Altered Ca^{2+} sensitivity of tension in single skeletal muscle fibres from *MyoD* gene-inactivated mice

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1. Single, fast glycolytic skeletal muscle fibres were isolated from wild-type ($MyoD^{+/+}$) and *MyoD* mutant mice ($MyoD^{-/-}$), which lack a functional copy of the *MyoD* gene. Fibres were chemically permeabilized to permit manipulation and control of the ionic environment of the otherwise intact myofilament apparatus.

2. Results show a fivefold greater variability in the [Ca^{2+}] required for half-maximum tension generation among individual $MyoD^{-/-}$ fibres in comparison with controls ($p < 0{\cdot}05$).

3. Consistent with this finding, Western blot analysis showed a sevenfold greater variability in the isoform expression pattern of the thin filament regulatory protein troponin T in $MyoD^{-/-}$ compared with control fibres ($p < 0{\cdot}05$).

4. Electrophoretic analysis of single-fibre segments indicated no apparent alteration in the isoform expression pattern of other regulatory and contractile proteins. In addition, other parameters of contractile function, including velocity of unloaded shortening, and maximum force production, were not significantly different between $MyoD^{-/-}$ and $MyoD^{+/+}$ fibres.

5. These findings indicate that the thin filament structure–function relationship is altered due to the *MyoD* mutation and suggest that *MyoD* plays a role in establishing and/or maintaining the differentiated phenotype of adult fast skeletal muscle fibres.

Ectopic expression of the *MyoD* gene is sufficient to activate the myogenic programme in several differentiated, non-muscle lineages (Weintraub, 1993). Based on this and other findings, it has been suggested that *MyoD* has a central function in skeletal muscle differentiation (Weintraub, 1993). To directly test this idea, Rudnicki, Braun, Hinuma & Jaenisch (1992) inactivated the *MyoD* gene in mice. Surprisingly, skeletal muscle formation and development appears to be normal in mice lacking a functional copy of the *MyoD* gene ($MyoD^{-/-}$). Muscle gene expression, sarcomere ultrastructure and fibre-type distribution in the $MyoD^{-/-}$ animals are comparable with wild-type controls. There is also no obvious impairment in mobility in these animals, suggesting that muscle function is not markedly affected in the $MyoD^{-/-}$ animals. Taken together, these findings indicate that skeletal muscle development and function is apparently normal in $MyoD^{-/-}$ mice (Rudnicki *et al.* 1992).

However, it is not known whether the *MyoD* mutation disrupts contractile function or structure at the level of the single skeletal muscle fibre. To address this possibility, we examined the Ca^{2+}-activated contractile properties and the myofilament protein isoforms of single fast skeletal muscle fibres isolated from $MyoD^{-/-}$ and wild-type $MyoD^{+/+}$ adult mice. The single fibres were chemically permeabilized to directly control the intracellular solution bathing the otherwise intact contractile apparatus, and to permit the determination of Ca^{2+}-activated contractile function. The expression pattern of contractile and regulatory proteins in control and $MyoD^{-/-}$ single fibres was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blots.

METHODS

Preparations

The generation of the *MyoD* mutant mice has been described previously (Rudnicki *et al.* 1992). Fast, glycolytic skeletal fibres (type II B) were isolated from the superficial region of the vastus lateralis (SVL) muscle of wild-type ($MyoD^{+/+}$) and $MyoD^{-/-}$ adult mice. All mice tested were males between 5 and 12 months old. Ca^{2+}-activated mechanical function was highly comparable among the control animals over this age range. Bundles of approximately

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fifty fibres were dissected from each muscle whilst in relaxing solution (see below) and tied with surgical silk to glass capillary tubes. Bundles were stored for up to 3 weeks at −20 °C in relaxing solution containing 50% (v/v) glycerol.

Experimental apparatus

Individual skeletal fibres were carefully pulled free from one end of the fibre bundle and mounted between a force transducer (model 400A, Cambridge Technology Inc., Watertown, MA, USA; noise level at the output equivalent to 1 mg of the signal peak to peak) and a high-performance moving-coil galvanometer (model 6350, Cambridge Technology Inc.). The attachment procedure involved placing the ends of the fibre in troughs that were made from 29-gauge stainless-steel tubing (Metzger *et al.* 1989). The fibre was viewed through an inverted microscope and its overall length was adjusted with a mechanical translator to set resting sarcomere length. Sarcomere length for both control and *MyoD*$^{-/-}$ fibres was set at 2·50–2·55 μm.

