Quantitation of Muscle Precursor Cell Activity in Skeletal Muscle by Northern Analysis of MyoD and Myogenin Expression: Application to Dystrophic (mdx) Mouse Muscle

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The regeneration of skeletal muscle is dependent upon proliferation and fusion of activated mononuclear muscle precursor cells. Early and specific markers of this population of activated cells are the transcription factors MyoD and myogenin. Northern analysis was used to determine levels of MyoD and myogenin mRNA in (i) muscles regenerating after experimental crush injury and (ii) in limb muscles of dystrophic mdx mice at various ages in comparison to controls. In crush-injured muscle, MyoD and myogenin mRNA increased at 24 h, peaked between 2 to 6 days, and returned to uninjured control levels by 15 days after injury. In both mdx and control mice, MyoD and myogenin mRNA levels were high in fetal muscles and decreased rapidly during the 2 weeks after birth. In mdx muscles, the mRNA levels increased significantly from about 21 days, remained high until around 40 days, and then decreased to a relatively constant yet elevated level when compared to control muscles. The elevated levels persisted to 420 days of age. The results show that this technique can be used to provide sensitive quantitative information on the size of the population of activated precursor cells in skeletal muscle. As such, it represents a novel and convenient means of measuring regenerative activity in vivo in whole muscles.

INTRODUCTION

The regenerative capacity of skeletal muscle is largely dependent on the proliferation of mononuclear muscle precursor cells (mpc), which subsequently fuse together to form multinucleated new muscle cells called myotubes or with damaged myofibers (1). To date it has been difficult to assess the extent of mpc proliferation in response to various types of trauma. Histological evaluation of myotube formation, e.g., Ref. (2), and autoradiographic analysis of replicating mpc labeled by tritiated thymidine injection (3) are techniques that are carried out on individual tissue sections. Such limited sampling may not be representative of the entire muscle and these methods are very time-consuming. In addition, injection of tritiated thymidine is not economically feasible in larger species. The procedure described in this paper, using the skeletal-muscle-specific genes MyoD and myogenin, provides a quantitative and specific method for readily estimating the regenerative activity of entire muscles and is applicable to all species.

The skeletal-muscle-specific genes MyoD (4) and myogenin (5) are expressed in activated mpc and are members of a gene family considered to be the nodal point during specification of the myogenic lineage, for review, see (6). Myogenin expression is first detected as early as 8.5 days and MyoD at 10.5 days in the rostral somites of the mouse embryo (7), begins to decline between 15 and 17 days of gestation, and falls to very low levels by the third week after birth (8). In situ hybridization studies show little or no transcription of these genes by quiescent mpc of mature, undamaged mouse muscle (9). However, in muscle regenerating after crush injury, mRNA sequences for MyoD and myogenin were detected in mononuclear cells by 6 h, peaked between 24 and 48 h, and declined to preinjury levels by 8 days after injury (9). The specific expression of these genes in activated, replicating, skeletal mpc permits mRNA levels for these genes to be used as a quantitative measure of growth and regeneration in skeletal muscle. In the present paper, the validity of this approach was first tested on the crush-injury model of muscle regeneration for which extensive histological and autoradiographical (3, 10) and in situ hybridization (9) data existed. Following these assessment studies, the technique was applied to the quantitation of muscle regeneration during the life span of the dystrophic mdx mouse.

The mdx mouse results from a spontaneous mutation of a laboratory mouse strain, first reported by Bulfield et al. (11), and is the most widely used model for the study
of the fatal X-linked human disease known as Duchenne muscular dystrophy (DMD). The defect is genetically and biochemically similar to DMD in that muscle fibers lack dystrophin (12). However, this model generally lacks the clinical and pathological characteristics of DMD patients, in whom the muscles are finally replaced by fatty and connective tissue (13). Although severe muscle necrosis and regeneration are observed in mdx mouse muscles at around 2–3 weeks postpartum (14, 15, 16), this is less conspicuous in older mice. Controversy exists regarding the long-term severity of the disease, although variations may reflect differences in colonies, sampling, and experimental method. Some reports indicate that muscle necrosis and regeneration are transient and that necrosis is less evident in aging animals (14, 17), whereas others report that the extent of necrosis and regeneration is essentially sustained or only slightly reduced with age in mdx muscles (2, 15, 16, 18). Northern analysis of MyoD and myogenin levels was used to resolve this controversy.

