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Rapid mapping by transposon mutagenesis of epitopes on the muscular dystrophy protein, dystrophin

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ABSTRACT

Antibody-binding epitopes in the central helical region of the muscular dystrophy protein, dystrophin, have been mapped using a new strategy of transposon mutagenesis. Tn*1000* transposons carrying translation termination codons were introduced randomly by bacterial mating into a large fragment of dystrophin cDNA in a pEX2 plasmid to produce a library of transformants expressing truncated dystrophin fusion proteins. Epitopes were progressively lost as the expressed sequences were shortened, enabling the epitopes recognised by 22 monoclonal antibodies to be placed in order along the dystrophin molecule without *in vitro* manipulation of DNA. The C-terminus of each truncated fusion protein was precisely located within the dystrophin sequence by direct sequencing of pEX2 transformants using transposon-specific primers. Sequences as short as 7 and 17 amino-acids have been identified as essential for antibody binding in this way. Nineteen of the 22 monoclonal antibodies had been selected for their ability to bind both native and SDS-denatured dystrophin and 15 of these bind to one sequence of 74 amino-acids (residues 1431 – 1505 of the 3684 residue sequence). This may be an area of high immunogenicity or of close structural similarity between native dystrophin and the SDS-treated recombinant fragment used for immunization.

INTRODUCTION

Dystrophin is the 423kD protein product of a gene on the human X-chromosome which is altered by mutation in both Duchenne and Becker muscular dystrophies [1]. Becker patients, in particular, often produce truncated forms of dystrophin when genetic deletions are expressed as in-frame deletions in the protein product [2]. Such deletions have been detected using antibodies raised against different dystrophin regions [3] and monoclonal antibodies, accurately mapped to specific epitopes, could provide a powerful means of characterizing the effects of genetic deletions at the protein level. To this end, we have mapped 22 monoclonal antibodies against dystrophin using a new strategy of epitope mapping with transposons.

Previously, epitopes have been mapped using fragmentation of the antigen by chemical or proteolytic cleavage [4], chemical synthesis of peptide fragments [5], competition between antibodies [6], protection by antibody against proteolytic cleavage [7] or against chemical modification [8] and natural variants in different tissues or species [9]. In a few cases, epitopes have been characterized by X-ray crystallography of antibody-antigen complexes [10]. With antigens expressed from cDNA in bacterial plasmids, antigenic fragments can be produced by deletion mutagenesis with restriction enzymes or exonucleases [11] and by construction of epitope libraries of random cDNA fragments [12,13]. Variants can be produced by site-directed mutagenesis [14]. These methods, however, involve extensive *in vitro* manipulations or are limited by available sites within the cloned sequence.

In a new strategy, we have found that epitopes on dystrophin can be located rapidly and efficiently using transposition mutagenesis. In a simple bacterial mating, the Tn*1000* transposon was inserted randomly [15] into a pEX2 expression vector carrying dystrophin cDNA. Translation termination codons at both ends of Tn*1000* and in all three reading frames resulted in the production of shortened dystrophin fusion proteins. Those fusion proteins which had lost epitopes were identified by direct antibody screening of bacterial colonies, so that no further DNA manipulation was needed to place the epitopes in a linear sequence. To locate the epitopes more precisely, the new C-termini of fusion proteins resulting from transposon insertion were determined by dideoxy sequencing of the cDNA using universal Tn*1000*-specific primers.

This simple strategy for epitope mapping by transposon mutagenesis both entirely confirms and greatly refines the results of chemical cleavage mapping and has obvious general applications.

MATERIALS AND METHODS

Plasmids

pEX2:Cf23b, which carries a 2.8kb *Eco*RI-*Pst*I segment of dystrophin cDNA from bases 2648 to 5457 [16], was generously provided by Drs D.R. Love and K.E. Davies (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford).

