DNA probe analysis for carrier detection and prenatal diagnosis of Duchenne muscular dystrophy: a standard diagnostic procedure

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SUMMARY Thirteen marker loci localised on the short arm of the X chromosome are available for use in genetic studies for Duchenne muscular dystrophy (DMD). This large number of probes detecting about 20 RFLPs encouraged us to set up a standard procedure using a sequence of selected probes and restriction enzymes for the diagnosis of DMD families. The application of DNA probe analysis for carrier detection and prenatal diagnosis, involving 61 pedigrees of both familial and isolated cases, has yielded the following results. Carrier detection using flanking markers was possible in more than 75% of the cases (104 out of 136 females) with a reliability of better than 98%. Prenatal diagnosis was possible in 95% of the cases (65 out of 68 proven carriers or women at risk). Twenty-three prenatal diagnoses were performed on male fetuses; 13 appeared to have a low risk for DMD (< 1%) and thus the pregnancies continued. Seven have since come to term and the male infants have normal CK levels. The genetic distances of the loci relative to the *DMD* locus and their order on the short arm of the X chromosome were deduced from our total DMD family material and are not significantly different from those reported earlier. For 754 (*DXS84*) we found a genetic distance of 5 cM with a lod score of +12.4 and 95% confidence limits between 2 and 12 cM. Similar data were obtained for pERT87 (*DXS164*), suggesting that in our family material both loci are tightly linked. Multiply informative recombinations showed that both 754 and pERT87 map proximal to the DMD mutations in the cases studied. The high frequency of DMD mutations and its relation to the observed instability in this part of the genome will be discussed. Unequal crossing over is proposed as one of the mechanisms contributing to the high mutation frequency.

Duchenne muscular dystrophy (DMD) is a severe, X linked recessive, neuromuscular disorder. The high incidence in the population (one in 3000 males¹) is caused by a high mutation rate of approximately 1 in 10 000. The observed instability of this locus, and the basic biochemical defect responsible for the Duchenne phenotype, are not yet understood. For many years, carrier detection for DMD has been based on pedigree analysis and on serum creatine kinase (CK) levels, even though CK determination displays both variability² and overlap between normals and carriers in around 30% of obligate carriers. Since Davies *et al*³ described the first RFLPs located in Xp and flanking *DMD*,

other RFLPs in Xp have been reported to be potentially useful for diagnosis of DMD.^{4 5} We have performed both carrier detection and prenatal diagnosis in a number of cases, using a total of 11 polymorphic loci flanking the *DMD* locus.⁶ Our experience with the many available RFLPs and the large number of DMD families have led us to develop a standardised diagnostic procedure.

Recently Kunkel *et al*⁷ described the selective cloning of deleted sequences from a DMD patient (BB, Francke *et al*⁸) leading to the cloning of sequences at the locus *DXS164* (pERT87). In about 8% of all DMD patients, this sequence (pERT87), or subclones from it, are deleted.^{9 10} It is likely that pERT87 is located within the *DMD* region itself, based upon a series of partial deletions at this locus,

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which appear to extend in either direction. The majority of the mutation sites for DMD, mapped by RFLP analysis, are located distal to pERT87. However, at least two X;autosome translocation breakpoints are located proximal to the locus *DXS164*, and the position of *DXS164* relative to all mutation sites is not clear. We have therefore used bridging markers in the DNA probe analysis and, when available, CK levels were used as corroborative evidence. Our results using DNA probes for the diagnosis of Duchenne muscular dystrophy and carrier detection, both pre- and postnatally, are presented in this paper. In addition, the genetic order of loci and linkage data of the DMD probes used, gathered from Dutch DMD families, are presented.

