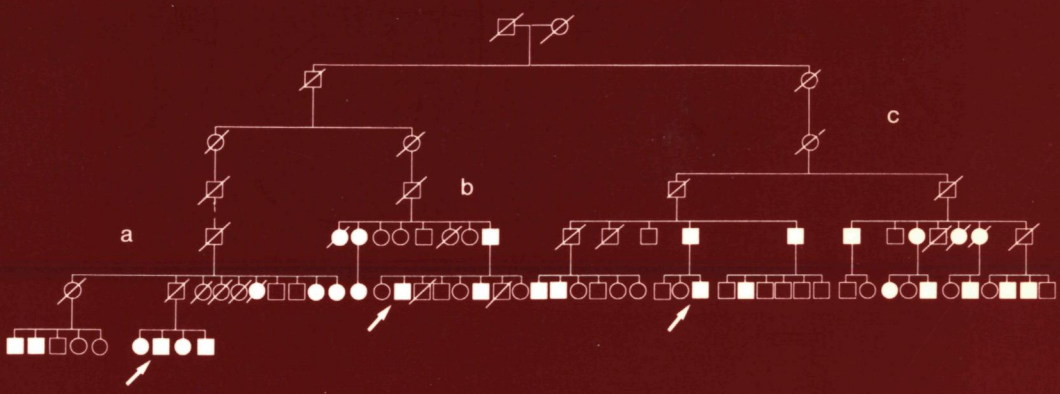
GENETIC STUDIES IN MYOTONIC DYSTROPHY



H.G. Brunner

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Publicatie van dit proefschrift is mede mogelijk gemaakt door giften van het FBW Anthropogenetica en het Klinisch Genetisch Centrum Nijmegen

GENETIC STUDIES IN MYOTONIC DYSTROPHY

Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen
in het bijzonder de Geneeskunde

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen
volgens besluit van het college van decanen
in het openbaar te verdedigen op
donderdag 2 december 1993
des namiddags te 1.30 uur precies

door

Henri Gerrit Brunner

geboren op 18 october 1956 te Rotterdam

Promotores: Prof. Dr. H.H. Ropers
Prof. Dr. B. Wieringa

CONTENTS

CHAPTER 1

General review

Myotonic dystrophy (DM) was first recognized as a disease entity at the beginning of the 20th century. Batten and Gibb (1909) and Steinert (1909) independently described a series of patients with atrophy and weakness of the face and distal limb muscles in combination with the inability to relax the muscles after forceful contraction (myotonia). Steinert reported detailed pathological studies in one of his patients with myotonic dystrophy, and stressed the occurrence of a "myopathic progressive dystrophy" (Steinert, 1909). Before these landmark publications, DM had been lumped together with different myotonic syndromes under the diagnosis of myotonia congenita or Thomsen disease (Thomsen, 1876). It is now known that myotonia occurs in several distinct genetic disorders, notably in myotonia congenita (various types), in paramyotonia congenita, in periodic paralysis (2 types) and in the Schwartz Jampel syndrome (Harper, 1989). DM is by far the most frequent of the inherited myotonias as judged from a survey performed by Becker and co-workers (Grimm, 1976; Becker, 1977). By 31 December 1960, this group had collected information on 562 patients with DM living in the Federal Republic of Germany and West Berlin, on 168 patients with recessive generalized myotonia, and on 116 cases of dominant myotonia. However, the authors recognized that underascertainment was still a major problem for each of the disorders in their sample. Another important early contribution to the study of DM was the description by Greenfield (1911), by Fleischer (1918), and by Vogt (1921) of ocular cataract in DM. Vogt (1921) commented on the characteristic finding of multiple, small, dustlike or gleaming opacities that were found exclusively in the cortex of the lens and that were intermingled with red or green (rarely with yellow or blue) crystals. He also showed that the apparent colour of the crystals was due to the dispersion of light from his slit lamp. These early descriptions of myotonia, muscle weakness, and cataract established what are now regarded as the most characteristic signs of myotonic dystrophy. However, even these early reports noted the involvement of other organ systems, specifically testicular atrophy and frontal balding in males, as well as reduced intelligence and lack of initiative (Curschmann, 1912; Curschmann, 1925). Many other symptoms may occur in myotonic dystrophy (table 1.1.2). A full discussion would be beyond the scope of this thesis and only a few other clinically relevant problems will be mentioned here.

Difficulty in swallowing is frequent in DM and may lead to aspiration. If the oesophagus is dilated, aspiration may occur during sleep, and the clinical picture may be that of unexplained recurring bronchopneumonia. Megacolon is also frequent, and occasional patients with intestinal pseudo-obstruction

have been reported. Cognitive impairment (especially in cases of childhood or congenital onset) and hypersomnolence (with or without central alveolar hypoventilation) are not uncommon. Thomasen (1948) studied 101 patients with DM and confirmed previous observations of reduced initiative and a satisfied care-free temperament. Although mental changes form a characteristic part of the clinical picture, little is known about the neuropathological substrate. Thalamic inclusion bodies, and intracytoplasmatic inclusion bodies in the putamen, caudate nucleus, substantia nigra and cerebral cortex have all been reported as well as minor abnormalities in gyral architecture (Ono et al. 1987). Studies using CT and MRI scanning of the brain have demonstrated cerebral atrophy, focal white matter changes, anterior temporal lobe lesions and occasional calcification of the basal ganglia (Huber et al. 1989; Avrahami et al. 1987). A recent report has emphasized the presenile occurrence of abundant neurofibrillary tangles, especially in the parahippocampal area of the brain. (Kiuchi et al. 1991). The exact relationship (if any) between the clinical changes and the neuroradiological and neuropathological changes is obscure.

General anaesthesia in DM patients carries increased risk because of the possibility of prolonged apnoea, increased sensitivity to central depressant medication, aspiration, succinylcholine induced muscle contracture, or cardiac rhythm disturbances followed by cardiac arrest (Aldridge, 1985). Cardiac illness in DM is infrequent, but subclinical cardiac involvement in DM is very common (Moorman et al. 1985). Sudden death occurred in 4% in one study (Moorman et al. 1985). Occasional patients may require pacemaker implantation. All DM patients should have regular review for cardiac symptoms as well as ECG testing (anonymous, 1992).

Based on experience with more than 50 DM families, I believe that specific symptoms cluster in some DM families. As a case in point, we have studied a large Dutch DM family, in which two siblings were repeatedly hospitalized for intestinal pseudo-obstruction (Brunner et al. 1992a). Another observation of intestinal pseudo-obstruction in siblings with DM has been made (C.J. Höweler, personal communication). Pleiomorphic adenoma of the salivary glands, which is another very rare complication of DM (Jóhannesson et al. 1978; personal observations), has also been observed in siblings (C. Höweler, personal communication). Perhaps the most persuasive evidence for an unusual familial presentation of DM is found in a large Dutch pedigree, where all clinically affected family members showed significant polyneuropathy (Spaans et al. 1986). Polyneuropathy was the sole symptom in 4 additional members of the family who had inherited the DM gene as judged from genetic linkage studies (Brunner et al. 1991b). Other possible examples of familial clustering of symptoms can be found in the literature (Brunner et al. 1992a). For many specific symptoms, the exact frequency in DM patients

is unknown. This renders it very difficult to assess whether an observed familial clustering of symptoms could be due to chance alone. A possible explanation for clustering of symptoms is the existence of modifier genes (Haldane, 1941; Ravin and Waring, 1939). In fact, the selection for genes that modify the deleterious effects of autosomal dominant mutations was invoked by Fischer (1931) as one of the main forces in evolution. The many possible interactions of the myotonic dystrophy gene product(s) at the cellular level (see Brook et al. 1992 for review) suggest that a mutation in other genes involved in the same metabolic pathway or cellular structure could set the stage for selective dysfunction of specific organs or cell types affected by the DM mutation.
Further clinical aspects of DM are reviewed extensively in a recent monograph on DM (Harper, 1989).

1.1.2 classification of DM in clinical subtypes; and anticipation

Age at onset is the single most important factor determining the clinical picture in myotonic dystrophy. This was first recognized by Fleischer (1918). Three main clinical forms of DM are often recognized (Dyken, 1969; Harper, 1989). Congenital onset DM typically presents with polyhydramnios during the 3rd trimester of pregnancy. At birth, severe hypotonia is found, with diminished respiration and swallowing (Vanier, 1960; Harper, 1975). Some cases show talipes or arthrogryposis. Respiratory function may or may not improve sufficiently to allow survival (Rutherford et al. 1989). Most individuals with congenital-onset DM subsequently show mild to moderate mental retardation (Harper, 1975) with poor prognosis for normal family life and ultimately gainful employment. Long term complications include recurrent otitis, articulation defects, persistent disabling talipes, recurrent gastro-intestinal malfunction, and possibly early death due to aspiration pneumonia (O'Brien and Harper, 1984).
Adult onset DM is characterized by onset in adolescence or early adulthood. This group of patients presents with the classical picture of myotonia and muscle weakness, with or without cataract or additional features as shown in table 1.1.2 (Höweler, 1988; Harper, 1989).
Late onset myotonic dystrophy is a very mild condition. In most cases, cataract is the only feature.
While most patients will fit into this simple classification scheme, some evidence exists that further groups should be defined. For instance, O'Brien and Harper (1984) noted that some of the siblings of their congenital-onset cases showed mental retardation without the typical congenital features of hypotonia, or breathing and swallowing problems. Such childhood-onset cases have been mentioned by others, too (Bundey, 1982; Höweler, 1986; Koch et al. 1991). Furthermore, Höweler (1986) and more recently Koch

and co-workers (1991) divided their adult onset cases into two subgroups, that is early adult and late adult (Höweler, 1986) or early adult and adult (Koch et al. 1991). This leads to the following classifications, mainly based on the age at onset:

TABLE 1.1.1

	Koch et al. 1991	Höweler, 1986
Congenital	< 1 year	at birth
Childhood	1-10 years	0-9 years
Early adult	11-20 years	10-29 years
Late adult	21-40 years	30-49 years
Senile		≥50 years
Mild	cataracts only	

Fleischer (1918) was the first to describe the progression of disease severity in families with myotonic dystrophy. DM may present with presenile or senile cataract in the first generation, while the next generation shows classical (i.e. adult onset) DM. In the third generation, patients will show either childhood onset or congenital onset of DM (Höweler, 1986). However, the number of liveborn children in this generation is decreased, due to infertility, celibacy and pregnancy loss (Fleischer, 1918; Klein, 1958; Höweler, 1986; Veillette et al. 1989; Koch et al. 1991). In fact, none of the childhood onset or early adult onset males in the series of Höweler (1986) and of Koch et al. (1991) had any offspring.
Fleischer noted that different families with DM were often related through common ancestors for whom no history of disease was known. He suggested that DM could be transmitted for many generations with no ill effect, with the possible exception of cataract (Fleischer, 1918). Fleischer's findings have been confirmed in pedigree studies in various countries (Mathieu et al. 1990; Lotz and van der Meyden, 1985; Klein, 1958; personal observations). Each of these studies showed that DM families often have distant common ancestors and therefore supported the concept of significantly lower expression in earlier generations than in later generations of a DM family. Bell (1947) noted that the disease presented earlier in subsequent generations, and that this antedating was accompanied by the extension of signs from one tissue to another, e.g. from the lens of the eye to muscular tissue, and perhaps to nervous tissue. She also noted an excess of mildly affected or asymptomatic fathers (59%) who had transmitted the disease (Bell, 1947). A similar excess of males among mildly affected transmitters of the disease was found by

Klein (1958).
The phenomenon of increasing severity and earlier onset with each generation, has become known as anticipation. Recently, extensive reviews of the history of anticipation in DM have been published (Höweler, 1986; Harper, 1989; Harper et al. 1992). In brief, Fleischer's original observation was widely accepted, until the geneticist Penrose pointed out that anticipation had not been documented in other species, and that selection biases, inherent to the study of human inherited disease might also account for the phenomenon (Penrose, 1948). These selection biases included ascertainment through severely affected individuals, as well as reduced fertility of severely affected individuals. Such biases would prohibit the ascertainment of mildly affected offspring born to a severly affected parent (complementary pairs), since these children would be either asymptomatic at the time of examination (and thus considered unaffected) or nonexistent, because of selective infertility. This view was adopted by most geneticists (with a few notable exceptions such as the study by Klein (1958)), until the study of Höweler showed that in 14 DM families, 60 of 61 parent-child pairs showed anticipation (Höweler, 1986; Höweler et al. 1989). This study has recently been duplicated in 40 American DM families with almost identical results (Ashizawa et al. 1992b). The molecular mechanisms involved in anticipation and in the congenital form of DM will be further discussed in sections 1.3.4 and 1.5.

TABLE 1.1.2

Organ systems that are affected in myotonic dystrophy, and major symptoms

ORGAN SYSTEM	SYMPTOMS
Muscles	Myotonia; atrophy and weakness of predominantly the facial, masticatory, neck, forearm, and lower leg musculature
Heart	Atrioventricular conduction disturbance; atrial fibrillation; sudden cardiac death
Lungs	Aspiration pneumonia; alveolar hypoventilation; respiratory insufficiency
Gastrointestinal	Swallowing defect; delayed emptying of oesophagus and stomach; crampy abdominal pain; diarrhoea or obstipation; megacolon; intestinal pseudo-obstruction
Uterus	Spontaneous abortion or premature birth; reduced voluntary muscle power during second stage of labour; postpartum haemorrhage; retained placenta
Eyes	Cataract; retinal degeneration; hypotonia
Endocrine	Testicular atrophy; irregular menses; peripheral insulin resistance
Adnexa	Premature balding; pilomatrixoma
Brain	Apathy and inertia; mental retardation; hypersomnolence; increased sensitivity to sedatives
Peripheral nerves	Reduced nerve conduction velocity; polyneuropathy
Heart, lung, brain	Anaesthetic complications

1.1.3 Biochemical studies into the pathogenesis of DM

Before the nature of the mutation became known, a large number of studies had been performed in order to define the basic biochemical defect in DM. These studies have provided several clues that point towards a generalized dysfunction of cell membranes. The myotonia itself probably reflects a disturbance of the normal process of depolarization and repolarization of the muscle membrane (Rüdel and Lehmann-Horn, 1985). This is supported by the finding that myotonia may be experimentally induced by activators of protein kinase C through lowering of the sarcolemmal chloride conduction (Brinkmeyer and Jockusch, 1987). However, abnormal chloride conduction is not the basic defect in DM (Rüdel and Lehmann-Horn, 1985). In fact, a report of tight linkage between a skeletal muscle chloride channel gene (CLC-1) on chromosome 7 and both autosomal dominant myotonia congenita (Thomsen disease) and autosomal recessive generalized myotonia (Becker disease) suggests that genetic defects involving this chloride channel cause generalized myotonia. This is further supported by a point mutation (Phe-to-Cys) in two families with autosomal recessive generalized myotonia (Koch et al. 1992). On the other hand, linkage and mutation analysis in families with paramyotonia congenita (PC), hyperkalemic periodic paralysis (HYPP) and acetazolamide-responsive myotonia congenita suggests that these disorders are caused by allelic defects in a skeletal muscle sodium channel gene on chromosome 17q (Ptacek et al. 1991; Ptacek et al. 1992; McClatchey et al. 1992a; McClatchey 1992b). A mutation in the homologous sodium channel gene has been demonstrated in myotonic mice (Steinmeyer et al. 1991) and in Quarter horses with periodic paralysis (Rudolph et al. 1992).

Other findings pointing to membrane abnormalities in DM include abnormal protein kinase activity in the erythrocyte membrane (Roses and Appel, 1973; Roses and Appel, 1974), abnormal results of electron spin resonance studies of erythrocytes (Butterfield et al. 1974), increased calcium influx in erythrocytes (Plisker et al. 1978), lowered temperature response of membrane phosphorylation in erythrocytes (Vickers et al. 1979), defective phospholipid metabolism in the erythrocyte (Grey et al. 1980), increased fragility of the band 3 membrane protein of erythrocytes (Tsuchiya et al. 1983), abnormal platelet function (Bornstein et al. 1988), whole body insulin resistance (Moxley et al. 1984), reduced insulin binding to cultured fibroblasts (Hudson et al. 1987), expression of the apamin receptor on muscle cell membranes (Renaud et al. 1986), abnormalities of the fast sodium current in cultured muscle cells (Rüdel et al. 1989), increased release of potassium after ischemic excercise (Wevers et al. 1990), and altered Ca^{2+} homeostasis of cultured muscle cells (Jacobs et al. 1991). The studies of Roses and Appel (1973; 1974) on abnormal protein kinase activity have recently received indirect support from the finding that the myotonic dystrophy mutation

involves the expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member (Brook et al. 1992; Fu et al. 1992; Jansen et al. 1992b). This subject will be discussed further in section 1.3.2.

1.2 Towards cloning of the DM gene

1.2.1 Genetic mapping of DM

1.2.1.1 Protein polymorphisms

Myotonic dystrophy was the first major autosomal disorder to show genetic linkage in man, when Mohr suggested linkage of DM to the Lewis and Lutheran blood groups (Mohr, 1954). At that time the relationship between the secretor status and the Lewis blood group was unclear. It was subsequently realized that the expression of the Lewis blood group antigen was dependent on the secretor status of an individual. Greenwalt (1961) confirmed that Lewis-Lutheran linkage was in fact secretor-Lutheran linkage. Studies by Renwick and coworkers (1971) and by Harper and coworkers (1972) confirmed linkage between DM and the secretor locus. It was not until several years later that the addition of the peptidase D gene (PEPD) (O'Brien et al. 1983) and the complement component 3 gene (C3) (Eiberg et al. 1983) to this linkage group allowed assignment of the DM gene to chromosome 19, since both C3 and PEPD had previously been assigned to this chromosome through the study of somatic cell hybrids (McAlpine et al. 1976; Whitehead et al. 1982).

1.2.1.2 Early DNA polymorphisms: C3 and APOC2

The detection of polymorphism at the DNA level in the form of restriction fragment length polymorphisms (RFLP's) has greatly facilitated the creation of genetic maps on which disease loci may be placed (Botstein, 1980). The first RFLP to be studied in DM was detected by using the gene for the third component of complement (C3) as a probe. Combining data from the C3 SstI RFLP and the C3 protein polymorphism in their sample with data from the previous study of Eiberg and colleagues (1983), Davies and coworkers (1983) arrived at a lod score of 3.36 at $\Theta = 0.05$ between DM and C3 in males. In females, no linkage could be established. The authors hypothesized that these discrepant results might be due to more frequent recombination in females than in males. That recombination frequency should be higher in females than in males had been previously suggested by Haldane based on data from various animal species (1922), and confirmed for specific human chromosomes by Keats (1982).

The next RFLP proved to be much closer to the DM locus. A study by Shaw et al. (1985) yielded a maximum lod score of 7.87 at $\Theta = 0.04$ (both sexes

combined) between DM and a set of RFLP's at the locus for Apolipoproteir C2 (APOC2). Close linkage between DM and APOC2 was subsequently confirmed by several groups (Hulsebos et al. 1985; Pericak-Vance et al. 1986; Bird et al. 1987). Compiling recently published linkage data (table 1.2.1), the DM-APOC2 lod score is now well over 90 at a recombination frequency of 0.02-0.03 (Le Beau et al. 1989).

TABLE 1.2.1: *Data on APOC2-DM linkage*

Reference	Zmax.	Θ
Brunner et al. (1989b)	19.91	0.02
Korneluk et al. (1989)	31.08	0.01
Yamaoka et al. (1990)	27.53	0.04
Harley et al. (1991b)	17.07	0.03

At first it was unclear whether the DM gene was on the short or on the long arm of chromosome 19. Linkage to C3 and PEPD appeared to indicate a genetic localization on 19p (Lusis et al. 1985; Lusis et al. 1986), while linkage with APOC2 was more consistent with a localization on 19q (Shaw et al. 1986; Hulsebos et al. 1985). This apparent discrepancy was resolved by the reassignment of PEPD to 19q (Davis et al. 1987) and the exclusion of DM from the C3-19cen interval on 19p (Friedrich et al. 1987).

1.2.1.3 Use of hybrid cell lines 908K1 and WILFM2 to generate clones from the DM region

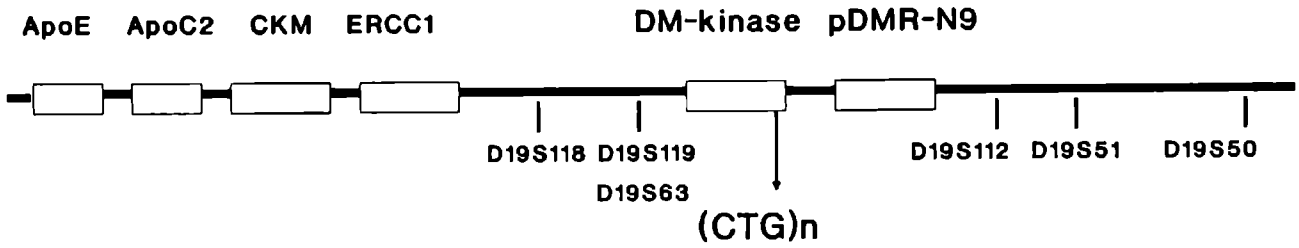
With all the data pointing towards a localization of DM on the proximal long arm of chromosome 19, two groups set out to generate a panel of somatic cell hybrids containing small pieces of chromosome 19. These somatic cell hybrids were used to isolate and to map genes and polymorphic DNA sequences that might be relevant to the search of the DM gene. As a starting point, both groups used a cell line (908K1 or WILFM2) containing a single der (19;X) chromosome with the breakpoint on chromosome 19 occurring at 19q13.2 (Hulsebos et al. 1986; Brook et al. 1986). These studies succeeded in accurately localizing a large number of genes and anonymous DNA fragments to specific intervals of the long arm of chromsome 19 (Schonk et al. 1989; Schonk et al. 1990; Brook et al. 1987; Brook et al. 1991; see also Schonk, 1991 for review). However, all polymorphic markers that were localized to the proximal long arm of chromosome 19 by this approach turned out to be further away from the DM gene than APOC2 (Shaw et al. 1986; Brunner et al. 1989a; Harley et al. 1991b). The first marker to appear as closely linked to DM as APOC2 was probe LDR152, that defines a MspI and a PstI polymorphism at locus D19S19. This probe had been isolated from a flow-sorted library that was enriched for human chromosome 19. A

iod score of 15.4 was reported without recombination (Bartlett et al. 1987). Somewhat surprisingly, this locus was later shown to be further away from DM than APOC2 (Yamaoka et al. 1990). Reinspection of the original linkage data by the same group of investigators suggests that the absence of recombination between DM and D19S19 in the original study may have been influenced by a preponderance of male meioses, the assumption again being that this segment of chromosome 19 has a smaller cross-over frequency in male meioses than in female meioses (Yamaoka et al. 1990). Other loci were described that appeared promising at first, but were subsequently shown to map further away from DM than APOC2. In particular, a clone (BCL3) from a lymphoma cell line translocation breakpoint, and a Na^+, K^+-ATPase α subunit gene (ATP1A3) did not bring the search any closer to the DM gene, although they helped in further ordering of the 19q12-q13.2 region (Korneluk et al. 1989; Harley et al. 1988). The APOE and APOC1 genes likewise were shown to be further from DM than the APOC2 gene (Smeets et al. 1988; Smeets et al. 1989; Brunner et al. 1989b; Walsh et al. 1990), although all three apolipoprotein genes are tightly linked, both physically (Smit et al. 1988a; Smit et al. 1988b) and genetically (Myklebost and Rogne, 1986; Myklebost and Rogne, 1988; Humphries et al. 1984). A critical step in the progress towards the DM gene was made when somatic cell hybrid studies showed that the gene for muscle type creatine kinase (CKM) maps physically close to the APOC2 gene in 19q13.2 (Stallings et al. 1988), and linkage analysis showed that the CKM gene could be as close to the DM gene as APOC2, or possibly closer (Brunner et al. 1989a). The demonstration that the distal breakpoint in the parental cell lines 908K1 and WILFM2 was within the CKM gene (Smeets et al. 1990; Brook et al. 1991) then targeted attention to the region of chromsome 19 just distal to the 19q13.2 endpoint of these somatic cell hybrid cell lines. Confirmation of a genetic localization of the DM gene distal to both APOC2 and CKM was subsequently obtained from the study of individual cross-over events (Brunner et al. 1989b; Johnson et al. 1989; Korneluk et al. 1989; Walsh et al. 1990; Yamaoka et al. 1990; Harley et al. 1991). The coding sequence of the CKM gene was investigated in a DM patient, but no translationally significant mutation was found (Bailly et al. 1991). It was shown that in a previously characterized cross over event with markers at the APOC2 and CKM loci, the DM gene also recombined with newly discovered polymorphisms at the ERCC1 locus (Smeets et al. 1991; Shutler et al. 1991). Since the ERCC1 and ERCC2 genes had been shown to lie distal to the 908K1 translocation breakpoint (Smeets et al. 1990), these findings excluded the DM gene from the previously constructed somatic cell hybrids (Smeets, 1991; Brook et al, 1991).

1.2.1.4 Genetic markers distal to ERCC1

Redefining the localization of the DM gene as 19q13.2-q13.3 (Smeets et al. 1991) led to the search for distal flanking markers from 19q13.3. Most efforts in this direction used the 20XP3542-1-4 cell line (Stallings et al. 1988) as the basis for further cloning of the DM genetic area. Markers distal to DM include the protein kinase C gene (Johnson et al. 1988) and the anonymous markers pEWRB1.1/4 (D19S50) (Korneluk et al. 1989), and p134c (D19S51) (Johnson et al. 1990; Tsilfidis et al. 1991a). The latter locus was shown to be closely linked genetically, at a recombination frequency of approximately .006 (Tsilfidis et al. 1991a). However, this locus has not yet been physically linked to the DM gene region (Buxton et al. 1992). Shutler and coworkers (1992) cloned 350 kb of genomic DNA extending from ERCC1 in a telomeric direction. In addition, they further characterized a previously reported DM-ERCC1 cross-over family (Smeets et al. 1991, Shutler et al. 1991). In this family, the recombinant event was shown to have occurred distal to D19S118 (Tsilfidis et al. 1991b). This excluded approximately 200kb of sequence between ERCC1 and D19S118 as the site of the DM gene. The segment of chromosome 19 that contains the DM mutation was reduced to at most 300 kb by the detection of a cross over event that placed the anonymous marker pX75b (D19S112) distal to DM (Jansen et al. 1992a). The cloning of the DM genetic region (Aslanidis et al., 1992; Figure 1.2.1.4) was completed with the identification of two overlapping yeast artificial chromosome (YAC) clones and several cosmids containing D19S118 and D19S119 (Korneluk et al. 1991) on the proximal side of DM and D19S112 on the distal side of DM.

FIGURE 1.2.1.4: *Schematic representation of the DM gene area in 19q13.*



Only the order of the loci on the chromosome is shown. The centromere is on the left.
Genes are designated by boxes.
Anonymous markers are designated according to the numbering system of the Human Gene Mapping Conference.
The location of the trinucleotide (CTG) repeat in the 3'-untranslated region of the DM chromosome is indicated.

1.2.2 Linkage disequilibrium

The first indication of linkage disequilibrium in DM was found in a study of French Canadian families from the Saguenay-Lac-Saint-Jean area (Laberge et al. 1985; Laberge, 1989). In these studies, the E4 allele of APOE was significantly overrepresented in DM patients in comparison with unaffected controls. The observed allelic association was attributed to a founder effect, since it is known that this population is descended from a small group of immigrants from France at the end of the 17th and the beginning of the 18th century. In fact, extended genealogical reconstruction has later shown that all of 746 DM patients in this area are descendant from a single ancestral couple who were married in 1661 (Mathieu et al. 1990). Subsequent studies of several APOC2 polymorphisms in 23 French-Canadian families from other parts of Canada again demonstrated linkage disequilibrium (MacKenzie et al. 1989). This group of investigators expanded their findings by performing extensive haplotype analysis with 10 RFLPs on a set of 87 DM chromosomes. The results indicated that most DM patients in the French Canadian population have one of two ancestral mutations (Korneluk et al. 1991b). In a small study of 15 Finnish families, significant linkage disequilibrium was again found between DM and APOC2, but not between DM and CKM (Nokelainen et al. 1990). Gennarelli and coworkers (1991) also did not detect linkage disequilibrium between DM and CKM in 59 DM families from Italy and Spain.

A very important step in the progress towards the DM gene was the identification of strong linkage disequilibrium between D19S63 (Brook et al. 1991) and DM in the "outbred" British population. The results suggested that at least 58% of the DM patients in the British population, as well as in the French-Canadian population have the same ancestral mutation (Harley et al. 1991a). Unfortunately, the authors did not further elaborate this hypothesis. However, at this point it was clear that the DM mutation behaved in an unusual fashion compared to other autosomal dominant mutations, since for a condition having such obvious negative consequences on fertility (Höweler, 1986) many new mutations would be required to maintain the gene frequency. The strong linkage disequilibrium between DM and D19S63 was quickly confirmed in other laboratories (Cobo et al. 1992; and our own unpublished data). Less marked linkage disequilibrium was also observed with the D19S112 marker, distal to DM (Jansen et al. 1992).

1.3 The DM mutation

1.3.1 Identification of the DM gene

Recently, several groups simultaneously reported the discovery of the DM mutation. Each of these groups relied on the genetic map generated over a period of 6 years as has been outlined in the previous section. Harley and

coworkers (1992a), Buxton and coworkers (1992a), and Aslanidis and coworkers (1992) screened genomic clones from the previously defined candidate region for expressed sequences. Each group reported that one of their cDNA clones detected a DNA fragment that is larger in DM patients than in normal subjects. Individual families showed increasing expansion of the unstable fragment in subsequent generations, suggesting a biological explanation for the anticipation phenomenon (see section 1.1.2). In addition, Harley and coworkers (1992a) reported that a second (Eco RV) polymorphism at this locus (D19S95) is in almost complete linkage equilibrium with DM, since the larger allele was found on 74/75 DM chromosomes, but only on 140/232 normal chromosomes. They suggested that it was likely that most cases of DM have a common origin, most likely a small insertion or duplication with an inherent ability to expand (Harley et al. 1992). Fu and coworkers (1992) arrived at the DM mutation by screening YAC clones from the previously defined DM genetic region (Jansen et al. 1992a; Shutler et al. 1992; Aslanidis et al. 1992) for trinucleotide repeats. This strategy was based on similarities in the unusual pattern of inheritance between DM and the fragile X syndrome, a point that had previously been made by Sutherland and coworkers (1991). They identified a cosmid (pMDY1) containing a CTG trinucleotide repeat sequence that showed expansion in DM patients. They further showed that the CTG trinucleotide repeat is part of the 3'-untranslated region of a gene that shows homology to protein kinase genes (Fu et al. 1992). Simultaneously, Brook and coworkers (1992) and Mahadevan and coworkers (1992b) reported that the expansion occurred in this CTG trinucleotide repeat sequence, and that the repeat was part of the 3'-untranslated region of a gene with possible serine-threonine protein kinase activity. Jansen and coworkers (1992b) showed that the CTG trinucleotide repeat sequence in the homologous mouse gene contains only 5 trinucleotides and is interrupted by two A residues. Alternatively spliced isoforms were found in both human and mouse cDNAs prepared from brain and heart mRNA. Whether these isoforms have different functions *in vivo* is unclear. Jansen and coworkers (1992b) further demonstrated that the DMR-B15 gene, which contains the CTG trinucleotide repeat is expressed at low levels in almost all tissues. High-level expression was observed in skeletal muscle, heart and various organs containing smooth muscle. Another gene (DMR-N9), which is located in close proximity to DMR-B15 is most strongly expressed in brain and testis. Thus, one may speculate that the expression of DMR-N9 is also affected by the expansion of the CTG trinucleotide repeat.

1.3.2 Function of the putative DM gene

Based on homology to conserved sequnece motifs, the putative DM gene product is considered to belong to the family of (serine-threonine) protein

kinases (Brook et al. 1992; Fu et al. 1992; Janssen et al. 1992b). It has been proposed that the putative protein kinase (or kinases) could play a significant role in the regulation of excitation-contraction coupling or maintenance of cellular physiology via regulation of protein-protein interactions in ion channels, or insulin-receptor signalling (Jansen et al. 1992b).
Brook and coworkers (1992) noted strong similarity to the cAMP-dependent protein kinase (cAPK) and suggested that some of the known functions of cAPKs fit well with the diverse defects exhibited by DM patients. Such cAPK functions include phosphorylation of ion channels, control of glycogen and lipid metabolism through cell signalling involving enzyme phosphorylation and modification of gene expression. Some members of the serine-threonine protein kinase family are known protooncogenes that are amplified and overexpressed in certain tumours. For example, the AKT2 gene, which maps to 19q13.1-q13.2 is amplified in ovarian carcinomas (Cheng et al. 1992). In this regard, it may be worthwhile to examine gene expression in tumours that show association with DM such as pleiomorphic adenomas of the salivary glands (Jóhannesson et al. 1978) and pilomatrixomas (Harper, 1972; Chiaramonti and Gilgor, 1978; Delfino et al. 1985).
It is to be expected that our knowledge of the biochemical basis of DM will greatly expand over the next few years. At present, only limited data are available. Questions concerning the cellular function and location of the putative protein kinase, the function of alternatively spliced isoforms (Jansen et al. 1992b), the possible involvement in DM of other genes from the CTG-containing region of the chromosome, or the tissue distribution of repeat sequence mosaicism in DM patients have not yet been answered. However, the answers to these questions are necessary before a meaningful comparison can be made between the clinical phenotype and the primary disturbance of cellular function in this condition. Many other questions will undoubtedly present themselves as this part of the DM story gets underway.

1.3.3 Origin of the DM mutation

Genealogical studies (Fleischer, 1918; Höweler, 1986; Mathieu et al. 1990; and unpublished results) suggest that in selected families the DM mutation may be at least several generations old. Based on the almost complete linkage disequilibrium between the expanded CTG trinucleotide repeat sequence and the D19S95 insertion polymorphism (Harley et al. 1992a), as well as with other markers in the DM gene area (Harley et al. 1992c) it has been suggested that all DM mutations are descended from a single ancestral mutation. Based on this assumption, one group has even attempted to correlate the distribution of DM in various countries and populations with the evolutionary tree of the human race (Ashizawa and Epstein, 1991). Stable transmission of expanded alleles over four generations

has been noted in individual families (Barceló et al. 1992). However, the suggestion that expanded alleles are often stably transmitted for several generations (Harper et al. 1992) is unlikely to be correct in view of their strong over-all tendency to expand further (e.g. Brunner et al. 1992b; and unpublished; see also paragraph 1.3.4). Rather, one would suspect that unstable expanded alleles are quickly eliminated from the population because of the reduced fertility of males with adult-onset disease (Höweler, 1986) and the reduced potential for procreation for childhood-onset and congenital-onset cases of both sexes (O'Brien and Harper, 1984). For linkage disequilibrium to exist, the unstable expanded repeat sequence should originate from a semi-stable premutated allele that can only be destabilized by a distinct event, either mutational or epigenetic. This theory has been further expanded in the case of the fragile X syndrome by Morton and MacPherson (1992). These authors suggested that 4 types of alleles exist at the fragile X locus: 5-60 repeats (N); 52-150 repeats (S and Z) and >200 repeats (L). In this model, the S and Z alleles are similar in size, but differ in state, the S allele being relatively stable, while the Z allele is highly unstable. Based on estimates in the paper by Morton and MacPherson (1992), Chakravarty (1992) calculated the following frequencies:

TABLE 1.3.3.1

Allele	Type	Frequency	Mean Age (generations)
N	Normal	0.9751	-
S	Stable insert	0.0225	90
Z	Unstable insert	0.0014	2
L	Mutation (symptomatic)	0.0010	1.4

The rate of conversion from S to Z, which is the critical step in this 3-step model was estimated as 1.1% per generation (Chakravarty, 1992). This model derives some support from the detection of limited linkage disequilibrium between larger "normal" alleles at the fragile X locus (36-50) and marker haplotypes that had previously been shown to be overrepresented in fragile X patients (Richards et al. 1992c).

It seems reasonable to assume that a very similar situation exists for DM. The fact that all chromosomes carrying the DM mutation have an insertion at locus D19S95, within an intron of the putative DM gene, whereas only 55% of normal chromsomes carry this insertion suggests that only a proportion of normal chromosomes has the potential to evolve into a DM chromosome. Indeed, Imbert and coworkers (1993) have suggested the presence of 3 distinct groups of normal chromosomes. Chromosomes with 5 CTG trinucleotide repeats always carry the D19S95 insertion, but chromosomes with 11-15 CTG trinucleotide repeats generally do not. Larger normal alleles of 16-40 CTG trinucleotide repeats also carry the insertion (table 1.3.3.2).

TABLE 1.3.3.2: *CTG-trinucleotide number and D19S95 polymorphism*

CTG trinucleotide number	Frequency*	D19S95 insert present	
		own data	Imbert et al. 1993
5	.45	8/8	104/104
11-15	.45	0/11	12/149
16-40	.10	6/6	34/34

*Based on 382 chromosomes (Brook et al. 1992; Brunner et al. 1992b)

It is presently unknown, which proportion of all chromosomes carrying the 1kb insertion at locus D19S95 (approximately 55% in the normal population) contain the presumed semistable premutated alleles (S in table 1.3.3.1). It may well be that only chromosomes with 16 or more CTG trinucleotides (10%) or a subgroup of these are at risk for further expansion. For instance, the frequency of alleles containing 20 or more CTG trinucleotide repeats is approximately 5% in caucasians (Brook et al. 1992; Brunner et al. 1992b). Given the suggestion that all DM chromosomes are derived from a common ancestral chromosome (Harley et al. 1992a; Harley et al. 1992c) it would seem that the gene pool containing semi-stable premutated alleles must be much larger than has been calculated for fragile X. Also, the DM mutation has to be much older than the 90 generations that have been calculated in the case of the fragile X syndrome (Chakravarty, 1992).
So far, it is unclear what constitutes the essential difference between the S class of semi-stable premutated alleles and the N class of normal alleles. At present, there are at least three possible scenarios:

1. The 1 kb insertion at D19S95 found in 55% of the normal population is itself the initiating factor for the destabilization of the trinucleotide repeat. This is unlikely, in view of the fact that approximately 50% of DM chromosomes carry the smallest allele for a PstI polymorphism at the D19S63 locus (table 1.3.3.3). Since this allele is found on only 20% of normal chromosomes, and not itself in linkage disequilibrium with D19S95, the DM premutation pool is probably significantly smaller and contains only a fraction of all chromosomes carrying the D19S95 insertion.
2. The 1 kb insertion at D19S95 is in linkage disequilibrium with a subgroup of alleles, presumably those with larger CTG trinucleotide repeats (e.g. those with 16 or more CTG trinucleotide repeats; Imbert et al. 1993). This hypothesis may be tested by comparing the distribution of
   alleles for D19S63 between chromosomes of different trinucleotide content.
   Premutated (S) alleles would be expected to show a significant excess of the 6.5kb allele for D19S63, as well as a reduced frequency of the 6.8kb allele (table 1.3.3.3).
   A single ancestral mutation would have caused overrepresentation of the 6.5 kb allele in combination with an equal diminution of the 6.8 and 7.1 kb alleles. Therefore, the overrepresentation of the 6.5 kb

allele *and* underrepresentation of the 6.8 kb allele of D19S63 does not *prima facie* seem compatible with a single ancestral chromosome from which all DM chromsomes have descended. A limited number (2-6) of ancestral DM chromosomes seems a more likely explanation.

Data obtained by Imbert and coworkers (1993) strongly support this model, since the distribution of alleles at the D19S112 locus for DM chromosomes and for larger normal alleles containing ≥19 CTG trinucleotides was virtually identical. The other large group of chromosomes containing the D19S95 insert are those with 5 CTG trinucleotides. These showed a very different distribution of alleles at the D19S112 locus, indicating that this allele is not a frequent origin of DM chromosomes. The authors suggested that between 1 and 4 ancestral mutations were probably responsible for all DM chromosomes and that alleles containing between 19 and 30 CTG trinucleotides (10% overall frequency) may constitute a reservoir for recurrent DM mutations.

3. An unknown number of ancestral mutations have occurred which can give rise to unstable premutated (Z) alleles only if the 1kb insertion at D19S95 is present on the same chromosome (cis-effect). Until the first *bona fide* expanded allele lacking the 1kb D19S95 insertion has been documented, this scenario remains possible. However, the number of ancestral mutations should not be very large, because of the observed linkage disequilibrium with D19S63 (table 1.3.3.3). It is theoretically possible that under this scenario a few frequent mutations coexist with a larger number of rare mutations, a situation reminiscent of that found in cystic fibrosis.

TABLE 1.3.3.3: *Linkage disequilibrium between DM and D19S63*[*]

D19S63 PstI alleles	DM (n=122)	non-DM (n=436)
1 (7.1 kb)	.44	.58
2 (6.8 kb)	.07	.25
3 (6.5 kb)	.49	.17

[*]Combined data from Harley et al. (1991a), from Cobo et al. (1992), and from our own laboratory (unpublished)

1.3.4 Anticipation revisited

As noted in previous sections, the number of CTG trinucleotidesin DM tends to increase further in successive generations and both the age at onset and the clinical phenotype correlate with the size of the mutation (Harley et al. 1992b, Hunter et al. 1992). These findings have provided a biological basis for the observation that the onset of DM occurs earlier in successive generations (Fleischer, 1918; Ravin and Waring, 1939; Bell, 1947; Klein, 1958; Höweler et al. 1989). However, the factors governing the transition from minimally expanded CTG trinucleotide repeats found in subjects who are asymptomatic or have cataract as their only feature, to larger mutations associated with muscle weakness and clinical myotonia have not yet been elucidated.

We studied 68 parent-child pairs (from 38 sibships) where the parent had less than 100 CTG trinucleotie repeats (Chapter 9). A correlaton between DM allele size in the parent and DM allele size in the offspring was found. A similar correlation has also been documented for the CCG trinucleotide expansion associated with the fragile X syndrome (Fu et al. 1991, Yu et al. 1991, Heitz et al. 1992).

An excess of mildly affected or asymptomatic male transmitters has previously been noted in DM families (Bell, 1948; Klein, 1958; Harper, 1989; Brunner et al. 1991). We demonstrated that this male excess is specifically associated with the first appearance of the clinical phenotype in the family (Brunner et al. submitted; Chapter 9).

The basis for this male transmission bias appears to be the different magnitude of the CTG expansion on male and female transmision. In our study of 68 parent-child pairs, the chance that a mutation of 60-79 CTG trinucleotides in the parent expanded to $\geq$100 CTG trinucleotides in the offspring was 48/52 for offsping of males and 7/16 for offspring of females ($p = 0.0002$), (Brunner et al. submitted; Chapter 9). Preliminary evidence for greater instability with male transmission was also obtained by others (Barceló et al. 1993).

If the rate of expansion is indeed greater with male than with female transmission, this raises a number of questions.

First, it is not clear, whther this male transmission bias is due to greater instability in male meiosis, in the mitotic divisions preceding meiosis, or in the mitotic divisions that occur in early embryogenesis. Alternatively, the apparent instability of paternally inherited DM mutations may simply reflect the larger number of cell divisions during male gametogenesis than during female gametogenesis (Edwards, 1989).

