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Alternative Transcriptional Initiation and Splicing of Mouse *Lamc2* Message

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To investigate the mechanism of two forms of messages (5.1 and 2.4 kb) in mouse *Lamc2*, a gene encoding for the g2 chain of epithelial cell-specific laminin 5, we analyzed approximately 40 kb of genomic DNA containing the sequences from the first intron to the 23rd exon to the 3¢ untranscribed region. With the 5¢ rapid amplification of cDNA end (RACE) and primer extension technique using RNA from mouse kidney and thymic epithelial cells, we found that the smaller message starts at the 2895th base of the cDNA within the 19th exon. Upstream sequences of this alternative start site showed the promoter activity in the reporter assay with the secreted form of alkaline phosphatase (SEAP). We also identified a novel alternatively spliced exon (exon 19B) that includes two stop codons. These results revealed two possible additional open reading frame that are different from the previously described alternative human g2 peptide. Therefore, the mechanism for generating smaller message and the event of alternative splicing are quite distinct between mouse and human *Lamc2*, although genomic organization is highly conserved. The significance of the presence of alternative message is discussed.

Keywords: Alternative Splicing; Alternative Transcriptional Initiation; Genomic Structure; Laminin 5; Premature Termination Codon; Promoter.

Introduction

The laminin family of heterotrimeric extracelluar matrix (ECM) glycoproteins consists of α, β, and γ chains (Tryggvason, 1993). Each of these chains is encoded by genetically distinct genes (Burgeson *et al*., 1994). Up to now, five α-subunit, three β-subunit, and three γ-subunit genes have been discovered (Koch *et al*., 1999). Combinatorial assembly of each subunit generates separate laminin isoforms, and so far 13 members in the laminin protein family have been reported (Koch *et al*., 1999). All three chains of laminin protein have globular domains separated by multiple epidermal growth factor (EGF)-like domains within short N-terminal arms. Their long-arm portions are composed of heptad repeats typical for α-helical coiled-coil proteins. In addition, the C-terminus of each α chain contains five globular domains (Engel, 1992). The main function mediated by the laminin family is related to cell attachment and migration (Gagnoux-Palacios, 1997; O'Toole, 1997). They also play a role as the extracellular ligands for integrin receptors that participate in cell anchorage and anchorage-dependent signal transduction. Cell migration is also enhanced when the matrix metalloprotease 2 (MMP2) cleaves the γ2 chain in ECM (Giannelli *et al*., 1997).

Laminin 5 consists of α3 (200 kDa), β3 (140 kDa), and γ2 (155 kDa) chains and is the only known isoform using the γ2 chain (Koch *et al*., 1999; Rousselle *et al*., 1991). It

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Abbreviations: ECM, extracelluar matrix; FTOC, fetal thymic organ culture; JEB, junctional form of epidermolysis bullosa; ORF, open reading frame; PTC, premature termination codon; RACE, rapid amplification of cDNA end; SCID, severe combined immunodeficiency; SEAP, secreted form of alkaline phosphatase.

is found in most epithelial basement membranes. From electron microscopy (EM) analysis of skin tissue, it was shown to be a component of hemidesmosome in lamina lucida, where α6β4 integrin co-localizes (Domloge-Hultsch *et al.*, 1992). It also interacts with α3β1 integrin in focal adhesion (Carter *et al.*, 1991; Wayner *et al.*, 1993). Mutation in any of the laminin 5 chains generates the junctional form of epidermolysis bullosa (JEB), a human autosomal recessive disease (Kon *et al.*, 1998; McGrath *et al.*, 1995; Takizawa *et al.*, 1998a; 1998b).

Unlike the γ1 chain, which is expressed in both epithelial and endothelial cells, the γ2 chain is believed to be expressed mostly in epithelial cells (Kallunki *et al.*, 1992; Sugiyama *et al.*, 1995); however, alternatively spliced forms of the message were detected in such non-epithelial tissues as the cortical area of the human brain (Airenne *et al.*, 1996).

Previously, the *Lamc2* cDNA clone that encodes γ2 chain of laminin 5 protein (TSO-2D1: TSO for thymic stroma originated) was isolated from a subtractive PCR-based cDNA library from mouse thymic stromal cells (Kim *et al.*, 1998). Mouse *Lamc2* showed two different message sizes (5.1 and 2.4 kb) in a cell line-specific manner (Kim *et al.*, 2000). The longer message was expressed in the thymus of normal and SCID mice and in 427.1.86 cells (mouse thymic nurse cell line), while the shorter message was also expressed in 1308.1.86 (mouse thymic cortical epithelial cell line) and 1307.6.17 cells (mouse thymic medullary epithelial cell line). In human *LAMC2*, two sizes (5.1 and 4.3 kb) of the messages were reported (Kallunki *et al.*, 1992; Mizushima *et al.*, 1996). These two messages in human are the results of alternative splicing and the different usage of the polyadenylation site. Some mRNA of human *LAMC2* do not go through a normal splicing event between exon 22 and 23 but include the sequences of intron 22 to generate the shorter message; however, the same mechanism does not seem to be applicable to the shorter messages of mouse *Lamc2*, because the lengths of the shorter message in human and mouse are different. In addition, the shorter message was detected only when a 3′ portion of cDNA was used as a probe in Northern blot analysis (Kim *et al.*, 2000). Therefore, either the different kind of alternative splicing from human *LAMC2* or alternative transcriptional initiation is a more feasible mechanism for generation of the second, shorter message of mouse *Lamc2*.

Using *in situ* hybridization, the longer message was seen to localize predominantly to the subcapsulary region (Kim *et al.*, 2000) where only early developing thymocytes are found (Penit, 1986). The signal is distributed not only in the area of the basement membrane but extends to a deeper subcapsulary region, indicating that laminin 5 protein may also play a role outside the hemidesmosome in the thymus. Furthermore, the laminin 5 protein seems to form a highly specialized structure in the thymic nurse cell line 427.1.86 (Kim *et al.*, 2000). Immunocytostaining with laminin 5-specific antibodies localized a stable structure in that particular part of the cell.

