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Cloning of the DMD Gene*

Louis M. Kunkel

Division of Genetics and the Howard Hughes Medical Institute, Children's Hospital Boston, Boston

Louis M. Kunkel

I must start by thanking Jane Gitschier for that very kind and thorough introduction. Would you believe I have tears in my eyes? At the onset, I must say that I am extremely honored to have received the 2004 William Allan Award from the American Society of Human Genetics. This honor was beyond my wildest dreams when I started my career in human genetics.

In my lecture, I will try to outline which parts of my career I feel contributed to my receiving this award. I'm here today to review a little bit about the cloning of the gene responsible for Duchenne muscular dystrophy (DMD) and to tell you a little bit about where things are currently going in muscular dystrophy research. But, before getting into the muscular dystrophy gene–cloning story, I'd like to tell you a little bit about the early work that I did with the Y chromosome, because it fits with the whole theme of what I've done with my life in research. I will follow the Y-chromosome remarks with the story behind the *DMD* gene cloning, its protein identification, and how this led to improved diagnosis of the muscular dystrophies. I will also discuss some of the biochemistry of the muscular dystrophies and the approaches we might take in therapy for the muscular dystrophies. I will close and go through a series of thanks and acknowledgments to many of the past lab members—because you're honoring not just me, but my laboratory over many years. You also honor all the scientists and physicians who have collaborated with me over the years. I would point out that probably as many as 300 people in the audience today will see their name somewhere through my talk or on one of my slides.

Now to the Y chromosome. The Y chromosome is a unique entity in the sense that it's isolated from the rest of the genome. It should have sequences on it that are specific to it. It is influential and plays the major role as to whether you are male or female. And Dr. David Page at MIT and a number of others have done very well in characterizing the Y chromosome.

I started as a graduate student, with Drs. Kirby Smith and Ned Boyer at Johns Hopkins University, with a hypothesis that I could isolate from the Y-chromosome DNA sequences that were present solely on the Y—that weren't present in female DNA. I used a subtractive-hybridization strategy to enrich for those sequences on the Y chromosome. And the Y-specific criteria would be that they should hybridize solely to male DNA and not to female DNA. You would expect that, if you had two Y chromosomes, there would be twice as many sequences. Our paper describing our characterization of these Y-chromosome sequences was kindly communicated to PNAS by Dr. Victor McKusick (Kunkel et al. 1977). The Y-specific sequences failed to hybridize to *E. coli* DNA and DNA isolated from a human female. These same sequences hybridized to DNA isolated from a human male. The reassociation kinetics to the male DNA showed perfect hybridization kinetics and nearly 100% hybridization. Individuals with two Y chromo-

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Address for correspondence and reprints: Dr. Louis M. Kunkel, Division of Genetics and the Howard Hughes Medical Institute, Enders 570, Children's Hospital Boston, 300 Longwood Avenue, Boston, MA 02115. E-mail: kunkel@enders.tch.harvard.edu

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somes or bearing an isochromosome of the long arm of the Y showed a 2-fold increase in hybridization rate. It's almost like an RT-PCR reaction where you're looking at a 2-fold increase in amplification rate. The differential subtractive hybridization described in his paper served as the basis for some of the things that I'll tell you later. But this paper was most quoted, not for the Y chromosome work, but for the method we used to prepare DNA from whole human blood. And it was one of my most-quoted papers over the history of my time in genetics research.

Now let's turn to Duchenne dystrophy and the isolation of the gene altered in Duchenne muscular dystrophy. I came to Boston from San Francisco with the intent to try to look at the X chromosome. The late Dr. Sam Latt was starting to sort the human X chromosome, and he wanted to make chromosome-specific recombinant libraries from those chromosomes. And I proposed to the Muscular Dystrophy Association in a fellowship application that I would use those sorted chromosomes to prepare DNA segments that I could use to try to map the DMD gene. DMD, as you all know, is a very severe X-linked recessive disorder and is one of the most common recessive disorders in the human population. It presents in boys at ~3–5 years of age, with patients showing progressive muscle weakness. The progressive muscle wasting leads to their being confined to wheelchairs, usually by the age of 9 or 10 years. One of the hallmarks of this progressive muscle-wasting disease is the histological appearance of the muscle. Normal muscle, when stained with hemotoxlin and eosin, is highly organized with regular-sized fibers. In contrast is the obviously very disorganized muscle of a patient with Duchenne dystrophy, showing the connective tissue infiltration and hypertrophy of myofibers. You also see some inflammation within the muscle and extensive deposits of connective tissue.

