Activation of the c-ski Oncogene by Overexpression

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The v-ski oncogene is a truncated version of the cellular proto-oncogene, c-ski, and lacks sequences coding for both the N- and C-terminal ends of the c-ski protein. In the region of overlap, v-ski and c-ski differ by only one amino acid. To determine whether these differences underlie v-ski's oncogenic activation, we have cloned cDNAs for several alternatively spliced c-ski mRNAs and introduced these cDNAs into replication-competent retroviral vectors. The biological activities of these c-ski constructs have been compared with those of v-ski. We found that all c-ski gene products, when expressed at high levels from the promoter in the retroviral long terminal repeat, can induce morphological transformation, anchorage independence, and muscle differentiation in avian cells. Cells that are susceptible to ski-induced transformation and myogenesis normally express endogenous c-ski at low levels. Thus, it appears that overexpression of ski is sufficient for oncogenic and myogenic activation.

A multitude of genetic changes have been shown to convert proto-oncogenes into fully transforming oncogenes. Transduction of proto-oncogenes by retroviruses and the subsequent expression of the resulting genes inevitably result in overexpression because transcription from the long terminal repeat is extremely efficient. However, full transforming activity often requires additional changes in the transduced gene. Point mutations (1, 25, 35), deletions (1, 18, 26, 28, 29), fusion to viral (11, 14, 16, 30) or other cellular proteins (2, 37), or multiple combinations of these changes have all been shown to contribute to the acquisition of oncogenic activity. In the specific case of nuclear oncogenes, activation often requires both overexpression and deletion of either coding or noncoding regulatory sequences (3, 5, 6, 10, 22, 23, 36).

V-ski encodes a nuclear protein that induces morphological transformation, anchorage independence, and terminal muscle differentiation in avian cells (8, 21, 32). Cloning of c-ski cDNAs has identified three different, alternatively spliced c-ski mRNAs (34) and has confirmed that v-ski is a truncated form of the c-ski proto-oncogene (31). All three of the predicted c-ski proteins contain both N- and C-terminal domains that are not encoded by the viral genome.

To investigate whether deletions of coding sequences from the cellular genome are responsible for either the transforming or the myogenic activities of v-ski, we have constructed nondefective retroviral vectors expressing the complete coding regions of the known cDNAs (33). We report here that all of these viruses possess v-ski's biological activities, suggesting that truncation of ski's coding sequences is not required for either oncogenic transformation or myogenesis.

MATERIALS AND METHODS

Viral constructs. We have previously described the construction of replication-competent avian retroviruses RCAS-27, -Δ29, and -29 (33). We have now constructed the correspoding retroviruses that lack ski's exon 2, a task simplified by the availability of earlier constructs in the ClaI2Nco adaptor plasmids (15). Briefly, a 531-bp KpnI-HindIII fragment from the ski cDNA FB2/29 (previously called FB28), which lacks exon 2 sequences (34), was used to replace the 632-bp KpnI-HindIII regions containing exon 2 sequences of FB27, FBΔ29, or FB29 in the ClaI2Nco vectors. These constructs, now lacking the exon 2 coding sequences, were excised as ClalI fragments and cloned into a replication-competent avian retrovirus named RCAS (15, 34). The same fragments were also inserted into a version of RCAS containing the polymerase gene from the Bryan high-titer virus (RCASBP [20]). The viruses encoding these constructs are called RCAS (or RCASBP/-2/27, -2Δ29, or -2/29. The structures of all the inserted cDNAs are diagrammed in Fig. 1. V-ski, supplied with an artificial ATG initiator codon, was also inserted into RCASBP and served as a positive control. For this purpose, an Ncol linker was ligated to a blunt-ended Xhol site 14 bases upstream from the gag-v-ski junction (31) to create the sequence [CCC ATG GGT CA GAA GAG GCG] with the Ncol linker in brackets. Thus, the amino acid sequence upstream of v-ski is changed from AAREQG to MGREEQ. The resulting gene was cloned into the ClaI2Nco adaptor plasmid and transferred into the virus as a ClaI fragment, exactly like the c-ski cDNAs.

Cell culture and transfections. Both chicken embryo cells (CECs) and quail embryo cells (QECs) were cultured as previously described (8). Plasmids containing the constructed proviruses were purified on CsCl gradients and introduced into CECs by using either calcium phosphate precipitation (7) or lipofection (12, 13), with identical results. CECs were passed three to four times following transfection to allow the spread of the virus and then scored for morphological transformation. Indirect immunofluorescence assays with anti-ski monoclonal antibodies were performed to demonstrate that infection was complete. Supernatants from these fully infected CECs were filtered and used to infect QECs and fresh CECs, and these secondary infected populations were allowed to become completely infected before
assaying for differentiation or anchorage-independent growth. Differentiation of QECs and soft-agar cloning of both transfected and infected CECs were assayed as previously described (8). Cells were seeded at two different densities (10^4 and 5 × 10^4 cells per 60-mm dish) for each soft-agar cloning assay.

