# The Major Laminin Receptor of Mouse Embryonic Stem Cells Is a Novel Isoform of the $\alpha_6\beta_1$ Integrin

Helen M. Cooper, Richard N. Tamura, and Vito Quaranta

The Scripps Research Institute, La Jolla, California 92037

*Abstract.* Laminin is the first extracellular matrix protein expressed in the developing mouse embryo. It is known to influence morphogenesis and affect cell migration and polarization. Several laminin receptors are included in the integrin family of extracellular matrix receptors. Ligand binding by integrin heterodimers results in signal transduction events controlling cell motility. We report that the major laminin receptor on murine embryonic stem (ES) cells is the integrin heterodimer $\alpha_6\beta_1$, an important receptor for laminin in neurons, lymphocytes, macrophages, fibroblasts, platelets and other cell types. However, the cytoplasmic domain of the ES cell $\alpha_6$ ($\alpha_{6B}$) differs totally from the reported cytoplasmic domain amino acid sequence of $\alpha_6$ ($\alpha_{6A}$). Comparisons of $\alpha_6$ cDNAs from ES cells and other cells suggest that the $\alpha_{6A}$ and $\alpha_{6B}$ cytoplasmic domains derive from alternative mRNA splicing. Anti-peptide antibodies to $\alpha_{6A}$ are unreactive with ES cells, but react with mouse melanoma cells and embryonic fibroblasts. When ES cells are cultured under conditions that permit their differentiation, they become positive for $\alpha_{6A}$, concurrent with the morphologic appearance of differentiated cell types. Thus, expression of the $\alpha_{6B}\beta_1$ laminin receptor may be favored in undifferentiated, totipotent cells, while the expression of $\alpha_{6A}\beta_1$ receptor occurs in committed lineages. While the functions of integrin $\alpha$ chain cytoplasmic domains are not understood, it is possible that they contribute to transferring signals to the cell interior, e.g., by delivering cytoskeleton organizing signals in response to integrin engagement with extracellular matrix ligands. It is therefore reasonable to propose that the cellular responses to laminin may vary, according to what $\alpha$ subunit isoform ($\alpha_{6A}$ or $\alpha_{6B}$) is expressed as part of the $\alpha_6\beta_1$ laminin receptor. The switch from $\alpha_{6B}$ to $\alpha_{6A}$, if confirmed in early embryos, could then be of striking potential relevance to the developmental role of laminin.

In mammalian embryonic development, morphogenic events are governed by several primary cellular processes including cell–cell and cell–substratum interactions, direction-specific migrations, and regulated proliferative events. These phenomena may be influenced by the extracellular matrix (ECM)[1] components in the immediate environment of the pluripotent embryonic cells (Ekblom et al., 1986; Edelman, 1988; Thiery, 1989).

A specialized ECM, the basement membrane, is the first ECM to appear during mammalian embryogenesis (Martin and Timpl, 1987). The major component of all basement membranes, laminin (Ln), is comprised of three large polypeptides (A, B1, and B2), and appears to influence cell behavior in a variety of ways (Ekblom et al., 1986; Martin and Timpl, 1987). To date, Ln has been shown to mediate cell adhesion, spreading and migration, proliferation, neurite outgrowth, and may also be effective in stimulating cellular differentiation (Edgar et al., 1984; Aumailley et al., 1987; Martin and Timpl, 1987; Klein et al., 1988; Goodman et al., 1989; Panayotou et al., 1989; Vukicevic et al., 1990). Ln is the first ECM component to be expressed in the developing mammalian embryo and is expressed widely throughout the embryonic tissues thereafter (Cooper and MacQueen, 1983; Ekblom et al., 1986). It is markedly enriched in regions of the embryo where epithelial histogenesis is occurring and is believed to be responsible for initiating and maintaining the polarized state of epithelial cells (Klein et al., 1988; Sorokin et al., 1990). It has been demonstrated that Ln is directly involved in the conversion of mesenchyme to epithelium in the developing murine kidney. Initially it was observed that Ln B chains were constitutively expressed in kidney mesenchyme while A chain expression was coincident with the onset of cell polarization as the cells converted to an epithelial phenotype (Klein et al., 1988). Moreover, addition of antibodies, specific for the distal end of the long arm of Ln (the E8 fragment), was found to inhibit the polarization of developing epithelial cells during conversion in embryonic kidney organ cultures (Klein et al., 1988). Thus, it appears that Ln is essential for epithelial development during kidney embryogenesis and that the timing of this event is orchestrated by the onset of Ln A chain synthesis.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; ES, embryonic stem; FN, fibronectin; LIF, leukemia inhibitory factor; Ln, laminin; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; Vn, vitronectin.

Over recent years a large family of cell surface receptors for many ECM components have been identified and termed integrins (Hynes, 1987; Ruoslahti, 1988; Albelda and Buck, 1990; Hemler, 1990; Springer, 1990). These receptors mediate cell adhesion and migration in a specific fashion via selected interactions with ECM components. Evidence is now accumulating that engagement of ligand by the integrin heterodimers results in signal transduction events leading to cell motility, proliferation, or activation (Hemler, 1990; Springer, 1990). Although there are several members of the integrin family that have been characterized as Ln receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$), most have been shown to have multiple specificities for other ECM components (Hemler, 1990). However, the $\alpha_6\beta_1$ integrin is specific only for Ln (Sonnenberg et al., 1988). Furthermore, the binding site of $\alpha_6\beta_1$ on Ln has been identified and is located within the E8 proteolytic fragment derived from the long arm of the Ln molecule (Hall et al., 1990; Sonnenberg et al., 1990). Recent evidence now suggests that the $\alpha_6\beta_1$ integrin may play a key role in mediating the effects of Ln during embryogenesis. Using the murine embryonic kidney mesenchyme/epithelial conversion model described above, Sorokin et al. (1990) demonstrated that expression of the $\alpha_6$ subunit and the Ln A chain was coordinately regulated at the point in development at which the nonpolarized mesenchymal cells were converting to polarized epithelial cells. In addition, in organ cultures, a monoclonal antibody specific for the $\alpha_6$ subunit also inhibited epithelial development (Sorokin et al., 1990). Therefore, the $\alpha_6\beta_1$ integrin plays an integral role in the induction of polarity during the differentiation of embryonic kidney mesenchyme into epithelium. Since Ln is expressed widely throughout the embryo, a general role may be hypothesized for Ln and its receptor, $\alpha_6\beta_1$, in many of the morphological events leading to the development of cell polarity during embryogenesis.

