A 3′ Enhancer Controls Snail Expression in Melanoma Cells

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Abstract

The *snail* gene encodes a transcriptional repressor that functions during animal development and in cancer progression to promote epithelial-mesenchymal transitions. Strict spatial and temporal boundaries of Snail expression in development imply precise transcriptional control, which becomes inappropriately activated in many cancer subtypes. To gain insight into the molecular mechanism(s) governing transcriptional control of Snail, we analyze chromatin structural changes associated with Snail transcription in melanoma cells. Regardless of transcriptional status, the Snail promoter displays three constitutive DNase hypersensitive sites (HS) and a moderate level of histone H3 Lys^4 dimethylation. A robust HS is found in the 3′ region of A375 melanoma cells, in which Snail is highly expressed, but is absent in cells not expressing Snail. This element is conserved throughout the mammalian lineage and strongly activates expression of a reporter in A375 and Colo829 melanoma cells, but not in keratinocytes or primary melanocytes. Activity of this enhancer is associated with enrichment of H3 Lys^4 dimethylation and H3 acetylation at both the enhancer and the promoter. Additionally, enhancer activity is associated with H3 Lys^4 trimethylation at the promoter. A physical interaction between the 3′ enhancer and promoter was observed in Snail-expressing cells, demonstrating a direct role for the enhancer in Snail expression. These results suggest a model in which the Snail promoter is constitutively packaged in a poised chromatin structure that can be activated in melanoma cells by a tissue-specific enhancer, which physically contacts the promoter. [Cancer Res 2007;67(13):6113–20]

Introduction

Melanoma is responsible for the majority of skin cancer mortality in the United States (1). The high mortality rate of malignant melanoma is a function of its propensity for invasion of neighboring tissue (2). The acquisition of invasive behavior in cancer cells of epithelial origin is due in part to a phenotypic switch referred to as epithelial-mesenchymal transition (EMT), in which epithelial cells lose contacts with their neighbors and assume migratory characteristics (3). EMT can be promoted in various developmental processes or in disease states by expression of the Snail family of transcriptional repressors (4–6).

Snail produces invasive cellular behavior by directly repressing the promoters of genes required for epithelial differentiation, including E-cadherin (5, 6), claudins, and occludin (7, 8). Expression of Snail also induces the mesenchymal genes matrix metalloproteinase 2, vimentin, and fibronectin (5, 9, 10). Malignant melanoma is associated with elevated Snail expression and concomitant down-regulation of E-cadherin (11), as are cancers of breast (12), colon (13), and hepatic (14) origins. Snail confers on cells the properties of decreased cell-cell adhesion, increased motility, and resistance to apoptosis (9, 15–18), all acquired properties of cancer cells (19). A detailed molecular description of aberrant snail regulation in cancer cells would yield insight into the metastatic process, as well as normal development.

The number of signaling pathways implicated in the regulation of Snail expression is as diverse as the developmental processes in which Snail plays a role. Snail expression is activated by signaling through the fibroblast growth factor receptor 1 in mammalian gastrulation (20), through transforming growth factor β (TGFβ) in hair bud morphogenesis (21), and by Notch in cardiac valve development (22). Studies in cultured cells have identified Ras/mitogen-activated protein (MAP) kinase, phosphoinositide-3-kinase (PI3K), integrin-linked kinase, and nuclear factor-κB (NF-κB) systems as capable of inducing Snail transcription (23–26). Several transcription factors have also been linked to Snail induction, including Gli1 (27), early growth response 1 (EGR1) (28), high mobility group AT-hook 2 (29), and NF-κB (24, 25). Response elements to some of the above pathways have been broadly identified in studies focusing exclusively on putative regulatory DNA at the *snail* locus in a proximal position 5′ of the transcription start site. Regulatory elements associated with the high levels of Snail found in melanoma cells have not been defined.

We have studied the roles of chromatin architecture and cis-acting elements in the control of Snail transcription in melanoma cells. We found multiple, constitutive DNase hypersensitive sites (HS) in the promoter, indicative of constitutively open chromatin structure. In contrast, the 3′ region of the Snail gene displayed a HS specifically in A375 melanoma cells, in which Snail is highly expressed. Local DNA sequence at the 3′ HS showed a high degree of conservation in mammals, indicating a fundamental biological function. Reporter gene expression was dramatically increased by the 3′ element specifically in melanoma cells expressing high levels of Snail. Chromatin immunoprecipitation (ChIP) assays at the Snail promoter revealed a significant level of histone H3 Lys^4 dimethylation surrounding the transcription start site and proximal promoter at the silent locus, supporting the notion of a poised chromatin structure. Activity of the 3′ enhancer was associated with H3 Lys^4 trimethylation and acetylation of H3 Lys^{18} at the proximal promoter. These marks of transcriptionally active chromatin were also enriched at the active 3′ enhancer but absent at the inactive enhancer. Finally, a physical interaction between the 3′ enhancer and promoter was shown by chromosome conformation capture (3C) assays. These results suggest that the Snail

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promoter is poised for transcription, and that activation in melanoma cells occurs through a 3′ enhancer that functions in part by physically interacting with the promoter.

Materials and Methods

Cells lines and RNA measurement. Spontaneous killer (SK) cells (30) were provided by Dr. C. Parkos (Emory University, Atlanta, GA). Primary melanocytes were obtained from Sciencell Research Laboratories. All other cell lines were obtained from the American Type Culture Collection. Primary melanocytes were grown in Medium 254 containing human melanocyte growth supplement (Cascade Biologics). Colo829 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone). All other cells were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone). Where indicated, cells were treated with 50 μmol/L SB415286 (Sigma-Aldrich) for 24 h. For the determination of mRNA levels, RNA was isolated from cells using TRIzol (Invitrogen Corporation). cDNA was prepared by reverse transcription using random hexamers, then used as a template in real-time PCR. Reverse transcription-PCR primer sequences were actin, 5′-CTCTTCCAGCCTTCCTTCCT-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′; Snail, 5′-ATCCGAAGCCACACACTG-3′ and 5′-CACTGGTACTTCTTGACATCTG-3′. Threshold cycles for Snail were normalized relative to actin threshold cycles.

