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The Problem of Duchenne Muscular Dystrophy [and Discussion]

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The problem of Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal X-linked muscular disorder. The biochemical defect remains unknown, but the gene responsible has been mapped to band Xp21. The gene has now been cloned in two laboratories solely from knowledge of its map location. L. M. Kunkel and his colleagues isolated genomic sequences (*PERT 87*) from within a large deletion causing DMD, whereas our group isolated genomic sequences (*XJ*) spanning the junction of an X-autosome translocation causing the disease. Chromosome walking by both groups has led to the isolation of over 400 kilobases of the *PERT 87* and *XJ* region. Subclones of *PERT 87* and *XJ* reveal restriction fragment length polymorphisms that segregate with the DMD gene in 95% of meioses, and fail to hybridize with DNA from about 8% of male patients. Selected subclones of *PERT 87* and *XJ* contain exons that hybridize to muscle-derived complementary DNA (cDNA) clones. The cDNA clones detect a large (16 kilobase) message. Analysis of deletions, mutations and translocations suggests a DMD gene of between two million and three million base pairs. The clones obtained so far are useful for attempts to generate antibody against the gene product and for carrier identification and prenatal diagnosis.

Introduction

Duchenne muscular dystrophy (DMD) is the most severe and the most frequent of the muscular dystrophies (Moser 1984). It follows an X-linked pattern of inheritance and, therefore, it is primarily males that are affected and two thirds of them inherit the disease gene from a carrier mother. The age of onset of the disease is at 3 or 4 years, when affected boys have difficulty climbing stairs and difficulty rising from a sitting position on the floor. As the disease progresses, the lower limbs become weaker and by the age of 6 or 7, leg braces are usually required. By the age of 10 or 11, most are confined to a wheelchair. During the teenage years, the muscles of the upper body become progressively weaker, leading to severe curvature of the spine and decreased pulmonary function. Death is common between the ages of 18 and 25.

One of the major difficulties for families with an affected child is the fact that there has been no prenatal diagnostic test, and carrier identification tests have been inconclusive. In addition, despite many years of research into the pathophysiology of this disease, the biochemical defect remains unknown. In 1983, when gene cloning work began, the only solid fact known was that the gene responsible for the disease mapped to the middle of the short arm of the X-chromosome in a dark staining band called Xp21. The two successful approaches to cloning the gene have depended entirely on knowing the map location and have by-passed the need to know anything about the gene product. The work to be described on the gene cloning has two readily

identifiable purposes. The first is to identify the gene itself and to use the gene sequences to generate the gene product, to isolate the gene product, and to determine its function and site of action in the body. The long-term goal is for complete understanding of the disease and, hopefully, new and rational approaches to therapy or a cure. The second goal is the development of DNA probes at the site of the gene that can be used in restriction fragment length polymorphism (RFLP) analysis for much improved carrier identification and for prenatal diagnosis.

Mapping of the DMD gene to Xp21

Three lines of evidence have indicated that the gene responsible for Duchenne muscular dystrophy and its milder counterpart, Becker muscular dystrophy (BMD), map to the short arm of the X-chromosome. The first evidence came from a group of females who were found to have the disease secondary to an X-autosome translocation (for review, see Boyd *et al.* (1986)). In these girls, the translocation exchange point in the X-chromosome is always in band Xp21, suggesting the possibility that the translocation might disrupt the gene and cause the disease. The disease is expressed in these girls because a secondary consequence of the translocation is nonrandom X inactivation, in which the normal X is inactivated in all or most cells of the body. The net result is the disruption of the gene on one chromosome and the inactivation of the gene on the other chromosome, resulting in full manifestation of the disease.

The second line of evidence came from a small group of males with Duchenne muscular dystrophy and other X-linked disorders, including chronic granulomatous disease, glycerol kinase deficiency, adrenal hypoplasia and retinitis pigmentosa (for review see Clarke *et al.* (1986)). These boys have multiple diseases as a consequence of small, but cytologically visible deletions in the Xp21 region, providing evidence that the genes for all of these conditions are located in this region. The third line of evidence came from family studies in which random segments of DNA from the short arm of the X-chromosome were used to identify RFLPs that were subsequently found to segregate with the DMD gene in families (Davies 1985).

