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The E-cadherin promoter: Functional analysis of a G·C-rich region and an epithelial cell-specific palindromic regulatory element

(epithelium-specific gene expression/initiator element/keratin promoter regulatory element/tumor differentiation/tumor invasion)

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ABSTRACT **The cell–cell adhesion molecule E-cadherin is specifically expressed in epithelia and is involved in the maintenance of the epithelial phenotype. Expression of E-cadherin is downregulated in many poorly differentiated carcinomas, which leads to higher motility and invasiveness of the cells. To examine the mechanisms that regulate tissue-specific expression, we have characterized the promoter of the E-cadherin gene. We found that an upstream fragment (positions −178 to +92) mediates strong expression of a chloramphenicol acetyltransferase reporter gene in epithelial cells (i.e., 60% of the level obtained with simian virus 40 promoter/enhancer constructs), whereas in nonepithelial cells this promoter was either inactive or much less active. By DNase I footprinting and gel retardation analysis as well as through functional dissection of the regulatory sequences, we identified two regions that contribute to tissue-specific activity of the promoter: (*i*) a G·C-rich region between −25 and −58 that generates basic epithelial promoter activity, most likely in combination with an "initiator" element present at the single transcription start site of the gene, and (*ii*) a palindromic sequence between −75 and −86 (named E-pal) that potentiates the activity of the proximal E-cadherin promoter and confers epithelial cell-specific activity on a simian virus 40 promoter. The E-pal sequence is homologous to cis regulatory elements active in keratin gene promoters and competes with these elements for nuclear factor binding. Interestingly, the activity of the E-cadherin promoter was reduced in dedifferentiated breast carcinoma cells, indicating that the identified elements are subject to negative regulation during tumor progression.**

Epithelial tissues are characterized by unique structural properties, the most prominent being the tight association of individual cells through various junctional organelles and the polarized distribution of cytoplasmic and cell surface components. As a functional consequence, epithelial cells are often less mobile than cells of mesenchymal origin, they form selective permeability barriers, and they carry out vectorial transport processes in tissues (1). Several of these epithelial characteristics are controlled by the function of the cell–cell adhesion molecule E-cadherin, which is a 120-kDa transmembrane glycoprotein specifically expressed in epithelial tissues (see ref. 2 for a review). Antibodies against the extracellular domain of E-cadherin disturb its function and thereby lead to the loss of the epitheloid morphology of the cells. This treatment also enhances the motility and reduces the tight junction-mediated resistance of the cells (3–5). Furthermore, forced expression of E-cadherin cDNA in nonepithelial cells generates epithelium-like monolayers that exhibit polarized distribution of certain marker molecules (6–8).

During tumor progression, a loss of E-cadherin expression can parallel dedifferentiation of human carcinomas *in vitro* and *in vivo*; i.e., E-cadherin is present in well-differentiated noninvasive carcinomas, but its expression is often downregulated in poorly differentiated invasive tumors (4, 9, 10). Furthermore, invasiveness of dedifferentiated carcinoma cells can be prevented by transfection with E-cadherin cDNA and restored by treatment of the transfected cells with dissociating anti-E-cadherin antibodies (9). From these studies it is evident that E-cadherin is a major determinant for the establishment and the maintenance of the normal epithelial phenotype and might act as an invasion suppressor in carcinomas.*

MATERIALS AND METHODS

A mouse genomic library in cosmid pWE15 (Stratagene) was screened for the putative E-cadherin promoter region with the ^{32}P-labeled 145-base-pair (bp) *Sac* I–*Acc* I fragment of the 5′ end of the mouse E-cadherin cDNA (6, 11). Primer extension analysis was performed with 2 μg of poly(A)$^+$ RNA hybridized to 10^5 cpm of two 5′ ^{32}P-labeled oligonucleotide primers (a, complementary to positions +88 to +107; b, complementary to positions +133 to +157). S1 nuclease mapping was performed according to Sambrook *et al.* (12).

