DNA binding and transcriptional activation by the 
Ski oncoprotein mediated by interaction with NFI

Pheruza Tarapore, Craig Richmond*, Guoxing Zheng¹, Steven B. Cohen¹, 
Bruce Kelder², John Kopchick², Ulrich Kruse³,§, Albrecht E. Sippel³, 
Clemencia Colmenares⁴ and Ed Stavnezer¹.*

Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of 
Medicine, Cincinnati, OH 45267, USA, ¹Department of Biochemistry, Case Western Reserve University, 
Cleveland, OH 44106, USA, ²Edison Biotechnology Institute, Ohio University, Athens, OH 45701, USA, 
³Institut für Biologie III, Albert-Ludwigs Universität, D-79104 Freiburg, Germany and ⁴Department of Cancer 
Biology, Cleveland Clinic Foundation Research Institute, Cleveland, OH 44195, USA

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ABSTRACT
The Ski oncoprotein has been found to bind non-
specifically to DNA in association with unidentified 
nuclear factors. In addition, Ski has been shown to 
activate transcription of muscle-specific and viral pro-
moters/enhancers. The present study was undertaken 
to identify Ski’s DNA binding and transcriptional 
activation partners by identifying specific DNA binding 
sites. We used nuclear extracts from a v-Ski-transduced 
mouse L-cell line and selected Ski-bound sequences 
from a pool of degenerate oligonucleotides with 
anti-Ski monoclonal antibodies. Two sequences were 
identified by this technique. The first (TGGC/
ANNNNNT/GCCAA) is the previously identified binding 
site of the nuclear factor I (NFI) family of transcription 
factors. The second (TCCCNNGGGA) is the binding 
site of Olf-1/EBF. By electrophoretic mobility shift 
assays we find that Ski is a component of one or more 
NFI complexes but we fail to detect Ski in Olf-1/EBF 
complexes. We show that Ski binds NFI proteins and 
activates transcription of NFI reporters, but only in 
the presence of NFI. We also find that homodimerization 
of Ski is essential for co-activation with NFI. However, the 
C-terminal dimerization domain of c-Ski, which is 
missing in v-Ski, can be substituted by the leucine 
zipper domain of GCN4.

INTRODUCTION
v-Ski is a 49 kDa nuclear protein that is truncated by 20 amino 
acids (aa) at its N-terminal end and 292 aa at its C-terminal end 
with respect to the full length c-Ski protein (1–3). Analysis of the 
deduced amino acid sequence of v-Ski reveals a number of 
sequence motifs which suggest that its biochemical activity may 
involve DNA binding and transcriptional regulation (2). These 
motifs include a proline rich region, a potential helix–loop–helix 
and several highly conserved histidine and cysteine residues that 
could constitute metal binding domains. In addition to these 
elements, the c-Ski protein contains a C-terminal dimerization 
domain that mediates Ski homodimerization as well as its 
hetrodimerization with the related protein SnoN (4,5). It has 
been suggested (5) that efficient dimerization mediated by this 
domain underlies c-Ski’s more potent transforming activity 
compared to v-Ski which is lacking this region (6).

Both v-ski and c-ski are capable of inducing transformation and 
myogenesis when overexpressed via retroviral vectors (6,7). In 
addition, published work implicates ski in the transformation of 
both erythroid and myeloid cells (8,9) and in the differentiation of 
megakaryocytes (10) and neuronal cells (11). It is not clear how 
ski influences these varied cellular processes but some published 
work suggests that it functions as a regulator of transcription. Ishii 
and co-workers have shown that purified, bacterially-expressed 
human c-Ski (hu-Ski) does not bind DNA cellulose unless it is 
mixed with a nuclear extract from Molt 4 cells (12). Kelder and 
co-workers (13) have extended these findings by showing that 
v-Ski expressed in mouse L-cells binds DNA cellulose and 
activates transcription from several viral enhancers. In studies 
related to ski’s role in muscle differentiation, Rosenthal and 
co-workers showed that Ski activates transcription from the 
muscle-specific myosin light chain enhancer (14). In this case, 
Ski appears to act in combination with the muscle regulatory 
protein, MyoD. Thus a simple explanation of Ski’s multiple 
activities is to propose that it interacts with a variety of 
transcription factors and alters their roles in transcriptional 
regulation.