One of the two cloning approaches utilized DNA from one of the translocation patients, whereas the other approach utilized DNA from one of the multiphenotype deletion patients.

Cloning of a DMD translocation junction

The approach to gene cloning in our own laboratory took advantage of a patient first brought to our attention by Dr Christine Verellen. This patient, born and still living in Belgium, began to have difficulty, with frequent falls, at the age of 2. By the age of 5 she showed some of the classical symptoms of the disease and, by the age of 8 a diagnosis of DMD was made. By the age of 16 it had become clear that the patient had a mild form of the disease more in keeping with a diagnosis of BMD. The patient's chromosomes showed an X-autosome translocation between the short arm of the X (breakpoint in Xp21) and the short arm of chromosome 21 (breakpoint in 21p12). Studies by Dr Verellen in our laboratory (Verellen-Dumoulin *et al.* 1984) demonstrated that the normal X-chromosome was inactive in most cells of the body. Study of the chromosomes with a silver-staining technique that revealed ribosomal RNA genes indicated that the exchange point in the short arm of 21 was within a block of genes encoding 18*S* and 28*S* ribosomal RNA.

Because the ribosomal genes had been cloned in the laboratory of Dr R. Schmickel, it became feasible to use ribosomal gene probes in an attempt to isolate a large fragment of DNA that spanned the translocation junction between chromosome 21 and the X-chromosome. One

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of the difficulties in this approach was the fact that there are nearly 400 copies of the ribosomal genes in a human cell and only one of these was expected to be disrupted by the translocation. To overcome this, cells from the patient were fused with mouse cells, and the resulting hybrid cell lines were allowed to lose all of their human chromosomes except for one of the two translocation chromosomes. One of these hybrids, carrying the X-derived half [der(X)] of the translocation (Worton *et al.* 1984), carried only three or four copies of the ribosomal gene sequence and one of these ribosomal genes was shown by Southern blot analysis to be disrupted, presumably at the site of the translocation (Worton *et al.* 1986).

To clone the translocation junction, DNA from the hybrid carrying the der(X) chromosome was digested and ligated to the arms of bacteriophage. The resulting DNA library was screened with one of the ribosomal gene probes, and a number of hybridizing phage were identified. One of these, called XJ 1, was examined in detail. Firstly, it was found to contain 620 base pairs (b.p.) of ribosomal gene at one end and sequences derived from the X-chromosome at the other end (Ray *et al.* 1985). Sequences from the X-chromosome part of this clone were used as probes to reveal RFLPs that were subsequently found to segregate with the DMD gene in family studies. Moreover, when the same probe was used against Southern blots of DNA from a number of male patients, about 6% of the patients failed to hybridize with the XJ 1 sequence. This suggested that 6% of male patients may have a deletion of the DMD gene, and that the deletion includes the region of XJ 1. Taken together, the indication was strong that the XJ 1 sequence mapped within or very close to the DMD gene.

Subsequently, the XJ 1 probe has been used to isolate overlapping phage clones that extend farther toward the centromere and telomere of the X-chromosome. These clones, called XJ 2 and XJ 3, were then broken down into smaller subclones and again utilized as hybridization probes to identify additional overlapping sequences. By chromosome walking in this manner, ten phage clones have been identified and found to contain approximately 140 kilobases of the X-chromosome. Before describing further experiments with the *XJ* region of the muscular dystrophy locus, we will describe the deletion approach to cloning that has led to the isolation of an adjacent region of the X-chromosome.