The −3000 and −1400 E-cadherin promoter/chloramphenicol acetyltransferase (CAT) gene constructs were prepared by cloning appropriate restriction fragments into the polylinker of the promoterless plasmid pCAT-Basic (Promega). The −800, −178, −58, and −21 promoter/CAT constructs were prepared by BAL-31 nuclease (Boehringer Mannheim) digestion of the −3000 deletion fragment. All deletion fragments had a common 3′ end at position +92. CAT activities of the various constructs were compared with those produced by CAT plasmids containing either the simian virus 40 (SV40) promoter/enhancer (pCAT-Control, Promega) or the Rous sarcoma virus promoter. Furthermore, two complementary oligonucleotides spanning the sequence from −92 to −69 (E-pal) were cloned via *Bam*HI linkers into the *Bgl* II site of the CAT plasmid containing the SV40 promoter (pCAT-Promoter, Promega) or via blunt-end ligation into the *Acc* I site upstream of the −58 promoter/CAT construct. CAT assays were performed and activities were quantified as described (13) except that cells were transfected in suspension. To control for variations in transfection efficiency, plasmid pCH110 (a gift of G. Ryffel, University of Essen), containing the SV40 promoter and the *Escherichia coli lacZ* gene, was cotransfected and the amounts of cell extracts were adjusted according to the β-galactosidase activity.

Nuclear extracts were prepared and gel retardation assays were performed as described (14, 15), except that 1 μg of poly(dI-dC) (Boehringer Mannheim) was used as nonspecific competitor. The following oligonucleotides and their complements were used: E-pal, 5′-GATCCGGCTGCCACCTG-CAGGTGCGTCCCG-3′; KER-1, 5′-GATCCAAGTG-

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Abbreviations: CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; nt, nucleotide(s).
*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M81449).

TAGCCTGCAGGCCCACACCG-3′ (16); AP-2, 5′-GATCCAAAGTCCCCAGGCTCCCCAG-3′ (17); KTF-1, 5′-TCGACAACAAACACCCTGAGGCTACGTAG-3′ (18). For DNase I footprinting, the −178/+92 bp promoter fragment was radioactively labeled at either the 5′ end of the coding strand (with phage T4 polynucleotide kinase) or at the 3′ end of the noncoding strand (with Klenow fragment of DNA polymerase I) and cleaved at position +17 with *Ksp* I. Footprinting assays were performed as described (19) with 100–150 μg of protein from crude nuclear extracts, 1 μg of poly(dI-dC), and 1–2 ng of labeled probe (specific activity, 2 × 10^7 cpm/μg). For the Sp1 footprinting assay, 15 ng of purified recombinant transcription factor Sp1 (Promega) was employed.

RESULTS

Structure of the 5′ Flanking Region of the Mouse E-Cadherin Gene. A 252-bp *Pst* I fragment of genomic cosmid clone m18-1 (Fig. 1*A*) was found to contain sequence representing the 5′ end of the previously characterized E-cadherin cDNA (position x in Fig. 1*B*; ref. 6). When two oligonucleotides (long arrows in Fig. 1*B*) were used for primer extension on poly(A)$^+$ RNA of CSG 120/7 mouse salivary gland carcinoma cells (which express E-cadherin; ref. 9), two elongation products of 107 and 157 nucleotides (nt) were obtained (Fig. 2, lanes a and b). When the 252-bp *Pst* I genomic fragment was hybridized to CSG poly(A)$^+$ RNA, a 170-nt fragment was protected from digestion by S1 nuclease (Fig. 2, lanes c and d). The transcription start site of the E-cadherin gene is thus located 127 nt upstream of the ATG translation start codon (+1 in Fig. 1*B*).