To construct the tension–pCa (pCa = −log [Ca^{2+}]) relationship, each fibre was transferred to a particular Ca^{2+}-activating solution and steady-state isometric tension was allowed to develop, after which the fibre was rapidly (< 1 ms) slackened, so that tension fell to zero (Fig. 1). The fibre was then relaxed by transferring it to pCa 9·0 solution. The difference between steady tension and the tension baseline following the slack step was measured as total tension. To obtain active tension, the resting tension measured at pCa 9·0 (about 1% of total tension) was subtracted from total tension. Tension–pCa relations were determined for each fibre by expressing tensions (P) at various submaximal Ca^{2+} concentrations as fractions of the maximum value, P_o, obtained in the same fibre at pCa 4·5. In general, every fourth contraction was at pCa 4·5 to check for any deterioration in fibre performance. Fibre cross-sectional area was calculated assuming an elliptical fibre geometry.

Shortening velocity was determined using the slack-test procedure whereby length changes of varying amplitude (ΔL) were applied to one end of the fibre and the time required for the fibre to take up the imposed slack was recorded (Δt; Edman, 1979; Metzger & Moss, 1988). The slope of the ΔL *versus* Δt relationship is unloaded shortening velocity.

Relaxing and activating solutions

Solutions consisted of (mM): 7 EGTA, 1 free Mg^{2+}, 4·42 total ATP, 14·5 creatine phosphate, 20 imidazole and sufficient KCl to yield an ionic strength of 180 mM; temperature was set at 15 °C; solution pH was 7·00 (Metzger *et al.* 1989).

Western blotting

The primary antibody was a mouse monoclonal anti-Troponin T (TnT) antibody (JLT-12, Sigma), and the secondary antibody was a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Fab specific). Detection was by enhanced chemiluminescence (ECL; Metzger *et al.* 1993). Each lane was loaded with a fibre segment length of approximately 10 mm. Because isolated fibre segments from this muscle averaged about 5 mm, typically two fibre segments were added per lane. Loading of 10 mm total fibre length was typically required to detect the four major TnT isoforms in these SVL fibres. Also, because functional studies were performed on fibre segments of about 2–5 mm in end-to-end length, we did not routinely determine function and TnT expression in the same preparation.

Silver staining was performed on the upper portion of the same gel that was used for the Western blot, to indicate that protein loading was comparable in each lane. The relative expression of TnT isoforms was determined in each Western blot by densitometry using a Bio-image (Millipore) system which utilizes an 8-bit black and white 2-D array detector with 1024 × 1024 resolution. Relative expression of TnT-4 was calculated by integrating the areas corresponding to TnT isoforms 1–4, and by setting the total area integrated to 100%. Thus the percentage of TnT-4 expressed is the percentage of TnT-4 relative to the total TnT isoform expression obtained in that lane. In some experiments we also used the gravimetric method to determine the relative expression of the TnT isoforms. Results using the gravimetric method were highly comparable with those obtained using the Bio-image system (data not shown).

To detect tropomyosin isoform expression, TnT antibody complexes were removed by exposing membranes to a stripping buffer (2% SDS, 62·5 mM Tris, 100 mM β-mercaptoethanol; pH 6·7) for 30 min at 50 °C. Stripping was verified using the ECL kit (Amersham) (results not shown). Blots were then blocked with 5% milk in Tris-buffered saline and reprobed with monoclonal anti-sarcomeric Tm antibody (CH1, Sigma) diluted 1 : 50 using the protocol described above. For determination of troponin I isoform expression, a monoclonal antibody (TI-4) which detects all troponin I isoforms was used (a kind gift from Dr S. Schiaffino). Troponin I expression was also determined using SDS gels (Fig. 3).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Isolated single skeletal fibres were placed in a 0·5 ml microfuge tube containing SDS sample buffer (10 μl mm^{-1} of segment length) and stored at −80 °C for subsequent analysis of contractile and regulatory protein content by SDS–PAGE and scanning densitometry, as described previously (Metzger *et al.* 1989).

Curve fitting and statistics

To derive values for the mid-point (termed pCa_{50} or K) and Hill coefficient (n_H) from the tension–pCa relationships, data were fitted using the Marquardt–Levenberg non-linear least-squares fitting algorithm, using the Hill equation in the form:

$$P_r = [Ca^{2+}]^{n_H}/(K^{n_H} + [Ca^{2+}]^{n_H}),$$

where $P_r = P/P_o$. Hartley's F_{max} test was used to test for heterogeneity of variances ($p < 0{\cdot}05$).