Second, irrespective of its cause, the greater instability of paternally inherited alleles contrasts with the (almost) exlusively maternal inheritance of the most severe forms of myotonic dystrophy. This may partly reflect a greater

reduction in fertility of affected males than of females with onset of clinical signs in early adulthood or in childhood (Höweler, 1986). However, those affected males that are fertile, still do not have offspring with congenital-onset DM. As discussed in paragraph 1.5, several explanations have been proposed for this phenomenon. Most of these explanations invoke a maternal factor other than the DM mutation itself. However, the finding that 11 cases of decreasing CTG repeat length were all paternally inherited (Ashizawa et al. 1992; Hunter et al. 1993; O'Hoy et al. 1993; Shelbourne et al. 1992; Brunner et al. 1993) suggests that a paternal factor may be acting directly on the size of the DM mutation in the offspring. In fact, a combination of male infertility and selection against individual sperm bearing large expansions at the DM locus may explain the exclusively maternal origin of the severe congenital form of DM. Recent data on sperm samples from males with different disease severity also support the hypothesis of selection against larger DM alleles in sperm (G. Jansen, personal communication).
Third, if CTG repeat expansion occurs not only during gametogenesis, but also in the (early) embryo, this raises the question whether genomic imprinting may be involved in the different rate of repeat expansion of paternally and maternally derived alleles. Data from two different experiments indicate that the DM-kinase gene is expressed from both parental chromosomes in both mouse and man (G. Jansen et al. 1993). While this does not completely rule out a differential imprint affecting secondary DNA structure, it shows that genomic imprinting in the usual sense (i.e. uniparental expression at the RNA level) is not involved in DM.
Further studies of trinucleotide repeat length in sperm from DM patients, but also in fetal tissues at different stages of embyogenesis, and possibly in tissue culture experiments should be of great value for further elucidation of the dynamics of the unstable mutation.

1.3.5 Similarities to other conditions with unusual inheritance patterns

1.3.5.1 Similarities to the fragile X syndrome and to X-linked spinal muscular atrophy

A number of reviews have discussed the similarities between the first three disorders in which the mutation was found to consist of an expanded trinucleotide repeat sequence (Richards and Sutherland 1992a, 1992b; Sutherland and Richards, 1992) Before the DM mutation was identified, expanded trinucleotide repeats had been identified in fragile X syndrome and in X-linked spinal and muscular atrophy (La Spada et al. 1991; Fu et al. 1991; Verkerk et al. 1991; Yu et al. 1991; Oberlé et al. 1991). Some of the similarities (and a few differences) are given in table 1.3.5.1. Since recently, at least 40 genes have been identified that contain polymorphic trinucleotide repeats, this list will most likely expand over the next few years (Riggins et

al. 1992). In fact, since the initial preparation of this text, three more disorders were found to be associated with expanded trinucleotide repeats, namely Huntington's disease (the Huntington's disease collaborative research group, 1993), spinocerebellar ataxia type 1 (Orr et al. 1993), and FRAXE mental retardation (Knight et al. 1993).

TABLE 1.3.5.1: *Trinucleotide repeat mutations in Fragile X syndrome, X-linked spinal and muscular atrophy and myotonic dystrophy*

	FRA X	XR-SMA	DM
TRINUCLEOTIDE	CCG	CTG	CTG
REGION OF GENE	5'-untranslated region of FMR-1	coding sequence of androgen receptor gene	3'-untranslated region of DM-kinase
NORMAL NUMBER	6-60	13-30	3-40
ASYMPTOMATIC CARRIERS	60-200		42-90
PATIENTS	200-2000	39-60	200->2000
METHYLATION OF EXPANDED ALLELE	YES	UNKNOWN	UNKNOWN
INSTABILITY (mitotic and meiotic)	YES	SLIGHT	YES
EFFECT ON TRANSCRIPTION	REDUCED/ABSENT	NONE	UNKNOWN
FOUNDER CHROMOSOMES	YES	NO?	SINGLE MUTATION ?
ANTICIPATION INFLUENCED BY SEX OF PARENT	YES (females only)	YES	(YES: COMPLEX)

1.3.5.2 Non-Mendelian patterns in Huntington's disease and in other disorders

Since Mendel's laws imply that genes are transmitted from one generation to the next essentially unaltered, anticipation and imprinting are important modifications of these laws. It may be worthwhile to examine the literature for other conditions which also show unexplained deviations from the expected situation under strictly Mendelian inheritance.

Both fragile X syndrome and DM show significant linkage disequilibrium between the disease mutation and polymorphisms in the adjacent DNA segments. This suggests a common origin of the mutation in different families (Richards et al. 1992; Harley et al. 1991a). The linkage disequilibrium in DM has been discussed in some detail in paragraphs 1.2.2 and 1.3.3. Another possible candidate for an unstable mutation was the gene for Huntington's disease (Harper et al. 1992a). Linkage disequilibrium (Skraastad et al. 1992) and anticipation with parental sex effect (Ridley et al. 1988) had both been demonstrated in Huntington's disease. Also, the early onset form of Huntington's disease which is asssociated with prominent rigidity rather than choreic movements is almost exclusively inherited from the father. Since the initial preparation of this text, the mutation that causes Huntington's disease has indeed been shown to consist of an expanded trinucleotide (CAG) repeat (the Huntington's disease collaborative reseach group, 1993).

Interesting observations have been reported for several disorders, that might turn out to be caused by an unstable mutation. Possible candidates include the finding of strong allelic association in Ashkenazi Jews between the gene for autosomal dominant torsion dystonia and RFLP markers on chromosome 9 (Risch et al. 1990; Ozelius et al. 1992). Also, two recent pedigree studies appear to show earlier onset (and more severe symptoms) in successive generations of a family with neurogenic scapuloperoneal amyotrophy (DeLong and Siddique, 1992) and of a family with cavernous hemangiomas of the brain (Steichen-Gersdorf et al. 1992). However, these reports concern single families that are rather small, and therefore some of the biases suggested by Penrose (1948) to account for the anticipation phenomenon may well be operative. Anticipation has also been suggested to occur in familial amyloid neuropathy (Sousa et al. 1991), in neurofibromatosis type 2 (Evans et al. 1992), and in myoclonic dementia (Little et al. 1986).

An effect of parental sex on expression in offspring has been noted for monogenic disorders such as spinocerebellar ataxia and amyotrophic lateral sclerosis (Leone et al. 1992; Harding, 1981). Interestingly, an expanding (CAG) trinucleotide repeat has now been detected in a subset of families with spinocerebellar ataxia that maps to chromosome 6 (Orr et al. 1993). An effect of parental sex on transmission has also been noted for disorders that

are usually considered to show multifactorial inheritance such as epilepsy (Ottman et al. 1988), congenital heart defects (Nora and Nora, 1988), psoriasis (Traupe et al. 1992), and neural tube defects (Mariman and Hamel, 1992; Chatkupt et al. 1992). In most of these conditions, genomic imprinting (Reik 1989) may be an alternative explanation, as it is in hereditary glomus tumors (van der Mey et al., 1989) or in familial Angelman syndrome (Meijers-Heijboer et al. 1992; Wagstaff et al. 1992). However, it should not be overlooked that genomic imprinting and unstable mutations caused by expanded trinucleotide repeat sequences are not mutually exclusive mechanisms. In fact, the extensive methylation of large expansions in the FMR-1 gene shows that dynamic mutations affecting the DNA sequence may induce epigenetic effects that can perhaps influence gene function (Oberlé et al. 1991; Richards and Sutherland, 1992b; Hansen et al. 1992; Sutcliffe et al. 1992).

1.3.6 Genotype-phenotype correlations

Many monogenic disorders show variation in severity, age at onset and type of symptoms, both within and between families. This is called variable expressivity. In those cases where symptoms are completely lacking, the term non-penetrance is commonly used. Consistent differences between families may indicate that different mutations - either allelic or non-allelic - are involved. However, differences within a single family - or between families carrying the same mutation - are more difficult to explain. Within the last few years, it has become apparent that such variability in expression may be due to any one of several mechanisms, usually involving mutations at additional loci, which together constitute the genetic background. Alternatively, the mutation at the main locus may be influenced directly, either through a second mutation (as in the case of unstable mutations), through influence of the homologous (normal) chromosome, or through the process of genetic imprinting, wich is defined as functional inequality of maternal versus paternal copies of a gene or gene region (Cattanach and Kirk, 1985). Table 1.3.6 shows a compilation of various mechanisms of variable phenotypic expressivity of single gene disorders. Some of these mechanisms have yet to be convincingly demonstrated in man (e.g. position effect variegation). On the other hand, it appears unlikely that this list should be complete in its present form. Rather, an increasing number of mechanisms, as well as combinations of these should be anticipated. Also, as indicated above, some of the mechanism listed are not mutually exclusive. For instance, it has been proposed that genetic imprinting is a function of sex-linked modifier genes (Sapienza, 1990). Others have stressed the possible role of heterochromatin formation in explaining both position effect variegation in Drosophila as well as genetic imprinting in man (Tartof and Bremer, 1990). Finally, Reik et al. (1989) postulated that anticipation in Huntington's disease could be caused by genomic imprinting.

TABLE 1.3.6.1:
Mechanisms of variable expressivity of monogenic disorders

MECHANISM	REFERENCES
Exogenous factors	Kappas et al. 1989
Heterogeneity of mutation	
Allelic	Byers et al. 1991
Non-allelic	Parfrey et al. 1990
Contiguous gene syndrome	Ballabio et al. 1990
Intrafamiliar variability at main locus	
Second mutation	Howell et al. 1991; Goldfarb et al. 1992
Unstable mutation	Verkerk et al. 1991
Imprinting	van der Mey et al. 1989
Allelic modification	Cepellini et al. 1955
Position effect variegation	Reuter et al. 1990
Anticipation	Ridley et al. 1988
Influences from other locus	
Non-allelic modifier	Maestri and Beaty, 1992
Sex-limited/sex-influenced trait	Egli et al. 1985
Somatic mosaicism	Edwards et al. 1990
Other	
Depletion of mitochondrial DNA	Moraes et al. 1991
Secondary mutation at other locus	Zeviani et al. 1989
Abnormal karyotype	Garcia-Dorado et al. 1990

In DM, the variability of the size of the unstable mutation appears to be the major factor determining the clinical phenotype (Harley et al. 1992b; Ashizawa et al. 1992b; Tsilfidis et al. 1992; Hunter et al. 1992; Hunter et al. 1993). The fact that correlations between the genotype (as determined from peripheral blood DNA samples) and the clinical phenotype are rather broad may be at least partly explained by the existence of somatic heterogeneity between different tissues of an individual. Other factors may also influence the pattern of expression of DM in individual cases. To some extent, this is supported by the finding of familial clustering of specific symptoms of DM (Brunner et al. 1992a). However, reports of this type of observation are strongly biased towards the unusual and only prospective studies can establish whether such familial clusters are truly nonrandom.
It may be noted that a recent study of neurofibromatosis (NF) type 1, also concluded that the expression of specific NF1 traits was similar in close relatives, and that this trait-specific clustering was best explained by the influence of modifying genes (Easton et al. 1993).

1.4 Presymptomatic diagnosis of DM

Myotonic dystrophy is one of the most frequent reasons for referral to our genetic counselling unit. Often, an apparently healthy family member is concerned about transmitting the disorder to future offspring. It has been shown repeatedly that detailed neurological examination, electromyography, and slit lamp examination will each detect highly specific signs in a small proportion of such asymptomatic individuals (Harper, 1989). Because of the many possible symptoms, several other clinical tests have previously been proposed for screening of at-risk individuals. No clear additional benefit has been demonstrated for any of these (reviewed in Harper, 1989). Nonetheless, when careful clinical testing has been performed, approximately 8% of asymptomatic young adults at 50% prior risk will still turn out to be gene carriers when examined by DNA analysis (Brunner et al. 1991a; Brunner et al. 1992b).

DNA analysis with closely linked markers has been widely used for presymptomatic as well as prenatal diagnosis (Meredith et al. 1986; Bird et al. 1987; Norman et al. 1989; Thibault et al. 1989; Yamaoka et al. 1990; Milunsky et al. 1991; Mulley et al. 1991; Nokelainen et al. 1991; Lavedan et al. 1991). If both clinical testing and the analysis of linked DNA markers suggest that the consultand is not affected, the risk of possessing the DM gene drops to less than 1% for adult individuals at 50% prior risk. Predictive testing using linked genetic markers has generally proven effective in DM (Reardon et al. 1992), and very few diagnostic errors have been recognized in retrospect (Brunner et al. 1992b; Shelbourne et al. 1992).

Current diagnostic protocols are all based on direct detection of the expanded CTG trinucleotide repeat sequence, although linked genetic markers are still used occasionally to confirm results from direct mutation analysis. Direct mutation analysis using PCR amplification of the expanded repeat sequence (Mahadevan et al. 1992b) has proven highly effective in excluding DM when the presence of two normal-sized CTG trinucleotide repeats can be demonstrated (Brunner et al. 1992b; HJM Smeets and W Nillesen, personal communications). This method has also shown good results for the detection of expanded alleles in most families (e.g. Smeets et al. 1992; Brunner et al. 1992b). However, in some cases the abnormal expanded allele is not visualized by PCR because of preferential amplification of the normal (smaller) allele. Therefore, in those cases where only a single normal allele can be seen after PCR, further testing is required. This may be done by Southern blotting of genomic fragments that contain the CTG trinucleotide repeat or by linkage analysis using closely linked polymorphic systems such as D19S112 (Jansen et al. 1992a). Other laboratories have decided to rely mainly on conventional Southern blotting of genomic fragments containing the repeat (Shelbourne et al. 1992; Myring et al. 1992). This circumvents the

problem of preferential amplification of the normal allele, at the cost of more laborious (and time-consuming) methodology.

1.5 Congenital myotonic dystrophy

Congenital myotonic dystrophy is characterized by reduced mobility and swallowing *in utero* (Pearse and Höweler, 1983). Reduced mobility causes positional limb deformities such as talipes equinovarus. Insufficient swallowing causes polyhydraminos, which may initiate premature birth. Following birth, severe respiratory problems are the rule (Rutherford et al. 1989). These may end in the demise of the affected child. In exceptional cases, the fetus may have severe lung hypoplasia caused by intrauterine immobility (personal observation). Surviving infants show a gradual increase in muscle strength over a period of weeks to months. The long-term prognosis is poor with regard to independent functioning. Mild to moderate mental retardation, in combination with residual muscular weakness and skeletal problems such as talipes often cause significant problems (O'Brien and Harper, 1984). After puberty, muscle weakness again progresses.

The pathogenesis of the congenital form of DM is unknown. Several authors have commented on the immature aspect of the skeletal muscle fibers (Sarnat and Silbert, 1976; Saghal et al. 1983; Iannaccone et al. 1986; Farkas-Bargeton et al. 1988) as well as of other organs (Young et al. 1981). In this regard, the observation that the DM gene product shows very high expression at the neurmuscular junction of type 1 fibers during innervation (B Wieringa, personal communication) suggests a possible pathogenetic mechanism underlying the severe muscle hypotonia in the neonatal period.

In 1972, Harper and Dyken discussed the striking preponderance of affected mothers compared to affected fathers of children with the congenital form of myotonic dystrophy (Harper and Dyken, 1972a). They suggested that a maternal environmental factor might interact with the presence of the DM gene in these children. The nature of this putative maternal factor is still unknown (see Harper, 1989 for review). Some authors have suggested the possibility that the mitochondrial genome (which is exclusively maternally inherited) might interact with the DM gene (Merrill and Harrington, 1985; Poulton, 1988; Poulton, 1992). However, determination of the complete mitochondrial DNA sequence in patients with congenital DM has excluded this possibilty (Thyagarajan et al. 1991). It has also been suggested that genomic imprinting might be involved in DM (e.g. Reik, 1989). This latter suggestion was particularly attractive because imprinting effects have been documented for the homologous region on chromosome 7 in mouse (Searle and Beechey, 1990; Cavanna et al. 1990; Saunders and Seldin, 1990). However, the DM-kinase gene is expressed from both chromosomes in both humans and mice (G. Jansen et al., 1993).

Koch et al. (1991) reported findings that appeared to strengthen the hypothesis of maternal metabolites acting on a heterozygous offspring. Points in favour of this hypothesis are:

- Congenital DM is exclusively maternally inherited.
- The severity of the clinical picture increases in subsequent siblings in sibships containing a congenital case (fraternal anticipation).
- The severe neonatal hypotonia improves with time (comparable to maternal myasthenia gravis)
- The occurrence of congenital DM in offspring correlates with the age at onset and severity of the symptoms in the mother, while carrier sisters also have a risk of having congenitally affected children appropriate to their disease status.

Koch and coworkers pointed out that these findings are not easily reconciled with either genomic imprinting or mitochondrial effects (Koch et al. 1991). A completely different view was taken by Höweler, who showed that fertility is severly reduced in males with age at onset in the 2nd or 3rd decade compared to similarly affected females (Höweler, 1986; Höweler et al., 1989). He therefore concluded, that the selective infertility in males causes a lack of paternal inheritance. In combination with continued anticipation on transmission through females, this at least partly explains the exclusively maternal inheritance of congenital DM.

It has been shown that, on average, individuals with congenital DM have larger CTG trinucleotide inserts than patients with noncongenital forms of the disease (Harley et al. 1992b, Tsilfidis et al. 1992). However, based on the partial overlap of CTG repeat lengths between cases with congenital and noncongenital forms of DM, Harley et al. (1992c) believe that the simple correlation between repeat length and clinical phenotype does not entirely explain the most severely affected class of congenitally affected individuals.

As discussed in paragraph 1.3.4, the chance of a severely affected offspring born to an affected male may be further reduced by contraction or even reversion of the mutation in the offspring. Therefore, we believe that selection against transmission of very large alleles may prevent males from having congenitally affected offspring. This view has also been taken by others (Mulley et al. 1993; Lavedan et al. 1993). Studies of CTG repeat length in various tissues from congenitally affected patients, as well as studies on sperm from affected males may further clarify this issue.

1.6 Outlook

1.6.1 Diagnosis

The discovery of the mutation underlying DM has already changed diagnostic practice for this disorder. Given the ease of diagnosis by DNA analysis, there is no doubt that this method will become the method of choice for

diagnosis as well as exclusion of DM. Moreover, the level of diagnostic accuracy obtained by DNA analysis of the CTG trinucleotide repeat expansion is clearly higher than that previously attained using a combination of clinical examination, slit lamp examination and EMG (Brunner et al. 1992b; Brunner et al. 1991a; Harley et al. 1992b; Tsilfidis et al. 1992; Shelbourne et al. 1992). It should be noted however, that each of these groups with extensive experience in the diagnosis of DM at the molecular level has encountered families in which an expanded CTG repeat sequence was not present. Although clinical misdiagnosis may explain at least some of these cases, detailed reexamination of such families is of great importance to determine whether other mutations of the DM gene exist that also lead to a DM phenotype. At least one group is currently examining the open reading frame of the DM gene for alternative mutations in a family with clinically diagnosed DM that does not show expansion of the CTG trinucleotide repeat (Mahadevan et al. 1992b). Analogous observations in fragile X syndrome may provide indirect support for the hypothesis of alternative mutations in DM (Wöhrle et al. 1992; Gedeon et al. 1992; De Boulle et al. 1993).

We have reported a family with an unusual combination of myotonic dystrophy and peripheral neuropathy that appeared a promising candidate for an allelic mutation (Spaans et al. 1986; Brunner et al. 1991a). Notwithstanding the highly unusual presentation with a polyneuropathy, affected subjects in this family have an expanded CTG trinucleotide repeat (W. Nillesen, personal communication). Although this apparently excludes an allelic mutation, it remains possible that the phenotype in this family is modified by a second mutation in the DM gene region.

An interesting example of such a two-mutation mechanism has been reported. Two distinct disease phenotypes (Creutzfeld-Jacob disease and familial fatal insomnia), have been reported in associon with a pathogenic Asn^{178} mutation in the proteolipid prion protein gene on chromosome 21. However, further analysis of this gene has shown that the clinical phenotype in each family depends on a common polymorphism in the same gene. This polymorphism changes a methionine to a valine at position 129 of the prion proteolipid protein, and is not itself considered pathogenic. The possibility of an interaction between the two mutations was raised to account for the difference in expression of the disease (Goldfarb et al. 1992).

Recently, two groups have stressed the importance of counselling potential DM mutation carriers before actual DNA testing is offered (Harper et al. 1992b; Laberge, 1991). Our own experience suggests that this is indeed an important issue, since we have already detected unstable expanded alleles in two clinically normal spouses of DM family members in the context of preparatory DNA testing aimed at later prenatal diagnosis.

1.6.2 Prognosis

A broad correlation has been observed between the size of the expanded CTG trinucleotide repeat and the severity of the clinical picture of DM (Harley et al. 1992b; Ashizawa et al. 1992b; Tsilfidis et al. 1992; Hunter et al. 1992). The fact that considerable overlap exists in DNA fragment length (assayed on peripheral blood cells) between individuals with markedly disssimilar clinical pictures shows that accurate prognosis cannot be provided by the current DNA diagnostic protocols. The exception to this rule may be the carriers who have less than 100 CTG trinucleotide repeats with little evidence for somatic mosaicism. Clinically relevant muscle involvement has not been noted in a sample of over 70 individuals with this type of expansion (HJM Smeets and H.T. Brüggenwirth, personal communication). Therefore it may be possible to reassure such individuals that cataract is the only complication that they are likely to develop as a result of being a DM mutation carrier. Extensive somatic mosaicism as well as differences in mean fragment length have been demonstrated in various tissues from affected individuals with severe forms of DM (G Jansen, B Wieringa, personal communications). This finding may explain some (and possibly most) of the discrepancies between clinical status and DNA findings in peripheral blood cells. However, presence or absence of specific symptoms in a given patient is most likely determined by a multitude of factors. These may not only include the size of the DM mutation, but also the possible modifying influences of other genes, as well as non-genetic influences from the environment (Ravin and Waring, 1939; Brunner et al. 1992a). For these reasons, a prognosis of disease progression based on DNA analysis alone is presently not very reliable and is unlikely to become accurate in the near future.

1.6.3 Epidemiology

Within the next few years, it can be anticipated that much more will be learned concerning the origins of the DM mutation, as well as the factors governing its transition from a putative semistable premutation (or protomutation; Barceló et al. 1993) to the highly unstable expansion of 40-100 CTG trinucleotide repeats found in subjects with late onset forms of the disease. This process most likely involves several distinct steps, each of which is controlled by a specific factor or set of factors. The hypothetical ancestral DM premutation from which all DM mutations are derived may involve all chromosomes that carry the 1kb insertion (D19S95; Harley et al. 1992a) that is present in an intron of the putative DM gene. Alternatively, the presence of this insertion in combination with allele lengths of 15 or more CTG trinucleotide repeats could constitute the original premutation (Imbert et al. 1993; paragraph 1.3.3). Finally, the influence of another mutation (either on the 1kb insertion fragment or in some other part of the gene) has not yet

been excluded as contributing to the instability of the DM trinucleotide motif.
As discussed in paragraph 1.3.4, transmission by a male is more likely to result in the transition from a minimal mutation of 40-100 CTG trinucleotide repeats to a larger (>100 CTG trinucleotides) expanded allele than transmission by a female. For subjects with adult onset of the disease, the situation is probably influenced by selection against larger mutations in the male germ line. Contraction or even complete reversal of a DM mutation may be more frequent among paternally inherited mutations (Brunner et al. 1993; Shelbourne et al. 1992; O'Hoy et al. 1993; Mettler et al. 1992; Hunter et al. 1993; Mulley et al. 1993; Lavedan et al. 1993; Ashizawa et al. 1992a; unpublished data). In contrast, female transmission is likely to result in further expansion of the mutation, possibly explaining the fact that congenital-onset DM is virtually limited to the offspring of females with adult-onset or childhood-onset DM (Mulley et al. 1993; Lavedan et al. 1993; Brunner et al. in press).
Comparison of allele size in various tissues from congenital and non-congenital DM with similar expansions in peripheral blood cells may at least determine whether the amplification of the DM allele is the decisive factor in determining clinical status. Determination of allele sizes in sperm from males with various clinical forms of DM could help to further clarify these issues. Analysis of unselected blood samples from populations with differences in the prevalence of DM may also help to determine the proportion of chromosomes at risk of eventually expanding to a DM mutation.

1.6.4 Prospects for therapy

With the discovery of the putative DM gene, and the recognition of similarities to genes encoding serine-threonine protein kinases (Brook et al. 1992; Fu et al. 1992; Jansen et al. 1992b), we enter a new era of studies aimed at characterizing, and eventually correcting, the abnormalities at the cellular level in DM. At this writing, the effect of the expanded CTG repeat on the function of the putative DM gene (DM-kinase, Jansen et al. 1992b; myotonin protein-kinase, Fu et al. 1992; C28, Brook et al. 1992) or on neighbouring genes such as DMR-N9 (Jansen et al. 1992b) is still unclear. Furthermore, the putative DM gene has been shown to encode differently spliced mRNAs in brain and heart (Jansen et al. 1992b). It may well be that the DM gene has different functions in different tissues. Whether this means that eventually, DM will be amenable to treatment at the molecular level cannot be predicted at this time. Recent advances in gene therapy by intramuscular injection of genetically engineered myoblasts (Barr and Leiden, 1991; Dhawan et al. 1991) or of specific DNA constructs (Acsadi et al. 1991) suggest that, at least in principle, it is possible to have a modified gene

product expressed in muscle *in vivo*, but whether this will be of therapeutic relevance to DM is not at all clear. Recent experience with the intramuscular injection of human dystrophin constructs in dystrophin-deficient mdx mice (Acsadi et al. 1991) has not offered great hope in this regard. Before gene therapy or even pharmacological modulation of DM can be envisaged, animal model systems will have to be established (Harper, 1989). Gene targeting in embryonic stem cells has been successfully employed to construct animal models for several other human gene defects, and the cloning of the mouse homologue of the putative DM gene (Jansen et al. 1992b) provides the first step in this direction (G. Jansen, B. Wieringa, personal communications).

REFERENCES

Acsadi G, Dickson G, Love DR et al. (1991) Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. Nature 352:815-818.

Aldridge LM (1985) Anaesthetic problems in myotonic dystrophy Br J Anaesth 57:1119-1130

Anonymous (1992) Editorial. The heart in myotonic dystrophy. The Lancet. 339:528-529

Ashizawa T,Dubel JR, Dunne CJ et al. (1992a) Anticipation in myotonic dystrophy. II. Complex relationships between clinical findings and structure of the GCT repeat. Neurology 42:1871-1877

Ashizawa T, Dunne CJ, Dubel JR et al. (1992b) Anticipation in myotonic dystrophy. I. Statistical verification based on clinical and haplotype findings. Neurology 42:1871-1877

Ashizawa T, Epstein HF (1991) Ethnic distribution of the myotonic dystrophy gene. Lancet 338:642-643

Aslanidis C, Jansen G, Amemiya C, et al (1992) Cloning of the essential myotonic dystrophy region and mapping of the putative defect. Nature 355:549-51

Avrahami E, Katz A, Bornstein N, Korczyn AD. Computed tomographic findings of brain and skull in myotonic dystrophy. J Neurol Neurosurg Psych 50:435-438, 1987

Bailly J, MacKenzie AE, Leblond S, Korneluk RG (1991) Assessment of a creatine kinase isoform M defect as a cause of myotonic dystrophy and the characterization of two novel CKMM polymorphisms. Hum Genet 86:457-462

Ballabio A, Bardoni B, Carrozzo R et al. (1989) Contiguous gene syndromes due to deletions in the distal short arm of the human X chromosome. Proc Natl Acad Sci, USA 86:10001-10005

Barceló J, Tsilfidis C, Earle-MacDonald M et al. (1993) Intergenerational stability of the myotonic dystrophy protomutation. Hum Mol Genet 2: 705-709.

Barr E, Leiden JM (1991) Systemic delivery of recombinant proteins by genetically engineered myoblasts. Science 254:1507-1509

Bartlett RJ, Pericak-vance MA, Yamaoka L, Gilbert J, Herbstreith M, Hung WY, Lee JE, Mohandas T, Bruns G, Laberge C, Thibault MC, Ross D, Roses AD (1987) A new probe for the diagnosis of myotonic muscular dystrophy. Science 235:1648-1650

Batten FE, Gibb HP (1909) Myotonia atrophica. Brain 32:187-205

Becker PE (1977) Myotonia congenita and syndromes associated with myotonia. Georg Thieme publishers, Stuttgart

Bell J (1947) Dystrophia myotonica and allied diseases. The Treasury of Human Inheritance 4:342-410

Bird TD, Boehnke M, Schellenberg GD, Deeb SS, Lipe HP (1987) The use of apolipoprotein CII as a genetic marker for myotonic dystrophy. Arch

Neurol 44:273-275
Bornstein NM, Zahavi M, Korczyn AD, Zahavi J (1988) Increased platelet aggregation and release reaction in myotonic dystrophy. J Neurol Sci 84:239-246
Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32:314-331
Brinkmeier H, Jockusch H (1987) Activators of protein kinase C induce myotonia by lowering chloride conductance in muscle. Biochem Biophys Res Comm 148:1383-1389
Brook JD, Harley HG, Walsh KV et al. (1991) Identification of new DNA markers close to the myotonic dystrophy locus. J Med Genet 28:84-88
Brook JD, McCurrach ME, Harley HG, et al. (1992) Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell 68:799-808
Brook JD, Skinner M, Roberts SH, Rettig WJ, Almond JW, Shaw DJ (1987) Further mapping of markers around the centromere of human chromosome 19. Genomics 1:320-328
Brunner HG, Hamel BCJ, Rieu P, Höweler CJ, Peters FTM (1992a) Intestinal pseudo-obstruction in myotonic dystrophy. J Med Genet 29:791-793
Brunner HG, Jansen G, Nillesen W, Nelen MR, de Die CEM, Höweler CJ, van Oost BA, Wieringa B, Ropers HH, Smeets HJM. Reverse mutation in myotonic dystrophy (1993) N Engl J Med. 328:476-480
Brunner HG, Korneluk RG, Coerwinkel-Driessen M, MacKenzie A, Smeets H, Lambermon HMM, Oost van BA, Wieringa B, Ropers HH (1989a) Myotonic dystrophy is closely linked to the gene for muscle-type creatine kinase (CKMM). Hum Genet 81:308-310
Brunner HG, Nillesen W, Oost van B, Jansen G, Wieringa B, Ropers HH, Smeets HJM (1992b) Presymptomatic diagnosis of myotonic dystrophy. J Med Genet 29:780-784
Brunner HG, Smeets H, Lambermon HMM, Coerwinkel-Driessen M, Oost van BA, Wieringa B, Ropers HH (1989b) A multipoint linkage map around the locus for myotonic dystrophy on chromosome 19. Genomics 5:589-595
Brunner HG, Smeets HJM, Nillesen W, Oost van B, Biezenbos van den JBM, Joosten EMG, Pinckers AJLG, Hamel BCJ, Theeuwes AGM, Wieringa B, Ropers HH (1991a) Myotonic dystrophy. Predictive value of normal results on clinical examination. Brain 114:2303-116
Brunner HG, Spaans F, Smeets HJM, Coerwinkel-Driessen M, Hulsebos T, Wieringa B, Ropers HH (1991b) Close linkage with chromosome 19 but not chromosome 17 in a family with myotonic dystrophy associated with hereditary motor and sensory neuropathy. Neurology 41:80-84
Bundey S (1982) Clinical evidence for heterogeneity in myotonic dystrophy. J Med Genet 19:341-348.
Butterfield DA, Roses AD, Copper ML (1974) A comparative spin resonance study of the erythrocyte membrane in myotonic muscular dystrophy.

Biochemistry 13:5078-5082.
Buxton J, Shelbourne P, Davies J, et al. (1992a) Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. Nature 355:547-8
Buxton J, Shelbourne P, Davies J, et al. (1992b) Characterization of a YAC and cosmid contig containing markers tightly linked to the myotonic dystrophy locus on chromosome 19. Genomics 13:526-531
Byers PH, Wallis GA, Willing MC (1991) Osteogenesis imperfecta: Translation of mutation to phenotype. J Med Genet 28:433-442
Cattanach BM, Kirk M (1985) Differential activity of maternally and paternally derived chromosome regions in mice. Nature 315:496-498
Cavanna JS, Greenfield AJ, Johnson KJ, Marks AR, Nadal-Ginard B, Brown SDM (1990) Establishment of the mouse chromosome 7 region with homology to myotonic dystrophy region of human chromosome 19q. Genomics 7:12-18
Cepellini R, Dunn LC, Turri M (1955) An interaction between alleles at the Rh locus in man which weakens the reactivity of the Rh_0 factor (D^u). Proc Natl Acad Sci 41:283-288
Chakravarti A (1992) Fragile X founder effect? Nature Genet 1:237-238
Chatkupt S, Lucek PR, Koenigsberger MR (1992) Parental sex effect in spina bifida: A role for genomic imprinting? Am J Med Genet 44:508-512
Cheng JQ, Godwin AK, Bellacosa A et al. (1992) AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci 89:9267-9271
Chiaramonti A, Gilgor RS (1978) Pilomatricomas associated with mytonic dystrophy. Arch Dermatol 114:1363-1365
Cobo A, Grinberg D, Balcells S, Vilageliu L, Gonzalez-Duarte R, Baiget M (1992) Linkage disequilibrium detected between myotonic dystrophy and the anonymous marker D19S63 in the Spanish population. Hum Genet 89:287-291
Curschmann H (1912) Ueber familiare atrophische Myotonie. Dtsch Z Nervenheilk 45:161-202
Curschmann H (1925) Zur Nosologie und Symptomatologie der myotonischen Dystrophie. Dtsch Arch Klin Med 149:129-144
Davies KE, Jackson J, Williamson R, Harper PS, Ball S, Sarfarazi M, Meredith L, Fey G (1983) Linkage analysis of myotonic dystrophy and sequences on chromosome 19 using a cloned complement 3 gene probe. J Med Genet 21:259-263
Davis MB, Schonk D, Monteiro M, Oerlemans F, Povey S, Wieringa B (1987) Localization of PepD to the long arm of chromosome 19. Ann Hum Genet 51:195-199
DeBoulle K, Verkerk AJMH, Reyniers E (1993) A point mutation in the FMR-1 gene associated with fragile X mental retardation. Nature Genet 3:31-35
Delfino M, Monfrecola G, Ayala F, Suppa F, Piccirillo A (1985) Multiple familial pilomatricomas: A cutaneous marker for myotonic dystrophy. Dermatologica 170:128-132

DeLong R, Siddique T (1992) A large New England kindred with autosomal dominant neurogenic scapuloperoneal amyotrophy with unique features. Arch Neurol 49:905-908

Dhawan J, Pan LC, Pavlath GK et al. (1991) Systemic delivery of human growth hormone by injection of genetically engineered myoblasts. Science 254:1509-1512

Dyken P (1969) The changing syndromes of dystrophia myotonica. Neurology 19:292

Easton DF, Ponder MA, Huson SM, Ponder BAJ (1993) An analysis of variation in expression of neurofibromatosis (NF) type 1 (NF1): Evidence for modifying genes. Am J Hum Genet 53:305-313

Edwards JH (1987) Familiarity, recessivity and germline mosaicism. Ann Hum Genet 53:33-47

Edwards MJ, Byers PH, Cohn DH (1990) Mild osteogenesis imperfecta produced by somatic mosaicism for a lethal mutation in a type 1 collagen gene. Am J Hum Genet 47:215

Egli CA, Rosenthal SM, Grumbach MM, Montalvo JM, Gondos B (1985) Pituitary gonadotropin-independent male-limited autosomal dominant sexual precocity in nine generarions: Familial testotoxicosis. J Pediatr 106:33-40

Eiberg H, Mohr J, Nielsen LS, Simonsen N (1983) Genetics and linkage relationships of the C3 polymorphism: discovery of the C3:Se linkage and assignment of LES-C3-DM-Se-PEPD-LU synteny to chromosome 19. Clin Genet 24:159-170

Evans DGR, Huson SM, Donnai D, et al. (1992) A genetic study of type 2 neurofibromatosis in the United Kingdom. I. Prevalence, mutation rate, fitness, and confirmation of maternal transmission effect on severity. J Med Genet 29:841-846

Farkas-Bargeton E, Barbet JP, Dancea S et al. (1988) Immaturity of muscle fibers in the congenital form of myotonic dystrophy: its consequences and its origin. J Neurol Sci 83:145-149

Fischer RA (1931) The evolution of dominance. Biol Rev 6:345-368

Fleischer B (1918) Ueber myotonische Dystrophie mit Katarakt. Albrecht von Graefes. Arch Klin Ophtalmol 96:91-133

Friedrich U, Brunner H, Smeets D, Lambermon E, Ropers HH (1987) Three-point linkage analysis employing C3 and 19cen markers assigns the myotonic dystrophy gene to 19q. Hum Genet 75:291-293

Fu Y-H, Kuhl DPA, Pizzuti A, et al. (1991) Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047-58

Fu Y-H, Pizzuti A, Fenwick RG et al. (1992) An unstable triplet repeat in a gene related to myotonic dystrophy. Science 255:1256-8

García-Dorado J, De Unamuno P, Fernández-López E, Salazar Veloz J, Armijo M (1990) Incontinentia pigmenti: XXY male with a family history. Clin Genet 38:128-138

Gedeon AK, Baker E, Robinson H et al. (1992) Fragile X syndrome without CCG amplification has an FMR deletion. Nature Genet 1:341-344

Genarelli M, Novelli G, Cobo A, Baiget M, Dallapicola B (1991) 3'creatine kinase (M-type) polymorphisms linked to myotonic dystrophy in Italian and Spanish populations. Hum Genet 87:654-656

Goldfarb LG, Petersen RB, Tabaton M et al. (1992) Fatal familial insomnia and familial Creutzfeldt-Jacob disease: Disease phenotype determined by a DNA polymorphism. Science 258:806-808

Greenfield JG (1911) Notes on a family of "myotonia atrophica" and early cataract, with a report on an additional case of myotonia atrophica. Rev Neur Psych (Edin) 9:169-181

Greenwalt TJ (1961) Conformation of linkage between the Lutheran and Secretor genes. Am J Hum Genet 13:69-88

Grey JE, Gitelman HJ, Roses AD (1980) Defective phospholipid metabolism in the erythrocyte plasma membrane. J Clin Invest 65:1478-1482

Grimm, T (1976) The age of onset and the age of death in patients with dystrophia myotonica, J Génét hum 23:301-308

Haldane JBS (1922) Sex ratio and unisexual sterility in hybrid animals. J Genet 12:101-109

Haldane JBS (1941) The relative importance of principal and modifying genes in determining some human diseases. J Genet 41:149-157

Hansen RS, Gartler SM, Scott CR, Chen SH, Laird CD (1992) Methylation analysis of CGG sites in the CpG island of the human FMR1 gene (1992) Hum Molec Genet 1:571-578

Harding AE (1981) Genetic aspects of autosomal dominant late onset cerebellar ataxia. J Med Genet 18:436-441

Harley HG, Brook JD, Jackson CL, Glaser T, Walsh KV, Sarfarazi M, Kent R, Lager M, Koch M, Harper PS, Levenson R, Housman DE, Shaw DJ (1988) Localization of a Human Na^+,K^+-ATPase α subunit gene to chromosome 19q21-13.2 and linkage to the myotonic dystrophy locus. Genomics 3:380-384

Harley HG, Brook JD, Shelley JF et al. (1991a) Detection of linkage disequilibrium between the myotonic dystrophy locus and a new polymorphic DNA marker. Am J Hum Genet 49:68-75

Harley HG, Brook JD, Rundle SA, et al (1992a) Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. Nature 355:-545-6

Harley HG, Rundle SA, Reardon W, et al (1992b) Unstable DNA sequence in myotonic dystrophy. Lancet 339:1125-28

Harley HG, Sandkuijl L, Rundle SA (1992c) Expansion of an unstable CTG repeat in myotonic dystrophy: relationship of phenotype to genotype and further evidence for a single ancestral mutation. Am J Hum Genet 51 Suppl A128

Harley HG, Walsh KV, Rundle S, et al (1991b) Localizaton of the myotonic dystrophy locus to 19q13.2-19q13.1 and its relationship to twelve polymorphic loci on 19q. Hum Genet 87:73-80

Harper PS (1972) Calcifying epithelioma of Malherbe. Association with myotonic muscular dystrophy. Arch Derm 106:41-44

Harper PS (1975) Congenital myotonic dystrophy in Britain. I. Clinical aspects. Arch Dis Child 50:505-513

Harper PS (1989) Myotonic Dystrophy, 2nd ed., W.B. Saunders Company, London

Harper PS, Dyken PR (1972) Early onset dystrophia myotonica-evidence supporting a maternal environmental factor. Lancet 2:53-55

Harper PS, Harley HG, Reardon W, Shaw DJ (1992a) Anticipation in myotonic dystrophy. New light on an old problem. Am J Hum Genet 51:10-16

Harper PS, Harley HG, Shaw DJ (1992b) Detection of minimum mutation carriers in myotonic dystrophy. Lancet 340:238-239

Harper PS, Rivas ML, Bias WB, Hutchinson JR, Dyken PR, McKusick VA (1972b) Genetic linkage confirmed between the locus for myotonic dystrophy and the ABH-secretion and Lutheran blood group loci. Am J Hum Genet 24:310-316

Heitz D, Devys D, Imbert G, Kretz C, Mandel J-L (1992) Inheritance of the fragile X syndrome: size of the fragile X premutation is a major determinant of the transition to full mutation. J Med Genet 29:794-801

Höweler CJ (1986) A clinical and genetic study in myotonic dystrophy. Thesis, Erasmus University, Rotterdam.

