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Genetics

Recovery of induced mutations for X chromosome-linked muscular dystrophy in mice

(*mdx* alleles/*N*-ethylnitrosourea mutagenesis/mouse models of human disorders)

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ABSTRACT **We have used elevated levels of plasma creatine phosphokinase activity to identify muscular dystrophy phenotypes in mice and to screen the progeny of chemical mutagen-treated male mice for X chromosome-linked muscular dystrophy mutations. We were not successful in identifying heterozygous carriers of these induced muscular dystrophy mutations in >8000 progeny. However, we were highly successful in identifying three additional alleles of the characterized *mdx* locus. These alleles of *mdx* were recovered from various mutagen-treated males and they occur on an X chromosome that carries flanking markers that allow us to follow the mutations in genetic crosses and in the development of corresponding mutant stocks. These alleles have been designated as mdx^{2Cv}, mdx^{3Cv}, and mdx^{4Cv}. Preliminary data show that mice with mdx^{2Cv} and mdx^{3Cv} mutations have muscular dystrophic phenotypes that do not grossly differ from the characterized *mdx* mutation. These additional *mdx* mutations expand the value of mouse models of X chromosome-linked muscular dystrophy and potentially define additional sites of mutation that impair dystrophin expression.**

A recessive X chromosome-linked muscular dystrophy mutation (*mdx*) has been identified in the laboratory mouse that is isogenic on the homozygous inbred mutant strain C57BL/10Sn.*mdx* (1). Mutant mice have pathological features that indicate that it is a true myopathy. The defect is expressed in all skeletal muscles of *mdx*/Y males and *mdx*/*mdx* females with extensive limb muscle degeneration, inflammation, and regeneration (2) as well as myocardial lesions (3). However, the affected mutant males and females do not show overt signs of wasting or impaired motor function, which is common to the human Duchenne–Becker muscular dystrophy mutation (4). They are also capable of breeding successfully so that the *mdx* mutation can be directly propagated. Heterozygous mutant carrier females +/*mdx* do not show physiological symptoms such as elevated levels of muscle enzyme in circulating blood or histopathology associated with the myopathic condition (1). The *mdx* mutation has been mapped between *Hprt* and *Pgk-1* on the mouse X chromosome (5, 6) and within the mouse *Dmd* locus that encodes dystrophin (7).

The chemical *N*-ethylnitrosourea (ENU) has been identified as a powerful mutagen in mice. Male mice treated with one intraperitoneal dose of ENU transmit germ-line mutations to progeny at a frequency as high as 1 mutation per 700 loci tested (8–11). In this report, we describe the results of our use of ENU to produce muscular dystrophy mutations in mice. In one series we screened a group of female progeny of ENU-treated males in an attempt to identify heterozygous carriers of muscular dystrophy mutations. No muscular dystrophy mutations were recovered from those progeny. However, in a second series, we screened for muscular dystrophy mutations when mutagen-treated males carrying variant alleles of *Hprt* and *Pgk-1* were mated with C57BL/10Sn.*mdx* females. The resulting female progeny of these matings were heterozygous for +/*mdx* and showed normal levels of muscle enzyme in circulating blood and normal muscle histology. Female progeny with the resulting muscular dystrophy mutations would be heterozygous for the flanking *Hprt* and *Pgk-1* markers and show elevated muscle enzymes in circulating blood. Six of 1563 generation 1 females were identified who had elevated levels of muscle enzyme in circulating blood plasma. These increased levels of muscle enzyme were heritable as X chromosome-linked traits and were subsequently identified as muscular dystrophy mutations. This report describes the recovery of these mutations and their initial characterizations.