RESULTS

Steady-state isometric tension was determined at eight to ten different pCa values (range 7·0–4·5) to construct the tension–pCa relationship in each single fibre. Results showed marked differences in Ca^{2+}-activated contraction in *MyoD*$^{-/-}$ compared with control skeletal muscle fibres (Fig. 1). Specifically, the variability in the distribution of individual pCa_{50} values was more than 5-fold greater among *MyoD*$^{-/-}$ compared with *MyoD*$^{+/+}$ control skeletal muscle fibres ($p < 0{\cdot}05$). This value was calculated by dividing the variance in the *MyoD*$^{-/-}$ pCa_{50} data by the variance obtained from controls (Fig. 1). In other words, the individual pCa_{50} values from control fibres clustered around the mean value, whereas the *MyoD*$^{-/-}$ pCa_{50} values were fairly evenly dispersed above and below the mean without evidence of clustering at a particular pCa_{50}.

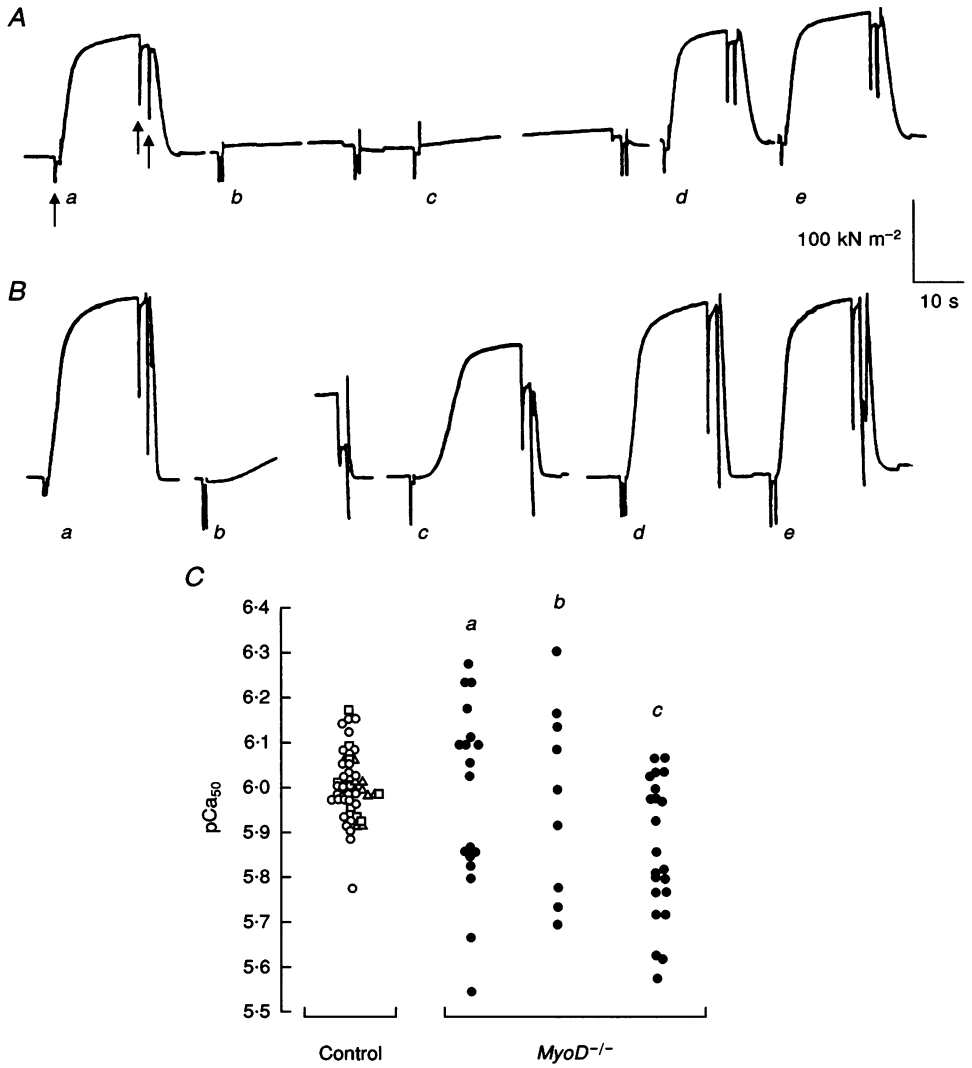

Figure 1. Ca^{2+}-activated contraction in single fibres from *MyoD*$^{-/-}$ mice

