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Sp1 Binding Plays a Critical Role in Erb-B2- and v-ras-Mediated Downregulation of α_2-Integrin Expression in Human Mammary Epithelial Cells

JIANPING YE,[1] RUO HUI XU,[1] JOYCE TAYLOR-PAPADIMITRIOU,[2] AND PAULA M. PITHA[1,3]*

Oncology Center[1] and Department of Molecular Biology and Genetics,[3] The Johns Hopkins University School of Medicine, Baltimore, Maryland, and Epithelial Cell Biology Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom[2]

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The human α_2-integrin gene is transcriptionally downregulated in a nontumorigenic human mammary epithelial cell line, MTSV1-7, and its clonal variant HB2, overexpressing the Erb-B2 oncogene. In this study, we have used deletion mutations within the α_2-integrin promoter inserted 5′ of the chloramphenicol acetyltransferase or luciferase reporter genes to identify the element that is responsible for the Erb-B2-mediated downregulation. The results of the transient-transfection assay showed that the Sp1 binding element located in the core region (positions −64 to +1) of the α_2-integrin promoter plays an essential role in the α_2-integrin promoter activity and its downregulation by Erb-B2. By gel shift assay, we have demonstrated that this element binds with a high degree of affinity not only to Sp1, but also to Sp3. The downregulation of the α_2-integrin promoter activity could also be achieved by overexpression of v-Hras (v-ras), suggesting that the signals generated by Erb-B2, which lead to downregulation of the α_2-integrin gene expression, may proceed through the ras pathway. Both the Erb-B2- and the v-ras-overexpressing cells exhibited a Sp1 DNA binding activity lower than that of the parental line, while the relative levels of Sp1 protein in these cells were not altered. The Erb-B2- and v-ras-mediated downregulation could be reversed by the overexpression of Sp1 and by a dominant negative variant of ras (rasN17), confirming the importance of Sp1 and the ras pathway. The inhibitory effects of Erb-B2 on transcriptional activity of the α_2-integrin promoter were observed in transient-cotransfection assays using α_2-integrin reporter plasmids and plasmids expressing the Erb-B2 or v-ras oncogene. The same effects were seen when an α_2-integrin reporter gene construct was transfected into MTSV1-7 or HB2 cells permanently overexpressing Erb-B2 or v-ras. The effects of Erb-B2 or v-ras on the transcriptional activity of the α_2-integrin promoter were observed in nontumorigenic luminal epithelial cell lines (MTSV1-7 and HB2) as well as in the breast cancer cell line T47D. These data suggest that in luminal epithelial cells and the breast cancers which develop from them, the Erb-B2 proto-oncogene signaling leads to inhibition of $\alpha_2\beta_1$-integrin gene expression and could contribute to the disruption of tissue architecture seen in breast cancers.

The disruption of tissue architecture that occurs in the development of invasive breast carcinomas is associated with the altered expression and function of adhesion molecules such as E-cadherin and integrins. In epithelial cells, E-cadherin is found in the adherens junctions and plays a major role in the maintenance of epithelial polarity through homotypic, calcium-dependent interactions between cells. The major function of the integrins is to mediate attachments to the extracellular matrix, but they can also be involved in cell-cell interactions (for a review, see reference 1). Integrins are heterodimeric transmembrane glycoproteins consisting of α and β subunits, of which 15 α and 8 β subunits have been cloned. Individual β subunits can heterodimerize with different α subunits, resulting in a large number of integrin receptors. Individual integrins can often bind more than one ligand, and individual ligands are often recognized by more than one integrin. Signals generated by the integrin-ligand interaction are only now being characterized and may be specific for a particular integrin and ligand (14). Since the ligands which bind integrins can also vary between cell types, it is important to consider integrin expression and function in the context of cell phenotype. In the epithelial cells of the mammary gland, the most abundantly expressed integrins are members of the β_1 family ($\alpha_2\beta_1$ and $\alpha_3\beta_1$) and the $\alpha_6\beta_4$ integrin, which is a component of hemidesmosomes. Of these, the $\alpha_2\beta_1$ integrin is the major integrin expressed by the luminal epithelial cells, from which the majority of breast cancers develop (1, 9).

A number of studies have reported a decrease in the expression of E-cadherin and of the $\alpha_2\beta_1$ integrin in carcinomas. In breast and prostate carcinomas, this decrease appears to be associated with an increase in invasiveness (5, 42, 50, 57). The alteration in expression of adhesion molecules observed in malignancies could involve several mechanisms. Mutations or deletions in the genes could occur, and mutations in the E-cadherin gene have in fact been observed in gastric carcinoma cell lines (41) and in lobular carcinomas of the breast (24). Changes in the integrin genes have not yet been reported. Inactivation of E-cadherin gene expression by methylation has also recently been shown to occur in different tumors, including primary breast cancers, in which methylation of the CpG cluster in the first exon of the E-cadherin gene correlates with the downregulation of E-cadherin gene expression (20, 56). Finally, oncogenes or proto-oncogenes such as *Erb-B2*, *bcl-2*, and c-*fos*, which can induce the epithelial mesenchyme conversion, can also control transcription of the E-cadherin and α_2-integrin genes (16, 17, 34, 45). Specifically, overexpression of the Erb-B2 gene in a nontumorigenic human mammary epi-

* Corresponding author. Mailing address: Oncology Center, The Johns Hopkins University, 418 N. Bond St., Baltimore, MD 21231. Phone: (410) 955-8871. Fax: (410) 955-0840. Electronic mail address: parowe@welchlink.welch.jhu.edu.

thelial cell line, MTSV1-7, has been found to induce downregulation of transcription of the genes coding for E-cadherin and the α_2-integrin subunit (17). This cell line, which was developed from cells cultured from human milk (3), exhibits the phenotype of the luminal epithelial cell and forms organized three-dimensional structures in collagen gels. In this system, the interaction of the $\alpha_2\beta_1$ integrin with the collagen is crucial for morphogenesis (8), which is altered in the Erb-B2 transfectants (16).

The Erb-B2 (p185c-erbB2) proto-oncogene belongs to the family of tyrosine kinase receptors which includes Erb-B1 (epidermal growth factor [EGF] receptor), Erb-B3, and Erb-B4 (32, 49). Overexpression of Erb-B2 and Erb-B3 (4, 32), resulting from gene amplification or transcriptional upregulation, has been found in some breast carcinomas and is associated with a poor prognosis (29, 46). Although no specific ligand for the Erb-B2 receptor has been identified, overexpression of Erb-B2 results in constitutive activation of its intrinsic kinase activity (6). Furthermore, EGF and heregulin ligands that bind to Erb-B1 and Erb-B3 or Erb-B4, respectively, induce heterodimerization of these receptors with Erb-B2 and its cross phosphorylation. Since Erb-B2 overexpression enhances the affinities of binding of both EGF and heregulin to their receptors, it has been suggested that Erb-B2 is an essential component of high-affinity EGF and heregulin receptors and plays an important role in the signaling pathway induced by these receptors (28). The signaling induced by binding of EGF to its receptor is coupled to the ras pathway (reviewed in references 49 and 53), and overexpression of Erb-B2 in breast carcinomas was also shown to amplify ras signaling (28).

In an attempt to determine the possible role of oncogenes in the observed changes in α_2-integrin levels detected in breast tumors, we examined the molecular mechanism by which Erb-B2 affects transcription from the α_2-integrin gene promoter in human mammary epithelial cells of luminal origin (17). In this study, we have focused on the role of a Sp1 binding site (α_2 Sp1 site) in the core promoter of the α_2-integrin gene in the human mammary epithelial cell lines. The Sp1 transcription factor is a DNA-binding protein that binds to GGGCGG sequences (GC boxes) present in a variety of cellular and viral promoters (7, 26, 27). A single Sp1 site is sufficient to activate transcription. The binding of Sp1 to DNA is determined by a zinc finger motif present in the C-terminal region of the Sp1 protein (27), while the glutamine-rich domains are essential for the transcriptional activation (15). Our results point to a critical role for the Sp1 binding element localized in the core α_2-integrin promoter region (positions -64 to $+1$) in the transcriptional activity of this promoter and in Erb-B2-mediated downregulation of the α_2-integrin promoter. Moreover, the data show that Erb-B2-mediated downregulation of the α_2-integrin promoter activity proceeds through the ras pathway and can be reversed by a dominant negative v-ras (ras-dn17). Similar results were obtained with the nontumorigenic mammary epithelial cell line MTSV1-7, its clonal derivative HB2, and a breast cancer cell line, T47D. All these cells exhibit the phenotype of the luminal epithelial cell. The data demonstrate that by stimulating v-ras activation in mammary epithelial cells, the Erb-B2 proto-oncogene can downregulate transcription of an adhesion molecule which is crucial for maintenance of the structure of the human mammary gland.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were purchased from GIBCO BRL Life Technologies. The synthesized single-strand oligonucleotides were deprotected and desalted, and complementary strands were denatured at 80°C for 5 min and annealed at room temperature. The double-stranded oligonucleotides were labeled with [^{32}P]dCTP (Amersham, Arlington Heights, Ill.) by using Klenow fragment (Bethesda Research Laboratories, Gaithersburg, Md.) and used as probes in the electrophoretic mobility shift assay (EMSA). Unlabeled double-stranded oligonucleotides were used as competitors. The oligonucleotides used in this study were as follows: a putative AP2-Sp1 site (positions -63 to -40) in the α_2-integrin promoter (59) (5′-GAGGGGCGGGCCGGGGCGGGCCCT-3′, containing a *Hin*dIII linker at the 5′ end and an *Xba*I linker at the 3′ end), the Sp1 binding site in the human immunodeficiency virus (HIV) long terminal repeat (LTR) (51) (5′-GGGAGGCGTGGCCTGGGCGGGACTGGGGAGTG GCGA-3′), an AP2 binding site from the simian virus 40 (SV40) enhancer (52) (5′-GGTGTGGAAGTCCCCAGGCTCCCCAGCAC-3′), and an NF-κB binding site from the interleukin-2 receptor α-chain promoter (47) (5′-GCAGGGG AATCTCCCTC-3′).