MATERIALS AND METHODS

Mice. An X chromosome marker stock carrying electrophoretic variants of the gene products for hypoxanthine phosphoribosyltransferase (HPRT) and phosphoglycerate kinase type 1 (PGK-1) was produced for these studies. The HPRT-A and PGK-1A allelic forms differ from the electrophoretic allotypes HPRT-B and PGK-1B found in all inbred strains of mice examined to date (12, 13). The $Hprt^a$ allele used for this marker X chromosome came from *Mus castaneus* (12) whereas the $Pgk\text{-}1^a$ allele came from *Mus musculus* (13). The $Pgk\text{-}1^a$ allele was transferred to the C3H/HeHa strain background by repeated backcrosses for 10 generations. The resulting C3H/HeHa.$Pgk\text{-}1^a$ congenic strain was crossed with *Mus castaneus* ($Hprt^a$, $Pgk\text{-}1^b$) to produce $Hprt^{a/b}$, $Pgk\text{-}1^{a/b}$ progeny. These progeny were backcrossed to the C3H/HeHa $Hprt^b$, $Pgk\text{-}1^a$ congenic strain and the recombinant $Hprt^a$, $Pgk\text{-}1^a$ progeny were identified and backcrossed to the C3H/$Pgk\text{-}1^a$ congenic for 10 generations to establish the X chromosome marker stock C3H.X_{25} that carries both variant X chromosome markers $Hprt^a$ and $Pgk\text{-}1^a$.

We obtained the C57BL/10Sn.*mdx* mutant from G. Bulfield (Institute of Animal Physiology and Genetics Research, Edinburgh, U.K.). These mice are maintained as a homozygous inbred breeding colony. Other inbred mice were obtained from the West Seneca Laboratories of Roswell Park Memorial Institute. These included C57BL/6Ros, DBA/Ha, B6D2F_1 hybrids.

Enzyme Assays. We have modified the coupled assay of creatine phosphoskinase (CK) to detect the accumulation of NADPH fluorimetrically. The change in fluorescence is determined for a 10-min period at 30°C in 5 μl of plasma. A semiautomated enzyme assay was used that employed a spectrofluorometer interfaced with a computer. The fluorescence signal from the reaction mixture was directly trans-

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Abbreviations: CK, creatine phosphokinase; ENU, *N*-ethylnitrosourea; HPRT, hypoxanthine phosphoribosyltransferase; PGK-1, phosphoglycerate kinase type 1.

formed to estimate the specific activity per μl of blood plasma (where 1 activity unit = μmol of NADPH per min per μl of plasma). Samples of 30 or more mice were assayed at each time and the mean and standard deviation were determined for each sample. Individuals with specific activities exceeding two standard deviations of the mean were compared with the activity levels of *mdx* controls and reassayed if they were comparably high to the levels observed in *mdx* mice. Mice that had plasma CK activity >7 units were set aside and blood was again collected after 1 week.

Blood Sampling. Substantial variation was observed among normal mice in levels of blood plasma CK activity possibly as a consequence of physical activity or stress. We attempted to minimize the variation in enzyme levels by leaving the mice undisturbed for 2 or 3 days prior to bleeding. Mice were lightly etherized and bled through heparinized capillaries from the retroorbital sinus. A uniform volume of 100 μl of blood was collected from each mouse. On average, 40–60 min were required to collect a replicate of 30–40 samples.

Electrophoretic Assays. PGK-1 allozyme phenotypes were determined on cellulose acetate by the method developed by Bücher *et al.* (27) as described by Chapman *et al.* (14). HPRT allozyme phenotypes were determined by isoelectric focusing as reported by Chapman *et al.* (14).

Mutagen Treatment. Male mice were treated with ENU (Sigma) at 10 μg/ml of 0.1 M sodium citrate/0.1 M sodium phosphate, pH 6.0. The concentration of ENU in the final solution was determined using a colorimetric assay for nitrite. In brief, the procedure involves reacting the nitrosyl group of the ENU with concentrated sulfuric acid, in the presence of excess phenol, to form indophenol, which under alkaline conditions gives a maximal absorption peak at 615 nm and a blue color. Mice received one injection i.p. of ENU at doses from 125 μg/g to 250 μg/g.

