THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 276, No. 29, Issue of July 20, pp. 27424–27431, 2001
*Printed in U.S.A.*

# A New Role for E12/E47 in the Repression of *E-cadherin* Expression and Epithelial-Mesenchymal Transitions*

Received for publication, January 29, 2001, and in revised form, March 30, 2001
Published, JBC Papers in Press, April 17, 2001, DOI 10.1074/jbc.M100827200

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**Down-regulation of *E-cadherin* expression is a determinant of tumor cell invasiveness, an event frequently associated with epithelial-mesenchymal transitions. Here we show that the mouse E12/E47 basic helix-loop-helix transcription factor (the *E2A* gene product) acts as a repressor of *E-cadherin* expression and triggers epithelial-mesenchymal transitions. The mouse E47 factor was isolated in a one-hybrid system designed to isolate repressors of the mouse *E-cadherin* promoter. Epithelial cells ectopically expressing *E47* adopt a fibroblastic phenotype and acquire tumorigenic and migratory/invasive properties, concomitant with the suppression of *E-cadherin* expression. Suppression of *E-cadherin* expression under stable or inducible expression of *E47* in epithelial cells occurs at the transcriptional level and is dependent on the E-boxes of the *E-cadherin* promoter. Interestingly, analysis of endogenous *E2A* expression in murine and human cell lines illustrated its presence in E-cadherin-deficient, invasive carcinoma cells but its absence from epithelial cell lines. This expression pattern is consistent with that observed in early mouse embryos, where *E2A* mRNA is absent from epithelia but strongly expressed in the mesoderm. These results implicate E12/E47 as a repressor of *E-cadherin* expression during both development and tumor progression and indicate its involvement in the acquisition and/or maintenance of the mesenchymal phenotype.**

Invasion of tumor cells into adjacent connective tissues represents the first step of metastasis in carcinomas. The invasion process involves the loss of cell-cell interactions together with the gain of proteolytic and migratory properties and is frequently associated with epithelial-mesenchymal transitions (EMTs)[1] (1, 2), a crucial process for the generation of different tissues during embryonic development (3). Strong cell-cell adhesion is mainly dependent on the E-cadherin/catenin adhesion system in both embryonic and adult epithelial tissues (4–7). Indeed, the loss of E-cadherin-mediated intercellular interactions is required for the acquisition of the invasive phenotype in epithelial tumors (8–10) and for EMT processes that take place during early embryonic development (5, 7). Therefore, characterization of the molecular mechanisms that control *E-cadherin* down-regulation is of prime importance for the understanding of both the tumor invasion process and normal embryonic development.

Different mechanisms have been proposed to participate in the silencing of *E-cadherin* expression during tumor progression, including genetic and epigenetic alterations of the *E-cadherin* locus (11–14) and changes in chromatin structure and transcriptional regulation (15–18). With regard to the latter hypothesis, previous studies on the mouse *E-cadherin* promoter have shown that a palindromic element, E-pal, containing two adjacent E-boxes, behaves as a strong repressor in *E-cadherin*-deficient carcinoma cells and fibroblasts (16, 19, 20).

Very recently, we have carried out a yeast one-hybrid screening to identify transcriptional repressors that interact with this E-pal element leading to the identification of the zinc finger transcription factor Snail as a direct repressor of *E-cadherin* expression (21). Here, a second factor isolated in the same screening process is described which corresponds to the mouse bHLH factor E47 (22–24). The E47 and E12 bHLH proteins are alternative splice products of the *E2A* gene (25), the founder member of the vertebrate class I bHLH genes (22, 25–27). A large body of evidence indicates that the *E2A* gene products play a central role in tissue-specific gene regulation, usually after their dimerization with tissue-specific class II bHLH proteins (28–31). Nevertheless, homodimers of E47 bHLH proteins are functionally active in B-cell lineages where they participate in the transcriptional regulation of several Ig genes (32). In fact the products of *E2A* gene (E12/E47) are required for B-cell differentiation and Ig gene rearrangements (33). In addition, targeted disruption of the *E2A* gene leads to thymic lymphomas, suggesting that *E2A* gene products can act as tumor suppressors (34, 35). The transcriptional activity of E2A proteins can be negatively regulated by their dimerization with another subclass of HLH factors, the Id proteins (36). Additional class I bHLH members are coded by independent genes such as *HEB* and *E2-2* (37, 38). Gene knockout and knock-in analysis indicates that these gene products can functionally cooperate with, or substitute for, *E2A* (39, 40).

Previous studies have shown that E12/E47 proteins are distributed widely in most adult tissues although at different

* This work was supported by Spanish Ministry of Education and Culture Grants SAF98-0085-C03-01 (to A. C.), DGICYT-PM98-0125 (to M. A. N.), and PB97-0054 (to F. P.), Comunidad Autónoma de Madrid Grants 08.1/0024.1/99 and 08.1/0055./2000 (to A. C. and F. P.), and European Union Grant FMXR-CT96-0065 (to M. A. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank*™*/EBI Data Bank with accession number(s) AF352579.*

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[1] The abbreviations used are: EMT(s), epithelial-mesenchymal transition(s); bHLH, basic helix-loop-helix; bp, base pair(s); GST, glutathione *S*-transferase; MDCK, Madin-Darby canine kidney; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; PARP, poly(ADP-ribose) polymerase.

levels of expression (26, 41). However, analysis of E12/E47 expression has not been undertaken in epithelial tissues or cell lines. Using a combination of expression studies, band-shift assays, promoter analysis, and gain-of-function experiments in epithelial cells, evidence of a novel role for E12/E47 is provided here. We show that E12/E47 participates in the repression of *E-cadherin* expression and in the EMT process, leading to the acquisition of invasive properties. These results further reinforce the significance of transcriptional repression as a mechanism for E-*cadherin* down-regulation during both development and tumor progression.

## EXPERIMENTAL PROCEDURES

*Plasmid Constructs and One-hybrid Screening*—The one-hybrid screen designed to detect transcription factors interacting with the wild type E-pal element of the *E-cadherin* promoter has been described recently (21). 41 of the isolated clones carried cDNA inserts coding for the mouse E47 bHLH transcription factor (22). The complete cDNA sequence of *E47* was subcloned into the pcDNA3 (Invitrogen) and the pMT-CB6 vectors (42) under the control of the cytomegalovirus and the sheep metallothionein I promoters, respectively.

*Generation of Recombinant Proteins*—The full cDNA coding sequence of mouse *E47* was amplified from the pACT2 vector using the following primers: forward, 5′-AGAATTCTGGATGATGAACC-3′; reverse, 5′-ATACTCGAGGGCTCACAGG-3′. The 1,972-bp product was then subcloned into the pGEX4T1 vector (Amersham Pharmacia Biotech) in-frame with the glutathione *S*-transferase (GST) protein. The sequence of the fusion construct was verified by automatic sequencing from both ends and using several internal oligonucleotides covering the full sequence. Production and purification of the recombinant GST-E47 protein were carried out following standard procedures.

