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Characterization of the Promoter for the α3 Integrin Gene in Various Tumor Cell Lines: Roles of the Ets- and Sp-Family of Transcription Factors

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Abstract The α3β1 integrin is an adhesion receptor for extracellular matrix proteins, including laminin isoforms, and plays crucial roles in the organization of epithelial and endothelial tissues. The aberrant expression of this adhesion molecule on tumor cells is associated with their invasive and metastatic potentials. In the present study, we analyzed the elements essential for α3 integrin gene expression in various tumor cell lines with different tissue origins by luciferase assay. An approximately 0.3 kb fragment of the 5′-flanking region of the mouse α3 integrin gene (−260/+84, relative to the major transcription start site) showed strong promoter activity in all six examined tumor cell lines. However, we found that these cell lines could be divided into two groups according to the level of dependency on the putative Ets-transcription factor binding motif located at −133. This motif was previously shown to be crucial for α3 integrin expression in MKN1 gastric carcinoma cells. The gene expression in one group of cell lines was upregulated mainly by the Ets motif, whereas that in the other group was less dependent on the Ets motif. We then postulated that additional regulatory elements were responsible for the expression of α3 integrin, and found that a GC-rich motif at −69 was another important element. An electrophoretic mobility shift assay using specific antibodies and a Western blot analysis of nuclear proteins revealed that the Sp3-transcription factor bound to this GC-rich motif. These results suggest that the Sp3 and Ets transcription factors cooperatively regulate α3 integrin gene expression and that the contribution of each element depends on the type of tumor cells. J. Cell. Biochem. 97: 530–543, 2006.

Key words: integrin; gene promoter; luciferase assay; Ets-transcription factor; Sp3

Integrins are a family of cell adhesion molecules that consist of non-covalently associated heterodimers (α and β subunits) and that mediate cell-extracellular matrix (ECM) and cell-cell interactions. The α3β1 integrin (VLA-3), a member of the β1 integrin subfamily, has been identified as a high affinity receptor for laminin isoforms, including laminin-5 and laminin-10/11 [Carter et al., 1991; Kikkawa et al., 1998]. This adhesion molecule also serves as a receptor for a variety of ECM proteins, such as fibronectin, collagen, laminin-1 (a prototype of laminin), and thrombospondin-1 [Guo et al., 2000; Kreidberg, 2000; Giannelli et al., 2002; Tsuji, 2004]. Moreover, intercellular adhesions mediated through homotypic interaction of the α3β1 integrin or heterotypic interaction between the α3β1 and α2β1 integrins have been reported [Sriramarao et al., 1993; Symington et al., 1993]. Thus, the α3β1 integrin has been regarded as a mysterious adhesion molecule with pleiotropic-binding specificities that is involved in a variety of physiologically important processes. The α3β1 integrin is widely distributed in various epithelial and endothelial tissues and plays crucial roles in the organization and maintenance of these tissues, and mice deficient in α3 integrin, die during the neonatal period with defects of the lungs, kidneys, and skin, suggesting that the α3 integrin plays essential

Abbreviations used: ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction.

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roles in their organogenesis [Kreidberg et al., 1996]. This integrin forms complexes with other cell-surface proteins, including transmembrane-4 superfamily (TM4SF) proteins, also referred to as tetraspanins. The functions of the integrin in cell adhesion, motility, and signaling have been suggested to be regulated by the complex formation [Berditchevski, 2001; Hemler, 2001]. The cDNA for the hamster-, human-, and mouse-α3 integrins has been cloned [Tsuji et al., 1990, 1991; Takada et al., 1991; Takeuchi et al., 1995]. The mouse α3 integrin gene was encoded by 26 exons spanning over 40 kb, and the alternative usage of exon 25 generated two cytoplasmic variants, the α3A and α3B subunits [Tamura et al., 1991; Tsuji et al., 1999]. The expression of the α3B subunit has been reported to be more restricted than that of the α3A subunit, which is widely distributed in various tissues; however, the difference in their functions has not been well characterized [de Melker et al., 1997].

The expression of the α3β1 integrin at both the protein and mRNA levels is increased in fibroblastic cells after the oncogenic transformation by SV40 or polyoma virus [Tsuji et al., 1991]. The enhanced expression of this integrin in the transformed cells is likely to be related to their oncogenic phenotypes. A number of studies have demonstrated that the aberrant expression of the α3β1 integrin in various tumor cells was associated with changes in their invasive and metastatic potentials. For instance, in melanoma [Natali et al., 1993; Schumacher and Schaumburg-Lever, 1999], gastric carcinoma [Nishimura et al., 1996; Ura et al., 1998], glioma [Kishima et al., 1999], and hepatocellular carcinoma [Giannelli et al., 2001], the expression levels of the α3β1 integrin were positively correlated with the degree of malignancy. Recent studies, including ours, have indicated that expression of the α3β1 integrin on gastric carcinoma cells was closely related to the formation of peritoneal metastasis [Ura et al., 1998; Takatsuki et al., 2004]. It was also reported that the α3β1 integrin mediated the activation signals for the production of matrix-degrading enzymes in tumor cells [Ito et al., 2001; Tsuji et al., 2002]. Therefore, the mechanism of the transcriptional regulation of the α3β1 integrin gene in tumor cells seems to be of considerable interest. We previously isolated the 5′-flanking region of the mouse integrin α3 subunit gene, and analyzed its promoter activity [Kato et al., 2002]. The results demonstrated that the putative binding site for Ets transcription factors located at 133 bp upstream of the major transcription start site of the integrin α3 subunit was crucial for the expression of the integrin α3 subunit gene in MKN1 gastric carcinoma cell line. However, it has not been clarified whether the expression of α3 integrin is regulated by a similar mechanism in other tumor cells with distinct tissue origins. In the present study, we characterized the transcriptional regulation of the integrin α3 gene by use of several tumor cell lines, including gastric carcinoma, bladder carcinoma, melanoma, and fibrosarcoma, and presented evidence for the involvement of a GC-rich motif located between the Ets-binding site and the major transcription start site. The results also suggested the possible cooperation of the Ets- and Sp-family of transcription factors.

MATERIALS AND METHODS

Reagents

Restriction endonucleases and modifying enzymes were purchased from TaKaRa (Osaka, Japan), Toyobo (Osaka, Japan), and Gibco BRL (Rockville, MD). p-Nitrophenyl β-D-galactopyranoside was from Sigma (St. Louis, MO). The Luciferase Assay System and Tfx-20^{TM} were purchased from Promega Corp. (Madison, WI). Oligonucleotides and [γ-^{32}P]-ATP were supplied by Amersham-Pharmacia Biotech (Tokyo, Japan). Anti-α3 integrin monoclonal antibody (clone SM-T1) was prepared in our laboratory [Takeuchi et al., 1994]. FITC-labeled anti-mouse IgG antibody was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Horseradish peroxidase-conjugated goat antibody to rabbit IgG was purchased from KPL (Gaithersburg, MD). Antibodies against the Sp-family of transcription factors (anti-Sp1, anti-Sp2, anti-Sp3, and anti-Sp4 antibodies) were products of Santa Cruz Biotechnology (Santa Cruz, CA).