Materials and methods

PROBES

In table 1 the probes used are listed with their main characteristics and their sources. Further information about these loci, such as vectors, insert sizes, allele sizes, minor allele frequencies, etc, can be found in the recombinant DNA committee report of the Eighth Human Gene Mapping Workshop held in Helsinki in 1985.¹²

DNA EXTRACTION AND ANALYSES

The DMD families were referred through the seven genetic centres in the Netherlands. Lithium-heparin blood samples were taken from family members and were sent to us within 24 hours. For prenatal diagnosis, chorionic villi taken at the 10th or 11th week of gestation were investigated. The villi of male fetuses were used immediately for DMD diagnosis. Fetal sexing was mainly performed in the cytogenetics unit in Rotterdam by direct chromosome analysis.¹³ Creatine kinase levels were deter-

mined in various regional hospitals. The method of Hofker *et al*⁵ was used to extract DNA from total blood and the same method was used to extract DNA from the chorionic villi samples, ranging from 30 to 100 mg. DNA samples of 5 to 10 µg were digested with the appropriate restriction enzymes (table 1, fig 1) according to the specifications of the manufacturers. Electrophoresis was performed on 0.7% agarose gels in Tris-acetate buffer, pH 7.7, for 17 hours at 1 volt/cm. Southern blotting on Gene Screen Plus (NEN) and hybridisation were carried out as described previously.⁶

DIAGNOSTIC PROCEDURE

Many RFLPs detected by the subclones (pERT87-1, pERT87-8, and pERT87-15) of pERT87 (kindly provided by L Kunkel) are tightly linked to *DMD* at approximately 5 cM and are highly informative in DMD family studies, as are the various deletions at this locus. Because of the many RFLPs involved (fig 1), we have chosen a standard diagnostic procedure which always starts with an initial digestion of DNA of all family members using five enzymes, followed by hybridisation with seven probes as shown in table 2 under stage I. These five initial enzymes were selected because of their relative cheapness and the possibility of re-using the blots. In order to obtain as much information as possible to perform carrier detection in the minimum time, we also combined probes into one hybridisation reaction, for example, C7 and pERT87-1, both polymorphic for *EcoRV*. The *EcoRV* RFLP for pERT87-1 has not yet been described. It detects a 13 kb major allele (70%) and a 12 kb minor allele. The first seven probes used were selected on their Xp localisation close to or flanking the *DMD* locus and also on their heterozygosity frequency. This initial combination gave enough information to establish carrier status in about 50% of the cases. Where necessary, the five initial blots were rehybridised with additional probes, up to a total of 14 RFLPs (underlined in fig 1), as shown in table 2 under stages I and II. If needed, additional digests were performed so as to make use of other RFLPs (stage III), thus supplying sufficient information to establish informativeness in over 95% of the cases.

LINKAGE ANALYSIS

Linkage analysis was carried out on a PDP 11 computer using a programme called Myotonia (W S Volkers, 1986, personal communication) which derives lod scores at intervals of 0.01 recombination fraction (θ) and is based on standard formulae. Recombination frequencies were checked by hand using the maximum likelihood method.¹⁴ From the individual pedigrees, crossovers between informative

TABLE 1 *Xp markers.*

Name of probe	Locus symbol (HGM8)	Genetic distance to DMD (cM)	RFLP restriction enzyme(s)	Overall heterozygote frequency	Probe source
RC8	<i>DXS9</i>	17 ¹¹	<i>TaqI</i>	0.23	Davies
782	<i>DXS85</i>	20 ¹¹	<i>EcoRI</i>	0.50	Hofker
pXUT23	<i>DXS16</i>	18 ¹¹	<i>BglIII</i>	0.42	Willard
pD2	<i>DXS43</i>	18 ¹¹	<i>PvuII</i>	0.40	Kunkel
99-6	<i>DXS41</i>	15 ¹¹	<i>PstI</i>	0.49	Kunkel
B24	<i>DXS67</i>	—	<i>MspI</i>	0.15	Kunkel
C7	<i>DXS28</i>	10 ¹¹	<i>EcoRV</i>	0.25	Mandel
pERT87	<i>DXS164</i>	2-5* ¹⁰	Many	0.9	Kunkel
XJ1-1	NA	—	<i>TaqI</i>	0.45	Worton
754	<i>DXS84</i>	5-12* ¹¹	<i>PstI/EcoRI</i>	0.66	Hofker
cX5-7	<i>DXS148</i>	—	<i>MspI</i>	0.46	Hofker
OTC	<i>OTC</i>	10 ¹¹	<i>MspI</i>	0.67	Fenton
L1-28	<i>DXS7</i>	20 ¹¹	<i>TaqI</i>	0.45	Bakker