Cloning sequences from within an Xp21 deletion

The approach of Kunkel and his colleagues at Boston Children's Hospital was to utilize DNA from one of the multiphenotype children with a cytological deletion of a major part of band Xp21. The rather clever strategy involved the 'Phenol Enhanced Reassociation Technique' (PERT) and resulted in several PERT clones from the region that was deleted in this patient (Kunkel *et al.* 1985). Briefly, a DNA library was prepared from the DNA of an individual with XXXXY chromosomes. DNA from this individual was used to obtain an enrichment for X-chromosomal sequences. Before preparing the library, however, this DNA was denatured and subjected to extensive reassociation with a 200-fold excess of sheared and denatured DNA from the deletion patient. The sheared molecules hybridized to DNA from the XXXXY patient, except that in the region of the Xp21 deletion, there were no competing sheared DNA molecules. Because the DNA from the XXXXY patient had been digested enzymically and, because DNA molecules consisting of a sheared fragment hybridized to an enzymically cut fragment are not easily cloned in plasmid vectors, the resulting library was enriched for DNA molecules in which two enzymically cut fragments had come together. This, in turn, was enriched for sequences from the deleted segment of Xp21.

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The key finding in Kunkel's laboratory was that one clone from this library, called *PERT 87*, failed to hybridize with DNA from about 6% of male patients (Monaco *et al.* 1985). The implication was that these patients had a deletion of the DMD gene and that the deletion included the *PERT 87* probe site. Moreover, the *PERT 87* probe was used to identify overlapping phage clones from a genomic library and chromosome walking was done until 220 kilobases of the X-chromosome had been isolated in 18 clones. Many subclones from this region were found to detect RFLPs and these were found to segregate with the DMD gene in families. Thus the evidence strongly suggested that the *PERT 87* sequence lay within, or very close to, the DMD gene.

The genetic map of the DMD locus

Once the first *PERT 87* probes had been isolated, subclones that detect RFLPs were sent to many laboratories around the world and, in a large collaborative study, over 1300 patients were examined for hybridization to the *PERT 87* probes. In this world-wide study, about 7% of male patients were discovered to have deletions of the *PERT 87* sequences (Kunkel *et al.* 1986). Subsequent analysis revealed that many of these patients have deletions that extend throughout the entire 220 kilobases of the *PERT* region and the entire 140 kilobases of the *XJ* region. Several other patients were found to have deletions that began within the *PERT 87* or *XJ* region and extended towards the telomere or the centromere. This indicated that *PERT 87* and *XJ* were close together on the chromosome. This was later confirmed by pulsed-field gel electrophoresis, when it was demonstrated that part of the *PERT 87* locus and all of the *XJ* locus lay on the same 840 kilobase *Sfi*1 fragment (van Ommen *et al.* 1986) and on the same 400 kilobase *Sal*1 fragment (Burmeister & Lehrach 1986). Chromosome walking from the *XJ* region has now joined *XJ* with *PERT 87* to form one continuous region of cloned sequence. Many subclones are available from the region and these detect deletions in patients, ranging in size from a few kilobases up to several hundred kilobases (Hart *et al.* 1986; Thomas *et al.* 1986). The size of the deletions and the diversity of their end-points indicates that the DMD gene might be very large. A selected set of deletions ascertained in our laboratory are shown in figure 1.

A further indication that the DMD gene might be large came from cytogenetic analysis of Boyd & Buckle (1986). They demonstrated by high resolution banding studies that some of the translocation exchange points in the X-autosome translocation females mapped in the upper part of band Xp21, whereas other translocation exchange points mapped in the lower part of Xp21. They estimated that the amount of DNA between the most distal and the most proximal exchange points was three to four million b.p. If the hypothesis was correct that these translocations acted by disrupting the gene, then a gene of three to four megabases was indicated. In further studies using *XJ* and *PERT 87* probes, Boyd *et al.* (1987) have demonstrated that the more distal translocation exchange points map distal to the *PERT 87* region and the proximal exchange points map on the centromeric side of the *XJ* region. The separation between these distal and proximal exchange points is a minimum of 450 kilobases (figure 1).