Plasmids and transfection. For reporter assays, the pGL2-Basic vector (Promega Corporation) was used to create reporter constructs. The Snrpn proximal promoter (515 bp) was cloned into pGL2-Basic using *Nhe*I/*Hin*dIII restriction sites and is described in ref. 31. Test enhancer fragments were generated by PCR, sequenced, and inserted upstream of the Snrpn promoter using *Kpn*I. Primer sequences used for these constructs were 1 to 859, 5′-GGTACCAAATGGCCTTTG-3′ and 5′-GGTACCCTCGAAACCCCTGA-3′; 1 to 296, 5′-GGTACCAAATGGCCTTTG-3′ and 5′-GGTACCTGACTCTGGGGCTCCGAAGC-3′; 92 to 674, 5′-GGTACCTGCTCCGAGGACGGCGGGAC-3′ and 5′-GGTACCGGGCTTGGTGACCTCAGGCAA-3′; 537 to 859, 5′-GGTACCTGCGGGTCCCTCTAGGTCG-3′ and 5′-GGTACCCTCGAAACCCCTGA-3′; 274 to 556, 5′-GGTACCTTCGGAGCCCCAGAGTCA-3′ and 5′-GGTACCGACCTAGAGGGACCCGCA-3′; 294 to 527, 5′-GGTACCGGCTGGGGGTGGGGGGA-3′ and 5′-GGTACCGAGCTCGACACAGTAGGCCTTCAGCTC-3′; 276 to 400, 5′-GGTACCTTCGGAGCCCCAGAGTCA-3′ and 5′-GGTACCGAGCTCAAGCTGCAAGTCATTAAGGGCTG-3′; 384 to 554, 5′-GGTACCTTAATGACTTGCAGC-3′ and 5′-GGTACCGAGCTCGACCTAGAGGGACCCGCAGG-3′; and 340 to 478, 5′-GGTACCTTCTTCTAAACAGGAAATGGTG-3′ and 5′-GGTACCGAGCTCCTTCCCTTCAGGGAGGCAGGC-3′. Transfection was done with LipofectAMINE 2000 (Invitrogen Corporation) according to the manufacturer's instructions, using six-well plates with 2 μg of DNA transfected per well. Primary melanocytes were transfected by electroporation using a Nucleofector (Amaxa Biosystems). After 2 days, luciferase activity was measured using the dual luciferase assay system (Promega), with a pRL-TK plasmid as a control for transfection efficiency.

DNaseI hypersensitivity assay. Hypersensitivity assays were done as described (32). Nuclei were isolated from 1×10^8 cells per titration series and treated with 0.05 to 2 μg/mL DNase I (Roche Applied Sciences) at 37°C for 5 min. DNA was purified by two extractions with phenol/chloroform followed by ethanol precipitation. For each DNase concentration point, 15 μg of DNA was restricted with *Pac*I and subjected to agarose gel electrophoresis and Southern blotting. Blots were hybridized with probes amplified by PCR using the following primers: 5′ analysis, 5′-GGAGCTCCGCAAGAGGGGAAGG-3′ and 5′-TCCTAAGTCCCCAGTCTCCAG-3′; 3′ analysis, 5′-CTGGTACGTGCCCCTCCAGGCG-3′ and 5′-TCTGCTTTGCCCATCTGCTTAGC-3′.

Chromatin immunoprecipitation. ChIP assays were conducted as described (33). Chromatin was prepared from 4×10^7 cells. One-tenth of this chromatin preparation was used in each immunoprecipitation. Chromatin was crosslinked with 1% formaldehyde, sheared by sonication, precleared with protein A beads, and incubated with 5 μg of the indicated antibodies overnight. Antibodies were obtained from Upstate Biotechnology as follows: anti–acetyl-Histone H3, which recognizes acetylation at Lys^9 and Lys^{14}; anti–acetyl-Histone H3 (Lys^{18}); anti–dimethyl-Histone H3 (Lys^4); and anti–trimethyl-Histone H3 (Lys^4). Immune complexes were recovered with protein A beads. Crosslinks were reversed at 65°C, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Real-time PCR was used to quantitate the amount of immunoprecipitated DNA, using a genomic DNA standard curve for each primer set. The amount of immunoprecipitated DNA was normalized to 1/30 of the input chromatin for each assay. Irrelevant antibody against T cell receptor was used as a negative control. Primer sequences were as follows: HS1, 5′-CCATACAATGAATAGTCCGCATCC-3′ and 5′-GAACCTCCACACTAACCTACACC-3′; promoter, 5′-GTGCGTTTCCCTCGTCAATGC-3′ and 5′-GCCGGGACACCTGACCTTC-3′; and enhancer, 5′-GAGCAGCCCTTAATGACTTG-3′ and 5′-CCCAACTCCCTAACTTCCC-3′.

