Skeletal muscle gene transfer: regeneration-associated deregulation of fast troponin I fiber type specificity

PATRICIA L. HALLAUER, GEORGE KARPATI, AND KENNETH E. M. HASTINGS
Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4

Hallauer, Patricia L., George Karpati, and Kenneth E. M. Hastings. Skeletal muscle gene transfer: regeneration-associated deregulation of fast troponin I fiber type specificity. Am J Physiol Cell Physiol 278: C1266–C1274, 2000.—Direct gene transfer into skeletal muscle in vivo presents a convenient experimental approach for studies of adult muscle gene regulatory mechanisms, including fast vs. slow fiber type specificity. Previous studies have reported preferential expression of fast myosin heavy chain and slow myosin light chain and troponin I (TnIslow) gene constructs in muscles enriched in the appropriate fiber type. We now report a troponin I fast (TnIfast) direct gene transfer study. We injected into the mouse soleus muscle plasmid DNA or recombinant adenovirus carrying a TnIfast/β-galactosidase (β-gal) reporter construct that had previously been shown to be expressed specifically in fast fibers in transgenic mice. Surprisingly, microscopic histochemical analysis 1 and 4 wk postinjection showed similar TnIfast/β-gal expression in fast and slow fibers. A low but significant level of muscle fiber segmental regeneration was evident in muscles 1 wk postinjection, and TnIfast/β-gal expression was preferentially targeted to regenerating fiber segments. This finding can explain why TnIfast constructs are deregulated with regard to fiber type specificity, whereas the myosin constructs previously studied are not. The involvement of regenerating fiber segments in transduction by plasmid DNA and recombinant adenoviruses injected into intact normal adult muscle is an unanticipated factor that should be taken into account in the planning and interpretation of direct gene transfer experiments.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
adeno viral TnIfast/β-gal constructs were expressed equally well in fast and slow fibers, i.e., expression was deregulated with respect to fiber type. Histoch emical analysis revealed the presence of regenerating muscle fiber segments 1 wk postinjection, although there had been no deliberate attempt to induce necrosis/regeneration. Moreover, regenerating fiber segments were preferentially targeted for both plasmid and adeno viral transduction, and this can account for the observed deregulated expression of TnIfast gene constructs in slow fibers. The unexpected involvement of regenerating segments in transduction following injection into normal adult muscle has implications for the use of direct gene transfer methods in muscle gene regulatory studies, particularly in the case of adult gene regulatory mechanisms that may be perturbed by regeneration.

MATERIALS AND METHODS

Animals. Normal (CD-1) and immunocompromised SCID (C.B-17/IcrCrL-scidBR) mice were obtained from Charles River Laboratories, St. Constant, Quebec, Canada. Transgenic mice carrying the TnlacZ21 transgene were from line 29 (15). Adult mice >6 wk old of both sexes were used. Animal housing and experimentation followed the guidelines of the Canadian Council for Animal Care in protocols approved by the McGill University Animal Care Committee.

TnIfast/β-gal gene constructs. We used the TnIfast/β-gal plasmid TnlacZ21 (15), which contains 530 bp of TnIfast 5′-flanking DNA, exon 1, intron 1, and the first (untranslated) part of exon 2 of the quail TnIfast gene, linked to a 5.1-kb Sma I/EcoRI fragment of pRSVZ (23) containing promoterless Escherichia coli lacZ sequences and SV40 splicing and polyadenylation sequences, in the vector pBR322. As a control for assessing plasmid uptake we used the β-gal parent plasmid pRSVZ, in which expression is driven by a 524 fragment of the Rous sarcoma virus (RSV) proviral genome including the 3′ long terminal repeat (LTR) (24). The recombinant adenovirus AdTnlacZ(1), previously referred to as AVTnlacZ (21), consists of a 5.7-kb TnlacZ(1) construct inserted by homologous recombinant into the E1 region of E1- E3-deleted human adenovirus type 5. The TnIfast/β-gal insert in AdTnlacZ(1) is identical to that of TnlacZ21, except that, because of adenovirus vector insert size constraints, the bovine growth hormone gene polyadenylation sequence [a 267-bp Xba I/BamHI fragment from pRc/RSV (Invitrogen)] was used in place of SV40 sequences.