Höweler CJ (1988) Nieuwe inzichten bij dystrophia myotonica. Ned Tijdschr Geneesk 132:1076-1078

Höweler CJ, Busch HFM, Geraedts JPM, Niermeijer MF, Staal A (1989) Anticipation in myotonic dystrophy: fact or fiction? Brain 112:779-797

Huber SJ, Kissel JT, Shuttleworth EC et al. Magnetic resonance imaging and clinical correlates of intellectual impairment in myotonic dystrophy. Arch Neurol 46:536-540, 1989

Hudson AJ, Huf MW, Wright CG, Silver MM, Lo TCY, Banerjee D (1987) The role of insulin resistance in the pathogenesis of myotonic muscular dystrophy. Brain 110:469-488

Hulsebos T, Brunner H, Wieringa B, Friedrich U, Smeets D, Oei T, Hustinx T, Scheres J, Wienker T, Humphries S, Myklebost O, Junien C, Haar ter B, Ropers HH (1985) Regional assignment of C3, GPI, APOC2, and β-HCG and their linkage relationships with DM and 19cen. Cytogenet Cell Genet 40:658

Hulsebos T, Wieringa B, Hochstenbach R, Smeets D, Schepens J, Oerlemans F, Zimmer J, Ropers HH (1986) Toward early diagnosis of myotonic dystrophy: construction and characterization of a somatic cell hybrid with a single human der(19) chromosome. Cytogenet Cell Genet 43:47-56

Humphries SE, Berg K, Gill L, Cumming AM, Robertson FW, Stalenhoef AFH, Williamson R, Børresen AL (1984) The gene for apolipoprotein C-II is closely linked to the gene for apolipoprotein E on chromosome 19: Clin Genet 26:389-396

Hunter A, Tsilfidis C, Mettler G, Jacob P, Mahadevan M, Surh L, Korneluk R (1992) The correlation of age of onset with CTG trinucleotide repeat

amplification in myotonic dystrophy. J Med Genet 29:774-779
Hunter A, Jacob P, O'Hoy K, MacDonald I, Mettler G, Tsilfidis C, Korneluk RG (1993) Decrease in the size of the myotonic dystrophy CTG repeat during transmission from parent to child: Implications for genetic counselling and anticipation. Am J Med Genet 45:401-407
the Huntington's disease collaborative research group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971-983
Iannaccone ST, Bove KE, Vogler C et al. (1986) Muscle maturation delay in infantile myotonic dystrophy. Arch Pathol Lab Med 110:405-411
Imbert G, Kretz C, Johnson K, Mandel J-L (1993) Origin of the expansion mutation in myotonic dystrophy. Nature Genet 4:72-76
Jacobs AEM, Benders AAGM, Oosterhof A, Veerkamp JH, van Mier P, Joosten EMG (1991) The calcium homeostasis and the membrane of cultured muscle cells from patients with myotonic dystrophy. Biochim Biophys Acta 1096:14-19
Jansen G, Bartolomei M, Kalscheuer V, Merkx G, Wormskamp N, Mariman E, Smeets D, Ropers H-H, Wieringa B (1993) Hum Mol Genet 2:1221--1227
Jansen G, de Jong PJ, Amemiya C, et al (1992a) Physical and genetic characterization of the distal segment of chromosome of the myotonic dystrophy area on chromosome 19q. Genomics 1992;13:509-17
Jansen G, Mahadevan M, Amemiya C et al (1992b) Characterisation of the myotonic dystrophy (DM) region predicts multiple protein kinase isoform encoding mRNAs that differ at their 3' ends. Nature Genetics 1:261-6
Jóhannesson G, Henriksson KG, Ödkvist L (1978) Coincidence of dystrophia myotonica and pleiomorphic adenoma of the parotid gland. Acta Neurol Scand 57:275-278
Johnson K, Shelbourne P, Davies J, Buxton J, Nimmo E, Anvret M, Bonduelle M et al. (1989) Recombination events that locate myotonic dystrophy distal to APOC2 on 19q. Genomics 5:746-751
Johnson K, Shelbourne P, Davies J, Buxton J, Nimmo E, Siciliano MJ, Bachinski LL, Anvret M, Harley H, Rundle S, Miki T, Brunner H, Williamson R (1990) A new polymorphic probe which defines the region of chromosome 19 containing the myotonic dystrophy locus. Am J Hum Genet 46:1073-1081
Johnson KJ, Jones PJ, Spurr N, Nimmo E, Davies J, Creed H, Weiss, Williamson R (1988) Linkage relationships of the protein kinase C gamma gene which exclude it as a candidate for myotonic dystrophy. Cytogenet Cell Genet 48:13-15
Kappas A, Sassa S, Galbraith RA, Nordmann Y (1989) The porpyrias. In: "The metabolic basis of inherited disease" 6th ed., Eds. Scriver CR, Beaudet AI, Sly WS, Valle D. McGraw Hill Book Company, New York:p.1305-1365
Keats B (1982) Genetic mapping: Chromosomes 6-22. Am J Hum Genet 34:730-742

Kiuchi A, Otsuka N, Namba Y, Nakano I, Tomonaga M. Presenile appearance of abundant Alzheimer's neurofibrillary tangles without senile plaques in the brain in myotonic dystrophy. Acta Neuropath 82:1-5, 1991

Klein D (1958). La dystrophie myotonique (Steinert) et la myotonie congenitale (Thomsen) en Suisse: Etude clinique, génétique, et démographique. J Genet Hum 7 (Suppl):1-328

Knight SJL, Flannery AV, Hirst MC, Campbell L, Christodoulou Z, Phelps SR, Pointon J, Middleton-Price HR, Barnicoat A, Pembrey ME, Holland J, Oostra BA, Bobrow M, Davies KE (1993) Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. Cell 74:127-134

Koch MC, Grimm T, Harley HG, Harper PS (1991) Genetic risks for children of women with myotonic dystrophy. Am J Hum Genet 48:1084-1091

Koch MC, Steinmeyer K, Lorenz C et al. (1992) The skeletal muscle chloride channel in dominant and recessive human myotonia. Science 257:797-800

Korneluk RG, MacKenzie AE, Nakamura Y, Dubé, Jacob P, Hunter AGW (1989) A reordering of human chromosome 19 long-arm DNA markers and identification of markers flanking the myotonic dystrophy locus. Genomics 5:596-604

Korneluk RG, Tsilfidis C, Shutler G, Mahadevan M, Baily J, Surh LC (1991a) A three allele insertion polymorphism is identified by the human chromosome 19q13.3 probe pKBE0.8 (D19S119). Nucleic Acids Res 19:1157

Korneluk RG, Tsilfidis C, Shutler G et al. (1991b) Two distinct chromosome 19 haplotypes show linkage disequilibrium with myotonic dystrophy in a French Canadian population. Am J Hum Genet 47 [Suppl] A186

Kourilsky P (1986) Molecular mechanisms for gene conversion in higher cells. Trends in Genet 1:60-63

Laberge C, Gaudet D, Morisette J, Moorjani S, Thibault M-J (1985) Linkage of myotonic dystrophy and APOE in a French Canadian isolate. Cytogenet Cell Genet 40:675

Laberge C (1989) Myotonic dystrophy in Quebec:Geographical distribution and concept of genetic homogeneity. Can J Neurol Sci 16:123-128

Laberge CM (1991) Ethics of a genetic program for myotonic dystrophy. Am J Hum Genet 48:1204-1206

La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77-79

Lavedan C, Hofmann H. Shelbourne P, Duros C, Savoy D, Johnson K, Junien C (1991) Prenatal diagnosis of myotonic dystrophy using closely linked flanking markers. J Med Genet 28:89-91

Lavedan C, Hofmann-Radvanyi H, Rabes JP, Roume J, Junien C (1993) Different sex-dependent constraints in CTG length variation as explanation for congenital myotonic dystrophy. Lancet 341:237

LeBeau MM, Ryan D, Pericak-Vance MA (1989) Report of the committee on the genetic constitution of chromosome 18 and 19. Cytogenet Cell Genet

51:338-357
Leone M (1991) Parental sex effect in familial amyotrophic lateral sclerosis. Neurology 41:1292-1294
Little BW, Brown PW, Rodgers-Johnson P, Perl DP, Gajdusek DC (1986) Familial myoclonic dementia masquerading as Creutzfeldt-Jacob disease. Ann Neurol 20:231-239
Lotz BP, Van der Meyden CH (1985). Myotonic dystrophy. Part I. A genealogical study in the northern Transvaal. SA Med J 67:812-814
Lusis AJ, Heinzmann C, Sparkes RS, Geller R, Sparkes MC, Mohandas T (1985) Regional mapping on chromosome 19: apolipoprotein E, apolipoprotein CII, low density lipoprotein (LDL) receptor, peptidase D, glucose phosphate isomerase. Cytogenet Cell Genet 40:683
Lusis AJ, Heinzmann C, Sparkes RS, Scott J, Knott TJ, Geller R, Sparkes MC, Mohandas T (1986) Regional mapping of human chromosome 19: Organization of genes for plasma lipid transport (APOC1, -C2, and -E and LDLR) and the genes C3, PEPD, and GPI. Proc Natl Acad Sci USA 83:3929-3933
MacKenzie AE, MacLeod HL, Hunter AGW, Korneluk RG (1989) Linkage analysis of the apolipoprotein C2 gene and myotonic dystrophy on human chromosome 19 reveals linkage disequilibrium in a French-Canadian population. Am J Hum Genet 44:140-147
Maestri NE, Beaty TH (1992) Predictions of a 2-locus model for disease heterogeneity: Application to adrenoleukodystrophy. Am J Med Genet 44:576-582
Mahadevan M, Sabourin LA, O'Hoy K et al. (1992a) Mutation analysis in myotonic dystrophy families exhibiting no CTG repeat amplification at the DM locus. Am J Hum Genet 51 Suppl A328
Mahadevan M, Tsilfidis C, Sabourin L, et al (1992b) Myotonic dystrophy mutation: an unstable CTG repeat in the 3'-untranslated region of the gene. Science 1253-5
Mariman ECM, Hamel BCJ (1992) Sex ratios of affected and transmitting members of multiple case families with neural tube defects. J Med Genet 29:695-698
Mathieu J, De Braekeleer M, Prévost C (1990). Genealogical reconstruction of myotonic dystrophy in the Saguenay-Lac Saint-Jean area (Quebec, Canada). Neurology 40:839-842
McAlpine PJ, Mohandas T, Ray M, Wang H, Hamerton JL (1976) Assignment of the peptidase D gene locus (PEPD) to chromosome 19 in man. Cytogenet Cell Genet 16:204-205
McClatchey AI, Lin CS, Wang J, Hoffman EP, Rojas C, Gusella JF (1992a) The genomic structure of the human skeletal muscle sodium channel gene. Hum Mol Genet 1:521-527
McClatchey AI, McKenna-Yasek D, Cros D et al. (1992b) Novel mutations in families with unusual and variable disorders of the skeletal muscle sodium channel gene. Nature Genet 2:148-152
Meijers-Heijboer EJ, Sandkuijl LA, Brunner HG et al. (1992) Linkage analysis

with chromosome 15q11-13 markers shows genomic imprinting in familial Angelman syndrome. J Med Genet 29:853-857
Meredith AL, Huson SM, Lunt PW, Sarfarazi M, Harley HG, Brook JD, Shaw DJ, Harper PS (1986) Application of a closely linked polymorphism of restriction fragment length to counselling and prenatal testing in families with myotonic dystrophy. Br Med J 293:1353-1356
Merril CR, Harrington MG (1985) The search for mitochondrial inheritance of human disease. Trends Genet 5:140-144
Milunsky JM, Skare JC, Milunsky A (1991) Presymptomatic and prenatal diagnosis of myotonic muscular dystrophy with linked DNA probes. Am J Med Sci 301:231-237
Mohr J (1954) A study of linkage in man. Copenhagen: Munksgaard
Moorman JR, Coleman RE, Packer DL et al. (1985) Cardiac involvement in myotonic muscular dystrophy. Medicine 64:371-387
Moraes CT, Shanske S, Tritschler H-J, et al. (1991) mtDNA depletion with variable tissue expression: A novel abnormality in mitochondrial disease. Am J Hum Genet 48:492-501
Morton NE, Maclean CJ, Lew R, Yee S. Multipoint linkage analysis. Am J Hum Genet 1986;38:868-83
Morton NE, MacPherson JN (1992) Population genetics of the fragile-X syndrome: Multiallelic model for the FMR1 locus. Proc Natl Acad Sci USA 89:4215-4217
Moxley RT, Corbett AJ, Minaker KL, Rowe JW (1984) Whole body insulin resistance in myotonic dystrophy. Ann Neurol 15:157-162
Mulley JC, Gedeon AK, White SJ, Haan EA, Richards RI (1991) Predictive diagnosis of myotonic dystrophy with flanking microsatellite markers. J Med Genet 28:448-452
Mulley JC, Staples A, Donnelly A, Gedeon AK, Hecht BK, Nicholson GA, Haan EA, Sutherland GR (1993) Explanation for exclusive maternal origin for congenital form of myotonic dystrophy. Lancet 341:236-237
Myklebost O, Rogne S, Olaisen B, Gedde-Dahl T jr, Prydz H (1984) The locus for apolipoprotein CII is closely linked to the apolipoprotein E locus on chromosome 19. Neurology 36:1418-1423
Myklebost O, Rogne S (1986) The gene for human apolipoprotein CI is located 4.3 kilobases away from the apolipoprotein E gene on chromosome 19. Hum Genet 73:286-289
Myklebost O, Rogne S (1988) A physical map of the apolipoprotein gene cluster on human chromosome 19. Hum Genet 78:244-247
Myring J, Meredith AL, Harley HG, Kohn G, Norbury G, Harper PS, Shaw DJ (1992) Specific molecular prenatal diagnosis for the CTG mutation in myotonic dystrophy. J Med Genet 29:785-788
Nokelainen P, Alanen-Kurki L, Winqvist R, Falck B, Somer H, Leisti J, Johnson K, Savontaus ML, Peltonen L (1990) Linkage disequilibrium detected between dystrophia myotonica and APOC2 locus in the Finnish population. Hum Genet 85:541-545
Nora JJ, Nora AH (1988) Maternal transmission of congenital heart diseases:

New recurrence risk figures and the questions of cytoplasmatic inheritance and vulnerability to teratogens. Am J Cardiol 59:459-463

Norman AM, Floyd JL, Meredith AL, Harper PS (1989) Presymptomatic and prenatal diagnosis for myotonic dysrtophy by means of linked DNA markers. J Med Genet 26:750-754

Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boué J, Bertheas MF, Mandel JL (1991) Instability of a 550-base pair DNA segment and abnormal methylation in fragile x syndrome. Science 252:1097-1102

O'Brien, Ball S, Sarfarazi M, Harper PS, Robson EB (1983) Genetic linkage between the loci for myotonic dystrophy and peptidase D. Ann Hum Genet 47:117-121

O'Brien TA, Harper PS (1984) Course prognosis and complications of childhood-onset myotonic dystrophy. Dev Med Child Neurol 26:62-67

O'Hoy K, Tsilfidis C, Mahadevan M, Neville CE, Barceló J, Hunter AGW, Korneluk RG (1993) Reduction in size of the myotonic dystrophy trinucleotide repeat mutation during transmission. Science 259:809-812O n o S, Inoue K, Mannen T et al. Neuropathological changes of the brain in myotonic dystrophy - Some new observations. J. Neur Sci 81:301-320, 1987

Orr HT, Chung M-y, Banfi S, Kwiatkowsi TJJr, Servadio A, Beaudet AL, McCall AE, Duvick LA, Ranum LPW, Zoghbi HY (1993) Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. Nature Genet 4:221-226

Ottman R, Annegers JF, Hauser WA, Kurland LT (1988) Higher risk of seizures in offspring of mothers than of fathers with epilepsy. Am J Hum Genet 43:257-264

Ozelius LJ, Kramer PL, de Leon D et al. (1992) Strong allelic association between the torsion dystonia gene (DYT1) and loci on chromosome 9q34 in Ashkenazi jews. Am J Hum Genet 50:619-628

Parfrey PS, Bear JC, Morgan J, et al. (1990) The diagnosis and prognosis of autosomal dominant polycystic kidney disease. New Engl J Med 323:1085--1090

Pearse RG, Höweler CJ (1979) Neonatal form of dystrophia myotonica. Five cases in preterm babies and a review of earlier reports. Arch Dis Child 54:331-338

Penrose LS (1948) The problem of anticipation in pedigrees of dystrophia myotonica. Ann Eugen (Lond) 14:125-232

Pericak-Vance MA, Yamaoka LH, Assinder RIF, Hung W-Y, Bartlett RJ, Stajich JM, Gaskell PC, Ross DA, Sherman S, Fey GH, Humphries S, Williamson R, Roses AD (1986) Tight linkage of apolipoprotein C2 to myotonic dystrophy on chromosome 19. Neurology 36:1418-1423

Plishker GA, Gitelman HJ, Appel SA (1978) Myotonic muscular dystrophy: altered calcium transport in erythrocytes. Science 200:323-325

Poulton J (1988) Mitochondrial DNA and genetic disease. Arch Dis Chil 63:83-885

Poulton J (1992) Congenital myotonic dystrophy and mtDNA. Am J Med Genet 50:651

Ptacek LJ, Tawil R, Griggs RC, Storvick MS, Leppert M (1992) Linkage of atypical myotonia congenita to a sodium channel locus. Neurology 42:431-433

Ptacek LJ, Trimmer JS, Agnew WS, Roberts JW, Petajan JH, Leppert M (1991) Paramyotonia congenita and hyperkalemic periodic paralysis map to the same sodium channel locus. Am J Hum Genet 49:851-854

Ravin A, Waring JJ (1939) Studies in dystrophia myotonica. 1. Hereditary aspects. Am J Med Sci 197:593-609

Reardon W, Floyd JL, Myring J, Lazarou LP, Meredith AL, Harper PS (1992) Five years experience of predictive testing for myotonic dystrophy using linked DNA markers. Am J Med Genet 43:1006-1011

Reik W (1989) Genomic imprinting and genetic disorders in man. Trends Genet 5:331-336

Renaud JF, Desnuelle C, Schmid-Antomarchi H, Hugues M, Serratrice G, Lazdunski M (1986) Expression of apamin receptor in muscles of patients with myotonic muscular dystrophy. Nature 319:678-680

Renwick JH, Bundey SE, Ferguson-Smith MA, Izatt MM (1971) Confirmation of linkage of the loci for myotonic dystrophy and ABH secretion. J Med Genet 8:407-416

Reuter G, Giarre M, Farah J, Gausz J, Spierer A, Spierer P (1990) Dependence of position-effect variegation in Drosophila on dose of a gene encoding an unusual zinc-finger protein. Nature 344:219-223

Richards RI, Sutherland GR (1992a). Heritable unstable DNA sequences. Nature Genetics 1:7-9

Richards RI, Sutherland GR (1992b). Dynamic mutations: a new class of mutations causing human disease. Cell 70:709-712

Richards RI, Holman K, Friend K, et al. (1992c) Evidence of founder chromosomes in fragile X syndrome. Nature Genet 1:257-260

Ridley RM, Frith CD, Crow TJ, Conneally PM (1988) Anticipation in Huntington's disease is inherited through the male line but may originate in the female. J Med Genet 25:589-595

Ridley RM, Frith CD, Farrer LA, Conneally PM (1991) Patterns of inheritance of the symptoms of Huntington's disease suggestive of an effect of genomic imprinting. J Med Genet 28:224-231

Riggins GJ, Lokey LK, Chastain JL, Leiner HA, Sherman SL, Wilkinson KD, Warren ST (1992) Human genes containing trinucleotide repeats. Nature Genet 2:186-191

Risch NJ, Bressmann SB, deLeon D (1990) Segregation analysis of idiopathic torsion dystonia in Ashkenazi Jews suggests autosomal dominant inheritance. Am J Hum Genet 46:533-538

Roses AD, Appel SH (1973) Protein kinase activity in erythrocyte ghosts of patients with myotonic muscular dystrophy. Proc Natl Acad Sci USA 70:1855-1859

Roses AD, Appel SH (1974) Muscle membrane protein kinase in myotonic

muscular dystrophy. Nature 250:245-247
Rousseau F, Heitz D, Biancalana V, et al (1991). Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 235:1673-81
Rüdel R, Lehmann-Horn F (1985) Membrane changes in cells from myotonia patients. Physiol Rev 65:310-357
Rüdel R, Ruppersberg JP, Spittelmeister W (1989) Abnormalities of the fast sodium current in myotonic dystrophy, recessive generalized myotonia, and adynamia episodica. Muscle & Nerve 12:281-287
Rudolph JA, Spier SJ, Byrns G et al Periodic paralysis in Quarter horses: a sodium channel mutation disseminated by selective breeding. Nature Genet 2:144-147
Rutherford MA, Heckmatt JZ, Dubowitz V (1989) Congenital myotonic dystrophy: respiratory function at birth determines survival. Arch Dis Child 64:191-195
Saghal V, Bernes S, Saghal S (1983) Skeletal muscle in preterm infants with congenital myotonic dystrophy. J Neurol Sci 59:47-55
Sapienza C (1990) Sex-linked dosage-sensitive modifiers as imprinting genes. Development Suppl:107-113
Sarnat HB, Silbert SW (1976) Maturational arrest of fetal muscle in neonatal myotonic dystrophy. Arch Neurol 33:466-474
Saunders AM, Seldin MF (1990) The syntenic relationship of proximal mouse chromosome 7 and the myotonic dystrophy gene region on human chromosome 19q. Genomics 6:324-332
Schonk D (1991) Cell genetic characterization of the chromosome 19q region carrying the myotonic dystrophy gene. Thesis, University of Nijmegen.
Schonk D, Coerwinkel-Driessen M, Dalen van I, Oerlemans F, Smeets B, Schepens J, Hulsebos T, Cockburn D, Boyd Y, Davis M, Rettig W, Shaw D, Roses A, Ropers H, Wieringa B (1989) Definition of subchromosomal intervals around the myotonic dystrophy region at 19q. Genomics 4:384-396
Schonk D, Dijk van P, Riegman P, Trapman J, Holm C, Willcocks TC, Sillekens P, Venrooy van W, Wimmer E, Geurts van Kessel A, Ropers HH, Wieringa B (1990) Assignment of seven genes to distinct intervals on the midportion of human chromosome 19q surrounding the myotonic dystrophy gene region. Cytogenet Cell Genet 54:15-19
Searle AG, Beechey (1990) Genomic imprinting phenomena on mouse chromosome 7. Genet Res Camb 56:237-244
Shaw DJ, Meredith AL, Sarfarazi M, Harley HG, Huson SM, Brook JD, Bufton L, Litt M, Mohandas T, Harper PS (1986) Regional localisations and linkage relationships of seven RFLPs and myotonic dystrophy on chromosome 19. Hum Genet 74:262-266
Shaw DJ, Meredith AL, Sarfarazi M, Huson SM, Brook JD, Myklebost O, Harper PS (1985) The apolipoprotein C2 gene: subchromosomal localization and linkage to the myotonic dystrophy locus. Hum Genet 70:271-273

Shelbourne P, Winqvist R, Kunert E et al. (1992) Unstable DNA may be responsible for the incomplete penetrance of the myotonic dystrophy phenotype. Hum Mol Genet 1:467-473

Shutler G, Korneluk RG, Tsilfidis C et al. (1992) Physical mapping and cloning of the proximal segment of the myotonic dystrophy gene region. Genomics 13:518-525

Shutler G, MacKenzie AE, Brunner H, Wieringa B, De Jong P, Lohman FP, Leblond S, Bailly J, Korneluk RG (1991) Physical and genetic mapping of a novel chromosome 19 ERCC1 marker showing close linkage with myotonic dystrophy. Genomics 9:500-504

Skraastad MI, Van de Vosse E, Belfroid R et al. (1992) Significant linkage disequilibrium between the Huntington disease gene and the loci D4S10 and D4S95 in the Dutch population. Am J Hum Genet 51:730-735

Smeets HJM (1991) A molecular genetic approach to myotonic dystrophy. Thesis, Nijmegen

Smeets B, Poddighe J, Brunner H, Ropers HH, Wieringa B (1988) Tight linkage between myotonic dystrophy and apolipoprotein E genes revealed with allele-specific oligonucleotides. Hum Genet 80:49-52

Smeets H, Bachinski L, Coerwinkel M, Schepens J, Hoeijmakers J, van Duin M, Grzeschik KH, Weber CA, de Jong P, Siciliano MJ, Wieringa B (1990) A long-range restriction map of the human chromosome 19q13 region: Close physical linkage between CKMM and the ERCC1 and ERCC2 genes. Am J Hum Genet 46:49-501

Smeets HJM, Hermens R, Brunner HG, Ropers HH, Wieringa B (1991) Identification of variable simple sequence motifs in 19q13.2-qter: Markers for the myotonic dystrophy locus. Genomics 9:257-263

Smeets HJM, Nillesen W, Los F et al. (1992) Prenatal diagnosis of myotonic dystrophy by direct mutation analysis. Lancet 340:237-238

Smeets HJM, Brunner HG, Ropers HH, Wieringa B (1989) Use of variable simple sequence motifs as genetic markers: application to study of myotonic dystrophy. Hum Genet 83:245-251

Smit M, Kooij-Meijs van der E, Frants RR, Havekes L, Klasen E (1988a) Apolipoprotein gene cluster on chromosome 19. Definite localization of the APOC2 gene and the polymorphic HpaI site associated with type II hyperlipoproteinemia. Hum Genet 78:90-93

Smit M, Kooij-Meijs van der E, Woudt LP, Havekes LM, Frants RR (1988b) Exact localization of the familial dysbetalipoproteinemia associated HpaI restriction site in the promoter region of the APOCI gene. Biochem Biophys Res Comm 152:1282-1288

Sousa A, Coelho I, Lobato L, Segueiros (1991) Parental transmission and anticipation of age at onset in FAP-I. Am J Med Genet 50 (Suppl):A483

Spaans F, Jennekens FGI, Mirandolle JF, Bijlsma JB, de Gast GC (1986) myotonic dystrophy associated with hereditary motor and sensory neuropathy. Brain 109:1149-1168

Stallings RL, Olson E, Strauss AW, Thompson LH, Bachinski LL, Siciliano MJ (1988) Human creatine kinase genes on chromosome 15 and 19, and

proximity of the gene for the muscle form to the genes for apolipoprotein C2 and excision repair. Am J Hum Genet 43:144-151

Steichen-Gersdorf E, Felber S, Fuchs W, Russeger L, Twerdy K (1992) Familial cavernous angiomas of the brain: observations in a four generation family. Eur J Pediatr 151:861-863

Steinert H (1909) Myopathologische Beiträge 1. Ueber das klinische und anatomische Bild des Muskelschwunds der Myotoniker. Dtsch Z Nervenheilkd 37:58-104

Steinmeyer K, Klocke R, Ortland C, Gronemeier M, Jockusch H, Gründer S, Jentsch TJ (1991) Inactivation of muscle chloride channel by transposon insertion in myotonic mice. Nature 354:301-304

Sutcliffe JS, Nelson DL, Zhang F et al. (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. Hum Molec Genet 1:397-400

Sutherland GR, Haan EA, Kremer E et al. (1991) Hereditary unstable DNA: a new explanation for some old genetic questions? Lancet 338:289-292

Sutherland GR, Richards RI (1992) Invited editorial: Anticipation legitimized: unstable DNA to the rescue. Am J Hum Genet 51:7-9

Tartoff KD, Bremer M (1990) Mechanisms for the construction and developmental control of heterochromatin formation and imprinted chromosome domains. Development Suppl:35-45

Thibault M-C, Mathieu J, Moorjani S, et al. (1989) Myotonic dystrophy: Linkage with apolipoprotein E and estimation of the gene carrier status with genetic markers. Can J Neurol Sci 16:134-140

Thomasen E (1948) Myotonia. Universitetsforlaget i Aarhus, Denmark

Thomsen J (1876) Tonische Krämpfe in willkürlich bewegende Muskeln in Folge von ererbter psychischer Disposition. Arch Psychiatr 6:702-718

Thyagarajan D, Byrne E, Noer S, Lertritt P, Utthanophol P, Kapsa R, Marzuki S (1991) Mitochondrial sequence analysis in congenital myotonic dystrophy. Ann Neurol 30:724-727

Traupe H, van Gurp PJM, Happle R, Boezeman J, van de Kerkhof PCM (1992) Psoriasis vulgaris, fetal growth, and genomic imprinting. Am J Med Genet 42:649-654

Tsilfidis C, MacKenzie AE, Mettler G, Barceló J, Korneluk RG (1992). Correlation between trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. Nature Genet 1:192-195

Tsilfidis C, MacKenzie AE, Shutler G, Leblond S, Bailly J, Johnson K, Williamson R, Siegel-Bartelt J, Korneluk RG (1991a) D19S51 is closely linked with and maps distal to the myotonic dystrophy locus on 19q. Am J Hum Genet 49:961-965

Tsilfidis C, Shutler G, Mahadevan M, Korneluk RG (1991b) A frequent HincII polymorphism identified by the human chromosome 19q13.3 probe pKEX0.8 (D19S118). Nucleic Acids Res 19:1157

Tsuchiya Y, Sugita H, Kuroiwa Y (1983) Myotonic dystrophy: Fragility of band 3 membrane protein upon resealing of erythrocyte ghosts and the normality of ATPase activity, sialic acid and spectrin extractibility of ghosts. Biochemical medicine 30:271-279

Van der Mey AGL, Maaswinkel-Mooy PD, Cornelisse CJ, Schmidt PH, van de Kamp JJP (1989) Genomic imprinting in hereditary glomus tumors: Evidence for new genetic theory. Lancet ii:1292-1294

Vanier TM (1960) Dystrophia myotonica in chldhood. Br Med J 2:1284-1288

Veillette S, Perron M, Mathieu J (1989) La dystrophie myotonique: II nuptialité, fécondité et transmission du gène. Can J Neurol Sci 16:114-118

Verkerk AJMH, Pieretti M, Sutcliffe JS et al. (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914

Vickers JD, McComas AJ, Rathbone MP (1979) Myotonic muscular dystrophy: Abnormal temperature response of membrane phosphorylation in erythrocyte membranes. Neurology 29:791-796

Vogt A (1921) Die Cataract bei der myotonischer Dystrophie. Schw Med Wschr 51:669-674

Wagstaff J, Knoll JHM, Glatt KA (1992) Maternal but not paternal transmission of 15q11-13-linked nondeletion Angelman syndrome leads to phenotypic expression. Nature Genet 1:291-294

Walsh KV, Harley HG, Brook JD et al. (1990) Linkage relationships of the apolipoprotein C1 gene and a cytochrome p450 gene (CYP2A) to myotonic dystrophy. Hum Genet 85:305-310

Wevers RA, Joosten EMG, van de Biezenbos JBM, Theewes AGM, Veerkamp JH (1990) Excessive plasma K^+ increase after ischemic exercise in myotonic muscular dystrophy. Muscle & Nerve 13:27-32

Whitehead AS, Solomon E, Chambers SP, Povey S, Bodmer WF (1982) Assignment of the structural gene for the third component of human complement to chromosome 19. Proc Natl Acad Sci USA 79:5021-5025

Wöhrle D, Kotzot D, Hirst MC. et al. (1992) A microdeletion of less than 250kb, including the proximal part of the FMR-1 gene and the fragile-X site, in a male with the clinical phenotype of fragile-X syndrome. Am J Hum Genet 51:299-306

Yamaoka LH, Pericak-Vance MA, Speer MC et al. (1990) Tight linkage of creatine kinase (CKMM) to myotonic dystrophy on chromosome 19. Neurology 40:222-226

Young RSK, Gang DL, Zalneraitis EL, Krishnamoorty KS (1981) Dysmaturation in infants of mothers with myotonic dstrophy. Arch Neurol 38:716-719

Yu S, Pritchard M, Kremer E, Lynch M, Nancarrow J, Baker E, Holman K, Mulley JC, Warren ST, Schlessinger D, Sutherland GR, Richards RI (1991) Fragile X genotype characterized by an unstable region of DNA. Science 252:1179-1181

Zeviani M, Servidei S, Gellera C, Bertini E, DiMauro S, DiDonato S (1989) An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. Nature 339:309-3110

CHAPTER 2

Friedrich U, Brunner H, Smeets D, Lambermon E, Ropers HH. Three-point linkage analysis employing C3 and 19cen markers assigns the myotonic dystrophy gene to 19q.

Human Genetics 75: 291-293, 1987.

SUMMARY.

In seven large families with myotonic dystrophy (DM) comprising 102 individuals, linkage studies were performed employing restriction fragment length polymorphisms in the complement component 3 gene and the 19cen C banding heteromorphism as genetic markers. Three-point linkage analysis excludes DM from the 19cen-C3 segment and strongly supports its assignment to the proximal long arm of chromosome 19.

INTRODUCTION

Linkage between the myotonic dystrophy (DM) locus, the Lewis (Le) and Lutheran (Lu) blood groups, and the secretor locus (Se) has been firmly established since the early 1970s through the pioneering work of Mohr (1951), Renwick et al. (1971), and Harper et al. (1972). The demonstration of linkage between Se, Lu, and the complement component 3 (C3) has assigned this linkage group to chromosome 19 (Eiberg et al. 1983). During the past S years numerous other genes have been added to this group, which now comprises some 40 gene loci (see Naylor et al. 1985, for compilation). Recently several nucleotide sequence polymorphisms have been detected on chromosome 19 employing cloned genes or random DNA sequences as probes, and a number of these have been used for linkage analyses in DM families, as compiled by Sherman et al. (1985). These and other studies suggest that the DM gene may be located near the middle of chromosome 19, but its location relative to the centromere is still unknown. Part of this uncertainty derives from the fact that two of the most closely linked genetic markers of DM, peptidase D and apolipoprotein C2 (APO C2), map to the short and the long arm of chromosome 19, respectively (Hulsebos et al. 1985; Lusis et al. 1985). Because the precise regional assignment of the DM gene is a prerequisite for the isolation of suitable diagnostic DNA markers and for attempts to characterize the DM gene itself, we have used the 19cen C banding heteromorphism (Friedrich 1985) and restriction fragment length polymorphisms detected with a genomic C3 probe to perform linkage studies in DM families. Three point linkage analysis of these data suggests that the DM locus maps on the proximal long arm of chromosome 19.

MATERIALS AND METHODS

Linkage studies were performed in seven large Dutch families, comprising 102 individuals. For the analysis of restriction fragment length polymorphisms (RFLPs) near the C3 gene, DNA was isolated from blood, cleaved

with the restriction enzymes SstI and TaqI, subjected to electrophoresis, blotted onto nitrocellulose filters, hybridized with a 32P-labeled 1.39 kb genomic C3 clone, and autoradiographed essentially as described previously (Wieacker et al. 1984). Analysis of the SstI and TaqI restriction patterns revealed no obvious linkage disequilibrium and as expected, no crossovers between these markers were detected. Therefore, the information from these two RFLP systems was combined to yield a complex C3 locus with four different haplotypes. In subsequent linkage analyses, these four haplotypes were treated as alleles at a single locus.

To evaluate the 19cen C-banding heteromorphisms, chromosome preparations were made from peripheral lymphocyte cultures for 72 h. The preparations were stained consecutively with Q- and C-banding methods. Ten metaphases per individual were evaluated independently by two observers. Both the size (small or large) and the localization of the heterochromatic blocks (p arm, cen, or q arm) were considered, which resulted in six different centromere types.

These heterochromatin blocks were transmitted in a Mendelian fashion and did not undergo conspicuous morphological changes during transmission. The potential use of the 19cen heteromorphism as a genetic marker has been emphasized earlier (Friedrich 1985). Three-point linkage analyses were performed employing the LINKAGE program package (version 3.5) which was kindly provided by Dr. Jürg Ott (Zürich, Switzerland). The available modification of this program was able to handle only up to four alleles at a single locus. To cope with this limitation, 19cen types had to be modified in a small branch of the largest DM family, which was possible without altering the genetic information. For DM we assumed a gene frequency of 0.0001, and non-affected first degree relatives of DM patients were subdivided into two different liability classes depending on their age and whether or not they had been carefully examined. Lod scores were calculated for various male recombination fractions assuming that in females genetic distances or the relevant segment of chromosome 19 are 2.5 times larger than in males.

RESULTS AND DISCUSSION

In six of the seven families studied segregation of discernible C banding heteromorphisms was observed, and four of these were informative with regard to linkage with DM. In two families conspicuously large markers were seen that extended towards the short arm of chromosome 19 and segregated with DM in the majority of cases. Four families were informative for DM and C3, and two families were informative for DM, 19cen, and C3.

Table 1. Two-point linkage data

Locus versus locus[a]	Male θ									
	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
DM–Cen	–	1.723	2.936	3.164	2.997	2.619	2.124	1.569	0.997	0.452
C3–Cen	–	2.252	3.069	2.752	2.123	1.484	0.956	0.556	0.264	0.074
DM–C3	–	0.074	0.762	0.915	0.893	0.787	0.631	0.449	0.263	0.104

[a] DM, Myotonic dystrophy gene; C3, C3-combined TaqI/SStI polymorphisms; Cen, 19 centromere heteromorphism

Positive lod scores were obtained for all of the three possible gene pairs with peak values at $\Theta=0.15$ (DM-19cen), $\Theta=0.15$ (DM-C3), and $\Theta=0.10$ (19cen-C3), respectively (Table 1). In view of the considerable genetic distances between the three markers, no attempt was made to localize the crossovers and to infer the probable gene order "by eye" because this was considered too unreliable. Instead, we decided to make use of the ILINK program of the LINKAGE package to assess the relative order and the most likely distances by three-point analysis. Results of this analysis are clearly in favor of the order C3-Cen-DM. The relative likelihood for this order is about 3 times that for the order DM-C3-Cen and almost 30 times that for C3-DM-Cen (Table 2). The C3 gene has been assigned to the distal half of 19p (Ball et al. 1984; Hulsebos et al. 1986). In males C3 and DM are clearly but not tightly linked and in females linkage is very loose (Sherman et al. 1985). In contrast, linkage between APO C2 and DM is very close in both sexes with peak lod scores of above 23 for all recombination fractions between 0.01 and 0.05 (Hulsebos et al. 1985; Meredith et al. 1985; Pericak-Vance et al. 1985). The APO C2 gene has been assigned to the long arm of chromosome 19 by two groups (Lusis et al. 1985; Hulsebos et al. 1985) and recently other closely linked DNA markers have been assigned to this region as well (Roses et al. 1986). In contrast, numerous distal short arm markers including the LDL-receptor and the insulin receptor are not closely linked to DM (see Naylor et al. 1985). Therefore all available evidence excludes the DM gene from the short arm region distal from the C3 gene. Our three-point linkage data indicate that a localization of the DM gene below the centromere is about 30 times more likely than a localization between 19cen and C3 (Table 2).

Table 2. Relative likelihoods for the gene orders C3–DM–Cen, DM–C3–Cen, and C3–Cen–DM, respectively, as determined by three point linkage analysis. Genetic distances (in cM) correspond to maximum likelihoods for any given order of genes. The maximum likelihood for the order C3–Cen–DM is about 30 times higher than that for C3–DM–Cen

Gene order	Relative likelihood
$C_3 \overset{12.6}{\text{———}} DM \overset{12.8}{\text{———}} Cen$	1
$DM \overset{17.2}{\text{———}} C_3 \overset{10.4}{\text{———}} Cen$	2.99
$C_3 \overset{10.6}{\text{———}} Cen \overset{15.4}{\text{———}} DM$	29.40

Taken together this implies that we have excluded the DM gene from the short arm of chromosome 19. The assignment of DM to (the proximal part of) 19q should facilitate the identification of additional diagnostic markers of the DM gene and thus may contribute to the reliable early diagnosis and prevention of this disorder. Combination of the estimated distance for C3-19cen and 19cen-DM gives a value of 25 cM for C3-DM. This is considerably higher than the distance that was measured by two-point linkage between C3 and DM in our material (15cM). This last value is in accordance with literature data as compiled by Sherman et al. (1985). Even more notable is the relatively large distance between DM and 19cen (15 cM, see two-point linkage data in Table 1), considering the fact that close linkage with DM has been reported for PEPD which is localized on 19p (O'Brien et al. 1983; Lusis et al. 1985) and in view of the relative scarcity of chiasmata in the pericentromeric region of chromosome 19 (Hulten 1974). Thus it may well be that the true distance between 19cen and DM may be smaller than our present estimate, which is supported by multi-point linkage analyses involving DM, 19cen, and various 19q markers (E. Lambermon, unpublished work). Therefore the 19cen heteromorphism may be useful as a diagnostic marker of DM. Follow-up linkage studies will be required for more reliable estimation of the distance between 19cen and DM. For the time being and irrespective of the outcome of such analyses, the 19cen heteromorphism is valuable as the most closely linked and most informative polymorphic marker known whose localization "north" of DM can be considered established.

Acknowledgements.
We thank Mrs. Bente Falkenberg who performed the chromosomal analysis and part of the double-blind study of the centromere markers. Thanks are also due to Dr. B. van Oost for reading, to Mrs.J.Jansson-Lankkamp for typing the manuscript, and to Dr. B. Wieringa for help and advice with the RFLP studies. This work was supported by the Dutch Praeventiefonds, grant 28-1165, and by the Deutsche Forschungsgemeinschaft, grant Ro 389/15-1 to H.-H. Ropers.

REFERENCES

Ball SR, Buckton KE, Corney G, Fey G, Montiero M, Noades JE, Pym B, Robson EB, Tippet P (1984) Mapping studies with peptidase D (pepD). (7th International Workshop on Human Gene Mapping) Birth Defects 20:411-412

Eiberg H, Mohr J, Staub-Nielsen L, Simonsen N (1983) Genetics and linkage relationships of the C3 polymorphism: discovery of C3-Se linkage and assignment of LES-C3-DM-Se-PepD-Lu synteny to chromosome 19. Clin Genet 24 :159-170

Friedrich U (1985) Centromere heteromorphism in chromosome 19. (Letter to the editor) Clin Genet 28: 358-359

Harper PS (1972) Genetic studies in myotonic dystrophy. Thesis for degree of DM, University of Oxford, UK

Hulsebos T, Brunner H, Wieringa B, Friedrich U, Smeets D, Oei T, Hustinx T, Scheres J, Wienker T, Humphries S, Myklebost O, Junien C, ter Haar B, Ropers HH (1985) Regional assignment of C3, GPI, ApoC2 and beta-HCG and their linkage relationships with DM and 19cen. Cytogenet Cell Genet 40: 658

Hulsebos T, Wieringa B, Hochstenbach R, Smeets D, Schepens J, Oerlemans F, Zimmer J, Ropers H-H (1986) Toward early diagnosis of myotonic dystrophy: construction and characterization of a somatic cell hybrid with a single human der(19) chromosome. Cytogenet Cell Genet 43: 47-56

Hulten M (1974) Chiasma distribution in males. Hereditas 76: 52-58

Lusis AJ, Heinzmann C, Sparkes RS, Geller R, Sparkes MC, Mohandas T (1985) Regional mapping on human chromosome 19: apolipoprotein E, apolipoprotein CII, low density lipoprotein (LDL) receptor, peptidase D, glucose D, glucose phosphate isomerase. Cytogenet Cell Genet 40: 683

Meredith AL, Shaw DJ, Harley HG, Sarfarazi M, Huson SM, Brook JD, Myklebost O, Harper PS (1985) Linkage of myotonic dystrophy to APOCII and other RFLP's on chromosome 19: an approximate localization for the DM gene. Cytogenet Cell Genet 40: 698

Mohr J (1951) Estimation of linkage between the Lutheran and Lewis blood groups. Acta Pathol Microbiol Scand 29: 339-344

Naylor S, Lalouel J-M, Shaw DJ (1985) Report of the committee on the genetic constitution of chromosomes 17, 18 and 19. Helsinki Conference 1985. Cytogenet Cell Genet 40: 242-267

O'Brien T, Ball S, Sarfarazi M, Harper PS, Robson EB (1983) Genetic linkage between the loci for myotonic dystrophy and peptidase D. Ann Hum Genet 47: 117-121

Pericak-Vance MA, Yamaoka L, Assinder R, Bartlett RJ, Ross D, Fey G, Humphries S, Williamson R, Conneally PM, Roses A (1985) Tight linkage of APOC2 with myotonic dystrophy on chromosome 19. Cytogenet Cell Genet 40:721-722

Renwick JH, Bundey SE, Ferguson-Smith MA, Izatt MM (1971) Confirmation of linkage of the loci for myotonic dystrophy and ABH secretion. J Med Genet

8: 407-416
Roses AD, Pericak-Vance MA, Ross DA, Yamaoka L, Bartlett RJ (1986) RFLPs at the D19S19 locus of human chromosome 19 linked to myotonic dystrophy (DM). Nucleic Acids Res 14: 55 69
Sherman SL, Ball SP, Robson EB (1985) A genetic map of chromosome 19 based on 2-point lod tables. Cytogenet Cell Genet 40: 742
Wieacker P, Davies K, Cooke HJ, Pearson PL, Williamson R, Bhattacharya S, Zimmer J, Ropers HH (1984) Towards a linkage map of the human X-chromosome: regional assignment of 16 cloned single copy DNA sequences employing a panel of somatic cell hybrids. Am J Hum Genet 36: 265-276

Note added in proof. Recent studies of somatic cell hybrids carrying various different fragments of chromosome 19 have provided unambiguous proof for location of the PEPD gene on 19q (M. Davis, D. Schonk et al., in preparation). This refutes earlier evidence for a location of the PEPD gene on 19p (Lusis et al. 1985) and corroborates the assignment of DM as described in this paper.

CHAPTER 3

Brunner HG, Korneluk RG, Coerwinkel-Driessen M, MacKenzie A, Smeets H, Lambermon HMM, Oost van BA, Wieringa B, Ropers HH.

Myotonic dystrophy is closely linked to the gene for muscle-type creatine kinase (CKMM).

Human Genetics 81: 308-310, 1989.

SUMMARY

We have studied genetic linkage between the gene for creatine kinase muscle type (CKMM) and the gene for myotonic dystrophy (DM). In a panel of 65 myotonic dystrophy families from Canada and the Netherlands,a maximum lod score (Zmax) of 22.8 at a recombination frequency (Θ) of 0.03 was obtained. Tight linkage was also demonstrated for CKMM and the gene for apolipoprotein C2 (ApoC2). This establishes CKMM as a useful marker in for myotonic dystrophy.