Chromosome conformation capture. 3C assays were done essentially as described (34). Briefly, cells (1×10^7 per sample) were incubated in DMEM containing 1% formaldehyde for 10 min. Glycine was added to a final concentration of 0.125 mol/L, and the cells were washed twice in cold lysis buffer [0.34 mol/L sucrose, 10 mmol/L Tris (pH, 7.9), 10 mmol/L NaCl, 1% NP40] containing protease inhibitors. The nuclei were collected by centrifugation, washed once with 1× *Pst*I restriction enzyme buffer, and resuspended in restriction buffer containing 0.1% SDS. Samples were incubated for 10 min at 37°C on a rotating wheel. Triton X-100 was added to a final concentration of 1%, and the nuclei were incubated for 10 min at

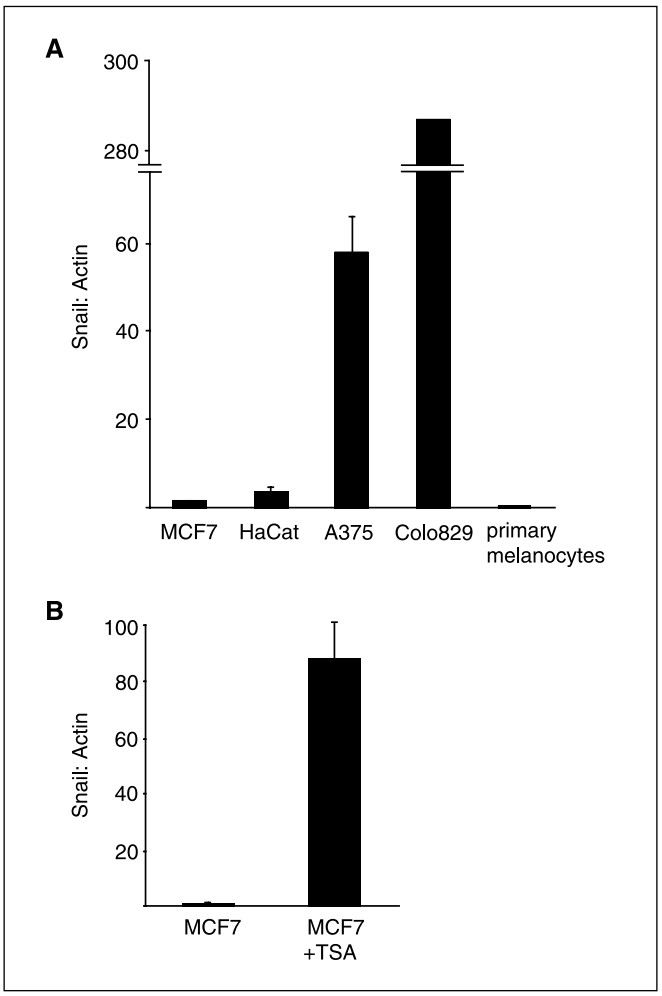

Figure 1. Relative levels of Snail mRNA in selected cell lines. *A,* Snail expression in epithelial cell lines (MCF7, HaCat), melanoma cell lines (A375, Colo829), and primary melanocytes. *B,* MCF7 cells were treated with vehicle or with 200 nmol/L TSA for 32 h. Total RNA was isolated from the indicated cells, reverse transcribed, and amplified by real-time PCR. Snail mRNA levels were normalized to actin and expressed as fold over MCF7. Data represent the average of at least two experiments ± 1 SD.