Thus, it is likely that Ln, and therefore $\alpha_6\beta_1$, are also directly involved in the events of preimplantation development. As was observed for the developing kidney epithelium, the Ln A, B1 and B2 chains are noncoordinately expressed. While both the B1 and B2 chains can be detected by immunohistochemistry at the 4-cell stage, the A chain polypeptide is not detected until the 8- to 16-cell stage after compaction has begun (Cooper and MacQueen, 1983).

Mouse embryonic stem cells (ES cells) are continuous cell lines that outgrow from the inner cell mass of cultured preimplantation mouse embryos, and that maintain the potential to support normal development of embryonic and extraembryonic structures when reinjected into blastocysts and implanted (Robertson et al., 1986). Here we demonstrate that the integrin, $\alpha_6\beta_1$, is the major Ln receptor on ES cells. We further show that the $\alpha_6$ subunit of this receptor is expressed in ES cells as an isoform with a cytoplasmic domain structurally distinct from that previously described for $\alpha_6$. In addition, in vitro differentiation of the pluripotent ES cells is accompanied by the induction of expression of $\alpha_6$ chains with the conventional cytoplasmic domain.

## Materials and Methods

### Cell Lines

The ES cell line, CCE (Schwartzberg et al., 1989) was initially cultured on murine embryonic fibroblasts (STO cells) to prevent differentiation. To study the expression and function of integrins in this ES cell line it was necessary to remove the STO cells from the culture system. Therefore, the CCE ES cell line was subcloned into leukemia inhibitory factor (LIF) ($10^3$ units/ml) (Amrad Co., Victoria, Australia) containing media (DMEM; 10% FCS, 100 $\mu$M $\beta$-mercaptoethanol, 2 mM glutamine). LIF has been shown to prevent ES cell differentiation (Moreau et al., 1988; Smith et al., 1988; Williams et al., 1988). The sublines were cultured on gelatin (0.1%) coated plates. Several subclones were expanded and continually cultured in LIF containing media. The subline ES1 was chosen for the studies described here. The D3 embryonic stem cell line was derived by Doetschman et al. (1985). D3 cells were cultured in LIF containing medium as described above except that 15% FCS was used. ES1 and D3 cells were allowed to differentiate on gelatin (0.1%) coated plates over a period of 8-9 d in the absence of LIF.

The murine B16Fl melanoma line was derived from a C57Bl/6 melanoma and cultured in DMEM, 5% FCS, 2 mM glutamine and penicillin-streptomycin (50 IU/ml-50 $\mu$g/ml).

### Antibodies and Extracellular Matrix Components

The rabbit polyclonal anti-$\alpha_{6A}$ cytoplasmic domain antiserum (6844) was raised against the last 15 amino acids (IHAQPSDKERLTSDA) of the reported human $\alpha_6$ sequence (Tamura et al., 1990), while the 382 antisera was raised to a synthetic peptide from the carboxy terminus of human $\alpha_{6B}$ (KDEKYIDNLEKKQWITKWNRNESYS) (Tamura et al., 1991). An additional cysteine residue was included at the $NH_2$ terminus for coupling peptides to a protein carrier (keyhole lympet hemocyanin) for immunization. The rat monoclonal antibody, GoH3, is specific for an extracellular epitope on both the human and murine $\alpha_6$ subunit (Sonnenberg et al., 1987). The isotype matched control antibody, B3B4, recognizes the B lymphocyte specific antigen, CD23. The anti-$\alpha_6$ specific monoclonal antibody, 135.13c, and the control antibody, 439.9b, specific for the human $\beta_4$ integrin subunit, have been described (Kennel et al., 1989). Anti-peptide antisera to the cytoplasmic domains of rat $\alpha_1$, chicken $\alpha_3$, human $\alpha_4$, human $\alpha_5$, and human $\beta_1$ sequences were shown to be cross-reactive with the respective mouse $\beta_1$ integrins by immunoprecipitation of B16Fl melanoma, STO fibroblast, and MMT carcinoma murine cell lines.

Human fibronectin (Fn), human vitronectin (Vn) and human type IV collagen (Col IV) were purchased from Telios (La Jolla, CA). Murine laminin (Ln) and the basement membrane preparation, Matrigel, were obtained from Collaborative Research, Inc., Waltham, MA.

### Cell Adhesion Assays

Cell adhesion assays were carried out as follows. Wells of 96-well plates (Linbro/Titertek; Flow Laboratories, Inc., McLean, VA) were coated with a variety of extracellular matrix components at a concentration of $1 \times 10^{-8}$ M over a period of 16–18 h at 4°C. The wells were then washed twice with Dulbecco's PBS containing $Mg^{2+}$ and $Ca^{2+}$ (DPBS) (Flow Laboratories, Inc.) and blocked for 30 min with 0.1% BSA in DPBS. After a final wash with DPBS, $2 \times 10^5$ cells were added per well and incubated for the given period of time at 37°C. Cells were added to the wells in 100 $\mu$l of DMEM containing 1% FCS, $10^3$ units/ml of LIF, 100 $\mu$M $\beta$-mercaptoethanol, 2 mM glutamine, penicillin-streptomycin (50 IU/ml-50 $\mu$g/ml), and 80 $\mu$g/ml gentamicin. In the case of antibody inhibition assays, $1 \times 10^5$ cells (50 $\mu$l per well; DMEM, 2% FCS, $2 \times 10^3$ units/ml LIF, 200 $\mu$M $\beta$-mercaptoethanol, 4 mM glutamine, penicillin-streptomycin (100 IU/ml-100 $\mu$g/ml), 160 $\mu$g/ml gentamicin) were plated per well and incubated for 8 h at 37°C in the presence of the diluted antibody supernatants (50 $\mu$l/well). Nonadherent cells were removed from wells by washing twice with DPBS. Adherent cells were fixed for 5 min in 3% paraformaldehyde, 2% sucrose at room temperature. Adherent cells were stained with 0.5% crystal violet in 20% methanol. The plates were then dried, the stain solubilized in 0.1 M citric acid in 50% ethanol (pH 4.2) and the OD at 550 nm determined using an EIA Elisa reader (Bio-Tek Instruments, Inc., Burlington, VT). Nonspecific adherence was determined by plating cells on BSA coated wells for the appropriate period of time. Specific adhesion to a given substrate was calculated by subtracting OD (550 nm) obtained with cells plated on BSA from that observed for cells plated on specific substrate. For adhesion inhibition assays with antibodies, results are expressed as "% Maximum Adhesion," where maximum adhesion represents the OD(550 nm) of cells plated in the absence of any antibody, minus nonspecific adhesion on BSA.