Identifying Muscular Dystrophy Phenotypes from Blood Levels of CK. A value of 8 fluorescence units of CK activity was used as a cutoff point to identify putative mutant or mutant carrier mice among the progeny of ENU-treated males. Controls with blood CK levels >7 units were retested after 1 week to ascertain whether the CK activity value observed in the first blood sample was reproducible in a second. Nonmutant control mice with plasma CK activities >7 units in the first sample had blood plasma CK activity levels of <7 units in the second sample (data not shown). Plasma CK activity levels were measured on mice between 6 and 12 weeks of age to avoid age effects on plasma CK activity levels (15–17).

RESULTS

Screening for Heterozygous Carriers of Muscular Dystrophy. Increased levels of muscle enzyme in blood have been used as a method of identifying heterozygous carriers of Duchenne muscular dystrophy with up to 95% success (4). We reasoned that we might expect to recover mouse mutations for muscular dystrophy by identifying these mutations in females that were heterozygous for the recovered mutations. For this work, we tested the generation 1 (G_1) progeny of both inbred and hybrid males treated with various dosages of ENU. We used these doses because previous work in our laboratory suggested that inbred mice would be more sensitive to the sterilizing effects of ENU.

We injected a total of 355 males of two strains, C57BL/6Ros and DBA/Ha, and an F_1 hybrid, B6D2F_1, with doses of ENU from 125 μg/g to 250 μg/g. The breeding records summarized in Table 1 show that inbred males were more sensitive to the highest dose of ENU (250 μg) with fewer males breeding after treatment and fewer progeny produced by C57BL/6 males compared with DBA/Ha. By contrast, the groups of hybrid males receiving ENU at 250 μg/g had significantly more males breeding after treatment and an increased number of female progeny produced. In subsequent experiments we used a decreased dose of ENU to increase the number of males breeding and the number of G_1 progeny available for testing. We observed that an ENU dose of 200 μg/g in C57BL/6 was the maximum dose that would not produce irreversible sterility. Overall, we produced and tested 8336 G_1 female progeny from 182 ENU-treated males. No heritable increases in circulating muscle enzyme levels were observed in this series of progeny (data not shown). However, a total of 13 morphological mutations were recovered, which included dominant white spotting, tail kinks, short tail, and grey coat color. The reduced fertility of males receiving higher doses of ENU and the recovery of morphological mutations at frequencies greater than those observed in other control populations (18) indicate that the ENU treatments given to males were reaching the testes and that mutational events were being produced in the germ cells.

Table 1. Female progeny tested from inbred (C57BL/6Ros and DBA/Ha) and B6D2F_1 males treated with various doses of ENU

Strain	ENU dose, μg/g	Males treated, no.	Treated males breeding, no.	G_1 female progeny, no.	Dominant morphological mutants, no.
C57BL/6	250	60	2	24	
	200	165	108	4217	10
	150	37	33	1579	2
DBA/Ha	250	47	6	398	
	125	10	9	1384	
B6D2F_1	250	36	24	734	1
Total		355	182	8336	13

Males in a second breeding group, which carried variant alleles of X chromosome genes, were treated with ENU (Table 2). We used either F_1 hybrid males (C3H.X_{25} × C57BL/6Ros) or their intercrosses to take advantage of the increased reproduction of the hybrids compared with the C3H.X_{25} congenic strain. We treated 154 males who carried the X chromosome marker alleles with ENU at either 250 or 200 μg/g. From these, 62 males were fertile and produced 2074 female progeny. Of these, 1597 G_1 female progeny were from C57BL/10Sn.*mdx* mothers (Fig. 1). We observed a significant difference in the distribution of plasma CK activity levels in heterozygous +/*mdx* females compared with wild-type (+/+) homozygotes [χ^2 (12df) = 392 (where df = degrees of freedom); $P < 0.005$) (Fig. 2). However, the CK activity levels of individual heterozygous carriers were not sufficiently high that we would detect a significant number of heterozygous carriers as being different from the wild-type phenotype.