The function of laminin 5 was tested during thymic development (Kim *et al.*, 2000). When the functionally blocking monoclonal antibody against laminin 5 (P3H9-2) was treated in fetal thymic organ culture (FTOC), the yield of thymocytes, especially of the $CD44^{+}CD25^{+}$ and $CD44^{-}CD25^{+}$ stage double negative ($CD4^{-}CD8^{-}$) thymocytes was severely reduced, although no significant change was observed in the case of non-blocking antibodies (P3E4) against the same protein. Moreover, P3H9-2 antibody treatment in FTOC increased the fraction of CD69 and apoptosis-specific marker annexin V positive cells in the early double negative thymocyte population (Kim *et al.*, 2000). Together, these results support the idea that the binding of thymocytes to the thymic extracellular matrix through laminin 5 plays an important role in early thymocyte survival as well as in adhesion and migration in the process of thymic development.

The results in this report provide the mechanism of generating alternatively sized mouse *Lamc2* message: an alternative transcriptional initiation that can yield C-terminally truncated peptides of the laminin γ2 chain. A novel alternative splicing site is identified that can lead to premature termination of full-length open reading frame (ORF).

Materials and Methods

Northern blot analysis Total RNA from different cell lines was prepared using the guanidinium isothiocyanate method (Ausubel *et al.*, 1998). Twenty μg of total RNA from various cell lines or tissues were separated on an 1% agarose gel containing 0.7% formaldehyde and transferred onto a nylon membrane, Nytran (Schleicher and Schuell, USA).

Screening of mouse genomic DNA library A mouse genomic DNA library cloned in λ FIX II vector (Stratagene, USA) was screened with probes prepared from the mouse *Lamc2* cDNA sequence (the region from *Eco*RI to *Xho*I of TSO-2D1-7A clone, full-length cDNA from 0 to 5.1 kb).

5′ rapid amplification of cDNA ends (5′RACE) An adaptor-ligated cDNA pool from the mouse kidney was purchased from Clontech (USA) or was synthesized with poly$(A)^{+}$ RNA from the 427.1.86 cell line and the thymii of SCID mice according to the protocol of Marathon™ cDNA Amplification Kit (Clontech) (Jo *et al.*, 2001; Kim *et al.*, 1999). PCR reactions were conducted with two adaptor primers and two gene-specific primers and Ex-Taq polymerase (Takara Shuzo Co., Japan). The first round of PCR was performed with adaptor primer 1 and gene-specific primer 1 (3504–3484 in the cDNA sequence; 5′ GAGGTGGTTCCTCTGCCGACG 3′) and the second round with adaptor primer 2 and gene-specific primer 2 (3471–3451; 5′

CAGGTCAGACATCAAGGGCCG 3′) (Table 1). PCR cycle conditions were according to manufacturer's instructions (Clontech).

RT-PCR Five μg of total RNA from the 1308.1.86 cell line was primed with a mixture of 0.25 μg random primer (Promega, USA) and 0.25 μg oligo (dT) 12-18 primer (Promega) incubated at 70°C for 10 min and cooled on ice. Reverse transcription was carried out with SuperScript II (Life Technologies, USA).

PCR was carried out with 10 pmol of 5′ primer (one of 5A, 2823-2845; 5B, 2886-2908; 5C, 2903-2926), 10 pmol of 3′ primer (3A, 3174-3153) (Table 1), 2 μl of the cDNA synthesized above, Ex-Taq polymerase. PCR was carried out with 30 cycles of the following program: 94°C for 30 s, 57.1°C for 90 s, and 72°C for 90 s. Finally, the reaction mixture was incubated at 72°C for 5 min.

Primer extension analysis Total RNA (1 or 5 μg) from the 1308.1.86 cell line were used in the primer extension analysis. The primer covering the cDNA sequence from the 3074th to 3053rd base (Table 1) was used because the expected band sizes were suitable.

Reporter assay Transfections for the reporter assay were performed with lipofectamine (Life Technologies). Secreted alkaline phosphatase (SEAP; Clontech) activities of each construct were measured according to the manufacturer's instruction 24 h after transfection. β-gal activities were measured according to the manufacturer's instruction (Promega) to compensate for variation of transfection efficiencies.

Cells and culture Thymic epithelial cell lines from transgenic mice expressing the SV40 T antigen are the kind gift of Barbara Knowles (Jackson Laboratory, USA). The 427.1.86, 1308.1.86, 1307.6.17, 6.1.1, and 1307.1.11 were mouse thymic epithelial cell lines originated from subcapsulary regional nurse cell, the cortical epithelial cell, the medullary epithelial cell, the medullary epithelial cell, and unidentified medullary cell, respectively (Faas *et al*., 1993). TN1, TN3, TN4, and Tst1 are thymic epithelial cell lines from $p53^{-/-}$ mice (generous gifts from S. Apasov, Laboratory of Immunology, NIAID, NIH). Y1, NIH 3T3, and A2B5 are from American Type Culture Collection (USA).

Results

Two sizes of Lamc2 message in mouse thymic epithelial cell lines To find the proper cell lines for the presence of *Lamc2* messages, additional cell lines were screened by Northern blot analysis. As shown in Fig. 1A, it is clear that two different sizes of *Lamc2* message are present in some thymic epithelial cell lines tested when the 2.2–3.5 kb region (*Bam*HI-*Eco*RI in Fig. 1B) of cDNA was used as a probe. The longer message (5.1 kb)

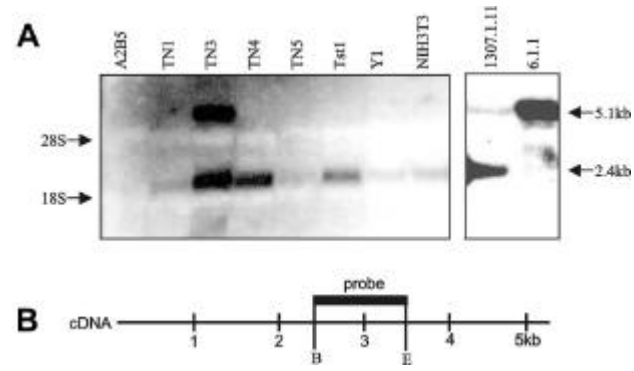

Fig. 1. Northern blot analysis of laminin γ2 chain in various cell lines. Panel A, the membrane was hybridized with a [α-^{32}P] dCTP-labeled probe. The 2.2 kb (*Bam*HI) ~3.5 kb (*Eco*RI) region of cDNA was used for the probe. A2B5, hybridoma cell line; TN1, TN3, TN4, TN5, and Tst1, thymic epithelial cell line from p53-/- mice; Y1, kidney epithelial cell line; 1307.1.11 and 6.1.1, thymic epithelial cell lines. Panel B, location of the probe used in Northern blot analysis (2.2–3.5 kb region of cDNA) compared with the cDNA of mouse *Lamc2*.