*Cell Culture and Generation of Tumors*—The origin, tumorigenic properties and expression of *E-cadherin* of the murine keratinocyte cell lines MCA3D, PDV, HaCa4, and CarB have been described previously (18, 20, 21, 43) and are summarized in Fig. 6*b*. Human cell lines derived from differentiated colon carcinoma (HT29P), differentiated and dedifferentiated mammary adenocarcinomas (MCF7 and MDA-MB435S), bladder transitional cell carcinoma (T24), and melanomas (A375P) were provided by Dr. A. Fabra (Institut de Recerca Oncologica, Barcelona, Spain). The characteristics of these human cell lines have been described previously (21) and are summarized in Fig. 6*c*. Cells were grown in Dulbecco's modified Eagle's medium (CarB, MDCK-II and NIH3T3) or Ham's F-12 medium supplemented with a complete set of amino acids (MCA3D, PDV, and HaCa4) or in Dulbecco's modified Eagle's medium:Ham's F-12 medium (1:1, Life Technologies, Inc) (human cell lines) supplemented with 10 μg/ml insulin for the mammary cells. Tumors were induced in athymic male nu/nu mice by subcutaneous injection as described previously (21). Animals were obtained from the animal production unit of IFA-CREDO factory (France) and maintained in sterile conditions according to institutional guidelines. Injected animals were observed every 2 days and sacrificed when the tumors reached a size of 1.5–2.0 cm, external diameter.

*Stable Transfections*—Transfections were carried out as described recently (21) using the LipofectAMINE Plus reagent (Life Technologies, Inc.). Stable transfectants were generated from MDCK cells after selection with 400 μg/ml G418. Five and six independent clones were isolated from pcDNA3-*E47* and from control pcDNA3 transfections, respectively. Stable transfectants were also generated from PDV cells with the pMT-CB6-*E47* vector and its corresponding control, also selected with 400 μg/ml G418. PDV-pMT-CB6 stable transfectant clones were grown in F-75 flasks to 40% confluence, and 100 μM $ZnSO_4$ was then added to the cultures to induce the expression of the metallothionein I promoter. Cells were collected at the indicated times and analyzed for *E-cadherin* and *E47* expression by RT-PCR.

*RT-PCR Analysis*—Poly $(A)^+$ mRNA was isolated from the different cell lines using Microfast Track isolation kit (Invitrogen). RT-PCR was carried out as described previously (21). Mouse and human PCR products were obtained after 25–30 cycles of amplification with an annealing temperature of 65–70 °C. Primer sequences were as follows. For mouse *E-cadherin*: forward, 5′-CGTGATGAAGGTCTCAGCC-3′; reverse, 5′-ATGGGGGCTTCATTCAC-3′ (amplifies a fragment of 616 bp). For mouse *E12/E47*: forward, 5′-TACCCCTCCGCCAAGACC-3′; reverse, 5′-TTGGGGGATAAGGCACTG-3′ (amplifies a fragment of 412 bp). For canine *E-cadherin* (kindly provided by Y. Chen, Harvard Medical School): forward, 5′-GGAATCCTTGGAGGGATCCTC-3′; reverse, 5′-GTCGTCCTCGCCACCGCCGTACAT-3′ (amplifies a fragment of 560 bp). For mouse and canine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′; reverse, 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (amplifies a fragment of 900 bp). For mouse *β-actin*: forward, 5′-TGGGCCGCTCTAGGCACC-3′; reverse, 5′-CTCTTTGATGTCACGCACG-3′ (amplifies a fragment of 540 bp).

*E-cadherin Promoter Analysis*—MDCK-mock and MDCK-*E47* cells were transiently transfected with 5 μg of the wt-178 construct, or the mE-pal construct, fused to the chloramphenicol acetyltransferase (*CAT*) reporter gene (16, 19) and 1 μg of the CMV-luciferase construct as a control of transfection efficiency. The activity of SV40-*CAT* reporter plasmid was also analyzed in parallel in each sample. CAT and luciferase assays were performed as described previously (18, 20) with the activity normalized to that of the wild type promoter detected in MDCK-mock cells.

*Nuclear Extracts and Band-shift Assays*—Nuclear extracts from the indicated cell lines were obtained as described previously (18, 20). Band-shift assays with the $^{32}P$-labeled wild type E-pal or the mutant E-pal probe were carried out with the recombinant GST-E47 protein or nuclear extracts as described previously (20) but using the following buffer: 25 mM Hepes, pH 7.9, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 10% glycerol. Incubations were performed for 30 min at room temperature. 1 μg of recombinant GST-E47 or GST control protein and 5 μg of nuclear extracts were used in the absence or presence of the indicated competitors. For supershift assays, 5 μg of mouse monoclonal Yae, or rabbit polyclonal anti-E2A antibody (E2A.E12, V-18) (Santa-Cruz Biotechnology), was added to the reaction buffer and incubated for 15 min at room temperature before addition of the labeled wild type E-pal probe.

*Immunofluorescence and Western Blot Analysis*—Staining for the different markers was performed on methanol-fixed cells as described previously (21). Preparations were visualized using a Zeiss Axiophot microscope equipped with epifluorescence. Western blot analyses were carried out on whole cell or nuclear extracts with the indicated antibodies as described previously (21). For detection of E12/E47 protein in murine cell lines and MDCK transfectant cells, the V-18 antiserum was used (1:200 for immunostaining and 1:500 for Western), whereas in human cell lines the mouse monoclonal Yae was used (1:2,000). The anti-poly(ADP-ribose) polymerase (PARP) antiserum (1:500) (provided by Dr. A. López-Rivas, Instituto López Neyra, Granada, Spain) was used as a loading control for the nuclear extracts and the monoclonal anti-α-tubulin N356 (Amersham Pharmacia Biotech) as a loading control of whole cell extracts.

*Migration and Invasion Assays*—Migration in wound assays and invasion analysis on collagen type IV gels were carried out as described previously (21).

*In Situ Hybridization of Mouse Embryos*—*In situ* hybridization analyses of whole mount embryos and vibratome slices were performed as described recently (21, 44). The mouse *E2A* probe corresponding to the complete *E47* cDNA sequence was used as a probe. The slices were photographed with a Leica DMR microscope under Nomarski optics.