A and *B*, slow time scale records of Ca^{2+}-activated isometric tension in permeabilized single fast skeletal muscle fibres obtained from a *MyoD*$^{-/-}$ mouse. pCa of the activating solution at points *a–e* in traces *A* and *B* were: 4·5, 6·3, 6·2, 5·0 and 4·5, respectively (typically, tensions were determined at 8–10 pCa values; only a subset of these records are shown in the figure). *Aa*, the first arrow indicates changing the pCa of the solution bathing the fibre from 9·0 to 4·5. At the second arrow, the fibre length was rapidly released (< 1 ms) to obtain tension baseline (not seen on these slow time scale recordings), and the solution changed back to pCa 9·0. At the third arrow, fibre length was re-extended to the pre-release length. *Ab* and *c*, the breaks in the tension record correspond to approximately 3 min. pCa_{50} was 5·73. End-to-end fibre length was 2·39 mm. Sarcomere length was 2·55 μm. *B*, pCa_{50} was 6·27. End-to-end fibre length was 1·6 mm. Sarcomere length was 2·50 μm. In record *b* the break in the tension record represents 1 min. *C*, distribution of pCa_{50} values obtained from control and *MyoD*$^{-/-}$ single skeletal muscle fibres. Each point represents a pCa_{50} obtained from an individual fibre. Control data were obtained from SVL fibres ($n = 49$) from five different adult *MyoD*$^{+/+}$ animals: two 129/Sv mice (○), two Balb/C mice (□) and one rat (△). No differences in pCa_{50} variability were apparent among the control fibres obtained from the different animals tested (values for Balb/C1, Balb/C2, 129/Sv1&2 mice and Sprague–Dawley rat (means ± S.D. (*n*, variance)) were: 6·06 ± 0·07 (5, 0·0049), 5·95 ± 0·03 (4, 0·0009), 6·00 ± 0·09 (29, 0·0081) and 5·99 ± 0·05 (11, 0·0024), respectively). In the *MyoD*$^{-/-}$ group, three different animals were studied: animals *a* and *b* were *MyoD*$^{-/-}$ (Balb/C) mice, and animal *c* was a *MyoD*$^{-/-}$ (129/Sv) mouse, and 49 fibres were tested in total. The means ± S.D. (*n*, variance) for *MyoD*$^{-/-}$animals *a*, *b* and *c* were: 5·97 ± 0·21 (9, 0·0441), 5·98 ± 0·21 (18, 0·0441) and 5·85 ± 0·16 (22, 0·0256), respectively. Variance is a measure of the variability among individual data points in a group (calculated as S.D. × S.D.). The pCa_{50} variability was greater in each *MyoD*$^{-/-}$ animal compared with the control animals. Overall, the *MyoD*$^{-/-}$ variance/control variance ratio was 5·64, a value which was significantly different from 1·00 ($p < 0·05$). For the ratio calculation, the mean variance was determined using all the individual data points (not group animal means) for each group (e.g. control or mutant).

