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Requirement for the SnoN Oncoprotein in Transforming Growth Factor β-Induced Oncogenic Transformation of Fibroblast Cells

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Transforming growth factor β (TGF-β) was originally identified by virtue of its ability to induce transformation of the AKR-2B and NRK fibroblasts but was later found to be a potent inhibitor of the growth of epithelial, endothelial, and lymphoid cells. Although the growth-inhibitory pathway of TGF-β mediated by the Smad proteins is well studied, the signaling pathway leading to the transforming activity of TGF-β in fibroblasts is not well understood. Here we show that SnoN, a member of the Ski family of oncoproteins, is required for TGF-β-induced proliferation and transformation of AKR-2B and NRK fibroblasts. TGF-β induces upregulation of *snoN* expression in both epithelial cells and fibroblasts through a common Smad-dependent mechanism. However, a strong and prolonged activation of *snoN* transcription that lasts for 8 to 24 h is detected only in these two fibroblast lines. This prolonged induction is mediated by Smad2 and appears to play an important role in the transformation of both AKR-2B and NRK cells. Reduction of *snoN* expression by small interfering RNA or shortening of the duration of *snoN* induction by a pharmacological inhibitor impaired TGF-β-induced anchorage-independent growth of AKR-2B cells. Interestingly, Smad2 and Smad3 play opposite roles in regulating *snoN* expression in both fibroblasts and epithelial cells. The Smad2/Smad4 complex activates *snoN* transcription by direct binding to the TGF-β-responsive element in the *snoN* promoter, while the Smad3/Smad4 complex inhibits it through a novel Smad inhibitory site. Mutations of Smad4 that render it defective in heterodimerization with Smad3, which are found in many human cancers, convert the activity of Smad3 on the *snoN* promoter from inhibitory to stimulatory, resulting in increased *snoN* expression in cancer cells. Thus, we demonstrate a novel role of SnoN in the transforming activity of TGF-β in fibroblasts and also uncovered a mechanism for the elevated SnoN expression in some human cancer cells.

Transforming growth factor β (TGF-β) inhibits the growth of epithelial, endothelial, and lymphoid cells but promotes proliferation of fibroblast cells (29, 36, 37, 51). Although this growth-promoting activity of TGF-β has been demonstrated in many fibroblast cell lines, its ability to induce oncogenic transformation has been shown only in murine AKR-2B and normal rat kidney (NRK) fibroblast cells (29, 37). The intracellular signal transduction pathways that lead to TGF-β-induced proliferation or transformation of these fibroblasts are not well understood. A recent report identified PAK2 as a mediator of TGF-β-induced transformation of fibroblasts in a Smad-independent pathway (53). However, the Smad-dependent pathway that mediates this activity has not been characterized. The Smad proteins are critical mediators of TGF-β signaling in both epithelial cells and fibroblasts (1, 10, 17, 27). Upon binding of TGF-β1, the type I TGF-β receptor kinase (TβRI) becomes activated by the type II receptor kinase and phosphorylates two highly related cytoplasmic Smad proteins, Smad2 and Smad3. The phosphorylated Smad2 and Smad3 then form heteromeric complexes with Smad4 and translocate into the nucleus. The heteromeric Smad complexes can bind to the Smad-binding element [SBE; CAG(A/T)CTG] or certain GC-rich sequences present on TGF-β-responsive promoters either alone or in collaboration with sequence-specific DNA binding cofactors such as the FoxH family of proteins (10, 17, 41, 49). Through interaction with transcriptional coactivators or corepressors on the TGF-β-responsive promoters, Smad complexes can activate or inhibit transcription of TGF-β target genes (10, 27).

Although Smad2 and Smad3 are highly homologous and can function cooperatively to activate the expression of some TGF-β-responsive genes including p15, p21, and PAI-1 (8, 13, 30, 44, 46), they also exhibit important structural and functional differences. Smad3, but not Smad2, binds DNA through its Mad homology 1 (MH1) domain. In addition, Smad3 has been shown to interact with many transcriptional factors, while the number of transcriptional factors bound to Smad2 may be more limited (27). Finally, Smad3, but not Smad2, has been shown to repress transcription of certain genes (12, 14, 20, 22, 23, 52).

The activity of the Smad proteins can be modulated by interaction with various cellular proteins (41, 49). We and others have previously identified the SnoN oncoprotein as an important negative regulator of the TGF-β pathway (47, 48). SnoN interacts directly with Smad2, Smad3, and Smad4 and represses their abilities to activate TGF-β target genes by disrupting the formation of an active heteromeric Smad complex, recruiting a transcription corepressor complex to the TGF-β-responsive promoters, and blocking the binding of transcriptional coactivator p300/CBP (16, 54). Overexpression of SnoN markedly impairs the ability of cells to undergo growth inhibition in response to TGF-β (47).

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SnoN is a member of the Ski family of oncoproteins. It was identified based on sequence homology with v-Ski, the transforming protein of the Sloan-Kettering virus (32–34). SnoN contains oncogenic activity because when overexpressed, it induces anchorage-independent growth of chicken and quail embryo fibroblasts (5). Elevated SnoN expression has also been detected in many types of human cancer (18, 32, 33, 56). Although the mechanism by which SnoN regulates cell transformation has not been fully understood, at least in chicken embryo fibroblasts, the ability of SnoN to antagonize TGF-β signaling may be required for its transforming activity. A mutant SnoN defective in binding to the Smad proteins failed to block TGF-β-induced growth arrest and did not induce oncogenic transformation of chicken embryo cells (16).

The expression of SnoN in normal cells and tissues appears to be tightly regulated. SnoN is expressed in most embryonic and postnatal tissues, albeit at a low level. Upregulation of SnoN expression occurs during certain stages of embryonic development in tissues with a high degree of proliferative activity (35), as well as in adult cancer cells, including those derived from breast cancer, esophageal cancer, lung cancer, and rhabdomyosarcoma (18, 32, 33, 56). The expression of SnoN has been shown to be regulated at the levels of gene amplification, transcriptional activation, and protein stability (3, 18, 45, 47). In esophageal cancer (18) and in many ovarian cancer cells (J. Gray, personal communication), amplification of the 3q26 region of the human chromosome that contains the *snoN* gene has been detected, and this may partially account for the increased production of SnoN. In many lung cancer cells, the increase in *snoN* expression occurs at the level of transcription (Q. Zhu and K. Luo, unpublished observation). We have shown previously that TGF-β is a potent regulator of SnoN expression at both the level of protein stability and the level of transcriptional activation. Within 30 min of TGF-β stimulation, SnoN is rapidly degraded by the ubiquitin-dependent proteasome in a Smad3-dependent manner, allowing activation of TGF-β target genes (3, 45, 48). Two hours after TGF-β addition, a marked increase in levels of both *snoN* mRNA and protein can be detected (47). The functional significance of this later phase of increased SnoN expression has not been determined. It may function to shut down Smad activity in a negative-feedback manner. It may also serve additional functions in regulating cellular growth and differentiation in a Smad-independent manner.

In this study, we investigated the molecular mechanism by which TGF-β activates *snoN* expression and the functional significance of this upregulation in the transforming activity of TGF-β. We show that activation of *snoN* transcription by TGF-β requires the binding of the Smad proteins to the *snoN* promoter, and we have determined the *cis*- and *trans*-acting elements involved. More importantly, we provide evidence that prolonged upregulation of SnoN expression plays a critical role in TGF-β-induced oncogenic transformation of fibroblast cells.

MATERIALS AND METHODS

Cell culture, antisera, and constructs. Mouse NIH 3T3 fibroblasts, NRK fibroblasts, and the rat intestinal epithelial cell line RIE-1 (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Hep3B human hepatoma cells (ATCC) were maintained in minimal essential medium supplemented with 10% FBS. The mouse embryonic cell line AKR-2B (ATCC) was grown in McCoy's 5A medium supplemented with 10% FBS.

Antibodies for phospho-Smad2 and phospho-Smad3 were generous gifts from Aristidis Moustakas. Antisera against Smad3 (FL425 and I-20), Smad4 (C-20), Smad2 (S-20), SnoN (H317), and p300 (N15) were purchased from Santa Cruz Biotechnology. Antisera against Smad2 were purchased from BD, Transduction Laboratories. The antibody against hexamethylene bisacetamide-induced protein 1 (HEXIM1) was a gift from Qiang Zhou.

All reporter constructs containing deletions, truncations, or mutations in the *snoN* promoter were generated by PCR and cloned into the pGL_2 basic vector (Promega). All mutations were verified by sequencing.

Transfection and transcriptional reporter assays. Hep3B cells were transiently transfected with the reporter constructs using LipofectAMINE Plus (Invitrogen) (16). For luciferase assays, a total of 2.5 μg of DNA (0.5 μg of the luciferase reporter construct and 2 μg of Smad proteins) was used for each transfection. Luciferase activity was measured 48 h later.

Northern blotting. Hep3B, RIE-1, NIH 3T3, NRK, and AKR-2B cells were serum starved for 16 h and then treated with 200 pM TGF-β1 in the presence or absence of 100 μg/ml cycloheximide or 1 μg/ml actinomycin D for the indicated times. Total RNA was prepared from these cells using the RNeasy Total RNA kit (QIAGEN). Twenty micrograms of total RNA was resolved on a 1% formaldehyde gel, transferred to a nylon membrane, and analyzed by Northern blotting with a human or mouse *snoN* cDNA probe. All of the *snoN* bands in the Northern blots were quantified by Image J.

