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Snail1 transcriptional repressor binds to its own promoter and controls its expression

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ABSTRACT

The product of *Snail1* gene is a transcriptional repressor of E-cadherin expression and an inductor of the epithelial–mesenchymal transition in several epithelial tumour cell lines. Transcription of *Snail1* is induced when epithelial cells are forced to acquire a mesenchymal phenotype. In this work we demonstrate that Snail1 protein limits its own expression: Snail1 binds to an E-box present in its promoter (at −146 with respect to the transcription start) and represses its activity. Therefore, mutation of the E-box increases Snail1 transcription in epithelial and mesenchymal cells. Evidence of binding of ectopic or endogenous Snail1 to its own promoter was obtained by chromatin immunoprecipitation (ChIP) experiments. Studies performed expressing different forms of Snail1 under the control of its own promoter demonstrate that disruption of the regulatory loop increases the cellular levels of Snail protein. These results indicate that expression of *Snail1* gene can be regulated by its product and evidence the existence of a fine-tuning feed-back mechanism of regulation of *Snail1* transcription.

INTRODUCTION

The Snail1 family members *Snail1 (Snail)* and *Snail2 (Slug)* are essential for triggering epithelial-to-mesenchymal transitions (EMTs) during embryonic development and tumour progression (1). Both genes codify for DNA-binding proteins with activity as transcriptional repressors. In mammals, Snail1 blocks E-cadherin expression by binding to specific 5′-CACCTG-3′ boxes in its promoter (2,3). Snail1-induced E-cadherin downregulation is necessary for early phases of embryonic development, since mice deficient in Snail1 expression fail to downregulate E-cadherin levels and to complete gastrulation (4). Repression of E-cadherin transcription is also particularly relevant in the transition from adenoma to carcinoma, since a causal relationship between loss of expression of this protein and the invasive properties of some tumours has been established (5,6).

Effects of Snail1 expression on epithelial cells are not limited to repress E-cadherin, since it induces a complete EMT (3,7), Accordingly, some other epithelial genes are directly repressed by Snail1 as MUC1 (7), and the tight junction proteins claudins and occludin (8). Other Snail1 targets are vitamin D_3 receptor (9), the β-subunit of the Na^+, K^+ ATPase (10), and p53 (11) and cycD2 (12), two genes presumably responsible for the resistance to apoptosis and decreased proliferation observed in cells transfected with Snail1. Moreover, Snail1 stimulates the expression of matrix proteases (13), Wnt5a factor (14), transcriptional factors Zeb1 and Lef-1 (7,14), and the mesenchymal markers vimentin and fibronectin (FN) (3,7).

Snail1 protein is composed by two well defined domains that interact with each other (15). The C-terminal domain is responsible for binding to the DNA and presents specificity for sequences with a 5′-CACCTG-3′ core. The N-terminal is required for transcriptional repression and can recruit histone deacetylase family members (16). Snail1 repressive activity can also be modulated by phosphorylation of a proline–serine-rich sequence situated in the regulatory domain. Two phosphorylation motifs have been allocated in this subdomain, one

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

involved in Snail1 export from the nucleus, and the other in its ubiquitinylation and degradation (15,17). GSK-3β kinase seems to be responsible for the modification of both motifs (17). Moreover, the C-terminus of Snail1 protein can be phosphorylated by PAK-1 kinase (18); in this case this modification maintains the protein in the nucleus. Subcellular distribution of Snail1 is also sensitive to the expression of the STAT3-target LIV1 Zn transporter (19).

Upregulated expression *SNAIL1* gene has been detected in several experimental conditions in which cells are forced to adopt a mesenchymal phenotype (20–26). In order to study the elements controlling *SNAIL1* gene expression, we have recently cloned and characterized a DNA fragment corresponding to the human promoter (26). The activity of this promoter (−869/+59, respect to the transcription start) mimics the expression of Snail1 during EMT and is greatly dependent on ERK2 and GSK-3β/NFκB pathways activity (26,27). Other researchers have demonstrated that PI3 kinase (PI3K) activity also controls *Snail1* transcription and promoter activity (24), probably acting on the same pathway than GSK-3β/NFκB. However, these pathways are also active in epithelial cells and do not enterely explain the specificity of expression of Snail1 in mesenchymal cells. In this article we describe the existence in this *SNAIL1* promoter of a functional 5′-CACCTG-3′ E-box that acts as a negative element. We also demonstrate that Snail1 binds to this element and therefore creates a negative loop controlling its own expression.

MATERIALS AND METHODS

Cell culture

Cells were grown in DMEM (Invitrogen) containing 10% FBS (Biological Industries) unless otherwise specified. The generation and properties of HT-29 M6 clones and SW-480 cells stably transfected with Snail1-HA has been described previously (2,9). Use of other cell lines (MiaPaca-2, RWP-1, SW-620, NIH-3T3) has been reported previously (26).