Cells

MKN1 (a human gastric carcinoma cell line) was supplied by RIKEN Cell Bank (Tsukuba, Japan). The KATO III human gastric carcinoma cell line, HT-1080 human fibrosarcoma cell line, EJ-1 human bladder carcinoma cell line, and A172 human glioma cell line were supplied by Health Science Research Resources Bank (Osaka, Japan). The A375 human melanoma

cell line was supplied by American Type Culture Collection (Manassas, VA). These cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) at 37°C under 5% CO_2.

Flow Cytometric Analysis

The expression of the α3 integrin was measured by a flow cytometer (FACS Calibur; BD Biosciences, San Diego, CA) using monoclonal anti-human α3 integrin antibody (SM-T1) and FITC-labeled anti-mouse IgG antibody as described previously [Takeuchi et al., 1994].

Construction of Reporter Plasmids

A series of deletion constructs (L0.3, L0.2, and L0.1) in a luciferase-containing plasmid, pGL3-basic (Promega), were prepared from the 4.0 kb DNA fragment of the 5′-flanking region of mouse α3 integrin as described previously [Kato et al., 2002]. Introduction of mutations into L0.3, L0.2, and L0.1 was performed by the PCR-based site-directed mutagenesis as described by Weiner et al. [1994] using *pfu* DNA polymerase (Stratagene, La Jolla, CA). Mutated constructs (mE, mS, mES, mS1, mS2, mS3, mS4A, and mS4B) were prepared by using L0.3 or L0.2 as a template and a double-stranded oligonucleotide as primers. The primers with the following sequences were used: 5′-TTTTCTCTTTCCC-CGTAAGGAAAGCA-3′ for the construction of mE and mES (mutation in the Ets-binding site at −133), 5′-CGGTGGGGATTAGGGGC-GCGCT-3′ for the construction of mS, mES, and mS4B (mutation in the Sp-binding site at −69), 5′-AAGGTGTTTAGGTGCGGGGCT-3′ for mS1 (mutation in the Sp-binding site at −97), 5′-AGGTGCGTTTCTGGGCGGT-3′ for mS2 (mutation in the Sp-binding site at −89), 5′-TGCGGGGCTGTTAGGTGGGGA-3′ for mS3 (mutation in the Sp-binding site at −81), and 5′-CGGTGGGGAGGCTTTGCGCGCT-3′ for mS4A (mutation in the Sp-binding site at −69) (mutated bases are underlined). The conditions for the PCR were as follows: 95°C, 1 min; 58°C, 1 min; 72°C, 8 min; 25 cycles. The products of PCR were sequentially treated with *Dpn* I and with *Kpn* I/*Sac* I. The digested fragments after electrophoretic separation on an agarose gel were subcloned into the *Kpn* I/*Sac* I site of pGL3-basic plasmid. The introduction of the mutation was confirmed by nucleotide sequencing with a DNA sequencer (Applied Biosystems model 377; Foster City, CA) using the BigDye™ terminator cycle sequencing method.

The 5′-flanking region of the human α3 integrin gene was amplified by PCR using genomic DNA from human gastric carcinoma cell line MKN45. A set of primers with the following sequences was used: 5′-ATAGGTA-CCTTTGGGGGTTGCCGACAGGT-3′ (forward primer) and 5′-ATAGAGCTCGACCTGTTCA-CCTGCTCCCCGC-3′ (reverse primer). The PCR conditions were as follows: 96°C, 2 min; 55°C, 1 min; 72°C, 2 min; 30 cycles. The PCR product (539 bp) was digested with *Kpn* I and *Sac* I, and the digested fragment was subcloned into the *Kpn* I/*Sac* I site of the pGL3-basic plasmid. The nucleotide sequence was confirmed by DNA sequencing (DDBJ/EMBL/GenBank databases; accession number AC002401).

Transfection and Luciferase Assay

Luciferase assay was conducted with a Luciferase Assay System (Promega) using reporter plasmids constructed in a pGL3-basic plasmid essentially as described previously [Kato et al., 2002]. Briefly, tumor cells were seeded in 6-well plates (5×10^5 cells/well) and cultured for 24 h. The cells were transfected with a reporter plasmid (3.0 μg) by the lipofection method using Tfx-20™ (Promega) for 60 or 90 min in serum-free media (ASF-104; Ajinomoto, Tokyo, Japan). pRSV-β-Gal plasmid (1.0 μg) was co-transfected as an internal control. After being cultured for 48 h in RPMI 1640/10% fetal bovine serum, the cell extracts were assayed for luciferase activity with a chemiluminometer. β-Galactosidase activity in the cell extract was determined by using 2 mM p-nitrophenyl β-D-galactopyranoside as a substrate in 20 mM sodium phosphate buffer (pH 7.5) to normalize the transfection efficiency in each sample.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were extracted from MKN1 cells and KATO III cells according to the method by Ko et al. [1999]. Double-stranded oligonucleotides containing the Ets- or Sp-family-binding site of the 5′-flanking region of the mouse α3 integrin gene were used as probes and competitors. The sequences of the oligonucleotides used in this study are as follows: 5′-TTTTCTCTTTCCCCGGAAGGAAAGCAGAG-3′ (W1, the wild-type containing the Ets-binding site); 5′-TTTTCTCTTTCCCCGTAAGGAAAG-CAGAG-3′ (m1, a mutant of W1); 5′-CGGTG-

GGGAGGCGGGGCGCGCT-3′ (W2, the wild-type containing the Sp-binding site); and 5′-CGGTGGGGATTAGGGGCGCGCT-3′ (m2, a mutant of W2). The ends of probes were labeled with [γ-^{32}P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (TaKaRa). ^{32}P-labeled probes (15,000 d.p.m) and nuclear extracts (5 μg protein) were mixed in 0.02 ml of 25 mM Tris-HCl buffer (pH 7.9) containing 65 mM KCl, 6 mM $MgCl_2$, 0.25 mM EDTA, and 10% glycerol (binding buffer) in the presence of dI-dC (1 μg or 400 ng), and incubated for 20 min at room temperature. For supershift assays, 2 μl of antibody against Sp1, Sp2, Sp3, or Sp4 (2 mg/ml) was added to the mixture and incubated at 4°C prior to the addition of the probe. The mixture was separated by polyacrylamide gel (6%) electrophoresis using 10 mM Tris-acetate (pH 7.8) containing 0.25 mM EDTA (0.25 × Tris/acetate/EDTA) as a running buffer.

