Heterodimers of the SnoN and Ski oncoproteins form preferentially over homodimers and are more potent transforming agents

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ABSTRACT

sno is a member of the ski oncogene family and shares ski’s ability to transform avian fibroblasts and induce muscle differentiation. Ski and SnoN are transcription factors that form both homodimers and heterodimers. They recognize a specific DNA binding site (GTCTAGAC) through which they repress transcription. Efficient homodimerization of Ski, mediated by a bipartite C-terminal domain consisting of five tandem repeats (TR) and a leucine zipper (LZ), correlates with efficient DNA binding and cellular transformation. The present study assesses the role of SnoN homodimerization and SnoN:Ski heterodimerization in the activities of these proteins. Unlike Ski, efficient homodimerization by SnoN is shown to require an upstream region of the protein in addition to the TR/LZ domain. Deletion of the TR/LZ from SnoN decreases its activity in transcriptional repression and cellular transformation. When co-expressed in vitro, c-Ski and SnoN preferentially form heterodimers. In vivo, they form heterodimers that bind the GTCTAGAC element. Tethered Ski:Sno heterodimers that lack TR/LZ domains are more active than either their monomeric counterparts, tethered Ski:Ski homodimers or full-length SnoN and c-Ski. This work demonstrates, for the first time, the differences between dimer formation by Ski and SnoN and underscores the importance of dimerization in their activity.

INTRODUCTION

v-Ski protein is a 438 amino acid nuclear protein and is missing 20 residues from the N-terminus and 292 residues from the C-terminus of c-Ski (reviewed in 1). This retroviral oncogene transforms both chicken embryo fibroblasts (CEFs) and quail embryo fibroblasts (QEFs) and it converts QEFs into myoblasts (2). Its myogenic activity has also been demonstrated in vivo by showing that transgenic mice expressing a truncated version of c-Ski, similar to v-Ski, have over-developed skeletal muscles (3).

c-Ski is a 750 amino acid nuclear protein that also induces cellular transformation and muscle differentiation of avian fibroblasts when highly over-expressed from an avian retroviral vector (4,5). c-Ski also causes morphological transformation of CEFs when expressed at lower levels with a less efficient vector, whereas v-Ski does not. This result shows that v-Ski’s transforming potential is not a result of c-Ski’s truncation, but is instead a function of the high level of expression of v-Ski from the avian retrovirus.

A high affinity dimerization domain at the C-terminal end of c-Ski is missing in v-Ski (6,7). This region is responsible for c-Ski’s increased transforming potency compared with v-Ski, suggesting that efficient dimer formation underlies this enhanced activity. Analysis of this domain has uncovered two structural motifs (6,8). The more N-terminal motif consists of an α-helix formed by five imperfect tandem repeats (TR) of 25 amino acids each. The second, more C-terminal motif consists of an α-helical leucine zipper (LZ) that participates in dimer formation (6,7). The TR and LZ act cooperatively in mediating dimerization, with the equilibrium constant of the combined domains (Kd = 2 × 10^-8/M) being significantly less than that of the TR alone (Kd = 4 × 10^-6/M) or of the LZ alone (Kd > 2 × 10^-3/M) (7). Upon dimerization the entire TR/LZ region forms an almost continuous α-helix that is relatively resistant to proteolysis (7). Although v-Ski lacks the TR/LZ domain, it is nonetheless capable of self-association, albeit at greatly reduced efficiency relative to c-Ski (6). v-Ski contains two domains that have been shown to have a role in dimerization. The stronger of the two, termed d2, is located at residues 250–325 and is highly conserved (unpublished data).

sno, the other member of the ski family, was discovered in a low stringency screen of human cDNA libraries with a chicken ski probe (9). Subsequently, sno has been cloned from chicken (10) and mouse (11) cDNA libraries. Four sno cDNAs, representing alternatively spliced forms, have been found in humans, including...
snoN (9), snoA (9), snoI (12) and snoN2 (13). In the mouse, two isoforms are seen, full-length snoN and sno-deE3 (also called snoN2). The latter is the result of alternative splicing from a 5′ splice site located within exon 3 of snoN (11,13). The 12 kb chicken sno gene contains six exons, the first of which is non-coding (14). Only the largest form, snoN, has been found in chickens (10).