Tissue culture. T47D breast tumor cells were cultured in Dulbecco modified Eagle medium (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL), 2 mM glutamine, and 50 μg of gentamicin (Quality Biological, Inc., Gaithersburg, Md.) per ml. The human mammary epithelial cell lines, MTSV1-7 and HB2, derived from epithelial cells cultured from human milk (3, 8, 9), were grown in the same medium supplemented with 5 μg of hydrocortisone (Sigma) and 10 μg of bovine insulin (Sigma) per ml. The ce-1 cell line developed from MTSV1-7 by overexpression of Erb-B2 (16) and HB2 cells, transfected with and overexpressing Erb-B2 or v-ras, were selected and grown in the same medium containing 800 mg of G418 per ml. The transfected clones reported here were assessed for Erb-B2 or v-ras expression by determination of the levels of respective mRNAs by Northern (RNA) blot analysis.

Antibodies and recombinant nuclear proteins. Antibodies to Sp1, Sp3, AP2, or YY1 used in the supershift assay were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Recombinant human Sp1 or AP2 nuclear proteins, a gift from H. Young, were purchased from Promega Corporation, Madison, Wis.

Preparation of nuclear extracts. Nuclear proteins were prepared as described recently (55). Briefly, 5×10^7 to 10×10^7 cells were trypsinized and washed twice in Hanks' solution. The cells were then lysed with 500 μl of lysis buffer (50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.8], 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 20 μg of aprotinin per ml, 100 μM DL-dithiothreitol) and kept on ice for 4 min. Nuclei were pelleted by centrifugation at 14,000 rpm for 1 min, and the supernatants were discarded. The nuclei were washed once with the same buffer without Nonidet P-40, and the nuclear proteins were extracted in 300 μl of extraction buffer (lysis buffer with 500 mM KCl and 10% glycerol). The protein concentrations were determined by using the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.), and the samples were diluted to 1 μg/μl with extraction buffer. The nuclear proteins were stored at −70°C.

EMSA. The DNA-protein binding reaction was conducted as described previously (55). Briefly, DNA-protein binding was carried out in a 24-μl reaction mixture containing 1 μg of poly(dI · dC) (Sigma), 3 μg of nuclear protein extract, 3 μg of bovine serum albumin, and 12 μl of 2× Y buffer (54). Where indicated, an unlabeled double-stranded oligomer was added as a competitor. The mixture was incubated on ice for 10 min. A total of 4,000 cpm of ^{32}P-labeled oligonucleotide probe was added, and the mixture was incubated for an additional 20 min at room temperature and then loaded onto a 5% acrylamide gel (GIBCO BRL) that had been prerun at 110 V for 30 min with 0.5× Tris-borate-EDTA buffer. For supershift assays, the mixture was incubated on ice with antibody for 20 min and then incubated at room temperature with radiolabeled probe for an additional 20 min. The DNA-protein complexes were separated by electrophoresis at 210 V for 90 min. The gels were dried and exposed to X-OMAT film overnight (Eastman Kodak, Rochester, N.Y.).

Plasmid vectors. Three types of reporter gene vectors were used in this study. (i) An α_2-integrin promoter-controlled chloramphenicol acetyltransferase (CAT) reporter was constructed from an HIV-CAT vector (37) by replacement of the HIV LTR with an α_2-integrin promoter fragment from positions -961 to $+55$ at *Xho*I-*Hin*dIII sites, generated by PCR from HeLa cell genomic DNA. Constructs containing -776, -549, -370, -294, and -92 fragments of the α_2-integrin promoter were generated by deleting the 5′ end of the α_2-integrin promoter by using unique restriction enzyme digestion sites *Acc*I, *Bgl*II, *Xma*I, *Nar*I, and *Xma*III, respectively, in combination with *Xho*I. The -64 α_2-integrin CAT vector was generated by inserting a fragment from positions -64 to $+55$ of the α_2-integrin promoter (generated by PCR) at *Xho*I-*Hin*dIII sites into the CAT vector. (ii) The plasmid containing the α_2-integrin Sp1 enhancer in front of the thymidine kinase (TK) promoter-controlled β-galactosidase reporter vector was developed by linking a TK promoter, which was obtained by *Hin*dIII-*Bgl*II digestion of pBL-CAT2 vector (13), to a β-galactosidase gene in the pEQ3 vector (54) at a *Hin*dIII-*Bgl*II site, which was then modified by replacement of the *Sma*I-*Xba*I fragment with a polylinker containing *Sma*I, *Hin*dIII, *Sal*I, and *Xba*I restriction sites. One copy of the α_2-integrin Sp1 element was inserted at the *Hin*dIII-*Xba*I sites. (iii) The -64 α_2-integrin luciferase reporter vector was generated by inserting the $-64/+55$ PCR product of the α_2-integrin promoter in front of the luciferase gene at *Xho*I-*Hin*dIII sites in the pGL2 enhancer vector (Promega), which contains the SV40 enhancer. The mutated -961 or -64 α_2-integrin promoters, with mutation in the α_2 Sp2 site, were generated by PCR using the mutated α_2 Sp1 oligonucleotide as a primer. The amplified fragments were then cloned in the pGL2 vector at the *Xho*I-*Hin*dIII site.

Two internal-control vectors were used to normalize transfection efficiency in the transient-transfection assay, the SV40 promoter-controlled luciferase expression vector pGL2 (Promega) and a cytomegalovirus (CMV) promoter-controlled β-galactosidase expression vector (Clontech Laboratories, Inc., Palo Alto, Calif.).

The expression vectors used in cotransfection assays were an SV40 promoter-controlled Erb-B2 expression vector, as previously described (3), and a CMV promoter-controlled Sp1 expression vector (a generous gift from Robert Tjian, Department of Biochemistry, University of California at Berkeley). The v-Hras (v-ras) expression vectors and the dominant negative ras expression vector were generous gifts from J. deClue and U. Rapp.

Transient-transfection assays. Approximately 5×10^5 cells were plated on a 60-mm-diameter culture dish 1 day before transfection. For transient transfection, 2.5 to 10 μg of the reporter vector was mixed with DEAE-dextran (600 μg/ml) in 1.5 ml of Optimal medium (GIBCO BRL). The plated cells were washed once with Optimal medium, incubated with the mixture of DNA and dextran for 2.5 h at 37°C, and then shocked with 15% glycerol for 30 s. The cells were then washed twice with Hanks' solution and cultured in 3 ml of the culture medium with 10% fetal calf serum. The culture medium was changed 24 h later, and after an additional 24 h, cells were collected and the expression of the reporter gene was assayed. In each assay, 0.5 μg of the control vector was used. When the CAT reporter or the luciferase reporter was used, the CMVβ-galactosidase expression vector was used as an internal control. For the β-galactosidase reporter, SV40-luciferase vector was used as an internal control. The final concentration of transfected DNA was kept constant in all cotransfection assays.

Assay of the reporter gene. The harvested cells were lysed by three freeze-thaw cycles. After centrifugation at 14,000 rpm for 10 min, the supernatant was collected and used for the reporter gene assay. The β-galactosidase, CAT, and luciferase assays used were described previously (54, 55). To compensate for the differences in transfection efficiency, the activities of the reporter genes were normalized to the constant levels of the internal control at each point. The data are mean values from at least three individual experiments.

