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Hypermethylation of a Small CpGuanine-Rich Region Correlates with Loss of Activator Protein-2α Expression during Progression of Breast Cancer

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ABSTRACT

The transcription factor activator protein-2α (AP-2α) has recently been implicated as a tumor suppressor protein that can be lost during tumor progression and that exhibits growth-inhibitory properties when overexpressed in cancer cell lines. We now demonstrate that hypermethylation of a discrete 5′ region within a promoter CpG island of the gene is associated in breast cancer with the loss of *AP-2α* expression. Multiple CpG sites within the island become hypermethylated during breast cancer evolution. However, only hypermethylation of the most CpG-rich region, a small, ~300-bp area at the 3′ end of exon 1, fully distinguishes neoplastic from normal breast tissue and correlates with transcriptional silencing. In cell culture, silenced *AP-2α*, associated with exon 1 hypermethylation, is re-expressed by 5-aza-2′deoxycytidine resulting in the restoration of a functional DNA sequence-specific binding protein. *In vivo*, as detected by a very sensitive nested PCR approach, methylation of the discrete AP-2α exon 1 region does not occur in normal breast epithelium and occurs in only 3 (16%) of 19 ductal carcinoma *in situ* (DCIS) lesions, but is present in 12 (75%) of 16 invasive breast tumors ($P < 0.001$; DCIS *versus* invasive cancers). Tumors unmethylated for this region expressed *AP-2α* protein throughout, whereas tumors with hypermethylation showed large areas of loss. Our studies then determine that hypermethylation of a small region of a CpG island correlates with silencing of *AP-2α* in breast cancer and suggest that inactivation of this gene could be a factor in, and a useful marker for, the progression of DCIS lesions.

INTRODUCTION

Activator protein-2α (AP-2α) is an important transcription factor that mediates essential events in the development of mammalian species (1–3). In adults, the protein is particularly important to epithelial cells and has been implicated in activities such as maintenance of homotypic cell–cell adhesion through direct activation of the E-cadherin (*E-cad*) gene (4), mediation of growth arrest through activation of the *p21* gene (5), and promotion of cell apoptosis through an interactive process with the c-*myc* gene (6). Several additional important genes have been reported to be activated or repressed through AP-2α action including estrogen receptor (*ER*), *c-erbB-2*, *growth hormone*, *c-kit*, *type IV collagen*, and *insulin receptor-like growth factor* (6).

Given the important normal roles of AP-2α noted above, loss of function of this protein could have important ramifications for the evolution of cancer, such as contribution to the loss of apoptosis and growth control, hormonal response to estrogens, and increased cellular invasion. Indeed, a mounting body of evidence indicates a candidate tumor suppressor role for AP-2α in human cancer. First, the *AP-2α* gene is located in chromosome position 6p22, a region of frequent loss of heterozygosity in breast and other cancers (7). Second, although N-Ras transformation promotes an increase in *AP-2α* mRNA, AP-2α function is actually diminished through the capacity of the gene product to down-regulate the activity of its own promoter (8). Third, the growth-promoting activity of the oncogenic virus SV40 includes the ability of the large T antigen to block the activity of AP-2α directly through inhibiting binding of the protein to DNA (9). Finally, loss of AP-2α protein has been reported for several human cancers, and there is evidence that such loss may predict for poor survival and enhancement of tumorigenicity (10–14). In this regard, Zeng *et al.* (5) have reported that insertion of an exogenous *AP-2α* gene into cancer cells can inhibit growth through the activation of *p21* expression.

Despite the importance of *AP-2α* function to normal biological processes, and a candidate tumor suppressor role for this gene, no definitive mechanism has been elucidated to account for loss of function of this protein during tumorigenesis. Genetic alterations of the *AP-2α* gene in cancer cells have not been reported. In the present study, we provide a mechanism by showing that an epigenetic change, absence, or decrease of the AP-2α protein in association with an abnormal increase of DNA methylation within exon 1 of the gene, is an extremely frequent event in human breast cancer. This type of gene dysfunction is frequently found in neoplasia and has been well documented from a functional standpoint, as an alternative to gene mutations for loss of function of several key tumor suppressor genes (15, 16). However, the gene silencing has heretofore been associated, in terms of fully separating normal from neoplastic tissues, with dense hypermethylation of broad promoter regions containing CpG islands. We now define a situation in which the classic proximal promoter region of AP-2α is a rather weak CpG island (17) in which methylation of only a small CpG-rich region in exon 1 fully separates neoplastic breast epithelial cells from normal breast epithelium and strongly correlates with decreased expression of the gene, including loss of functional protein. Furthermore, whereas hypermethylation of this discrete region characterizes the majority of invasive breast cancers, it appears much less frequent in ductal carcinoma *in situ* (DCIS) and, thus, may constitute an important event in the progression of breast malignancy.

MATERIALS AND METHODS

Cell Lines and DNA. Human breast tumor and cosmetic reduction mammoplasty samples were obtained from the Department of Pathology at Johns Hopkins Hospital, the Clinica Puerta de Hierro (Madrid, Spain), and the Hospital Santa Cristina Madrid (18). DNA from purified normal breast epithelial tissue was obtained as described previously (19). DCIS samples and

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invasive adenocarcinomas were obtained at the time of surgical resections for early-stage breast cancer. MCF-7 and MDA-MB-231 breast cell lines were grown in 5%-FCS-supplemented DMEM. MDA-MB-435 cells were grown in RPMI, and HBL-100 cells in McCoy's medium, under the same conditions.

MSP. The standard methylation-specific PCR (MSP) assay, as described previously (20), was used to analyze the methylation status of the upstream AP-2α CpG-rich region in bisulfite-treated DNA from cultured cells, paraffin slides, and frozen tissues. The primers for standard MSP are as follows: unmethylated upstream primer AP2–5′-UN-S (5′-GGTTTTTATATTTGTGTTGGAGTGGTT), unmethylated downstream primer AP2–5′-UN-AS (5′-AAAACTCCCATATACACTTAAAAATCTCC), methylated upstream primer AP2–5′-ME-S (5′-TTTATATTTGTGTCGGAGCGGTC), and methylated downstream primer AP2–5′-ME-AS (5′-AAACTCCCGTATACGCTTAAAAATC). This corresponds to bp −134 relative to the transcription start site (GenBank accession no. X77343.2). PCR conditions were 35 cycles at 95°C for 30 s, 55°C for 50 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Nested PCR analysis (21) of the upstream CpG-rich region used non-CpG-containing flanking primers. An initial amplification step is performed with the flanking primers followed by the standard MSP amplification step. Primers used for the first reaction were: AP2-FLANK-UP (5′-AAGGTTTTATTAGTTTYGTAGGTTG-GAG-3′) and AP2-FLANK-DOWN (5′-CCTCATTAACATATCAACAATA-ATCCAATTA-3′). PCR conditions of 35 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 8 min were used. Internal primers were as follows: unmethylated upstream primer AP2-UN-S (5′-AGGTTGG-AGTGGTTTTAGAAGTAAGTTTGT-3′), unmethylated downstream primer AP2-UN-AS (5′-ACCAAATACCACATCTACACCAACCA-3′), methylated upstream primer AP2-ME-S (5′-TTGGAGTGGTTTTAGAAGTAAGTTCGC-3′), and methylated downstream primer AP2-ME-AS (5′-AAATACCACATC-TACGCCGACCG-3′). PCR conditions were identical to first reaction except that the annealing step was 60°C for 30 s.