was detected in the TN3, 1307.1.11, and 6.1.1 cell lines. In contrast, shorter message (2.4 kb) was detected in many other cell lines, such as the TN1, TN3, TN4, Tst1, 1308.1.86, and 1307.6.17 thymic epithelial cell lines, the Y1 kidney epithelial cell line, and the NIH 3T3 fibroblasts. In the case of the 6.1.1 thymic epithelial cell line, only larger message was detected; however, neither of the two bands was seen in the A2B5 hybridoma cell line. Therefore, the longer message is highly restricted in its expression even among the epithelial cell lines, while the shorter message is more widely expressed in epithelial cells as well as in fibroblasts.

Screening the mouse genomic library and analyzing isolated clones We screened the mouse genomic DNA library using the *Lamc2* cDNA as a probe. As shown in Fig. 2, the lambda clones are aligned relative to the cDNA of *Lamc2* after Southern blot analysis and PCR using the internal primers (Table 1). These clones are well aligned in accordance with the published genomic structure of human *LAMC2* (Airenne *et al*., 1996).

Exon-intron boundary sequences and the sizes of each exon were determined (Table 2) by nucleotide sequencing. Although the genomic organization patterns of mouse and human *Lamc2* genes are nearly identical, there are some differences in size between human and mouse *Lamc2*. The sizes of exon 8, 12, 13, 16, 18, and 21 in mouse *Lamc2* are 116, 146, 160, 150, 150, and 156 bp, versus 113, 143, 157, 156, 153, and 159 bp in human *LAMC2* (Airenne *et al*., 1996). The changes in the length of each exon occurred by 3 or 6 bp, exactly one or two codons. The homologies of splicing donors and acceptors

Table 1. Primers used in this study.

Name	Location	Orientation	Sequence	Purpose
2-5	176-157	antisense	5′-TGTCGATGGAGCTCCTGATC	S, PCR
2-3	225-242	sense	5′-GGTTCACTGCGAGCGGTC	S, PCR
3-5	425-408	antisense	5′-GTGCATCCAGCATCGGTG	S, PCR
3-3	338-355	sense	5′-GACAATGCAGGTGTAAGC	S, PCR
4-5	516-497	antisense	5′-TGGTTTGCAGACACATCGGC	S, PCR
4-3	476-494	sense	5′-TCTCTGGACCCTGTGATTC	S, PCR
5-5	646-629	antisense	5′-TGAAGTCGGCAGAGGCGT	S, PCR
5-3	555-572	sense	5′-TGACTATCATCTGGACCG	S, PCR
6-5	778-761	antisense	5′-CTGATCTTCGGGCAGAAC	S, PCR
6-3	680-703	sense	5′-ATGTGGATGGTTGGAAGGCGGTTC	S, PCR
7-5	891-874	antisense	5′-GGCAGACGGCTGTCTACC	S, PCR
7-3	880-897	sense	5′-CAGCCGTCTGCCTACGAT	S, PCR
8-5	1071-1052	antisense	5′-TGTGAGGTTCCGCAGTAACC	S, PCR
8-3	1041-1060	sense	5′-CGAATATCGAAGGTTACTGC	S, PCR
9-5	1307-1290	antisense	5′-CCCTCCCCTTGGCAGTTA	S, PCR
9-3	1220-1239	sense	5′- CCAGGAATGTGCTTCTGGTT	S, PCR
10-5	1364-1345	antisense	5′-TCAATGTCAGGATTCTCGTC	S, PCR
10-3	1337-1354	sense	5′-ACTCGGGGGACGAGAATC	S, PCR
11-5	1600-1583	antisense	5′-TGCATTGGCAGCGTTGAC	S, PCR
11-3	1649-1666	sense	5′-GCAGATGCTTGAAATGTA	S, PCR
12-5	1879-1858	antisense	5′- AAGCAGGACAACTGGTTAGGGC	S, PCR
12-3	1791-1810	sense	5′-AGAGTGTCGAGGTGATGGCA	S, PCR
13-5	2063-2042	antisense	5′-CCTTCTGAAATCTGAGCTTCTC	S, PCR
13-3	1984-2005	sense	5′-CCAGTGCAGCTGGAAGGCAGGA	S, PCR
14-5	2128-2107	antisense	5′-GGGTCTTGTAGTCGTTCTCTTG	S, PCR
14-3	2188-2205	sense	5′-AACAGAGTTCAGGATACG	S, PCR
15-5	2346-2328	antisense	5′-GTCTGCCTTTCTTGTAGCC	S, PCR
15-3	2274-2293	sense	5′-CCATTCTTCTGAGCACTACG	S, PCR
16-5	2493-2474	antisense	5′-CATAAGACCTTGTACCACGG	S, PCR
16-3	2387-2406	sense	5′-GGGAAACTGAGGACTACTCC	S, PCR
17-5	2565-2546	antisense	5′-AGCTTCAATGTCGGCTTGGG	S, PCR
18-5	2742-2725	antisense	5′-GTTCCCCAGATTGTTTCG	S, PCR
18-3	2650-2671	sense	5′- GCAAAGAGGATCAGACAAAAGG	S, PCR
19-5	2866-2849	antisense	5′-CATTGCCCATACTTAGCG	S, PCR
19-3	2832-2851	sense	5′-AAACAGAGCCCAAGAAGCAGC	S, PCR
20-5	3074-3053	antisense	5′-CTAGCTGCGTTCTTTGCCCGTT	S, PCR, probe, pr ex
20-3	3063-3079	sense	5′-AAAGAACGCAGCTAGGGAGG	S, PCR
21-5	3245-3226	antisense	5′-TCCGTGTCAAACTCCAGCTC	S, PCR, probe
21-3	3108-3129	sense	5′-GGAGATAGGGAGTCTGAACTTG	S, PCR
22-5	3360-3343	antisense	5′-GAGGTGTAGGATGCCGTC	S, PCR
22-3	3309-3329	sense	5′-CGGAGTTACCATCCAAGACAC	S, PCR
23-5	3472-3451	antisense	5′-CCAGGTCAGACATCAAGGGCCG	S, PCR, RACE
RT-5A	2823-2845	sense	5′-CCTTGCTAAAAACAGAGCCCAAG	RT-PCR
RT-5B	2886-2908	sense	5′-GAACATCCTGAAGAACCTCCGAG	RT-PCR
RT-5C	2903-2926	sense	5′-TCCGAGGTTAGTGCTGTGTGATCC	RT-PCR
RT-3A	3174-3153	antisense	5′-TATCTCGCTGCTGATCTCCAGG	RT-PCR
RACE-1	3504-3484	antisense	5′-GAGGTGGTTCCTCTGCCGACA	RACE