METHODS

Animals

All procedures were approved by the Animal Welfare Committee of the University of Western Australia. Inbred dystrophic (mdx) mice and the control parental C57Bl/10Sn strain were supplied by the Australian Neuromuscular Research Institute, University of Western Australia, and additional mdx mice were a generous gift from Dr. L. Austin of the Department of Biochemistry, Monash University, Melbourne, Victoria. Inbred BALB/c mice aged 8 weeks were obtained from the Animal Resources Centre of Western Australia. Time-pregnant females were provided by the ANRI Animal Resources Centre. Male and female animals were caged overnight and conception was considered to have occurred the following morning when a coagulation plug was observed. This was designated Day 0 of pregnancy and is accurate to within ±8 h (19). All mice received a standard diet of mouse pellets and were maintained in a controlled environment.

Crush Injury and Muscle Sampling

The mid region of the tibialis anterior (TA) muscles in both legs of 20 BALB/c mice were crush-injured with artery forceps while the mice were under fluothane anesthesia, as described by McGaechie and Grounds (3). The TA is about 15 mm in length and the site of the lesion was about 3 mm wide and 4 mm long and extended throughout the depth of the muscle. The skin wound overlying the lesion was closed and sutured. Two animals were sacrificed at each time point from 1 to 15 days after injury. The entire TA muscle from each leg was removed and processed for Northern analysis. Muscles were sampled from a total of 200 mdx and 150 control C57Bl/10Sn mice at 15 and 19 days of gestation and postnatally from 3 to 420 days of age. Muscles from the hind limbs of 7 to 10 fetal and neonatal mice, and from 3 young mice up to 10 days old, were pooled for each time point. Samples from two littermates were combined for the later time points.

Probes

The MyoD probe (4) used was a 1.8-kb full-length cDNA clone (pVZC11a) isolated from the mouse, and the myogenin probe (5) was a 1.5-kb cDNA clone isolated from the rat (pBU65#7). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (1.27 kb) was isolated from the rat (20). The GAPDH probe was used as an internal standard, which enabled variations in total RNA concentration between samples to be accounted for.

Preparation of Total RNA and Northern Analysis

Mice were killed by CO2 asphyxiation, and entire TA muscles were removed aseptically and immediately placed in ice-cold 50 mM sodium acetate buffer, containing 6.0 M lithium chloride, 3 M urea, 2% 2-mercaptoethanol, and 1% sarcosyl. Samples were homogenized immediately and the RNA was extracted by the method of LeMeur et al. (21).

Total RNA (20 μg) was analyzed by Northern blot hybridization (22). Gels were stained with ethidium bromide and photographed under uv illumination and then vacuum blotted onto HyBond N* membrane in 10× SSC buffer, fixed in 40 mM sodium hydroxide for 10 min, and rinsed in 2× SSC. Prehybridization of membranes was carried out overnight in a solution of 5× SSPE, 5× Denhardt’s solution (2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) PVP), 0.5% (w/v) sodium dodecyl sulfate, 50% formamide, 10% dextran sulfate, and 0.02 mg/ml salmon sperm DNA (sheared by sonication and denatured by heating at 95–100°C). Membranes were then hybridized under the same conditions to the appropriate 32P-random-primer-labeled cDNA probe (sp act ~106 cpm/μg) for 18–24 h. All samples (prepared from crush injury, mdx, and control C57Bl/10Sn mice) to be hybridized with a particular probe were analyzed at the same time, with the same batch of labeled probe. This eliminated possible variations in specificity of probe and hybridization efficiency. The membranes were placed in X-ray cassettes fitted with Quanta 3 intensifying screens in contact with Cronex X-ray film (Du Pont) and exposed at −70°C. Membranes probed with MyoD and myogenin cDNAs were exposed for 9 days, after which time they were stripped, reprobed with GAPDH cDNA, and exposed for 6–8 h.

Densitometry

The autoradiographic signals obtained for MyoD, myogenin, and GAPDH expression were analyzed by laser densitometry (Epson Colour Imaging Scanner 4000). The absorbance of each band was corrected for background
FIG. 1. RNA from crush-injured muscle samples and a normal liver sample. Total RNA was prepared from TA muscles of BALB/c mice at the times indicated after crush injury. RNA samples (20 μg), prepared from two mice for each time point, were analyzed by Northern blot hybridization and autoradiography. Membranes were probed with 32P-labeled MyoD, myogenin, and GAPDH cDNA (upper panels). A sample of normal liver RNA is included as a negative control for MyoD and myogenin expression. The ethidium bromide stained gel is shown and the positions of the 18S and 28S ribosomal RNA bands are indicated (lower panel).

and MyoD and myogenin values were normalized with respect to GAPDH expression.

