MATN and LAPTM Are Parts of Larger Transcription Units Produced by Intergenic Splicing: Intergenic Splicing May Be a Common Phenomenon

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Abstract

Intergenic splicing, the joining of exons from separate genes, has been observed only rarely in mammals. While the matrilin (MATN) and lysosomal-associated protein transmembrane (LAPTM) genes comprise distinct gene families, we have demonstrated intergenic splicing between two sets of family genes, the matrilin-3 (MATN3) and lysosomal-associated protein transmembrane 4α (LAPTM4A), and the matrilin-2 (MATN2) and lysosomal-associated protein transmembrane 4β (LAPTM4B). The expression pattern and sub-cellular localization of the MATN–LAPTM hybrid transcripts differ from those of the original genes, suggesting unique functions for the products. Our observations indicate that intergenic splicing is a common and well-regulated phenomenon and underscore the fundamental challenges in defining the gene (transcriptional unit). Given these findings, the number of gene in the human genome may be smaller than present estimates suggest.

Key words: MATN3; LAPTM4A; intergenic splicing; hybrid mRNA; fusion protein

1. Introduction

Intergenic splicing is the splicing together of exons from distinct genes. It appears to be a rare phenomenon, as only seven examples of intergenic splicing have been well characterized in mammals.1–7 Close proximity or a particular orientation of source genes are not thought to drive intergenic splicing; for example, in the case involving translin-associated factor X (TRAX) and disrupted in schizophrenia 1 (DISC1), the two genes are separated by 60 kb of genomic DNA.4 It has been speculated that intergenic splicing might serve as an efficient mechanism for generating new multi-domain proteins and could therefore have major evolutionary implications.3

Matrilins (MATNs) are a family of non-collagenous extracellular matrix (ECM) proteins that consist at least of four related proteins, termed matrilin-1 through -4. All four are expressed in the developing skeletal system, showing varied patterns of tissue expression.3 MATNs are composed of one or two von Willebrand factor A (vWFA) domains, separated by a variable number of epidermal growth factor (EGF)-like repeats, followed by the coiled-coil domain.8–10 Matrilin-3 (MATN3) expression is restricted to developing cartilage and is postulated to function in bridging protein–protein and cell–protein networks in the ECM.8,9

Lysosomal-associated protein transmembrane (LAPTM) family proteins reside in the lysosomal membrane and are thought to function as transporters or channels. There are three LAPTM genes comprising LAPTM4α (LAPTM4A), LAPTM4β (LAPTM4B) and LAPTM5 (LAPTM5). The two LAPTM4 proteins have four predicted transmembrane domains, whereas LAPTM5 has five.11–13 LAPTM4A is thought to regulate intracellular compartmentalization of amphipathic solutes and possibly mediates the cell’s sensitivity to anthracyclines, antibiotics, ionophores, nucleobases and organic cations.12

The MATN and LAPTM families are entirely different with regard to localization and putative function. While characterizing the transcription start site of MATN3,
however, we unexpectedly identified a novel intergenic splicing product that joined portions of the MATN3 and LAPTm4A transcripts. The hybrid transcript showed a specific expression pattern and sub-cellular localization that was different from the original genes and is further alternatively spliced. Alignments of the MATN and LAPTm family genes on the genome suggested that this composite structure may also be characteristic of other MATN and LAPTm family gene pairs. This speculation is confirmed for the MATN2 and LAPTm4B pair.

2. Materials and Methods

2.1. RNA extraction and reverse transcription

Total RNAs were extracted using ISOGENTM (NIPPON GENE, Tokyo, Japan) and were purified with RNasy Mini Kit or RNasy Lipo Tissue Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Total RNAs (500 ng) were reverse-transcribed by MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamers, according to the manufacturer’s protocol.

