Transcriptional Defects Underlie Loss of E-Cadherin Expression in Breast Cancer[1]

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Abstract

Decreased expression of E-cadherin (E-cad), a calcium-dependent cell adhesion molecule, has been seen in many different epithelial cancers. Although somatic mutations in the *E-cad* gene have been identified in a small subset of tumors, in the majority of cancers, the mechanisms underlying loss of *E-cad* expression are poorly understood. We have cloned the human *E-cad* promoter and defined its critical components in functional assays. In eight human breast cancer cell lines, there was a striking correlation between endogenous *E-cad* gene expression and the *E-cad* promoter activity observed following the introduction of reporter gene constructs into the lines. These and other observations suggest that defects in *trans*-acting pathways regulating *E-cad* expression are the primary basis for the loss of its expression in most breast cancers. The results have significant implications for understanding the gene expression differences that underlie tumor heterogeneity and progression events in breast and other epithelial cancers.

Introduction

E-cad,[3] a calcium-dependent transmembrane protein of roughly M_r 120,000, regulates epithelial cell-cell interactions at specialized regions of the plasma membrane called adherens junctions (1, 2). The function of E-cad depends critically upon its ability to link to the submembrane cytoskeletal matrix through its interactions with other proteins, such as α-, β-, and γ-catenin/plakoglobin (1–5). Alterations in the structure or expression of E-cad or the catenins have been found to promote aberrant cell-cell interactions *in vitro* (1, 2, 5, 6). Decreased or undetectable levels of E-cad expression have frequently been seen in immunohistochemical studies of many different epithelial cancers (reviewed in Ref. 2). In some cancers, loss of E-cad expression has been associated with the loss of differentiated features in tumor cells and/or increased propensity of the cells to invade and metastasize to distant sites. In addition, the restoration of E-cad expression following *E-cad* gene transfer has been shown to inhibit the invasive and metastic properties of the cells in *in vitro* and animal model systems (7–9)

The *E-cad* gene is located on chromosome 16q in a region that is frequently affected by allelic loss in several cancer types (10–13). Somatic mutations in the *E-cad* gene have been identified in more than 30% of gastric cancers of diffuse subtype, about 5–10% of endometrial and ovarian cancers, and about 5–10% of breast cancers, particularly those of lobular type (2, 14–17). The mutations identified include missense, nonsense, and splice mutations, as well as deletions. Nevertheless, in the majority of cancers in which *E-cad* expression is altered, the mechanisms accounting for its reduced or absent expression are poorly understood.

Two recent studies have presented apparently discordant conclusions on the mechanisms underlying loss of *E-cad* expression in cancer. A study by Graff *et al.* (18) concluded that *E-cad* expression was silenced in breast and prostate cancers by hypermethylation of the *E-cad* promoter sequences, whereas the findings of Hennig *et al.* (19) implied that the silencing of *E-cad* promoter activity in several different cancer types was due to loss of factor binding and/or chromatin rearrangement in the regulatory region. We report here the results of our studies to address the mechanisms underlying the loss of *E-cad* expression in breast cancer. Our findings of the human *E-cad* promoter suggest that defects in *trans*-acting pathways regulating *E-cad* gene expression are the primary mechanisms underlying loss of *E-cad* expression in breast cancer.

Results

Analysis of Human E-cad Promoter Activity. Previous studies have identified the transcription start sites for the murine and human *E-cad* genes (20, 21). In addition, several elements in the murine *E-cad* gene that regulate its expression in epithelial cells have been defined, including a 5′ promoter region, located within the 100-bp region immediately upstream of the transcription start site, and an enhancer region in the first intron (19, 20, 22, 23). Within the murine 5′ promoter region, a CCAAT-box and two candidate AP-2 binding sites in a GC-rich region have been characterized. In addition, a 12-bp palindromic element, located in the 5′ promoter region and called E-Pal, appears to be critical in directing epithelial-specific expression of *E-cad* (22, 23). Although the GC-rich region and CCAAT box are well con-

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[3] The abbreviations used are: E-cad, E-cadherin; AzaC, 5′-aza-2′deoxycytidine; Luc, luciferase; CMV, cytomegalovirus.