Analysis of Sequence-Specific DNA-Binding Proteins

The proteins bound to the GC-rich motif upstream of the α3 integrin gene were analyzed by a combination of affinity purification and Western blotting. The affinity resin with an oligonucleotide containing a tandem repeat of the GC-rich sequence was synthesized essentially by the method of Kadonaga and Tjian [1986]. A pair of complementary 22-mer oligonucleotides with the following sequences was designed to have cohesive ends after one of its oligonucleotides is annealed to the other: 5′-GGGAGGCGGGGCGCGCTCGGTG-3′ (W3) and 5′-AGCGCGCCCCGCCTCCCCACCG-3′ (W4) (complementary sequences are underlined). W3 and W4 oligonucleotides were annealed and ligated to form an oligomer, and the double-stranded oligonucleotide products were subcloned into a T-easy vector (Promega) after Taq DNA polymerase treatment. For the control experiment, a mutated double-stranded oligonucleotide was also prepared by ligation of the following complementary oligonucleotides (mutated bases are underlined): 5′-GGGATTAGGGGCGCGCTCGGTG-3′ (m3, a mutant of W3) and 5′-AGCGCGCCCCTAATCCCCACCG-3′ (m4, a mutant of W4).

Subsequently, a biotinylated double-stranded oligonucleotide was prepared by PCR using a plasmid containing a tandem octamer of the W3/W4 or m3/m4 oligonucleotide pair as a template and a set of primers with the following sequence: 5′-(biotin)-ATGGCGGCCGCGGGAATT-3′ and 5′-AGGCGGCCGCGAATTTCACTA-3′. The conditions for PCR were: 95°C, 1 min; 56°C, 1 min; 72°C, 4 min; 30 cycles. The PCR product was conjugated with streptavidin-Dynabeads M-280 (DYNAL, Oslo, Norway).

The DNA beads, thus prepared, were mixed with the nuclear extracts (100 μg protein) from KATO III cells in 0.6 ml of binding buffer used for EMSA containing dI-dC (10 μg), and the mixture was incubated for 60 min at 4°C. After the beads were washed with binding buffer three times, the proteins bound to the beads were eluted by treatment with 0.2 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.02% bromophenol blue at 95°C for 2 min. The DNA-binding proteins were separated on 7.5% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blotted membranes were then probed with anti-Sp3 antibody, and protein bands were visualized with horseradish peroxidase-conjugated goat antibody to rabbit IgG secondary antibody and enhanced chemiluminescence reagent (ECL; Amersham Biosciences).

RESULTS

Transient Expression Analysis of the 5′-Flanking Region of the Mouse α3 Integrin Gene in Various Tumor Cells

We previously reported that an approximately 0.3 kb fragment upstream of exon 1 of mouse α3 integrin gene (L0.3, −260/+84) (Fig. 1) showed strong promoter activity in gastric carcinoma MKN1 cells and that a putative binding sequence for Ets-transcription factors present in this region was crucial for the activity [Kato et al., 2002]. In this study, we employed six tumor cell lines derived from different tissues (Table I) to examine whether the promoter that was assigned in the previous study is also active in these cells. The flow cytometric analysis indicated that these cells showed different levels of α3 integrin expression (Fig. 2). The introduction of L0.3 into these cells resulted in the levels of luciferase activity that were higher than the background levels in all tested cell lines (Table I). The level of luciferase activity induced by the transfection of L0.3 into each cell line was roughly correlated with the level of α3 integrin expression as measured by flow cytometry, suggesting that the 0.3 kb fragment contains elements that upregulate the

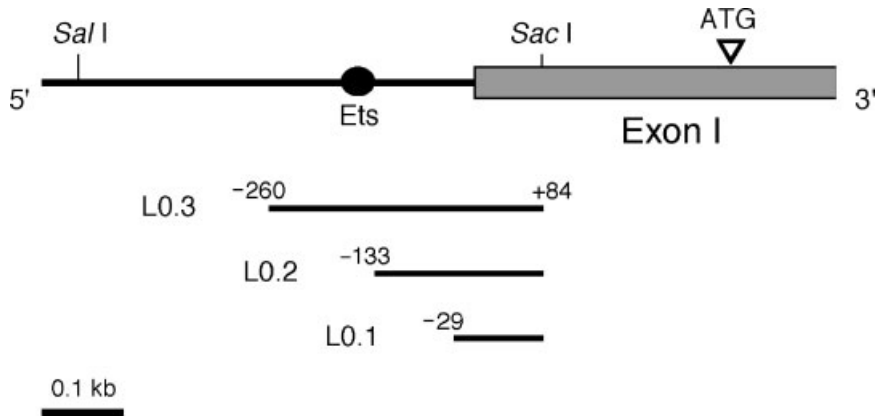

Fig. 1. Structure of the 5′-flanking region of the mouse α3 integrin gene. The map shows exon 1 and positions for restriction endonucleases (*Sal* I and *Sac* I) as well as the location of the putative Ets transcription factor-binding sequence. The translation initiation site is indicated by *ATG*. The positions of the fragments inserted in the deletion constructs (L0.3, L0.2, and L0.1) are also shown.

expression of the α3 integrin gene in these tumor cell lines.

Effects of Deletion and Mutation on the Promoter Activity

To specify the region essential for promoter activity leading to the expression of α3 integrin, we prepared two 5′-deletion constructs, L0.2 (−133/+84) and L0.1 (−29/+84), and analyzed their effects on the level of luciferase activity (Fig. 3). In A172, MKN1, and A375 cells, the level of luciferase activity induced by L0.2 was markedly lower than that induced by L0.3, and in fact was as low as the control level. By contrast, in EJ-1, HT-1080, and KATO III cells, L0.2 induced significant increases in luciferase activity relative to that in the control cells, and the difference between the levels induced by L0.2 and those induced by L0.3 was moderate; the ratios of L0.2-induced activity to L0.3-induced activity were 69% for EJ-1, 49% for HT-1080, and 54% for KATO III. However, the transfection with L0.1 yielded only basal-level activity in all six cell lines. According to the profiles of the luciferase activities induced by L0.3 and L0.2, the six cell lines could be divided into two groups. The first group consisted of A172, MKN1, and A375 cells, in which the elements essential for the promoter activity were located between −260 and −133. The second group was made up of EJ-1, HT-1080, and KATOIII cells, in which the region essential for the promoter activity was assigned to a wider range (−260/−29).

We next examined the role of the putative Ets-binding site at −133 (Fig. 1). As shown in Figure 4, the introduction of a mutation into the Ets-binding sequence (mE, CGGAA to CGTAA) decreased promoter activity in all tested cell lines. However, decreases in promoter activity by the mutation in the second group (EJ-1, HT-1080, and KATOIII) were less prominent than those in the first group (A172, MKN1, and A375). The mutation almost completely abolished promoter activity in the first group of cell lines, whereas significant levels of promoter activity were induced in the second group of cell lines by the mutated L0.3 (mE). The differential inhibitory effects of the mutation on promoter activity in the two groups of cell lines were in good agreement with the results of the deletion experiments (Figs. 3 and 4).

TABLE I. Transient Expression Analysis of α3 Integrin Gene Promoter Activity in Tumor Cell Lines

Cell line		α3 integrin expression[a]	Relative luciferase activity[b]
Name	Type		
EJ-1	Bladder carcinoma	225.0	7384 ± 3021
HT-1080	Fibrosarcoma	115.0	3025 ± 310
A172	Glioma	58.1	2087 ± 667
MKN1	Gastric carcinoma	38.9	1097 ± 121
A375	Melanoma	15.0	503 ± 82
KATO III	Gastric carcinoma	6.7	584 ± 157

[a]The expression of α3 integrin was measured by flow cytometric analysis with a monoclonal anti-α3 integrin antibody (Fig. 2), and the mean fluorescence intensities are shown.
[b]Values (mean ± SD) are expressed in relation to the luciferase activity of pGL3-basic, which was taken as 1.0, and normalized by β-galactosidase activity.