*This study.

—No data available.

FIG 1 Regional distribution of 13 loci along the short arm of the X chromosome detecting a total of 21 RFPLs. The 14 restriction enzymes underlined indicate the 14 RFPLs covered by the five initial blots (see table 2 stages I and II).

TABLE 2 Diagnostic procedure for each DMD family.

	RFLP markers used			Restriction enzymes used
	Proximal side	DMD region	Distal side	
Stage I				
Initial blots	OTC		99-6†	<u>MspI</u>
	<u>754</u>	<u>87-1*</u>	<u>C7</u>	<u>PstI</u>
			D2	<u>EcoRV</u>
		87-15/		<u>PvuII</u>
		XJ1.1‡		<u>TaqI</u>
Stage II		87-1		<u>MspI</u>
Re-use blots	CX5.7	87-8		<u>MspI</u>
	<u>L1.28</u>		RC8	<u>TaqI</u>
			B24	<u>MspI</u>
Stage III	<u>754-11</u>		782	<u>EcoRI</u>
Additional blots and re-use		87-1/15		<u>XmnI</u>
		87-8	pXUT23	<u>BglII</u>
		87-1		<u>BstNI</u>
				<u>BstXI</u>

*A new RFLP for 87-1 (EcoRV).

†A subclone of 99-6 was used to lower the background.

‡XJ1.1 only used in the later pedigrees in combination with 87-15.

The probe name is underlined when it is used in a combined hybridisation (eg C7 and 87-1).

markers relative to other markers or the *DMD* locus were scored to establish the most likely order on the X chromosome.

Results

CARRIER DETECTION AND PRENATAL DIAGNOSIS

From the 61 pedigrees investigated (30 familial and 31 isolated cases), we were able to assess carrier status in 75% of the 136 females at risk (table 3) using bridging markers at a maximum distance of 15 cM on either side of the *DMD* locus. For 68 females we demonstrated non-carrier status with more than 98% reliability and for 36 females the *DMD* carrier status on the basis of bridging markers was proven. In 33 of these carriers, there was enough information to offer prenatal diagnosis. Fig 2 (DL12) shows

TABLE 3 DMD carrier detection using bridging markers.

61 DMD families (30 proven familial) (31 isolated cases)	Carrier detection		Prenatal diagnosis possible
	Reliable (>98%)	Not conclusive	
136 females at risk	68 non-carrier 36 carrier 104 cases (>75%)	32 cases	33 cases 32 cases 65 cases (>95%)

FIG 2 Family DL12, an example of a carrier detection. Subject II.2 is an obligate carrier informative for F(99-6), M(87-1), and C(754). Subjects III.1 and III.2 are not carriers for DMD (>99% reliability) on the basis of F, M, and C. Subjects III.3 and III.4 are DMD carriers on the basis of F, M, and C (>99% reliability).