SNAIL1 promoter fragments and other DNA constructs

Cloning of the human *SNAIL1* promoter (−869/+59) in pGL3 basic (Promega), was described previously (26). Note that a putative snail binding site of the plasmid was eliminated, and therefore named pGL3*. The human *SNAIL1* promoter constructions −194/+59, −125/+59 and −78/+59 have also been reported. Mutant promoters in the Ebox3 (−869/+59 Mut E1 and −194/+59 Mut E1) were obtained using the QuickChange™ site-directed mutagenesis kit (Stratagene). The sense oligonucleotide sequence was 5′-CCAGCAGCCGGCG**A**ACCT**A**CTCGGGGAGTG-3′ and the antisense was 5′-CACTCCCCGAG**T**AGGT**T**CGCCGGCTGCTGG-3′, mutated oligonucleotides are displayed in bold. Preparation and use of Snail1-P2A and Snail1 ZnF mutants has been reported (2).

Depletion of *SNAIL1* mRNA levels by micro-interference RNA (miRNA)

Cloning of a human Snail specific miRNA in pPRIME-CMV-GFP vector was performed as described (28). An oligonucleotide containing two specific human SNAIL1 sequences (5′-CGATGTGTCTCCCAGAACT-3′ and 5′-GACCGATGTGTCTCCCAGAACT-3′) was amplified and cloned in pKS plasmid. Positive clones were sequenced and cloned in EcoRI/XhoI sites of pPRIME-CMV-GFP vector. A scrambled sequence was used as control. Plasmids were transfected to RWP-1 cells using Lipofectamine-Plus Reagent according to manufacturer's instructions (Invitrogen). Transduced (GFP+) cells were sorted by fluorescence activated cell sorter (FACS) and two pools of transfected cells were used for further assays.

Analysis of the expression of Snail1 controlled by its own promoter

Snail1-HA and Snail1-P2A-HA forms were cloned in pGL3* −194/+59 SNAIL1 promoter or pGL3* −194/+59 (MUT E1) vectors using XbaI/HindIII sites present in pGL3*.

pGL3* −194/+59 SNAIL1 prom-Snail1-HA, pGL3* −194/+59 (Mut E1) SNAIL1 prom-Snail1-HA, pGL3* −194/+59 SNA1 prom-Snail1-P2A-HA, pcDNA3-Snail1-HA or pcDNA3-Snail1-P2A-HA were transfected together with 70 ng of peGFP as internal control to RWP-1 and SW-480 cells. Protein expression was analyzed by western blot 48 h after transfection. Cell extracts for western blotting analysis were done in SDS buffer [25 mM Tris–HCl (pH 7.6), 10 mM KCl, 1 mM EDTA, 1% SDS]. Equal amounts of total cellular extracts were subjected to 15% SDS–PAGE and transferred to a nitrocellulose membrane. Blots were analyzed with antibodies anti-HA (rat mab, Roche) or anti-enhanced green fluorescent protein (EGFP) (mouse mab JL-8, Clontech).

Luciferase reporter assays

Reporter assays were performed using 250 ng of the indicated human *SNAIL1* promoter. Cells were cotransfected with 0.1, 1 and 10 ng of expression plamids encoding Snail1 wild-type or mutant proteins. SV40–*Renilla* luciferase plasmid (1 ng) was cotransfected to control the efficiency of transfection. Expression of Firefly and *Renilla* luciferases were analyzed 48 h post-transfection, according to manufacturer's instructions.

Chromatin immunoprecipitation assay (ChIP)

NIH-3T3, SW-480 and SW-480-Snail1 or HT-29 M6 and HT-29 M6-Snail1 (4×10^6 cells) were cross-linked with 1% formaldehyde. Cell lysates were prepared in IP buffer [16.7 mM Tris (pH 8), 167 mN NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS] for SW-480 cells and HT-29 M6 cells or in lysis buffer [50 mM Tris (pH 8), 10 mM EDTA and 1% SDS] for 10 min at room temperature. Cell lysates were sonicated to generate 200–1500 bp DNA. Immunoprecipitation of ectopic Snail1-HA (SW-480 and HT-29 M6 transfectants) was carried out with antibodies against HA epitope (Roche) in IP buffer 1. NIH3T3 endogenous Snail1 was immunoprecipitated with a specific monoclonal antibody (Mab) rose against the Snail1 protein (29) in IP buffer. Precipitates were then re-extracted with lysis buffer and re-immunoprecipitated for 3 h at 4°C; then, the samples where treated with elution buffer (100 mM Na_2CO_3, 1% SDS) and incubated at 65°C overnight to reverse formaldehyde cross-linking. Samples were treated with proteinase K and RNAse and DNA purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham). Promoter regions