DNase I footprinting. A DNase I footprinting reaction was performed as described in the protocol of the "Core Footprinting System" (Promega). One microgram of a fusion protein consisting of glutathione *S*-transferase (GST) and Smad3 or Smad4 was preincubated on ice for 10 min in binding buffer (50 mM Tris, pH 8.0, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol), following by a 20-min incubation on ice with a ^{32}P-labeled DNA probe (1×10^6 cpm/μg). The samples were then treated with 5 μl DNase I (1 U/μl; Promega) for exactly 2 min at room temperature and analyzed on a 6% denaturing polyacrylamide gel. The relative positions of the protected bands on the gel were determined by a G reaction using the protocol of Maxam and Gilbert (28).

EMSA. DNA fragments containing regions of the *snoN* promoter between kb −2.8 and −2.4 (−2.8/−2.4) or between kb −2.4 and −2.0 (−2.4/−2.0) were amplified by PCR, end labeled with T4 polynucleotide kinase, and gel purified. The ability of recombinant Smad3, Smad4, or nuclear extracts to bind to these DNA fragments was analyzed by electrophoretic mobility shift assays (EMSA) as described previously (15, 46).

ChIP. Hep3B cells were stimulated with 200 pM TGF-β1 and cross-linked with formaldehyde for 10 min, and chromatin immunoprecipitation (ChIP) was carried out as described previously (4) with the following modifications. The chromatin fractions from 2×10^7 cells were precleared with protein A-Sepharose for 30 min at 4°C and then subjected to immunoprecipitation with 5 μg specific antibodies (anti-Smad3 [I-20], -Smad4 [C-20], -Smad2 [S-20], and -p300 [N15]) at 4°C. For PCR, 3 μl from a 30-μl immunoprecipitate or 3 μl of a 1:30 dilution from a 60-μl input sample was used as a template. The primer pairs used to amplify TGF-β-responsive regions of the *snoN* and PAI-1 promoters are as follows: for the *snoN* promoter, 5′-CTCGAGGAAGGAAGGAGGG-3′ (forward) and 5′-TACACACAGCCTCTGACGTC-3′ (reverse); for the PAI-1 promoter, 5′-CCAGCACACCTCCAACCTC-3′ (forward) and 5′-CCCTGCAGCC AAACACAGC-3′ (reverse).

RNA interference. Small interfering RNAs (siRNAs) were designed to specifically target either murine *snoN*, murine *smad3*, or human *smad3* and *smad2* in accordance with the guidelines provided (OligoEngine). Oligonucleotides carrying the siRNAs are as follows (sense strands shown): murine *snoN*, 5′-CTCCA TTCTGCAGAGGAAG-3′; human *smad3*-a, 5′-CGTCAACACCAAGTGCAT C3′; human *smad3*-b, 5′-GGCCATCACCCAGAAC-3′; human *smad2*, 5′-ACA GGACGATTAGATGAGC-3′; negative-control sequence, 5′-CACCTGTACA GTTGCTAGT-3′. The oligonucleotide pairs were cloned into the pSUPER vector (OligoEngine).

Generation of AKR-2B cell lines stably expressing a siRNA. Oligonucleotide pairs encoding the siRNA for murine *snoN* or an unrelated sequence in the pSUPER.retro.puro vector (OligoEngine) were transfected into Phoenix-Eco cells with LipofectAMINE Plus (Invitrogen). Forty-eight hours posttransfection, a total of 3×10^5 AKR-2B cells were infected with the viral supernatant. Forty-eight hours later, the infected cells were switched into a medium containing 1.5 μg/ml puromycin to start selection. To confirm the expression of the siRNA, individual clones of infected cells were treated with 100 pM TGF-β1 for 1 h. Total RNA was extracted from these cells, and the levels of *snoN* mRNA were analyzed by Northern blotting.

Soft-agar assay. A 2-ml volume of growth medium containing 0.66% Bacto agar (Difco) was poured into a 6-well dish to form the bottom layer. A total of 5,000 AKR-2B cells or 50,000 NRK cells were suspended in 2 ml of medium containing 0.4% agar and 2 nM (for AKR-2B cells) or 10 ng/ml (for NRK cells) epidermal growth factor (EGF), with or without 100 pM TGF-β1, and this suspension was overlaid on the hardened bottom layer. A 1-ml volume of fresh medium containing 0.4% agar and 2 nM or 10 ng/ml EGF, with or without 100 pM TGF-β1, was added to the dish every week. After 3 weeks of incubation, colonies were visualized by staining with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) and scanned on a Hewlett-Packard ScanJet 5500C to visualize the colonies. For treatment with the TβRI inhibitor SB-431542, 1 ml of medium with or without 10 μM SB-431542 (Tocris) was added on top of the soft agar at the indicated time point post-TGF-β1 addition. The medium was replaced every 2 days after weekly soft-agar feeding.

Immunoprecipitation and Western blotting. Endogenous Smads were analyzed by Western blotting as described previously (26). The densities of bands in Western blots were quantified by Image J. For immunoprecipitation of endogenous SnoN, a polyclonal antibody to SnoN was raised against a peptide located at the N terminus of human SnoN (DDGSPPAKKMITDIH). The anti-peptide antibody was covalently coupled to protein A-Sepharose and incubated with nuclear extracts from AKR-2B cells stimulated with or without TGF-β1. SnoN was analyzed by Western blotting with anti-SnoN (Santa Cruz).

RESULTS

TGF-β induces prolonged *snoN* expression in two fibroblast cell lines that are transformed by TGF-β1. We have shown previously that in Hep3B cells, TGF-β induces a marked increase in *snoN* gene expression within 2 hours of stimulation (47). To determine whether this is a general phenomenon that also occurs in other cell types, especially in AKR-2B and NRK fibroblasts that are transformed by TGF-β either alone or together with EGF (29, 37), a time course of TGF-β1 treatment was performed for several cell lines including two epithelial cell lines, RIE-1 (Fig. 1A) and HaCaT (data not shown), and three fibroblast cell lines, NIH 3T3, NRK, and AKR-2B (Fig. 1A). Like Hep3B cells, RIE-1 and HaCaT cells undergo cell cycle arrest in response to TGF-β. In contrast, the growth of the three fibroblast cell lines is stimulated by TGF-β. Total RNA was prepared from these cells, and the levels of *snoN* mRNA were measured by Northern blotting. As shown in Fig. 1A, induction of *snoN* mRNA by TGF-β was observed in all cell lines. The increase in *snoN* mRNA levels occurred within 1 h of TGF-β1 stimulation and peaked by 2 h. However, the duration and magnitude of the induction differ from cell line to cell line. The *snoN* mRNA level remained high for at least 8 to 24 h in the two TGF-β-transformed fibroblast lines, while it rapidly decreased within 2 to 4 h in NIH 3T3 cells and within 4 to 6 h in the epithelial cell lines (Fig. 1A and data not shown). Interestingly, the strongest and longest induction of *snoN* expression was observed in AKR-2B cells, which display a strong transforming phenotype in response to TGF-β (as evidenced by the formation of large numbers of fast-growing colonies on soft agar). In NRK cells, where TGF-β induces a moderate level of transformation, the duration of induction of *snoN* expression was correspondingly shorter.

To confirm that the upregulation of *snoN* mRNA resulted in an increase in SnoN protein, Western blot analysis was carried out using an antibody that recognizes mouse SnoN. In AKR-2B cells, TGF-β induced an increase in the SnoN protein level at 2 h after stimulation, and the SnoN level remained high for at least 8 h after stimulation (Fig. 1B). Taken together, our data indicate that induction of *snoN* expression by TGF-β occurs in all cell lines examined, but only those cell lines that are transformed by TGF-β appeared to exhibit a stronger and prolonged induction.

TGF-β activates *snoN* gene expression at the level of transcription. To examine whether the induction of *snoN* expression, especially at later time points, in AKR-2B and NRK cells requires protein synthesis, cells were pretreated with the protein synthesis inhibitor cycloheximide. The induction of *snoN* gene expression by TGF-β in either AKR-2B (Fig. 1C) or Hep3B and NRK (data not shown) cells in the presence or absence of cycloheximide was then examined by Northern blotting. As shown in Fig. 1C, pretreatment with cycloheximide did not affect the levels of *snoN* mRNA at any time after TGF-β stimulation in AKR-2B cells or other cell lines (data not shown), indicating that this upregulation is due to direct activation of TGF-β signaling pathways.

We next examined *snoN* mRNA expression following TGF-β stimulation in the absence or presence of the transcription inhibitor actinomycin D. As shown in Fig. 1D, induction of *snoN* expression was effectively blocked by actinomycin D, suggesting that TGF-β activates *snoN* expression mainly at the level of transcription. To determine whether alteration in the stability of SnoN contributes to the prolonged induction of SnoN, AKR-2B cells were treated with TGF-β1 for 2 h to induce SnoN expression. Cycloheximide was then added, and the treatment (with both TGF-β1 and cycloheximide) was allowed to continue for another 6 h. As shown in Fig. 1E, cycloheximide completely blocked SnoN induction by TGF-β after 8 h, suggesting that induction of SnoN expression is entirely due to the increase in SnoN synthesis. Consistent with this, in a pulse-chase assay, the half-life of TGF-β-induced, newly synthesized SnoN was not altered after 6 additional hours of TGF-β treatment in AKR-2B cells (data not shown).

To map the TGF-β-responsive elements in the *snoN* promoter, a 4.8-kb murine *snoN* genomic DNA fragment containing sequences immediate upstream of the translation start site was cloned upstream of the luciferase reporter gene. When transfected into Hep3B or AKR-2B cells, this 4.8-kb fragment was sufficient to mediate TGF-β-induced transcriptional activation (Fig. 1F). Because Hep3B cells are easier to transfect than AKR-2B cells, we carried out the major part of the promoter-mapping experiments with Hep3B cells. The key experiments were later repeated with AKR-2B cells.

