from nonconsanguineous marriages, or for individuals from consanguineous marriages where the marker locus was heterozygous, both marker alleles were counted.

Haplotype analysis

Haplotype analysis was performed with the markers D9S1878, D9S1817, D9S2177, D9S2178, D9S2179, D9S2180, D9S2181, D9S1804, D9S1791, D9S1859, and D9S1874, in the order suggested by the human genome sequence database of the Sanger Centre (http://www.sanger.ac.uk/HGP/Chr9/). The haplotypes for the disease-bearing chromosomes, as well as those for healthy chromosomes, were derived from inspection of the family data. The most-likely haplotypes were constructed manually using the minimum recombination strategy. The phase of the *NM* region was determined by genotyping the patients and their parents. When parents were not available, haplotypes were inferred from children.

Results

Excess of homozygosity

In autosomal recessive disorders, affected individuals from consanguineous families would be expected to be homozy-

Table 1. New markers used in this study

Locus	Primer sequences[a] (5′-3′)	Product size (bp)	Repeat	Heterozygosity
D9S2177	GCCTTGAGTGTAAATGCCAA GGGCACTGAGTGAAAAGAA	200	$(CTTT)_{16}$	0.82
D9S2178	CTCAAACTCCTGGCCTCAAG CCATCACCATCATTAAGGCA	148	$(CA)_{26}$	0.93
D9S2179	CTCACTCCCTTCGTGGTCAT CTCCAGGCAGATAGACAGCC	200	$(GTCT)_{13}$	0.68
D9S2180	CAGCTAGGGACTTGGAAGGA TATCGACCCAGCCTCTCATT	173	$(AG)_{18}(TG)_{22}(GA)_{6}$	0.8
D9S2181	CTTGTTCAAAGGGGTTGGTC TCTGATCCTCTAGCCCATGC	160	$(GT)_{21}$	0.52

[a] 58°C annealing temperature was used in all polymerase chain reactions

gous for an allele at the disease locus because of identity by descent from a common progenitor. We analyzed excess of homozygosity in the patients having NM using 18 microsatellite markers, which cover a region of 15 cM on chromosome 9p1.

Eight of 18 polymorphic markers showed significant excess of homozygosity in consanguineous patients (Table 2). The patient in family 4 was heterozygous at D9S2178, and those in families 3 and 7 were heterozygous at D9S1874. All of the NM patients from consanguineous families were homozygous within the region between markers D9S2179 and D9S1859. One NM patient from a nonconsanguineous family was also homozygous in the region between markers D9S2179 and D9S1804.

Table 2. Homozygosity for 13 markers in the *NM* region

	Homozygosity					
	Patients		Relatives			
Locus[a]	No.	%	No.	%	χ^2	*P*
D9S1845	7	87.5	0	0	12.5	<0.01
D9S1878	7	87.5	1	8.3	9.5	<0.01
D9S1817	7	87.5	4	33.3	3.7	NS
D9S2177	7	87.5	1	8.3	9.5	<0.01
D9S2178	7	87.5	2	16.7	7.1	<0.01
D9S2179	8	100.0	5	41.7	4.8	<0.05
D9S2180	8	100.0	0	0.0	16.1	<0.01
D9S2181	8	100.0	6	50.0	3.6	NS
D9S1804	8	100.0	6	50.0	3.6	NS
D9S1791	8	100.0	1	8.3	12.8	<0.01
D9S1859	8	100.0	5	41.7	6.3	<0.05
D9S1874	6	75.0	3	25.0	1.9	NS
D9S1862	2	28.6	2	22.2	0.1	NS

NS, not significant
[a] The order of marker loci is based on previously published data (Dib et al. 1996; http://webace.sanger.ac.uk/)

Linkage disequilibrium analysis

Allele distributions for 18 polymorphic markers were compared with estimated frequencies for both the patients and their parents. Allele "199-bp" of D9S2179 was overrepresented on *NM* chromosomes, although there were 7 alleles observed at this locus and this particular allele occurred in only 4.5% of Japanese control chromosomes (Table 3). When alleles on *NM* chromosomes were compared with those on the healthy chromosomes of the parents, a significant linkage disequilibrium was detected in 199 bp at this locus ($\chi^2 = 9.0$; $P < 0.005$). For D9S1859, the "107-bp" allele was overrepresented ($\chi^2 = 7.8$; $P < 0.01$), despite being the second most common allele in the control population (21.4% in Japanese). Other alleles significantly associated with *NM* chromosomes are shown in Table 4.