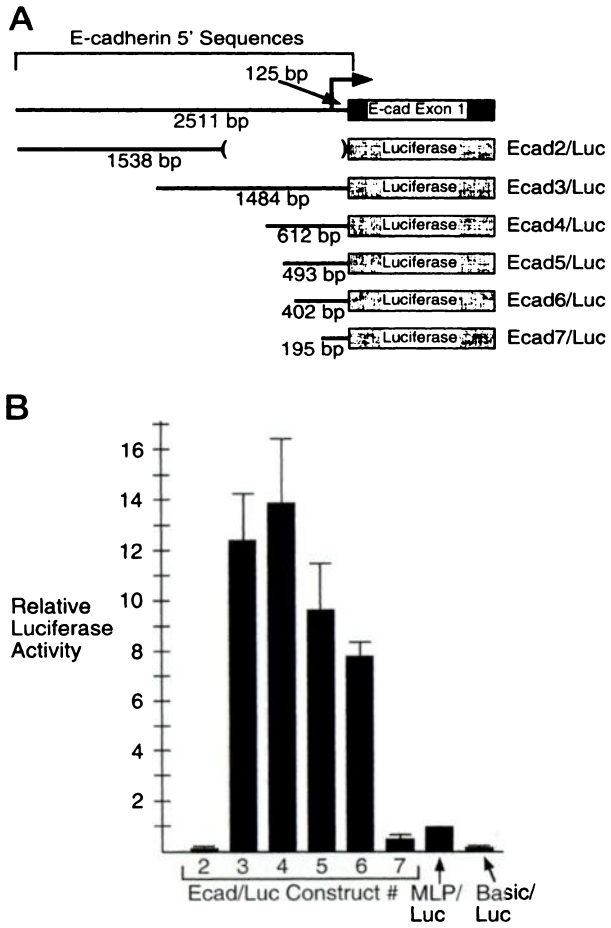

Fig. 1. Localization of promoter activity in human *E-cad* 5′ flanking sequences. *A*, for each *Luc* reporter gene construct, the extent of sequences 5′ to the *E-cad* initiating methionine is indicated. The proximal sequences deleted in the Ecad2/Luc construct are also indicated. The human *E-cad* transcriptional start site has been localized 125 bp upstream of the initiating methionine codon. In all reporter gene constructs, the *E-cad* initiating methionine codon has been destroyed, and a 28-bp flanking sequence separates the *E-cad* sequences from the initiating methionine of the *Luc* gene. *B*, relative Luc activity of the Ecad/Luc constructs and control constructs in MCF-7 breast cancer cells. Luc activities were determined by triplicate transfections of MCF-7 breast cancer cells with the indicated Luc constructs and a SV40-LacZ control construct. All Luc activities were normalized for β-galactosidase activity. The activity of an adenoviral late promoter Luc construct was assigned a value of one in each experiment. *Columns*, mean of the normalized Luc activities for all other constructs; *bars*, SD.

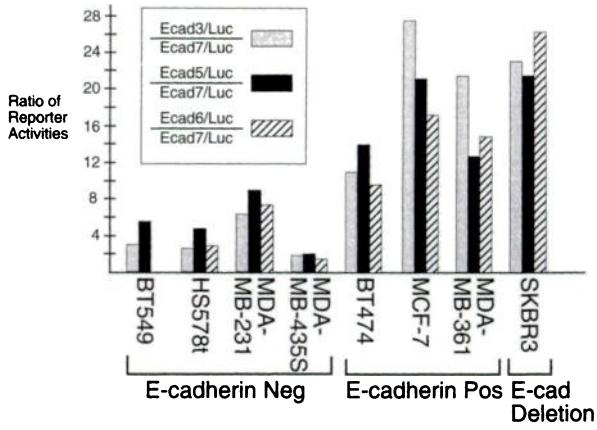

Fig. 2. Sequences between −70 and −277 in the human *E-cad* promoter are critical for its activity in breast cancer cell lines. The relative Luc activities of four *E-cad* Luc constructs were assessed in the eight breast cancer cell lines indicated. The Luc activities of the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs were compared to that of the Ecad7/Luc construct, and the mean ratio of the activities is indicated. The Luc activity of each construct was determined by three or more independent experiments, and all Luc activities were normalized for β-galactosidase activity. The *E-cad* expression status of the cell lines is indicated. The SKBR3 line lacks *E-cad* expression because of a homozygous deletion in the *E-cad* gene (16).

served in the human *E-cad* promoter region, the E-pal element is less well conserved (Ref. 21 and data not shown).