RESULTS

Suppression of α_2-integrin promoter activity by the Erb-B2 oncogene. Expression of the adhesion molecule α_2 integrin is downregulated in human mammary epithelial cells overexpressing Erb-B2 (16), and this downregulation occurs at the level of gene transcription. In an effort to identify the Erb-B2 response element in the promoter region of the α_2-integrin gene, deletion analysis of the 5′ region of the α_2-integrin promoter region was carried out. The initial analysis was done with a fragment of the 5′-flanking region (−961/+55) which was generated by PCR from HeLa cell genomic DNA. Additional deletions of this promoter fragment (Fig. 1A) were obtained by restriction enzyme digestion or PCR as stated in Materials and Methods. The α_2-integrin promoter (−961/+55 bp) and its deletion mutants were inserted upstream of the CAT reporter, and these α_2-integrin promoter-controlled CAT gene plasmids were cotransfected with or without an Erb-B2 expression vector into the MTSV1-7 and T47D cell lines. The results from transient-transfection assays showed that all of the 5′-deletion constructs of the α_2-integrin promoter tested were expressed in these two cell lines, but with different efficiencies. In the human mammary cell line MTSV1-7 (Fig. 1B) and in the T47D cells (Fig. 1C), the promoter activities of the −961 and −766 α_2 CAT plasmids were higher than the activities of the −549, 370, or −370 α_2-integrin CAT deletion mutants. This indicates that the sequences localized between positions −961 and −549 contribute to the efficient constitutive expression of this promoter, possibly because of the presence of enhancing elements. In contrast, the −294 and −92 α_2-integrin CAT plasmids were expressed about four- to fivefold more efficiently than the −549 and −370 deletion constructs in MTSV1-7 cells, but not in T47D cells. These data indicate that the region between positions −549 and −294 (or −549 and −92) of the α_2-integrin promoter may contain cell-specific silencing elements. The activity of the −64 α_2-integrin CAT plasmid in both cell lines was about three- to fourfold lower than the activity of the −92 α_2-integrin CAT plasmid in MTSV1-7 and T47D cells. It is interesting that all the 5′-deletion mutants of the α_2-integrin promoter tested were downregulated by the overexpression of Erb-B2. The Erb-B2-mediated downregulation could be detected even when only 64 bp of the 5′-flanking region of the α_2-integrin promoter region was inserted upstream of the CAT gene. This suggests that the Erb-B2 response element may be located in the proximal promoter region (designated α_2 core promoter) of the α_2-integrin promoter. This suppression was observed in the two mammary epithelial cell lines used (MTSV1-7 and T47D), indicating that the Erb-B2-mediated downregulation of α_2-integrin promoter activity is a general phenomenon in luminal epithelial cells regardless of whether they are derived from normal or malignant breast tissue.

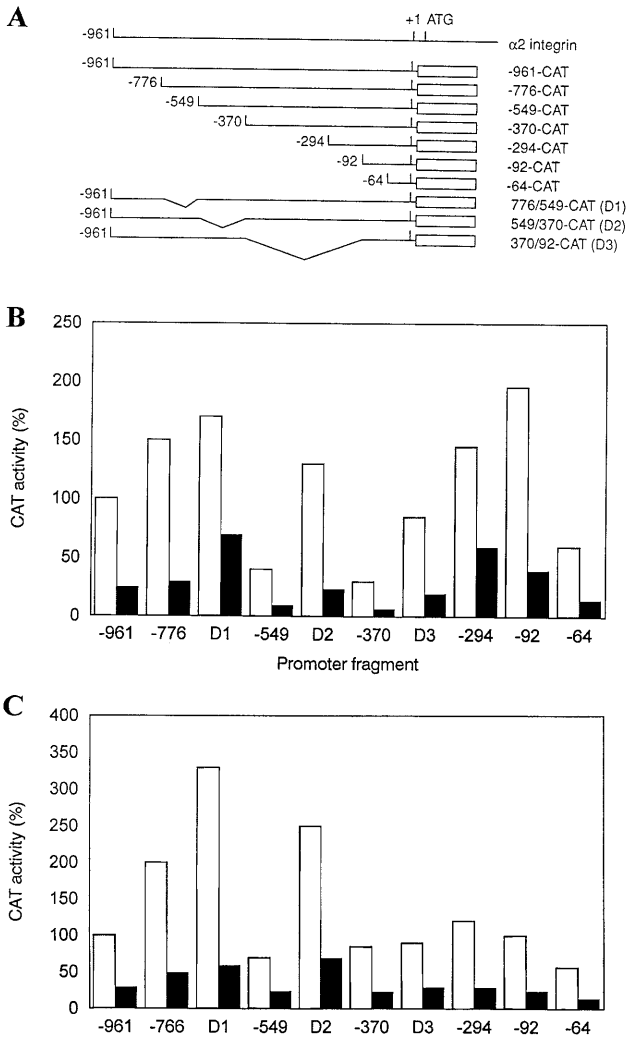

FIG. 1. Suppression of the α_2-integrin promoter by the Erb-B2 oncogene in cotransfection assays. (A) Schematic diagram of 5′ and internal deletions of α_2-integrin promoters controlling the CAT reporter. (B and C) Five micrograms of the indicated α_2-integrin promoter-CAT hybrid plasmid was cotransfected with the Erb-B2 expression plasmid (10 μg) (black bars) or with a SP72 control plasmid (white bars) into MTSV1-7 and T47D cells, respectively. CAT activity was analyzed 48 h after the transfection and normalized to the β-galactosidase internal control as described in Materials and Methods. Each bar represents a mean value of three individual transfections with a standard deviation under 10%.

The Sp1 binding site in the α_2 core promoter. In light of the results from the above mapping study, we directed our attention to the 64-bp proximal region of the α_2-integrin promoter,

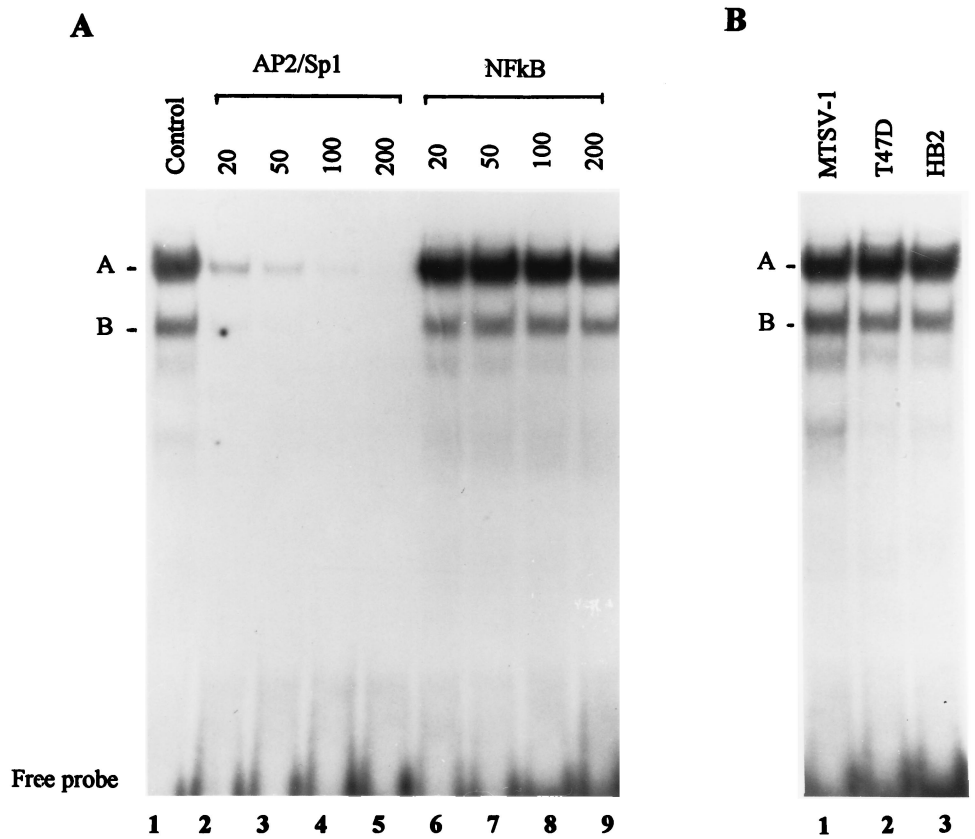

FIG. 2. Binding of nuclear proteins from mammary epithelial cells to the Sp1-AP2-Sp1 site. (A) Nuclear extracts were prepared from HB2 cells and incubated with a radiolabeled Sp1-AP2-Sp1 oligonucleotide probe (see Materials and Methods), and DNA-protein complexes were resolved on 5% polyacrylamide gels. Unlabeled Sp1 or NF-κB oligodeoxynucleotides were used as competitors in the concentrations indicated above the lanes (in nanograms). (B) EMSA analysis of the activities of the nuclear extracts from T47D breast cancer cells or MTSV1-7 and HB2 mammary epithelial cells of binding to the radiolabeled Sp1-AP2-Sp1 oligomer. The positions of complexes A and B are indicated to the left of the gels.

the core promoter. DNA sequence analysis of this region indicated the presence of a putative Sp1-AP2-Sp1 binding sequence in positions −61 to −42 of the core promoter. The oligonucleotide fragment representing this putative binding sequence was used as a radiolabeled probe to analyze the binding of nuclear proteins from the nontumorigenic human mammary epithelial cell lines MTSV1-7 and HB2 as well as proteins from the breast cancer tumor line, T47D, in an EMSA. The results showed the formation of two specific DNA-protein complexes with different mobilities. The formation of these complexes was inhibited in a dose-dependent manner by the unlabeled probe but was not affected by an oligomer corresponding to the NF-κB binding sequence in the interleukin-2 receptor α-chain promoter (Fig. 2A). The abundances of the two complexes were similar in all three cell lines (Fig. 2B).

