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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 gene.

To investigate whether deletions of coding sequences from the cellular gene 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 corresponding retroviruses that lack *ski*'s exon 2, a task simplified by the availability of earlier constructs in the Cla12Nco adaptor plasmids (15). Briefly, a 531-bp *Kpn*I-*Hin*dIII fragment from the *ski* cDNA FB2/29 (previously called FB28), which lacks exon 2 sequences (34), was used to replace the 632-bp *Kpn*I-*Hin*dIII regions containing exon 2 sequences of FB27, FBΔ29, or FB29 in the Cla12Nco vectors. These constructs, now lacking the exon 2 coding sequences, were excised as *Cla*I 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, -2/Δ29, 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 *Nco*I linker was ligated to a blunt-ended *Xho*I site 14 bases upstream from the *gag*-v-*ski* junction (31) to create the sequence [CCC ATG GG]T CGA GAG GAA CAG, with the *Nco*I linker in brackets. Thus, the amino acid sequence upstream of v-*ski* is changed from AAREEQ (in *gag-ski*) to MGREEQ. The resulting gene was cloned into the Cla12Nco adaptor plasmid and transferred into the virus as a *Cla*I 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

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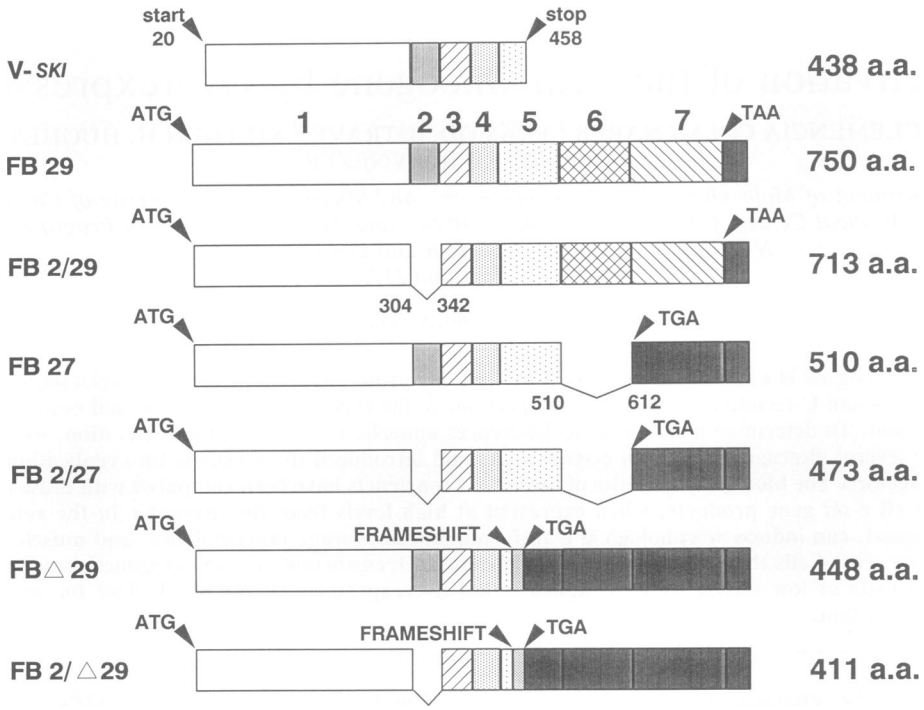

FIG. 1. Structure of v-*ski* and c-*ski* cDNAs. FB29, FB2/29, and FB27 cDNAs have been cloned from a chick embryo body wall library (34); the other cDNAs are in vitro constructs. Boxes with different shading represent the known *ski* coding exons and are numbered above the full-length cDNA, FB29. Both initiation and termination codons for c-*ski*s are indicated; all ATGs are the authentic c-*ski* initiators. v-*ski* begins 20 codons downstream of the c-*ski* initiator and required an artificial ATG, as described in Materials and Methods; this is therefore labeled "start." Numbers given at splice sites, or at v-*ski* start and stop codons, refer to the codon numbers in the full-length c-*ski* cDNA (34); numbers in the right-hand column indicate the total number of amino acids predicted for the product of each coding region.