TnlacZ21 plasmid DNA was injected into nontransgenic adult mouse soleus muscles, and gene expression was analyzed by β-gal histochemistry on cryostat cross sections. Muscle fiber types were identified by myosin ATPase histochemistry or immunohistochemistry on serial sections. The soleus muscle was selected for this study because it contains about equal numbers of fast and slow muscle fibers thoroughly interspersed (Ref. 42; and see Fig. 1), with few hybrid fibers containing both fast and slow myosin (~4% by immunohistochemistry, data not shown), and because its small size, simple cylindrical shape, and parallel fiber orientation permit a thorough microscopic analysis of the entire fiber population. By comparing whole muscle cross sections taken at ~0.5-mm intervals, we could track individual fibers along the muscle. This allowed us to accurately count the total number of β-gal-expressing...
fibers in each muscle and also to examine changes in morphology and in β-gal expression levels along the lengths of individual fibers.

When we examined TnI LacZ1 plasmid DNA-injected muscles after 7 days, we observed β-gal-expressing fibers were not stained from end to end. β-Gal staining was usually limited to fiber segments ~1–2 mm in length and was graded in intensity; along the lengths of individual fibers, weakly staining segments were often found between strongly stained and unstained segments. On quantitation by microdensitometry (Fig. 4), the staining intensities of fast and slow fiber profiles did not differ significantly (Mann-Whitney U-test, P > 0.05).

These results indicated that direct-transferred TnIfast/β-gal constructs, either as plasmid DNA or as recombinant adenovirus, were expressed about equally well in fast and slow fibers.

To address the possibility that an inherent preferential expression in fast fibers might be obscured by a countervailing preferential plasmid DNA uptake by slow fibers, we injected the plasmid pRSVZ, in which β-gal expression is driven by the general promoter, the Rous sarcoma virus LTR. We found that pRSVZ, like TnILacZ1, was expressed in similar numbers of fast and slow fibers after injection into mouse soleus muscle.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Days Postinjection</th>
<th>No. of Muscles</th>
<th>Total</th>
<th>Slow</th>
<th>Fast</th>
<th>Not typeable</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnIfast/β-gal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid 7</td>
<td>16</td>
<td>74</td>
<td>31</td>
<td>27</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Plasmid 28*</td>
<td>8</td>
<td>16</td>
<td>5</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Adenovirus 7</td>
<td>20</td>
<td>21</td>
<td>7</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>pRSVZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid 7</td>
<td>16</td>
<td>47</td>
<td>18</td>
<td>16</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>TnI slow/β-gal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid 7</td>
<td>4</td>
<td>26</td>
<td>14</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Numbers of β-galactosidase (β-gal)-expressing fibers were determined by serial reconstruction of whole muscles following X-Gal histochemistry on cross sections at multiple sectioning levels. Fiber numbers shown are cumulative totals over several experiments involving a total number of soleus muscles indicated (No. of Muscles). Fibers were classified as fast or slow if they showed typical morphology/ATPase histochemistry at any sectioning level within the β-gal-expressing fiber segment, regardless of whether atypical/regenerating morphology was observed at other sectioning levels. Fibers showing atypical/regenerating morphology throughout the β-gal-expressing segment were classified as not typeable. Note that, in addition to these entirely untypeable fibers, regenerating segments were also observed within fibers that could be typed as fast or slow at other sectioning levels. By χ² analysis, the distribution of slow and fast fibers in each row was not significantly different (P > 0.05) from 1:1, the fast-to-slow ratio of the soleus muscle, except for the last row, TnI slow/β-gal plasmid, which showed a significant excess of slow fibers (P < 0.05). *Long-term (28-day) experiments used SCID mice.
and slow fibers (Table 1) and gave X-Gal-staining optical densities that were not significantly different in the two fiber types (data not shown). We conclude that fast and slow fibers have similar transducibility and that the equivalent expression of TnILacZ1 in both fiber types directly reflects transcriptional capabilities uncomplicated by differential plasmid DNA uptake.

Preferential transduction of regenerating segments. In cross sections of plasmid- or adenovirus-injected muscles 7 days postinjection, most fibers had the typical microscopic appearance and ATPase histochemical reactions of normal fast or slow muscle fibers. However, a minority, ranging up to \( \sim 15\% \), were morphologically/histochemically atypical. Many of these had the characteristics of regenerating muscle fibers; they were small, had centrally, rather than peripherally, located nuclei, and expressed the embryonic isoform of myosin heavy chain (Fig. 5). Their ATPase histochemical staining was intermediate between the strong staining of typical slow fibers and the extremely weak staining of typical fast fibers and often showed the myotube appearance characteristic of newly formed or regenerated muscle fibers. Regenerating fibers were found throughout the muscles, scattered individually or in small clusters.