Chicken SnoN is a 76 kDa protein that has been shown to localize in the nucleus (10). SnoN has also been shown to bind to the Ski GTCT element (15) and thereby repress transcription in reporter gene assays (16). When expressed at high levels from the RCASBP(A) retroviral vector, SnoN induces clonal growth of要好好用则的co-immunoprecipitations (co-IP) all constructs were cloned into the TM1 vector, a gift of Dennis Templeton. When the EE epitope was needed, 5′EETM1 was used. When the tag was not needed, 3′EETM was used and the inserts contained a stop codon before the EE tag. Alternatively the 5′NSL-TM1 vector was used. This is a modified version of 5′ETM1, that contains the SV-40 nuclear localization signal in place of the EE tag. The Ski-TR/LZ plasmid was made from p265NM (NcoI/XbaI), which contains the coding region of the c-Ski TR and LZ, and EETM1 (NcoI/SpeI). EE-SnoN was made by inserting a NcoI–SalI fragment containing the entire cdNA into EES(TM1 (NcoI/SalI)). G8 SnoN was made by inserting a PstI fragment of c-ski encoding residues 15–119 into TM-Sno digested with NsiI. SnoN-TR/LZ was made by PCR amplification of the region encoding residues 511–690 of SnoN. The 5′ primer was designed to include an NcoI site and the PCR fragment (NcoI–SalI) was ligated into EETM1 (NcoI/SalI). SnoN TR was made from SnoN-TR/LZ by cutting with NheI and BglII, followed by filling in the resulting ends (Klenow). The plasmid was then ligated closed, which resulted in a stop codon five amino acids downstream. SnoN-LZ was made from EE-SnoN by NheI/NcoI digestion, followed by filling in the resulting ends (Klenow). The plasmid was then ligated closed. The SnoN(292–690) construct was made from EE-SnoN by digesting first with NcoI and then a partial digest with AccI. The correct size fragment was blunted (Klenow) and ligated closed. The LZ was removed to create SnoN(292–638) as described above for SnoN-TR. The SnoN(368–690) and SnoN(368–638) pair were made in a similar manner. SnoN(1–432) was made from a SnoN fragment (NcoI–HindIII blunt) that was ligated into EE-TM1 cut with NcoI and Stul. Ski(Sno-TR/LZ) was constructed by PCR of SnoN using a 5′ primer that incorporated an NdeI site and includes residues 511–690 of SnoN. This fragment was digested with NdeI/SalI and was cloned into EE-Ski (NdeI/SalI). Ski(Sno-TR) was made by digesting Ski(TR/LZ) with NheI/SalI and inserting a fragment of SnoN generated via PCR that contained Ski-LZ. SnoN(Ski-LZ) was made from a fragment of TM-Ski (SalI/SalI), which was ligated into EE-TM-SnoN (NheI, blunt with Klenow/SalI).

Chicken retroviral versions of various SnoN forms were made by cloning the SnoN segments of the above constructs as BsiWI–XbaI fragments into RCAS(A)BP X/S (BstWI/SpeI). This vector is a derivative of RCAS(A)BP (20,21) that has a multicloning site inserted into the unique ClaI site (Sharpay and Stavnezer, unpublished results). Full-length chicken c-ski cdNA was also cloned into the murine retroviral vector, pB Abe puro (22). This vector was packaged into infectious xenotropic virus by transfection of the PA317 cell line (23).

MATERIALS AND METHODS

Recombinant vectors for expression of SnoN and Ski proteins

For in vitro co-immunoprecipitations (co-IP) all constructs were cloned into the TM1 vector, a gift of Dennis Templeton. When the EE epitope was needed, 5′ETM1 was used. When the tag was not needed, 3′EETM was used and the inserts contained a stop codon before the EE tag. Alternatively the 5′NSL-TM1 vector was used. This is a modified version of 5′ETM1, that contains the SV-40 nuclear localization signal in place of the EE tag. The Ski-TR/LZ plasmid was made from p265NM (NcoI/XbaI), which contains the coding region of the c-Ski TR and LZ, and EETM1 (NcoI/SpeI). EE-SnoN was made by inserting a NcoI–SalI fragment containing the entire cdNA into EES(TM1 (NcoI/SalI)). G8 SnoN was made by inserting a PstI fragment of c-ski encoding residues 15–119 into TM-Sno digested with NsiI. SnoN-TR/LZ was made by PCR amplification of the region encoding residues 511–690 of SnoN. The 5′ primer was designed to include an NcoI site and the PCR fragment (NcoI–SalI) was ligated into EETM1 (NcoI/SalI). SnoN TR was made from SnoN-TR/LZ by cutting with NheI and BglII, followed by filling in the resulting ends (Klenow). The plasmid was then ligated closed, which resulted in a stop codon five amino acids downstream. SnoN-LZ was made from EE-SnoN by NheI/NcoI digestion, followed by filling in the resulting ends (Klenow). The plasmid was then ligated closed. The SnoN(292–690) construct was made from EE-SnoN by digesting first with NcoI and then a partial digest with AccI. The correct size fragment was blunted (Klenow) and ligated closed. The LZ was removed to create SnoN(292–638) as described above for SnoN-TR. The SnoN(368–690) and SnoN(368–638) pair were made in a similar manner. SnoN(1–432) was made from a SnoN fragment (NcoI–HindIII blunt) that was ligated into EE-TM1 cut with NcoI and Stul. Ski(Sno-TR/LZ) was constructed by PCR of SnoN using a 5′ primer that incorporated an NdeI site and includes residues 511–690 of SnoN. This fragment was digested with NdeI/SalI and was cloned into EE-Ski (NdeI/SalI). Ski(Sno-TR) was made by digesting Ski(TR/LZ) with NheI/SalI and inserting a fragment of SnoN generated via PCR that contained Ski-LZ. SnoN(Ski-LZ) was made from a fragment of TM-Ski (SalI/SalI), which was ligated into EE-TM-SnoN (NheI, blunt with Klenow/SalI).