2.2. 5’ RACE

5’ RACE for the human MATN3 gene was performed using BD Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s protocol. Sequences of the first and second RACE primers were 5’-CCAGAGTGTCCATTATCCGGGAGACA-3’ (nucleotides 378–403 of NM_002381) and 5’-TGATCAAGGTTCTACACCCCG-3’ in exon 2 of MATN3, LAPTm4A–MATN3 nested PCR: 5’-ACCGGTCTCAGCACCCTCCTC-3’ in exon 1 of LAPTm4A and 5’-GCCCTACGCCAGTCTACAGCA-3’ in exon 1 of LAPTm4B and 5’-GTGCCAGAGGAGAAGCTCAGCTC-3’ in exon 2 of MATN3, (ii) LAPTm4B–MATN2: 5’-ACCCGCTTCTACTCAACCAG-3’ in exon 1 of LAPTm4B and 5’-TGTCACAGATGAACTCCTG-3’ in exon 3 of MATN2 and (iii) LAPTm5–MATN1: 5’-AGACCTGCTGCTGCTCATT-3’ in exon 1 of LAPTm5 and 5’-ACCGAGAAACTCCTGCTTC-3’ in exon 2 of MATN1, respectively. PCR conditions were as follows: initial denaturation (94°C, 2 min) followed by 20–40 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), extension (72°C, 30 s) and final extension (72°C, 7 min). PCR products were electrophoresed on 2–3% agarose gels.

2.3. Expression analysis of the hybrid transcripts

Expression of LAPTm4A, MATN3 and the LAPTm4A–MATN3 hybrid transcripts in various cell lines was examined using chondrogenic and non-chondrogenic cell lines. The chondrogenic cell lines included CS-OKB (gift from Dr Chano, Shiga University of Medical Science, Japan) and UOMS27 (IFO Animal Cell Bank); the non-chondrogenic cell lines included HuH7 (JCRB Cell Bank), HeLa (JCRB Cell Bank) and HEK293 (Clontech). The sources of the human cell lines were CS-OKB and UOMS27, chondrosarcoma; HuH7, hepatoma; HeLa, cervical cancer; and HEK293, embryonic kidney. Expression of the LAPTm4A–MATN3; hybrid transcripts in various tissues was examined using the BD MTC Panels (Human I and II, Clontech).

2.4. Cloning of the LAPTm4A–MATN3 fusion gene

Full-length of the LAPTm4A–MATN3 cDNA was amplified by the Takara LA Taq system (Takara Shuzo) according to the manufacturer’s instructions using a set of primers 5’-AAAACGCCGTTGACTTTGGT- TTGG-3’ in 5’-UTR of LAPTm4A and 5’-TCAGG- TGAGAAATTTGGAGCA-3’ in 3’-UTR of MATN3. Reactions were carried out in a total volume of 25 μl, using as templates several ng of cDNA. Primer sequences for amplification are (i) LAPTm4A–MATN3: 5’-CAGTGACCGTTCTACACGCA-3’ in exon 1 of LAPTm4A and 5’-GTGATGCTCAGCACCCTCCTC-3’ in exon 2 of MATN3, LAPTm4A–MATN3 nested PCR: 5’-ACCGGTCTCAGCACCCTCCTC-3’ in exon 1 of LAPTm4A and 5’-GCCCTACGCCAGTCTACAGCA-3’ in exon 1 of LAPTm4B and 5’-GTGCCAGAGGAGAAGCTCAGCTC-3’ in exon 2 of MATN3, (ii) LAPTm4B–MATN2: 5’-ACCCGCTTCTACTCAACCAG-3’ in exon 1 of LAPTm4B and 5’-TGTCACAGATGAACTCCTG-3’ in exon 3 of MATN2 and (iii) LAPTm5–MATN1: 5’-AGACCTGCTGCTGCTCATT-3’ in exon 1 of LAPTm5 and 5’-ACCGAGAAACTCCTGCTTC-3’ in exon 2 of MATN1, respectively. PCR conditions were as follows: initial denaturation (94°C, 2 min) followed by 20–40 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), extension (72°C, 30 s) and final extension (72°C, 7 min). The resulting PCR product was gel-purified and was cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). The obtained clones were confirmed by sequencing and were cloned into pcDNA3.1 Directional TOPO expression Kit (Invitrogen).
2.5. Cellular localization of the hybrid transcripts