To study the downstream (exon 1) CpG-rich region by nested PCR, flanking primers X95235 flank-S (5′-AGTATTTTGTGTTTATTTAGAGAGTAGT-TTTATTTGGG-3′) and X95235 flank-AS (5′-AAAAAAAAATCAAACT-CAAAACCTATAACC-3′) were used under the conditions 35 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 8 min. First-round PCR samples were quantified using Kodak image analysis software. Using the calculated DNA concentration, first-round PCR products were brought within the effective range of the assay by serial dilution with distilled water, 1:500 for paraffin tissue and 1:40,000 for frozen tissue and cell line samples. The internal amplification step was then performed. Internal primers were as follows: unmethylated upstream primer X95235 UM-S (5′-GTAGT-TTTATTTGGGTGTGAGATTGAG-3′), unmethylated downstream primer X95235 UM-AS (5′-ACACAAATAATCAAACCAACATCACA-3′), methylated upstream primer X95235 ME-S (5′-GTTTTATTTGGGTGCGAG-ATCG-3′), and methylated downstream primer X95235 ME-AS (5′-AATA-ATCGAACCGACGTCGCG-′3). PCR conditions were 31 cycles at 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 8 min. Negative controls contained no DNA, and positive controls were performed for each PCR reaction. Standards were normal peripheral blood lymphocytes and a reaction containing no DNA template.

Bisulfite-Modified Genomic Sequencing. Sequencing of bisulfite-modified DNA was carried out by solid-phase DNA sequencing (22). Sense primer (AP2biseq-S 5′-GAAGTGTTAGAAGTTGGGTTTTAGG-3′) and antisense primer (AP2biseq-AS 5′-TTCCCTTTTCCAACTCTTTACC-3′) were used to amplify a 347-bp region of the *AP-2α* promoter (bp −868 to −22; Fig. 1*B*), and the downstream exon 1 region (bp 598–776 from GenBank accession no.

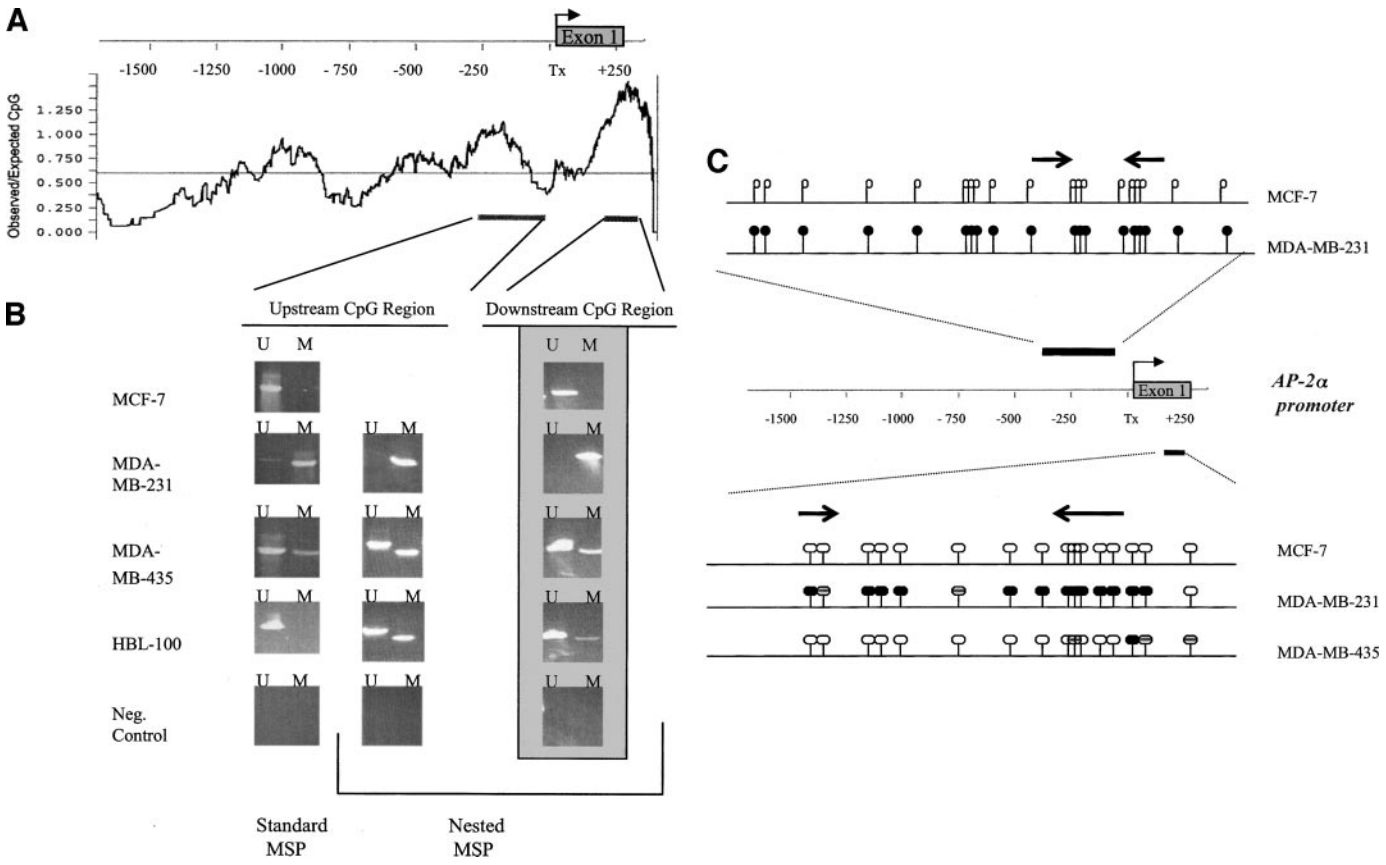

Fig. 1. Analysis of the methylation status of the *AP-2α* promoter region in cell lines. *A*, a CpG density analysis of the *AP-2α* promoter region. The only highly enriched CpG content occurs in a small region at the end of exon 1. *Solid bars*, areas of the *AP-2α* promoter and flanking regions studied by methylation-specific PCR (MSP). *B*, MSP analysis of bisulfite-treated DNA, using primers for the regions depicted in *A*, from MCF-7, MDA-MB-231, MDA-MB-435 breast cancer cells, and HBL-100 immortalized breast epithelial cells. *U*, unmethylated signal; *M*, methylated signal. Notable are the unmethylated status of MCF-7 cells upstream and downstream but complete methylation of the MDA-MB-231 cells. Cell lines MDA-MB-435 and HBL-100 retain significant unmethylated alleles. *C*, sequencing of bisulfite-treated DNA. *Each lollipop*, a single CpG site. ●, methylated sites; ○, unmethylated sites. *Striped lollipops*, sites with variable methylation. For the upstream CpG dense region, six cloned alleles from MCF-7 cells, and four from the MDA-MB-231 cells were analyzed, and all yielded the results shown. For the downstream region, a minimum of eight clones for MCF-7, MDA-MB-435, and MDA-MB-231 cells were analyzed. *Arrows above the lollipops*, the location of nested MSP primers.

X95235) was amplified using sense flanking primer X95235 flank-S and antisense primer X95235 flank-AS. The PCR product was then cloned into the TA cloning vector according to the manufacturer's (Invitrogen) instructions and sequenced using the T7 and M13 primers.

Reverse Transcription-PCR. Total RNA was isolated using Trizol Reagent. (Life Technologies, Inc.) and cDNA was prepared as described previously (23). PCR conditions were a hot start of 98°C for 3 min followed by 35 cycles of 98°C for 30 s, 60°C for 1 min, and 72°C for 1 min using *Taq* Polymerase. A 72°C for 10-min extension period completed the reaction. The upstream primer AP2ART-UP 5′-TGGGTGAGAGACCGAGAGGG-3′ and downstream primer AP2ART-DOWN 5′-GGCTCTGCCTCTGGCCG-3′ spanned at least one intron/exon boundary. E-cad was amplified using upstream primer 5′-GGAAGTCAGTTCAGACTCCAGCC-3′ and downstream primer 5′-AGGCCTTTTGACTGTAATCACACC-3′. Eighteen cycles of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reverse transcription-PCR served as the internal control.