**Protein analysis.** Cell labeling with [35S]methionine, immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western transfers, and immunofluorescence assays were all performed as described previously (33). All analyses were performed on fully infected populations; for those viruses which proved to be transforming, these populations were, by this point, also fully transformed morphologically.

**RESULTS**

c-ski transcripts have been shown to undergo alternative splicing (34). cDNAs corresponding to the complete coding region (FB29) or lacking exon 2 (FB2/29, formerly called FB28) or exon 6 (FB27) have been identified. To compare the transforming and myogenic potentials of c-ski and v-ski, we have constructed replication-competent retroviral vectors encoding each of the three known alternatively spliced c-ski mRNA species, as well as several cDNA variants constructed in vitro. We have also inserted into the same vector v-ski, devoid of different sequences and provided with an artificial initiation codon (see Materials and Methods). All the inserts tested are diagrammed in Fig. 1. The construct lacking both exons 2 and 6 (FB2/27) was constructed in vitro and has not been detected in chicken mRNA. In addition, we tested a cDNA derived from FB29 which was found to contain a frameshift mutation at codon 436 of c-ski and therefore terminated at codon 448 (FB2/29). This construct was included because it encodes the 19 N-terminal amino acids that are deleted in v-ski, yet it otherwise encodes a protein very similar to v-ski (which ends at codon 458).

The first set of constructs were assembled in the RCAS nondefective retroviral vector (15). However, subsequent results indicated that vectors expressing the polymerase gene from the Bryan high-titer virus (RCASBP) yielded fully infected, fully transformed cells more rapidly than RCAS. Therefore, the final set of constructs (those lacking exon 2) were inserted only into RCASBP. To distinguish the viruses from the cDNAs, we have included the name of the vector (RCAS or RCASBP) before the number identifying the cDNA.

**All viral constructs encode the expected proteins.** CECs were transfected with each of the constructs described above. Each population was then serially passaged to allow the virus to spread. Within two passages (or fewer, in the case of RCASBP-based viruses), morphological transformation was evident in all cultures transfected with ski-encoding viruses; within three or four passages, all cell populations appeared fully transformed. These cells were used to analyze the ski proteins encoded by the various constructs, either by immunoprecipitation following [35S]methionyl labeling of cells or by Western blotting (immunoblotting) (Fig. 2). As previously shown (33), the major protein species encoded by the full-length cDNA in RCAS-29 and RCASBP-29 (Fig. 2A, lanes 2 and 3, respectively) have apparent molecular masses of approximately 90 kDa, al-
though the predicted molecular mass is 84 kDa. Western blotting also detected a major species of 90 kDa in cells infected by RCASBP-29 (Fig. 2B, lane 3). This size discrepancy and the presence of additional protein species in the 85- to 90-kDa range in the immunoprecipitates possibly reflect different levels of phosphorylation (33). The fastest-migrating form (Fig. 2A, lane 3) corresponds closely to the predicted molecular mass of the unmodified species encoded by these viruses. Interestingly, the abundance of the ski proteins produced by RCASBP-29 is about one-fourth of that produced by RCASBP-29 (compare lanes 2 and 3 in Fig. 2A).

As previously shown (33), the RCASBP-27 virus encodes a protein with an apparent molecular mass of about 60 kDa (Fig. 2A, lane 4, and 2B, lane 2). Bands corresponding to the predicted size of 56 kDa are also present in both the immunoprecipitates and the Western blot.

Western blotting was used to compare the protein products encoded by viruses with or without exon 2. The data in Fig. 2B show that the c-ski products of RCASBP-2/29, -2/29, and -2/27 (lanes 4, 5, and 6, respectively; see arrowheads) are slightly smaller than those from the corresponding viruses that contain exon 2; the difference in mobility (about 4 kDa) is as expected for proteins that differ by 37 amino acids (34).

