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High resolution deletion breakpoint mapping in the DMD gene by whole cosmid hybridization

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ABSTRACT

The locus DXS269 (P20) defines a deletion hotspot in the distal part of the Duchenne Muscular Dystrophy gene. We have cloned over 90 kilobase-pairs of genomic DNA from this region in overlapping cosmids. The use of whole cosmids as probes in a competitive DNA hybridization analysis proves a fast and convenient method for identifying rearrangements in this region. A rapid survey of P20-deletion patients is carried out to elucidate the nature of the propensity to deletions in this region. Using this technique, deletion breakpoints are pinpointed to individual restriction fragments in patient DNAs without the need for tedious isolation of single copy sequences. Simultaneously, the deletion data yield a consistent restriction map of the region and permit detection of several RFLPs.

A 176 bp exon was identified within the cloned DNA, located 3′ of an intron exceeding 150 Kb in length. Its deletion causes a frameshift in the dystrophin reading frame and produces the DMD phenotype. This exon is one of the most frequently deleted exons in BMD/DMD patients and its sequence is applied in a pilot study for diagnostic deletion screening using Polymerase Chain Reaction amplification.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder with a progressive, muscle wasting course and a fatal outcome before the age of thirty (review by Emery 1988 [1], Moser 1984 [2]). It affects one in about 3500 live-born males, one third of whom are considered to be due to a new mutation. DMD is allelic with the milder and rarer Becker muscular dystrophy (BMD) [1, 3, 4]. The DMD gene maps to chromosome band Xp21.2 [5] and spans 2,300 kilobasepairs (Kb) [6]. Overlapping cDNAs of its 14 Kb transcript have been cloned [3] and were found to code for a 430 kD membrane protein called dystrophin [4]. Variations of protein presence, abundance and size are clearly associated with BMD and DMD [7, 8]. Its mode of action is still unclear and its synthesis and location are presently under study [9, 10, 11, 12]. Due to the enormous size of the DMD gene [13, 14, 15], a high mutation rate might be expected, but the nature of the mutations is exceptional, as more than 60% of the patients have major deletions or duplications of parts of the gene [16]. Several independent studies, involving field inversion gel electrophoresis (FIGE) [16, 17], cDNA hybridizations [3, 16, 18, 19] and intragenic probes [20, 21], have shown a clustering of deletion breakpoints in a major deletion hotspot in the distal half of the gene and a minor hotspot in the 5′ part of the gene. This suggests a structural propensity of specific regions of the gene to rearrange. Given the high mutation rate, detailed characterization of these hotspots is warranted, both for diagnostic purposes

and to unravel the underlying causes of the rearrangements, as this may provide more general insight into genomic instability. We have set out to characterize the major deletion hotspot in the distal half of the gene. The use of standard procedures for studying genomic DNA by subcloned unique probes is impractical, given the size of the region under study. For a fast and complete coverage of long stretches of DNA, we applied competitive DNA hybridization using whole cosmids as probes [22, 23]. We find this method to be a versatile tool for locating deletion breakpoints, ordering restriction fragments and detecting RFLPs.

EXPERIMENTAL PROCEDURES

Cosmid library construction

A cosmid library was constructed essentially as described [24], using partially MboI-digested DNA from a 49,XXXXY lymphoblastoid cell line (GM1202, Human cell line repository, Camden NJ.). DNA fragments with a mean size of 40−50 Kb were dephosphorylated and ligated into the BamHI site of c2RB [24]. Cosmids were packaged and transduced into *Escherichia coli* strain 1046 at an efficiency of 1.9×10^5 cosmids per μg DNA. A library of 7.5×10^5 cosmids was plated on nylon membrane (Genescreen Plus, NEN Research Products) to a density of 30,000 colonies in 86 mm Petri dishes. From these masterplates two sets of replica filters were made for screening.