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Bacteria

MH1498 is F+ *srl*::Tn*10 recA1 deoC* (λcI857). MH1512 was used as recipient in bacterial matings and is *srl*::Tn*10 recA1 rpsL lac* Z_{am} *Δ(bio-uvrB) trpE* (λNam7-*N*am53 *c*I857 *ΔH*1), a recombination-deficient derivative of M72 [17]

Antibodies

Monoclonal antibodies were produced from two mice immunized with the fusion protein expressed by pEX2:Cf23b after purification by gel filtration in SDS [16]. Three antibodies not described previously, MANDYS21, 23 and 24 came from the same mice.

Bacterial culture

Routine growth was at 32°C to maintain repression of the λp_L promoter of the pEX2 vector. L broth or L agar were used throughout with 5μg/ml thymine for growth of the donor strain MH1498. Cells were transformed by the $CaCl_2$ method [18]. Care was taken to use purified single colony isolates of transformants and transposed stocks produced by mating.

Transposition protocol

The target plasmids were introduced into MH1498 and transformants were selected for ampicillin resistance. For mating, 10ml cultures of log phase donor and recipient cells were centrifuged, gently resuspended together in 10ml of antibiotic-free medium and transfered to a large (1 litre or larger) flask pre-warmed to 32°C. After 1h at 32°C without shaking, the mixture was centrifuged and washed twice in an equal volume of 10mM $MgSO_4$ to remove exogenous b-lactamase. 10−100μl samples were spread on plates containing 100μg/ml streptomycin, 50μg/ml ampicillin and 50μg/ml methicillin (Sigma Chemical Co.). Surplus mixture was stored at 4°C for later plating, if necessary. Individual colonies from the selective plates were restreaked on streptomycin-ampicillin-methicillin agar.

Screening with antibodies

Up to 100 ampicillin/streptomycin-resistant colonies were steaked onto replica plates and grown overnight at 32°C. Each plate was screened with two antibodies. The colonies were lifted onto dry nitrocellulose circles (Schleicher & Schull, BA85) which were placed on Whatman 3MM paper soaked in L/ampicillin broth preheated to 32°C. Expression of pEX2 fusion protein was induced by incubation for 2.5 hours at 42°C. A second nitrocellulose circle was placed on top to form a sandwich. Lifts were placed on Whatman 3MM paper soaked with 5% SDS and incubated for 30 min at 90°C to lyse the cells [19].

The nitrocellulose sandwich was placed between Whatman 3MM paper and subjected to electrophoresis in 192mM glycine-25mM Tris pH 8.3 for 5mins in each current direction at 400mA. Lifts were then separated and subjected to electrophoresis for a further 25 mins at 400mA with the colonies facing the cathode.

Lifts were blocked in 3% skimmed milk powder in incubation buffer (0.05% Triton X-100 in PBS). After two 5min. washes in PBS, lifts were incubated with monoclonal antibody culture supernatant (1/100 dilution in incubation buffer/1% horse serum/1% foetal calf serum/0.3% bovine serum albumin) for 1hr at 20°C. After three 5min washes with PBS, blots were incubated with peroxidase-labelled rabbit anti-(mouse Ig) (DAKOpatts; 1/1000 in the same buffer) for 1hr. After four 5min washes with PBS, substrate was added (0.4mg/ml diaminobenzidine (Sigma) in 25mM phosphate-citrate buffer pH 5.0 with 0.012% H_2O_2).

To screen with larger numbers of antibodies (11−28), a 'miniblotter' apparatus (Immunetics or BioRad) was used. A sheet of nitrocellulose, cut to fit the miniblotter, was placed on L/ampicillin agar. Using a ruler, test clones were drawn with a sterile toothpick across the full width of the sheet as horizontal lines 2mm apart and grown overnight at 32°C. Subsequent steps were as above, except that the monoclonal antibodies were applied as vertical lanes across all clones in the miniblotter. On some occasions, clones were prescreened for transposon insertions within the plasmid cDNA inserts by digesting with *Eco*RI and *Pst*I and checking for a size shift in the 2.8kbp Cf23b fragment on agarose gels.