INTRODUCTION

Myotonic dystrophy (DM) is the commonest disorder within the myotonias, a genetically heterogeneous group of disorders characterized by delayed muscle relaxation (Becker, 1977). The disease was delineated as a separate entity at the beginning of this century (Steinert, 1909 Batten and Gibb, 1909) and has since been recognized to be the commonest muscular dystrophy of adult life (Harper, 1979). Apart from myotonia and wasting of skeletal muscles, associated symptoms are common and include cardiac and smooth muscle involvement, ocular cataracts, frontal balding, testicular atrophy, whole body insulin resistance and CNS involvement. Clinical diagnosis may be difficult because of the highly variable age of onset. The fundamental defect underlying myotonic dystrophy is not known and therefore presymptomatic diagnosis is crucially dependent on reliable genetic markers. The myotonic dystrophy gene has been mapped to chromosome 19 by demonstration of linkage to the third component of human complement C3 (Eiberg et al. 1982) and subsequent localization of C3 on chromosome 19 by somatic cell hybrid studies (Whitehead et al. 1982). Regional localization of the DM gene on proximal 19q has been suggested because of tight linkage to the gene for apolipoprotein CII (Shaw et al. 1985; Pericak-Vance et al. 1986; Hulsebos et al. 1985), which maps to the proximal long arm of chromosome 19 (Hulsebos et al. 1985, Lusis et al. 1985) and by exclusion of the DM gene from proximal 19p (Friedrich et al. 1987).

Several polymorphic markers have been described from chromosome 19. RFLPs from the ApoC2 locus are now widely used for presymptomatic and prenatal DM testing (Meredith et al. 1986; Pericak-Vance et al. 1986). However, due to linkage disequilibrium between the different polymorphisms at the ApoC2 locus (Wallis et al. 1984, MacKenzie et al. 1988, van Oost, unpublished data) a proportion of families remain uninformative for ApoC2, precluding DNA diagnosis. We have studied two RFLPs from the gene for

creatine kinase muscle type (CKMM) in a sample of 65 families with myotonic dystrophy from Canada and the Netherlands, comprising approximately 600 individuals. Our results indicate that CKMM is closely linked to DM and that probes from this locus should be useful in both diagnosis and research of myotonic dystrophy.

MATERIALS AND METHODS

Patients

A total of 65 families comprising approximately 600 individuals were studied. A person was considered affected if the family history was positive for myotonic dystrophy and at least one of the following was found:

1. Myotonia
2. Specific lens opacities on slit lamp examination
3. Congenital myotonic dystrophy
4. Transmission of the disorder through the person in question

DNA isolation and southern blotting procedure

Chromosomal DNA from peripheral blood was isolated according to Aldridge et al.(1984) with minor modifications. Chromosomal DNA (10-15μg) was digested with the appropriate restriction enzymes under conditions specified by the manufacturer and then resolved by electrophoresis on a 0.7% (w/v) agarose gel. Gels were soaked in 0.25 M HCl for 10 min to partly depurinate the DNA. DNAs were subsequently denatured in 0.4 M NaOH and transferred onto Biotrace (Gelman Sciences) or Gene Screen TM Plus (New England Nuclear) membranes in the same solution over 6-12 hrs. DNA blots were washed in 2xSSC and dried at room temperature.

DNA probes

A 3.2 kbp human genomic fragment (BamHI-Sau3A) of the 3' untranslated and 3' flanking region of the CKMM gene (Coerwinkel et al. 1988) was used to identify CKMM alleles. This
probe identifies a TaqI RFLP with alleles of 4.3 and 4.2 kbp (Putney et al. 1984), and a NcoI RFLP with alleles of 3.3kbp and 2.3+1.0kbp, respectively (Coerwinkel et al. 1988).
Two probes from the ApoC2 region were used in this study: a 530bp PstI

fragment from the ApoC2 cDNA (Myklebost et al. 1984), which recognizes a 3.8/3.2kbp TaqI RFLP (Humphries et al. 1983), a 14.5/11.5kbp NcoI RFLP (Frossard et al. 1986), a 2.5/1.6 kbp BanI RFLP (Frossard et al. 1987), a 6.0/4.0 kbp BamHI RFLP (Meredith et al. 1986) and a 6,0/4.0 kbp AvaII RFLP (Korneluk et al. 1987). In addition, the pSC11 genomic subclone, located 1kbp to the 5' end of the gene was used to detect a 12/9kbp BglI RFLP (Wallis et al. 1984).
DNA inserts were excised from their vector plasmids, isolated on low-gel temperature agarose gels (Biorad) and labeled with Klenow DNA polymerase I in the presence of ^{32}P-dCTP (Amersham, UK) and random sequence d(N)6 hexamer primers (Pharmacia, P-L Biochemicals) (Feinberg and Vogelstein 1984).

Prehybridization and hybridization procedures
Prehybridization and hybridization was performed at 65°C for 16 hrs in 6xSSC, 5xDenhardts, 0.2% (w/v) SDS, 10% (w/v) dextran sulphate and 500μg/ml denatured herring sperm DNA. Washing was done at a final striongency of 65°C at 0.1xSSC. All other conditions were as described earlier (Hulsebos et al. 1986). For re-use filters were stripped in 0.2 M NaOH (10 min) followed by neutralization in 2xSSC (30 min). For autoradiography filters were exposed to Kodak XR-Omat X-ray film for 1-4 days.

Linkage analysis
Two-point linkage analysis was performed using the MLINK program of the LINKAGE program package (Lathrop and Lalouel 1984) version 4.7., assuming a gene frequency of 1 in 10,000 for the mutant DM allele. Persons at-risk for DM were assigned to liability classes depending on their age and the clinical examinations performed, as described previously (Friedrich et al, 1987). For unaffected persons under age 20 years the DM genotype was considered unknown.
For both the APOC2 and CKMM loci, information from different polymorphic systems was combined to yield haplotypes which were subsequently treated as alleles at a single locus. For reasons explained in the text, recombination frequencies were assumed equal for males and females in all calculations.

RESULTS

Linkage analysis

Two-point linkage analyses between DM and CKMM was performed in 65 pedigrees, comprising approximately 600 individuals.The maximum lod score (Zmax) for DM-CKMM was 22.8 at a recombination fraction (Θmax) of 0.03. (see Table l). Linkage relationships with ApoC2 were determined as well yielding a lod score of 44.0 at a Θmax of 0.00. The DM-ApoC2 relationship was studied in a subset of 20 families from the Netherlands generating a Zmax of 14.2 at Θmax=0.02.

In three families, a cross-over was observed between DM and CKMM that could not be explained by non-penetrance for the disease gene. Owing to lack of phase information it was not possible to establish whether the recombination had taken place in a male or a female meiosis. Therefore, no attempt was made to calculate male and female recombination frequencies separately.

Informativity of CKMM locus

In 61 unrelated Dutch individuals, allele frequencies for the TaqI 4.3/4.2 kbp RFLP (Tt) were 0.24 and 0.76. For the NcoI 3.3/ 2.3+1.0 kbp RFLP (Nn) the allele frequencies were 0.375 and 0.625, respectively. Haplotypes were constructed at the CKMM locus for 20 unrelated individuals. The haplotype frequencies were 2.5% for TN, 25% for Tn, 37.5% for tn and 35% for tN. The chi-squared value for these allelic associations between the NcoI and TaqI polymorphic sites did not reach statistical significance (chi square=3.686, $p \geq 0.05$ df=1). Thus, no significant linkage disequilibrium between the two RFLPs was observed, although the sample was relatively small. The PIC value calculated from these haplotype frequencies is 0.616, which is close to the value obtained under the hypothesis of no linkage disequilibrium.

Table 1. Lod scores at different recombination frequencies between DM, ApoC2, and CKMM. Male and female recombination frequencies were assumed equal in all calculations

Locus vs locus	Recombination frequencies											
	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10	Z_{max}	θ_{max}
DM vs CKMM	22.29	22.75	22.82	22.72	22.51	22.24	21.91	21.55	21.15	20.73	22.82	0.03
ApoC2 vs CKMM	43.10	42.19	41.26	40.31	39.36	38.40	37.43	36.45	35.46	34.47	44.00	0.00
DM vs ApoC2	9.74	14.05	14.22	14.13	13.98	13.80	13.60	13.37	13.13	12.87	14.22	0.02

DISCUSSION

These results show that both DM and ApoC2 are closely linked to the gene for creatine kinase muscle type (CKMM). CKMM has been localized to 19q13.2-19q13.3 on the basis of somatic cell hybrid and in situ hybridisation studies (Nigro et al. 1987). Both ApoC2 and DM have been localized to proximal 19q (Hulsebos et al. 1985. Lusis et al. 1985. Friedrich et al. 1987). Therefore these loci appear to be close to each other both physically and genetically.

The recombination rate of 0.03 between DM and CKMM observed in this study is similar to the recombination rate of 0.02 between DM and ApoC2, the latter being in agreement with data taken from the literature (Meredith et al. 1986. Pericak-Vance et al. 1986). The large LOD score and absence of recombination between CKMM and ApoC2 strongly suggests that the two loci are situated on the same side of DM. The precise ordering of the three loci as well as the establishment of their polarity on chromosome 19 is currently being established by multipoint linkage analysis in recombinant families (Brunner et al., Korneluk et al., unpublished).

The low rate of recombination observed in this study (0.03) shows that probes from the CKMM locus may be applied profitably in presymptomatic diagnosis of myotonic dystrophy. In view of the small or absent linkage disequilibrium between the CKMM-TaqI and CKMM-NcoI RFLPs, a substantial proportion of families should be informative for CKMM-TaqI/CKMM-NcoI. This is particularly important as substantial linkage disequilibrium is found between the various ApoC2 RFLPs as noted by us and by others (MacKenzie et al. 1988; van Oost unpublished; Wallis et al. 1984). This explains why ApoC2 is uninformative in about 50% of families. D19S19, another closely linked marker (Bartlett et al. 1987) has allele frequencies of 0.15/0.85 and thus is informative in only a small proportion of families. However, if all three markers are tested, approximately 85% of families should be informative for at least one of the RFLPs, stressing the value of this approach.

In conclusion, we suggest that the gene for creatine kinase muscle form (CKMM) is one of the most tightly linked markers of DM known to date and may be used for diagnostic as well as research purposes. Obviously, flanking markers are of great importance to improve diagnostic accuracy in DNA diagnosis of DM. Studies aiming at the construction of a complete genetic map around the myotonic dystrophy locus are currently underway (Korneluk et al. in preparation; Brunner et al. in preparation) and should soon reveal whether ApoC2 and CKMM map to the same side of, or flank the DM locus.

Acknowledgements:
We thank M.Lathrop for providing the LINKAGE program package and J. Schepens, P. van Zandvoort and D.Lahey for expert technical assistance.
This work was supported by grant RO 389/15-1 of the Deutsche Forschungsgemeinschaft, by Grant 87-2694 of the Dutch Prinses Beatrixfonds, by the Dutch Praeventiefonds Grant 28-1165 to H.H.R and by a grant to R.G.K. from the Muscular Dystrophy Association of Canada. A.M. is a Post-Doctoral Fellow of the Canadian Heart Foundation.

REFERENCES

Aldridge J, Kunkel L, Bruns G, Tantravahi U, Lalande M, Brewster T, Moreau E, Wilson M, Bromley W, Roderick T, Latt SA (1984) A strategy to reveal high-frequency RFLPs along the human X chromosome. Am J Hum Genet 36:546-564

Bartlett RJ, Pericak-Vance MA, Yamaoka L, Gilbert J, Herbstreith M, Hung WY, Lee JE, Mohandas T, Bruns G, Laberge C, Thibault M-C, Ross D, Roses AD (1987) A new probe for the diagnosis of myotonic muscular dystrophy. Science 235:1648-1650

Batten FE, Gibb HP (1909) Myotonia Atrophica. Brain 32:187-205

Becker PE (1977) Myotonia Congenita and syndromes associated with myotonia. Thieme, Stuttgart

Coerwinkel-Driessen M, Schepens J, van Zandvoort P, van Oost B, Mariman E, and Wieringa B (1988) NcoI RFLP at the creatine kinase-muscle type gene locus (CKMM chromosome 19). Nucl Acid Res 16:8743

Eiberg,H., Mohr, J., Staub-Nielsen, L., Simonsen, N. (1982). Linkage relationship between the locus for C3 and 50 polymorphic systems: assignment of C3 to the DM-Se Lu linkage group; confirmation of C3-Les linkage; support of Les-DM synteny. In: Proc. VIth Int. Cong. Hum. Genet., Jerusalem 1981. New York A.Liss.

Feinberg AP, Vogelstein B (1984) A technique for radiolabeling restriction endonuclease fragments to high specific activity. Anal Biochem 137:266-267

Friedrich U, Brunner H, Smeets D, Lambermon E, and Ropers HH (1987) Three-point linkage analysis employing C3 and 19cen markers assigns the myotonic dystrophy gene to 19q. Hum Genet 75:291-293

Frossard PM, Coleman RT, and Assmann G (1986) NcoI RFLP at the human apolipoprotein CII gene locus. Nucl Acid Res 14:5120

Frossard PM, Coleman RT, Funke H, Assmann G (1987) Dimorphic markers for the human apolipoprotein CII locus. Gene 51:103-106

Harper PS (1979) Myotonic Dystrophy. Philadelphia Saunders.

Hulsebos T, Brunner H, Wieringa B, Friedrich U, Smeets D, Oei T, Hustinx T, Scheres J, Wienker T, Humphries S, Myklebost O, Junien C, ter Haar B,

Ropers HH (1985) Regional assignment of C3, GPI, ApoC2 and beta-HCG and their linkage relationships with DM and 19 cen. Cytogenet Cell Genet 40:658

Hulsebos T, Wieringa B, Hochstenbach R, Smeets D, Schepens J, Oerlemans F, Zimmer J, and Ropers H-H (1986) Toward early diagnosis of myotonic dystrophy: construction and characterization of a somatic cell hybrid with a single human der(19) chromosome. Cytogenet Cell Genet 43:47-56

Humphries SE, Jowett NI, Williams L, Rees A, Vella M, Kessling A, Myklebost O, Lydon A, Seed M, Galton DJ, and Williamson R (1983) A DNA polymorphism adjacent to the human apolipoprotein CII gene. Mol Biol Med 1:463-471

Korneluk RG, MacLeod HL, Leblond SC, Monteith NL, Baralle FE, Hunter AGW (1987) AvaII RFLP at the human Apolipoprotein CII (Apo CII) gene locus. Nucl Acid Res 15:6769

Lathrop CM, Lalouel JM (1984) Easy calculations of lod scores and genetic risks on small computers. Am J Hum Genet 36:460-465

Lusis AJ, Heinzmann C, Sparkes RS, Geller R, Sparkes MC, and Mohandas T (1985) Regional mapping on chromosome 19: Apolipoprotein E, apolipoprotein CII, low density lipoprotein (LDL) receptor, peptidase D, glucose phosphate isomerase. Cytogenet Cell Genet 40:683

MacKenzie AE, MacLeod HL, Hunter AGW, and Korneluk RG (1988) Linkage analysis of the Apolipoprotein C2 gene and myotonic dystrophy on human chromosome 19 reveals linkage disequilibrium in a French Canadian population. Am J Hum Genet 44:140-147

Meredith AL, Huson SM, Lunt PW, Sarfarazi M, Harley HG, Brook JD, Shaw DJ, Harper PS (1986) Application of a closely linked polymorphism of restriction fragment length to counselling and prenatal testing in families with myotonic dystrophy. Br Med J 293:1353-1356

Myklebost O, Williamson B, Markham AF, Myklebost SR, Rogers J, Woods DE, and Humphries SE (1984) The isolation and characterization of cDNA clones for human apolipoprotein C2. J Biol Chem 259:4401-4404

Nigro JM, Schweinfest CW, Rajkovic A, Pavlovic J, Jamal S, Dottin RP, Hart JT, Kamarck ME, Rae PMM, Carty MD, and Martin-DeLeon P (1987) cDNA cloning and mapping of the human creatinine kinase M gene to 19q13. Am J Hum Genet 40:115-125

Pericak-Vance MA, Yamaoka LH, Assinder RIF, Hung W-Y, Bartlett RJ, Stajch JM, Gaskell PC, Ross DA, Sherman S, Fey GH, Humphries S, Williamson R, and Roses DA (1986) Tight linkage of apolipoprotein C2 to myotonic dystrophy on chromosome 19. Neurology 36:1418-1423

Putney S, Herlihy W, Royal N, Pang H, Aposhian HV, Pickering L, Belagaje R, Biemann K, Page D, Kuby SA, and Schimmel P (1984) Rabbit muscle creatinine phosphokinase. J Biol Chem 259:14317-14320

Shaw DJ, Meredith AL, Sarfarazi M, Huson SM, Brook JD, Myklebost O, Harper PS (1985) The apolipoprotein CII gene: subchromosomal localization and linkage to the myotonic dystrophy locus. Hum Genet 70:271-273

Wallis SC, Donald JA, Forrest LA, Williamson R, and Humphries SE (1984) The isolation of a genomic clone containing the apolipoprotein CII gene and the detection of linkage disequilibrium between two comman DNA polymorphisms around the gene. Hum Genet 68:286-289

CHAPTER 4

Brunner HG, Smeets H, Lambermon HMM, Coerwinkel--Driessen M, Oost van BA, Wieringa B, Ropers HH.

A multipoint linkage map around the locus for myotonic dystrophy on chromosome 19.

Genomics 5: 589-595, 1989

SUMMARY

We have performed a genetic linkage study in 26 families with myotonic dystrophy, employing 16 polymorphic DNA markers as well as the chromosome 19 centromere heteromorphism. Fourteen of these markers had been assigned previously to one of five different intervals of the 19cen-19q13.2 segment by using somatic cell hybrids (Schonk et al. 1989, in press). For the long arm of chromosome 19, a genetic map has been constructed that encompasses 9 polymorphic markers and the DM gene. Our studies indicate that the DM and CKMM genes map distal to the ApoC2-ApoE gene cluster and to the anonymous polymorphic markers D19S15 and D19S16, but proximal to the D19S22 marker. The orientation of DM and CKMM remains to be determined.

INTRODUCTION

Myotonic dystrophy (DM) is an autosomal dominant multisystem disease characterized by progressive muscle weakness, myotonia, ocular cataracts and other symptoms. With an estimated incidence of 1 in 8000 it is the commonest of adult muscular dystrophies (Harper 1979). The myotonic dystrophy gene has been mapped to chromosome 19 through demonstration of linkage to complement component 3 (C3) (Eiberg 1982) and the assignment of C3 to chromosome 19 by somatic cell hybrid analysis (Whitehead et al, 1982). Recently, we and others have established that unlike C3, which maps to 19p13.2 (Ball et al., 1984), the DM gene is located on the long arm of chromosome 19. This is supported by tight linkage to ApoC2 (Hulsebos et al, 1985; Shaw et al, 1985; Pericak-Vance et al, 1986) which has been localized to proximal 19q (Hulsebos et al, 1985; Lusis et al, 1985), by the re-assignment of the peptidase D gene, another closely linked DM marker, to 19q (Davis et al., 1987) and by exclusion of the DM gene from proximal 19p (Friedrich et al, 1987).

In order to generate a genetic map around the DM locus, we have performed a linkage study in families with myotonic dystrophy, employing 16 regionally assigned, chromosome 19-specific RFLP markers as well as the centromeric C banding heteromorphism. This has enabled us to map the DM gene to, or near, band 19q13.2 and to establish gene orders in the DM gene region.

MATERIALS AND METHODS

Patients

A total of 26 families were studied comprising approximately 300 individuals, and representing 190 potentially informative meioses. In accordance with guidelines, set forth by an international working group on the molecular defect in myotonic dystrophy (Griggs et al., 1989), a person was considered affected if he or she had transmitted the gene, or if the family history was positive and myotonia and muscular weakness or congenital myotonic dystrophy were found.

DNA isolation and Southern blotting procedure

Chromosomal DNA from peripheral blood was isolated according to Aldridge et al.(1984), with minor modifications. Chromosomal DNA (10-15μg) was digested with the appropriate restriction enzymes as specified by the manufacturer and separated by electrophoresis on a 0.7% (w/v) agarose gel. Gels were soaked in 0.25 M HCl for 10 min to partly depurinate the DNA. DNAs were subsequently denatured in 0.4 M NaOH and transferred onto Biotrace (Gelman Sciences) or Gene Screen TM Plus (New England Nuclear) membranes as described (Brunner et al., 1989). DNA blots were washed in 2xSSC (0.3 M NaCl, 30 mM sodium citrate) and dried at room temperature.

Chromosome 19 centromere polymorphism

Variation in size and location of the constitutive heterochromatin of the centromeric region of chromosome 19 was used to define alleles at the 19cen locus, as described previously (Friedrich et al., 1987).

TABLE 1

Markers Used for Linkage Analysis in DM Families

Probe name	Locus	Interval	RFLP enzyme	Alleles (kb)	Allele frequencies	Ref.
C3	C3	p13.3–13.2	*Taq*I	2.5/1.8	0.9/0.1	(9)
			*Sst*I	12/9 + 3	0.78/0.22	(9)
	Cen	1	Cytogenetic polymorphism		0.15/0.3/0.25/0.3	(13)
pPM17.4	D19S29	2	*Msp*I	5.5/3.5	0.55/0.45	H. Smeets, unpublished
p4.1H2	D19S7	2	*Msp*I	9/8	0.32/0.68	(40)
pHW60	D19S13	2	*Taq*I	5.5/2.6	0.68/0.32	(18)
p20B18	D19S30	2	*Msp*I	3.0/2.5	0.63/0.37	H. Smeets, unpublished
pIJ2	D19S9	3	*Eco*RI	9.0/5.0	0.13/0.87	(5)
p5B18	D19S28	3	*Taq*I	2.7/1.7	0.57/0.43	H. Smeets, unpublished
pPM6.7	D19S18	3	*Eco*RI	40/12 + 15	0.65/0.35	(42)
			*Msp*I	5.0/3.5	0.7/0.3	(42)
Cyp2A	Cyp2A	3	*Sst*I	9.3/5.2 + 4.1	0.55/0.45	(45)
pJSB11	D19S16	4	*Taq*I	8.5/6.5	0.36/0.64	(37)
pJSB6	D19S15	4	*Taq*I	6.0/6.5	0.038/0.962	(36)
			*Taq*I	4.5/2.7	0.116/0.884	(36)
			*Taq*I	3.3/1.6	0.077/0.923	(36)
pLDR152	D19S19	4	*Pst*I	19/11	0.14/0.86	(34)
ApoE	ApoE	4	Allele-specific oligos		0.89/0.11	(43)
ApoC2	ApoC2	4	*Taq*1	3.8/3.5	0.55/0.45	(20)
			*Nco*I	14.5/11.5	0.3/0.7	(14)
			PCR method many 78–102 bp			H. Smeets, unpublished
cCKMM3'	CKMM	4	*Taq*I	4.3/4.2	0.24/0.76	(33)
			*Nco*I	3.3/2.3 + 1.0	0.32/0.68	(7)
pEFD4.2	D19S22	5	*Taq*I	2.2/2.0	0.62/0.38	(27)

Note. Probe assignments to intervals 2 (19q12–q13.1), 3 (19q13.1), 4 (19q13.2), and 5 (19q13.2–qter) refer to data of Schonk *et al* (38). Cen refers to the chromosome 19 centromere heteromorphism (13).

Probes and RFLP's

RFLP's used in this study are given in table 1. Fourteen probes had previously been regionally assigned to one of five intervals on the long arm of chromosome 19 (Schonk et al., 1989). The D19S22 marker has subsequently been assigned to the 19q13.2-qter region defined as interval 5 (D.Schonk, personal communication). All probes were subcloned into either pSP64, pSP65, pGEM3 or pGEM4 vector plasmids (Promega Biotec) and grown in E.coli HB101. For details of insert size and cloning sites see Schonk et al. (1989). Insert DNA fragments were excised from their vector plasmids, isolated and subsequently labeled by primed synthesis with Klenow DNA polymerase I in the presence of ^{32}P-dCTP (Amersham, UK) and random sequence d(N)6 hexamer primers (Brunner et al., 1989).

Prehybridization and hybridization was performed at 65°C for 16 hrs in 6xSSC, 5xDenhardts, 0.2% (w/v) SDS, 10% (w/v) dextran sulphate and 500μg/ml denatured herring sperm DNA. Washing was done at 65°C and 0.1xSSC final stringency. All other conditions were as described earlier (Hulsebos et al., 1986b). For re-use, filters were stripped in 0.2 M NaOH (10 min) followed by neutralization in 2xSSC (30 min). For autoradiography, filters were exposed to Kodak X-Omat S X-ray film for 1 to 4 days.

For ApoE typing, ϵ3 and ϵ4 specific oligonucleotides were employed as described previously (Smeets et al., 1988). For detection of a hypervariable (TG)m(AG)n tandem repeat in the first intron of the ApoC2 gene (Das et al., 1987), DNA from the relevant portion of the gene was amplified by polymerase chain reaction (Saiki et al., 1988), resolved on 10% (w/v) sequencing gels, blotted onto Gene Screen TM Plus (New England Nuclear) membranes and hybridized with a ^{32}P-dCTP labeled 15-mer [$(TG)_4(AG)_3A$] oligonucleotide. Details of this procedure will be presented elsewhere (H.Smeets et al., manuscript in preparation).

Linkage analysis

Two-point linkage analysis was performed using the LIPED program (Ott, 1976) and the MLINK and LODSCORE programs of the LINKAGE program package (Lathrop and Lalouel, 1984) version 4.7 . For multipoint linkage analysis, the ILINK program of the LINKAGE program package was used. This program calculates the maximum likelihoods for different gene orders by an iterative procedure that optimizes recombination frequencies.

Healthy persons at risk for DM were assigned to one of two liability classes (with penetrances of 0.95 and 0.80, respectively) depending on their age and whether or not clinical examination had been complete. For unaffected persons under age 20 years the DM genotype was considered unknown. For DM, a gene frequency of 0.0001 was assumed.

For the C3, ApoC2, D19S18, D19S15 and CKMM loci, which are charac-

terized by more than one RFLP, information from different polymorphic systems was combined to yield haplotypes which were subsequently treated as alleles at a single locus. Haplotypes were constructed through careful inspection of the pedigrees. For the ApoC2 locus, significant linkage disequilibrium exists for the TaqI and NcoI polymorphisms (H.Brunner and B.A. van Oost, unpublished observations). Haplotype frequencies were estimated as 0.55, 0.35 and 0.10 for the 3.8/11.5kb, the 3.5/14.5kb and the 3.8/11.5kb TaqI/NcoI haplotypes respectively. The 3.5/11.5kb haplotype was not observed in our sample. For the ApoC2 hypervariable repeat sequence (VSSM), no information on allele frequencies is available, since this method has been used in only a small number of families. We therefore decided to use the same haplotype frequencies for the extended haplotypes obtained with the VSSM method. For the CKMM TaqI and NcoI polymorphisms, the degree of linkage disequilibrium is small, with estimated haplotype frequencies of 0.375, 0.35, 0.25 and 0.025 for the 4.2/2.3+1.0kb, the 4.2/3.3kb, the 4.3/2.3+1.0kb and the 4.3/3.3kb TaqI/NcoI haplotypes (Brunner et al., 1989). For D19S15 the frequencies of the different alleles is given in table 1. These polymorphisms appear to be in full linkage equilibrium ($p=0.95$ by chi-square with 8 degrees of freedom). For C3, empirical haplotype frequencies of 0.50, 0.40, 0.05 and 0.05 were assigned to the 2.5/12kb, the 2.5/9+3kb, the 1.8/12kb and the 1.8/9+3kb TaqI/SstI polymorphisms. For D19S18, due to strong linkage disequilibrium, only a small amount of information was gained from the MspI polymorphism in two families that were uninformative for the EcoRI polymorphism. We therefore entered the information in these families under the same allele frequencies as the EcoRI polymorphism.
Equal recombination frequencies were assumed for males and females in all calculations, except where indicated otherwise.

RESULTS

Two-point linkage relationships between DM and various chromosome 19 markers are given in table 2. Loose linkage was found for C3, which maps to 19p13, for 19cen and for markers from interval 2 (19q12-19q13.1). In contrast, linkage is close for D19S18 from interval 3 (19q13.1) and for all markers from interval 4 (19q13.2). For D19S22 (interval 5, 19q13.2-qter) a maximum lod score of 0.52 at $\Theta=0.26$ was obtained (male and female meioses combined). For recombination fractions up to 6% linkage between DM and D19S22 was excluded by a Lod score <-2. None of the differences between male and female recombination fractions was significant at the 5% level using Fisher's exact test. For DM-C3 and DM-Cen a higher female

than male recombination rate was suggested ($p=0.09$ and $p=0.07$ respectively). Thus, no obvious difference between male and female recombination rates was detected, with the exception of the pericentromeric region which may be characterized by a marked excess of female recombination. Therefore, equal male and female recombination rates were assumed for all subsequent calculations aiming at the delineation of gene orders in interval 3, 4 and 5.

TABLE 2

Linkage Relationships between DM and 17 Polymorphic Markers from Chromosome 19

	$\theta_m = \theta_f$		Sex-specific analysis		
Locus	θ_{max}	Z_{max}	$\theta_{m,max}$	$\theta_{f,max}$	Z_{max}
C3	0.17	0.76	0.06	0.50	1.87
19cen	0.20	2.02	0.09	0.50	3.31
PM17.4	0.13	1.23	0.11	0.14	1.24
D19S7	0.18	1.49	0.16	0.26	1.57
D19S13	0.18	1.76	0.14	0.24	1.85
20B18	0.10	2.12	0.13	0.07	2.17
D19S9	0.17	1.06	0.19	0.00	1.25
5B18	0.12	2.82	0.11	0.13	2.83
D19S18	0.08	4.75	0.05	0.17	5.10
Cyp2A	0.10	2.94	0.10	0.10	2.94
D19S16	0.07	8.82	0.00	0.17	10.19
D19S15	0.07	7.66	0.06	0.08	7.68
D19S19	0.00	4.99	0.00	0.00	4.99
ApoE	0.02	7.97	0.00	0.08	8.50
ApoC2	0.02	19.91	0.02	0.01	19.98
CKMM	0.00	11.26	0.00	0.00	11.26
D19S22	0.26	0.51	0.21	0.50	0.91

Note. Pedigrees were analyzed under the assumption of equal (left) or independent (right) male and female recombination rates. Marker loci are ordered in groups as shown in Table 1. 19cen refers to the chromosome 19 centromere heteromorphism (13).

A primary map of the relevant portion of 19q was obtained by performing four-point linkage analyses involving DM and three marker loci, D19S16, ApoC2 and D19S18. In a previous study (Schonk et al., 1989) we had assigned D19S18 to interval 3 and both D19S16 and ApoC2 to interval 4 (see table 1). Likelihood estimates for all gene orders that are compatible with these assignments indicate that ApoC2 and DM map distal to D19S16, with odds against all other possible orders exceeding 10^3:1 (Table 3). The nuclear genetic map generated in this way, (cen-)D19S18-D19S16-DM/ApoC2(-ter), was subsequently expanded by the addition of other loci, as outlined below.

D19S15 was excluded from the DM-ApoC2 interval by three-point analysis (odds $<1:10^4$). Four-point analysis with ApoC2, D19S16 and D19S18 indicates that D19S15 is located between D19S16 and ApoC2 (relative likelihood >70:1).

Four-point analysis with Cyp2A, an interval 3 marker, and ApoC2 and D19S16 from interval 4 suggests that DM is distal to ApoC2 since the likelihood for Cyp2A-D19S16-ApoC2-DM was found to be approximately 70 times that for the alternative order Cyp2A-D19S16-DM-ApoC2 (Table 4).

Recently, ApoE and ApoC2 were shown to be only 50kb apart (Myklebost and Rogne, 1988). The close physical linkage of these loci explains why recombination between ApoC2 and ApoE is very rare (Myklebost et al.1984; Humphries et al., 1984; Berg et al., 1985; Smeets et al., 1988). Still, in one family we have observed such a recombination event, and this has enabled us to determine the orientation of the ApoC2-ApoE gene cluster. Four-point analysis indicates that ApoE is located proximal to ApoC2 (relative likelihood 250:1, see Table 4).

Finally, a relative likelihood of less than $1:10^{10}$ in a three-point analysis with ApoC2 excludes DM from a position distal to D19S22. This establishes the following map: (cen-)D19S18-D19S16-D19S15-ApoE-ApoC2-DM-D19S22(-qter).

D19S19 was only informative in a small number of families. Analysis of individual cross-overs suggests that this marker is most likely located between D19S15 and ApoC2 (figure 2).

For the highly informative CKMM marker, very tight linkage was found with DM, ApoC2 and ApoE. Recent physical mapping data argue for CKMM being distal to ApoC2, as discussed below.

For D19S9, an interval 3 marker, a position proximal to D19S18 was favoured by odds of approximately 95:1 (Table 4). The other interval 3 markers could not be positioned on this map since three- and four-point maximum likelihood calculations failed to favour specific gene orders. For interval 2 markers, the situation is similar. Each of these markers displays tight genetic linkage to all other markers from this group (data not shown),

TABLE 3

Relative Likelihoods for Permutated Orders of DM and the Marker Loci ApoC2, D19S18, and D19S16

Order	Relative likelihood
D19S18–D19S16–ApoC2–DM	1
D19S18–D19S16–DM–ApoC2	2.93×10^{-1}
D19S18–DM–ApoC2–D19S16	3.25×10^{-4}
D19S18–ApoC2–DM–D19S16	7.68×10^{-5}
DM–D19S18–D19S16–ApoC2	2×10^{-8}
D19S18–ApoC2–D19S16–DM	1×10^{-8}
D19S18–DM–D19S16–ApoC2	9×10^{-9}
DM–D19S18–ApoC2–D19S16	2×10^{-9}

Note. Only those permutations that are compatible with the physical data shown in Table 1 were evaluated.

TABLE 4

Relative Likelihoods for Gene Orders Involving Other Loci from 19q13

Order	Relative likelihood
D19S18–D19S16–D19S15–ApoC2	1
D19S18–D19S15–D19S16–ApoC2	1.35×10^{-2}
D19S18–D19S16–ApoC2–D19S15	1.54×10^{-3}
Cyp2A–D19S16–ApoC2–DM	1
Cyp2A–D19S16–DM–ApoC2	1.44×10^{-2}
D19S16–ApoE–ApoC2–DM	1
D19S16–ApoC2–ApoE–DM	3.98×10^{-3}
D19S16–D19S15–ApoE–ApoC2	1
D19S16–ApoE–D19S15–ApoC2	2.43×10^{-3}
D19S9–D19S18–D19S16–ApoC2	1
D19S18–D19S9–D19S16–ApoC2	1.04×10^{-2}

but all are only loosely linked to DM. Therefore, no extensive calculations were carried out to establish their order. A genetic map encompassing the 19cen to D19S22 segment is shown in Figure 1. Its total length is 54 cM in males and 130 cM in females, with approximately equal male and female recombination rates distal to interval 2. Under this gene order, no double recombinants in interval 3 and 4 are required to explain the segregation patterns observed (see figure 2).

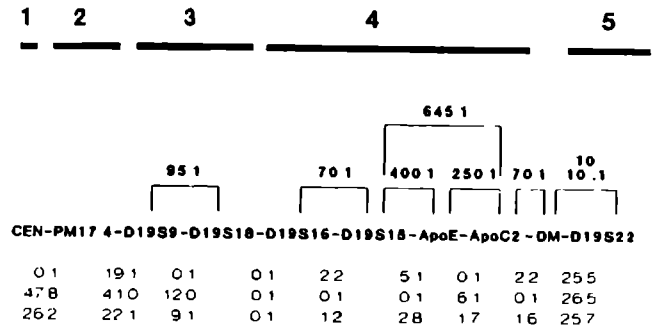


FIG. 1. Genetic map of 19q. For designation of physically defined intervals, see Table 1. Genetic distances are given in centimorgans for independent and for equal male and female recombination rates, assuming Haldane's mapping function. For various pairs of loci, odds are presented for the most likely and inverted gene orders. Genetic and physical data support localization of D19S19 between D19S15 and ApoE. CKMM could not be separated by recombination from either ApoC2 or DM. Physical data map this locus distal to ApoC2 (see text).

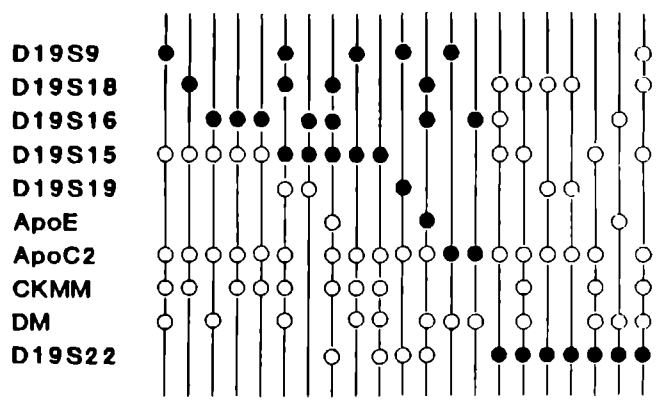


FIG. 2. Schematic representation of crossovers between various 19q13 loci. The order of DM and CKMM remains to be determined.

DISCUSSION

This study provides a detailed genetic map of the DM gene region on the long arm of chromosome 19 and defines marker loci that flank the DM gene. On the proximal side, ApoC2, ApoE, D19S15, D19S16 and D19S18 are all tightly linked to DM which renders them suitable for diagnostic use. Taken together, this array of proximal markers should prove informative in virtually all families with a suitable pedigree structure. D19S22, the distally flanking marker on this map, appears to be very loosely linked to DM. On the other hand, recent data from another group (Nakamura et al, 1988) argue for a distance of 11 cM between D19S22 and ApoC2. This may indicate that the true genetic distance between DM and D19S22 is somewhat smaller than suggested by our study. Obviously, the identification of additional closely linked distal markers will be essential for both accurate genetic diagnosis and for progress towards isolation of the DM gene proper.

As stated above, due to a very low degree of informativity of the D19S19 marker, we are presently unable to unambiguously position this locus on the map presented here. However, as shown in figure 2, our data strongly suggest that it may lie in the interval between D19S15 and ApoC2. Two lines of evidence confirm that this locus is proximal to both ApoC2 and DM. First, a cross-over has been reported that separates the DM gene from ApoC2 and D19S19, indicating that these loci are on the same side of DM (Johnson et al, 1987). Secondly, recent experiments indicate that D19S19, D19S15 and D19S16 are absent from a somatic cell hybrid line (Stallings et al., 1988) carrying a 19q fragment that partly overlaps interval 4 and has retained ApoC2, ApoE and CKMM (M. Siciliano, personal communication; D. Schonk, personal communication). Therefore, D19S19 is located in the proximal portion of interval 4 in the vicinity of D19S15, which implies that it may be less closely linked to DM than previously assumed (Bartlett et al, 1987).

In contrast, a recent study indicates that the CKMM gene is very closely linked to DM (Brunner et al., 1989). In this study, which was performed on a combined set of 65 Dutch and Canadian families, the maximum lod score (Zmax) was 22.8 at a recombination fraction (Θ) of 0.03 (both sexes combined). No recombination was detected between CKMM and ApoC2 in a large number of meioses (Zmax = 44.0 at Θ=0.00), indicating that these markers are probably on the same side of DM. CKMM has been assigned to 19q13.2-q13.3 by in situ hybridisation (Nigro et al, 1987).

Evidence from our laboratory indicates that the CKMM gene overlaps the distal breakpoint of a 19cen-q13.2 chromosome fragment that carries all interval 4 markers (Wieringa et al, to be published). This implies that CKMM is the most distal marker of this group and consequently, DM may

be located in interval 5, just distal to the 19q13.2 breakpoint. Confirmation of this gene order should come from analysis of recombination events separating DM and CKMM.

Three markers in the genetic map presented here have been included in a map presented by Nakamura et al. (1988). Our data support the order D19S9-ApoC2-D19S22, that was proposed by these authors. D19S7 and D19S9 were placed on opposite sides of ApoC2 by Donis-Keller et al. (1987). However, our data support a localization of both D19S9 and D19S7 proximal to ApoC2, which is in accordance with the findings of Nakamura et al. (1988). Comparison of our map with that proposed by Sherman et al. (1985) is limited by differences in methodology and the markers that were used. The larger female than male distance of our final genetic map is in accordance with previous findings (Sherman et al., 1985; Donis-Keller et al., 1987; Nakamura et al., 1988).

It might be argued, that a genetic map generated in disease families may not be applicable to the general population if the disease is caused by a structural rearrangement, such as a deletion or inversion. However, comparison of DM patients and normal controls by cytogenetic studies and long range physical mapping of the putative DM gene region has so far not produced any evidence in favour of such a hypothesis (D.Smeets, B.Wieringa, unpublished results). Thus, present data suggest that the putative DM gene mutation, is not caused by an abnormality within the resolution of the genetic map presented here.

So far, attempts to isolate genes from neighbouring, closely linked markers has met little success. In fact, all (four) human genes that have been cloned on the basis of their known chromosomal location could be identified through chromosomal aberrations that were associated with recognizable clinical phenotypes (Orkin et al., 1986; Page et al, 1987). In the absence of specific translocations or deletions on chromosome 19 that are associated with myotonic dystrophy, there is no alternative to the genetic approach involving the search for, and analysis of, rare cross-over events between DM and very closely linked markers to direct the search towards the site of the DM gene. The genetic map presented here may provide a framework for future studies of this kind.

Acknowledgements:

We would like to thank J.Schepens for skillful technical assistance, and Dr J.-M. Lalouel for providing the LINKAGE program package. We are grateful to Drs. R. Bartlett, Y.Nakamura, I. Phillips, and D. Shaw for kind donation of probes. This study was supported by grant 28-1165 from the Dutch Prevention Fund, by grant Ro 389/15-2 from the Deutsche Forschungsgemeinschaft to H.H.Ropers and by the American Muscular Dystrop-

hy Association, under the Association's Task Force on Genetics Grant Program.

REFERENCES

Aldridge, J., Kunkel, L., Bruns, G., Tantravahi, U., Lalande, M., Brewster, T., Moreau, E., Wilson, M.,. Bromley, W., Roderick, T., Latt, S.A. (1984). A strategy to reveal high-frequency RFLPs along the human X chromosome. Am. J. Hum. Genet. 36:546-564.

Ball, S.R., Buckton, K.E., Corney, G., Fey, G., Montiero, M., Noades, J.E., Pym, B., Robson, E.B., and Tippet, P. (1984). Mapping studies with peptidase D (PEPD). (HGM7) Birth Defects: Original Article Series 20:411-412. March Of Dimes Birth Defects Foundation. White Plains, NY.

Bartlett, R.J., Pericak-Vance, M.A., Yamaoka, L., Gilbert, J., Herbstreith, M., Hung, W.Y., Lee, J.E., Mohandas, T., Bruns, G., Laberge, C., Thibault, M.-C., Ross, D., Roses, A.D. (1987). A new probe for the diagmosis of myotonic muscular dystrophy. Science 235:1648-1650.

Berg,K., Pedersen, J.C., Börresen, A.L., Heiberg, A., Solaas, M.H., Humphries, S.E. (1985). Concerning some chromosome 19 markers in families with hypercholesterolemia with xanthomatosis. Cytogenet. Cell Genet. 40:582.

Brook, J.D., Shaw, D.J., Meredith, A.L., Bruns, G.A.P., Harper, P.S. (1984). Localisation of genetic markers and orientation of the linkage group on chromosome 19. Hum. Genet. 68:282-285.

Brunner, H.G., Korneluk, R.G., Coerwinkel-Driessen, M., MacKenzie, A., Smeets, H., Lambermon, H.M.M., van Oost, B.A., Wieringa, B., Ropers, H.-H. (1989). Myotonic dystrophy is closely linked to the gene for muscle type creatine kinase. Human Genetics 81:308-310.

Coerwinkel-Driessen, M., Schepens, J., van Zandvoort, P., van Oost, B., Mariman, E., and Wieringa, B. (1988). NcoI RFLP at the creatinine kinase-mucle type gene locus (CKMM chromosome 19). Nucl.Acids Res. 16:8743.

Das, H.K., Jackson, C.L., Miller, D.A., Leff, T., and Breslow, J.L. (1987). The human apolipoprotein CII gene sequence contains a novel chromosome 19-specific minisatellite in its third intron. J.Biol.Chem. 262:4787-4793.