For immunostaining with antibody to the V5 tag, HeLa cells were cultured at 37°C under 5% CO₂ in DMEM-high glucose supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum (FBS). OUMS27 cells were cultured at 37°C under 5% CO₂ in DMEM-high glucose supplemented with kanamycin (50 μg/ml) and 10% FBS. Twenty-four hours after plating in a 2-well glass slide at a density of 1 × 10⁵ cells/well, cells were transfected with 1 μg of a pcDNA3.1D/V5-His-TOPO (Invitrogen) containing full-length cDNA of the fusion gene, using FuGENE6 (Roche Applied Science, Mannheim, Germany). Forty-eight hours after transfection, the cells were fixed with 100% methanol for 5 min and examined for immuno-fluorescence. The antibodies used for markers of intracellular organelles were as follows: for LAMP1 (a marker of lysosome), mouse anti-LAMP1 monoclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-mouse IgG TExAS RED (Santa Cruz Biotechnology); for Mannosidase II (a marker of Golgi), anti-rabbit Mannosidase II polyclonal IgG (Abcam, Cambridge, UK) and Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probe, OR, USA); for PDI [a marker of endoplasmic reticulum (ER)], anti-rabbit PDI polyclonal IgG (Abcam) and Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probe).

Cells were washed three times with phosphate-buffered saline (PBS) and incubated at room temperature for 1 h with 10% BSA in PBS to block non-specific binding of antibodies. Primary antibodies were diluted in 3% BSA (1/100–200) and incubated with the fixed cells for 1 h at room temperature. Cells were washed three times with 1 ml of PBS. Secondary antibodies were diluted in 3% BSA (1/500) and incubated with cells for 1 h at room temperature. Cells were washed three times with PBS. Cells were incubated with 10% BSA in PBS to block non-specific binding of antibodies. The rabbit anti-MATN3 polyclonal antibody¹⁴,¹⁵ (a gift from Dr Raimund Wagener) was diluted in 3% BSA and incubated with fixed cells for 1 h at room temperature. Cells were washed with 1 ml of PBS three times. The goat anti-rabbit IgG fluorescein-conjugated secondary antibody (Chemicon International, Temecula, CA, USA) was diluted in 3% BSA and incubated with cells for 1 h at room temperature. Cells were washed with 1 ml of PBS three times and examined using a fluorescence microscope equipped with a FITC filter.

3. Results

3.1. Identification of a novel fusion transcript between LAPTM4A and MATN3

To examine the 5′ region of the human MATN3 gene, we obtained several clones extending the previous 5′ end using 5′ RACE with 3′ primers in exon 2 of MATN3 (Fig. 1). Sequence analysis extended the longest clone 128 bp beyond the 5′ end of the MATN3 reference sequence (GenBank accession no. NM_002381), and subsequent BLAST analysis identified the extended sequence as exons 1 and 2 of the LAPTM4A gene (GenBank accession no. NM_014713). In this clone, exon 1 of MATN3 was skipped. Examination of genomic sequence (GenBank accession no. NT_015926) revealed that LAPTM4A is located ~20 kb 5′ to MATN3 on the same strand (Fig. 1), with no genes between the two. Exons 1 and 2 of LAPTM4A reside 45.4 and 34.7 kb apart from exon 2 of MATN3, respectively.

We confirmed the presence of the novel transcript through an exon-connection experiment, amplifying the entire coding region of the fusion gene by RT–PCR, using primers that flank the 5′-UTR of LAPTM4A and the 3′-UTR of MATN3. This experiment verified the identity of an intergenic splicing product consisting of exons 1 and 2 of LAPTM4A and exon 2 to the last exon (exon 8) of MATN3. As the two open reading frames (ORFs) are fused in frame, translation of the hybrid mRNA, starting at the LAPTM4A initiation codon, are predicted to produce a LAPTM4A–MATN3 fusion protein containing the first transmembrane domain of LAPTM4A and all major domains of MATN3 (Fig. 2).