Hybridization

For a standard filter hybridization 10 ng probe DNA was labeled to a specific activity of 8×10^7 dpm/μg with [α-^{32}P]dCTP using the Multiprime DNA labelling System (Amersham). For library screenings, 25 ng probe DNA was labeled. Filter prehybridization (5 min.) and hybridization (16 hrs.) were carried out at 65°C in a hybridization mixture containing 0.125 M Na_2HPO4 (pH=7.2), 0.25 M NaCl, 1 mM EDTA, 7% SDS and 10% PEG (Polyethyleneglycol 6000, BDH) [25], using a probe concentration of 0.5 ng per ml. Competitive DNA hybridization involved 1.5 hr preassociation at 65°C, in which 1 volume (500 μl) of 10mM Tris/0.1mM EDTA (pH 7.4) containing 5 ng labeled, denatured cosmid DNA and 100 μg of placental DNA, sheared to 500−800 bp and denatured was added to 4 volumes (2.0 ml) of hybridization mix (see above). Subsequently, this preassociation mix was added to prehybridized filters and the hybridization was continued. Hybridized filters were washed at 65°C from 2×SSC/0.1%SDS down to 0.3×SSC/0.1%SDS. Autoradiography took 18 hours at −70°C for single copy probes and 4 to 8 hours when using preassociated whole cosmid probes.

Gel electrophoresis

Field Inversion Gel Electrophoresis (FIGE) was carried out essentially as described [16]. For the separation of fragments resulting from partially digested cosmids we used a setting of 12 V/cm, a pulse time ramp of 0.5−5.0 seconds and a backward-forward ratio of 1:2.

Sequencing

Fragments to be sequenced were subcloned in pKUN1 [26]. DNA sequencing was done with the dideoxy chain termination method on double stranded DNA using Sequenase protocols (United States Biochemical Corporation).

PCR amplification

To identify P20-region deletions in patient DNA's the polymerase chain reaction [27] was carried out on 0.125 μg template DNA using 100 pmol P20-region and 6.25 pmol J66-region [13] primers and 1 unit of Taq DNA polymerase. Amplification was carried out for 32 cycles, each consisting of 1 minute denaturation at 99°C and 3 minutes annealing and extension at 60°C.

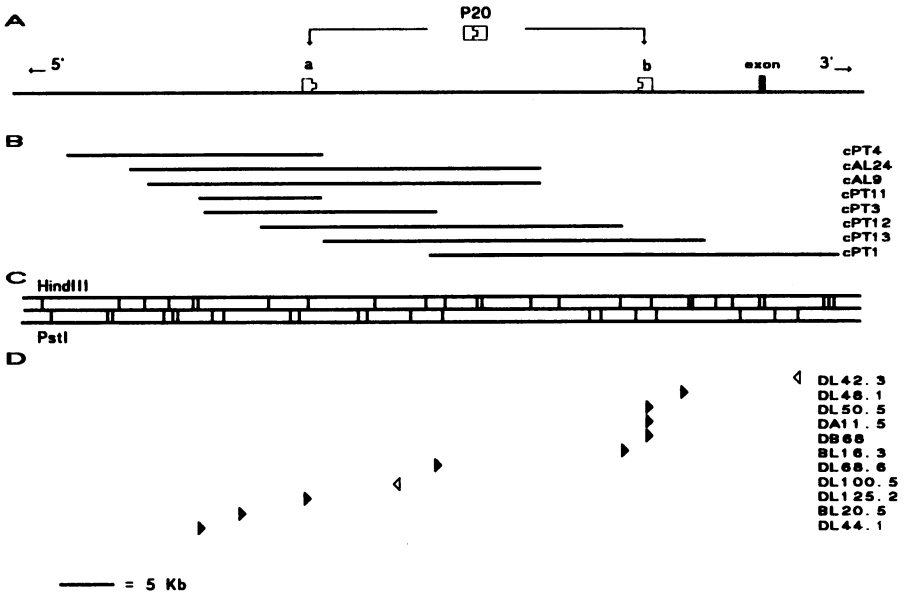

Figure 1. Characterization of 90 Kb of cloned genomic DNA around P20.
A: Probe P20 consists of 2 genomically separated segments (P20a & P20b) located 32 Kb apart. An exon is located 10.5 Kb distal to P20b.
B: Cosmid walk covering 90 Kb of genomic DNA in 8 different cosmids. The entire region can be covered using 3 cosmids: cPT4, cAL24/cAL9/cPT12 and cPT1.
C: Physical map of the cloned region, showing the HindIII and PstI restriction sites.
D: Location of the mapped deletion breakpoints using whole cosmid probes. Arrow heads pointing to the right denote the start of a deletion, extending distally. Arrow heads pointing to the left denote the start of a deletion, extending proximally.