Immunoprecipitation and Western Blotting. Cells (2×10^6) were plated in T-175 flasks and were allowed to grow to 50% confluence and then were treated with 5-aza-2′deoxycytidine for 48 h. At the end of 48 h, cells were trypsinized and pelleted, and nuclear extracts were prepared according to the manufacturer's directions (Pierce). Protein concentration was determined (Bio-Rad), and lysates were stored at −70°C until use. Nuclear protein (100 μg) was immunosorbed with nonspecific antibody by the addition of 1 μg of control rabbit serum together with 20 μl of Protein A/G Agarose on a rotator for 1 h at 4°C. Nonspecific activity was removed by centrifugation at 2500 rpm for 5 min. AP-2α was immunoprecipitated with specific antibody (C-18, Santa Cruz Biotechnology) overnight at 4°C. Protein-conjugated antibody was recovered by centrifugation at 2500 rpm. Samples were washed three times in cold PBS, repeating the centrifugation step. Supernatant was removed, and pellet was suspended in 40 μl of electrophoresis sample buffer and was heated at 70°C for 10 min. Protein samples were separated on a 4–12% Bis-Tris gel in 3-(N-morpholino) propane sulfonic acid (MOPS) buffer (Novex) at 200 V for 50 min and were transferred to nitrocellulose membrane using the Nu-PAGE electrophoresis system (Novex). The membrane was blocked for 1 h in 10% nonfat milk powder/0.05% Tween and was probed with 2 μg/ml AP-2 α/β monoclonal antibody (Serotec). Signal was visualized with horseradish peroxidase-conjugated secondary antibody using the ECL Chemiluminescence kit (Amersham).

EMSA. Double stranded oligonucleotides were synthesized (Life Technologies, Inc.) corresponding to a putative AP-2α binding site in the E-cad promoter. E-cad primers 965 EMSA-F 5′-GCGGTACGGGGGGCGGT-GCTCCGGGGCTACCTGGC-3′ and 965 EMSA-R 5′-GCCAGGTGAGC-CCCGGAGCACCGCCCCCCGTACCGC-3′ were annealed. Alternatively, oligonucleotides corresponding to the consensus AP-2α and OCT-1 sites were purchased (Promega). Oligonucleotides (15 pmol) were labeled with γ-^{32}P using T4 polynucleotide kinase. Anti-AP2α polyclonal antibody (2 μg; Santa Cruz Biotechnology) was added to indicated reactions for 20 min at room temperature to supershift the AP-2α-DNA complexes. An electrophoretic mobility shift assay (EMSA) was performed on each oligonucleotide using 10 μg of nuclear extract from indicated cell lines, 1 μg of poly(deoxyinosinic-deoxycytidylic acid [poly(dI-dC)] and reaction buffer (5 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 5% glycerol) up to a total volume of 15 μl. The reactions were incubated on ice for 20 min and resolved on a 5% polyacrylamide gel and were fixed, dried, and developed. AP-2α protein was synthesized *in vitro* using a T′N′T kit (Promega) and was used at 4 μl in a 10-μl reaction.

Immunostaining of Paraffin Sections. Paraffin-embedded tissue section slides were deparaffinized in successive dilutions of ethanol and deionized in water. Endogenous peroxidase activity was quenched with 2.5% hydrogen peroxide in methanol for 30 min. Slides were hydrated in water and antigen was unmasked by microwave treatment for 2–5 min in 9 mM sodium citrate (pH 6). After rinsing twice in PBS, samples were blocked with 1.5% goat serum in PBS for 30 min. Tissue sections were incubated with primary monoclonal antibody 3B5 (3, 24) supernatant or a 1:500 dilution of mouse monoclonal E-cad antibody (Transduction Labs) in PBS overnight at 4°C in a humidifying chamber. This antibody has been shown to have AP-2α specificity and recognizes AP-2 protein in stained paraffin-embedded samples (3, 11, 24). After rinsing in 0.01% Triton/PBS and three times with PBS, samples for AP-2 staining were incubated with DAKO Envision System for 1 h. Slides were washed twice in PBS, developed for 3–8 min with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma), slightly counterstained with Mayer's hematoxylin, dehydrated, and mounted with Cytoseal XYL (Stephens Scientific). Expression of AP-2α in tumor cells was compared with normal epithelial cells within the same sample.

RESULTS

Patterns of CpG Methylation, and Correlation with Expression, in the *AP2-α* Promoter in Cultured Breast Cancer Cells. To determine whether an epigenetic mechanism might mediate loss of AP-2α function in cancer cells, we first studied the methylation status of the canonical promoter of this gene in a panel of established cultured breast cancer cells and the immortalized, nontumorigenic mammary epithelial cell line, HBL-100. We created a plot of the *AP-2α* promoter region CpG density by combining accession numbers X95235 and X77343. The results revealed that the classic proximal promoter region of *AP-2α*, from approximately 1 kb upstream of the transcription start site and flanking exon 1, averages just under 0.7 for observed- over expected-CpG content (Fig. 1*A*) to qualify as a CpG island (17). Within the island, a small, exceptionally CpG-rich region (~300-bp) area overlaps the 3′ end of exon 1 (Fig. 1*A*).

The MSP assay, used in a standard, non-nested manner, revealed in MCF-7 breast cancer cells and HBL-100 immortalized breast epithelial cells that the region upstream of exon 1 in *AP-2α* is fully unmethylated whereas some methylated alleles are detected in MDA-MB-435 cells. A more sensitive nested MSP approach also detects some methylated alleles in the MDA-MB-435 cells and, in addition, in the HBL-100 cells but not in MCF-7 cells (Fig. 1*B*). In contrast, MDA-MB-231 cells are completely methylated in this region. A closer examination of the *AP-2α* promoter methylation pattern using bisulfite genomic sequencing (Fig. 1*C*) confirmed the MSP results seen in MCF-7 and MDA-MB-231 cells. In MCF-7 cells, all 19 of the upstream CpG sites analyzed by sequencing showed no methylated *AP-2α* alleles ($n = 6$ individual clones sequenced). In contrast, the CpG sites in all of the alleles ($n = 4$) from the MDA-MB-231 cells were methylated.

Nested MSP of the exon 1 CpG-rich region in cell lines shows a methylation pattern that is similar to that found in the upstream CpG-region (Fig. 1*B*). MCF-7 cells are fully unmethylated, MDA-MB-435 and HBL-100 cells contain some methylated alleles, and MDA-MB-231 cells are completely methylated in exon 1. Bisulfite genomic sequencing performed on MCF-7 cells corroborated the exon 1 MSP data (Fig. 1*C*). All 16 of the CpG sites, including the region studied by MSP, are unmethylated in MCF-7 cells. The limited exon 1 methylation seen in MDA-MB-435 cells by nested MSP is evident as scattered sites of CpG methylation by bisulfite sequencing, and virtually all CpG sites are methylated in MDA-MB-231 cells.