an example of the diagnosis of two carriers and two non-carriers, using the markers 754, pERT87-1 (*Msp*I), and 99-6. In 32 out of the 136 cases no conclusive statement could be made on the carrier status because (a) there was a detected recombination between the nearest informative bridging markers (17 cases). Fig 3 shows an example (DL85); (b) there was no informative marker on one side of the DMD locus (five cases); and (c) the phase of the 'DMD' X chromosome was unknown, since neither a patient nor healthy male sibs were available (10 cases). However, in those 32 females who had a 50% risk for DMD carriership, there was sufficient information to discriminate between the potential DMD chromosome and the healthy paternal chromosome and thus to offer prenatal diagnosis (in 95% of cases). To date, 23 prenatal diagnoses on male fetuses have been performed (table 4). In 16 cases, we were able to provide a diagnosis on the basis of bridging markers. Three were found to be affected and five unaffected with a greater than 98% reliability (fig 4, DL56). In eight of the pregnan-

TABLE 4 Prenatal diagnosis for DMD using bridging markers.

Diagnosis on male fetuses at risk (23 cases)	DMD status of fetus	DMD carrier status of mother (confirmed by this test)	Pregnancies
16 cases (reliability >98%)	13 unaffected	1 carrier 4 carrier (>98%) 8 non-carrier† (>98%)	Continued 13 cases†
7* cases (not conclusive)	3 affected	3 carrier (>98%) 5 carrier (>98%) 2 at risk‡	Terminated 10 cases

*Due to crossover, uninformative markers, etc (see text).
†In seven cases healthy boy born with normal CK levels.
‡In these cases prenatal diagnosis would not have been necessary, if the family material had been sent before the start of the pregnancy.

cies, which had come to our attention in an advanced stage, the prospective mothers proved not to be carriers of the DMD mutation. Seven of the 13 pregnancies diagnosed as unaffected have come to term and all male infants have normal CK levels. In seven out of the 23 prenatal cases we could not draw any conclusions about the DMD risk to the fetus because of recombination between the nearest informative flanking markers (four cases), one possible mosaic Klinefelter case (XXY), insufficient family data (one case), or no informative markers (one case). The latter two groups, as well as the eight in which the mother appeared not to be a carrier, were all cases that came to our attention at an advanced stage of pregnancy. In these cases prenatal diagnosis would not have been necessary had the DNA analysis on the family members been performed earlier. In our material, we detected a deletion in the *DXS164* locus in eight out of 67 independent DMD patients. Clearly, in those families, carrier detection and prenatal diagnosis is highly reliable using the pERT87 probe alone.

LINKAGE MAP OF THE DMD LOCUS
Linkage data and physical order of the markers were established from 40 pedigrees of proven familial DMD cases. We obtained lod scores for the Xp markers used relative to the DMD locus. In table 5 the recombination fractions (θ) at the maximum lod scores are listed with the 95% confidence limits and the number of informative meioses, sub-divided into known and unknown phase. For the locus *DXS84*, recognised by the probes 754 and 754-11, a lod of +12.44 at a distance of 5 cM was found, based on four recombinants in 77 informative meioses. Also on the proximal side of the DMD mutation site(s), the locus *DXS164* was found to have a lod of +10.46

FIG 3 Family D185, an example of carrier detection. Subject II.2 is an obligate carrier, informative flanking markers C7(J), 754-11(T), and OTC(G). Subject II.1 is a healthy male, crossover between 754 and OTC detected. Subject II.5 is a carrier (>99% reliability). Crossover detected between F(99-6) and J(C7) does not interfere with the result. No conclusive result was obtained for subjects II.4 and II.6. In both cases a recombinant was found between C7 and 754-11. Subject II.4 shows repeatedly raised CK levels, II.6 has normal CK levels. Prenatal diagnosis using flanking markers is possible in all four cases (for the proven carriers II.2 and II.5 using C7 and 754-11; for female at high risk II.4 using 99-6 and 754-11; for female at risk II.6 using C7 and OTC).

at 5 cM (two recombinants in 51 informative meioses). On the distal side, the locus *DXS28* or probe C7 was found to be the closest marker at 7 cM with a lod of +4.31 (two recombinants in 31 informative meioses). The order of the loci on Xp was established by three point crosses (table 6). Of

the four recombinants detected for 754 and *DMD*, three were also informative for pERT87. Two of them occurred between 754, pERT87 and *DMD* and one occurred between 754 and pERT87, *DMD*, providing the order 754-pERT87-*DMD*.

