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Detection of Linkage Disequilibrium between the Myotonic Dystrophy Locus and a New Polymorphic DNA Marker

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Summary

We have examined the linkage of two new polymorphic DNA markers (D19S62 and D19S63) and a previously unreported polymorphism with an existing DNA marker (ERCC1) to the myotonic dystrophy (DM) locus. In addition, we have used pulsed-field gel electrophoresis to obtain a fine-structure map of this region. The detection of linkage disequilibrium between DM and one of these markers (D19S63) is the first demonstration of this phenomenon in a heterogeneous DM population. The results suggest that at least 58% of DM patients in the British population, as well as those in a French-Canadian subpopulation, are descended from the same ancestral DM mutation. We discuss the implications of this finding in terms of strategies for cloning the DM gene, for a possible role in modification of risk for prenatal and presymptomatic testing, and we speculate on the origin and number of existing mutations which may result in a DM phenotype.

Introduction

Myotonic dystrophy (DM) is the commonest adult form of muscular dystrophy, with an incidence of at least 1/10,000. It is a dominantly inherited disorder with a highly variable age at onset and degree of severity. Although characterized by myotonia and progressive muscle weakness and wasting, a wide range of tissues apart from muscle may also be affected. The disease is caused by a single gene that has been shown, by extensive linkage analysis, to be located on the long arm of chromosome 19 (Harper 1989). Both the nature of the gene and its protein product are completely unknown. For a review of DM, see the work of Harper (1989).

The cloning of the DM gene will enable its structure and function to be investigated, allow the molecular pathology of the disease to be analyzed, and provide the means for prenatal diagnostic testing and carrier detection based (in many cases) on direct mutational analysis. Previous studies have concentrated on defining the smallest human chromosome 19 region containing the DM locus. These studies have been carried out in three different ways:

1. Construction and characterization of somatic cell hybrid lines containing (*a*) contiguous human chromosome 19 portions translocated onto either another human chromosome or a rodent chromosome (Brook et al. 1984, 1987, and in press; Hulsebos et al. 1986; Stallings et al. 1988; Schonk et al. 1989) or (*b*) human chromosome 19 fragments stably retained in a background of rodent chromosomes (Brook et al. 1986; Schonk et al. 1989). These lines have been used to localize new and existing DNA, protein, and cell surface markers to small regions of chromosome 19 and thus provide a consensus map of the whole chromosome (Le Beau et al. 1989).
2. Construction of lambda libraries and cosmid libraries to identify new polymorphic DNA markers linked to the DM locus, to produce a genetic map of the relevant region of chromosome 19 (Brunner

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et al. 1989; Johnson et al. 1989, 1990; Korneluk et al. 1989; Harley et al., in press).
3. Use of pulsed-field gel electrophoresis (PFGE) to produce fine-structure maps in the region of DNA markers shown to be within 2–3 cM of the DM gene (Shaw et al. 1989; Smeets et al. 1990).

All three approaches have been employed in our efforts to clone the DM gene, which has now been localized to a small region of chromosome 19—i.e., 19q13.2–13.3 (Brunner et al. 1989; Korneluk et al. 1989; Schonk et al. 1989; Johnson et al. 1990; Brook et al., in press; Harley et al., in press).

We have used a recently identified *Bst*EII polymorphism with an existing DNA marker, ERCC1 (J. Gusella and B. Seizinger, personal communication). ERCC1 is known to be the marker closest to DM on the proximal side (Smeets et al. 1990). We have also isolated two new polymorphic DNA markers (D19S62 and D19S63) which map distal to the CKM locus, as shown by their presence/absence in somatic cell hybrids and by analysis of a family in which DM, D19S62, and D19S63 have recombined with CKM and CYP2B (Brook et al. 1990*a*, 1990*b*, 1991). With respect to CKM, D19S62 and D19S63 are therefore either closer to or flanking the DM gene. We report here a study to determine the order of D19S62 and D19S63 in relation both to CKM, ERCC1, and DM and to other long-arm markers, together with an analysis of linkage disequilibrium between these markers and the DM locus. The distances of the new markers, relative to each other and to ERCC1 and CKM, were established by PFGE. The construction of the long-range restriction map was facilitated by the use of *Not*I end clones and linking clones. The combined use of PFGE and linkage disequilibrium analysis has enabled us to identify and define physically the region in which the DM gene is most likely to be.