S, nucleotide sequencing; PCR, PCR for determining the size of intron; probe, probe for Southern blot analysis; pr ex, primer extension; RACE, rapid amplification of cDNA end.

Table 2. The exon/intron junctional sequences and the sizes of each intron in the mouse *Lamc2*[a,b].

Exon	Splicing donor MAG**GT**RAGT	Intron size[c] (kb)	Splicing acceptor YYYYYYNY**AG**G	Exon
1	N.D.[d]	N.D.	ttttccct**ag**T	2
2	AGG**gt**aggt	7.3	tgggccac**ag**G	3
3	ACT**gt**aagc	4.4	gttcactc**ag**A	4
4	TAG**gt**cagt	5.2	tctgctgc**ag**G	5
5	AGG**gt**aaag	1.3	tgtcttgc**ag**A	6
6	CTG**gt**atgt	1.7	catttctc**ag**C	7
7	CAG**gt**aaaa	3.2	aattatgc**ag**A	8
8	ATA**gt**aagt	1.2	tctattgc**ag**G	9
9	CGG**gt**gagt	0.6	tacgctcc**ag**G	10
10	CAG**gt**aggg	2.0	tccttccc**ag**G	11
11	GAG**gt**agta	1.7	ttatctct**ag**C	12
12	CAG**gt**atgc	2.8	gtgctttc**ag**A	13
13	AAG**gt**ccgg	0.4	tcgtgtgc**ag**G	14
14	ACT**gt**atgt	1.9	tgttccct**ag**A	15
15	CAG**gt**aaac	1.2	atgtcttc**ag**C	16
16	AAA**gt**acgt	1.3	ctcgccac**ag**C	17
17	ACG**gt**gagg	1.1	gtgtccac**ag**G	18
18	CAG**gt**acct	2.4	ctatttac**ag**C	19
19	GAG**gt**tagt	0.7	ttcattct**ag**A	20
19B	ATG**gt**gggt	0.7	ttcattct**ag**A	20
20	CAG**gt**aaag	1.6	attctccc**ag**G	21
21	CTG**gt**gagt	0.3	tcgtcctc**ag**G	22
22	TAG**gt**ctgt	2.1	cctaaaac**ag**A	23

[a] M, A or C; R, A or G; Y, C or T.
[b] Exon sequences are in uppercase and intron in lowercase.
[c] The sizes of introns are approximated by gel resolution of PCR products.
[d] N.D., not determined.

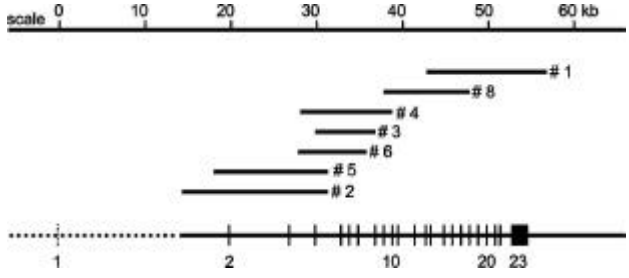

Fig. 2. Alignment of isolated lambda clones. Based on Southern blot analysis and the electrophoresis pattern of PCR products using internal primers and sequencing, each lambda clone was aligned against human *LAMC2* genomic structure. Scale is shown at the top of the figure.

between the two species are 85.2 and 78.5%, respectively, if the number of matching base pairs is divided by that of all consensus sequences (MAGGTRAGT: splicing donor, YYYYYYNYAGG: splicing acceptor) (Mount, 1982). The sizes of all introns are shown in Table 2. Overall, intron are 0.3 to 7.4 kb long and are very similar to their human counterparts (Airenne *et al*., 1996).

From these results, we concluded that the genomic organization of *Lamc2* is highly conserved between human and mouse.

The shorter message starts within the 19th exon Next we employed 5′ RACE to find the 5′ end of the 2.4 kb shorter message seen in Northern blot. To amplify the 5′ portion of the shorter message, we designed two gene-specific primers corresponding to the nucleotide positions in 3504–3484 and 3472–3451 in cDNA (RACE-1 and 23-5 primer in Fig. 3A and Table 1), respectively. Consequent RACE reaction with cDNA synthesized from mouse kidney mRNA yielded multiple products (Fig. 3B). The correct RACE products were selected by Southern blot analysis using the sequences 3226–3245 (21-5 primer in Fig. 3A and Table 1) as a probe (Fig. 3C). In Southern blot analysis, four different-sized products were positively identified: RACE #1, 2, 3, and 4 (Figs. 3B and 3C); however, the RACE #1 band failed to reproduce the same band, while the other three bands were well amplified. We then determined the nucleotide sequences of a total of ten RACE clones. Six of eight independent clones from the largest RACE product (band #2) contained the 5′ sequence that starts at the 2895th base located in the 19th exon of *Lamc2* cDNA (RACE #2-2, 4, 5, 6, 7, and 8; Fig. 3D). This site was considered a strong candidate for the 5′ end of the shorter message, because it was from the largest RACE product and because the expected size of the RNA transcribed from this site matches well with that of the shorter band seen in Northern blot analysis (2.4 kb).