We next addressed how the above methylation profiles correlated with the expression status of the *AP-2α* gene. By reverse transcription-PCR analysis, all of the unmethylated, or only partially methylated cell lines, tested, including HBL-100, MCF-7, and MDA-MB-435 cells, express *AP-2α* at the RNA level (Fig. 2*A*). In contrast, MDA-MB-231 cells, which have a heavily methylated *AP-2α* promoter region, and are the only cells fully methylated at the CpG-rich region at the end of exon 1, do not demonstrate detectable *AP-2α* transcript by reverse transcription-PCR. Further evidence for the role of methylation in transcriptional silencing of *AP-2α* in the MDA-MB-231 cells was evidenced by the fact that treatment of these cells with 0.5 μM demethylating agent 5-aza-2′deoxycytidine resulted in the induction of *AP-2α* mRNA in 48 h (Fig. 2*B*). The MCF-7 cell line, completely free of *AP-2α* promoter region 1 methylation, and the HBL-100 and MDA 435 cells, which have methylated and unmethy-

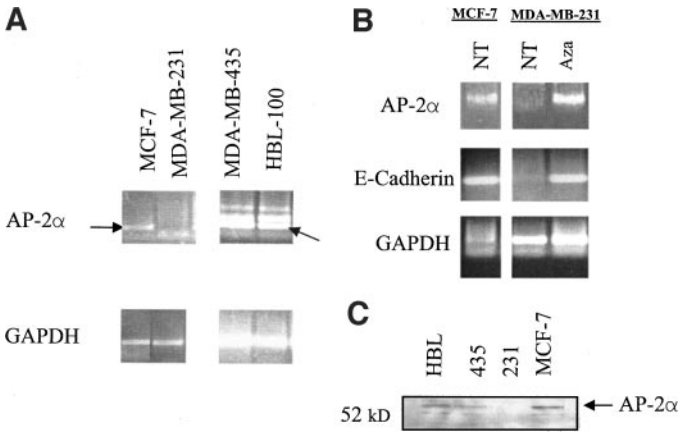

Fig. 2. Reverse transcription-PCR and Western blot analyses of *AP2–2α* and *E-cad* expression in various cell lines. *A, arrow*, the position of the *AP-2α* transcript seen in MCF-7, MDA-MB-435, and HBL-100 cells, but not in MDA-MB-231 cells. The higher molecular weight bands in the 435 and HBL-100 cells represent the position predicted for known *AP-2α* splice variants, which are further defined in the "Discussion" section. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts serve as a loading control. *B*, reactivation of *AP-2α* RNA expression using the demethylating agent 5-aza-2′deoxycytidine (5-Aza-dC; in the image, *Aza*). MCF-7 cells basally express the *AP-2α* and *E-cad* (*E-Cadherin*) transcripts. In the 231 cell line, *AP-2α* and *E-cad* are not expressed at the RNA level. Inhibition of methylation by 0.5 μM 5-Aza-dC effectively facilitates reactivation of the *AP-2α* and *E-cad* transcripts in MDA-MB-231 cells within 48 h of treatment. GAPDH serves as a control for RNA loading. *NT*, non-treated. *C*, Western blot analysis of AP-2α protein in the cell lines. Approximately 150 μg of nuclear extract were analyzed for each cell line. A specific AP-2α band is noted in the unmethylated cell lines MCF-7 and HBL-100 (*HBL*), as well as in the partially methylated line MDA-MB-435 (*435*). The methylated cell line MDA-MB-231 (*231*) does not express AP-2α protein. *kD, M_r* in thousands.

lated alleles, all produce AP-2α protein as analyzed by Western blot. The MDA-MB-231 cell line, which contains only methylated alleles, does not produce AP-2α protein (Fig. 2*C*).

Functional Consequences of *AP-2α* Methylation in Breast Cancer Cells. We next determined how the transcriptional activity mediated by *AP-2α* might correlate in breast cancer cells with the methylation status of the promoter for this gene and with 5-aza-2′deoxycytidine-induced re-expression of the gene. In nuclear extracts of MCF-7 cells, which are unmethylated for, and express, *AP-2α*, as examined by EMSAs, there was a distinct binding complex demonstrable to a consensus AP-2α oligomer and to a sequence from the E-cad promoter that contains an AP-2α binding site (Fig. 3, *Lanes 4* and *5*). These complexes correlated exactly to the position produced by the binding of the AP-2α oligonucleotide probe incubated with *in vitro* translated *AP-2α* cDNA (*Lanes 1–3*). Furthermore, with the addition of a specific AP-2α antibody, a distinct supershift was produced for the binding complex induced by MCF-7 cell extract (*Lane 6*), whereas no change was seen with a control antibody to the transcription factor Sp1 (*Lane 4*).

In contrast to the MCF-7 cells, nuclear extracts from untreated MDA-MB-231 cells, which are fully methylated for the *AP-2α* promoter and fail to express the gene, showed minimal-to-absent binding to either the consensus AP-2α oligonucleotide, or the *E-cad* promoter oligonucleotide (Fig. 3, *Lanes 7* and *11*). A dramatic increase in binding to both oligonucleotides was seen on incubation of the MDA-MB-231 cells with 5-aza-2′deoxycytidine (*Lane 9*). This binding included, in addition to the specific band for AP-2 α, two additional gel shift bands indicating, interestingly, induction of more than one binding factor. However, for both oligonucleotides, only the specific AP-2α band was reduced and, simultaneously, was supershifted with the AP-2α antibody precisely as seen in the MCF-7 cells (*Lanes 10* and *12*). We, thus, conclude that silencing in association with full promoter methylation disrupts *AP-2α* promoter-binding activity in breast cancer cells, and this function can be restored by demethylation and reactivation of the gene.

AP-2α Promoter CpG Regions Are Differentially Methylated in Normal and Neoplastic Cells. We next studied the methylation of the 5′region of *AP-2α* in three different sources of normal breast including purified mammary epithelium ($n = 4$), whole breast tissue taken at the time of reduction mammoplasty procedure ($n = 3$), and histologically normal tissue distal to DCIS ($n = 9$). MSP analysis, as studied by comparing results for the standard and nested MSP assays, revealed that normal cells have various levels of protection from methylation for the CpG-enriched region just upstream from exon 1. By standard MSP (Table 1; examples in Fig. 4*A*), 15 of 16 normal samples showed no methylation. Importantly, however, using the more sensitive nested approach, the number of samples methylated in this region increased to 6 (38%) of the 16 samples, including samples from each of the sample types of normal breast studied (Table 1; examples in Fig. 4*A*).

In contrast to the finding of methylation in the more upstream region in some normal breast samples, even by the nested procedure,