Davies, K.E., Jackson, J., Williamson, R., Harper, P.S., Ball, S., Sarfarazi, M., Meredith, L., Fey, G. (1983). Linkage analysis of myotonic dystrophy and sequences om chromosome 19 using a cloned complement component 3 gene probe. J. Med. Genet. 20:259-263.

Davis, M.B., Schonk, D., Monteiro, M., Oerlemans, F., Povey, S., Wieringa, B. (1987). Localization of PEPD to the long arm of chromosome 19. Ann. Hum. Genet. 51:195-199.

Donis-Keller, H., and 32 others (1987). A genetic linkage map of the human genome. Cell 51:319-337.

iberg, H., Mohr, J., Staub-Nielsen, L., Simonsen, N. (1982). Linkage relation-

ship between the locus for C3 and 50 polymorphic systems: assignment of C3 to the DM-Se-Lu linkage group; confirmation of C3-Les linkage; support of Les-DM synteny. In: Proc. VIth Int. Cong. Hum. Genet., Jerusalem 1981. New York A.Liss.

Friedrich, U., Brunner, H. Smeets, D., Lambermon, E., and Ropers, H.H. (1987). Three-point linkage analysis employing C3 and 19cen markers assigns the myotonic dystrophy gene to 19q. Hum. Genet. 75:291-293.

Frossard, P.M., Coleman, R.T., and Assmann, G. (1986). NcoI RFLP at the human apolipoprotein CII gene locus. Nucl. Acids Res. 14:5120.

Griggs, R.C., Wood, D.S. and the working group on the molecular defect in myotonic dystrophy. (1989). Criteria for establishing the validity of genetic recombination in myotonic dystrophy. Neurology in press.

Harper, P.S. (1979). Myotonic dystrophy. Philadelphia Saunders.

Hulsebos, T., Brunner, H., Wieringa, B., Friedrich, U., Smeets, D., Oei, T., Hustinx, T., Scheres, J., Wienker, T., Humphries, S., Myklebost, O., Junien, C., ter Haar, B., Ropers, H.H. (1985). Regional assignment of C3, GPI, ApoC2 and beta-HCG and their linkage relationships with DM and 19 cen. Cytogenet. Cell Genet. 40:658.

Hulsebos, T., Westphal, H., Peek, R., Coerwinkel, M., and Wieringa, B. (1986a). pHW60 an anonymous single copy clone from the proximal portion of the long arm of chromosome 19 (HGM8 assignment no. D19S13).Nucl.Acids Res.14:7137.

Hulsebos, T., Wieringa, B., Hochtsenbach, R., Smeets, D., Schepens, J., Oerlemans, F., Zimmer, J., and Ropers, H.-H. (1986b). Toward early diagnosis of myotonic dystrophy: construction and characterization of a somatic cel hybrid with a single human der (19) chromosome. Cytogenet. Cell Genet. 43:47-56.

Humphries, S.E., Jowett, N.I., Williams, L., Rees, A., Vella, M., Kessling, A., Myklebost, O., Lydon, A., Seed, M., Galton, D.J., and Williamson, R. (1983). A DNA polymorphism adjacent to the human apolipoprotein CII gene. Mol. Biol. Med. 1:463-471.

Humphries, S.E., Berg,K., Gill, K., Cumming, A.M., Robertson F.W., Stalenhoef, A.F.H., Williamson, R., Börreson, A.-L. (1984). The gene for apolipoprotein C2 is closely linked to the gene for Apolipoprotein E on chromosome 19. Clin. Genet. 26:389-396.

Johnson,K., Jones, P., Nimmo, E., Weiss, M., Miki, T., Savontaus, M.L., Anvret, M., Williamson, R. (1987). A recombination event in a myotonic dystrophy patient that locates the two most tightly linked markers on the same side of the defect locus on chromosome 19. Cytogenet. Cell Genet. 46:634-635.

Lathrop,C.M., Lalouel, J.M.(1984). Easy calculations of lod scores and genetic risks on small computers.Am.J.Hum.Genet.36:460-465.

Lusis, A.J., Heinzmann, C., Sparkes, R.S., Geller, R., Sparkes, M.C., and Mohandas, T. (1985). Regional mapping on chromosome 19: Apolipoprotein E, apolipoprotein CII, low density lipoprotein (LDL) receptor, peptidase D,

glucose phosphate isomerase. Cytogenet. Cell Genet. 40:683.

Myklebost, O., Rogne, S.,, Olaissen, B., Gedde-Dahl, T., Prydz, H.(1984). The locus for apolipoprotein C2 is closely linked to the Apolipoprotein E locus on chromosome 19 in man. Hum. Genet. 67:309-312.

Myklebost, O., and Rogne, S. (1988). A physical map of the apolipoprotein gene cluster on human chromosome 19. Hum. Genet. 78:244-247.

Nakamura, Y., Lathrop, M., O'Connell, P., Leppert, M., Lalouel., J.-M., and White, R. (1988). A primary map of ten DNA markers and two serological markers for human chromosome 19. Genomics 3:67-71.

Nigro, J.M., Schweinfest, C.W., Rajkovic, A., Pavlovic, J., Jamal, S., Dottin, R.P., Hart, J.T., Kamarck, M.E., Rae, P.M.M., Carty, M.D., DeLeon, P.M. (1987). cDNA cloning and mapping of the human creatine kinase M gene to 19q13. Am. J. Hum. Genet. 40:115-125.

Orkin, S.H. (1987). Reverse genetics and human disease. Cell 47:845-850.

Ott, J. (1976). A computer program for linkage analysis of general human pedigrees. Am. J. Hum. Genet. 28:528-529.

Page, D.C., Mosher, R., Simpson, E.M.C., Mardon, G., Pollack, J., McGillavray, B., de la Chapelle, A., Brown,. L. (1987). The sex-determining region of the human Y chromosome encodes a finger protein. Cell 51: 1091-1104.

Pericak-Vance, M.A., Yamaoka, L.H., Assinder, R.I.F., Hung, W.-Y., Bartlett, R.J., Stajch, J.M., Gaskell, P.C., Ross, D.A., Sherman, S., Fey, G.H., Humphries, S., Williamson, R., and Roses, D.A. (1986). Tight linkage of apolipoprotein C2 to myotonic dystrophy on chromosome 19. Neurology 36:1418-1423.

Putney, S.,Herlihy, W., Royal, N., Pang, H., Aposhian, H.V., Pickering, L., Belagaje, R., Biemann, K., Page D., Kuby, S.A., and Schimmel, P. (1984). Rabbit muscle creatinine phosphokinase. J.Biol.Chem. 259:14317-14320.

Roses, A.D., Pericak-Vance, M.A., Ross, D.A., Yamaoka, L., and Bartlett, R.J. (1986). RFLPs at the D19S19 locus of human chromosome 19 linked to myotonic dystrophy (DM). Nucl. Acids Res. 14:5569.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlish, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Schepens, J., Hulsebos, T., Smeets, H., Coerwinkel, M., Brunner, H., Ropers, H.H., and Wieringa, B. (1987a). A locus at 19cen-q13.2 (D19S15) containing three RFLPs linked to myotonic dystrophy (DM) is recognized by probe pJSB6. Nucl. Acids Res.15:3193.

Schepens, J., Smeets, H., Hulsebos, T., Brunner, H., and Wieringa, B. (1987b). Isolation of a polymorphic DNA sequence pJSB11 (D19S16) from the human chromosome 19 cen-q13.2 region linked to the myotonic dystrophy (DM) gene. Nucl. Acids Res.15:3192.

Schonk, D., Coerwinkel-Driessen, M., van Dalen, I., Oerlemans, F., Smeets, B., Schepens, J., Hulsebos, T., Boyd, Y., Davis, M., Rettig, W., Shaw, D., Brook, D., Bartlett, R., Roses, A., Ropers, H., Wieringa, B. (1989).

Definition of five subchromosomal intervals around the myotonic dystrophy region at 19cen-q13.2. Genomics 4:384-396.

Shaw, D.J., Meredith, A.L., Sarfarazi, M., Huson, S.M., Brook, J.D., Myklebost, O., Harper, P.S. (1985). The apolipoprotein CII gene: subchromosomal localization and linkage to the myotonic dystrophy locus. Hum. Genet. 70:271-273.

Shaw, D.J., Meredith, A.L., Sarfarazi, M., Harley, H.G., Huson, S.M., Brook, J.D., Bufton, L., Witt, M., Mohandas, T., and Harper, P.S. (1986). Regional localisations and genetic relationships of seven RFLPs and myotonic dystrophy on chromosome 19. Hum. Genet. 74:262-266.

Sherman, S.L., Ball, S.P., Robson, E.B. (1987). A genetic map of chromosome 19 based on family linkage data. Ann. Hum. Genet. 49:181-187.

Smeets, H., Markslag, P., Bril, J., Hulsebos, T., Brunner, H., Schonk, D., Ropers, H.H., and Wieringa, B. (1987). EcoRI RFLP at 19cen-19q13.2 identified by the anonymous DNA sequence pPM6.7 (D19S18). Nucl. Acids Res. 15:8120.

Smeets, B., Poddighe, J., Brunner, H., Ropers, H.H. and Wieringa, B. (1988) Tight linkage between myotonic dystrophy and apolipoprotein E genes revealed with allele-specific oligonucletides. Hum.Genet. 80:49-52.

Stallings, R.L., Olson, E., Strauss, A.W., Thompspon, L.H., Bachinski, L.L., Siciliano, M.J. (1988). Human creatine kinase genes on chromosomes 15 and 19 and proximity of the gene for the muscle form to the genes for apolipoprotein c2 and excision repair. Am. J. Hum. Genet. 43:144-151.

Wainwright, B.J., Watson, E.K., Shephard, E.A., Philips, I.R. (1985). RFLP for a human cytochrome P-450 gene at 19q13.1 -qter. Nucl. Acids Res. 13:4610.

Whitehead, A.S., Solomon, E., Chambers, S., Bodmer, W.F., Povey, S., and Fey, G. (1982). Assignment of the structural gene for the third component of human complement to chromosome 19. Proc. Natl. Acad. Sci. 79:5021-5025.

CHAPTER 5

Brunner HG, Smeets HJM, Nillesen W, Oost van B, Biezenbos van den JBM, Joosten EMG, Pinckers AJLG, Hamel BCJ, Theeuwes AGM, Wieringa B, Ropers HH.

Myotonic dystrophy. Predictive value of normal results on clinical examination.

Brain 114: 2303-2311, 1991.

SUMMARY

Myotonic dystrophy (DM) is well-known for its highly variable clinical picture, including the age at which symptoms are first detected. In order to assess the proportion of asymptomatic gene carriers at different ages, we have used linked DNA markers to study individuals at 50% genetic risk of DM, in whom neurological examination, slit lamp examination and EMG had failed to show diagnostic signs. A total of 139 asymptomatic offspring of DM patients were studied. Our analyses identified 11 out of these 139 as probable gene carriers. Our data show that penetrance of the DM gene increases with age. After correction for the possibility of genetic recombination between the DM gene and the DNA markers used, we calculated the residual chance of carrying the DM gene as 8.3% for clinically normal offspring aged between 20 and 39 years.

We evaluated several factors that might influence this figure. Neither the sex of the propositus nor that of the affected parent modified the risk of carrying the DM gene. Presence of aspecific lens opacities did also not correlate with the risk of having inherited the DM gene.

Since a significant proportion of DM gene carriers are not detected by neurological examination, including slit-lamp examination and EMG, these results confirm the need for DNA analysis in asymptomatic offspring of DM patients.

INTRODUCTION

Myotonic dystrophy (DM) is an autosomal dominant multisystem disorder with variable expressivity and incomplete penetrance. A number of studies have documented that the proportion of symptomatic patients increases with age, the median age at onset of symptoms being 20-25 years (Harper, 1989). EMG studies and slit lamp examination are useful for the detection of subclinically affected individuals (Polgar et al., 1972). It has been suggested that the use of these ancillary investigations will allow the detection of most, if not all, gene carriers either by young adulthood (Harper, 1973; 1989), or by age 40 (Bundey, 1974). Nevertheless, it is likely that occasional gene carriers remain undetected. This is supported by reports of obligate gene carriers who failed to show diagnostic abnormalities at an advanced age (Höweler, 1986; Streib et al, 1987; personal observations).

The assignment of the DM gene to chromosome 19, and the subsequent development of closely linked genetic markers have created the possibility of using DNA linkage analysis for predictive testing in DM families. We here report the use of DNA analysis to assess the predictive value of a full clinical evaluation, including EMG and slit lamp examination in a large

cohort of individuals at 50% risk of developing DM.

MATERIALS AND METHODS

Clinical data

We have used data from a previous linkage study (Brunner, 1989b) and from the current diagnostic DNA service at our department to examine the value of a negative clinical examination in persons at risk for DM. We examined the DNA data for all individuals who fulfilled the following criteria:

1. Prior risk of DM 50%.
2. Absence of clinical or electrical myotonia, significant muscle weakness, multicoloured lens opacities or congenital myotonic dystrophy.

In all cases, age given corresponds to the age at clinical examination. If clinical examination had been performed at different ages, only the age at first examination was used.

With rare exceptions, individuals in our study had been examined clinically at one of the 8 Dutch university hospitals.

DNA data

DNA analysis was performed using previously described methods (Brunner et al., 1989a; 1989b; Smeets et al., 1989; 1991). Closely linked markers that were used defined the following loci: BCL3, APOC1, APOC2, CKM, ERCC1, D19S63, D19S112, D19S51, D19S50, KLK and RRAS. Recombination frequencies used in risk calculations were as published elsewhere (Le Beau et al., 1989; Korneluk et al., 1989a; Johnson et al., 1990), with the following modifications: For APOC2 and APOC1, recombination frequencies were taken as 2% and 3% respectively.

For D19S63, a recombination frequency of $\leq 1\%$ was used, based on our own data as well as on data from other laboratories. So far, no recombination has been observed between the DM gene and this locus (Brook et al., 1991). For D19S112, we used an estimated recombination frequency of 2% (H. Smeets, unpublished data).

For both KLK and RRAS, we assumed a recombination frequency of 12%, based on map position and two-point linkage analysis (Smeets et al., 1991; and unpublished data).

Linkage phase (i.e. the marker allele with which the DM gene segregates within a particular family) was always established through examination of affected individuals in accordance with published criteria (Griggs et al., 1989).

TABLE 1. MARKERS USED FOR DNA DIAGNOSIS

Probe name/ method	*Locus*	*Location relative to DM*	*Recombination fraction*	*Reference*
pα1.4P	BCL3	Proximal	4%	Korneluk *et al.* (1989*a*)
PCR-assay	APOC1	Proximal	3%	Nillesen *et al.* (1990)
VSSM	APOC2	Proximal	2%	LeBeau *et al.* (1988); Smeets *et al.* (1989)
pSC11	APOC2	Proximal	2%	LeBeau *et al.* (1988)
pE15	APOC2	Proximal	2%	LeBeau *et al.* (1988)
pCKMM3′	CKM	Proximal	1%	LeBeau *et al.* (1988)
VSSM	ERCC1	Proximal	1%	LeBeau *et al.* (1988); Smeets *et al.* (1991)
pE0.8	ERCC1	Proximal	1%	LeBeau *et al.* (1988); Shutler *et al.* (1991)
pD10	D19S63	Unknown	≤1%	Brook *et al.* (1991)
pX75b	D19S112	Distal	2%	Unpublished data
p134c	D19S51	Distal	2½%	Johnson *et al.* (1990)
pEWRB1.1	D19S50	Distal	9%	Korneluk *et al.* (1989*b*); LeBeau *et al.* (1988)
pEWRB1.4	D19S50	Distal	9%	Korneluk *et al.* (1989*b*); LeBeau *et al.* (1988)
pHGK	KLK	Distal	12%	Hermens *et al.* (1990); Smeets *et al.* (1991)
VSSM	RRAS	Distal	12%	Smeets *et al.* (1991)

*All probes detect conventional restriction fragment length polymorphisms (RFLPs), except: VSSM = variable simple sequence motifs; PCR = polymerase chain reaction.

Statistical evaluation

In order to calculate the chance of a false negative diagnosis on clinical examination, we derived the following formulas.

1. NOTATIONS

DM^+	Myotonic Dystrophy gene present
DM^-	Myotonic Dystrophy gene absent
DNA^-	Low risk marker allele
DNA^+	High risk marker allele
$CLIN^-$	Clinical examination negative
$CLIN^+$	Clinical examination positive

2. ASSUMPTIONS

a. The prior risk of DM equals 50% for all individuals in the study.
$P[DM^+] = 0.5$

b. Using linked DNA markers, the chances of a false positive and of a false negative diagnosis are symmetric and known.
$P[DNA^- \mid DM^+] = P[DNA^+ \mid DM^-] = q$

c. DNA diagnosis and clinical diagnosis are conditionally independent.
$P[DNA^+, CLIN^+ \mid DM^+] = P[DNA^+ \mid DM^+] \times P[CLIN^+ \mid DM^+]$

d. The specificity of a positive clinical diagnosis of DM is 1.
$P[CLIN^- \mid DM^-] = 1$

e. Call $p = P[CLIN^+ \mid DM^+]$ (the sensitivity of the clinical examination) the unknown parameter.

3. FORMULA

Using simple probability rules:

$$P[DNA^- \mid CLIN^-] = \frac{P[DNA^-, CLIN^-]}{P[CLIN^-]}$$

$$= \frac{P[DNA^-, CLIN^- \mid DM^+] \times P[DM^+] + P[DNA^-, CLIN^- \mid DM^-] \times P[DM^-]}{P[CLIN^- \mid DM^+] \times P[DM^+] + P[Clin^- \mid DM^-] \times P[DM^-]}$$

$$= \frac{q \cdot (1-p) \cdot \frac{1}{2} + (1-q) \cdot 1 \cdot \frac{1}{2}}{(1-p) \cdot \frac{1}{2} + \frac{1}{2}} = \frac{q - qp + 1 - q}{1 - p + 1} = \frac{1 - qp}{2 - p}$$

4. ESTIMATION OF SENSITIVITY AND NEGATIVE PREDICTIVE VALUE

We estimated p from the equation:

$$\frac{1-qp}{2-p} = \text{sample fraction } DNA^- = \frac{n-d}{n}$$

Where:

d = number of individuals with a high risk marker genotype
n = number of clinically normal individuals tested with DNA markers

Also:

$\frac{1}{2-p}$ is the negative predictive value of the clinical examination.

Using Taylor series expansion an approximation of the standard error can be calculated (Mood et al., 1974).

Assumption a and b are met, because genetic recombination frequency is not dependent on clinical expression of the DM gene.
Assumption c is met if strict diagnostic criteria are used (Griggs et al., 1989).
Assumption d is correct because DM shows regular autosomal dominant inheritance, and only offspring of known gene carriers were included in this study.

All comparisons were performed by X^2-test.

RESULTS

A total of 151 fully investigated, clinically normal individuals were tested, using the DNA marker systems shown in table 1. A recombination event in the vicinity of the DM gene precluded accurate phase determination in 1 nuclear family. This family was excluded from subsequent analyses. Other reasons for exclusion were: non-paternity shown by DNA analysis (4 individuals in 2 families), and non-informativity of the DNA analysis, defined as >5% chance of incorrect genotype assignment (6 individuals in 5

families). The final analysis included 139 individuals (66 males and 73 females) in 69 families. All individuals were at 50% prior risk of DM. None had unequivocal clinical or electrical myotonia, specific multicoloured lens opacities, significant muscle wasting or weakness, or signs of congenital myotonic dystrophy. DNA analysis was usually informative for either the CKM, ERCC1 or D19S63 locus. On average, the diagnostic reliability of the DNA analysis was 98.44% (range 96% - >99%).

In 11 individuals, DNA analysis indicated a high probability (mean 98.82%; range 98% - >99%) of carrying the DM gene (table 2). The number of expected false positive diagnoses due to genetic recombination in our sample equals only 2.16 (139 x 0.0156). Therefore, approximately 9 (= 11 - 2.16) individuals tested should be asymptomatic gene carriers.

TABLE 2. RESULTS OF DNA ANALYSIS IN 139 CLINICALLY NORMAL OFFSPRING OF PATIENTS WITH DM

	DNA analysis	
Age	*Low risk genotype**	*High risk genotype**
0–9	3	0
10–19	12	2
20–29	60	4
30–39	25	5
40–49	11	0
50–59	12	0
60–69	5	0
Total	128	11

*High and low risk genotypes were defined as a >95% and a <5% chance of carrying the DM gene, respectively.

Using the equations given in the Materials and Methods section, we calculated the negative predictive value (i.e. the probability of not carrying the DM gene for a person with a prior risk of 50% and a negative clinical diagnosis). For the complete study, this was 93.4% (± 2.5%). In order to derive estimates that can be used for genetic counselling purposes, we then calculated the negative predictive value for persons <20 years, between 20 and 39 years, and >40 years of age. In these age groups, the negative predictive value was calculated as 89.4% (± 8.9%), 91.7% (± 3.5%), and 99.8% (± 2.6%), respectively.

We also considered the possibility that other factors, such as sex of the propositus or of the affected parent, might influence the penetrance of the DM gene. Moreover, we compared individuals with high and low risk genotypes with regard to the presence of nondiagnostic signs, such as atypical cataract or mild muscular weakness without myotonia. As can be seen in tables 3 and 4, these parameters were equally distributed among persons with and without the DM gene.

TABLE 3. DISTRIBUTION OF HIGH AND LOW RISK GENOTYPES ACCORDING TO OTHER VARIABLES THAT WERE ANALYSED

	Low risk genotype (n = 128)*	*High risk genotype* (n = 11)*	*P-value by χ^2-test*
Propositus male/female	59/69	7/4	0.42
Mother/father affected	51/77	5/6	0.96
Non-specific features present/absent	24/104	3/8	0.77

*High and low risk genotypes were defined as a >95% and a <5% chance of carrying the DM gene, respectively.

TABLE 4 FEATURES THAT WERE CONSIDERED SUGGESTIVE BUT NOT DIAGNOSTIC OF DM

	Low risk genotype (n = 128)*	*High risk genotype* (n = 11)*
Non-specific lenticular opacities	14	2
Ptosis	1	1
Mild muscular weakness	2	0
Equivocal clinical myotonia/normal EMG	4	1
ECG abnormalities[+]	2	0
Mental retardation	1	1
EMG abnormalities other than myotonia	1	0
Total present	24[a]	3[b]
Absent	104	8

*High and low risk genotypes were defined as a >95% and a <5% chance of carrying the DM gene, respectively [+]ECG abnormalities were not searched for systematically [a]One individual with both aspecific lens abnormalities and equivocal clinical myotonia. [b]One individual with both aspecific lens abnormalities and ptosis and one individual with both aspecific lens abnormalities and equivocal clinical myotonia.

In order to further validate our results, we tried to obtain updated clinical data for patients with a high risk DNA genotype. Additional data were collected for 5 of the 11 individuals that had been indicated by DNA marker studies as putative carriers of the DM gene.

A 39 year old male was found to carry the high risk allele for the flanking markers APOC2 and for D19S51. Upon clinical examination, EMG and slit lamp examination he was completely normal. Four years later, he is still completely asymptomatic. However, one of his sons now shows action and percussion myotonia and muscular weakness, thereby identifying him as an oligate gene carrier.

EMG examination was repeated in 4 asymptomatic individuals identified as probable DM gene carriers by DNA analysis. In two females, EMG and slit lamp examination remained negative at ages 27 and 37 respectively, 3 years after first examination. In another female, EMG showed electrical myotonia at age 41 , 5 years after the first examination had been negative. In a male, clinical examination, including EMG was normal at age 33 and 35 years. However, another EMG at age 37 showed unequivocal myotonia.

Follow-up data have thus confirmed the presence of the DM gene in 3 of 5 individuals with a high risk genotype.

DISCUSSION

Previous studies of penetrance in DM have relied largely on the age at which the expected 50% ratio of affected individuals was reached. These studies have suggested that most (if not all) DM gene carriers should be detectable by young adulthood (Harper, 1973; 1989) or by age 40 (Bundey, 1974). However, not all relatives were studied, and EMG studies were performed in only a minority of patients. Therefore, estimates of penetrance from these studies were necessarily imprecise. Another similar study (Schubert et al., 1980) is difficult to interpret, since probands may have been included in the analysis (Bundey, 1982). Published curves of age at onset (Harper, 1989) refer to self-reported symptoms, and therefore lead to overestimation of the age at which the disorder becomes recognizable by clinical examination.
All individuals in the study reported here had been previously examined clinically by an experienced neurologist and ophthalmolgist for signs of myotonic dystrophy. It is possible that a higher percentage of gene carriers might have been detected if all individuals had been investigated by a single investigator, including standardized EMG of muscles such as the Orbicularis Ori and Masseter (Streib and Sun, 1983). Nonetheless, we feel that our data give a realistic estimate of the diagnostic accuracy that is attained in tertiary care university hospitals. This is supported by the fact, that individuals with a high risk genotype appeared evenly distributed among the referring centers.

TABLE 5. SENSITIVITY OF CLINICAL DIAGNOSIS OF DM AND PROBABILITY THAT A CLINICALLY NORMAL INDIVIDUAL DOES NOT CARRY THE DM GENE (NEGATIVE PREDICTIVE VALUE)

Age (yrs)	*Number of individuals tested (n)*	*Number with high risk genotype (d)*	*Sensitivity of clinical diagnosis (p) (%)*	*Negative predictive value [1/(2−p)] (%)*
0–19	17	2	88.2 ± 10.0	89.4 ± 8.9
20–39	94	9	91.0 ± 3.8	91.7 ± 3.5
40–69	28	0	99.8 ± 2.6	99.8 ± 2.6
All ages	139	11	93.0 ± 2.7	93.4 ± 2.5

Overall sensitivity of the clinical assessment was 93% in our sample. Diagnostic accuracy increased with age. Since the number of individuals less than 20 years old is relatively small, the estimated negative predictive value for this age group (89.4% $\pm$ 8.9) is very tentative with wide confidence limits. Others have noted that a significant proportion of young DM gene carriers may be missed by clinical examination (including EMG and slit lamp examination) especially before 10 years of age (Harper, 1973). However, in sibships in which an older sibling has congenital myotonic dystrophy, early detection may be less difficult (Bundey, 1974; O'Brien et al., 1983). No sibs of congenitally affected individuals were studied here.

Between the ages of 20 and 39 years, the negative predictive value of a normal clinical examination was 91.7% ($\pm$ 3.4%). This figure is of particular importance, since genetic counselling is most often requested by individuals in this age group. Of 28 individuals aged 40 years or older, none had a high risk genotype. It is likely, that less cases are missed over the age of 40 years than under the age of 40 ($P = 0.08$), but more data are needed to derive an accurate estimate of penetrance in the older age groups.

The excess of affected fathers in our series (table 3) is largely due to individuals older than 40 years of age. For this age group, the father was affected in 21 out of 27 individuals tested. An excess of paternal transmission of DM was found in another recent study (Veilette et al., 1989). As suggested by others (Harper, 1989), this may reflect a tendency for milder involvement in the offspring of affected males, thus bringing their families to medical attention at a relatively advanced age. This phenomenon is the reverse of the well-known overrepresentation of maternal transmission among cases of congenital myotonic dystrophy. In spite of these influences of parental sex on gene expression in DM, we found no differences in the proportion of high risk genotypes in the offspring of male and female DM patients (6/83 and 5/56, $P > 0.10$). Sex of the propositus also had no apparent effect, 4/73 females and 7/66 males being identified as probable DM gene carriers ($P > 0.10$).

In a study, reporting on diagnostic DNA analysis in DM, Norman et al. (1989) found that 4 out of 34 at-risk individuals carried a high risk genotype, as determined by studies with linked DNA markers. Although their results appear similar to ours (11 out of 139 positive by DNA analysis), more detailed comparison with that study is not possible, since clinical tests, age at diagnosis, prior risk, and sex of patients and affected parents were not specified.

Some studies have emphasized the significance of additional symptoms that are compatible with a diagnosis of DM, but not specific for this disorder (e.g. Pryse-Philips et al., 1981). Our data suggest that the significance of such a "partial syndrome" is limited, since the frequency of nonspe-

cific abnormalities was similar for those with a positive and a negative diagnosis of DM by DNA studies (3/11 and 24/128 respectively, $P > 0.10$, see tables 3 and 4). The same conclusion was reached in a recent study, which reported follow-up on 44 patients with nonspecific features of DM. After an average period of 2.4 years, 30 still showed only nonspecific signs, 8 showed a typical form of DM, while 6 no longer presented any identifiable anomaly (Mathieu et al., 1989).

In conclusion, our data show that for offspring of patients with DM, the risk of possessing the myotonic dystrophy gene is approximately 8% if upon complete clinical examination in young adulthood no abnormalities can be demonstrated. The likelihood of normal clinical findings in a DM gene carrier may be less after age 40 years, but more data are needed to confirm this. We suggest that DNA analysis should be part of the diagnostic work-up of persons at risk for myotonic dystrophy.

Acknowledgements:

We thank Marcel Nelen and Marga Coerwinkel for technical assistance, We are grateful to the many clinicians who referred their patients to us, and provided additional clinical data.

We thank Drs Shaw (Cardiff, UK), Korneluk (Ottawa, Canada) and Johnson (London, UK) for kind donation of probes.

This work was supported by the Deutsche Forschungsgemeinschaft (Grant No. Ro389/15-4 to H.H.R.), The Beatrix Fonds (Grant No.89/9-29) ,the Muscular Dystrophy Association And the Associaton Francaise contre les Myopathies.

REFERENCES

Brook JD, Harley HG, Walsh KV, Rundle SA, Siciliano MJ, Harper PS, Shaw DJ (1991) Identification of new DNA markers close to the myotonic dystrophy locus. Journal of Medical Genetics, 28, 84-88.

Brunner H, Coerwinkel-Driessen M, Smeets B, Schonk D, Schepens J, Oerlemans F, Hamel B, Ropers H, Wieringa B (1989a) Definition of subchromosomal intervals around the myotonic dystrophy gene. In: Genetics of neuromuscu lar disorders, C. Bartsocas , editor. Alan R. Liss Inc., New York, pp. 107 -114.

Brunner HG, Smeets H, Lambermon HMM, Coerwinkel-Driessen M, van Oost BA, Wieringa B, Ropers HH (1989b) A multipoint linkage map around the locus for myotonic dystrophy on chromosome 19. Genomics, 5, 589-595.

Bundey S (1974) Detection of heterozygotes for myotonic dystrophy. Clinical Genetics, 5, 107-109.

Bundey S (1982) Letter to the editor; Myotonic dystrophy - Early detection and

genetic counselling. Journal of Neurology, 226, 295.
Griggs RC, Wood DS, the working group on the molecular defect in myotonic dystrophy (1989) Criteria for establishing the validity of genetic recombi nation in myotonic dystrophy. Neurology, 39, 420-421.
Harper PS (1973) Pre-symptomatic detection and genetic counselling in myotonic dystrophy. Clinical Genetics, 4, 134-140.
Harper PS (1989) Myotonic dystrophy. 2nd edition. London, WB Saunders Co.
Hermens R, Coerwinkel M, Trapman J, Riegman PHJ, Vlietstra RJ, Smeets H, Wieringa B (1990) An MspI RFLP detected by the human glandular kallikrein gene (hGK) on chromosome 19q. Nucleic Acids Research, 18, 208.
Höweler CJ (1986) A clinical and genetic study in myotonic dystrophy. Thesis, Rotterdam p. 66.
Johnson K, Shelbourne P, Davies J, Buxton J, Nimmo E, Siciliano MJ, Bachinski LL, Anvret M, Harley H, Rundle S, Miki T, Brunner H, Williamson R (1990) A new polymorphic probe which defines the region of chromosome 19 containing the myotonic dystrophy locus. American Journal of Human Genetics, 46, 1073-1081.
Korneluk RG, MacLeod HL, McKeithan TW, Brooks JD, MacKenzie AE (1989a) A chromosome 19 clone from a translocation breakpoint shows linkage and linkage disequilibrium with myotonic dystrophy. Genomics, 4, 146-151.
Korneluk RG, MacKenzie AE, Nakamura Y, Dubé I, Jacob P, Hunter AGW (1989b) A reordering of human chromosome 19 long-arm DNA markers and identification of markers flanking the myotonic dystrophy locus. Genomics, 5, 596-604.
Le Beau MM, Ryan D Jr, Pericak-Vance MA (1989) Report of the committee on the genetic constitution of chromosomes 18 and 19. Cytogenetics Cell Genetics, 51, 338-357.
Mathieu J, Simard M, De Braekeleer M, Boily C, Deschênes A (1989) Partial syndrome of myotonic dystrophy: Clinical presentation and follow-up. the Canadian Journal of Neurological Sciences, 16, 99-103.
Mood AM, Graybill FA, Boes DC (1974) Introduction to the theory of statistics. 3rd Edition, McGraw-Hill, Tokyo.
Nillesen WM, Smeets HJM, van Oost BA (1990) Human AoC1 Hpa1 restriction site polymorphism revealed by the polymerase chain reaction. Nucleic Acids Research, 18, 3428.
Norman AM, Floyd JL, Meredith AL, Harper PS (1989) Presymptomatic detection and prenatal diagnosis for myotonic dystrophy by means of linked DNA markers. Journal of Medical genetics, 26, 750-754.
O'Brien T, Newcombe RG, Harper PS (1983) Outlook for a clinically normal child in a sibship with congenital myotonic dystrophy. the Journal of Pediatrics, 103, 762-763.
Polgar JG, Bradley WG, Upton ARM, Anderson J, Howat JML, Petito F, Roberts DF, Scopa J (1972) The early detection of dystrophia myotonica. Brain, 95, 761-776.

Pryse-Phillips W, Johnson GJ, Larsen B (1982) Incomplete manifestations of myotonic dystrophy in a large kinship in Labrador. Annals of Neurology, 11, 582-591.

Schubert T, Jerusalem F, Martenet AC, Metaxas M, Meyer M (1980) Myotonic dystrophy - Early detection and genetic counselling. Journal of Neurology, 223, 13-22.

Shutler G, MacKenzie AE, Brunner H, Wieringa B, de Jong P, Leblond S, Bailly J, Korneluk RG (in press) Physical and genetic mapping of a novel chromosome 19 ERCC1 marker showing close linkage with myotonic dystrophy. Genomics.

Smeets HJM, Brunner HG, Ropers HH, Wieringa B (1989) Use of variable simple sequence motifs as genetic markers: Application to the study of myotonic dystrophy. Human Genetics, 83, 245-251.

Smeets HJM, Hermens R, Brunner HG, Ropers HH, Wieringa B (1991) Identification of variable simple sequence motifs in 19q13.2-qter: Markers for the myotonic dystrophy locus. Genomics, 9, 257-263.

Streib EW, Fine B, Aita JF (1987) Myotonic dystrophy sine myotonia: Normal EMG in two obligate gene carriers of advanced age. Electromyography and Clinical Neurophysiology, 27, 443-446.

Streib EW, Sun SF (1983) Distribution of electrical myotonia in myotonic muscular dystrophy. Annals of Neurology, 14, 80-82.

Veillette S, Perron M, Mathieu J (1989) Myotonic dystrophy: II Marriage, fertility and gene transmission. the Canadian Journal of Neurological Sciences, 16, 114-118.

CHAPTER 6

Brunner HG, Spaans F, Smeets HJM, Coerwinkel-Driessen M, Hulsebos T, Wieringa B, Ropers HH.

Close linkage with chromosome 19 but not chromosome 17 in a family with myotonic dystrophy associated with hereditary motor and sensory neuropathy.

Neurology 41: 80-84, 1991

SUMMARY

We identified a large kindred that shows classical myotonic dystrophy (MyD), together with hereditary motor and sensory neuropathy (HMSN) in some individuals and HMSN alone in others. A previous study of this family has shown cosegregation of the MyD and HMSN phenotypes with the Lutheran and Secretor loci in some branches of this family, indicating linkage to chromosome 19. We reanalysed this family with two recombinant DNA marker systems from the ApoC2 locus on chromosome 19. Our results demonstrate that all affected individuals have inherited a unique ApoC2 haplotype that was not found in their clinically and electrophysiologically normal sibs. We also obtained evidence against involvement of the HMSN1 locus on chromosome 17. In this family a moderately severe neuropathy may be the only clinical sign of MyD for many years. Our results are consistent with an unusually neuropathic mutation at the MyD gene. However, involvement of two closely linked genes (one for MyD and the other for HMSN) can also explain our findings.

INTRODUCTION

Myotonic dystrophy (MyD) is an autosomal dominant disorder which is closely linked to the gene for Apolipoprotein C2 (ApoC2) on chromosome 19. Hereditary motor and sensory neuropathy (HMSN) is a genetically heterogeneous disorder. Classification into several subtypes is based on the pattern of inheritance, peripheral nerve conduction velocity and associated clinical features. The main locus for HMSN1 is on chromosome 17, but a subset of families show linkage to the Duffy blood group locus on chromosome 1.

We have performed a genetic linkage study in a large kindred with features of both myotonic dystrophy (MyD) and hereditary motor and sensory neuropathy (HMSN) that was previously described by Spaans and colleagues [1]. In that study, out of 31 at-risk family members examined, 13 showed signs of HMSN, 8 of whom also showed signs of MyD. Results of nerve biopsies performed in 2 patients were suggestive of HMSN1. No patient was found with MyD alone. The pedigree structure suggested a single mutational event for both disorders in the deceased great-grandparents since upon examination neither MyD nor HMSN was found in several other branches of their respective families. Genetic linkage studies were performed with marker systems for chromosome 1 and chromosome 19. Although only partly informative, haplotype construction from the Secretor and Lutheran data indicated cosegregation of the HMSN phenotype with these chromosome 19 loci.

No linkage was demonstrated with the chromosome 1 markers. Based on these results, the authors proposed that the syndrome could be caused by an allelic form of the 'common' MyD gene or by two closely linked loci on chromosome 19. The significance of these results was limited by the fact that the Lutheran and Secretor loci ecombine with the MyD gene locus in approximately 10-20% of cases and in some 10% with each other [2]. We therefore decided to reanalyze this family using recombinant DNA methods, focussing our attention on chromosome 19 (MyD) and chromosome 17 (HMSN).

MATERIALS AND METHODS

Subjects

The family we studied has been described previously [1]. In the affected family branch, out of 31 investigated family members, 13 persons were found to have impaired peripheral nerve conduction. The mean values of the median and peroneal nerve conduction velocities were 62 and 56% respectively of the mean values found in the unaffected family members. Median and sural nerve sensory action potentials were either not recordable or showed reduced amplitudes and increased latencies. Sural nerve biopsies in two individuals showed a considerable decrease in myelinated nerve fibre density. Many myelinated nerve fibres were surrounded by small concentric onion bulb-like structures.

Of the 13 persons with a HMSN phenotype, 8 showed unequivocal signs of MyD. Electrical myotonia and cataract were found in all of them. Four individuals showed the characteristic multicoloured crystalline lens opacities. Other signs of MyD such as testicular atrophy, fromtal balding, myopathic facies and mental dullness were found in a few patients. None of the patients has any known cardiac involvement.

The 18 other at-risk individuals showed no signs of either MyD or HMSN upon careful clinical examination including EMG and slitlamp examination [1].

Since the previous study 4 affected subjects were deceased (II-25, II-28, III-7 and III-12), whereas one affected person who refused participation in the previous linkage study (II-24), could be included this time (compare the present Fig. 2 with Fig. 7 in the article by Spaans and colleagues [1]).

Physical examination, slit lamp and EMG studies

Clinical studies were repeated in the 5 affected subjects who had shown no signs of MyD in the previous study (III-1, III-16, IV-19, IV-21, IV-22). In II-31 and IV-20 these studies were performed for the first time. These individuals had a full neurological examination, slit lamp examination, motor

and sensory nerve conduction studies, and concentric needle EMG of at least two muscles (interosseous I and extensor digitorum brevis). In the median nerve, motor and sensory conduction was measured in the segment elbow-wrist. Motor conduction in the peroneal nerve was measured in the segment knee-ankle and sensory conduction in the sural nerve between the ankle and the lower part of the calf (12 cm). The calculated conduction velocities were corrected to a skin temperature of 32°C (2 m/s per deg. C). At this temperature conduction velocities are abnormal if below 50 m/s in the median nerve and below 40 m/s in the leg nerves. Absence of sensory nerve action potentials is always considered abnormal.

DNA analysis

Blood samples for DNA analysis were taken from 10 affected and 18 unaffected family members and from 7 spouses (see Fig. 2).

DNA was extracted from leukocytes using standard procedures as described [3]. Our analyses used DNA polymorphisms from the ApoC2 gene region, which has been shown to be about 2cM proximal to the MyD gene [3,4]. To increase informativity, a novel method for the detection of polymorphism within the ApoC2 gene [5], was used in conjunction with the well-known ApoC2-TaqI RFLP system [6]. For the ApoC2-TaqI RFLP [6], DNA was cut with the restriction enzyme TaqI,

electrophoresed on 0.7% (w/v) agarose gels and blotted onto nylon membranes. Hybridization was performed with a α-^{32}P-dCTP labeled 300bp probe from the 3'end of the ApoC2 gene as described [3]. For detection of polymorphism in a highly variable [TGnAGm] repeat sequence (Variable Simple Sequence Motif, VSSM) in the first intron of the ApoC2 gene, DNA was amplified by the polymerase chain reaction [7] using oligonucleotides for the regions flanking the repeat. Amplified DNA was run on 10% sequencing gels, electroblotted on a nylon membrane and probed with aan end-labeled internally hybridizing (TG_7T) oligonucleotide [5]. Autoradiography was performed for 2 hours, using double intensifying screens.

Two markers were chosen, because they are closely linked to a gene for HMSN1 on chromosome 17 [8].For the pEW301 probe which defines the D17S58 locus on chromosome 17, the TaqI and BglII polymorphisms were scored. For the pUC10-41 probe (D17S71), we tested the PvuII and MspI polymorphisms.

Linkage analysis

For linkage analysis, the latest version (5.03) of the LINKAGE program package [9] was used. Penetrance was assumed to be complete for the HMSN phenotype in all individuals in this study. For HMSN a gene frequency of 0.0001 was used. Allele frequencies for the different RFLP

systems were as described [3,8]. No recombination was detected between the ApoC2 TaqI RFLP and the internal [TGnAGm] repeat polymorphism. The two-point lod score between the two ApoC2 polymorphisms was 7.09 without recombination, confirming that they indeed represent the same locus. Therefore, information from these polymorphic systems was combined to yield haplotypes, which were subsequently treated as alleles at a single locus. All haplotypes generated by this method have a low frequency in the population, but their exact frequency remains to be determined. In our calculations, we arbitrarily estimated the frequency of each of the 7 haplotypes observed as 0.1 .

RESULTS

Physical examination, slit-lamp and EMG studies

The relevant findings in the 7 newly investigated family members are given in table 1. For the previous findings in the reinvestigated subjects, as well as for the findings in the other affected members we refer to the article by Spaans and colleagues [1].

Only subject II-31 had no clinical or electrophysiologic abnormalities. All others had distinct nerve conduction disturbances, which were most prominent in IV-20.

Weakness was limited to the toe extensors in III-16, IV-20 and IV-21, atrophy in these cases was limited to the intrisic foot muscles. The most serious nerve conduction abnormalities were found in IV-20 and IV-21. In the latter they were somewhat more pronounced than 6 years before. Subject IV-19 showed a distinct paresis of foot- and toe-extension and pronounced atrophy of the intrinsic hand muscles, lower leg and intrinsic foot muscles. III-16 lacked one ankle jerk whereas the other was normal.

Nonspecific cataract was present in an early stage in III-1. III-16 had been operated for cataract of the right eye and showed a distinct cataract of the left eye. On both sides the cataract never showed a specific myotonic character.

Of the 5 subjects with HMSN and without signs of DM at the previous EMG-examinations, III-16 and IV-19 now showed a number of typical myotonic runs, which were found only in the intrinsic hand muscles.