Figure 2A shows that some of the proteins immunoprecipitated with our monoclonal antibodies from uninfected QECs or CECs are identical in size to those expressed by the virally encoded c-ski mRNAs and may represent the major c-ski products in these cells. QECs (Fig. 2A, lanes 1 and 1A) express mostly a 60-kDa form, although a small amount of a 90-kDa species is visible in lane 1A. CECs express a protein of approximately 50 kDa, as well as minor species in the 90-kDa range (Fig. 2A, lanes 5, and Fig. 5A).

c-ski can transform CECs in culture. The transforming potential of the viruses shown in Fig. 3 was measured in both transfected CECs and in fresh CECs infected with viruses produced by these transfected cultures. In both cases, cells were passaged to allow the virus to spread. After three or four passages, indirect immunofluorescence with anti-ski antibodies showed high levels of ski protein in the nuclei of all cells, except for control cells infected with the vector alone (RCASBP), which were indistinguishable from uninfected cells (data not shown). The transforming activity of the viruses was measured by their ability to induce both clonal growth in soft agar and the epithelioid morphological appearance characteristic of v-ski-transformed cells.

As shown in Fig. 3, all of the viruses expressing c-ski cDNAs were capable of inducing soft-agar growth, at levels comparable to those observed for v-ski (8, 21, 32). The presence of the 19 N-terminal amino acids deleted in v-ski had no effect on ski's transforming activity. This is shown most clearly by RCASBP-29, which encodes the c-ski N terminus but expresses a truncated protein otherwise very similar to v-ski (31) and which is comparable to v-ski in terms of both agar cloning efficiency (Fig. 3) and transformed morphology (Fig. 4C and E). Viruses encoding proteins which terminate at the end of exon 5 (RCASBP-27 and RCASBP-2/27) were also indistinguishable from v-ski (data not shown). However, viruses which included both exons 6 and 7 and which expressed the highest levels of the longest c-ski proteins (RCASBP-29 and RCASBP-2/29) appeared to have more potent transforming activity than the other viruses, including those expressing v-ski. Both induced the appearance of highly refractile "foci" in monolayers (Fig. 4F), and both produced more large, rapidly growing soft-agar colonies than the other viruses (data not shown), although the percent cloning efficiency was approximately equal for all ski-containing viruses (Fig. 3). Cells infected by the vector alone, or by the vector with a c-ski insert in the wrong orientation, were indistinguishable from uninfected cells (Fig. 4A and B).
Morphological transformation was evident in the cultures by two passages after transfection and was efficiently induced by all of the c-ski viruses. In addition, the change in morphology appeared more rapidly and was more dramatic in viruses derived from the RCASBP vector, which encodes the polymerase gene of the Bryan high-titer strain of Rous sarcoma virus (20), than in viruses derived from RCAS (compare Fig. 4D and E). This difference did not appear to result from more efficient virus spread, because it was evident even after multiple cell passages and in cell populations which showed no differences in the efficiency of soft-agar cloning. RCASBP vectors that carry other inserts (e.g., chloramphenicol acetyltransferase) express the inserts more efficiently than do the parental RCAS vectors. This increase is not due to an increased number of proviruses per cell, but it does involve an absolute increase in the level of expression (14a). We observe a similar difference in the levels of ski expression (Fig. 2A, lanes 2 and 3), as discussed above.

**c-ski induces muscle differentiation.** Virus produced by the infected CECs was used to infect early-passage QECs. As shown in Fig. 3 and in the examples in Fig. 5, all versions of c-ski induced muscle differentiation in QECs. We have not precisely measured the effects of c-ski on the growth rate of QECs, but routine culture of these cells indicates that the c-ski viruses, like the v-ski viruses (8), enhance the rate of proliferation of QECs.

**DISCUSSION**

The viral oncogene v-ski is a truncated version of one of the c-ski mRNAs. As a retroviral gene, v-ski is expressed at levels more than 100 times higher than the proto-oncogene (21). The goal of these studies was to determine how the truncation of c-ski and its increased levels of expression contribute to its conversion into an oncogene. We have constructed retroviruses which express each of the differentially spliced c-ski cDNAs identified so far (34), as well as an in vitro recombinant lacking both exons 2 and 6. Correct expression of the various c-ski proteins has been confirmed by both immunoprecipitation and Western analysis (33) (Fig. 2). Immunostaining of the infected cells has shown that all of these forms of ski have the expected nuclear localization (Fig. 2) (data not shown).

Expression of c-ski mRNAs has been demonstrated in CECs, albeit at low levels (21), and has been observed in QECs as well (7a). We now show that uninfected CECs and QECs express proteins that can be precipitated by anti-ski monoclonal antibodies and have apparent molecular masses identical to those of proteins encoded by the virally expressed cDNAs (Fig. 2). A 90-kDa form is the same as that expressed by the full-length mRNA encoding exons 1 to 7 (FB29), while a 60-kDa species may be the product of the message lacking exon 6 (FB27). Both of these mRNAs have been identified by cDNA cloning, and the detection of proteins which may correspond to their encoded products is...
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not surprising. On the other hand, the major protein immunoprecipitated from CECs has an apparent molecular mass of 50 kDa; such a protein could be encoded by a doubly spliced mRNA lacking both exons 2 and 6 (FB2/7; Fig. 2B, lane 6). However, this mRNA has not been detected by either cDNA cloning or S1 nuclease protection analyses (34), suggesting that it is relatively rare. It is therefore puzzling to find it expressed in CECs at higher levels than other ski products. The low levels of these putative c-ski proteins agree with the low levels of c-ski mRNAs detected in

