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-*ski* 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 IIa, 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 MLC1CAT transgene predominates in fast Type IIb fibers (10). Thus, the MLC regulatory sequences contained in the MLC1CAT 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

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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 (1×10⁵ cells for myoblasts and 4×10⁵ cells for myotubes). NIH3T3 mouse fibroblast cells were propagated in DMEM with 10% FCS and seeded for transfection at 1×10⁶ 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 ¹⁴C-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 5×10⁵ c.p.m. of probe. The hybridizations were incubated overnight at 50°C in 80% formamide and 1× buffer (5× hybridization buffer is 0.2 M PIPES, pH 6.4, 2 M 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. Sutcliffe 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 FBΔ29, 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 β-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 (6) 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 *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

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).

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 3. The MCK promoter/enhancer region is activated by *c-ski* in C₂C₁₂ cells. An MCK reporter (pMCKCAT4800) was transfected with a *c-ski* expression vector, FB29 (black bars) or with pMEX expression vector (shaded bars). Uninduced myoblasts were set to one. Value for each bar is the average of at least three experiments (with standard error).

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

Figure 4. Transactivation of the MLC enhancer by *c-ski* is dependent on the presence of specific E boxes. The pMLCCAT173 reporter with the wildtype MLC enhancer or mutations in each of three E boxes (Am, Bm, Cm) was cotransfected into C₂C₁₂ myoblasts with (black bars) or without (shaded bars) the FB29 *c-ski* expression vector. MLC wildtype enhancer activity without *c-ski* was set at 100. Values are the average of at least three experiments (with standard error).

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, FBA29 (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 (MLC1CAT920). This transgenic line exhibits muscle-specific CAT activity (9) exclusively in fast skeletal muscle fibers (10). To generate MLC1CAT/*c-ski* bigenic mice, a homozygous MLC1CAT920 transgenic mouse was mated with the MSV-*ski* heterozygous transgenic mouse (line 8566) which exhibits fast IIB muscle fiber hypertrophy (21). Approximately half of the resulting progeny carried both transgenes, while the other half carried only the MLC1CAT transgene. The muscles isolated from MLC1CAT/*c-ski* bigenic progeny had an average of three-fold greater CAT activity than the muscles of their MLC1CAT/wt littermates lacking the *c-ski* transgene (Fig. 6). The increase in CAT activity in the bigenic animals demonstrates that the induction of MLC enhancer activity by *c-ski* cotransfection observed in tissue culture can be reproduced *in vivo*. This increase in CAT activity was not detected in the soleus, a predominantly slow muscle. Notably, the soleus is phenotypically normal and does not express Ski at the same

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 transcripts in MSV/*ski* mice RNase protection assays were performed, 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 MLC1CAT reporter in fast muscles of transgenic mice. The transactivation of MLC1CAT 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[N₆₋₇]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 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 littermates (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.

significant changes in MyoD or myogenin expression levels were observed (P. Suttrave 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 IIb 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 IIb 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. Suttrave and S.H. Hughes, unpublished observations) and MLC (Fig. 7). A possible clue may lie in the induction pattern

myogenic conversion and myogenic factor expression by parallel pathways. Moreover, in the MSV-*ski* transgenic mice, no

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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