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Characterization of Mouse Integrin α3 Subunit Gene[1]

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Integrin α3β1 (VLA-3) is an adhesion receptor for extracellular matrix proteins including various isoforms of laminin. We have isolated mouse genomic clones encoding the integrin α3 subunit and deduced the exon/intron organization. The mouse integrin α3 subunit gene is encoded by 26 exons spanning 40 kb. The exon/intron structure of the integrin α3 subunit gene resembles that of the integrin α6 subunit gene, but differs somewhat from those of other members of the integrin family. We have demonstrated that the cytoplasmic domain splicing variants of the α3 subunits (α3A and α3B) are generated by alternative exon usage. We also cloned the 5′-flanking region and performed a preliminary analysis of its promoter activity in various tumor cell lines with different degrees of integrin α3 expression. Following transfection, activity in the luciferase assay was found to be roughly correlated with the expression level of integrin α3 as measured by flow cytometry. Furthermore, the luciferase assay was performed with normal and SV-40- or polyoma virus-transformed fibroblasts. In mouse, human, and hamster fibroblasts, higher levels of luciferase expression were observed in transformed cells than in normal cells. This result is consistent with our previous finding that integrin α3 expression at both the protein and mRNA levels is enhanced upon oncogenic transformation of fibroblasts by tumor viruses.

Key words: integrin, genomic cloning, oncogenic transformation, adhesion molecule, very late antigen.

Members of the integrin superfamily play a role in mediating cell-matrix and cell-cell adhesion. Integrins are transmembrane glycoproteins consisting of a non-covalently associated heterodimer, comprising α and β subunits. The α3β1 integrin (VLA-3) functions as an adhesion receptor for isoforms of laminin, *i.e.* laminin-5 (epiligrin/nicein/kalinin/ladsin) and laminin-10/11 (*1-4*), and for intercellular adhesion (*5-8*). Several reports have suggested that α3β1 integrin forms complexes with other cell-surface proteins, including transmembrane-4 superfamily (TM4SF) proteins, a transferrin receptor, and an amino acid transporter, and that these complexes may play key roles in cell adhesion, motility, signaling, transport, and other cell membrane functions (*9-15*). The cDNA for the hamster, mouse, and human integrin α3 subunit have been cloned (*16-19*). A variant of the integrin α3 subunit with a different cytoplasmic sequence has been detected (*20*), and its specific tissue distribution has also been reported (*21*). We have previously found that the expression of α3β1 integrin is increased following the oncogenic transformation of fibroblasts (*16, 17*). The effect of the enhanced expression of this integrin on transformed cells is likely to be related to their oncogenic phenotypes. A number of studies have demonstrated the aberrant expression of α3β1 integrin in various tumor cells in association with changes in their invasive and metastatic potentials (*22-30*). Thus, the regulatory mechanism for α3β1 integrin expression seems to be of considerable interest. In this study, we attempted to isolate mouse genomic clones encoding the integrin α3 subunit and its 5′-flanking region, and to analyze its promoter activity in normal and SV-40-transformed mouse fibroblasts. We also characterized the exon/intron structure, finding evidence that the cytoplasmic variants of the integrin α3 subunit are generated by alternative exon usage.

MATERIALS AND METHODS

Cells—BALB/3T3 (murine embryonal fibroblasts, ATCC CCL-163, JCRB9005), SV-T2 (SV-40-transformed BALB/3T3, ATCC CCL-163.1, JCRB9099), WI-38 (human lung fibroblasts, ATCC CCL-75, JCRB9017), WI-38

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Abbreviations: VLA, very late antigen; SV-40, simian virus-40; D-MEM, Dulbecco's modified MEM.

VA-13 (SV-40-transformed WI-38, ATCC CCL-75.1, JCRB9057), HT-1080 (human fibrosarcoma, ATCC CCL-121, JCRB 9113), T24 (human bladder carcinoma, ATCC CRL-1619, JCRB 0711), and KATO III (human gastric carcinoma, ATCC HTB-103, JCRB 0611) were supplied by the Health Science Research Resources Bank (Osaka). A375 (human melanoma, ATCC CRL-1619) was supplied by American Type Culture Collection. These cell lines were cultured in Dulbecco's modified MEM (D-MEM) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. K562 (human erythroblastoid leukemia, ATCC CCL-243, RCB0027) and U937 (human histiocytic lymphoma, ATCC CRL-1593, RCB0435) were supplied by Riken Gene Bank (Wako, Saitama), and cultured in RPMI 1640/10% fetal bovine serum.