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solutions were mixed at 4°C for at least 1 h. An aliquot of 50 μl of protein A–agarose was added (50% suspension, previously washed with XL+) and the suspension was again mixed for at least 1 h at 4°C. The protein A–agarose was pelleted via centrifugation and washed four times with 200 μl of XL. The precipitates were resuspended in sample buffer (24), heated at 100°C for 5 min and cleared by centrifugation. The supernatants were analyzed by SDS–PAGE (24) or Tris–tricine SDS–PAGE (25). The gels were fixed (45% methanol, 10% acetic acid), soaked in Enlightening (Du Pont), dried and the bands were visualized using fluorography.

Cell culture, transfection and soft agar cloning

Transient and stable transfections were performed using DOTAP according to the manufacturer’s instructions (Boehringer Mannheim Corp.). The chicken embryo fibroblast cell line UMN-SAH/DF#1 (DF1) was a gift of D. Foster of the University of Minnesota. Cell culture and viral infection were performed as previously described (21), except that DF1 cells were cultured in DMEM with 10% fetal bovine serum. Cells were passed three to four times post-transfection to allow the spread of virus. Soft agar cloning was done as previously described (21).

Crosslinking

Proteins generated by in vitro translation as described above were reacted with 0.6 mg/ml of the homo-bifunctional crosslinking reagent bis(sulfosuccinimidyl) suberate (BS3) as described previously (6,7). After 15 min at room temperature (RT), 10 μl of 1 M glycine was added to stop the reaction. Samples were analyzed by SDS–PAGE as described above except that gels were not treated with Enlightening. Bands on the resulting autoradiographs were visualized using fluorography.

Glutathione S-transferase (GST) pull-down assays

Construction of pGEXv-Ski, its use to generate a GST–v-Ski fusion protein and the use of the protein in GST pull-down assays have been described previously (6). For use in these binding assays, v-Ski was labeled with [35S]methionine by coupled in vitro transcription and translation as described previously (6). The GST fusions with segments of Ski were created in pGEX2T by replacement of v-ski with deleted forms truncated at either a KpnI site (codon 214) or a BamHI site (codon 325). The deletion of codons 138–203 was created by recombining two previously described mutants (ΔAH2 and ΔAH4) using the NheI sites inserted at the position of each mutation (26).

Reporter assays

The reporter constructs and assay have been described (15,16). For transient transfection the indicated constructs were expressed from RSVPL.

Electrophoretic mobility shift assay (EMSA)

EMSA were done as previously described (15,16).

Construction of the forced Sno:Ski dimer

A PCR fragment encoding amino acids 46–326 of c-Ski (ski-bb) was generated by primer pair ski1 ((gggacctagcactgggtatcagaaaaagttga) and ski2 ((gggacctagcactgggtatcnctcggctcagctgg). Another PCR fragment encoding amino acids 1–389 of SnoN (sno-bb) was generated by primer pair sno1 (gggacctagcactgggtatcagaaaaagttga) and sno2not (ataagttgcgcacgttactcggctcagctgg). To generate Ski:Sno, the ski-bb fragment was digested with BglII and ligated with the sno-bb fragment digested with BamHI. The ligated fragment was digested with SpeI and NotI and inserted into the cog site of the RCAS-BP(A)SX vector. The same strategy of cutting ski-bb with either BanHI or BglII followed by ligation was used to generate Ski:Ski. The ligated dimer was then cut with SpeI and ClaI and inserted into RCAS-BP(A)SX at the SpeI and BsrBI sites. Monomeric forms were generated by cutting ski-bb with SpeI and Clal and sno-bb with SpeI and NotI. These fragments were then cloned into RCAS-BP(A)SX at the appropriate sites. The resultant plasmids are referred to as SX/ski, sx/ski, SX/ski and SX/sno.

RESULTS

The domains required for SnoN homodimerization are not the same as for Ski homodimerization

Previous work on homo- and heterodimer formation by c-Ski and SnoN employed either chemical crosslinking or high concentrations of bacterially expressed proteins in GST pull-down assays. That work had demonstrated that the TR and LZ domains of Ski mediate high affinity dimer formation (6–8). For the present work, we felt more sensitive assays of dimerization could be performed at low protein concentration by performing co-IP of in vitro co-translated proteins. The co-IP was performed using an antibody that recognizes only one of the potential dimerization partners. This method requires that the interaction between the proteins be stable enough to hold up to repeated washing. On the other hand, it does not require interactions that juxtapose specific types of residues, as is required in chemical crosslinking (e.g. primary amines or sulfhydryls).

In order to validate our co-IP protocol, we first examined the previously described homodimer formation mediated by the TR/LZ of c-Ski (6). In this case, the isolated TR/LZ domain of c-Ski is co-translated with an internally deleted form of c-Ski (SkiAEi) that retains the C-terminal TR/LZ domain and the N-terminal epitope for the G8 mAb. SkiAEi is translated very efficiently and has been shown to dimerize in a similar manner to that of the full-length c-Ski (6). In agreement with earlier work, the results show that the Ski TR/LZ protein co-IPs with SkiAEi (Fig. 1A).