ISOLATED CASES

We studied families containing an isolated case of *DMD*. In 12 of these sporadic cases we have information on the origin of the mutation using flanking RFLPs.¹⁵ In four cases the paternal chromosome was affected and in eight cases either the maternal or grandmaternal chromosome was affected. In four of the eight female origins, meiotic recombination had occurred between pERT87 and the closest informative distal flanking marker, suggesting that mutational events such as unequal crossing over contribute to the relatively high rate of *DMD* mutation, as was suggested by Winter and Pembrey,¹⁶ and that there may be a preferential

TABLE 5 Lod scores for *DMD* and marker loci.

Marker	Recombinants phase known	Recombinants phase unknown	Z max	θ max	95% confidence limits
RC8	2 in 7	0 in 4	+0.75	0.18	0.03-0.49
782	2 in 15	10 in 34	+2.88	0.17	0.08-0.31
pXUT23	4 in 8	0 in 4	+0.03	0.37	0.12-0.50
pD2	6 in 25	5 in 21	+2.27	0.23	0.13-0.37
99-6	3 in 23	4 in 24	+3.85	0.16	0.07-0.29
C7	0 in 10	2 in 21	+4.31	0.07	0.01-0.19
pERT87	2 in 40	0 in 11	+10.46	0.05	0.01-0.12
754	4 in 35	0 in 42	+12.44	0.05	0.02-0.12
OTC	2 in 16	1 in 15	+3.85	0.10	0.03-0.24
L1-28	6 in 23	1 in 16	+2.59	0.18	0.08-0.33

TABLE 6 Order of marker loci determined by use of three point crosses (for example, see fig 3 subject II.2 crossover between L1.28, OTC and 754; subject III.3 crossover between L1.28 and OTC, 754, confirming the order -----L1.28-OTC-754-----).

RC8 782 XUT23	D2 99-6 C7	B24 DMD 87 754	5-7	OTC 1-28	Number of cross overs
RC8//782		DMD			1×
782//XUT23	D2				1×
782	D2//99-6				3×
	D2 99-6//C7				2×
	99-6	B24// 87			1×
	C7	//DMD 754			2×
		DMD//87 754			2×
		87//754			1×
	D2	DMD 754	//	OTC	3×
		DMD		OTC//1-28	3×

RC8-782-XUT23; D2-99-6-C7; B24-DMD-87-754; 5-7; OTC-1-28-cen.

FIG 4 Family DL56, an example of prenatal diagnosis. Subject I.3 is an obligate carrier not informative for available DNA markers except for 782. CK levels not raised. Subject II.2 is a DMD carrier on the basis of repeatedly raised CK levels. First pregnancy (male fetus III.1, 1984) terminated in the 11th week after sex determination. In 1985 the RFLPs of pD2(E), pERT87-1(M), and 754(C) were found to be informative in subject II.2. In subject III.2 (male fetus) the grandpaternal X chromosome was detected (E,k,C); therefore he was not affected, the pregnancy continued, and a boy was born in 1986 with normal CK levels.

mutation site distal to pERT87. This is supported by the appearance of a deletion mutation associated with a recombination event occurring in the same meiosis¹⁵ (fig 1D). The sample size is too small to draw conclusions on whether the male and female contribution to mutation differs. However, the observed 1:2 male:female ratio is approximately what one would expect with equal mutation rates in male and in female derived X chromosomes.^{17 18}

Discussion

MAP OF THE DMD REGION

The physical order of loci and the linkage data we

obtained in this study (table 5) are not significantly different from published data,¹¹ although the genetic distance of DMD from DXS84 (754) reported by various groups seems to vary considerably: from the 5 cM we and other continental European groups have found up to 19 cM. Taking into account all reports, as summarised in the Eighth Human Gene Mapping Report (Helsinki), it seems that the average genetic distance of DXS84 is about 12 cM. A revaluation of all the data available is therefore clearly indicated, to see if there is heterogeneity of recombination and whether some families contribute far more to the recombination frequency than others.

