Activation of a muscle-specific enhancer by the Ski proto-oncogene

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ABSTRACT

In transgenic mice, muscle-specific expression of the c-ski oncogene induces hypertrophy exclusively in a subset of fast muscle fibers. Here we report that regulatory elements from two genes expressed in fast fibers, myosin light chain 1/3 (MLC) and muscle creatine kinase (MCK), were activated when co-transfected with c-ski expression vectors in myoblasts. The expression from the MLC enhancer was reduced when the c-ski oncogene was cotransfected with MyoD into NIH3T3 fibroblasts. Activation of the MLC enhancer by Ski also occurred in vivo, since bigenic progeny generated by mating MLC-CAT and MSV-sW transgenic mice displayed higher CAT activity in their muscles than did the MLC-CAT parental line. Identification of gene targets for the fiber-specific action of the c-ski gene product provides a molecular model that could be used for the further dissection of Ski-induced hypertrophy, both in tissue culture and in vivo.

INTRODUCTION

The growth and differentiation of skeletal muscle has been a highly productive model of tissue-specific gene regulation. However, the diversification of muscle fiber types, a central problem of cellular differentiation, is not understood at the molecular level. Some studies have suggested that the diversity of muscle fibers present in the adult may be inherited from intrinsically different embryonic myoblast populations (1,2). Other studies have suggested that myoblasts are pluripotent with respect to fiber type and that exogenous factors can determine myoblast differentiation (3). In either case, the molecular basis of this heterogeneity remains obscure. The slow progress in this field has been due chiefly to a shortage of well-characterized gene targets and candidate regulatory molecules involved in the fiber determination process and to the lack of appropriate cell culture or in vivo systems to monitor the fate of specific fiber types.

Muscle fibers have been traditionally classified according to their myosin content. Fast myosins such as those in Type Ila, Iib and IIx fibers are typically found in muscles which contract for relatively brief periods of rapid movement, whereas slow myosins, such as those in Type I fibers, are associated with muscles used for postural movements. The regulatory determinants of fiber type may therefore be elucidated by defining the control mechanisms operating on fiber-specific genes, such as those encoding different myosin heavy chain and light chain isoforms. These mechanisms would involve both the development and maintenance of fiber type.

We have previously characterized the regulation of the rat myosin light chain (MLC) 1/3 locus (4–6), which is expressed predominantly in fast fibers (7). The locus has an unusual configuration, with two widely spaced promoters (MLC1 and MLC3) and a potent muscle-specific enhancer element 25 kb downstream of the MLC1 promoter (8). In tissue culture, these promoters, in combination with the enhancer, are active only in differentiated myotubes and not in myoblasts. Transgenic mice carrying the MLC1 promoter and MLC enhancer, linked to a chloramphenicol acetyl transferase (CAT) reporter gene, express the CAT transgene exclusively in skeletal muscle (9). Expression of the MLC1/CAT transgene predominates in fast Type Iib fibers (10). Thus, the MLC regulatory sequences contained in the MLC1/CAT transgene comprise a genetic target for putative fiber-specific regulatory factors.

Detailed analysis of the MLC enhancer has identified binding sites for multiple regulatory proteins, including the MyoD family of myogenic factors (4–6), the MAPF proteins (11) and the MEF2/MADS gene family of regulatory factors (12). Although the expression patterns of several of these proteins remains to be determined, the ubiquitous distribution of the myogenic factor transcripts in embryonic muscles (13–16) suggests that they are not responsible for the initial establishment of different fiber types in developing muscles. Other evidence suggests that they may be involved in the maintenance of fiber type in the adult (17).