The third indication of a large gene came from family studies in which RFLP markers from the *PERT 87* (Fischbeck *et al.* 1986; Bertelson *et al.* 1986) and *XJ* (Thompson *et al.* 1986) region were found to segregate with the DMD gene in only 95% of meioses. In 5%, a recombination event took place in the chromosome interval between the site of mutation and the site of the probe. Because the *PERT 87* and *XJ* probes are clearly located within the DMD

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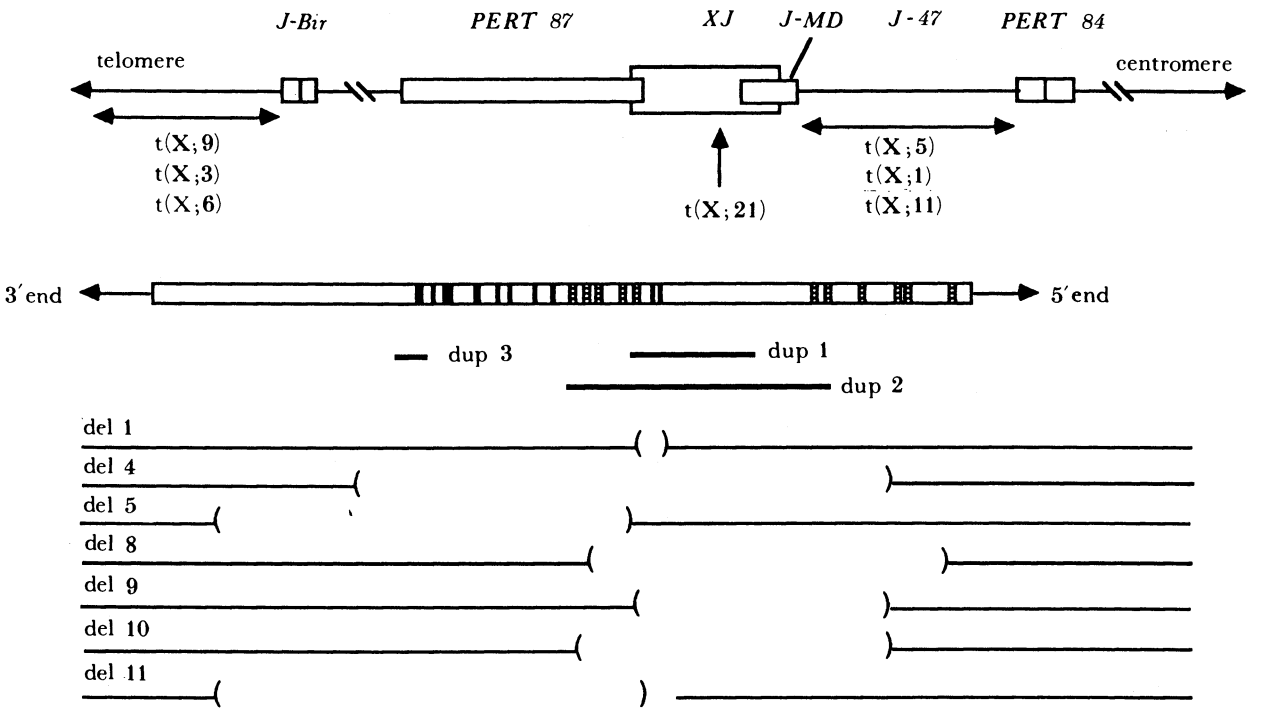

FIGURE 1. Schematic diagram of the DMD gene. The top line shows the *PERT 87* region (220 kilobases) and the overlapping *XJ* region (140 kilobases). Also shown are the cloned regions *PERT 84*, *J-MD* and *J-Bir*, the last two cloned by isolating deletion junctions from two patients with deletions from *PERT 87* to the centromeric side (*J-MD*) and the telomeric side (*J-Bir*). Below this, the position of several translocation exchange points is indicated. The block in the centre is an exon map. The exons marked as black bars (the first eight in the *PERT 87* region plus two in the *XJ* region) have been accurately mapped. Those marked as stippled bars represent the approximate location (five in the centromeric end of *PERT 87*, six on the centromeric side of *XJ*) of exons that, so far, have not been precisely mapped. We have seen three patients with tandem duplications, and the duplicated region of each is indicated. Patient 3 (*dup* 3) has the mild Becker form of muscular dystrophy and duplicates only one exon. A selected set of deletions ascertained in our laboratory is shown at the bottom of the figure. Each deletion removes a different set of exons, and the numbers correspond to the lane numbers in figure 2.

gene (see below) and, because a 1% recombination frequency corresponds, on average, to about 1000 kilobases of DNA, the result suggests that some of the mutations may occur up to 5000 kilobases from the *PERT 87–XJ* region.