In this report we describe studies undertaken to investigate this 
possibility by determining whether, on its own or in combination 
with associated nuclear proteins, Ski possesses specific DNA

*To whom correspondence should be addressed. Tel: +1 216 368 3353; Fax: +1 216 368 3419; Email: exs44@po.cwru.edu

Present addresses: + Department of Genetics, University of Wisconsin, Madison, WI, USA and § Department of Molecular and Experimental Medicine, The 
Scripp Research Institute, La Jolla, CA, USA

The authors wish to be known that, in their opinion, the first two authors should be regarded as joint First Authors.
binding activity. To accomplish this goal, we have employed cyclic amplification and selection of targets technique (CASTing) (15,16) which is a modification of the method developed by Oliphant et al. (17). In this technique one starts with a pool of oligonucleotides containing a central region of random sequence and performs repetitive cycles of DNA binding and PCR amplification of bound sequences to identify specific DNA binding sites. We have carried out CASTing with the use of immunoffinity chromatography to purify the Ski-bound oligonucleotides. This approach has allowed us to identify one of Ski’s partners and to assess its ability to regulate transcription in vivo as a consequence of these DNA and protein interactions. We show that the major binding site identified is identical to that previously found for the NFI family of transcription factors (18–21). We present evidence for the involvement of Ski in protein complexes that bind this site and we show that Ski binds NFI proteins and activates transcription of a reporter gene with upstream NFI binding sites. Our data show that Ski dimerization is essential for cooperation with NFI, and this requirement is discussed with regard to the proposed role for dimerization in cellular transformation.

MATERIALS AND METHODS

Ski-specific monoclonal antibody affinity matrix

The v-Ski-specific monoclonal antibodies (mAbs), G8 and M6, were covalently linked to protein A agarose using the Immunopure IgG orientation kit (Pierce) according to the protocol supplied by the manufacturer. Ascites fluid (1 ml) containing either mAb G8 or M6 was used per 2 ml of protein A agarose. The protein A agarose-coupled mAbs were shown to specifically immunoprecipitate v-Ski from [35S]methionine labeled cell lysates (data not shown). The binding capacity of 1:1 mixture of the two matrixes was measured by competitive immunoprecipitation using a bacterial GST-v-Ski competitor (5) and found to be ~150 ng v-Ski/µl matrix (data not shown).

Oligonucleotides

The oligodeoxynucleotides (oligos) used for identification of v-Ski binding sites contain 22 bases of random sequence (CASTing oligo). On the 5’-end, this sequence is flanked by 16 bases of known sequence including the recognition site for the restriction enzyme BamHI. The 3’ flank consists of 14 bases of known sequence including a terminal 10 base self complementary sequence and the site for the restriction enzyme PstI. The primer used for PCR amplification of Ski bound oligos was identical to the 16 base sequence at the 5’-end of the CASTing oligo. The sequences of these oligos and of those used as probes and competitors in electrophoretic mobility shift assays are shown below. CASTing oligo: ggcggatccacctaca(n22)gtgcactgcagtg; PCR primer: ggcggatccacctaca(n22)gtgcactgcagtg; PCR probe: ggcggatccacctaca(n22)gtgcactgcagtg; 

Western blotting and immunofluorescent detection of v-Ski expression

A 1.5 kb BgII fragment from plasmid pcRpolski containing the entire v-ski coding region plus 145 bp of downstream gag sequence (2) was expressed in a clonal line (P28#3) of transfected mouse L-cells (13) or in chicken embryo fibroblasts (CEF) infected with a recombinant avian retrovirus RCASBPA (22). Lysates of these cells and of control L-cells were analyzed for v-Ski production by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting with anti-v-Ski monoclonal antibodies and the chemiluminescent detection system of Tropix (6). The relative amounts of v-Ski in these lysates were determined by densitometry of the exposed film. The intracellular location of v-Ski in P28#3 cells was determined by indirect immunofluorescence as described previously (23).