The c-ski gene was first identified by its homology to a viral oncogene, v-ski, which was carried by a class of acute transforming retroviruses (18,19). Perhaps the most intriguing aspect of the c-ski gene is not its weak oncogenic activity, but rather its myogenic potential (20,21). The c-ski gene product is located in the nucleus (22,23) and its overexpression induced cells isolated...
from the body wall of quail embryos (QECs) to differentiate into muscle (20). Moreover, multiple independent lines of mice carrying different c-ski cDNAs driven by the transcriptional regulatory sequences (LTR) of the murine sarcoma virus (MSV) displayed a distinct muscular phenotype (21). Molecular analysis of these transgenic lines revealed high levels of c-ski transgene expression in skeletal muscles. The muscles of these transgenic mice demonstrated no hyperplasia but there was a pronounced increase in the diameter of a subset of the muscle fibers: fast glycolytic muscle fibers (Type IIb) underwent extensive hypertrophy, whereas fast and slow oxidative fibers (Type Iia and I) were unaffected (21,24). The pattern of fiber hypertrophy induced by the MSV-ski transgene is therefore indicative of a fast-fiber specific bias. Elucidating the action of the c-ski oncogene on the expression of specific genes could provide a potentially powerful tool to investigate the molecular mechanisms underlying the establishment and maintenance of diverse fiber types in developing muscles.

The present study documents precocious activation of the MLC enhancer by c-ski in myoblast cotransfections and the involvement of myogenic factor binding sites in this activation. c-ski suppresses activity from the MLC enhancer in transfected fibroblasts (and even antagonizes transactivation by MyoD in these cells), indicating that other components of the myogenic cell background are required for ski-mediated activation. In MSV-ski × MLC1CAT bigenic mice the CAT transgene is induced in fast but not in slow muscles. The identification of regulatory elements that are responsive to the muscle-specific activity of Ski in vivo provides a means for identifying putative cofactors involved in fiber-specific gene expression.

MATERIALS AND METHODS

Tissue culture

The C2C12 mouse myoblast line was originally derived from adult satellite cells (25) and later selected for fast fusion (26). Myoblasts were cultured in DMEM supplemented with 20% fetal calf serum (FCS), penicillin and streptomycin (Gibco, CAT #600-5070AG). C2C12 cells were seeded for transfections on 100 mm plates (1×10^6 cells for myoblasts and 4×10^5 cells for myotubes). NIH3T3 mouse fibroblast cells were propagated in DMEM with 10% FCS and seeded for transfection at 1×10^5 cells/100 mm plate. The following day the media was changed 3 h prior to transfection.

Plasmids, transfections and CAT assays

MLC1CAT expression plasmids and corresponding enhancer mutants were described previously (6). MCKCAT constructs (27) were a gift from E. Olson. The c-ski chicken cDNAs (FB29 and FBΔ29) have been previously described (28). The MSV-MyoD expression vector (29) was a gift from A. Lassar and H. Weintraub. All plasmids used for transfections were purified over two cesium chloride gradients. CAT expression plasmids were transfected along with an SV40 β-galactosidase plasmid (Promega CAT #E1081) into C2C12 and NIH3T3 cells by calcium phosphate coprecipitation. The media was changed after 16 h (20% FCS for myoblasts and 2% horse serum for myotubes). Forty-eight hours later, cells were harvested according to published protocols (30). Protein assays or β-galactosidase enzymatic assays were performed to normalize the amount of cell extract used in the CAT assays. Protein concentration was measured with Bio-Rad protein assay dye reagent (Cat. #500-0006). β-Galactosidase assays were performed with fixed volumes (30 μl) of cell extract. CAT enzymatic assays were performed according to a published protocol (31). Using L-[3H]chloramphenicol as substrate and butyryl CoA as donor, the reaction mixtures were incubated for 2 h at 37°C and were extracted into 300 μl of xylene and then back extracted twice with 100 μl of 0.25 M Tris–Cl (pH 8.0). Radioactivity in 200 μl of the organic phase was quantitated in a scintillation counter. Mock transfections always yielded less than 300 c.p.m. and transfections yielded between 1000 and 80 000 c.p.m. For CAT assays from transgenic mouse tissues, muscles were dissected, the tissues were frozen in dry ice and cell extracts were prepared by successive cycles of freezing and thawing. The amount of extract used for the CAT assays was normalized to total protein content. CAT assays were performed as described above but with a 20 min incubation. Each experimental set (a wildtype and c-ski littermate) was normalized to the wildtype EDL muscle (average EDL = 82 145 c.p.m.).