Having validated our method, we set out to investigate dimerization of SnoN using the co-IP assay. Because we do not possess an antibody for the SnoN protein that can be used for this purpose, we constructed two epitope-tagged versions of SnoN. The first construct (G8-SnoN) contains the epitope for the G8 antibody, which resides in the first 100 amino acids of c-Ski, fused to SnoN residues 309–690. Since this N-terminal portion of c-Ski does not participate in dimerization (6), its presence does not affect the protein associations being examined. The second construct (EE-SnoN) contains the polyglutamate synthetic peptide epitope (EE) at the N-terminal end of full-length SnoN (27).
SnoN contains a C-terminal domain related to the c-Ski TR/LZ that has been implicated in both hetero- and homodimer formation (6,8). We therefore focused on this region of SnoN by performing co-IPs with G8-SnoN. In a systematic analysis, we have examined a series of overlapping constructs that retain the LZ alone, the TR/LZ or the TR alone and extend towards the N-terminus. We find that neither the TR nor the LZ domain alone perform co-IPs with G8-SnoN. In a systematic analysis, we have focused on this region of SnoN by showing that the SnoN(1–432) does not support recombination of its own because SnoN(1–432) does not support co-IP in the absence of the TR domain (data not shown).

Having identified the domains required for SnoN homodimerization in vitro, we wanted to determine whether efficient dimerization plays a role in its activity in vivo. We have used two different approaches to address this question. The first involves assays of transcriptional repression of a reporter with upstream copies of the Ski/SnoN binding site (GTCTAGAC). Previous work has shown that both Ski and SnoN repress transcription of this reporter (15,16). Here we compare the repression activity of EE-SnoN, EE-SnoN(1–638), which lacks the LZ domain, and EE-SnoN(1–432), which lacks both the TR and LZ but contains the upstream dimerization element. The results show that all three constructs repress expression of the reporter, but the two deleted forms, EE-SnoN-LZ and EE-SnoN(1–432), are only about half as potent as full-length EE-SnoN which repressed transcription to 15% of the control (Fig. 3). These results suggest that efficient dimerization increases the repression activity of SnoN. However, it is surprising that the assay failed to detect a significant difference between EE-SnoN(1–638) and EE-SnoN(1–432) as the presence of the TR on the former should increase the efficiency of dimer formation.

The second assay employed to assess the effect of dimerization in vivo is measuring cellular transformation. Both c-Ski and SnoN have been previously shown to transform infected CEFs as measured by clonal growth in soft agar (10,21). The same three forms of SnoN used in the reporter assay, EE-SnoN, EE-SnoN(1–638) and EE-SnoN(1–432), were cloned into the retroviral vector RCASBP(A). CEFs infected with these viruses were then assayed for clonal growth in soft agar (Fig. 3). As previously reported (16), EE-SnoN supports clonal growth at the same efficiency (2.4%) as unmodified SnoN (10). The two deleted forms give lower values, EE-SnoN(1–638) (1.8%) and EE-SnoN(1–432) (1.5%), that are similar to each other and correlate well with the results of the reporter assays described above.

The two experiments show that high affinity dimerization, mediated by the entire tripartite dimerization domain, is advantageous for maximum repression and transformation by SnoN. However, there is not a linear relationship between these in vivo activities and dimerization affinity as measured by co-IP in vitro. This follows from the observation that SnoN(1–638), which contains the combination of the TR and the 292–367 upstream regions fused in-frame with residues 309–690 of SnoN. IN, input control showing 10% of the amount of the co-translated protein used in the co-IP; BG, background binding of the singly translated target protein to the protein A and mAb used in the co-IP; IP, co-IP of co-translated proteins. Input and precipitated proteins were analyzed by SDS–PAGE and fluorography.

(A)–(D) contain the indicated 35S-labeled proteins.

Figure 1. SnoN requires the TR and residues 292–368 for homodimerization. co-IP assays were performed using 35S-labeled, in vitro translated proteins and either the G8 (A and B) or EE mAb (C and D) as described in Materials and Methods. The G8 mAb binds SkiAEI or G8-Sno and the EE mAb precipitates the EE-tagged SnoN. G8-Sno contains an N-terminal G8 epitope tag from c-Ski (residues 16–119) fused in-frame with residues 309–690 of SnoN. IN, input control showing 10% of the amount of the co-translated protein used in the co-IP; BG, background binding of the singly translated target protein to the protein A and mAb used in the co-IP; IP, co-IP of co-translated proteins. Input and precipitated proteins were analyzed by SDS–PAGE and fluorography.

Figure 2. The regions of SnoN that are required for homodimer formation. A series of SnoN constructs were tested for their ability to form dimers with SnoN by co-IP, as described in the legend to Figure 1.