We observed 82 G_1 progeny who had levels of plasma CK activity that were >8 units on the first test. These females were retested after being allowed to rest for 1 week and we observed that 10 of these 82 G_1 females had CK activity levels

Table 2. Female progeny tested from X chromosome-marked males mated with either *mdx* or +/+ females

Male genotype	ENU dose, μg/g	Males treated, no.	Treated males breeding, no.	G_1 female progeny, no.	
				+*	*mdx*†
(C3H.X_{25} × BL/6)F_1	250	72	38	411	854
(C3H.X_{25} × BL/6)Int‡	200	82	24	66	743
Total		154	62	477	1597

*+, G_1 progeny from +/+ mothers.
†*mdx*, G_1 progeny from *mdx* mothers.
‡Int, intercrossed stocks at the second backcross generation to produce a *Hprt*^{a}, *Pgk-1*^{a} homozygous and hemizygous stock.

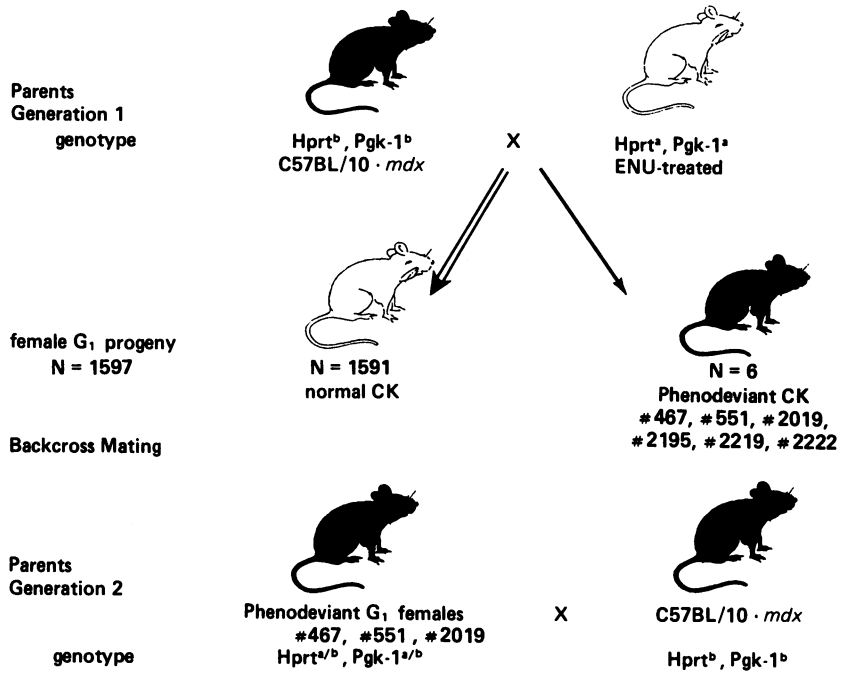

FIG. 1. Mating protocol used to produce progeny of ENU-treated males mated with C57BL/10.*mdx* females. Black mice symbolize dystrophic phenotypes with elevated CK activity levels in circulating blood. Also shown are generation 2 backcross matings to follow the transmission of putative *mdx* mutants among backcross progeny. The classes of backcross progeny are shown in the table for females 467, 551, and 2019. The asterisk (*) indicates the X chromosome carrying the putative mutation. The relative frequency of progeny in each class (*N*) is indicated next to the mean CK activity in the plasma of those progeny. *Hprt*^{a} (*/*mdx*), *Pgk-1*^{b} and *Hprt*^{b} (*/*mdx*), *Pgk-1*^{a} are recombinational events and the resulting X chromosome could carry the induced mutation or the preexisting *mdx* allele. No progeny of these or subsequent matings carrying the *Hprt*^{a}, *Pgk-1*^{a} chromosomes showed wild-type CK activity levels in blood. CK levels reported are as units/μl of plasma.