RESULTS

MyoD and Myogenin Expression in Crush Injured Muscle

Northern analysis with 32P-labeled MyoD, myogenin, and GAPDH cDNA probes (Fig. 1) was carried out on total RNA prepared from regenerating TA muscles at 1 to 15 days after crush injury. Densitometric analyses of the MyoD and myogenin hybridizations were normalized against GAPDH expression and the results are presented in Figs. 2a and 2b, respectively. By Day 2 after injury, mRNA levels for MyoD and myogenin were approximately eight- and three-fold higher, respectively, than control levels in uninjured muscle (not shown). In the case of MyoD, this elevation was already apparent on Day 1 postinjury. The peak of MyoD expression was reached between Days 3 and 4. The peak for myogenin expression appeared at the same time but extended out to Day 5. MyoD and myogenin mRNA levels returned to control values sometime after 8 days and before 15 days postinjury.

MyoD and Myogenin Expression in Muscles of mdx and Control C57Bl/10Sn Mice

Muscle samples from mdx and C57Bl/10Sn mice strains were taken from fetuses at 15 days of gestation to 420 days of age and analyzed by Northern analysis as described above.

MyoD expression. The levels of MyoD mRNA in skeletal muscles of the mdx and C57Bl/10Sn (control) mice from Embryonic Day 15 to around 420 days (13 months) of age are shown in Fig. 3a. In both mdx and control mice, MyoD mRNA levels were high in fetal muscles and dropped rapidly during the 2 weeks after birth. MyoD levels in control mice decreased to a low constant background level by 24 days postpartum and essentially remained at this level in all adult ages studied.

In contrast, in mdx mice after the initial perinatal decrease, the MyoD mRNA levels increased from around 21 days of age and remained elevated to a varying extent throughout the time points examined. The highest levels of MyoD expression appeared to be between 21 and 40 days, with relatively reduced but sustained expression af-

FIG. 2. Densitometric analysis of mRNA levels in regenerating TA muscles of BALB/c mice. Total RNA was prepared from TA muscles of BALB/c mice at the times indicated after crush injury. Samples (20 μg), prepared from two mice for each time point, were analyzed by Northern blot hybridization. MyoD (a) and myogenin (b) mRNA levels, relative to GAPDH, were determined by densitometry.
FIG. 3. MyoD and myogenin mRNA levels in skeletal muscles of mdx and C57Bl/10Sn (control mice). RNA samples prepared from mdx (▲, △) and C57Bl/10Sn (●, ○) mice at the ages indicated were analyzed by Northern blot hybridization. MyoD (a) and myogenin (b) mRNA levels, relative to GAPDH expression, were determined by densitometry. Each point represents a single RNA sample prepared from muscle of 2 (adult animals) to 10 (fetal animals) mice.

MyoD and myogenin expression patterns were different. MyoD expression was increased in the control strain up to 20 days of age, and decreased sharply at birth. Myogenin expression peaked later in embryonic development and decreased sharply around birth. This initial pattern was also observed for the control strain; however, after this time, expression in the mdx and the control mice differed. There was a decline in myogenin levels in the control strain, reaching background (adult) levels by 24 days of age. This rapid postnatal decrease was not seen in the mdx mouse in which the myogenin levels decreased gradually until 17 days and then increased again at Day 21. After this time, myogenin expression remained elevated throughout the time points tested and, like MyoD, appeared to be highest between about 3 to 6 weeks of age.

DISCUSSION

MyoD expression was increased in the control strain up to 20 days of age, and decreased sharply at birth. Myogenin expression peaked later in embryonic development and decreased sharply around birth. This initial pattern was also observed for the control strain; however, after this time, expression in the mdx and the control mice differed. There was a decline in myogenin levels in the control strain, reaching background (adult) levels by 24 days of age. This rapid postnatal decrease was not seen in the mdx mouse in which the myogenin levels decreased gradually until 17 days and then increased again at Day 21. After this time, myogenin expression remained elevated throughout the time points tested and, like MyoD, appeared to be highest between about 3 to 6 weeks of age.
in situ hybridization studies (9). The present study demonstrates that Northern analysis of MyoD and myogenin mRNA levels in skeletal muscle can provide very early, specific, and quantitative information about the level of mpc activation in regenerating muscle. Also, since the whole muscle is sampled, a representative picture of the total mpc population can be attained, whereas only a fraction of the muscle is sampled by light microscopic examination of tissue sections analyzed by in situ hybridization with MyoD and myogenin probes (9). This is not to say that in situ studies of muscle tissue are not informative—on the contrary they provide essential information about the number, distribution, and nature of the cells transcribing these genes. However, the plane of section examined may not be wholly representative of the total mpc population within a muscle.