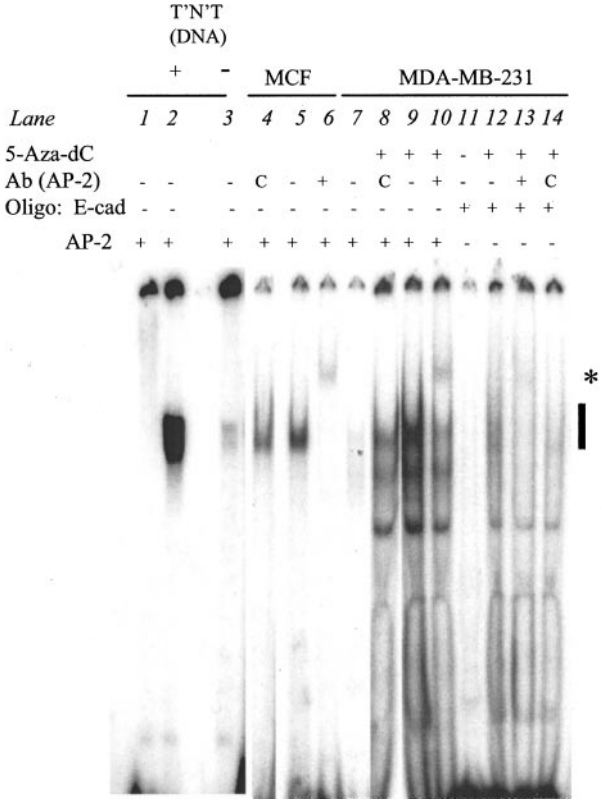

Fig. 3. Electromobility gel shift analysis (EMSA) of the binding of AP-2α protein, from various extracts, to either an oligonucleotide (for sequences see "Materials and Methods") containing the AP-2α consensus sequence (oligonucleotide-AP2) or a defined AP-2α binding site in the *E-cad* gene proximal promoter (oligonucleotide, *E-cad*). *Lane 1*, labeled probe no AP-2α protein; *Lanes 2* and *3*, extracts from a TNT (*T′N′T*) reaction to which a cDNA for AP-2α was transcribed and translated (+) or omitted (-). A marked increase in binding to a single band (position marked by *vertical bar at the far right of the gel*) is observed in the + extract. *Lanes 4—6*, nuclear extract from MCF7 cells that express an unmethylated *AP-2α* gene. Notable is the specific binding in the same band as seen in the +TNT extract, and a supershift of this major band (*position marked by the ∗ at the far right of the gel*) with preincubation of an AP-2α antibody [*Ab (AP-2)*], but not with SP1 antibody (*Lane marked C*) with the extract. *Lanes 7–14*, results for extracts from the 231 cells that contain a nonexpressed, hypermethylated *AP-2α* gene. Binding for extracts from untreated cells to either the AP-2α or the E-cad oligonucleotides (*Oligo*) is minimal to absent, but there is a distinct increase in three bands, especially for the AP-2α oligonucleotide, for cells treated with 5 Aza dC. The top of these latter bands is in the position for the AP-2α-specific band for the TNT (*T′N′T*) and MCF7 (*MCF*) extracts, and this band is reduced for the AP-2α oligonucleotide, and eliminated for the E-cad oligonucleotide, when the AP-2α (*Lane 13*), but not the SP1 antibody (*Lane* marked C) is added. The other bands are not altered by either antibody.

Table 1 *Summary of methylation-specific PCR (MSP) analysis of normal DNA from individuals without breast cancer and distal-to-tumor tissue from women with ductal carcinoma in situ (DCIS)*

Samples	Upstream		Exon 1
	Standard MSP	Nested MSP	Nested MSP
Purified Epithelium			
N1[a]	U	U	U
N2	U	U	
N3	U	U	
N4	U	M	U
Whole breast			
N5	U	M	U
N6	U	U	U
N7	U	M	U
Peripheral to DCIS			
DCIS N8	M	M	U
DCIS N9	U	U	U
DCIS N10	U	U	U
DCIS N11	U	U	U
DCIS N12	U	U	U
DCIS N13	U	M	U
DCIS N14	U	M	U
DCIS N15	U	U	U
DCIS N16	U		U

[a] N numbers, preparations of breast cells taken from different women at time of a mammoplasty procedure; U, unmethylated; M, methylated.

no methylation of the discrete exon 1 CpG-rich region was found in any normal tissue (Table 1; examples, Fig. 4*A*). These data illustrate that, in normal-appearing breast epithelial cells and whole breast tissue, there is full protection from methylation only within the exon 1 CpG-rich region. This also suggests that the exon 1 methylation found, above, in the MDA-MB-231 cells occurs during the process of tumorigenesis.

AP-2α Hypermethylation at Exon 1 Occurs Infrequently Early in Tumorigenesis, Is Highly Prevalent in Invasive Breast Cancer, and Contributes to a Marker Panel Covering the Genome of Breast Cancer. We next sought to determine the significance of *AP-2α* gene hypermethylation to breast cancer *in vivo*. To use DNA from small sections of fixed tissues containing DCIS lesions, and because this assay is best to completely rule out methylation in normal tissues, we exclusively used the nested MSP analysis for studies of the upstream and exon 1 regions in invasive tumors and the DCIS lesions. The upstream CpG region from 27 DCIS lesions demonstrated methylation in 12 (44%) of 27 cases (data not shown). However, for 19 samples of DCIS that were available for further study by nested MSP at exon 1, only 3 (16%) contained hypermethylated exon 1 alleles (example, Fig. 4*B*).

For 16 cases of invasive cancers, we analyzed both the upstream and downstream CpG-dense regions for methylation status and compared these data with AP-2α protein expression, as detailed later below. In these invasive tumors, by nested assays, there was high concordancy for methylation of the upstream region and exon 1. Seventy % (11 of 16) of these cancers contained methylation in the upstream region, whereas 12 (75%) of 16 were methylated in the downstream region (examples Fig. 4*B*). This frequency for a single promoter hypermethylation ranks with that for *PAX5α* (25) as the highest we have seen for invasive breast cancer (26). Furthermore, the difference between the hypermethylation of the downstream region between the invasive tumors and DCIS (above) is highly significant ($P < 0.001$). Exon 1 bisulfite genomic sequencing analysis was also performed on several of the noncultured breast tumor samples, and normal peripheral blood lymphocyte DNA was sequenced as a control (Fig. 4*C*). Normal cells show no methylation at any of the 16 CpG sites, and a representative unmethylated invasive tumor is completely unmethylated by sequencing. However, invasive tumor 41, which had fairly equal densities of methylated and unmethylated alleles by MSP, shows the same 1:1 ratio of methylated to unmethylated alleles by sequencing. Many of the unmethylated clones probably represent contaminating normal cells.

We have recently stressed that promoter hypermethylation provides a potentially powerful molecular marker system for cancer detection because panels consisting of relatively few markers can provide wide coverage for virtually every tumor type (26). The high frequency for *AP-2α* hypermethylation found in the present study suggests that this change could enrich such a hypermethylation panel for breast cancer. Indeed, in a previously determined panel (26) consisting of *ER*, *E-cadherin*, *GSTπ*, *$p16^{INK4A}$*, *RAR-β* and *BRCA1*, aberrant methylation was found in at least 1 gene in 29 (88%) of 33 of the tumors examined for all of the genes. Addition of *AP-2α* methylation to the panel, as performed in the same cancer specimens, provides a hypermethylation mark for at least one of the genes in 32 (97%) of 33 of the tumors.

AP-2α Hypermethylation Correlates With Loss of Protein in Primary Breast Cancers. To establish whether the correlation between *AP-2α* methylation and loss of expression observed *in vitro* is present in clinical breast tissue, immunohistochemical analyses were conducted. In past studies of this type to evaluate the AP-2α content of various cancer types, difficulties have arisen in interpretation because staining has been observed in the cytoplasm rather than solely in the nucleus using a commercially available polyclonal antibody (10–14). We then, as defined in "Materials and Methods," took great care to work out the specificity of our staining techniques by first testing our procedures on cell lines for which the AP-2α expression pattern has been published, such as MCF-7 and MB-231 cells. When these cells are embedded in paraffin, nuclear AP-2α staining was only detected in the former cell line, which expresses an unmethylated gene (data not shown).

By immunohistochemistry, normal breast epithelium, unmethylated at the downstream region, and whether methylated at the upstream region or not, exhibits nuclear AP-2α expression that is heterogeneously distributed among the ductal cells (Table 2; example, Fig. 5*A*). Stromal areas did not express AP-2α protein. Interestingly, as seen best in a slightly hyperplastic duct from a patient with DCIS at another site (Fig. 5*B*), the epithelial cells immediately lining the lumen contained positive, albeit heterogeneously distributed, nuclear staining, whereas the outer cells in the position of mesodermally programmed myo-epithelial cells, were uniformly negative (Fig. 5*B*).

Immunohistochemistry was performed on 8 of the 19 aforementioned DCIS cases in which upstream and downstream *AP-2α* methylation status had been determined. Three of seven tumors that were unmethylated at both the upstream and downstream CpG-enriched regions showed expression of protein as did two tumors methylated at the upstream but not the downstream region (Table 2; examples, Fig. 5, *C* and *E*). As shown, unmethylated DCIS tissue often showed stronger AP-2α expression than did normal tissue, and an occasional tumor actually overexpressed AP-2α (Fig. 5*E*). In most protein-positive tumors, there was heterogeneity for cells with nuclear staining, suggesting cell-to-cell variability in gene expression.