### Cell Labeling and Immunoprecipitations

Undifferentiated ES cells (1–2 $\times$ $10^7$ cells) were surface labeled with

$Na^{125}I$ using the lactoperoxidase procedure (Roth, 1975). Differentiated ES cells proved to be significantly more fragile than undifferentiated ES cells and did not survive the more rigorous washing steps required during the iodination procedure. Therefore, differentiated ES cells were metabolically labeled with [$^{35}$S]methionine as described previously (Kajiji et al., 1989). Preparation of nonionic detergent cell lysates, immunoprecipitations and analysis by SDS-PAGE were performed as described by Kajiji et al. (1989).

### Flow Cytometry

ES1 cells were detached using 10 mM EDTA while in log phase of growth. $1 \times 10^6$ cells were stained with a 1:100 dilution of ascites from the $\alpha_6$ specific monoclonal antibody, 135.13c, or the isotype-matched control antibody, 439.9b, for 30 min at 4°C. After washing three times with DPBS, cells were incubated in the presence of the secondary anti-rat IgG antibody labeled with fluorescein isothiocyanate (Boehringer Mannheim Biochemicals, Indianapolis, IN) (30 min, 4°C). Cells were then washed three times with DPBS and fixed with 0.1% paraformaldehyde in DPBS before analysis. Cells were analyzed on a FACS® 440 (a registered trademark of Becton Dickinson and Company for a fluorescence-activated cell sorter).

### Cloning of $\alpha_6$ cDNA Fragments by Polymerase Chain Reaction (PCR) and cDNA Sequencing

Poly(A)$^+$ RNA was isolated from both differentiated and undifferentiated cell lines using the Invitrogen Fastrack Kit (Invitrogen, La Jolla, CA). Single-stranded cDNA was then synthesized from 10 $\mu$g of mRNA using AMV reverse transcriptase (20 U; Molecular Genetics Resources, Tampa, FL) and 1 $\mu$g of random hexamer primers (Pharmacia Inc., Piscataway, NJ). The cDNAs were then ethanol precipitated and resuspended in 50–70 $\mu$l of water. 1 $\mu$l of cDNA was amplified per 50 $\mu$l PCR reaction mixture (2.5 mM $MgCl_2$, 50 mM KCl, 10 mM $\beta$-mercaptoethanol, 66 mM Tris-HCl, pH 8.3) using 0.1 $\mu$M oligonucleotide primers, 0.25 mM each of dATP, dTTP, dCTP, and dGTP, and 1.25 U of Taq 1 polymerase (AmpliTaq; Perkin-Elmer Corp., Cetus, CA). The PCR program consisted of two steps. (*a*) 40 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C with a 5 s/cycle extension on the 72°C segment; (*b*) 10 min at 72°C and a final shift to 4°C. Nested pairs of PCR primers were used to ensure that $\alpha_6$ specific fragments were amplified. Both sets were derived from the human cDNA sequence as determined by Tamura et al. (1990). The first set corresponded to basepairs 2,918–2,937 (primer 1,157) and 3,454–3,473 (primer 1,156) of the human $\alpha_6$ sequence while the nested primer pair corresponded to basepairs 2,942–2,960 (primer 1,681) and 3,433–3,452 (primer 2,002). Second round PCR was carried out on 1 $\mu$l of the reaction mixture generated from the first round PCR.

Amplified $\alpha_{6A}$ and $\alpha_{6B}$ fragments from first round PCR (primer pair 1,156/1,157) were purified using GeneClean (Bio 101, La Jolla, CA), treated with DNA polymerase I and T4 polynucleotide kinase, again purified with Gene Clean, and subcloned into Bluescript-pKS+ (Stratagene, La Jolla, CA). Positive clones were sequenced manually (Sequenase kit; U.S. Biochemical Corp., Cleveland, OH) using T3 and T7 polymerase vector primer sequences. Sequences were analyzed on a VAX-VMS, version 5.2 computer, with programs of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

## Results

### The Major Laminin Receptor of ES Cells Is the $\alpha_6\beta_1$ Integrin

A variety of embryonic stem cell lines (ES cells) have been described (Baribault and Kemler, 1989) and proposed as model systems with which to study morphogenesis in early development (Doetschman et al., 1985; Rossant, 1990). Therefore, to explore the role of $\alpha_6\beta_1$ in preimplantation mouse embryos, we have examined the expression and function of this Ln receptor in ES cells.

Initially, the adhesive phenotype of the ES1 cell line was determined. Cell adhesion assays were carried out in 96-well tissue culture plates which had been coated with various ECM components. ES1 cells ($2 \times 10^5$ cells/well) were incubated for the indicated time and the number of adherent cells quantitated by staining with crystal violet after fixing with paraformaldehyde. ES1 cells displayed strong avidity for Ln, Fn, and Matrigel (a basement membrane extract containing Ln, Fn, collagen IV, and proteoglycans) after 2 h and maintained this level of adhesion over the full 24-h incubation period (Fig. 1). In contrast, no adhesion to vitronectin or type IV collagen was observed.

To determine whether the ES1 cells expressed the integrin Ln receptor, $\alpha_6\beta_1$, immunoprecipitations were carried out on detergent lysates from ES1 cells surface labeled with $^{125}$I. Autoradiographs of 5% SDS-PAGE gels revealed that the $\alpha_6$ subunit-specific monoclonal antibodies GOH3 (Fig. 2) and 135.13c (not shown) precipitated two proteins of 130 and 150 kD under non-reducing conditions. The mobilities of these protein bands corresponded to those expected for the integrin $\beta_1$ and $\alpha_6$ subunits, respectively, indicating that the $\alpha_6\beta_1$ heterodimer was present at the cell surface.

Fluorescence-activated flow cytometry demonstrated that the 135.13c monoclonal antibody brightly stained a single homogeneous population of ES1 cells (Fig. 3). Thus, the ES1 cell line is comprised of a single homogeneous population of $\alpha_6\beta_1$ positive cells.