A series of 5′ deletion mutants of the *snoN* promoter was generated from the 4.8-kb *snoN* genomic fragment and tested for ability to mediate TGF-β responses (Fig. 1G). Deletions of sequences from kb −4.8 to −2.8 had no effect on TGF-β-induced transcriptional activation of the *snoN* promoter (Fig. 1G). However, further deletions to kb −2.4 completely abrogated TGF-β responsiveness, suggesting that the TGF-β-responsive element is located between kb −2.8 and −2.4 of the *snoN* promoter. In agreement with this, when cloned upstream of a heterologous promoter, this 400-bp fragment (sno2.8/2.4) was able to mediate TGF-β-induced activation of the reporter construct to an extent comparable to that exhibited by the natural 2.8-kb *snoN* promoter fragment (Fig. 1G).

TGF-β induces prolonged phosphorylation of Smad2 in AKR-2B and NRK cells. Since Smad proteins are critical mediators of TGF-β signaling, we next examined whether they are involved in TGF-β-induced *snoN* expression. For this purpose, the inhibitory Smad, Smad7, was cotransfected with sno2.8 into

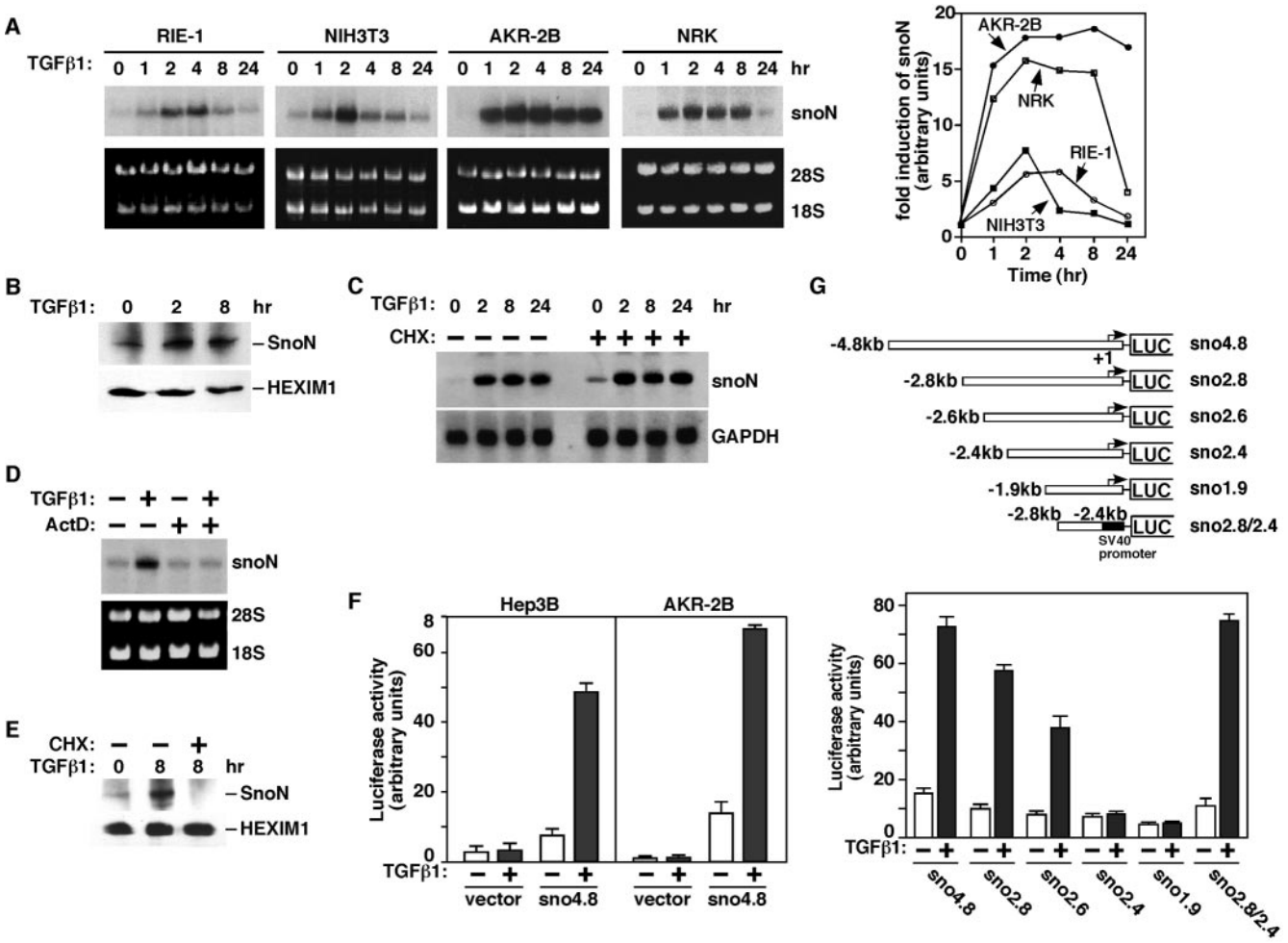

FIG. 1. TGF-β induces a prolonged upregulation of *snoN* expression in AKR-2B and NRK fibroblasts at the level of transcription. (A) TGF-β induces *snoN* gene expression in multiple cell types. RIE-1, NIH 3T3, NRK, and AKR-2B cells were serum starved for 16 h and stimulated with 100 pM TGF-β1 for various periods of time as indicated. Total RNA was isolated from these cells, and *snoN* expression was analyzed by Northern blotting. 28S and 18S RNAs are shown as loading controls. Quantification of the Northern blot was carried out using Image J. The density of the *snoN* signal was normalized to the density of 18S RNA, and the induction of *snoN* expression by TGF-β in each cell line is shown in the graph. (B) TGF-β upregulates SnoN protein expression in AKR-2B cells. AKR-2B cells were serum starved for 20 h and stimulated with 100 pM TGF-β1 for various amounts of time as indicated. Nuclear extracts were prepared from these cells, and endogenous SnoN was isolated by immunoprecipitation with an anti-SnoN peptide antiserum and analyzed by Western blotting with anti-SnoN (Santa Cruz). As a loading control, nuclear extracts were analyzed by Western blotting with an antibody against nuclear protein HEXIM1. (C) Protein synthesis is not required for TGF-β-induced expression of *snoN*. AKR-2B cells were treated with or without 100 μg/ml cycloheximide (CHX) in the absence or presence of 100 pM TGF-β1 at the indicated time points. *snoN* expression was analyzed by Northern blotting. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for equal loading. (D) TGF-β induces *snoN* gene expression at the transcriptional level. AKR-2B cells were treated with or without actinomycin D (ActD; 1 μg/ml) in the absence or presence of 100 pM TGF-β1. Fifteen micrograms of total RNA was analyzed by Northern blotting. (E) The prolonged induction of SnoN in AKR-2B cells is not due to reduction of protein degradation. AKR-2B cells were stimulated with TGF-β1 for 2 h followed by treatment with or without 100 μg/ml CHX in the presence of TGF-β1 for another 6 h. Endogenous SnoN was isolated and analyzed as described for panel B. HEXIM1 levels in the nuclear extracts were measured as a loading control. (F) TGF-β induces activation of the *snoN* promoter. Hep3B cells or AKR-2B cells were transfected with either the sno4.8 reporter plasmid, containing 4.8 kb of the *snoN* promoter sequence, or the pGL_2basic vector control and were stimulated with 50 pM TGF-β1 for 16 h. Luciferase activity was measured 48 h after transfection. (G) Identification of a TGF-β-responsive region in 4.8 kb of the *snoN* promoter. Hep3B cells were transfected with the luciferase reporter constructs listed in the diagram at the top and were stimulated without (open bars) or with (closed bars) 50 pM TGF-β1 for 16 h.

Hep3B cells. Overexpression of Smad7 efficiently blocked TGF-β-inducible transcription from the *snoN* promoter (Fig. 2A), suggesting that this upregulation is mediated by a Smad-dependent pathway.

To examine whether the difference in the duration of *snoN* induction reflects a difference in the activities of the Smad proteins, we next measured the relative amounts of active Smad2 and Smad3 in various cell lines. AKR-2B, NRK, NIH 3T3, and RIE-1 cells were treated with TGF-β1 for various periods. The phosphorylation of Smad2 and Smad3 was detected by Western blotting with antibodies specific for phospho-Smad2 or phospho-Smad3. In all cell lines, TGF-β1 in-