Unexpectively, five of six clones from the RACE #2 products initiating at the 2895th base (RACE #2-2, 4, 5, 6, and 7; Fig. 3D) contained an additional 72 bp, while the remaining clone (RACE #2-8; Fig. 3D) did not. This region was named exon 19B, because it is from the 5′ 72 bp of the 19th intron. It is likely that a fraction of mRNA undergoes an alternative splicing event that passes the junction between the 19th exon and the 19th intron. Instead, a secondary splice donor site located at the 3′ end of exon 19B (arrow, Fig. 3D) was used in this message. Furthermore, two novel stop codons are included in exon 19B: a UAG codon at the third base and a UAA codon at the 24th base (boxed in Fig. 3E). These stop codons are in-frame with the ORF in the 5.1 kb message (see **Discussion**).

To test the start of the shorter message at the 2895th base identified by 5′ RACE with the kidney sample and the presence of exon 19B in other tissue types, primer extension analysis was performed using total RNA from the 1308.1.86 thymic epithelial cell line (Faas *et al*., 1993) using the primer covering 3074–3053 bases of the cDNA sequence (20–5 primer in Table 1). The 1308.1.86 cell line was chosen because this cell was previously shown to express only the smaller message (Kim *et al*.,

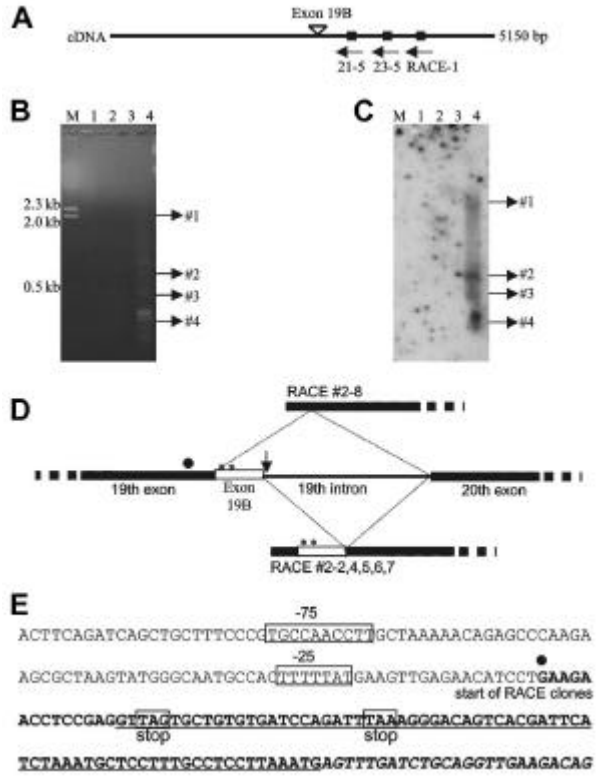

Fig. 3. The sequences of RACE clone #2 show alternative transcriptional initiation and splicing. The adaptor-ligated mouse kidney cDNA pool was sequentially amplified with two gene-specific primers (3504–3484, 3472–3451) and two adaptor primers to determine the 5′ sequence of the short-form *Lamc2*. The nucleotide sequences of the RACE product were determined and compared with those of cDNA and mouse genomic *Lamc2*. Panel A, experimental scheme for the 5′ RACE to identity the 5′ end of short message. Two of three primers (RACE-1, 23-5) were used for RACE reaction (Table 1). RACE-1 was used in primary PCR reaction and 23-5 for nested PCR reaction. The 21-5 primer (Table 1) is located in the 21st exon and was used in the Southern blot analysis (Panel C) to verify the specificity of RACE products. Panel B, RACE products were separated on 1.5% agarose gel. Lane M, lambda-*Hin*dIII DNA size marker; lanes 2 and 3, primary PCR results; lanes 4 and 5, nested PCR results; lanes 2 and 4, cDNA synthesized from 427.1.86 cell mRNA was used for template; lanes 3 and 5, cDNA synthesized from mouse kidney mRNA was used for template. Panel C, PCR products seen in Panel B were transferred onto the Nylon membrane and probed with [γ-^{32}P]-labeled 21-5 oligonucleotide. Panel D, relative alignment of the sequence of RACE clones and the genomic structure of mouse *Lamc2*. RACE #2-2, 4, 5, 6, and 7 are composed of the 3′ portion of exon 19, exon 19B, and exon 20, whereas RACE #2-8 is the 3′ portion of exon 19 and exon 20. Arrow indicates the splicing donor site located at the 3′ end of exon 19B. ●, start site of short message; *, two stop codons located in exon 19B. Panel E, the nucleotide sequences around exons 19, 19B, and 20. The 19th exon sequences that are not transcribed are indicated as normal letters; those transcribed are indicated as bold letters. The nucleotides corresponding to exon 19B are shown in bold and italicized, and those corresponding to the 20th exon are bold.

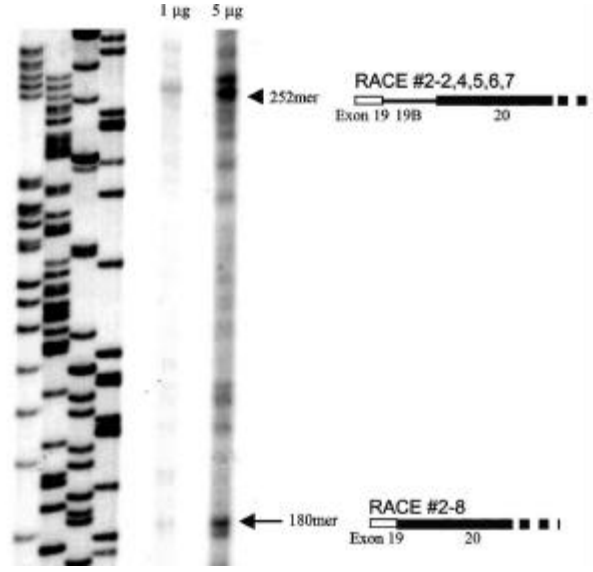

Fig. 4. Primer extension analysis shows alternative transcriptional initiation and alternative splicing. Total RNA (1 and 5 μg) from the 1308.1.86 cell line were reverse-transcribed with [γ-^{32}P]-labeled primer (3074–3052) and electrophoresed on 6% acrylamide gel. The bands with expected sizes are marked; arrow indicates 180 bp size, and solid triangle indicates 252 bp size.