were detected by PCR amplification with the following specific primers: *SNAIL1* human promoter (GI: 9 650 757) 5′-GGCGCACCTGCTCGGGGAGTG-3′ and 5′-GCCGATTCGCGCAGCA-3′, corresponding to sequences 20 603–20 623 and 20 811–20 796, respectively; *Snail1* mouse promoter (GI: 2 105 424) 5′-CGCACCTGCTCCGGTCTCAG-3′ and 5′-CTACGATCCCCTAGCAGCAG-3′, corresponding to sequences 683–703 and 802–822, respectively *CDH1* (E-Cadherin) promoter (GI: 29 568 028) 5′-ACTCCAGGCTAGAGGGTCAC-3′ and 5′-CCGCAAGCTCACAGGTGCTTTGCAGTTCC-3′ (80 636–80 655 and 80 853–80 825, respectively); human negative Control (*Cyclophilin A*, GI: 5 882 164): 5′-ATGGTCAACCCCACCGTG-3′ and 5′-TGCAATCCAGCTAGGCATG-3′ (137–154 and 800–782, respectively) and murine negative control (*RNA pol II*, GI: 45 501 362) 5′-ACTCCAGGCTAGAGGGTCAC-3′ and 5′-TAGGTGCTCAGACCTCGTCA-3′.

Gel retardation assays

Assays were performed essentially as described previously (2) using recombinant proteins glutathione *S*-transferase (Snail1-GST and GST as a control) and a ^{32}P-labelled double stranded oligonucleotide corresponding to the −173/−125 sequence of human *SNAIL1* promoter.

Semi quantitative and quantitative RT–PCR analysis

Total RNA was extracted using Gen Elute Mammalian total RNA kit (Sigma). Semi-quantitative analysis of exogenous murine *Snail1* or endogenous human *SNAIL1* RNAs was performed as described previously (7) using 28, 29 and 28 cycles, respectively. FN and E-cadherin (CDH1) RNA levels were determined as described previously (7). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was analyzed using oligonucleotides 5′-GGCCAGACTTTGTTGGATTTG-3′ and 5′-TGCGCTCATCTTAGGCTTTGT-3′ for 29 cycles. Quantitative determination of RNA leves was performed in triplicate using QuantiTect SYBR Green RT–PCR (Qiagen) and the same oligonucleotides. RT–PCR and data collection were performed on ABI PRISM 7900HT. All quantitations were normalized to an endogenous control Cyclophilin or HPRT. The relative quantitation value for each target gene compared to the calibrator for that target is expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to Cyclophilin or HPTR).

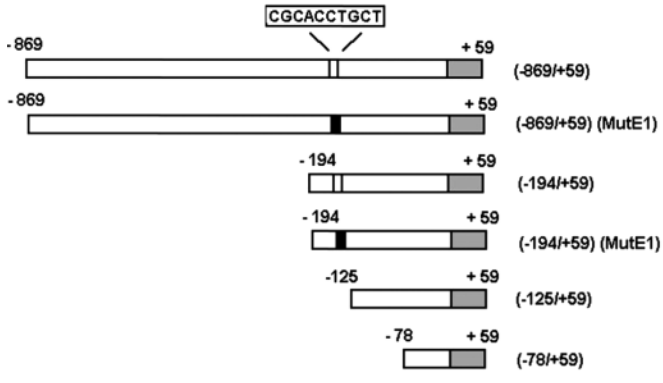
RESULTS

A 5′-CACCTG-3′ box in *SNAIL1* promoter represses its activity

We have cloned and characterized a DNA fragment corresponding to human *SNAIL1* promoter (−869/+59). This promoter was active in all cell lines analyzed although it presented greater activity in cells with higher levels of Snail1 mRNA (26). A diagram of the different fragments of this promoter used in this work is shown in Figure 1. Analysis of the activity of these constructions revealed the existence of an inhibitory sequence located between nucleotides −194 and −125, since deletion of this sequence increased the activity of the promoter in most cell lines (compare values for −194/+59 and −125/+59 promoters, Figure 1). A 5′-CACCTG-3′ E-box, corresponding to the sequence bound by Snail1 in other promoters, was present between nucleotides −149 and −144 (both included). To check the relevance of this element, the 5′-CACCTG-3′ was mutated to 5′-AACCTA-3′, a sequence unable to bind Snail1 (see below). Mutation of this element significantly increased the activity of −194/+59 promoter in RWP-1, SW-480, SW-620, NIH-3T3 (Figure 1) and MiaPaca-2 (data not shown) cells. In cells presenting low levels of Snail1 (HT-29 M6) (2), the effect of this mutation was minimal. Identical results were obtained when the mutation was performed on the −869/+59 promoter.

Snail1 represses *SNAIL1* promoter and downregulates *SNAIL1* mRNA levels

We analyzed the ability of Snail1 to inhibit the activity of *SNAIL1* promoters in cells presenting low levels of this protein.