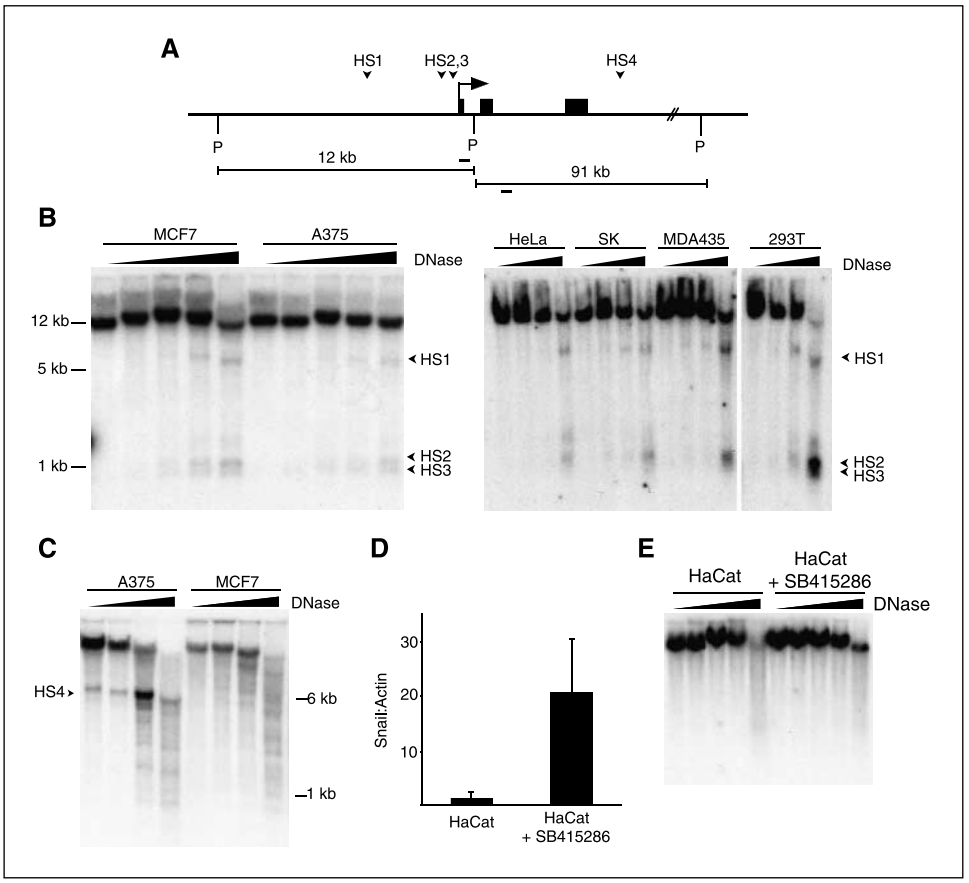

Figure 2. DNase HSs in the Snail region. *A,* a diagram depicting the *snail* locus with exons (*blocks*), transcription start site (*arrow*), and *Pac*I sites (*P*) defining the parent fragments that were monitored in the DNase hypersensitivity assays. The position of the probes used are indicated, as are the positions of HSs (*HS1, HS2, HS3*, and *HS4*). *B,* Southern blot showing the parent 5′ *Pac*I fragment and DNase HSs. Nuclei from cells (*left,* MCF7 or A375 cells; *right,* HeLa, SK, MDA435, and 293T cells) were treated with increasing concentrations (*triangles*) of DNase I. DNA was extracted, restricted with *Pac*I, and subjected to Southern blotting. HeLa, SK, MDA435, and 293T cell lines express low levels of Snail and were derived from cervical carcinoma, colon carcinoma, breast carcinoma, and embryonic kidney, respectively. *C,* a Southern blot comparing DNase hypersensitivity of the 3′ *Pac*I fragment depicted in (*A*) in A375 or MCF7 cells identified a HS associated with expression. *D,* HaCat cells were treated with 50 μmol/L SB415286 for 24 h. RNA was extracted from cells, reverse transcribed, and amplified by real-time PCR. Snail mRNA levels were normalized to actin and expressed as fold over untreated. Data represent the average of three experiments ± 1 SD. *E,* HaCat cells were treated as in (*D*) and analyzed for DNase hypersensitivity of the 3′ *Pac*I fragment as in (*C*). No 3′ HS was observed following treatment in HaCat cells.

37°C to sequester the SDS. Cross-linked DNA was digested overnight with 200 units *Pst*I. The restriction enzyme was heat inactivated by incubation at 65°C for 20 min. Samples were then diluted 50-fold in T4 DNA ligase buffer (New England Biolabs, Inc.) containing 1% Triton X-100 and incubated for 10 min at 37°C. T4 DNA ligase (4,000 units) was added, and the samples were incubated overnight at 16°C followed by a 30-min incubation at room temperature. After adding proteinase K (10 μg/mL), crosslinks were reversed by incubation overnight at 65°C. DNA was extracted in phenol/chloroform and precipitated in ethanol. The samples were dissolved in water, and 50 and 100 ng of 3C DNA was amplified by PCR. The PCR primer sequences represented in Fig. 5 were P1, 5′-CCTAGCGAGTGGTTCTTCTG-3′; P2, 5′-CCAACTCCCTAACTTCCCTTC-3′; NC1, 5′-AGTGTGATGAGACCT-GAACC-3′; NC2, 5′-GGTTCAGGTCTCATCACACT-3′; loading control-1, 5′-GGCAAGCAACTTAACCTCTC-3′; loading control-2, 5′-CTCCCTCAACTGT-CACTCTC-3′. The entire set of reactions was done twice with identical results.

Alignments. Multiple genomic alignments to the May 2004 assembly of the human genome were done by the University of California at Santa Cruz (UCSC) Genome Bioinformatics Group using the Blastz (35) and Multiz (36) algorithms, and conservation scores were assigned using PhastCons (37). The results were displayed using the UCSC Genome Browser (38).[3]

Results

Model cell lines. To model the active state of the *snail* gene in melanoma, Snail mRNA expression was surveyed in A375 and Colo829 melanoma cells and compared with primary melanocytes and the epithelial cell lines HaCat (keratinocyte) and MCF7 (breast carcinoma; Fig. 1*A*). A375 and Colo829 cells expressed high levels of Snail, whereas primary melanocytes, HaCat, and MCF7 cells displayed low levels of Snail transcripts. Therefore, A375 and Colo829 cells were selected to model the transcriptionally active *snail* locus, and HaCat and MCF7 cells were chosen to represent the inactive state.

Previous studies in breast cancer cells revealed a role for the Mi2/MTA3 chromatin remodeling complex in repression of the *snail* gene (31). To confirm the importance of histone acetylation in regulating transcription of Snail, MCF7 cells were treated with Trichostatin-A (200 nmol/L), an inhibitor of histone deacetylases (Fig. 1*B*). Snail transcripts were induced to levels comparable to those found in A375 cells, suggesting that local chromatin architecture is a critical determinant in the control of Snail expression.

Three constitutive HSs localize to the Snail 5′ region. The human *snail* gene is comprised of three exons and is located in a relatively gene-poor region of chromosome 20. The nearest neighboring genes are located >30 kb in the centromeric direction and >90 kb in the telomeric direction from the *snail* locus. Cis-acting regulatory sequences can often be visualized as sites in chromatin that are hypersensitive to DNase I cleavage. To identify elements regulating Snail transcription, we compared the patterns of DNase hypersensitivity at the *snail* locus in cell lines representing the active or inactive transcriptional states. To date, all cis-elements regulating Snail expression are proposed to be located in the 5′ proximal promoter region. Therefore, we monitored DNase

[3] http://genome.ucsc.edu/

hypersensitivity in chromatin 5′ of the *snail* gene, represented by a 12-kb *Pac*I fragment (Fig. 2*A*). Indirect end-labeling of this fragment's DNase cleavage products revealed three HSs (Fig. 2*B*). The first site (HS1) maps to ~4.5 kb upstream of transcription start; the remaining sites (HS2, HS3) are positioned at the proximal promoter at approximately −100 and −300 bp relative to transcription start. Remarkably, the same DNase hypersensitivity profile was found at both the active and repressed loci. HS1-3 were also present in four other unrelated cell lines (Fig. 2*B*: HeLa, SK, MDA435, and 293T), all of which express low levels of Snail (data not shown), supporting the classification of these HSs as constitutive.