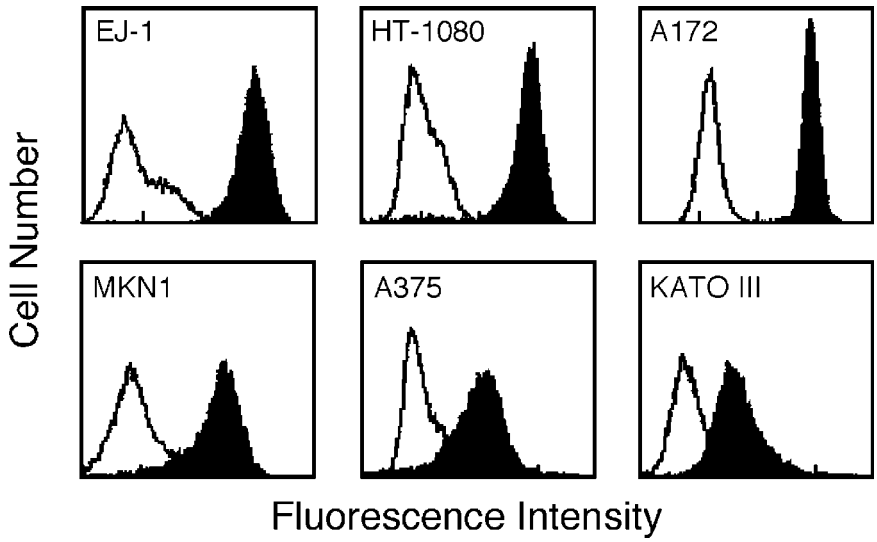

Fig. 2. Flow cytometric analysis of the expression of α3 integrin in tumor cell lines. Six tumor cell lines (EJ-1, HT-1080, A172, MKN1, A375, and KATO III) were stained with anti-α3 integrin monoclonal antibody (SM-T1) and FITC-labeled anti-mouse IgG antibody, and analyzed by flow cytometry. Profiles of control experiments without anti-α3 antibody are also shown by thin lines.

Since the luciferase assay using deleted and mutated constructs suggested the presence of additional sites for the upregulation of the α3 integrin expression in the second group of cell lines, we attempted to specify the elements essential for the promoter activity within −133/−29. The TRANSFAC database search [Wingender et al., 2001] for the sequence of this

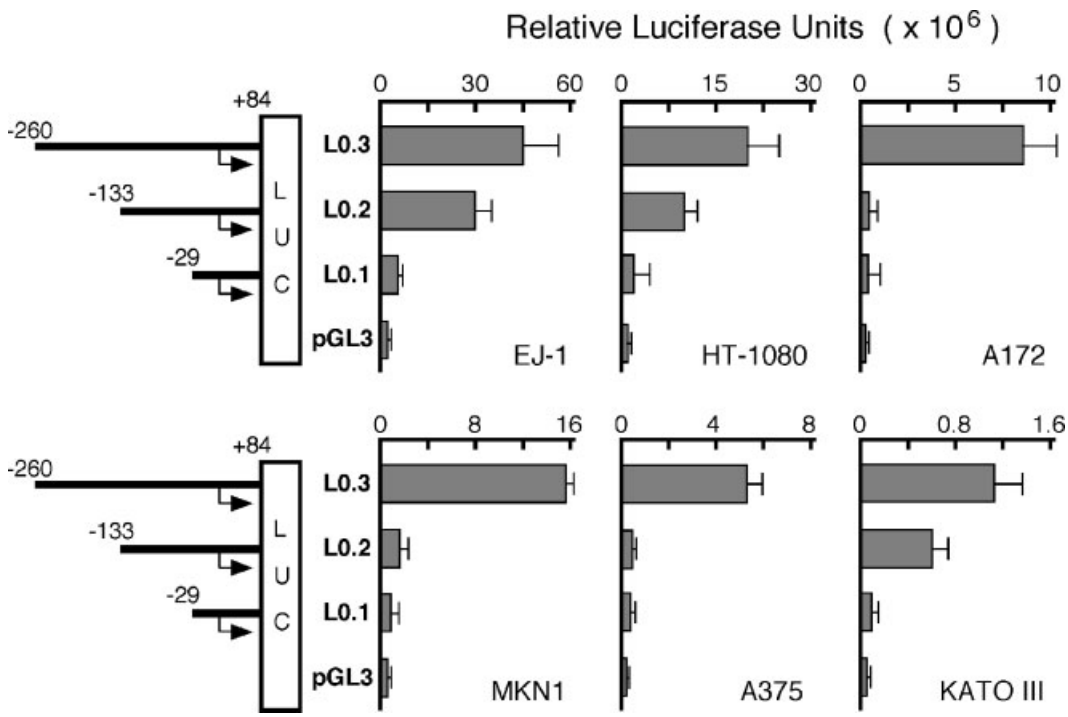

Fig. 3. Promoter activity of serial deletion constructs of the 5′-flanking region of the mouse α3 integrin gene. Relative luciferase activity was determined following the introduction of reporter constructs (L0.3, L0.2, or L0.1) into EJ-1, HT-1080, A172, MKN1, A375, and KATO III cells. The activity was normalized to β-galactosidase activity induced by co-transfection with pRSV-β-Gal plasmid. The assays were carried out in triplicate, and the error bars indicate the standard deviation.

