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Isolation of a conserved sequence deleted in Duchenne muscular dystrophy patients

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SUMMARY
We have isolated a DNA sequence (HIP25) by subtraction- hybridisation which is deleted in a number of Duchenne muscular dystrophy (DMD) patients. HIP25 is conserved in evolution and hybridises to human fetal and adult muscle mRNA. HIP25 is absent in human fetal fibroblast mRNA. Physical mapping data localise this sequence within Xp21 between the breakpoints of X;autosome translocations found in two females suffering from the disease. HIP25 is a candidate exon sequence for the basic defect in DMD boys deleted at this locus.

INTRODUCTION

Duchenne muscular dystrophy is an X-linked recessive disorder affecting 1 in 3000 newborn males (see reference 1 for review). Affected boys are normally wheelchair-bound by the age of 11 and die in their late teens. A milder X-linked muscular dystrophy, Becker muscular dystrophy (BMD), shows similar clinical features. Affected boys are wheelchair-bound much later than for DMD and in some cases the condition can be very mild indeed with patients living a normal life span. Both DMD and BMD have been mapped to the same region of the human X chromosome within Xp21 using restriction fragment length polymorphisms (RFLPs) and genetic linkage analysis in affected families (for review see reference 2). This has led to improved carrier status determinations in females and also to prenatal diagnosis[3]. Twelve cases of the clinical manifestation of DMD/BMD in females have been published where the females possess balanced X;autosome translocations with the breakpoint on the X chromosome within Xp21. In these patients the translocated X chromosome is active and the normal X chromosome is preferentially inactivated (for review see reference 4). Cytogenetic analysis of the breakpoints on the X chromosome suggests that they occur in different sub-bands of Xp21[5]. Recently, two DNA sequences (pERT87[6] and XJ1.1[7]) have been isolated which are deleted in approximately 7% of affected boys. However, both of these sequences recombine with the DMD locus in 5% of the meioses so far studied[8]. These data suggest that the DMD and BMD

loci are extremely large (at least a megabase) and complex (for review see reference 9). More recently, Monaco et al[10] have isolated a candidate gene sequence from the pERT87 region and found that it hybridises to a large 16kb mRNA species in fetal muscle. The relationship of this sequence to the development of the Becker and Duchenne phenotypes remains to be determined.

We have adopted several strategies for isolating candidate genes for DMD and BMD within the Xp21 region. One approach has been to use subtraction hybridisation to isolate sequences lying within small deletions of Xp21. This method was first used for analysing Y chromosome sequences by Lamar and Palmer[11] and was the technique used by Kunkel and colleagues[12] for isolating the pERT87 locus. In this paper we describe the application of a modification of these methods which we have used to isolate a conserved sequence mapping between translocation breakpoints in affected DMD females.

METHODS

HIP reaction

Subtraction hybridisation was carried out in 2.4M phosphate buffer (1.2M Na_2HPO_4, 1.2M NaH_2PO_4 pH 6.8) at 76°C for 18 hours. These conditions were obtained from Avery et al[13].

DNA extraction and Southern blotting

Blotting was carried out as described by Davies et al[14]. Washing was carried out at 0.1 X SSC at 65°C; exposure 2 days at -70°C.

RNA preparation

Dot analysis was performed by spotting RNA onto hybond filters (Amersham) and hybridised as described for Northerns[15]. RNA was isolated by homogenising tissue in 8 volumes of 4M LiCl, 8M urea. The RNA was left to precipitate for 48 hours, and then centrifuged. The resultant pellet was resuspended in 8M urea, phenol and chloroform extracted and finally ethanol precipitated using 1/15 vol 3M NaAc + 2.5 vols EtOH. Yields 0.02-0.05% of sample weight.