2000). As shown in Fig. 4, a band was observed at the 180 bp size (arrow, Fig. 4) and another band at the 252 bp size (solid triangle, Fig. 4). This difference is consistent with the difference introduced by alternatively spliced exon 19B. cDNA in the 252 bp band seems to have the sequences of exon 19B plus those of cDNA of the 180 bp band. The 180 bp band (arrow, Fig. 4) seems to correspond to RACE clone #2-8 (Fig. 3D) and the 252 bp band (solid triangle, Fig. 4) to RACE clones #2-2, 4, 5, 6, and 7 (Fig. 3D).

Next, to test the possibility of promoter activity in the upstream region of the 2895th base, the nucleotide sequence of this region was screened for potential binding sites for transcription factors using the MatInspector ver. 2.2/TRANSFAC 3.5 database (http://dot.imgen.bcm.tmc.edu:9331). There is no obvious TATA box found; there is, however, a TATA-like sequence (TTTTTAT) (boxed in Fig. 5) in the -25 bp region and a CAAT-like sequence (TGCCAACCTT) in the -75 bp region. There are also two putative AP1 binding sites (-427, -195), three AP4 binding sites (-631, -184, -92), five TCF11 binding sites (-589, -495, -424, -192, -156), two GATA-1 binding sites (-610, -234), one STAT-1 (-275), NFY-1 binding site, and a CAAT box (-572) (Fig. 5). The presence of these potential transcription factor binding sites strengthen the idea that this region can function as a internal promoter.

To test the promoter activity of the upstream region of the internal start site, the reporter activity of this region

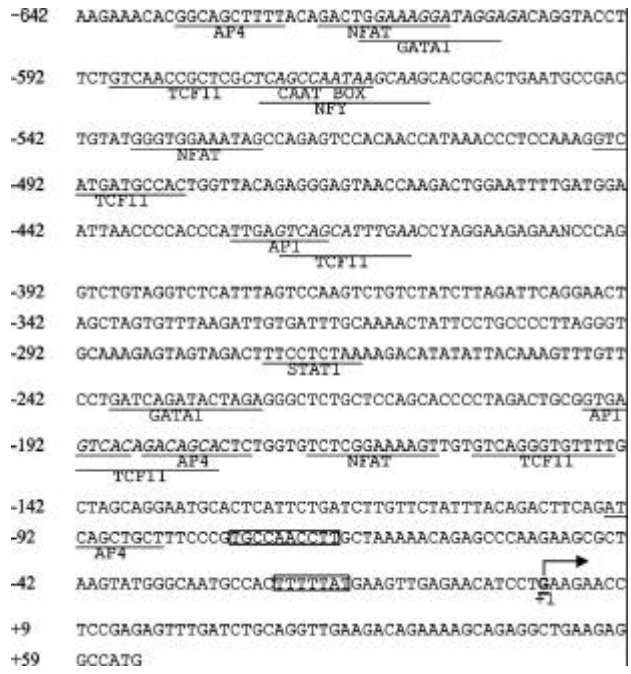

Fig. 5. The nucleotide sequences of the secondary promoter located in the 19th exon. Sequences from the secondary promoter identified from the 5′ RACE and primer extension analysis are presented. Potential binding sites for various transcription factors are indicated. The putative transcription start site (G) is marked with an arrow and bold.

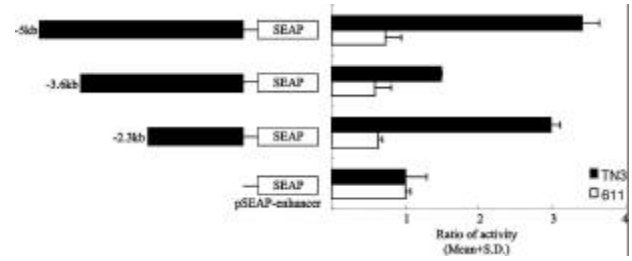

Fig. 6. Reporter assay showing the promoter activity of the upstream region of the 2895th base. A series of deletion constructs containing -2.3 to -5 kb upstream from the 2895th base is shown along with the result of the SEAP reporter assay in TN3 and 6.1.1 cells. The SEAP light unit was divided by the activity of β-gal, which was co-transfected with each deletion construct to compensate for the variation in transfection efficiency. pSEAP enhancer vector was used as a negative control. Each experiment was repeated two or three times with at least triplicate in one trial.

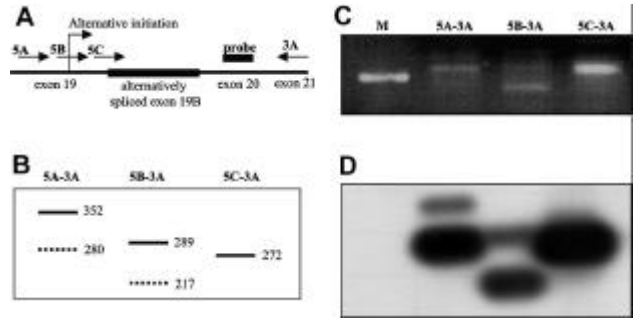

Fig. 7. RT-PCR and Southern blot analysis showing the alternatively spliced transcripts in the cell. Panel A, location of the primers used in RT-PCR (5A, 2823–2845; 5B, 2886–2908; 5C, 2903–2926; and 3A, 3174–3153) and in Southern blot (3074–3053). Panel B, expected electrophoresis pattern and size of RT-PCR product. Panel C, total RNA (5 μg) from the 427.1.86 cell line were reverse-transcribed, amplified with 5A, 5B, 5C, and 3A primers, and the products electrophoresed on 2% agarose gel. A band seen in lane M represents a 234-bp fragment of *Hae*III-digested φX174 DNA size marker. Panel D, PCR products in Panel C were blotted and probed with [γ-^{32}P]-labeled oligonucleotide indicated in Panel A.

was measured using the SEAP system. Three different sizes of upstream region were placed in the pSEAP2-enhancer vector, which has no endogenous promoter activity. As shown in Fig. 6, the SEAP reporter activity increased approximately 4 fold relative to the promoterless vector when the upstream 5 kb sequence was used in the TN3 thymic epithelial cell line, but the degree of enhancement decreased to 2-fold basal level with the 3.6 kb sequence. When the upstream 2.3 kb region was used for transfection, the fold induction was recovered to a level comparable to that of the 5 kb sequence. In the case of the 6.1.1 thymic epithelial cell line, which does not express the smaller message (Fig. 1), promoter activities of 5, 3.6, and 2.3 kb were generally lower than the counterparts of TN3 cell, and there is no difference observed in all three constructs. These data strongly support the possibility that the shorter message observed in Northern blotting arises from the promoter localized in the upstream region of 2895th base.