In an effort to further define the elements in the human *E-cad* promoter that are responsible for its transcriptional activity, we generated a panel of reporter gene constructs in which human *E-cad* 5′ flanking sequences of various extents were cloned upstream of the firefly *Luc* gene (Fig. 1*A*). We then characterized the Luc activities generated by these constructs following their transfection into MCF-7 and MDA-MB-361 cells, two breast cancer cell lines with high levels of endogenous *E-cad* expression. Similar activity profiles were obtained with the panel of constructs following transfection into each of the cell lines, although only the results for the MCF-7 cell line are shown in Fig. 1*B*. The constructs Ecad3/Luc, Ecad4/Luc, Ecad5/Luc, and Ecad6/Luc all generated Luc activities much greater than the reporter construct lacking any insert (*i.e.*, pGLBasic/Luc; Fig. 1*B*). The activities of most *E-cad* reporter constructs were even greater than the activity of a positive control vector containing the major late promoter of adenovirus (pMLP/Luc). The Ecad2/Luc vector lacks the *E-cad* transcription start site, and as expected, it failed to yield detectable levels of Luc following transfection (Fig. 1*B*). We also found that the E-cad7/Luc construct had weak activity. This observation implied that although the CCAAT and GC-rich elements present in the 70-bp region upstream of the *E-cad* transcription start site were sufficient for promoter activity, the elements conferred relatively weak activity compared to those present in constructs containing more 5′ *E-cad* sequences. We also noted that an *E-cad* reporter construct containing roughly 2.5 kb of *E-cad* 5′ flanking sequences had very reduced promoter activity (data not shown), suggesting that inhibitory elements may be present upstream of the more proximal E-cad promoter elements.

Activity of E-cad Promoter Constructs Parallels Endogenous Gene Activity. Studies were undertaken with a subset of the Ecad/Luc constructs in a panel of eight breast cancer cell lines to further assess the relationship between reporter gene activity and endogenous *E-cad* expression in the lines. As noted above, the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs had considerably more activity than the E-cad7/Luc construct in MCF-7 cells and MDA-MB-361 cells. As shown in Fig. 2, these findings were confirmed and extended to an additional breast cancer cell line with moderate to high levels of endogenous E-cad expression (BT474; Ref. 16). However, in four breast cancer lines lacking endog-