A 3′ HS correlates with Snail expression. The absence of a correlation between DNase hypersensitivity and transcriptional activity in the 5′ region prompted us to examine the introns and 3′ region, encompassed by a 91-kb *Pac*I fragment (Fig. 2*A*). A HS (HS4) located 3′ of the *snail* gene was found in A375, but was absent in MCF7 cells and in HaCat cells (Fig. 2*C* and *E*). The correlation of HS4 with high Snail expression suggested that HS4 sequences might constitute a cis-regulatory element.

The HS4 sequence within the *snail* locus is conserved. Because Snail serves many conserved functions during animal development, the elements that direct its tissue-specific expression are also likely to be conserved. If HS4 in fact represents such an enhancer, one would predict conservation in its sequence. Using the multiple sequence alignments available from the UCSC genome browser[3] (38), sequence conservation was plotted relative to genomic position (Fig. 3*A*). This analysis showed a high degree of conservation at the HS4 position, with significant similarity in all available mammalian genomes. In nonmammalian vertebrate lineages, conservation at HS4 seemed to be lost, implying either that the enhancer is present but in a different position, or that the enhancer is unique to mammals. Conservation throughout the mammalian lineage constitutes evidence that the HS4-conserved region serves an important biological function.

Transcriptional enhancement is mediated by HS4. To determine whether HS4 possesses gene regulatory activity, reporter assays were done. An 850-bp *Kpn*I fragment coincident with the position of HS4 (Fig. 3*A*) was cloned by PCR and inserted upstream of the enhancerless Snrpn promoter driving a luciferase reporter

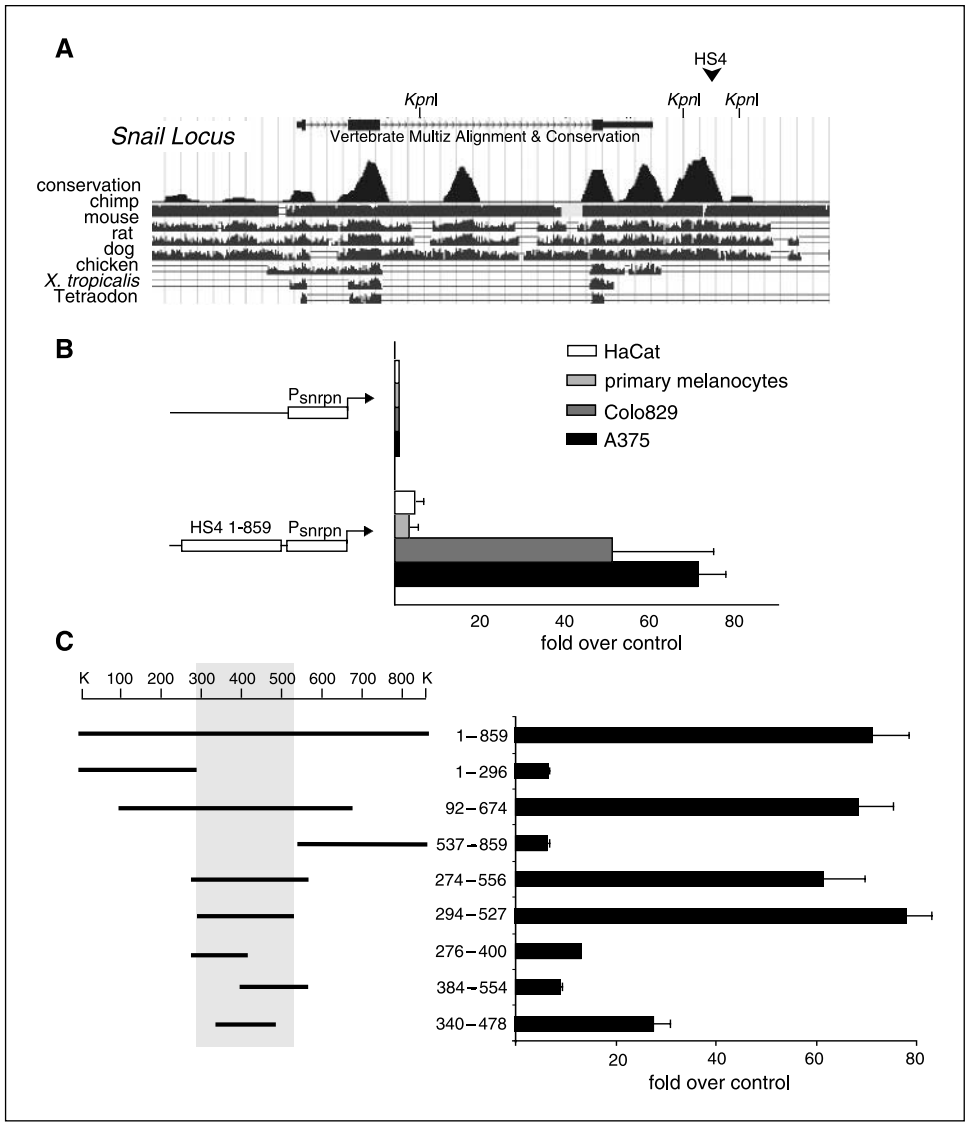

Figure 3. HS4 enhances transcription from a heterologous promoter in a cell type–specific manner. *A,* conservation of HS4 was observed in mammalian genomes but not in lower vertebrates. The human Snail locus is shown with a histogram plot showing the degree of conservation across multiple vertebrate genomes (*conservation, blue*). *Green,* conservation scores for each pairwise alignment of a given genome relative to the human genome. Positions of HS4 and *Kpn*I sites are also indicated. Alignments were done and analyzed by the UCSC Genome Bioinformatics Group and displayed by the UCSC Genome Browser. *B,* HS4 is active in Snail-expressing melanoma cells. Primary melanocytes, HaCat, Colo829, or A375 cells were transfected for 2 d with a luciferase reporter driven by the Snrpn promoter alone or coupled to a 859-bp *Kpn*I fragment encompassing HS4. Data were normalized for transfection efficiency by cotransfection of a *Renilla* reporter plasmid and expressed as fold over Snrpn promoter activity alone. Data represent averages ± 1 SD of at least two experiments. *C,* a series of subfragments from the 859-bp *Kpn*I fragment in (*A*) were linked to the Snrpn reporter plasmid and tested for enhancer activity by transfecting A375 cells as in (*A*). Data are presented as fold over Snrpn promoter activity and are averages of at least two experiments ± 1 SD.