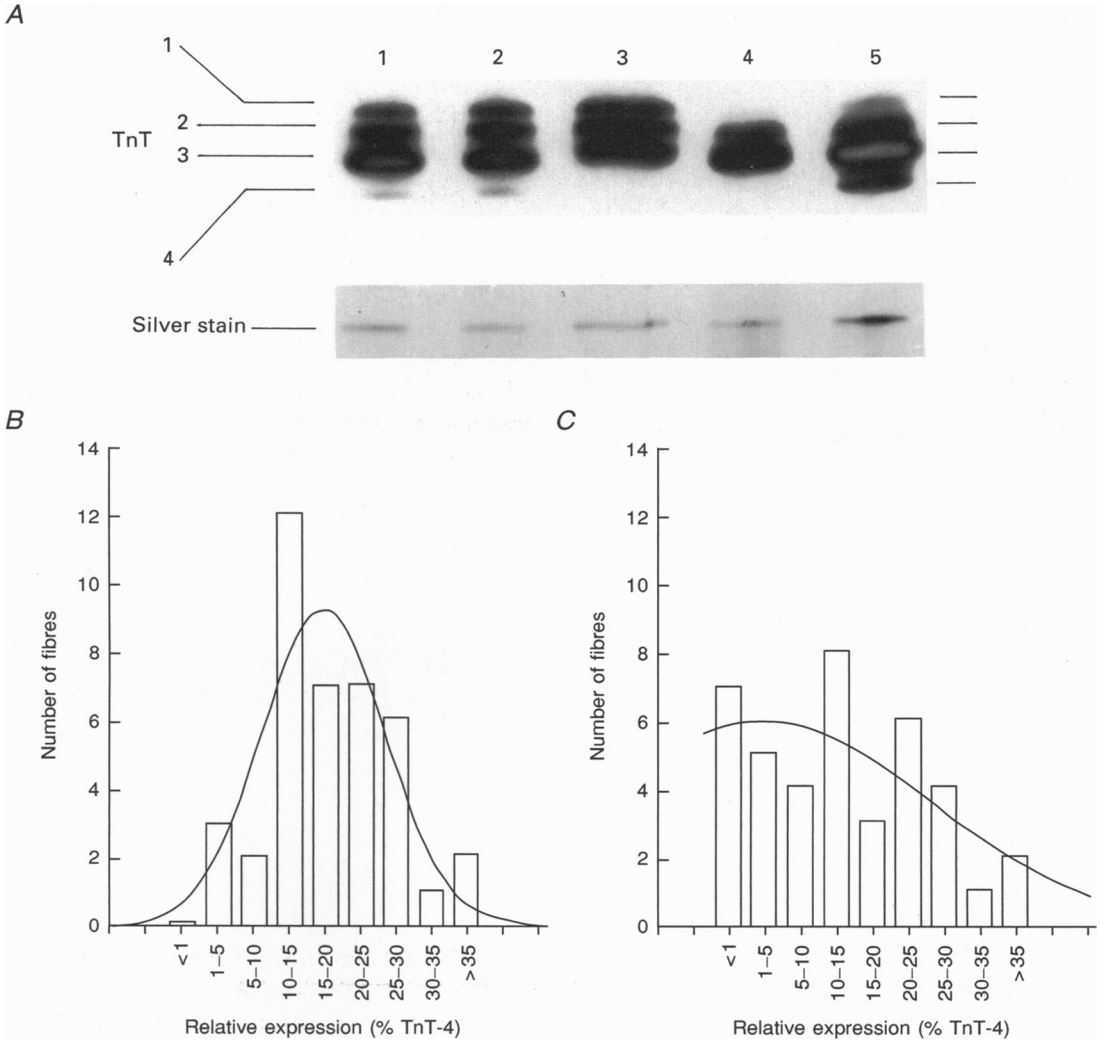

Figure 2. Troponin T isoform expression in control and *MyoD*$^{-/-}$ single skeletal muscle fibres

A, Western blot detection of troponin T (TnT) isoforms in control and *MyoD*$^{-/-}$ fast skeletal muscle fibre segments. TnT isoform detection was by enhanced chemiluminescence. Lanes 1–2 are control fibre segments. Lanes 3–5 are *MyoD*$^{-/-}$ fibre segments. Relative expression of TnT isoforms (% of total) was:

	Lane	1	2	3	4	5
TnT isoform	1	20	18	27	0	9
	2	32	35	30	43	30
	3	44	45	43	57	42
	4	4	2	0	0	19

Total expression in each lane equals 100%. Approximately 10 mm of a fibre segment length was loaded per lane. Silver staining was performed on the upper portion of the gel and indicated comparable protein loading per lane. *B* and *C*, summary of the relative expression of the TnT-4 isoform in control (*B*) and *MyoD*$^{-/-}$ (*C*) fibre segments. Each individual relative TnT-4 expression value was placed into one of nine bins which ranged from < 1 to > 35% expression, with each successive bin incremented by 5%. The best fit normal curve is shown for each plot. S.D. of the fit was 8·7 in *B* and 23·4 in *C*. Variance is significantly greater for *MyoD*$^{-/-}$ compared with control data ($p < 0{\cdot}05$). Forty fibre samples were analysed per group. *MyoD*$^{-/-}$ results obtained from *MyoD*$^{-/-}$ (Balb/C) mice.

The increased variability in pCa_{50} was evident in populations of single skeletal fibres isolated from three *MyoD*$^{-/-}$ mice, in which the genetic background was either Balb/C-129 (*a–b*, Fig. 1*C*) or 129/Sv (*c*, Fig. 1*C*). This indicates that differences in the genetic background among the *MyoD*$^{-/-}$ mutant mice tested did not alter the main finding. The control fast skeletal muscle fibres used in this analysis were obtained from two Balb/C mice (used in generating chimeric mice and maintenance of the mutant strain) and two 129/Sv mice (from which the embryonic stem cells for gene targeting were derived; Rudnicki *et al.* 1992). There were no significant differences in pCa_{50} variability between the two control inbred strains of mice and those from an adult rat SVL muscle (Fig. 1). Interestingly, other parameters of contractile function, including maximum Ca^{2+}-activated tension (P_o) and maximum velocity of unloaded shortening, were not different in terms of the mean value and distribution (i.e. variance) between *MyoD*$^{-/-}$ and *MyoD*$^{+/+}$ fast skeletal fibres (Table 1). The mean values for these contractile parameters are in general agreement with those obtained previously in adult type II B skeletal muscle fibres (Metzger *et al.* 1989; Metzger & Moss, 1990).