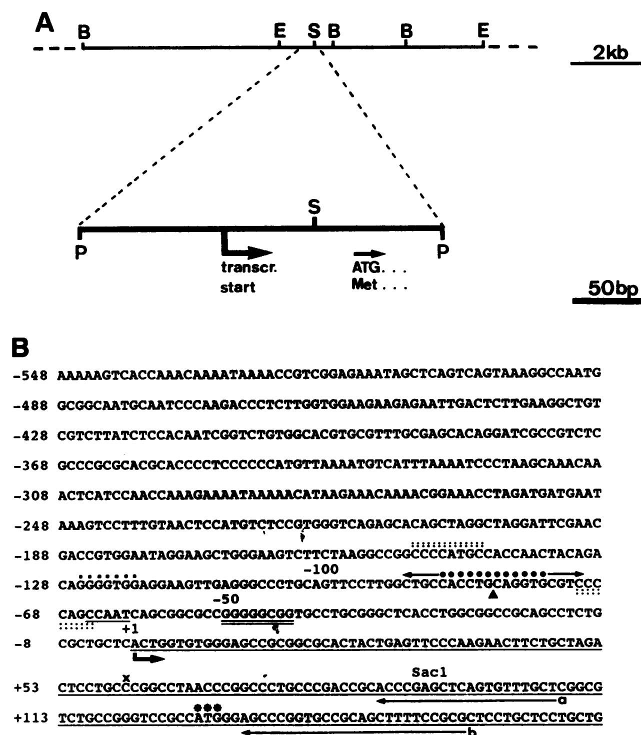

FIG. 1. Restriction map and partial nucleotide sequence of the 5′ region of the mouse E-cadherin gene. (*A*) Restriction map of part of the m18-1 cosmid insert. B, *Bam*HI; E, *Eco*RI; S, *Sac* I; P, *Pst* I. (*B*) DNA sequence of the genomic region surrounding the transcription start site (position +1). The translation initiation codon ATG (***) and the 5′ end (x) of the previously published mouse E-cadherin cDNA sequence (6) are indicated. Oligonucleotides (a and b) used for primer extension mapping (see Fig. 2) are represented by long arrows. Potential transcription factor binding sites are indicated: AP-2 (double dotted line), CAAT box (single underline), Sp1, (double underline), and variant Sp1, (single dotted line). The palindromic element E-pal (dots between short arrows) with the flanking inverted repeats (short arrows) is also indicated.

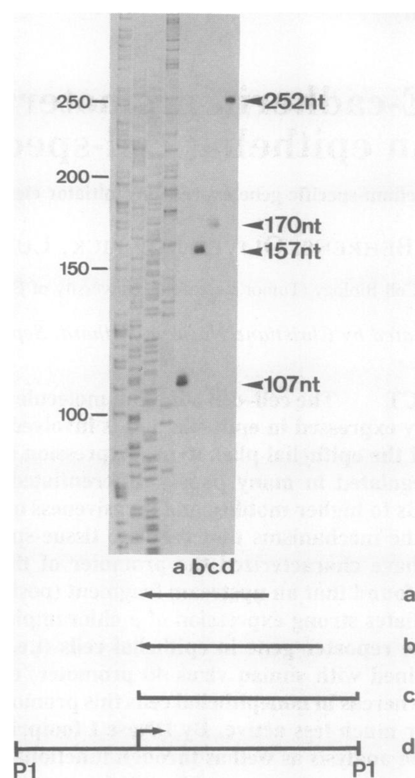

FIG. 2. Mapping of the E-cadherin mRNA transcription start site by primer extension and S1 nuclease analysis. Lanes a and b, primer extension products using antisense primers a and b [indicated in Fig. 1*B* by long arrows; difference in length of the extension products (50 nt) corresponds to the 50-bp distance between the two primers in the E-cadherin sequence]; lane c, protected fragment obtained after hybridization of the 252-bp *Pst* I–*Pst* I probe to total RNA and digestion with S1 nuclease; lane d, the *Pst* I–*Pst* I probe. The four lanes at left show products of sequencing reactions, used as size markers. P1, *Pst* I.