Since the oligodeoxynucleotide probe used in the EMSA contains overlapping sequences that have homology with consensus Sp1 and AP2 binding sequences, the two complexes detected may be formed by binding of Sp1 and AP2 factors. To identify the binding proteins, we used the authentic Sp1- and AP2-binding oligodeoxynucleotides as competitors in an EMSA and Sp1 and AP2 antibodies in a supershift assay. The results showed that the formation of both complexes was prevented by the authentic, unlabeled Sp1 oligodeoxynucleotide (Fig. 3A, lane 2). In contrast, the oligodeoxynucleotide representing the AP2 binding site did not reduce the formation of either complex (Fig. 3A, lane 3). These results suggest that proteins involved in formation of the two complexes share DNA binding specificity with the nuclear factor Sp1 but not with AP2. Furthermore, results of the supershift analysis with the Sp1-specific antibody showed that complex A was recognized by the Sp1 antibody, while complex B was less affected (Fig. 3A, lane 5), indicating that the proteins present in these two complexes are immunologically distinct. In agreement with the oligodeoxynucleotide competition result, the AP2 antibody and unrelated YY1 antibody did not reduce or supershift either complex (Fig. 3A, lanes 6 and 7), implying that neither AP2 nor an antigenically related protein was present in these complexes. Thus, these data strongly suggest that complex A is formed by binding of the Sp1 nuclear factor and that complex B is formed by binding of a protein that has the same DNA binding specificity as Sp1. The nuclear factor AP2 is not involved in formation of either complex A or complex B.

To further support our conclusions, the binding of recombinant human Sp1 and AP2 proteins to the α_2-integrin Sp1 radiolabeled probe was analyzed by EMSA. The results show that two complexes were formed with the Sp1 protein. The major complex (A in Fig. 3B) had a mobility similar to that of complex A that is formed in the nuclear extract (data not shown). The minor band exhibits a slower mobility (Fig. 3B, lanes 1 to 3). The specificity of complex A was confirmed by incubation with the Sp1 antibody (Fig. 3B, lane 2). Although we did not detect a supershift of the minor complex, we cannot

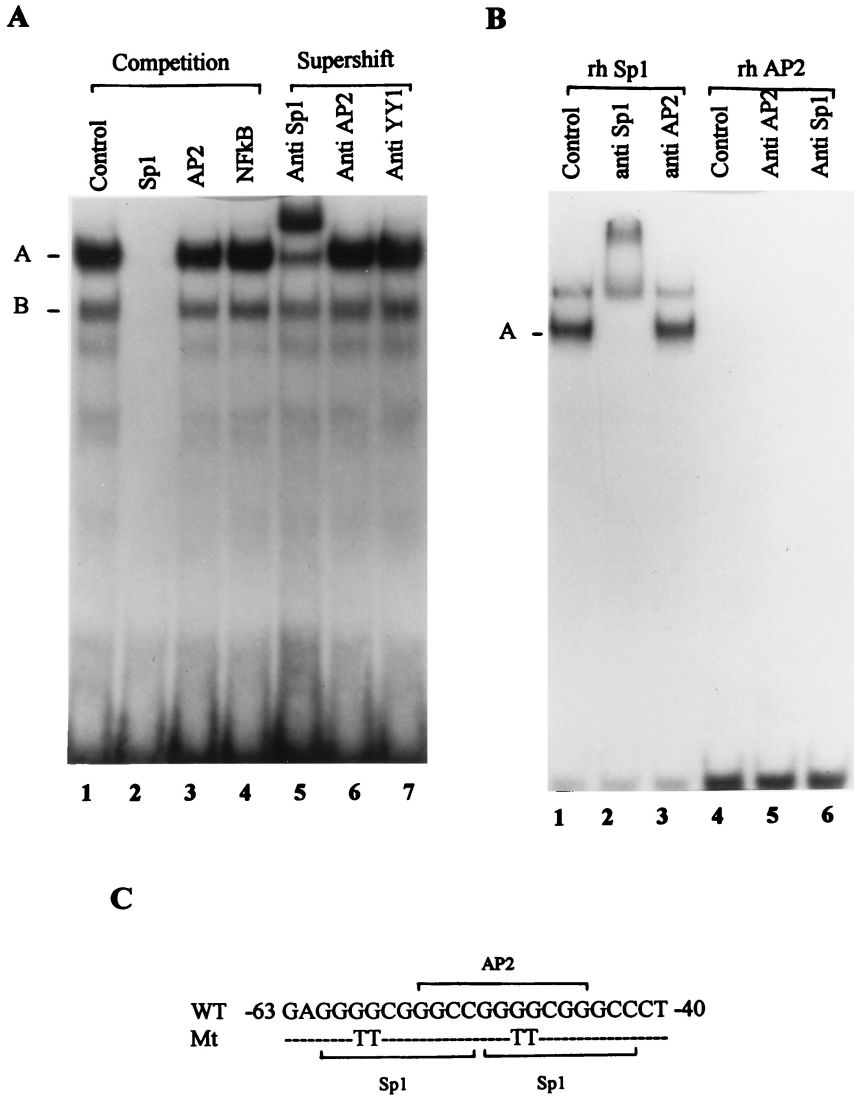

FIG. 3. Sp1, but not AP2, binds to the α_2-integrin Sp1-AP2-Sp1 site. (A) Binding of nuclear extracts from HB2 cells to a radiolabeled Sp1-AP2-Sp1 oligodeoxyribonucleotide probe was analyzed by EMSA as described for Fig. 2. The authentic Sp1, AP2, and NF-κB oligomers (100 ng) were used as competitors. Polyclonal Sp1, AP2, or YY1 antibodies (200 μg) were used in the supershift assay as described in Materials and Methods. (B) Binding of the purified human Sp1 or AP2 protein (5 ng) to the radiolabeled Sp1-AP2-Sp1 oligomer. The Sp1- and AP2-specific antibodies were used to confirm specificity of the complexes. rh, recombinant human. (C) DNA sequences of the wild-type (WT) and mutated (Mt) α_2-integrin Sp1-AP2-Sp1 site.

eliminate the possibility that the band detected in the position of the minor complex (lane 2) may represent a supershifted complex A and that the uppermost band represents supershifted minor complex (compare with Fig. 3A, lanes 1 and 5). The AP2 protein that forms an AP2 complex with the authentic AP2 oligodeoxynucleotide (data not shown) was not able to bind the α_2-integrin Sp1 oligodeoxynucleotide probe (Fig. 3B, lanes 4 to 6). On the basis of these results, we conclude that the putative Sp1-AP2-Sp1 site in the α_2-integrin promoter is a Sp1 binding site. There is no experimental evidence that this sequence can also bind to the AP2 protein. Thus, we designated this promoter element the α_2-integrin Sp1 (α_2 Sp1) site in the following study.

Binding of Sp3 to the α_2-integrin Sp1 binding site. The above results indicate that complex A, identified in an EMSA with nuclear extracts from human mammary epithelial cells, is formed by binding of the Sp1 protein, while the nature of complex B remains unclear. The formation of both complexes A and B could be inhibited by the Sp1 oligodeoxynucleotide (Fig. 3A, lane 2) and abrogated by mutation of the Sp1 binding sequence in the α_2 Sp1 probe (Fig. 3C and 4A, lane 2), indicating that the two complexes show similar DNA binding specificities. Interestingly, the formation of complex B was much weaker when the Sp1 site present in the HIV type 1 (HIV-1) LTR was used as a probe (Fig. 4B, lane 1), suggesting that the α_2-integrin Sp1 site has a higher affinity for the protein present in complex B. Since the Sp1 family has three additional members that share DNA binding specificity with Sp1 (24, 30), our data suggest that complex B may be formed by another member of the Sp1 family (30). Sp3 has the same cell distribution as Sp1 (30). It has been reported previously that the HIV Sp1 site binds Sp3 and forms a DNA-protein complex that migrates faster than the Sp1 complex (35). Since the complex B formed with the α_2-integrin Sp1 probe comigrated with the fast-moving complex formed with the HIV Sp1 probe (Fig. 4B), we assumed that the unknown protein in complex B may be nu-

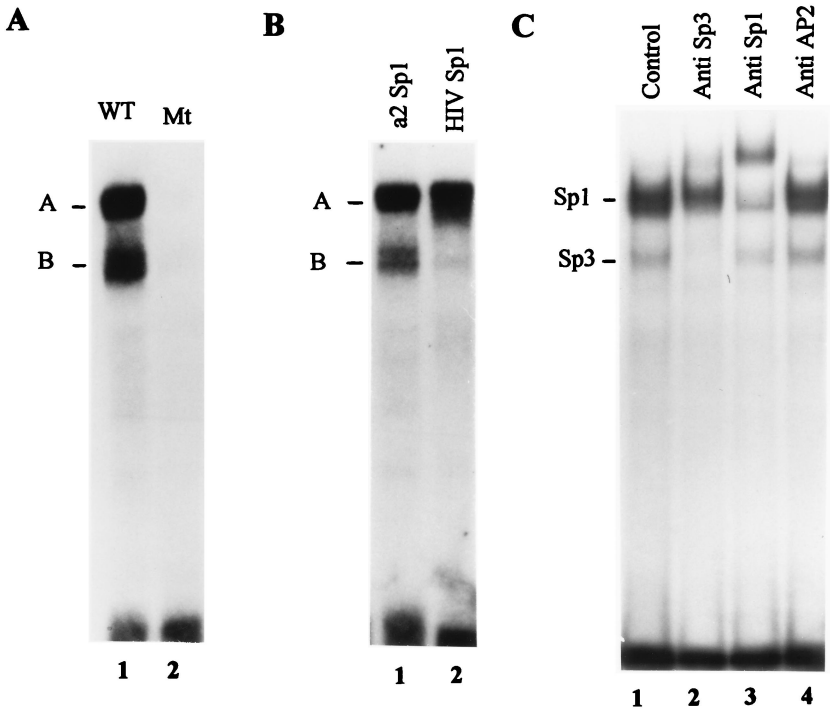

FIG. 4. Binding of Sp3 to the α_2-integrin Sp1-AP2-Sp1 site (α_2 Sp1 site). (A) Binding of nuclear extracts from HB2 cells to a radiolabeled α_2 Sp1 probe (WT) and its mutant (Mt) analyzed by EMSA. (B) Comparison of the binding of nuclear extracts from HB2 cells to a radiolabeled α_2 Sp1 oligomer and to Sp1 oligomer corresponding to the binding site in the HIV-1 LTR. (C) Identification of proteins present in complexes A and B with Sp3-, Sp1-, and AP2-specific antibodies (200 μg).

clear factor Sp3. To test this hypothesis, we used the anti-Sp3 antibody in a supershift assay and showed that the complex B was specifically and completely removed by the Sp3 antibody (Fig. 4C, lane 2). Thus, on the basis of DNA binding specificity and antigenicity, we conclude that the unknown protein in complex B is the nuclear factor Sp3. However, the possibility that this protein is not an authentic Sp3 but an antigenically related protein with the same DNA binding specificity cannot be completely excluded.