Material and Methods

General Techniques

Isolation of DNA, Southern transfer, and DNA hybridization were performed according to standard procedures (Maniatis et al. 1982). Labeling of probes was done by the method of Feinberg and Vogelstein (1983) or by nick translation, using commercial kits (Amersham International) to specific activities of >1 $\times 10^8/\mu g$ DNA. Blots were washed to stringencies appropriate for each probe and were autoradiographed for 1–7 d at −70°C.

Table 1

RFLPs Used in Study

Locus Name (probe name/gene symbol) and RFLP	Allele	Allele Size (kb)	Allele Frequency[a]
Excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1):			
*Bst*EII	A1	12	.84
	A2	10	.16
D19S62 (pD8):			
*Hin*dIII	A1	4.8	.23
	A2	2.5	.77
D19S63 (pD10):[b]			
*Pst*I	A1	8.0	.57
	A2	7.2	.27
	A3	7.0	.16
*Pvu*II	A1	6.0	.57
	A2	5.6	.27
	A3	5.4	.16
*Hin*cII	A1	>20	.78
	A2	15	.22

[a] Derived from the set of DM families used in the two-point linkage analyses.
[b] The *Pst*I and *Pvu*II RFLPs are due to the same insertion/deletion polymorphism.

DNA Probes

The DNA probes, the polymorphisms they detect, and the allele frequencies in our DM families are described in table 1 (for D19S62, D19S63, and ERCC1), and in the work of Harley et al. (in press) (for D19S7, D19S8, D19S9, D19S19, D19S22, APOC1, APOC2, ATP1A3, BCL3, CKM, CYP2A, and PRKCG). Also see the work of LeBeau et al. (1989) and Brook et al. (1990*a*, 1990*b*).

Family Material and Linkage Analysis

Forty-five of the DM families used in the present study have formed the basis of a previous study (Harley et al. 1988, and in press; Walsh et al. 1990). The families are all of European origin. Clinical criteria included electromyogram and ophthalmological examination when necessary. Only these 45 families were used in calculating the recombination fraction (θ) between each DNA locus and the DM gene and between pairs of DNA loci. Only individuals whose status could be unequivocally determined were used for analysis of linkage of DM to the DNA loci. Therefore age at onset did not need to be taken into account for calculations of linkage involving DM, and com-

plete penetrance of the DM gene could be assumed. Since the new markers used in the present study were known from their physical localization to be very close to DM, it was not thought necessary to obtain the maximum possible lod score (Z) information from the families, and therefore it was decided not to use at-risk individuals in this part of the analysis. All family members were used to estimate the θ value between each pair of DNA loci. θ Values were calculated without regard to sex, a procedure which is not invalid for the relatively small intervals under study, and the mutation rate of the DM gene was assumed to be zero. Analysis of DNA typings by using the HOMOG program provided strong evidence for homogeneity with respect to the disease locus (H. G. Harley, unpublished results). Each pedigree was inspected for recombinant individuals. The LINKAGE package of programs (version 5.03; Lathrop et al. 1984) was used to calculate the θ value between each DNA locus and the DM gene.

In addition, an extra 50 DM families were used for the calculation of linkage disequilibrium. These families consisted minimally of an affected parent, his or her normal spouse, and one affected offspring. However, if available, data on any unrelated, normal individual from both sets of pedigrees was included in the calculations. The clinical criteria were as stringent as those used for the families in the linkage analysis. Linkage disequilibrium calculations were done according to the formula of Hill and Robertson (1968), and the significance of the disequilibrium was tested using standard χ^2 formulas, with the Yates correction if the size of any of the expected classes was below five.