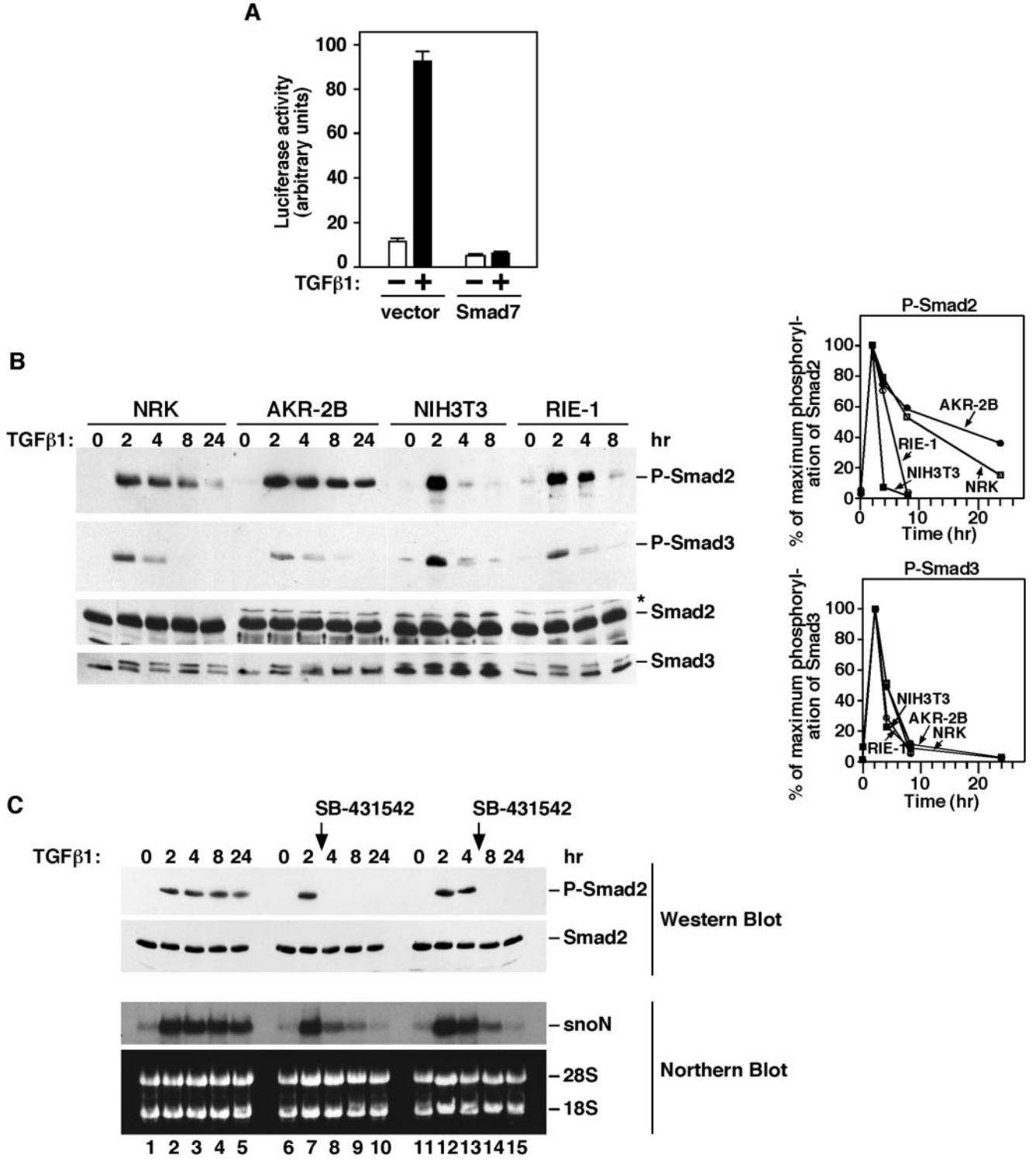

FIG. 2. Prolonged *snoN* expression depends on the activation of Smad2. (A) TGF-β-induced *snoN* expression is Smad dependent. Hep3B cells were transfected with 0.5 μg of sno2.8 alone or together with 2.0 μg of Smad7 and were stimulated without (open bars) or with (closed bars) 50 pM TGF-β for 16 h. (B) Phosphorylation of the R-Smads in response to TGF-β in various cell lines. Cells were treated with 100 pM TGF-β1 for the indicated periods of time. Equal amounts of total-cell lysates prepared from each sample were analyzed by Western blotting using antibodies against phospho-Smad2 (P-Smad2) (top panel), phospho-Smad3 (P-Smad3) (middle panel), or anti-Smad2 and anti-Smad3 (bottom panel). The intensities of phospho-Smad2 and phospho-Smad3 bands in the Western blots were quantified and calculated as percentages of the maximal level of phosphorylation upon TGF-β stimulation (at 2 h) (graphs). Asterisk indicates a nonspecific band recognized by the anti-Smad2 antibody. (C) AKR-2B cells were stimulated with 100 pM TGF-β1 for the indicated times. At 3 h (lanes 8 to 10) or 6 h (lanes 14 and 15) after TGF-β1 addition, dimethyl sulfoxide or 10 μM SB-431542 was added to the culture. Equal amounts of total-cell lysates were analyzed by Western blotting with anti-P-Smad2 or anti-Smad2 (top panels). The levels of *snoN* mRNA in these cells were analyzed by Northern blotting (bottom panels).

duced a rapid increase in phosphorylation of Smad2 and Smad3 (Fig. 2B). In NIH 3T3 and RIE-1 cells, levels of phospho-Smad2 reached a maximum at 1 h after stimulation and declined back to the basal level after 4 h (Fig. 2B), while levels of phospho-Smad2 remained high in AKR-2B and NRK cells for 8 to 24 h (Fig. 2B and C). In contrast, the duration of phospho-Smad3 was not significantly different in the four cell lines (Fig. 2B). This suggests that phosphorylation of Smad2 and phosphorylation of Smad3 are regulated separately in these cells and that a longer induction of *snoN* expression by TGF-β correlates with a prolonged phosphorylation and activation of Smad2.

To further strengthen the notion that the sustained activation of Smad2 is responsible for the prolonged induction of *snoN* expression by TGF-β in AKR-2B and NRK cells, a specific inhibitor of the TβRI kinase, SB-431542, was employed (7, 19, 21). AKR-2B (Fig. 2C) or NRK (data not shown) cells were stimulated with TGF-β for various periods. SB-431542 was added to the culture at 3 h or 6 h after TGF-β addition. The levels of phospho-Smad2 were then measured by Western blotting with anti-phospho-Smad2, and *snoN* expression was examined by Northern blotting. As shown in Fig. 2C, SB-431542 effectively shortened the duration of receptor-mediated phosphorylation of Smad2. Interestingly, the induction of *snoN* expression by TGF-β also showed a marked decrease upon addition of SB-431542, and the timing of the inhibition of *snoN* induction correlated with that of the inhibition of Smad2 phosphorylation (Fig. 2C and data not shown). This finding confirms that in AKR-2B and NRK-2B cells, the sustained Smad2 phosphorylation in response to TGF-β, instead of the stability of mRNA, is responsible for the prolonged induction of *snoN* expression. It also suggests that the sustained phosphorylation of Smad2 is likely due to the activity of TβRI kinase, not to a reduced phosphatase activity in these cells.

Binding of Smad complexes to the TGF-β-responsive elements is essential for TGF-β-induced activation of *snoN* transcription. Since induction of *snoN* expression correlated with activation of Smad2 (Fig. 2C), we next examined whether the Smad2/Smad4 complex binds directly to the 400-bp TGF-β-responsive region in the *snoN* promoter to activate *snoN* expression. To locate the binding sites of Smads within this TGF-β-responsive promoter region, DNase I footprinting was carried out using two 200-bp end-labeled DNA probes, containing sequences from kb −2.8 to −2.6 and from kb −2.6 to −2.4 of the *snoN* promoter, respectively. Recombinant Smad4 generated four protected regions within the 400-bp TGF-β-responsive region (Fig. 3A). All of them contain a CAGA or TCTG core sequence that was previously identified as the SBE (42). In an EMSA using the four Smad4-protected sequences as probes, Smad4 bound to all four SBEs (Fig. 3B). These binding activities were disrupted by point mutations that changed the core SBE sequences. Interestingly, although all four probes contain the same core SBE sequence, the affinities of Smads for the four regions differed, indicating that the flanking sequences of SBE also affect binding of the Smad proteins.

To determine the importance of these Smad binding elements in TGF-β-induced *snoN* expression, mutations altering the four SBEs either individually or together were introduced into the *snoN* promoter. In a luciferase assay (Fig. 3C), mutations of individual SBEs decreased TGF-β inducible transcription only slightly. In contrast, alteration of all SBEs markedly abrogated TGF-β-induced *snoN* transcription but did not affect the level of basal transcription. This indicates that all four Smad binding sites together mediate TGF-β-induced transcription of the *snoN* gene.

Finally, to confirm that this 400-bp TGF-β-responsive region in the *snoN* promoter mediates binding of the endogenous Smad proteins in vivo, ChIP assays were carried out. A pair of primers spanning the 400-bp TGF-β-responsive region was employed in the PCR to measure the endogenous proteins bound to this region of the *snoN* promoter. As an example of a typical TGF-β-responsive promoter, the plasminogen activator inhibitor type I (PAI-1) promoter was analyzed in parallel. Primers flanking the 800-bp fragment from the PAI-1 promoter that contains the TGF-β-responsive elements were included in the PCRs. In the absence of TGF-β, Smad4, as well as very low levels of R-Smads, was found to bind to the TGF-β-responsive regions of both *snoN* and PAI-1 promoters (Fig. 3D). Treatment of cells with TGF-β1 for 1 h resulted in an increase in the binding of Smad2 and the coactivator p300/CBP to the two promoter sequences. Surprisingly, association of Smad3 with the *snoN* promoter was not detected in the presence of TGF-β. In contrast, the binding of Smad3 to the PAI-1 promoter was dramatically increased by TGF-β treatment. Thus, Smad2 and Smad3 may play different roles in regulation of *snoN* transcription, and the effect of Smad3 on *snoN* gene expression may differ from that on the expression of typical TGF-β-responsive genes such as PAI-1.

Smad2 and Smad3 play opposite roles in regulation of *snoN* expression. To investigate the effects of Smad2 and Smad3 on the activation of *snoN* expression, Smad2 or Smad3 was cotransfected with Smad4 and the sno2.8 reporter into Hep3B or AKR-2B cells. As a control, pLUC800, which contains the 800-bp TGF-β-responsive element from the PAI-1 promoter, was tested in a parallel experiment. As shown in Fig. 4A, expression of Smad2 and Smad4 caused a marked increase in transcription from both the *snoN* and PAI-1 promoters, even in the absence of TGF-β1. Surprisingly, overexpression of Smad3 and Smad4 inhibited activation of the *snoN* promoter induced by TGF-β in both cell lines (Fig. 4A). This inhibition appears to be specific to the *snoN* promoter, because expression of Smad3 greatly activated transcription from the PAI-1 promoter both in the presence and in the absence of TGF-β. Thus, Smad2 enhances *snoN* gene expression, while Smad3 blocks it.