In the one tested DCIS lesion with hypermethylation of the downstream exon 1 region, nuclear AP-2α protein was markedly diminished with only occasional heterogeneously distributed cells expressing small amounts of the protein (Fig. 5*D*). However, two of the seven unmethylated tumors also had loss of expression that was actually virtually complete. This loss could be an indication of an alternative pathway for AP-2α dysregulation in tumors in which the gene is not hypermethylated. In summary, for DCIS, AP-2α was absent in three of eight tested DCIS cases, one of which was hypermethylated at exon 1.

We also studied, by immunohistochemistry, the relationship be-

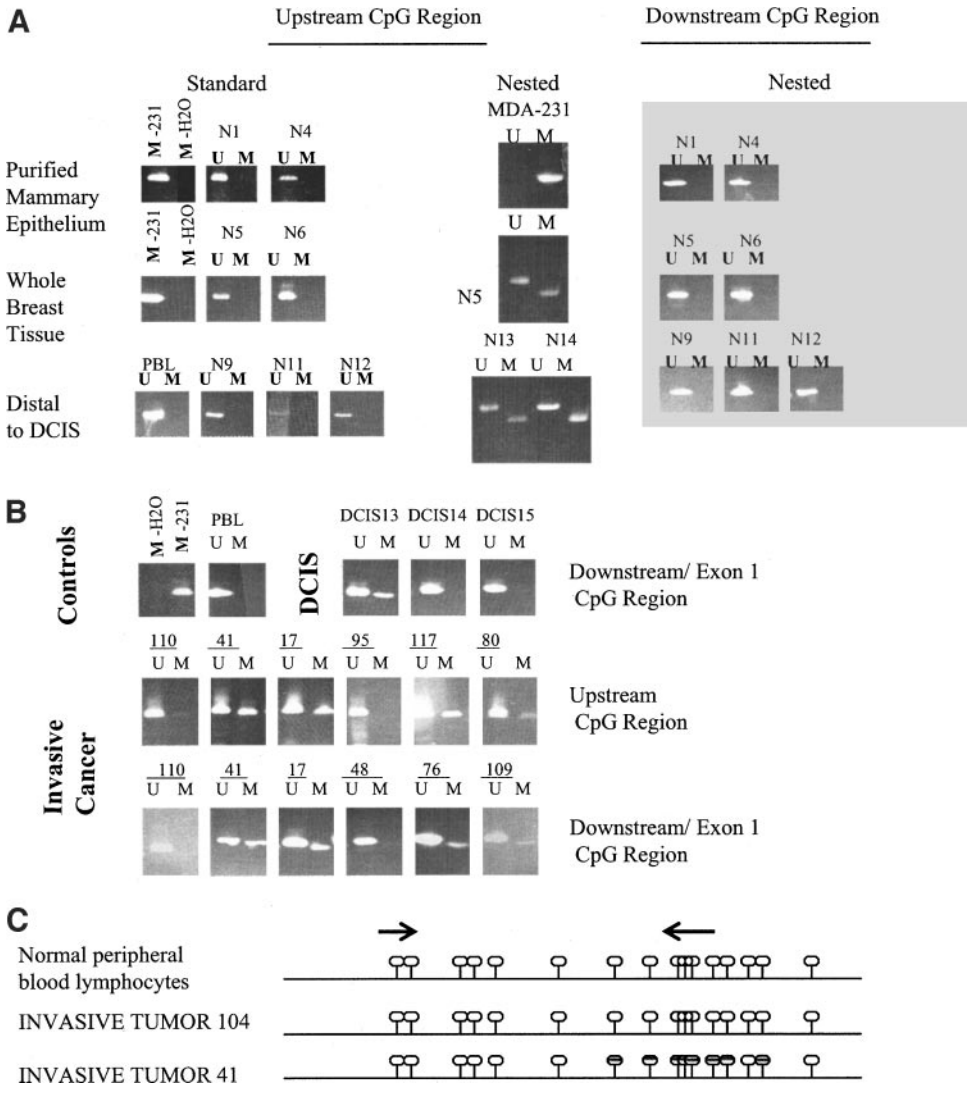

Fig. 4. *A*, standard and nested methylation-specific PCR (MSP) determination of the methylation status (*U*, unmethylated; *M*, methylated) of the *AP-2α* gene promoter in normal breast epithelium and normal-appearing tissue distal-to-ductal carcinoma *in situ* (DCIS; in the image, *Distal to DCIS*). Controls are DNA from the MDA-MB-231 (*M-231*) cell line as a sample known to be methylated at both the upstream and downstream regions (see Fig. 1*B*) and peripheral blood lymphocytes (*PBL*) from a normal individual and a sample containing no DNA. *Lanes N1* and *N4*, DNA from preparations of purified breast epithelial cells from different women taken at the time of a mammoplasty procedure. Notable is the absence of exon 1 methylated alleles for standard and nested assays of the upstream and downstream regions. Most whole breast tissue from women without breast cancer (*N5* and *N6*), and tissue distal to DCIS, *N9*, *N11*, and *N12*, are free of *AP-2α* methylation (*U*) by standard and nested assays at the upstream region; but *N5*, *N13*, and *N14* are methylated (*M*) by the nested assay at these sites. No normal tissues were methylated at the downstream region by the nested assay (*far right panel*). *B*, nested MSP analysis of the *AP-2α* promoter in DCIS and invasive cancer specimens. Of the DCIS samples shown, DCIS 13 is one of the 3 of 19 having *AP-2α* methylation in exon 1, and DCIS 14 and 15 are typical of those that do not. The methylation detected in the DCIS lesion (*DCIS 13*) is absent in the matching histologically normal peripheral tissue, DCIS N13 (see Table 1). Upstream and downstream CpG regions were compared, and representative MSP results are shown for invasive cancers. *AP-2α* methylation is a frequent event in invasive breast cancer at both the upstream and the downstream regions (samples *41, 17, 117,* and *80*). One invasive tumor is methylated for the upstream but not for the downstream region (sample *110*), and one is unmethylated at both (sample *95*). Primers identical to those for Fig. 1 were used. *C*, bisulfite genomic sequencing analysis of tumor samples. Normal peripheral blood lymphocyte DNA was sequenced as a control. Normal cells show no methylation at any of the 16 CpG sites. A representative unmethylated invasive tumor is completely unmethylated by sequencing. However, invasive tumor 41, which had fairly equal densities of methylated and unmethylated alleles by MSP, shows the same 1:1 ratio of methylated to unmethylated alleles by sequencing. *Arrows above the lollipops,* the location of nested MSP primers. M-H_2O refers to water controllance (ODNA).

tween exon 1 methylation status and protein expression in 12 invasive breast cancers. In four tumors unmethylated at the downstream exon 1 region, whether or not the upstream region was methylated, AP-2α protein expression was present throughout the tumor, with some cytoplasmic staining but with dense staining confined to the nucleus (Table 2; examples, Fig. 6, *A* and *C*). In contrast, in nine tumors with methylation at the downstream exon 1 site, there was distinct regional loss of protein expression as seen by large areas with absent protein staining (Fig. 6*E*).

AP-2α Methylation Status Compared with That of Downstream Target Genes. As stated in the "Introduction," two genes frequently hypermethylated in breast cancer, *ER* and *E-cad*, are downstream targets for modulation of transcription by AP-2α. We, thus, investigated in the primary breast cancers presently studied, how the incidence of *AP-2α* hypermethylation compared with that for *E-cad* and *ER*. We observed a strong correlation between *AP-2α* and *E-cad* methylation (21 of 49 tumors methylated for both genes; $P = 0.015$) and between *AP-2α* and *ER* methylation (20 of 29 tumors methylated for both genes; $P = 0.030$). It is also interesting to note that methylation of E-*cad* and *ER* predominantly occurred in those samples in which *AP-2α* is methylated. This non-random association of the hypermethylation for the above three genes is further apparent in that a comparison in 34 of the presently studied primary breast cancers for aberrant methylation of *AP-2α* with other genes frequently hypermethylated in breast cancer, including *GSTπ*, *$p16^{INK4A}$*, *RARβ*, and *BRCA1*, showed no evidence for concordance (data not shown).