Identification of transposon insertion sites by DNA sequencing

Plasmid DNA was prepared by alkaline lysis [18] and subjected to dideoxy sequencing [20] using Sequenase v.2.0 protocols (United States Biochemicals, Cleveland, Ohio) with α-[^{35}S]-dATP (Amersham International plc) and 6% polyacrylamide-urea gels at 55°C. Sequencing primers specific to the γ and/or δ ends of Tn*1000* can be used. The δ primer, AGGGGAACTGAGAGCTCTA, is homologous to bases 86 to 68 of Tn*1000* and the 3′ end of the γ primer, CAGCTACAA-CATACGAAAG, is 70bp from the γ terminus of Tn*1000* [21]. Gels were soaked in 10% acetic acid for 30 min to remove urea before drying and exposing to Kodak XAR-5 film.

RESULTS

The approach to epitope mapping using the bacterial Tn*1000* transposon is outlined diagramatically in Fig. 1. Transposition into the dystrophin coding sequence invariably results in premature translation termination and a truncated protein product. Epitopes on dystrophin can be ordered because shorter truncated proteins will be recognised by fewer antibodies. Precise location of the Tn*1000* insertion by DNA sequencing then allows more detailed mapping of the epitopes. In practice, the mutant protein will carry between 2 and 36 transposon-encoded amino-acids at its C-terminus, depending on the reading frame. (Table I).

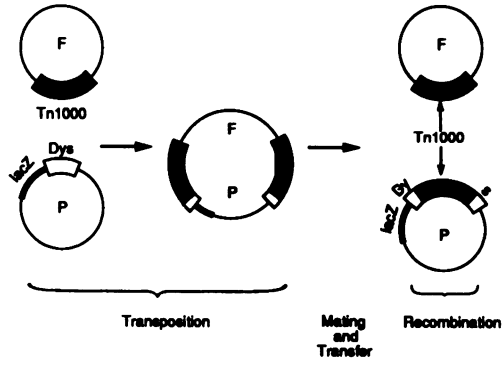

Fig. 1. Transposition mechanism for Tn*1000* mutagenesis of plasmid DNA. Tn*1000* on sex factor F tranposes into the target plasmid P, duplicating the transposon and linking F and P together in the co-integrate. The co-integrate is transferred to a female cell where site-specific recombination in Tn*1000* restores F and leaves P mutated by a Tn*1000* insertion.

Tn1000 transposition mutagenesis – rationale

Tn*1000* is a natural part of the *E. coli* F sex factor and transposes into other plasmids co-habiting the same male bacterium at low frequency (about 10^{-6} detectable events per cell per generation). During transposition, Tn*1000* is duplicated in a transitory linkage of F and target plasmids and hence mating transfers a composite of F joined with the target plasmid. Once in the female or recipient, this co-integrate molecule undergoes a site-specific recombination reaction to restore F and release a target plasmid with a transposon insertion (Fig. 1) [22].

The first step is to transform the donor strain, MH1498, with the target plasmid. MH1498 has a *recA1* mutation to improve plasmid stability and carries the λ*c*I857 prophage to repress expression from the λ promoter of pEX2. The *rpsL*$^+$ and *deoC* markers of MH1498 confer streptomycin sensitivity and thymine dependency respectively and are used to counter-select against the donor after mating. Other similar donors would be equally useful but F′ donors derived from strains such as JM103 and DH5α are unsuitable because duplication of Tn*1000* sequences in an F′ leads to multiple rearrangements of the target plasmids. The presence of streptomycin and lack of thymine prevents growth of donors after mating and only those streptomycin-resistant recipients which received the target plasmid during mating can grow in the additional presence of ampicillin. Methicillin improves selectivity by preventing exogenous β-lactamase from rescuing Amp^s cells and is especially recommended with dense mixtures of conjugating cells. Since the only means of target plasmid transfer is via a transposition event, all surviving recipients on the selective plates contain tranposed target plasmids.

Table 1. Position of translational stop codons in ends of Tn*1000*.

Tn*1000* end	frameshift[1]	stop codon / bases from end	additional amino acids
δ	0	TGA / 7-9	2
	1	TGA / 108-110	36
	2	TAA / 35-37	12
γ	0	TGA / 7-9	2
	1	TGA / 51-53	17
	2	TGA / 107-109	36

[1] number of bases between last complete codon of target sequence and start of Tn*1000*.