Analysis of serial sections revealed that individual muscle fibers contained both normal-appearing and regenerating segments (e.g., cells 10 and 11, Fig. 2, E-H, and cells 1 and 2, Fig. 3). Approximately one-fourth of the fibers that showed regenerating morphology on a reference section could be identified as typical slow or fast fibers on sections cut 0.5 mm above or below; extrapolation over the \( \sim 7\)-mm length of the

Fig. 2. Expression of direct-transferred TnIfast/ß-gal construct TnILacZ1 in slow as well as in fast muscle fibers 7 days following plasmid DNA injection into mouse soleus muscle. Each pair of panels shows X-Gal histochemical reaction for ß-gal (left) and the corresponding areas of serial sections reacted for myosin ATPase histochemistry following preincubation at pH 4.6 (right) as in Fig 1. Numbered arrows point out the same fibers in left and right panels. A-E: different examples of ß-gal-expressing fibers. Fibers 1, 2, and 6 are typical fast fibers, and 3 and 8 are typical slow fibers, and 4, 5, 7, and 9 are atypical/regenerating fibers. E-H: same set of fibers at different levels (\( \sim 0.5\) mm apart) along the length of the muscle. Note that fibers 10 and 11 appear as typical slow fibers in E but as atypical/regenerating fibers in F and G (fiber 11) or G and H (fiber 10).
soleus muscle suggests that most or all regenerating fibers represent segments of otherwise normal-appearing muscle fibers.

A large fraction (38–81%) of TnI fast/β-gal-expressing muscle fibers included β-gal-expressing regenerating segments demonstrable by sectioning at 0.5-mm intervals (e.g., fibers 3, 4, 7, and 9–11, Fig. 2; fibers 1, 2, and 4, Fig. 3; and see also Fig. 6). In some such fibers (those counted as “not typeable” in Table 1) the entire β-gal-expressing segment showed regenerating features, but in many others it included both normal and regenerating segments. Regenerating fiber profiles were approximately fourfold overrepresented among the β-gal-expressing subset of fiber profiles by comparison with their overall representation in the muscle sections (27–66% vs. 7–15%). Thus, in both plasmid DNA- and adenovirus-injected muscles, TnI fast/β-gal expression was preferentially targeted to regenerating segments.

Long-term expression of TnILacZ1 plasmid in fast and slow fibers. Because our results at 7 days postinjection revealed preferential transduction of regenerating fiber segments, we wished to determine whether fast-fiber-specific expression of TnI fast/β-gal might emerge in longer-term experiments that would permit the transduced regenerating fiber segments to undergo additional maturation. However, very little β-gal expression was detected in longer-term experiments in normal mice. In an extensive experimental series in CD1 mice at 3 wk postinjection, we found only a single β-gal-expressing fiber when we should have expected to see scores. Others have observed that plasmid- or adenovirus-mediated gene expression is transient in normal animals, apparently due to immunological reaction against the reporter gene polypeptide (9, 37, 40), and that longer-term expression can be attained using immunocompromised animals (2, 27). We repeated the experiments in genetically immunodeficient SCID mice and then readily detected TnI fast/β-gal-expressing fibers 4 wk postinjection. None of the β-gal-expressing fibers expressed detectable embryonic myosin heavy chain, indicating that regeneration/maturation had proceeded beyond the stage observed at 7 days postinjection. Nonetheless, similar numbers of fast and slow fibers (Table 1, and see Fig. 6) expressed β-gal, and the...
β-gal-staining optical densities of fast and slow fiber profiles were similar (data not shown). Thus we did not see evidence that regenerated slow muscle fibers repress TnIfast/β-gal expression on further maturation, although it is possible that stable β-gal protein or mRNA synthesized early during regeneration could obscure a subsequent transcriptional repression.

Expression of a TnIslow construct in fast and slow fibers. The TnI gene family includes members expressed specifically in both fast (TnIfast) and slow (TnIslow) muscle fibers. Previous direct gene transfer studies of TnIslow constructs, based on biochemical assay of reporter enzymes in rat muscle homogenates, showed approximately sixfold greater expression of direct-transferred TnIslow constructs in soleus muscle than in extensor digitorum longus muscle at 5 days postinjection, indicating slow fiber preferential expression (11). To assess fiber-type-specific expression of TnIslow constructs at the individual cell level, we injected construct BW188Z in which β-gal expression was driven by human TnIslow enhancer and promoter elements that are known by microscopic analysis to drive slow-fiber-specific expression in transgenic mice (11). When we examined expression at 7 days postinjection, we detected β-gal expression in both fast and slow fibers (Table 1). However, there was preferential expression in slow fibers; almost three times as many slow fibers as fast fibers showed β-gal-staining (significant by χ² test, P < 0.05), and their average staining intensity was twice as great (significant by Mann-Whitney U-test, P < 0.025). Thus the TnIslow construct showed some deregulated expression in the inappropriate fiber type, although it differed from TnIfast, in that fiber type deregulation was not so extensive as to completely override preferential expression in the appropriate fiber type.