Immunoprecipitations of $^{125}$I surface-labeled ES1 cell lysates with antisera to peptides corresponding to the cytoplasmic domains of the integrin subunits $\alpha_1$, $\alpha_3$, $\alpha_4$, $\alpha_5$, and $\beta_1$ revealed that ES1 cells also expressed the $\alpha_5\beta_1$ and $\alpha_3\beta_1$ integrins on their surface (data not shown). No other $\beta_1$ integrins were detectable in these immunoprecipitation experiments (note, however, that no anti-mouse $\alpha_2$ antibodies were available). The integrin, $\alpha_5\beta_1$, has been shown to be specific for Fn only (Ruoslahti, 1988), while $\alpha_3\beta_1$ has been demonstrated to have multiple specificities for Fn, Ln, and collagen (Elices et al., 1991). Therefore, the specificity pattern of ES1 cell adhesion observed in Fig. 1 correlated well with the expression of $\alpha_6\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$.

Cell adhesion assays were carried out in the presence of the monoclonal antibody, GoH3, which is specific for the $\alpha_6$ subunit. Fig. 4 demonstrates that GoH3 culture supernatant, at a dilution of 1:50, inhibited ES1 cell adhesion to Ln by >95% compared to control. In contrast, at the same concentration, GoH3 inhibited ES1 cell adhesion to Fn by <20% of control. The control was the isotype-matched antibody B3B4, which had little or no effect on ES1 cell adhesion to either Ln or Fn, compared to wells with no antibody. Specific inhibition of Ln adhesion by GoH3 was reproducibly observed in five independent assays, indicating that $\alpha_6\beta_1$ is the major integrin, if not the only one, used as a Ln receptor by ES1 cells. This would imply that the $\alpha_3\beta_1$ integrin does not function as a Ln receptor on these cells, at least in our culture and/or assay system.

### Embryonic Stem Cells Express a Structurally Different Form of the $\alpha_6$ Integrin

Immunoprecipitations of $^{125}$I-labeled ES1 lysates revealed that a polyclonal rabbit antiserum (6844), raised against the last 15 amino acid residues of the human $\alpha_6$ subunit, did not precipitate the $\alpha_6\beta_1$ heterodimer from ES1 cell lysates (Fig. 2). However, this antiserum did precipitate $\alpha_6\beta_1$ from $^{125}$I-labeled lysates of a murine melanoma line (B16Fl) (Fig. 2) indicating that this antiserum did cross-react with the murine $\alpha_6$ subunit. In addition, the polyclonal anti–cytoplas-

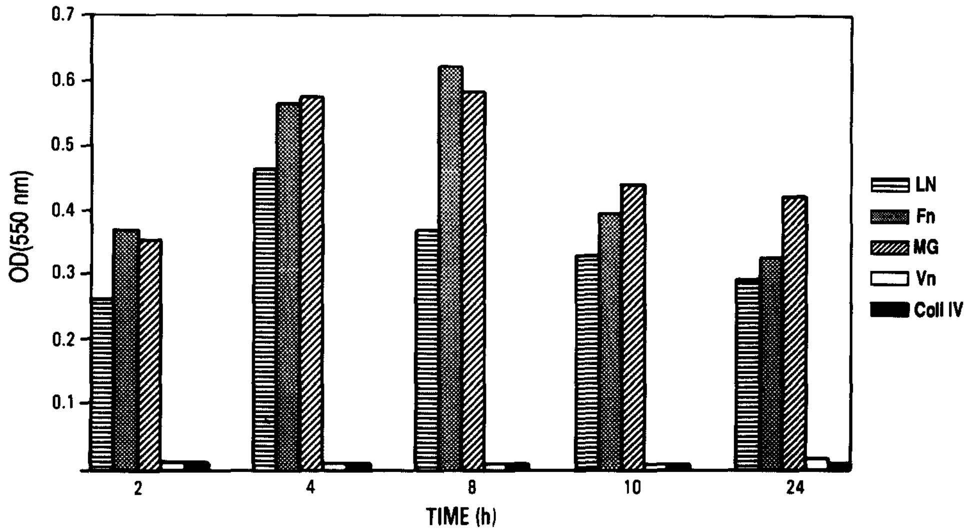


*Figure 1.* Time course of ES1 cell adhesion to extracellular matrix components. Cells were incubated in wells coated with laminin (*LN*), fibronectin (*Fn*), Matrigel (*MG*), vitronectin (*Vn*), or type IV collagen (*Coll IV*) for the indicated length of time. Adherent cells were fixed and then quantitated by staining with crystal violet followed by solubilization and determination of the optical density at 550 nm in an automated ELISA reader.

mic domain antiserum also precipitated the $\alpha_6$ subunit from both a murine embryonic fibroblast line (STO) and a murine mammary tumor cell line (MMT) (data not shown). Immunoprecipitations were also performed on $^{125}$I-labeled lysates from the D3 ES cell line (Doetschman et al., 1985). As was observed for ES1 lysates, the GoH3 monoclonal antibody, but not the 6844 polyclonal antiserum, immunoprecipitated the $\alpha_6\beta_1$ heterodimer (not shown). Therefore, the $\alpha_6$ subunit expressed by ES cells lacked the epitope within the $\alpha_6$ cytoplasmic domain that is recognized by the 6844 antiserum.