Cell line				
HT-29 M6	RWP-1	SW-480	SW-620	NIH-3T3
0.57 ± 0.14	0.78 ± 0.3	0.59 ± 0.1	0.74 ± 0.3	0.53 ± 0.2
0.51 ± 0.2	1.4 ± 0.3	1.1 ± 0.1	1.6 ± 0.1	1.1 ± 0.2
1	1	1	1	1
0.75 ± 0.1	1.5 ± 0.3	1.7 ± 0.3	1.9 ± 0.2	1.6 ± 0.3
ND	1.6 ± 0.3	1.6 ± 0.6	1.8 ± 0.3	1.8 ± 0.2
0.62 ± 0.3	1.5 ± 0.3	0.52 ± 0.2	ND	1.7 ± 0.4
Promoter Activity				

Figure 1. *SNAIL1* promoter contains an E-box that binds a repressor. This figure depicts the length and activity of the indicated *SNAIL1* promoters, either wild-type or bearing a mutation in the E-box (MutE1), determined as described in Materials and Methods. The table shows the average ± SD of four or five experiments performed; values were also referred to that obtained for −194/+59 wild-type *SNAIL1* promoter in each cell line. ND, not determined.

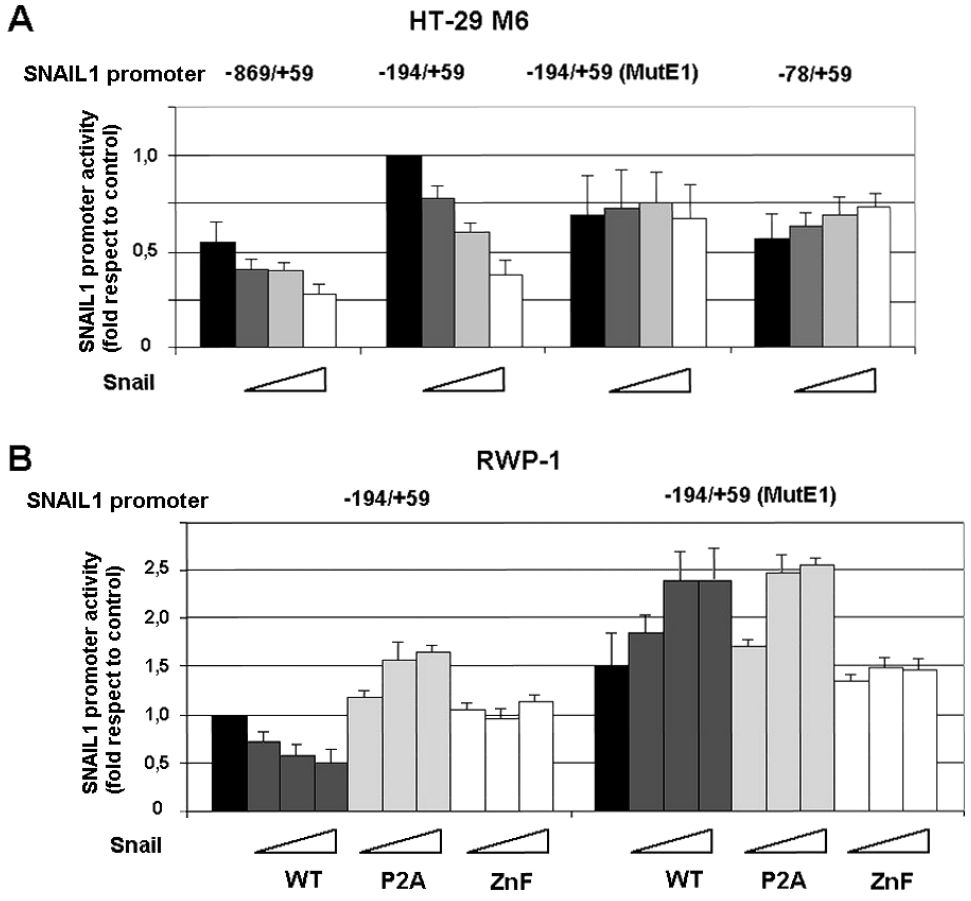


Figure 2. Snail1 represses the activity of *SNAIL1* promoter. Activity of the different promoter fragments was determined in HT-29 M6 (**A**) and RWP-1 cells (**B**) by transient transfection; when indicated, wild-type Snail1 or P2A or ZnF mutant cDNAs were cotransfected at several concentrations. The three mutants were expressed at similar levels (data not shown). Black bars correspond to activity of each promoter in the absence of Snail1 cotransfection. The table shows the average ± SD of three experiments performed.