RNase protection assays

RNase protection assays were performed according to the protocol of Melton et al. (32). MLC1, MLC3 and GPDH (to demonstrate equal loading) probes were hybridized simultaneously. Approximately 10 μg of total RNA was hybridized with 5×10^5 c.p.m. of probe. The hybridizations were incubated overnight at 50°C in 80% formamide and 1x buffer (5× hybridization buffer is 0.2 M PIPES, pH 6.4, 2 mM NaCl, 5 mM EDTA). After hybridization the samples were diluted in ribonuclease digestion buffer (10 mM Tris–Cl, pH 7.5, 0.3 M NaCl, 5 mM EDTA) and treated with RNase T1 at a concentration of 1 U/μl for 60 min at 30°C. The RNase digestions were stopped by addition of 10 μl of 20% SDS and 4 μl of proteinase K (stock 10 mg/ml) and incubation at 37°C for 15 min. The digested samples were extracted with phenol-chloroform (1:1 mixture) and ethanol-precipitated with carrier tRNA. The pellet was rinsed with 70% ethanol, dried, dissolved in formamide containing bromophenol blue and xylene cyanol dyes. The samples were denatured at 100°C and separated on a 6% polyacrylamide gel containing 7.5 M urea.

RESULTS

An MLC1CAT reporter is activated in C2C12 cells by overexpression of the Ski protein

To determine whether the Ski protein could induce muscle-specific gene expression in tissue culture, we cotransfected mouse C2C12 myoblasts with an MLC1CAT reporter plasmid and a c-ski expression vector. The reporter, pMLC1CAT538 (6), contains the 400 bp MLC1 promoter and 538 bp of the MLC enhancer linked to a CAT gene. Two MSV-ski expression vectors were tested: FB29, a full length cDNA and FBΔ29, a truncated form approximately equal in length to v-ski (23,28). FB29 codes for a 90 kDa protein while FBΔ29 codes for a 50 kDa protein. The 90 kDa protein appears to have additional phosphorylation sites but both forms of the Ski proteins have similar activities in avian cells (33) and in transgenic mice (P. Sutrave and S. H. Hughes, unpublished observations). As seen in Figure 1, both c-ski cDNA expression vectors stimulated expression from the cotransfected
Figure 1. Ski transactivates the MLC promoter/enhancer in C2C12 myoblasts (a–c) and myotubes (d–f). The MLC1CAT538 reporter was cotransfected with either control vector (pMEX) (a,d) or FB29, a full length c-ski cDNA expression vector (b,e) or FBA29, a truncated c-ski cDNA expression vector (c,f). The activity of the pMLC1CAT538 reporter in myotubes without c-ski was set to 100 for each experiment. Activity was normalized to cotransfected p-galactosidase. Values are the average of three experiments (with standard error).

pMLC1CAT538 plasmid, both in myoblasts (9-fold) and in myotubes (2-fold). CAT expression was more strongly induced by c-ski cotransfection in myoblasts, presumably because in myotubes activation of the MLC enhancer by myogenic factors obscured further induction by the Ski protein.

Activation of the MLC reporter by the Ski protein requires the MLC enhancer element

Previous deletion analysis was used to define a 173 bp region of the MLC enhancer which contains four E boxes and possesses strong muscle-specific activity in tissue culture (6). E boxes (CANNTG) are transcription factor binding sites and a subset of E boxes have been shown to play an important role as targets for myogenic factors in many myogenic regulatory regions including those of the MLC1/3 locus. In order to delineate the cis-acting elements responsible for the activation of pMLC1CAT538 by c-ski, a CAT reporter plasmid (pMLC1CAT173) that contained a 400 bp MLC1 promoter fragment and the 173 bp downstream MLC enhancer fragment was cotransfected into C2C12 myoblasts together with the FB29 c-ski expression vector. pMLC1CAT173, though not as responsive as pMLC1CAT538, was consistently activated 2-fold in the presence of this c-ski cDNA expression vector (Fig. 2).