Isolation of NotI *End CLones and Linking Clones*

A *Not*I end clone library was constructed from the cell line 20XP-3542-1-4, a human-hamster hybrid containing a human chromosome 19 fragment that retains the proximal markers most closely linked to DM (Stallings et al. 1988), in order to facilitate the construction of the long-range restriction map. DNA from the cell line was first digested to completion with *Not*I and then was partially digested with *Mbo*I to an average length of 15–20 kb. The DNA in the correct size range was purified by low-melting-point agarose-gel electrophoresis, agarase treatment, phenol/chloroform extraction, and ethanol precipitation and was ligated into the vector lambda NB4, which is a lambda EMBL3 derivative in which the right-hand *Bam*HI cloning site has been replaced with a *Not*I site. After in vitro packaging by using a commercial system (Stratagene), the phage were plated on McrA/McrB-deficient *Escherichia coli* strains, and clones containing human inserts were identified by plaque hybridization with labeled total human DNA.

Linking clones were obtained by screening a total human *Mbo*I partial digest library in lambda EMBL3 by using single-copy fragments from the *Not*I end clones. A *Not*I linking clone will contain DNA sequences from both sides of a *Not*I site.

PFGE

Pulsed-field gels were run on a CHEF apparatus (Chu et al. 1986). Samples were prepared according to a method described elsewhere (Shaw et al. 1989), were digested with *Not*I, *Mlu*I, or *Not*I plus *Mlu*I, and were electrophoresed for 46 h at 5.5 V/cm, with a pulse time ramped from 60 to 300 s.

Results

Linkage and Disequilibrium Analysis

The results of the two-point linkage analysis of each marker to DM and to each other marker are presented in tables 2 and 3. In this analysis haplotypes were constructed for those probes detecting multiple RFLPs. Inspection of the pedigrees revealed no obli-

Table 2

Two-Point *Z* Values for Each Probe to DM

		Z at θ of											
PROBE	POLYMORPHISM	.00	.05	.10	.15	.20	.25	.30	.35	.40	.45	θ_{max}	Z_{max}
D19S62 ..	*Hin*dIII	8.68	10.06	9.02	8.07	6.57	5.27	3.96	2.67	1.47	.51	.02	10.46
D19S63 ..	*Pst*I	12.51	18.60	15.58	14.82	11.94	9.45	6.98	4.63	2.53	.90	.03	18.74
D19S63 ..	*Hin*cII	3.00	7.13	6.60	5.86	4.75	3.70	2.66	1.68	.84	.25	.04	7.15
ERCC1 ..	*Bst*EII	2.10	1.89	1.66	1.43	1.19	.94	.70	.48	.27	.11	.00	2.10