As observed in Figure 2A, Snail1 transfection to HT-29 M6 cells repressed the basal activity of −869/+59 and −194/+59 promoters but was inactive on −194/+59 fragment when the E-box was mutated (−194/+59 Mut E1), or on fragments lacking the 5′-CACCTG-3′ box (−78/+59). Same dependence on the E-box for the inhibition by Snail1 was also observed in RWP-1 cells (Figure 2B). Analysis of the effect of different Snail1 mutants on Snail1 promoter indicated that depletion of the entire N-terminus (in the ZnF mutant) or substitution of Pro2 to Ala (P2A) prevented the inhibitory effect (Figure 2B). Both mutants are inactive on E-cadherin promoter, as it has been published previously (2). Curiously, although ZnF mutant was totally inactive, P2A Snail1 seemed to increase the activity of the promoter. A similar slight stimulation was observed when the effect of wild-type Snail1 was studied on the −194/+59 fragment bearing the mutated E-box (Figure 2B).

Next, we checked whether the Snail1 repression of its own promoter correlated with a decrease of endogenous *SNAIL1* RNA levels. Taking advantage that murine *Snail1* RNA can be easily distinguished from the human one, the effect of the ectopic expression of Snail1 on endogenous *SNAIL1* RNA levels was analyzed by RT–PCR. As presented in Figure 3A, murine Snail1 transfection to HT-29 M6 cells clearly downregulated endogenous *SNA1* mRNA. Inhibition of endogenous *SNAIL1* by ectopic expression of *Snail1* cDNA in several HT-29 M6 clones was determined to be between 40 and 60% by quantitative RT–PCR (qRT–PCR) (Figure 3B). A pool of RWP-1 cells transfected with Snail1 also showed a similar decrease in endogenous *SNAIL1* mRNA (Figure 3B).

Downregulation of Snail1 endogenous levels confirmed that this transcription factor was repressing the activity of the endogenous promoter. Expression of a miRNA specific for *SNAIL1* diminished the levels of this mRNA in RWP-1 cells (Figure 4A). A control miRNA did not exert any effect. Activity of *SNAIL1* promoter was upregulated by 50–60% in cells transfected with this miRNA respect to the control (Figure 4B), a similar increase to that detected after the mutation in the E-box (see Figure 1).

Snail1 protein binds to *SNAIL1* promoter

Binding of Snail1 to *SNAIL1* promoter was verified by Gel-shift and ChIP assays. Gel-shift assays indicated that recombinant Snail1 fused to GST-Snail1 binds efficiently to an oligonucleotide including the E-box sequence (Figure 5A). Presence of the shifted band was competed with an excess of

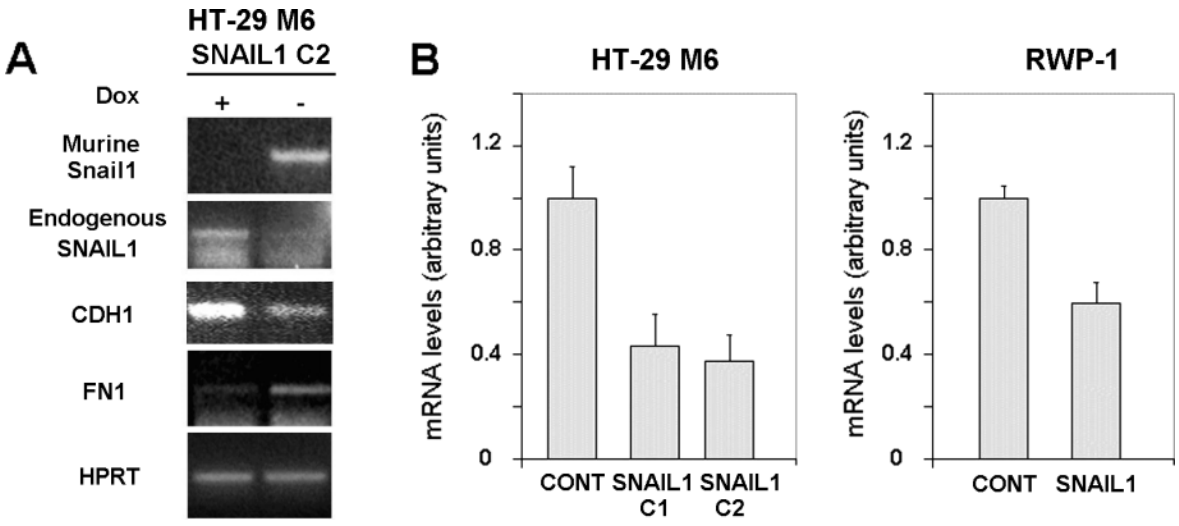


Figure 3. Snail1 downregulates *SNAIL1* mRNA. RNA was obtained from control HT-29 M6 cells or clones expressing murine Snail1-HA SNAIL1 (clones C1 and C2), grown in the absence of doxyciclin unless indicated. Note that these cells express ectopic murine Snail-HA only in the absence of doxyciclin. RNA was also obtained from a pool of RWP-1 cells stably transfected with pcDNA3-Snail1-HA or with empty plasmid. Levels of endogenous human Snail1 *(SNAIL1)* RNA were determined as indicated by semi-quantitative (**A**) or quantitative RT–PCR (qRT–PCR) (**B**). The analysis of murine Snail1 (*Snail1*), E-cadherin (*CDH1*), fibronectin (*FN1*) and an internal control *(HPRT)* is also shown.