A novel alternative splicing occurs in the 19th intron

We employed RT-PCR to verify the existence of the message containing exon 19B in the cells. Four primers were designed as in Fig. 7A: 5A, 5B, 5C, and 3A. The 5A and 5B primers are located in the 19th exon and used to amplify the two different-sized messages that may or may not include exon 19B. The 5C primer, designed to bind to the 3′ region of the 19th exon and 5′ region of exon 19B at the same time, can only amplify the message containing exon 19B. The 3A primer is designed to be in the 21st exon in order to exclude the possibility of genomic DNA contamination RT-PCR. The expected band sizes of each PCR reaction are 352 and 280 bp in the reaction using 5A and 3A primer, 289 and 217 bp using 5B and 3A primer, and 272 bp with 5C and 3A primer, respectively (Fig. 7B).

The RT-PCR results were exactly as expected (Figs. 7B and 7C). All bands tested positive for the internal nucleo-

tide sequences by Southern blot analysis (Figs.7A and 7D). The intensities of the bands with insertion of the novel exon were always weaker (a tenth of that without insertion). This alternative exon is also present in the larger message (5.1 kb), because binding sites for primer 5A are present only in the larger message.

Discussion

The structure of laminin genes has been determined in detail in only a limited numbers of cases. The genomic structures of human LAMB1 (Vuolteenaho *et al*., 1990), mouse LAMB1 (Vasios *et al*., 1989), human LAMB3 (Pulkkinen *et al*., 1995), human LAMC1 (Kallunki *et al*., 1991), Drosophila LAMC1 (Chi *et al*., 1991), and human *LAMC2* (Airenne *et al*., 1996) have been reported. Generally, the sizes of the genes encoding the members of the known laminin family are large (Ekblom and Timple, 1996). The human LAMB1 gene is over 80 kb in size and contains 34 exons (Vuolteenaho *et al*., 1990). The human LAMC1 gene is approximately 60 kb and has 28 exons (Kallunki *et al*., 1991), and the human *LAMC2* gene is 55 kb and possesses 23 exons (Airenne *et al*., 1996). Therefore it is not surprising that mouse *Lamc2* is also spread over 40 kb. The size of the mouse *Lamc2* gene from exon 2 to exon 23 in this study is very similar to that of human *LAMC2* (Airenne *et al*., 1996) (Table 2). The sizes of 15 of 21 exons (total 23 exons minus first and last exons) are identical between human and mouse *Lamc2*. Even in exons showing different sizes, the difference is only 3 or 6 bp: one or two codons. The differences in exon size are reflected in the numbers of corresponding amino acids originating from each exon. One more amino acid is present in exons 8, 12, and 13 of mouse *Lamc2*, and one more amino acid in exons 18 and 21 and two more in exon 16 of human *LAMC2*. The homologies of splicing donor and acceptor sequence are 85.2 and 78.5%, respectively, indicating that the evolutional pressure for selecting the function of this protein is very similar. Therefore, it is surprising to find that the alternative message is generated by a completely different mechanism that may yield alternative proteins.

It is generally accepted that expression of the laminin γ2 chain is limited in epithelial cells; however, from the result of Northern blot analysis shown in Fig. 1 and our previous data (Kim *et al*., 2000), the full-length message is expressed in a more restricted manner even among thymic epithelial cell lines. Besides the 427.1.86 thymic nurse cell line, we showed that TN3, 1307.1.11, and 6.1.1 cell lines also express the 5.1 kb message. In contrast, the shorter message seemed more widely distributed than we originally thought. All the mouse thymic epithelial cell lines tested (except 6.1.1), kidney epithelial cell (Y1), and fibroblasts (NIH 3T3) expressed the 2.4 kb message. The expression of short *Lamc2* message in fibroblasts was a surprise, because since its expression is generally restricted to epithelial cells.

It is not clear whether the expression of the two messages is regulated by factors in the extracellular environment or is an intrinsic feature of each cell line. In addition, it is possible that expression of the larger message is not stable, because we found the changes in the expression pattern in older cultures of same cell lines (data not shown). Other researchers (Sugiyama *et al*., 1995) did not notice the existence of the smaller message, probably because they used the probe from 1.5 to 2.2 kb of *Lamc2* cDNA, which does not include the portion of the smaller message. The second band is not a cross hybridization of the message of LAMC1 (Durkin *et al*., 1988) or recently cloned LAMC3 (Koch *et al*., 1999), as no obvious homology between them was found in the 3′ region (2.1–5.1 kb) of *Lamc2*.

The results of the 5′ RACE, primer extension analysis, and reporter assay are consistent with the idea that the second message is generated by an alternative transcriptional initiation within the 19th exon. The results from two independent methods in two different tissue types seem to suggest that a smaller message starts at the same site and contains the alternatively spliced exon (see below).

In addition to the 5′ RACE and primer extension analysis, the reporter assay also provides direct and clear evidence that the second message of mouse *Lamc2* is generated by alternative transcriptional initiation within the 19th exon. The promoter activity behaves in a cell type-dependent manner. Only the cell lines expressing the smaller message showed promoter activity with a range of 2 to 4 fold relative to the promoterless reporter. This fold difference is within the range of promoter activity. In addtion, the profile of reporter activities (Fig. 6) suggests that more complex regulation may occur with the 5 kb upstream region, such as repression within the -2.3 ~ -3.6 kb region.