Screening of the Mouse Genomic Library—A mouse (BALB/c) genomic library constructed in λEMBL3 (8×10^5 independent clones) was screened with the entire cDNA for the mouse integrin α3 subunit (*19*), labeled with ^{32}P by the random priming method (*31*) using the Rediprime™ DNA labeling system (Amersham, Buckinghamshire, UK).

Subcloning and Sequencing—DNA from positive clones was isolated and analyzed by restriction enzyme mapping and Southern hybridization using the mouse integrin α3 subunit cDNA. The restriction fragments obtained by digestions with *Bam*HI, *Eco*RI, and/or *Hin*dIII were subcloned into pBluescript SK (Stratagene, San Diego, CA, USA) and sequenced with a DNA sequencer (Applied Biosystems model 373A, Foster City, CA, USA). By comparing these sequences with the cDNA sequences, the exon/intron structure was determined.

Transfection and Luciferase Assay—The luciferase assay was conducted using a Dual-Luciferase™ reporter system along with pGL3-Basic and pRL-CMV vectors (Promega, Madison, WI, USA). BALB/3T3 and SV-T2 cells (1.5×10^5 cells) were seeded in 6-well plates and cultured for 20 h. The cells were then transfected with a mixture of the plasmid construct in pGL3 vector (3.6 μg) and pRL-CMV (0.7 μg) (used as an internal control) by the lipofection method using Tfx-50™ (Promega) in serum-free medium for 1 h, and subsequently cultured for 40 h in D-MEM/10% fetal bovine serum. The cells were then harvested, and the cell extracts were assayed for luciferase activity with a luminometer (Lumat LB 9507, Berthold Japan, Tokyo) according to the manufacturer's instructions.

Flow Cytometric Analysis—The expression of integrin α3 was measured by flow cytometry (Cyto ACE-150, Jasco, Tokyo) using a monoclonal anti-human integrin α3 antibody (SM-T1) as described previously (*7*).

RESULTS AND DISCUSSION

Genomic Cloning and Structure of the Mouse Integrin α3 Subunit Gene—Screening of 8×10^5 clones of a mouse genomic library by plaque hybridization using mouse integrin α3 subunit cDNA (*19*) as a probe resulted in the isolation of six positive clones. These clones were characterized by restriction mapping, Southern blotting analysis, and sequencing. Based on these results, the genomic structure of the mouse integrin α3 subunit was determined.

The gene is over 40 kb in length and is composed of 26 exons arranged in five clusters (Fig. 1). The exonic sequences were found to be identical to the cDNA sequence (*19*). All the sequences at the exon/intron boundaries (Table I) are in agreement with the consensus rule for splicing (*32*). The transmembrane domain is encoded by exon 24, and the extracellular domain is encoded by exons 1-23. There is no obvious relationship between the exon/intron structure and the seven homologous repeats present in the extracellular domain (*17*). A variant of the mouse integrin α3 subunit (α3B) whose cytoplasmic sequence is distinct from that of the authentic α3 subunit (α3A) has been previously identified (*20*). The characterization of the exon/intron structure of the gene demonstrated that these variants are generated by alternative exon usage (Fig. 2A). The entire cytoplasmic domain of the α3A subunit was found to be encoded by exon 25, in which a TGA stop codon is present following the coding sequence for 36 amino acids (Fig. 2B). On the other hand, this exon is skipped in the mRNA of the α3B subunit, and exon 26, instead, encodes 51 amino acids in the cytoplasmic domain of the α3B subunit preceding another TGA stop codon. In both the α3A and α3B subunits, however, the borders of the transmembrane and cytoplasmic domains (*i.e.* the beginning of exon 25 or 26) have similar sequences; with out of five amino acid residues identical (CGFFK for α3A and CDFFK for α3B) (Fig. 2B). The GFFKR motif, as in the α3A subunit, is highly conserved among integrin α subunits and plays a crucial role in the interaction with calreticulin, a multifunctional calcium-binding protein