Selection of Ski bound oligos

Nuclear extracts were prepared from P28#3 cells using the method of Dignam (24). An aliquot (100 µl) of ‘Dignam extract’ (DE) was added to 600 µl buffer C (100 mM NaCl, 50 mM Tris–HCl pH 7.9, 5 mM MgCl2, 1 mM DTT) containing 2.5 µg double stranded CASTing oligo, 5 µg yeast tRNA, 5 µg Poly (dl-dC) and 100 µg/ml BSA. This reaction was incubated for 1 h at 4°C with gentle mixing. Then 200 µl of a 70% suspension of anti-Ski mAb affinity beads (prepared with an equal mixture of Ski-specific monoclonal antibodies G8 and M6), was added and incubation continued for 1 h at 4°C with gentle mixing. The mixture was transferred to a chromography column and the buffer solution allowed to drain out. The resulting column was then washed with 25 column vol of ice cold buffer C. Bound oligos were eluted with 2.5 column vol of buffer C containing 1 M NaCl. The eluted oligos were extracted with phenol/chloroform, diluted to 0.25 M NaCl and precipitated with 2 vol of ethanol in the presence of 10 µg glycogen. The precipitate was dissolved in H2O, adjusted to 0.3 M NaOAc and reprecipitated with ethanol.

One half of the oligonucleotide DNA eluted from the mAb affinity matrix was amplified by PCR using the PCR primer oligo. Amplified DNA was resolved on a 5% polyacrylamide gel and the full length reaction product was excised from the gel. Gel slices were crushed and incubated in 1 M KAc overnight at 37°C. The eluted DNA was precipitated twice with ethanol and used for a second round of selection. This purified product was digested with PstI and subjected to a final round of binding and affinity purification.

Cloning and sequencing of selected oligonucleotides

Oligonucleotide DNA isolated as described above was digested with BamHI and cloned into a modified pUC18 vector which had been previously digested with PstI and BamHI. The vector modification entailed digesting with HindIII, blunting the sticky ends with the Klenow fragment of DNA polymerase and religating. This produced a translational reading frame shift which rendered the pUC-encoded α-peptide of β-galactosidase non-functional. Insertion of the isolated oligos restores the correct reading frame and thus provides a screen for recombinants (17). When plated on bacterial plates containing X-gal and IPTG,
colonies which contain plasmid with inserted oligo DNA will have a blue color and those lacking inserts will remain white. Positive colonies (blue) were picked at random and the inserted DNA sequenced by the dideoxy chain termination method (25). We also cloned and sequenced oligos from the starting pool and confirmed that the central degenerate region consisted of random nucleotide sequences with no consensus and no relatedness to the sequences selected (data not shown).

**Electrophoretic mobility shift assay (EMSA)**

Binding reactions (20 µl) containing 3–6 µg P28#3 nuclear extract were performed in Dignam buffer D (20 mM HEPES pH 7.9, 100 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 20% glycerol) containing 2 µg poly (dl-dC), 300 µg/ml BSA and 50,000 c.p.m. (0.5–1 ng) ^32^P-labeled oligonucleotide probe. Binding reactions were incubated at room temperature for 20 min and loaded directly onto a 5% polyacrylamide gel which had been pre-electrophoresed for 90 min at 150 V. Electrophoresis was continued at the same voltage for 3 h. The gel and chamber buffers used were either 50 mM Tris, 0.38 M glycine and 2 mM EDTA (TGE) or 90 mM Tris, 90 mM boric acid and 2.5 mM EDTA (TBE) as indicated in the figure legends. Following electrophoresis the gel was dried and autoradiographed overnight at −70°C with an intensifying screen. For competition assays the binding conditions were as described above except that unlabeled oligonucleotide competitors (amounts described in figure legends) were added and incubated for 15 min at room temperature prior to addition of the ^32^P-labeled probe. Monoclonal anti-Ski antibodies G8, G37 or anti-β-galactosidase mAb B20 (2 µl ascites fluid) were added to binding reactions after addition of all other components and the reactions were then incubated and analyzed as above.

**Plasmid construction**

The chloramphenicol acetyl transferase (CAT) reporter plasmids were derived from RSV-CAT (26) by replacing its Ndel to EcoRI (in RSV-LTR) fragment with the Ndel to EcoRI fragment from pUC18 containing the multi-cloning site, to generate pENCAT. This deletes the RSV-LTR enhancer, and places the pUC multi-cloning site sequence immediately upstream of the minimalLTR promoter. A reporter (pNF(CAT)) with seven tandem copies of the Ski (NFI) binding site was generated by cloning self-ligated oligonucleotide competitors (amounts described in figure legends) were added and incubated for 15 min at room temperature prior to addition of the ^32^P-labeled probe. Monoclonal anti-Ski antibodies G8, G37 or anti-β-galactosidase mAb B20 (2 µl ascites fluid) were added to binding reactions after addition of all other components and the reactions were then incubated and analyzed as above.