The big question when I started this work in the early 1980s was how we could go about identifying the gene responsible for this disorder without a known biochemical defect. We had a genetic disorder, but we had no defective protein, as many had had before for diseases such as the hemoglobinopathies and Lesch-Nyan syndrome. We did know that DMD resided on the human X chromosome, because it was inherited as an X-linked recessive trait.

Knowing that it was an X-linked disorder, the gene responsible for Duchenne dystrophy must fall somewhere on the X chromosome. So, just by the process of elimination, we've eliminated essentially 95% of the genome. But where on the X was the Duchenne gene? Well, as I said, I joined Dr. Latt's lab to do chromosome sorting. The fluorescent-sort pattern of chromosomes from a 46XY individual—that is, an individual with one X chromosome—compared with that of an individual with five X chromosomes, is quite different at the peak in which the X falls. The peak containing the X chromosome is much higher when there are five X chromosomes. We were able to take segments of that peak, create an X chromosome–specific phage recombinant DNA library, and prepare DNA segments from that library (Kunkel et al. 1982).

Unbeknownst to me, at the same time I was doing this, Dr. Kay Davies at Saint Mary's in London was doing the same thing. And she actually published well before me the cloning of a representative cDNA library from which she derived segments of X-chromosome DNA (Davies et al. 1981). We followed up with our publication the following year, with Dr. Latt as senior author (Kunkel et al. 1982). We also used very similar methods that I had used before with the Y chromosome, to show that we could regionally localize the X-chromosome segments on the human X. Dr. Umadevi Tantravahi was able to produce beautiful blots by using variant X-chromosome rearrangements, with different chromosome-arm copy numbers to localize the cloned segments.

We used the cloned X chromosome–DNA segments to identify those that detected restriction-fragment–length polymorphisms. Using a similar approach, Dr. Davies' group was able to show that the Duchenne gene showed linkage to markers from the short arm of the X chromosome and that it could be flanked by two markers (Davies et al. 1983). Thus, the *DMD* gene was in the short arm of the human X. That was followed by Dr. Peter Harper's group showing that you could actually map the less-common and milder form of the disease, Becker muscular dystrophy, to the same region of the X chromosome (Kingston et al. 1984). These clones that detected restriction-fragment–length polymorphisms were used to do prenatal diagnosis of patients and of women who were at risk of having kids with Duchenne dystrophy (Bakker et al. 1985).

A second, independent way that the Duchenne gene was localized was done by a very astute set of geneticists, mapping X-chromosome translocations in girls with Duchenne dystrophy. For one girl, Dr. Ron Worton and Dr. Christine Verellen were able to show that the X chromosome was translocated to the region of chromosome 21 that contained the ribosomal genes (Verellen-Dumoulin et al. 1984). The normal X chromosome was inactive in almost all the cells of this girl. The abnormal, disrupted chromosome was the active chromosome, and it was thought that the disruption of that X chromosome was the reason why this girl had muscular dystrophy. The positioning of the *DMD* gene to that break point within the ribosomal gene cluster led Dr. Ron Worton to reason that he could use the junction fragment of the translocation break point—a piece of the X chromosome joined to a piece of a ribosomal gene—as a way to obtain a portion of the *DMD* gene (Worton et al. 1984). He

could use a ribosomal gene probe to try to pull out a piece of the X chromosome. And presumably, if it was broken in the Duchenne gene, he would pull out a piece of the Duchenne gene at the same time. Numerous other translocations that apparently disrupted the DMD gene in females were described, and these were quite heterogeneous in their break-point position.