Table 3

Two-Point *Z* Values for Each Pairwise Combination of Markers

Marker Pair[a]	θ_{max}	Z_{max}	1 *Z* Support Interval
D19S62-D19S7	.28	.70	.11–.50
D19S9	.12	1.46	.02–.36
CYP2A	.15	1.48	.02–.39
ATP1A3	.06	3.89	.01–.21
D19S8	.03	5.46	.00–.14
D19S19	.23	.44	.03–.50
BCL3	.00	6.04	.00–.08
APOC1	.00	3.92	.00–.09
APOC2	.02	8.42	.00–.10
CKM	.03	8.25	.00–.12
ERCC1	.05	3.24	.00–.22
D19S63	.00	9.36	.00–.07
D19S22	.09	1.82	.02–.31
PRKCG	.26	.56	.11–.50
D19S63-D19S7	.23	1.61	.14–.38
D19S9	.12	4.52	.05–.24
CYP2A	.13	8.06	.08–.21
ATP1A3	.05	11.66	.02–.11
D19S8	.11	10.57	.06–.19
D19S19	.23	0.87	.17–.29
BCL3	.02	12.52	.00–.08
APOC1	.06	9.62	.02–.14
APOC2	.02	14.00	.00–.08
CKM	.03	31.42	.01–.08
ERCC1	.06	1.48	.00–.32
D19S22	.12	5.72	.05–.23
PRKCG	.18	4.25	.10–.29
ERCC1-D19S7	.38	.19	.24–.50
D19S9	.14	.90	.01–.50
CYP2A	.00	1.86	.00–.25
ATP1A3	.06	3.75	.01–.20
D19S8	.13	1.65	.02–.35
D19S19	.00	1.63	.00–.34
APOC1	.00	1.70	.00–.18
APOC2	.00	6.39	.00–.09
BCL3	.04	4.64	.00–.16
CKM	.00	11.10	.00–.04
D19S22	.00	.88	.00–.50
PRKCG	.28	.42	.12–.50

[a] The order in which the second markers are listed corresponds to their order on the chromosome, in the direction 19cen-19qter.

gate recombinants between any of the markers (D19S62, D19S63, and ERCC1) and the DM locus (also see Brook et al. 1991). However, the *Z* values for neither D19S62 nor D19S63 were at a maximum at zero recombination. This is because, for individuals whose genotypes cannot be determined directly, the LINKAGE program infers genotypes from population frequencies. In such cases the possibility of recombination cannot be excluded. The θ values between markers are what would be expected on the basis of their localization to the same small region of chromosome 19, but it was not possible to determine the order of D19S62, D19S63, or ERCC1 in relation either to each other or to DM, as no definitive, multiply informative recombinants were detected between any of these markers and the DM locus. The order of the DNA loci was established by PFGE mapping (see below).

The distributions of alleles for the markers D19S62, D19S63, and ERCC1, both in DM chromosomes and in normal chromosomes, are shown in table 4.

Table 4

Analysis of Linkage Disequilibrium between D19S62, D19S62, ERCC1, and DM Locus

Marker and Enzyme	Allele	No. of DM Chromosomes	No. of Normal Chromosomes	D[a]	χ^2
D19S62:					
*Hin*dIII	1	4	28	−.110	1.95[b]
D19S63:					
*Pst*I/*Pvu*II	1	20	121		
	2	6	58		
	3	37	36	−.399	44.93[c]
*Hin*cII	1	31	174		
	2	28	30	−.330	28.60[c]
ERCC1:					
*Bst*EII	1	28	102		
	2	4	20	.044	.30[b]

[a] Calculated according to the formula of Hill and Robertson (1968).
[b] Not significant ($P > .05$).
[c] Significant ($P < .001$).

D19S63 is the only marker in strong disequilibrium with DM: allele 3 of the *Pst*I RFLP and allele 2 of the *Hin*cII RFLP are inherited with the DM chromosome more often than would be predicted from allele frequencies in the normal population and are therefore in disequilibrium with the DM locus (both results are significant at the 99.9% level).

The results were also assessed for linkage disequilibrium between the various RFLPs at the D19S62, D19S63, and ERCC1 loci. There was only one pair that showed significant disequilibrium—namely, the *Hin*cII and *Pst*I RFLPs at D19S63.

Nine of the families used in the present study were from the Sanguenay region of Quebec and are of French origin (Laberge et al. 1985; Laberge 1989). In this set of families, allele 3 of the D19S63 *Pst*I polymorphism is associated with the mutant DM gene, as it is in the majority of families of European origin. Since it is known that all of these families have a common and relatively recent origin, it was decided that, for the purpose of estimating linkage disequilibrium they should be treated as one large family, and therefore only one French-Canadian DM chromosome was used in the calculations. Inclusion of one chromosome from each of the nine French-Canadian families would merely have resulted in a higher level of significance of the observed linkage disequilibrium.