High efficiency dimerization is required for maximum repression and transformation

Results obtained in a repeat of this experiment using EE-SnoN agree with those obtained with G8-Sno by showing that the SnoN TR/LZ in combination with the upstream region in SnoN(292–690) efficiently forms dimers with EE-SnoN (Fig. 1C). When the LZ was removed, leaving residues 292–638, dimers form with EE-Sno at reduced efficiency (Fig. 1D). The results with both G8-Sno and EE-Sno demonstrate that high affinity SnoN homodimerization requires the TR/LZ domain plus an upstream region (amino acids 292–367). This region is not sufficient for dimerization on its own because SnoN(1–432) does not support co-IP in the absence of the TR domain (data not shown).

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ΔSnoN LZ alone is able to heterodimerize with c-Ski studies (Fig. 4 A). However, neither the SnoN TR alone nor the contains the 292–367 region identified in the SnoN homodimer are able to dimerize very efficiently although neither protein with c-Ski is restored (Fig. 4 D). However, the 292–367 region of 292–367 region in SnoN(292–637), the ability to heterodimerize (data not shown).

c-Ski TR/LZ is required for high efficiency heterodimer formation with the C-terminal subdomains of c-Ski also show that the entire and C). Additional co-IP experiments using G8-Sno in combination with either the 292–367 region or the LZ domain.

Having mapped the domains involved in heterodimer formation, we wanted to compare their relative affinity to form homodimers and heterodimers. To accomplish this goal, we used covalent protein crosslinking because it allows the detection and quantitation of the three potential dimer combinations (Ski:Ski, Ski:SnoN and SnoN:SnoN). Our only assumption is that crosslinking should affect the equilibria of all forms of dimers equally. The smaller Ski:AEi protein is used in this assay, because it facilitates separation and identification of all predicted species by SDS–PAGE.

To assess the relative affinities of Ski and SnoN, we crosslinked mixtures containing the proteins at different ratios and compared the observed ratios of homodimers and heterodimers with theoretical values calculated assuming identical affinities of all forms. If the affinity to form heterodimers and homodimers is identical, crosslinking mixtures containing equal amounts of SnoN and c-Ski would yield the following ratios of dimers: 25% SnoN:SnoN, 50% SnoN:Ski and 25% Ski:Ski. Instead of this 1:2:1 ratio, we observe a 1:4.5:1 ratio (Fig. 5 A and B, lanes 4). Instead of this 1:2:1 ratio, we observe a 1:4.5:1 ratio (Fig. 5 A and B, lanes 4). When the proteins were expressed at a 2:1 Ski:SnoN ratio (lanes 3) or a 2:1 SnoN:Ski ratio (lanes 5) the amount of the heterodimer of the more abundant protein should equal the heterodimer. However, in both cases the quantity of heterodimer was at least twice that of the homodimers (compare Ski:Sno with Ski:Ski in lane 3 or with Sno:Sno in lane 5). These results show a preference for the formation of Ski:Sno heterodimers versus their respective homodimers in vitro.

The tandem repeat of SnoN is responsible for the preferential formation of SnoN:Ski heterodimers

Having demonstrated that SnoN and Ski preferentially form heterodimers, we wanted to know which of the three dimerization subdomains are responsible for this preference. For this work a series of chimeric constructs were used in which the TR/LZ domains of SnoN and c-Ski were swapped. The first chimera [Ski*ΔTR/LZ/SnoN] contained c-Ski (residues 1–457) and the TR/LZ of SnoN (residues 501–690) resulting in the c-Ski TR/LZ domain and is incapable of co-IP (data not shown).

The C-terminal regions of c-Ski and SnoN contain the necessary domains for heterodimerization

Previous work employing methods that do not require high affinity interactions implicated the TR and LZ domains of SnoN and c-Ski in heterodimerization (6,8). The present work implicated an additional segment (residues 292–367) in homodimerization of SnoN and this region is reasonably well conserved in Ski. We have therefore re-examined heterodimer formation using the co-IP assay. As shown in Figure 4A, c-Ski:AEi and SnoN-TR/LZ are able to dimerize very efficiently although neither protein contains the 292–367 region identified in the SnoN homodimer studies (Fig. 4A). However, neither the SnoN TR alone nor the SnoN LZ alone is able to heterodimerize with c-Ski:AEi (Fig. 4B and C). Additional co-IP experiments using G8-Sno in combination with the C-terminal subdomains of c-Ski also show that the entire c-Ski TR/LZ is required for high efficiency heterodimer formation (data not shown).

Interestingly, when the SnoN TR is supplemented with the 292–367 region in SnoN(292–637), the ability to heterodimerize with c-Ski is restored (Fig. 4D). However, the 292–367 region of SnoN alone is not sufficient for this interaction because SnoN(1–432) does not co-IP with c-Ski (Fig. 4E). This shows that high affinity heterodimerization with c-Ski requires the TR of SnoN in combination with either the 292–367 region or the LZ domain.

c-Ski and SnoN form heterodimers preferentially

Having mapped the domains involved in heterodimer formation, we wanted to compare their relative affinity to form homodimers versus heterodimers. To accomplish this goal, we used covalent protein crosslinking because it allows the detection and quantitation region that is sufficient for co-IP (Fig. 1D), is only slightly more active than SnoN(1–432), which is missing the entire TR/LZ domain and is incapable of co-IP (data not shown).

