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Transformation-Defective v-*ski* Induces MyoD and Myogenin Expression but Not Myotube Formation

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The *ski* oncogene induces muscle differentiation in otherwise nonmyogenic quail embryo cells (C. Colmenares and E. Stavnezer, Cell 59:293–303, 1989). Here we report that v-*ski* induces both MyoD and myogenin expression, suggesting that activation of these muscle regulatory genes may be a critical step in *ski*-induced myogenesis. We also describe a transformation-defective mutant of v-*ski* (tdM5i) that fails to induce myotube formation, although it induces the expression of many muscle-specific genes, including the MyoD and myogenin genes. Therefore, if activation of MyoD and myogenin expression is a necessary component of the myogenic program triggered by *ski*, it is clearly insufficient to account for complete muscle differentiation.

Specific pathways of cellular differentiation are thought to be regulated by lineage-specific changes in gene transcription. In the case of muscle differentiation, the MyoD family of transcriptional regulators appears to play a pivotal role in both cellular determination and induction of muscle-specific gene expression (5, 7, 19, 25, 29). Like these genes, the *ski* oncogene encodes a nuclear protein that induces determination to the myogenic lineage (6). Because *ski* stimulates rapid cellular proliferation of myogenic cells, we had suggested that it might act upstream of the MyoD family, if indeed these genes participate in a common pathway of muscle differentiation. We further reasoned that if *ski*'s myogenic activity required only activation of the MyoD family, then nonmyogenic mutants of *ski* should fail to induce expression of these genes. We have recently identified a mutation in *ski* that allowed us to test this model.

To construct *ski* mutations, we inserted oligonucleotides encoding glycines and prolines in order to disrupt predicted alpha-helical regions of v-*ski*. The mutation shown in Fig. 1, an in-frame insertion of four codons at position 145, is predicted to destabilize one of two potential amphipathic helices (AH1 and AH2). To test the effects of this disruption, the mutant and the wild-type v-*ski* were inserted into a replication-competent avian retroviral vector (13, 16) to create viruses called tdM5i and SRA-SKV, respectively. tdM5i failed to induce myotube formation in quail embryo cells (QECs) (Table 1 and Fig. 2a to c). In addition, tdM5i-infected QECs (tdM5i-QECs) did not show the morphological transformation, anchorage-independent growth, or increased growth rate characteristic of *ski*-transformed QECs (SKV-QECs). By these criteria, tdM5i-QECs are indistinguishable from uninfected QECs. Both show a large, flat morphology, a slow growth rate, and a complete inability to clone in soft agar.

To verify that lack of activity in tdM5i does not result from drastically reduced expression or mislocalization of the *ski* protein, we examined the intracellular location of *ski* in tdM5i-QECs by immunostaining with anti-*ski* monoclonal antibodies. The *ski* product was found to be located in the nucleus in both tdM5i-QECs and terminally differentiated SKV-QECs (Fig. 2d to f), as previously shown for *ski*-transformed chicken cells (2). Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed a protein of the predicted size in tdM5i-infected cells (data not shown). The intensity of the signal obtained with tdM5i, in both immunofluorescence and immunoprecipitation, was well within the range observed with fully active v-*ski* viruses (compare panels e and f in Fig. 2). Thus, the mutation in tdM5i appears to have altered the function of the protein rather than its abundance or intracellular location.

The results obtained in the assay for myotube formation (Table 1) indicated that tdM5i was totally defective for myogenesis. However, previous studies have shown that myoblast fusion and expression of muscle-specific genes can be uncoupled by the use of inhibitors (10, 12, 22) or as a result of mutations (1, 23). Therefore, we looked for muscle-specific gene expression in tdM5i-QECs by staining cells with antibodies to muscle-specific proteins. Desmin expression, an early marker of the myogenic lineage (15, 17), was completely absent in QECs but easily detectable in most cells infected by either SRA-SKV or tdM5i (Fig. 2g to i). Myosin expression, a marker of terminal differentiation, was also observed in tdM5i-QECs, but the pattern of staining was