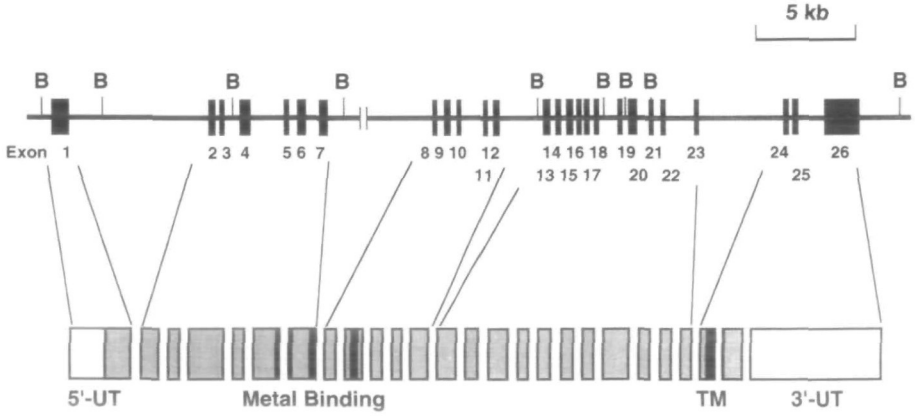

Fig. 1. **Genomic organization of the mouse integrin α3 subunit.** A schematic diagram of the 26 exons encoding the mouse integrin α3 subunit is shown (upper line and solid boxes). B indicates the restriction sites for *Bam*HI. The lower boxes indicate the corresponding domains of the mouse integrin α3 subunit. The shadowed boxes represent coding exons, and the empty boxes represent the 5'- and 3'-untranslated regions (5'-UT and 3'-UT). The metal-binding sequences and the transmembrane domain (TM) are indicated by solid boxes.

(*33*). Since calreticulin has been reported to be a modulator of integrin acivity (*34*), it is possible that the functions of the integrin α3 variants are regulated in different ways by this protein. The cytoplasmic domains of integrins also interact with the cytoskeleton and other molecules related to signal transduction in many cellular events. Thus, it is important to clarify the regulatory mechanism for the splicing of the integrin α3 subunit mRNA. It has been shown that each of the α subunits of other laminin-binding integrins, α6β1 and α7β1, also has two cytoplasmic variants, A and B (*35, 36*), and that the expressions of these variants are regulated differently during embryogenesis (*37*).

The number of exons in the mouse integrin α3 subunit gene is similar to that of other integrin α subunit genes (*38-40*), especially the integrin α6 subunit gene, which is composed of 26 or 27 exons (*41*). However, the exon/intron structures of these genes differ somewhat. Obvious differences are, for example, found in the exons encoding the metal-binding sequences (Fig. 3). In the α subunits of the β2 integrin family (αM and αX subunits), each metal-binding sequence is encoded by a single exon and is located in the middle of the exon. In contrast, the first metal-binding sequence in the integrin α3 or αIIb subunit is encoded by two separate exons, and the second sequences in the α3 and αIIb subunits are located at the 3′-ends of exons 7 and 12, respectively. The third sequence in the α3 subunit is, however, located in the middle of exon 9, as in the case of the αM and αX subunits, whereas that in the αIIb subunit is present at the 3′-end of exon 13. The variations in the distribution of these homologous sequences among individual integrins may provide important information for phylogenic analyses of the integrin genes. Recently, the genomic organization of the human integrin α3 subunit gene was characterized by a search of the GenBank/EMBL database (*42*). The genomic structure of the mouse integrin α3 subunit gene is similar to that of its human counterpart in the number of exons, but different in the sizes of several exons (*e.g.* exons 3, 5, 6, 10, 11, and 16), the length of most of the introns, and the cluster formation of exons, which does not exist in the human gene.