To further confirm this, we employed the siRNA duplex specific for either human Smad2 or human/murine Smad3 and introduced it into Hep3B or AKR-2B cells using the pSUPER vector (6). In transfected Hep3B or AKR-2B cells, the siRNA duplexes specifically reduced the expression of Smad proteins (Fig. 4B, bottom panels). When introduced together with sno2.8, the siRNA for Smad2 decreased *snoN* transcription in response to TGF-β, while expression of Smad3 siRNAs resulted in a marked increase in *snoN* transcription (Fig. 4B). As a control, expression of an unrelated siRNA did not affect the expression of Smad proteins or TGF-β-induced *snoN* transcription (Fig. 4B). Taken together, these results indicate that Smad2 and Smad3 exert different effects on *snoN* gene expression.

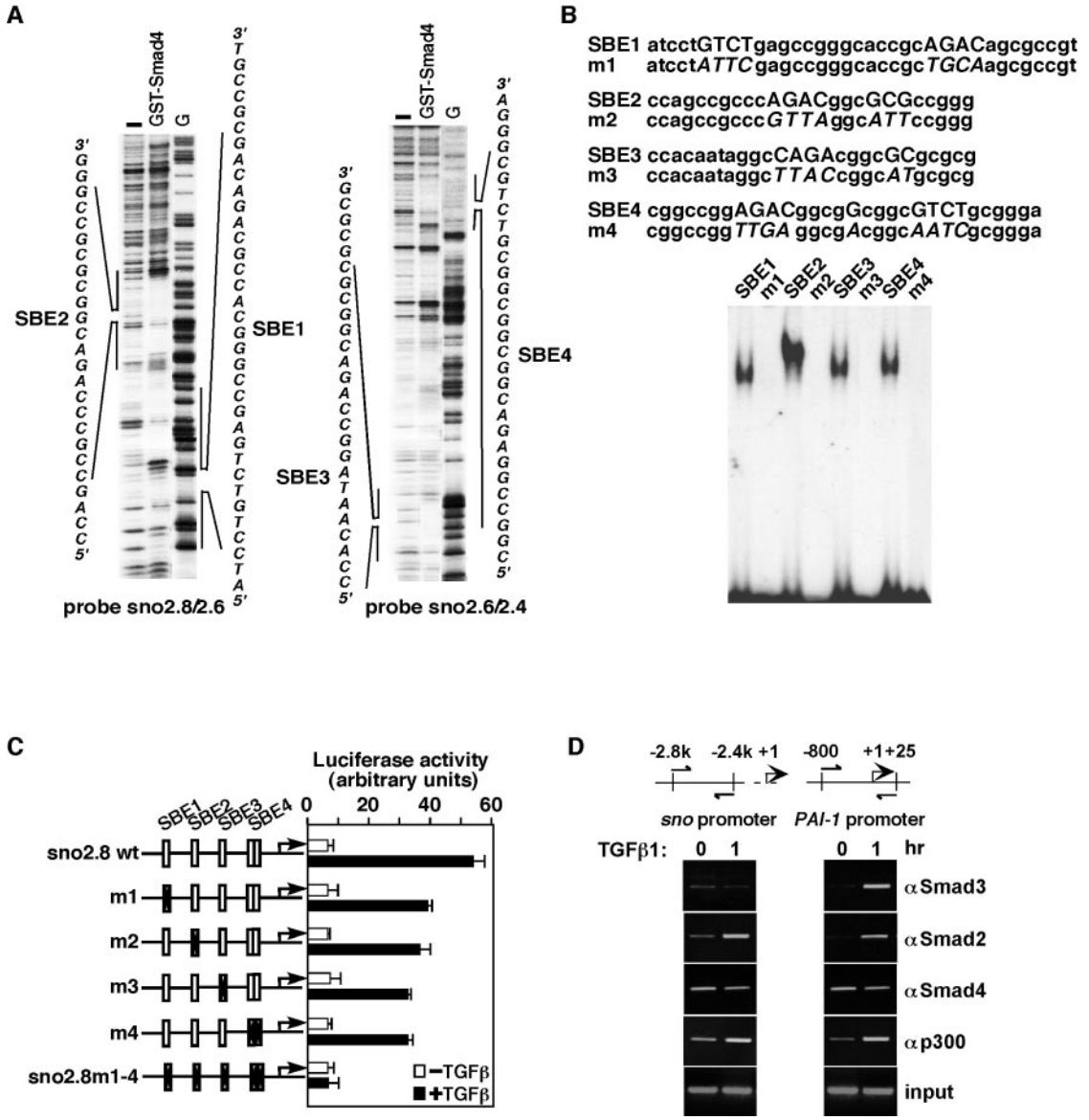

FIG. 3. Binding of Smad proteins to the *snoN* promoter is required for the activation of *snoN* transcription in response to TGF-β1. (A) DNase I footprinting assay. GST-Smad4 was incubated with the indicated ^{32}P-labeled *snoN* promoter fragments, followed by digestion with DNase I. The regions protected by Smad4 are labeled as SBE1, SBE2, SBE3, and SBE4. The nucleotide sequences of these protected regions were determined by the Maxam-Gilbert G sequencing method. (B) EMSA. (Top) Oligonucleotide probes containing wild-type and mutant Smad binding sites (SBE1 to -4). The WT SBE motifs are capitalized and boldfaced. The mutant sequences are italicized. (Bottom) GST-Smad4 (1 μg) was incubated with ^{32}P-labeled probes in an EMSA. Protein/DNA-binding complexes were visualized by autoradiography. (C) WT or mutant *snoN* promoter fragments containing mutations in the SBE motifs were cloned into the pGL$_2$ basic vector and transfected into Hep3B cells. Luciferase activity was measured 16 h after TGF-β stimulation. (D) Chromatin immunoprecipitation assay. Soluble chromatin was prepared from Hep3B cells treated with or without TGF-β1 for 1 h. The chromatin immunoprecipitation assay was carried out using various antibodies as indicated. Promoter fragments present in the immunoprecipitates were amplified by PCR using pairs of primers that cover the TGF-β-responsive regions in the *snoN* and PAI-1 promoters as indicated at the top and detected by agarose gel electrophoresis.

The DNA binding activity and oligomerization of Smad3 are required for inhibition of TGF-β-induced *snoN* transcription by Smad3. In order to understand how Smad3 negatively regulates *snoN* gene expression, we next examined whether the DNA binding and oligomerization activities of Smad3 are required for this inhibition. A mutant Smad3 (R74A) deficient in binding to DNA (42) was cotransfected with Smad4 and sno2.8 into Hep3B cells. While wild-type (WT) Smad3 effectively blocked activation of *snoN* transcription by TGF-β, Smad3R74A failed to do so (Fig. 4C). Not only was this mutant Smad3 incapable of inhibiting *snoN* expression; it actually activated transcription both in the absence and in the presence of TGF-β, as did Smad2 (Fig. 4C). This result suggests that the DNA binding ability of Smad3 is critical to its inhibitory activity.