While Ron Worton was attempting to clone the translocation break point, my lab was working with a patient who was first seen by Dr. Roberta Pagon, in Seattle, in Dr. Hans Och's group. The patient, B.B., had four X-linked disorders: Duchenne muscular dystrophy, chronic granulomotous disease, retinitis pigmentosa, and the rare red-cell phenotype McLeod. Roberta reasoned that this could be a contiguous-gene–deletion syndrome and asked Dr. Christine Disteche, a cytogeneticist who had actually just recently left a fellowship in Dr. Latt's laboratory, whether she could call this a "deletion." Christine said she felt she couldn't but recommended one of the best cytogeneticists in the country at the time, Dr. Uta Francke. They sent cells from the patient to Dr. Francke, then at Yale.

Dr. Francke felt very strongly that this was a deletion, and a number of us—including Dr. Worton, Dr. van Ommen and me—sent probes to her from the X-chromosome short arm to test against DNA isolated from various X-chromosome deletions, including those from B.B., to see whether she could indeed document a deletion of the short arm of the X chromosome that might be the cause of B.B.'s four genetic disorders (Francke et al. 1985). In a parallel effort, I reasoned that we might be able to use this DNA sample from this patient with a deletion in the same way I did the Y-chromosome work. That is, the patient should be missing DNA sequences that were present on a normal X chromosome, and I should be able to set up an experiment that would allow me to enrich for segments that were missing from this child's DNA.

DNA isolated from B.B. was sheared and mixed with a small amount of DNA isolated from an individual who had four X chromosomes. The "tracer DNA," as it is called, was cleaved with *Mbo*I, which left sticky ends that were compatible for cloning with a *Bam*H1 site. The DNA from the patient was sheared, so it had blunt ends and would not be compatible for cloning with a *Bam*HI site. We mixed them in a 200:1 ratio, and we used a hybridization technique that Dr. Kirby Smith had been working on while I was a graduate student at Hopkins, first described by Dr. David Kohne, called "phenol-enhanced reassociation." This was a way of getting the unique sequence-tracer molecules in the mixture to self-reassociate in the presence of this excess DNA when they found no complementary strand in the excess DNA of B.B.. Three types of hybridized molecules would form those with both strands sheared—which was the majority of the molecules, molecules with one strand sheared and the other *Mbo*I ended; these are molecules that found a complementary sequence in B.B.'s DNA. There would also be a very minor population of *Mbo*I/*Mbo*I-ended hybridized molecules that should be compatible with closing within a vector cleaved with *Bam*HI. These were *Mbo*I-cleaved molecules that found no complement in the deletion DNA but were able to self-hybridize to their own complement.

While these experiments were developing, Dr. van Ommen visited the lab in Boston and told us about a result that he had found in collaboration with Dr. Uta Francke. They had found a clone by the name of "754" that was missing from B.B.'s DNA (Francke et al. 1985). We, at the same time, were in the process of putting together the publication that described the specific cloning of DNA segments from the DNA isolated from the boy (Kunkel et al. 1985). The seven so-called pERT clones were completely absent from the DNA of the deletion patient. So we now have segments of DNA from the X chromosome that presumably are nearby or within the four genes causing the complex phenotype of B.B.

How do you move from there? I had proposed to the Muscular Dystrophy Association, in my first grant application, that we might use these deletion clones to identify the DMD gene. B.B.'s DNA had this large deletion, and we now had eight clones that were absent: seven identified in our analysis and the 754 clone identified by Drs. van Ommen and Francke. We knew from other X-linked disorders that ~5% of patients who had an X-linked disorder had a small deletion as the cause of that disorder. Some portion of the gene was missing. So the idea was, why not take these clones absent from the DNA isolated from the patient with the larger deletion and scan 50 or so DMD patients for deletions in their DNA at one of these loci. A deleted clone would likely be the closest to the Duchenne gene. We gathered the DMD patient DNA samples from our neuromuscular clinic and those collected by Dr. Fischbeck, then at Penn, and Dr. Roses, then at Duke. We were able to detect deletions with the pERT 87 clone but with none of the other clones (Monaco et al. 1985). This was the work of a very talented graduate student, Dr. Tony Monaco.