Experimental (P20-exon) primer set	a) 21 mer, 5'-CTGGAGCTAACCGAGAGGTGC-3'
	b) 20 mer, 5'-CATTCCTATTAGATCTGTCG-3'
Control (J66-region) primer set	a) 20 mer, 5'-CTGCAGGCATTGACTGACTT-3'
	b) 20 mer, 5'-CCTCCAAAGAACCTCTTGGA-3'

Materials

Restriction endonucleases and T4 DNA ligase (Pharmacia), calf intestinal alkaline phosphatase (Boehringer Mannheim GmbH), Taq DNA polymerase (Cetus) and nylon membranes (NEN) were used as specified by the manufacturer.

RESULTS

Cosmid isolation

Probe P20 consists of two segments which were previously shown to map less than 120 Kb and possibly only 30−40 Kb apart [20]. As the parental cosmid from which it was derived was only 7 Kb long, it was postulated to be the result of a deletion event within a cosmid [20]. Strikingly, P20 not only detects a major deletion hotspot, but also for a significant number of deletions the proximal breakpoint occurs within or between the P20 segments [20]. To obtain a set of cosmids covering the entire P20 region, we screened

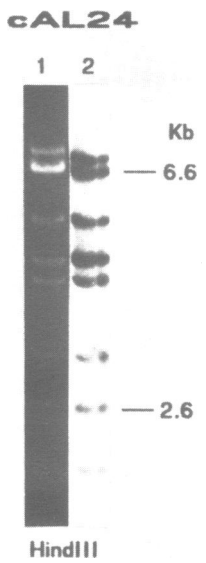

Figure 2. Comparison of the HindIII fragment pattern of cosmid cAL24 (lane 1) with competitive hybridization of cosmid cAL24 to HindIII-digested placenta DNA (lane 2). The patterns match, with the exception of a second 6.6 Kb band, visible in cAL24 but not after hybridization, and a 2.6 Kb band detected by hybridization only. The latter is the genomic fragment corresponding to the 3′ end of cAL24, present as a 6.6 Kb junction fragment, containing 1.4 Kb genomic DNA and 5.2 Kb vector DNA.

two independently constructed cosmid libraries with P20. This yielded 8 different and partially overlapping clones, together yielding a contiguous restriction map of 90 Kb (fig. 1a & 1b). Although the distance between the two P20 segments was found to be 32 Kb, none of the cosmids contained sequences of both P20 segments. Six cosmids contain the proximal segment of P20 (P20a) and two the distal segment (P20b). This supports our ideas for the origin of P20 and suggests that also in *E. coli* the sequences around P20 may be instable.

Competitive DNA hybridization

Hybridization of the cosmids in the presence of excess human competitor DNA to a panel of DMD deletions showed 7 out of 8 cosmids to originate from a single genomic location at DXS269 (P20). One cosmid (cPT11) however contained unique sequences both outside and inside DMD deletions and must therefore be a ligation artefact. Cosmid cPT3, although hybridizing to DXS269, has a total insert length of 29 Kb and altered vector fragments, suggesting secondary internal rearrangements. For each cosmid, the overall validity of using whole cosmids in a competitive DNA hybridization assay was confirmed by comparing the cosmid restriction pattern with that arising from the hybridization to a genomic DNA digestion. Allowing for altered fragment lengths at the MboI cloning sites, the patterns matched (fig. 2).

Cosmid mapping by Field Inversion Gel Electrophoresis

SalI-linearized cosmids were partially digested with HindIII or PstI. The resulting fragments were separated on a FIGE gel. Subsequent hybridization of these partials to sequences flanking the linearization site reveals the fragment orders in one lane, while restriction sites for the different enzymes are ordered relative to each other from adjacent lanes. The