gene (Fig. 3*B*). Transient transfection of A375 or Colo829 cells with these constructs showed that the 850-bp *Kpn*I fragment was able to direct high-level expression of the reporter. Conversely, primary melanocytes and HaCat cells showed low reporter gene expression. A series of deletion constructs were created to define the minimal sequences responsible for this activity (Fig. 3*C*). The shortest fragment that maintained full activity is a 230-bp sequence containing multiple potential transcription factor binding motifs. These results revealed the existence of a cell type–specific enhancer located 3′ of the *snail* gene whose activity correlates with DNase hypersensitivity.

To determine whether HS4 is inducible by agents known to increase Snail transcription, we treated HaCat cells with the GSK3β inhibitor, SB415286 (25). This treatment enhanced Snail transcription (Fig. 2*D*), but failed to induce HS4 formation (Fig. 2*E*). This result suggests that induction of Snail can be accomplished by multiple independent pathways.

Histone modifications across the *snail* locus. Because repression of Snail is dependent on histone deacetylase activity (Fig. 1*B*), the mechanism of HS4-dependent enhancement of Snail transcription may be expected to operate in part through opposing histone modifications associated with transcriptionally active chromatin. We conducted ChIP assays to characterize the histone modification status at key regulatory positions across the *snail* locus, comparing the transcriptionally active and inactive states (Fig. 4). Positions of interest were the upstream HS (HS1), the proximal promoter (HS2, HS3) and the 3′ enhancer (HS4). At each of these positions, nucleosomes were relatively depleted of histone H3 acetylated at Lys^9 and Lys^{14} in HaCat cells (Fig. 4). Likewise, antisera against acetyl-Lys^{18} of H3 (Fig. 4) showed that this residue is deacetylated across the locus in HaCat cells. In contrast, A375 and Colo829 cells displayed enrichment of H3 acetylation (Lys^9, Lys^{14}, and Lys^{18}) at the promoter and enhancer, but not at the upstream HS1. Thus, H3 acetylation at Lys^9, Lys^{14}, and Lys^{18} accompanies Snail promoter activity. The high level of H3 lysine acetylation at the active 3′ enhancer shows that chromatin remodeling at the enhancer itself involves acetylation of histone residues in addition to the formation of a DNase HS.

Another histone modification associated with accessible chromatin is the dimethylation of H3 Lys^4 (39). This modification was markedly enriched in A375 and Colo829 at both the promoter and the enhancer (Fig. 4). Interestingly, the repressed promoter in HaCat cells contained a significant amount of H3 Lys^4 dimethylation. Full Snail promoter activity is associated with H3 Lys^4 trimethylation (Fig. 4), a mark of actively transcribing promoters (40). This modification is absent at the active enhancer, consistent with its promoter specificity.

The 3′ enhancer interacts with the Snail proximal promoter. A key question of enhancer function concerns how enhancers communicate with promoters to recruit polymerase. One proposed model for enhancer action at a distance is that enhancer-bound proteins physically interact with the promoter-bound factors, resulting in looping out of the intervening DNA (41). To determine whether the active 3′ enhancer physically interacts with the Snail promoter, the 3C assay was employed (34). In this assay, interactions are preserved by formaldehyde crosslinking of chromatin. The crosslinked chromatin is restriction enzyme–digested, diluted greatly, and then ligated under conditions that favor intramolecular products. If the regions of interest were held together by crosslinking, then a