The Sp1 binding site is required for the activity of the α_2-integrin promoter. The α_2-integrin promoter is a TATA-less promoter (59). In such promoters, a proximal Sp1 binding site can act as a transcription initiator (36, 43). In deletion analysis of the α_2-integrin promoter, we observed that the −64 promoter fragment still retained transcriptional activity, although at a reduced level, indicating activity of the α_2-integrin Sp1 site. To determine whether the Sp1 binding site plays a role in the activity of the α_2-integrin promoter, we introduced a mutation (Fig. 3C) which abrogates the Sp1 binding activity (Fig. 4A, lane 2) into the α_2 Sp1 site in the context of the −961 and −64 promoter fragments (Fig. 1A). The mutant promoters, as well as the wild-type promoters, were linked to a luciferase reporter gene. The activities of the α_2-integrin promoter luciferase reporter vectors were examined by transient gene transfection assays with HB2 cells. The results showed that the wild-type −961 or −64 promoter was transcriptionally active, and the promoter activity was inhibited by overexpressed Erb-B2 (Fig. 5A, WT). This is consistent with the results obtained by using the CAT reporter vectors (Fig. 1B). In contrast, the mutation in the α_2-integrin Sp1 site which abrogated binding activity abolished the transcriptional activity of both the −961 and the −64 α_2-integrin promoters (Fig. 5A, Mt). Similar results were obtained in a transfection assay with T47D cells (data not shown). These results indicate that the Sp1 binding activity at the α_2-integrin Sp1 site is required for the transcriptional activity of the α_2-integrin promoter. Since the α_2-integrin Sp1 site is very close to the transcription initiation site (about 40 bp away), it may act as a transcriptional initiator in the TATA-less α_2-integrin gene promoter.

To further confirm that the activity of the core promoter depends on the Sp1 activity, we cotransfected an Sp1 expression vector with the −64 α_2-integrin luciferase vector into the HB2 mammary cell line. The results showed that overexpressed Sp1 significantly increased α_2-integrin core promoter activity in HB2 cells (Fig. 5B), while it did not affect the activity of a control SV40 promoter (Fig. 5B). Similar results were obtained with T47D cells (not shown). However, when the α_2-integrin Sp1 sequence from the α_2-integrin promoter was linked to the basic TK promoter in a β-galactosidase vector, the basal level of the TK promoter was increased about threefold, and the overexpression of Sp1 further increased the transcriptional activity of this promoter (Fig. 5C). In contrast, when the mutated α_2-integrin Sp1 site was linked to the TK promoter, no increase in transcriptional activity was observed. These data strongly suggest that the Sp1 binding site is required for the activity of the α_2-integrin core promoter.

The Sp1 binding site is responsible for Erb-B2 suppression of the α_2-integrin promoter. The result of the 5′-deletion study of the α_2-integrin promoter using a CAT reporter vector shows that even the core promoter (position −64) responds to Erb-B2 downregulation. By using −64 α_2-integrin promoter-controlled luciferase gene expression (luciferase was used because of the increased sensitivity of the assay), it was possible to show that the activity of the −64 α_2-integrin core promoter was inhibited by overexpression of Erb-B2 in a dose-dependent fashion (Fig. 6A). To test whether the Sp1 site is responsible for this downregulation, we examined the activity of the Sp1–TK–β-galactosidase hybrid plasmid in a cotransfection assay

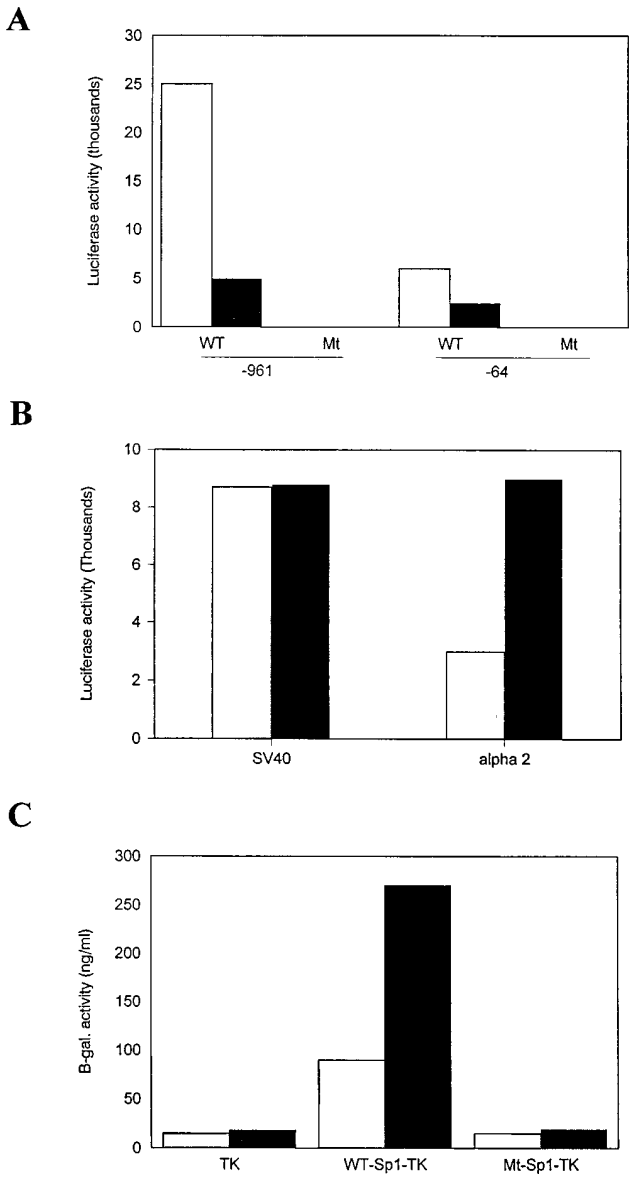

FIG. 5. Sp1 binding activity is required for the transcriptional activity of the α_2-integrin promoter. (A) Mutant (Mt) derivatives of the α_2-integrin -961 and -64 promoters, in which Sp1 binding activity was abrogated (as shown in Fig. 3C), were linked to the luciferase gene as described in Materials and Methods. The activities of the wild-type (WT) and mutant promoters were examined by a cotransfection assay with the control vector (pSP72) (white bars) or Erb-B2 overexpression vector (black bars) in HB2 cells. (B) The activity of the -64 α_2-integrin promoter was used to examine the effect of Sp1 overexpression. The reporter was cotransfected into HB2 cells with pSP72 vector (white bars) or the Sp1 expression vector (black bars). The ratio of the DNA for the reporter vector and the Sp1 expression vector or control vector pSp72 was 1:2. An SV40 early promoter-controlled luciferase vector was employed as a control. The activity shown for the SV40 promoter represents only 10% of the observed activity. (C) One copy of the wild-type (WT-Sp1-TK) or mutant (Mt-Sp1-TK) α_2 Sp1 oligodeoxyribonucleotide was inserted into a TK promoter–β-galactosidase hybrid vector as described in Materials and Methods. The activity of the TK promoter was examined in the absence (white bars) or presence (black bars) of the Sp1 expression vector in HB2 cells. In this experiment, the ratio of reporter to Sp1 expression vector used was 1:2. The reporter activity shown has been normalized to the internal control. Each bar represents a mean value of four individual transfections.

with the Erb-B2 expression vector. The results (Fig. 6B) show that while the activity of the TK promoter alone was not affected by Erb-B2 overexpression, the insertion of the α_2-integrin Sp1 site element conferred responsiveness to Erb-B2 and suppressed β-galactosidase gene expression. In contrast, the vector containing the mutated Sp1 element that had lost Sp1 binding activity did not respond to the Erb-B2 downregulation (Fig. 6B). These data suggest that the Sp1 site is an Erb-B2 response element in the α_2-integrin promoter.