Transient Expression Analysis of the 5′-Flanking Region—We also cloned the 5′-flanking region of the mouse integrin α3 subunit gene. The restriction map for this region is shown in Fig. 4. To examine the promoter activity of the 5′-flanking region, we prepared a chimeric construct

TABLE I. **Exon-intron boundaries of the mouse integrin α3 subunit gene.**

Exon	Intron/Exon/Intron	Exon length (bp) (coding sequence)
1	ATGGGC-------CTACCT/gtaagt	206
2	ttccag/CCTTCT-------AGAAAA/gtgaga	128
3	ttacag/GTGACC-------GTCCTG/gtaagt	80
4	gcctag/GTCTGT-------GGAAAG/gtggga	250
5	ctgcag/GAAACA-------ATATTG/gtaagc	87
6	tcccag/GGTACA-------TGATGG/gtgaga	211
7	gtgcag/GTGGCA-------TCCAGG/gtagga	197
8	ccccag/ACATCG-------CAGCAG/gtatga	89
9	cttcag/ATAATC-------GCTGCG/gtgagc	137
10	ctatag/GGCCCG-------CTCCTG/gtaaga	87
11	caacag/TGTTCA-------ACATCA/gtaagt	68
12	ccacag/CCCTGG-------CTGATG/gtgaga	137
13	ccccag/GACAAT-------ACTGAG/gtgagc	150
14	ccccag/GTCCAC-------AAGCAG/gtggta	98
15	cttcag/GCTCCA-------CGCCCG/gtgagt	148
16	cctcag/TCTGGG-------CAGAGG/gtgaga	72
17	tgctag/ATGGAG-------GTCCAC/gtgagt	80
18	tctcag/GTCGAG-------CAGCTT/gtgggt	78
19	atccag/AATGAA-------TTCCAG/gtaagg	103
20	ccacag/GTGAGC-------CTCTCT/gtaagt	183
21	cttcag/GACCCT-------GTGTTG/gtgagt	123
22	cctcag/ACCTGC-------ATTGAG/gtaagt	114
23	tggtag/GACTAC-------ACATGG/gtgagt	99
24	ctgcag/TTCTCT-------TGGAAG/gtagaa	126
25	ctccag/TGCGGC-------ACCCGG/gtaaca	108
26	ccccag/TGTGAC-------	153

