The transcription factor Slug represses *E-cadherin* expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors

Victoria Bolós[1], **Hector Peinado**[1], **Mirna A. Pérez-Moreno**[1,*], **Mario F. Fraga**[2], **Manel Esteller**[2] and **Amparo Cano**[1,‡]

[1]Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), Arturo Duperier, 4, 28029 Madrid, Spain
[2]Centro Nacional de Investigaciones Oncológicas, Melchor Fernández Almagro, 4, 28029 Madrid, Spain

*Present address: Laboratory of Mammalian Cell Biology and Development, The Rockefeller University. 1230 York Avenue, Box 300. New York, NY 10021, USA
‡Author for correspondence (e-mail: acano@iib.uam.es)

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Summary

Transcriptional repression mechanisms have emerged as one of the crucial processes for the downregulation of *E-cadherin* expression during development and tumour progression. Recently, several E-cadherin transcriptional repressors have been characterized (Snail, E12/E47, ZEB-1 and SIP-1) and shown to act through an interaction with proximal E-boxes of the *E-cadherin* promoter. We have analyzed the participation of another member of the Snail family, Slug, and observed that it also behaves as a repressor of *E-cadherin* expression. Stable expression of Slug in MDCK cells leads to the full repression of *E-cadherin* at transcriptional level and triggers a complete epithelial to mesenchymal transition. Slug-induced repression of *E-cadherin* is mediated by its binding to proximal E-boxes, particularly to the E-pal element of the mouse promoter. Detailed analysis of the binding affinity of different repressors to the E-pal element indicates that Slug binds with lower affinity than Snail and E47 proteins. These results, together with the known expression patterns of these factors in embryonic development and carcinoma cell lines, support the idea that the in vivo action of the different factors in *E-cadherin* repression can be modulated by their relative concentrations as well as by specific cellular or tumour contexts.

Key words: Slug, E-cadherin, Epithelial to mesenchymal transition (EMT)

Introduction

Maintenance of stable cell-cell contacts and cell polarity is an essential requirement for the functionality and homeostasis of epithelial tissues in the adult organism. This strict tissue organization is lost during the progression of epithelial tumours (carcinomas) and is particularly evident at the invasion stage when tumour cells dissociate from the primary tumour and acquire the ability to traverse the basement membrane that separates the epithelial tissues from the adjacent connective tissues (Behrens et al., 1992; Stetler-Stevenson et al., 1993). The E-cadherin–catenin complexes represent the main adhesion system responsible for the maintenance of cell-cell contacts in epithelial tissues (Takeichi, 1995; Huber et al., 1996). Downregulation of E-cadherin expression or functional perturbations of the E-cadherin–catenin complexes have been found to occur very frequently during the progression of carcinomas (Takeichi, 1993; Birchmeier and Behrens, 1994; Christofori and Semb, 1999). Indeed, loss of E-cadherin expression has been shown to be responsible for the loss of intercellular adhesion occurring during invasion (Perl et al., 1998). As a consequence, during the invasive process, tumour cells not only lose their cell-cell adhesion properties but also frequently undergo profound changes in their phenotype known as epithelial to mesenchymal transitions (EMTs) (Behrens et al., 1992; Christofori and Semb, 1999). The invasive process is reminiscent of the EMTs that occur during defined stages of embryonic development, such as during mesoderm formation at the primitive streak and the delamination of the neural crest cells from the neuroectoderm (Bellairs, 1987; Burdsal et al., 1993). The EMTs that occur both during development and tumour invasion are associated with the functional loss of E-cadherin. The molecular bases of the E-cadherin downregulation during tumour progression have started to be elucidated in the past years. The present evidence indicates that silencing of E-cadherin expression may involve genetic and epigenetic changes (Christofori and Semb, 1999). Among them, hypermethylation of the *E-cadherin* promoter and transcriptional repression are emerging as predominant mechanisms in most carcinomas (Risinger et al., 1994; Yoshiura et al., 1995; Henning et al., 1996; Giroldi et al., 1997; Hajra et al., 1999; Rodrigo et al., 1999; Tamura et al., 2000; Cheng et al., 2001). Several independent factors, Snail, E47, ZEB-1 (δEF-1) and SIP-1 (ZEB-2), have been recently characterized as transcriptional repressors of *E-cadherin* acting through interaction with specific E-boxes of the proximal promoter (Cano et al., 2000; Batlle et al., 2000;

Perez-Moreno et al., 2001; Grooteclaes and Frisch, 2000; Comijn et al., 2001). Interestingly, some of these E-cadherin repressors were previously characterized as important regulators during embryonic development. The role of Snail in triggering EMT during development of diverse species from *Drosophila* to mammals is now firmly established (for a review, see Nieto, 2002); SIP-1 shows specific expression during early neural development in *Xenopus* (Van Grunsven et al., 2000); and the expression pattern of the *E2A* gene (coding for E12/E47) in early mouse embryo is compatible with its participation in EMTs (Perez-Moreno et al., 2001).

The zinc finger factor Snail belongs to the Snail superfamily of transcriptional repressors (Hemavathy et al., 2000; Nieto, 2002), in which other relevant members are found, such as Slug. Mouse Snail and Slug share a high degree of homology both at the N-terminal region, with the SNAG transactivation domain, and the C-terminal region containing four and five zinc fingers, respectively (Manzanares et al., 2001). However, they differ in the intermediate P-S rich region, with Slug members containing a specific 29 amino-acid sequence, called the Slug domain (Manzanares et al., 2001). Gain- and loss-of-function studies have indeed established the role of Slug in triggering EMTs in defined regions of the chick and *Xenopus* embryos (Nieto et al., 1994; Carl et al., 1999; La Bonne and Bronner-Fraser, 2000; Del Barrio and Nieto, 2002). These evidences suggest that Slug could also participate in the repression of *E-cadherin* expression. However, other observations have not supported such a repressor role for Slug, since overexpression of Slug in rat bladder carcinoma cells was not able to repress *E-cadherin* but instead induced desmosome dissociation (Savagner et al., 1997) and our previous analysis in a collection of mouse epidermal keratinocyte cell lines did not show any correlation between *E-cadherin* and *Slug* expression profiles (Cano et al., 2000). These apparent discrepancies can either indicate intrinsic functional differences between Slug and Snail factors in relation to E-cadherin regulation or reflect the specific contribution of different cellular contexts in which both factors could act as repressors.

In order to get further insights into the function of Slug and Snail factors in relation to *E-cadherin* expression, we have analyzed the potential role of Slug as a repressor in parallel to Snail using the prototypic epithelial cell system of MDCK cells. Here we show that stable expression of Slug in MDCK cells leads to a full EMT associated with the complete repression of *E-cadherin* expression, increased expression of mesenchymal markers and acquisition of a highly migratory behaviour. The phenotypic effects induced by ectopic Slug expression in MDCK cells are apparently independent of the endogenous *Snail* expression as no significant changes in *Snail* mRNA levels or in *Snail* promoter activity were detected in MDCK-*Slug* transfected cells. Binding analysis indicates that Slug binds specifically to the E-boxes of the E-pal repressor element of the mouse *E-cadherin* promoter although, interestingly, with lower affinity than Snail and E47 repressors. These results indicate that Slug and Snail are functionally equivalent as *E-cadherin* repressors and that both factors can contribute to EMTs and/or the maintenance of the mesenchymal/migratory phenotype depending of their relative concentrations and/or the specific cellular and tissue context.

Materials and Methods

Plasmid constructs and generation of recombinant proteins

The complete cDNA sequence of mouse *Slug* was obtained by introducing the UGA stop codon from the previously described *mSlug* cDNA (Sefton et al., 1998) by PCR and was subcloned into the pcDNA3 expression vector (Invitrogen) under the control of the cytomegalovirus promoter. To obtain the GST-mSlug fusion construct, the 843 bp coding sequence of m*Slug* was restriction excised from the pcDNA3 construct and subcloned into the pGEX4T1 vector (Pharmacia Biotech) in frame with the gluthatione-S-transferase (GST) protein. Similarly, the full cDNA sequence of mouse *Snail* (Cano et al., 2000) was cloned in the pGEX4T1 vector. The sequences of the fusion constructs were verified by automatic sequencing from both ends using several internal oligonucleotides covering the full sequence. The generation of GST-mE47 construct has been recently described (Perez-Moreno et al., 2001). Production and purification of the recombinant GST-fusion proteins was carried out following standard procedures.

Generation of anti-Slug and anti-Snail sera

Polyclonal antibodies against GST-Snail and GST-Slug recombinant proteins were generated by injection into rabbits following standard procedures. The sera obtained from both kinds of injection were purified by affinity chromatography using the corresponding recombinant proteins linked to sepharose CNBr-columns (Pharmacia Biotech.).