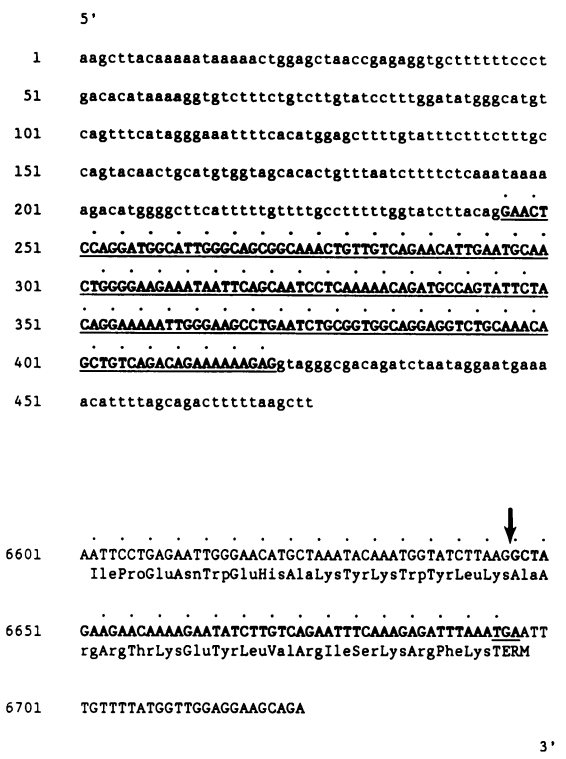

Figure 3. Analysis of the P20 exon.
A: Sequence of the 475 bp exonic HindIII fragment, located 10.5 Kb distal to P20b. The 176 bp exonic sequence, position 6,647−6,822 bp of the cDNA, is underlined with dots marking the reading frame. Intronic sequences are in lower case.
B: Relevant stretch of 124 bases of cDNA sequence generated in patients missing the P20 exon. The arrow marks the deletion site. The continued reading frame in the next exon is shown until reaching a translational stopcodon (TGA).

absolute lengths of the various fragments were calculated from ethidium bromide stained complete digestions of the cosmids. Since the large overlapping regions between the various cosmids provided an additional control, the presence of SalI sites in the cloned region, precluding the complete reading of the fragment orders, did not hamper the ordering. Figure (fig. 1c) shows the entire genomic DNA region cloned, characterized for HindIII and PstI restriction sites.

Presence of exonic sequences

Hybridization of HindIII digested cosmids with cDNA probe 5b-7 [3] showed the presence of a 0.5 Kb exon containing fragment in cosmid cPT1 (data not shown), located 10.5 Kb distal to P20b. This is consistent with our prior placing of P20 proximal to a 0.5 Kb exonic HindIII fragment [20]. The exon contained therein is the first exon deleted in a large majority of distal BMD/DMD deletions and thus far proves to be one of the most frequently deleted exons in the DMD gene [3, 6]. In the 80 Kb of cloned sequence proximal to it, no exons are found. FIGE data indicate that this intron, containing P20 and the majority of deletion breakpoints, spans 160−180 Kb [6]. Subcloning and sequencing of the 0.5 Kb HindIII

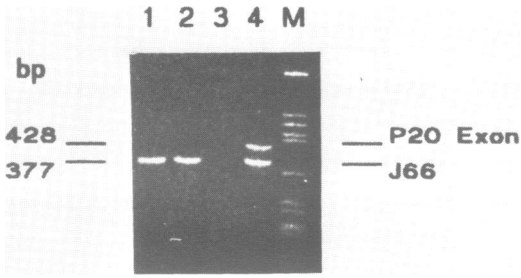

Figure 4. PCR amplification of DNA segments of a DMD patient with a combined set of P20 and J66 amplimers (see exp. proc.), lane 1. Control lanes represent known P20 exon negative, J66 region positive (lane 2), P20 exon and J66 region negative (lane 3) and P20 exon and J66 region positive (lane 4) DNAs. The patient DNA in lane 1 is deleted for the P20 exon.