Figure 3. Efficient dimerization of SnoN correlates with maximal repression and transformation. DF1 cells were co-transfected with GTCT/2×2tkLuc (500 ng) and the RSVPL expression plasmid encoding the indicated SnoN segment (500 ng). At 36–48 h post-transfection, cells were harvested, lysed and their luciferase activity was determined. The data are expressed as relative activity, which equals the ratio of the activity detected with the indicated SnoN domains divided by that detected with empty RSVPL. Data shown are the averages of six replicates. Transformation is assayed by counting colonies in soft agar of primary CEFs infected with a RCASBP(A) retroviral vector expressing the indicated SnoN segment. Equal numbers of cells (1.2 × 10⁵) were cultured in suspension in 0.35% agar. Colonies were counted at 30 days and the results are given as the percentage of cells plated that gave rise to colonies containing >16 cells.

Figure 4. The C-terminal portion of SnoN is required for heterodimerization with Ski. Co-IPs were performed using the G8 mAb as described in the legend to Figure 1. (A–E) contain the indicated 35S-labeled proteins.
In order to establish if either the TR or LZ is individually responsible for this preference, two additional chimeric forms were analyzed. SnoN*(Ski-LZ) consisted of all of SnoN except the LZ domain (residues 1–638) fused to the LZ domain of c-Ski (residues 501–638). The acquisition of Ski’s LZ domain did not alter dimerization by this protein. SnoN*(Ski-LZ) still preferentially formed dimers with Ski, while it formed dimers with SnoN at the theoretical ratio for equal affinity (Table 1). The second chimera [Ski*(SnoN-TR)] contained all of c-Ski except that its TR subdomain alone is responsible for SnoN’s preference to form heterodimers with c-Ski.

### Table 1. The SnoN TR domain dictates preferential heterodimer formation with Ski

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<th>Cotranslated Proteins</th>
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<th>Dimer Ratio Actual</th>
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<td>1: 4.5: 1</td>
<td>1: 2: 1</td>
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<td>Sno</td>
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<td>TR/LZ + Sno</td>
<td>0.87</td>
<td>1: 2: 1</td>
<td>1: 2.3: 1.3</td>
</tr>
<tr>
<td>TR/LZ + Ski</td>
<td>1.0</td>
<td>1: 4.1: 1.2</td>
<td>1: 2: 1</td>
</tr>
<tr>
<td>TR/LZ + Sno</td>
<td>1.1</td>
<td>1: 2.7: 1</td>
<td>1: 2.1: 1.1</td>
</tr>
<tr>
<td>TR/LZ + Ski</td>
<td>0.77</td>
<td>1: 6: 1</td>
<td>1: 1.5: 0.6</td>
</tr>
</tbody>
</table>

Heterodimers are functional in vivo

Knowing that c-ski and sno are co-expressed in most cells and having shown that SnoN and Ski preferentially heterodimerize in vitro, we wanted to see if they form functional heterodimers in vivo. We chose not to perform co-IP experiments because a positive result would show only that the two proteins form heterodimers intracellularly but would not provide evidence of heterodimer function. Because each protein individually is able to transform CEFs, induce myogenesis in QEFs and repress transcription via the GTCT binding site, these assays cannot be used to assess the functionality of SnoN:Ski heterodimers. However, both c-Ski and SnoN bind the GTCT site (15) and our previous work showed that c-Ski binds this site as a dimer. It therefore appeared likely that we could detect DNA binding by Ski:Sno heterodimers by using a probe with one copy of this binding site in EMSAs combined with antibody supershifts. This is a reasonable way to determine not only that heterodimers exist in vivo but that they are functional DNA binding proteins.

To perform these EMSAs required co-expression of Ski and SnoN proteins in chicken fibroblasts because these cells provide the necessary co-binding proteins. To co-express the proteins, we first infected DF1 chicken fibroblasts with PBabe-puro-c-Ski recombinant retroviruses produced by PA317 amphotropic packaging cells. To introduce snoN, puromycin-resistant DF1(c-Ski) cells or uninfected DF1 cells were infected with a chicken retrovirus expressing EE-SnoN [RCASBP(A)-EE-SnoN]. DF1 cells expressing both c-Ski and EE-SnoN, only c-Ski or only EE-SnoN, as verified by western analysis (Fig. 6A), were used to prepare nuclear extracts.