Stable transfections

MDCK-II cells, grown in DMEM medium (Gibco BRL) in the presence of 10% FBS, 10 mM glutamine and antibiotics were transfected with 3 μg of pcDNA3-m*Slug* or control pcDNA3 vector as recently described (Cano et al., 2000; Perez-Moreno et al., 2001) using the Lipofectamine Plus reagent (Gibco BRL). Stable transfectants were generated after selection with 400 μg/ml G418 during three to four weeks. Four independent clones were isolated from pcDNA3-*Slug*, one of which was further subcloned by limited dilution, and six independent clones were isolated from control pcDNA3 transfections. The generation of MDCK-*Snail* cells has been previously reported (Cano et al., 2000).

RT-PCR analysis

Total RNA was isolated from the different cell lines, and RT-PCR analyses were carried out as previously described (Cano et al., 2000; Perez-Moreno et al., 2001). Mouse and canine PCR products were obtained after 30-35 cycles of amplification with an annealing temperature of 60-65°C. Primer sequences were as follows. For mouse *Slug*, forward: 5′ CGCGAATTCCCGCCCGCAGCCACC 3′; reverse: 5′ ACTCTCGAGCTAGTGTCAATGGGCGAC 3′ (amplifies a fragment of 843 bp). For canine *E-cadherin* (sequence kindly provided by Y. Chen, Harvard Medical School), forward: 5′ GGAATCCTTGGAGGGATCCTC 3′; reverse: 5′ GTCGTCCTCGC-CACCGCCGTACAT 3′ (amplifies a fragment of 560 bp). For canine *Snail*, forward: 5′ CCCAAGCCCAGCCGATGAG 3′; reverse: 5′ CTTGGCCACGGAGAGCCC 3′ (amplifies a fragment of 200 bp). For mouse and canine *glyceraldehyde-3-phosphate dehydrogenase* (GADPH*)*, forward: 5′ TGAAGGTCGGTGTGAACGGATTTGGC 3′; reverse: 5′ CATGTAGGCCATGAGGTCCACCAC 3′ (amplifies a fragment of 900 bp).

E-cadherin and *Snail* promoter analysis

The mouse *E-cadherin* promoter sequences (–178 to +92) in its wild-type and mutant Epal (mEpal) version were excised by *Xba*I restriction from the chloramphenicol acetyltransferase (*CAT*) reporter gene (Behrens et al., 1991) and cloned into the *Nhe*I site of pGL2 vector (Invitrogen) fused to a firefly *Luciferase* reporter gene (–178 wild-type and mEpal-luciferase constructs, respectively). A 900 bp fragment of the mouse *Snail* 5′ upstream sequences (containing nucleotides 8 to 905 of the reported proximal sequences of the mouse *Snail* gene) (Jiang et al., 1997) was amplified by PCR from a 5 kb genomic clone (a gift of T. Gridley, Jackson Laboratories, USA) using specific oligonucleotides linked to *Bam*HI and *Kpn*I restriction sites and cloned into the same restriction sites in the pXP1 vector fused to the *Luciferase* reporter gene. A mutation into an E-box element located at the –221 position of the mouse *Snail* gene (Jiang et al., 1997) was introduced by three cycles of PCR-directed mutagenesis using specific primers carrying the specific mutations (5′ CACCTG 3′ to 5′ **TG**CCTG 3′). To determine the activity of the *E-cadherin* and *Snail* promoters, cells were transiently transfected in 24 well plates with 200 ng of the wild-type or mutant reporter constructs and 20 ng of TK-Renilla construct (Promega) as a control for transfection efficiency. Where indicated, cotransfections were carried out in the presence of the indicated amounts of pcDNA3-*Slug* and pcDNA3-*Snail* vectors. Luciferase and renilla activities were measured using the Dual-Luciferase Reporter assay kit (Promega) and normalized to the wild-type promoter activity detected in mock-transfected cells. Alternatively, MDCK-mock and MDCK-m*Slug* cells were transiently transfected in P-60 dishes with 5 μg of the –178 wt construct or the mE-pal construct, fused to the *CAT* reporter gene (Behrens et al., 1991) and 2 μg of CMV-*luciferase* construct as a control for transfection efficiency. CAT and luciferase assays were performed as previously described (Faraldo et al., 1997; Rodrigo et al., 1999), with the activity normalized to that of the wild-type promoter detected in MDCK-mock cells.

Electrophoretic mobility band-shift assays (EMSA)

Band-shift assays with the ^{32}P-labeled wild-type E-pal probe were carried out with recombinant GST-Slug, GST-Snail and/or GST-E47 protein. Briefly, the incubation buffer used was: 20 mM Hepes pH 7.9, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.4 mM $ZnSO_4$, 40 μM $ZnCl_2$ and 10% glycerol. Incubations were performed for 30 minutes at room temperature. The indicated amounts of the different recombinant GST fusion or GST control proteins were used in the absence or presence of the indicated competitors. As an irrelevant competitor poly(dI-dC) (Amersham) was utilized. For supershift assays, 5 μg of rat monoclonal anti-mouse Slug (Liu and Jessell, 1998) (Hybridoma bank, Iowa University), rabbit polyclonal anti-Snail antibody or the corresponding control IgGs were added to the reaction buffer and incubated for 15 minutes at room temperature before addition of the labeled probe. Sequences of the oligonucleotides used as probes and/or competitors were: wild-type E-pal, 5′ GGCTGC**CACCTGCAGGTG**CGTCCC 3′ (E-boxes indicated in bold) and mutant E-pal, 5′ GGCTGC**CACCT**<u>**TT**</u>**AGGTG**CGTCCC 3′ (mutated nucleotides underlined).

Capillary electrophoresis mobility shift assay (CEMSA)

DNA-protein binding affinities were calculated by capillary electrophoresis using 5′ fluorescent modified oligonucleoties (6-FAM oligos) (obtained from Isogen) as recently described (Fraga et al., 2002). A neutral coating capillary (Beckman Coulter S.A.) (32.5 cm × 75 μm, effective length 20 cm) was used in a P/ACE MDQ capillary electrophoresis system (Beckman Coulter S.A.) connected to a Karat Software® data-processing station. The running buffer (40 mM Tris-borate, 0.95 mM EDTA, pH 8.0) was chosen to provide a low current when working at high voltage (30 kV, 923 V/cm) in order to maintain the stability of protein-DNA complexes during separation. Laser-induced fluorescence (LIF) was detected by excitation at 488 nm (3-mW Argon ion laser), and emissions were collected through a 520 nm emission filter (Beckman Coulter S.A.). Samples were injected under low pressure (0.2 psi) for 2 seconds and the run temperature was maintained at 20°C. The run was performed at 30 kV voltage with reverse polarity. Before each run, the capillary was conditioned by washing with running buffer for 2 minutes. Buffers and running solutions were filtered through 0.2 μm pore-size filters. Three replicates of each concentration were prepared and each was run twice. Binding reactions were performed in a modification of the binding buffer previously described (Wade et al., 1999). Increasing amounts of all proteins were added to 6-FAM-labeled DNAs in binding buffer (10 mM Tris HCl, pH 8.0, 3 mM $MgCl_2$, 50 mM NaCl, 0.4 mM $ZnSO_4$, 40 μM $ZnCl_2$, 0.1 mM EDTA, 0.1% NP-40, 2 mM DTT, 5% glycerol and 0.4 mg/ml BSA) and incubated overnight at 4°C. Binding affinities were quantified by Scatchard analyses using GraFit 3.1 software. In brief, the saturation of the oligonucleotide (R=[complex]/([complex]+[protein])) was plotted against increasing quantities of each protein. The concentration required for 50% saturation of binding ($R_{1/2}$) was then calculated, seeking the best fit of the data to different binding models/curves.

Immunofluorescence and western blot analysis

Cells grown to confluence on coverslips were fixed in methanol (–20°C, 30 seconds) and stained for the various epithelial and mesenchymal markers as previously described (Cano et al., 2000; Perez-Moreno et al., 2001). For F-actin staining, cells were fixed in 3.7% formaldehyde-0.1% Triton X-100 (30 minutes at room temperature), followed by incubation with FITC-phalloidin (Sigma Chemical Co.) for 30 minutes at room temperature and washed (4×) in excess PBS. Slides were mounted on Mowiol, and the preparations were visualized using a Zeiss Axiophot microscope equipped with epifluorescence. For detection of mSlug protein in MDCK transfectant cells, rabbit polyclonal anti-mouse Slug was used (1:50). Western blot analyses were carried out on whole-cell extracts with the indicated antibodies as previously described (Cano et al., 2000; Perez-Moreno et al., 2001). The antibodies used included: rat monoclonal anti-E-cadherin ECCD-2 (1:100) (provided by M. Takeichi, Kyoto University, Japan), mouse monoclonal anti-β-catenin (1:200) and mouse monoclonal anti-plakoglobin (1:500) (Transduction Lab.) and mouse monoclonal anti-vimentin (1:200) (Dako). Mouse monoclonal anti-α-tubulin (1:2000) (Sigma Chemical Co.) was used as a loading control. Western blot analysis of purified recombinant proteins was carried out with anti-GST (Sigma Chemical Co.) and anti-E47 (E2A.V18) (Santa Cruz Biotech.) polyclonal antibodies.