Table 2 *Summary of promoter region methylation status in normal and in ductal carcinoma in situ (DCIS) and invasive breast cancer tissue correlated with protein expression*[a]

	Upstream	Downstream	Expression status	Prevalence[b]
Normal	U/M[c]	U	+	
DCIS	U	U	+	5/8
	M	U	+	2/8
	M	M	−	1/8
Invasive	U	M	−	2/12
	M	U	+	2/12
	M	M	−	7/12
	U	U	+	2/12

[a] The designation of a tumor as positive for methylation, M, means a distinct band for the methylated MSP product was observed as shown in figure 4.
[b] Prevalence for composite changes (number of samples demonstrating specified characteristics/total number of samples).
[c] U, unmethylated; M, methylated.

We then compared the methylation and expression status of *AP-2α* and *E-cad* in some of the same tumors to see how patterns of protein expression might correlate. Three general patterns emerged. First, in invasive lesions in which neither gene was hypermethylated, both proteins were expressed throughout the whole regions examined (Fig. 6, *A* and *B*). Second, in those few invasive tumors in which *AP-2α* is unmethylated and E-cad is methylated, most areas were positive for the former protein but heterogeneously negative for the latter (Fig. 6, *C* and *D*). Finally, when both genes were hypermethylated as is often the case, a coincident heterogeneous loss pattern is apparent (Fig. 6, *E* and *F*). Thus, whereas hypermethylation and loss of protein for the two genes can occur independently, these events often involve the genes simultaneously and could contribute additively to tumor progression.

DISCUSSION

We now define that, in human breast cancer, there is a correlation between loss of expression of the important transcription factor, AP-2α, and increased DNA methylation involving a CpG island in the 5′ region of the gene. In this setting, frequent loss or diminished expression of *AP-2α* correlates most closely with hypermethylation of a discrete CpG-rich region of exon 1. Although increased methylation tends to involve all regions of the CpG island in tumors *versus* normal breast tissues, this exon 1 region is the only one that, even when assessed with very sensitive methylation assays, is never methylated in normal breast. It will be important to learn more about the function and chromatin constitution of this small area. The findings also point out the importance of comprehensive examination of entire 5′ regions of a gene to identify the areas truly correlating with epigenetically

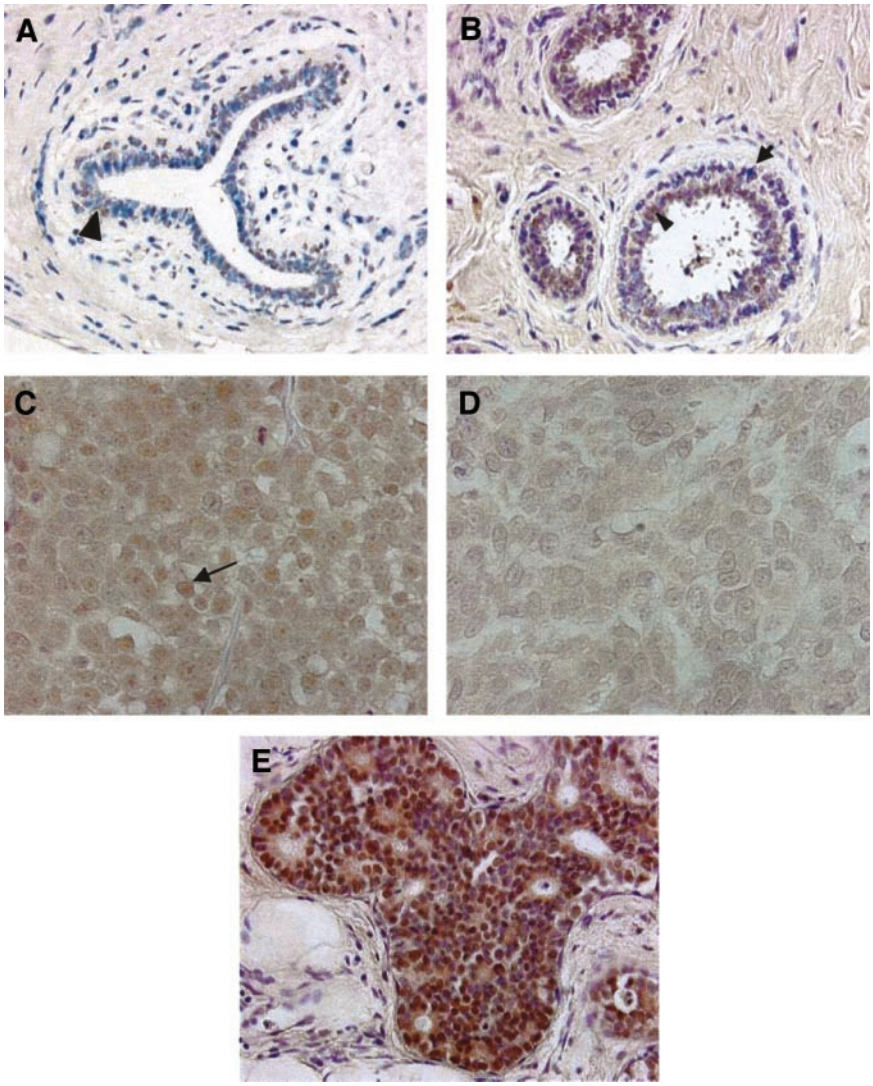

Fig. 5. Examples of immunohistochemistry studies of AP-2α in normal breast samples and ductal carcinoma *in situ* (DCIS). *A*, histologically normal breast epithelium distal to breast carcinoma containing a cell population heterogeneous for nuclear expression (*arrowhead*) of AP-2α. *B*, a slightly hyperplastic duct from a patient with concomitant DCIS showing heterogeneous staining of AP-2α in the epithelial cells lining the lumen (*arrowhead*). Expression is absent in the more mesodermally programmed myo-epithelial cells (*small arrow*). C, sample DCIS 15 (shown in Fig. 4 to be unmethylated) contains positive nuclear AP-2α staining throughout. *At arrow*, robust signal. *D*, nuclei in DCIS 13, which is densely methylated in exon 1, are mostly negative for AP-2 protein. *E*, a tumor that was not hypermethylated for exon 1 actually overexpressed AP-2α in virtually all cells. ×20.