## RESULTS

*Mouse bHLH Factor E47 Interacts with the E-pal Element in the E-cadherin Promoter*—We have described recently a one-hybrid yeast system designed to isolate transcriptional repressors interacting with the E-pal element of the mouse *E-cadherin* promoter. This screening led to the isolation of the zinc finger transcription factor Snail and its characterization as a strong repressor of *E-cadherin* expression (21). A second factor isolated in high abundance (32% of the clones) in the same screen showed identity with the reported C-terminal sequence of mouse *E47* cDNA (22). The full *E47* cDNA (2,631 nucleotides), isolated in several clones, encodes an open reading frame of 648 amino acids starting from a methionine at nucleotide 133, which corresponds to the initiator methionine identified in the human E12 protein (23). It also includes the 558 nucleotides of the 3′-untranslated region. The deduced amino acid sequence for mouse E47 shows high similarity with the previously described partial amino acid sequences of the mouse E47 protein (amino acids 1–153 and 323–478) (45) with the unique exception of the absence of residue Gln-387, which is also absent in the deduced amino acid sequences of the human (23, 24) and rat (46) E12/E47 cDNAs. Other specific

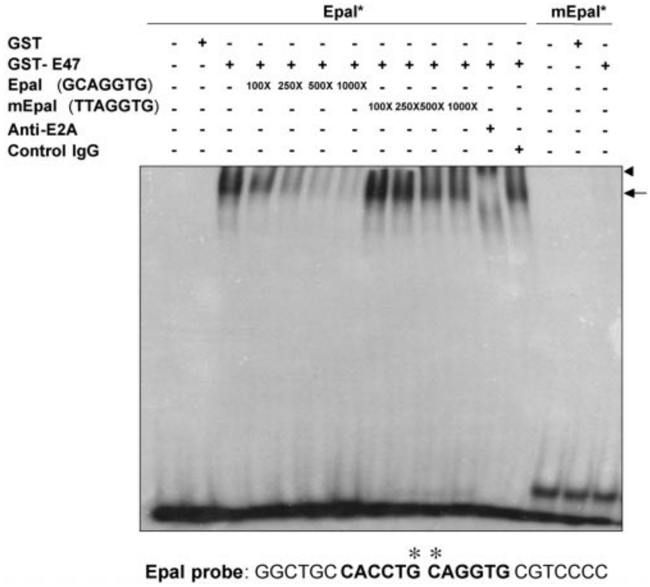


FIG. 1. **The bHLH transcription factor E47 binds to the E-pal element of *E-cadherin* promoter through the E2-boxes.** Recombinant GST and GST-E47 proteins (1 μg) were incubated with the $^{32}$P-labeled wild type E-pal (*Epal**) or mutant E-pal (*mE-pal**) probe in the absence or presence of the indicated cold probes used at 100-, 250-, 500- and 1,000-fold molar excess or in the presence of 5 μg of anti-E2A Yae mouse monoclonal or control mouse IgG. The retarded complexes detected are indicated by an *arrow* and the supershifted complex by an *arrowhead*. The complete sequence of the E-pal probe is indicated at the *bottom* of the figure with the E2-boxes showed in *black letters*. The specific nucleotides mutated in the mE-pal oligonucleotide are indicated by *asterisks* and also shown in *parentheses* in the *upper part* of the figure. The *first 13 lanes* from the *left* correspond to the samples incubated with the Epal* probe and the *last three lanes* to those incubated with the mEpal* probe, as indicated in the *upper part* of the figure.

changes detected in the amino acid sequence of the full mouse E47 protein were the insertion of two Ala residues (Ala-311, Ala-312), the absence of Ala-168, present in the rat E47 isoform (46), and the lack of several amino acid insertions uniquely present in the human E12/E47 proteins (GSSS at 260, Ala-295, His-510) (23, 24).

The specific binding of mouse E47 to the E-pal element of the *E-cadherin* promoter detected in the yeast one-hybrid screen was confirmed by band-shift assays using a recombinant fusion protein, GST-E47. As shown in Fig. 1, GST-E47 interacts with the wild type E-pal probe giving rise to two closely migrating specific retarded complexes that are competed efficiently by the unlabeled wild type probe but only partly competed by an excess (×500 and ×1,000) of the cold mutant E-pal probe (mE-pal). An unrelated oligonucleotide corresponding to the CCAAT-box of the *E-cadherin* promoter showed no competition (data not shown). The apparent partial competition observed with the mE-pal would suggest a weak interaction of the GST-E47 protein with the mE-pal probe. However, no interaction of GST-E47 protein could be detected in band-shift assays using the mE-pal as labeled probe (Fig. 1, *right lanes*), indicating that mutation of the two central nucleotides of the E-pal element strongly decreases the binding affinity of the GST-E47 protein. These results are consistent with the fact that the two point mutations of the mE-pal probe abolish the two E2-boxes consensus sequence (16, 21). They are also in agreement with the observed binding *in vivo* of E47 in the yeast one-hybrid system. Here, binding was detected for all E47 isolated clones in the yeast strain carrying the selection gene (*HIS3)* under the control of the wild type E-pal element, but not in the corresponding control yeast strain carrying the mutant E-pal (21). The binding specificity of GST-E47 to the E-pal probe was confirmed by supershift assays using an anti-E2A antibody. The addition of anti-E2A Yae monoclonal antibody led to the disappearance of the major complexes and the appearance of a slowly migrating supershifted complex (Fig. 1). These results indicate that mouse E47 interacts with the E-pal element of the *E-cadherin* promoter through the E2-boxes both *in vivo* in the yeast system and in *in vitro* binding assays, supporting a role for E47 in the transcriptional control of *E-cadherin*.

*Stable Expression of E47 in MDCK Cells Represses E-cadherin Expression and Induces EMT and an Invasive Phenotype*—To gain insights into the putative role of E47 in the regulation of *E-cadherin* expression, gain-of-function studies were performed in the prototypic epithelial cell line MDCK. Cells were stably transfected with pcDNA3 (mock) or pcDNA3-*E47* (E47) vectors. Whereas no changes were observed in the morphology of MDCK-mock transfectants, a dramatic conversion to a fibroblastic phenotype was observed in five independently isolated clones after transfection with the *E47* expression vector (Fig. 2*A*, *a* and *g*). The cells apparently lost all epithelial characteristics and acquired a spindle appearance. This phenotypic change was associated with a loss of E-cadherin expression (Fig. 2*A*, *h*), redistribution of other epithelial markers such as plakoglobin (Fig. 2*A*, *i*) and desmogleins (not shown), and increased organization of the mesenchymal markers vimentin (Fig. 2*A*, *j*) and fibronectin (Fig. 2*A*, *k*). Ectopic expression of the E47 protein was observed in the nuclei of the transfected cells (Fig. 2*A*, *l*). The qualitative changes of the various markers observed by immunofluorescence were confirmed by Western blot analysis (Fig. 2*B*) of whole cell extracts. This analysis confirmed the absence of E-cadherin and an increase in levels of vimentin and fibronectin in the *E47*-transfected cells. *E47* mRNA transcripts were detected in these cells by RT-PCR analysis (Fig. 2*D*), and Western blot analysis of nuclear extracts (Fig. 2*C*) confirmed the expression of a protein of relative molecular weight ($M_r$) 70,000 in MDCK-*E47* transfectants, corresponding to the expected size of the ectopic protein. Analysis of *E-cadherin* expression by RT-PCR showed a complete absence of endogenous *E-cadherin* transcripts in MDCK-*E47* transfectant cells (Fig. 2*D*). These results indicate that stable overexpression of *E47* in MDCK cells leads to the full repression of *E-cadherin* expression and induces a dramatic EMT.