Migration assays

The migratory/motility behavior of transfectant cells was analyzed by the wound assay. Monolayers of confluent cultures were lightly scratched with a Gilson pipette tip and, after washing to remove detached cells, the cultures were observed at timely intervals as previously described (Cano et al., 2000; Perez-Moreno et al., 2001).

Results

Slug interacts with the E-pal element in the mouse *E-cadherin* promoter

To analyze the potential of Slug to interact with the *E-cadherin* promoter, band-shift studies (EMSA) were performed using the E-pal element of the mouse promoter containing two adjacent E-boxes (Behrens et al., 1991) as the labeled probe. When tested at high concentration (0.5-1 μg), recombinant GST-Slug protein bound specifically to the E-pal element, generating three retarded complexes (Fig. 1A). The three complexes were competed by an excess (×500) of the cold E-

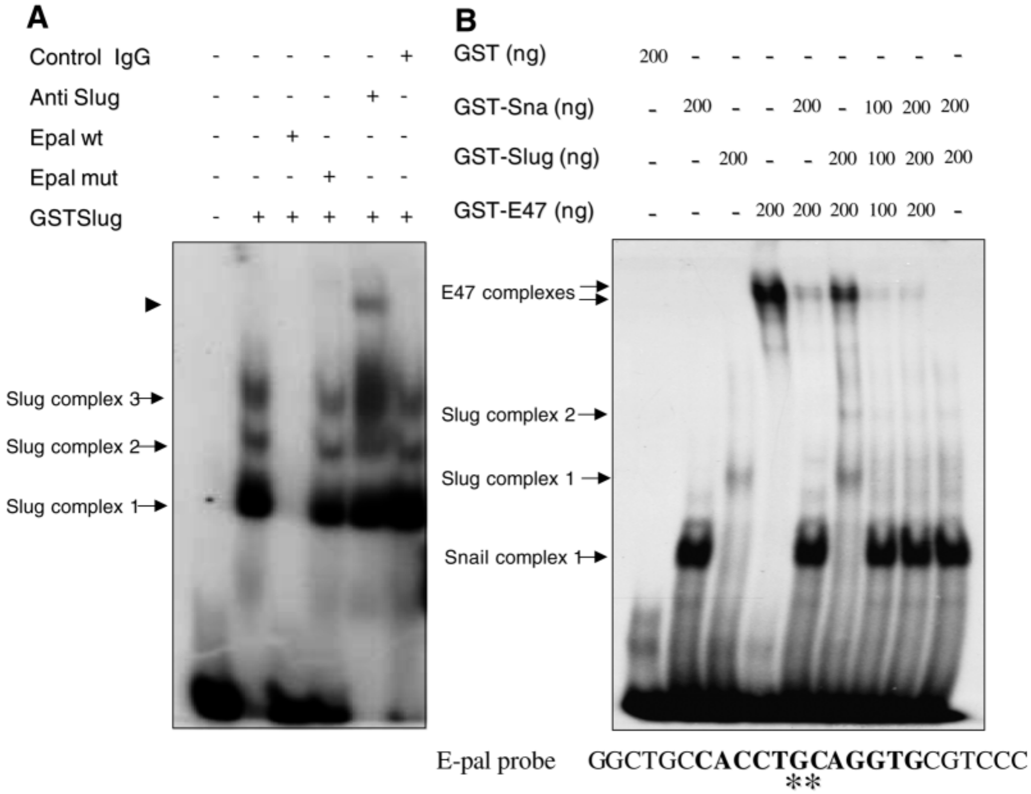

Fig. 1. The Slug transcription factor binds to the E-pal element of *E-cadherin* promoter through the E-boxes. (A) Recombinant GST-Slug protein (1 μg) was incubated with the ^{32}P-labeled E-pal probe in the absence or presence of 500-fold molar excess of wild-type or mutant cold oligonucleotides or in the presence of 5 μg of anti-Slug monoclonal antibody or control mouse IgG. The retarded complexes are indicated by arrows and the supershifted complex by an arrowhead. (B). Recombinant GST, GST-Snail, GST-Slug and GST-E47 proteins were incubated at the indicated combinations with the ^{32}P-labeled wild-type E-pal probe. The different retarded complexes detected are indicated by arrows. The complete sequence of the E-pal probe is indicated at the bottom of the figure with the two E-boxes showed in black letters. Asterisks indicate the position of the mutated nucleotides in the mEpal oligonucleotide.

pal oligonucleotide but uncompeted by the mutant E-pal version, carrying two point mutations in the central nucleotides that abolish the two E-boxes and the repression of this element (Behrens et al., 1991; Faraldo et al., 1997; Rodrigo et al., 1999). The specificity of the Slug complexes was further confirmed by the use of the anti-Slug monoclonal antibody, which leads to the appearance of a supershifted complex (arrowhead in Fig. 1A). Two recently described repressors of *E-cadherin*, Snail and E47 bHLH, also interact specifically with the E-pal element of the mouse *E-cadherin* promoter (Cano et al., 2000; Perez-Moreno et al., 2001). The binding of Slug, Snail and E47 fusion proteins to the E-pal element was then compared. As shown in Fig. 1B, when the three factors were tested independently, at 200 ng, Snail showed the highest affinity for E-pal binding, followed by E47, whereas Slug apparently bound more weakly than the former two factors, generating only the highest mobility complex (Slug complex 1). The higher affinity of Snail for the E-pal element compared with the other two factors was also detected when different combinations of the three factors were tested (Fig. 1B). When GST-Snail protein was present together with a stoichiometric amount of the other two factors, the Snail complex always predominated over the E47 and/or Slug complexes. However, Slug complexes could be detected when GST-Slug protein was tested in the presence of GST-E47 protein alone (Fig. 1B). The relative affinity of Snail and Slug factors for the E-pal element was analyzed in concentration-dependent band-shift assays performed for both factors (Fig. 2). As can be observed, GST-Snail saturated the binding of the labeled probe at 50-100 ng, generating a main fast mobility complex (Snail complex 1) and a minor slower mobility complex (Snail complex 2) (Fig. 2, right panel). At higher GST-Snail concentrations, the abundance of the second lower mobility complex increased slightly. By contrast, a weak binding of GST-Slug protein was detected at 50-100 ng (Fig. 2, left panel), generating only the highest mobility complex (Slug complex 1), and saturation of the probe was only observed at higher concentrations (250-500 ng) when the two additional slowest mobility complexes (Slug complexes 2 and 3) were detected. The complexes generated by either GST-Snail and GST-Slug factors were effectively competed by an excess (×1000) of the cold wild-type oligonucleotide but uncompeted by a similar excess of the cold mutant E-pal oligonucleotide. The results presented in Figs 1 and 2 also show a different mobility for the retarded complexes generated by GST-Snail and GST-Slug proteins that can not be explained by their molecular mass (Sefton et al., 1998). Instead they suggest that the recombinant Slug protein binds preferentially to the E-pal probe in a dimeric or higher multimeric molecular form, whereas recombinant Snail might bind preferentially in a monomeric form.

The specific binding affinities of the different GST-fusion factors for the E-pal element were analyzed by quantitative capillary electrophoresis mobility shift assays (CEMSA) (Fraga et al., 2002). The integrity of the different fusion proteins used was analyzed by western blot using anti-GST or anti-E47 antibodies (Fig. 3D). All of the GST-fusion proteins exhibited a high integrity, although the presence of intact GST protein could also be detected in all samples (Fig. 3D; data not shown). The relative amount of intact GST-fusion proteins (containing the DNA-binding domain at the C-terminal region in all cases) present in each preparation was estimated (by comparison with standard protein loadings) and used for calculation of the actual concentration of intact proteins used in the subsequent capillary electrophoresis assays (Fig. 3A-C).