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 [^{35}S]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* mRNAs, as well as several cDNA variants constructed in vitro. We have also inserted into the same vector v-*ski*, devoid of other viral 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 (FBΔ29). 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 [^{35}S]methionine 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-

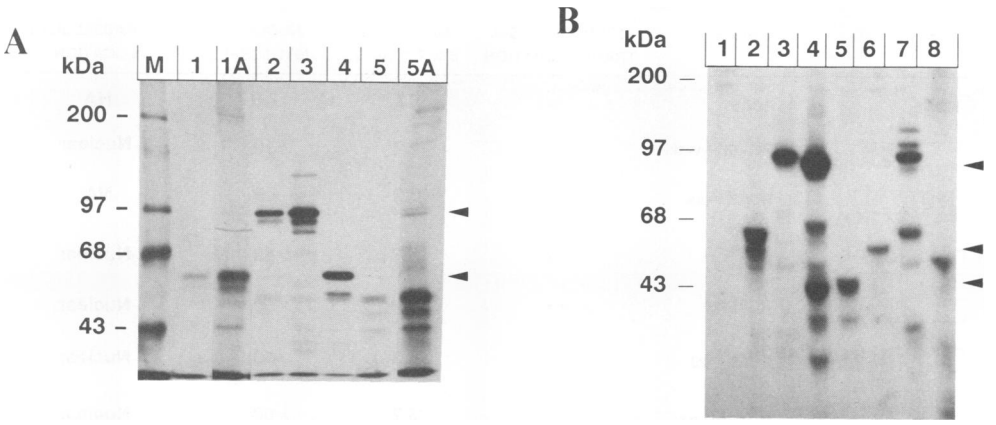

FIG. 2. Analysis of the proteins encoded by c-*ski* and the c-*ski* viruses. (A) Uninfected CECs, or virus-infected cells, were labeled with [^{35}S]methionine. Cells were lysed, and proteins were immunoprecipitated with anti-*ski* monoclonal antibodies. For uninfected CECs and QECs, an amount of lysate equal to 15×10^6 cpm was used for immunoprecipitation, while for infected cells the amount of lysate was only 2×10^6 cpm. Lanes: M, markers; 1, uninfected QECs; 1A, sixfold longer exposure of lane 1; 2, CECs infected by RCAS-29; 3, CECs infected by RCASBP-29; 4, CECs infected by RCASBP-27; 5, uninfected CECs; 5A, sixfold longer exposure of lane 5. Arrowheads on the right indicate the major virally encoded c-*ski* proteins precipitated from cells infected by RCASBP-29 (90 kDa) or RCASBP-27 (60 kDa). (B) Lysates of uninfected CECs, or of cells infected with the indicated viruses, were run on an SDS-polyacrylamide gel. After transfer to nitrocellulose, the proteins were detected by the binding of rabbit antiserum to *ski* (33) followed by ^{125}I-protein A. The proteins derived from viruses which lack exon 2 are indicated by arrowheads and have apparent molecular masses of 45, 56, and 87 kDa, each slightly smaller than the corresponding ones encoding exon 2, as expected. Lanes: 1, uninfected CECs; 2 to 8, cells infected by the viruses RCASBP-27 (lane 2), RCASBP-29 (lane 3), RCASBP-2/29 (lane 4), RCASBP-2/Δ29 (lane 5), RCASBP-2/27 (lane 6), RCASBP-27 and -29 (lane 7), and RCASBP-Δ29 (lane 8).

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 RCAS-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/Δ29, 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-Δ29, 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).