Nearly coincident with our work, Dr. Peter Ray, with Dr. Worton, was successful in cloning the ribosomal-junction fragment from the translocation carrier female and was also able to detect deletions with a clone from a region that they termed the "XJ region" (Ray et al. 1985). So we had two independent clones from the X chromosome that detected deletions.

These deletion-detecting clones were detecting the primary mutation in these boys with DMD, and the world scientific community heard about this well before publication of the results. Duchenne muscular dystrophy is such a common disorder, and physicians and patients were

anxious for the ability to do diagnostics of DMD. We made available the pERT 87 clone and surrounding subclones of DNA to many investigators around the world, some of whom are here in the audience. They contributed results, each of them, on a set of patient DNA samples in which they did deletion analysis. Overall, >1,300 DMD patients were analyzed for deletions at the pERT 87 locus, of which ~8% were deleted for the pERT 87 clone (Kunkel 1986). One of the conditions that we used for allowing the clones to go to other people was that they would send us the DNA samples of patients who were detected as having deletions. We were then able to map the deletion break points in the ever-growing chromosome walk that Tony Monaco was performing around the pERT 87 (designated the "*DX164*") locus. We found that there were patients who had the telomeric pERT 87-27 subclone present and the remainder of the *DX164* locus missing. There were also patients who had the centromeric side of the *DX164* locus present but the remainder of the locus missing. Some of these centromeric deletions were also not deleted for XJ clones. Many of the *DX164* subclones detected restriction-fragment–length polymorphisms, and these were being used to track the *DMD* gene in families. Somewhat surprisingly, some of these families exhibited recombination between the marker and the disease phenotype. This indicated that the mutation being followed was someplace outside the *DX164* locus.

We were finding recombinants that indicated that the mutations were outside and in either direction from our location on the X chromosome. We also knew that the *XJ* locus was completely independent of the *DX164* locus. So either we were in the wrong place or this was a very large gene spaced over a very large distance. So how does one find that large gene within the chromosome walk? Throughout the entire walk, we were looking for direct evidence of transcription by testing the unique sequence clones against cDNA libraries and northern blots—to no avail.

We then decided to use a very systematic approach, looking at sequence conservation among species, knowing that, for most cloned segments of genes, there is a sufficient nucleotide homology between the species and we should be able to pick up, by Southern-blot hybridization, the cross-species homology. Two segments of DNA within this *DX164* locus, PERT 87-4 and PERT 87-25, detected restriction fragments in DNA samples isolated from bovine, cebus, hamster, mouse, and even chicken. Both clones were sequenced by Tony Monaco in both directions, in both mouse and human. A small open reading was found in both species that was surrounded by consensus splice sites. He used the clone as a hybridization probe against a muscle-cDNA library and was able to pick out a cDNA that hybridized eight *Hin*dIII restriction fragments in human DNA. All the hybridizing *Hin*dIII fragments were completely missing from DNA isolated from B.B., and they were spaced over the entire 210-kb phage walk of the *DX164* locus (Monaco et al. 1986).

On northern blots, the transcript that was detected by this cDNA was 14 kb in size. Given the size of the transcript and the spacing of exons in the *DX164* locus, the prediction was that this was a large locus. A similar result was found by Dr. Ron Worton's group. Arthur Burghes used chromosome walking and sequence conservation to identify a second set of cDNAs that was nonoverlapping with those of the *DX164* cDNAs, again implying a very large locus (Burghes et al. 1987). During this time, a number of people were also doing pulse-gel electrophoresis and were documenting that this was an enormous locus and that the Duchenne gene was therefore spaced over a very large area (Burmeister et al. 1986; van Ommen et al. 1986). Tony Monaco later, after leaving my laboratory, cloned the entire locus in overlapping cosmids and was able to show that it was 2.5 million bp from the first exon to the last exon of the gene. In our era of the complete human sequence, the *DMD* gene is annotated from 30 to 32.5 Mb on the X-chromosome map. In most cases, if you looked at an interval of 2.5 Mb on the Web browser of genes within an interval, you would expect to see 10–15 genes within the interval. In the case of the Duchenne gene, only one gene is in the interval. The *DMD* gene is the largest mammalian locus encoding a single protein.