The upstream region of the E-cadherin gene contains several potential binding sites for transcription factors—e.g., a G·C-rich region harboring a putative Sp1 site at position −50 and a CAAT box at position −65 (Fig. 1*B*). No obvious TATA box homology is present; however, the sequence surrounding the transcription start site (5′-CTCACTGG-3′) is similar to the initiator sequence (5′-CTCANTCT-3′) described in a TATA-less promoter (20, 21; the underlined nucleotide A represents the transcription start site). Interestingly, a 12-bp palindromic sequence (5′-CACCTGCAGGTG-3′, marked by ▲ in Fig. 1*B*) flanked by two 4-bp inverted repeats was identified at positions −75 to −86 (E-pal). This palindromic sequence is identical in 8 of 10 positions to the KER-1 binding site (5′-GCCTGCAGGC-3′) and in 7 of 12 positions to the KTF-1 binding site (5′-CACCCTGAGGCT-3′), which are regulatory elements present in keratin gene promoters of human and *Xenopus*, respectively (16, 18).

Promoter Activity of the 5′ Flanking Region of the E-Cadherin Gene. When constructs containing 3000, 1400, and 800 bp of upstream sequence in front of the CAT gene were transfected into CSG epithelial cells, CAT activities 10-15% that of the SV40 promoter/enhancer CAT construct were obtained (Fig. 3). However, when the sequence upstream of position −178 was deleted, CAT activity increased 5-fold—i.e. to 60% of that of the SV40 promoter/enhancer construct. Further reduction of the upstream region (deletion −58) led to

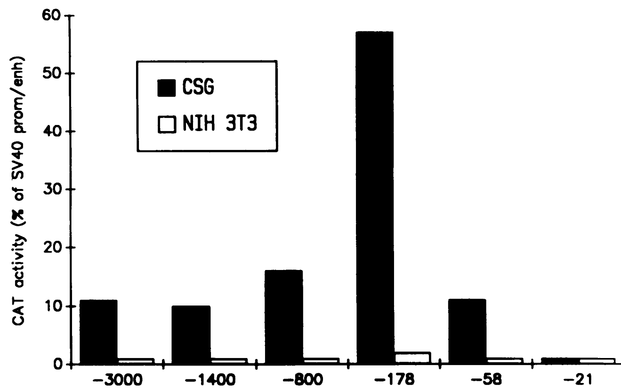

FIG. 3. Promoter activity of the E-cadherin 5′ flanking sequences in CSG epithelial cells and in NIH 3T3 fibroblasts. CAT activities were determined after transfection of various E-cadherin promoter deletion/CAT constructs into CSG cells (filled columns) and 3T3 cells (open columns). Numbers below the bars indicate the 5′ ends of the various deletion fragments. Similar cell-type specificity was observed when the data were based on the activity of a Rous sarcoma virus promoter/CAT construct. Correct initiation of transcription at the authentic start site was verified in the case of the −178 E-cadherin promoter/CAT construct by primer extension analysis (data not shown). prom/enh, Promoter/enhancer.

a decrease of CAT activity to 10% of that of the SV40 promoter/enhancer. The activity dropped to below 2% when the G·C-rich region with the putative Sp1 site was also removed (deletion −21). In 3T3 mouse fibroblasts (which do not express E-cadherin), CAT activity was barely detectable with all constructs tested. We also tested the −178 and −58 deletion/CAT constructs in other E-cadherin-positive and -negative cell lines (Table 1). Both deletion fragments exhibited activity in differentiated epithelial cells but not in dedifferentiated epithelial cells or in fibroblast and smooth muscle cells.

Binding of Nuclear Factors to the −178/+17 E-Cadherin Promoter Fragment. We performed gel retardation assays with nuclear extracts of CSG epithelial cells, using a −178/+17 fragment and portions of it as probes (Fig. 4*A*). Specific bandshifts were seen with probe (b) ranging from −58 to +17 and the complete probe (c), but not with probe a (Fig. 4*B*). The E-pal oligonucleotide also formed specifically retarded complexes (Fig. 4*B*).