Expressed sequences from the DMD locus

Once DNA sequences were identified from the site of the DMD locus, as previously described, several subclones from the region were utilized as hybridization probes in an attempt to identify sequences that are expressed in muscle tissue. Complementary copy DNA (cDNA) clones have now been isolated from muscle cDNA libraries, utilizing probes from both the *PERT 87* and *XJ* regions. A 1 kilobase cDNA clone from the *PERT 87* region was found to contain eight exons of the DMD gene (Monaco *et al.* 1986) Most of these exons were found to map within the *PERT 87* region. One cDNA clone of 2 kilobases in size, detected with an *XJ* region probe, was found to contain 13 exons (Burghes *et al.* 1987). Five of these exons map within the *PERT 87* region, two map within the XJ region and six map on the centromeric side of *XJ*. Altogether, over 20 exons of the DMD gene have now been identified, and they map over a region of the X-

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chromosome that is at least 350 kilobases in size (figure 1). Both cDNA clones referred to previously are found to hybridize to muscle mRNA of approximately 16 kilobases in size. This would indicate that only about one fifth of the entire cDNA has been isolated, and the entire gene might therefore be expected to map over a region five times larger than the 350 kilobases examined so far†.

In further studies, it has been possible to demonstrate that of the 13 genomic fragments detected by our 2 kilobase cDNA probe, five of these fragments are derived from the segment of the X-chromosome that has been translocated to 21, and six of the fragments are derived from that portion of the X-chromosome on the centromeric side of the translocation junction (Burghes *et al.* 1987). This confirms that the X;21 translocation has indeed split the DMD gene, distributing exons of the gene onto two different chromosomes. A complete characterization of the two translocation chromosomes at the nucleotide sequence level has confirmed that there is no major deletion or secondary rearrangement at the site of the exchange (Bodrug *et al.* 1987) and has proven that the translocation occurred in the germ line of the patient's father (Kean *et al.* 1986). This is, to our knowledge, the first demonstration of a translocation that has disrupted a gene to cause a genetic disease.

As stated earlier, one of the major objectives is to utilize DNA sequence as a starting point for the production and isolation of the gene product. To this end, we and others are now attempting to subclone the cDNA into expression vectors, so as to generate peptides corresponding to a portion of the DMD gene product. These will then be used as immunogens to raise antibodies, and the antibodies will be used as reagents to identify and purify the natural product of the normal DMD gene. It should be possible to identify the size and the subcellular localization of the DMD gene product. Ultimately, it is anticipated that it will be possible to determine the structure and function of the gene product and to determine the nature of the basic defect in the disease†.

Diagnostic testing

The second major objective of the research is to be able to utilize fragments of DNA from the DMD gene as diagnostic tools for carrier identification and for prenatal diagnosis. The easiest approach to diagnosis is in the case of deletions. In this regard, a multiplicity of genomic probes has been of great value in detecting deletions of various regions of the DMD gene. However, now that cDNA clones are available, it is clear that they are much more valuable because one cDNA clone of 2 kilobases in size reveals as many as 13 fragments of the gene, each containing one or more exons, on a single Southern blot. It is therefore possible to analyse 200–300 kilobases of the gene with a single probe. This is shown in figure 2, where it is clear that each of the patients is missing a different subset of fragments, corresponding to deletions of different exons of the gene. Where it is known that one affected child in the family has a deletion of a portion of the gene, it becomes feasible to test females in the family to determine if they have a similar deletion on one of their two X-chromosomes. It also becomes possible to do prenatal testing in subsequent pregnancies utilizing DNA from chorion villous biopsy or from amniotic fluid cells†.