**Cloramphenicol acetyltransferase (CAT) assays**

*Drosophila* Schneider Line 2 (SL2) cells were seeded at a density of 3 × 10^6^ cells per 35 mm plate in Ex-cell 400 medium supplemented with 6.8 mM glutamine and cultured overnight at 25°C prior to transfection. Triplicate plates were co-transfected with each combination of CAT reporter (600 ng) and the indicated expression plasmid (activator plus empty pac5c-pl vector to total 600 ng) by the DEAE dextran (100 µg/ml) method for 4–5 h at 25°C (35). Transfection medium was removed, the cells washed, re-fed with 2 ml of medium and cultured for 72 h. Cells were then harvested into 1 ml TNE (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) and spun for 1 min at 13 000 r.p.m. in a microcentrifuge. The cell pellet was resuspended in 150 µl of 0.25 M Tris–HCl pH 7.8 and lysed by three freeze–thaw cycles using an ethanol-dry ice bath and a 37°C water bath. Following cell lysis the sample was heated at 65°C for 10 min, cooled on ice, clarified by centrifugation at 13 000 r.p.m. for 5 min and the supernate used to determine CAT activity. Where indicated samples of each lysate were used to determine the amount of protein and these values, which never varied by more than 10%, were used to normalize the CAT activity data. We did not employ an internal control for transfection efficiency because our earlier
work had shown that Ski activates expression of all of the commonly used control plasmids (13).

CAT assays were performed by the liquid scintillation method (36) in 100 µl reactions containing 50 µl cell lysate plus 50 µl 0.2 M Tris–HCl pH 8.0, 0.5 mg/ml butyryl CoA and 0.2 µCi [3H]chloramphenicol. Reactions were incubated at 37°C for 2–3 h and extracted with 200 µl xylene. After two back extractions with 10 mM Tris–HCl pH 7.5, 1 mM EDTA, the xylene phase was added to 4 ml scintillation fluid (Eco-Lite) and counted to determine the amount of butyrylated chloramphenicol formed.

**In vitro translation, co-immunoprecipitation and GST-fusion protein binding**

[35S]methionine labeled Ski and NFI proteins were produced by coupled transcription–translation in vitro using the p5'EETM1 or pRP0.5 plasmids described above according to the suppliers protocol (Promega). Binding of in vitro translated proteins to GST-v-Ski was performed and analyzed as described previously (5). GST binding studies were also performed in the presence of 100 and 200 µg/ml ethidium bromide to eliminate DNA–protein interactions (37). For studies involving co-immunoprecipitation two expression plasmids were employed in a single transcription–translation reaction. Following translation a sample was set aside for analysis of the total translation of the two proteins while the remainder was subjected to immunoprecipitation in buffer containing 50 mM Tris–HCl pH 7.8, 250 mM NaCl, 0.1% NP-40 and 0.1 mM PMSF. The untreated translation products and immunoprecipitates were analyzed by SDS–PAGE and fluorography. The ability of anti-NFI anti-serum to co-precipitate Ski protein was used to estimate protein–protein association. To assess the efficiency of the anti-serum to precipitate NFI and its background binding of the Ski protein, the two proteins were produced individually in separate transcription–translation reactions and subjected to analysis by immunoprecipitation and SDS–PAGE. To test the ability of an added LZ to affect dimerization of v-Ski, a plasmid (pEETMΔskiGCN4) encoding a v-Ski-like protein containing the GCN4 LZ but deleted of the region encoding the epitope for the G8 mAb was co-transcribed–translated with either plasmid pEESki-dN which encodes the undeleted v-Ski-like protein lacking the LZ or plasmid p5'EESki-GCN4 which encodes the same Ski segment plus the LZ. Background binding of G8 mAb to the ΔskiGCN4 protein was determined by performing and analyzing an equal amount of this protein alone produced in a separate translation.