The process of EMT induced by overexpression of *E47* in MDCK cells prompted analyses of the migratory/invasive properties of control and *E47*-transfected cells. The migratory properties of the transfectants were first analyzed in a wound culture assay (21) where MDCK-*E47* cells showed a highly migratory behavior, beginning to enter the wound after just 4 h postincision (Fig. 3*d*). Approximately 70% of the wound surface was colonized by *E47*-expressing cells 6 h after the wound was made (Fig. 3*f*), whereas at this time the mock-transfected cells had not yet started to migrate (Fig. 3*e*). The invasive properties of the MDCK-*E47* transfectants were analyzed further by invasion assays in collagen type IV gels. In these experiments, MDCK-*E47* cells were able to invade and migrate through the collagen gels (1.5% of the seeded cells emigrated through the gel matrix and filter after 12 h), whereas mock-transfected cells were not invasive at all. The tumorigenic properties of the transfectants were analyzed by subcutaneous injection into athymic nu/nu mice (Table I). MDCK-*E47* cells gave rise to tumors with a high growth rate at all injection sites (10 out of 10). 70% of the tumors induced by *E47* transfectants reached an external diameter of 1 cm 10–12 days postinjection with the rest achieving this size 15 days postinjection. In fact, the tumors induced by MDCK-*E47* cells grew at a very high rate, and the animals had to be sacrificed 18 days postinjection, when all

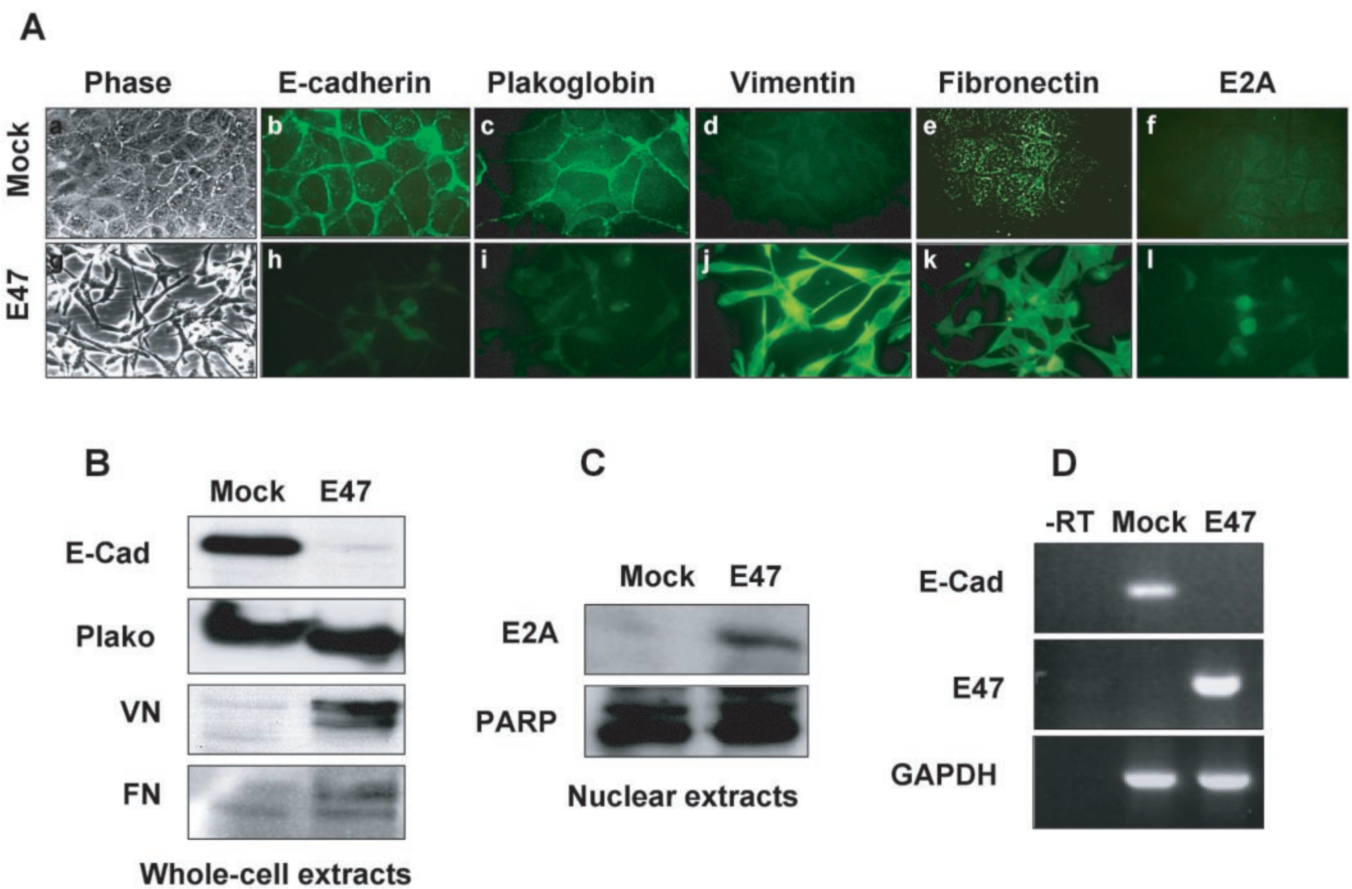


FIG. 2. **Stable transfection of *E47* into MDCK cells induces an epithelial-mesenchymal conversion concomitant with a loss of epithelial markers and the gain of mesenchymal markers.** *Panel A*: *a* and *g*, phase-contrast images of living, subconfluent cultures of a mock-transfected clone (*a*) and an *E47*-transfected clone (*g*); *b–f*, and *h–l*, immunofluorescence images of mock (*b–f*)- and *E47*-transfected (*h–l*) cells showing the localization and organization of E-cadherin (*b* and *h*), plakoglobin (*c* and *i*), vimentin (*d* and *j*), fibronectin (*e* and *k*), and E2A (*f* and *l*) proteins. *Panels B* and *C*, Western blot analysis of whole cell (*B*) and nuclear (*C*) extracts of the indicated proteins in mock- and *E47*-transfected clones. Detection of nuclear PARP levels was used as a loading control for nuclear extracts. *Panel D*, the presence of *E2A* and *E-cadherin* transcripts in mock- and *E47*-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 reverse transcriptase. Mock-transfected cells apparently do not express endogenous *E2A* gene, and endogenous *E-cadherin* expression was repressed in *E47*-transfected clones.

tumors had reached an external diameter of 1.5–2.0 cm. These results indicate that overexpression of the transcription factor E47 induces an extremely aggressive tumorigenic and migratory phenotype in MDCK cells.