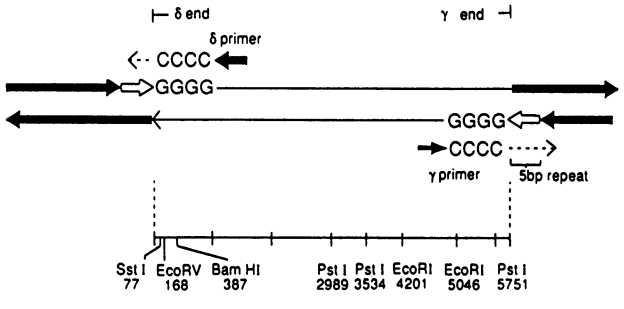

Fig. 2. Structural features of Tn*1000* used in determining points of insertion in target sequences. The transposon is shown inserted in the δ/γ orientation, but it could equally well be γ/δ. Sequences extending from γ and δ primers show a common CCCC tract before entering the target sequence. Transposition occurs via a 5bp staggered cut which leads to a 5bp duplication of target sequence (indicated by arrow heads). Restriction enzyme sites useful in positioning and orientating Tn*1000* are based on earlier work [15] and the Tn*1000* sequence, kindly provided by Dr. R. Reed.

Screening for Tn1000 insertional mutations

The 8.6kbp pEX2:Cf23b plasmid makes a large fusion protein consisting of a 116kD β-galactosidase derivative with the cDNA insert-encoded polypeptide at its C-terminal end [16]. Clearly, only those transposon insertions which interrupt the cDNA insert are useful for epitope mapping so the proportion of useful transposon mutants will depend on the size of the insert relative to pEX2. In the case of the 2.8kbp Cf23b dystrophin insert , about 30% of transposed derivatives contained Tn*1000* within