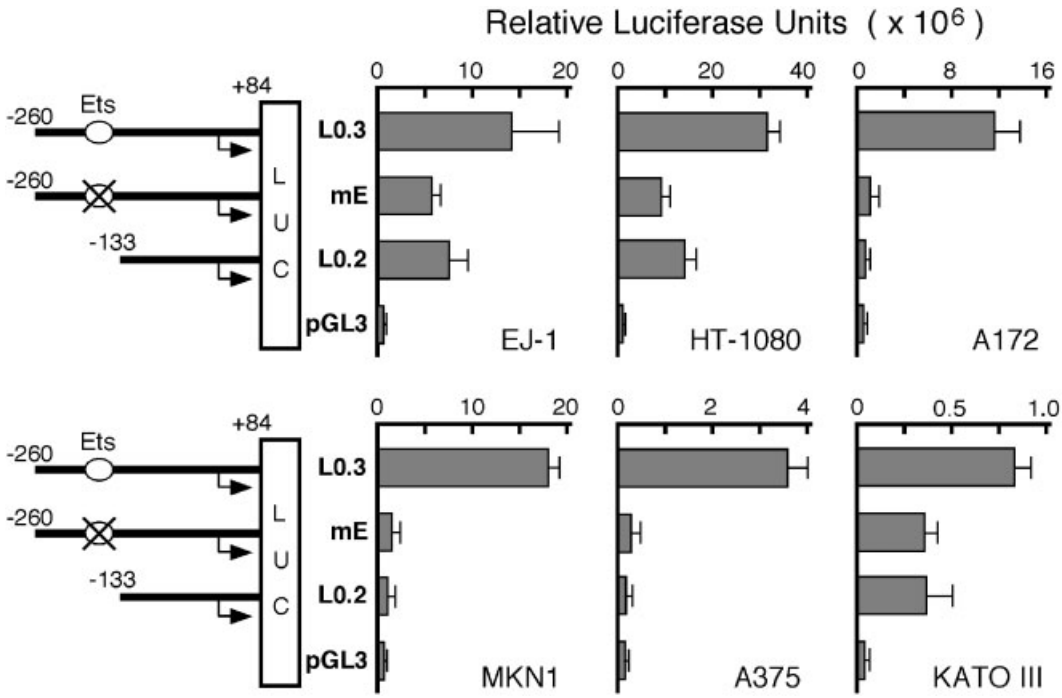

Fig. 4. Effects of mutation in the Ets-binding sequence of the mouse α3 integrin gene on the promoter activity. The wild-type L0.3 (−260/+84) or the mutated construct was introduced into EJ-1, HT-1080, A172, MKN1, A375, and KATO III cells. Relative luciferase activity was determined in triplicate, and data were normalized to β-galactosidase activity.

region revealed that four GC-rich sequences, which are known as binding sites for the Sp-family of transcription factors, are clustered in the region −97/−58 (Fig. 5A). We then prepared several constructs with a mutation in these Sp-binding sequences from L0.2 (mS1, mS2, mS3, mS4A, and mS4B), and analyzed their promoter activity in KATO III cells (Fig. 5B). The introduction of a mutation into the Sp-binding site at −69 (mS4A, GGCGGGGC to GGCTTTGC; mS4B, GGCGGGGC to TTAGGGGC) abolished the promoter activity of L0.2, whereas mutations of other Sp-binding sites at −97 (mS1), −89 (mS2), or −81 (mS3) showed no substantial effect on the activity in KATOIII cells. Similar results were obtained when these mutated constructs were assayed in EJ-1 and HT-1080 cells (data not shown). These results strongly suggested that the transcription of the α3 integrin gene in some tumor cells is regulated by the Sp-family of transcription factors as well as the Ets-family of transcription factors.

We then evaluated the contribution of these two elements, the Ets-binding site at −133 and the Sp-binding site at −69, to the promoter activity for the expression of the α3 integrin gene in each group of cell lines. The mutation was introduced into either the Ets-site or the Sp-site of L0.3 (−260/+84), and the mutated constructs were assayed for the promoter activity in MKN1 and KATO III cells. The activity of L0.3 with mutation in the Ets-site at −133 (mE) was more markedly decreased in MKN1 cells than in KATO III cells (Fig. 6A, left panels), as was consistent with the results shown in Figure 4. On the other hand, the luciferase activity in KATO III cells was more severely affected by the mutation in the Sp-site at −69 (mS) than that in MKN1 cells. The double mutant at both the Ets- and Sp-sites (mES) showed almost no activity in either MKN1 or KATO III cells.

A homology search between the human and mouse α3 integrin genes revealed that the putative Ets-binding site at −133 and the Sp-binding site at −69 are well conserved in these species (Fig. 6B), and that these elements are present at 460 bp and 397 bp upstream, respectively, of the translation initiation ATG in the human α3 integrin gene. We then examined whether mutation of the corresponding Ets- and Sp-binding sites in the human gene

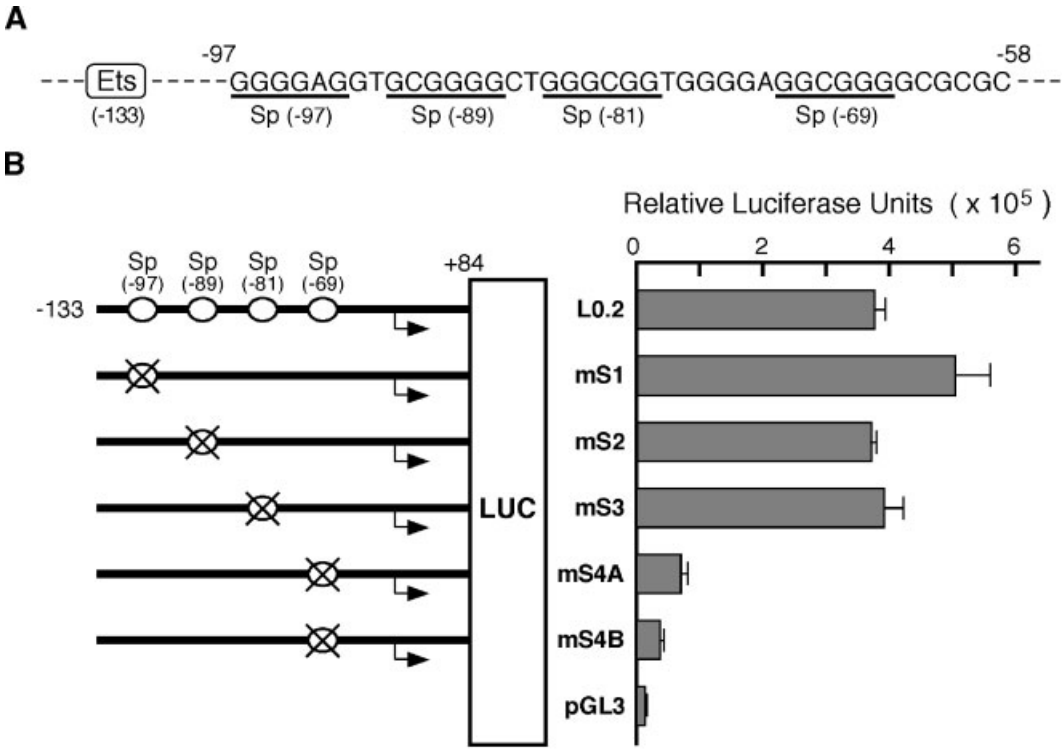

Fig. 5. Effects of mutation in the Sp-binding sequences of the mouse α3 integrin gene on promoter activity. **A**: The GC-rich sequence between −97 and −58 of the mouse α3 integrin gene is shown. The TRANSFAC database search [Wingender et al., 2001] revealed the presence of four putative Sp-binding sites. **B**: KATO III cells were transfected with wild-type L0.2 (−133/+84) or mutated constructs, and relative luciferase activity after culture for 48 h was determined. The assays were carried out in triplicate, and the data were normalized to β-galactosidase activity.

caused similar suppression of the promoter activity in MKN1 and KATO III cells. As shown in Figure 6A (right panels), the inhibition profile resulting from the mutation of each site in the human gene was similar to that in the mouse gene. These results suggested that the expression of α3 integrin in both MKN1 and KATO III cells depends, at least in part, on the Sp-family of transcription factors, but that the expression in MKN1 cells is less dependent on these factors. Both Ets- and Sp-binding elements are likely to cooperatively upregulate the α3 integrin expression in most tumor cells, and each element appears to contribute differently to the expression depending on the types of tumor cells.