Recently, an alternatively spliced form of Smad2 that lacks

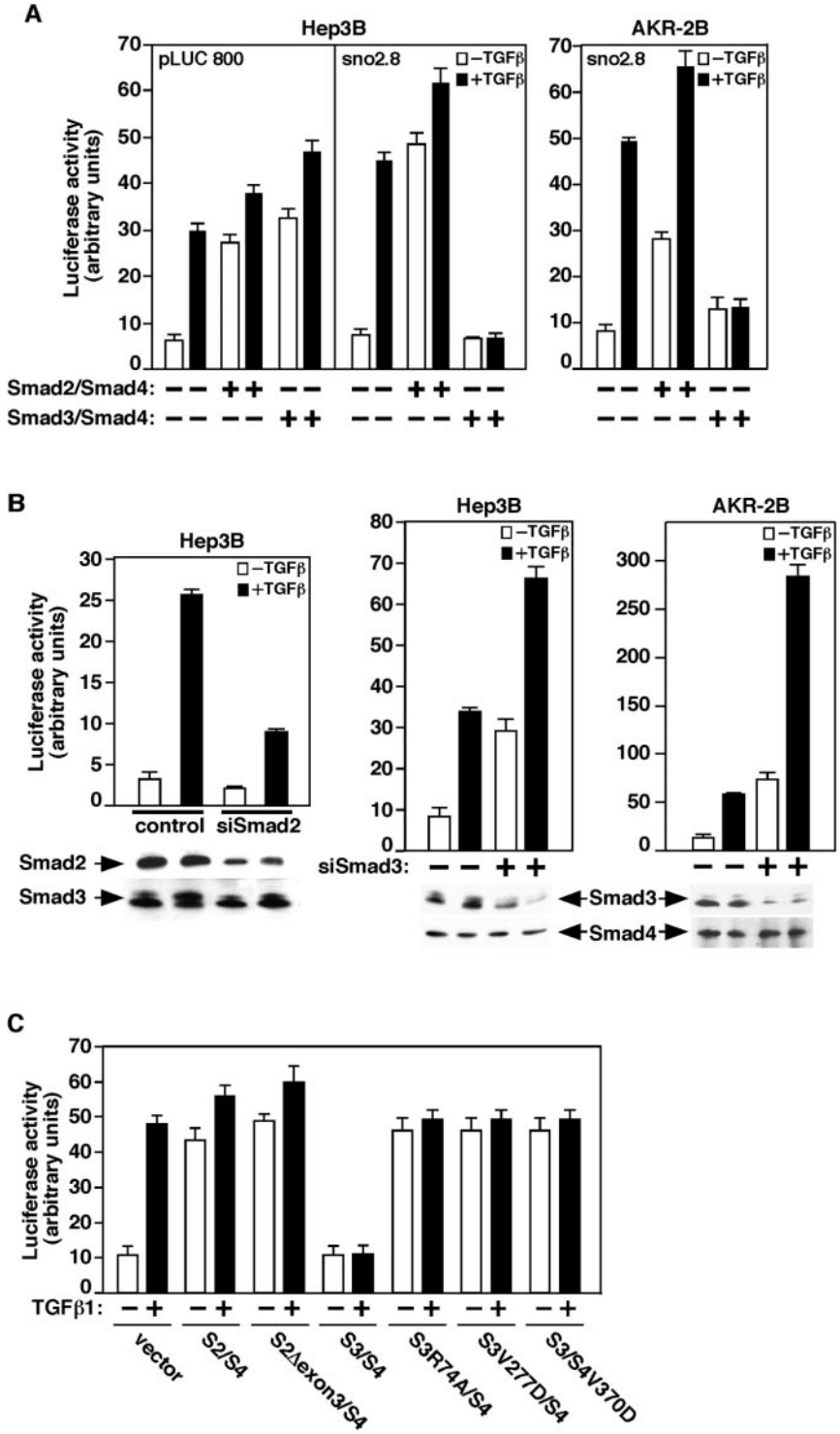

FIG. 4. Smad2 and Smad3 play opposite roles in regulation of *snoN* expression. (A) Hep3B or AKR-2B cells were cotransfected with the indicated Smad complexes and sno2.8 or pLUC800, containing 800 bp of the TGF-β-responsive region from the PAI-1 promoter, in the absence (open bars) or presence (closed bars) of 50 pM TGF-β1 for 16 h. (B) Effects of reduction of Smad2 or Smad3 expression on *snoN* transcription. Hep3B or AKR-2B cells were transfected with sno2.8 and the pSUPER vector expressing either human Smad2 siRNA, Smad3 siRNAa, a control siRNA (in Hep3B cells), or human Smad3 siRNAb, which cross-reacts with mouse Smad3 (in AKR-2B cells). *snoN* transcription was examined by the luciferase assay (graphs). The expression levels of Smad2, Smad3, and Smad4 in these luciferase reactions were measured by Western blotting. (C) The DNA binding activity of Smad3 and oligomerization of Smad3 with Smad4 are required for the inhibition of TGF-β-induced *snoN* transcription. WT Smad4 together with a Smad3 mutant deficient in DNA binding (S3R74A) or defective in hetero-oligomerization (S3V277D), or WT Smad3 together with mutant Smad4 defective in hetero-oligomerization (S4V370D), was cotransfected with sno2.8 into Hep3B cells. As a control, either WT Smad2/Smad4, Smad2(Δexon3)/Smad4, or WT Smad3/Smad4 was cotransfected with sno2.8. Luciferase activity was measured as described for Fig. 1F. Abbreviations: S2/S4, WT full-length Smad2/Smad4; S2(Δexon3)/S4, WT short isoform of Smad2 missing exon3/Smad4; S3/S4, WT Smad3/Smad4 complexes.

exon 3, Smad2(Δexon3), has been shown to mediate most of TGF-β signaling events during embryonic development (11, 55). Smad2(Δexon3) contains the DNA binding activity and has been speculated to function in manner similar to Smad3 (55). We therefore tested the role of Smad2(Δexon3) in the regulation of *snoN* induction by TGF-β. When Smad2 (Δexon3) was cotransfected with Smad4 and sno2.8 into either AkR-2B or Hep3B cells, it transactivated *snoN* promoter as efficiently as full-length Smad2 (Fig. 4C). This indicates that, at least in this case, Smad2(Δexon3) functions like full-length Smad2 [Smad2(FL)], not Smad3, and that the DNA binding ability of Smad is necessary but not sufficient for the inhibitory function. In addition, the expression levels of Smad2(Δexon3) in all cell lines described in this study are much lower than those of Smad2(FL) (data not shown). This suggests that Smad2(FL) plays a major role in regulation of *snoN* expression in these cell lines.

Oligomerization of Smad3 with Smad4 has been shown to be essential for the transcription activity (40). To examine whether this ability is required for the inhibition of *snoN* expression by Smad3, mutant Smad3 (S3V277D) or Smad4 (S4V370D) defective in oligomerization was examined in the luciferase reporter assay (Fig. 4C). When introduced together with WT Smad4 or Smad3, both mutants failed to inhibit *snoN* expression (Fig. 4C). Rather, they activated transcription from the *snoN* promoter as efficiently as Smad2. This suggests that hetero-oligomerization of Smad3 with Smad4 is essential for its inhibitory activity. Interestingly, some of the mutations of Smad4 found in human cancer cells disrupt the ability of Smad4 to heterodimerize with Smad3 (40). Our results suggest that in these cancer cells (39, 50), Smad3 activates, instead of inhibiting, *snoN* expression. This may account for the elevated level of *snoN* expression in these cancer cells.

A Smad-inhibitory element (SIE) mediates inhibition by Smad3. Internal deletions within the 2.8-kb *snoN* promoter fragment were generated and used to determine the region in the *snoN* promoter that mediates the inhibitory function of Smad3. As shown in Fig. 5A, deletion of sequences up to kb −2.0 had no effect on the inhibitory response to Smad3. In contrast, further deletion to kb −2.4 or an internal deletion of sequences between kb −2.0 and −2.4 abolished the suppressive effects of Smad3/Smad4. These two deletion mutants also exhibited reduced basal transcription but still retained the ability to respond to TGF-β. This suggests that the region between kb −2.4 and −2.0 is critical for both basal transcriptional activity and Smad3-dependent transcriptional inhibition.

Since the DNA binding activity of Smad3 was important for its inhibition of *snoN* transcription, we speculated that Smad3 may bind directly to a specific inhibitory element in this region of the *snoN* promoter. A DNase I footprinting assay was therefore carried out to determine the Smad3 binding site using the promoter fragment from kb −2.4 to −2.0 promoter fragment as a probe. A protected region with the sequence 5′-CGGCG ACGGCGACGGCGACGGCG-3′ was identified at bp −2179 to −2201 (Fig. 5B). This sequence is a triple-repeat of the CGACGG box that displays no resemblance to the typical SBE motif. In an EMSA, purified recombinant Smad3 and Smad4 both bound to the WT but not the mutant triple CGACGG sequence (Fig. 5C), confirming that this sequence can indeed mediate Smad binding.

To determine whether this novel Smad binding element can mediate inhibition of *snoN* expression by Smad3/Smad4, the triple CGACGG box was mutated in sno2.8. When this mutant was cotransfected with Smad3 and Smad4 into Hep3B cells, transcription from the mutant *snoN* promoter (SIEm) was not repressed by the Smad3/Smad4 complex (Fig. 5D). These results demonstrate that this CGACGG box is a novel Smad3-dependent inhibitory element (SIE) and that Smad3/Smad4 inhibits TGF-β-induced transcription of the *snoN* gene by binding to the SIE.

Taken together, our molecular analysis of *snoN* transcription indicates that the Smad2/Smad4 complex binds directly to the SBE sequences in the *snoN* promoter to activate its expression, while Smad3/Smad4 inhibits *snoN* expression through binding to an inhibitory element. Thus, Smad2 and Smad3 play opposite roles in the regulation of *snoN* expression, and this also provides an explanation for our earlier observation that the duration of *snoN* induction by TGF-β correlates positively with activation of Smad2 but not with that of Smad3.

Induction of *snoN* expression by TGF-β is required for TGF-β-induced transformation of AKR-2B and NRK fibroblasts. TGF-β has been shown to promote the proliferation of many fibroblast cell lines (2). However, only AKR-2B and NRK cells undergo anchorage-independent growth in response to TGF-β1 (29, 37). We showed earlier that induction of *snoN* expression in these two cell lines has a longer duration and a higher intensity than in other cell lines (Fig. 1A). To determine whether this induction is required for TGF-β-induced transformation of AKR-2B cells, we first introduced a siRNA specific for the murine *snoN* gene stably into AKR-2B cells to reduce *snoN* expression. As a negative control, an unrelated siRNA was also stably introduced into AKR-2B cells (control cells). Several stable clones that showed a significant decrease in both basal and TGF-β-induced *snoN* expression were obtained (Fig. 6A and data not shown). Two of these clones, sisnoN#6 and -#13, were analyzed further.

In the absence of TGF-β, the sisno cells grew more slowly than AKR-2B cells and control cells (data not shown), suggesting that SnoN acts to promote cell proliferation. Interestingly, TGF-β stimulated the proliferation of both the parental AKR-2B and control cells but inhibited that of sisno cells (Fig. 6B). To examine the effect of reducing *snoN* expression on the ability of TGF-β to induce anchorage-independent growth of AKR-2B cells, sisno cells were subjected to a soft-agar assay. Cells were suspended in 0.4% agar medium in the absence or presence of 100 pM TGF-β1 and overlaid on the bottom agar. After 3 weeks, while both the parental AKR-2B and control cells readily formed soft-agar colonies in the presence of TGF-β, the two stable cell lines expressing the *snoN* siRNA failed to do so (Fig. 6C). In a parallel control experiment, none of the cell lines grew in the soft agar in the absence of TGF-β (Fig. 6C). These results indicate that SnoN expression is required for TGF-β-induced oncogenic transformation of AKR-2B fibroblasts. Thus, SnoN is an important mediator of the transforming activity of TGF-β.