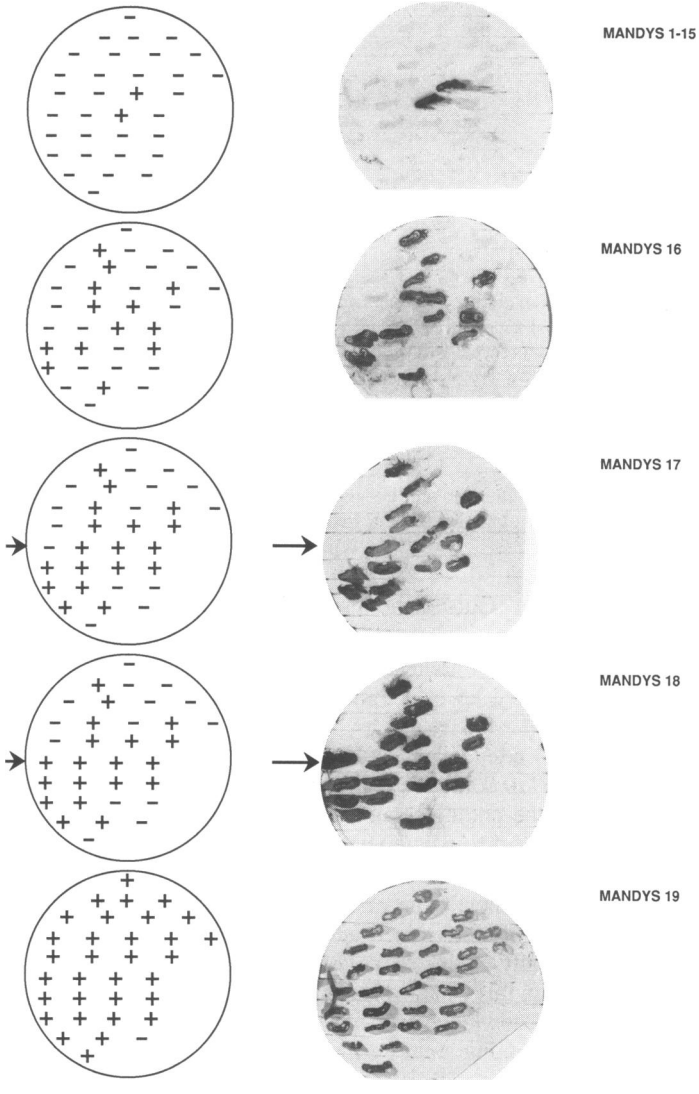

Fig. 3. Colony lift method of screening clones for monoclonal antibody binding. *E. coli* clones with transposon-carrying plasmids (pre-screened in this example for a transposon within the cDNA insert) were grown on replica plates, lifted onto nitrocellulose circles and tested with different monoclonal antibodies as described in Methods. The layout of clones on the plate and their reaction with each antibody is shown diagramatically alongside actual lifts. Antibodies which bind closest to the C-terminus of the recombinant fusion protein (MANDYS 1–15 inclusive) react with fewest clones (those producing the longest fusion proteins), whereas N-terminal antibodies (e.g MANDYS19) also react with clones producing shorter fusion proteins. Arrows indicate the single clone which clearly carries the MANDYS18 epitope but not the MANDYS17 epitope.

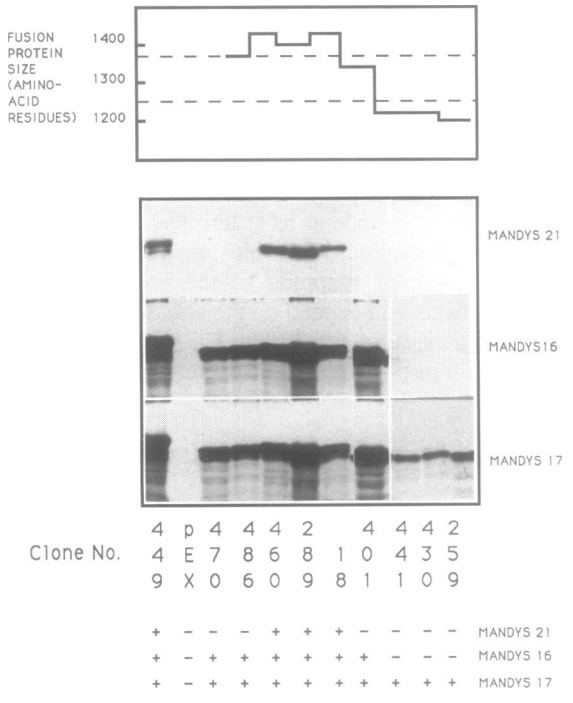

Fig. 4. Examples of colony screening by SDS-PAGE and Western blotting. Total protein extracts of *E. coli* transformants were analysed on 7% polyacrylamide gels and blots were reacted with antibodies as described in Methods. The upper panel shows the actual size, determined by DNA sequencing, of each fusion protein in the gel lanes of the lower panel (except 449 and 470, which have not been sequenced); dotted lines show the minimum lengths required for binding MANDYS21 and 16. Three examples of antibodies binding to a series of truncated fusion proteins are shown. Apart from the fusion protein and its degradation products, the only other protein is aggregated material at the top of the gel. Antibody binding results are summarized in the bottom panel.

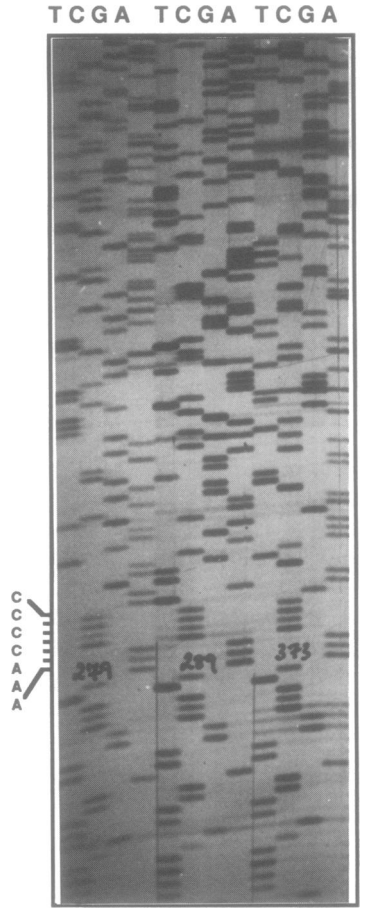

Fig. 5. DNA sequencing to determine transposon insertion position. The polymerase initially copies the transposon sequence up to AAACCCC; subsequently the dystrophin cDNA sequence at the transposon insertion position differs for each clone: AAGAATG... for 279; TATTGCA... for 289; GAACTCA... for 373.

the insert cDNA. Outside the insert cDNA, transposons inserted into the *lacZ* region of the fusion protein will produce clones negative for all antibodies, while insertions elsewhere in the plasmid will have no effect and clones will be positive for all antibodies.