Erb-B2 overexpression reduces DNA binding activity of Sp1 but not Sp3 in mammary epithelial cells. In the study described above, we identified Sp1 and Sp3 as factors binding to the α_2-integrin Sp1 site. Since Sp1 is a transcription activator and Sp3 can act as a transcription repressor (23), the outcome of the transactivation activity of the Sp1 element may depend on a relative ratio between these two factors. To be able to analyze the DNA binding activity of Sp1 and Sp3 under conditions in which Erb-B2 is overexpressed from an endogenous gene, we examined the DNA binding activity of these two nuclear factors in MTSV1-7 and HB2 cell lines that were permanently transfected with an Erb-B2-expressing vector. The MTSV1-7 cell line, ce-1, has previously been described (16). The Erb-B2-transfected HB2 cell line was developed by the transfection of HB2 cells with an Erb-B2 expression vector (16) which also contained a neomycin resistance gene. The transfected clones were selected by growth in G418, and the expression of Erb-B2 was determined by Northern blot analysis. The clone expressing the highest levels of Erb-B2 mRNA

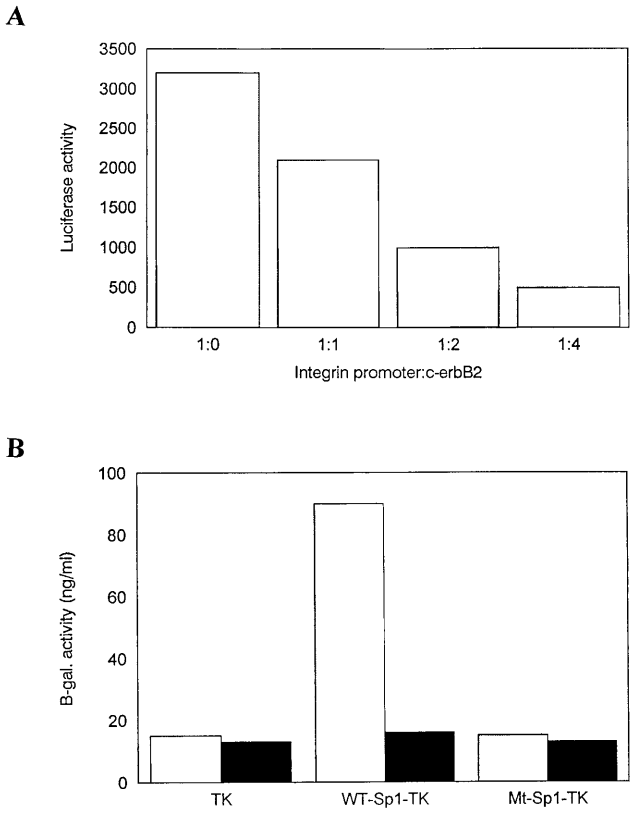

FIG. 6. The Sp1 site is critical for Erb-B2-mediated suppression. (A) The -64 α_2-integrin promoter luciferase gene construct (5 μg) was used in a cotransfection assay with different amounts of the Erb-B2 expression vector in HB2 cells. The pSP72 vector was used to keep the total amount of transfected DNA constant in each transfection. The ratios between reporter and Erb-B2 expression vector are indicated. (B) The TK–β-galactosidase vectors (5 μg) containing the α_2 Sp1 (WT-Sp1-TK) elements were cotransfected with 10 μg of the Erb-B2 expression vector (black bars) or pSP72 control vector (white bars) into HB2 cells. Each bar represents a mean value of three independent transfections. Activity of the reporter gene was normalized to the internal control.

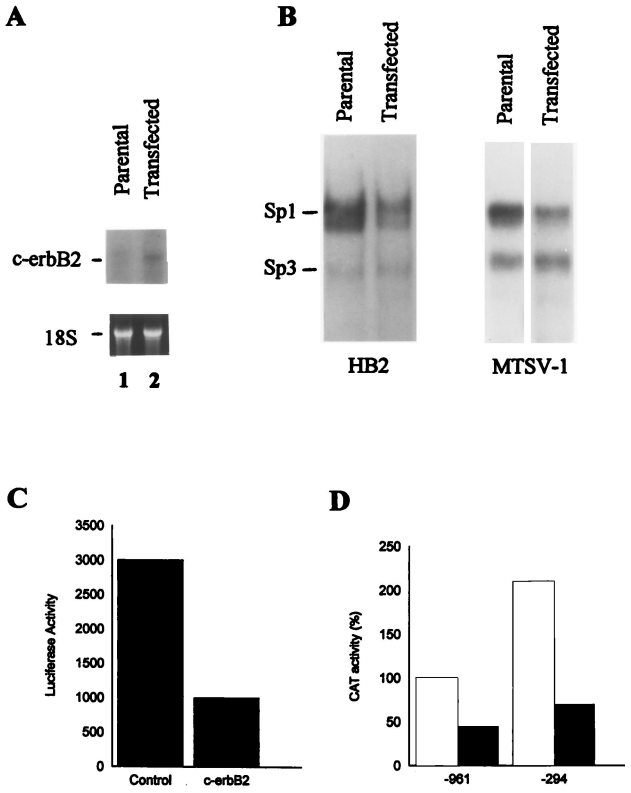

FIG. 7. Downregulation of the α_2-integrin promoter activity in Erb-B2-overexpressing cells is associated with reduced Sp1 DNA binding activity. (A) Northern blot analysis of an Erb-B2 mRNA in HB2 cells permanently transfected with the Erb-B2 expression plasmid. (B) Sp1 DNA binding activity was reduced in two Erb-B2-overexpressing cell lines. The activity of nuclear extracts from Erb-B2-overexpressing cell lines (transfected HB2 and MTSV1-7 cells) and from parental cells of binding to the α_2 Sp1 probe is shown. (C) The -64 α_2-integrin promoter luciferase vector was transfected into parental (Control) and Erb-B2-overexpressing (c-erbB2) HB2 cells. (D) Two α_2-integrin promoter (bp -961 and -294)-CAT hybrid plasmids (see Fig. 1) were transfected into parental (MTSV1-7) (white bars) and Erb-B2-expressing (black bars) MTSV1-7 cells. In panels C and D, the mean values of three independent experiments, normalized to the internal control, are given.

(Fig. 7A) was selected for further studies (HB2-Erb-B2). Nuclear extracts from Erb-B2-expressing cells were then analyzed for activities of binding of Sp1 and Sp3 to the α_2-integrin Sp1 probe (Fig. 7B). The results of the EMSA show that nuclear extract from the HB2-Erb-B2 cells exhibited a lower Sp1 binding activity than the parental HB2 cells, while the binding of Sp3 was identical in the parental and Erb-B2-expressing cells (Fig. 7B, left panel). A similar difference in the binding was obtained when nuclear extracts from the previously established ce-1 cells and the parental line, MTSV1-7, were compared. Transcriptional activity of the α_2-integrin promoter was also analyzed in these two Erb-B2-overexpressing cell lines. In correlation with the change in the Sp1 binding activity, the expression of the α_2-integrin promoter (-64 α_2 luciferase vector) showed a lower transcriptional activity in the HB2-Erb-B2 cell line than in the parental HB2 cells (Fig. 7C). The downregulation of the α_2-integrin promoter was not unique to this cell clone, since the downregulation of -961 and -294 α_2 promoters was also seen in Erb-B2-overexpressing MTSV1-7 cells (Fig. 7D).

Reversion of Erb-B2 suppression by overexpressed Sp1. We reasoned that if the loss of DNA binding activity of Sp1 is a mechanism by which Erb-B2 inhibits α_2-integrin transcription, then overexpression of Sp1 protein may reverse the Erb-B2-mediated suppression. The HB2 cells were therefore cotransfected with a plasmid containing the -64 α_2-integrin core promoter controlling the luciferase gene with a constant amount of the Erb-B2-expressing vector and a variable amount of the Sp1 expression vector. The results show that Erb-B2 suppressing activity was overcome by transfection with Sp1 when the ratio of Erb-B2- to Sp1-transfected plasmids was 1:2, and the levels of expression were markedly increased when the Erb-B2 and Sp1 ratio was 1:4 (Fig. 8). These results further support the model that in cells overexpressing Erb-B2, suppression of α_2-integrin gene expression is caused by a reduction in Sp1 activity.

The downregulation of α_2-integrin gene expression by the Erb-B2 oncogene is mediated by the ras-raf pathway. The Erb-B family of receptors contain a tyrosine phosphorylation site that can bind to Grb2, which then binds to SOS and activates the ras pathway (reviewed in references 18 and 53). To determine whether the Erb-B2-mediated downregulation of α_2-integrin promoter activity proceeds through the ras pathway, we have examined the effect of active ras (v-ras) on the activity of the α_2-integrin promoter in a transient-transfection assay. The human mammary epithelial cell line MTSV1-7 was cotransfected with α_2-integrin CAT vector or its 5′-deletion mutants together with the vectors overexpressing the v-ras gene, and the levels of CAT activity were measured at 48 h posttransfection. The results (Fig. 9A) showed that overexpression of v-ras downregulates the transcriptional activity of the α_2-integrin promoter to the same extent as overexpression of Erb-B2 (threefold). The same effect was seen in T47D cells (data not shown). Furthermore, suppression by activated v-ras required the same *cis* elements in the α_2-integrin promoter as the suppression by Erb-B2. The expression of all the deletion mutants of the α_2-integrin promoter was suppressed as efficiently by v-ras as by Erb-B2.