To determine whether the MLC enhancer was necessary for induction by c-ski, we cotransfected C2C12 cells with a CAT reporter plasmid containing the 400 bp MLC1 promoter but lacking the enhancer element (pMLC1CAT), along with the FB29 c-ski expression vector. The c-ski expression vector failed to induce the enhancerless pMLC1CAT reporter (Fig. 2), indicating that the precocious activation of the MLC1CAT reporter by Ski in myoblasts involves the same cis-acting elements demonstrated to be necessary for activation of MLC1CAT expression during myogenic differentiation. The myosin light chain locus has two differentially regulated promoters, MLC1 and MLC3 (34,35), so we tested the response of the MLC3 promoter to c-ski cotransfection. A reporter in which the CAT gene was driven by the MLC3 promoter without the enhancer (pMLC3CAT) showed no increase in CAT activity in the presence of the c-ski cDNA (Fig. 2). However, inclusion of the 173 bp MLC enhancer fragment (pMLC3CAT173) in the cotransfected plasmid, resulted in an induction by Ski of over 2-fold in myoblasts (Fig. 2) and over 1.5-fold in myotubes (data not shown). This demonstrates that the MLC enhancer, rather than either of the two MLC promoters, is required for induction by c-ski cotransfection. In addition, Ski appears to affect gene expression through elements in the MLC enhancer that lie outside the 173 bp fragment, previously described as sufficient for full enhancer activity in cell culture (6).

MCK regulatory elements are also transactivated by the Ski protein in C2C12 cells

Since many muscle-specific genes share common cis-acting elements in their regulatory regions, we asked whether another muscle-specific regulatory region was responsive to c-ski cotransfection. One of the best characterized of these regions lies immediately upstream of the muscle creatine kinase (MCK) gene (27,36) which also contains fiber-specific regulatory elements (M.P. Wenderoth and S.D. Hauschka, pers. comm.). Like the MLC gene, the MCK regulatory region contains multiple E boxes (27,36). To determine whether the Ski protein could activate transcription from the MCK regulatory elements, we cotransfected C2C12 cells with a c-ski cDNA expression vector (FB29) together with a CAT reporter driven by the MCK promoter and upstream enhancer (the 4800 bp 5' of the transcription start site) (pCK4800CAT) (27). Coexpression of c-ski induced pCK4800CAT expression an average of 3-fold in myoblasts and only slightly increased expression in myotubes (Fig. 3). The

![Figure 2. Transactivation of the MLC1 and MLC3 promoters by c-ski is dependent on the presence of the MLC enhancer. The activity of each reporter in C2C12 myoblasts cotransfected with a c-ski expression vector (FB29) is shown relative to activity in the absence of c-ski (transfection with pMEX control vector) which was set to one for each experiment. The data shown is the average of five experiments (with standard error).](image-url)
activation of the MCK regulatory elements by c-ski cotransfection is therefore similar to the response of the 173 bp MLC enhancer, but less marked than the response of the 538 bp MLC enhancer. In all cases though, the response to Ski was greater in myoblasts than in myotubes.

**E boxes are required for transactivation of the MLC enhancer by c-ski cotransfection**

To further define the cis-acting elements required for the precocious activation of MLC-CAT reporters by c-ski cotransfection in myoblasts, we exploited a set of well characterized mutants of the 173 bp MLC enhancer. Mutational analysis of three E boxes within this 173 bp MLC enhancer (sites A, B and C) demonstrated that a pair of these E boxes is necessary for MLC enhancer activity in C2C12 myotubes (6). Mutation of either site A (mA) or site B (mB) resulted in a 50% reduction in MLC enhancer activity in transfected C2C12 myotubes whereas mutation of site C (mC) abolished the ability of the enhancer to activate expression from the MLC1 promoter. Site C is necessary but not sufficient for myogenic responsiveness. Simultaneous mutation of both sites A and B reduced CAT activity to nearly the level observed from the promoter alone. The transactivation of these mutant MLC enhancers by myogenic factors in NIH3T3 fibroblasts displayed a different pattern: the MLC enhancer could not be transactivated if either site B or C was mutated, while mutation of site A resulted in a 50% reduction in activity. A similar pattern was observed in c-ski transactivation of pMLC1CAT173 mutants in C2C12 myoblasts. In summary, mutation of sites B and C abrogated Ski-mediated activation of the MLC enhancer while mutation of site A had no effect (Fig. 4). Sites B and C together are therefore necessary, both for transactivation by myogenic factors in a non-muscle background as well as for stimulation by c-ski cotransfection in myoblasts.