To investigate possible structural differences within the cytoplasmic domain of the $\alpha_6$ subunits expressed by ES1 and B16F1 cells, amplification of $\alpha_6$ cDNA by reverse transcription–polymerase chain reaction (RT-PCR) was carried out

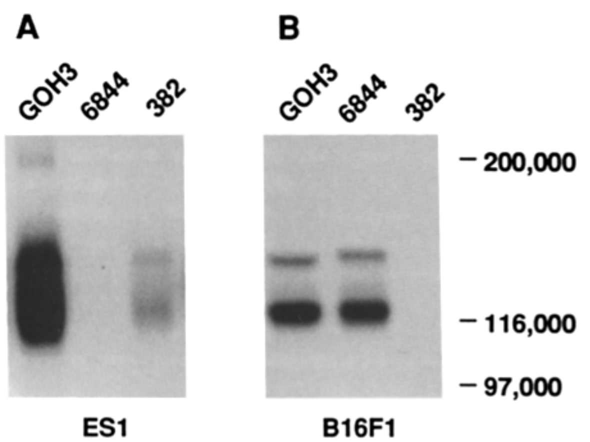


*Figure 2.* Immunoprecipitations of $\alpha_6\beta_1$ from $^{125}$I-labeled detergent lysates of the embryonic stem cell lines ES1 (*A*) and the murine melanoma line, B16F1 (*B*) using the monoclonal antibody GoH3, specific for an epitope on the extracellular domain of $\alpha_6$ or the polyclonal antisera, 6844, specific for the cytoplasmic domain of $\alpha_{6A}$ and 382, specific for the cytoplasmic domain of $\alpha_{6B}$. Immunoprecipitations were analyzed by 5% SDS-PAGE under nonreducing conditions. The upper band (~150,000 D) corresponds to $\alpha_6$, the lower band (~130,000 D) corresponds to $\beta_1$. Molecular mass markers on the right are in daltons.

on mRNA from these cells. A nested set of PCR primers (primer pairs 1,157/1,156 and 1,681/2,002), derived from the human $\alpha_6$ sequence (Tamura et al., 1990), were used to ensure specificity of the reaction. Fig. 5 *a* shows the PCR products obtained. The RT-PCR fragment amplified from B16F1 melanoma mRNA corresponded to the size expected (510 bp) for the murine homologue of the human $\alpha_6$ (Fig. 5 *a*, lane 2). However, the PCR fragment obtained from the amplification of the ES1 cell cDNA was smaller (~380 bp). Additional amplifications from four independent ES1 mRNA preparations yielded only the 380-bp fragment and never the larger fragment amplified from B16F1 melanoma.

The RT-PCR fragments from the ES1 and B16F1 cells were subcloned and sequenced. The sequence of the larger B16F1 fragment (Fig. 6) was 89% identical to the human $\alpha_6$ sequence (Tamura et al., 1990) at the nucleotide level and 91% identical at the amino acid level, indicating that it likely represents the murine homologue of the $\alpha_6$ subunit. The B16F1 RT-PCR fragment (Fig. 6) encoded the carboxyterminal portion of the extracellular domain as well as the transmembrane and cytoplasmic domains of the $\alpha_6$ subunit.

The sequence of the smaller RT-PCR fragment (Fig. 6) was identical to the B16F1 sequence except that an internal deletion of 130 bp was observed. Tamura et al. (1991) have recently described a second form of the human $\alpha_6$ subunit mRNA ($\alpha_{6B}$) in which the segment encoding the cytoplasmic domain of the published sequence ($\alpha_{6A}$) was absent. In-

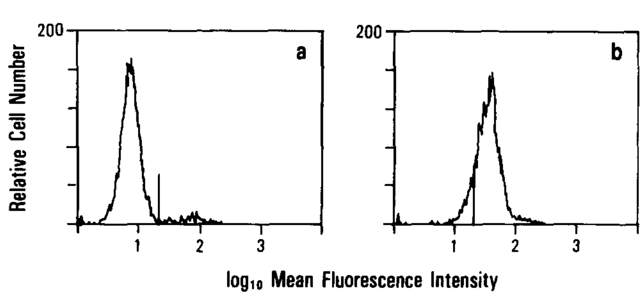


*Figure 3.* Fluorescence-activated flow cytometry of ES1 cells labeled with (*a*) the isotype-matched control monoclonal antibody, 439.9b, or (*b*) the $\alpha_6$ subunit-specific monoclonal antibody, 135.13c.

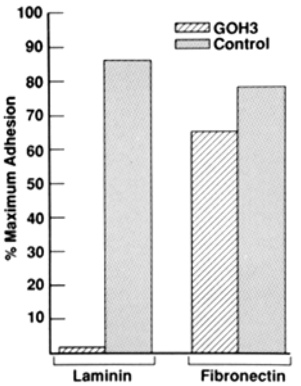


*Figure 4.* Inhibition of ES1 cell adhesion to laminin. ES1 cells were incubated in laminin- or fibronectin-coated wells in the presence of the anti–$\alpha_6$ monoclonal antibody, GoH3, or the isotype-matched antibody, B3B4. After 8 h incubation, cells were fixed, stained, solubilized, and quantitated by determining optical density at 550 nm. Results are expressed as percentage of maximum adhesion, which is represented by wells in which no antibody was added. Antibodies were used as 1:50 dilution of hybridoma supernatant (~0.2 μg/ml). Control antibody was the rat monoclonal B3B4.

stead, a reading frame from the 3′ untranslated region of the $\alpha_{6A}$ sequence encoded a novel cytoplasmic domain. The human $\alpha_{6B}$ cDNA was found to be 130 bp shorter than the $\alpha_{6A}$ isoform. The location of the 130-bp deletion observed in the ES1 $\alpha_6$ PCR fragment exactly matched that of the human $\alpha_{6B}$ sequence. Therefore, it appeared that ES1 cells could express the murine equivalent of the $\alpha_{6B}$ isoform. To verify this possibility, immunoprecipitations were carried out with an antiserum (382) raised against a synthetic peptide corresponding to the sequence of the cytoplasmic tail of human $\alpha_{6\beta}$.

Fig. 2 shows that antiserum 382 precipitated protein bands virtually identical to those reactive with anti–$\alpha_6$ monoclonal GOH3, indicating that ES1 cells do express $\alpha_{6B}$ protein,

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 1157 →
gactcttaactgtagcgtgaacgtgaggtgtgtgaacatcaggtgcccactgcgagggct   2976
gactcttaactgtagcgtgaacgtgaggtgtgtgaacatcaggtgcccactgcgagggct
 T  L  N  C  S  V  N  V  R  C  V  N  I  R  C  P  L  R  G  L

ggacagcaaggcctctctcgttcttcgttccaggttgtggaacagcacatttctagagga   3036
ggacagcaaggcctctctcgttcttcgttccaggttgtggaacagcacatttctagagga
 D  S  K  A  S  L  V  L  R  S  R  L  W  N  S  T  F  L  E  E