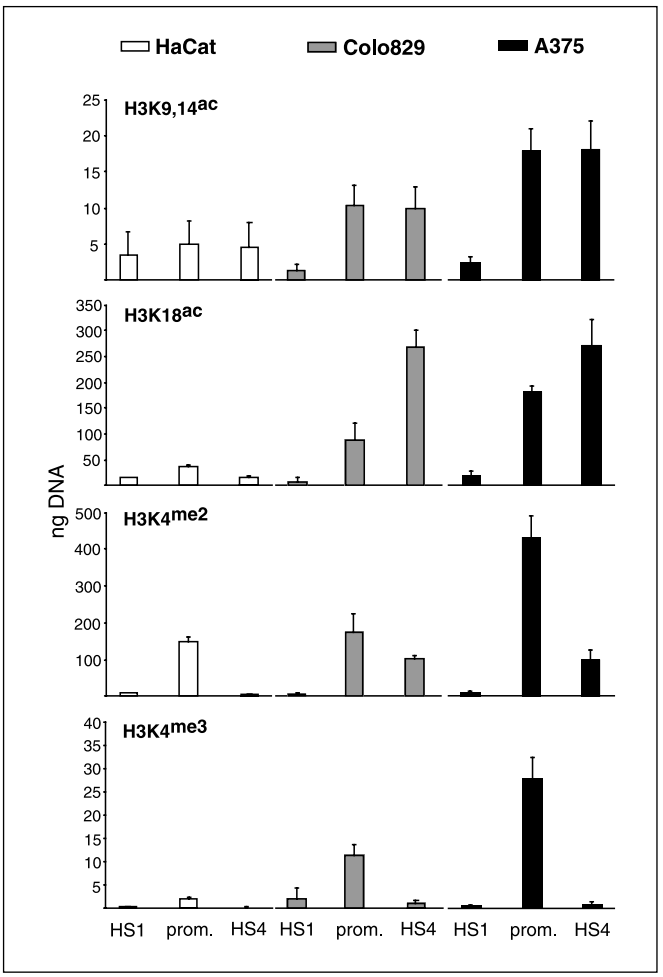

Figure 4. Active histone H3 modifications are enriched at the active Snail promoter and enhancer, H3 Lys^4 dimethylation is enriched at both the active and inactive Snail promoter, and H3 Lys^4 trimethylation is enriched specifically at the active promoter. Histone modification profiles were analyzed at three positions across the *snail* locus by ChIP assays using antibodies against histone H3 acetylated at Lys^9 and Lys^{14} (*top*), acetylated H3 Lys^{18} (*second*), and dimethylated H3 Lys^4 (*third*), and trimethylated H3 Lys^4 (*bottom*). Immunoprecipitated DNA was normalized to input and quantified by real-time PCR. Data represent averages of three experiments ± 1 SD.

novel ligation product between the crosslinked DNA sequences is favored and can be detected by PCR. The 3C assay was done on Snail-expressing (A375, Colo829) and nonexpressing (MCF7, primary melanocyte) cells (Fig. 5). The results showed that a PCR product created by ligation between the 3′ enhancer and promoter proximal DNA was formed in A375 and Colo829, but not in MCF7 or primary melanocytes. Detection of the 3C product was dependent on the inclusion of formaldehyde and DNA ligase. Moreover, the novel 3C product was not detected in naked genomic DNA treated under identical conditions, demonstrating a need for chromatin context. Importantly, no 3C products were detected with either of the fragments that lie between the enhancer and the promoter (PC1/NC1 or PC2/NC2), demonstrating that the interactions are specific. A loading control showed that all of the samples contained a similar amount of input DNA/chromatin. These data suggest that the 3′ enhancer regulates the Snail promoter by a mechanism involving physical interaction between the enhancer and promoter.

Discussion

Transcriptional regulation results from the dynamic interaction of transcription factors, cis-elements, and chromatin architecture. Although considerable attention has been placed on the roles of various signaling pathways in regulating Snail expression, the contributions of specific cis-elements and chromatin architecture are undefined. We have examined the native chromatin structure in the region surrounding the Snail locus, and correlated chromatin structure with gene activity. This approach revealed the presence of a tissue-specific Snail enhancer located 3′ of the gene that has been conserved through mammalian evolution.

In all cell lines examined, the 5′ region of the *snail* gene displays a uniform DNase hypersensitivity pattern, regardless of Snail transcriptional status. Hypersensitive sites 2 and 3 map to the proximal promoter, where elements responsive to MAP/extracellular signal-regulated kinase kinase (MEK)/Ras, NF-κB, and EGR1 are located (24, 28). Constitutively accessible chromatin structure at the silent promoter, in addition to the intermediate level of H3 Lys^4 dimethylation, suggest that the promoter is poised for activation (Fig. 6). This result is similar to previous data showing significant H3 Lys^4 dimethylation in transcriptionally permissive globin genes before their activation in embryonic erythrocytes, whereas this mark was absent in most other silent genes (42). A poised promoter state enables a rapid transition between activation and repression (43, 44) and may explain the rapid kinetics of Snail activation and deactivation seen in response to TGFβ2 in the hair follicle (21). A constitutively accessible promoter architecture raises the question of what structural features, if any, distinguish the active from the silent state. A pivotal role for acetylation at *snail* was suggested by previous work showing derepression of Snail transcription upon knockdown of the HDAC-containing MTA3 complex in breast epithelial cells (31). We now corroborate this result by discovering a clear correlation between histone H3 acetylation and Snail promoter activity. Together, these data point to H3 acetylation as a potential switch in conversion of the poised to the active promoter.