In families where the affected child has no detectable deletion, it then becomes necessary to use RFLP markers within, or near, the gene and to follow the segregation of these markers from mother to child. By knowing which markers are carried on the same chromosome as the DMD

† See note added in proof.

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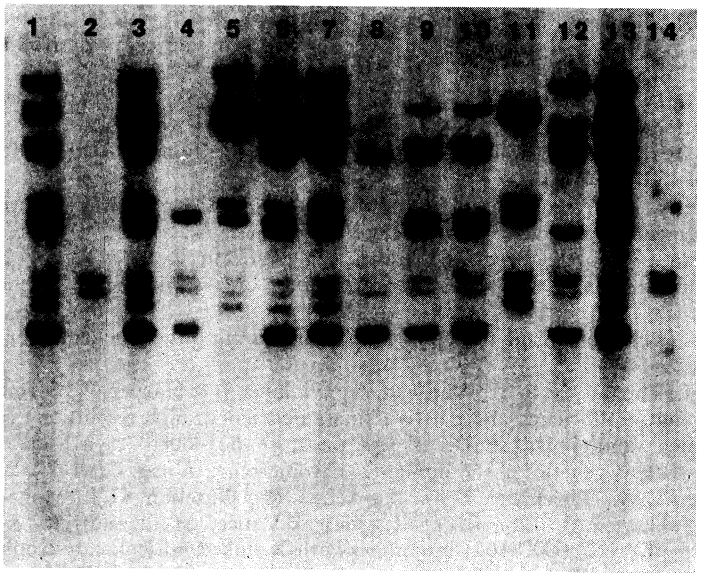

FIGURE 2. Southern blot analysis of 14 patients with DMD. Thirteen bands can be visualized on the *Hin*dIII digest, each band presumably containing a DNA fragment that carries an exon of the DMD gene and therefore hybridizes with the 2 kilobase cDNA probe. The cDNA was isolated by Burghes *et al.* (1987) from a cDNA library prepared by Lloyd *et al.* (1985). Patient 1 (lane 1) has deleted only one *Hin*dIII band (relative to a control in lane 6) and this is the fragment that carries the two *XJ* region exons (see figure 1). The remaining patients have deletions that include different sets of exons, and were useful for constructing the exon map in figure 1.

gene in mother, it becomes possible to examine daughters of a known carrier to determine if they also carry the markers that are linked to the DMD gene. Prenatal diagnosis may also be done by testing DNA from the fetus for the presence or absence of the linked markers. For this type of testing, not only are the markers from the *PERT 87* and *XJ* region of considerable value, but a number of other probes outside the gene, flanking it on both sides, are also of great value. In fact, because the diagnostic error can be greatly reduced through the use of flanking markers, in many instances the flanking markers are more valuable than the internal markers derived from the *PERT 87* and *XJ* locus.

In summary, the past three years have seen remarkable achievements in the study of DMD. Starting from the known location of the gene in band Xp21 of the X-chromosome, it has been possible to isolate DNA sequences from the site of the gene. These sequences have been used as probes to identify cDNA clones that contain multiple exons of the gene. Both the genomic fragments and the cDNA clones are of tremendous value in identifying deletions in male patients and in identifying RFLP markers for carrier identification and prenatal diagnosis. In addition, the availability of cDNA clones holds great promise that it will soon be possible to prepare antibody directed against the product of the DMD gene and this should, ultimately, lead to complete understanding of the disease. Clearly, such an understanding is a prerequisite to any attempt at a rational therapy or cure for this common and lethal genetic disease.

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Discussion

M. Bobrow (*Guys Hospital Medical School, London, U.K.*). Professor Worton described the loss of 70 b.p. at the site of the X;21 translocation. Is the deletion relevant, and is there an exon in this vicinity?

R. G. Worton. The deletion is not relevant. There is no exon in the vicinity so that no coding sequences are lost or gained in the formation of the translocation. The disease is caused in our patient, not by the loss of coding sequences, but rather by the disruption of the gene with the 5′ exons remaining on the der(X) chromosome and the 3′ exons being translocated onto the der(21) chromosome.