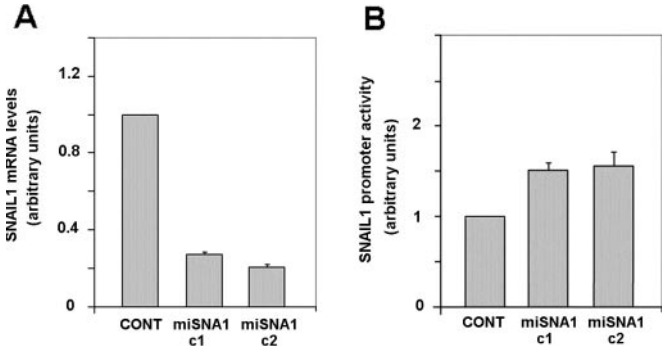


Figure 4. Interference in the expression of Snail1 increases *SNAIL1* promoter activity. RWP-1 cells expressing a control pPRIME-GFP plasmid or two pools containing a miRNA specific for human SNAIL1 (miSNAIL c1 and c2) were obtained as described in Materials and Methods and grown in standard conditions. RNA was isolated and the expression of SNAIL1 was determined by qRT–PCR as indicated above (**A**). The three populations were transfected with 25 ng of (−869/+59) SNAIL1 promoter and the activity of this promoter determined as indicated (**B**). The figure shows the average ± SD of two experiments performed in triplicate.

unlabelled oligonucleotide but not with a version containing the mutant E-box described above, indicating that Snail1 presents the same requirements for binding to this sequence than to those described previously in *CDH1* promoter (2). Association of Snail1 protein to *SNAIL1* promoter was also demonstrated by ChIP analysis. HA-tagged Snail1 was immunoprecipitated with a HA Mab from clones stably expressing Snail1 in HT-29 M6 cells or SW-480 cells, and presence of *SNAIL1* or *CDH1* promoter sequences were determined by PCR. This analysis confirmed that Snail1 protein binds to native *SNAIL1* promoter *in vivo* (Figure 5B). A similar association was detected with *CDH1* promoter, a well-established target of Snail1 protein (Figure 5B).

Binding of endogenous Snail1 to *Snail1* promoter was also verified using a specific Mab for Snail1 protein (29). *SNAIL 1* promoter sequences were present in the immunoprecipitated Snail1 protein from NIH-3T3 fibroblasts (Figure 5C).

A Snail1 inhibitory feed-back control is active in cell culture

In order to determine the relevance of this feed-back loop, Snail1-HA cDNA was expressed in RWP-1 cells under the control of a fragment of its own promoter (−194/+59). The constructions used in this experiment are shown in Figure 6. We reasoned that the interruption of this inhibitory loop, either by using a Snail1 mutant unable to repress (P2A mutant) or a promoter version with the E-box mutated (−194/+59 Mut E1), should produce higher levels of expression of the ectopic protein, detected with the HA antibody. As observed in Figure 6, the levels of Snail1-HA protein were clearly higher when the Mut E1 promoter was used, or when Snail1 P2A protein was expressed, with respect to the control, carrying the wild-type promoter and Snail1 cDNA. This increase was reproducibly detected in three experiments and cannot be attributed to differences in the transfection efficiency or by a higher stability of P2A protein (Figure 5). As expected, expression of (194/+59) SNAIL1 Prom-Snail1 (P2A) did not affect activity of *CDH1* promoter, since this mutant is inactive, whereas both (194/+59) SNAIL1 Prom-Snail1 WT and (194/+59) Mut E1 SNAIL1 Prom-Snail1 WT repressed it (data not shown).

Similar results than in RWP-1 were observed in SW-480 cells: P2A protein was detected at higher levels than wild-type Snail1 when expressed under the control of *SNAIL1* promoter, but not when under a constitutive cytomegalovirus (CMV) promoter. Thus, also in these cells, Snail1 controls the activity of its own promoter. Therefore, these results indicate the existence of an inhibitory feed-back mechanism that controls Snail1 expression, dependent on the repressive activity of the protein and the integrity of the 5′-CACCTG-3′ element in the *SNAIL1* promoter.