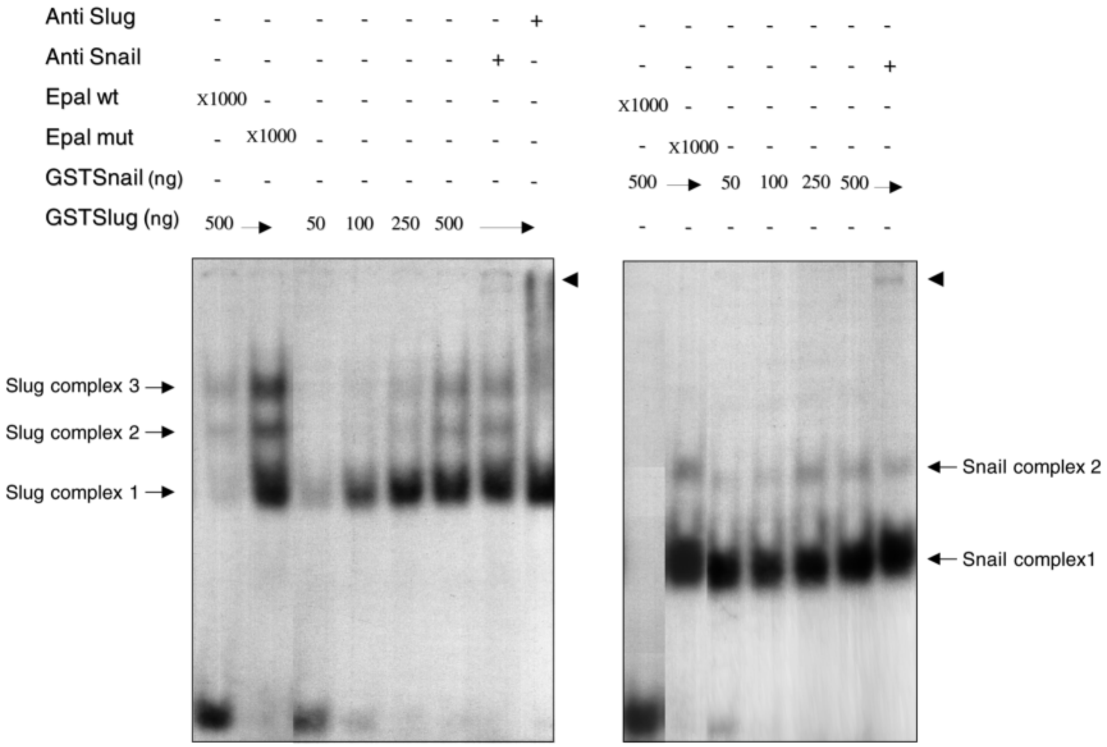

Fig. 2. Differential binding of Snail and Slug to the E-pal element. Recombinant GST-Slug (left panel) and GST-Snail (right panel) proteins were incubated at the indicated concentrations (50-500 ng) with the ^{32}P-labeled wild-type E-pal probe in the presence or absence of 1,000-fold molar excess of the wild-type or mutant cold oligonucleotides or in the presence of 5 μg of anti-Slug monoclonal antibody (left panel) or anti-Snail polyclonal antibody (right panel). The different retarded complexes are indicated by arrows and the supershifted complexes by arrowheads. Wild-type E-pal and mE-pal oligonucleotides are as shown in Fig. 1.

At 1 nM concentration, GST-Snail protein exhibited a main electrophoretic mobility complex eluting at 5 minutes 4 seconds and a very minor peak that was slightly retarded; GST-Slug protein showed almost no binding activity, and GST-E47 showed a slower eluting complex (at 5 minutes 8 seconds) of lower intensity than the GST-Snail complex (Fig. 3A). Control GST protein did not exhibit any binding to the 6-FAM E-pal probe (Fig. 3A, upper panel), and no binding of the various GST-fusion factors was obtained with the 6-FAM mutant E-pal oligonucleotide (data not shown). At 1 μM concentration, the three GST-fusion factors exhibited saturating binding to the 6-FAM wild-type E-pal probe, generating complexes of different sizes as estimated from their specific elution profiles (Fig. 3B). Interestingly, at saturating concentrations GST-Slug generated several complexes of apparent sizes larger than the main one generated by GST-Snail, and GST-E47 also generated two large complexes of similar intensity (Fig. 3B). These results are in close agreement with the results obtained in the EMSA assays showed in Fig. 1 and Fig. 2. Nevertheless, it should be noted that the capillary electrophoresis assay is optimized for resolution of DNA samples and, therefore, the size of the different DNA-protein complexes can not be accurately estimated (Fraga et al., 2002). Kinetic assays performed with the capillary electrophoresis system allowed quantitative determination of the binding affinities for the different GST-fusion factors from the $R_{1/2}$ parameter (concentration required for 50% saturation of binding) (Fraga et al., 2002). As shown in Fig. 3C, the estimated $R_{1/2}$ for GST-Snail ($5.4\pm0.08\times10^{-10}$ M) is two orders of magnitude higher than the $R_{1/2}$ for GST-Slug ($2.2\pm3.73\times10^{-8}$ M) and one order of magnitude higher than the $R_{1/2}$ for GST-E47 ($6.07\pm0.82\times10^{-9}$ M).

Taken together, the results of the band-shift analyses indicate that the binding affinity of the three analyzed factors for the E-pal element is: GST-Snail>GST-E47>GST-Slug. The relative binding affinity of the three factors deduced from the band-shift assays is also in agreement with our recent in vivo binding analysis for the E-pal element using the one-hybrid approach, in which out of 130 isolated clones, 49% corresponded to Snail, 32% to E47 and one single clone to Slug (Cano et al., 2000; Perez-Moreno et al., 2001). Although a different representation of the three factors in the NIH3T3 library used for the one-hybrid analysis can not be excluded, this possibility seems unlikely since similar levels of *Snail* and *Slug* mRNA were observed in NIH3T3 cells (data not shown). These results, therefore, support the idea that the in vitro binding affinities of the recombinant factors detected in the band shift assays reflect the relative affinities of the different factors in vivo.

Slug represses *E-cadherin* promoter and induces EMT upon stable expression in MDCK cells

The ability of Slug to bind to the E-pal element prompted us to analyze its effect on the *E-cadherin* promoter activity. Transient transfection assays in the prototypic epithelial cell line MDCK in the presence of pcDNA3-*Slug* showed that Slug is able to repress the *E-cadherin* promoter activity in a dose-dependent manner, with a 50% inhibition achieved in the presence of 240 ng (Fig. 4A). Comparative analysis of the effect of Snail on the activity of the *E-cadherin* promoter, inducing 70% repression at 50 ng in this cell line (Fig. 4A), suggested a lower repressor activity of Slug on the mouse *E-cadherin* promoter. Similar results were obtained with the epidermal keratinocyte PDV cells, where Slug induces only a 30-40% inhibition of the *E-cadherin* promoter activity at concentrations (250-500 ng) at which Snail induces 60-80% inhibition (data not shown) (Cano et al., 2000). Although a

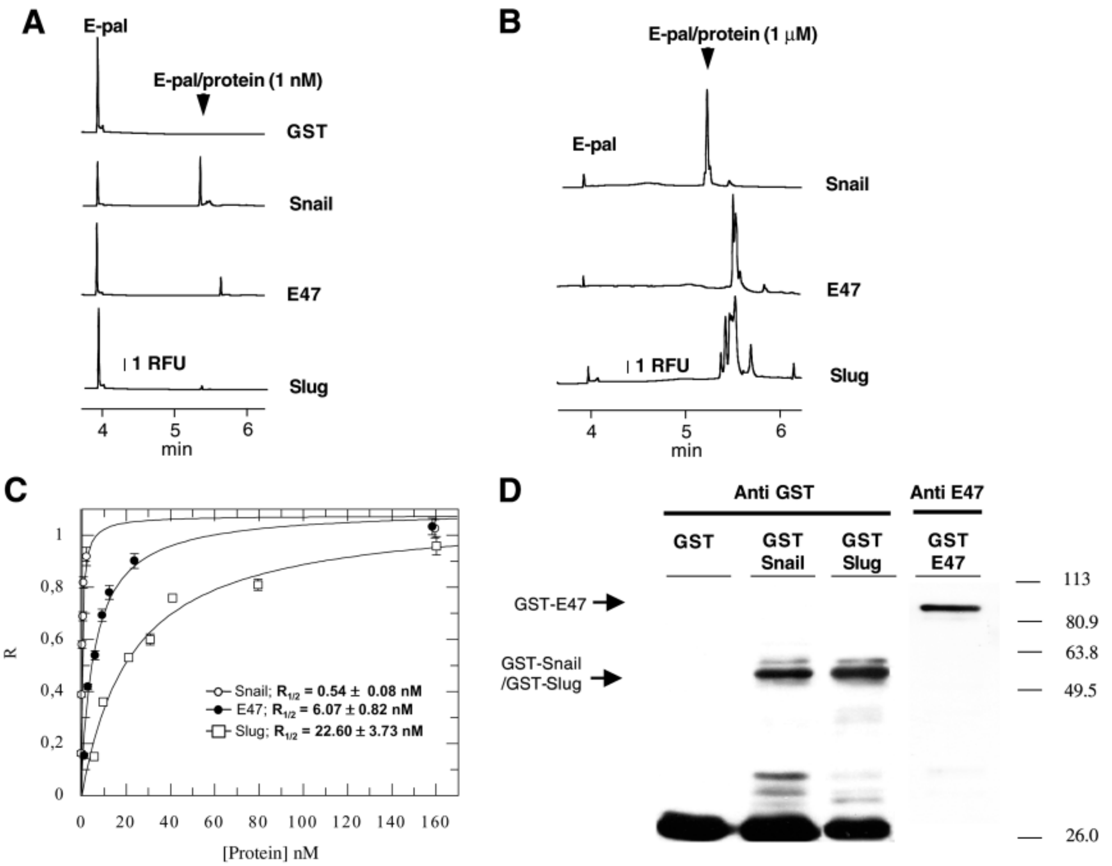

Fig. 3. Binding affinity of Snail, E47 and Slug recombinant proteins to E-pal synthetic oligonucleotide estimated by capillary electrophoretic mobility shift assays (CEMSA). (A,B) Electropheregrams for mixtures of 6-FAM-labeled wild-type E-pal oligonucleotide (24 nM) and **(**A**)** 1 nM of control GST, GST-Snail, GST-E47 and GST-Slug or (B) 1 μM of GST-Snail, GST-E47 and GST-Slug, subjected to CEMSA analysis as described in Materials and Methods. RFU, relative fluorescence units; min, elution time in minutes. The elution of the main E-pal oligo–GST-Snail complex is indicated by arrows (E-pal/protein) in both panels. (C) Concentration-dependent binding of GST-Snail (white circles), GST-E47 (black circles) and GST-Slug (white squares) to the 6-FAM wild-type E-pal probe. The indicated concentrations of the different recombinant proteins were analyzed by CEMSA, in duplicated samples, in three independent experiments. The single-site ligand-binding fit was performed using GraFit 3.1 software. R, saturation ([complex]/[complex]+[DNA]). [protein], protein concentration of intact GST-factors. Results are expressed as mean±s.d. (D) Western blot analysis of purified recombinant proteins. 50 μg of the purified recombinant proteins analyzed with anti-GST or anti-E47 polyclonal antibodies, as indicated. Migration of the molecular weight markers (in kDa) and of the different intact recombinant proteins is indicated at the side of the panel.