Electrophoretic Mobility Shift Assay Using Ets- and Sp-Consensus Sequences

Because the luciferase assay suggested that both the Ets-binding site at −133 and the Sp-binding site at −69 were involved in the promoter activity of the mouse α3 integrin gene, we analyzed protein binding to these sites by an electrophoretic mobility shift assay (EMSA). We synthesized several oligonucleotides with the wild-type or mutated sequences corresponding to these two regions. An oligonucleotide containing the Ets-binding site (W1, −147/−119) was used as a probe to detect proteins in a nuclear extract from MKN1 cells that interact with the oligonucleotide. As shown in Figure 7A (lanes 1–6), we detected three specific DNA-protein complexes (indicated by arrowheads; Ea, Eb, and Ec), which were not detected with the oligonucleotide with a mutated sequence (m1, GGAA to GGTA). These bands were competed by a 100- or 200-fold molar excess of unlabeled wild-type (W1) but not by the mutant oligonucleotide (m1). When the mixture of the oligonucleotide with the sequence of the Sp-binding site (W2, −78/−57) and a nuclear extract from KATOIII cells was subjected to EMSA, four specific bands were observed (indicated by arrowheads; Sa, Sb, Sc, and Sd) (Fig. 7A, lanes 7–11), which disappeared in the

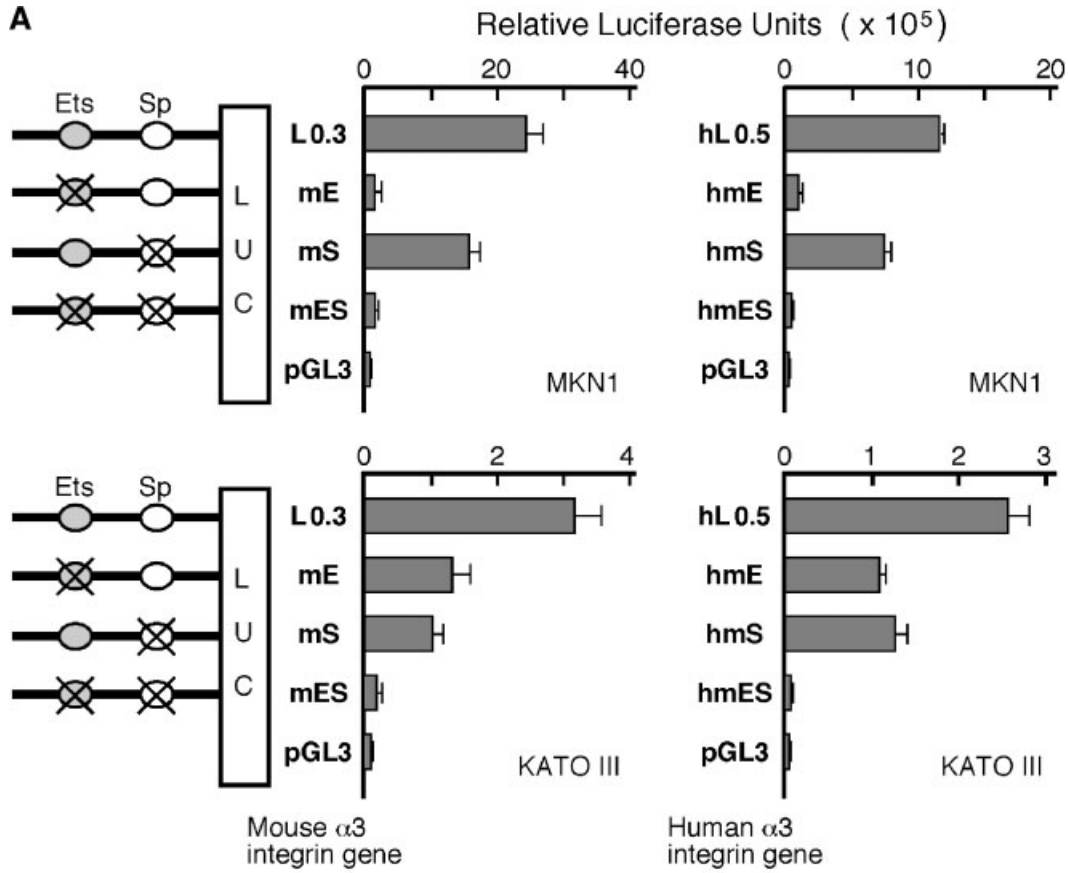

Fig. 6. Effects of mutation in Ets- and/or Sp-binding sites of the mouse- and human-α3 integrin genes on promoter activity. **A**: The wild-type L0.3 (−260/+84) or mutated constructs (the Ets-binding site at −133 and/or Sp-binding site at −69) of the mouse α3 integrin gene were introduced into MKN1 cells (**upper left panel**) or KATO III cells (**lower left panel**). After the cells were cultured for 48 h, the luciferase activity was determined. Similarly, the wild-type (hL0.5) and mutated constructs (the Ets-binding site and/or Sp-binding site) derived from the human gene were introduced into MKN1 cells (**upper right panel**) or KATO III cells (**lower right panel**), and the promoter activity was measured. The assays were carried out in triplicate, and the data were normalized to β-galactosidase activity. **B**: The nucleotide sequences of the promoter regions for human- and mouse-α3 integrin genes are aligned (DDBJ/EMBL/GenBank databases; accession numbers AC002401 and AB080229). The identical nucleotides in the human and mouse genes are indicated by asterisks. Numbers indicate the nucleotide residues from the major transcription start site (mouse) or from the first ATG (human). The putative Ets- and Sp-binding sequences are underlined.