SKI INSERT	VIRUS	MORPHOLOGICAL TRANSFORMATION	CLONING IN SOFT AGAR (%)	NUCLEI IN MYOTUBES (%)	INTRACELLULAR LOCATION
NONE	RCAS	-	0.5	<5	NA
	RCASBP-v-skI	++	4.2	>50	Nuclear
	RCAS-v-lks	-	0.3	<5	NA
	RCAS-27	+	3.7	>50	Nuclear
	RCAS-29	++	2.8	>50	Nuclear
	RCAS-△29	+	5.3	>50	Nuclear
	RCASBP-27	++	5.2	>50	Nuclear
	RCASBP-29	+++	3.8	>50	Nuclear
	RCASBP-△29	++	3.3	>50	Nuclear
	RCASBP-2/27	++	4.5	>50	Nuclear
	RCASBP-2/29	+++	7.1	>50	Nuclear
	RCASBP-2/△29	++	6.4	>50	Nuclear

FIG. 3. Structure of the c-*ski* cDNAs tested and biological activity of the c-*ski* viruses. v-*ski* or the c-*ski* cDNAs diagrammed were inserted into either the RCAS or RCASBP retroviral vectors (15), as described in Materials and Methods. Details of the pictured coding regions are provided in Fig. 1. CECs or QECs infected with the viruses produced from these constructed proviruses were assayed for transformation and myogenesis, as described in Materials and Methods. Morphological transformation was scored as negative (−), intermediate (+), transformed (++), or highly transformed and focus forming (+++). Examples of these phenotypes are shown in Fig. 4A to F. The number of clones in soft agar was obtained by counting all groups of more than approximately 16 cells; percentages represent the average of two or three experiments. After two days in differentiation medium, QECs were fixed, stained, and counted as described previously (8). Examples of myogenic and undifferentiated cells are shown in Fig. 4. NA, not applicable.

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 (33) (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