different efficiency in expression of Snail and Slug vectors after transient transfection can not be formally excluded, the above results suggest that Slug might exhibit a lower repression activity than Snail on the mouse *E-cadherin* promoter. This suggestion would also be in agreement with the lowest binding affinity of Slug for the E-pal element demonstrated in the binding assays.

To gain further insights into the role of Slug in the regulation of *E-cadherin* expression, gain-of-function studies were performed in MDCK cells. Cells were stably transfected with pcDNA3 (mock) or pcDNA3-*Slug* (*Slu*) vectors. Although no changes were observed in the morphology of MDCK-mock transfectants, a dramatic conversion to a fibroblastic phenotype was observed in four independent clones and three subclones isolated after transfection with the *Slug* expression vector. The results obtained for four of the selected clones are shown in Fig. 5 and compared to control-mock and MDCK-*Snail* cells, as recently described (Cano et al., 2000). The *Slug*-transfected cells apparently lost all epithelial characteristics and acquired a spindle appearance (Fig. 5b-e), similar to *Snail*-transfected cells (Fig. 5f). This phenotypic change was associated with a loss of E-cadherin expression (Fig. 5h-k), apparent loss and redistribution of other epithelial markers such as plakoglobin (data not shown) and increased organization of the mesenchymal markers fibronectin (Fig. 5n-q) and vimentin (Fig. 5t-w). The overall changes observed in the different markers in the *Slug*-transfectants are very similar to those recently described for MDCK-*Snail* cells (Cano et al., 2000) and are shown in Fig. 5 (panels l, r and x) for comparison. The qualitative changes in the various markers observed in the different *Slug*-transfectant clones by immunofluorescence were confirmed by western blot analysis of whole-cell extracts (Fig. 6A). This analysis confirmed the absence of E-cadherin and an increase in levels of vimentin and fibronectin in the *Slug*-transfected cells (Fig. 6A) (data not shown), as previously reported in MDCK-*Snail* and MDCK-*E47*-transfected cells

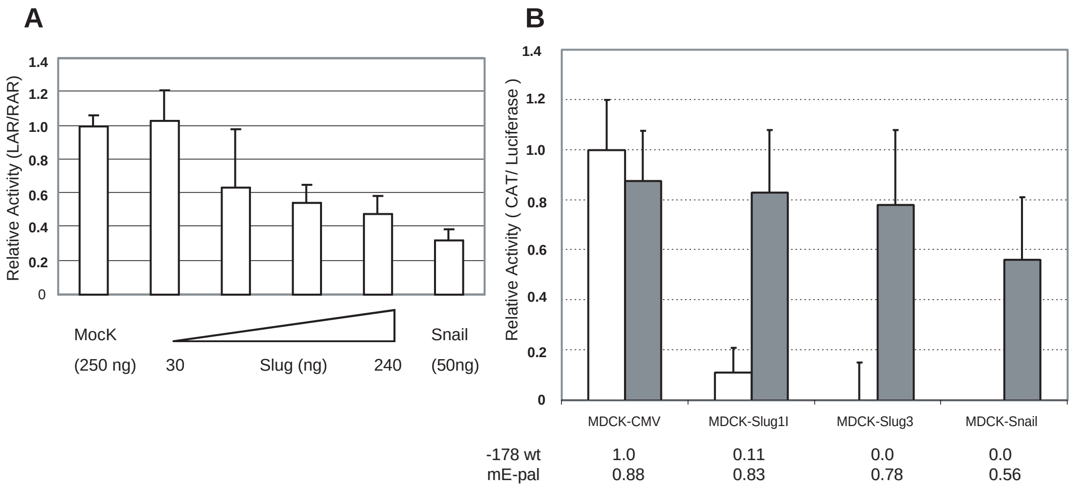

Fig. 4. *Slug* represses the activity of the mouse *E-cadherin* promoter both in transient and stable MDCK transfectants. (A) MDCK cells were transiently cotransfected with 200 ng of the –178 wild-type *E-cadherin* promoter construct fused to the *luciferase* reporter gene in the presence of the indicated amounts of pcDNA3 (Mock), pcDNA3-*Slug* or pcDNA3-*Snail* vectors. Luciferase and renilla activities were determined 24 hours after transfection. The activity of the promoter is expressed relative to that obtained in the mock-transfected cells. Results represent the mean±s.d. of at least two independent results. (B) The activity of the –178 wild-type *E-cadherin* promoter is strongly reduced or completely silenced in *Slug*- and *Snail*-expressing cells. MDCK-mock, two independent MDCK-*Slug* clones and MDCK-*Snail* cells were transiently transfected with the –178 wild-type (white bars) or mE-pal (grey bars) *E-cadherin* constructs fused to the *CAT* reporter gene. Luciferase and CAT activities were determined 24 hours after transfection. The activity of the promoter constructs is represented relative to that of the –178 wt construct detected in the mock-transfected clone. Results represent the mean±s.d. of two independent experiments. The relative levels of activity for both constructs in each cell line are also indicated at the bottom. Slu1I represents one subclone isolated from an original Slu1 clone; Slu3 represents an independently isolated clone.

(Cano et al., 2000; Perez-Moreno et al., 2001). In addition, inmunoblot analysis of plakoglobin and β-catenin in MDCK-*Slug* cells (Fig. 6A) showed reduced expression of the two catenins in most clones, a differential feature compared with MDCK-*Snail* cells and MDCK-*E47*, where no changes in plakoglobin levels were observed (Cano et al., 2000; Perez-Moreno et al., 2001). Analysis of *E-cadherin* expression by RT-PCR showed a complete absence of endogenous *E-cadherin* transcripts in MDCK-*Slug*-transfected cells (Fig. 6B). Since neither mSlug monoclonal nor polyclonal antibodies are useful for western blot detection, expression of exogenous mouse *Slug* mRNA transcripts was analyzed by RT-PCR. This analysis showed the expression of m*Slug* transcripts at various levels in the different *Slug* clones by RT-PCR (Fig. 6B). In addition, ectopic expression of the Slug protein could be observed in the nuclei of the transfected cells (Fig. 6Cb,c, compare with mock cells shown in panel a). The repression of *E-cadherin* expression in the *Slug* transfectants was further analyzed at the promoter level. As shown in Fig. 4B, the activity of the exogenous wild-type *E-cadherin* promoter was fully suppressed or reduced to 10% of the activity detected in MDCK-mock cells, as exemplified here for two independent MDCK-*Slug* clones. By contrast, the activity of the mutant E-pal construct was very similar in both MDCK-*Slug* and mock cells, indicating that Slug repression of the *E-cadherin* promoter is mediated through the E-pal element. The activity of the exogenous *E-cadherin* promoter constructs in MDCK-*Slug* transfectants is also very similar to that exhibited by MDCK-*Snail* cells (Fig. 4B).