*E47 Represses E-cadherin Promoter Activity*—E47 induces a dramatic EMT in MDCK cells, an event associated with repression of *E-cadherin* expression (Fig. 2*D*). This suggests a role for E47 in the down-regulation of *E-cadherin* promoter activity. To extend these observations and to analyze directly its effect on *E-cadherin* expression over time, the protein was transiently expressed in the epidermal keratinocyte cell line PDV using an inducible system in which *E47* expression was driven by the $Zn^{2+}$-inducible metallothionein promoter (Fig. 4*a*). Expression of *E47* mRNA started to be detected 6 h postinduction and increased steadily up to 24 h followed by a slight decrease at 48 h. A small decrease in the endogenous *E-cadherin* mRNA was observed 12 h after induction followed by a clear reduction (60%) at 24 h and its complete disappearance 48 h postinduction.

To support further the role of E47 as a repressor of *E-cadherin* expression, the activity of an exogenous *E-cadherin* proximal promoter was analyzed in mock- and *E47*-transfected MDCK cells. As indicated in Fig. 4*b*, the wild-type promoter construct exhibited a robust activity in MDCK-mock cells, similar to that of a SV40-CAT control construct, whereas this activity was almost undetectable in MDCK-*E47* cells (3% of the activity observed in MDCK-mock cells). The mutant construct (mE-pal), in which the E2-boxes of the E-pal element are abolished, showed 50% activity relative to that of the wild type promoter in MDCK-mock cells. In contrast, this mE-pal construct showed a 7.5-fold increase in activity over that of the wild type promoter in MDCK-*E47* cells (Fig. 4*b*). The activity of the wild type and mE-pal constructs in MDCK-Snail transfectant cells, reported recently (21), showed a behavior similar to that of MDCK-*E47* cells (data not shown), in agreement with results reported previously in other E-cadherin-deficient dedifferentiated carcinoma cells (16, 20). These results indicate that transcriptional repressor(s) interact with the E-pal element in *E47*-transfected cells. This was confirmed by bandshift assays against the E-pal probe using nuclear extracts obtained from MDCK-mock and MDCK-*E47* cells. Two specific retarded complexes of a similar intensity were detected in the MDCK-*E47* extracts (Fig. 5), which were competed efficiently by the unlabeled probe but competed weakly by an excess of the mE-pal probe. The partial competition observed with the mE-pal oligonucleotide probably indicates the interaction of additional factors present in the nuclear extracts of MDCK-*E47* cells with the E-pal element but apparently independent of the E2-boxes. Addition of anti-E2A antiserum led to the total disappearance of the slowest migrating complex and the appearance of a weak supershifted band. In contrast, very weak complexes were detected when using the nuclear extracts from

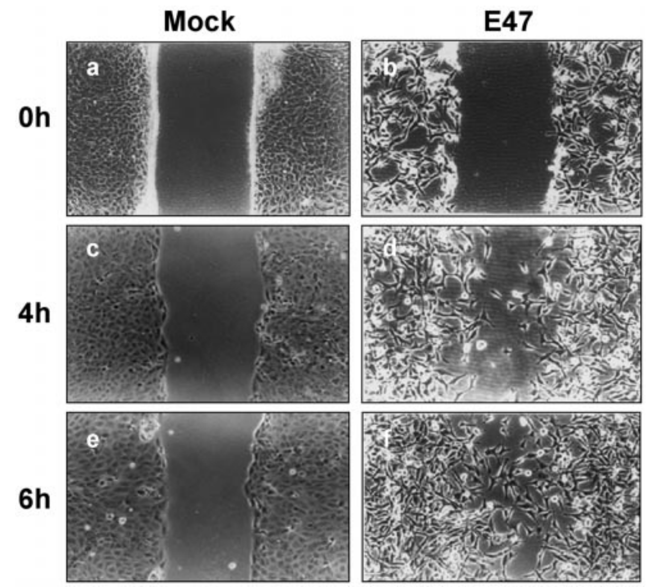


FIG. 3. ***E47* expression in epithelial cells induces a migratory phenotype.** The motility/migratory behavior of mock-transfected (*a*, *c*, and *e*) and *E47*-transfected (*b*, *d*, and *f*) MDCK cells was analyzed in an *in vitro* wound model. Confluent cultures of the mock clones and *E47*-transfected clones were gently scratched with a pipette tip to produce a wound. Photographs of the cultures were taken immediately after the incision (*a* and *b*) and after 4 h (*c* and *d*) and 6 h (*e* and *f*) in culture.

TABLE I
*Tumorigenicity of MDCK-E47 cells in nude mice*

$1 \times 10^6$ cells each of the indicated cell lines were injected subcutaneously into the two flanks of Balb/c nu/nu mice, and the animals were checked every 2 days for the appearance of tumors.

| | Tumors/ injection site | Latency to reach 1 cm[a] | | |
|---|---|---|---|---|
| | | 10–12 | *days* | 13–15 |
| MDCK-mock | 0/10 | 0/0 | | 0/0 |
| MDCK-*E47* | 10/10 | 7/10 | | 3/10 |

[a] The total number of tumors induced by the transfectants that had reached an external diameter of 1 cm (latency) at the indicated days after injection.

MDCK-mock cells (Fig. 5). The supershifts obtained after the addition of anti-E2A antibodies to nuclear extracts from MDCK-*E47* cells are similar to those obtained from nuclear extracts of diverse origins in which the disappearance of specific E2A complexes is detected easily, but the supershifted complexes are frequently very weak (32, 47).

*E12/E47 Factor Is Expressed in E-cadherin-deficient Cells and in the Embryonic Mesoderm*—Once demonstrated that ectopic expression of *E47* is able to induce a dramatic phenotypic change in prototypical epithelial cells in culture, we decided to analyze the expression of *E2A* in a panel of mouse epidermal keratinocyte cell lines which ranged from well differentiated (MCA3D) to fully dedifferentiated spindle carcinoma cells (CarB). These cell lines have been characterized previously with regard to *E-cadherin* expression and tumorigenic and invasive properties (43). The fibroblastic NIH3T3 cell line was also included in the study. RT-PCR analysis (Fig. 6*a*) was carried out using oligonucleotides designed to amplify a conserved region between *E47* and *E12* transcripts. *E2A* transcripts could be amplified from E-cadherin-deficient HaCa4 and CarB cells as well as from NIH3T3 fibroblasts, but not from the E-cadherin-positive MCA3D and PDV cells. The expression of E12/E47 was confirmed by Western blot analysis of nuclear extracts obtained from the different cell lines (Fig. 6*b*). In agreement with RT-PCR results, E12/E47 protein could be detected in HaCa4, CarB, and NIH3T3 cells but was absent in