As a control, in *MyoD*$^{-/-}$ fibres which demonstrated reduced Ca^{2+} sensitivity of contraction (e.g. Fig. 1), exogenous purified skeletal troponin C was added to the bathing media (Metzger *et al.* 1989), to test whether Ca^{2+} desensitization resulted from sub-stoichiometric troponin C content of the thin filament which would manifest functionally as a reduced pCa_{50} (Brandt, Diamond & Schachat, 1984; Moss, Giulian & Greaser, 1985). However, Ca^{2+}-activated tension was unchanged following exogenous troponin C treatment. In addition, maximum Ca^{2+}-activated tension development

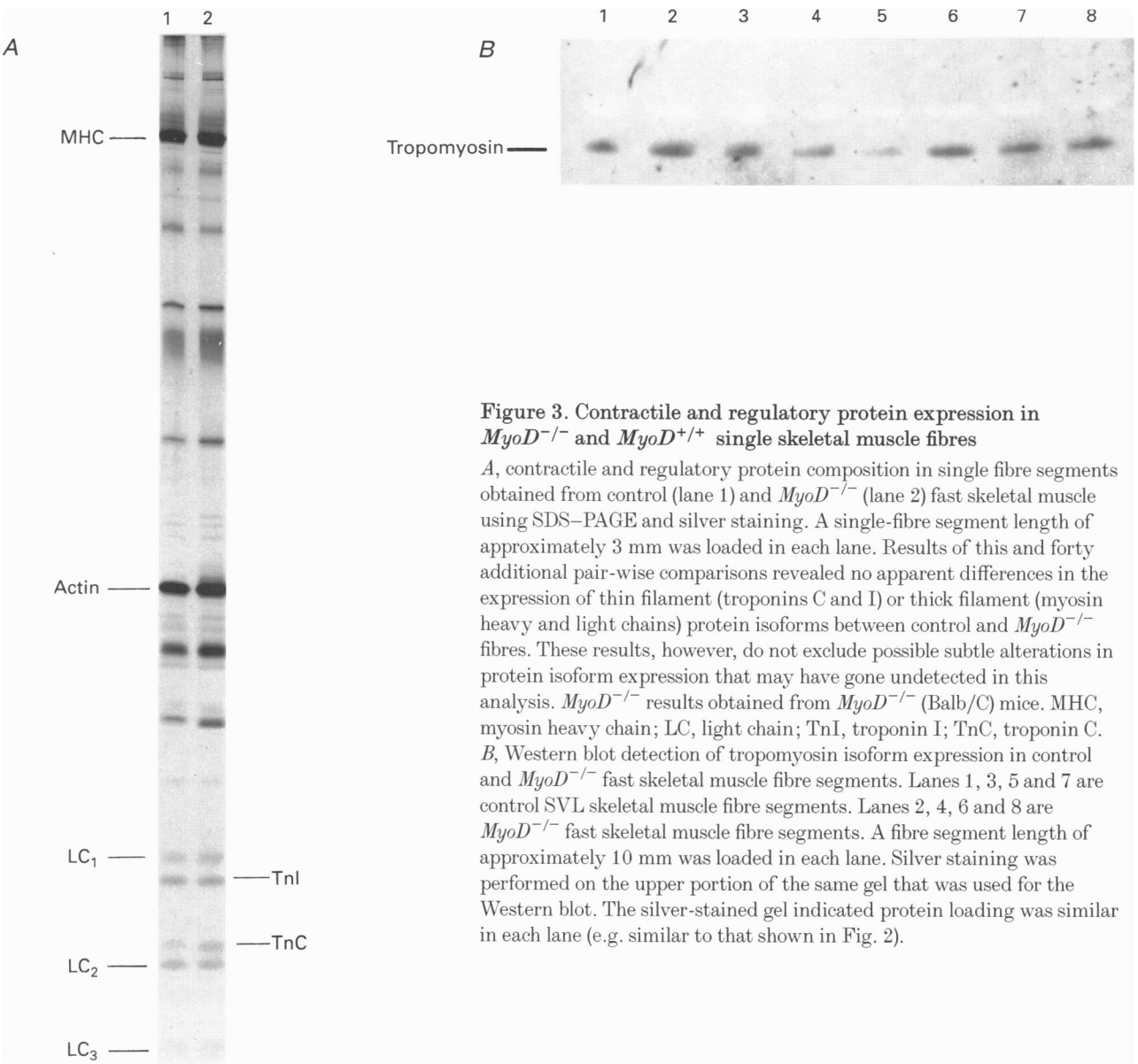

Figure 3. Contractile and regulatory protein expression in *MyoD*$^{-/-}$ and *MyoD*$^{+/+}$ single skeletal muscle fibres

A, contractile and regulatory protein composition in single fibre segments obtained from control (lane 1) and *MyoD*$^{-/-}$ (lane 2) fast skeletal muscle using SDS–PAGE and silver staining. A single-fibre segment length of approximately 3 mm was loaded in each lane. Results of this and forty additional pair-wise comparisons revealed no apparent differences in the expression of thin filament (troponins C and I) or thick filament (myosin heavy and light chains) protein isoforms between control and *MyoD*$^{-/-}$ fibres. These results, however, do not exclude possible subtle alterations in protein isoform expression that may have gone undetected in this analysis. *MyoD*$^{-/-}$ results obtained from *MyoD*$^{-/-}$ (Balb/C) mice. MHC, myosin heavy chain; LC, light chain; TnI, troponin I; TnC, troponin C. *B*, Western blot detection of tropomyosin isoform expression in control and *MyoD*$^{-/-}$ fast skeletal muscle fibre segments. Lanes 