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The Pax3-Cre Transgene Exhibits a Rostrocaudal Gradient of Expression in the Skeletal Muscle Lineage

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Summary

Pax3-Cre (P3Pro-Cre) transgenic mice have been used for conditional gene deletion and/or lineage tracing in derivatives of neural crest, neural tube, metanephric mesenchyme, and ureteric mesenchyme. However, the extent of its expression in skeletal muscle has not been reported. We investigated the expression of P3Pro-Cre in the skeletal muscle lineage using the R26R reporter and found an unexpected rostrocaudal gradient of expression. By X-gal staining, head, neck, forelimb, diaphragm, and most of the chest wall muscles did not show evidence of Cre expression, whereas all muscle groups posterior of the diaphragm stained blue. Intercostal muscles exhibited a rostrocaudal gradient of staining. The consistency of this expression pattern was demonstrated by using P3Pro-Cre to mutate a conditional dystroglycan allele. The result was loss of dystroglycan from caudal muscles, which exhibited the histological signs of muscle fiber injury and regeneration characteristic of muscular dystrophy. The lack of dystroglycan in regenerating myofibers suggests that the P3Pro-Cre transgene is active in satellite cells and/or in their precursors. In contrast, rostral muscles, including feeding and breathing muscles, maintained dystroglycan expression and were spared from disease. Accordingly, the mutants were viable for over a year. Its unique gradient of activity makes the P3Pro-Cre transgene a previously unappreciated yet powerful tool for manipulating gene expression in skeletal muscle and its precursors.

Keywords

Pax3; Cre; Dystroglycan; Muscular dystrophy

The Cre/lox system has become one of the cornerstones of contemporary biomedical research. Although there are many Cre mouse lines available, some are likely incompletely characterized in terms of the exact sites of Cre expression. We have discovered a previously unreported but very useful distribution of Cre expression driven by the Pax3-Cre (P3Pro-Cre) transgene (Li *et al.*, 2000). This transgene utilizes the proximal 1.6 kb of the mouse *Pax3* promoter to direct the expression of Cre recombinase. P3Pro-Cre was originally reported to be widely expressed in neural crest cell derivatives (such as cardiac outflow tract and dorsal root ganglia) and in rib cartilage, and then subsequently shown to also be expressed in metanephric and ureteric mesenchyme that give rise to nephrons and ureteric smooth muscle, respectively (Chang *et al.*, 2004; Cheng *et al.*, 2007; Grieshammer *et al.*, 2005; Liu *et al.*, 2006; Poladia *et al.*, 2006). However, there has been little description of its expression in the skeletal muscle lineage.

To investigate the distribution of P3Pro-Cre expression in skeletal muscle, we mated the transgene onto the *Gt(ROSA)26Sor^{tm1Sor}* (R26R) *lacZ* reporter mouse background (Soriano, 1999) and stained the whole body and individual muscles at different ages with X-gal (Fig. 1). We observed differential staining of muscle groups in a pattern that generally increased in an anterior to posterior fashion. All head muscles, neck muscles, forelimb muscles, and the diaphragm were unstained. In the trunk, large muscles (pectoral and back muscles) were unstained, but intercostals exhibited an anterior to posterior gradient of blue staining: the most rostral intercostals did not stain, but beginning at intercostal six towards thirteen there were increasing numbers of blue muscle fibers. Below the diaphragm, all muscles were stained blue, including those of the hindlimbs. We also assayed knockin mice expressing Cre from the endogenous *Pax3* promoter (Engleka *et al.*, 2005) rather than from a transgene. The major difference compared to the P3Pro-Cre transgenic mice was the lack of the obvious rostrocaudal gradient; all muscles, except for those of the head and neck, were stained blue (data not shown). These data indicate that 1) the P3Pro-Cre transgene lacks an element directing expression in rostral muscles, in agreement with previous studies (Brown *et al.*, 2005), and 2) rostral muscles of the R26R mice in our colony are capable of expressing *lacZ* after Cre-mediated recombination.

We also used the P3Pro-Cre transgene to mutate the dystroglycan (DG) gene (*Dag1*) using a conditional *Dag1* allele, *Dag1^{lox}* (Cohn *et al.*, 2002; Moore *et al.*, 2002). P3Pro-Cre; *Dag1^{lox/lox}* and P3Pro-Cre; *Dag1^{lox/-}* mice (mutants) were born at the expected frequencies and seemed healthy at birth. By 2 weeks of age, however, their rate of weight gain lagged behind that of sex-matched control littermates. After one month of age mutants weighed 30–40% less than controls. Mutant mice began to show signs of hindlimb muscle weakness after 2 months, and by 5–6 months there was notable spinal kyphosis and contracture of the hindlimbs, which progressed eventually to severe dystrophy of hindlimb muscles. Nevertheless, mutants lived for more than a year, at which time they were euthanized. At this age mutant animals exhibited a hybrid phenotype, with normal head, neck and upper body, but with severely dystrophic lower trunk and hindlimb muscles (Fig. 2). Repeated attempts to breed mutant males and females were unproductive.