DNase I footprinting analysis with the −178/+17 fragment and nuclear extracts from mouse and human epithelial cells and fibroblasts revealed two major protected areas, the G·C-rich region (from −25 to −58) and the E-pal element

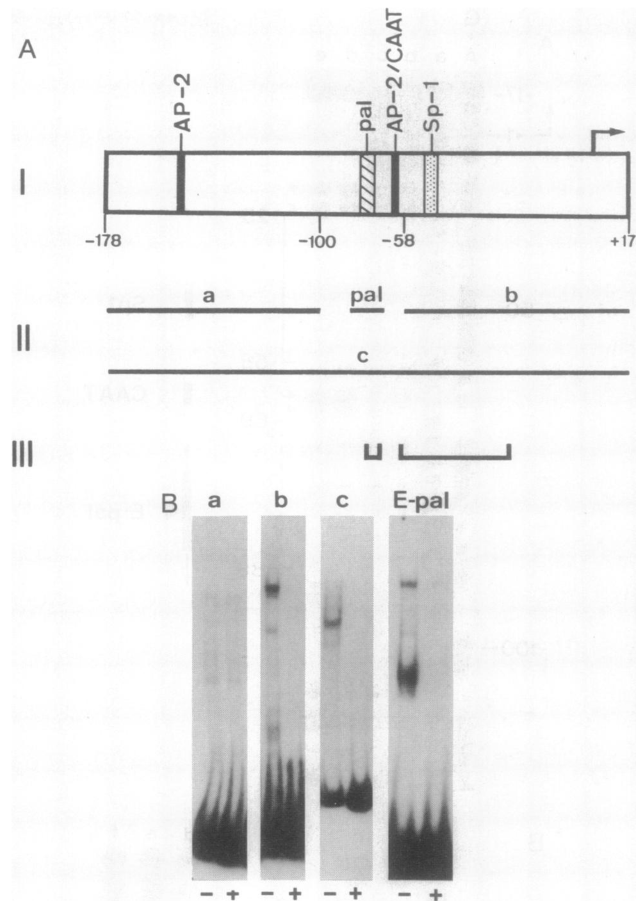

FIG. 4. Binding of nuclear factors to the −178/+17 E-cadherin genomic fragment. (*A*) Schematic representation of the −178/+17 fragment showing putative transcription factor binding sites (I), the probes used in gel retardation experiments (II), and the regions protected by nuclear factors in the DNase I footprinting assays (III). (*B*) Gel retardation assays with probes a, b, and c, and the E-pal oligonucleotide using nuclear extracts from CSG epithelial cells. +, Preaddition of a 50-fold molar excess of the corresponding unlabeled probe.

(from −69 to −88) (Fig. 5 *A* and *B*). No significant differences in the footprints were seen with nuclear extracts from epithelial cells and fibroblasts. We found that protection of the G·C-rich region cannot be solely due to binding of Sp1, since purified factor produced a more restricted protection pattern (Fig. 5*C*).

Table 1. Activity of the −178 and −58 E-cadherin promoter fragment and the E-pal element in epithelial and nonepithelial cells

Cell line	Origin of cells	Activity*			SV40 promoter activity increase with E-pal†	E-cadherin expression‡
		−178	−58	−58/E-pal		
MCF-7	Epithelial (human)	72	12	46	7	+
MDA-MB 361	Epithelial (human)	30	ND	ND	20	+
CSG	Epithelial (mouse)	55	12	15	2	+
MDA-MB 436§	Epithelial (human)	6	2	2	1	−
MRC-5	Fibroblast (human)	2	1	ND	0.3	−
NIH 3T3	Fibroblast (mouse)	2	1	ND	0.6	−
AOSMC	Smooth muscle (pig)	3	3	2	0.2	−

ND, not determined.
*CAT activities of the −178 and −58 E-cadherin promoter/CAT constructs and of a −58 E-cadherin promoter construct containing two copies of E-pal are presented as percentage of activity of the SV40 promoter/enhancer/CAT construct.
†Ratios of CAT activities obtained of the E-pal (four copies)/SV40 promoter/CAT construct to those of the construct without insertion of the E-pal sequences.
‡As determined in ref. 9.
§A dedifferentiated breast carcinoma cell line (9).