**RESULTS**

**Source of v-Ski protein for DNA binding**

Human c-Ski protein was shown to require additional factors present in nuclear extracts to bind DNA cellulose (12). We have confirmed this finding with both v-Ski (13) and chicken c-Ski (data not shown). We therefore chose to use nuclear extracts from cells over-expressing v-Ski rather than using purified bacterially expressed Ski to isolate DNA binding sites. To this end, we chose a cell line established from clone P28#3 of transfected L-cells that expresses v-Ski at approximately five times the level seen in CEFs infected with an avian retrovirus expressing v-Ski (Fig. 1, compare CEFvsks, 20 µg to P28#3, 7 µg). Western blotting also shows that the v-Ski expressed in P28#3 cells is the correct apparent molecular mass of 55 kDa and that our antibody is specific for this protein as no v-Ski-like protein is detectable in extracts from control L-cells (Fig. 1). Immunofluorescence shows that v-Ski in P28#3 cells is located in the nucleus but is excluded from the nucleolus (Fig. 1). This is identical to the localization seen in chicken embryo fibroblasts (CEF) infected by a v-ski retrovirus (1).

**v-Ski binds two DNA consensus sites**

We have found that all of the v-Ski protein in nuclear extracts of P28#3 cells binds DNA cellulose and is eluted at NaCl concentrations >0.1 M (13). This result indicates that cellular factors which are required for Ski to bind DNA (12) are expressed at non-limiting levels in P28#3 cells. To determine if this result reflects the potential for sequence-specific DNA binding by v-Ski-containing complexes, we have employed P28#3 nuclear extracts in the CASTing technique to purify Ski bound sequences from a pool of synthetic, random-sequence oligonucleotides as described in Materials and Methods. The sequences of 35 Ski-bound oligos are presented in Figure 2. No two of the 35 sequenced clones contain the same insert indicating that selective protein binding and not selective PCR amplification is responsible for the enrichment of the isolated sequences. These sequences readily fall into two distinct groups that possess either of two consensus sequence motifs (Fig. 2). Fourteen clones contain a close match to the motif 1A sequence (TCGGC/ AXXXXT/GCCAgA) which has perfect dyad symmetry and is the previously identified binding site for the NFI family of transcription factors (18–21). Another five clones contain motif 1B which is a NFI half site. Five clones contain the second consensus sequence TCCCCxGGGA (TC3 dyad) which also possesses dyad symmetry and has been previously identified as the DNA binding site for the transcription factor, Olf-1/EBF (38,39). An additional eight clones contain the motif 2B sequence (ATCCC) which is probably a half site of motif 2A.

To examine the specificity of binding to these sequences we have performed competition binding experiments using the
Figure 2. Sequences isolated by CASTing with anti-Ski mAbs and P28\#3 nuclear extracts. Sequences of 35 randomly selected clones containing oligonucleotides isolated after three rounds of CASTing from a degenerate-sequence oligonucleotide pool as described in Materials and Methods. Only the sequence of the 22 base degenerate region is shown here. Consensus sequences of each motif are shown underlined below each group. The segment of each sequence related to the consensus is bolded.

Figure 3. Electrophoretic mobility shift assay (EMSA) of v-Ski binding sites. (A) A competition EMSA was performed by incubating 6 µg P28\#3 nuclear extract with increasing amounts (10, 20, 50 and 100 ng) of unlabeled oligonucleotide cognate competitor (same as probe) or mutant competitor (TTTCcaAAAA) for 15 min prior to addition of 20,000 c.p.m. 32P-labeled oligo probe (NFI, left; TC3 right). Following addition of probe, reactions were incubated an additional 15 min. Complexes were analyzed by EMSA and autoradiography. Arrows indicate the major gel shift complexes which are only competed with a specific competitor. (B) NFI binding sites do not compete for TC3 dyad binding and vice versa. Binding reactions were performed as above. For each competitor indicated, 200 ng was used per reaction. TC3 and T refer to TCCCacGGGA; NFI and N refer to TGGCacgTGCCA where the sequence given is the variable region in competitor DNAs, (0) indicates that no competitor was added.