The apparent similarity in the phenotype and molecular markers exhibited by the *Slug* and *Snail* transfectants raised the possibility that the effects observed in *Slug*-overexpressing cells could be due to increased expression of endogenous Snail rather than the direct effect of exogenous Slug. To investigate this specific point, the expression of endogenous *Snail* mRNA in different *Slug*-clones was analyzed by RT-PCR, using specific primers for canine *Snail*. As previously reported (Comijn et al., 2001), low levels of endogenous *Snail* mRNA were detected in control MDCK-mock cells, and no significant changes were observed in the *Snail* mRNA levels in the different MDCK-*Slug* clones (Fig. 6B) (variations from 0.7- to 1.2-fold of the level found in mock cells were estimated from the semiquantitative RT-PCR analysis). To further investigate the potential influence of Slug in the regulation of *Snail* expression, the activity of a *Snail* promoter construct carrying 900 bp of the 5′ upstream region of the mouse *Snail* gene (Jiang et al., 1997) was analyzed in MDCK-mock and selected *Slug*-transfected cell lines. As shown in Fig. 6D, this promoter construction exhibited a similar activity both in MDCK-mock and *Slug*-transfectant cells as well as in MDCK-*Snail* cells. Indeed, the slight variations in the *Snail* promoter activity observed in the different MDCK-*Slug* clones are very similar to the relative levels of endogenous *Snail* mRNA detected by RT-PCR (compare Fig. 6D with 6B, d*Sna* panel). Interestingly, mutation of a proximal E-box located at –221 position of the mouse *Snail* gene (Jiang et al., 1997) reduced the activity of the *Snail* promoter to about 50% in most of the analyzed cell lines (Fig. 6D, grey bars), suggesting a contribution of this E-box in the regulation of *Snail* expression. The results obtained in the RT-PCR analysis and *Snail* promoter studies strongly

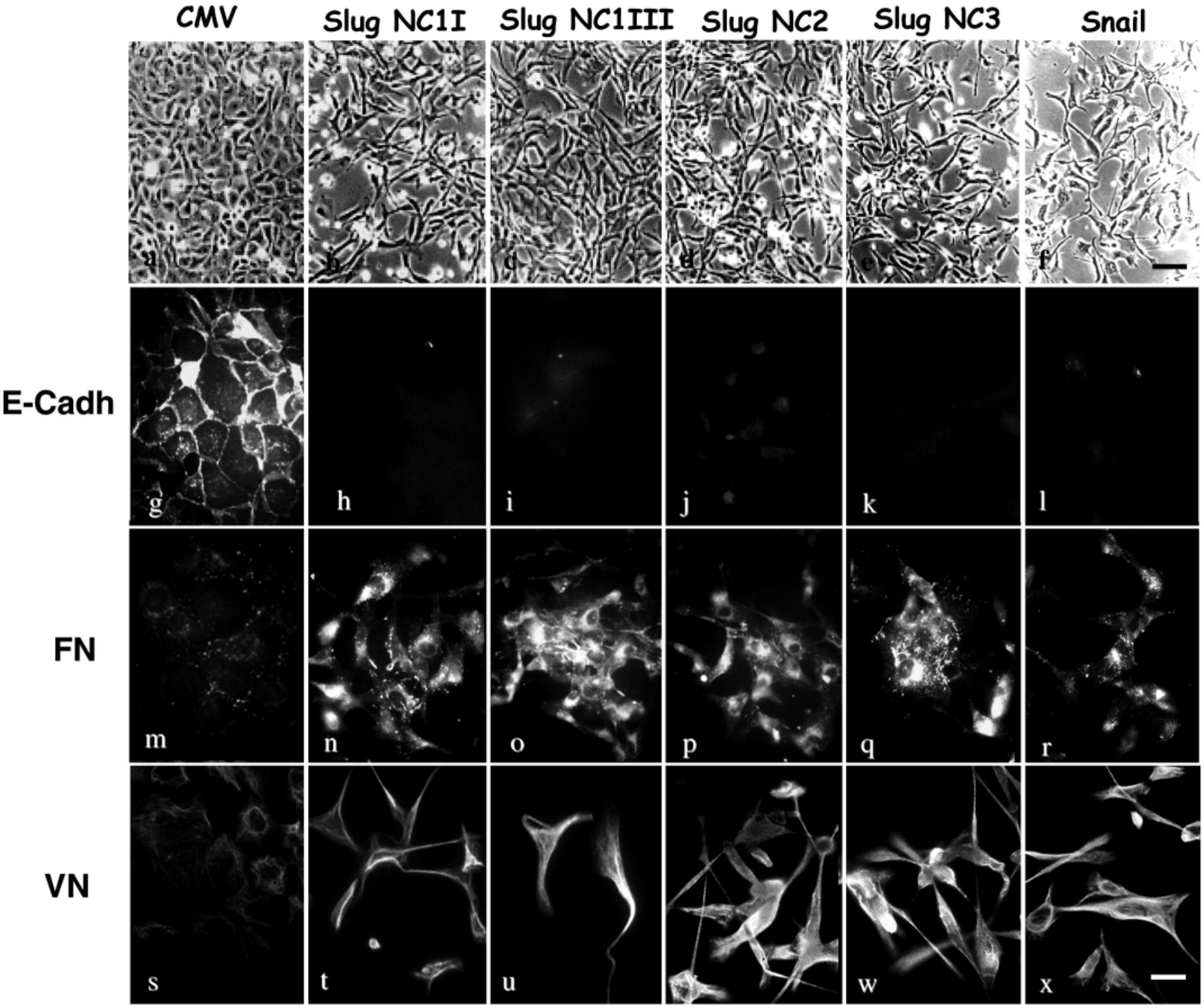

Fig. 5. Stable transfection of *Slug* into MDCK cells induces epithelial to mesenchymal conversion concomitantly with the loss of epithelial markers and the gain of mesenchymal markers. (a-f) Phase-contrast images of living, subconfluent cultures of a mock-transfected clone (a), four Slug-transfected clones (b,c,d,e*)* and Snail-transfected cells (f). (g-x) Immunofluorescent images of the indicated cell lines showing the localization and organization of E-cadherin (g-l), fibronectin (m-r) and vimentin (s-x). See the loss of E-cadherin stain and the increased expression and fibrous organization of fibronectin and vimentin in the *Slug* and *Snail* transfectants. Slu2 and Slu3 represent independent clones; Slu1I and Slu1 III represent two subclones isolated from an original Slu1 clone. Bars, 40 μm (a-f); 20 μm (g-x).

suggest that Slug overexpression does not contribute significantly to the regulation of *Snail* expression, at least in MDCK cells. Taken together, the above results indicate that stable overexpression of Slug in MDCK cells leads to the full repression of *E-cadherin* expression and to induction of a dramatic EMT, apparently independently from the level of endogenous *Snail* expression.

Slug expression induces a highly migratory behaviour

The process of EMT induced by overexpression of Slug in MDCK cells prompted the analyses of the migratory/motility properties of control and *Slug*-transfected cells in wound-culture assays. The results obtained with two of the selected *Slug* clones are shown in Fig. 7A, where it can be clearly observed that MDCK-*Slug* cells exhibited a highly migratory behaviour, beginning to enter the wound in a random fashion 6 hours post-incision (Fig. 7Ae,h). Approximately, 80-90% of the wound surface was colonized by Slug expressing cells 9 hours after the wound was made (Fig. 7Af,i), whereas at this time the mock-transfected cells had started to colonize the wound by coherent unidirectional migration (Fig. 7Ac). The migratory ability of MDCK-*Slug* cells in the wound assays is similar to or even higher than that of MCDK-*Snail* cells, which