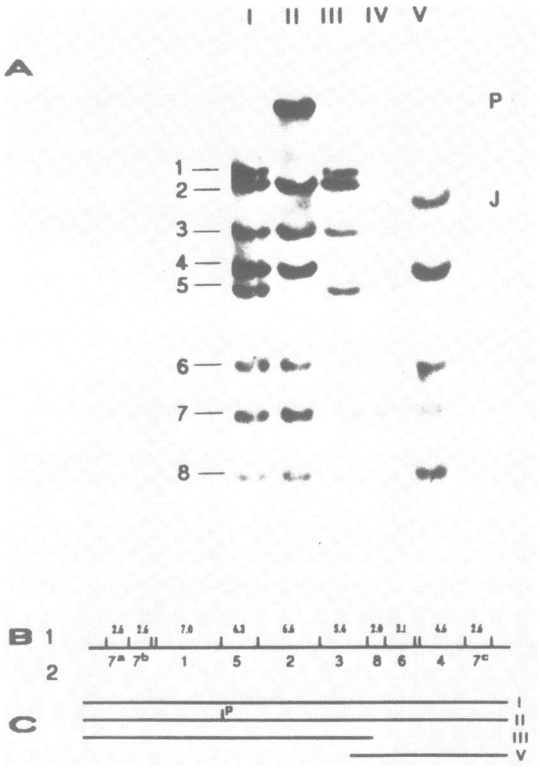

Figure 5. Competitive DNA hybridization analysis.
A: Competitive hybridization of cosmid cAL24 to DMD patient DNAs digested with HindIII. The various lanes show DNA not deleted (I, II), completely deleted (IV) and partially deleted (III & V) respectively. In lane II the bands 1 (7.0 Kb) and 5 (4.3 Kb) are replaced by a new band at 11.3 Kb (P), due to a polymorphic HindIII site.
B: HindIII restriction map of cAL24. B1 = fragment size, B2 = relative fragment order as seen on hybridization of lane I–V.
C: Mapping of deletion breakpoints (P marks polymorphic HindIII site).

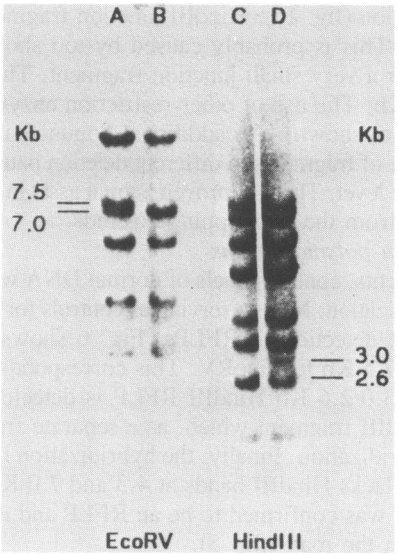

Figure 6. Polymorphic EcoRV and HindIII bands detected by cosmid cPT13 in placenta DNA. Markers indicate the polymorphic fragments.

fragment revealed a 176 bp exon (fig. 3), located at 6,647−6,822 bp of the dystrophin cDNA sequence [27]. The 5′ and 3′ borders of this exon map respectively flush with and within a coding triplet [28]. Furthermore, the proximal end of the exon coincides with the border between internal repeats R18 and R19 identified within the dystrophin protein sequence [28].

PCR amplification screening

The sequence information of the exonic fragment and its frequent deletion in muscular dystrophy permit the use of region-specific amplimers for use in deletion screening by PCR amplification [27]. For each reaction we used two sets of primers; one set straddling the P20 exon, resulting in a 428 bp fragment and a second set from the vicinity of the J66 probe (DXS268) [6, 13] and giving a 377 bp fragment. This last sequence is located 22 Kb distal to an exon which covers the cDNA from 9,146−9,292 bp [6]. This region of the DMD gene is known to be deleted in BMD/DMD patients in less than 1% of the cases [20]. It serves as a positive control. Pilot screening of a random set of 25 BMD/DMD patients revealed 10 deletions of the P20 region. Of these, five deletions were tested and confirmed to miss the P20 exon by cDNA hybridizations, thus demonstrating the application of PCR amplification as a potentially highly rewarding diagnostic tool (fig. 4).

Mapping of deletion breakpoints

Using whole cosmids as probes on HindIII digested patient DNA we attempted a further characterization of the major deletion hotspot around P20. For screening we used a panel of DMD deletion patients known to have a deletion breakpoint within the P20 intron (fig. 5). We successfully localized the deletion breakpoints for 11 out of 19 patients tested. Eight of these were previously found to map between the segments of the P20 probe or distal to them [20]. Two more breakpoints were mapped proximal of P20a and one was mapped distal of the exon. The remaining 8 patients have deletion breakpoints mapping

proximal of the cloned region (fig. 1d). HindIII junction fragments were not detected for all identified breakpoints. This is probably caused by too short a hybridizing sequence in the junction fragment or a very small junction fragment. The lower detection limit of our method is below 1.0 Kb. The use of other restriction enzymes, however, has shown junction fragments (data not shown). An additional bonus of this mapping procedure is that the absence or presence of fragments in different deletion patients unambiguously orders these fragments on genomic level. This has proven a great asset in constructing the restriction map of the entire region from the overlapping cosmids.