Ski participates in an NFI site binding complex

To confirm that v-Ski protein is present in the complexes which bind to either the TC3 dyad or the NFI site, we have attempted to either supershift the complexes or inhibit their formation using Ski specific mAbs. We first screened a set of v-Ski mAbs generated in this lab for their ability to alter complex formation or mobility. This screen has identified two mAbs, G8 and G37, that produce specific supershifted bands in the NFI EMSA (Fig. 4A). The two mAbs produce a supershifted band of similar mobility suggesting that they are recognizing Ski in the same complexes. To be certain that the production of supershifted complexes with the G8 mAb is due to interaction with Ski in specific DNA–protein complexes, we performed two controls. In the first, we determined whether the supershifted species was subject to specific competition by an unlabeled NFI oligo. In the second, we used an anti-β-galactosidase mAb (B20), which is the same isotype as G8 and G37 (γI) and was generated simultaneously with the other mAbs as a result of immunizing against a β-galactosidase-v-Ski fusion protein. The absence of the supershifted band in the presence of excess competitor and in the presence of B20 mAb supports the conclusion that this band results from specific interactions with the NFI binding site and with Ski protein (Fig. 4A). On the other hand, we failed to identify an anti-Ski mAb that has a reproducible effect on the mobility or
Figure 4. v-ski specific mAbs supershift an NFI DNA binding complex. DNA binding reactions with the NFI probe were performed with P28#3 nuclear extract as in Figure 3. The indicated samples received 2 ml mAb ascites fluid after addition of labeled probe. G8 and G37, ski specific mAbs; B20, anti-β-galactosidase mAb; – indicates no Ab was added. Specific competitor was same as NFI competitor from Figure 3. The supershifted band is indicated by the triangle.

formation of complexes with the TC3 oligo and we have also failed in attempts to detect Ski in these complexes by western analysis (data not shown). Because of this uncertainty, and because the TC3 sequence was under-represented in the pool of bound sequences, we have limited the remainder of the analyses reported here to the studies involving the NFI site.

Both v-Ski and c-Ski bind NFI proteins

The EMSA results suggest that Ski is associated with a specific NFI binding site complex, but do not establish that this occurs by direct interaction between Ski and NFI proteins. To establish this, we have asked whether Ski proteins bind directly with members of the NFI family. As shown in Figure 5A (lanes 2 and 5) we find that GST-v-Ski binds in vitro translated NFI-A1 and NFI-B2. The efficiency of binding the NFI proteins is about the same as the binding of v-Ski to itself (Fig. 5A, lane 8). The binding of these three proteins by GST alone is detectable (Fig. 5A, lanes 3, 6 and 9) but insignificant compared to that of GST-v-Ski. To test whether these associations are direct or mediated by binding of the proteins to contaminating DNA, we performed similar assays in the presence of ethidium bromide (37). As shown in Figure 5B, GST–NFI-A1 binds efficiently to itself and to c-Ski in the presence of ethidium bromide at either 100 or 200 µg/ml. These results indicate that if our reticulocyte lysate contains DNA it is not responsible for the association of Ski and NFI-A1.

We have also assayed association of c-Ski and NFI by in vitro co-translation and co-immunoprecipitation (Fig. 5C). The results show that a fraction of the c-Ski protein is co-immunoprecipitated by the NFI antiserum when it is co-translated with NFI-A1 (Fig. 5C, lane 5) but not when it is translated on its own (Fig. 5C, lane 4). Although not immediately apparent from the signal intensity, this result is more impressive than the GST binding data in demonstrating the association between these proteins. That is because the co-immunoprecipitation assay employs only in vitro translated c-Ski and NFI proteins whose concentrations are estimated to be in the nanomolar range whereas the GST binding assays are performed at micromolar concentrations of the fusion protein.

Ski enhances transcription activation by NFI

Having found that Ski and NFI proteins participate in both protein–protein and protein–DNA interactions, we sought to investigate the effect of these interactions on transcriptional regulation. Initial experiments using NFI reporters in both L-cells and chicken fibroblasts have been inconclusive, probably due to the expression of endogenous Ski as well as multiple forms of NFI (data not shown). To surmount these problems we have turned to Drosophila SL2 cells as they lack both Ski and NFI but support transcription activation by exogenous NFI (40).
Co-transfection of SL2 cells with either paccski or pacvski does not result in transcriptional activation of a reporter containing upstream NFI sites (pNf7-CAT) above that seen with the empty pac5c-pl vector (Fig. 6A). However, co-transfection of pacNF1-A1 or pacNF1-B2 with the pNf7-CAT reporter results in an impressive 40- or 10-fold activation of CAT expression, respectively (Fig. 6A). This activation is dependent on the upstream NFI binding sites in the reporter since CAT expression from the same reporter lacking the binding sites is not increased by NFI or Ski (Fig. 6A).