From the gene, I would like to now turn to the protein. And this is a place where I actually could have used the help of my dad, who had died just before all of this research happened. He was an immunologist, and I had to now become an immunologist. Eric Hoffman joined the lab as a postdoctoral fellow and started to work with making fusion peptides, expressing pieces of the *DMD* gene and putting them into both rabbits and sheep, and creating antibodies against these fusion peptides. He used two fusion peptides, one of 30 kDa and a second of 60 kDa. These were injected into rabbits and were used to look for the *DMD* gene–encoded protein on western blots. While he was doing this, the group at Columbia had heard that this was a huge gene with a very large transcript and that there was a known gene expressed in muscle, *nebulin,* which encoded a 600-kDa protein, and proposed *nebulin* as the candidate for the Duchenne gene (Wood et al. 1987). Just after that report, Eric Hoffman was able to show that it wasn't a 600-kDa protein; it was actually a 400-kDa protein, which we named "dystrophin" (Hoffman et al. 1987*a*). The name was based on the fact that we had used patient samples from kids with muscular dystrophy as a source of the DNA to identify this gene and the encoded protein. Most muscle-expressed proteins ended with "in," so "dystroph-in."

Once you have antibodies, you can ask, where is the protein? Eric Hoffman was forced to use western blots and membrane purifications, because the antibodies crossreacted with α-actinin. In collaboration with Dr. Kevin Campbell, we showed that dystrophin copurified with membranes and that those membranes were supposedly most enriched with the triad structures of muscle (Hoffman et al. 1987*b*). Well, it turns out that dystrophin was not localized to the triads, but it was localized to the plasma membrane, which contaminated our membrane preps. This sarcolemmal localization was very elegantly shown by Drs. Elizabeth Zubrzycka-Gaarn and Kichi Arahata independently, sadly neither of whom is with us any longer (Arahata et al. 1988; Zubrzycka-Gaarn et al. 1988). They were able to localize dystrophin to the plasma membrane of a muscle cell. We, working with Dr. Edwardo Bonilla, were able to show the same thing a few months later (Bonilla et al. 1988).

Dr. Michael Koenig joined the laboratory and was responsible for cloning the entire 14-kb transcript as overlapping cDNA clones (Koenig et al. 1987). He sequenced it manually, not with a machine. And it took him 6 mo to sequence that entire 14-kb transcript. It was predicted to be a rodlike structure and, because it underlay the plasma membrane, he felt it played a role in membrane stability under contraction and relaxation of muscle (Koenig et al. 1988).

The work outlined here led to improved diagnostics. The dystrophin gene, being so big, is an enormous target for deletion mutations. Dr. Jeff Chamberlain, working with Dr. Tom Caskey at Baylor, was able to develop a deletion-screening procedure that used multiplex PCR (Chamberlain et al. 1988). And Dr. Alan Beggs in my group was able to elaborate on that and show that we could pick up, by multiplex PCR, ~98% of all deletion mutations at the dystrophin locus (Beggs et al. 1990). This led to a very rapid and inexpensive molecular test for the most commonly deleted exons.

This is the method that's still in use today for diagnosis of ~65% of patients with Duchenne dystrophy. This leaves many patients without a molecular diagnosis, which is a serious drawback and somewhat unfair to the patients with muscular dystrophy. It's just recently that Dr. Kevin Flanigan has actually worked out a very high-throughput robotic sequencing strategy to look at all mutations in the dystrophin gene (Flanigan et al. 2003). We'll see later that some therapies being developed are mutation specific. We are working with Applied Biosystems to work on a modification of his protocol to look at point mutations at the dystrophin locus in a very high-throughput, rapid, robust way (Bennett et al. 2004). We are also targeting all the genes involved in all the different types of muscular dystrophy. I believe improved DNA diagnostics is one of the great unmet needs in muscular dystrophy research.