EMSAs performed with extracts of singly or doubly infected DF1 cells and a 32P-labeled oligonucleotide containing a single copy of the GTCTAGAC binding site are shown in Figure 6B. A single complex was formed with the EE-SnoN extract that was much stronger than a similar complex detected with the c-Ski extract (compare lanes 1 and 7). This difference is consistent with the western analysis that showed that EE-SnoN expression was about five times higher than c-Ski (Fig. 6A). The complex in lane 7 was not supershifted by the EE mAb (lane 8) but was shifted by the M6 Ski-specific mAb (lane 9), establishing that this complex contained a Ski homodimer. Addition of the EE mAb to the EE-SnoN EMSA produced a supershifted complex containing the vast majority of the EE-SnoN complex (lane 2). The c-Ski-specific M6 mAb supershifted only ~10% of the complex (lane 3). Since virtually all of the complex was shifted by the EE mAb and therefore contained EE-SnoN, the fraction supershifted by M6 mAb apparently represented a complex formed by heterodimers of EE-SnoN and endogenous c-Ski.

The results obtained with the extract from EE-SnoN/c-Ski co-infected cells support this conclusion. The complex produced in the absence of Ab (lane 4) was supershifted almost completely by the EE mAb (lane 5). When the M6 mAb was used, the resulting supershifted complex now accounted for almost 50% of the shifted species (lane 6). Because most of the complex contained EE-SnoN, the majority of the M6 supershifted complex...
be approached by co-expressing the Sno:Ski dimer has transforming activity. This question cannot be answered by co-expressing proteins. We were therefore very interested in knowing whether the N-terminal transformation domain of Ski (7) was fused to a nuclear localization signal. It was derived from a region of Ski (amino acids 305–326) that is protease sensitive (26) and is therefore likely to be flexible and long enough for a face to face positioning of the two monomers. We suspected that this would be the orientation because both segments contain the upstream dimerization domain (residues 292–367 in SnoN and 226–301 in Sno).

Figure 6. Co-expressed c-Ski:SnoN heterodimers bind to the GTCT element. (A) Western analysis of nuclear extracts of DF1 CEFs infected with either pBABEpuro-c-Ski, RCASBP-EESnoN or both viruses was performed as described in Materials and Methods. The level of c-Ski expression is ~20% that of SnoN. (B) EMSAs and antibody supershifts with a 32P-labeled oligonucleotide probe containing a single copy of the GTCTAGAC binding site were performed as described in Materials and Methods. Antibody supershifts were performed with affinity-purified mAbs. M6 is specific for c-Ski; EE is specific for the Glu-Glu epitope tag on EE-SnoN.

Biological activity of the Sno:Ski heterodimer

All of the data presented suggest that heterodimers of Ski and Sno are likely to be the dominant functional intracellular form of these proteins. We were therefore very interested in knowing whether the Sno:Ski dimer has transforming activity. This question cannot be approached by co-expressing sno and ski because this would result in the production of not only the Sno:Ski heterodimer, but also the Sno:Sno and Ski:Ski homodimers. Because each of the homodimers is capable of cellular transformation, it is obvious that with co-expressed proteins it would be difficult to clearly attribute any activity to the Sno:Ski heterodimer. To overcome this problem, we engineered a tethered Ski:Sno dimer in which the N-terminal transformation domain of Ski (7) was fused to a homologous N-terminal domain of SnoN. As controls we also generated constructs in which Ski and Sno were each linked in tandem to themselves. However, we could not detect expression of the Sno:Sno tethered form so only the results with the Ski:Ski dimer are included here.

The sequence that links the tethered monomers contained a nuclear localization signal. It was derived from a region of Ski (amino acids 305–326) that is protease sensitive (26) and is therefore likely to be flexible and long enough for a face to face positioning of the two monomers. We suspected that this would be the orientation because both segments contain the upstream dimerization domain (residues 292–367 in SnoN and 226–301 in Sno).

Figure 7. The upstream dimerization domain of Ski binds both Ski and SnoN. The indicated segments of v-Ski were expressed as GST fusion proteins in bacteria, purified and used in GST pull-down assays with 35S-labeled, in vitro translated v-Ski or SnoN as described in Materials and Methods. Input and bound proteins were detected by fluorography after SDS–PAGE (7.5%). Lane 1 contains 10% of the amount of labeled proteins used in the binding assays given in lanes 2–6; lane 2, GST; lane 3, GST–Ski(1–137;204–213); lane 4, GST–Ski(1–137;204–324); lane 5, GST–Ski(325–458); lane 6, GST–v-Ski(1–435).

Confident that the tethered dimers could associate as expected, we tested their ability to transform CEFs by forced expression via RCASBP(A) recombinant viruses. The results given in Figure 8A show that the tethered Ski:Sno heterodimer was extremely potent in cellular transformation. The agar cloning efficiency of Ski:Sno-transformed cells (11%) was ∼2.5–4 times higher than that of full-length c-Ski or SnoN alone and >10 times higher than that of the truncated monomeric forms. Because retrovirally over-expressed c-Ski or SnoN exist predominantly as homodimers in cells (15), this result suggests that the Ski:Sno heterodimer is a more potent transforming agent than either Ski:Ski or Sno:Sno homodimers. This conclusion is bolstered by the fact that agar colony formation induced by the Ski:Ski tethered dimer was less than half that of the Ski:Sno form.