Gametes Observed In Backcross Progeny

Genotype			Serum CK Levels					
			Progeny of 467		Progeny of 551		Progeny of 2019	
			♂ (N)	♀ (N)	♂ (N)	♀ (N)	♂ (N)	♀ (N)
Hprt^a	*	Pgk-1^a	19.2 (3)	18.75 (2)	16.1 (2)	15.2 (5)	22.8 (2)	17.1 (3)
Hprt^a	(*/mdx)	Pgk-1^b	15.7 (2)		13.5 (1)		35.7 (2)	26.3 (1)
Hprt^b	(*/mdx)	Pgk-1^a		27.8 (2)			17.9 (1)	6.2 (1)
Hprt^b	mdx	Pgk-1^b	16.6 (3)	23.2 (2)	13.2 (4)	18.7 (4)		27.1 (3)
		Total	N = 8	6	7	9	5	8

>8 units in a second test. Two of these G_1 females (mice 467 and 551) were from separate ENU-treated males, and 4 (mice 2019, 2195, 2219, and 2222) were from a third ENU-treated male. Four additional G_1 females were observed in the progeny of breeding group 2. One was a sibling to the group of 4 (mice 2019, 2195, 2219, and 2222) and did not breed and 3 females failed to express the *Hprt*^{a} and *Pgk-1*^{a} alleles from the ENU-treated father. The latter were presumptive X0 phenotypes expressing the maternal *mdx* genotype.

Transmission of the mdx Phenotype. The six phenodeviant G_1 females were mated with C57BL/10Sn.*mdx* males to establish the transmission of elevated plasma CK levels with the *Hprt*^{a}, *Pgk-1*^{a} chromosome. Backcross male progeny of these matings would carry the *Hprt*^{b}, *mdx*, *Pgk-1*^{b} from the original *mdx* mother and *Hprt*^{a}, *, *Pgk-1*^{a} from the ENU-treated father. If we produced another *mdx* mutation, the *Hprt*^{a}, *Pgk-1*^{a} backcross males should have uniformly high plasma CK activity levels. Conversely, the backcross females should show elevated levels of plasma CK activity if a resulting *mdx* mutation failed to complement the *mdx* allele from the *mdx* father of the backcross test progeny. In each case, the backcross progeny carrying the *Hprt*^{a}, *Pgk-1*^{a} chromosome show elevated levels of plasma CK activity consistent with the inheritance of a mutant allele for *mdx* (Fig. 1 *Lower*). The *Hprt* and *Pgk-1* loci are ≈25 cM (1 cM = 1% recombination) apart on the X chromosome, and the frequency of either *Hprt*^{a}, *Pgk-1*^{b} and *Hprt*^{b}, *Pgk-1*^{a} gametes did not differ from that expected recombination (10/43 = 0.23 ± 0.06). Test crosses of mice 2195, 2219, and 2222 also produced backcross progeny with similar results (data not shown). We conclude that the elevated plasma CK activity levels in the six phenodeviant G_1 progeny are heritable as alleles of the X chromosome-linked *mdx* locus and that these alleles fail to complement the existing *mdx* mutation.

Histology of Putative *mdx* Mutants. We compared the histology of skeletal and cardiac muscle of generation 2 (G_2) HPRT-A, PGK-1A males from females 467 and 551 with wild-type and *mdx* tissues to determine whether the elevated muscle enzyme levels in circulating blood were a consequence of a true myopathy. The G_2 male progeny of both females 467 and 551 showed elevated blood plasma CK activity levels and an electrophoretic analysis of CK activity in blood demonstrated that the elevated activity was predominantly of the muscle isozyme form (Table 3). Both male progeny also show pseudomyotonia in electromyographic tests. The muscle histology of progeny of females 467 and 551 did not differ from C57BL/10Sn.*mdx* in that all three types of mice showed pronounced alterations in fiber regeneration and degeneration, central nuclei, and phagocytosis of the cytoplasm by invading macrophages (Fig. 3). Additional tests are required to ascertain the onset of the altered histology and whether different mutant lines show differences in pathology.