Restriction fragment length polymorphisms

Parallel to the deletion patients, control panels of normal DNA were digested and subjected to competitive DNA hybridization. Next to serving as controls for a correct fragment pattern, these panels permitted the detection of RFLPs. Fig. 6 shows cosmid cPT13 detecting variable bands at 7.0 and 7.5 Kb for EcoRV. This corresponds to the RFLP detected by P20b [20]. In addition a 3.0/2.6 Kb HindIII RFLP is detected (fig. 6), suggesting the presence of a 0.4 Kb HindIII fragment which, as a separate fragment, is not detected in our competitive DNA hybridization. Finally, the hybridization results with cosmid cAL24 show that patient DL50.5 lacks HindIII bands at 4.3 and 7.0 Kb, but has a new 11.5 Kb band instead (fig. 5). This was confirmed to be an RFLP and independently places these two fragments adjacent on the map (fig. 5).

DISCUSSION

In this paper we have studied the major deletion hot spot, located centrally in the DMD gene by cosmid cloning, mapping and hybridization, monitoring 90 Kb of genomic DNA around P20 (DXS269). The only exon located in this region was identified and analyzed. We show that labelled, entire cosmids after competitive hybridization with excess human placenta DNA are excellent probes for the rapid and convenient detection of deletion breakpoints and RFLPs in Southern blots of DMD patients. Even when using relatively frequent cutters like HindIII and PstI, the band pattern arising after competitive DNA hybridization of whole cosmids is simple. All fragments present in the cosmids are detected in genomic blots, except fragments below 1.0 Kb. Generally, we find stronger signals using cosmid competition hybridization than with single copy probes, permitting shorter exposure times (see exp. proc.). A possible explanation can be found in the length effect of hybridizing with cosmids. Only a small portion of the sequences will hybridize to membrane bound fragments, while most sequences will probably have reassociated with other stretches of randomly primed fragments of the same cosmid in the liquid phase. Clearly, this may proceed over much greater length for a cosmid than for a plasmid or a purified restriction fragment, thus enhancing the signal eventually bound to the filter-bound fragment. Above a fragment length of approx. 1 Kb we see a rather constant signal intensity. Below this size the intensity decreases, suggesting some sort of threshold in the hybridization conditions we use. Finally, the same clear fragment patterns are produced by many other cosmids, originating from chromosomes 4 and 16 or other regions of the X-chromosome (M.I. Skraastad, M.H. Breuning and our unpublished observations). This indicates that successful application does not depend on a possible favorable repeat distribution or repeat content in the P20 region. So far, any cosmid of which we were unable to generate unique subclones, could be hybridized in this way. In conclusion, the use of whole cosmids as probes not only obviates the tedious and laborious isolation of single copy probes, but, by monitoring a large area with one contiguous probe, also increases

the chances of directly hitting a mutation including the detection of several widespread RFLPs. Thus this technique may have considerable potential in diagnostic applications, such as junction fragment detection in carrier females and RFLP screening in haplotype analysis.

The P20-exon

The exon we have isolated lies on HindIII fragment 33 in the restriction map of the complete cDNA [3, 6]. Its 176 bp length implies it to be a 'frameshifting' exon when deleted. Inspection of the cDNA sequence shows that the P20-exon begins with an undisrupted triplet, coinciding with the border of protein repeats R18 and R19 [28]. The exon ends at position 2 of a coding triplet. The frameshift resulting from deleting this exon generates a TGA stopcodon at position 6,871−6,873 bp, 48 nucleotides into the next exon. Deletion of the P20-exon thus generates a truncated dystrophin protein. We find 6 patients in our family material deleted only for this exon and all of them have DMD; BMD patients with deletions starting with the P20 exon lack at least one additional exon, so the deletion of this next exon should restore the register of the reading frame and thus the code for the remainder of the dystrophin protein [6, 29, 30, Koenig et al.,manuscript submitted]. Considering the position of the P20-exon relative to the predicted protein structure, its sequence is part of the second, rod-shaped domain. At first sight, it seems tempting to seek an association between the repetitive structure of this part of the transcript and the propensity of this region to rearrange. However, the wide genomic spreading of the deletion breakpoints and the absence of any exons in 160−180 Kb proximal to P20 [6], refutes this idea upon further consideration. It remains to be seen whether repetitions are present on intronic level.

