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

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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)

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‡‡ The abbreviations used are: EMTs, epithelial-mesenchymal transitions; 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.

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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 E47 was amplified through the following primers: forward, 5'-AGAATTTTGAGTGATGACCC-3'; reverse, 5'-ATACTCGAGGGTCACAGG-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. 6b. Human cell lines derived from differentiated colon carcinoma (HT29P), differentiated and dedifferentiated mammary adenocarcinomas (MCF7 and MDA-MB435S), bladder transitional cell carcinoma (ST24), 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. 6c. Cells were grown in Dulbecco’s modified Eagle’s medium (CarB, MDCCK-II and NH3T3) 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 Transformations**—Transformations were carried out as described recently (21) using the LipofectAMINE Plus reagent (Life Technologies, Inc.). Stable transformants were generated from MDCCK cells after selection with 400 μg/ml G418. Five and six independent clones were isolated from pcDNA3-E47 and from control pcDNA3 transformations, respectively. Stable transformants 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 transformant clones were grown in F-75 flasks to 40% confluence, and 100 μM ZnSO4 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 are as follows. For mouse E-cadherin: forward, 5'-CGTCTGAGAATGCTTCCAGCC-3'; reverse, 5'-AGGCGGCTTTCTTTGATGTCACGCACG-3' (amplifies a fragment of 616 bp). For mouse E-cadherin (kindly provided by Y. Chen, Harvard Medical School): forward, 5'-GAAATTTTGAGTGATGACCC-3'; reverse, 5'-ATACTCGAGGGTCACAGG-3' (amplifies a fragment of 412 bp). For canine E-cadherin (kindly provided by E. Yaffe, Harvard Medical School): forward, 5'-GAAATTTTGAGTGATGACCC-3'; reverse, 5'-GAAATTTTGAGTGATGACCC-3' (amplifies a fragment of 412 bp). For mouse and canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5'-TGGGCGCTGGTGTTGAGGGATCCTC-3'; reverse, 5'-CATGGAGGAGGATTG5GAGG-3' (amplifies a fragment of 900 bp). For mouse β-actin: forward, 5'-TGGGCCGCTCTTAGGCACCC-3'; reverse, 5'-CTCCTTTGATGATGACCCAGG-3' (amplifies a fragment of 540 bp).

**E-cadherin Promoter Analysis**—MDCCK-mock and MDCCK-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 MDCCK-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 [α-32P]-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 Heps, 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 competitor. For supershift assays, 5 μg indicated competitors. For supershift assays, 5 μg indicated competitors. For supershift assays, 5 μg indicated competitors. For supershift assays, 5 μg indicated competitors. For supershift assays, 5 μg indicated competitors.

**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 Axiopt microscope equipped with epi-fluorescence. 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 MDCCK transfectants, the V-18 antisera 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) antisera (1:500) (provided by Dr. A. Lopez-Rivas, Instituto Lopez 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 assays 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 encodes the C58 CAG codons 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 Glu-387, which is also absent in the deduced amino acid sequences of the human (23, 24) and rat (46) E12/E47 cDNAs. Other specific
with the mEPal* probe, as indicated in the upper part of the figure. The first 13 lanes were incubated with the Epal* probe and the last three lanes with the arrowhead control yeast strain carrying the mutant E-pal (21). The binding of the wild type E-pal element, but not in the corresponding mutant of the mE-pal probe abolish the two E2-boxes con-

The specific binding of mouse E47 to the E-pal element of the E-cadherin promoter detected in the yeast one-hybrid screen (23, 24). 

Stable Expression of E47 in MDCK Cells Represses E-cadherin Expression and Induces EMT and an Invasive Pheno-
type—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. 

The specific binding of mouse E47 to the E-pal element of the E47 probe was observed in the nuclei of the transfected cells (Fig. 2A, I). The qualitative changes of the various markers observed by immunofluorescence were confirmed by Western blot analysis (Fig. 2B) 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. 2D), and Western blot analysis of nuclear extracts (Fig. 2C) confirmed the expression of a protein of relative molecular weight (Mr) 70,000 in MDCK-E47 transfectedants, 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 transfected cells (Fig. 2D). 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 transfectedants 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. 3d). Approximately 70% of the wound surface was colonized by E47-expressing cells 6 h after the wound was made (Fig. 3f), whereas at this time the mock-transfected cells had not yet started to migrate (Fig. 3e). The invasive properties of the MDCK-E47 transfectedants 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 transfectedants 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 transfectedants 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
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. 2D). 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 Zn²⁺-inducible metallothionein promoter (Fig. 4a). 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. 4b, 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. 4b). The activity of the wild type and mE-pal constructs in MDCK-Snail transfected 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 band-shift 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

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**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.
incision (wound. Photographs of the cultures were taken immediately after the transfected clones were gently scratched with a pipette tip to produce a wound. Confluent cultures of the mock clones and in vitro

reached an external diameter of 1 cm (latency) at the indicated days after injection.

Table 1

Tumorigenicity of MDCK-E47 cells in nude mice

<table>
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<th>Tumors/injection site</th>
<th>Latency to reach 1 cm(a)</th>
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<tr>
<td></td>
<td></td>
<td>10–12</td>
</tr>
<tr>
<td>MDCK-mock</td>
<td>0/10</td>
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<tr>
<td>MDCK-E47</td>
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\(a\) 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. 6a) 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. 6b). In agreement with RT-PCR results, E12/E47 protein could be detected in HaCa4, CarB, and NIH3T3 cells but was absent in 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. 6c) 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. 6c). 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 matrices, although it does show down-regulated E-cadherin expression (Fig. 6c) (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
E12/E47 and E-cadherin Repression

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 μg of nuclear extracts from each sample was incubated with the 32P-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 μg 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.

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, a–f), the heart primordium (Fig. 7b) and the extraembryonic membranes except for the allantos (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 tumour 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 highlighted 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 (28, 33, 41). In relation to this, the expression of the E2A gene products in all embryonic cell lines is 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 post-coitum (dpc; panel c), and transverse vibratome sections of 10.5 days post-coitum (dpc) 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.

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 E-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), Mesod (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 achaete-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 MDCK-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. 7f), 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 develop-
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.

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