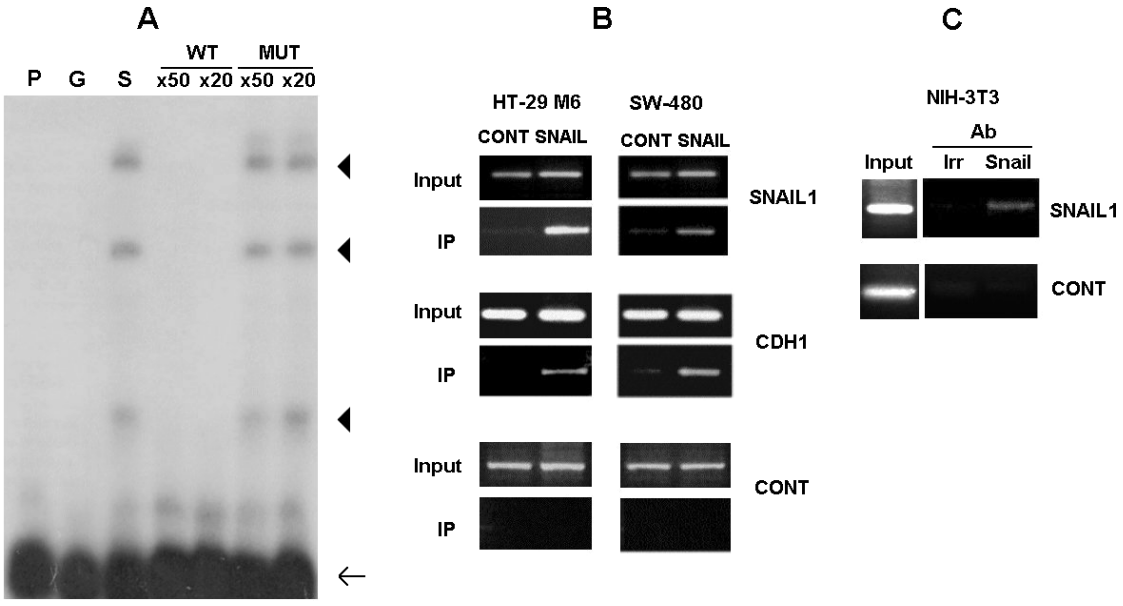


Figure 5. Snail1 binds to *SNAIL1* promoter. (**A**) Affinity-purified GST–Snail1 fusion protein or GST protein was incubated with double-stranded P^{32}-labelled oligonucleotides corresponding to the E-box of *SNAIL1* promoter. P, no protein added; G, 150 ng of GST. In the reminder lanes, binding experiments were carried out with 150 ng of GST–Snail1 without competitor (S) or competing with a 20- or 50-fold excess of unlabelled wild-type (WT) or mutant (MT) oligonucleotide. Arrow, free probe; arrowhead, specific shifted band. (**B**) ChIP analysis was performed as indicated in Materials and Methods using control or Snail1-HA transfected HT-29 M6 or SW-480 cells. Analysis of E-cadherin (CDH1) and Cyclophillin (CONT) was performed as positive and negative controls. (**C**) ChIP was performed immunoprecipitating endogenous Snail1 from NIH-3T3 fibroblasts with a specific anti-Snail1 MAb or an irrelevant IgG. Presence of Snail1 promoter-specific sequences was determined as indicated in Materials and Methods. Genomic sequences of RNA pol II gene were analyzed as negative control.

DISCUSSION

In the last five years, the essential role of Snail1 in the control of EMT has been supported by new evidences (30). Therefore, the study of the mechanisms that control Snail1 expression is a matter that deserves special attention. Recent results have demonstrated that Snail1 gene expression requires the activity of ERK2 and PI3K signalling pathways (24,26). However, activity of these two pathways is not specific of mesenchymal cells. Therefore, we have investigated additional mechanisms of control of Snail1 transcription. We show in this article that Snail1 can directly repress its own expression, creating a self-inhibitory loop by binding to an E-box sequence present in its promoter. It is worth indicating that this E-box is conserved in the Snail1 promoters sequenced in mouse, rat, macaque and bovine; and also in *Drosophila melanogaster* and zebrafish.

Existence of feed-back loops has been described previously, and they seem to be particularly relevant in cell pathways implicated in embryo development (31). This mechanism provides the possibility of buffering, allowing corrections of the cell system when it is perturbed. In the case of Snail1, it is also possible that this negative feed-back loop may contribute to the oscillatory pattern of expression of this gene during somitogenesis that has been recently described (32). Our RNA interference experiments also indicate that the existence of this self-repression is significant for controlling *SNAIL1* expression in epithelial cells, avoiding that transient increases in ERK2 and PI3K induce a sustained activation of Snail1 protein and the subsequent phenotypic changes. Therefore, this loop would be responsible for controlling the stability of Snail1 expression.