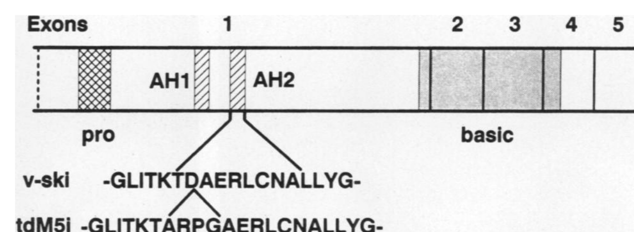

FIG. 1. Map of the tdM5i mutation with respect to potentially functional regions of v-*ski*. After insertion of an oligonucleotide encoding the indicated amino acids, the mutated *ski* was cloned into a nondefective avian retroviral vector (13, 16). Exons refer to the corresponding exons of c-*ski* and are separated by solid lines; dotted lines indicate the amino- and carboxy-terminal ends of the 435-amino-acid v-*ski* sequence (27). AH1 and AH2, Potential amphipathic helices, from residues 116 to 125 (AH1) and 142 to 151 (AH2); pro, proline-rich region; basic, region with a net basic charge.

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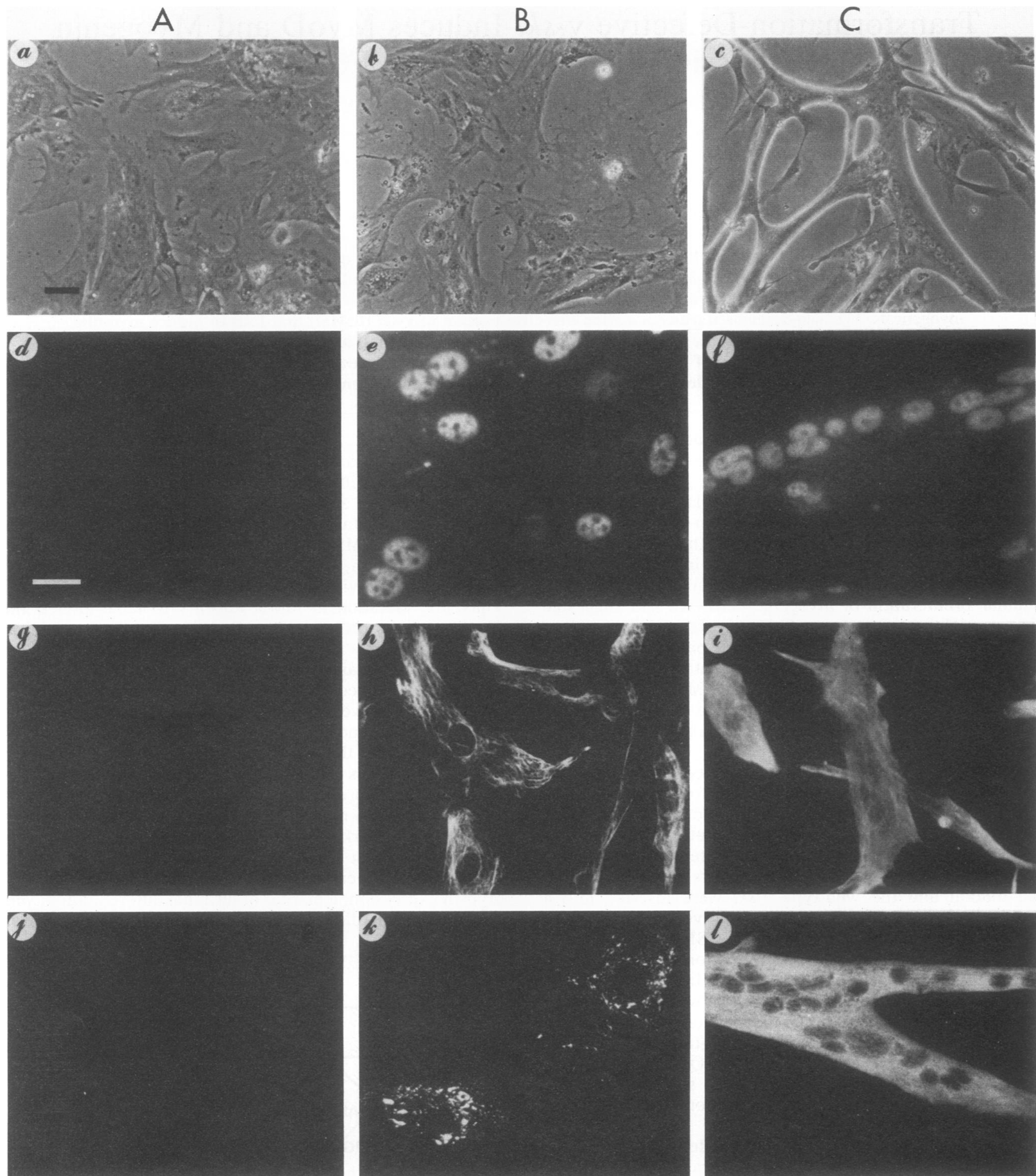