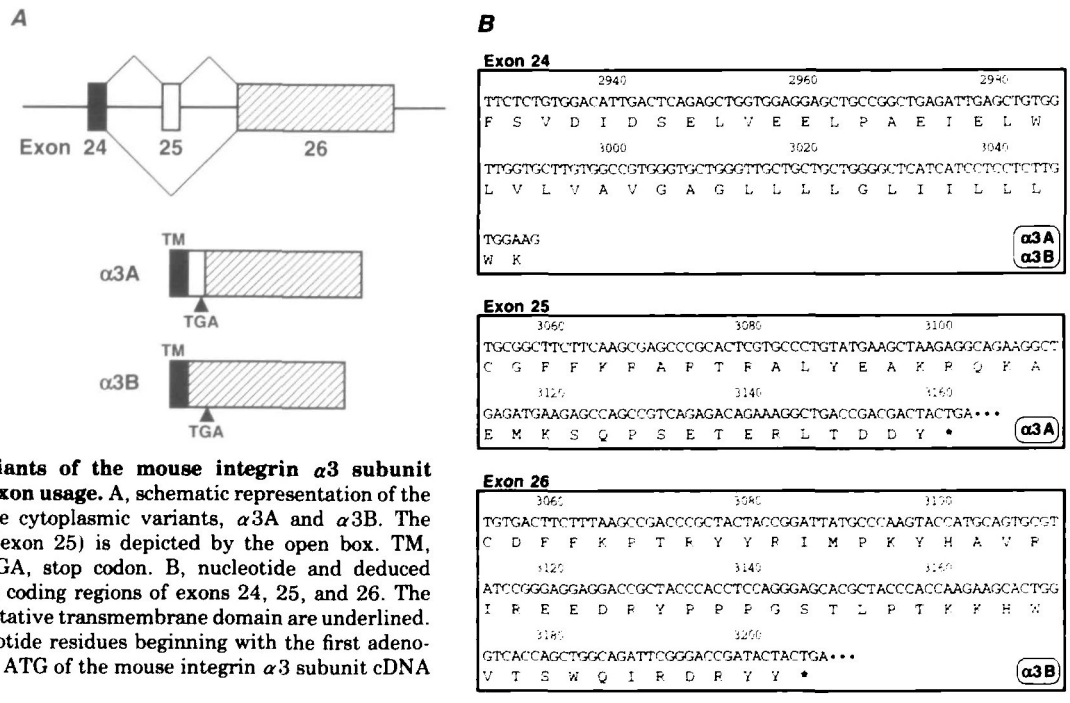

Fig. 2. **Cytoplasmic variants of the mouse integrin α3 subunit generated by alternative exon usage.** A, schematic representation of the exon/intron structure of the cytoplasmic variants, α3A and α3B. The alternatively spliced exon (exon 25) is depicted by the open box. TM, transmembrane domain; TGA, stop codon. B, nucleotide and deduced amino acid sequences of the coding regions of exons 24, 25, and 26. The amino acid residues in the putative transmembrane domain are underlined. Numbers indicate the nucleotide residues beginning with the first adenosine residue of the initiation ATG of the mouse integrin α3 subunit cDNA (*19*).

in which the 4.0 kb *Eco*RI/*Sac*I fragment upstream of exon 1 is inserted into the luciferase gene-containing plasmid pGL3-Basic. Luciferase expression was measured following transfection of the constructs into mouse, human, and hamster fibroblasts (BALB/3T3, WI-38, and NIL, respectively) and their SV-40- or polyoma virus-transformants (SV-T2, WI-38 VA13, and NILpy). When the construct containing the upstream fragment (pGL-ES) was introduced into BALB/3T3 and SV-T2 cells, it promoted higher levels of luciferase activity than the background levels in both types of cells (Table II). Furthermore, the relative luciferase activity induced by pGL-ES in the transformed cells was about 4-times higher than that in normal cells. Similar results were obtained when the construct was introduced into human and hamster fibroblastic cells. In both cases, higher levels of luciferase activity were detected in the transformed cells than in normal cells; 3.5-times higher in human fibroblasts transformed with SV-40 than in normal fibroblasts (WI-38 VA13 *vs.* WI-38) and 2.6-times higher in hamster fibroblasts transformed with polyoma virus than in normal cells (NILpy *vs.* NIL). We previously reported that the expression of the integrin $\alpha 3$ subunit in fibroblastic cells at both the protein and mRNA levels is enhanced upon oncogenic transformation by tumor viruses such as SV-40 and polyoma virus (*16, 17*). The results of the luciferase assays are consistent with our previous findings. The data strongly suggested that the 5′-flanking region includes elements that upregulate the expression of the integrin $\alpha 3$ subunit gene in oncogenically transformed fibroblastic cells.

We next performed transient expression analysis with various tumor cell lines. Six cell lines, including fibrosarcoma, gastric and bladder carcinoma, melanoma, and leukemia, were examined for the expression of the integrin $\alpha 3$ subunit. Flow cytometric analysis of these cells revealed that they express this integrin differently on their cell surfaces. HT-1080 (fibrosarcoma) and T24 (bladder carcinoma) show high expression levels of integrin $\alpha 3$ (Fig. 5, A and B), whereas K562 (erythroblastoid leukemia) and U937 (histiocytic lymphoma) express this integrin at low levels (Fig. 5, E and F). Integrin $\alpha 3$ expression on KATO III (gastric carcinoma) and A375 (melanoma) is moderate (Fig. 5, C and D). As shown in Table III, the relative luciferase activity induced by transfection roughly parallels the levels of integrin $\alpha 3$ expression in these cells. Taken

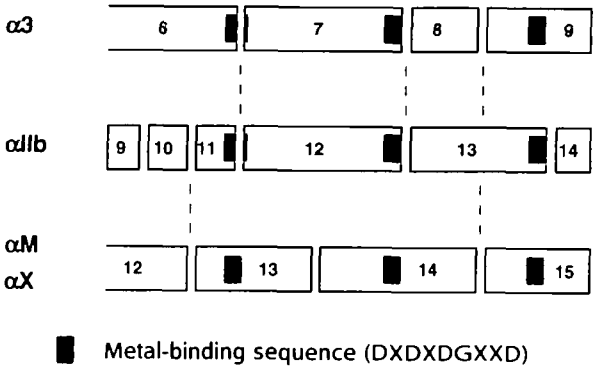

Fig. 3. **Comparison of the mouse integrin $\alpha 3$ subunit with other integrin α subunits in exon organization of the metal-binding regions.** The schematic diagrams demonstrate the alignment of exons encoding the metal-binding regions of the integrin $\alpha 3$, αIIb, αM, and αX subunits. Number in the box, exon number; solid box, metal-binding sequence.