**Ski does not transactivate the MLC enhancer in non-muscle cells and inhibits transactivation by MyoD**

In transient transfection assays, the four myogenic factors are able to activate, to varying degrees, expression from muscle-specific regulatory elements in fibroblasts (37,38) including the MLC enhancer (5,6). To determine if the Ski protein could transactivate the MLC enhancer in non-muscle cells in the absence of myogenic factors, we cotransfected the NIH3T3 fibroblast cell line with the MLC1CAT538 reporter and a c-ski cDNA expression vector. As seen in Figure 5, the c-ski cDNA expression vector did not transactivate the MLC1CAT538 reporter in NIH3T3 cells. To test the possibility of synergism between the Ski protein and MyoD, we cotransfected the NIH3T3 fibroblast cell line with MyoD expression vector and the MLC1CAT538 reporter and a c-ski cDNA expression vector. As seen in Figure 5, the MyoD expression vector did not transactivate the MLC1CAT538 reporter in NIH3T3 cells. To test the possibility of synergism between the Ski protein and MyoD, we cotransfected the NIH3T3 fibroblast cell line with MyoD expression vector and the MLC1CAT538 reporter and a c-ski cDNA expression vector. As seen in Figure 5, the MyoD expression vector did not transactivate the MLC1CAT538 reporter in NIH3T3 cells. To test the possibility of synergism between the Ski protein and MyoD, we cotransfected the NIH3T3 fibroblast cell line with MyoD expression vector and the MLC1CAT538 reporter and a c-ski cDNA expression vector. As seen in Figure 5, the MyoD expression vector did not transactivate the MLC1CAT538 reporter in NIH3T3 cells.
is phenotypically normal and does not express Ski at the same
in the soleus, a predominantly slow muscle. Notably, the soleus
This increase in CAT activity was not detected
reproduced
c-ski
activity by
cotransfection observed in tissue culture can be
transgene (Fig. 6). The increase in CAT activity in the bigenic
the muscles of their MLClCAT/wt littermates lacking the
c-ski
transgene. The muscles isolated from MLClCAT/c-sfa' bigenic
both transgenes, while the other half carried only the MLC 1 CAT
transgenes. One parent was a transgenic
c-ski
bigenic mice, a homozygous MLC1CAT920 transgenic
in vivo,
we generated bigenic mice carrying both
enhancer in myoblasts.
The MLC enhancer is activated by Ski in transgenic mice
In this study we demonstrated that the Ski protein activates
the MLC and MCK regulatory elements in myoblast cultures and also
induces an MLClCAT reporter in fast muscles of transgenic
mice. The transactivation of MLClCAT reporters by the Ski
protein in C2C12 myoblasts is mediated through a 538 bp
cis-acting regulatory region. A subset of this region that is still
responsive to Ski (the 173 bp enhancer) appears to be the same as
one that was previously characterized as necessary for activation
in C2C12 myotubes in the absence of coexpressed Ski protein (6).
In addition, specific E boxes located in the MLC enhancer appear
to be necessary for the precocious activation of the 173 bp MLC
enhancer in myoblasts by the Ski protein. Regulatory elements
associated with muscle genes, such as MLC1/3 and MCK, which
have specific regions shown to confer fast-fiber specificity in
transgenic mice (M.P. Wenderoth and S.D. Hauschka, pers.
comm.), could therefore constitute targets for induction by the
transgenic c-ski gene product.
Since the Ski protein is localized primarily in the nucleus
(22,23), the transcriptional activation of the MLC and MCK
genes may result from direct binding of Ski to DNA motifs within
their regulatory elements. A binding site for NF-1
(TGG[NGG-7]CCAA), a nuclear factor, has been implicated as a
possible DNA target for the Ski protein (C. Richmond, P.
Tarapore and E. Stavnezer, pers. comm.). However, there are no
complete NF-1 consensus sites in the regulatory regions of the
MLC gene. Alternatively, the interaction between myogenic
factors and their targets may be facilitated by the Ski protein.
In addition, the differential interaction of the transgenic c-ski gene
product with each of the myogenic factors could generate
fiber-specific transcriptional regulation in muscle. This possibility
is supported by work demonstrating that the myogenic factors are
differentially expressed according to fiber type in adult rat and are
involved in the transition from fast to slow fibers caused by
denervation and thyroid hormone treatment (17).
It is possible that the effects of Ski on myogenic differentiation
are mediated through induction of the myogenic factor genes
themselves. In QECs infected with a retroviral vector carrying
c-ski, the expression of both MyoD and myogenin was induced
(39). However, infection with a mutated version of v-ski did not
result in myotube formation although MyoD and myogenin were
still activated. These results suggest that v-ski induces full