This capability of Snail1 protein to bind its own promoter has also been detected in cells with a mesenchymal phenotype. Mutation of the E-box in *SNAIL1* promoter increased the activity of this promoter in these cells, indicating that the feed-back control is also active. We speculate that this self-limitation of Snail1 transcription might be relevant to prevent undesired effects of this transcription factor, as those related with inhibition of cell growth, an effect observed when Snail1 is over-expressed in several cell lines (12).

A recent report, published while this article was under revision, demonstrates that another member of the Snail family, Snail2 can also bind to an E-box present in its own promoter (33). Unexpectedly, on this promoter Snail2 does not act as a repressor but as an activator. According to our results, Snail2 did not affect *SNAIL1* promoter activity (S. Peiró, M. Escrivà and A. G. de Herreros, unpublished data). In any case, these results provide new evidence indicating self-regulation of their own promoters may be a general property of the Snail family.

It also should be remarked that, respect to other cell targets of Snail1 repression described so far, *SNAIL1* promoter is the only one than contains just one E-box in the 600 bp upstream the transcription start. This is probably the reason that originates that repression of *SNAIL1* promoter by Snail1 is more modest than those previously measured on other promoters. We know that this feed-back control, although restricts *SNAIL1* transcription, can be at least over-run in cells receiving a very potent stimulation of ERK2 and PI3K pathways, that would cause a substantial activation of *Snail1* transcription and the subsequent EMT. Therefore, we suggest that this feed-back control of Snail1 expression might be responsible

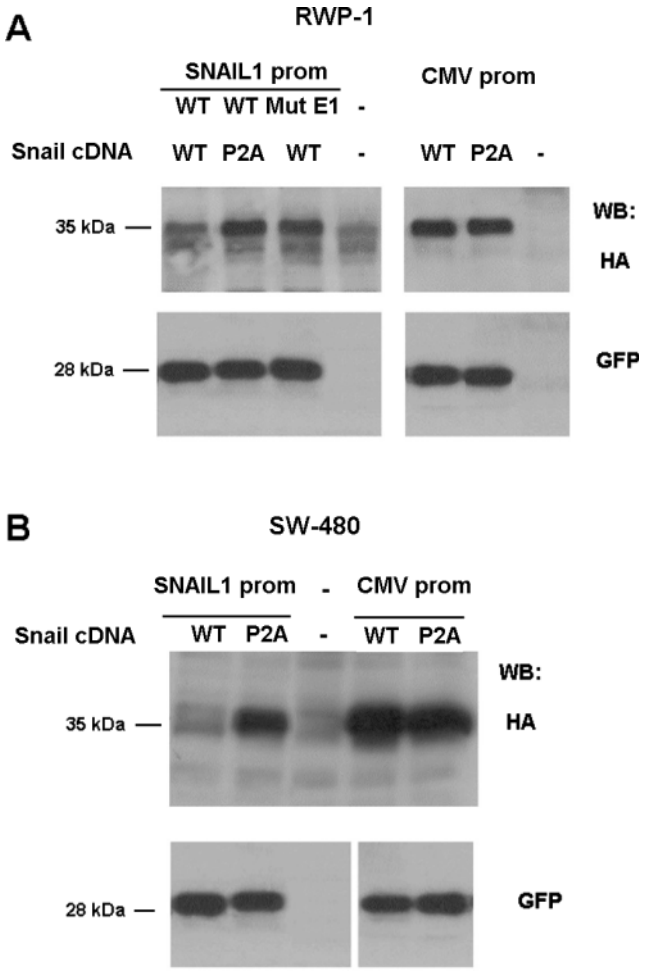


Figure 6. Snail1 represses its own expression. Wild-type or P2A mutant Snail1-HA cDNA were inserted in pGL3 plasmid substituting the luciferase reporter gene, and placed under the control of *SNAIL1* (−194/+59) promoter, either wild-type or mutated in the E-box (mut E1). Therefore the following constructions were used pGL3(−194/+59 SNAIL1 prom)-Snail1-HA, pGL3(−194/+59 (Mut E1) SNAIL1 prom)-Snail1-HA, pGL3(−194/+59 SNA1 prom)-Snail1-P2A-HA. These plasmids were transfected to RWP-1 (**A**) or SW-480 cells (**B**) and expression of ectopic Snail1-HA was analyzed two days later by western blot using the HA antibody. GFP was cotransfected to check that differences in the expression were not due to distinct efficiency of transfection. In parallel, WT or P2A Snail1 was expressed under the control of CMV promoter in order to check that the two proteins present a similar stability in these cells. A control with not-transfected cells was also included. The panels show the results of representative experiments of three performed in each cell line. The estimated sizes of Snail-HA and GFP are shown.

for the establishment of a threshold for the levels of the signals that cause a sustained expression of Snail1. In any case, the existence of this feed-back pathway helps to understand the intrinsic cell networks controlling EMT during early embryo development and provide new insights to explain the induction of this transition during tumour invasion.

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