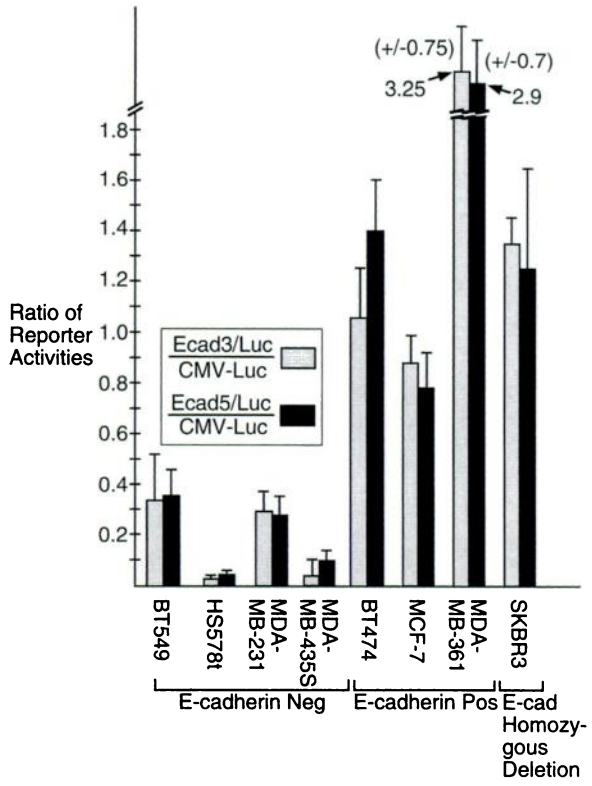

Fig. 3. E-cad promoter activity is correlated with endogenous *E-cad* activity in breast cancer cell lines. The relative Luc activity of two different *E-cad* Luc constructs (Ecad3/Luc and Ecad5/Luc; see Fig. 1) in eight breast cancer cell lines is indicated. The Luc activities of the two *E-cad* reporter constructs were compared to the Luc activity of a control CMV-driven reporter construct (pUHC-13-3). Luc activities in each cell line were determined by three to five independent experiments with the *E-cad* and CMV-Luc constructs and a SV40-LacZ control construct. All Luc activities were normalized for β-galactosidase activity. *Columns,* mean ratio of the luciferase activities; *bars,* SD.

enous *E-cad* expression and without evidence for mutational inactivation of the *E-cad* gene (BT549, HS578t, MDA-MB-231, and MDA-MB-435S; Ref. 16), the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc vectors had considerably less activity (Fig. 2).

SKBR3 cells lack *E-cad* expression because a substantial portion of the *E-cad* gene is affected by homozygous deletion (16). Given that the homozygous deletion in SKBR3 is clearly sufficient for complete inactivation of *E-cad*, the pathways and transacting factors regulating *E-cad* promoter activity might be expected to be intact in SKBR3. Consistent with this prediction, following transfection into the SKBR3 cell line, we found that the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs all had considerably greater activity than the Ecad7/Luc construct (Fig. 2). Additional studies comparing the activities of the Ecad3/Luc and Ecad5/Luc constructs with that of a control CMV-Luc vector further established that the *E-cad* promoter activities closely paralleled endogenous E-cad activity in the eight cell lines (Fig. 3). *In toto*, our studies indicate that *trans*-acting factors that interact with sequences between 70 and 277 bp upstream of the E-cad transcriptional start site are likely to be critical in regulating E-cad expression in breast epithelial cells. Defects in the expression or regulation of one or more of these transacting factors appear to be a major contributor to the loss of E-cad expression in a number of breast cancer cell lines.

AzaC Treatment Fails to Reactivate E-cad Expression. As discussed above, the previous studies of Graff *et al.* (18) had shown that hypermethylation of the *E-cad* proximal promoter region was correlated with decreased *E-cad* expression in a panel of breast and prostate cancer cell lines. In addition, these authors reported that treatment of selected breast and prostate cancer cell lines with the demethylating agent AzaC reactivated *E-cad* expression. In particular, using Western blot and immunofluorescence studies, the authors found minimal reactivation of *E-cad* expression in the MDA-MB-231 and HS578t breast cancer cell lines following exposure of the cells to 0.5 μM AzaC for 3 days (18).

We sought to assess E-cad expression by Western blot analysis in five *E-cad*-negative breast cancer cell lines following exposure of the cells to various levels of AzaC. All five lines lacked detectable *E-cad* mutations (16). As shown in Fig. 4, we failed to detect E-cad expression by Western blot analysis in any of the *E-cad*-negative breast cancer cell lines treated with 1 or 3 μM AzaC for 5 days, including the two breast lines (*i.e.*, MDA-MB231 and HS578t) studied by Graff *et al.* Two factors complicate definitive interpretation of the effects of AzaC treatment on endogenous E-cad expression. First, our Western blot analysis may have been somewhat less sensitive than the Western blot and immunofluorescence studies of Graff *et al.* (18). Second, similar to the findings of Graff *et al.* (18), we found that AzaC treatment had essentially no detectable effects on the methylation status of the proximal *E-cad* promoter in the breast cancer cell lines (data not shown). Although these caveats should be borne in mind, our results clearly demonstrate that E-cad expression cannot be reactivated to the levels seen in *E-cad*-positive breast cancer cell lines by brief AzaC treatment (Fig. 4).