A summary of the CK activity levels, electrophoretic profile of CK in blood plasma, and the histopathology of the cardiac and skeletal muscle is shown in Table 3. We conclude from the transmission of elevated plasma CK activity and the muscle histology of male progeny of G_1 females 467 and 551 that we have recovered additional mutations of the *mdx* locus from these ENU-treated males. The recovery of these mutations on X chromosomes with the flanking markers *Hprt*^{a} and *Pgk-1*^{a} provides a direct method of following these mutations in subsequent crosses and in the development of congenic strains. We propose the designation of these mutations as alleles of *mdx* with the nomenclature of *mdx*^{2Cv} (our

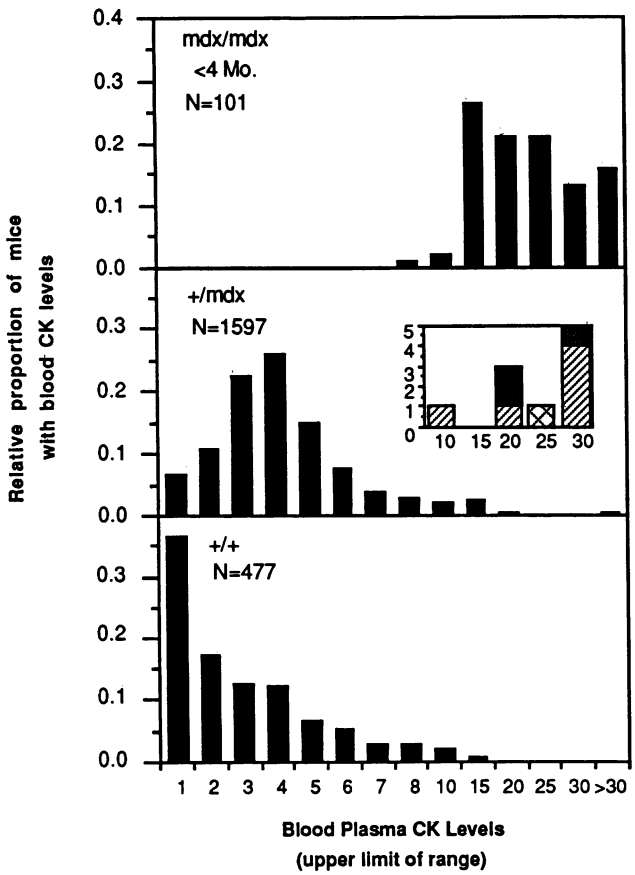

FIG. 2. Frequency distribution of mice with blood plasma CK activity levels (units/μl of plasma) in increasing levels of specific activity. The numbers at the bottom represent the upper limits for each class such that 1 is 0–1, 2 is 1.01–2, etc. (*Top*) The plasma activity of *mdx/mdx* mutants <4 months of age. (*Middle*) The distribution of activities observed in the group 2 G_1 progeny (Table 1) from C57BL/10.*mdx/mdx* mothers. (*Insert*) The 10 phenodeviants that had high CK activity levels in the second blood sample tested. The hatched bars indicate G_1 progeny that bred as our induced *mdx* mutants and the stippled bars were the X0 progeny. The crosshatch bar represents the sibling of 2019 that failed to breed. (*Bottom*) G_1 progeny that carry the $Hprt^a$, $Pgk\text{-}1^a$ marker chromosome in matings of ENU-treated males with C57BL/6J mothers.

laboratory substrain designation) and mdx^{3Cv} for the mutations from G_1 females 467 and 551, respectively. At this time