Physical Mapping of the CKM-ERCC1-D19S62-D19S63 Region

The human *Not*I end clones from the 20XP-3542–1-4 cell line were first localized by using a somatic cell hybrid mapping panel. This included the cell line 5HL94, which retains a single human 19 (Stallings et al. 1988), and the cell lines G35F3B and GM89A99c7B, which retain the regions 19pter-q13.2 and 19q13.2-qter, respectively (Brook et al. 1984). Three clones were localized to the regions 19q13.2-qter, distal to CKM. These clones were designated NE15, NE16, and NE17. For NE16 and NE17, overlapping clones were obtained from a total human library. These were shown to contain *Not*I sites and may therefore be used as linking clones to identify adjacent *Not*I fragments by PFGE analysis.

All of these clones together with D19S62 (pD8), D19S63 (pD10), CKM, and ERCC1 were hybridized to CHEF pulsed-field blots of *Not*I-, *Mlu*I-, and *Not*I plus *Mlu*I-digested DNAs from a variety of sources, including 20XP-3542-1-4 and other chromosome 19-containing hybrid cell lines, human white blood cells, and human lymphoblastoid cell lines. In all cases, discrete fragments whose sizes could be estimated were obtained. Several sites were identified that were differentially digested, according to the cell type used. All of the information was combined to form a long-range restriction map, shown in figure 1.

It has already been demonstrated that ERCC1 and CKM are on the same 300-kb *Not*I fragment (Smeets et al. 1990). The *Not*I and clone NE17 was found to hybridize to the same fragment. The sequence from the other side of the *Not*I site was obtained from a linking clone and was shown to hybridize to the same small *Not*I fragment as did clone NE15. NE15 and D19S63 were shown to be on the same *Mlu*I partial

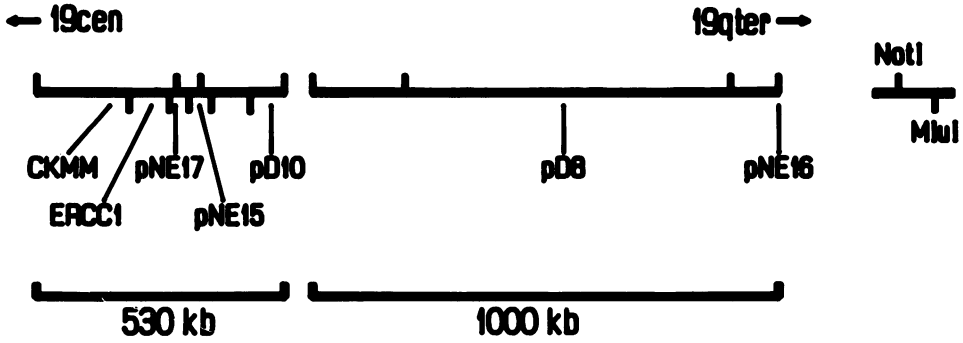

Figure 1 Long-range restriction map of region of chromosome 19q distal to CKM locus. The positions of the CKM, ERCC1, D19S62 (pD8), and D19S63 (pD10) loci are shown together with the *Not*I end and linking clones (NE15, NE16, and NE17). The positions of the *Mlu*I sites in the right-hand part of the map cannot yet be defined relative to the *Not*I sites and are therefore not included. There is an unmapped region between the two "islands" of DNA; this is discussed in the text. (CKM and CKMM are both symbols for the creatine kinase, muscle form, gene.)

digest fragment; hence the markers CKM, ERCC1, NE17, NE15, and D19S63 can all be assigned to a 530-kb "island" of DNA, as shown in figure 1.