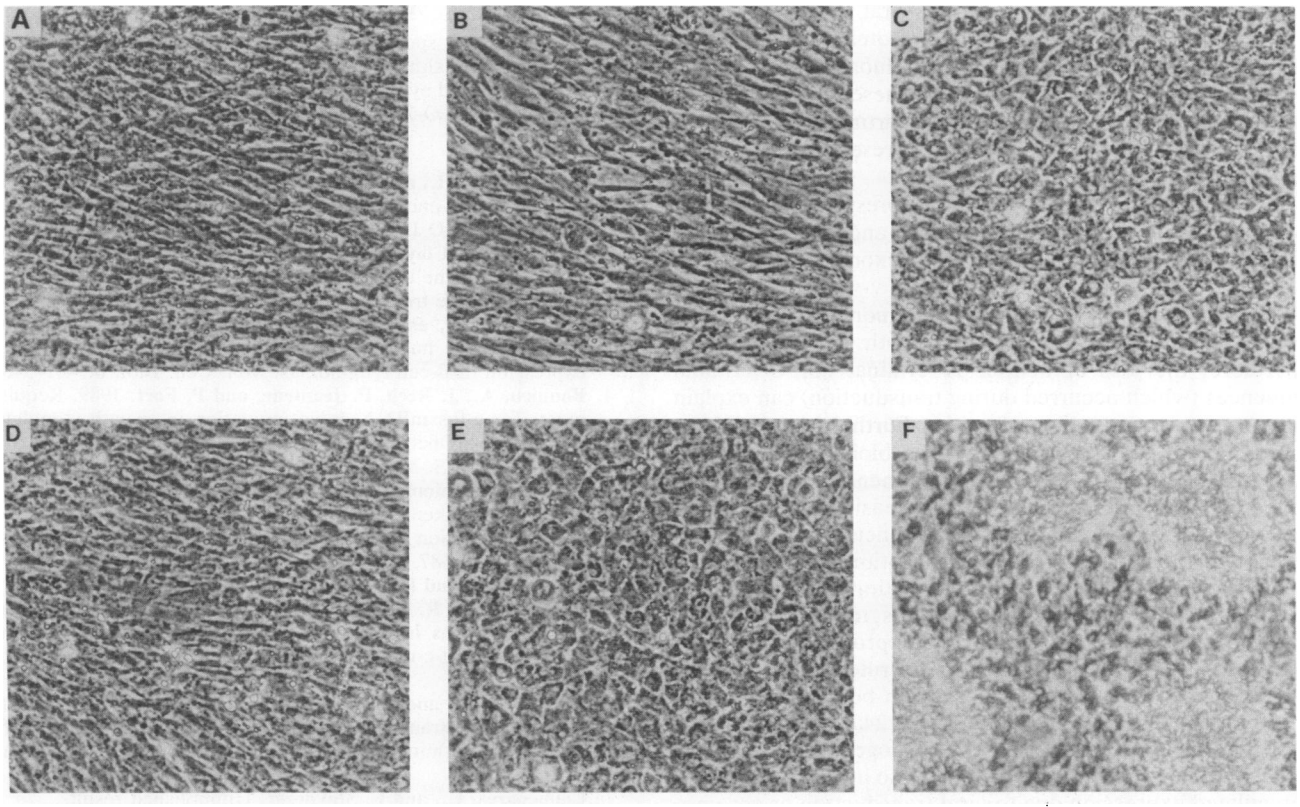

FIG. 4. Morphology of cells transformed by the c-*ski* viruses. CECs were infected with the indicated viruses and passaged three or four times to allow virus spread. All cells were allowed to reach confluence and photographed 1 or 2 days later. (A) uninfected CECs; (B) CECs infected by RCASBP; (C) CECs infected by RCASBP-v-*ski*; (D) CECs infected by RCAS-Δ29; (E) CECs infected by RCASBP-Δ29; (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.

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 (FB2/27; 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

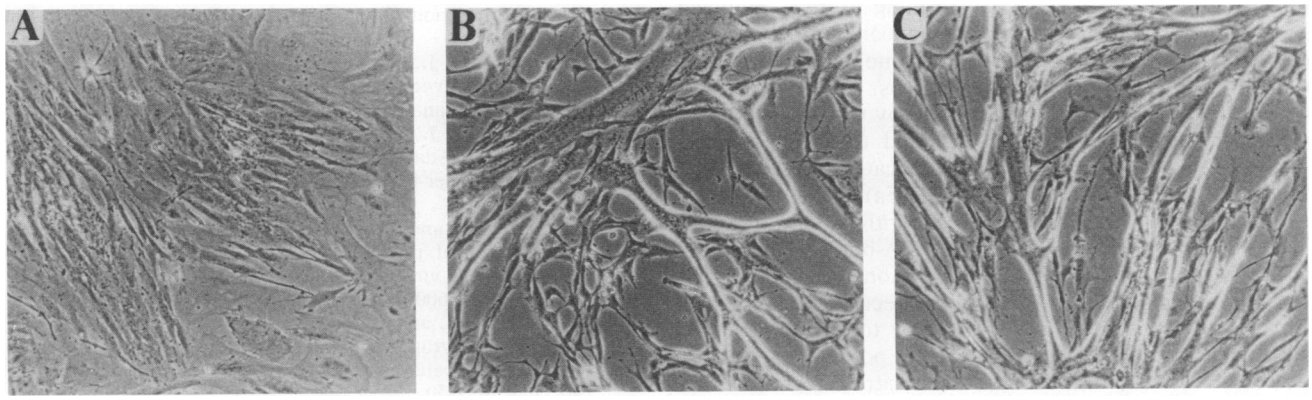

FIG. 5. c-*ski* viruses induce muscle differentiation. Uninfected QECs (A) or QECs infected by RCASBP-v-*ski* (B) or RCASBP-29 (C) were photographed after 2 days in differentiation medium. All other c-*ski* viruses were equally myogenic.

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 *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, 24a, 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 ATTTA motifs that have been implicated in rapid mRNA turnover (19, 22, 27). The 3′ noncoding region of c-*ski* cDNAs also contains several ATTTA 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 FBΔ29 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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