Figure 5. Transactivation of the MLC enhancer by MyoD in fibroblasts is
repressed by c-ski. The pMLC1CAT538 reporter was cotransfected into
NIH3T3 fibroblasts alone (a) or with the c-ski expression vector, FB\Delta29 (b) or
a MyoD expression vector (c) or both (d). Activity in (c) was set to 100 for each
experiment. Values are the average of at least three experiments (with standard
error).

myogenic factors present in myoblasts, we cotransfected MyoD
and c-ski expression vectors simultaneously with the
pMLC1CAT538 reporter into NIH3T3 cells. Unexpectedly, c-ski
expression vectors antagonized the transactivation of the
pMLC1CAT538 reporter by MyoD (Fig. 5). This result argues
against a simple synergistic model in which the Ski protein acts
in conjunction with myogenic factors to induce the MLC
enhancer in myoblasts.

The MLC enhancer is activated by Ski in transgenic mice
In order to examine if the c-ski gene could activate the MLC
enhancer in vivo, we generated bigenic mice carrying both
MLC1CAT and c-ski transgenes. One parent was a transgenic
mouse carrying a 1500 bp MLC1 promoter fragment with 920 bp
of the MLC enhancer and the CAT gene (MLClCAT920). This
transgenic line exhibits muscle-specific CAT activity (9) exclusively
in fast skeletal muscle fibers (10). To generate MLClCAT/
c-ski bigenic mice, a homozygous MLClCAT920 transgenic
mouse was mated with the MSV-ski heterozygous transgenic
to line 8566 which exhibits fast fiber hyper-

high levels as other muscles in the MSV-ski transgenic line (24).
In addition, only a minor increase was seen in the adult EDL
muscle, a muscle that has a high level of c-ski expression in the
transgenic line (24). Therefore, though the expression of c-ski can
vary by as much as 7-fold (D.P. Lana, S.H. Hughes, J.M.
Leferovich and A.M. Kelly, manuscript in preparation) the level
of c-ski does not appear to be the sole determinant of the
variability of MLC1CAT induction in these mice.
To investigate the relative levels of endogenous MLC tran-
scripts in MSV/ski mice RNase protection assays were per-
formed, using MLC1/3 probes, on mRNA from adult tissues of a
c-ski transgenic mouse and a normal littermate. The c-ski
transgenic adult mice displayed no increase in the steady state
levels of either MLC1 or MLC3 mRNA over that of the wildtype
littermate (Fig. 7). Therefore, the endogenous levels of MLC
mRNA do not parallel the increase in CAT transgene activity seen
in the adult bigenic mice.