The in situ hybridization studies of Grounds et al. (9) report maximum levels of MyoD and myogenin expression by 2 days postinjury and a rapid decrease to uninjured, control levels by 8 days. Northern analysis, which also suggests coexpression of these genes, shows a peak of MyoD and myogenin expression around 4 days and the levels are still somewhat elevated at 8 days postinjury. This apparent discrepancy in peak expression times may simply reflect the different methods of sampling the muscle tissue, as for Northern analysis, the entire TA muscle was sampled, whereas in situ hybridization procedures examine individual sections of muscle. An additional reason for the apparently sustained expression of these genes as detected by Northern analysis is the short-term persistence of some MyoD and myogenin mRNA within newly formed myotubes. Such expression could not be detected by in situ hybridization with 35S- or 125I-labeled riboprobes (9) where labeling levels within myotubes were similar to background labeling. However, subsequent experiments using digoxigenin-labeled riboprobes (K. L. Garrett, unpublished observations) did demonstrate the persistence of low levels of MyoD and myogenin transcripts within newly formed myotubes in muscles regenerating up to 12 days after crush injury. The Northern analysis used in the present study would include this low level of mRNA in myotubes.

A comparison of the Northern results with autoradiographic data (3, 10) shows that the onset and peak of mpc activity corresponds closely with that of mpc replication. In normal (3, 10) and mdx (10) mice, mpc replication begins around 30 h, peaks at about 3 days, and is greatly reduced by 5 days after crush injury. This supports the proposal that Northern analysis can accurately quantitate the regenerative activity of skeletal muscles.

Northern analysis of MyoD and myogenin mRNA levels was applied to the X-linked dystrophic mdx mouse model. MyoD expression and myogenin expression in the mdx mouse demonstrate two distinct phases. The first peak was seen in both mdx and control mice late in embryonic development and decreased before birth. Similar coexpression of MyoD and myogenin in mice was observed by Eftimie et al. (8). They reported a peak of expression at 17 days of gestation, followed by a perinatal decrease. This peak corresponds to the rapid growth of skeletal muscle late in embryonic development and in neonatal mice and it is proposed that down-regulation is related to innervation of newly formed myotubes (8, 23). In mdx mice, the postnatal decrease was followed at about 21 days of age by elevated MyoD and myogenin expression. This time has been widely documented as the onset of muscle necrosis and regeneration in the limb muscles of mdx mice (2, 11, 14–18, 24). Thus, the postnatal onset of elevated mRNA levels for MyoD and myogenin described here correspond closely to the time at which regenerative activity (in response to necrosis) is first observed in these dystrophic muscles.

Similarly, in a study using the techniques of DNA amplification and in situ hybridization, myogenin mRNA was detected in muscles of mdx and control mice up to 14 days postpartum, but at 21, 28, and 70 days was only present in mdx muscles (M. J. Skynner and G. R. Coulton, manuscript in preparation). Furthermore, studies using tropomyosin phosphorylation as a measure of regenerative activity appear to show a similar pattern; the phosphorylation levels begin to drop from birth in both mdx and control animals, then rise in mdx muscles to peak at 3 weeks, and are always higher than those of age-matched controls throughout adulthood (25).

The apparent peak of MyoD and myogenin gene expression seen in the present study between 3 and 7 weeks of age is associated with the time when sequential necrosis and regeneration is reported to be most pronounced (18). It is of interest that this peak corresponds with a striking peak of mpc replication seen in autoradiographic studies of mdx mice aged from 15 to 300 days (26). The postnatal levels of MyoD and myogenin mRNA in mdx muscle did not fall to those observed in the control mouse strain, but remained higher throughout the time points tested. These maintained levels of expression indicate that regeneration, and, by inference, necrosis, of the skeletal muscles of adult and old mdx mice is continuous and is not transient and nonprogressive as suggested by some reports.

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REFERENCES