About 500 independent isolates of pEX2:Cf23b mutated at random with Tn*1000* were collected. Screening for insertion of Tn*1000* into the coding sequence was performed in two ways. The first involved restriction endonuclease digestion of plasmid DNA to identify transpositional disruption of the 2.8kb *Eco*RI-*Pst*I coding fragment. The orientation of Tn*1000* in the target DNA can also be determined at this stage by the judicious use of asymmetrically-cutting enzymes, such as *Bam*HI, *Sst*I or *Eco*RI (Fig.2), though this will also become clear after DNA sequencing and advance knowledge of orientation is unnecessary.

A faster screening strategy involved identification of mutant clones which react with some antibodies but not others, since these must arise by transposon insertion between epitopes. Fig.3 shows direct antibody screening of 33 clones, which for this example were preselected from earlier screens. This experiment alone determines the order of epitopes along the dystrophin molecule, since antibodies which bind to a greater number of clones will recognise epitopes closer to the N-terminus of the fusion protein.

Colony blot results were confirmed by SDS-PAGE of *E.coli* extracts, which can also reveal size differences between different truncated fusion proteins, even though these differences are small compared with the size of the fusion proteins (Fig.4).Clone 449 binds all antibodies and produced the largest fusion protein. The pEX2 plasmid control, which expresses the *lacZ* gene only, shows that antibody binding is completely dependent on the presence of a cDNA insert. The critical clones for mapping the MANDYS21 epitope are illustrated; clone 486 is the longest which fails to express the epitope and 289 is the shortest which does express it. Also shown is the shortest which binds MANDYS17, clone 259.

Mapping sites of Tn1000 insertion

The precise site of transposon insertion was determined by DNA sequencing with primers complementary to unique sequences in the γ and δ ends of Tn*1000* (Fig.2). A typical sequencing gel with three clones which read 'downstream' into the dystrophin cDNA 3′ to the tranposon (Fig.5) shows the terminal AAACCCC of Tn*1000* bases in the extending strand after which dystrophin sequences start. Since Tn*1000* can insert in either orientation, a single primer will read 'upstream' into the non-coding cDNA strand of the double-stranded plasmid in 50% of cases. It is necessary, therefore, to compare both the direct sequence and also the reverse complementary sequence with the known dystrophin cDNA sequence and one of the two should match. An alternative approach is to determine the orientation in advance