A large amount of aspecific spontaneous muscle fiber activity was found in several muscles of both IV-19 and IV-20, but typical myotonic discharges were not found in IV-20. However, because of a normal interference pattern on volition and the absence of atrophy in the relevant muscles, it is unlikely that the spontaneous activity was due to denervation.

Table 1. Relevant findings on physical examination and EMG in the 5 reinvestigated and 2 not previously investigated family members

	II-31	III-1	III-16	IV-19	IV-20	IV-21	IV-22
Age (yrs) at examination	63*	51†	44†	20†	17*	12†	10†
Sex	M	F	F	M	M	M	F
Weakness	—	—	+	+ + +	+	+	—
Muscular atrophy	—	—	+	+ + +	+	±	—
Clinical myotonia	—	—	—	+	—	—	—
Distal sensory disturbance	—	—	+	+ +	+	—	—
Absent knee jerks	—	—	—	—	—	+	—
Absent ankle jerks	—	—	±	+	+	+	+
Pes cavus	—	—	±	+ +	±	—	—
Cataract	—	±	+	—	—	—	—
Myotonic discharges	—	—	+	+	—	—	—
Small polyphasic MUPs	—	—	—	+	—	—	—
High voltage MUPs	—	—	+	—	—	—	—
Median nerve MCV	52	48	44	39	25	26	44
Peroneal nerve MCV	46	44	33	NR	16	NR	26
Median nerve SCV	53	49	43	NRE	NR	NR	42
Sural nerve SCV	42	NR	NR	NR	NR	NR	NR

* First examination.
† Reexamined (for previous examination see reference 1).
\+ Abnormal sign present.
MUPs Motor unit action potentials.

MCV Motor conduction velocity (m/sec).
SCV Sensory conduction velocity (m/sec).
NR No potential recorded.
NRE No potential recorded with stimulation at the elbow.

DNA analysis

Examples of the respective hybridization patterns of the ApoC2-TaqI and the ApoC2-VSSM polymorphisms are shown in Fig. 1.

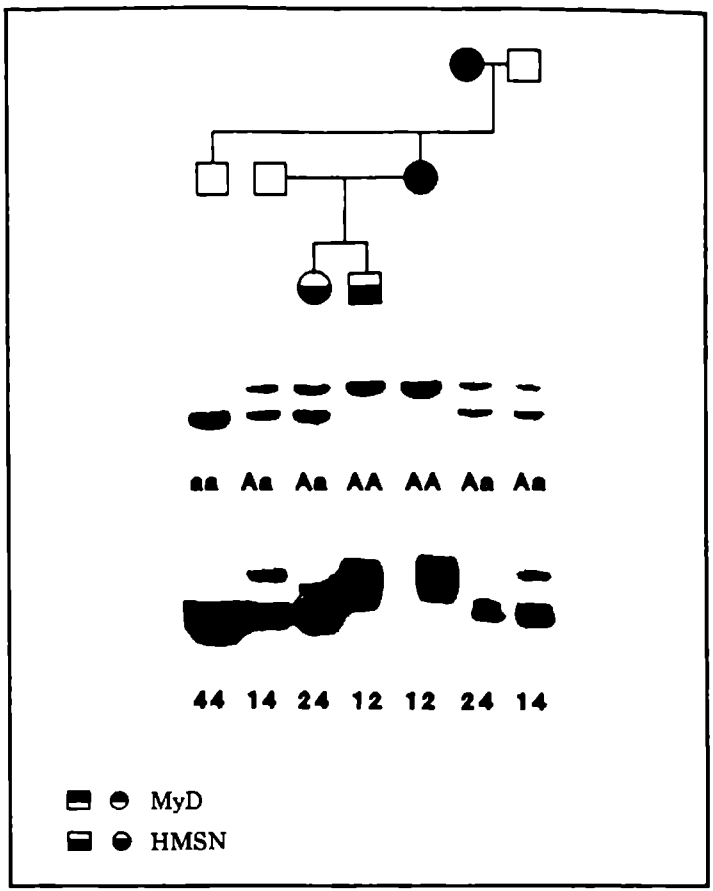


Figure 1. Inheritance of the ApoC2 Taq *I RFLP (upper panel) and the ApoC2 VSSM polymorphism (lower panel) in 1 branch of the family. From left to right, data are shown for individuals III-14, spouse of III-17, IV-22, IV-21, III-17, II-27, and spouse of II-27. Band sizes are: 3.8 and 3.5 kbp for the ApoC2* Taq *I A and a alleles and approximately 108, 104, and 96 bp for the 1, 2, and 4 ApoC2 VSSM alleles. The gene that causes HMSN cosegregates with the A and 2 alleles for these 2 marker systems respectively (haplotype B). Circles represent females and squares represent males.*

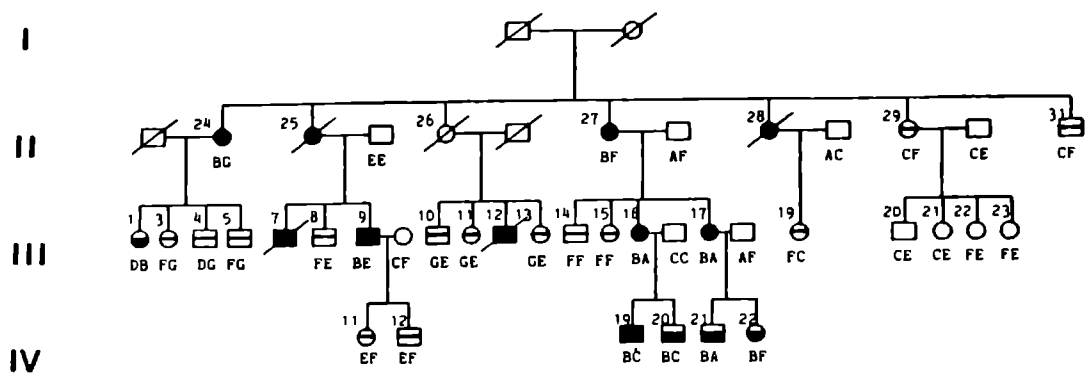


Figure 2 Pedigree of the affected family branch. Only the subjects who took part in the present linkage study and their parents are depicted here To facilitate comparison with the study of Spaans et al,[1] the previously used number coding has been adopted Letters A to G denote ApoC2 haplotypes constructed from the ApoC2-Taq I and ApoC2-VSSM polymorphisms. Haplotype symbols are as follows A = 3 8 kbp (Taq I) and 108 bp (VSSM),B = 3 8 kbp and 104 bp, C = 3 8 kbp and 100 bp, D = 3 8 kbp and 96 bp, E = 3 5 kbp and 100 bp, F = 3 5 kbp and 96 bp, G = 3 5 kbp and 92 bp

Results of the DNA analysis of the complete pedigree are shown in Fig. 2. All persons with reduced nerve conduction velocities shared a single haplotype (designated B in the pedigree), that was not found in their unaffected relatives. The maximum Lod score was 7.04 without recombination, confirming the tight genetic linkage between ApoC2 and the HMSN phenotype in this family. As expected from the ApoC2 haplotype results, no recombination was detected with either the ApoC2 TaqI or the ApoC2 VSSM polymorphism (Lod scores of 3.76 and 6.92 respectively).
Analysis of the chromosome 17 markers does not support linkage with HMSN in our family. The pEW301 BglII polymorphism and both pUC10-41 polymorphisms were not informative. Evidence against linkage was obtained with the pEW301 TaqI polymorphism. At least three recombinations have occurred with this locus. Lod scores for ApoC2 and for pEW301 (D17S58) are given in table 2.

DISCUSSION

The findings presented here indicate that all individuals in this family who have evidence of a polyneuropathy have inherited the same segment of chromosome 19, thereby confirming and extending the results of Spaans and colleagues [1]. The use of an extremely informative new polymorphic system within the ApoC2 gene has enabled us to completely elucidate the segregation of the ApoC2 gene region in this family. The ApoC2 gene has been shown to map at only 2cM proximal to the MyD gene [3,4]. Because recombination between MyD and ApoC2 is very rare it is highly likely that those individuals who show only the HMSN phenotype have in fact inherited the MyD gene.
In 2 of 5 individuals (III-16 and IV-19) who previously showed signs of polyneuropathy only, myotonic discharges were demonstrated this time. Similarly, in our previous study of this family, one 35-year-old subject (III-17) showed myotonic signs that had been absent 5 years earlier [1]. Therefore, in this family, HMSN may be diagnosed at a very early age whereas signs of MyD appear much later.
Four persons in the present study show only signs of HMSN.
One 17-year-old boy (IV-20) who was not tested electrophysiologically before, showed severe slowing of nerve conduction (peroneal nerve MCV about 30% of normal, median nerve MCV less than 50% of normal). No typical myotonic discharges were recorded. Similar results to six years ago were obtained in a ten-year-old girl (IV-22), and her twelve-year-old brother (IV-21). In view of their young age and the results of the genetic linkage study, these individuals may be expected to develop signs of MyD at a later age.

Nonpenetrance of the MyD gene at the age of 51 (individual III-1) is unusual, but by no means impossible [10,11].

Given the apparent association between these two disorders, it appears, that in this family the finding of reduced nerve conduction velocities is a reliable indicator of the presence of both MyD and HMSN. Conversely, family members with normal nerve conduction velocities should not carry any of the two genes. Our DNA studies confirm this, since out of 14 at-risk persons with normal conduction velocities, none has inherited the ApoC2 haplotype that is associated with the MyD gene in this family.

As discussed by Spaans and colleagues [1], a number of studies have shown mild or moderate peripheral nerve involvement in MyD. One recent publication demonstrated increased heat and/or cold thresholds of the skin over the ankle in 79% of 24 MyD cases. Slowing of sural sensory conduction and peroneal motor conduction was found in 17% and 25% respectively, whereas median and ulnar sensory conduction velocities were not significantly decreased [12]. In another study, sural nerve biopsies showed reduced myelinated fibre density in 11 of 13 myotonic dystrophy patients with preferential loss of large myelinated fibers. The findings were considered consistent with a chronic axonopathy of moderate severity [13]. In these studies the differences in peripheral nerve conduction velocity between MyD patients and controls were small.

The family reported here is unique because of the early expression of markedly reduced nerve conduction velocity in all 14 individuals that have inherited the MyD gene. Clearly, the polyneuropathy observed in the younger patients predates the onset of the classical features of MyD by many years.

Several studies have shown that although some families with autosomal dominant HMSN show linkage to chromosome 1 markers [14-18], most families apparently do not [19-23]. It has recently been established that the large majority of families with HMSN1 map to chromosome 17 [8,24,25]. Our study shows no indication that a locus on chromosome 17 is responsible for the neuropathy in this family. The results are therefore compatible with an unusually neuropathic mutation of the MyD gene itself. For the neuropathy in MyD the situation may be similar to what has been suggested for the cardiac involvement [26]. This feature of MyD is frequently found in some families, but is absent in others, suggesting that although all MyD families are due to mutations in a single gene on chromosome 19, the exact type of mutation may determine the clinical picture in that particular family. Obviously, other explanations are possible, such as modifying influences from other genes or environmental factors. However, these are less likely to produce such a constant picture throughout a large family.

Table 2. Lod scores for linkage of the neuropathy (HMSN) versus the ApoC2 locus from chromosome 19 and versus the D17S58 locus from chromosome 17

	0.00	0.01	0.05	0.10	0.20	0.30	0.40
HMSN vs ApoC2	7.04	6.94	6.51	5.95	4.72	3.34	1.83
HMSN vs D17S58	$-\infty$	−2.85	−1.42	−0.83	−0.32	−0.12	−0.02

Finally, HMSN is genetically heterogeneous and therefore genes on several chromosomes other than chromosomes 1 and 17 may be capable of inducing a hereditary neuropathy. Thus, the involvement of two closely linked genes on chromosome 19 (one for MyD, the other for HMSN) can also explain our findings. We suggest that chromosome 19q remains a candidate region for the mutation in those HMSN families that are not linked to either chromosome 17 or chromosome 1.

Acknowledgements.
We thank M.Lathrop for providing the LINKAGE program package. This work was supported by grant RO 389/15-2 of the Deutsche Forschungsgemeinschaft to H.-H.R., by grant 87-2694 of the Dutch Beatrixfonds, by the Dutch Praeventiefonds grant 28-1165 and by the American Muscular Dystrophy Association and the Piton Foundation.

REFERENCES

1. Spaans F, Jennekens FGI, Mirandolle JF, Bijlsma JB, de Gast GC. Myotonic dystrophy associated with hereditary motor and sensory neuropathy. Brain 1986;109:1149-1168.
2. Gedde-Dahl T, Olaisen B, Teisberg P, Constant Wilhelmy M, Mevåg B, Helland R. The locus for apolipoprotein E (ApoE) is closely linked to the Lutheran (Lu) blood group locus on chromosome 19. Hum Genet 1986; 67:178-182.
3. Brunner HG, Smeets H, Lambermon HMM, et al. A multipoint linkage map around the locus for myotonic dystrophy on chromosome 19. Genomics 1989;5:589-595.
4. Le Beau MM, Ryan D, Percak-Vance MA. Report of the committee on the genetic constitution of chromosomes 18 and 19, Human Gene Mapping 10 (1989):Tenth International Workshop on Human Gene Mapping. Cytogenet Cell Genet 1989;51:338-357.
5. Smeets HJM, Brunner HG, Ropers H-H, Wieringa B. Use of variable simple sequence motifs as genetic markers: Application to study of myotonic dystrophy. Hum Genet 1989;83:245-251.
6. Humphries SE, Jowett NI, Williams L et al. A DNA polymorphism adjacent to the human apolipoprotein CII gene. Mol Biol Med 1983;1:463-471.
7. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487-491.

8. Vance JM, Nicholson GA, Yamaoka LH, et al. Linkage of Charcot-Marie-Tooth neuropathy type 1a to chromosome 17. Exp. Neurology 1989;104:186-189.
9. Lathrop CM, Lalouel JM. Easy calculations of lod scores and genetic risks on small computers.Am J Hum Genet 1984;36:460-465.
10. Harper PS. Myotonic dystrophy. 1989 Philadelphia Saunders.
11.Meredith AL, Huson SM, Lunt PW et al. Application of a closely linked polymorphism of restriction fragment length to counselling and prenatal testing in families with myotonic dystrophy. Br Med J 1986;293:1353-1356
12.Jamal GA, Weir AI, Hansen S, Ballantyne JP Myotonic dystrophy: A reassessment by conventional and more recently introduced neurophysiological techniques. Brain 1986;109:1279-1296.
13.Cros D, Harnden P, Pouget J, Pellissier JF, Gastaut JL, Serratrice G. Peripheral neuropathy in myotonic dystrophy: A nerve biopsy study. Ann Neurol 1988;23:470-476.
14.Bird TD, Ott J, Giblett ER. Evidence for linkage to Charcot-Marie-Tooth neuropathy to the Duffy locus on chromosome 1. Am J Hum Genet 1982; 34:388:394.
15.Stebbins NB, Conneally PM. Linkage of dominantly inherited Charcot Marie Tooth neuropathy to the Duffy locus in an Indiana family (abstract). Am J Hum Genet 1982;34:195A.
16.Guiloff RJ, Thomas PK, Contreras M, Armitage S, Schwarz G., Sedgwick EM. Evidence for linkage of type I hereditary motor and sensory neuropathy to the Duffy locus on chromosome 1. Ann Hum Genet 1982;46:25-27.
17.Dyck PJ, Ott J, Moore SB, Swanson CJ, Lambert EH. Linkage evidence for genetic heterogeneity among kinships with hereditary motor and sensory neuropathy, type I. Mayo Clin Proc 1983;58:430-435.
18.Ionasescu V, Anderson R, Burns TL, Searby C, Ionasescu R, Ferrel R. Evidence for linkage of Charcot-Marie-Tooth neuropathy (CMT1) to Apolipoprotein A2 (Apo-A2). Am J Hum Genet 1988;42:74-76.
19.Bird TD, Ott J, Giblett ER, Chance PF, Sumi SM, Kraft GH. Genetic linkage evidence for linkage heterogeneity in Charcot-Marie-Tooth neuropathy (HMSN Type I). Ann Neurol 1983;14:679-684.
20.Rossi A, Paradiso C, Cioni R, Rizzutto N, Guazzo G. Charcot-Marie-Tooth disease: study of a large kinship with an intermediate form. J Neurol 1985;232:91-98.
21.Raeymakers P, de Jonghe P, Backovens H et al. Absence of genetic linkage of Charcot-Marie-Tooth disease (HMSN Ia) with chromosome 1 gene markers. Neurology 1989;39:844-846.
22.Middleton-Price HR, Harding AE, Berciano J, Pastor JM, Huson SM, Malcolm S. Absence of linkage of hereditary motor and sensory neuropathy type I to chrososome 1 markers. Genomics 1989;4:192-197.
23.Griffiths LR, Zwi MB, McLeod JG, Nicholson GA. Chromosome 1 linkage studies in Charcot-Marie-Tooth neuropathy type 1. Am. J. Hum. Genet. 1988;42:756-771.
24.Middleton-Price HR, Harding AE, Monteiro C, Berciano J, Malcolm S.

Linkage of hereditary motor and sensory neuropathy type 1 to the pericentromeric region of chromosome 17. Am J Hum Genet 1990;46:92-94.
25. Raeymaekers P, Timmerman V, De Jonghe P et al. Localization of the mutation in an extended family with Charcot-Marie-Tooth neuropathy (HMSN 1). Am J Hum Genet 1989;45:953-958.
26. Hawley RJ, Gottdiener JS, Gay JA, Engel WK. Families with myotonic dystrophy with and without cardiac involvement. Arch Int Med 1983; 143:2134-2135.

CHAPTER 7

Brunner HG, Nillesen W, Oost van B, Jansen G, Wieringa B, Ropers HH, Smeets HJM

Presymptomatic diagnosis of myotonic dystrophy.

J Med Genet 29: 780-784, 1992

SUMMARY

The discovery of an expanded $(CTG)_n$ repeat sequence in myotonic dystrophy (DM) has greatly improved our ability to detect DM gene carriers who have few or none of the classical signs of this disorder. We here report our experience with two such groups of gene carriers. We used a PCR-based analysis that should be especially sensitive to small increases in CTG triplet number, that might escape detection by conventional Southern blot analysis. Our analyses show that on 100 non-DM chromsomes, the number of CTG triplets ranged from 5 to 37. We then studied 17 obligate gene carriers aged 55 years and over, who showed no muscle weakness. All of the gene carriers in this group showed a relatively small increase in the number of CTG triplets (52 to 90 CTG triplets) with limited somatic mosaicism. We subsequently studied 11 individuals (aged 19 to 36 years) who had previously been identified as gene carriers by genetic linkage studies, but who lacked diagnostic signs. In this prospectively studied group, 9 individuals showed an expanded allele, confirming the earlier prediction from linked genetic markers. The other 2 individuals showed only 2 normal alleles, and an expanded allele was not detected. Revision of the clinical data casts doubt on the original diagnosis of DM in their families. Preferential amplification of the normal non-expanded allele was noted for 3 asymptomatic gene carriers in this study (as well as for 2 of their clinically affected relatives). We caution that at least in our hands, the DM mutation can be confidently excluded by this PCR-based method only if both normal alleles have been identified. If an expanded allele is not seen, and only a single normal allele is visualized on 6% PAGE, confirmatory testing with linked genetic markers or conventional Southern blotting of the $(CTG)_n$ repeat is advocated.

INTRODUCTION

Myotonic dystrophy (DM) shows a wide range of clinical expressivity in affected individuals [1]. Severity of the disease is at least partly correlated with the number of CTG trinucleotide repeat units found in the 3' untranslated region of a protein kinase gene located in band q13.3 of chromosome 19 [2-10]. There is a marked tendency for an increase in CTG triplet number from one generation to the next, which is paralleled by earlier onset of the disease in successive generations [11,12], a phenomenon known as anticipation. Genealogical studies in DM families [11-15] suggest that in earlier generations additional asymptomatic gene carriers may have been present, presumably with only a minor expansion of the CTG motif. However, a premutation, not involving expansion of the CTG trinucleotide repeat, is

another possibility. This situation is highly reminiscent of the CCG-repeat expansion seen in the fragile X syndrome [16-18]. Our previous study using linked DNA markers has suggested that a considerable proportion of siblings and offspring of DM patients are asymptomatic in early adulthood [19]. Therefore, asymptomatic individuals may not be confined to the early generations of a DM family.
We studied these issues in detail by assaying the CTG triplet number in 17 obligate carriers of the DM gene as defined by pedigree analysis and genealogical studies. We further studied a group of asymptomatic offspring of DM patients who had been identified previously as probable gene carriers by the application of closely linked DNA markers.

METHODS

Families were ascertained through the DNA diagnostic service from our department. As of January 1992, this service has performed diagnosis with linked DNA markers in 164 DM families, of which 76% were referred by other genetic departments. Care was taken to assure that all families met previously defined diagnostic criteria [20]. For the purpose of this study, we selected 13 families that contained 1 or more obligate gene carriers aged 55 and over who showed no muscular weakness. Obligate gene carriers were identified by pedigree analysis or through genealogical studies that linked independently ascertained pedigrees. There were 14 males and 3 females in this group, ranging in age from 55 years to 92 years (mean 69.8 years). Among 17 obligate gene carriers, 7 had undergone cataract surgery. The other 10 were considered asymptomatic. EMG and slitlamp examination had been performed in 6/10. Typical lenticular opacities were noted in 3/6. EMG was normal in all 6. In addition, 11 individuals from 9 families were restudied, for whom our previous study [19] had indicated a high probability of carrying a DM mutation in spite of normal results on clinical examination. We used data from 50 normal Dutch individuals (100 chromosomes) who had married into DM families to establish the normal range of CTG triplet number for controls.
Chromosomal DNA was isolated from peripheral blood [21]. Genetic marker systems flanking the DM mutation have been described previously [22-26]. Using a recently described PCR assay [7], we tested the expansion of the CTG repeat in genomic DNA. The CTG repeat was amplified with flanking primers 406 and 409 reported by Mahadevan et al. [7]. The resulting DNA fragments were resolved on 1% and 4% agarose gels. A Southern blot, made from the 1% gel on Gene Screen Plus, was screened with a ^{32}P end-labeled

$(CTG)_{10}$ oligonucleotide as a probe, and the hybridizing fragments were subsequently visualized by autoradiography. Alleles containing up to approximately 140 CTG triplets could also be identified on a 6% polyacrylamide / 7 M Urea sequencing gel after ^{32}P end-labeling one of the amplification primers.

RESULTS

Normal controls

Data from 100 normal chromsomes are summarized in figure 1. Allele lenghts were found to range from 5 to 37 CTG triplets. The most common alleles in this sample were 5, 13 and 14 CTG triplets (38, 22 and 15% respectively).

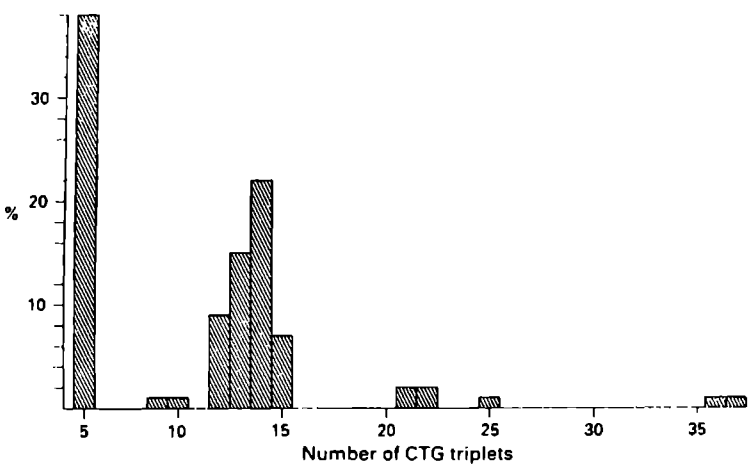


Figure 1:
Distribution of allele sizes for 100 non-DM chromosomes. Allele sizes are given as the number of CTG triplets.

Obligate carriers

All of the 17 obligate gene carriers (14 males and 3 females) showed an expanded allele with multiple bands and weaker signals on 6% polyacrylamide gels, different from normal alleles at this locus (5-37 CTG triplets). Allele size (estimated from the strongest signal among these multiple bands) ranged from 52 to 90 CTG triplets (Figure 2).

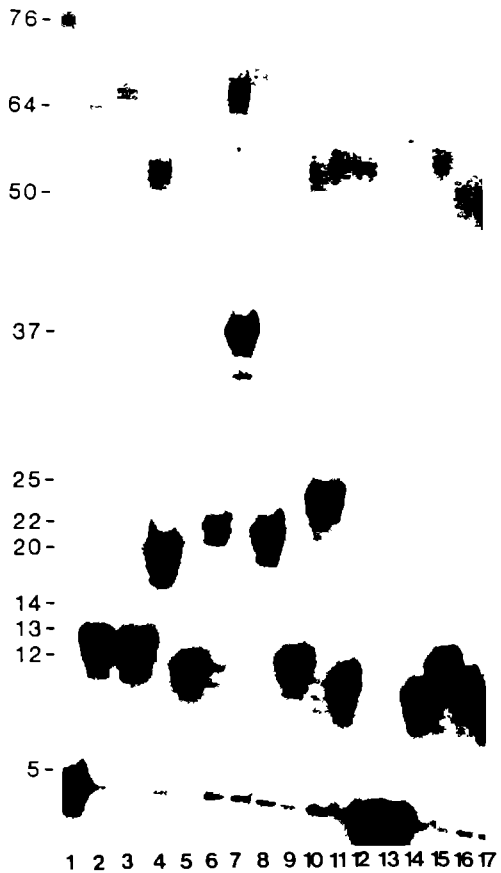


Figure 2:

CTG repeat analysis of obligate DM gene carriers (lanes 1-5, 7, 8, 10-12 and 14-17) by 6% denaturing polyacrylamide gel electrophoresis. Expanded PCR-amplified alleles range from 52 to 76 CTG triplets. The larger alleles show up less well, probably due to differences in the efficiency of the PCR reaction. Lane 7 represents a 72-year old male who shows bilateral cataract as his only clinical feature. This individual has an expanded allele of 74 CTG triplets and a normal allele of 37 CTG triplets.

The apparent instability of the expanded allele could represent an in vitro artifact of the PCR reaction. However, in view of the strikingly different appearance of the 37 and 52 CTG triplet signals, it is more likely, that the CTG repeat shows somatic mosaicism in vivo above a threshold of approximately 40-50 CTG triplets. With 1 exception (II-7, figure 3), CTG repeats were found to be larger in offspring than in these obligate carriers, confirming that the expanded repeat sequence was unstable.

A striking example of anticipation is shown in figure 3. In this family, 2 individuals (I-3 and II-7) aged 90 and 53 respectively are asymptomatic on full examination, including EMG and slit lamp examination, except for a few typical lenticular opacities in I-3. A third individual (I-1) is also completely normal at age 85, but no EMG has been performed. These obligate heterozygotes showed CTG triplet numbers of 52, 60 and 52 respectively, based on results of 6% PAGE (lanes 16, 15 and 17, figure 2). The 1% agarose Southern blot of the PCR product shows the expected band of approximately 0.2 kb for these individuals. Above this band, a smear can be detected, which is frequently observed for alleles of this size, and which may be due to heteroduplex formation of normal and expanded alleles, rather than to somatic mosaicism. Symptomatic disease has been diagnosed in the descendents of these obligate carriers (III-1,2,3; figure 3). These individuals all have expanded alleles exceeding 200 CTG triplets.

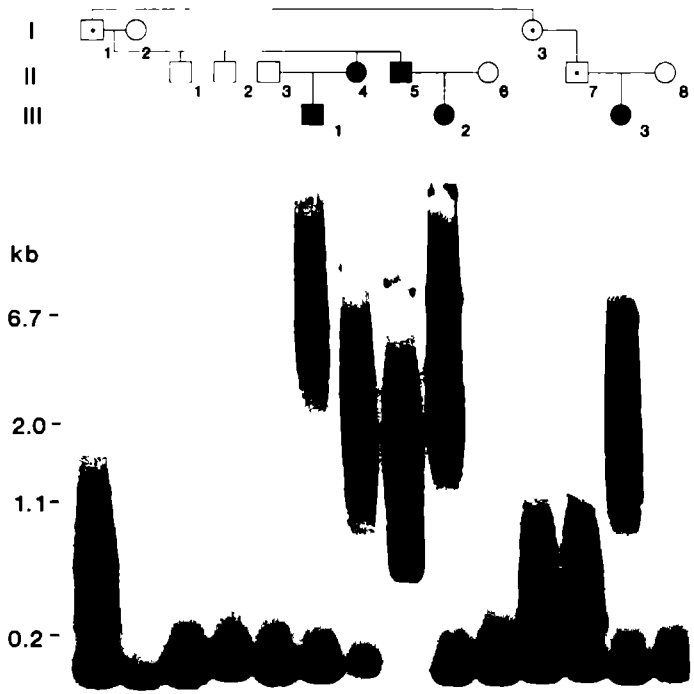


Figure 3:
Southern blot analysis of PCR-amplified CTG repeat sequence run on a 1% agarose gel in a DM family.
Transmission of DM through 3 clinically asymptomatic individuals (I-1, I-3, II-7), aged 90 years, 85 years and 53 years, who show only mild expansion in this assay. CTG triplet numbers for these obligate carriers (determined from 6% polyacrylamide gel electrophoresis) were 52, 52 and 60 respectively. Clinically affected individuals (II-4, II-5, III-1, III-2, III-3) show more extensive expansion of the CTG repeat.

Probable gene carriers identified by linkage analysis

An expanded CTG repeat was demonstrated in 9/11 asymptomatic offspring, who carried the high risk haplotype for closely linked DNA markers (table 1).

These individuals ranged in age from 19 to 36 years. In 4/9 the size of the expanded allele fell within the range previously observed for asymptomatic (or cataract-only) individuals aged 55 years and older. In the other 5 asymptomatic DM gene carriers, allele sizes were found to be considerably larger >200 CTG triplets).

In the final 2 cases, a clinical diagnosis of DM in a key relative was not supported by the DNA studies. In the first family, the 34 year-old proband had received a clinical diagnosis of myotonic dystrophy on the basis of electromyographical myotonia, typical multicoloured cataract, and muscle weakness (albeit in an unusual distribution for DM). This individual carries 2 normal alleles for the CTG trinucleotide repeat. Electromyographical myotonia and multicoloured lens opacitites were reported for her father, who also showed a normal number of CTG triplets on both chromosomes. Either this family shows an unusal form of the disease, not associated with expansion of the CTG repeat, or the original clinical diagnosis was incorrect. If the latter applies, the sister, who carries the same haplotype for markers flanking the DM locus (individual 8 in table 1) is probably not at risk of the disease. In the other family, several affected individuals showed a characteristic smear of expanded repeats. However, in a female in whom DM had been diagnosed based on mild muscular weakness and electromyographical myotonia, an expanded allele was not found. This puts the clinical diagnosis in doubt for this woman and indicates that her son (individual 6 in table 1) is probably not at risk of DM.

Preferential amplification of the normal allele

An expanded allele was not initially detected in 3 individuals with the primer pair (406 and 409 from Mahadevan et al.[7]) used in this study in spite of multiple (up to 5) attempts. These individuals had previously been predicted to be gene carriers by linkage analysis. Since only a single normal allele was seen on 6% poylacrylamide gel electrophoresis, we asssumed that an abnormal allele was present but undetectable. By choosing another primer combination (primer 406 from Mahadevan et al. [7] and a new primer: GGC.ACA.GAA.GCC.CGG.CCC.ACC, nt position 11-32) the presence of an expanded allele was confirmed in 2 individuals. In the third individual an expanded allele could only be demonstrated by direct restriction analysis and Southern blotting of the genomic fragment containing the CTG repeat sequence.

TABLE 1: RESULTS OF CTG ANALYSIS IN 11 ASYMPTOMATIC INDIVIDUALS CONSIDERED TO BE AT HIGH RISK OF CARRYING A DM MUTATION BY LINKED DNA MARKERS

INDIVIDUAL[1]	AGE	SEX	AFFECTED PARENT	CLINICAL EXAMINATION[2]	CTG ANALYSIS[3]	REMARKS
1	19	M	FATHER	NORMAL	EXPANDED SMEAR (>200 CTG TRIPLETS)	MENTAL RETARDATION
2	19	M	FATHER	ATYPICAL LENS CHANGES EQUIVOCAL CLINICAL MYOTONIA / NORMAL EMG	EXPANDED SMEAR (>200 CTG TRIPLETS)	
3	33	M	FATHER	NORMAL[4]	EXPANDED SMEAR (>200 CTG TRIPLETS)	
4	33	F	FATHER	NORMAL	APPROXIMATELY 74 CTG TRIPLETS	
5	24	F	MOTHER	NORMAL	APPROXIMATELY 60 CTG TRIPLETS	
6	29	M		NORMAL	NORMAL	NOT AT RISK DIAGNOSIS IN RELATIVE IN DOUBT
7	36	F	FATHER	SLIGHT PTOSIS ATYPICAL LENS CHANGES[4]	EXPANDED SMEAR (>200 CTG TRIPLETS)	
8	28	F		NORMAL	NORMAL	NOT AT RISK DIAGNOSIS IN RELATIVES IN DOUBT
9	33	M	MOTHER	NORMAL	APPROXIMATELY 61 CTG TRIPLETS	
10	38	M	MOTHER	NORMAL[5]	APPROXIMATELY 88 CTG TRIPLETS	
11	21	M	FATHER	NORMAL	EXPANDED SMEAR (>200 CTG TRIPLETS)	

1. Individuals at high risk were identified in a previous study of 139 asymptomatic offspring of DM patients [19].
2. Clinical examination included EMG, neurological examination and slitlamp examination. Aspecific signs (such as non-coloured opacities or mental retardation) were found in 3/9 individuals (33%), compared to 24/128 (19%) of those at low risk.
3. Based on results of 1% agarose gel electrophoresis and 6% polyacrylamide gel electrophoresis of PCR-amplified products.
4. Following the DNA marker analysis, repeated EMG examination was performed, and myotonia was demonstrated.
5. Following the DNA marker analysis, DM was diagnosed in a son.

DISCUSSION

We show here that asymptomatic carriers of the DM gene mutation can be diagnosed by PCR-based analysis of the CTG trinucleotide repeat. This method is especially helpful in determining the origin of the mutation when both parents are clinically unaffected and the family history is negative [27].
Among 17 cases aged 55 years and over without muscle weakness, none showed an allele exceeding 90 CTG triplets. Larger alleles may not be compatible with absence of muscular symptoms in middle and old age. However, more data is needed before predictions are possible for individual cases.
Our results confirm the paucity of new mutations in DM suggested by genealogical studies [11-15], since individuals identified as obligate gene carriers always carried an expanded allele. This is consistent with the results of studies showing linkage dysequilibrium in various populations [28-30]. In fact, the finding of linkage dysequilibrium in DM is difficult to explain, unless we assume that either the penetrance of the DM gene is virtually zero for many generations as suggested by the anticipation model, or that carriers of the DM mutation have increased fecundity, or both. The finding that all of the asymptomatic obligate carriers defined by pedigree analysis or genealogical studies showed only a small increase in CTG triplet number (52-90 CTG triplets) supports the anticipation model of DM.
In spite of anticipation, the DM mutation may occasionally remain clinically silent, even among siblings and offspring of symptomatic cases. In five of our patients, (aged 19, 19, 21, 33 and 36 respectively) careful clinical examination including EMG and slitlamp examination failed to show any definite sign of DM. This may appear surprising since each showed an expanded allele which clearly exceeded 200 CTG triplets (individuals 1, 2, 3, 7 and 11 in table 1). Follow-up studies are required to establish whether these individuals will develop muscular symptoms in later life. So far, repeated EMG studies (performed after the results from the DNA analysis were known), have demonstrated myotonic discharges in 2 of them (individuals 3 and 7, table 1). Neither clinical myotonia, nor significant muscle weakness was detected on re-examination. In the other asymptomatic cases, CTG triplet numbers of 60, 61, 74 and 88 were within the range found for obligate gene carriers without muscular symptoms after age 55.
We currently estimate the chance that a clinically normal sibling or offspring of a DM patient carries a mutated gene to be 7.8% for those aged between 20 and 39 years of age. This figure is very similar to our previous estimate in the same sample of DM families (8.3%) [19]. We have now excluded 2 individuals because of unreliable diagnosis, while we had formerly anticipated the reclassification as low-risk of 2 individuals because of genetic

recombination with the marker loci [19]. In addition, our study confirms that at the molecular level anticipation is the rule in DM, since in our sample of 9 asymptomatic offspring we always found a CTG triplet number equal to or exceeding that of the affected parent. Finally, we do not know the explanation for the family in which expansion of the CTG repeat was not detected in either clinically affected member. Such families have been noted by others [7]. It remains to be seen whether the results in these families are due to diagnostic error, or to allelic mutation. The existence of allelic mutations, not causing expansion of the CCG repeat sequence, has been suggested for the fragile X syndrome [31].

Finally, we want to stress that in individuals with only a single normal band by PCR, the DM mutation should not be considered excluded, because of the possibility of preferential amplification of the normal allele. In such instances, additional studies are required, using either alternative primer combinations, conventional Southern blotting of genomic fragments, or linked genetic markers. Only when such studies confirm the presence of normal alleles only, can the consultand be reassured that future offspring will not be at risk.

Acknowledgements:

We wish to thank Drs van de Biezenbos, van Essen, Snoek, Sterringa, Niermeijer, Busch, Höweler, De Die-Smulders and Hennekam for providing additional clinical information. We thank Marcel Nelen and Hennie Bruggenwirth for skillful technical assistance. This study was supported by the American Muscular Dystrophy Association, the Prinses Beatrixfonds, Grant 87-2694 and by Grant Ro 389/15-4 from the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Harper PS. Myotonic Dystrophy, 2nd Ed. London. WB Saunders Co 1989.
2. Harley HG, Brook JD, Rundle SA, et al. Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. Nature 1992; 355:545-6.
3. Aslanidis C, Jansen G, Amemiya C, et al. Cloning of the essential myotonic dystrophy region and mapping of the putative defect. Nature 1992;355:549-51.
4. Buxton J, Shelbourne P, Davies J, et al. Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. Nature 1992;355:547-8.
5. Brook JD, McCurrach ME, Harley HG, et al. Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell 1992;68:799 -808.

6. Fu Y-H, Pizzuti A, Fenwick R, et al. A sequence scanning method identifies an unstable triplet repeat in myotonic dystrophy. Science 1992;255:1256-8.
7. Mahadevan M, Tsilfidis C, Sabourin L, et al. Identification and characterization of an unstable trinucleotide repeat at the myotonic dystrophy locus. Science 1992;1253-5.
8. Jansen G, Mahadevan M, Amemiya C et al. Characterisation of the myotonic dystrophy (DM) region predicts multiple protein kinase isoform encoding mRNAs that differ at their 3' ends. Nature Genetics (in press).
9. Harley HG, Rundle SA, Reardon W, et al. Unstable DNA sequence in myotonic dystrophy. Lancet 1992;339:1125-28.
10. Tsilfidis C, MacKenzie AE, Mettler G, Barceló J, Korneluk RG. Correla tion between trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. Nature genetics 1992;1:192-195.
11. Höweler CJ, Busch HFM, Geraedts JPM, Niermeijer MF, Staal A. Anticipation in myotonic dystrophy: Fact or fiction. Brain 1989;112:779-97.
12. Fleischer B, Ueber myotonischer Dystrophia mit Katarakt. Albr von Graefes Arch Klin Ophthalmol 1918;96:91-133.
13. Mathieu J, De Braekeleer M, Prévost C. Genealogical reconstruction of myotonic dystrophy in the Saguenay-Lac Saint-Jean area (Quebec, Canada). Neurology 1990;40:839-842.
14. Klein D. La dystrophie myotonique (Steinert) et la myotonie congenitale (Thomsen) en Suisse: Etude clinique, génétique, et démographique. J Genet Hum 1958;7 (Suppl):1-328.
15. Lotz BP, Van der Meyden CH. Myotonic dystrophy. Part I. A genealogical study in the northern Transvaal. SA Med J 1985;67:812-814.
16. Verkerk AJMH, Pieretti M, Sutcliffe JS, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 1991; 65:905-14.
17. Fu Y-H, Kuhl DPA, Pizzuti A, et al. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 1991;67:1047-58.
18. Rousseau F, Heitz D, Biancalana V, et al. Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 1991;235:1673-81.
19. Brunner HG, Smeets HJM, Nillesen W, et al. Myotonic dystrophy. Predic tive value of normal results on clinical examination. Brain 1991; 114:2303-11.
20. Griggs RC, Wood DS, the working group on the molecular defect in myotonic dystrophy. Criteria for establishing the validity of genetic recombination in myotonic dystrophy. Neurology 1989;39:420-421.
21. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for

extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16:1215.
22. Brunner HG, Smeets H, Lambermon HMM, et al. A multipoint linkage map around the myotonic dystrophy locus on chromosome 19. Genomics 1989; 5:589-95.
23. Korneluk RG, MacKenzie AE, Nakamura L, Dube I, Jacob P, Hunter AGW. A re-ordering of human chromosome 19 long-arm DNA markers and identification of markers flanking the myotonic dystrophy locus. Genomics 1989;5:596-604.
24. Smeets HJM, Hermens R, Brunner RG, Ropers HH, Wieringa B. Identification of variable simple sequence motifs in 19q13.2-qter: markers of the myotonic dystrophy locus. Genomics 1991;9:257-63.
25. Jansen G, de Jong PJ, Amemiya C, et al. Physical and genetic characterization of the distal segment of chromosome of the myotonic dystrophy area on chromosome 19q. Genomics (in press).
26. Harley HG, Walsh KV, Rundle S, et al. Localizaton of the myotonic dystrophy locus to 19q13.2-19q13.1 and its relationship to twelve polymorphic loci on 19q. Hum Genet 1991;87:73-80.
27. Smeets HJM, Nelen MR, Los F et al. Myotonic dystrophy: Prenatal diagnosis using direct mutation analysis. the Lancet (in press).
28. MacKenzie AE, MacLeod HL, Hunter AGW, Korneluk RG. Linkage analysis of the apolipoprotein C2 gene on human chromosome 19 reveals linkage and linkage dysequilibrium in a French-Canadian population. Am J Hum Genet 1989;44:140-147.
29. Nokelainen P, Alanen-Kurki, Winqvist R, Falck B, Somer H, Leisti J, Johnson K, M-L Savontaus, Peltonen L. Linkage dysequilibrium detected between dystrophia myotonica and ApoC2 locus in the Finish population. Hum Genet 1990;85:541-545.
30. Harley HG, Brook JD, Floyd J, Rundle SA, Crow S, Walsh KV, Thibault M-C, Harper PS, Shaw DJ. Detection of linkage dysequilibrium between the myotonic dystrophy locus and a new polymorphic DNA marker. Am J Hum Genet 1991;49: 68-75.
31. Nakahori Y, Knight SLJ, Holland J, Schwartz C, Roche A, Tarleton J, Wong S, Flint TJ, Froster-Iskenius U, Bentley D, Davies KE, Hirst MC. Molecular heterogeneity of the fragile X syndrome. Nucl Acid Res 1991;19:4355-4359.

CHAPTER 8

Brunner HG, Jansen G, Nillesen W, Nelen MR, de Die CEM, Höweler CJ, van Oost BA, Wieringa B, Ropers HH, Smeets HJM.

Brief report: Reverse mutation in myotonic dystrophy.