Protein-based diagnostics were developed first by Dr. Eric Hoffman in collaboration with a group of referring physicians. He was able to show that patients with Duchenne dystrophy made virtually no or very small amounts of very-abnormal-sized protein, whereas patients with milder Becker dystrophy made an internally truncated protein of near-normal abundance (Hoffman et al. 1988). These protein-based tests, both by western blotting and immunohistochemistry, have become the standard of care for diagnosis of X-linked dystrophy. Carrier females can also be detected and are mosaics for dystrophin deficiency, with some fibers missing dystrophin where the normal X has been inactivated and some fibers where dystrophin is present. This is quite diagnostic of carrier females. Tony Monaco, in a paper that was published in the January issue of *Genomics,* with a received date in February, explained our hypothesis about the difference between a deletion yielding a patient with Duchenne dystrophy and a deletion in a patient with Becker dystrophy (Monaco et al. 1988). The deletions in patients with Becker dystrophy retained the reading frame of the protein, whereas the deletions of patients with Duchenne dystrophy disrupted it.

Now dystrophin doesn't work alone, and the identification of dystrophin led to a complete set of unique membrane proteins that had previously been unrecognized. Much of this work was accomplished by pulling dystrophin out of muscle with an antibody and by asking what else came along with it. This work was first really pioneered by our early collaborator, Dr. Kevin Campbell; he showed that there was an integral lack of a protein named "dystroglycan" that was associated with dystrophin (Campbell et al. 1989). Campbell et al. showed that there are actually a number of other proteins that interacted with dystrophin, and they proposed a model of this interaction, termed the "dystrophin-associated protein complex," or DAPC (Ervasti and Campbell 1991).

At the same time, Dr. Ozawa's group in Japan was doing similar fractionations of dystrophin and its associated proteins and was able to show that the dystrophin-associated proteins actually separated into two classes: those that were of the dystroglycan complex and those that were of the sarcoglycan complex (Yoshida and Ozawa 1990; Yamamoto et al. 1993). The sarcoglycans were quite interesting, because the first one cloned, encoding a 50-kDa protein, was one that was localized by Dr. Campbell's group and was found to be completely missing from the muscle of a patient with limb-girdle dystrophy, which was endemic within the Tunisian population (Matsumura et al. 1992). That dystrophy was mapped, by Dr. Jeff Vance's group in collaboration with Dr. Ben Hamida, to chromosome 13 (Othmane et al. 1992). The 50-kDa protein was actually encoded by a locus on chromosome 7.

So this made things a bit confusing, but it was clear

that there was a form of autosomal recessive dystrophy on chromosome 7 (Roberds et al. 1994). The second of the sarcoglycans was cloned independently by both Dr. Campbell's group in collaboration with Dr. Charles Jackson and by us in collaboration with Dr. Ozawa. This protein was named "β-sarcoglycan" after these two papers went into *Cell* at the same time and were rejected as redundant with each other. The reviewers asked us to give the protein the same name. Dr. Jackson had identified Amish families that were segregating a recessive dystrophy that mapped to chromosome 4, a location to which the β-sarcoglycan clone mapped (Lim et al. 1995). Dr. Bonnemann used a panel of patients with dystrophy, provided to us by Dr. Eric Hoffman, to look for a patient who might have a mutation in the β-sarcoglycan gene (Bonnemann et al. 1995). This work was followed by the cloning of the chromosome 13 gene, which encoded the γ-sarcoglycan gene, work Dr. Elizabeth McNally in my group did in collaboration with Dr. Ozawa (Noguchi et al. 1995). Subsequently, Dr. Vincenzo Nigro was able to use a chimeric cDNA clone and to show that the γ-sarcoglycan gene had a homolog—which he called "δ-sarcoglycan"—on chromosome 5, a location to which Mayana Zatz had just mapped a familial dystrophy locus. I actually reviewed both papers and believe that I indirectly showed the two groups that one has the gene and one has the locus. The groups got together, and that led to their identification of mutation in the δ-sarcoglycan gene in this recessive form of muscular dystrophy (Nigro et al. 1996).