To exclude the possibility that the Erb-B2- and v-ras-mediated effects on the α_2-integrin promoter proceed through two different pathways, we examined whether the overexpression of a dominant negative mutant v-ras N17 (dnRas) abolished the effect of Erb-B2. The results of the transient-cotransfection assay showed that the expression of the -64 α_2-integrin luciferase vector could be fully restored in cells cotransfected with Erb-B2 in the presence of dnRas (Fig. 9B). The expression of dnRas also enhanced the α_2-integrin promoter activity in the

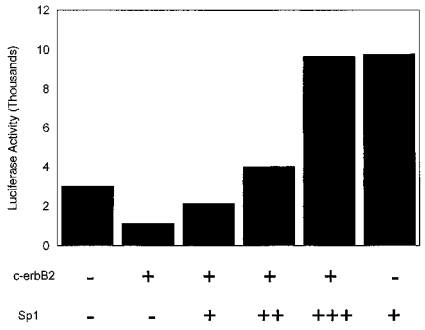

FIG. 8. Reversion of Erb-B2 suppression activity by Sp1. The -64 α_2-integrin promoter-controlled luciferase vector (2 μg) was transfected into HB2 cells with 4 μg of the Erb-B2 expression vector in the presence of different amounts of the Sp1 expression vector. Each plus sign represents 4 μg of plasmid DNA. The pSP72 vector was used to keep the total amount of DNA constant. Luciferase activity was determined and normalized to the internal control as stated in Materials and Methods. The means of three independent experiments are shown.

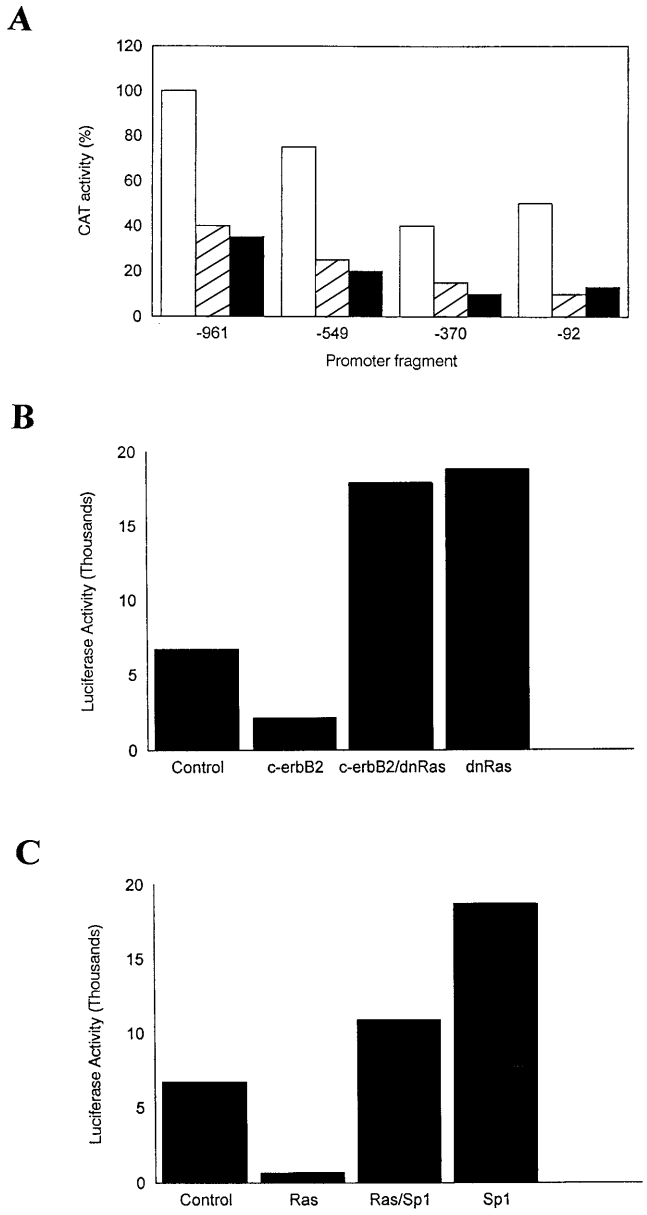

FIG. 9. Erb-B2 signaling proceeds through the ras pathway. (A) The α_2-integrin promoter-controlled CAT vectors (5 μg) were cotransfected into T47D cells with 10 μg of the pSP 72 control vector (white bars), the Erb-B2 expression vector (hatched bars), or the v-ras expression vector (black bars). (B) Reversion of Erb-B2 suppressive activity by a dominant negative ras (dnRas). The −64 α_2-integrin promoter-controlled luciferase vector (5 μg) was cotransfected either with the pSP72 vector (control) or with the Erb-B2 expression vector (c-erbB2; 10 μg) with or without the dominant negative v-ras (dnRas) expression vector (10 μg) into HB2 cells. Luciferase activity was determined as stated in Materials and Methods. (C) Reversion of ras suppressive activity by Sp1. The −64 α_2-integrin luciferase vector used in panel B was cotransfected either with SP72 (control) or with ras and Sp1 expression vectors or with a combination of both of these expression vectors (Ras/Sp1). Each bar represents a mean value of three independent transfections, and the activity shown was normalized to the internal control.

control cells, presumably by downregulation of the endogenous v-ras levels in these cells. The above results strongly suggest that the overexpressed Erb-B2 oncogene utilizes the v-ras transduction pathway to alter the activity of the Sp1 transcription factor and consequently downregulates (sixfold) the activity of the α_2-integrin promoter region. Accordingly, the downregulation of expression of the −64 α_2-integrin promoter by v-ras could be completely reversed by cotransfection with Sp1 (Fig. 9C). Thus, overexpression of Sp1 can reverse both the Erb-B2- and the v-ras-mediated downregulation of the α_2-integrin core promoter. The observed downregulation of the α_2-integrin promoter activity by v-ras could also be observed in the HB2 cell line permanently transduced by v-ras (Fig. 10A). Expression of the transfected −64 α_2-integrin luciferase vector was substantially reduced in cells overexpressing v-ras (3-fold) compared with that in the parental cell line. The downregulation of α_2-integrin promoter activity correlated with the results of the EMSA analysis. The binding properties of nuclear extracts from HB2 v-ras-transfected cells, constitutively expressing v-ras, showed a decreased binding of Sp1 to the α_2-integrin Sp1 probe compared with that of the parental line (Fig. 10B).

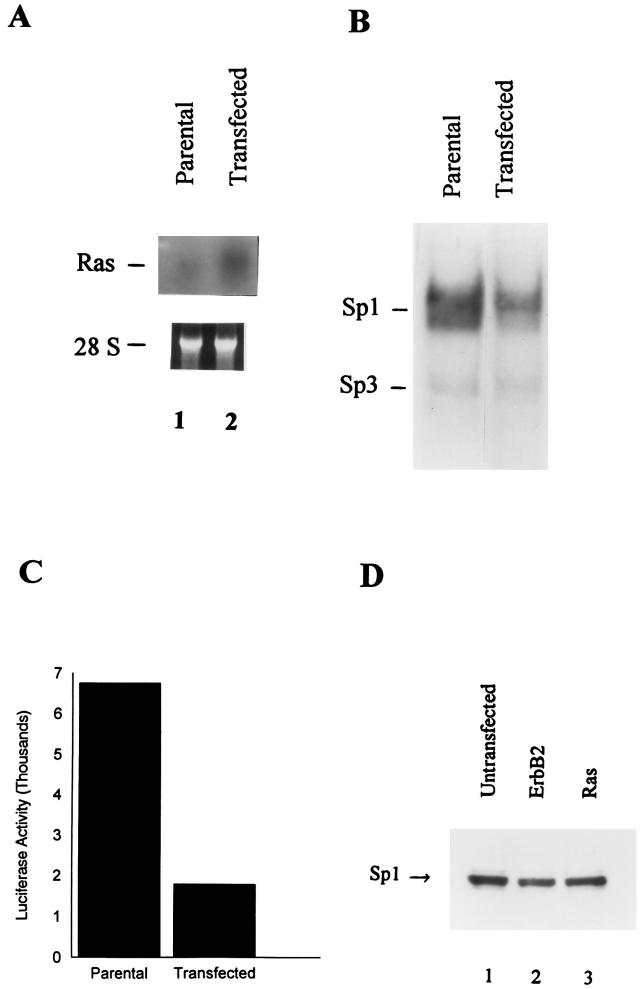

FIG. 10. Suppressive activity of ras is associated with a reduced Sp1 DNA binding activity. (A) Northern blot analysis of ras mRNA in ras-transfected HB2 cell line and parental HB2 cells. (B) Binding of nuclear extracts from the parental HB2 and HB2 transfected cells to the α_2 Sp1 probe analyzed by the EMSA. (C) The transcriptional activity of the −64 α_2-integrin promoter is reduced in v-ras-transfected HB2 cells. The α_2-integrin −64 promoter-controlled luciferase gene was transfected into HB2 or HB2 v-ras-expressing cells (transfected), and luciferase activity was determined and normalized to the internal control as stated in Materials and Methods. Mean values of three independent transfections are given. (D) Relative levels of Sp1. Nuclear proteins (10 μg) from the parental HB2 cells (lane 1) and Erb-B2-transfected (lane 2) and v-ras-transfected (lane 3) HB2 cells were separated on a sodium dodecyl sulfate gel and detected by Western blot hybridization with Sp1 antibody.