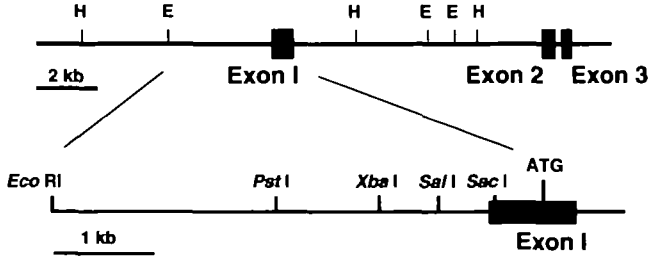

Fig. 4. **Strucuture of the 5′-flanking region of the mouse integrin $\alpha 3$ subunit gene.** The map (upper line) shows the cloned region with the positions of the exons and the restriction sites for *Hin*dIII (H) and *Eco*RI (E). The restriction map for the 5′-flanking region (lower line) is also shown at a higher magnification. The translation initiation site is indicated by ATG.

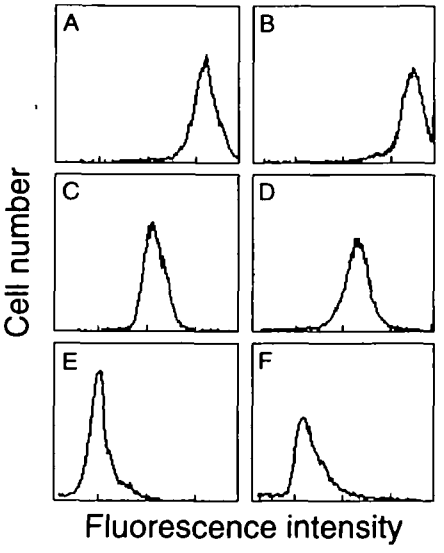

Fig. 5. **Flow cytometric analysis of the expression of integrin $\alpha 3$ in various tumor cell lines.** The cells were stained with a monoclonal anti-integrin $\alpha 3$ antibody (SM-T1) and FITC-labeled anti-mouse IgG antibody, and analyzed by flow cytometry (Cyto ACE-150, Jasco, Tokyo). (A), HT-1080; (B) T24; (C), KATO III; (D), A375; (E), K562; (F), U937.

TABLE II. **Transient expression analysis of integrin $\alpha 3$ subunit gene promoter activity in normal and transformed fibroblasts.**

Cells	Relative luciferase activity[a]
BALB/3T3	6.4
SV-T2	26.4
WI-38	5.3
WI-38 VA13	18.6
NIL	13.9
NILpy	35.5

[a]Luciferase activity induced by the introduction of pGL-ES (a chimeric construct with the upstream 4.0 kb fragment) is expressed in relation to the activity of pGL3-Basic taken as 1.0. Duplicate transfections were performed in each experiment; the variation between duplicates did not exceed 10% of the mean for the experiment.

TABLE III. **Transient expression analysis of integrin α3 subunit gene promoter activity in various tumor cell lines.**

Host cell line		Integrin α3 expression[a]	Relative luciferase activity[b]
Name	Type		
HT-1080	Fibrosarcoma	++	33.1
T24	Bladder carcinoma	++	29.7
KATO III	Gastric carcinoma	+	18.1
A375	Melanoma	+	15.0
K562	Erythroblastoid leukemia	±	3.2
U937	Histiocytic lymphoma	±	4.8

[a]The expression of integrin α3 was measured by flow cytometric analysis using a monoclonal anti-integrin α3 antibody (Fig. 5). [b]Values are expressed in relation to the activity of pGL3-Basic taken as 1.0. Duplicate transfections were performed in each experiment; the variation between duplicates did not exceed 10% of the mean for the experiment.

together, these data indicate that the 5′-flanking region contains sequences that control the expression of the integrin α3 gene. Further characterization of the promoter region and transcription factors involved in the regulation of the integrin α3 gene is necessary to elucidate the mechanism of its expression.

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