FIG. 2. Expression of v-*ski* and muscle-specific proteins in SKV-QECs and tdM5i-QECs. (A) Uninfected QECs; (B) tdM5i-QECs; (C) SKV-QECs. Cells were transferred from growth medium to differentiation medium (6) and either photographed (a to c) or fixed (d to l) 48 h later. After fixation, cells were immunostained for *ski* (d to f), desmin (g to i), or myosin (j to l). Cells were fixed in 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and then treated with 5 mM glycine for 10 min; incubation with monoclonal antibodies to *ski* (G8), desmin (D33; Dako), and myosin (MF20) was for 1 h and was followed by treatment with fluorescein-labeled goat anti-mouse antibody. Samples were examined and photographed on a Zeiss IM35 microscope. Scale bars: a to c, 40 μm; d to l, 20 μm.

TABLE 1. Growth and differentiation characteristics of tdM5i-QECs[a]

Cells	Doubling time (h)[b]	Cloning in agar (%)	Nuclei in myotubes (%)
tdM5i-QECs	38 ± 5	0	1–2
QECs	46 ± 2	0	0
SKV-QECs	12 ± 1	5	69

[a] Assays for doubling time, soft agar cloning, and myotube formation were performed as described previously (6).

[b] Cells were plated in triplicate; numbers represent averages ± standard errors.

highly abnormal; the protein appeared to concentrate in patches, and only a fraction of the cells were positive (Fig. 2j to l). Therefore, although tdM5i-QECs do express muscle-specific genes, both cell fusion and the organization of myosin into contractile filaments are defective under the influence of the altered *ski* gene.

To determine the effect of *ski* on MyoD and myogenin expression, we performed Northern analysis of RNA from uninfected and infected QECs. We found no trace of either MyoD or myogenin mRNA in uninfected QECs (Fig. 3) even after extensive overexposure (data not shown). These results, as well as the lack of desmin expression (Fig. 2g to i), confirm our previous findings which indicated that uninfected QECs are not myogenic cells (6). However, expression of v-*ski* in QECs led to the production of both MyoD and myogenin mRNAs (Fig. 3a and c). These results indicate that *ski* is acting upstream of MyoD and myogenin in the