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atattccaaactgaactacttggacattctcctgagggcttccatagatgtcaccgctgc
 Y  S  K  L  N  Y  L  D  I  L  L  R  A  S  I  D  V  T  A  A

tgctcagaatatcaagctcctcaccgccggcactcaggttcgagtgacggtgtttccctc   3156
tgctcagaatatcaagctcctcaccgccggcactcaggttcgagtgacggtgtttccctc
 A  Q  N  I  K  L  L  T  A  G  T  Q  V  R  V  T  V  F  P  S

aaagactgtagctcagtattcaggagtagcttggtggatcatcctcctggctgttcttgc   3216
aaagactgtagctcagtattcaggagtagcttggtggatcatcctcctggctgttcttgc
 K  T  V  A  Q  Y  S  G  V  A  W  W  I  I  L  L  A  V  L  A

cgggattctgatgctggctctattagtgtttttactgtggaa..................   3276
cgggattctgatgctggctctattagtgtttttactgtggaagtgtggcttcttcaagag
 G  I  L  M  L  A  L  L  V  F  L  L  W  K [C  G  F  F  K  R]

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aaataagaaagatcattacgatgccacctatcacaaggctgagatccatactcagccgtc
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tgataaagagaggcttacttccgatgcatagtattgatctacttccataattgtgtggat
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tctttaagcgctctaggtacgatgacagcattccccgataccatgcggtgcggatccgga   3456
tctttaagcgctctaggtacgatgacagcattccccgataccatgcggtgcggatccgga
 F  K  R] S  R  Y  D  D  S  I  P  R  Y  H  A  V  R  I  R  K
       1156
aagaagagcgagagat   3516
aagaagagcgagagat
  E  E  R  E
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*Figure 6.* Nucleotide sequence of the $\alpha_6$ fragments (see Fig. 5) amplified by RT-PCR from ES1 (top line) and B16F1 (bottom line). The arrows indicate the position of the 1,157 and 1,156 primers. The numbers at the right correspond to the published human $\alpha_6$ sequence (Tamura et al., 1990). The gap in the ES1 $\alpha_{6B}$ sequence (top line) is marked with a dotted line. Translation is shown under the nucleotide sequences, in the one letter amino acid code. The asterisk indicates a stop codon. The sequence GFFKR, which is conserved in all integrin α chain cytoplasmic domains, is in shaded boxes.

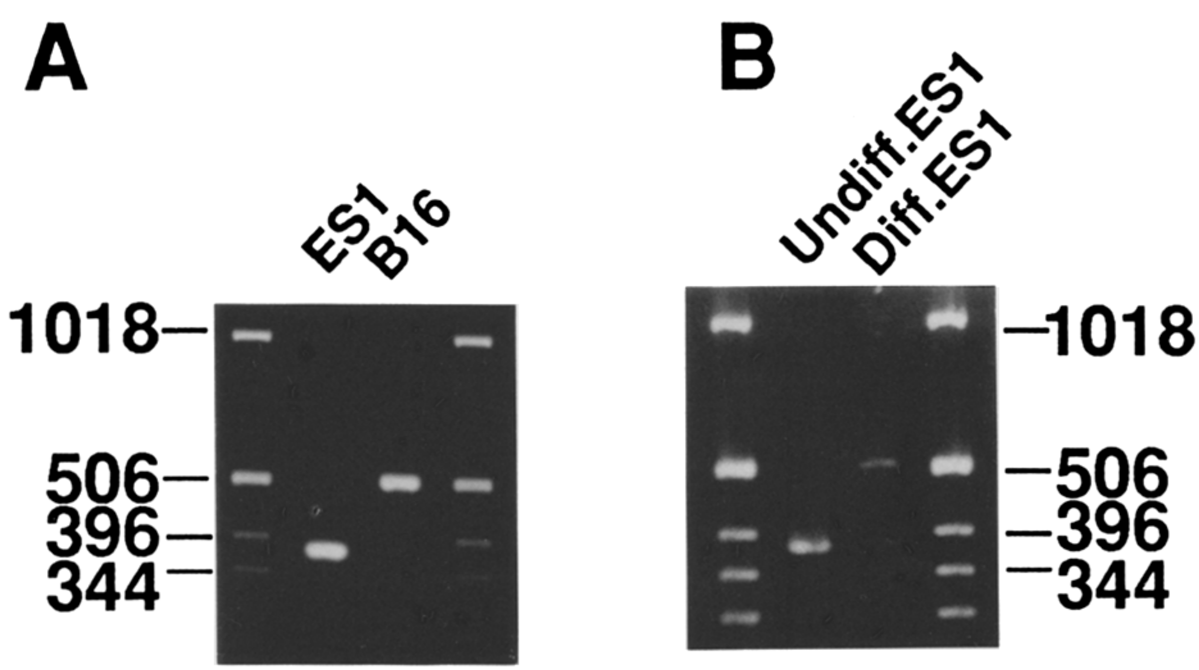


*Figure 5.* RT-PCR amplification of (*A*) ES1 and B16F1 mRNAs and (*B*) undifferentiated and differentiated ES1 mRNAs using the $\alpha_6$ specific primer pairs 1157/1156 (see Fig. 6 for position of these primers in the $\alpha_6$ sequence), and the nested primer set 1681/2002. The upper band (~510 bp) derives from $\alpha_{6A}$ mRNA, the lower band (~380 bp) derives from $\alpha_{6B}$ mRNA. See text for further explanations. Size of migration standards (outer lanes) is indicated in basepairs.