RESULTS

The technique we employed is based on the rapid reassociation of DNA molecules in the presence of a high concentration of inorganic phosphate ("HIP" reaction)[13]. The DNA used was from a cell line derived from a patient, BB, who has a deletion of Xp21.2 and suffers from DMD, CGD, RP and the McLeod syndrome[16]. Sonicated DNA from patient BB was denatured and allowed to reassociate in the presence of DNA from the X library obtained

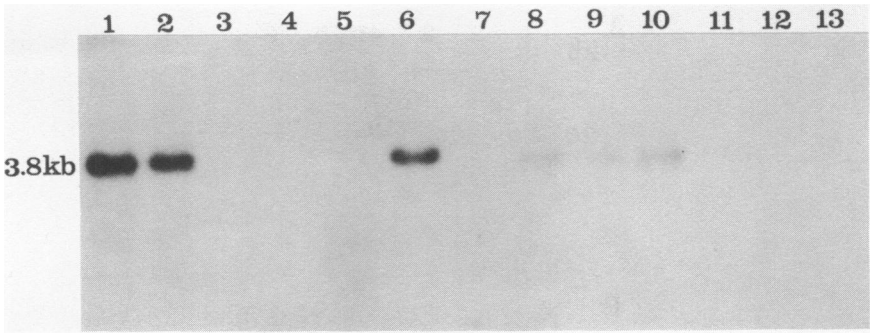

Figure 1 Physical mapping of HIP25. Southern blot analysis of Hinc II-digested DNA from (1) human female DNA; (2) human male; (3) patient BB; (4) hybrid cell line containing Xp11.0-Xqter; (5) hybrid cell line containing Xp21.1-Xqter; (6) hybrid line containing Xpter-Xqter; (7) hybrid containing Xp21-Xqter, derivative X chromosome of the X;11 translocation; (8) hybrid cell line containing Xpter-Xcen; (9) hybrid cell line containing Xpter-Xqter; (10) hybrid cell line containing the derivative X chromosome of the X;21 translocation; (11) hybrid cell line containing the derivative chromosome 21 of the X;21 translocation; (12) Chinese hamster DNA; (13) mouse DNA. 5μg of DNA per track, washing at 0.1 X SSC, 65°C; exposure 3 days at -70°C.

from the Life Sciences Division, Los Alamos National Laboratory. The X-library DNA had been grown up in bulk and the inserts excised with the enzyme Eco RI. The purified inserts were then further digested to completion with the enzyme Sau 3AI before being used in the reassociation experiments. The reassociated DNA was cloned into a Bam HI-cut plasmid vector to select for those molecules mapping within the BB deletion. Forty colonies were analysed of which six independent clones had inserts suitable for labelling. Of these, two were moderately repeated sequences, two were single-copy sequences on the X chromosome but not localised within Xp21, and one was a single-copy sequence localised within the BB deletion. One failed to give a signal in the hybridisation because its insert was too small. The clone which mapped within the BB deletion was given the laboratory acronym HIP25.

Figure 1 illustrates the physical mapping of HIP25 within the region Xp21 using a hybrid panel. HIP25 gives a negative signal both for BB DNA (track 3) and in the hybrid DNA originally thought to contain Xp11.3-Xqter described by Wieacker *et al*[17] but now thought to have a breakpoint within Xp21 in the BB deletion (track 5)[11]. A positive signal was obtained in the hybrid with the derivative X chromosome of the X;21 translocation (track 10) but HIP25 did not hybridise to the DNA from the derivative X chromosome of the X;11 translocation (track 7). Hybrids in tracks 4, 5, 6 and 7 are

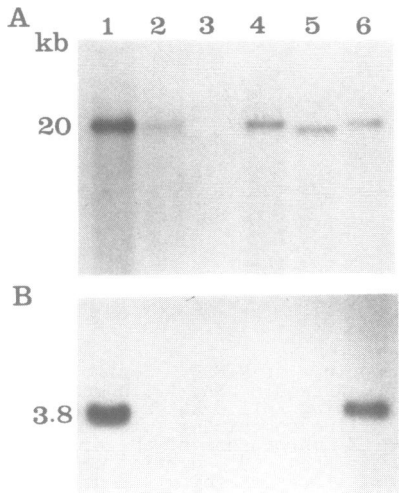

Figure 2 Analysis of patients with HIP25: (1) normal female; (2)-(5) DMD patients; (6) normal male. Pst I digests of 5µg DNA samples. A: hybridisation to pERT84; B: hybridisation to HIP25. Blotting was carried out as described by Davies et al[19]. Washing was carried out at 1 X SSC at 65°C; exposure 2 days at -70°C.