Factors Regulating E-cad Promoter Activity. Although our transfection studies with unmethylated report gene constructs indicate that *trans*-acting defects are likely to be the predominant mechanism underlying the loss of *E-cad* promoter activity in breast cancers, we sought to explore the possibility that methylation of CpG dinucleotide sites in the *E-cad* promoter region might cooperate with transcriptional defects to further extinguish *E-cad* expression. We compared the Luc activities of an unmethylated *E-cad* reporter gene vector to the Luc activities generated by the vector following its *in vitro* methylation with purified bacterial *Hha*I methylase or *Hpa*II methylase. High levels of Luc activity were generated by the unmethylated Ecad5/Luc vector in the *E-cad*-positive MDA-MB-361 cell line, whereas *in vitro* methylation of the vector with *Hha*I or *Hpa*II methylase markedly decreased Luc activity (Fig. 5). In the *E-cad*-negative cell line MDA-MB-435S, no significant effects on promoter activity were seen when the unmethylated Ecad5/Luc reporter construct was compared to the same vector methylated *in vitro* with *Hha*I or *Hpa*II methylase.

Hennig *et al.* (19, 23) have provided evidence that AP-2 or an AP-2-related factor binds to two tandem sites in the

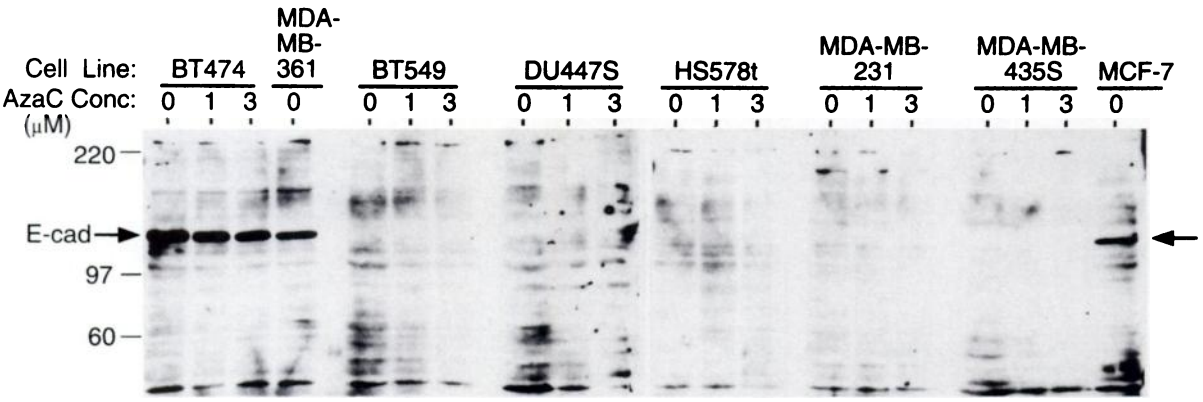

Fig. 4. E-cad expression in breast cancer cell lines is not reactivated by treatment with AzaC. Western blot analysis was carried out to assess E-cad expression in cell lines following a 5-day treatment with AzaC at 0, 1, or 3 μM. *Top,* identity of the cell lines; *arrow,* relative mobility of E-cad; *left,* molecular weight markers (in thousands). The cell lines BT474, MDA-MB-361, and MCF-7 are *E-cad*-positive, and the other five cell lines are *E-cad*-negative.