3.2. Expression of MATN3, LAPTM4A and the hybrid transcript

Expression of the LAPTM4A–MATN3 hybrid transcript in various tissues and cell lines was examined by RT–PCR. The hybrid transcript showed specific tissue expression restricted to placental (Fig. 3A), and.

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**Figure 2.** Schematic presentation of the predicted fusion protein resulting from intergenic splicing between LAPTM4A and MATN3 (hybrid transcript A). Asterisk, the transmembrane region; black bar, signal peptide; arrows, the EGF-like domain; horizontal arrows, coiled-coil domain. Open and shaded boxes indicate exons of LAPTM4A and MATN3, respectively.
expression was observed in HuH7 and OUMS27 cells (Fig. 3B). Expression of the hybrid transcript was not observed in cartilage and bone (Fig. 3B). We also compared the expression of the source genes, **LAPTM4A** and **MATN3**, with that of the hybrid transcript (Fig. 3). **LAPTM4A** was ubiquitously expressed in all tissues and cell lines examined. In contrast, **MATN3** was expressed in a more limited fashion; it was expressed relatively strongly in the placenta, lung, kidney, pancreas, testis and ovary. Among the cell lines, **MATN3** was expressed strongly in HuH7 and OUMS27 cells but weakly in HEK293 and HeLa cells. Thus, the expression patterns of the two source transcripts and the hybrid transcript differed greatly. In addition, we observed several different patterns of intergenic splicing products (hybrid transcripts B–D; Fig. 4).
3.3. Similar conserved genomic structures for the LAPTM and MATN family genes

Other LAPTM and MATN family genes showed genomic co-localization patterns similar to that of LAPTM4A and MATN3, with gene pairs aligning in head-to-tail orientation (Figs 5 and 6). No LAPTM family gene co-localizes with MATN4. Localization of LAPTM and MATN genes is preserved during evolution; in particular, co-localization of LAPTM4A and MATN3 is preserved among the mouse, rat, chicken and fugu genomes (ECR Browser: URL http://ecrbrowser.dcode.org/).

3.4. Identification of hybrid transcripts in other pairs of LAPTM and MATN family genes

We scanned for hybrid transcripts joining other pairs of LAPTM and MATN family genes using exon-connection experiments with primers specific for exon 1 of LAPTM genes and exon 2 or 3 of MATN genes. This analysis identified two LAPTM4B–MATN2 hybrid transcripts: the longer transcript skipped exon 7 (the last exon) of LAPTM4B, while the shorter transcript skipped exons 2–7 of LAPTM4B (Fig. 6). The longer form was predicted to be translated to LAPTM4B lacking only the amino acid residues encoded by the last exon. The shorter form was also predicted to be containing a single LAPTM transmembrane region. In both forms, exon 1 of MATN2 was skipped and the MATN2 component began at –26 from the original translation start site of MATN2 in exon 2. Both forms had a stop codon in the original 5′-UTR of MATN2 (–5 from the translation start site), which resulted in the total absence of the original MATN2 amino acid sequence and addition of unique seven amino acid residues to their C-terminals (Fig. 6). The LAPTM4B–MATN2 hybrid transcripts that showed specific tissue expression: the longer form was found in HuH7; the shorter form was found in heart and HEK293 (Fig. 7). No hybrid transcript between LAPTM5 and MATN1 was identified.

3.5 Cellular localization of the MATN3–LAPTM4A hybrid transcript

The sub-cellular localization of the LAPTM4A–MATN3 fusion protein was examined by immuno-fluorescence analysis of HeLa and OUMS27 cells transiently transfected with a C-terminal V5/His-tagged fusion gene. In both cells, the fusion protein was detected in the cytoplasm (Fig. 8A, D and G), and sub-cellular localization was confirmed by immunocytochemistry using a MATN3 antibody. Double-staining with antibodies for markers of lysosome, Golgi and ER revealed that the fusion protein did not localize within those organelles (Fig. 8).
4. Discussion

Here, we have shown that transcripts of two previously characterized genes can join via intergenic splicing to create a single transcriptional unit. Intergenic splicing has been reported for seven mammalian genes, however, this case is unusual in that most exons in the 5' gene component (LAPTM4) are skipped. In other instances,