New Engl J Med 328: 476-480, 1993

Myotonic dystrophy is a multisystem disorder that shows autosomal dominant inheritance and is characterized by muscular weakness and atrophy, clinical and electromyographical myotonia, ocular cataract and various other abnormalities, such as cardiac conduction disturbances, smooth muscle involvement, testicular atrophy in males, premature balding, increased anaesthaetic risk, and mental retardation in early onset cases[1]. It is the most common inherited muscular dystrophy in adulthood, with an incidence of approximately 1 per 7500. The clinical expression of myotonic dystrophy is variable, ranging from neonatal lethality to complete absence of symptoms. Recently, the disorder has been shown to be caused by an increased number of Cytosine-Thymidine-Guanine (CTG) trinucleotide repeats in the 3' untranslated region of a protein kinase gene located in the q13.3 band of chromosome 19[2-8]. The normal gene has between 5 and 40 CTG trinucleotide repeats, while DM alleles have from approximately 50 to several thousand CTG trinucleotide repeats. The severity of the clinical symptoms of myotonic dystrophy usually increases with transmission to subsequent generations, a phenomenon that has been termed anticipation[9]. This is paralleled by an increase in the length of the CTG repeat
sequence [2,5,7]. It has been suggested that the progressively increasing severity of myotonic dystrophy eventually leads to the extinction of the disease from a given pedigree[10].

Genetic theory assumes that equilibrium exists between the incidence of new deleterious gene mutations and their subsequent loss from the population through reduced viability and fertility of carriers of the mutated gene. No allowance is made in human genetic epidemiology for mechanisms that change the mutation itself from abnormal to normal. Reverse mutation, i.e. the spontaneous correction of a deleterious mutation and its subsequent transmission to unaffected offspring, has not been reported in humans, although it has been observed at low frequencies in bacteria and cultured mammalian cells.

We here report two families with myotonic dystrophy in which a reverse mutation has occurred. In the first family, an expanded CTG trinucleotide repeat found in a clinically affected male decreased to a normal allele of 24 CTG trinucleotide repeats in his healthy infant daughter. Similarly, in the second family the expanded allele found in the clinically affected father decreased to a normal-sized allele containing 19 CTG trinucleotide repeats in his healthy 25-year old son. The normalization of the mutated myotonic dystrophy gene in these offspring can be explained by mitotic and (possibly) meiotic instability of the expanded CTG repeat sequence.

CASE REPORTS

Family 1.

Prenatal diagnosis was requested by a 25 year old woman (Subject II-4, Family 1, Fig.1) and a 27 year old man (Subject II-3). Myotonic dystrophy had been diagnosed in the man 2 years earlier on the basis of mild muscular weakness, clinical and electrical myotonia, and a positive family history. The family was investigated with genetic markers closely linked to the myotonic dystrophy gene. All clinically affected family members carried the same haplotype for the ApoC2-VSSM and X75b-VSSM markers, which flank the myotonic dystrophy locus. The proband was heterozygous for these markers. Therefore, prenatal diagnosis was considered feasible. The first pregnancy (Subject III-1) was terminated after DNA marker analysis performed on a chorionic villus biopsy indicated that the fetus had inherited the DM mutation. Examination of fetal tissues confirmed the results. The second pregnancy (Subject III-2) was terminated when intrauterine death was diagnosed by ultrasonography, 6 days after a transcervical chorionic villus biopsy had been performed. DNA analysis indicated that this fetus would have been unaffected. In the third pregnancy, genetic marker analysis again indicated that the fetus (Subject III-3) had received the abnormal DNA marker haplotype. In view of the small genetic distances between markers in the myotonic dystrophy gene region[11-13], the chance that the fetus carried the myotonic dystrophy mutation was estimated to be greater than 99%. Mutation analysis[7] was subsequently performed to determine the size of the CTG repeat in the myotonic dystrophy gene of this fetus in order to obtain more reliable prognostic information.

Family 2.

A 23-year old man (Subject II-2, Family 2, Fig.1), and his 24-year old sister (Subject II-1) were examined for signs of myotonic dystrophy because of a history of the disease in several family members, including their father (Subject I-1). Clinical examination, including electromyography and slit lamp examination, was normal in both Subject II-1 and Subject II-2. However, analysis with genetic markers flanking the myotonic dystrophy gene locus showed that the son (Subject II-2) had inherited the paternal chromosome 19 that carries the myotonic dystrophy gene in this family. Because Subject II-2 was now considered to be a carrier of the myotonic dystrophy gene, repeated clinical examination, including electromyography of 10 muscles, was performed at age 25. This reexamination again did not reveal any signs of myotonic dystrophy. Because of the discrepancy between the clinical findings and the results of the DNA analysis, final diagnosis was deferred until direct detection of the myotonic dystrophy mutation was possible.

MATERIALS AND METHODS

Chromosomal DNA was isolated from peripheral blood cells, cultured fibroblasts or chorionic villi as described[14]. Spermatozoa were isolated from semen using single-layer Percoll centrifugation. DNA was isolated from the sperm pellet as described[15]. Genetic markers flanking the myotonic dystrophy mutation and their respective detection methods have been described previously [11-13]. All markers were tested at least twice. Using a recently described Polymerase Chain Reaction (PCR) assay[7], we tested the expansion of the CTG trinucleotide repeat in genomic DNA. The CTG trinucleotide repeat was amplified with flanking primers and the resulting DNA fragments were separated by electrophoresis on 1 percent and 4 percent agarose gels. A Southern blot, made from the 1 percent gel, was probed with a ^{32}P end-labeled $(CTG)_{10}$ oligonucleotide and the hybridizing fragments were visualized by autoradiography. Normal alleles were identified by the same PCR assay, using a ^{32}P end-labeled amplification primer. The PCR amplification product was separated by electrophoresis on a 6 percent polyacrylamide - 7 M urea sequencing gel and visualized by autoradiography. For purposes of paternity testing, several highly polymorphic loci were tested on chromosomes 3, 15, 17 and 19 [12,17-23]. Allele sizes were determined for each locus by comparison with control samples of known size, as well as by measurement on an automated sequencing system (ABI 373A), using the Gene Scanner™ software (Applied Biosystems, Foster City, Ca). An internal-lane size standard (Gene Scan- 2500 Rox) was added as a reference control for aligning peak data. The paternity index (I) and the probability of paternity (W) were calculated as described [24]. The research plan was approved by the medical ethics committee of the Nijmegen University Hospital.

RESULTS

In Family 1, genetic markers flanking the myotonic dystrophy mutation indicated that the third fetus (Subject III-3; Figure 1) had received the abnormal chromosome 19 from the father. Analysis of the causative myotonic dystrophy mutation showed an abnormal expanded allele in the father, but not in a chorionic villus sample from this fetus (Figure 1). This fetus had 2 normal alleles (Figure 2), the larger of which (24 CTG trinucleotide repeats) was apparently inherited from the father. However, this allele was clearly different from the father's normal chromosome 19, which carried an allele of 11 CTG trinucleotide repeats. This suggested that a reverse mutation had occurred through which the abnormal expanded CTG trinucleotide repeat in the father (approximately 150 to 600 CTG trinucleotide repeats; Fig. 1) had decreased to a normal-sized allele of 24 CTG trinucleotide

repeats in this fetus. After the birth of a normal girl, identical results were obtained on a sample of cord blood and on fibroblasts from the umbilical cord. Attempts to detect mosaicism for the reverted allele in other tissues from the father were unsuccessful. Additional reverted alleles were not found on analysis of DNA from either cultured skin fibroblasts or sperm from Subject II-3 (Figure 3). Nonpaternity could be excluded using several highly polymorphic systems from chromosomes 3, 15, 17 and 19 . The probability that Subject II-3 was the biological father of Subject III-3 was calculated as greater than 0.99998 (Table 1).

In Family 2, an asymptomatic 25 year old man (Subject II-2), had inherited the abnormal chromosome 19 from his father. However, expansion of the CTG trinucleotide repeat could not be demonstrated (Figure 1). Instead, 2 normal alleles were present (Figure 2), the larger of which (19 CTG repeat units) was inherited from the affected father. Therefore, the father's mutated myotonic dystrophy gene, which contained approximately 150 to 500 CTG trinucleotide repeats had changed to a normal-sized allele of 19 CTG trinucleotide repeats in the son. The reverted allele was present also in skin fibroblasts and semen from Subject II-2 (Figure 3). Abnormal expanded alleles were not present in these tissues. Thus, no evidence for either somatic or germline mosaicism was found in this subject. Nonpaternity was excluded using several highly polymorphic DNA markers from chromosome 3, 15, 17 and 19. The probability that Subject I-1 was the biological father of Subject II-2 was calculated as greater than 0.99999 (table 1).

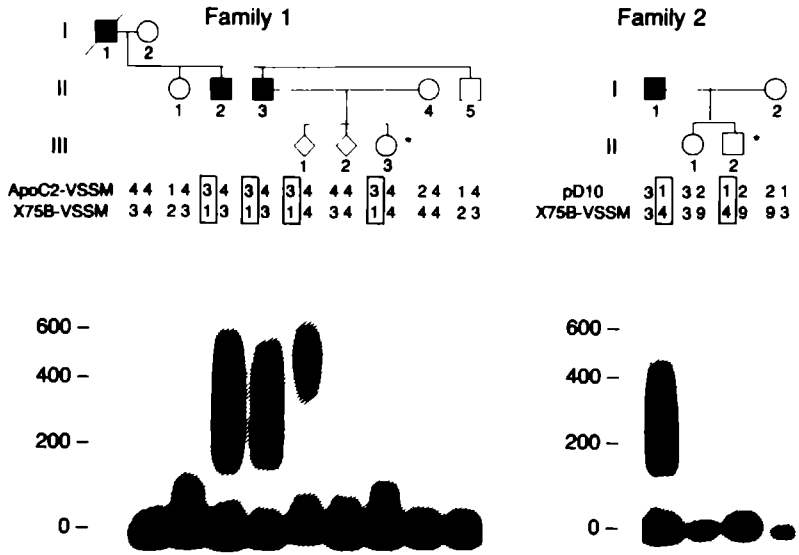


Figure 1. Linkage Analysis and Mutation Analysis in Two Families with Myotonic Dystrophy.

The squares denote male family members, and the circles female family members The slash denotes a deceased family member Prenatal diagnoses are indicated by diamonds. The shaded symbols indicate clinically affected subjects. Subjects carrying a reverted myotonic dystrophy gene are marked by an asterisk. In Family 1, all the affected subjects carry the 3 allele for the APOC2-VSSM marker and the 1 allele for the X75b-VSSM marker at locus D19S112 (boxed) Subject III-3 has also received these alleles from her father In Family 2, Subject II-2 has inherited the 1 allele for the pD10 marker at locus D19S63 and the 4 allele for the X75b-VSSM marker at locus D19S112 This haplotype (boxed) carries the myotonic dystrophy mutation in several affected family members, including the father (Subject I-1). Mutation analysis (lower panel) detected both normal alleles (with 5 to 40 CTG trinucleotide repeats) and abnormal alleles (with >50 CTG trinucleotide repeats). The size of the alleles, expressed as the number of CTG trinucleotide repeats, is shown beside the blots In Family 1, Subject III-3 does not have an expanded allele However, the marker haplotype suggests that she has inherited the myotonic dystrophy mutation from her father In Family 2, Subject I-1 has an abnormal expanded allele of 150 to 500 CTG trinucleotide repeats, whereas his son (Subject II-2) does not have an expanded allele, although this would be expected on the basis of the marker haplotype

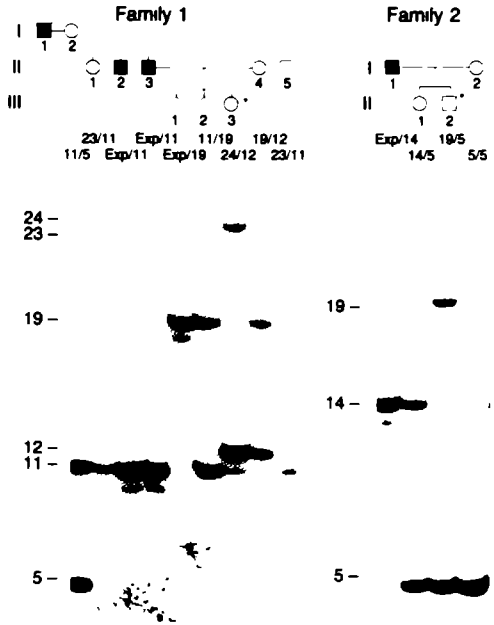


Figure 2. Precise Sizing of CTG Trinucleotide Repeats in Two Families with Myotonic Dystrophy.

Only normal alleles (with 5 to 40 CTG trinucleotide repeats) are visualized on 6 percent polyacrylamide-gel electrophoresis. The abnormal expanded (Exp) alleles fall outside the range of fragments detected in this assay. Affected subjects (Subjects II-2, II-3, and III-1 in Family 1 and Subject I-1 in Family 2) have only a single band, representing their single normal chromosome 19. Normal subjects (Subjects I-2, II-1, II-4, II-5, and III-2 in Family 1 and Subject II-1 in Family 2) have two bands; Subject I-2 in Family 2 has only a single band, since she inherited identical normal alleles of five CTG trinucleotide repeats from both her parents. In Family 1, Subject III-3 inherited a new normal allele of 24 CTG trinucleotide repeats from her affected father. In Family 2, the mutated allele has decreased in size, from approximately 150 to 500 CTG trinucleotide repeats (Fig. 1) in the affected father (Subject I-1) to 19 CTG trinucleotide repeats in his son (Subject II-2). The pedigree symbols are as described in Figure 1.

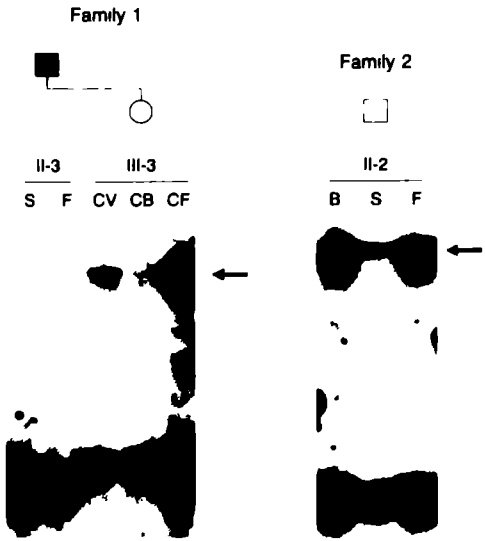


Figure 3 Tissue Testing in Two Families with Reversal of the Myotonic Dystrophy Mutation

The pedigree symbols are as described in Figure 1 The reverted (normal) allele is indicated by arrows In Family 1, the reverted allele was not found in either sperm (S) or cultured fibroblasts (F) from the father (Subject II-3) In both families the reverted allele was present in various tissues from the healthy offspring These included a chorionic-villus sample (CV), umbilical-cord blood (CB), and umbilical-cord fibroblasts (CF) from Subject III-3 in Family 1 and peripheral-blood cells (B), cultured skin fibroblasts, and sperm from Subject II-2 in Family 2

Table 1. Paternity Testing in Two Families with a Reversal of the Myotonic Dystrophy Mutation.

Locus	Reference	Family 1			Family 2		
		Nucleotides in Paternally Transmitted Allele	Allele Frequency	Paternity Index	Nucleotides in Paternally Transmitted Allele	Allele Frequency	Paternity Index
D17S513	Oliphant et al.[18]	183	0.36	1.4	193	0.33	1.5
IL2RB	Brewster et al.[16]	149	0.18	2.4	163	0.09	5.6
D3S11	Brett et al.[17]	135	0.15	3.3	135	0.15	6.7
HOX2B	Deinard et al.[19]	130	0.1	5	132 or 136	0.37 or 0.15	1.3
APOC2	Smeets et al.,[20] Fornage et al.[21]	80	0.1	5	96	0.04	12.5
D15S11	Mutirangura et al.[22]	253	0.07	7.6	243	0.5	2
GABRB3	Mutirangura et al.[23]	199	0.05	10	183	0.09	5.7
D19S112	Jansen et al.[12]	132	0.19	2.6	128	0.05	10
Probability of paternity			>0.99998			>0.99999	

DISCUSSION

Recently, a new class of genetic disease mutations has been described that is characterized by amplification of preexisting trinucleotide repeat units[25]. Apart from myotonic dystrophy[2-8], the Fragile X syndrome[26-27], and X-linked spinal and bulbar muscular atrophy[28] have been shown to be associated with this type of mutation. In both fragile X syndrome and myotonic dystrophy, the length of the respective trinucleotide repeats tends to increase in subsequent generations, and there is a positive correlation between the length of the repeat and the severity of the disease. Although transmission of the fragile X mutation is occasionally accompanied by a reduction in repeat length[27], this has never resulted in an allele of normal size. In our analysis of over 100 carriers of the myotonic dystrophy mutation, a decreasing repeat length has been found in only a single further family. Therefore, complete reversal of the mutated allele, yielding a CTG trinucleotide repeat sequence of normal size must be an exceptional event.

The mechanism causing the change from an abnormal expanded allele to a normal-sized allele is unknown. Single genetic recombination is excluded in these cases, since this would change the DNA marker haplotype surrounding the mutation. Double recombinants are also highly unlikely in view of the small genetic distances in this segment of chromosome 19. Either gene conversion (substitution of one parental allele for the other[29]) or direct deletion of the expanded repeat could explain the mutation reversal that we found in these two families.

We considered the possibility that the instability of the expanded CTG trinucleotide sequence in somatic tissues was also present in the fathers' germline, in the form of a large array of different sized alleles that included some normal-sized alleles. However, DNA analysis of cultured skin fibroblasts and of a sperm sample of the father (Subject II-3) in family 1 showed a similar (though not identical) distribution of expanded repeats as was found in his peripheral blood. Moreover, this analysis failed to detect the presence of reverted alleles (Figure 3), indicating that if germline mosaicism is present in Subject II-3, the frequency of reverted myotonic dystrophy mutations is very low. Alternatively, the reversal of the mutation in his daughter (Subject III-3) may have occurred in the early embryo.

The fact that the allele lengths in the 2 subjects reported here are entirely within the range found in normal subjects suggests that they should not develop myotonic dystrophy in the future. Thus, they represent true reverse mutations, rather than non-penetrance of an abnormal gene. Yet, it remains to be established whether their chromosomes have regained the normal stable state, since it is presently unknown whether the expanded trinucleotide repeat sequence is the only cause of the DNA instability in myotonic dystrophy.

Analysis of cultured skin fibroblasts and of a sperm sample of individual II-2 in family 2 showed 5 and 19 CTG trinucleotide repeats, identical to those detected in his peripheral blood cells (Figure 3). This suggests that the revertant allele containing 19 CTG trinucleotide repeats is stable in this subject's somatic tissues as well as in his germline.

In conclusion, the two subjects reported here are examples of complete spontaneous corrections of myotonic dystrophy mutations. These results should be taken into account when using flanking DNA markers for genetic diagnosis[30] since it is possible that other

genetic conditions in which the phenotype is highly variable will also prove to be associated with inheritance of unstable DNA sequences[31].

Acknowledgements:

We thank Dr RJ van Kooy and Dr GCML Christiaens (Department of Obstetrics and Gynecology, University Hospital, Utrecht, the Netherlands) for obtaining sperm and chorionic villus samples. Dr. We thank Dr. F. Spaans (Department of Clinical Neurophysiology, University Hospital Maastricht, the Netherlands) for performing additional neurophysiological examinations. This study was supported by the American Muscular Dystrophy Association, the Prinses Beatrixfonds, Grant 87-2694 and by the Deutsche Forschungsgemeinschaft, grant No. Ro 389/15-4.

REFERENCES

1. Harper PS. Myotonic Dystrophy, 2nd Ed. London. WB Saunders Co 1989.
2. Harley HG, Brook JD, Rundle SA, et al. Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. Nature 1992;355:545-6.
3. Aslanidis C, Jansen G, Amemiya C, et al. Cloning of the essential myotonic dystrophy region and mapping of the putative defect. Nature 1992;355:549-51.
4. Buxton J, Shelbourne P, Davies J, et al. Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. Nature 1992;355:547-8.
5. Brook JD, McCurrach ME, Harley HG, et al. Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell 1992;68:799-808.
6. Fu Y-H, Pizzuti A, Fenwick R, et al. A sequence scanning method identifies an unstable triplet repeat in myotonic dystrophy. Science 1992;255:1256-8.
7. Mahadevan M, Tsilfidis C, Sabourin L, et al. Identification and characterization of an unstable trinucleotide repeat at the myotonic dystrophy locus. Science 1992;255:1253-5.
8. Jansen G, Mahadevan M, Amemiya C et al. Characterisation of the myotonic dystrophy region predicts multiple protein isoform-encoding mRNAs. Nature

Genetics 1992;1:261-6.
9. Höweler CJ, Busch HFM, Geraedts JPM, Niermeijer MF, Staal A. Anticipation in myotonic dystrophy: Fact or fiction. Brain 1989;112:779-97.
10. Fleischer B, Ueber myotonischer Dystrophia mit Katarakt. Albr von Graefes Arch Klin Ophthalmol 1918;96:91-133.
11. Smeets HJM, Hermens R, Brunner HG, Ropers HH, Wieringa B. Identification of variable simple sequence motifs in 19q13.2-qter: markers of the myotonic dystrophy locus. Genomics 1991;9:257-63.
12. Jansen G, de Jong PJ, Amemiya C, et al. Physical and genetic characterization of the distal segment of chromosome of the myotonic dystrophy area on chromosome 19q. Genomics 1992;13:509-17.
13. Harley HG, Brook JD, Shelley JF et al. Detection of linkage disequilibrium between the myotonic dystrophy locus and a new polymorphic DNA marker. Am J Hum Genet 1991;49:68-75.
14. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16: 1215.
15. van Kooij RJ, van Oost BA. Determination of sex ratio of spermatozoa with a DNA probe and quinacrine staining: a comparison. Fertil Steril 1992; 58:384-7.
16. Brewster ES, Brennan MB, Vissing H. Dinucleotide repeat polymorphism in the IL-2Rß gene. Nucleic Acids Res 1991;19:4022.
17. Brett PM, Melmer G, Gurling HMD. A microsatellite polymorphism at the D3S11 locus. Nucleic Acids Res 1991;19:6978.
18. Oliphant AR, Wright EC, Swensen J, Gruis NA, Goldgar D, Skolnick MH. Dinucleotide repeat polymorphism at the D17S513 locus. Nucleic Acids Res 1991;19:4794.
19. Deinard AS, Ruano G, Kidd KK. A dinucleotide repeat polymorphism at the HOX2B locus. Nucleic Acids Res 1992;20:1171.
20. Smeets HJM, Brunner HG, Ropers H, Wieringa B. Use of variable simple sequence motifs as genetic markers: Application to the study of myotonic dystrophy. Hum Genet 1989;83:245-51.
21. Fornage M, Chan L, Siest G, Boerwinkle E. Allele frequency distribution of the $(TG)_n(AG)_m$ microsatellite in the apolipoprotein C-II gene. Genomics 1992;12:63-8.
22. Mutirangura A, Kuwano A, Ledbetter SA, Chinault AC, Ledbetter DH. Dinucleotide repeat polymorphism in the Angelman/Prader-Willi region (AS/PWS) of chromosome 15. Nucleic Acids Res 1992;1:139.
23. Mutirangura A, Ledbetter SA, Kuwano A, Chinault AC, Ledbetter DH. Dinucleotide repeat polymorphism at the $GABA_A$ receptor ß3 (GABRB3) locus in the Angelman/Prader-Willi region (AS/PWS) of chromosome 15. Hum Mol Genet 1992;1:67.
24. Nijenhuis LE. Paternity case analysis. In: R.H. Walker, Ed. Inclusion probabilities in parentage testing. American Association of Blood Banks, Arlington, Virginia. 1983:551-7.

25. Richards RI, Sutherland GR. Heritable unstable DNA sequences. Nature Genetics 1992;1:7-9.
26. Verkerk AJMH, Pieretti M, Sutcliffe JS, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 1991;65:905-14.
27. Rousseau F, Heitz D, Biancalana V, et al. Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 1991; 235:1673-81.
28. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fishbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 1991;352:77-9.
29. Kourilsky P. Molecular mechanisms for gene conversion in higher cells. Trends in Genet 1986;2:60-3.
30. Antonorakis SE. Diagnosis of genetic disorders at the DNA level. N Engl J Med 1989;320:153-163.
31. Sutherland GR, Haan EA, Kremer E, et al. Hereditary unstable DNA: a new explanation for some old genetic questions? Lancet 1991;338:289-92.

CHAPTER 9

Brunner HG, Brüggenwirth HT, Nillesen W, Jansen G, Hamel BCJ, Hoppe RLE, de Die CEM, Höweler CJ, van Oost BA, Wieringa B, Ropers HH, Smeets HJM

Influence of sex of the transmitting parent as well as of parental allele size on the CTG expansion in myotonic dystrophy.

Am J Hum Genet (in press)

SUMMARY

In patients with myotonic dystrophy (DM), the severity of clinical signs is correlated with the length of a $(CTG)_n$ trinucleotide repeat sequence. This sequence tends to expand in subsequent generations. In order to examine the kinetics of this process and in particular the influence of the mutant allele size and the sex of the transmitting parent, we have studied $(CTG)_n$ repeat lengths in the offspring of 38 healthy carriers with small mutations (<100 CTG trinucleotides, mean length $[CTG]_{67}$). In these studies, we found a weakly positive correlation between the size of the mutation in the carrier parents and their offspring. Furthermore, we observed that in the offspring of male transmitters, repeat lengths exceeding 100 CTG trinucleotides were much more frequent than in the offspring of carrier females (48 out of 52 [92%] vs. 7 out of 16 [44%], p=0.0002). Similarly, in genealogical studies performed in 38 Dutch DM kindreds, an excess of non-manifesting male transmitters was noted, which was most conspicuous in the last generation preceding phenotypic expression of DM. Thus, two separate lines of evidence suggest that the sex of the transmitting parent is an important factor determining DM allele sizes in the offspring. On the basis of our data we estimate that when both parents are asymptomatic, the odds are approximately 2:1 that the father carries the DM mutation. Because expansion of the CTG repeat is more rapid with male transmission, negative selection during spermatogenesis may be required to explain the exclusive maternal inheritance of severe congenital-onset DM.

INTRODUCTION

The mutation underlying myotonic dystrophy (DM) has recently been characterized as an expanding CTG trinucleotide repeat sequence in the 3' untranslated region of a protein kinase gene (Harley et al. 1992a; Buxton et al. 1992; Aslanidis et al. 1992; Mahadevan et al. 1992; Brook et al. 1992; Fu et al. 1992; Jansen et al. 1992). Repeats containing over 40 CTG units tend to expand further in successive generations and both age at onset and the clinical phenotype correlate with the size of the mutation (Harley et al. 1992b, Hunter et al. 1992). These findings have provided a biological basis for the progressively earlier onset of DM in consecutive generations, i.e. anticipation (Fleischer, 1918; Ravin and Waring, 1939; Bell, 1947; Klein, 1958; Höweler et al. 1989). However, the factors governing the transition from minimally expanded CTG trinucleotide repeats found in subjects who are asymptomatic or have cataract as their only feature, to larger mutations associated with muscle weakness and clinical myotonia have not yet been elucidated. We performed DNA analysis in DM families to evaluate whether the size of the CTG trinucleotide repeat sequence in the parent might influence the size of the mutation in the offspring. A parental size effect had already been documented by several groups for the CCG trinucleotide expansion associated with the fragile X syndrome (Fu et al. 1991, Yu et al. 1992, Heitz et al. 1992). Because there appears to be an excess of mildly affected or asymptomatic male transmitters in DM families (Bell, 1948; Klein, 1958; Harper, 1989; Brunner et al., 1991), we performed genealogical studies in 38 DM families and analysed previous reports of such studies to examine whether this male excess is specifically associated with the first appearance of the clinical phenotype in the family. We also studied the molecular basis for this phenomenon by analysing the influence of the sex of the transmitting parent on the size of the DM mutation in their offspring.

MATERIALS AND METHODS

Genealogical studies:
Over a 5-year period (1987 through 1991), all patients with myotonic dystrophy (DM) seen at the Nijmegen clinical genetics unit were asked to complete a pedigree form that included names, dates and places of birth and of marriage of their parents, grandparents and great-grandparents. Pedigrees were constructed which included all affected family members known to the proband. Whenever possible, this information was confirmed by reviewing the medical records or by clinical examination of family members. Affected individuals were considered to belong to a single pedigree if they were linked together through no more than 3 apparently unaffected individuals.

For technical reasons, we limited our studies to pedigrees which originated wholly or partly from the Province of North-Brabant. There has been no major emigration from, or immigration into, this area over the last 200 years. Its population (approximately 300.000 in 1800 and 2 million in 1991) shows little evidence of inbreeding, similar to the Dutch population as a whole (van Straaten, 1986). A total of 38 pedigrees were studied. For each particular pedigree, genealogical studies started at the common ancestral pair and were extended to approximately 1780 (6-8 generations). Sex and age at death were recorded for the obligate carriers that were thus identified.

Obligate gene carriers were considered asymptomatic if no muscle weakness or cataract had been noted until at least age 50. In the normal population between age 45 and 55 years, approximately 12% of individuals have lens opacities, and approximately 2½% have lens opacities causing visual loss (Leske and Sperdato, 1983). Therefore, subjects who developed cataract as the only symptom after the age of 50 years were also classified as asymptomatic. Generally, medical records or reliable clinical information or both were available for 2 to 3 generations preceding the appearance of clinical DM in a family. However, for individuals born prior to 1880 documentation was generally lacking.

In addition, data from 4 published clinical and genealogical studies of DM were included in our analysis (Fleischer, 1918; Klein, 1958; Höweler, 1986; Mathieu et al., 1990). For the study of the sex ratio, we excluded families that showed multiple alternative links.

Statistical significance was tested using either the normal approximation to the binomial distribution (two-tailed) or the X^2-test where appropriate.

DNA studies:

Families were ascertained through the DNA diagnostic service of our department. Care was taken to ensure that all families met previously defined diagnostic criteria [Griggs et al. 1988]. Chromosomal DNA was isolated from peripheral blood (Miller et al. 1988). Using a recently described PCR assay (Mahadevan et al. 1992) we determined the length of the CTG repeat in genomic DNA. The CTG repeat was amplified with flanking primers 406 and 409 (Mahadevan et al. 1992). Alleles containing up to approximately 90 CTG triplets were resolved on a 6% polyacrylamide / 7 M Urea sequencing gel after ^{32}P end-labeling one of the amplification primers (Brunner et al. 1992). Expanded alleles appeared on the polyacrylamide gel as a mosaic pattern of DNA fragments differing by one or a few trinucleotide repeats. The size of the most prominent band was estimated by comparison with a known sequencing ladder. Accurate sizing was also possible using fluorescently labeled PCR primers and subsequent analysis on an automated sequencer (ABI, 373), using Genescanner software and internal lane stand-

ards (Genescan 2500 ^{TR}ROX). Size estimates for larger alleles were obtained from analysis of PCR products on 1% and 2% agarose gels blotted onto Gene Screen Plus filters and hybridized with a ^{32}P end-labeled $(CTG)_{10}$ oligonucleotide. In general, the PCR products produced a smear and the size of the small end of the smear was determined by comparison with a mixture of lambda x HindIII and Phi X 174 x Hae III digestions as a marker. Finally, unsizable, large smears, partly resulting from heteroduplex formation in the PCR products, were checked by conventional Southern blot analysis of Hind III digested genomic DNA, using probe pGB2.6 (Mahadevan et al. 1992). The same size marker was used.
For statistical evaluation, Fischer's exact test, the X^2-test, Spearman correlation coefficient, Student's t-test and Wilcoxon's rank sum test were used where appropriate.

RESULTS

Genealogical studies:
We found that 22 out of 38 DM pedigrees (56%) were linked to at least one other DM pedigree. In 8 instances, 2 families were linked together. in 2 instances, 3 families were linked by the genealogical studies. Genealogical links involved between 4 and 10 apparently unaffected individuals (average 6.6 individuals). Genealogical linkage of 3 independently ascertained pedigrees is shown in figure 1. Analysis of our data indicated that genealogical links involve males more frequently than females (62 males vs. 38 females; $p=0.02$). Analysis of published DM pedigrees (Fleischer, 1918; Klein, 1958; Höweler, 1986; Mathieu et al., 1990) shows an excess of male transmission in each study. For all studies combined, genealogical links involved 387 males and 267 females ($p<0.001$). As shown in table 1, this difference is caused by a disproportionate excess of males in the last asymptomatic generation. Comparison of the sex ratio between individuals in the last asymptomatic generation and those in all preceding generations shows a highly significant difference (177/90 vs. 210/177, respectively; $p<0.005$). This was still true when a large French Canadian pedigree study (Mathieu et al., 1990) was excluded ($p<0.01$). This study classified individuals only as affected or unaffected without further details and identified a common ancestral pair for 746 DM patients from a highly inbred area. Inbreeding increases the chance of finding spurious genealogical links, although this is unlikely to be relevant in this case of extensive and detailed genealogical studies of a very large number of patients.

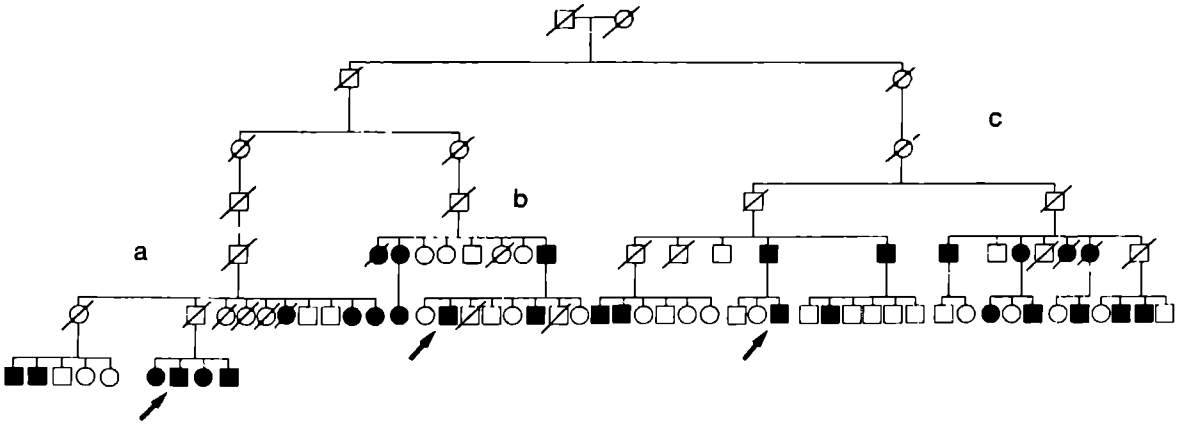


Figure 1:
Genealogical connections between 3 independently ascertained DM kindreds. Only DM patients and their siblings and offspring are shown. Probands are indicated. Shaded symbols denote clinically affected individuals. Clinically unaffected individuals were counted as belonging to the last asymptomatic generation if they had a clinically affected offspring. All other unaffected individuals that linked together different pedigrees were counted only once as belonging to the previous generations.

PATERNAL/MATERNAL TRANSMISSION OF THE DM GENE

	Fleischer	Klein	Höweler	Mathieu et al	This study	Total	p-Value*
	1918	1958	1986	1990			
Last asymptomatic generation	7/1	25/ 6	15/5	99/ 65	31/13	177/ 90	$p<0.001$
Previous Generations	6/8	31/16	7/4	135/124	31/25	210/177	$p=0.103$
Total	13/9	56/22	22/9	234/189	62/38	387/267	$p<0.001$

TABLE 1: *Comparison of Paternal versus Maternal Transmission of the DM Gene in 5 Studies.*

* p-Values were calculated for the ratio of paternal to maternal transmission compared to the expected 50% segregation by using the normal approximation to the binomial distribution (two-tailed).

The last asymptomatic generation shows an excess of male transmission compared to previous generations ($X^2=9.45$; $p<0.005$)

DNA studies:

We studied the offspring of 38 subjects (28 males and 10 females) with a CTG repeat size of less than 100 (range 41-95, mean 67 CTG trinucleotide repeats) and their offspring. Carriers of this type of mutation usually have cataract as their only feature, or no symptoms at all (Brunner et al. 1992, Reardon et al. 1992, and H.T.Brüggenwirth, unpublished results). These 38 subjects (only 3 of whom were included in the genealogical studies) had transmitted the DM mutation to a total of 68 offspring. In all but 2, the CTG repeat had increased in size upon transmission, confirming the general tendency for anticipation in this disorder (Fleischer, 1918; Höweler et al. 1989; Ashizawa et al. 1992). As shown in table 2, in 55/68 offspring (81%) the DM mutation had undergone expansion to at least 100 CTG trinucleotides. As expected, the size of the mutation in offspring correlated with the size of the parental mutation when all data were analysed together ($r=0.278$, $P=0.02$ by Spearman's rank correlation). However, results were not significant when paternal and maternal inheritance were considered separately (father-child, $r=0.129$, $p=0.36$; mother-child, $r=0.626$, $p=0.094$).

Apart from this weak effect of parental allele size, the sex of the parent was more important in determining the size of the mutated allele in the offspring. In the complete sample, a large mutation in the offspring.

To rule out the possibility that genetic factors other than the sex of the transmitting parent were responsilble for similar allele sizes in siblings, we repeated these calculations, this time restricting our analysis to the transmitting parents and the oldest offspring carrying the mutation. In this analysis, 23/28 offspring of males and 2/10 offspring of females had inherited a mutation ≥ 100 CTG trinucleotides ($P=0.0017$, Fischer's exact test). Given the effect of parental allele size on the size of the mutation in their offspring, we next examined whether the apparent effect of parental sex was caused by a difference in allele size between fathers and mothers in our study. However, the number of CTG trinucleotides was not different between male and female transmitters (66.9 ± 10.2 vs. 66.9 ± 8.8, not significant). Moreover, when we restricted our analysis to parental alleles of 60-79 CTG trinucleotides, there was again a significant difference between paternal and maternal transmission (41/42 vs. 3/11 offspring large mutation, $p<0.0001$ by Fischer's exact test, table 2). A significant difference between paternally and maternally inherited mutations was also found when we used a threshold of 200 or 300 CTG trinucleotide repeats to define large expansions

(table 2). When the absolute size of the repeat sequence was compared, paternally inherited DM mutations were found to be larger than maternally inherited DM mutations (310 $\pm$ 29.9 vs. 105 $\pm$ 40.7 CTG trinucleotide repeats, $p=0.0008$ by Student's T-test, 2-sided). While the sex of the parent appeared to influence the CTG expansion, no difference in CTG repeat size was noted when male and female sex of the offspring were compared ($X^2= 0.14$, not significant).

CTG repeat of parent	CTG repeat of offspring	Sex of transmitting parent Male	Female	p-Value*
40 - 59	≥100	6/9	1/2	n.s.
	≥200	5/9	0/2	n.s.
	≥300	1/9	0/2	n.s.
60 - 79	≥100	41/42	3/11	<0.0001
	≥200	36/42	2/11	0.0001
	≥300	26/42	2/11	0.019
80 - 99	≥100	1/1	3/3	n.s.
	≥200	1/1	1/3	n.s.
	≥300	1/1	1/3	n.s.
TOTAL	≥100	48/52	7/16	0.0002
	≥200	42/52	3/16	<0.0001
	≥300	28/52	3/16	0.026

TABLE 2: *Proportion of offspring with a DM mutation exceeding 100, 200, and 300 CTG trinucleotide repeats.*

* p-Values for male versus female transmission were calculated by Fischer's exact test (2-tailed).

DISCUSSION

We have performed genealogical studies as well as DNA studies in an attempt to elucidate some of the factors that are associated with the transition of small CTG expansions at the myotonic dystrophy (DM) locus (with limited phenotypic effect other than ocular cataract) to larger CTG expansions that cause more severe disease.

First, we confirmed previous reports (Lavedan et al., 1993; Harley et al., 1993; Redman et al., 1993) that the size of the parental allele correlates with the size of the DM mutation in the offspring.

Second, our study shows that the onset of clinical expression in DM families is preferentially associated with paternal transmission of the DM mutation. In the genealogical studies, an excess of males was found among subjects that linked independently ascertained DM kindreds. Male transmission was especially increased in the last generation preceding onset of clinical symptoms (177 of 267 transmitters male, $p < 0.005$ for last asymptomatic generation versus all previous generations combined, table 1). This apparent effect of the sex of the transmitting parent was also observed in the DNA studies. A marked difference in CTG trinuclotide repeat size in offspring was found when we compared the proportion of mutations exceeding an arbitrary threshold of 100 CTG trinucleotide repeats in offspring of male and female mutation carriers (table 2). The differential influence of paternal and maternal transmission persisted when we restricted our analysis to the firstborn affected offspring, or to offspring of carriers of mutations between 60 and 79 CTG trinucleotide repeats. The same conclusions were reached when the threshold separating small and large mutations was set at of 200 or 300 CTG trinucleotide repeats. We conclude that, although an increase in CTG trinucleotide repeat size is the rule for both sexes, the degree of this expansion is clearly greater for paternally transmitted mutations of this size range.

Our data do not allow to estimate in absolute figures the risk that a small mutation will evolve into a large mutation. However, both the results of the genealogical studies as well as the DNA studies suggest that expansion from a DM mutation < 100 CTG trinucleotide repeats, causing only cataract and no muscular disease (Brunner et al. 1992, Reardon et al. 1992; H.T.Brüggenwirth, unpublished data) to a mutation ≥ 100 CTG trinucleotide repeats, usually causing muscular symptoms, is approximately 2 times as frequent with paternal as with maternal transmission.

Although we have taken great care to exclude measurement errors, the somatic heterogeneity of the DM mutation precludes complete accuracy of allele size measurements. Nevertheless, a significant difference for maternally and paternally derived mutations was evident in each of our analyses. For this reason, the difference in CTG repeat size in paternally

versus maternally transmitted minimal DM mutations most likely constitutes a true biological phenomenon.

Our finding that the increase in CTG repeat size is larger with paternal than with maternal transmission of minimal DM mutations explains previous observations of an excess of males in the earliest generations of a clinically ascertained DM pedigree (Bell, 1947; Klein, 1958; Harper, 1989; Brunner et al. 1991; Harley et al. 1993). In our total DM family sample of more than 200 families, when both parents of a DM patient are clinically normal, the odds are approximately 2:1 that the father is the transmitting parent (unpublished data).

If alleles <100 CTG trinucleotides enlarge to a greater extent on paternal than on maternal transmission, then only the last asymptomatic generation should show an exces of male transmissions, and the previous generations should show a small excess of female transmissions. As shown in table 1, a small (but not significant) excess of male transmissions was found instead in the previous generations. This discrepancy may reflect biases that are inherent to these genealogical studies. Most importantly, if indeed paternal transmission of small alleles is associated with greater instability, then the number of transmissions will be larger if many transmissions are through females. Such long genealogical chains would obviously have a smaller chance of being detected in these studies. Also, our own study, and those of Höweler (1986) and of Mathieu et al. (1990) included all ancestors of a patient. In contrast, Fleischer (1918) and Klein (1958) most likely extended their genealogical studies preferentially along the male line once the same surname had been detected in two families. This may account for the relatively large excess of male transmission in previous generations found by Klein (1958). However, we emphasize that this male bias should be much stronger for the previous generations than for the last asymptomatic generation. Consequently, this bias cannot explain (and indeed is at variance with) our finding of a significant excess of male transmission in the last asymptomatic generation versus previous generations.