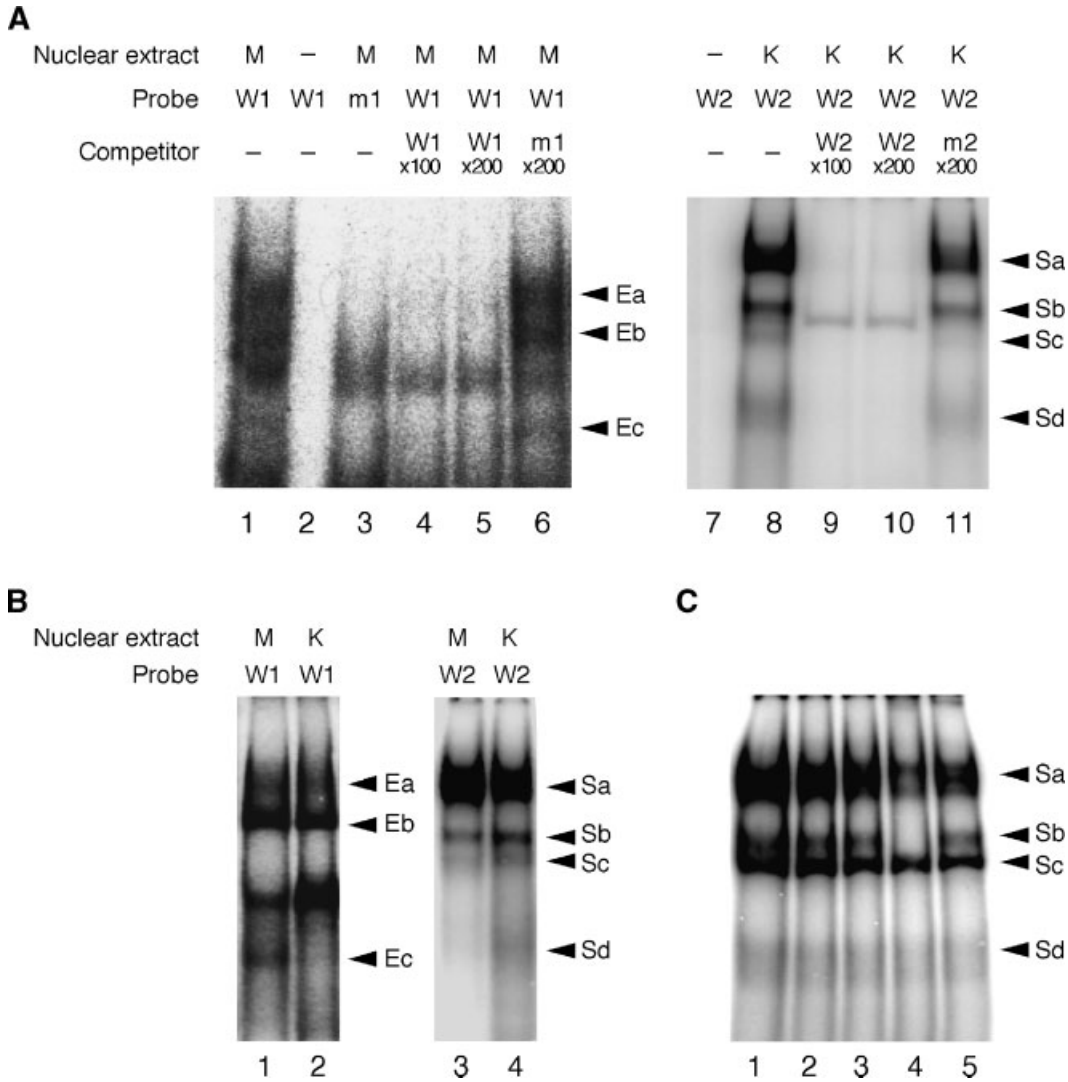

Fig. 7. Electrophoretic mobility shift assay (EMSA) using probes containing the putative Ets- or Sp-binding sequence. ^{32}P-labeled oligonucleotide probes were incubated with nuclear extracts from MKN1 cells (*M*) or KATO III cells (*K*). For competition analysis, a 100- or 200-fold molar excess of unlabeled oligonucleotides was added before the incubation. W1, the oligonucleotide probe (−147/−119) including the Ets-binding site; m1, a mutant of W1; W2, the oligonucleotide probe (−78/−57) including the Sp1-binding site; m2, a mutant of W2 (**A** and **B**). Effects of antibodies against the Sp-family of transcription factors on profiles of EMSA using W2 as a probe and KATO III cells as a source of nuclear extract were also examined as described in the MATERIALS AND METHODS (**C**). **Lane 1**, no antibody; **lane 2**, anti-Sp1 antibody; **lane 3**, anti-Sp2 antibody; **lane 4**, anti-Sp3 antibody; **lane 5**, anti-Sp4 antibody.

presence of a 200-fold molar excess of the unlabeled wild-type oligonucleotide (W2) but not in the presence of the mutant oligonucleotide (m2, GGCGGG to TTAGGG).

Because the α3 integrin gene expression in the two groups of tumor cell lines was suggested by luciferase assay to be differentially regulated by Ets- and Sp-related factors, we compared the EMSA profiles using an oligonucleotide containing the Ets- or Sp-binding sequence as a probe, and MKN1 or KATO III cells as a source of nuclear proteins. In the EMSA using W1 (the oligonucleotide for the Ets-site) as a probe, one band (Ec) was of an undetectable level when nuclear extracts from KATO III cells were used, although two other bands (Ea and Eb) were detected in comparable intensities irrespective of the source of the nuclear proteins (Fig. 7B, lanes 1 and 2). A similar assay was conducted with nuclear extracts from both cell lines using W2 (the oligonucleotide for the Sp-binding site) as a probe (Fig. 7B, lanes 3 and 4). One band (Sa) was detected in comparable intensities in both lanes, whereas the remaining three bands (Sb,

Sc, and Sd) derived from the mixture with nuclear proteins from MKN1 cells were less prominent than those from KATO III cells. These results suggest that the expression profiles of the binding proteins for the Ets- and Sp-sites vary depending on tumor cells and that the difference in the expression levels of these proteins affects the regulation of the transcription of the α3 integrin gene.

To identify the proteins bound by the Sp-site in W2, we conducted a supershift assay using antibodies against the Sp-family of transcription factors (Sp1, Sp2, Sp3, and Sp4). When anti-Sp3 antibody was added to the reaction mixture of EMSA, one of the bands (Sb) was specifically abrogated (Fig. 7C, lane 4), suggesting that the Sp3-transcription factor is a component of the protein–oligonucleotide complex. However, the other antibodies showed no substantial effect on the profiles of EMSA (Fig. 7C, lanes 2, 3, and 5). The addition of anti-Sp4 antibody apparently reduced the intensity of the Sa band; however, the result was not reproducible and we cannot conclude that Sp4 was bound by the W2 probe at present. These results indicate that the Sp3-transcription factor binds to the putative Sp-binding sequence at −69 and is crucial for the α3 integrin expression.

Analysis of Nuclear Proteins Bound to the Sp-Consensus Sequences

To confirm that Sp3-transcription factor binds to the Sp-binding sequence at −69, nuclear proteins that bound to this sequence were analyzed by Western blotting. The Western blot analysis of the whole nuclear proteins from KATO III cells with anti-Sp3 antibody gave three bands, including doublet bands with higher mobility (Fig. 8, lane 1). This was consistent with the previous report that three Sp3 isoforms were generated by differential translational initiation [Kennett et al., 1997]. When the proteins bound to and eluted from DNA beads with a tandem repeat of the 22-mer oligonucleotide containing the Sp-consensus sequence were probed with anti-Sp3 antibody, we detected a band of approximately 70 kDa (Fig. 8, lane 4), which is likely to correspond to the upper band of the doublet short type of Sp3-transcription factor (indicated by open arrowhead in Fig. 8). In contrast, we detected no band when we used control beads without DNA or those conjugated with mutated DNA (Fig. 8, lanes 2 and 3). These results again indicate that the GC-rich motif at −69 was specifically bound by Sp3 transcription factor.