DISCUSSION

In this study we demonstrated that the Ski protein activates
the MLC and MCK regulatory elements in myoblast cultures and also
induces an MLClCAT reporter in fast muscles of transgenic
mice. The transactivation of MLClCAT reporters by the Ski
protein in C2C12 myoblasts is mediated through a 538 bp
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still activated. These results suggest that v-ski induces full
Figure 6. A c-ski transgene activates the MLC-CAT transgene in the fast muscles of bigenic mice. Mice heterozygous for the c-ski transgene were mated to mice homozygous for the MLC1CAT transgene and resulting littersmates (5–12 months old) were analyzed. Various skeletal muscle tissues and hearts were dissected and CAT assays were performed on cell extracts. Values from each experiment are normalized to the expression of CAT in wildtype EDL. Black bars are MSV-ski/MLC-CAT bigenic mice, shaded bars are littermates that carry the MLC-CAT transgene but not the MSV-ski transgene. Values are the average of three animals (with standard error), except for tongue values which are from one animal each. IC = intercostal muscles.

Figure 7. Endogenous MLC transcripts are not increased by the presence of c-ski. RNase protection analysis was performed on RNA isolated from MSV-ski transgenic mice with the muscular phenotype (lane 6) and an MSV-ski transgenic line not displaying the phenotype (lane 5) and a wildtype mouse (lane 4). RNA was hybridized to a single probe containing the GAPDH and MLC1 and MLC3 cDNAs cloned into KS Bluescript vector. Protected fragments are indicated by arrows at right. Lane 1 shows radiolabeled Msp-digested pBR322 DNA as a marker. Lane 2 is the undigested probe. Lane 3 is the probe hybridized with tRNA.

myogenic conversion and myogenic factor expression by parallel pathways. Moreover, in the MSV-ski transgenic mice, no significant changes in MyoD or myogenin expression levels were observed (P. Sutrave and S.H. Hughes, unpublished observations).

The Ski protein need not bind directly to DNA sequences, or may need a cofactor to do so. We currently favor this last hypothesis, for the following reasons. First, previous studies demonstrated that a factor present in nuclear extracts may be required to enable a recombinant Ski protein to bind DNA (40). Secondly, the Ski protein alone failed to activate the MLC enhancer in fibroblasts but rather repressed the transactivation of the MLC enhancer by MyoD. Finally, in MSV-ski transgenic mice, muscle hypertrophy was limited to fast Ib fibers. The action of Ski may require an additional factor(s) that is expressed exclusively in certain myogenic subsets and that is also expressed in the C2C12 cell line. This would explain why type Ib fibers of MSV-ski transgenic mice undergo hypertrophy in response to Ski over-expression while other c-ski-expressing fibers do not.

How does the induction of reporter genes by c-ski cotransfection in muscle cells relate to its action in vivo? The molecular mechanism by which Ski induces fiber-specific hypertrophy remains to be determined and it is perplexing that in adult skeletal muscle of c-ski transgenic mice, no increase was seen for several endogenous muscle-specific transcripts including myogenic factors (P. Sutrave and S.H. Hughes, unpublished observations) and MLC (Fig. 7). A possible clue may lie in the induction pattern...
of the MLC and MCK reporters by c-ski cotransfection, which is greater in myoblasts than in myotubes. Endogenous myogenic factors in C2C12 myotubes, which alone are capable of activating these reporters during myogenic differentiation, may obscure the activation by exogenous Ski in myotubes. However, ski-mediated activation is not additive with that of endogenous myogenic factors, suggesting a precocious induction of the myogenic pathway in myoblasts. Based on these results in tissue culture, it is possible that Ski induces precocious differentiation and fusion of satellite cells destined for fast-fiber formation during postnatal development, resulting in an initial net increase in contractile protein production in the resulting myotubes, but that unlike the MLC1CAT transgene, endogenous muscle-specific transcripts are not permanently induced in the adult. This possibility is supported by preliminary observations that MLC1CAT induction by c-ski in bigenic 19 day old muscles is several-fold higher than in the same muscles of the adult (J. Engert, unpublished results).

In summary, we have identified muscle-specific regulatory elements that respond to the Ski protein, which is consistent with its ability to induce myogenic differentiation in vitro (20) and reflects the fiber-specific restriction of Ski-induced hypertrophy in vivo (21,24). The MLC and MCK regulatory sequences comprise the first identified genetic targets for the Ski protein, which will allow a molecular dissection of the mechanisms by which this proto-oncogene selectively activates muscle gene expression programs in vivo.

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