Over the years, the dystrophin-associated–protein complex, DAPC, has evolved as more and more proteins are shown to associate in it, and at least half the members of this complex are themselves involved in forms of muscular dystrophy; most were identified by biochemistry. That is, they were identified as part of this complex and then were tested in patients with different forms of muscular dystrophy. And this has been the work of many, many people. We believe this is the complex that stabilizes the plasma membrane of muscle and that, when it contracts and relaxes, the membrane is inherently unstable when it is compromised by mutation in these proteins. The membrane tears and allows calcium to influx into the cell, causing proteolytic degradation via calcium-activated proteins. There is still a lot of work to be done on the pathogenesis, and we still need to explain how all these different forms of muscular dystrophy—some of which aren't really immediately biochemically related to one another—actually give rise to similar muscular wasting seen in patients.

I would like to end the overview session with therapeutic approaches to muscular dystrophy. We've learned a lot, but it's been 18 years since the Duchenne gene was cloned, yet there's still no therapy other than the anabolic steroid, prednisone, that's useful for these kids. Now that's really a travesty—that we've gone that long and we still don't have therapies. But I should say, there is an enormous number of possibilities coming. I didn't talk at all about utrophin because I just really didn't have time, but much of it is the work of Dr. Kay Davies and a past student of mine, Dr. Teji Khurana, and it's really fantastic. Dr. Davies has shown that utrophin will actually correct for absent dystrophin, and upregulation of utrophin could correct for the absence of dystrophin (Tinsley et al. 1998).

I will touch on exon skipping, antibiotic use, and some gene- and cell-based therapies, ending with the cell-based therapies, something that my group has been involved with from the very beginning. We need an animal model of muscle disease, and there is a model. There are actually five alleles of that model now, the *mdx* mouse first published as a point mutation in exon 23 (Sicinski et al. 1989). The mouse makes no dystrophin yet lives a normal life span with minimal muscle weakness. The effort is on to correct for absent dystrophy. Dr. Jeff Chamberlain's group very elegantly showed that if you transgenically overexpress dystrophin in this animal model, it could completely restore dystrophin expression and it could actually restore functional characteristics (Cox et al. 1993). In addition, overexpression wasn't toxic to the muscle cell. So, if you put the dystrophin back, you'd actually correct the problem. So that's what all of us have been trying to do, correct the problem by restoring dystrophin expression by some means.

Dr. Lee Sweeney's group, working with antibiotics, was able to show that if you treated *mdx* mice with G418, an antibiotic, you could actually get some restoration of dystrophin expression to the membrane and actually get some improvement in the serum creatine-kinase levels (Barton-Davis et al. 1999). This has actually led to an effort at a company to find additional molecules that will do this much more efficiently. And Lee has talked about a compound by the name of "PTC124," which actually looks as if it works better than the antibiotics. That is now going to toxicity testing. This is very promising but will work on only point mutations that result in a premature stop codon.

A number of groups have looked at exon skipping. It's something we talked about, Tony Monaco and I, many, many years ago—about trying to fool the splicing machinery into removing exons that contained a mutation. Dr. Steve Wilton, along with Dr. Terry Partridge, has been able to show that you can actually restore dystrophin expression when the mouse muscle is exposed to an oligonucleotide against one of the spliced sites (Mann et al. 2001). In Europe, they are also starting human experiments to actually look and see whether they can do this read-through in human patients.

Myostatin is a negative regulator of muscle development. It has been shown by Dr. Teji Khurana, a past

student of mine, in collaboration with Wyeth, that if you downregulate or if you reduce the levels of myostatin, you actually build up muscle (Bogdanovich et al. 2002). Myostatin-null mice and myostatin-null cattle have about twice the muscle mass of a normal mouse or a normal cow. Wyeth is actually in human trials to try to knock myostatin levels down in three forms of muscular dystrophy.

Lastly, Dr. Jeff Chamberlain has just recently reported a very nice systemic delivery of microdystrophin to the muscle of the *mdx* mouse (Gregorevic et al. 2004). The work was presented in a platform session on Saturday, during the ASHG annual meeting, by Paul Gregorick; he's been able to systemically deliver a microdystrophin in AAV6 that actually restores function to the muscle. This vector appears to nearly completely restore dystrophin expression in these animals. This is an amazing amount of expression, and they're actually gearing up to do human trials with these vectors and microdystrophin.