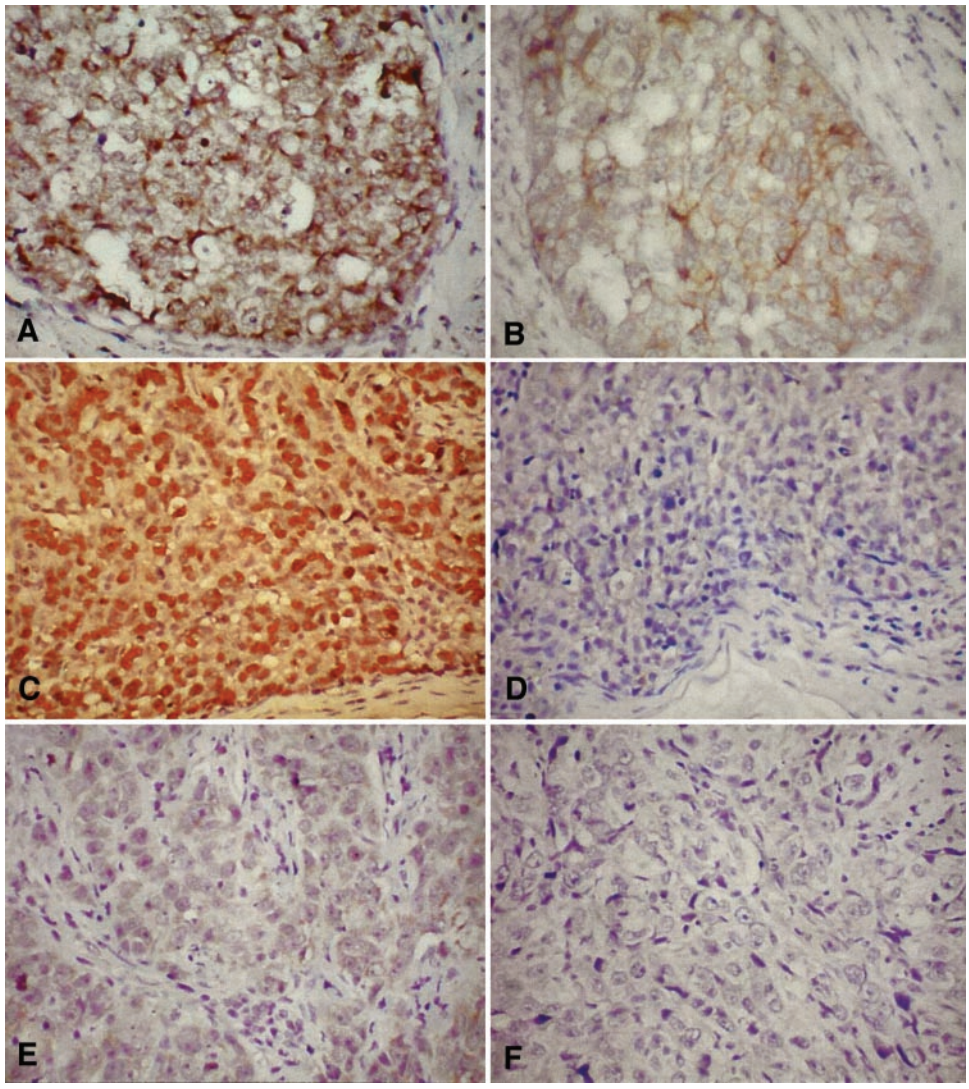

Fig. 6. Immunohistochemical analysis of AP-2α and E-cad expression in breast cancer. *A*, ductal carcinoma *in situ* (DCIS) showing uniform AP-2α expression throughout a tumor unmethylated for *AP-2α* at exon 1. *B*, serial section of tumor in *A* showing moderate E-cadherin (E-cad) expression when *E-cad* is unmethylated. *C*, invasive carcinoma metastatic to the lymph node and unmethylated at *AP-2α* exon 1. Expression of AP-2α throughout the tumor is manifest as prominently staining nuclei. *D*, the same tumor as *C*, methylated for E-cad, showing loss of the protein except for light staining in a few cells. *E*, a primary invasive ductal tumor methylated at AP-2α exon 1 and E-cad. AP-2α and E-cad proteins are virtually absent. ×20.

mediated loss of gene function in cancer and for use in molecular marker studies aimed at separating normal and neoplastic cells.

One confounding possibility for the regulatory significance of our methylation findings is that different transcripts for the *AP-2α* gene have been reported in which an alternative first exon, which includes sequences from intron 1, is involved (27–31). However, it is not known whether a different promoter region from the one we have outlined drives the alternative transcript, and the region shown in Fig. 1*A* is the only functioning promoter defined to date. The methylation events that we now describe fit with full control of the expression, and for silencing of the gene, by the region we have analyzed. The full paradigm is found for this epigenetic route of gene inactivation, as described for classic tumor suppressor genes, including a lack of the hypermethylation pattern in cells of origin of the tumor, decreased or absent expression in concert with the promoter change, and reactivation of the gene expression with drug-induced demethylation in cultured cells (16). Importantly, the gene silencing appears to correlate with the loss of both alternative forms of the transcripts, and the protein, in the 231 cells in which the 5′ region is fully methylated, although cells that are unmethylated can express a single protein form and both transcripts (Fig. 2). Importantly, the re-expression of *AP-2α* is associated in breast cancer cells with a restoration of DNA sequence-specific binding activity of the protein. Such direct demonstration of the functional consequences for promoter hypermethylation has been made for only a few genes in cancer, including restoration of repair activity for the *MLH1* gene (32), apoptosis signaling after re-expression of the *DAP-kinase* (33) and *SOCS 1* genes (34), and normalization of the mdm2 cellular distribution after reactivation of the *p14* gene (35).

Our present findings for AP-2α have importance for both biological and clinical aspects of breast cancer. For the former, we must first consider how the loss of transcriptional function of this gene could influence progression of the disease. The effects for loss of AP-2α expression in breast cancer must be considered from the perspective of downstream transcription targets for this gene. As noted in the "Introduction," AP-2α is known to have transcriptional activation capacity for multiple genes important to epithelial *versus* mesenchymal differentiation, and for the progression of breast cancer. This includes *E-cad*, for which epigenetically involved loss is frequently observed (23, 26, 36). Our present studies have implications for both the timing and the nature of the gene-silencing events that we have observed for *AP-2α* in context for those involving *E-cad*, during tumor progression.

Promoter hypermethylation of, and loss of protein for, the two genes can occur independently and heterogeneously (Fig. 6). Others have observed a progressive heterogeneous loss of AP-2α during tumor progression (10–12, 14, 37). We now report a similar finding of heterogeneity for protein loss in some invasive breast cancers.

Interestingly, hypermethylation for *E-cad* also is known to occur in a heterogeneous pattern throughout breast and other tumors, and this heterogeneity of methylation can vary in density from allele to allele of the promoter (23, 38), and protein levels can vary from cell to cell and, and even more so regionally, within invasive tumors (39). Our correlation of heterogeneous loss of both gene products to the same tumor regions in breast cancers now suggests that these simultaneous events could provide an important impetus for tumor progression.

How might one explain why the loss of function for both upstream and downstream genes in pathways involving AP-2α and targets such as *ER* and *E-cad* would need to occur within the same tumors? In this regard, it is important to remember that *AP-2*α is a master control transcriptional factor that modulates, but does not solely control, multiple target genes in its epithelial cell regulatory pathway. In our hands, this factor has only a modest activation of a gene like *E-cad* (data not shown); and other activators such as Rb and c-myc (4) and repressors such as Snail (40) and SIP1 (41) probably also control the overall expression status of this gene. Similarly, the regulation of *ER* is complex at the promoter level (42). Loss of AP-2α function might then yield only a partial loss of tumor suppressor function for the *ER* and *E-cad* genes that would become much more severe during tumor progression if these two downstream genes were also silenced directly by local promoter change. This could again vary from cell to cell. Indeed, it has previously been demonstrated for *E-cad* and *ER* (23, 36) that such heterogeneity of methylation can occur not only within tumors but also can occur between individual preinvasive breast tumors in the same patient (23). Thus, a variable degree of loss of function mediated by a series of factors, including promoter hypermethylation, could facilitate different levels of loss of function within the AP-2α pathway. This would result in ongoing selective advantage between cell groups within a tumor that all might synergize to yield various degrees of invasive and/or metastatic potential, in the case of E-cad, and hormonal responsiveness, for ER. We have recently defined a similar series of upstream and downstream hypermethylation changes for the GATA-4 and GATA-5 transcription factors and targets in gastrointestinal tumors (43).

From a clinical standpoint, our current data suggest an important potential contribution for use of *AP-2* α hypermethylation as a marker for enhancing molecularly based strategies for diagnosing and monitoring the prognosis of breast cancer. From the data in this first small study, hypermethylation of exon 1 is a late finding for breast cancer progression, or common to only some pathways to progression, because, as mentioned, it was found in only 3 of 19 DCIS samples. In addition to the potential importance of this finding for the biology of the disease, the data suggest that monitoring of the methylation status of *AP-2* α exon 1 could be an important parameter for predicting which DCIS lesions may be at risk for developing subsequent invasive disease. This possibility warrants further investigation in clinical studies.

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