When the assays are performed with the pNF7-CAT reporter plus both NFI and c-ski expression vectors ~2-3-fold greater activation is observed than with either NFI-A1 or NFI-B2 alone (Fig. 6A). A dose–response study shows that the increase in expression is directly proportional to the amount of c-ski expression vector used over the range of 150–600 ng; being ~3-fold with 300 ng and 7-fold with 600 ng (Fig. 6B). These results, and those of the DNA and protein binding experiments presented above, suggest that Ski binds to NFI sites and activates transcription only through its association with NFI proteins.

Somewhat surprisingly, when pacvski is co-transfected with the pNF7-CAT reporter plus NFI-A1 or NFI-B2 no increase in expression is observed relative to that seen with either NFI alone (Figs 6A and 7B). In earlier work we had noted that c-Ski possesses a high affinity dimerization domain that is deleted from the transduced viral protein (5). It seems possible that the absence of this domain in v-Ski might be responsible for its failure to co-activate transcription with NFI. To test this possibility we have added the LZ dimerization domain of GCN4 to the C-terminal end of a v-Ski-like protein. As shown by co-immunoprecipitation of an epitope-deleted Ski protein (Fig. 7A, lane 6), addition of the GCN4-LZ promotes dimerization of in vitro translated Ski protein, which are incapable of self association at these low protein concentrations in the absence of an added dimerization domain (Fig. 7A, lane 3). A small background precipitation of the probe by G8 mAb is visible (Fig. 7A, lanes 2 and 5) but <10% of that co-precipitated with the LZ-containing protein.

Having restored efficient dimerizing ability to v-Ski, we next tested the ability of this protein to co-activate transcription with NFI in reporter gene assays (Fig. 7B). As controls we see that once again v-Ski (at either 300 or 600 ng) shows no activation over that of NFI-B2 alone while c-Ski produces 5- and 12-fold activation at 300 and 600 ng, respectively. As expected, the v-Ski-like protein, SkidN also fails to co-activate transcription with NFI. However, this protein with the added GCN4-LZ dimerization domain (Ski-GCN4) yields 3- (300 ng) and 5-fold (600 ng) activation over that of NFI-B2 alone. These results indicate that dimerization of Ski is essential for its co-activation of transcription with NFI.

**DISCUSSION**

We have shown that, in association with other nuclear proteins, v-Ski binds to DNA as a result of sequence-specific interactions. Using nuclear extracts as the source of v-Ski and monoclonal antibodies to Ski to purify bound sequences we have identified two DNA consensus sequences bound by Ski-containing complexes. One of these is the previously identified binding site of the NFI proteins. Electrophoretic mobility shift assays with an NFI binding site probe reveal multiple specific complexes reflecting the expression of several NFI proteins which are known to bind the same site as both homodimers and heterodimers. The ability of a Ski-specific mAb to elicit a supershift of at least one of these complexes, suggests that v-Ski is a component of that complex. The observation that pure Ski protein does not bind the NFI site (data not shown) indicates that NFI proteins are required for Ski to bind this sequence and the resulting complexes consist of NFI dimers plus Ski.

The proposal that Ski binds the NFI site by virtue of its interaction with NFI is supported by our results showing that GST fusion proteins of v-Ski and NFI-A1 bind in vitro translated Ski and NFI proteins. The affinity of these interactions is apparently physiologically reasonable because it is also detected in co-translation/immunoprecipitation studies where both proteins