DISCUSSION

The TnILacZ1 construct is expressed in fast but not slow muscle fibers in the soleus and other mouse muscles when present as a germ line chromosomal transgene (Fig. 1 and Ref. 15). Thus all the cis regulatory elements required to direct fast-fiber-specific expression are present in the TnIfast/β-gal plasmid and adenovirus constructs used in the present study. However, despite the presence of these regulatory elements, we found that the TnIfast constructs were expressed with similar efficiencies in slow and fast fibers when direct-transferred by intramuscular injection in adult animals. Slow and fast fibers were present in the β-gal-expressing muscle fiber subpopulation in about the same ~1:1 ratio as they are found in the soleus muscle as a whole, and quantitative levels of β-gal expression were similar in the two fiber types.

Because of the intrinsic inefficiency of the direct-transfer method, the average numbers of β-gal-expressing fibers per muscle in our study were small, on the
order of 1–10. However, there is no reason to doubt that the β-gal-expressing fibers observed, though few in number in any individual muscle, are typical of the fibers that are responsible for reporter gene expression in other published studies using the direct-transfer method in normal adult muscle. By studying a large number of muscles, we accumulated sufficient data to reliably establish the character of the β-gal-expressing fiber population.

The unexpected deregulated expression of TnI fast/β-gal constructs in slow fibers in our study is likely related to inadvertent muscle fiber segmental necrosis/regeneration. We found a low but significant level of segmental regeneration in plasmid- and adenovirus-injected muscles and preferential targeting of TnI fast/β-gal gene expression to regenerating fiber segments. A large fraction of β-gal-expressing fibers showed features of regeneration, and regenerating fiber profiles were considerably (~4-fold) overrepresented in the β-gal-expressing fiber subpopulation compared with their abundance in the muscle as a whole. The presence of regenerating fibers in plasmid DNA-injected muscles has been observed before (12, 27), although their segmental nature and the preferential targeting of transferred gene expression have not been noted. Preferential transduction of regenerating fiber segments is consistent with studies showing that transduction by plasmid DNA or recombinant adenovirus is dramatically increased by conditions that induce extensive muscle fiber regeneration (1, 38, 39). Presumably, activated satellite cells and myoblasts, and/or newly formed fibers, are better able to take up and express plasmid/viral constructs than are fully mature muscle fibers.

Preferential transduction of regenerating segments can account for at least some of the deregulated expression of TnI fast/β-gal constructs in slow as well as in fast fibers. The endogenous TnI fast gene is known to be activated during myoblast differentiation (16, 20) and to be expressed in regenerating fast and slow muscle (35). Thus regenerating segments of both fast and slow fibers would be expected to contain transcription factors capable of driving TnI fast/β-gal gene expression. We have observed a weak but detectable activation of germ line transgene Tn ILacZ1 expression in regenerating slow muscle fiber segments in soleus muscle injected with a non-β-gal-encoding plasmid (data not shown).

Segmental regeneration implies muscle fiber segmental necrosis and repair, a process frequently observed in muscle pathology. We found that sham injection of vehicle only (0.15 M NaCl) induced similar levels of regeneration as did DNA injection (data not shown), so that physical disruption associated with the introduction of the injection needle and significant liquid volumes appears to be the predominant cause of necrosis/regeneration. Whatever the mechanism, necrosis occurs early on and after several weeks postinjection regenerating fiber segments are no longer found. Other studies have established that, while actively regenerating fibers are evident at 1 wk following acute muscle injury, regeneration is essentially complete by 3–4 wk (41).

Despite the transient nature of regeneration, we continued to find β-gal-stained slow muscle fibers 4 wk after injection of TnI fast/β-gal constructs. The continued presence of β-gal in slow fibers could reflect the presence of stable β-gal mRNA and/or protein synthesized during an earlier phase of regeneration. Alternatively, there may be ongoing deregulated TnI fast/β-gal gene expression in mature, fully regenerated slow fibers, perhaps as a persistent juvenile feature akin to the central nucleation that permanently marks regen- erated rodent muscle fibers (19).