1, 3, 5 and 7 are control SVL skeletal muscle fibre segments. Lanes 2, 4, 6 and 8 are *MyoD*$^{-/-}$ fast skeletal muscle fibre segments. A fibre segment length of approximately 10 mm was loaded in each lane. Silver staining was performed on the upper portion of the same gel that was used for the Western blot. The silver-stained gel indicated protein loading was similar in each lane (e.g. similar to that shown in Fig. 2).

Table 1. Summary of Ca^{2+}-activated contractile properties in control and $MyoD^{-/-}$ single fast skeletal muscle fibres

	P_o	V_{max}	pCa_{50}	Hill coefficient
Controls	134 ± 90	3·6 ± 1·2	6·00 ± 0·08	3·4 ± 1·0
$MyoD^{-/-}$ (Balb/C-129)	151 ± 70	3·4 ± 1·5	5·97 ± 0·21*	3·3 ± 1·4
$MyoD^{-/-}$ (129/Sv)	126 ± 82	n.d.	5·85 ± 0·16*	3·2 ± 1·4

Values are means ± S.D. All data are from mouse SVL fibres. * indicates that variance is significantly greater in the $MyoD^{-/-}$ group compared with control ($p < 0·05$). P_o (kN m^{-2}) is the maximum isometric tension developed at pCa 4·5. V_{max} is the maximum velocity of unloaded shortening and was determined using the slack-test method (Edman, 1979). V_{max} values are reported as muscle fibre lengths per second. The Hill coefficient reports the steepness of the tension–pCa relationship. Number of animals and fibres studied per group is given in Fig. 1. n.d., not determined. The distribution (i.e. variance) of the P_o, V_{max} and Hill coefficient data was similar in the control and $MyoD^{-/-}$ groups.

was comparable in the $MyoD^{-/-}$ and control fibres (Table 1). Taken together, these results indicate that the varied Ca^{2+} sensitivity was not due to alterations in troponin C content among the $MyoD^{-/-}$ fibres.