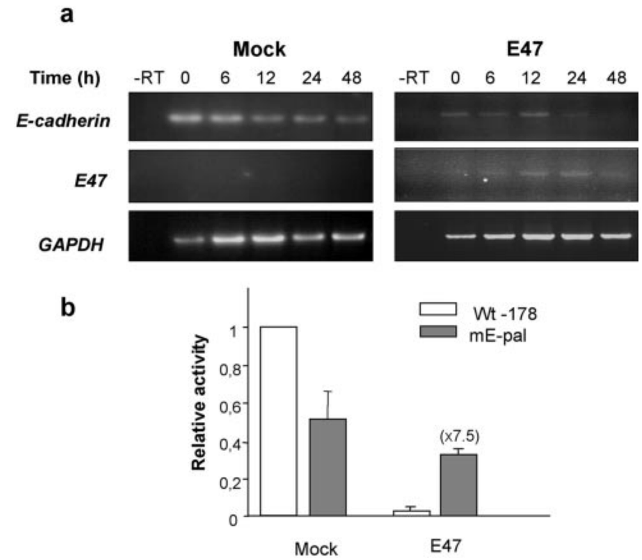


FIG. 4. **E47 represses the activity of the *E-cadherin* promoter in epithelial cell lines.** *Panel a*, PDV clones obtained by stable transfection with pMT-CB6 (*Mock*) and pMT-CB6-*E47* (*E47*) were grown to 40% confluence, and 100 $\mu$M ZnS04 was then added to the cells in fresh culture medium. Cells were collected at the time points indicated and analyzed by RT-PCR for *E-cadherin* and *E47* expression. The expression of *GAPDH* was analyzed in the same samples as a control for the amount of cDNA present in each sample. *Panel b*, the activity of the *E-cadherin* promoter is completely silenced in *E47*-expressing cells. Mock-transfected and *E47*-transfected MDCK clones were transiently transfected with the wild type (*Wt-178*; *white bars*) or mutant (*mE-pal*; *gray bars*) *E-cadherin* promoter fused to the *CAT* reporter gene. Luciferase and CAT activities were determined 24 h after transfection. The activity of the promoter constructs is represented relative to that of the wt-178 construct detected in the mock-transfected clone. Results represent the mean + S.D. of two independent experiments, each performed with duplicate samples. The fold increase detected in the mE-pal promoter activity relative to the wt-178 construct in *E47*-transfected cells is indicated by a *number* above the *error bar*.

MCA3D and PDV cells. To investigate further the relationship between E-cadherin and E2A, the expression of both proteins was analyzed in a panel of human carcinoma cell lines. The carcinoma cell lines chosen include epithelial and dedifferentiated cells derived from tumors of different etiologies, including breast (MCF-7 and MDA-MB435S), colon (HT29P), and bladder (T24) carcinomas, and melanomas (A375P cells), all of which have been described previously (21). This latter analysis (Fig. 6*c*) confirmed the inverse correlation between E-cadherin and E12/E47 in the human cell lines because significant levels of E12/E47 protein were detected in E-cadherin-deficient MDA-MB435S, A375P, and T24 cells, but the absence of E12/E47 was observed in E-cadherin-positive MCF7 and HT29P cells (Fig. 6*c*). In addition, those murine and human carcinoma cell lines that expressed E12/E47 showed invasive and metastatic properties (Fig. 6, *b* and *c*). The only exception to this was the bladder transitional cell carcinoma T24 cell line, which shows no invasive properties when analyzed on artificial gel matrixes, although it does show down-regulated *E-cadherin* expression (Fig. 6*c*) (21). However, *E-cadherin* down-regulation in T24 cells is caused by hypermethylation of the promoter (13), suggesting that a distinct molecular mechanism is operating in this case.

To explore the relationship between *E-cadherin* expression and the distribution of *E2A* gene products *in vivo*, we analyzed the expression of *E12/E47* mRNA during early mouse development. Previous studies have described the expression of the rat homolog in sections of embryos (48) ranging from 12 to 18 days postcoitum. We have carried out *in situ* hybridization analysis in whole mounted mouse embryos from 7.5 to 10.5

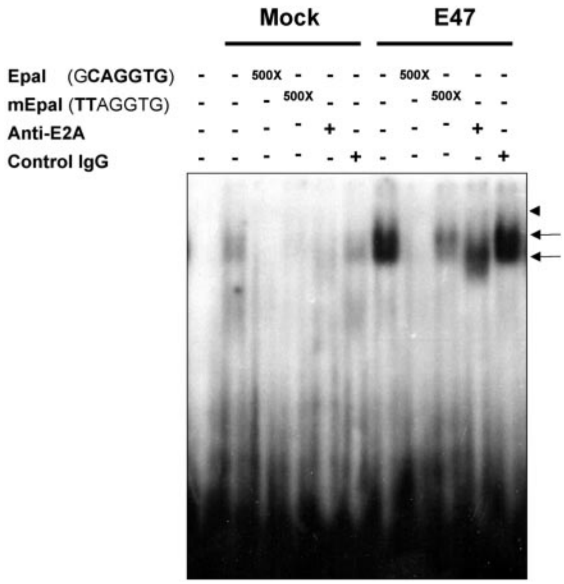


FIG. 5. **Endogenous bHLH protein E47 binds to the E-pal element of the *E-cadherin* promoter through the E2-boxes.** Nuclear extracts of mock- and *E47*-transfected MDCK cells were analyzed in band-shift assays. 5 $\mu$g of nuclear extracts from each sample was incubated with the $^{32}$P-labeled E-pal probe in the absence or presence of the indicated cold probes used at 500-fold molar excess or in the presence of 5 $\mu$g of anti-E2A antibody or control rabbit IgG. The retarded complexes detected are indicated by *arrows* and the supershifted complex by an *arrowhead*. The complete sequence of the E-pal probe and the specific mutated nucleotides in the mE-pal oligonucleotide are as indicated in Fig. 1. MDCK-*E47* transfectants contain E2A-specific nuclear complexes interacting with the E-pal element of the *E-cadherin* promoter, whereas those complexes are absent or present in very low amounts in epithelial MDCK-mock cells.