I'll end my talk by discussing cell transplantation. Muscles are a regenerative tissue, and I've felt all along that you should be able to take the mononuclear cells called "myoblasts" from a normal individual and introduce them into the muscle of a patient with muscular dystrophy. These should fuse to pre-existing fibers and produce absent gene products, such as dystrophin. My group has been working on this for many years, along with Dr. Terry Partridge and a number of others. With Dr. Partridge, we were able to convert *mdx* fibers from dystrophin-negative to -positive by introduction of fetal mouse–muscle progenitor cells (Partridge et al. 1989).

This work led to a series of human experiments with so-called myoblast transfer; all of which really didn't work (Gussoni et al. 1992; Huard et al. 1992; Karpati et al. 1993; Mendell et al. 1995). This led us to go back to the drawing board and ask whether there were additional ways one could identify cells that had a much higher fusion index. We took advantage of our close proximity to Dr. Richard Mulligan, who was using Hoechst dye to sort bone-marrow stem cells. This was actually a dye we had used many years before in our chromosome sorting. The stem cells of the marrow, when stained, were able to efflux the Hoechst dye, leaving a less brightly stained group of cells, termed "side population" (SP). These same cells isolated from muscle were able to restore the hematopoetic compartment and could systemically traffic from the circulation into muscle and that could contribute to muscle regeneration (Gussoni et al. 1999). We are working to improve this trafficking and, in collaboration with Dr. Jeff Chamberlain, we have transduced the cells with a lentivirus expressing a microdystrophin. Only SP cells are recruited into damaged muscle, indicating that they were enriched and represented some kind of a progenitor cell. By this intravenous delivery system, we were able to produce human microdystrophin in the mouse muscle; a bit <1% of the fibers expressed the human protein (Bachrach et al. 2004)—far below what would be considered therapeutically useful.

A second delivery method, developed by Dr. Giulio Cossu, is to introduce the cells intra-arterially—unlike intravenous delivery where the cells would be filtered through various different organs of the animal. The cells go directly to the muscle with arterial delivery. And the arterial delivery gives you a much more substantial ingraftment of these cells. Indeed, in Dr. Cossu's hands, using a model of α-sarcoglycan muscular dystrophy, he could actually restore function with arterial delivery of a stem cell, which bodes very, very well for therapy (Sampaolesi et al. 2003). Actually, I've heard that, in Italy, they're actually contemplating human trials of interarterially delivered stem cells. Indeed, we presented a patient a few years ago, in collaboration with Dr. Ken Weinberg, who had been transplanted with bone marrow and who still has residing within his muscle—many years later—donor cells from that transplant (Gussoni et al. 2002). Those donor cells, though, were so inefficient at producing dystrophin that they are not at all corrective. This first serendipitous use of bone-marrow transplant in DMD is interesting and does indicate that cells can be recruited from the circulation, albeit at very low efficiency.

Where are we now? What do we need? I think we need very-much-improved DNA diagnostics. We are doing a disservice to patients who don't carry a molecular diagnosis. I think we're getting close to newborn screening, something I feel is important, because, if you remember that muscle cross section of DMD muscle that I showed you, the damage is so great, even in the muscle of a 5 year old, that whatever treatment we use will need to be done sooner, before the muscle damage occurs. We need to understand the pathogenesis of the disease. As I talked about earlier, small-molecule and gene stem-cell therapy are all moving forward.

In ending, I'd like to acknowledge the Muscular Dystrophy Association for their support over the many years, including that first grant, which came from them to get me started. The NIH, NICHD, NINDS, NIAMS, NIA, and the Institute of Aging are actually unrelated to this. This is my longevity work. I have been hugely fortunate to be an Investigator in the Howard Hughes Medical Institute for many years. I've also been fortunate to have the support of many, many families of patients with muscular dystrophy.

I will close with a few slides of my lab over the years, giving tribute to the late Dr. Sam Latt, who mentored me through much of this work. I think he would have been up here well before me receiving this award, because his thoughtfulness and creativity were enormous. I thank all past members of the lab whom I may not have

acknowledged during my talk. Lastly, I'd like to thank my family, who has been so supportive over these years. And I also thank you all for listening.

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