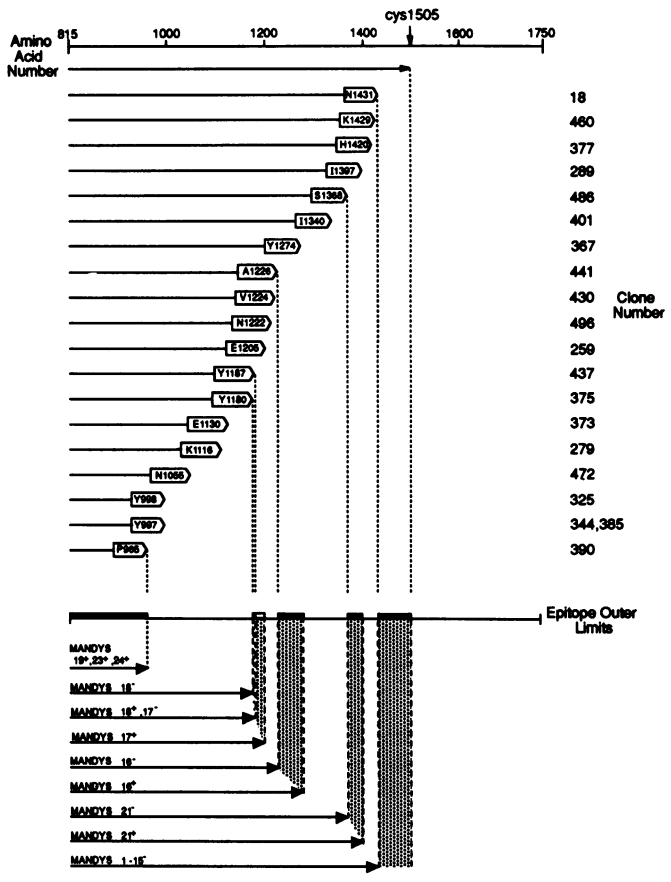

Fig. 6. Epitope map of dystrophin. The lengths of truncated dystrophin fusion proteins produced from clones with Tn*1000* insertions are shown in the upper section with C-terminal dystrophin amino-acid residues indicated in the boxed arrows. The reactivity of these clones to monoclonal antibodies is shown in the lower section, with broken vertical lines delineating sequences essential for antibody binding.

and use different primers for each orientation (Fig.2). When Tn*1000* transposes, it duplicates five bases of the target DNA at the point of insertion [22]. Thus, when sequencing 'downstream', the first five bases are actually duplicates of the five bases up to the point of insertion. Consequently, the truncated dystrophin coding sequence on the other side of the transposon is 5 bases longer than might be naively expected (Fig.2). This minor correction does not apply, of course, to 'upstream' sequencing into the non-coding strand.

Epitope mapping of dystrophin

The epitope map of dystrophin (Fig.6) confirms that the linear sequence of epitopes corresponds to a linear order of transposon interruptions. The epitope order was determined from Western blots (Figs 3 and 4) and the points of interruption of dystrophin sequences were obtained by DNA sequencing of 21 transposon-containing plasmids. The N-terminus and C-terminus of the mapped area are defined by the *Eco*RI cloning site at amino-acid 815 and a cysteine cleavage site, Cys 1505.

Insertion 390, terminating at P965, gave a positive response with MANDYS19, 23 and 24, but not with any other antibodies. Insertions 344, 385, 325, 472, 279, 373 and 375 would produce progressively longer fusion proteins but were still only recognised by the same three antibodies. However, insertion 437, seven

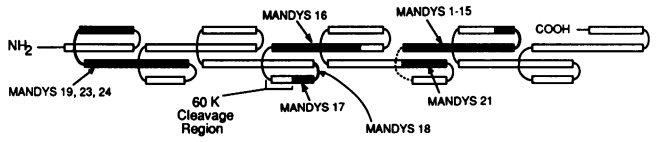

Fig. 7. Position of epitopes within the 108kD central rod fragment of dystrophin. The particular triple-helical array shown is that proposed in [24]. The 'short' H2 helix on this model is shown followed by a dotted line as 'linker' and the approximate position is shown of a proteolytic cleavage between MANDYS 16 and 17 which removes a 60kD dystrophin fragment from the fusion protein [16].

amino-acids longer than 375, reacted additionally with MANDYS18 (see arrow in Fig.3) showing that these amino-acids are essential for the MANDYS18 epitope. A synthetic peptide which included these 7 amino-acids (MTQAEEEYLERDFEYK) was found to bind MANDYS18 specifically in an elisa assay performed as in [9] (results not shown). The next insertion,259, was recognised by MANDYS17 as well as earlier antibodies, suggesting that the MANDYS17 epitope lies between Y1187 and E1205. The same reasoning was applied to identify residues essential for MANDYS16 (A1226 to Y1274), MANDYS21 (S1368 to I1397) and the MANDYS1−15 group (N1431 to C1505).