![Figure 6. Transcriptional activation by NFI and Ski. (A) Drosophila SL2 cells were transfected with CAT reporter plasmids (600 ng) lacking (pENCAT) or containing (pNF7-CAT) seven upstream NFI binding sites as indicated. Co-transfections with these reporters were performed with pac5c-pl, pacNF1-A1 (10 ng), pacNF1-B2 (2 ng), paccski29 (300 ng) and pacvski (300 ng) either separately or in combinations as indicated. All transfections were performed in triplicate and assayed for CAT activity as described in Materials and Methods. Values for fold activation were calculated relative to those obtained with pNF7-CAT plus pac5c-pl which ranged from 285 to 472 c.p.m. As shown, values obtained with pENCAT were not effected by co-transfection of pacNF1 or paccski and were the same as those obtained with pNF7-CAT plus pac5c-pl. Although, as discussed in Materials and Methods, we do not normalize to the activity of an internal control, our results are highly reproducible. For example, the data given for pNF7-CAT plus 10 ng pacNF1-A1 is the average of three experiments performed in triplicate which yielded fold activation values of 37, 40 and 34. (B) Drosophila SL2 cells were co-transfected with pNF7-CAT plus pacNF1-A1 (2 ng) and the indicated amounts of paccski29 plus pac5c-pl (total of 600 ng). Values are relative to that obtained with pNF7-CAT plus pac5c-pl (472 c.p.m.). All values are averages of triplicate transfections adjusted to constant protein amounts, and the average variation from the mean is indicated by the error bars. Note that in this experiment co-transfection of 300 ng pacski29 produced a 3.3-fold activation over that of 2 ng pacNF1-A1 alone, which is very similar to the 2.9-fold obtained with the LZ-containing protein. The proposal that Ski binds the NFI site by virtue of its expression with NFI. However, this protein with the added GCN4-LZ dimerization domain (Ski-GCN4) yields 3- (300 ng) and 5-fold (600 ng) activation over that of NFI-B2 alone. These results indicate that dimerization of Ski is essential for its co-activation of transcription with NFI.](image)
are expressed at sub-micromolar concentrations. The ability of Ski to bind both NFI-A1 and NFI-B2 suggests that the Ski binding site on these proteins is in the conserved N-terminal region that is required for both dimerization and DNA binding. However, Ski has no discernible homology with this or any other region of the four chicken NFI proteins (31,32). It therefore appears unlikely that Ski interferes with NFI dimerization by forming heterodimers via this NFI domain but suggests that Ski may associate with preformed NFI dimers to form mixed multimers.

The conclusion that Ski binds the NFI site through protein–protein interactions with NFI is also consistent with the results of our reporter gene assays. Using Drosophila cells which do not appear to express homologs of either Ski or NFI, we find that transactivation of an NFI reporter by c-Ski requires co-expression of NFI. With either NFI-A1 or NFI-B2 alone transcription is activated up to 40-fold and Ski increases this by 2–10-fold in a concentration-dependent fashion. With v-Ski we do not detect activation even in the presence of co-expressed NFI. This observation is at first disturbing because the NFI binding site on these proteins is in the conserved N-terminal region that is required for both dimerization and DNA binding. However, Ski has no discernible homology with this or any other region of the four chicken NFI proteins (31,32). It therefore appears unlikely that Ski interferes with NFI dimerization by forming heterodimers via this NFI domain but suggests that Ski may associate with preformed NFI dimers to form mixed multimers.

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Figure 7. Dimerization and transcriptional activation by Ski-GCN4. (A) Ski proteins, similar to v-Ski by lack of a C-terminal dimerization domain, were engineered with (SkidN-GCN4) and without (SkidN) an added LZ dimerization domain from GCN4. In addition, a deleted form of SkidN-GCN4 was produced which lacks the epitope for the G8 anti-Ski mAb (D1Ski-GCN4). The D1Ski-GCN4 protein was in vitro translated alone (lanes 2 and 5) or co-translated with either SkidN (lanes 1 and 3) or SkidN-GCN4 (lanes 4 and 6) in the presence of [35S]methionine. Of each translation reaction, 10% was set aside as an input control (lanes 1 and 4) while the remainder was immunoprecipitated with G8 mAb (lanes 2, 3, 5 and 6) as described in Materials and Methods. Controls and precipitated samples were analyzed by SDS–PAGE and fluorography. (B) Drosophila SL2 cells were co-transfected with pNF-CAT plus pacNFI-B2 (2 ng) and either 300 or 600 ng of the indicated pacski plasmid. Fold activation (Total) refers to the increase in CAT activity relative to that of pNF-CAT plus pac5c-p. Fold activation by Ski refers to the increase in CAT activity relative to that of pNF-CAT plus pacNFI-B2. The data were normalized to constant protein amounts as described in Materials and Methods.

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