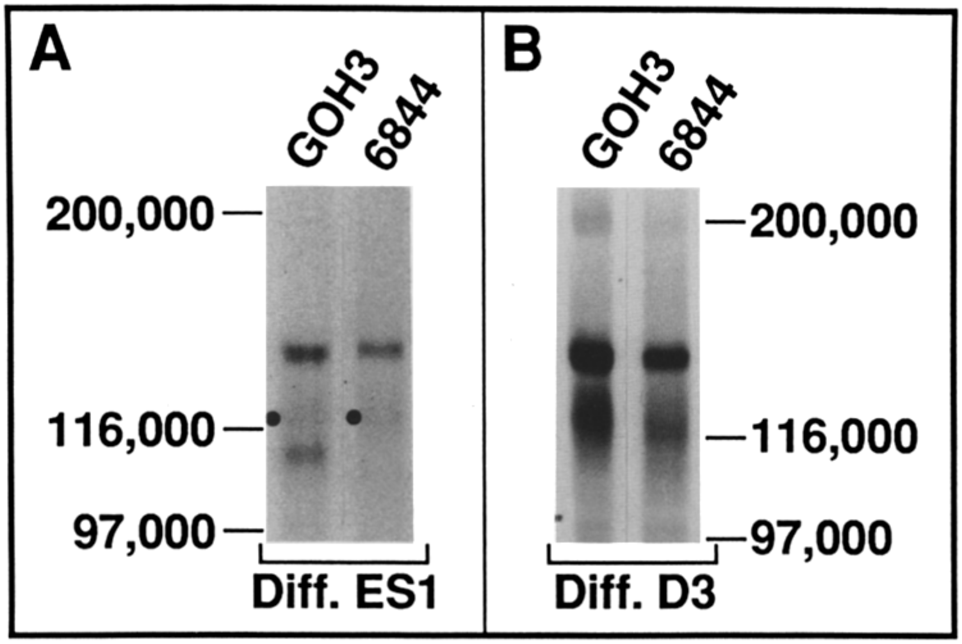


*Figure 7.* Immunoprecipitations of $\alpha_{6A}\beta_1$ and $\alpha_{6B}\beta_1$ from $^{35}$S-labeled detergent lysates of differentiated embryonic stem cell lines ES1 (*A*) and D3 (*B*) using the monoclonal antibody, GoH3, specific for $\alpha_6$, and the polyclonal antiserum, 6844, specific for the cytoplasmic domain of $\alpha_{6A}$. Immunoprecipitations were analyzed by 5% SDS-PAGE under nonreducing conditions. The upper band (~150,000 D) corresponds to $\alpha_6$, while the lower band (~130,000 D) corresponds to $\beta_1$. Note that in ES1 cells an immature form of $\beta_1$ (~105,000 D) preferentially incorporates [$^{35}$S]methionine under the radiolabeling conditions used. The position of mature $\beta_1$ is indicated by black dots. For unknown reasons, immature $\beta_1$ was consistently found in GOH3, but not 6844 immunoprecipitates, while mature $\beta_1$ is present in immunoprecipitations with either antibodies. Molecular mass markers on the right are in daltons.

probably complexed with $\beta_1$. Similar results were obtained with another ES cell line, D3 (not shown). Note that antiserum 382 is unreactive with the melanoma cell line B16F1, which expresses instead the $\alpha_{6A}$ isoform (Figs. 2 and 5). Conversely, anti-$\alpha_{6A}$ rabbit antiserum 6844 is negative with ES1 cells and positive with B16F1.

Bands immunoprecipitated by 382 antiserum are much weaker than those precipitated by GOH3 (Fig. 2). This, however, is probably due to low affinity of 382 for mouse $\alpha_{6B}$. Depleting ES1 lysates by four rounds of immunoprecipitations with 382 almost completely removed reactivity of GOH3, while depleting the lysates with normal rabbit serum had no effect (not shown). It is therefore unlikely that other forms of $\alpha_6$ (i.e., carrying the GOH3 but not the 382 epitope) are expressed in ES1 cells, although better reagents are necessary to rule out this possibility formally.

### *The Expression of the $\alpha_{6A}$ Isoform Is Initiated upon Differentiation of Pluripotent Cells*

To determine whether the expression of $\alpha_{6A}$ could be initiated upon differentiation, ES1 cells were allowed to differentiate over a period of 8–9 d in the absence of LIF. The morphology of the differentiated cells was dramatically different from that of undifferentiated ES1 cells maintained in LIF. Primary and nested PCRs were then carried out as described above. As expected, PCRs on cDNA from undifferentiated ES1 cells, using $\alpha_6$ specific primers, produced the 380-bp fragment corresponding to the $\alpha_{6B}$ cytoplasmic sequence (Fig. 5 *b*, lane *1*). However, similar amplification of cDNA from the differentiated cells produced two distinct fragments of 510 and 380 bp (Fig. 5 *b*, lane *2*) shown by nucleotide sequencing to be the $\alpha_{6A}$ and $\alpha_{6B}$ isoforms, respectively. Moreover, Fig. 7 *a* demonstrates that, in contrast to the immunoprecipitation data from undifferentiated ES1 cells (Fig. 2), the anti-cytoplasmic domain polyclonal antiserum, 6844, could immunoprecipitate the $\alpha_{6A}$ isoform from [$^{35}$S]-methionine-labeled lysates obtained from differentiated ES1 cells or D3 cells. Thus, differentiation of ES cells is accompanied by the induction of expression of the $\alpha_{6A}$ isoform.

## *Discussion*

In this paper we show that the major, if not the only, laminin receptor expressed by mouse ES cells is a member of the integrin family, $\alpha_6\beta_1$. Furthermore, we show that mouse $\alpha_6$ can exist in two versions, $\alpha_{6B}$ and $\alpha_{6A}$, which contain structurally distinct cytoplasmic domains. ES cells express exclusively $\alpha_{6B}\beta_1$ in the undifferentiated, pluripotent state. Upon differentiation, they begin expressing also the $\alpha_{6A}$ isoform of $\alpha_6$.

Murine embryonic stem cells have proven to be a valuable model for studying biochemical and morphological events occurring in early mammalian development. These cells closely resemble the pluripotent cells within the inner cell mass of the blastocyst from which they are derived. Moreover, in vitro differentiation of ES cells parallels the normal developmental events which give rise to all embryonic tissues (Evans and Kaufman, 1981; Doetschman et al., 1985). Furthermore, when these cells are injected into blastocysts, they are capable of contributing to 80–90% of the cells in the resulting chimeric animal, including the germline (Robertson et al., 1986; Baribault and Kemler, 1989). Therefore, ES cells provide a useful model for studying cell–ECM interactions mediated by integrins during early embryogenesis.

The data presented here indicate that integrins are expressed and used by ES cells. In comparison to most cell lines, the ES1 cell line expressed a limited repertoire of $\beta_1$ integrins. Immunoprecipitations of radiolabeled lysates from ES1 cells demonstrated that they express significant levels of the $\alpha_6\beta_1$ Ln receptor on the cell surface (Figs. 2

and 3). They also express the Fn receptor, $\alpha_5\beta_1$, and the multi-specific integrin, $\alpha_3B_1$ (Ln, Fn, and collagen). The expression of integrin Ln and Fn receptors on ES cells is in accordance with immunocytochemical data which shows that by the 8-cell-stage of preimplantation development both Ln and Fn are expressed at significant levels (Fleming and Johnson, 1988; Kimber, 1990). Interestingly, the pluripotent F9 teratocarcinoma line, also used as a model for early embryonic development (Hogan et al., 1981; Grover et al., 1983) expresses the same $\beta_1$ integrin repertoire of $\alpha_6\beta_1$, $\alpha_5\beta_1$, and $\alpha_3\beta_1$ (data not shown). While it is possible that ES cells may express other as yet unidentified integrin heterodimers, their limited integrin repertoire agrees well with their adhesive phenotype. ES1 cells specifically adhere to Ln and Fn but not to vitronectin and type IV collagen (Fig. 1).