Recently Kunkel *et al*¹⁰ reported a major collaboration in which 86 DXS164 deletions from 1201 independent DMD patients were described. The deletions appeared to extend both distally and proximally from pERT87. From three point segregation data, DXS164 maps proximal to the majority of the DMD mutations. However, cases are also reported where p87 maps distal to the mutation site (recombinations, translocation breakpoints, or deletion directions). Therefore, pERT87 might be considered as part of the 'DMD locus'. Alternatively, mutations on one side of pERT87 may act through affecting an activity on the opposite side. The same arguments hold true for the X;21 translocation junction clones (pXJ series) reported by Ray *et al*,¹⁹ located between DXS84 and DXS164. As long as uncertainty exists about the position of the DMD mutations relative to DXS164 and XJ1-1, markers should be used that are certain to be flanking the mutation site, except when deletions are detected by either pERT87 or pXJ. As flanking markers, 754 and C7 currently provide the best combination.

Using pulse field electrophoresis, van Ommen *et al*²⁰ have estimated that the genomic map of the region involved in muscular dystrophies of the Becker and Duchenne types contain between 1 and

4.2×10^6 base pairs. A generalised mapping function would suggest that a recombination frequency of 1% is equal to a physical distance of one million base pairs at the chromosome level. However, around the *DMD* locus, a 1% recombination distance apparently corresponds to only 1×10^5 base pairs. The observed high recombination frequency, the deletions,¹⁰ some of which are correlated with crossing over,¹⁵ and the X;autosome translocations¹⁹ clearly indicate the genetic instability of the Xp21 region. A number of other examples of genetic instability have been described: in the human α and β globin gene clusters^{21,22} where unequal crossing over gives rise to deletion mutants; the human LDL receptor locus²³ where Alu sequence mediated recombination causes deletions and duplications; and in the mouse T/t complex²⁴ where short chromosomal inversions cause illegitimate recombinations.

CARRIER DETECTION AND PRENATAL DIAGNOSIS

Both carrier detection and prenatal diagnosis have proved to be possible with greater than 98% reliability in about 75% of cases (pERT87 deletions not included). DNA analysis using bridging markers alone gives a reliable result in approximately 75% of cases; this is the inherent risk when using bridging markers between which recombination can occur in a significant proportion of cases. For example, when bridging markers both have a recombination distance of approximately 10 cM with *DMD*, such as *DXS84* (754) and *DXS28* (C7), then one in five offspring will show recombination. Creatine kinase levels may be extremely helpful, especially where crossovers within the *DMD* region are detected or no phase information is present.

Conclusions

We have standardised the procedure for diagnosing *DMD* with RFLPs by setting up a particular sequence of probes and enzymes with a view to obtaining optimal information with as few restriction enzymes and probes as possible, for example, by rehybridising the blots with more probes to achieve a relatively quick diagnosis (two to three weeks). Assuming that *DXS164* is located within the *DMD* region, the *DXS84* locus (754) should be considered to be the closest proximal marker and *DXS28* (C7) to be the closest distal marker. Extension of the cloned sequences at the loci bridging the *DMD* deletion/mutation region, or increasing the heterozygosity at those loci, is needed to improve diagnostic efficiency, which is at present 75% due to lack of informative RFLPs and recombination events.

We anticipate that cloning of the gene(s) responsible for the Duchenne phenotype will not essentially change the diagnosis of *DMD*, given that the mutations vary in both size and position. Thus, unless methods are developed to identify rapidly the precise nature of individual mutations in each family affected, the use of flanking RFLP markers will remain the primary diagnostic method, at least for the next few years.

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