Fiber-type-specific gene expression. Previous direct gene transfer studies of fiber type regulation have shown apparently proper fast/slow regulation of constructs based on fast myosin heavy chain IIB (36) and on slow myosin light chain (MLC-1s/v [18] and MLC2s [23]) and troponin I (TnI slow [11]) genes. In contrast, our results show similar expression of TnI fast constructs in slow and fast fibers. This difference reflects, in part, gene-to-gene variation in the extent to which fiber-type-specific expression is deregulated during muscle regeneration. Endogenous genes encoding MLC-1s/v (also called MLC1s b) and MLC2s are known to maintain slow muscle specificity throughout the regeneration process and are not expressed, even transiently, in regenerating fast muscle (13, 35). The endogenous fast myosin heavy chain IIB gene shows only an exceedingly transient deregulated expression (< 24 h duration) during slow muscle regeneration (17). Direct-transferred constructs based on these genes would be expected to show little or no regeneration-associated deregulation compared with TnI fast, which is expressed in regenerating slow muscle over a period of ~10 days (13). Indeed, the MLC-1s/v and MLC2s gene transfer studies showing slow muscle-enriched expression were carried out not in intact adult muscles but in regenerating muscles following toxin-induced total necrosis, to increase the transduction efficiency (18, 23). It is likely that different kinds of regulatory mechanisms control regeneration-deregulated (e.g., TnI fast) and regeneration-resistant (e.g., MLC-1s/v, MLC2s) fiber-type-specific gene expression.

Regarding TnI slow, the endogenous gene is known to be expressed in regenerating fast as well as slow muscle (13, 35) so some deregulated expression of direct-transferred TnI slow constructs in fast fibers might be expected. In our experiments we did in fact observe significant TnI slow/β-gal expression in fast as well as in slow fibers; however, preferential expression in slow fibers was nonetheless evident, consistent with the results of Corin et al. (11). Thus, although it is affected, the TnI slow gene appears to be less susceptible to regeneration deregulation than is the TnI fast gene. This different susceptibility could reflect either differences between the genes themselves in their responses to regeneration-associated transcription factors or differences in the regeneration process between fast and slow fibers.

Implications for use of the direct transfer technique. Our results reveal an unanticipated aspect of direct gene transfer that has important implications for its
experimen-tal use, namely, that intramuscular injection of plasmid DNA or recombinant adeno-virus does not strictly introduce genes directly into mature adult muscle fibers. Even in the absence of deliberate at-tempts to induce regeneration, injection of plasmid DNA or adeno-virus into adult muscle by itself induces segmental re-gen-eration. Although regenerating seg-ments are a minority, we find they are transduced more efficiently than mature fiber segments, so that much of the transferred gene’s tran-scriptional activity may be in re-gen-erated segments. Moreover, diffusion within the muscle fiber of reporter gene mRNA or protein into adjacent normal segments may give a misleading im-pression of transferred-gene expression in normal adult fibers.

The impact of regeneration varies greatly among muscle genes and is a factor that should be taken into ac-count in planning or interpreting direct gene transfer experiments. In the case of regulatory mechanisms that are relatively unperturbed by regeneration, direct gene transfer by intramuscular injection of plasmid or recomb-inant adeno-virus presents an effective experi-mental alternative to germ line transgenesis for molec-ular genetic studies such as cis-element mapping. How-ev-er, the same is not true for mechanisms that are ex- tensively perturbed by regeneration, such as those that drive fiber-type-specific expression of the TnI fast gene, and perhaps other aspects of adult muscle gene regulation as well. Direct gene transfer methods that induce and target regenerating segments are not appli-cable to the functional analysis of fiber type regulatory elements of the TnI fast gene. Transgenic mice remain an alternative approach; however, the development of con venient direct gene transfer methods that do not induce regeneration, or that do not preferentially tar-get regenerating fibers, would facilitate progress. Re-combinant adeno-associated virus vectors merit atten-tion in this regard, as recent reports suggest efficient direct gene targeting of adult muscle fibers (14, 22). Electrically augmented plasmid direct transfer (3, 25) may provide an additional avenue, if regeneration is found to play a less prominent role in this process than is the case in conventional plasmid direct transfer.

Bob Wade provided the TnIsUSE-95X1nucZ construct. He also provided inspiration and a fine example, both in science and in life. He will be missed. We thank Peter Merrifield and Stefano Schiaffino for kindly supplying antibodies.

This work was supported by a research grant from the Medical Research Council of Canada to K.E. M. Hastings, a Killam Fellow of the Montreal Neurological Institute.

Address for reprint requests and other correspondence: K. E. M. Hastings, a Killam Fellow of the Montreal Neurological Institute, McGill Univ, 3801 University St., Montreal, Quebec, Canada H3A 2B4 (E-mail: oxph@musica. mcgill.ca).

Received 14 June 1999; accepted in final form 13 January 2000.

REFERENCES