Since AKR-2B and NRK cells displayed a prolonged activation of *snoN* expression by TGF-β and can be transformed by TGF-β, we next asked whether this prolonged induction of *snoN* expression is responsible for the transformation of these two cell lines. For this purpose, the TβRI inhibitor SB-431542

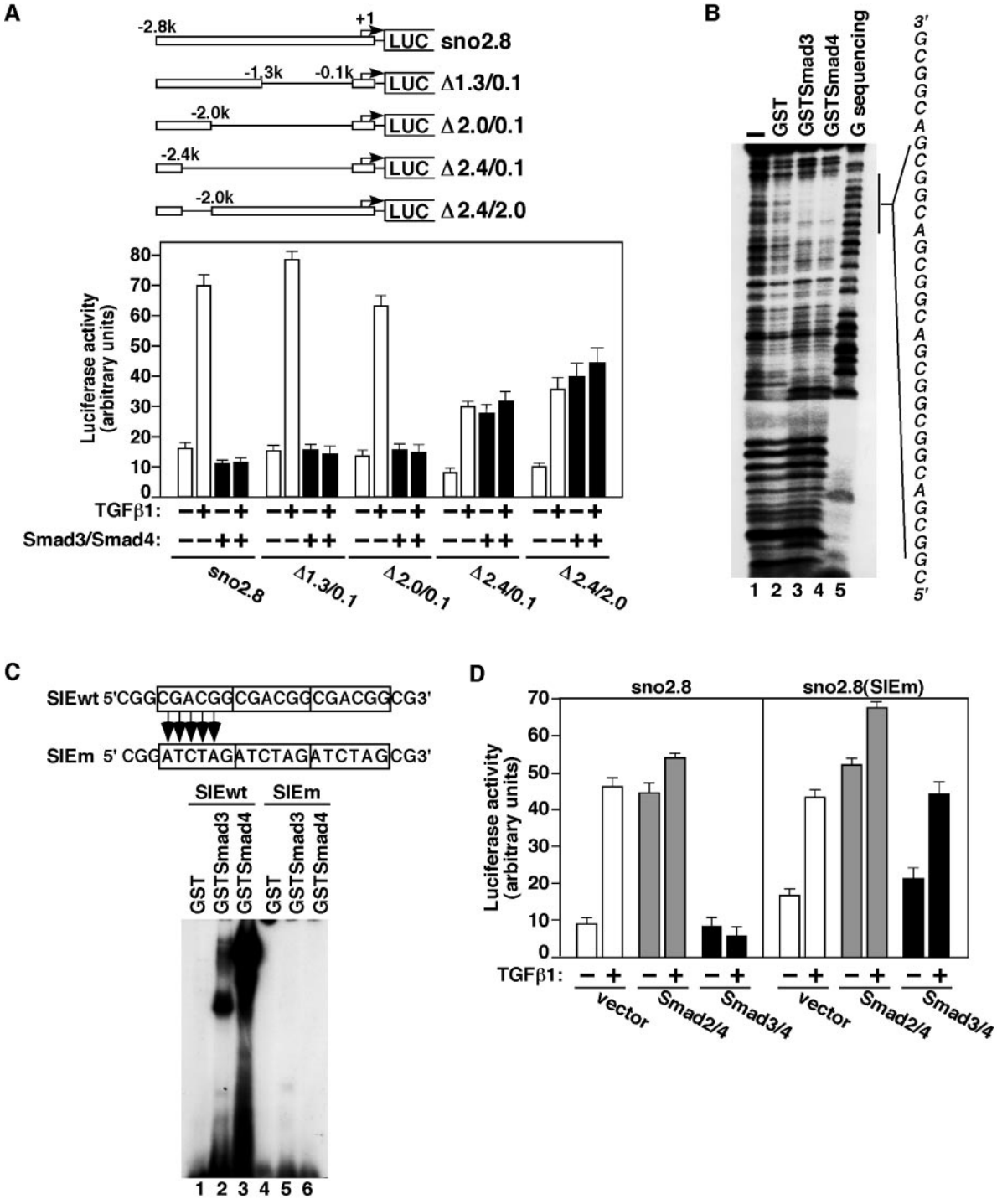

FIG. 5. Smad3/Smad4 binds to the Smad-inhibitory element and blocks TGF-β-induced *snoN* expression. (A) Identification of the SIE. (Top) Internal deletions of the *snoN* promoter were generated and introduced into Hep3B cells. (Bottom) Luciferase activity was measured in the absence or presence of Smad3/Smad4 and without (open bars) or with (closed bars) 50 pM TGF-β1. (B) Mapping of Smad3 binding site by a DNase I footprinting assay. One microgram of bacterially expressed GST (lane 2), GST-Smad3 (lane 3), or GST-Smad4 (lane 4) was incubated with a ^{32}P-labeled *snoN* promoter fragment containing the region from kb −2.4 to −2.0, followed by digestion with DNase I. The region protected by Smad3 and Smad4 is shown. (C) EMSA. ^{32}P-labeled oligonucleotide probes containing wild-type or mutant SIE sequences (top) were incubated with 1 μg of GST (lanes 1 and 4), GST-Smad3 (lanes 2 and 5), or GST-Smad4 (lanes 4 and 6) in an EMSA reaction (bottom). (D) SIE mediates inhibition of *snoN* transcription by Smad3. A luciferase reporter construct containing WT or mutant SIE was transfected into Hep3B cells together with Smad2 and Smad4 (gray bars) or Smad3 and Smad4 (black bars). *snoN* transcription induced by TGF-β was analyzed by the luciferase assay as described for panel A.

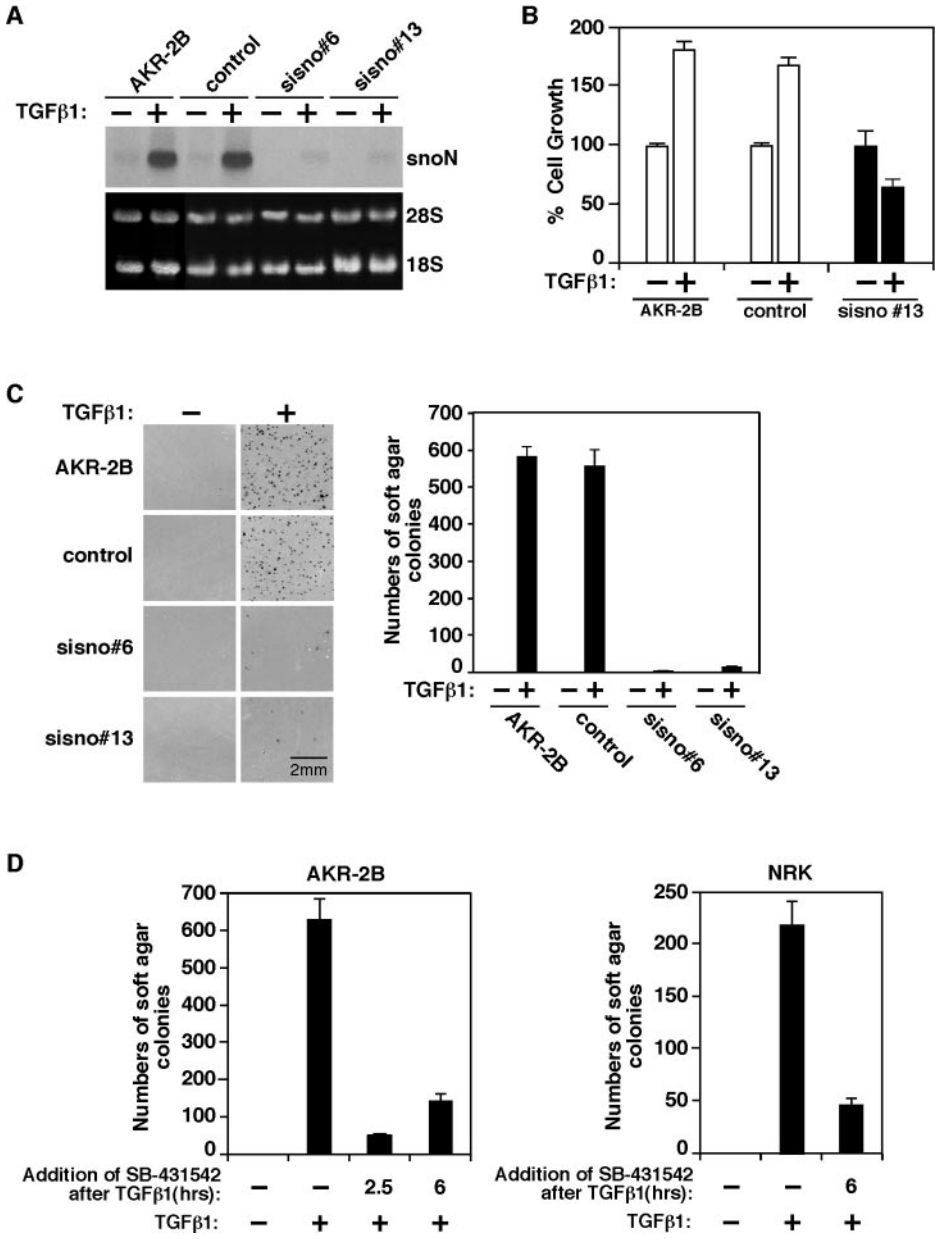

FIG. 6. *snoN* expression is required for TGF-β-induced oncogenic transformation of AKR-2B and NRK cells. (A) Reduction of *snoN* expression in AKR-2B cells by siRNA. Stable AKR-2B cell lines expressing either the siRNA for murine *snoN* or an unrelated control siRNA were generated as described in Materials and Methods. The levels of *snoN* in two representative stable clones (sisnoN#6 and -#13) in the absence or presence of 100 pM TGF-β1 were measured by Northern blotting and compared with levels in parental cells and control siRNA cells. (B) Effects of TGF-β on the growth of parental AKR-2B, control siRNA, and sisnoN cells. A total of 8×10^4 cells were incubated for 4 days in the presence or absence of 100 pM TGF-β1. The growth of cells was quantified by cell counting and normalized to the growth of cells in the absence of TGF-β1. (C) Soft-agar colony assay. A total of 5,000 parental AKR-2B cells or those expressing control siRNA or sisno were subjected to a soft-agar colony assay in the presence of TGF-β1 as described in Materials and Methods. The soft-agar plates were stained with 0.5 mg/ml MTT and scanned. A representative 5-mm^2 area of each 6-well plate is shown on the left. The number of soft-agar colonies in each plate was quantified, and the results are summarized in the graph on the right. (D) Prolonged induction of *snoN* expression by TGF-β is required for the transformation of both AKR-2B and NRK cells. A total of 5,000 AKR-2B cells or 5×10^4 NRK cells were set up in the soft-agar assay as described for panel C. One milliliter of medium with or without 10 μM SB-431542 was added on top of the soft agar at the indicated times post-TGF-β treatment.