Two studies have concluded that female transmission results in greater enlargements, even for small alleles (Lavedan et al. 1993; Redman et al. 1993). However, in these studies the data were not analysed separately for parental alleles of less than 100 CTG trinucleotides (0.3kb). Therefore it cannot be stated that the greater instability on paternal transmission noted by us for these small alleles was or was not present in their data. Moreover, close inspection of the data presented in these papers suggests that the proportion of parental alleles of <0.3kb (100 CTG trinucleotides) that enlarged by less than 0.5 kb in the offspring may in fact be larger for maternal transmission in both studies. Another study that reported that increases in size of the CTG trinucleotide repeat are not different between

male and female carriers of a DM mutation summarized data on the transmission of mutations of all sizes (Tsilfidis et al. 1992).
Harley et al. (1993) have recently suggested that the excess of minimally affected males might be due to a greater tendency to initial instability with male transmission. This suggestion is supported by our findings. However, data supporting that hypothesis were not presented, and the authors concluded that increase in repeat size is similar for both paternal and maternal transmissions when the increase is expressed as a proportion of the parental repeat size (Harley et al. 1993). The data in that paper are presented in a way that does not allow direct comparison between maternal and paternal alleles between 60-79 CTG trinucleotides; i.e. the group that showed the largest difference in our study.
We would like to suggest that the conflict between the conclusions in our study and those of others may be more apparent than real, and may reflect the fact that the other studies did not specifically compare maternal and paternal inheritance of alleles less than 100 CTG trinucleotides that were matched for size.
We postulate that the situation could be different for these categories because the transmission of larger paternal mutations may be under negative selection during spermatogenesis. It is obvious that there is selection against transmission of very large mutations by males, since males with onset of muscular weakness in early adulthood are usually infertile or celibate or both (Klein, 1958; Höweler et al. 1989). In addition, we believe that negative selection could also operate at the level of individual sperm cells, thereby effectively limiting the range of
mutated alleles to those with relatively smaller expansions. Indirect support for this hypothesis comes from studies documenting a decrease in CTG trinucleotide number from parent to child. In the large majority of cases of decreasing CTG repeat length the transmitting parent has been the father (Ashizawa et al. 1992; Hunter et al. 1993; Shelbourne et al. 1992; O'Hoy etal. 1993; Brunner et al. 1993; Lavedan et al. 1993; Harley et al. 1993; Redman et al. 1993; Abeliovich et al. 1993). It is tempting to speculate that a combination of male infertility and selection against individual sperm bearing large expansions at the DM locus could be involved in the exclusively maternal origin of the severe congenital form of DM. In this regard, it is of interest that in the segregation distorter (SD) system in Drosophila allele-specific male gamete dysfunction is dependent on an expanded repeat sequence (Lyttle, 1993).
Most of the dynamics of the CTG trinucleotide repeat expansion in DM are presently unknown. It is possible that the increased instability associated with male transmission noted in our study simply reflects the larger number of cell divisions in spermatogenesis than in oogenesis. On the other hand, it is

equally possible that expansion of the DM mutation is predominantly a postzygotic event. This would then suggest the possibility that the differential influence of maternal and paternal transmission on trinucleotide repeat length is influenced by genetic imprinting (Reik, 1988). In both humans and mouse, the DM-kinase gene is expressed from both chromosomes (Jansen et al. in press). This finding does not, however, exclude another type of imprinting characterized by differential mutability.

Finally it should be noted that in Fragile X syndrome the risk of transition from a small parental mutation ("premutation") to a large mutation in offspring also depends on the size of the parental trinucleotide repeat but that a large increase in allele size is virtually restricted to maternally inherited mutation (Fu et al. 1991, Yu et al. 1992, Heitz et al. 1992), again suggesting negative selection during spermatogenesis, genetic imprinting or both. Studies of trinucleotide repeat length in sperm from DM and fragile X patients should be of great value for further elucidation of the dynamics of these unstable mutations.

Acknowledgements

The authors are grateful to Nellie Peer, Anja Koster and Anke Otten, Department of Medical Statistics, University Hospital, Nijmegen, the Netherlands, for performing statistical calculations. Dr. R.C.M. Hennekam, Clincal Genetics Centre Utrecht and the Dutch Muscular Dystrophy Society (VSN) are gratefully acknowledged for their help with the initiation of the genealogical studies. Supported in part by the Deutsche Forschungsgemeinschaft.

REFERENCES

Abeliovich D, Lere I, Pahut-Lavon I, Shmueli E, Raas-Rothschild A, Frydman M (1993) Negative expansion of the myotonic dystrophy unstable sequence. Am J Hum Genet 52:1175-1181

Aslanidis C, Jansen G, Amemiya C, Shutler G, Mahadevan M, Tsilfidis C, Chen C et al. (1992) Cloning of the essential myotonic dystrophy region and mapping of the putative defect. Nature 355:549-51.

Ashizawa T, Dubel JR, Dunne PW, Dunne CJ, Pizzuti A, Caskey CT, Boerwinkle E et al. (1992a) Anticipation in myotonic dystrophy. II. Complex relationships between clinical findings and structure of the CGT repeat. Neurology 42:1877-1883.

Ashizawa T, Dunne CJ, Dubel JR, Perryman MB, Epstein HF, Boerwinkle E, Hejtmancik JF. (1992b) Anticipation in myotonic dystrophy. I. Statistical verification based on clinical and haplotype findings. Neurology 42:1871--1877.

Bell J (1947) Dystrophia myotonica and allied diseases. The Treasury of Human

Inheritance 4:342-410
Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H, Hunter K et al. (1992) Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell 68:799-808
Brunner HG, Smeets HJM, Nillesen W, Van Oost BA, Van Den Biezenbos JBM, Joosten EMG, Pinckers AJLG et al. (1991) Myotonic dystrophy. Predictive value of normal results on clinical examination. Brain 114:2303-11
Brunner HG, Nillesen W, Van Oost BA, Jansen G, Wieringa B, Ropers HH, Smeets HJM. (1992) Presymptomatic diagnosis of myotonic dystrophy. J Med Genet 29:780-784.
Brunner HG, Jansen G, Nillesen W, Nelen MR, de Die CEM, Höweler CJ, Van Oost BA et al. (1993) Reverse mutation in myotonic dystrophy. New Engl J Med 328:476-480
Buxton J, Shelbourne P, Davies J, Jones C, Van Tongeren T, Aslanidis C, de Jong P et al. (1992) Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. Nature 355:547-8.
Fleischer B (1918) Ueber myotonischer Dystrophia mit Katarakt. Albr von Graefes Arch Klin Ophthalmol 96:91-133
Fu Y-H, Kuhl DPA, Pizzutti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJMH et al. (1991) Variation of the CGG repeat at the Fragile X site results in genetic instability: Resolution of the Sherman paradox. Cell 67:1047-1058
Fu Y-H, Pizzuti A, Fenwick R, King J, Rajnarayan S, Dunne PW, Dubel J et al. (1992) A sequence scanning method identifies an unstable triplet repeat in myotonic dystrophy. Science 255:1256-8
Griggs RC, Wood DS, the working group on the molecular defect in myotonic dystrophy (1989) Criteria for establishing the validity of genetic recombination in myotonic dystrophy. Neurology 39:420-421
Harley HG, Brook JD, Floyd J, Rundle SA, Crow S, Walsh KV, Thibault M-C, Harper PS, Shaw DJ (1991) Detection of linkage disequilibrium between the myotonic dystrophy locus and a new polymorphic DNA marker. Am J Hum Genet 49:68-75
Harley HG, Brook JD, Rundle SA, Crow S, Reardon W, Buckler AJ, Harper PS et al. (1992a) Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. Nature 355:545-6
Harley HG, Rundle SA, Reardon W, Myring J, Crow S, Brook JD, Harper PS et al. (1992b) Unstable DNA sequence in myotonic dystrophy. Lancet 339: 1125-28
Harley HG, Rundle SA, MacMillan JC, Myring J, Brook JD, Crow S, et al. (1993) Size of the unstable CTG repeat sequence in relation to phenotype and parental transmission in myotonic dystrophy. Am J Hum Genet 52:1164 -1174.
Harper PS (1989) Myotonic Dystrophy, 2nd Ed. London. WB Saunders Co
Heitz D, Devys D, Imbert G, Kretz C, Mandel J-L (1992) Inheritance of the fragile X syndrome: size of the fragile X premutation is a major determinant

of the transition to full mutation. J Med Genet 29:794-801
Höweler CJ (1986) A clinical and genetic study in myotonic dystrophy. Thesis, Erasmus University, Rotterdam
Höweler CJ, Busch HFM, Geraedts JPM, Niermeijer MF, Staal A (1989) Anticipation in myotonic dystrophy: Fact or fiction. Brain 112:779-97
Hunter A, Tsilfdis C, Mettler G, Jacob P, Mahadevan M, Surh L, Korneluk R (1992) The correlation of age at onset with CTG trinucleotide repeat amplification in myotonic dystrophy. J Med Genet 29:774-779
Hunter A, Jacob P, O'Hoy K, MacDonald I, Mettler G, Tsilfidis C, Korneluk RG (1993) Decrease in the size of the myotonic dystrophy CTG repeat during transmission from parent to child: Implications for genetic counselling and anticipation. Am J Med Genet 45:401-407
Jansen G, Mahadevan M, Amemiya C, Wormskamp N, Segers B, Hendriks W, O'Hoy K et al. (1992) Characterisation of the myotonic dystrophy (DM) region predicts multiple protein kinase isoform encoding mRNAs that differ at their 3' ends. Nature Genetics 1:261-266
Jansen G, Bartolomei M, Kalscheuer V, Merkx G, Wormskamp N, Mariman E et al. (1993) No imprinting involved in the expression of the DM-kinase mRNAs in mouse and human tissues. Hum Mol Genet (in press)
Klein D (1958) La dystrophie myotonique (Steinert) et la myotonie congenitale (Thomsen) en Suisse: Etude clinique, génétique, et démographique. J Genet Hum 7 (Suppl):1-328
Lavedan C, Hofmann-Radvanyi H, Shelbourne P, Rabes J-P, Duros C, Savoy et al. (1993) Myotonic dystrophy: Size- and sex-dependent dynamics of CTG meiotic instability, and somatic mosaicism. Am J Hum Genet 52:875-883
Leske MC, Sperduto RD (1983) The epidemiology of senile cataracts: a review. Am J Epidemiol 118:152-165
Lyttle TW (1993) Cheaters sometimes prosper: Distortion of mendelian segregation by meiotic drive. Trends Genet 9:205-210
Mahadevan M, Tsilfidis C, Sabourin L, Shutler G, Amemiya C, Jansen G, Neville C et al (1992) Identification and characterization of an unstable trinucleotide repeat at the myotonic dystrophy locus. Science 1253-5
Mathieu J, De Braekeleer M, Prévost C (1990) Genealogical reconstruction of myotonic dystrophy in the Saguenay-Lac Saint-Jean area (Quebec, Canada). Neurology 40:839-842
Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215
O'Hoy K, Tsilfidis C, Mahadevan M, Neville CE, Barceló J, Hunter AGW, Korneluk RG (1993) Reduction in size of the myotonic dystrophy trinucleotide repeat mutation during transmission. Science 259:809-812
Ravin A, Waring JJ (1939) Studies in dystrophia myotonica. 1. Hereditary aspects. Am J Med Sci 197:593-609
Reardon W, Harley HG, Brook JD, Rundle SA, Crow S, Harper PS, Shaw DJ (1992) Minimal expression of myotonic dystrophy: A clinical and molecular analysis. J Med Genet 29:770-773

Redman JB, Fenwick RG, Fu Y-H, Pizzutti A, Caskey CT (1993) Relationship between parental trinucleotide GCT repeat length and severity of myotonic dystrophy in offspring. J Am Med Assoc 269:1960-1965

Reik W (1989) Genomic imprinting and genetic disorders in man. Trends Genet 5:331-336

Shelbourne P, Wingqvist R, Kunert E, Davies J, Leisti J, Thiele H, Bachmann H et al. (1992) Unstable DNA may be responsible for the incomplete penetrance of the myotonic dystrophy phenotype. Hum Mol Genet 1:467-473

Tsilfidis C, MacKenzie AE, Mettler G, Barceló J, Korneluk RG (1992) Correlation between trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. Nature Genetics 1:192-195

Van Straaten A (1986) Het verloop van het aantal consanguïene huwelijken in Nederland (1906-1982). Ned Tijdschr Geneesk 130:1984-1986

Yu S, Mulley J, Loesch D, Turner G, Donnelly A, Gedeon A, Hillen D et al. (1992) Fragile-X syndrome: Unique genetics of the heritable unstable element. Am J Hum Genet 50:968-980

CHAPTER 10

Brunner HG, Hamel BCJ, Rieu P, Höweler CJ, Peters FTM

Intestinal pseudo-obstruction in myotonic dystrophy.

J Med Genet 29: 791-793, 1992

SUMMARY

We describe 4 myotonic dystrophy (DM) patients who developed recurrent intestinal pseudo-obstruction. Some episodes were associated with gastroenteritis, while abdominal crowding may have occurred in 1 case during the 3rd trimester of pregnancy. In most instances, however, no apparent cause could be identified. Intestinal pseudo-obstruction may occur at any stage of DM. In 1 of our cases intestinal pseudo-obstruction preceded significant muscle weakness by 15 years. Intestinal pseudo-obstruction is usually treated effectively with conservative measures. These include restriction of oral intake, intravenous fluids and multiple enemas or colonoscopy. Improved intestinal function was noted in 1 case treated with the prokinetic agent cisapride. A partial sigmoid resection was performed in 3 cases with dolichomegacolon. No abnormalities were reported on histological examination. Since intestinal pseudo-obstruction is a rare complication of DM, it is of interest that 2 of our cases are siblings. Review of the literature reveals several reports of familial occurrence of specific complications. These include cardiac conduction disturbances, focal myocarditis, mitral valve prolapse, pilomatrixomas, polyneuropathy, normal pressure hydrocephalus and dilatation of the urinary tract. Myotonic dystrophy may show a tendency to familial clustering of organ-specific involvement.

INTRODUCTION

In myotonic dystrophy (DM), smooth muscle involvement can be demonstrated in most patients. Swallowing difficulties are a major concern and may lead to aspiration [1]. Symptomatic disturbance of colonic motility may occur with colicky abdominal pain similar to spastic colon. X-ray studies may be normal, or show frank megacolon. Occasional patients develop a severe disturbance of intestinal motility such as volvulus or intestinal pseudo-obstruction [2-6]. We here report on 4 DM patients who developed this complication.

CASE REPORTS

Case 1:

This man was born in 1941. He first noted myotonia at age 15. He was a conscript in the army at age 18, when he developped acute lower abdominal pain with abdominal distension. A temporary colostomy was performed. During the following year he experienced several episodes of abdominal

pain. X-ray studies showed a megasigmoid. On laparotomy, about 30 cm of sigmoid colon was resected. Microscopic examination showed normal ganglion cells without other abnormalities. At age 34 he presented with distal muscle weakness. Percussion myotonia of the thenar muscles and of the tongue was noted. EMG showed electrical myotonia and mild conduction delay. A diagnosis of myotonic dystrophy was made. At age 42, he developed fever, abdominal distension and pain. Plain abdominal X-ray showed marked dilatation of many small bowel loops. The patient was admitted to the hospital. The erythrocyte sedimentation rate increased from 12 to 42 mm.
Cultures of peripheral blood and of faeces were negative. The patient improved with conservative measures. At age 43 he was again admitted with vomiting, abdominal distension and bowel dilatation. The clinical picture again resolved with conservative measures. A contrast barium enema showed loss of haustra throughout the colon. Several subsequent episodes of constipation and crampy abdominal pain have led to 2 more admissions to the hospital. No further abdominal surgery has been necessary.

Case 2:
This woman was born in 1947. She is the sister of case 1. Her first pregnancy was complicated by polyhydramnios. A son was born, who died of respiratory insufficiency. A cardiac defect was suspected, but no abnormality was identified at autopsy. The patient became pregnant again, and delivered a normal daughter. At age 28, myotonic dystrophy was diagnosed during family studies. During her 3rd pregnancy at age 29, she developed acute ileus at 28 weeks, believed due to pressure of the uterus against the rectosigmoid. A temporary colostomy was performed. She delivered a healthy girl on the same evening. The child weighed 1880 grams and subsequently did well. The colostomy was closed 2 weeks after delivery. At age 30 years, she was admitted with crampy abdominal pain, constipation and vomiting, which resolved spontaneously. X-ray studies showed megacolon. A rectal biopsy showed no evidence of Hirschsprung's disease. Two years later, she was readmitted with ileus thought due to a volvulus of the sigmoid. A laparotomy was performed during which the obstruction was relieved. X-ray studies 2 months after operation showed an elongated sigmoid. A sigmoid resection was performed. The resected segment showed a normal anatomy, with ganglion cells in both Auerbach's and Meissner's plexuses. At age 37, the patient was again admitted because of subileus. Plain abdominal X-ray showed dilataton of multiple small bowel loops with air-fluid levels. She was treated with intravenous fluids and multiple enema's. At age 39, an episode of gastro-enteritis necessitated another admission for intestinal pseudo-obstruction. At age 41 years, she experienced her 6th episode of

intestinal pseudo-obstruction, which again resolved with conservative measures.

Case 3:
This man was born in 1963. At age 14, myotonic dystrophy was diagnosed on the basis of myotonia and positive family history. Both his father and paternal aunt have classical myotonic dystrophy. His paternal grandfather is mildly affected. The patient works in a sheltered environment. At age 19 years, he experienced several periods of abdominal pain. Evaluation at age 20 years showed reduced oesophageal motility and gastritis. Oesophageal manometry did not register a high pressure zone at the level of the lower oesophageal sphincter. Abdominal ultrasound studies were reported normal. No diagnosis was made. At age 25 years the patient was admitted because of abdominal pain and constipation of 1 week's duration. The abdomen was distended with high-pitched bowel sounds. Plain abdominal X-ray showed a dilated descending colon. Colonoscopy showed a distended rectum and colon, connected by a collapsed segment of approximately 10 cms. Following endoscopy, normal intestinal motility was restored. X-ray studies of the colon showed dilatation and elongation of the sigmoid. Pressure recording of the anal sphincter was normal. Rectal biopsy showed presence of ganglion cells. A partial sigmoid resection was performed 6 months later. No abnormalities were noted on histological examination.

Case 4:
This woman was first diagnosed as having myotonic dystrophy at age 20 years during family studies. She had been admitted as an infant because of hypotonia, insufficient respiration and swallowing difficulties. Her motor and mental development were delayed and she did not learn to read and write during 12 years of special schooling. She is considered to have the congenital form of DM. Since the age of 9 she had alternating diarrhea and constipation with abdominal cramps. No cause was identified during a pediatric examination. At the age of 21 she was admitted to the surgical department because of bilious vomiting and abdominal pains for 3 days. The abdomen was distended with high-pitched bowel sounds. A diagnosis of ileus was made. After 24 hours of conservative treatment, a laparotomy was performed. Both the small and large bowels were distended. No obstruction was found. Her postoperative course was complicated by pneumonia, necessitating artificial respiration for 8 days. She was discharged from the hospital after 4 weeks. With cisapride medication, there has been normal daily defecation for 6 months.

DISCUSSION

In DM, abnormal motility has been demonstrated of the oesophagus, stomach, small intestine, colon and anal sphincter [7]. Swallowing difficulties are common, as well as disturbances of oesophageal motility which put these patients at increased risk of aspiration [1]. Also common are attacks of abdominal pain, accompanied by constipation or diarrhoea, caused by reduced colonic motility [1]. However, only a few reports exist of major abdominal problems in DM patients. The patients reported here, developed signs and symptoms of intestinal obstruction as a complication of myotonic dystrophy. In case 1, intestinal problems predated significant muscle weakness by 15 years. Reduced intestinal motility is a likely factor that leads to this complication. Furthermore, elongation and distension of the sigmoid colon may have predisposed our patients to volvulus and invagination, and thus contributed to the risk of ileus. Other predisposing factors in our patients were bacterial gastroenteritis and pregnancy. Routine histological examination of resected colon showed no abnormalities in our patients, but special studies were not performed. Yoshida et al. [8] reported pathologic studies in a DM patient in whom a hemicolectomy was performed because of a megacolon. They found normal smooth muscle but marked abnormalities of the myenteric plexus, indicating a possible neuropathic origin of the intestinal motility. However, smooth muscle abnormalities of small and large intestine have been reported by others [9].

Conservative measures were usually successful in the treatment of episodes of intestinal pseudo-obstruction in our cases. Treatment consisted of restriction of oral intake, intravenous fluids, and multiple enemas or colonoscopy. If abdominal surgery cannot be avoided, care should be taken to prevent the possible complications of general anaesthesia in DM patients [10], as exemplified by our case 4. Maintenance therapy with prokinetic agents may be considered. Cisapride has been shown to stimulate gastric and colonic motility in DM patients [11], and was effective in our case 4.

Intestinal pseudo-obstruction appears to be a rare complication of DM. A few case reports can be found in the literature [1-6]. We have seen this complication in 3/130 patients (2.3%) in the course of genetic linkage studies. In view of the low frequency of this complication it is striking, that our cases 1 and 2 are sibs. Another sib pair with prominent gastrointestinal involvement has been described [6]. An interesting model that may explain such clustering of apparently rare complications was recently proposed by Beggs et al. [12], who found deletions in the dystrophin gene in 3 out of 23 patients with a clinical diagnosis of Fukuyama congenital muscular dystrophy (FCMD). They suggested that the FCMD phenotype in these patients should

be explained on the basis of an interaction of heterozygosity for FCMD with hemizygosity for the dystrophin mutation. Similarly, our cases could have a specific genetic susceptibility for intestinal pseudo-obstruction, which is only uncovered by the additional presence of the DM mutation. Interaction of a major gene mutation and the genetic background may explain familial clustering of specific phenotypes in several other disorders [13-18], and could well turn out to be an important determinant of variable expressivity in inherited disease.

Familial occurrence of specific complications of DM has been reported before [19-23,27,28,30-32] (table). However, a number of these reports concerned features that may be more common in DM than was originally realized. This is certainly the case for mitral valve prolapse [22], and for cardiac conduction abnormalities [24], and could even apply to pilomatrixomas and ureteral dilatation, since their prevalence in DM patients is not known. The siblings with normal pressure hydrocephalus, reported by Christensen [28] were incompletely documented, while a report of focal myocarditis in a mother and son [27] could represent a relatively aspecific inflammatory reaction due to many possible causes, including infection. On the other hand, the finding of markedly decreased conduction velocities in a large family with otherwise classical DM [20,21] may represent an unusual allelic mutation, since all 14 carriers of the DM mutation in this family also showed a polyneuropathy.

In conclusion, both allelic mutations of the DM gene proper and unlinked modifying genes may influence the clinical picture in individuals with DM. It may be worthwhile to not only be aware of the many possible complications of DM in general, but also to document unusual complications in a given pedigree. Such complications may show a tendency to recur, especially in close relatives.

Acknowledgements:
We wish to thank Drs. Ferdinandusse, de Bevere, and van der Linden for providing clinical details about the patients described in this report.

TABLE:

Complications that have been reported in multiple relatives with DM

FEATURE	FAMILY RELATION	REFERENCE	FREQUENCY IN DM
INTESTINAL PSEUDO-OBSTRUCTION	BROTHER AND SISTER	THIS REPORT	LOW
DILATATION OF URETERS	2 BROTHERS	[19]	UNKNOWN
CARDIAC CONDUCTION DISTURBANCE (CCD)	8 AFFECTED IN 4 DM FAMILIES WITH CCD 22 UNAFFECTED IN 14 DM FAMILIES WITHOUT CCD	[23]	HIGH [24]
NEUROPATHY	14/14 INDIVIDUALS IN A SINGLE PEDIGREE	[20-21]	COMMON, BUT LESS SEVERE [20]
MITRAL VALVE PROLAPSE	9/11 INDIVIDUALS IN A SINGLE PEDIGREE	[22]	30% [22]
PILOMATRIXOMA	2 SISTERS, MOTHER AND SON BROTHER AND SISTER 4 AFFECTED IN SINGLE PEDIGREE BROTHER AND SISTER	[30] [31] [32] PERSONAL OBSERVATION	AT LEAST 4% [30]
NORMAL PRESSURE HYDROCEPHALUS	BROTHER AND SISTER	[28]	LOW [29]
FOCAL MYOCARDITIS	MOTHER AND SON	[27]	PROBABLY LOW [26]

REFERENCES

1. Harper PS. Myotonic Dystrophy. 2nd Edition. W.B.Saunders Co. London, Philadelphia, Toronto, Sydney, Tokio 1989.
2. Kohn NN, Faires JS, Rodman T. Unusual manifestations due to involvement of involuntary muscle in dystrophia myotonica. New Engl J Med 1964;271: 1179-1183
3. Dabaghi RE, Scott LD. Intestinal pseudo-obstruction in a patient with myotonic dystrophy. Texas Medicine 1982;82:42-44
4. Chiu VSW, Englert E. Gastrointestinal disturbances in myotonia dystrophica. Gastroenterology 1962;42:745-746
5. Bertrand L. Le megacolon dans la maladie de Steinert. Rev Neurol 1949; 81:480-486
6. Lenard HG, Goebel HH, Weigel W. Smooth muscle involvement in congenital myotonic dystrophy. Neuropädiatrie 1977;8:42-52
7. Nowak TV, Ionasescu V, Anuras S. Gastrointestinal manifestations of the muscular dystrophies. Gastroenterology 1982;82:800-810
8. Yoshida MM, Krishnamurty S, Wattchow DA, Furness JB, Schuffler MD. Megacolon in myotonic dystrophy caused by a degenerative neuropathy of the myenteric plexus. Gastroenterology 1988;95:820-827
9. Pruzanski W, Huvos AG. Smooth muscle involvement in primary muscle disease. Arch Pathol 1967;83:229-233
10. Aldridge LM. Anaesthetic problems in myotonic dystrophy - A case report and review of the Aberdeen experience comprising 48 general anaesthetics in a further 16 patients. Br J Anaesth 1985;57:1119-1130
11. Horowitz M, Maddox A, Wishart J, Collins PJ, Shearman DJC. The effects of cisapride on gastric and colonic emptying in dystrophia myotonica. J Gastroenterol Hepatol 1987;2:285-293
12. Beggs AH, Neumann PE, Arahata K, Arikawa E, Nonaka I, Anderson MDS, Kunkel LM. Possible influences on the expression of dystrophin abnormalities by heterozygosity for autosomal recessive Fukuyama congenital muscular dystrophy. Proc Natl Acad Sci 1992;89:623-627
13. Santis G, Osborne L, Knight RA, Hodson ME. Independent genetic determinants of pancreatic and pulmonary status in cystic fibrosis. Lancet 1990; 336:1081-1084
14. Silverman EK, Province MA, Campbell EJ, Pierce JA, Rao DC. Variability of pulmonary function in alpha-1-antitrypsin deficiency: residual family resemblance beyond the effect of the Pi locus. Hum Hered 1990;40:340-355
15. Cobben JM, Breuning MH, Schoots C, ten Kate LP, Zerres K. Congenital hepatic fibrosis in autosomal-dominant polycystic kidney disease. Kidney Int 1990;38:880-885
16. Vilkki J, Ott J, Savontaus M-L, Aula P, Nikoskelainen EK. Optic atrophy in Leber hereditary optic neuroretinopathy is probably determined by an X-chromosomal gene closely linked to DXS7. Am J Hum Genet 1991;48:486-491

17. Neumann HPH, Wiestler OD. Clustering of features of von Hippel-Lindau syndrome: evidence for a complex genetic locus. Lancet;337:1052-1054.
18. Schneider M, Obringer AC, Zackai E, Meadows AT. Childhood neurofibromatosis: risk factors for malignant disease. Cancer Genet Cytogenet 1986; 21:347-354.
19. Bundschu HD, Hauger W, Lang HD. Myotonische dystrophie Curschmann-Steinert. Urologische Besonderheiten und histochemische Befunde an der Muskulatur. Dtsch Med Wschr 1975;100:1337-1341
20. Spaans F, Jennekens FGI, Mirandole JF, Bijlsma JB, de Gast GC. Myotonic dystrophy associated with hereditary motor and sensory neuropathy. Brain 1986;109:1149-1168
21. Brunner HG, Spaans F, Smeets HJM, Coerwinkel-Driessen M, Hulsebos T, Wieringa B, Ropers HH. Genetic linkage with chromosome 19, but not chromosome 17 in a family with myotonic dystrophy associated with hereditary motor and sensory neuropathy. Neurology 1991;41:80-84
22. Winters SJ, Schreiner B, Griggs RC, Rowley P, Nanda NC. Familial mitral valve prolapse and myotonic dystrophy. Ann Int Med 1976;85:19-22
23. Hawley RJ, Gottdiener JS, Gay JA, Engel WK. Families with myotonic dystrophy with and without cardiac involvement. Arch Int Med 1983;143: 2134-2136
24. Hawley RJ, Milner MR, Gottdiener JS, Cohen A. Myotonic heart disease: A clinical follow-up. Neurology 1991;41:259-262.
25. Streib EW, Meyers DG, Sun SF. Mitral valve prolapse in myotonic dystrophy. Muscle & Nerve 1985;8:650-653
26. Moorman JR, Coleman RE, Packer DL, Kisslo JA, Bell J, Hettleman BD, Stajich J, Roses AD. Cardiac involvement in myotonic muscular dystrophy. Medicine 1985;64:371-387
27. Rausing A. Focal myocarditis in familial dystrophia myotonica. Br Heart J 1972;34:1292-1294
28. Christensen PB. Normal pressure hydrocephalus in myotonic dystrophy. Eur Neurol 1988; 28:285-287
29. Riggs JE, Rubenstein MN, Gutmann L. Myotonic dystrophy and normal-pressure hydrocephalus. Neurology 1985;35:1535
30. Harper PS. Calcifying epithelioma of Malherbe. Association with myotonic muscular dystrophy. Arch Dermatol 1972;106:41-44
31. Chiaramonti A, Gilgor RS. Pilomatricomas associated with myotonic dystrophy. Arch Dermatol 1978;114:1363-1365
32. Delfino M, Monfrecola G, Ayala F, Suppa F, Picirillo A. Multiple familial pilomatricomas: A cutaneous marker for myotonic dystrophy. Dermatologica 1985;170:128-132

CHAPTER 11

SUMMARY

Myotonic dystrophy (DM) is an autosomal dominant multisystem disorder with a number of features that are not usually observed in other genetic conditions. Earlier age at onset in successive generations, called anticipation, as well as effects of the sex of the transmitting parent on the disease severity in offspring are examples of such features. In order to study the genetic basis of myotonic dystrophy, several groups, including our own, embarked upon projects aimed at isolating the myotonic dystrophy gene. Cloned polymorphic markers that had been assigned to specific loci on the physical map of chromosome 19 were used to localize the DM gene to 19q13 (paragraph 1.2 and chapters 2-4). A few crucial recombinant families and strong linkage disquilibrium then sufficiently narrowed the physical localization of the DM gene to allow its identification (paragraph 1.3). The unstable mutation underlying myotonic dystrophy consists of an expanded CTG trinucleotide repeat sequence in the 3'-untranslated region of a gene encoding a serine-threonine protein kinase family member. This gene has been termed DM-kinase. The expansion of the CTG repeat sequence presumably changes the activity of the DM-kinase. However, a change in activity of other genes in this area has not yet been excluded.

The myotonic dystrophy mutation can be demonstrated directly by Southern analysis of genomic fragments, or by PCR amplification of the unstable repeat. This method is now the preferred diagnostic test for myotonic dystrophy, especially since previous strategies based on clinical, ophthalmological, and electromyographical examination failed to identify approximately 8% of mutation carriers (Chapter 5).

The application of direct mutation analysis has explained phenomena that have puzzled geneticists and clinicians alike. The DM mutation is unstable and tends to expand further in successive generations. This explains the previous observations of earlier onset in successive generations (anticipation) in this condition.

The myotonic dystrophy mutation is in complete linkage disequilibrium with a 1kb insertion in an intron of the DM kinase gene. This linkage disequilibrium probably results from a shared ancestry of many (if not all) DM mutations. This suggests that new mutations rarely, if ever occur in myotonic dystrophy. Consistent with this hypothesis is the finding of common ancestors for independently ascertained DM families by genealogical studies (paragraph 1.3.4; chapter 9). Also, a small expansion of the CTG trinucleotide repeat is consistently found in asymptomatic obligate carriers (Chapter 7). Starting with such a small increase in CTG number, further expansion is more rapid with male transmission compared to female transmission. This finding explains the excess of asymptomatic male transmitters in genealogical studies of DM (paragraph 1.3.4; chapter 9). Reductions in the size of the DM repeat sequence occur only rarely. Occasionally, they may result in a

complete reversal of the myotonic dystrophy mutation (Chapter 8). The fact, that reductions in the size of the CTG trinucleotide repeat sequence occur predominantly with male transmission may point to selection against alleles containing large CTG trinucleotide expansions, either during spermatogenesis or in the early zygote (paragraph 1.3.4; chapter 9). Negative selection may also help explain the exclusively maternal transmission of the congenital form of myotonic dystrophy (paragraph 1.5).

Preliminary evidence for aditional genetic factors that modify the clinical expression of the DM mutation has been obtained in a family in which each of 14 affected subjects presented with a demyelinating neuropathy (Chapter 6). The occurrence of a rare complication such as intestinal pseudo-obstruction in siblings with DM also points in the direction of modifying factors, possibly of genetic origin (Chapter 10).

On the one hand, the story of the myotonic dystrophy mutation illustrates the successful application of molecular genetics to inherited disease. On the other hand, we should now recognize that clinical observations in the first half of this century had accurately predicted many of the surprising genetic features of the DM mutation. Such observations may yet uncover new aspects of genetic theory.

Samenvatting

Myotone dystrofie (DM) is een autosomaal dominant erfelijke aandoening die vele orgaansystemen kan treffen. De efelijkheid van de ziekte vertoont enkele bijzondere kenmerken die bij de meeste genetische aandoeningen niet worden gevonden. Zo is er het fenomeen van de anticipatie, waarmee wordt bedoeld dat de ziekte zich in opeenvolgende generaties op jongere leeftijd manifesteert. Daarnaast is de ernst van de aandoening mede bepaald door het geslacht van de ouder waarvan de ziekte is geerfd. Meerdere onderzoeksgroepen, waaronder de Nijmeegse, zijn daarom rond 1985 begonnen met projecten gericht op het ophelderen van de genetische basis van de myotone dystrofie. Met behulp van gecloneerde DNA merkers, die eerst aan een specifiek gedeelte van chromosoom 19 waren toegewezen, kon worden vastgesteld dat het gen voor de myotone dystrofie moest liggen in band q13 van chromosoom 19 (paragraaf 1.2 en hoofdstuk 2-4). Op grond van enkele zeldzame families waarin er recombinatie op trad tussen het DM gen en de dichtst gekoppelde DNA merkers, kon dit gebied vervolgens zodanig worden ingeperkt, dat het mogelijk bleek om het DM-gen te isoleren (paragraaf 1.3). De (instabiele) mutatie die aan het ziektebeeld van de myotone dystrofie ten grondslag ligt bestaat uit een toename in het aantal CTG trinucleotiden in het 3' uiteinde van een gen dat codeert voor een eiwit dat gerelateerd is aan de serine-threonine proteine kinases. Dit gen wordt wel DM-kinase genoemd. Men veronderstelt dat de toename van het aantal CTG trinucleotiden leidt tot een verandering van de activiteit van het DM-kinase. Het is echter ook mogelijk dat de mutatie de activiteit van andere genen in dit gebied eveneens beïnvloedt.

De myotone dystrofie mutatie kan zichtbaar worden gemaakt met behulp van Southern analyse, dan wel door PCR amplificatie van de instabiele repeterende sequentie. Dit onderzoek geldt nu als de meest betrouwbare diagnostische test voor de aandoening. Uit eerder onderzoek was gebleken dat klinisch neurologisch onderzoek van mogelijke gendragers, in combinatie met oogheelkundig en electromyografisch onderzoek, bij ongeveer 8% van de gendragers geen afwijkingen aantoonde (hoofdstuk 5).

Het toepassen van directe mutatie-detectie heeft enkele van de eerder genoemde bijzonderheden in de overererving van de myotone dystrofie opgehelderd. Zo is de DM-mutatie instabiel, en blijkkt het aantal CTG trinucleotiden in opeenvolgende generaties toe te nemen. Dit verklaart de klinische waarneming van de anticipatie, dat wil zeggen het in opeenvolgende generaties vroeger optreden van de aandoening.

De DM-mutatie erft altijd samen over met een 1 kb insertie in een intron van het DM-kinase gen. Het is aannemelijk dat dit berust op een gezamenlijke afstamming van alle DM-mutaties in de populatie. Als dat inderdaad zo is, dan zou dat

betekenen dat nieuwe mutaties zelden of nooit voorkomen bij de myotone dystrofie. Inderdaad blijken verschillende families met myotone dystrofie vaak af te stammen van dezelfde voorouders (paragraaf 1.3.4; hoofdstuk 9). Bovendien is bij de gezonde personen die als verbindende schakels tussen deze families fungeren altijd een (zij het geringe) toename van het aantal CTG trinucleotiden aantoonbaar (hoofdstuk 7). Beginnend met zo een geringe toename in het aantal CTG trinucleotiden is er juist dan sterke een sterke verdere toename van het aantal CTG tripletten in de volgende generatie als de mutatie door een man wordt doorgegeven. Dit betekent dat in de laatste generatie vóór er klinische verschijnselen optreden meer mannen dan vrouwen worden gevonden. Dit verklaart de relatief frequente paternale overerving bij stamboom-onderzoek van myotone dystrofie.
Vermindering van het aantal CTG trinucleotiden komt weliswaar voor, maar slechts in een minderheid van de gevallen. In uitzonderlijke gevallen gaat het om een zodanige afname, dat het aantal CTG trinucleotiden bij het kind in het normale bereik komt te liggen (5-37 CTG trinucleotiden). Dan is de DM-mutatie teruggemuteerd naar een normaal allel van het DM-kinase (hoofdstuk 8). Dat verminderingen in het aantal CTG trinucleotiden vooral optreden wanneer de mutatie door een man wordt overgedragen, zou kunnen wijzen op een negatieve selectie gericht tegen de allelen met het grootste aantal CTG trinucleotiden tijdens de spermatogenese of in de eerste celdelingen na de bevruchting (paragraaf 1.3.4; hoofdstuk 9). Deze negatieve selectie zou mede kunnen verklaren waarom de meest ernstige (congenitale) vorm van de aandoening zich uitsluitend voordoet bij transmissie via een vrouw (paragraaf 1.5).
Er zijn voorzichtige aanwijzingen dat het klinisch beeld van de myotone dystrofie niet alleen afhankelijk is van het aantal CTG trinucleotiden, maar eveneens van bijkomende genetische factoren. De waarneming van een familie waar de DM-mutatie steeds samen gaat met een demyeliniserende polyneuropathie wijst in deze richting (hoofdstuk 6). Het optreden van een zeldzame complicatie als de intestinale pseudo-obstructie (paralytische ileus) bij een broer en zuster met myotone dystrofie kan eveneens verklaard worden door aan te nemen dat het klinisch beeld van de ziekte wordt beinvloed door bijkomende factoren van mogelijk genetische aard (hoofdstuk 10).
Enerzijds is het verhaal van de DM-mutatie een illustratie van de succesvolle toepassing van moleculair genetische technieken bij de opheldering van een erfelijk bepaalde ziekte. Anderzijds doen wij er goed aan om ons te realiseren dat de verrassende eigenschappen van de DM-mutatie in belangrijke mate al voorspeld waren door klinische observaties in de eerste helft van deze eeuw. Dergelijke observaties kunnen ook nu nog leiden tot de ontdekking van nieuwe elementen in de genetica.

DANKWOORD

Medisch genetisch onderzoek is geen zaak meer voor eenlingen. Dat heb ik in de afgelopen 9 jaar wel geleerd. De vele mede-auteurs van de in dit proefschrift opgenomen publicaties tonen dat nog eens aan.
Het uiteindelijke bouwsel kon slechts ontstaan op de fundamenten die waren gelegd door Dominique en Bert (later Gert) en met behulp van vele stenen en steentjes die door anderen werden aangedragen. Ik hoop jullie (en al die anderen) hiervoor nog persoonlijk te bedanken.

Ben ter Haar gaf mij in 1984 de kans om een opleiding tot klinisch geneticus te combineren met wetenschappelijk onderzoek in het kader van het chromosoom 19 project. Hij was een inspirerend voorbeeld van hoe een goede dokter moet zijn. Zijn overlijden betekent een gemis.

Mijn direkte collega's van de klinische genetica moeten mijn preoccupatie met het wetenschappelijk onderzoek wel eens lastig hebben gevonden. Ze hebben het echter nooit laten merken, en daarvoor ben ik ze dankbaar.

Tot slot ben ik veel dank verschuldigd aan de patienten, hun families, en de Vereniging Spierziekten Nederland. Hun medewerking was een noodzakelijke voorwaarde voor het slagen van de hier beschreven onderzoeken.

CURRICULUM VITAE

Han Brunner werd geboren op 18 october 1956 te Rotterdam. Na het behalen van het diploma Gymnasium-ß aan het Praedinius Gymnasium te Groningen, werd in 1975 aangevangen met de studie Geneeskunde aan de Rijksuniversiteit aldaar.
In 1979 werd het kandidaatsexamen behaald. In 1984 werd de studie afgesloten met het behalen van het artsexamen. In de periode 1984-1988 was hij in opleiding tot klinisch geneticus bij de afdeling Anthropogenetica van de Katholieke Universiteit te Nijmegen (hoofd Prof. Dr. H.H. Ropers). Als opleider fungeerde in eerste instantie Drs. B.G.A. ter Haar. Na diens overlijden in 1986 werd de opleiding voortgezet onder leiding van Drs. B.C.J. Hamel. In 1988 volgde inschrijving in het specialisten register. Vanaf 1988 is hij als klinisch geneticus verbonden aan de afdeling Anthropogenetica (sectie klinische genetica) van de Katholieke Universiteit te Nijmegen.

STELLINGEN

behorend bij het proefschrift
"Genetic studies in myotonic dystrophy"
van H.G. Brunner

I

Het klinisch gedefinieerde begrip "anticipatie" is afdoende verklaard door de in opeenvolgende generaties toenemende lengte van de CTG repeat.

II

Dat er meer mannen dan vrouwen worden gevonden onder mild aangedane DM patienten wordt veroorzaakt door ascertainment bias, in combinatie met een grotere instabiliteit van de DM mutatie bij overerving via de man.

III

Dat bij de congenitale vorm van myotone dystrofie de mutatie vrijwel altijd van de moeder is geërfd, is ten dele een gevolg van de infertiliteit van mannen met de ernstiger vormen van deze aandoening. Belangrijker is echter de invloed van negatieve selectie tijdens de spermatogenese of de fertilisatie.

IV

De waarneming, dat een teruggemuteerd allel van 19 CTG eenheden stabiel overerft naar de volgende generatie, suggereert dat de instabiliteit van de DM mutatie in eerste instantie afhangt van de lengte van de CTG repeat.

V

Negatieve selectie tegen langere allelen op het DM locus tijdens spermatogenese/fertilisatie leidt tot een situatie die vergelijkbaar is met het "segregation distorter" systeem bij Drosophila.

VI

Door aan te nemen dat de DM mutatie onderhevig is aan segregatie distortie kan afdoende verklaard worden dat één enkele schadelijke mutatie met zo een hoge frequentie wereldwijd voor komt.

VII

Het samengaan in één grote familie van myotone dystrofie en een demyeliniserende polyneuropathie kan berusten op het gelijktijdig voorkomen van de geëxpandeerde CTG trinucleotide repeat en een modificerende mutatie elders in het DM-kinase gen.

VIII

Het Carney complex berust op een mutatie die een G-eiwit-effector keten stimuleert.

IX

Het syndroom van de hypofysaire resistentie tegen schildklierhormoon kan ook verklaard worden door aan te nemen dat de TRH-receptor bovenmatig actief is.

X

Voor het syndroom van de autosomaal dominant erfelijke leiomyomatose is het COL4A4 gen een goede kandidaat.

XI

Aangezien bijna de helft van alle publicaties in wetenschappelijke tijdschriften nooit zal worden geciteerd, is de ermee verbonden ijdelheid niet zelden misplaatst.

XII

Er zijn geen onsterfelijke wetenschappers. En ook de wetenschappelijke vondst overleeft maar zelden zijn schepper.

XIII

Het verdient overweging, om naast het reguliere toernooi om de wereldkampioenschappen atletiek nog een toernooi voor niet gedrogeerde atleten te organiseren.