Five of six marker loci between markers D9S2179 and D9S1859 were exclusively associated with *NM* chromosomes, suggesting that markers D9S2178 and D9S1804 flank the polymorphism on the distal and proximal sides of the *NM* region, respectively.

Haplotype analysis

The haplotypes of the *NM* chromosomes are shown in Table 5. One predominant haplotype was present on affected chromosomes and was comprised of the alleles significantly associated with *NM*. The core haplotype, 199-160-154-109-178-107 (marker order: D9S2179-D9S2180-D9S2181-D9S1804-D9S1791-D9S1859), was present on 50.0% of the affected chromosomes and on 0.0% of parent chromosomes, and was exclusively associated with *NM* chromosomes ($\chi^2 = 9.3$; $P < 0.005$).

Two chromosomes had the haplotypes 199-160-154-109-180-107 and 199-160-154-109-176-107, both of which could be derived from the core haplotype as a result of historical

Table 3. Allele sizes and frequencies of three new markers associated with the *NM* region

D9S2179		D9S2180		D9S2181	
Allele size (bp)[a]	Frequency[b]	Allele size (bp)[a]	Frequency[b]	Allele size (bp)[a]	Frequency[b]
191	0.034	153	0.068	150	0.023
199	0.045	155	0.034	152	0.15
203	0.56	157	0.39	154	0.22
207	0.10	160	0.080	158	0.49
211	0.10	162	0.068	160	0.080
215	0.11	164	0.045	162	0.045
218	0.045	166	0.080		
		168	0.023		
		174	0.023		
		176	0.011		
		178	0.023		
		181	0.011		
		183	0.023		
		185	0.045		
		189	0.057		
		194	0.023		

[a] Each number shows the fragment size of the polymerase chain reaction (PCR) product
[b] Estimated from 88 chromosomes from Japan

recombinational events, placing the *NM* gene centromeric to marker D9S1791. Five historical recombinations were found between the *NM* region and marker locus D9S2178, a marker on the immediate telomeric side of the core haplotype. Therefore, D9S2178 defined the telomeric boundary of the *NM* region.

The results demonstrated that 8 of 11 (72.7%) NM families, including both consanguineous and nonconsanguineous marriages, carried the haplotype 199-160-154-109. This haplotype was exclusively associated with NM ($\chi^2 = 12.6$; $P < 0.001$) (Table 6). These eight families originated from different geographic areas of Japan. The presence of a core haplotype, 199-160-154-109, may suggest the existence of a common progenitor in Japan.

Discussion

The basic molecular defects resulting in NM are still elusive. In our previous study (Ikeuchi et al. 1997), the *NM* gene was localized to a 23.3-cM region defined distally by marker D9S319 and proximally by marker D9S276, based on linkage analysis. This is, however, too large a region to use when attempting to clone the gene using a positional cloning strategy. In this study, we have pursued an alternative strategy based on homozygosity and linkage disequilibrium analysis.

Homozygosity mapping is a powerful technique with which to map the genes responsible for recessive diseases (Lander and Botstein 1987). This strategy has typically been applied to diseases in consanguineous pedigrees (Pollak et al. 1993; Scheffield et al. 1994; Hillaire et al. 1994). In this study, we managed to apply homozygosity mapping to narrow the *NM* region to approximately 3 cM on chromosome 9p13 because three patients from three different consanguineous families happened to have informative recombinations on *NM* chromosomes. The positional cloning strategy including homozygosity mapping is indeed powerful, but is often hampered by lack of informative recombinations. A larger number of consanguineous families would be required to narrow the *NM* region more precisely by homozygosity mapping.