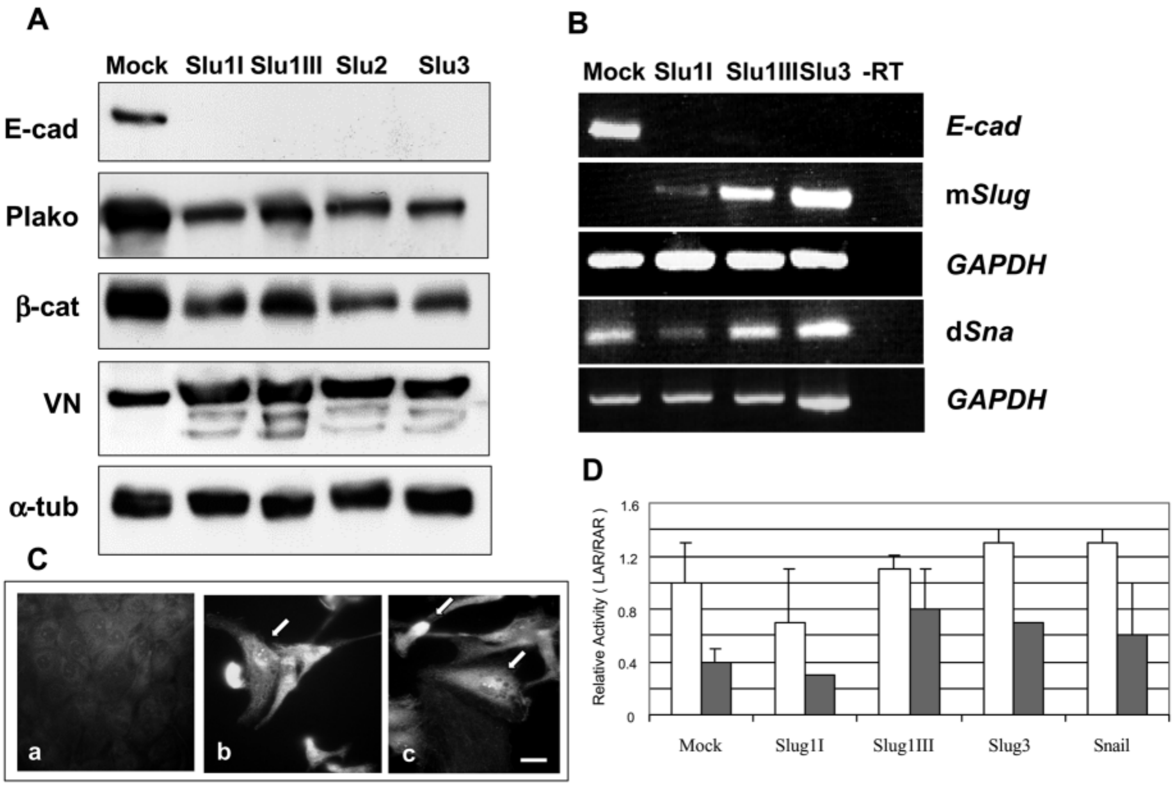

Fig. 6. The phenotypic effects induced by ectopic Slug expression in MDCK cells are associated with a full repression of E-cadherin expression and are independent of endogenous Snail expression. (A) Western blot analysis of whole cell extracts of the indicated proteins in mock- and *Slug*-transfected clones. E-cad, E-cadherin; Plako, plakoglobin; β-cat, β-catenin; VN, vimentin. Detection of α-tubulin (α-tub) levels was used as a loading control. (B) The presence of canine *E-cadherin*, mouse *Slug* and canine *Snail* transcripts in mock- and *Slug*-transfected clones was analyzed by RT-PCR. The expression of *GAPDH* was analyzed in the same samples as a control for the amount of cDNA present in each sample. The –RT lane shows the results of amplification in the absence of template. (C) Inmunofluorescence analysis for Slug expression in mock (a) and Slu 3 clone (b,c). Ectopic expression of the Slug protein was observed in the nuclei of the *Slug*-transfected cells (arrows in b and c). Bar, 20 μm. (D) The activity of the *mSnail* promoter in the *Slug*-transfectants corresponds to the endogenous *Snail* mRNA levels. The indicated cell lines were transiently transfected with the wild-type mouse *Snail* promoter construct (white bars) or with the mutant E-box (at –221) construct (grey bars) fused to a *luciferase* reporter gene. Luciferase and renilla activities were determined 24 hours after transfection. The activity of the promoter is expressed relative to that obtained in the mock-transfected cells with the wild-type construct. Results represent the mean±s.d. of at least two independent experiments.

require about 15 hours to completely heal the wound (Cano et al., 2000), and similar to the behaviour of the recently described MDCK-*E47* transfectants (Perez-Moreno et al., 2001) in this kind of assay. The organization of the actin cytoskeleton in *Slug*-transfectant cells is also compatible with its high migratory behavior. F-actin is organized in abundant stress fibres and lamellipodia-like structures in *Slug*-transfectants (Fig. 7Bb,c), in contrast to the cortical F-actin organization present in MDCK-mock cells (Fig. 7Ba). These studies also show that F-actin organization of MDCK-*Slug* transfectants is similar although not identical to that of MDCK-*Snail* cells, which exhibit a higher abundance of membrane protrusions and lamellipodia-like structures (Fig. 7Bd). The high migratory behaviour exhibited by *Slug* and *Snail* transfectants as compared to control mock cells cannot be attributed to their proliferation potential. In fact, both MCDK-*Snail* and -*Slug* transfectants exhibit a lower proliferation potential than control MDCK cells, with duplication times of 16-18 hours for *Slug* and *Snail* transfectants and 12 hours for mock cells.

Discussion

Downregulation of *E-cadherin* expression is a leading event during the progression of carcinomas into the metastatic cascade and is particularly required for the initial invasion stage. A great insight into the molecular mechanisms underlying *E-cadherin* silencing has been provided in recent years, with the finding that genetic and epigenetic mechanisms participate in different types of tumours and cancer cell lines (Christofori and Semb, 1999; Cheng et al., 2001). Analysis of the gene regulatory elements in the human and mouse *E-cadherin* genes has greatly supported the notion that repressors bound to proximal E-boxes of the *E-cadherin* promoter are major players in transcriptional repression in many different cellular contexts (Henning et al., 1996; Giroldi et al., 1997; Faraldo et al., 1997; Hajra et al., 1999; Rodrigo et al., 1999). Indeed, several *E-cadherin* transcriptional repressors have been characterized in the past two years that interact with the proximal E-boxes of the promoter (Cano et al., 2000; Batlle et al., 2000; Grooteclaes and Frisch, 2000; Perez-Moreno et al., 2001; Comijn et al., 2001). Of these, Snail was the first one

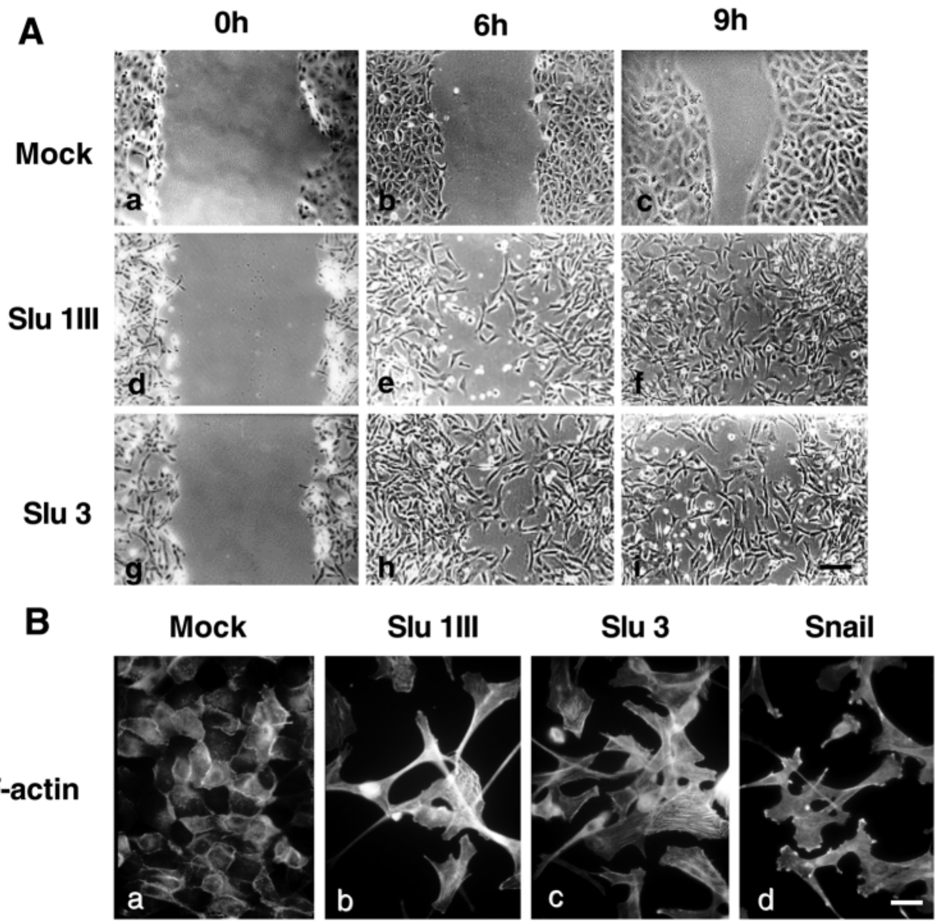

Fig. 7. Slug expression in epithelial cells induces a migratory and motile phenotype. (A) The motility/migratory behaviour of mock- (a-c) and *Slug*-transfected (d-i) cells was analyzed in an in vitro wound model. Confluent cultures of the mock and *Slug*-transfected clones were gently scratched with a pipette tip to produce a wound. Photographs of the cultures were taken immediately after the incision (a,d,g) and after 6 hours (b,e,h) and 9 hours (c,f,i*)* in culture. Bar, 40 μm. (B). The organization of F-actin was analyzed in fixed and permeabilized cells by incubation with FITC-phalloidin. Fluorescence images of mock (a), two *Slug*-transfected cell lines (b,c), and *Snail*-transfected cells (d). F-actin is organized in abundant stress fibres and lamellipodia-like structures in Slug-expressing cells. Bar, 20 μm.

described (Cano et al., 2000; Batlle et al., 2000). The Snail superfamily of zinc-finger transcription factors has emerged in the past years as important regulators of EMTs and other developmental processes, such as neural crest specification and pattern formation (for a review, see Nieto, 2002). Snail has now been firmly established as a repressor of *E-cadherin*, in early development of both *Drosophila* and mouse (Oda et al., 1998; Carver et al., 2001; Nieto, 2002) and in different murine and human carcinoma and melanoma cell lines and tumours (Cano et al., 2000; Batlle et al., 2000; Cheng et al., 2001; Poser et al., 2001; Yokoyama et al., 2001; Blanco et al., 2002). The role of Slug, another member of the Snail superfamily (Hemavathy et al., 2000; Nieto, 2002), as a potential *E-cadherin* repressor has remained uncertain. Previous studies in a rat bladder carcinoma cell line (Savagner et al., 1997) and in several mouse keratinocyte cell lines (Cano et al., 2000) did not support such a repressor role for Slug. In addition, *Slug* is not expressed in sites of EMT in the developing mouse embryo, explaining the lack of phenotype of the *Slug* mutant mice in these tissues (Jiang et al., 1998). However, it is expressed in EMT regions in both chick and *Xenopus* embryos, where it is able to drive EMTs (Nieto et al., 1994; Carl et al., 1999; LaBonne and Bronner-Fraser, 2000; Del Barrio and Nieto, 2002). The efficient role of Slug as inducer of EMT in chicken embryos most probably relies on its specific expression at those regions and the absence of Snail from EMT areas in this species (Sefton et al., 1998). A similar situation might occur in *Xenopus* embryos. The switching expression pattern between Slug and Snail family factors at EMT regions, observed in early chicken and mice embryos (Sefton et al., 1998), is probably due to the presence of differential control regulatory elements for both genes in the different species (Manzanares et al., 2001). Interestingly, Snail and Slug can be functionally equivalent when overexpressed in chick embryos (Del Barrio and Nieto, 2002). Therefore, Slug is a potential repressor of *E-cadherin*, at least in those specific cellular contexts. Moreover, the specific expression of *Slug* in migratory neural crest and mesodermal cells of the mouse embryo (Sefton et al., 1998; Cano et al., 2000) supports its involvement in the maintenance of the non-epithelial phenotype.