A. E. H. Emery (*The Medical School, University of Edinburgh, U.K.*). There is a danger, with the euphoria over the new technology, that parents may be encouraged to embark on a pregnancy with the occasional resultant birth of an affected son, as a consequence of the false negatives due to recombination with a linked DNA marker. Would Professor Worton comment on this problem?

R. G. Worton. This is a significant problem. As Professor Emery points out, many carriers have in the past elected not to have more children because of the high risk of an affected child. Now they enter prenatal diagnosis programmes in the anticipation that DNA markers can reduce their risk to zero. Of course, when flanking markers are informative we can come close to this ideal, but when only one marker is available a risk of 5% is the best we can offer. Five out of 100 such cases will result in the birth of an affected boy who might not have been born if the DNA tests were not available. We have had one such case to date, but fortunately the baby was normal. I certainly agree that this is a problem that should be kept in mind by anyone entering a testing programme.

A. Ryder-Cook (*Molecular Neurobiology Unit, University of Cambridge, U.K.*). How does Professor Worton explain finding cDNAs from libraries made by poly(A) priming which do not map on to the 3′ end of the DMD gene?

R. G. Worton. It is true that a cDNA library made by poly(A) priming would be expected to contain clones that come preferentially from the 3′ end of the DMD gene. However, one has to remember that the library was screened with genomic probes that came from the 5′ end of the gene, and so the only clones we would have any hope of finding would be those from the 5′ end. These clones were in quite low abundance in the library, and it clearly must have been primed at random sites along the message. Presumably, if we had had genomic probes from the 3′ end we would have been able to find a number of cDNA clones containing sequences also from the 3′ end.

A. Ryder-Cook. Would Professor Worton comment on the small size of the exons found in DMD spread over a very large area of the human genome? Is there any precedent for such a gene?

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R. G. WORTON. So far, we have only examined two exons and they seem to be quite small, less than 200 b.p. Kunkel's laboratory have examined a couple of others and also find them to be quite small. The introns have ranged in size from a few kilobases up to an intron in the *XJ* region of greater than 100 kilobases. Certainly there have been no other genes that have been examined that contain such a large number of small exons spread over such a large region. Whether this genomic structure turns out to have some biological basis, such as a repeating domain in the protein, remains to be determined.

Note added in proof (1 *February* 1988). Since the presentation of these results, new information has been quickly forthcoming. First, the isolation of the complete cDNA has been reported (Koenig *et al.* 1987) and when used as a probe the cDNA detects deletions in over 50% of DMD patients. There is a 'hot spot' for deletions toward the 3′ end of the gene. The cDNA derives from a 14 kb message encoding a muscle protein of about 425 kDa; the protein bears sequence homology to α-actinin and contains a potential actin binding domain (Hammonds 1987).

Second, cDNA sequences have been cloned into expression vectors, the expressed protein has been used to raise antibody against the gene product, and the antibody has been used to identify a muscle protein of about 400 kDa (Hoffman *et al.* 1987*a*). Similar results have been obtained in our laboratory by Elizabeth Zubrzycka-Gaarn and Dennis Bulman. Biochemical fractionation of muscle has led to the suggestion that the DMD protein (called 'dystrophin' by the Kunkel group) is preferentially located in the triads, the area of the muscle fibre controlling Ca^{2+} release and therefore muscle contraction (Hoffman *et al.* 1987*b*). In our own laboratory we have had somewhat contradictory results, and it is too early to pass judgement on the correctness of this subcellular localization. With the speed at which the field is moving, the function of the protein and therefore the biochemical cause of the disease may well be known by the time this article appears.