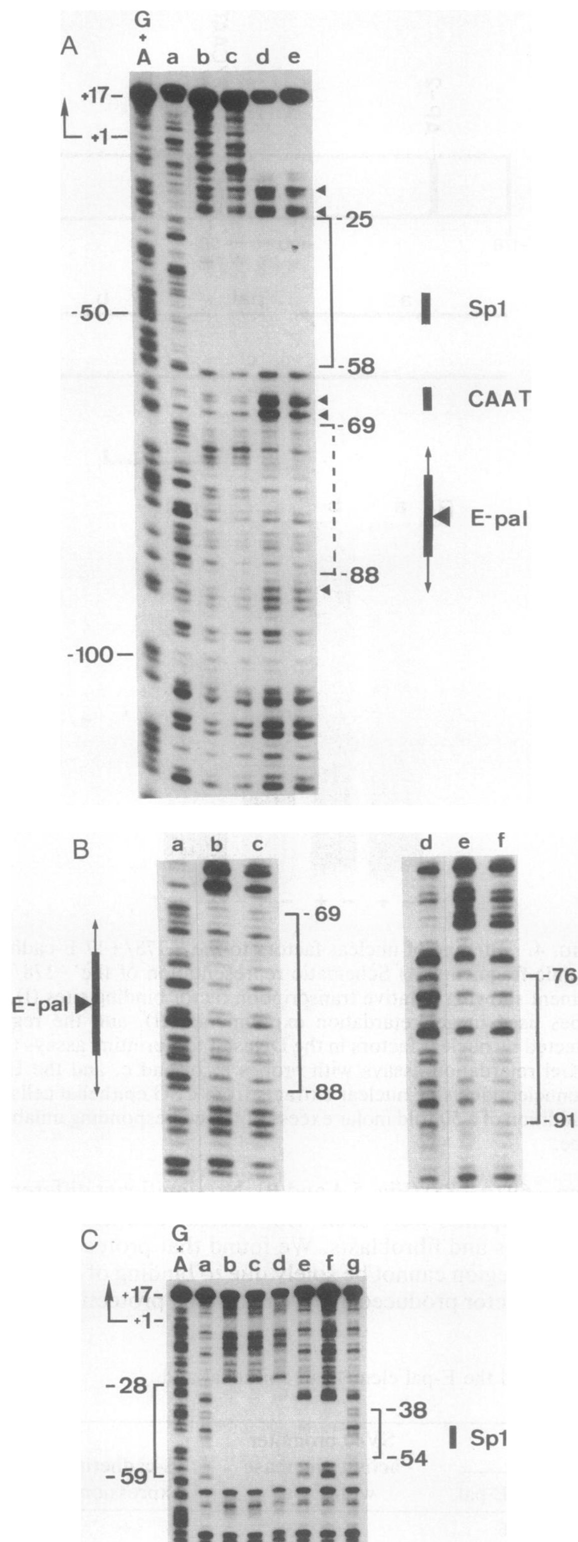

FIG. 5. Footprint analysis. (*A*) DNase I footprinting analysis of the −178/+17 fragment labeled with ^{32}P at the 5′ end of the coding strand. Shown are digestion patterns in the absence of nuclear extract (lane a) or in the presence of nuclear extract from CSG epithelial cells (lane b), 3T3 fibroblasts (lane c), MCF-7 carcinoma cells (lane d), or MDA-MB 435 carcinoma cells (lane e). Lane G+A shows products of sequencing reactions of the −178/+17 fragment. (*B*) DNase I protection of the E-pal sequence in the −178/+17 fragment labeled on the coding strand (lanes a–c) and on the noncoding strand (lanes d–f). Lanes a and d, no added extracts; lanes b and e, MCF-7 nuclear extract; lanes c and f, MDA-MB 435 nuclear extracts. (*C*) Binding of purified Sp1 to a subarea of the G·C-rich region shown by DNase I digestion of the noncoding strand in the absence (lane a) or presence (lanes b–d) of nuclear extract protein and in the presence of purified Sp1 (lanes e–g). In lanes c and e, 10 ng of an oligonucleotide containing two Sp1-binding sites of the SV40 promoter was included; in lanes d and g, 30 ng of this competitor was added.