Linkage disequilibrium mapping is also a powerful tool for localizing the disease gene for Mendelian disorders that are relatively rare in the population, especially in studies such as this one, which use a limited number of families. (Farrall et al. 1987; Kerem et al. 1989; Huntington's Disease Collaborative Research Group 1993). In an ideal situation, most disease chromosomes descend from a single ancestral mutation, and the mutation is old enough for recombinations to have made the region of strongest linkage disequilibrium small (Kolehmainen et al. 1997). In this study, significant linkage disequilibrium was detected between the *NM* region and several marker loci. The findings support the hypothesis that a major contribution was made from one founding *NM* mutation in Japan. Furthermore, the observed large genetic region (2.8 Mb) involved in significant linkage disequilibrium may suggest that the number of generations since the mutation occurred is small (Hoglund et al. 1995).

Haplotype analysis by a historical recombinational event is also useful for fine-mapping the location of the offending

Table 4. Summary of allelic association data

Locus	Allele[a]	No. of chromosomes: Patients	No. of chromosomes: Relatives	χ^2	*P*
D9S1878	289	11	1	7.4	0.0066
D9S1817	264	9	6	0.2	0.63
D9S2177	168	10	6	0.3	0.61
D9S2178	153	8	4	0.3	0.54
D9S2179	199	13	1	8.9	0.0028
D9S2180	160	13	2	7.2	0.0072
D9S2181	154	15	5	4.4	0.037
D9S1804	109	17	11	1.1	0.29
D9S1791	178	11	3	4.0	0.045
D9S1859	107	15	4	7.8	0.0051
D9S1874	198	11	4	1.9	0.17

[a] Each number shows the fragment size of the PCR product

Table 5. Multilocus haplotypes for polymorphic markers near the *NM* gene in 11 patients

	Haplotypes in patients																					
	Consanguineous families												Nonconsanguineous families									
Locus	1-4		3-3		4-4		6-3		7-2		11-1		2-4		5-4		8-2		9-3		10-4	
D9S1878	289	289	289	289	270	287	289	289	289	289	292	292	272	285	270	289	289	289	270	287	270	289
D9S1817	264	264	264	264	264	268	270	270	270	270	263	263	244	264	264	270	270	270	266	266	264	270
D9S2177	168	168	217	217	168	209	200	200	200	200	209	209	200	172	168	168	197	197	168	172	168	205
D9S2178	153	153	126	126	126	151	165	165	165	165	128	128	165	155	153	153	165	165	153	151	153	140
D9S2179	**199**	**199**	215	215	203	203	**199**	**199**	**199**	**199**	219	219	**199**	203	**199**	203	**199**	**199**	**199**	203	**199**	215
D9S2180	**160**	**160**	183	183	153	153	**160**	**160**	**160**	**160**	157	157	**160**	157	**160**	157	**160**	**160**	**160**	157	**160**	185
D9S2181	**154**	**154**	158	158	158	158	**154**	**154**	**154**	**154**	152	152	**154**	154	**154**	158	**154**	**154**	**154**	158	**154**	154
D9S1804	**109**	**109**	109	109	119	119	**109**	**109**	**109**	**109**	111	111	**109**	109	**109**	109	**109**	**109**	**109**	109	**109**	109
D9S1791	178	178	184	184	178	178	178	178	178	178	174	174	178	186	178	162	178	180	176	182	178	182
D9S1859	107	107	109	109	107	107	107	107	107	107	109	109	107	109	107	109	107	107	107	107	107	109
D9S1874	198	198	198	206	198	198	198	198	192	194	194	194	200	202	192	198	198	198	204	206	198	194

The boxes indicate chromosomal regions associated with *NM*
Each haplotype is described by the fragment size of the PCR product

Table 6. Haplotypes associated with *NM* and normal chromosomes

Haplotypes[a] D9S2179–D9S2180–D9S2181–D9S1804	No. of Chromosomes	
	NM	Healthy
199-160-154-109	12	0
203-157-158-109	2	2
203-157-154-109	1	0
203-157-152-111	1	0
203-153-158-119	1	0
215-183-158-119	1	0
215-185-154-109	1	0
219-157-152-111	1	0
Others	0	15
Total	20	17

[a] Each number shows the fragment size of the PCR product

gene (Toda et al. 1996; Wang et al. 1997; Kolehmainen et al. 1997). In this study, one predominant haplotype (199-160-154-109-178-107) composed of six significantly associated alleles was found on 50.0% of the *NM* chromosomes. This haplotype was exclusively associated with the *NM* chromosome and is likely to represent the ancestral haplotype in Japan. The historical recombination events were detected just distal to D9S2179 and just proximal to D9S1804, suggesting that the *NM* gene lies between marker loci D9S2181 and D9S1791.