Previous results have suggested that transformation by Ski and Sno is quite sensitive to their levels of expression. To determine whether the potent transforming ability of the Ski:Sno tethered dimer might be explained by its relative over-expression, we compared protein expression by western analysis. As shown in Figure 8B, the Ski:Sno tethered form is not expressed at higher levels than half that of the Ski:Sno form.
levels than any of the other forms. Thus the enhanced transforming ability of the Ski:Sno dimer is not due to high expression but is an inherent property of the protein.

**DISCUSSION**

We report here that the sequence requirements for efficient dimerization of SnoN are somewhat different than for c-Ski. Homodimerization of Ski requires a C-terminal bipartite domain consisting of a TR region and a LZ (7,8). These two domains form an extended α-helix that results in a high affinity parallel coiled coil interaction between the two nonomers. Formation of high affinity homodimers by SnoN involves a tripartite domain that includes not only its TR and LZ domains, but also an upstream region in the segment between residues 292 and 367. This region of SnoN shares 50% amino acid sequence identity with a region in the core transforming domain of v-Ski (dn2) that is involved in v-Ski self-association (unpublished data). Within this region, the proteins share a predicted amphipathic α-helix (28) that could provide a hydrophobic surface for protein–protein interaction.

Our results show that high affinity dimerization of SnoN is important for cellular transformation and transcriptional repression. Deletion of SnoN’s LZ domain reduces high affinity dimerization and diminishes both its transforming efficiency and its repression activity. This effect on dimerization is in agreement with previously published work on c-Ski showing that removal of the LZ results in an increase in the $K_d$ by almost two orders of magnitude (7). On the other hand, it is surprising that SnoN (1–432), which lacks the TR/LZ domain and is completely defective in high affinity dimerization, is not a less active transforming agent than Sno-LZ. This result is reminiscent of the comparison of v-Ski and c-Ski. v-Ski, which lacks the TR/LZ and dimerizes only at relatively high concentration, is nonetheless only marginally less potent as a transforming protein and one-third less potent as a repressor than c-Ski. However, as would be predicted, if dimerization ability is related to transforming potency, v-Ski must be expressed at relatively high levels to induce morphological transformation.

The differences between the transforming abilities of v-Ski and c-Ski can be overcome by a 5-fold change in their relative expression levels (21). Although dimerization domain deletions of both Ski and SnoN reduce their biological activity, the magnitude of the reduction in repression and transformation activity is not a simple function of the reduction in high affinity dimerization as measured in these studies. Despite the lack of a simple relationship, the present work confirms the importance of efficient dimerization in achieving maximal transformation and repression and extends this model to the activity of SnoN.

In this work we also show that SnoN and c-Ski form heterodimers through the association of their TR/LZ domains. It is likely that this interaction produces a bipartite coiled coil similar to that demonstrated for Ski homodimers (7). Heterodimers of SnoN and Ski are shown to form preferentially compared with formation of the respective homodimers. Thus the affinity of these proteins for each other is very high, with a $K_d$ in the nanomolar range based on the measured value for c-Ski homodimer formation (7). It is interesting that the preference for heterodimer formation is mediated by the TR domain because the SnoN and Ski sequences of this region are more distantly related (29% identity) than those of the LZ domains (51% identity).

Our results show that SnoN and Ski form heterodimers in vivo that bind to the specific GTCTAGAC DNA sequence. In addition, using a single chain SnoN:c-Ski heterodimer, we find that this protein transforms CEFs more dramatically than either wild-type SnoN or c-Ski. This is of interest especially when examining the overlapping expression patterns of these two genes in most tissues and in murine embryos (11,17,18). Transcripts of both c-ski and sno are seen at day 9.5 in neural tissues and neural crest cells and they rise during days 11.5–14.5 p.c. in developing brain and in skeletal muscle of the body wall and limbs. In addition, the two genes are co-expressed in all adult tissues examined. The preferential high affinity interaction between Ski and SnoN demonstrated in this work predicts that, when both proteins are expressed even at low concentrations, the majority will exist as heterodimers. In cases where the expression of either SnoN or c-Ski increases disproportionately, the respective homodimer would predominate. In fact, sno expression has been shown to be induced in both fibroblasts and myoblasts under conditions which do not alter c-ski expression (13,19).

A model that ascribes a unique role for heterodimers versus the respective homodimers is not uncommon. The Fos protein, which does not form homodimers efficiently and therefore fails to bind DNA detectably, can preferentially form heterodimers with Jun. The Fos:Jun heterodimer binds DNA efficiently and has a role in transcriptional regulation of a variety of genes (29,30). The Myc family of proteins also form heterodimers and homodimers that bind the same DNA site with different efficiencies and different outcomes (31–35). Myc forms homodimers very poorly and only binds DNA at very high Myc concentrations (36–38). The Myc:Max heterodimer binds DNA efficiently and is a transcriptional activator. The Mad:Max and Max:Max dimers also bind DNA well but function as transcriptional repressors (39). Changes in the ratios of these proteins can therefore act like a switch and this could also be true for SnoN and Ski. The biological role of the SnoN:Ski heterodimer versus the respective homodimers will be the subject of future work.

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REFERENCES