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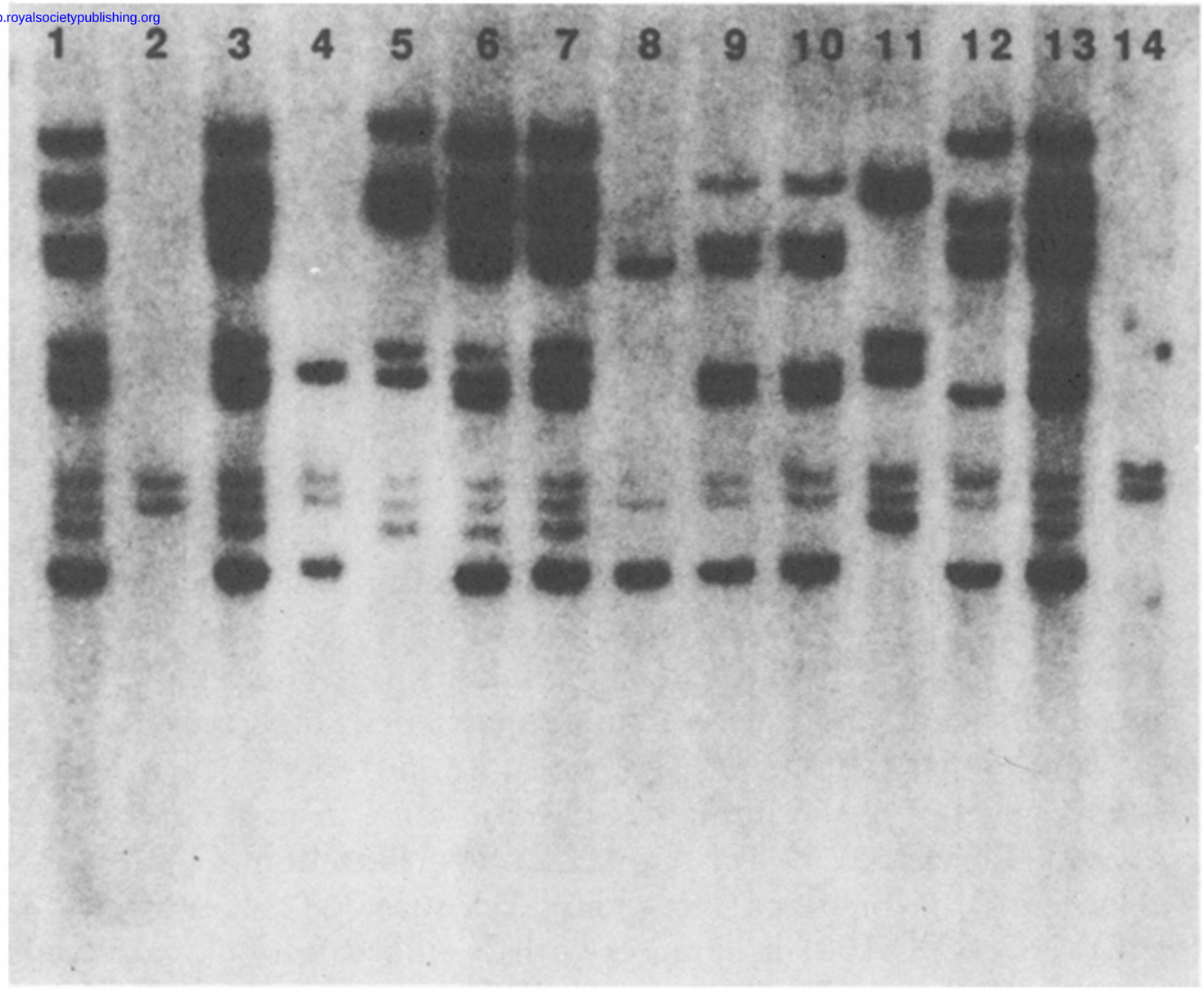

FIGURE 2. Southern blot analysis of 14 patients with DMD. Thirteen bands can be visualized on the *Hin*dIII digest, each band presumably containing a DNA fragment that carries an exon of the DMD gene and therefore hybridizes with the 2 kilobase cDNA probe. The cDNA was isolated by Burghes *et al.* (1987) from a cDNA library prepared by Lloyd *et al.* (1985). Patient 1 (lane 1) has deleted only one *Hin*dIII band (relative to a control in lane 6) and this is the fragment that carries the two *XJ* region exons (see figure 1). The remaining patients have deletions that include different sets of exons, and were useful for constructing the exon map in figure 1.