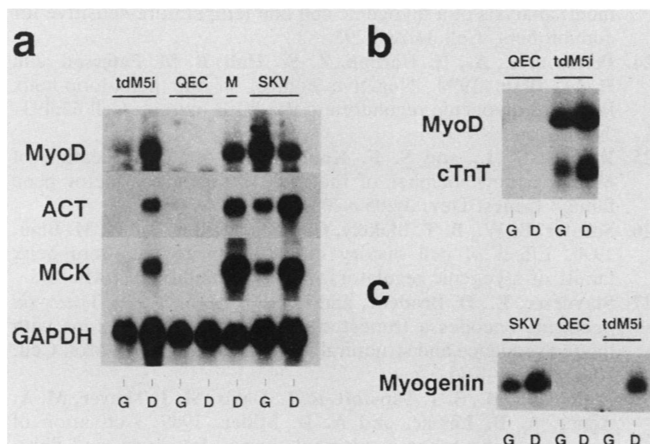

FIG. 3. Expression of MyoD, myogenin, and other muscle-specific mRNAs in SKV-QECs and tdM5i-QECs. Cells were harvested either from growth medium (G) or after 48 h in differentiation medium (D), and RNA was prepared as described previously (6). For each sample, 10 μg of total RNA was glyoxalated and run on a 1% agarose gel; Northern analysis was performed as previously described (6). (a) The same blot was hybridized sequentially with probes to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1.4 kb), quail MyoD (1.8 kb) (9), alpha-skeletal actin (ACT; 1.6 kb), and MCK (1.5 kb). Lane M, Uninfected quail myoblasts, isolated from embryonic skeletal muscle and allowed to differentiate in vitro. (b) An independent isolate of the same mutation, verified by DNA sequence analysis to be identical to tdM5i, was inserted into the same retrovirus; QECs infected by this virus were processed, and RNA was prepared as described above. The blot was probed simultaneously for MyoD and cardiac troponin T (cTnT; 1.3 kb). (c) The blot shown in panel b was stripped and reprobed with a cDNA of chicken myogenin (1.3 kb).

determination of these cells to the muscle lineage and suggest that *ski*'s ability to induce muscle differentiation is at least partly mediated through its induction of these genes. The high level of MyoD expression by SKV-QECs in growth medium (Fig. 3a) is consistent with results obtained with other myoblasts (5, 8, 24). Expression of the other muscle-specific mRNAs, including myogenin mRNA, in growth medium reflects the premature onset of terminal differentiation in a fraction of these cells as a result of local growth factor depletion; however, as previously shown (6), differentiation medium causes a strong induction of all of these mRNAs.

Surprisingly, the tdM5i gene product also induced MyoD and myogenin mRNA expression, as well as expression of many other muscle-specific mRNAs, such as those for cardiac troponin T, alpha-actin, and the muscle form of creatine kinase (MCK) (Fig. 3). The expression of MCK mRNA in both SKV-QECs and tdM5i-QECs (Fig. 3a) suggests that functional MyoD and/or myogenin proteins are present in these cells, since both MyoD and myogenin have been shown to be positive transcriptional regulators of MCK (11, 18). The phenotype induced by the tdM5i mutant shows that *ski*'s ability to activate transcription of muscle-specific genes, including the MyoD and myogenin genes, can be unlinked from its ability to transform cells and promote myotube formation. Furthermore, this functional segregation indicates that induction of the complete myogenic program in QECs requires activities of *ski* that are defective in tdM5i and cannot be supplied by the two muscle regulatory genes.

Although members of the MyoD family convert a variety of cell types to the myogenic lineage (28), their activity is not always sufficient for myogenic conversion in cells of nonmesodermal origin (26). Our results show that even in QECs, which have demonstrated myogenic potential, transcriptional activation of both MyoD and myogenin is not sufficient to lead to fusion and a complete muscle phenotype. Instead, myotube formation requires a fully functional *ski* protein. Perhaps two *ski* domains are necessary for full function; one might activate transcription of the MyoD, myogenin, and other muscle-specific genes, while the other, which is disrupted in tdM5i, is required for myoblast fusion. The second domain may also function in causing enhanced growth and transformation. In an alternative model, tdM5i might function as a dominant negative mutation, effectively counteracting the activity of MyoD or myogenin. Preliminary experiments suggest that this is not likely to be the case, since tdM5i does not seem to inhibit differentiation of normal quail myoblasts (unpublished results). The phenotype of tdM5i-QECs resembles one described for a temperature-sensitive mutant of L6E9 myoblasts (ts3b-2), which at the nonpermissive temperature undergo activation of muscle-specific genes but not fusion or commitment (1, 23). It is tempting to speculate that the lesion in ts3b-2 cells could have occurred in *ski* itself or in a member of the fusion pathway activated by *ski*.

The altered activity of tdM5i suggests that the predicted amphipathic helix which would be disrupted in this mutant may be important for *ski*'s function. This helix is one of a pair of amphipathic helices (AH1 and AH2 in Fig. 1) that were identified by structural analogy, and limited sequence relatedness, with helix-loop-helix (HLH) motifs of known regulatory proteins (3, 20, 21); however, *ski* has no adjacent basic region. The existence of a potential HLH structure within a domain of *ski* which can so drastically affect its myogenic potential suggests the intriguing possibility of

protein-protein interactions between the *ski* protein and other members of the HLH family, such as Id (3). Further analysis of tdM5i and other site-directed mutations in this region should help to establish whether this putative HLH constitutes a functional domain. Additional studies may reveal genes induced by *ski* but not by tdM5i, thereby allowing dissection of the separate pathways activated during muscle differentiation.

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