DISCUSSION

The dystrophin epitope order and location obtained by transposon mutagenesis considerably extends that previously obtained by cleavage of the fusion protein at cysteine residues [16]. The new transposon approach has separated the three closely spaced, MANDYS16−18 epitopes and has identified sequences of 48, 17 and 7 amino-acids respectively which are essential for the binding of each antibody. Previously, MANDYS16−18 were not separated by cysteine cleavage, though a proteolytic cleavage showed that MANDYS16 is closer to the C-terminus than the other two [16]. In contrast, the failure of transposons to separate the MANDYS1−15 group mirrors earlier results and suggests that their epitopes lie very close together. They are not identical antibodies, however, since three of them do not recognise chicken dystrophin, only one recognises dystrophin in fish and they belong to 3 different Ig subclasses (unpublished data). A 30kD dystrophin fragment (L1181 to F1388), the immunogen for a widely-used polyclonal antiserum against dystrophin, was found to be much more immunogenic than other recombinant fragments examined [1] and our preliminary chemical cleavage mapping left open the possibility that the MANDYS1−15 epitopes might also map to the same 30kD region [16]. More precise mapping with transposons, however, now shows that the MANDYS1−15 antibodies are novel in that they recognise a different part of dystrophin, closer to the C-terminus.

The high immunogenicity of the MANDYS1−15 region could be intrinsic to the amino-acid sequence or a consequence of protein structure. One model for the N1431-C1505 sequence [23] suggests a shortened main helix (H1) with non-helical linkers at each end. Linkers with relatively little conformation are possible binding sites for antibodies which recognise both native and denatured dystrophin. In a slightly different model for dystrophin structure, the MANDYS1−15 epitopes would follow an unusual 'short' H2 helix (Fig.7) which leaves surrounding helices more exposed [24]. While this may be co-incidental, a fusion protein immunogen which refolded poorly after SDS treatment might

elicit more antibodies against exposed dystrophin helices than against the mainly triple-helical structures of native dystrophin, thus explaining the preponderance of antibodies against this region. MANDYS21, 23 and 24 (Fig.7) bind very strongly to recombinant fusion protein but not at all to native dystrophin (results not shown), which is consistent with the suggestion that only parts of the recombinant fragment can refold like the native protein. Further narrowing of the outer limits of this 74 amino-acid sequence may favour one or other of the two models.

Other explanations of the uneven distribution of the epitopes shared by native and recombinant dystrophin (Fig.7) cannot be ruled out. These include differences between native dystrophin *in situ* and recombinant dystrophin due either to post-translational modification or to protein-protein interactions *in situ* which obscure parts of the dystrophin rod. Eighteen of the 22 hybridomas were obtained from one mouse and four (MANDYS11, 12, 18 and 23) from a second mouse. Antibodies from both mice recognize each of the three epitope regions in Fig.7, so their distribution cannot be explained as a fortuitous immune response of individual mice.

Transposon mutagenesis is ideally suited to mapping large numbers of antibodies and epitopes. It is easier to identify insertions which separate epitopes than insertions close to the outer limits of the epitope map. The outer limits in Fig.6 are determined by the *Eco*RI site and a cysteine cleavage site. The method is subject to the caveat, common to a number of mapping techniques, that structural consequences of deletions may, in some cases, cause loss of antibody binding. Under these circumstances, the sequences we have identified might be essential for maintaining the epitopes without being in direct contact with the antibody, the actual contact amino-acids being closer to the N-terminus than those required for maintaining their structure. This problem, however, is more significant for highly-conformational epitopes which are unique to correctly-folded antigen and these, unlike the MANDYS epitopes, rarely survive drastic treatments with SDS and β-mercaptoethanol. It could be obviated to a considerable extent by using transposons to introduce new translation initiation sites as well as premature termination sites and so define both N-terminal and C-terminal limits to the epitope (cf. DNAse I fragment epitope libraries [13]) and some progress towards constructing suitable transposon derivatives has already been made [25].

In summary, these experiments have shown the general usefulness of transposons in epitope mapping because of the ease of producing a nested series of insertional mutations containing premature translational stop signals. Tn*1000* was chosen because it can be used to make and select a population consisting entirely of transposed plasmids in one simple, inexpensive step, using genetically unsophisticated bacteria which are already widely circulated and generally available. DNA sequencing from Tn*1000* primers has been independently described [21,26,27,28]. Other tranposons could also be used, remembering that elements such as Tn*10* would be unsuitable because, with their long terminal repeats, there would be no unique regions for priming DNA sequencing reactions.

ACKNOWLEDGEMENTS

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