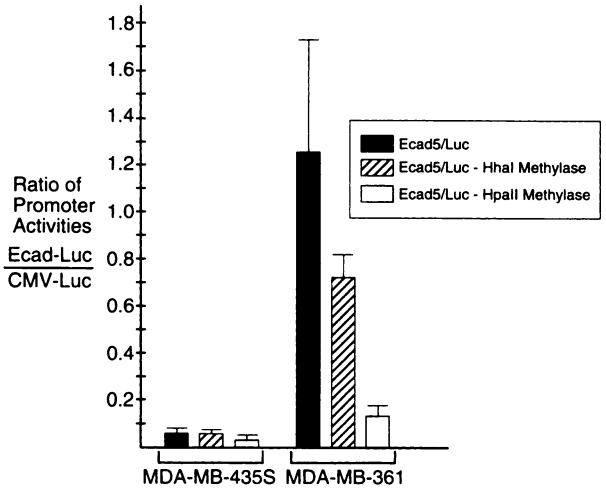

Fig. 5. Methylation of the *E-cad* promoter inhibits its activity. The relative Luc activity of the *Ecad5/Luc* reporter gene construct was assessed in the MDA-MB-435S and MDA-MB-361 cell lines following *in vitro* methylation with either *Hha*I or *Hpa*II methylase. Because transfection efficiencies differed among the lines, the Luc activity generated by the Ecad5/Luc construct was compared to the Luc activity of a control CMV-Luc reporter construct (pUHC-13-3). Luc activities were determined by three independent experiments, and all Luc activities were normalized for β-galactosidase activity. *Columns,* mean ratio of the luciferase activities; *bars,* SD.

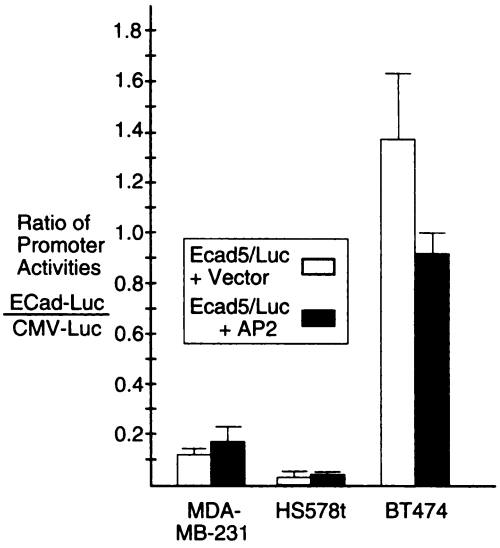

Fig. 6. E-cad promoter activity is not activated by AP-2 transfection. Shown in the figure are the relative Luc activities generated following cotransfection of three different breast cancer cell lines with the *Ecad5/Luc* reporter gene construct and either a pcDNA3 expression vector with an AP2 cDNA or the empty expression vector. Because transfection efficiencies varied among the cell lines, the Luc activities generated by the Ecad5/Luc construct were compared to the Luc activity of a control CMV-Luc reporter construct (pUHC-13-3). Luc activities were determined by three independent experiments, and all Luc activities were normalized for β-galactosidase activity. *Columns,* mean ratio of the luciferase activities; *bars,* SD.

GC-rich region of the murine *E-cad* promoter and that this region is critical in the regulation of *E-cad* expression, although their findings also suggest that the GC-rich region functions together with the CCAAT-box and E-pal elements to confer tissue-specific expression of E-cad. On the basis of the studies of AP-2 expression in breast cancer cell lines carried out thus far (24), there is little evidence for a correlation between *E-cad* expression and AP-2 expression, although only a limited number of cell lines have been studied. Nevertheless, to more directly assess the role of AP-2 in regulating *E-cad* expression in breast cancer, we transfected an AP-2 cDNA together with the Ecad5/Luc vector into two *E-cad*-negative cell lines. The AP-2 cDNA failed to activate the E-cad promoter in either the MDA-MB-231 or HS578t cell lines (Fig. 6). A small, but reproducible, decrease in *E-cad* promoter activity was seen in the BT474 E-cad-positive cell line following transfection of the AP-2 cDNA. The findings imply that loss of AP-2 activity is not likely to underlie the frequent loss of *E-cad* expression in breast cancer.