The nucleotide sequence of the internal promotor shown in Fig. 5 suggests multiple potential binding sites for transcription factors including AP1, AP4, TCF11, GATA-1, and STAT-1. Although there are no conventional TATA and CAAT boxes, TATA-like (TTTTTAT) and CAAT-like (TGCCAACCTT) sequences are found in the expected locations, -25 and -75, respectively. In eukaryotic genes, the TATA box is usually located 25–30 bp upstream of the transcription initiation site and directs accurate transcriptional initiation through interaction with TATA-binding protein (TBP). Transcription initiation is also regulated by another element, Initiator, overlapping with the transcriptional initiation site (Grosschedl and Birnstiel, 1980; Smale, 1997). The upstream region of the 2895th base contains no conventional initiator sequence. Therefore, multiple bands observed in the primer extension analysis

can be ascribed to the lack of a conventional strong TATA box (Fig. 4).

For translational initiation, the modified scanning model predicts the most 5′ AUG is favored (Kozak, 1980; 1984; 1986a). The most favorable consensus sequence for translational initiation is defined as (-6)GCC A/G CCAUG G/A(+4) (Kozak, 1986b), with positions –3 and +4 being the most critical. The uppermost 5′ ATG in the short message transcribed from the 2895th base exists as a (–6)GAG G CCATG A(+4), which matches the demand of Kozak's rule and is exactly the same as that for the longer message. This suggests that the shorter message can contain alternative ORF. It could use the 973rd Met located in the 20th exon as a start codon and the same stop codon (1193rd) with full-length ORF. Consequently, it can encode 220 amino acids corresponding to the C-terminal 1/3 portion of domains I and II, which form a coiled-coil structure by interacting with the α3 and β3 chains of laminin 5; however, the protein from this ORF lacks the signal sequence seen at the N-terminal region of the full-length protein. Therefore, it seems improbable that this fragment can be secreted into the ECM or bind to the α3 and/or β3 chain. It will take further studies to prove the existence of protein from this small ORF and to verify the actual role of this peptide in cells.

Mouse *Lamc2* message can also generate an alternative splicing in the 5′ splicing donor site of the 19th intron at least in the thymic cortical epithelial cell line 1308.1.86. In this cell line, the alternatively spliced form is present as much as the original form is, as seen in the primer extension experiment (Fig. 4). The resulting new exon, exon 19B, is actually a part of intron 19 and contains two in-frame stop codons located in the 3rd and 24th bases of exon 19B. Both of these stop codons are in-frame with the AUG codon of the beginning of full-length ORF. The original ORF of mouse *Lamc2* starts from the AUG codon at the 40th base and ends at the UGA codon at the 3618th base, which can encode 1192 amino acids. Hence, the alternative splicing event with the inclusion of the novel exonal sequence leads to premature termination and, therefore, results in truncated ORF of N-terminal 957 amino acids.

In contrast to the mouse *Lamc2*, the longer human ã2 transcript is expressed in all epithelial cells of tissues tested, while the shorter γ2* mRNA is expressed in more restricted areas such as the cerebral cortex, lung, and distal tubules of the kidney. Human *LAMC2* also generates the smaller message by alternative splicing fashion, but with completely different results (Airenne *et al*., 1996). The 5′ splicing donor site from intron 22 is not used during splicing. Therefore, the alternative splicing events in human and mouse *Lamc2* seem to be different in two main aspects. First, only the human case behaves as a mechanism to generate different-sized messages by including two alternative polyadenylation signals in exon 22* (corresponding to intron 22 in the long message); however, in the mouse, an alternative exon is present in both messages, and they are not involved in generating the distinguishably sized messages. Second, the mouse mechanism generates completely different ORFs. The alternative human *LAMC2* message encodes 1119 amino acids with C-terminal truncation, while the mouse message (5.1 kb) can encode 957 amino acids. These two alternative ORFs insert different amino acid sequences in different locations of the γ2 chain; however, the presence of truncated proteins is not formally verified in both systems (We failed to detect truncated protein by Western blotting; data not shown, and see below). Therefore, it is difficult to address their biologic roles at this time. Our effort to produce tagged protein by introducing constructs with an alternative exon in the cell line is now in progress.

Comparing nucleotide sequences in the alternative slicing sites can give us a clue to whether the event of one species can occur in the other. First, to test the possibility that exon 19B is present in human, the sequence of the splicing donor and 5′ region of intron 19 was compared. All nine consensus bases (MAGGTRAGT) for the splicing donor are perfectly conserved, and 70% of the first 20 bases (the number of bases from Airenne *et al*., 1996) of intron 19 are conserved between human and mouse. Of interest, in intron 19 of human *LAMC2*, the first stop codon observed in exon 19B of mouse *Lamc2* is also present, although it is not clear whether a second stop codon is also present due to a lack of sequence information. Therefore, it is possible for the similar alternative splicing events to exist in the human system and, if so, full-length ORF will be prematurely terminated as in the mouse. Second, the possibility of the alternative splicing substituting the exon22* for exon22 of human *LAMC2* was examined in the mouse. In intron 22, only seven of nine bases are conserved, and 45% of the first 20 bases are conserved between both species. Therefore, it is less likely that the alternative splicing event of human intron 22 occurs in the mouse. This is consistent with the fact that we never found this form of message in our cDNA library screening and Northern blot analysis.

Recently, many mutations occurring in one chain of laminin 5 have been demonstrated to be the molecular cause of the lethal (Herlitz) variant of JEB. Some nonsense mutations resulted in premature termination as well as instability of the message (Aberdam *et al*., 1994; McIntosh *et al*., 1993; Pulkkinen *et al*., 1994a; 1994b). Similarly, a premature termination codon (PTC) generated by an alternative splicing such as we observated may behave as a message destabilizer. It has been well demonstrated that the predominant consequence of a PTC by a mutation is not the production of a truncated protein but rather a severe reduction in the level of mRNA possessing the alteration (McIntosh *et al*., 1993). Undetectable amounts of LAMB3 and *LAMC2* mRNA were observed in

patient samples having nonsense mutation on both alleles (Aberdam *et al*., 1994; Pulkkinen *et al*., 1994b). Therefore, it is possible that the message containing exon 19B with two stop codons is unstable in a way similar to that having a nonsense mutation, and it is not surprising that efforts to detect the truncated protein failed. We suggest that the alternative splicing in the mouse system may be another regulatory mechanism of *Lamc2* message level in addition to the control by the promoter. This also raises the possibility of another mechanism for the human H-JEB if the percentage of messages containing exon 19B is dysregulated.

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