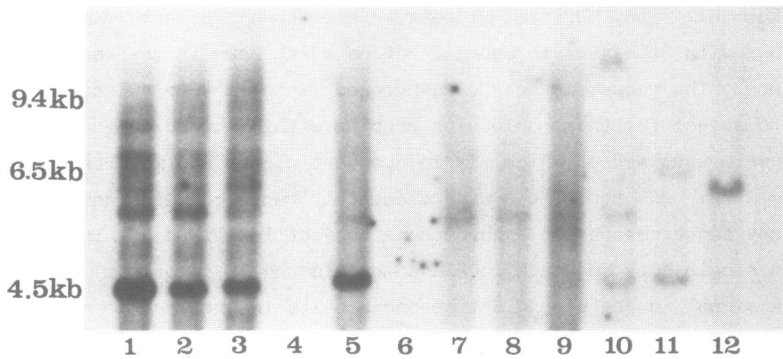

Figure 3 Hybridisation of HIP25 to DNA from different species. 5µg of DNA per track digested with Eco RI. Hybridisation at 30% formamide, 42°C. Washing at 1 X SSC, 65°C exposure 4 days. Track 1: female control; track 2: male control; track 3: chimpanzee; track 4: blank; track 5: hybrid containing Xpter-Xqter (see track 6 in figure 1); track 6: blank; track 7: mdx mouse; track 8: normal mouse; track 9: Chinese hamster; track 10: Xenopus laevis; track 11: Xenopus borealis; track 12: chick.

described in Wieacker et al[17], the hybrid in track 9 is HORL9.X42.2[18] and the X;21 translocation hybrids are described in Ray et al[7]. On the same hybrid panel, the clone 754 gave a positive signal for both derivative X

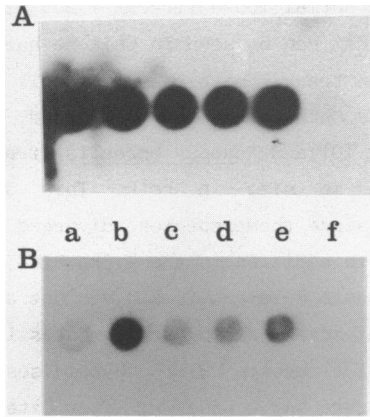

Figure 4 RNA dot analysis of HIP25 at 3 X SSC, 65°C (A) and 0.1 X SSC, 65°C (B). Tracks: (a) 100μg of total human adult fibroblast RNA; (b) 3μg of human fetal muscle poly(A)$^+$ mRNA; (c) 3μg of human adult muscle poly(A)$^+$ mRNA; (d) 3μg of rat adult muscle poly(A)$^+$ mRNA; (e) 10μg of mouse teratocarcinoma cell line total RNA; (f) 5μg of yeast tRNA. Dot analysis was performed by spotting RNA onto hybond filters (Amersham) and hybridised as described for Northerns[19]. A: washing at 3 X SSC, 65°C; B: washing at 0.1 X SSC, 65°C.

chromosomes which is consistent with previous localisations of 754[16,19]. This places HIP25 between the translocation breakpoints of the patients described by Verellen-Dumoulin et al[20] and Greenstein et al[21], with X;21 and X;11 translocations respectively. Since XJ1.1 is the cloned junction fragment of the X;21 translocation[7], this places HIP25 centromeric to this locus.

Figure 2B shows the mapping of HIP25 in DMD patients that are deleted for the pERT87 locus[8]. All the patients are deleted for HIP25. When the same blot depicted in figure 2A was hybridised to pERT84, which lies centromeric from pERT87, a positive signal was seen for all the samples except for the patient in track 3 (figure 2A). Thus the centromeric breakpoint of the deletions in most patients lies between pERT84 and HIP25. All patients are positive for the locus 754. In the patient in track 3 the breakpoint must lie between 754 and pERT84. We have also identified four patients who are not deleted for the pERT87 region but are deleted for HIP25[22].