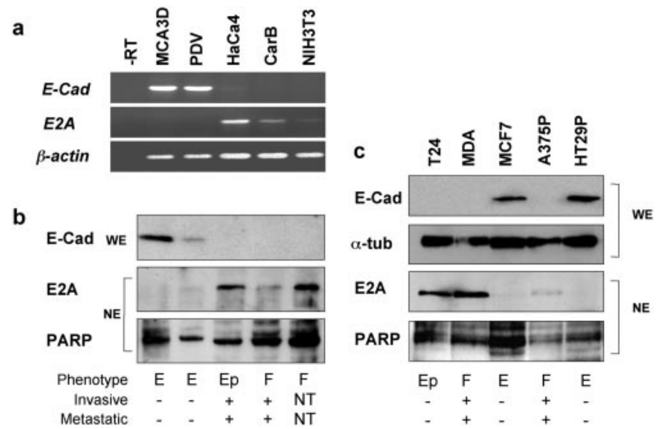


FIG. 6. **Endogenous E2A factors are present in mouse and human invasive cell lines and in fibroblasts.** *Panel a*, the expression of *E-cadherin* and *E2A* was analyzed by RT-PCR in a panel of mouse epidermal keratinocytes, ranging from well differentiated nontumorigenic cells to dedifferentiated and highly aggressive spindle carcinoma cells, and in NIH3T3 fibroblasts. The expression of $\beta$-actin was analyzed in the same samples as a control for the amount of cDNA present in each sample. *E2A* mRNA products were amplified only in the cell lines that showed repressed *E-cadherin* expression. *Panel b*, the inverse correlation between *E-cadherin* and *E2A* expression in the murine cell lines was also observed at the protein level. E-cadherin and E2A proteins were detected by Western blot analysis of whole cell extracts (*WE*) and nuclear extracts (*NE*) of the different cell lines, respectively. E12/E47 proteins were detected at varying levels in the E-cadherin-deficient cell lines. *Panel c*, the inverse correlation between *E-cadherin* and *E2A* was also detected in human carcinoma cell lines of various etiologies. Expression of E12/E47 was detected in *E-cadherin*-deficient A375, MDA-MB435S, and T24 cells. Detection of PARP nuclear protein and $\alpha$-tubulin was used as a loading control for the amount of total protein present in the nuclear extracts and whole cell extracts, respectively. The morphology of the different cell lines in culture is indicated as: *E*, epithelial; *Ep*, epithelioid; *F*, fibroblastoid. The invasive and metastatic properties of the cell lines are indicated as − and + symbols; *NT*, not tested. With exception of T24 cell line, expression of E12/E47 correlates directly with the invasive properties of the murine and human cell lines.

days postcoitum with a full-length *E47* probe, recognizing both *E47* and *E12* mRNAs (Fig. 7). At the stages analyzed, expression is detected in many different tissues throughout the embryo, but it is absent from the non-neural ectoderm (Fig. 7, *c–f*), the heart primordium (Fig. 7*b*) and the extraembryonic membranes except for the allantois (Fig. 7, *a* and *b*). The mesenchymal distribution of *E2A* products was maintained in 10.5 days postcoitum embryos, in which the complete absence of expression from the epithelia is clearly observed in vibratome sections (Fig. 7, *d–f*). Interestingly, the expression of *E-cadherin* at the same developmental stages follows an inverse pattern, being absent from all mesodermal tissues and strongly expressed in embryonic and extraembryonic epithelia regardless of their origin (21). These results support the role of the *E2A* gene products as repressors of *E-cadherin* expression and as factors involved in the acquisition and/or maintenance of the mesenchymal phenotype.

## DISCUSSION

Loss of E-cadherin mediated cell-cell adhesion is one of the hallmarks of the invasion process which occurs during the initial stages of the metastatic cascade. A large body of evidence points to *E-cadherin* as an invasion suppressor gene. This has stimulated investigation into the molecular mechanisms responsible for *E-cadherin* down-regulation during tumor progression. The recent identification of the transcription factor Snail as a powerful direct repressor of *E-cadherin* expression in carcinoma cell lines (21, 49) has highlighted the importance of transcriptional repression as a mechanism to silence *E-cadherin*. Previous studies on the regulation of *E-cadherin* by *Snail* indicate that during epithelial-mesenchymal transitions the same molecules and regulatory mechanisms are utilized for the same cellular processes during normal embryonic development and in pathological events in the adult such as cancer progression (21). The results presented here demonstrate that a second transcription factor, the class I bHLH E12/E47 factor, coded by the *E2A* gene, is also involved in the suppression of *E-cadherin* expression and in EMTs. The specific importance of E47, a member of a large family of bHLH transcription factors which could potentially interact with the E-pal element of the mouse *E-cadherin* promoter (31), was initially highligthed by its identification in the one-hybrid screen (41 out of 130 clones). Other bHLH factors were not identified in this screen with the exception of a product of the *E2-2* gene which represented a much smaller proportion of the isolated clones (to be reported elsewhere). The ability of E47 to interact specifically with the E2-boxes of the E-pal element was confirmed further in band-shift assays carried out both with a recombinant E47 protein and with nuclear extracts of *E47*-expressing cells. Interestingly, the products of *E2A* gene are not expressed in epithelial cell lines, whereas they are strongly expressed in *E-cadherin*-deficient, invasive cell lines. This observation is discrepant with the previous assumption that the *E2A* gene is expressed ubiquitously (26, 33, 41). In relation to this, the *in situ* hybridization analysis in mouse embryos presented here clearly illustrates the absence of *E2A* gene products in all embryonic epithelia, in contrast to its high expression in the mesoderm of early embryos. The inverse relationship observed between *E2A* and *E-cadherin* expression in early embryonic development argues in favor of a role for *E2A* gene products in the down-regulation of *E-cadherin* expression and thus in the generation and/or maintenance of the

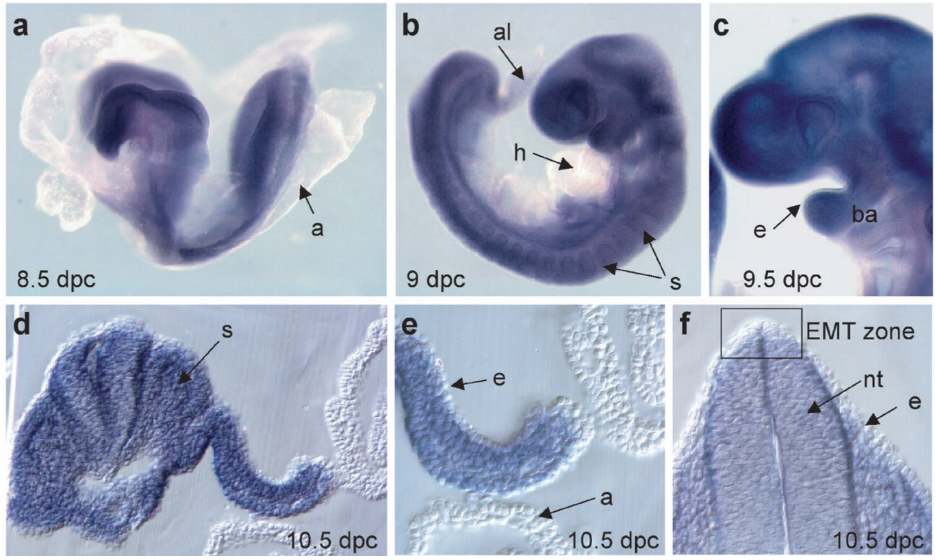


FIG. 7. **Expression of *E2A* in mouse embryos.** *E2A* is not expressed in embryonic epithelia. Whole mount *in situ* hybridization of mouse embryos at 8.5 (*panel a*), 9 (*panel b*), and 9.5 days postcoitum (*dpc*; *panel c*), and transverse vibratome sections of 10.5 days postcoitum embryos taken at the level of the posterior (*panels d* and *e*) and anterior trunk (*panel f*). *E2A* expression is detected in many different tissues throughout the embryo including the mesoderm and the neural tube, but it is absent from the non-neural epithelia (*panels c–f*), the heart primordium (*panel b*), and the extraembryonic membranes except for the allantois (*panel b*). Note that the region of the neural tube undergoing EMT (*panel f*, *EMT zone*) only expresses low levels of *E2A*. *a*, amnion; *al*, allantois; *ba*, branchial arch; *e*, ectoderm; *h*, heart; *nt*, neural tube; *s*, somites.

mesenchymal phenotype. In this context, it is important to consider that the lack of embryonic defects observed in *E2A* null mice can probably be explained by functional complementation by *E2–2* and *HEB* gene products (39, 40).