Discussion

A sizable fraction of breast cancers have been found to have reduced or absent *E-cad* expression, although mutations in the *E-cad* gene have only been identified in a very small

subset of the *E-cad*-negative cancers (2, 16, 17). In an effort to further understand the mechanisms underlying loss of *E-cad* expression, we have cloned the human *E-cad* promoter and have undertaken sequence and functional analysis of its elements in breast cancer cells. We found that the Ecad7/Luc reporter construct, although it contains the proximal 70 bp of *E-cad* sequence upstream of the transcription start site and the previously defined *E-cad* regulatory elements (*i.e.*, CCAAT box, two candidate AP-2 binding sites, and E-pal element), had only weak promoter activity. In contrast to the weak activity seen with the Ecad7/Luc construct, the Ecad6/Luc construct, containing a 275-bp fragment of *E-cad* proximal promoter sequences, had nearly the promoter activity of constructs containing considerably larger *E-cad* 5′ fragments of 500–1350 bp (*e.g.*, Ecad3/Luc and Ecad5/Luc). Thus, the findings imply that critical regulatory elements in the human *E-cad* promoter reside in the approximately 200-bp region immediately upstream of the previously defined minimal promoter elements. Furthermore, we noted a striking relationship between the endogenous *E-cad* gene activity in breast cancer cell lines and the promoter activities of the Ecad3/Luc, Ecad5/Luc, and Ecad6/Luc constructs when they were introduced into the cells. Our studies strongly suggest that defects in signaling pathways or *trans*-acting transcription factors that regulate *E-cad* expression are likely to be the primary mechanisms underlying the loss of *E-cad* expression in breast cancers.

Our results are consistent with those of two previous studies in which murine *E-cad* promoter activity was correlated with endogenous *E-cad* expression in several human cancer cell lines, including a total of four breast cancer lines (19, 22). Studies of the activity of a minimal human *E-cad* promoter in two prostate cancer cell lines have also suggested a correlation between endogenous *E-cad* expression and activation of *E-cad* promoter elements (21), although we have not found as clear-cut a relationship in our preliminary studies in prostate cancer cell lines.[4] Consistent with the results of the promoter activity studies, *in vivo* footprinting analyses have demonstrated protection of several distinct elements in the minimal *E-cad* promoter in *E-cad*-positive cancer cell lines but not in *E-cad*-negative lines (19).

Others have suggested that methylation of the *E-cad* promoter region may be responsible for the loss of *E-cad* expression in cancer, and increased methylation of the *E-cad* proximal promoter region has been identified in cancers and cancer cell lines lacking E-cad expression (18), an observation that we also confirmed in the lines studied here (data not shown). In a previous study, treatment of selected breast and prostate cancer cell lines with the demethylating agent AzaC was reported to reactivate *E-cad* expression in a minimal fraction of the treated cells (18). However, we failed to detect E-cad protein expression in any of five *E-cad*-negative breast cancer cell lines treated with AzaC, demonstrating that *E-cad* expression cannot be reactivated to the levels seen in *E-cad*-positive breast cancer cell lines by brief AzaC treatment. By studying *E-cad* promoter constructs in which CpG dinucleotides had been methylated *in vitro* by purified methylases, we did obtain support for the proposal that methylation of CpG dinucleotide sites in the *E-cad* promoter region might cooperate with transcriptional defects to further extinguish *E-cad* expression.

The binding of AP-2 or an AP-2-related factor to two tandem sites in the GC-rich region of the murine and human *E-cad* promoter has been suggested to regulate *E-cad* expression (23). However, we found that transfection of an AP-2 cDNA failed to activate the *E-cad* promoter in either of two *E-cad*-negative cell lines. Hence, loss of AP-2 is not likely to underlie the frequent loss of *E-cad* expression in breast cancer. Others have suggested that overexpression of HER-2/neu may cause a decrease in *E-cad* expression (25), although we failed to demonstrate a correlation between *E-cad* and HER-2/neu expression in breast cancer in our previous studies (16). Therefore, although the present evidence supports the proposal that defects in signaling and/or transcription factor pathways are the predominant mechanisms underlying altered *E-cad* expression in breast cancer, additional studies are clearly needed to elucidate the specific nature of these defects. Further characterization of these defects should provide new and important insights into the pathogenesis of breast cancer and the mechanisms underlying tumor cell heterogeneity and progression.