Cosmid characterization

When examining the isolated cosmids we found 2 out of 8 cosmids to be rearranged in the cloning process, both involving the region around P20. Although plausibly explainable as ligation artifacts, the fact that the original P20 cosmid was a recombined cosmid, as well as the absence in the libraries of cosmids extending over both P20 regions, suggests that the instability of the genomic region is somehow reflected in the cosmid libraries. As to general mishaps in the construction process of these specific libraries: other cosmid clones isolated from the same libraries, originating from chromosomes 4, 16 and X, show no obvious anomalies in vector size or genomic composition (personal communications, M.H. Breuning, F.P.M. Cremers).

Positions of breakpoints

Fig. 1d clearly shows that the deletion breakpoints are dispersed throughout the whole area studied. On the basis of a completely random distribution for the deletion breakpoints within the P20 intron, one would arrive at a 150 Kb intron size, as 10 out of 18 breakpoints (i.e. 56%) occur in 80 Kb of intronic sequence studied. This fits well with the intron size of 160−180 Kb found by FIGE analysis [6]. Nevertheless, we note that, while the whole examined area, including the 3′-adjacent intron, covers 90 Kb, 5 out of 11 breakpoints map within 20 Kb around the proximal part of P20 and five more breakpoints are confined to 7 Kb around the distal part of P20. Of the latter cluster, 3 breakpoints map within 2.5 Kb of each other and of the original P20 rearrangement breakpoint. Additional data are required to establish whether this apparent clustering within the deletion hotspot is significant. For the acquisition of such data we have initiated a multicenter study involving worldwide patient material. As the P20 exon is the first exon deleted in the mutation hotspot, the preponderance of distally orientated deletions is predictable, thus defining a sharp border

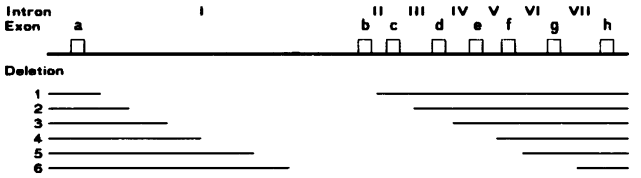

Figure 7. Schematic drawing of a fictive intragenic region in which several deletions with similar sizes start in the same large intron (I) and extend over a different number of consecutive exons.

for an exonic deletion hotspot. The large size of the P20 intron, however, combined with the much closer spacing of the subsequent exons [6], is responsible for the misleading suggestion that the proximal genomic breakpoints are more homogeneous than the distal ones. As illustrated by the schematic drawing in figure 7, the observed difference in breakpoint homogeneity at the exon level of the 5′ and 3′ ends of the deletions, can be completely accounted for by differences in exon spacing. All the deletions drawn would begin with the same exon and end with different consecutive exons. Our breakpoint mapping results bear out the validity of this model for the proximal breakpoints while the finding of a fairly conserved deletion size around 200 kb [6] suggests an overall validity of this schematic drawing. The further molecular analysis of the distal breakpoints awaits the complete cloning of the region distal to the presently cloned area. A final point of consideration is why a deletion-prone region, which appears to consist of nothing more than an intron, has not been lost in evolution, as predicted by its high risk for generating pathology which strongly decreases viability. A plausible explanation seems that this large intron may harbour other indispensible functions, such as sites for chromosomal scaffold attachment or nuclear matrix association or even other genes, possibly unrelated to dystrophin expression [31]. As it is conceivable that other putative functions may be somehow related to the observed deletion-sensitivity a further study of this region should yield interesting findings concerning chromosomal instability more in general. As such, DMD is one of the few natural model systems for which enough independent mutations are available to study these phenomena in precise detail.

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