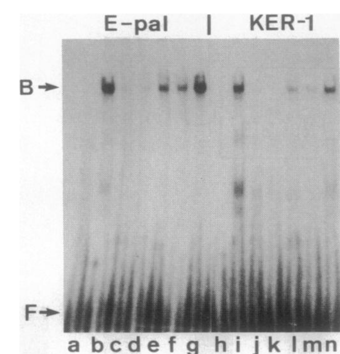

FIG. 6. Competition of the E-pal bandshift by other elements. Gel retardation assays were performed with nuclear extracts of HeLa cells (which contain KER-1; ref. 16) and oligonucleotides containing either the E-pal sequence (lanes a–g) or the KER-1 binding site of the human K14 keratin gene promoter (lanes h–n). Lanes a and h, absence of nuclear proteins; lanes b–g and i–n, presence of nuclear proteins. Competitors; 100-fold (lanes c and j) and 200-fold (lanes d and k) molar excess of the E-pal oligonucleotide; 100-fold (lanes e and l) and 200-fold (lanes f and m) molar excess of the KER-1 oligonucleotide; and 200-fold molar excess of an oligonucleotide containing the AP-2 binding site of the SV40 enhancer (lanes g and n). B, bound probe; F, free probe.

Binding Specificity of the E-Cadherin Palindromic Element. Oligonucleotides containing the E-pal sequence or the KER-1 binding site (see *Materials and Methods*) produced retarded complexes with similar mobility in gel retardation assays (Fig. 6, lanes b and i). Formation of these complexes could be blocked by an excess of either the unlabeled E-pal (lanes c, d, j, and k) or the unlabeled KER-1 (lanes e, f, l, and m) oligonucleotides. We confirmed that an oligonucleotide containing the AP-2 binding site of the SV40 promoter blocked formation of the KER-1 complex by ≈50% (16, 17); it did not, however, affect formation of the E-pal complex (lanes g and n). Conversely, E-pal blocked the AP-2 complex formation (data not shown).

Transcription Stimulation by the E-pal Sequence. We cloned the E-pal oligonucleotide upstream of the SV40 promoter/CAT and the −58 E-cadherin promoter/CAT constructs. When constructs with four copies of E-pal in front of the SV40 promoter were transfected, CAT activity in epithelial cells was stimulated 2- to 20-fold, whereas it was inhibited by a factor of 2–5 in fibroblasts and smooth muscle cells (Table 1). E-pal also stimulated the −58 E-cadherin promoter fragment in epithelial cells. Stimulation of activity of the SV40 promoter in the differentiated human breast carcinoma cell lines MCF-7 and MDA-MB 361 depended on the number of inserted E-pal copies (Fig. 7). E-pal did not, however, affect CAT expression in the dedifferentiated breast carcinoma cell lines MDA-MB 436 and 435.

DISCUSSION

The analysis of various deletion fragments of the E-cadherin promoter revealed that the regulatory region is composed of various cis-acting elements that contribute positively or negatively to the promoter activity. Apparently, sequences upstream of position −178 may contain silencing elements, since their removal increased activity of the remaining promoter. Further deletion of the 5′ flanking region (deletion −58) decreased activity by a factor of 5, which seems to be a consequence of removal of the E-pal sequence. It should be