The other two markers used in the present study, NE17 and D19S62, were shown to hybridize to the same 1,000-kb *Not*I partial digest fragment and to define a second island of DNA (fig. 1). At present there remains the possibility that there is more unmapped DNA between the two islands. The orientation of the map (fig. 1) on the chromosome is known from the localization of the various markers by using hybrids including G35F3B (19pter-q13.2) and GM89A99c7B (19q13.2-qter).

Discussion

Linkage analysis suggests a single locus for DM, on 19q13.2-q13.3. The most recently isolated markers are very closely linked to the DM gene, and the recombination detected in our families has not been sufficient to allow us to order these markers by conventional linkage analysis. The detection of linkage disequilibrium may enable us to pinpoint the DM gene by using haplotype analysis. The highly significant allelic association of D19S63 (allele 3 *Pst*I and allele 2 *Hin*cII) with DM suggests that one or a small number of ancestral mutations may be involved in the DM phenotype in these families. This result is entirely consistent with previous population studies, which have indicated a very low mutation rate in DM (Harper 1989). It is likely that our British study population's 58% of DM chromosomes with allele 3 of the D19S63 *Pvu*II polymorphism, as well as those in the French-Canadian sample, are all descended from a single ancestor. This is because these chromosomes all carry the allele that is rarest in the normal population, and separate DM mutations are unlikely to have arisen on the same genetic background. The DM mutation in the French-Canadian population is thought to have been introduced by one of the original founders over 300 years ago (Mathieu et al. 1990) and may have originated in northern Europe before the spread of this population to the British Isles. The remaining 42% of DM chromosomes may include some that have the same mutation—which has become associated with different D19S63 alleles by recombination—together with one or more other DM mutations. It may be possible to throw more light on this matter by the analysis of further RFLPs in the vicinity of D19S63 and by the construction of haplotypes; these studies are in progress.

The high degree of statistical significance seen in the results for D19S63 was obtained because, with both the *Pvu*II polymorphism and the *Hin*cII polymorphisms, the allele that is the rarest in the normal population is the one inherited with DM more often than would be expected by chance. With both D19S62 and ERCC1, DM is more frequently inherited with the common allele, making it difficult to detect a significant level of disequilibrium.

Linkage disequilibrium between other closely linked markers (APOC2, CKM, and BCL3) and DM was not observed in our population of DM families (Harley et al., in press). This is in marked contrast to results obtained in the French-Canadian population, in which strong linkage disequilibrium was observed

between DM and the loci APOE (Laberge et al. 1985), APOC2 (MacKenzie et al. 1989), BCL3 (Korneluk et al. 1989), and CKM (A. MacKenzie and R. G. Korneluk, personal communication). These observations can be explained by a founder affect in the French-Canadian population (Laberge 1989). Our DM families are derived from a much broader European population that is of mixed origin and therefore is not subject to such an effect. When there is a low or null mutation rate, as appears to be the case for DM, the use of a population such as ours provides a much more stringent test for linkage disequilibrium than does an isolated or inbred population.

The D19S63 marker will be very useful for prenatal diagnosis and carrier status testing in DM families. In affected parents, the degree of heterozygosity of the *Pst*I RFLP will be about 70%, when the observed linkage disequilibrium is taken into account. This could be increased by use of the *Hin*cII RFLP as well. Furthermore, in families in which, because of missing samples, the marker phase cannot be established, it should be possible to use the disequilibrium to improve estimates of risk for asymptomatic or unborn individuals.

The markers used in the present study have been mapped by PFGE. The distance between ERCC1 and D19S63 is about 300 kb, and that between D19S63 and D19S62 at least 250 kb and possibly as much as 1,000 kb. From the linkage disequilibrium analysis it appears that D19S63 is the marker closest to DM. In our attempts to isolate the DM gene, we are employing chromosome walking and other techniques to identify conserved and expressed sequences and HTF islands in the vicinity of D19S63. Further disequilibrium analysis should enable us to determine more exactly the location of the DM mutation(s) in relation to D19S63 and will also play an important role in the testing of candidate genes.

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