FIG. 4. Morphology of cells transformed by the c-ski viruses. CECs were infected with the indicated viruses and passaged three or four times to allow virus spread. All cells were allowed to reach confluence and photographed 1 or 2 days later. (A) uninfected CECs; (B) CECs infected by RCASBP; (C) CECs infected by RCASBP-v-ski; (D) CECs infected by RCAS-D29; (E) CECs infected by RCASBP-D29; (F) CECs infected by RCASBP-29. Note that the epithelioid pattern typical of v-ski-transformed cells (C) is evident in CECs infected with vectors encoding the Bryan polymerase gene (E) but is only partially visible in cells infected by the virus carrying the same c-ski gene but lacking the Bryan polymerase (D). Immunofluorescence indicated that all cells in both populations expressed the nuclear ski protein at high levels (data not shown). Cells infected by the virus encoding the FB29 (F) and FB2/29 (data not shown) cDNAs grow to higher densities and form foci in a monolayer.

FIG. 5. c-ski viruses induce muscle differentiation. Uninfected QECs (A) or QECs infected by RCASBP-v-ski (B) or RCASBP-29 (C) were photographed after 2 days in differentiation medium. All other c-ski viruses were equally myogenic.
uninfected cells (less than 1% of the viral RNA levels [21]) and with the observation that c-ski proteins in uninfected cells cannot be detected by immunofluorescence (9, 33). While the sizes and low abundance of these proteins suggest their authenticity, further analysis by partial proteolysis will be required to show whether they represent real ski products.

Our results show that all viruses expressing the different forms of c-ski can transform CECS and induce muscle differentiation in QECs. c-ski viruses expressing either the N-terminal 19 amino acids missing from v-ski or the variable C-terminal exons, or both, can cause morphological transformation, anchorage-independent growth, and myogenesis. Therefore, the loss of neither N-terminal nor C-terminal sequences (which occurred during transduction) can explain the oncogenic activation of c-ski. Furthermore, cDNAs lacking exon 2 possess all of v-ski's biological activities, indicating that this 37-amino-acid sequence, which is encoded by v-ski and is part of a highly basic domain (31), is not required for any of c-ski's known functions. The results presented here rule out protein truncation and alternative splicing as mechanisms of v-ski's activation and suggest that both its transforming potential and its myogenic activity result from overexpression of ski. Inappropriate or ectopic expression, although not conclusively ruled out, is an unlikely possibility, since ski mRNAs can be detected at low levels in virtually all cell types (21, 24, 26, 34).

Activation of other nuclear proto-oncogenes, such as myc (3, 6), fos (23), jun (5), and myb (26), also appears to involve deregulated expression due to viral transduction or chromosomal rearrangements. However, in many cases truncation plays an important role as well. In the case of v-fos, for example, overexpression is obtained by both transcriptional and posttranscriptional mechanisms (4, 17, 19, 22). Activation of v-fos involves the truncation of 3' noncoding sequences which contain repeated ATTAA motifs that have been implicated in rapid mRNA turnover (19, 22, 27). The 3' noncoding region of c-ski cDNAs also contains several ATTAA motifs. The constructs described here lack this region, as well as upstream noncoding sequences, so it is possible that other regulatory alterations contribute to the activation of c-ski.

ski proteins that have the normal c-ski N-terminus but lack the C-terminal region encoded by exons 6 and 7 (proteins expressed by all viruses containing FB27 or FBA29 cDNA) are comparable to v-ski in their transforming and myogenic potential. However, the largest c-ski protein (90 KDa) appears to be a more potent transforming protein, inducing larger colonies in soft agar and the appearance of refractile foci in culture. This phenotype, commonly seen in cells transformed by many oncogenic viruses, has not been observed previously in cells transformed by v-ski. The 90-KDa protein is more highly phosphorylated than the shorter, 60-KDa product, while neither the truncated c-ski nor v-ski shows any detectable modification (33, 5a). Presumably, the major site(s) of phosphorylation is within exon 6 or 7. Experiments that demonstrate the DNA-binding activity of the human c-ski product show that deletion of the C-terminal region, which is highly conserved between the chicken and human genes, reduces hu-c-ski's ability to bind DNA (24). Taken together, these data suggest the possibility that this C-terminal domain represents a regulatory region which modifies both ski's potential DNA binding and its transforming activity.

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