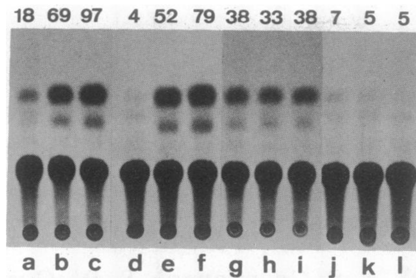

FIG. 7. Transcriptional activation of SV40 promoter/CAT expression mediated by the E-pal sequence. CAT activities in the differentiated breast carcinoma cell lines MCF-7 (lanes a–c) and MDA-MB 361 (lanes d–f) and in the dedifferentiated breast carcinoma cell lines MDA-MB 436 (lanes g–i), and MDA-MB 435 (lanes j–l) were measured after transfection of SV40 promoter plasmids (lanes a, d, g, and j), the plasmid containing two E-pal copies (lanes b, e, h, and k), and the plasmid containing four copies of E-pal (lanes c, f, i, and l). Radioactivity (cpm $\times 10^{-2}$) of acetylated chloramphenicol is indicated above each lane.

noted, however, that the −58 deletion fragment still retained appreciable epithelial specific activity. Finally, deletion of the G·C-rich area down to position −21 reduced promoter activity to near background levels. The E-cadherin gene promoter thus exhibits a modular structure (22, 23), and the control of epithelium-specific expression of E-cadherin might result from the combination of activity of the various regulatory elements.

The E-pal sequence stimulates epithelial cell-specific transcription directed by the heterologous SV40 promoter and by the proximal basic E-cadherin promoter in a dose-dependent manner. In nonepithelial cells, the E-pal element repressed activity of the SV40 promoter. E-pal thus seems to act as a tissue-specific positive or negative regulatory element, dependent on the nuclear factors present in the particular cell type. What kind of transcription factors might bind to the E-pal sequence? E-pal bears homology to palindromic elements found in promoters of keratin genes that are also specific markers for epithelial cells (24). In particular, the central 8 nt of E-pal and the KER-1 recognition sequence of the human keratin K14 gene (16) are identical, and the two sequences compete for binding to nuclear factors in gel retardation assays. It is therefore an attractive possibility that the expression of both types of epithelium-specific markers is regulated in a similar fashion. Interestingly, the E-pal sequence also competed in gel retardation experiments with oligonucleotides containing binding sites for the transcription factor AP-2 (25), indicating that an AP-2-like factor is a candidate for an E-pal-binding protein. It is also intriguing that both half-sites of E-pal harbor the CANNTG consensus sequence for binding of helix–loop–helix transcription factors (26). It is therefore tempting to speculate that the E-pal-binding protein(s) might be members of this family of transcription factors, which has been implicated in other cases of tissue-specific gene expression (26).

In footprint experiments, we detected efficient binding of nuclear factors to the G·C-rich region that surrounds the Sp1 site. However, the protection pattern extends beyond the area covered by purified Sp1 alone, suggesting that additional factors might bind to this region. We therefore assume that the combination of the G·C-rich region with the initiator element leads to transcriptional initiation at a single start site, and that the basic epithelial specificity of the E-cadherin promoter is produced by the interaction of other factors, possibly in combination with Sp1, with the G·C-rich region. Nuclear factors that bind to G·C-boxes have been discovered—e.g., ETF and GCF-1 (27, 28)—but there is no indication that these factors are involved in epithelial cell-specific gene regulation.

We did not observe any significant difference in nuclear factor binding to the here-identified E-cadherin promoter elements when extracts of epithelial and nonepithelial cells were compared, in spite of the differential activity of the promoter in these cells. There are several possibilities to explain this phenomenon: (i) both stimulating and inhibitory factors might bind to the regulatory elements in an indistinguishable manner; (ii) additional factors (e.g., coactivators) might generate the specificity by protein–protein interaction; (iii) specific activation or suppression of E-cadherin transcription might be created by differential modification of these factors in various cell types.

An important facet of E-cadherin function is shown by our finding that poorly differentiated carcinoma cells do not express the molecule, which results in increased invasive potential of these cells (4, 9, 10). Accordingly, the E-pal sequence stimulated transcription in differentiated breast carcinoma cells but was silent in their poorly differentiated counterparts. It will now be important to determine whether the here-identified E-cadherin regulatory elements are in fact targets for the action of oncogenes or tumor-suppressor genes, some of which are directly involved in transcriptional regulation.

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