The involvement of E47 in EMTs and repression of *E-cadherin* expression is supported by ectopic expression studies in a prototypic epithelial cell line. Stable expression of *E47* in MDCK cells induces a dramatic EMT, characterized by a complete suppression of *E-cadherin* expression and an increased expression and reorganization of mesenchymal markers. Significantly, stable expression of *E47* also leads to the acquisition of migratory/invasive and tumorigenic properties in MDCK cells. Additionally, in PDV cells, transient expression of *E47* from an inducible expression vector causes a reduction in *E-cadherin* expression (as observed by RT-PCR). These results, together with the inverse correlation between the endogenous *E2A* and *E-cadherin* expression in carcinoma cell lines and embryos, support the hypothesis that E12/E47 participate in the repression of *E-cadherin* expression. With regard to the specific mechanism leading to the repression of *E-cadherin* by E2A factor, our studies on the exogenous *E-cadherin* promoter in MDCK-*E47* cells, together with *in vitro* binding assays and the *in vivo* yeast system, indicate a direct interaction of E47 with the E2-boxes of the E-pal element of the *E-cadherin* promoter. Transcriptional regulation by *E2A* products is usually mediated by specific heterodimers formed by their combination with tissue-specific class II bHLH factors (25, 28–31). Thus, it is likely that a specific bHLH partner cooperates with E47 in the repression of *E-cadherin* expression. Potential partners for E47 could be the mesodermal bHLH factors described in various systems such as Twist (50), Meso1 (51), or Paraxis (52). However, we cannot exclude the possibility that E47 homodimers may be functionally active as *E-cadherin* repressors because our band-shift assays using the recombinant E47 protein and the *in vivo* yeast analysis indicate that this factor is able to interact with the E-pal element as an homodimer. Finally, the data presented here do not exclude that repression of the *E-cadherin* promoter by E12/E47 could also involve the association with additional transcription factors. In this context, it is relevant to mention that zinc finger factors such as Snail (21, 49, 53), Slug (21, 54), or ZEB (55), some of which have been characterized recently as *E-cadherin* repressors, also bind to the E-boxes of the *E-cadherin* promoter. Alternatively, or in addition, the repression mechanism of E12/E47 could also involve its interaction with other coregulators in macromolecular complexes, as has been described in the regulation of *achute-scute* complex in *Drosophila* (56) and in genes involved in hematopoiesis in erythroid cells (47). In any case, the interaction of E12/E47 with putative specific bHLH partners and/or additional regulators will ultimately depend on the cellular context.

Because very recently we and others have demonstrated an important role for the transcription factor Snail in the repression of *E-cadherin* expression (21, 49) and in the EMT events that occur at tumor invasion and development (21), it is pertinent to compare these studies with the present report. Both *Snail* and *E47* are able to trigger EMT upon stable ectopic expression in MDCK cells. However, a closer examination of MDCK-*E47* and MDCK-*Snail* transfectant cells reveals important differences in their behavior. In particular, MDCK-*E47* cells exhibited increased migration in wound assays, starting to migrate into the wound much faster than MDCK-*Snail* cells (see Fig. 3 and Ref. 21). In addition, the tumors induced by MCDK-*E47* cells show a higher rate of proliferation than those induced by MDCK-*Snail* cells. These observations suggest that although both factors are capable of triggering EMT and of inducing an invasive and tumorigenic phenotype they may operate in distinct aspects of these processes. Consequently, it will be important to analyze and compare the influence of both factors in other important tumor progression events, such as angiogenesis.

With regard to their role in EMT during embryonic development, the comparison of the expression patterns of *Snail* and *E2A* in early mouse embryos shows that *Snail* is highly expressed in the regions undergoing EMTs (mainly the precursors of the neural crest cells and the primitive streak) (21, 44), whereas *E2A* transcripts are detected throughout the mesoderm and the neural tube (Fig. 7). Interestingly, *E2A* transcripts are detected only at very low levels in regions undergoing EMT such as the neural crest cells delaminating from the neural tube (Fig. 7*f*), which show high levels of *Snail* expression (21, 44). In contrast, the expression pattern of *Slug*, another member of the Snail family, overlaps with that of *E2A* (21, 44). As mentioned above, Slug binds to the same E-boxes (54) as E47 and Snail, making it a more likely candidate to cooperate with E47. Indeed, Slug has been shown to participate in desmosome dissociation in rat bladder epithelial cells (57) and suggested to cooperate with Snail in the maintenance of the mesenchymal phenotype (21). Because repression of *E-cadherin* occurs in regions undergoing EMTs and it is maintained in the resulting mesenchyme (5, 21), it could be postulated that *Snail* and *E2A* play distinct roles in the repression of *E-cadherin* expression in embryonic development. *Snail* may function by rapidly repressing *E-cadherin* expression at specific EMT sites, and *E2A* may then contribute to the maintenance of this repression in the embryonic mesenchyme. It is tempting to speculate that a similar scenario could operate during the invasion process in which the two transcription factors could act in a coordinated or sequential action. Thus, Snail could be responsible for the initial down-regulation of *E-cadherin* expression at the invasion front while E2A, alone or in cooperation with other repressors, could contribute to the maintenance of *E-cadherin* repression and the invasive mesenchymal phenotype further away from the invasion front. Further studies addressed to identify additional target genes for *Snail* and *E2A* and their putative partners, together with a detailed analysis of their expression patterns in tumor biopsies are needed to confirm this hypothesis.

In summary, the results presented in this paper clearly show a novel role for the bHLH transcription factor E12/E47 as a repressor of *E-cadherin* expression and as an inducer of EMTs, concomitant with the acquisition of an invasive phenotype. They also reinforce the significance of transcriptional repression as a major mechanism involved in E-*cadherin* down-regulation. The next challenge will be the identification of specific partners for E12/E47 which may cooperate in the regulation of these important processes both during normal embryonic development and in tumor progression.

*Acknowledgments*—We thank A. Montes for technical assistance, A. Fabra for the human cell lines, M. Takeichi for ECCD-2 antibody, J. Behrens for *E-cadherin* promoter constructs, F. J. Rauscher for the pMT-CB6 vector, A. López-Rivas for anti-PARP antibody, M. Manzanares for helpful suggestions and critical reading of the manuscript, and L. Holt for editorial assistance.

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**A New Role for E12/E47 in the Repression of *E-cadherin* Expression and Epithelial-Mesenchymal Transitions**

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*J. Biol. Chem. 2001, 276:27424-27431.*
*doi: 10.1074/jbc.M100827200 originally published online April 17, 2001*