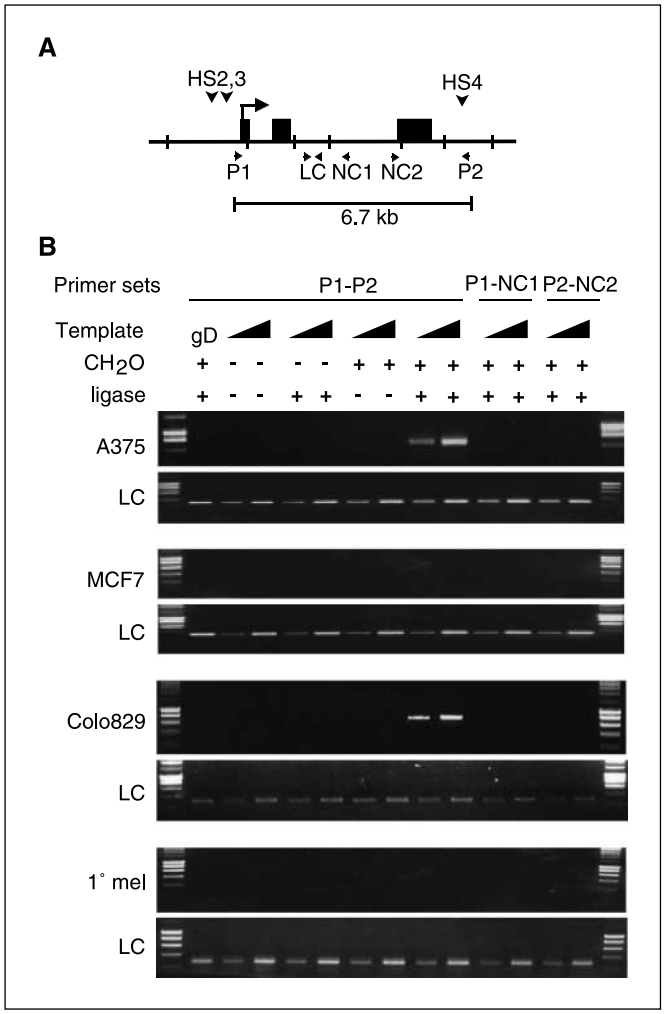

Figure 5. The 3′ enhancer interacts with the Snail proximal promoter. *A,* schematic of the *snail* gene with the position and orientation of the 3C primer pairs indicated. Primers P1 and P2 were designed to amplify a novel ligation product formed between the restriction fragments that encode the promoter region and enhancer DNA, respectively. Nonspecific DNA fragment primers NC1 and NC2 as well as a loading control (*LC*) are shown. *B,* 3C shows the presence of a specific (*P1-P2*) 3C product under conditions in which formaldehyde crosslinking and ligation of the samples was carried out. No product is observed with primer pairs P1-NC1 or P2-NC2. No 3C product was observed when 100 ng of naked genomic DNA was used (*gD*). The loading control primers (*LC*) showed that all of the samples contained similar amounts of DNA. *Triangle,* about 50 and 100 ng of template DNA were used in each of the reactions.

Transcriptional activity of the *snail* gene is correlated with increases in acetylation of H3 and in the trimethylation of H3 Lys^4 at the promoter, suggesting that dynamic regulation of these histone residues is a key step in the activation mechanism. In A375 and Colo829 melanoma cells, Snail promoter activity is also correlated with chromatin remodeling at the 3′ enhancer. These changes include the formation of a DNase HS, the formation of a DNA loop allowing physical interaction between the enhancer and the promoter, and the acquisition of active histone modifications at both the enhancer and promoter. Our findings suggest a model in which the enhancer recruits transcriptional activators, which then interact with the promoter to induce activation, in part through histone modification (Fig. 6). This may be similar to the mechanism revealed by a distal NF-κB enhancer for the MCP-1 gene, which is activated by the tumor necrosis factor. In that system, the distal enhancer recruits coactivating histone acetyltransferases (HATs) to acetylate the proximal promoter region chromatin (41).

Known activators of Snail transcription include NF-κB, TGFβ, PI3K, and MEK signaling pathways (23–25). Manipulation of each of these pathways failed to affect the activity of the Snail 3′ enhancer in reporter assays (data not shown), and we have shown that the induction of Snail by GSK3β inhibition failed to induce HS4 formation. This is not surprising given that response elements to the above signals localize to the Snail promoter (23–25). The wide assortment of pathways and factors capable of regulating Snail indicates that distinct signaling pathways are employed in different cell types. We present evidence that the 3′ enhancer operates in a melanoma/melanocyte-specific manner, as shown by specificity of DNase hypersensitivity and reporter activity. This specificity, in addition to evolutionary conservation of the underlying sequences at the enhancer, raises the question of the enhancer's function *in vivo*. Because Snail is an early marker for the neural crest (45), of which melanocytes are a derivative, an attractive hypothesis is that the 3′ enhancer directs the expression of Snail in the developing neural crest. The observation that melanoma cells up-regulate Snail through a conserved enhancer also underscores the ability of cancer cells to reactivate normal developmental gene expression programs to enhance their metastatic spread and survival (2).

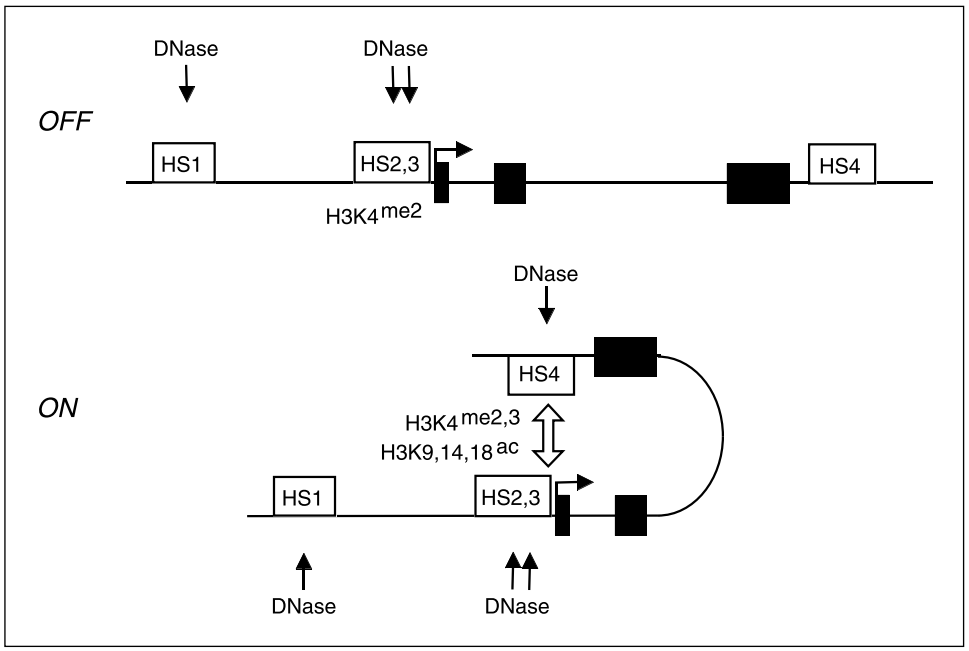

Figure 6. Model of Snail regulation by the 3′ enhancer. In cells that do not express Snail, DNase hypersensitivity at the 5′ end of the gene and histone methylation levels suggest that Snail is in a poised state for transcription. In Snail-expressing cells, the 3′ enhancer is activated, leading to the appearance of DNase HS4 and the formation of an interaction between the proximal promoter region and the enhancer. Expression of Snail is concomitant with the detection of histone acetylation and methylation associated with gene activation.

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