We have demonstrated that the $\alpha_6\beta_1$ integrin is the major Ln receptor on ES1 cells (Fig. 4). This is also true for the F9 teratocarcinoma (data not shown). It is likely that the $\alpha_5\beta_1$ integrin is the primary Fn receptor since in most cell types bearing $\alpha_5\beta_1$, it is the predominant Fn receptor (Albelda and Buck, 1990; Hemler, 1990). In contrast, the ECM adhesive activity of $\alpha_3\beta_1$, which is specific for Ln, Fn, and collagen, has been shown to be weak in the presence of other integrins with similar specificity (Elices et al., 1991). It is possible that in ES cells $\alpha_3\beta_1$ may be involved in cell-cell interactions rather than cell-ECM interactions as has been suggested for other cell types (Carter et al., 1990; Larjava et al., 1990). Specific antibodies capable of inhibiting murine $\alpha_3$ and $\alpha_5$ function would be useful in addressing these points. However, such reagents are currently unavailable.

Of major interest is the fact that $\alpha_6\beta_1$ expressed by ES cells possesses a novel $\alpha_6$ cytoplasmic domain that is structurally distinct from that of the published $\alpha_6$ subunit (Tamura et al., 1990). This conclusion is based on several lines of evidence. First, although specific monoclonal antibodies demonstrated that $\alpha_6\beta_1$ was expressed at the cell surface (Figs. 2 and 3), polyclonal antisera specific for the human $\alpha_6$ cytoplasmic domain did not immunoprecipitate $\alpha_6\beta_1$ integrins from ES1 detergent lysates (Fig. 2). Secondly, the nucleotide sequence of $\alpha_6$ cDNA fragments amplified by PCR from ES1 cells contained an internal deletion of 130 bp, as compared to $\alpha_6$ sequences amplified from mouse melanoma cells. The ES1 $\alpha_6$ sequence was found to be equivalent to the human $\alpha_{6B}$ isoform recently described by Tamura et al. (1991). Thirdly, an antiserum to the human $\alpha_{6B}$ cytoplasmic domain reacted with complexes resembling $\alpha_6\beta_1$ from ES1 radiolabeled detergent lysates. Since the same observations were repeated with another ES cell line, D3, it seems plausible to postulate that ES cells, and by inference the pluripotent embryonic cells of the inner cell mass, express exclusively the $\alpha_{6B}$ isoform.

Our data with ES cells grown in the absence of LIF show that, upon differentiation, the other isoform of $\alpha_6$, $\alpha_{6A}$, is induced. This agrees with preliminary findings indicating that many differentiated cell types express $\alpha_{6A}$. However, the fact that ES cells express solely the $\alpha_{6B}$ isoform suggests that this isoform may have specialized functions suited to the dynamics of the cells of the inner cell mass during preimplantation embryogenesis.

Based on their structures, it is likely that the $\alpha_{6B}$ and $\alpha_{6A}$ mRNAs arise by alternative splicing of primary transcripts. The ES cell expression data suggest that this alternative splicing event may be developmentally regulated. A precedent in this regard is the integrin gene $\alpha_{PS2}$, in which an exon encoding part of the extracellular domain is alternatively spliced during fly development (Brown et al., 1989).

The biological consequence of the existence of two $\alpha_6$ isoforms bearing distinct cytoplasmic domains is as yet unknown. It has been demonstrated that integrins provide a physical link between the ECM and the cytoskeletal network of the cell through interactions with talin and $\alpha$-actinin (Horwitz et al., 1986; Otey et al., 1990). Evidence is accumulating that integrin heterodimers also participate in signal transduction events (Sinigaglia et al., 1989; Werb et al., 1989; Matsuyama et al., 1990). Since the cytoplasmic domains of $\alpha_{6A}$ and $\alpha_{6B}$ display no homology at the amino acid level it is possible that they interact with distinct sets of cytoskeletal components. Alternatively, or in addition to differential cytoskeletal interactions, their potential for phosphorylation may be different and therefore may potentiate distinct signalling pathways, or differentially attenuate the same pathway. It has been demonstrated that the $\alpha_6$ subunit can be phosphorylated upon stimulation with phorbol esters (Shaw et al., 1990).

Evidence from several laboratories indicates that many integrins, including $\alpha_6\beta_1$, exist in a low affinity state that can be converted to a high affinity state in response to cell activation events (Dustin and Springer, 1989; Adams and Watt, 1990; Shimizu et al., 1990). Therefore, it is possible that the $\alpha_{6A}\beta_1$ and $\alpha_{6B}\beta_1$ receptors differ in their affinity for Ln, or alternatively, that their affinity for Ln can be differentially modulated.

It is now evident that the interaction between Ln and its integrin receptor, $\alpha_6\beta_1$, plays an integral role in primary morphological transformations during embryogenesis. Sorokin and colleagues (Sorokin et al., 1990) have clearly demonstrated that $\alpha_6\beta_1$ is directly involved in the conversion of embryonic kidney mesenchyme to epithelium by ensuring that the mesenchymal cells undergo the initial process of polarization. Cells of the inner cell mass undergo many similar morphological transformations before and during gastrulation (Fleming and Johnson, 1988; Kimber, 1990). As $\alpha_{6B}\beta_1$ is present in cell lines derived from the cells of the inner cell mass it is likely that this integrin may be involved in polarization events that occur during this time. Also, the first cellular polarization process of embryogenesis occurs during compaction (Fleming and Johnson, 1988; Kimber, 1990), the time at which the Ln A chain is first detected (Cooper and MacQueen, 1983). Thus, $\alpha_{6B}\beta_1$ may also play a role in driving the formation of the blastocyst. Ongoing studies in our laboratory are addressing these questions.

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