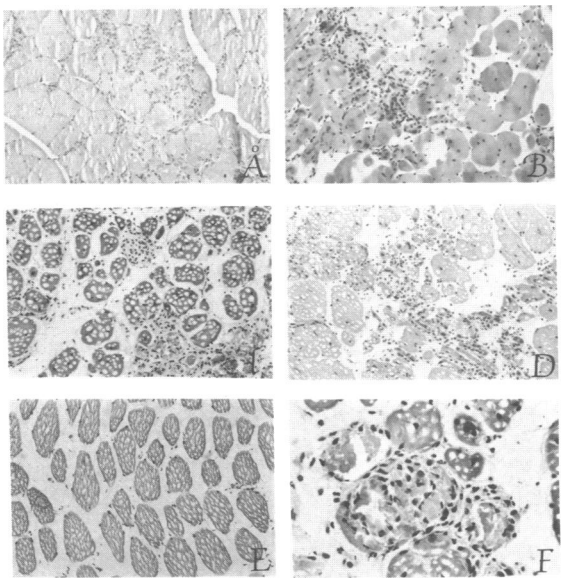

FIG. 3. Cross-sections of skeletal muscle of male mice with various mutations that show invasion of cells causing disruption of central nuclei in fibers and unequal fiber size. (*A*) C57BL/10.*mdx*. (*B*) Backcross male progeny from female 467 (mdx^{2Cv}). (*C*) Backcross male progeny from female 551 (mdx^{3Cv}). (*D*) Backcross male progeny from female 467 (mdx^{2Cv}). (*E*) C57BL/6J. (*F*) Fiber degeneration with phagocytosis.

we cannot determine whether the four mutations recovered in the G_1 females 2019, 2195, 2219, and 2222 are recurring instances of the same *mdx* mutation or more than one mutation in the same ENU-treated male. We are tentatively assuming a single mutational event with female 2019 as the representative carrier and designate this allele as mdx^{4Cv}.

DISCUSSION

The major finding of this work is that it is possible to recover specific mutations of biological interest from the progeny of ENU-treated males, in this case for the mouse X-linked muscular dystrophy locus *mdx*. These mutations occur on an X chromosome with the flanking markers $Hprt^a$, $Pgk\text{-}1^a$ and they do not complement with the established *mdx* mutant described by Bulfield *et al.* (1). Preliminary histological work indicates that the putative mutations from G_1 female 467 (mdx^{2Cv}) and from female 551 (mdx^{3Cv}) produce male progeny that have muscle pathology that does not differ from the

Table 3. Characterization of mice with muscular dystrophy

Description	CK expression: Total activity, units	% MM	% MB	% BB	EMG	Biopsy: Heart	Biopsy: Skeletal
Normal	191	39.2	8.3	52.5	Normal	Normal	Normal
G_2 mdx^{2Cv} (655)	1244	12.3	78.8	8.8	Pseudomyotonia	Focal fibrosis	Regeneration and fibrosis
G_2 male mdx^{2Cv} (657)	6210	100	—	—	Pseudomyotonia	Minimal fibrosis	Striking regeneration and degeneration
G_2 male mdx^{3Cv} (613)	1952	95.8	4.2	—	Pseudomyotonia	Some fibrosis	Fiber degeneration
C57BL/10Sn.*mdx*	1244	90.3	9.7	—	Pseudomyotonia	Fiber degeneration with phagocytosis	Fiber degeneration with phagocytosis

Mouse 613 is a G_2 male $Hprt^a$,$Pgk\text{-}1^a$ from 551 (mdx^{3Cv}) × C57BL/10Sn.*mdx*. Mice 655 and 657 are G_2 males $Hprt^a$,$Pgk\text{-}1^a$ from 467 (mdx^{2Cv}) × C57BL/10Sn.*mdx*. Total CK activity was determined using a spectrophotometric method in which units of CK are measured as mol of NADPH ($\times 10^{-6}$) per min at 37°C. These are the units commonly measured in clinical determinations of CK activity. These activity assays were performed independently at Baylor College by a different method than the general screening, which was performed at Roswell Park Memorial Institute. Percent MM, MB, and BB refer to electrophoretic separation of muscle (MM), liver (MB), and brain (BB) isozyme forms on cellulose acetate and the subsequent densitometric scanning of gels. Male 655 had elevated CK activity in blood, but the predominant electrophoretic form was MB. By contrast, the electrophoretic forms for males 657 and 613 as well as C57BL/10Sn.*mdx* were predominantly the muscle isozyme (MM) form. EMG, electromyographic tests.

C57BL/10Sn.*mdx* and is consistent with the expression of a true myopathy. However, the relative severity of the dystrophy present in each mutant can only be grossly determined from the individual G_2 males studied to date.