Immunostaining for DG was consistent with the X-gal histochemical results: X-gal-positive muscles in P3Pro-Cre; R26R mice lacked DG in P3Pro-Cre; *Dag1^{lox/lox}* mice; X-gal-negative muscles maintained apparently normal DG expression levels (Fig. 3). Affected muscles also lacked DG at the neuromuscular junction, which caused synaptic defects (Nishimune *et al.*, 2008). Moreover, muscles lacking dystroglycan showed the expected disruption of the dystrophin-glycoprotein complex (DGC) (data not shown), as previously reported in other mice with impaired DG expression (Cohn *et al.*, 2002; Cote *et al.*, 1999). But in contrast to a previous report (Cohn *et al.*, 2002), here there was no muscle hypertrophy (Fig. 2) and no evidence of expression of dystroglycan in regenerating muscle fibers, regardless of age (Fig. 4). This latter finding suggests that the P3Pro-Cre transgene was expressed in satellite cells in the affected muscles and/or in satellite cell precursors, which normally express *Pax3* (Montarras *et al.*, 2005; Relaix *et al.*, 2005; Schienda *et al.*, 2006). Consistent with this, Pax7-positive satellite cells could be located at the appropriate sites in muscles lacking DG (data not shown).

Histopathological analyses revealed the muscular dystrophy phenotype only in caudal muscle groups, with the appearance of regenerating myofibers with central nuclei and eventually widespread fibrosis (Fig. 5). These data reinforce DG's already demonstrated importance for stabilizing the DGC and maintaining muscle fiber integrity (Cohn and Campbell, 2000; Cohn *et al.*, 2002; Cote *et al.*, 1999; Durbeek and Campbell, 2002; Michele and Campbell, 2003; Parsons *et al.*, 2002). However, our finding that DG-null satellite cells are capable of driving regeneration differs from the conclusion of a previous report (Cohn *et al.*, 2002). That study showed that DG-null satellite cells in *MORE-DG* mice cannot support muscle fiber

regeneration after an acute pharmacological injury (cardiotoxin injection). In contrast, our mice have a chronic, less severe injury—disruption of the DGC—rather than an acute injury. These very different modes of injury may explain the discrepancy.

Herein we have documented the unique distribution of Cre recombinase in the skeletal muscle lineage of P3Pro-Cre transgenic mice. We note that there are other reports of transgenes displaying a rostrocaudal gradient (Donoghue *et al.*, 1991a; Donoghue *et al.*, 1991b; Ngo-Muller *et al.*, 2003; Spitz *et al.*, 2002). Interestingly, the original description of the P3Pro-Cre transgenic mice reported no somitic and no skeletal muscle expression (Li *et al.*, 2000). There are a few possible explanations for this. First, P3Pro-Cre expression may vary from one strain background to another; the original P3Pro-Cre transgenic mice were reported to have a B6/SJL background, whereas our mice have a primarily B6/CBA background. Another possibility is that the intense X-gal staining of the neural tube and skin in the R26R reporter mice (Li *et al.*, 2000) obscured staining of the caudal skeletal muscles. Finally, the transgene's activity may have changed over the course of many generations due to epigenetic influences. In any event, the transgene has behaved consistently in our hands for approximately seven generations over a three year period with respect to two different floxed alleles.

In summary, skeletal muscle expression of the P3Pro-Cre transgene is restricted primarily to caudal muscles but in general exhibits a rostrocaudal gradient. An important feature is the lack of expression in heart, diaphragm and feeding muscles. Thus, when used to mutate a conditional *Dag1* allele, there was severe muscular dystrophy in caudal muscles in the context of an otherwise normal animal with a long lifespan. This and similarly induced disease models should be very useful for studying potential treatments for muscular dystrophy, because both diseased and normal (control) muscles will be present in the same animal. In addition, we believe that utilizing the P3Pro-Cre transgene for conditional mutation represents a powerful new tool to study the functions of genes whose mutation in all skeletal muscles might either be incompatible with life or result in premature lethality because of diaphragm involvement. But we note that due to extramuscular expression of the P3Pro-Cre transgene in neural crest, neural tube, urogenital tissues (Chang *et al.*, 2004; Grieshammer *et al.*, 2005; Li *et al.*, 2000) and perhaps elsewhere, caution must be exercised both in choosing experimental questions and in interpreting outcomes. In this regard, the hypaxial muscle-specific MCre transgene that uses an enhancer from the upstream *Pax3* regulatory region (Brown *et al.*, 2005) might be a better choice in some cases. However, MCre expression in the diaphragm and forelimb muscles could be disadvantageous relative to the original P3Pro-Cre transgene analyzed here.