was employed in the soft-agar assay. AKR-2B or NRK cells were suspended in 0.4% agar medium in the presence of 100 pM TGF-β1 and overlaid on the bottom agar. After 2.5 h or 6 h of stimulation with TGF-β1, a medium containing 10 μM SB-431542 was added on top of the soft agar. As shown previously (29, 37), AKR-2B and NRK cells formed colonies in soft agar in the presence of TGF-β1. Addition of SB-431542 after 2.5 h or 6 h of TGF-β1 treatment markedly reduced the formation of soft-agar colonies (Fig. 6D). Since the addition of SB-431542 at these times allowed only transient activation of

Smad2 and upregulation of *snoN* expression (Fig. 2C), similar to that observed in other fibroblast and epithelial cell lines, these results again support the notion that prolonged induction of *snoN* expression is required for TGF-β-induced oncogenic transformation of AKR-2B and NRK cells. However, this result should be interpreted with caution, since treatment of cells with SB-431542 may also shorten the activation of other Smad-independent pathways of TGF-β (53). This could also contribute to the impaired transforming phenotype.

DISCUSSION

Although TGF-β has been shown to stimulate the growth of many fibroblast cell lines, only murine AKR-2B and NRK fibroblasts undergo anchorage-independent growth in response to TGF-β (29, 37). So far, the intracellular signaling pathway that mediates this transforming activity in the two fibroblast cell lines has not been clearly defined. Here we showed that SnoN is a critical mediator of TGF-β-induced transformation in AKR-2B and NRK fibroblast cells. TGF-β, through the action of the Smad2/Smad4 complex, induces a strong and prolonged upregulation of *snoN* expression in these fibroblast cells that lasts for more than 8 to 24 h. This sustained activation of *snoN* expression may be necessary for the anchorage-independent growth of these fibroblasts in response to TGF-β1, since reduction of *snoN* expression by siRNA abolished TGF-β-induced transformation of AKR-2B cells and shortening of the duration of *snoN* induction by a pharmacological inhibitor markedly impaired TGF-β-induced transformation of AKR-2B and NRK fibroblasts. This is the first demonstration of a critical role of SnoN in the transforming activity of TGF-β. In other fibroblast cell lines that exhibit only a transient and moderate upregulation of *snoN* expression, TGF-β induces only proliferation, not transformation, of cells. Thus, our results are consistent with the model that the proper duration of TGF-β signaling plays an important role in determining the specificity of downstream responses in a given cell type (31a).

SnoN is considered an oncoprotein due to its ability to induce transformation in chicken embryo fibroblasts and its high level of expression in cancerous but not normal human cells (24, 25). Our finding that the induction of *snoN* is required for TGF-β-induced transformation of AKR-2B and NRK cells is consistent with its function as a transforming protein. The mechanisms by which SnoN induces transformation in these cell types have not been well defined. We found that in chicken embryo fibroblasts that undergo growth arrest upon activation of the Smad proteins by TGF-β, the transforming activity of SnoN is dependent on its ability to bind and antagonize the Smad proteins (16). However, it is not clear whether this is also true in AKR-2B and NRK cells, because TGF-β stimulates the proliferation of these cells. Interestingly, reducing *snoN* expression in AKR-2B cells converted the growth response of the cells to TGF-β from stimulatory to inhibitory (Fig. 6B) but did not affect the phosphorylation and activation of the R-Smads (data not shown). We therefore speculate that the Smad proteins may still mediate growth-inhibitory signals in AKR-2B cells as they do in most of the epithelial cells and some fibroblasts but that this activity is antagonized efficiently by the strong and prolonged expression of SnoN in response to TGF-β. This could result in strong proliferative activity in the presence of TGF-β and could, in combination with the Smad-independent pathway (53), contribute to cell transformation. Alternatively, high levels of SnoN in these cells may induce transformation in a manner independent of its activity to antagonize Smad proteins. Future research will determine the molecular mechanism by which SnoN induces transformation of cells.

In this paper, we have further investigated the molecular mechanism of *snoN* gene induction by TGF-β in different cell types. We showed that induction of *snoN* transcription by TGF-β occurs through a mechanism common to both epithelial and fibroblast cells and that this induction is mediated by the Smad proteins. Interestingly, Smad2 and Smad3 play different roles in regulation of *snoN* transcription through direct binding to the positive and negative regulatory elements in the *snoN* promoter. This differential ability of Smad2 and Smad3 to regulate gene expression has been reported previously only for the regulation of mouse *goosecoid* expression during embryogenesis (20, 31). This is the first example of an exclusively Smad2 dependent TGF-β-target gene in adult cells. The mechanism of Smad3-mediated inhibition of *snoN* expression is different from that reported for the *goosecoid* promoter. In the case of the *goosecoid* promoter, it has been proposed that Smad3 competes with Smad4 for binding to the same sequence in the *goosecoid* promoter and prevents the access of the Smad2/Smad4 complex to DNA and other coactivators, including FAST2. In contrast, Smad3 inhibits *snoN* expression through direct binding to the SIE sequence separate from the TGF-β-responsive element that Smad2/Smad4 recognizes. This DNA binding ability of Smad3 is necessary but not sufficient for the inhibition of *snoN* expression, because the short isoform of Smad2, Smad2(Δexon3), which contains the DNA binding activity, enhances transcription from the *snoN* promoter as efficiently as full-length Smad2. Indeed, we found that the ability of Smad3 to hetero-oligomerize with Smad4 is also required for repression of *snoN* expression, suggesting that it is the Smad3/Smad4 complex that binds to SIE and inhibits *snoN* transcription. Interestingly, many mutations of Smad4 found in human cancer cells, including those tested in this study, disrupt the oligomerization activity of Smad4 protein. We found that these mutations can convert the activity of Smad3 on *snoN* expression from repressive to stimulatory. Thus, in human cancer cells with these Smad4 mutations, the *snoN* promoter may be activated to a higher level in the presence of TGF-β due to the lack of negative regulation by Smad3/Smad4 and an additional stimulation by Smad3. Since many tumor cells also display an elevated level of TGF-β1 as they progress to a more malignant stage (9, 38, 43), this may be a possible regulatory mechanism for the elevated expression of SnoN in those epithelial cancer cells.

Induction of *snoN* expression by TGF-β occurs in multiple epithelial and fibroblast cell lines as well as primary fibroblasts (Zhu and Luo, unpublished). However, the duration of the induction differs from cell line to cell line and seems to correlate with the duration of Smad2 phosphorylation and activation. Interestingly, the duration of Smad3 phosphorylation is similar in all cell lines examined and much shorter than that of Smad2. This suggests that phosphorylation of Smad2 and Smad3 may be regulated by different mechanisms in AKR-2B

and NRK cells. Such differential regulation of Smad2 and Smad3 phosphorylation has not been reported before. We speculate that Smad2 and Smad3 may be phosphorylated by different receptor subunits in these cells or may require different adaptor proteins to be recruited to the TGF-β receptor complex for phosphorylation, or they could be dephosphorylated by different phosphatases. Because the Smad adaptor proteins and phosphatases have not been fully identified, it is difficult at this moment to test which of the scenarios is responsible for the observed difference in regulation of R-Smad phosphorylation. It is also not clear how the level of phospho-Smad2 remains high for an extended period of time in the two fibroblast lines. It could be due to a higher level of active TβRI kinase or due to reduced phosphatase activity. Because SB-431542 that directly inhibits TβRI kinase readily blocked Smad2 phosphorylation, this sustained phosphorylation of Smad2 is mostly likely due to increased activity of TβRI.

SnoN is a corepressor of the Smad proteins in epithelial cells. Upon induction by TGF-β, the newly synthesized SnoN may inhibit the transcriptional activity of the Smad proteins and turn off the expression of some TGF-β target genes in a negative-feedback manner. However, the molecular details of this negative feedback mechanism have not been understood. In particular, it is not clear whether this negative feedback inhibition occurs to all or just some of TGF-β target genes. We noticed that in AKR-2B and NRK cells, the later phase of *snoN* transcription (8 to 24 h) was apparently not inhibited by the newly synthesized SnoN. At the moment, the reason for this lack of self-inhibition is not known. It is also not clear whether this phenomenon is specific for AKR-2B and NRK cells or is true for all cell lines. One could argue that this later phase of *snoN* induction is mediated by a Smad-independent mechanism. However, the strong correlation between the phospho-Smad2 level and *snoN* expression suggests that the Smad2 protein is very likely to be directly involved. It is possible that the newly synthesized SnoN in AKR-2B and NRK cells is subjected to modification by an unidentified factor and becomes inactive in inhibiting its own transcription. Clearly, more experiments are needed to fully address these questions.

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