However, no changes in the binding of Sp3 between parental and v-ras-overexpressing cell lines were observed. This binding pattern is nearly identical to that seen in Erb-B2-expressing HB2 cells. To examine whether the decrease in Sp1 binding is a result of lower Sp1 levels in v-ras (and Erb-B2)-overexpressing cells, the relative levels of Sp1 in parental and transfected cells were determined by Western blot (immunoblot) analysis with Sp1-specific antibodies. The results do not show any changes in the abundance of Sp1 protein in the transfected cells and thus indicate that the reduced DNA binding of Sp1 to the α_2-integrin Sp1 site in transfected cells is not due to the decrease of total levels of Sp1 protein.

DISCUSSION

A downregulation of expression of α_2-integrin and E-cadherin in human mammary epithelial cells overexpressing Erb-B2 has previously been demonstrated, and it has been shown that this downregulation occurs at the transcriptional level (16, 17). The present study has focused on the identification of the *cis* and *trans* elements of the α_2-integrin promoter that confer responsiveness to Erb-B2. We have shown that a single Sp1 binding element located within −61 to −44 bp upstream of the α_2-integrin mRNA start site (59) is sufficient to respond to Erb-B2-mediated downregulation and that the transcription factor Sp1 is an important component of this response.

The expression of the α_2-integrin gene displays both cell and differentiation specificity (9, 58), and a 961-bp fragment of the 5′-flanking region can confer cell-type-specific expression (59). The promoter of the α_2-integrin gene does not contain a TATA box but does contain five putative Sp1 and six AP2 sites in the first 311 bp of the 5′-flanking region, while the more distant region (800 bp) contains two estrogen receptor binding sites and two AP2 binding sites as well as a GPu box. The Erb-B2-responsive Sp1 binding element at positions −61 to −44 has the potential to bind members of the Sp1 family (23, 30) and the AP2 transcription factor (52). However, our in vitro analysis showed that this sequence does not bind AP2 but does bind two members of the Sp1 family of transcription factors, namely, Sp1 and Sp3. AP2 has been shown to be involved in the protein kinase C and cyclic AMP signaling pathway (25) and involved in the regulation of the Erb-B2 gene itself in mammary epithelial cells (10), but it does not appear to play a role in the downregulation of expression of the α_2-integrin subunit induced by Erb-B2.

The Sp1 transcription factor activates transcription by association with one of the coactivators associated with the TATA-binding protein (TBP) in the TFIID complex. Interaction between glutamine-rich activation domains of Sp1 and the TBP-associated factor dATF is an important component of the Sp1 transactivation (19). Since the TFIID complex and TBP-associated cofactors also play an essential role in the activation of TATA-less promoters, the mechanism by which Sp1 activates these two types of promoters may be based on common features (43). In a majority of the promoters containing Sp1 binding elements, Sp1 appears to provide a basal level of transcription, but, when acting in conjunction with other transcriptional activators or regulatory proteins, small changes in Sp1 activity can cause a significant alteration in gene expression. Sp1 plays a critical role in cytokine-stimulated expression of the vascular cell adhesion molecule gene (39). The myeloid-cell-specific expression of the CD11c gene was shown to be due to the binding of Sp1 to the CD11c promoter region and its interaction with Ap1 (40). In contrast, the Sp3 factor was found to suppress Sp1-mediated transcriptional activation by competitively binding to Sp1 consensus elements (23, 35), as well as to stimulate transcription by functional interaction with the retinoblastoma protein (48).

Our data suggest that the Erb-B2-mediated downregulation of the α_2-integrin promoter is a result of a lower activity of binding of Sp1 to the α_2-integrin Sp1 site. Mammary epithelial cells overexpressing Erb-B2 have exhibited decreases in both DNA binding activity of the Sp1 factor and transcriptional activity of the α_2-integrin promoter. The intensity of Sp3 binding to the α_2-integrin Sp1 site was not altered in Erb-B2-overexpressing cells. Although we have not determined the nature of the Erb-B2-mediated alteration of Sp1 that decreases its DNA binding capacity, we assume that the binding of Sp3 to the α_2-integrin Sp1 site may also contribute to the downregulation of the transcriptional activity of the α_2-integrin promoter in Erb-B2-overexpressing cells. Sp3 has been reported to function as a transcriptional repressor by competing with Sp1 binding (23, 35), and thus the transcriptional activity of the α_2-integrin Sp1 site may be determined by a balance between binding of the activator, Sp1, and the repressor, Sp3. Further experiments are required to verify this hypothesis. However, it is also likely that in Erb-B2-overexpressing cells, the interaction of Sp1 with the components of the TBP complex or its association with the other enhancer proteins (12, 36) may be impaired and contribute to the downregulation of α_2-integrin promoter activity.

Two features of the Sp1-mediated downregulation of the α_2-integrin promoter are of interest. The first is the inhibition of this downregulation by the overexpression of Sp1 protein. This observation suggests that in Erb-B2-overexpressing cells, Sp1 may be bound to a negative inhibitor that affects its transcriptional activity, such as Sp1-I, by reducing its DNA binding capacity (12). Reversion of the inhibition would then occur because the inhibitor is eliminated by competition with an excess of Sp1. The second interesting feature of the Erb-B2-mediated downregulation through Sp1 is its specificity for the α_2-integrin Sp1 site in the α_2-integrin promoter. This could be partially explained by the fact that the Sp1 site close to the transcription initiation site plays a critical role in the transcriptional activity of the TATA-less promoter, such as the α_2-integrin promoter, while the Sp1 site present in promoters containing the TATA box, such as the TK promoter, functions as a true enhancer and does not directly participate in transcription initiation (43). However, the fact that insertion of the α_2-integrin Sp1 site upstream of the TK promoter conferred the sensitivity of this promoter region to the Erb-B2 effect indicates that the α_2-integrin Sp1 site may be recognized by members of the Sp1 family different from those that recognize the other Sp1 sites. The higher affinity of the Sp3 (or Sp3-related) factor for binding to the α_2-integrin Sp1 site than to the HIV-1 LTR Sp1 site may indicate that a unique feature of the α_2-integrin Sp1 site is its high degree of affinity for Sp3.

The exact mechanism by which overexpressed Erb-B2 contributes to the more aggressive phenotype of breast carcinoma is not known. Overexpression of Erb-B2 results in a high level of autophosphorylation and induction of signaling pathways, involving both c-src and ras (18, 38, 44), that lead to activation of mitogen-activated protein kinase and stress-activated protein kinase and to induction of c-fos expression (21). Our results have shown that activated v-ras downregulates the expression of the α_2-integrin promoter, suggesting that the Erb-B2-mediated inhibition of α_2-integrin transcription proceeds through the ras-raf pathway. The finding that the dominant negative mutant of ras (dn17) reverses the Erb-B2 effect further indicates that Erb-B2 signaling proceeds through the ras pathway. It has been recently shown that the ErbB family receptors form heterodimers between Erb-B2 and either Erb-

B1, Erb-B3, or Erb-B4 (21, 28). Thus, although no ligand for Erb-B2 has been identified, the Erb-B2 receptor, when overexpressed, may augment binding of EGF and heregulin ligands to Erb-B1 and Erb-B3 or Erb-B4, respectively. While the early events in signaling from the Erb-B2 receptor are being clarified, the genes which are regulated by Erb-B2 signaling in human mammary epithelial cells have been less well characterized. In addition to the decreased expression of α_2 integrin, we have observed downregulation of the E-cadherin gene (unpublished results) and the epithelial-cell-specific MUC1 promoter (31). Upregulation of expression of intracellular adhesion molecule 1 (2) by heregulin has also been observed, and increased transcription of urokinase by Erb-B2 overexpression was seen in lung cancer cells (22). While it is not yet known whether all of these genes are regulated by the ras-induced pathway, this pathway clearly affects the expression of the α_2-integrin gene. Although the activation of the ras pathway generally results in the activation of gene expression, there are at least two examples that the activated ras-raf pathway also leads to silencing of genes. In medullar thyroid carcinoma cells, activated Raf-1 signaling silences expression of the Ret gene and induces differentiation (11). Also, transformation of fibroblast cells by activated ras results in suppression of the smooth-muscle α-actin promoter (33). The molecular mechanisms of these suppressive effects are unknown.

In this study, we have identified an element in the α_2-integrin promoter which responds to Erb-B2-generated signals operating through the ras pathway in human mammary epithelial cells. The $\alpha_2\beta_1$ integrin is a major integrin expressed at the basolateral surface of the luminal epithelial cells from which breast cancer develops and undoubtedly is a central component in maintaining the integrity of the epithelial layer (1). It is highly likely that a reduction in the level or function of the integrins is required for the loosening of the cell matrix and the cell-cell contacts necessary for cell division and morphogenesis. Thus, the downregulation of expression of the α_2-integrin gene in response to growth factor signaling through the ras pathway may represent a reversible event in dividing cells undergoing morphogenesis. In breast cancer, the ras pathway may be constitutively activated (38), and the downregulation of the expression of the α_2-integrin gene could become irreversible (20). This would result in or contribute to the disruption of tissue architecture seen in primary breast cancers (1).

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J.Y. and R.H.X. contributed equally to this project.

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