The cardiomyopathy observed in our examination of C57BL/10Sn.*mdx* and the mdx^{2Cv} and mdx^{3Cv} is overtly different from the detailed descriptions of the *mdx* mutant phenotype reported by Torres and Duchen (19) but similar to the cardiomyopathy observed by Bridges (3). In addition, the general histopathology of skeletal muscles is very similar between our study and those reported by Torres and Duchen (19) and by Tanabe *et al.* (20). It will be essential to examine the age of onset and the range of phenotypic effects in mdx^{2Cv} and mdx^{3Cv} to determine whether the recovered *mdx* mutations identify additional features of the *mdx* gene function or if all of the mutations described in this work produce the same pathogenesis. Overtly, the mild dystrophy of *mdx* and the recovered *mdx* mutations are suggestive of the relatively mild dystrophy conditions observed for Becker muscular dystrophy in humans.

The recessive expression of the mouse *mdx* gene in heterozygous females is consistent with the results reported by Bulfield *et al.* (1). However, we were able to establish that there is a slight but significant increase in plasma CK levels in heterozygous females when we compare the distribution of plasma CK activities for a large sample of $+/mdx$ females with $+/+$, which is consistent with the values reported by Ryder-Cook *et al.* (7). The high levels of plasma CK activity in control and $+/mdx$ carrier populations and the extensive overlap of the distribution of plasma CK activity levels between these genotypes probably account for our failure to find *mdx* mutations in the 8336 G_1 progeny of $+/+$ females.

The successful cloning of the human Duchenne muscular dystrophy gene has provided cDNA probes that have been used to recover mouse probes for the dystrophin gene product (21). In addition, polyclonal antibodies have been developed against fusion proteins derived from Duchenne muscular dystrophy cDNA sequences (22).

Skeletal and cardiac muscle of *mdx* and mdx^{2Cv} (mouse 467) was compared with normal mice for the dystrophic protein by using these antibody reagents. No detectable levels of dystrophin were observed in either *mdx* mutant with these antibodies, but the limits of detection are difficult to quantitate. In parallel experiments, the level of dystrophin transcript was assessed in normal, *mdx*, mdx^{2Cv} (mouse 467), and mdx^{3Cv} (mouse 551) (23). Chamberlain *et al.* (23) observed reduced levels of dystrophin transcripts within skeletal muscles and brain in these mutants. In both instances, the induced *mdx* mutations gave the same transcript and protein phenotype as that observed in the original *mdx* mutation from C57BL/10Sn. The dystrophin protein and transcripts were measured in mutant males and thus the results are consistent with *mdx* being the mouse homologue for the human *DMD* locus. However, the results could also represent a disruption of normal dystrophin expression for both transcript and protein in degenerating/regenerating muscles of affected mice.

The *mdx* was localized on the mouse X chromosome between *Hprt* and *Pgk-1* (6) and subsequent work on the mapping of the dystrophin locus placed it in the same chromosomal region (24–26). The specific mapping of *mdx* relative to the *Dmd* locus has been achieved and three recombinants in a series of >200 backcrosses place the *mdx* locus within *Dmd*. Thus, these results directly indicate that the decreased levels of dystrophin protein and mRNA in *mdx* and in two of our mutants, mdx^{2Cv} and mdx^{3Cv}, are probably the result of mutations within the dystrophin locus.

The *mdx* mutations recovered in this report add to the value of the previously identified *mdx* mutation. Moreover, since ENU has been most frequently associated with point mutations rather than deletions or chromosomal rearrangements, it will be fruitful to ask whether the induced mutations identify similar mutational effects at the molecular level or whether dystrophin gene function is impaired differently in each mutant. The recovery of three more mutant alleles among 1600 progeny is relatively high compared with ENU-induced mutations recovered for other loci (8, 11). However, this frequency may be a reflection of the relatively large size of the dystrophin locus and its 14-kilobase transcript. These findings further suggest that additional *mdx* mutations in the mouse can be recovered relatively easily using the testing methodologies we have devised.

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