Interestingly, the haplotype 178-107-198 for markers D9S1791, D9S1859, and D9S1874 was found in patient 4-4 (Table 5). If that haplotype was part of the ancestral mutation, the *NM* gene would be assigned to the region between the markers D9S1804 and D9S1791. The clinical course of the patient (described in Subjects and methods), however, differed slightly from those of other patients with the ancestral haplotype. Based on our entire set of data, we feel confident concluding that *NM* lies between marker loci D9S2178 and D9S1791 (Fig. 1).

Mitrani-Rosenbaum and Argov et al. (1996) showed that the gene for hereditary inclusion body myopathy in Persian Jews is localized proximal to marker D9S165, and recent extended work confirmed that the hIBM gene is located in an approximately 1-Mb region between D9S1791 and D9S50 (Eisenberg et al. 1999). That region is just proximal to that of *NM* as defined in this study, and there is no overlapped interval. One problem is that the order of markers is based on the draft sequence data of the human chromosome. We have completed the construction of a bacterial artificial chromosome (BAC) contig, which covers the *NM* region defined in this study. A physical map of the region between D9S2178 and D9S50 will disclose whether the disorders NM and HIBM result from allelic mutation of the same gene.

In conclusion, the homozygosity and linkage disequilibrium mapping applied in this study refined the localization of the *NM* gene to be the 1.5-Mb region between D9S2178 and D9S1791. Linkage disequilibrium analysis also supported the hypothesis that a major contribution is made from one founding *NM* mutation in Japan. We have already completed a BAC contig that covers the region of interest. Newly established polymorphic markers such as a single-nucleotide polymorphism will facilitate the refinement of the localization of the *NM* locus and make possible a positional cloning strategy to clone the *NM* gene.

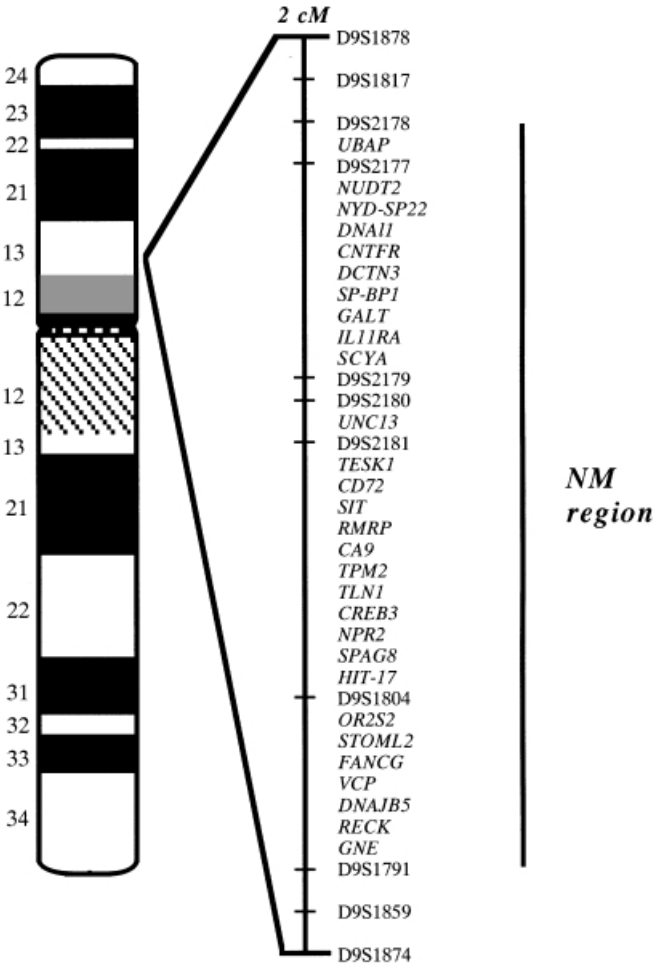

Fig. 1. Genetic and transcription map of the chromosome 9p13 region, showing the location of the *NM* gene Oistances are based on previously published data (Dib et al. 1996; http://webace.sanger.ac.uk/)

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