We provide here evidence for the repressor effect of Slug on the mouse *E-cadherin* promoter, and we have analyzed its relative contribution to this event compared with Snail and E47 repressors. The gain-of-function studies performed on the epithelial MDCK cell line indicate that overexpression of Slug fully represses endogenous *E-cadherin* expression and induces a dramatic EMT with all the leading characteristics of the process: increased expression and organization of mesenchymal markers and a high motility and migratory behaviour. The Slug-induced repression of *E-cadherin* in MDCK cells is exerted at the transcriptional level and dependent on the integrity of the two E-boxes of the E-pal element of the mouse promoter, as confirmed by the analysis of mRNA levels, promoter activity and band-shift assays. All these data support the idea that when overexpressed Slug can behave as a potent repressor of *E-cadherin* in epithelial cells. These data are also in agreement with a recent report indicating that Slug is a repressor of *E-cadherin* in breast carcinoma cell lines (Hajra et al., 2002). Moreover, our present studies clearly show that Slug is able to induce a complete EMT and provide additional information about the potential relative contribution of Snail and Slug to the downregulation of *E-cadherin*. Our quantitative binding studies with recombinant GST-fusion proteins clearly indicate that although both zinc factors are able to bind specifically to the E-boxes of the E-pal element, the affinity of Snail protein for this DNA element is two orders of magnitude higher than that of Slug. Indeed, of the three independent repressors of *E-cadherin* analyzed here (Snail, Slug and E47), Slug showed the lowest binding affinity for the E-pal element (Fig. 3). The binding assays also indicate that Slug binds preferentially to the E-pal element in a multimeric form, whereas Snail does it as a monomer (Figs 2 and 3). This differential behaviour can reside in the divergent intermediate P-S-rich region of both factors; the specific 29 amino-acid Slug domain could favour oligomerization of Slug. Alternatively, or complementarily, the whole conformation of both factors can influence the differential oligomerization and/or binding properties of both factors, a fact that here could not be anticipated on the basis of their similar zinc-finger-binding domains.

It should be noted that the *E-cadherin* promoter from different species contains several E-boxes with differential localization. The human promoter contains three E-boxes at –79, –30 and +22 nt position (Hennig et al., 1995; Giroldi et al., 1997; Batlle et al., 2000), the mouse promoter contains two adjacent E-boxes at –86 and –80, inside the E-pal element, and the proximal E-box at –31, but lacks the downstream E-box at +22 (Behrens et al., 1991; Rodrigo et al., 1999), whereas the canine promoter has been reported to be similar to the human promoter at the –79 and –30 E-boxes (Comijn et al., 2001). In this context, it is worth mentioning that the proximal –30 E-box of the mouse *E-cadherin* promoter did not show specific binding for either Snail of Slug, although it effectively binds to E12/E47 and other bHLH factors (L. Holt and A.C., unpublished). Therefore, the full repression of endogenous *E-cadherin* observed in MDCK-*Slug* and MDCK-*Snail* cells strongly suggest that the –79 E-box would be sufficient to mediate repression of the endogenous promoter by both Snail family factors, at least in canine cells. These observations, and our previous studies, also support the suggestion that the repression exerted by Slug and Snail on the mouse *E-cadherin* promoter is mainly driven through the E-boxes of the E-pal element. In agreement with the lowest binding affinity for the E-pal exhibited by Slug, transient transfection assays suggest that Slug may have a reduced repressor activity compared with Snail in MDCK and mouse keratinocyte cells. These results seem to differ from those recently reported for breast carcinoma cells, in which Snail and Slug showed similar repressor activities (Hajra et al., 2002), and may be due to differences in the sensitivity of the transfection assays, in expression efficiencies of the Snail and Slug constructs or to the cell systems analyzed. Hajra et al. also suggest that Slug is a more likely in vivo repressor of *E-cadherin* in breast carcinomas. The specific organization of the E-boxes in the mouse and human *E-cadherin* promoters could provide additional clues for differential repressor mechanisms for Snail and Slug factors in both species. Although further studies are required to clarify this specific issue our present results suggest that the relative concentrations of Snail and Slug, as well as that of other repressors and potential coregulators, is indeed important for their participation as *E-cadherin* repressors in a determined cellular context. This proposal is also supported by our previous analysis of *Snail* and *Slug* expression in several mouse epidermal keratinocyte cells showing no correlation between *Slug* and *E-cadherin* expression. In fact, some of the keratinocyte cell lines exhibit high levels of endogenous *Slug* expression while maintaining elevated expression of *E-cadherin* and high promoter activity, and only those which expressed *Snail* showed repression of *E-cadherin* and low promoter activity (Faraldo et al., 1997; Cano et al., 2000). In addition, our recent studies in human breast cancer biopsies in fact indicate a strong correlation between *Snail* expression, reduced *E-cadherin* expression and invasive grade of the tumours (Blanco et al., 2002), supporting a direct role for Snail as an *E-cadherin* repressor in in vivo tumour progression of breast carcinomas.

The present evidence from the recently characterized *E-cadherin* repressors (Snail, Slug, E12/E47, SIP1, ZEB-1) indicates that all of them are able to participate in the downregulation of *E-cadherin* expression in many different cell systems. The specific role of each factor, or their potential co-operation, in specific cellular contexts or in different types of carcinomas is not yet fully understood. In addition, the relative contribution of epigenetic mechanisms, mainly promoter hypermethylation, and trans-acting repressors in *E-cadherin* downregulation during tumour progression is presently unknown. It is plausible that both kinds of mechanism can operate in a coordinated fashion in defined cellular or tumour contexts, in a modified version of the two-hit hypothesis for tumour suppressors, as discussed recently for breast carcinomas in an elegant work (Cheng et al., 2001).

One important aspect to be considered when discussing E-cadherin downregulation in tumour progression is the fact that in most carcinomas this is a transient and dynamic event. Dynamic expression is supported by the frequently observed re-expression of E-cadherin in secondary metastatic foci and even in some lymph node metastasis (Takeichi, 1993; Gamallo et al., 1996; Christofori and Semb, 1999; Graff et al., 2000). Dynamic regulation of E-cadherin expression is a tightly regulated process during embryonic development in which E-cadherin is lost when EMTs occur but is re-expressed in the reverse situation: the establishment of epithelial lineages from mesoderm layers (Takeichi, 1995; Huber et al., 1996). In this context, it is tempting to speculate on the potential of the different *E-cadherin* repressors at different stages of the metastatic cascade. The initial invasion stage probably requires a rapid and effective repression of *E-cadherin*, which can be accounted for by the presence of repressors with high binding affinity for E-boxes of the promoter, like Snail. However, maintenance of the dedifferentiated and motile phenotype during the subsequent migration of invaded tumour cells can be achieved by weaker but more widely expressed repressors, such as Slug, or the formerly described repressors E12/47, ZEB-1, and SIP-1 (Perez-Moreno et al., 2001; Grooteclaes and Frisch, 2000; Comijn et al., 2001). In relation to Snail, Slug and E47, the expression pattern of the three factors in mouse development strongly supports the above hypothesis, as *Snail* is specifically expressed at the EMTs areas whereas *Slug* and *E12/E47* are excluded from them but present in the already migratory cells (Sefton et al., 1998; Cano et al., 2000; Perez-Moreno et al., 2001). The binding affinity data of the three factors for the E-pal element presented here also support the suggestion that Snail will predominate in the binding of the E-boxes of the *E-cadherin* promoter over Slug, and even E12/E47 factors. Although further experimental work is needed to test the above hypothesis, in particular, the analysis of the different repressors in human tumour biopsies, our present results strengthens our previous notion that similar mechanisms and molecules can be operating in EMTs and in the maintenance of the mesenchymal phenotype during development and in tumour progression.

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