The basis of altered Ca^{2+} regulation of contraction in $MyoD^{-/-}$ fibres could arise from a disruption in the normal expression of contractile and regulatory protein isoforms in these fibres. Indeed, a correlation has been reported previously between the Ca^{2+} sensitivity of contraction and the pattern of troponin T (TnT) isoform expression in normal striated muscle fibres (Schachat, Diamond & Brandt, 1987; McAuliffe, Gao & Solaro, 1990; Nasser, Malouf, Kelly, Oakeley & Anderson, 1991; Reiser, Greaser & Moss, 1992). We therefore investigated whether the pattern of TnT expression is altered in $MyoD^{-/-}$ fibres. Comparison of the TnT expression pattern between control and $MyoD^{-/-}$ fibres (forty observations per group) indicated an alteration in the TnT expression pattern in $MyoD^{-/-}$ fibres (Fig. 2). TnT-4, the fastest migrating TnT isoform on one-dimensional SDS gels, showed a 7-fold greater variability of expression in $MyoD^{-/-}$ compared with control fibre segments (calculated as $MyoD^{-/-}$ variance/control variance, $p < 0·05$). The variability in distribution of TnT isoforms 1, 2 and 3 was not significantly different between the control and $MyoD^{-/-}$ fibres (data not shown). Based on earlier results indicating a relationship between TnT isoform expression and contractility (Schachat *et al.* 1987; McAuliffe *et al.* 1990; Nasser *et al.* 1991; Reiser *et al.* 1992), our finding of altered TnT isoform expression could provide, at least in part, the basis for the disruption in Ca^{2+} sensitivity of contraction observed in the $MyoD^{-/-}$ fibres (Fig. 1).

In comparison, analysis of myofibrillar protein composition by SDS–PAGE and silver staining showed that the phenotype of the myosin heavy chain, myosin light chains 1, 2 and 3, and troponin I and troponin C was not altered in isolated single $MyoD^{-/-}$ fibre segments (Fig. 3). In other experiments, troponin I and tropomyosin isoform expression was determined by Western blot analysis, and the expression pattern in $MyoD^{-/-}$ fibres was found to be identical to control (Fig. 3; troponin I Western blot results were consistent with that shown in the SDS gels in Fig. 3).

DISCUSSION

This is the first report of a contractile phenotype due to the *MyoD* gene mutation. A single skeletal fibre functional assay was used to determine contractile structure and function in adult skeletal muscle fibres obtained from $MyoD^{-/-}$ mice. In comparison with controls, the isolated $MyoD^{-/-}$ fibres display a significantly greater variability in the Ca^{2+} concentration required for half-maximal activation of isometric tension. In keeping with this finding, the distribution of the TnT-4 isoform is significantly greater in $MyoD^{-/-}$ compared with $MyoD^{+/+}$ fibres.

Functionally, the observed alteration in thin filament structure and function in isolated single $MyoD^{-/-}$ fibres could be manifested as an alteration in muscle function in the living animal. In this respect, it is interesting to note that two-thirds of the $MyoD^{-/-}$ offspring from $MyoD^{+/-}$ parents died before weaning, whereas the offspring from $MyoD^{-/-}$ parents are of apparent normal litter size (Rudnicki *et al.* 1992). The basis for this apparent difference in survival is unknown. One possibility is that the alteration in Ca^{2+} sensitivity observed in $MyoD^{-/-}$ mice could impair muscle function compared with that in $MyoD^{+/-}$ or $MyoD^{+/+}$ mice. In that case the $MyoD^{-/-}$ mice would be at a disadvantage compared with littermates which have at least one functional copy of the *MyoD* gene.

The possibility of additional phenotypes due to *MyoD* gene inactivation is not excluded by these findings. For example, it is not known whether the oxidative/glycolytic energy pathways, or specific events in the excitation-coupling

cascade including membrane excitation or Ca^{2+} release from the sarcoplasmic reticulum are also altered in these fibres.

An interesting question emerging from this study is the mechanism by which TnT expression is altered in *MyoD*$^{-/-}$ fibres. Troponin T gene expression is both tissue specific and developmentally regulated, with the multiple TnT isoforms generated by alternative splicing. The mechanism underlying regulation of alternative splicing of mammalian genes is not known. Models of alternative splicing suggest there is an evolutionary hierarchy of alternative splicing mechanisms, ranging from stochastic (primitive form), to constitutive, to regulated alternative splicing (Smith, Paton & Nadal-Ginard, 1989). It is possible that in *MyoD*$^{-/-}$ fibres the normal regulated control of TnT gene alternative splicing is disrupted, resulting in altered TnT isoform expression in these fibres.

It was shown recently that members of the *MyoD* family are expressed in a fibre-type-dependent manner in adult animals: the *MyoD* gene appeared to be preferentially expressed in fast, glycolytic fibres, whereas Myogenin transcripts were more abundant in slow fibres (Hughes, Taylor, Tapscott, Gurley, Carter & Peterson, 1993). The differential expression of myogenic transcription factors in adult fast and slow muscle fibres, together with our finding of altered thin filament structure and function in single fast skeletal fibres, provides evidence that *MyoD* gene expression may play an important role in establishing and/or maintaining the differentiated state of adult fast skeletal muscle.

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