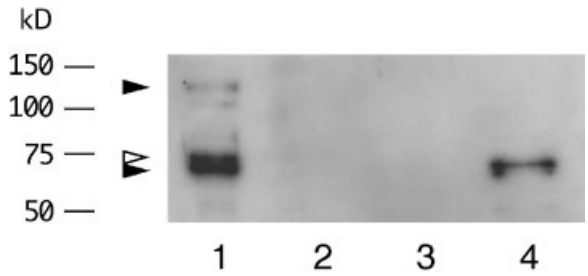

Fig. 8. Analysis of nuclear proteins bound to the Sp-binding sequence by Western blotting. Nuclear proteins from KATO III human gastric carcinoma cells were incubated with streptavidin beads conjugated with a biotinylated tandem repeat of double-stranded oligonucleotide containing the Sp-binding sequence, and the proteins bound to the beads were analyzed by Western blotting with anti-Sp3 antibody. **Lane 1**, whole nuclear extract; **lane 2**, eluate from control beads without DNA; **lane 3**, eluate from DNA beads with mutated oligonucleotide; **lane 4**, eluate from DNA beads with the wild-type oligonucleotide. Arrowheads indicate three isoforms of Sp3 transcription factor.

DISCUSSION

The α3β1 integrin has been thought to play essential roles in various physiological and pathological processes, including cellular proliferation, differentiation, development, wound healing, angiogenesis, transformation, and apoptosis [Kreidberg, 2000; Giannelli et al., 2002; Tsuji, 2004]. On the other hand, a number of studies have suggested a relationship between the aberrant expression of this integrin on tumor cells and their malignant behavior, such as invasion and metastatic potentials. We previously identified the consensus sequence for the Ets-family of transcription factors located at −133 in the 5′-flanking region as an essential element for the α3 integrin gene expression in the MKN1 gastric carcinoma cell line [Kato et al., 2002]. In the present study, we evaluated whether this element also plays a role in the gene expression of this integrin in various tumor cell lines. The results of our luciferase assay conducted with six tumor cell lines derived from various tissues suggest that the Ets-binding site at −133 is responsible for the upregulation of α3 integrin gene expression in all examined tumor cells. However, the six cell lines could be divided into two groups according to the level of dependency on the Ets-transcription factors; the first group consisted of MKN1, A375, and A172 cells, in which the deletion or

the mutation of the Ets-site almost completely abolished the promoter activity; while the second group was made up of KATOIII, HT-1080, and EJ-1 cells, in which the Ets-transcription factors played a rather limited role. We then postulated that additional regulatory elements were responsible for the α3 integrin gene expression in the second group of cell lines, and found that the putative binding site for the Sp-family of transcription factors at −69 was another important element by using deleted reporter constructs devoid of the Ets site. The introduction of mutations into the Sp-binding site resulted in almost complete inhibition of the Ets-independent promoter activity. Furthermore, the same mutation in the Sp-site of the Ets-containing plasmids also reduced the promoter activity in both the first group and the second group of cell lines, suggesting the cooperation of Ets- and Sp-transcription factors in the α3 integrin gene expression. The contribution of each transcription factor to the gene expression thus, seems to depend on the type of tumor cells. However, we found no relationship between the dependence of each factor and the tissues from which the tumor cells were derived, the epithelial/non-epithelial origin, or the level of expression of α3 integrin.

The putative Ets-binding sequence at −133 and the Sp-binding sequence at −69 were well conserved in the human counterpart (Fig. 6B), suggesting the importance of these consensus sites in regulating α3 integrin gene expression. An EMSA, using an oligonucleotide with the Sp-binding sequence demonstrated that the Sp3-transcription factor is a component of the nuclear protein–oligonucleotide complex (Fig. 7). Moreover, Western blot analysis revealed that Sp3-transcription factor specifically binds to the Sp-consensus sequence (Fig. 8). These results suggest that Sp3 is a strong candidate for a transcription factor involved in the α3 integrin expression in cooperation with Ets transcription factors.

Sp3 is a member of the Sp-family of transcription factors and shares more than 90% sequence homology with Sp1, which is also a member of this family [Suske, 1999; Li et al., 2004]. Although these transcription factors are thought to bind to the same cognate GC-rich motif, Sp3 but not Sp1 was shown in this study to specifically bind to the Sp-site at −69 in the 5′-flanking sequence of the mouse α3 integrin gene. It has been suggested that these structurally related transcription factors differentially function in transcriptional regulation. In the regulation of PKR protein kinase, for instance, the binding of Sp1 to the promoter is constitutive, whereas the binding of Sp3 is dependent on the stimulation by interferon, suggesting that Sp1 is involved in the basal expression of the gene and that Sp3 is responsible for the interferon-inducible transcription [Ward and Samuel, 2003]. In the expression of several genes, however, Sp3 acts as a repressor for the gene expression by competing the binding of Sp1 to the promoter [Birnbaum et al., 1995; Dennig et al., 1996; Yu et al., 2003]. In contrast, recent reports have shown that Sp3 is a transcriptional activator of the promoters for the c-*myc* gene [Majello et al., 1997] and cyclin-dependent kinase inhibitor (p21/WAF1/Cip1) gene [Sowa et al., 1999, Gartel et al., 2000]. The activity of Sp3 as a regulator for transcription is likely to depend on the cell-types, promoters, and cellular context. It has been reported that Sp3 has three isoforms generated by differential translational initiation [Kennett et al., 1997]. The Sp3 molecule bound by the Sp-site of the α3 integrin promoter was suggested to be of a short type (approximately 70 kDa) by Western blot analysis (Fig. 8). The short isoforms have been suggested to function as transcriptional repressors for several genes, including the dihydrofolate reductase gene (*DHFR*) and multidrug resistance gene (*MDR-1*) [Kennett et al., 2002], but are suggested to positively regulate the α3 integrin expression.

Recent reports have shown that the Ets-family of transcription factors cooperate with the Sp1 transcription factors to activate the promoter of several integrin genes, which include αV integrin expression in melanoma cells [Tajima et al., 2000], β2 integrin expression in myeloid cells [Rosmarin et al., 1998], and αIIb integrin expression in megakaryocytic cells [Block et al., 1996]. More recently, it was reported that the expressions of α5 integrin in corneal epithelial cells [Larouche et al., 2000] and α6 integrin in prostate carcinoma cells [Onishi et al., 2001] were also regulated by Sp1. In contrast, the expression of α3 integrin in most tumor cells is likely to involve the Sp3-transcription factor. The involvement of Sp3 may account, at least in part, for the specific tissue distribution of α3 integrin. The expression of α5 integrin, which served as a subunit of major fibronectin receptor (α5β1 integrin), in glioma

cells was reported to be regulated by the Ets-1-transcription factor [Kita et al., 2001]. However, we have so far obtained no evidence showing the involvement of Ets-1 in the α3 integrin gene expression, suggesting that the expressions of individual integrins are regulated by distinct mechanisms. The identification of the Ets-transcription factor responsible for α3 integrin gene expression remains to be clarified in a future study. In conclusion, the results obtained in the present study strongly suggest that the two elements for the binding of Ets and Sp3 transcription factors cooperatively regulate the α3 integrin gene expression and that the contribution of each element depends on the type of tumor cells.

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