We investigated the expression of HIP25 in muscle tissue and tested for evolutionary conservation. Figure 3 shows the result of the hybridisation of HIP25 to DNA from different species. At a stringency of 1 X SSC at 65°C, cross-hybridisation is observed with several species. A single main band

was seen both in mdx and normal mouse (tracks 7 and 8) as well as in chick (track 12). Three bands can be seen in Chinese hamster (track 9), one of which can also be seen in track 5 with a hybrid cell line containing the human X chromosome on a Chinese hamster background. Two bands can be seen in Xenopus laevis (track 10) and Xenopus borealis (track 11) although only one band is identical in size in both. This difference may reflect evolutionary divergence since these species diverged eight million years ago. The extra bands, in addition to the 4.5kb HIP25 band normally seen at high stringency in the human male and female, do not all dose with the X chromosome indicating autosomal homologous sequences in the human genome.

In an RNA dot blot assay, HIP25 hybridises strongly to mRNA from several tissue types (figure 4A). However, after extensive washing at 0.1 x SSC at 65°C, a strong signal was observed for human fetal muscle tissue but only weak hybridisation was seen for mRNA from human adult muscle and adult rat muscle mRNA (figure 4B). HIP25 does not hybridise to mRNA derived from fetal fibroblasts. Perhaps of interest is the hybridisation of HIP25 to mouse teratocarcinoma cell line RNA. Apart from the cell line RNA, which contained five times less mRNA, the RNA dots hybridise uniformly to a cDNA clone encoding glyceraldehyde 3'-phosphate dehydrogenase. Hybridisation of HIP25 to Northerns gives a smear at 3 x SSC, 65% and only faint hybridisation at 0.1 x SSC at 65°C with no discernible band (results not shown). This may be due to the degradation of a very large mRNA species or the cross-hybridisation of HIP25 to several mRNA species. The former may be more likely in view of the fact that HIP25 hybridises as a single copy sequence in Southern blots and the hybridisation extends all the way up the track with greater intensity to RNA larger than 28S RNA. Screening of cDNA libraries with HIP25 has so far failed to reveal a cDNA clone. This may be due to the fact that the gene is large and that HIP25 corresponds to the the 5'-end of a gene which would be underrepresented in an oligo-dT primed library. We are currently sequencing HIP25 in order to synthesise an oligomer that could be used as a primer for a new cDNA library.

CONCLUSIONS

The evidence presented here shows that a sequence, HIP25, mapping between the breakpoints in the X;11 and X;21 female translocations, is deleted in some Duchenne muscular dystrophy boys. Previous studies have localised pERT87 telomeric from the XJ1.1 and pERT84[23] loci. The order of sequences

on the short arm of the human X chromosome relative to HIP25 therefore is:

Xpter - pERT87 (DXS164) - XJ1.1 - HIP25 - pERT84 (DXS142) - 754 (DXS84) - Xcen

Since all but one of the deletions found in DMD boys in our laboratory have a breakpoint between HIP25 and pERT84, this may define a very important region for the expression of the DMD phenotype. Further studies on more patients with chromosome walks from HIP25 are being carried out to analyse this question.

Investigation of HIP25 in RNA Northern blots and dot assays shows it to hybridise strongly to mRNA from human fetal muscle. This observation, together with the sequence conservation of HIP25 suggest that this sequence may play an important role in muscle function. Further investigations are required to determine whether this region codes for a protein causally related to the DMD phenotype.

All of the patients in our laboratory deleted for XJ1.1 are also deleted for HIP25. Three patients are deleted for HIP25 but not pERT87-1, -8, -15, or 30. Thus the deletion of a region of Xp21 centromeric to pERT87 can also lead to the DMD phenotype. Pulsed-field gel analysis shows HIP25 and pERT87 to be on the same 430 kb Sac II fragment[24]. Thus, HIP25 could correspond to an exon towards the 5' end of the gene from the coding region of Monaco et al[10].

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