Materials and Methods

Plasmid Constructs. Genomic clones containing human *E-cad* exons 1 and 2, as well as 5′ flanking sequences, were isolated from a human genomic DNA library generously provided by Dr. Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, MD) using a human *E-cad* cDNA probe and multiple rounds of hybridization selection. An approximately 2.5-kb *Sal*I-*Nco*I fragment extending 5′ from the initiating methionine codon in exon 1 was identified and subcloned into pBluescriptII (Stratagene, La Jolla, CA). Deletions of varying extent in the *E-cad* sequences were generated using exonuclease III and mung bean nuclease (Stratagene). A series of *Luc* reporter gene constructs containing *E-cad* 5′ flanking sequences of various extents was generated by subcloning the *E-cad* sequences into the *Sac*I and *Hin*dIII sites of the pGL2-Basic vector (Promega Corp., Madison, WI) immediately upstream of the coding region of the firefly *Luc* gene. During the subcloning of each *E-cad* fragment, the *E-cad* initiating methione codon was destroyed. The identities of the *E-cad* sequences present in the vectors were confirmed by sequence analysis. The control Luc vectors pUHC-13-3 and pMLP/Luc, containing CMV and adenovirus major late promoter elements, respectively, have been described previously (26, 27). The pCH110 plasmid, containing a functional *LacZ* gene expressed under control of the SV40 early promoter, was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). The vector pcDNA3-AP2 was constructed by subcloning a 1.6-kb *Hin*dIII-*Eco*RI murine AP-2 cDNA fragment (kindly provided by Dr. Trevor Williams, Yale University) into the *Hin*dIII and *Eco*RI sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). All plasmid DNAs were isolated using reagents from QIAGEN, Inc. (Chatsworth, CA). *In vitro* methylation of the plasmid vector pEcad5/Luc was carried out on 20 μg of DNA using purified *Hha*I or *Hpa*II methylase (New England Biolabs, Inc., Beverly, MA) and the manufacturer's recommended reaction conditions.

Cell Culture. All cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in the recommended growth media. Cultures were incubated at 37°C with 5% CO_2 except for those maintained in Lebovitz's L-15 medium, which were grown at 37°C without CO_2. Selected cell lines were treated with 1 or 3 μM AzaC (Sigma Chemical Co., St. Louis, MO) for 5 days.

Transfections and Luc and β-Galactosidase Reporter Assays. Cell lines growing at roughly 70% confluence were transfected in six-well plates using 1 ml of Opti-MEM reduced serum medium (Life Technologies,

[4] X. Ji, E. R. Fearon, and R. Morton, unpublished observations.

Inc., Grand Island, NY), 4 μl of Lipofectin reagent (Life Technologies, Inc.), 0.8 μg of the indicated pGL2 or the pUHC-13-3 control Luc plasmid, and 0.8 μg of pCH110. Transfections to assess the affect of AP-2 were undertaken as described above, except that 8 μl of Lipofectin and 0.8 μg of pEcad5/Luc, 0.8 μg of pCH110, and 0.8 μg of pcDNA3-AP2 or pcDNA3 plasmid without a cDNA insert were used. Cell extracts were prepared 36–40 h posttransfection using reporter lysis buffer (Promega). One-fifth and one-half of the lysate were used for the Luc and β-galactosidase assays, respectively. Both assays were carried out as recommended by the manufacturer (Promega). Luc activities were measured in a luminometer (model TD-20E, Turner Corp., Mountain View, CA). All transfections were repeated three or more times.

Western Blot Analysis. Lysates were prepared in radioimmunoprecipitation assay buffer with protease inhibitors, as described previously (16). Approximately 40 μg of total protein per sample was separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA) by semi-dry electroblotting (Transblot, Bio-Rad, Hercules, CA). Western blot analysis of E-cad was carried out using the DECMA-1 rat monoclonal antibody (Sigma) and a horseradish peroxidase-conjugated goat antirat antibody (Pierce). AP-2 was detected with the AP-2 (C18) affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a horseradish peroxidase-conjugated goat antirabbit secondary antibody (Pierce). Antibody complexes were detected with the ECL Western kit blot (Amersham Corp., Arlington Heights, IL) and exposure to X-OMAT film.

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