METHODS

Mice

Dag1^{lox/lox} mice (Cohn *et al.*; Moore *et al.*, 2002) were provided by Kevin Campbell. Pax3-Cre transgenic (P3Pro-Cre) mice (Li *et al.*, 2000) were provided by Jonathan Epstein. Pax3-Cre knockin mice (Engleka *et al.*, 2005) and R26R indicator mice (Soriano, 1999) were obtained from The Jackson Laboratory. To generate animals for analysis, the Cre transgene was always transmitted through the sire because of undesirable Cre activity sometimes detected in the female germline. Mice were genotyped by PCR using genomic DNA extracted from tail biopsies. The primer pairs used were as follows. Cre: 5'-CGGTCGATGCAACGAGTGATGAG-3' and 5'-ACGAACCTGGTCGAAATC-3'; R26R: 5'-AAAGTCGCTCTGAGTTGTTAT-3' and 5'-GCGAAGAGTTTGTCTCAACC-3'; *Dag1^{lox}*: 5'-GGAGAGGATCAATCATGG-3' and 5'-CAACTGCTGCATCTCTAC-3'; *Dag1⁺*: 5'-GAGAGGAGCTGAACATGTCAG-3' and 5'-CCAGCTTAGTACCTTGCCGCAC-3'. *Dag1⁻* (fully recombined by Cre): 5'-CGAACACTGAGTTCATCC-3' and the second *Dag1^{lox}* primer above. All animal

experiments were approved by the Animal Studies Committee of Washington University School of Medicine.

X-gal Staining, Immunofluorescence, and Histology

X-gal staining was performed according to published methods (Chang *et al.*, 2004). A monoclonal antibody to the COOH-terminus of β -dystroglycan (MANDAG2; clone 7D11) (Pereboev *et al.*, 2001) was purchased from the Developmental Studies Hybridoma Bank (University of Iowa) and used for immunofluorescence after urea-glycine treatment of fresh frozen sections (Miner and Sanes, 1994). Anti-Pax7 was also purchased from the Developmental Studies Hybridoma Bank and used to stain frozen sections. Alexa 594-conjugated anti-mouse IgG1 antibody was purchased from Invitrogen (Carlsbad, California). For light microscopy, muscles were dissected, fixed in 10% buffered formalin, washed, dehydrated, embedded in paraffin, sectioned, and stained with H&E by standard methods.

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Fig. 1. X-Gal staining of P3Pro-Cre; R26R mice reveals a rostrocaudal gradient of Cre expression in skeletal muscle. **A:** X-gal staining of 1 day old (P1) whole pups with skin removed. Left, control; right P3Pro-Cre; R26R. **B:** Composite image of 20 µm sections of a P3Pro-Cre; R26R P1 pup showing the lack of staining of most muscles above the diaphragm (arrow) and involvement of most muscles below it. **C:** Higher magnification of the chest wall shows the intercostal muscle gradient. Note the lack of expression in the diaphragm (arrow). H, heart. L, lung.

Fig. 2. P3Pro-Cre-mediated deletion of DG induces severe muscular dystrophy in caudal but not rostral muscles. Upper mouse, mutant; lower, control. Mutant mice are viable and by a year of age resemble a hybrid consisting of: **A:** normal head, neck, upper body and forelimbs; **B:** severely dystrophic lower body and hindlimbs.

Fig. 3.

P3Pro-Cre-mediated loss of dystroglycan (DG) in caudal skeletal muscles at P1. **A:** Normal DG expression in control forelimb muscles. **B:** Mutant forelimb muscles with normal DG expression. **C:** Mid-thorax section from a control showing normal DG expression in diaphragm (Dia), intercostal (IC) and chest wall (CW) muscles. R, rib. **D:** Anterior thorax section from a mutant showing normal DG expression in IC and CW muscles. **E:** Mid-thorax section from a mutant showing almost complete absence of DG from IC muscles, but preserved DG expression in Dia and CW muscles. **F:** Posterior thorax section from a mutant showing a greater reduction in the number of DG-positive muscle fibers in the IC muscle. SC, subcutaneous muscle. **G:** Abdominal wall muscle from the control shows normal DG expression. **H:** Mutant abdominal

wall muscle lacks DG. **I:** Control hindlimb muscles show normal DG expression. **J:** DG is undetectable in mutant hindlimb muscles.

Fig. 4. Anti-DG staining of quadriceps from 11 months old control (A) and mutant (B) littermates. DG deletion using P3Pro-Cre was permanent; there was no reexpression of DG in regenerating skeletal muscle fibers.

Fig. 5. Histology reveals tissue alterations characteristic of muscular dystrophy in mutant caudal muscles only. Shown are H&E stained sections from rostral muscles (**A–D**) and from caudal muscles (**E–L**) of 3 month old control (left two columns) and mutant (right two columns) littermates. Note the central myofiber nuclei and inflammation in mutant caudal muscles. **A,C:** masseter. **B,D:** biceps brachii. **E,G:** abdominal wall. **F,H:** psoas. **I,K:** quadriceps. **J,L:** gastrocnemius.