**Figure 7.** Expression of LAPTM4B and MATN2 hybrid transcripts in various tissues (A) and cell lines (B). (A) Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, small intestine; lane 15, colon; lane 16, peripheral blood leukocyte; lane 17, negative control (water only). Expression of the hybrid transcript (the shorter form) was observed only in heart. (B) Lane 1, HuH7; lane 2, HeLa; lane 3, HEK293; lane 4, OUMS27; lane 5, CS-OKB; lane 6, negative control (water only). Expression of the longer hybrid transcript was observed in HuH7, and the shorter form was expressed in HEK293.

**Figure 8.** Sub-cellular localization of the LAPTM4A–MATN3 fusion protein in HeLa cells. Immunostaining for the fusion protein, showing cytoplasmic localization (A, D and G). Immunostaining for a lysosomal marker LAMP-1 (B), a Golgi marker Mannosidase II (E) and an ER marker PDI (H). Merge (C, F, I).
only the final exon in the 5' gene is skipped, with the exception of the TRAX–DISC1 hybrid, which omits the last two exons.\textsuperscript{4} The genomic distances between the source gene pairs characterized here are the second and third greatest among known examples.

This is the first example of intergenic splicing between two pairs of genes that belong to the same families. We were unable to identify a fusion transcript joining a third pair, \textit{MATN1} and \textit{LAPTM5}, using RT–PCR. Such a fusion may elude identification if its hybridization pattern differs from that of other pairs or if hybrid transcripts are alternatively spliced in a tissue-specific or cell type-specific manner. Hybridization of transcripts between multiple pairs of family genes suggests that intergenic splicing is more common than previously thought and that similar scenarios might exist among other gene families. Our observation illustrates the fundamental challenges in defining the gene, or the transcriptional unit. In the most recent version of the published human genome sequence (NCBI Human Build 35.1), the gene pairs \textit{MATN3–LAPTM4A} and \textit{LAPTM4B–MATN2} are classified as distinct genes. If intergenic splicing is more common than we presently know, then the current estimate of gene number in the human genome\textsuperscript{16} might be too high.

The role and function of intergenic splicing remain unclear. Combining various modular structures from distinct genes can increase the complexity of protein products, as seen with immunoglobulins. The biological and/or physiological significance of the additional minor transcripts is also unclear, although they could further increase complexity and the number of protein products generated from a set of genes. Alternatively, the fusion genes may have functional roles beyond creating genetic diversity. The specific expression of the \textit{MATN–LAPTM} hybrid transcripts suggests that this is a regulated phenomenon with a precise function. For comparison, a putative functional link exists between the tumor necrosis factor (TNF) ligands \textit{TWEAK} (\textit{TNFSF12}) and \textit{APRIL} (\textit{TNFSF13}) and the fusion protein they create. \textit{TWEAK} encodes a transmembrane protein expressed on the cell surface, and \textit{APRIL} encodes a secreted factor.\textsuperscript{7} The \textit{TWEAK–APRIL} fusion localizes to the cell membrane and presents the receptor-binding domain of \textit{APRIL} on the cell surface.\textsuperscript{7} It is also possible that the products of intergenic splicing might influence expression of the source genes in a tissue-specific and developmentally regulated fashion via transcriptional interference.

Although the function of the LAPTM4A–MATN3 fusion protein is unclear, its cellular and tissue expression patterns as well as its sub-cellular localization differ from those of the original source genes, suggesting a unique and specific role. Tandemly arranged tyrosine-containing motifs in the C-terminal domain are necessary and sufficient for localization of LAPTM4A to the lysosome.\textsuperscript{12} These motifs, along with the signal peptide of MATN3, are absent from the fusion protein, likely altering its sub-cellular localization. MATN3 contains a single N-terminal vWFA domain followed by four EGF repeats and the coiled-coil domains.\textsuperscript{8} These functional domains are retained in the fusion gene, while most of the putative domains of \textit{LATMP4A} are deleted. It is therefore likely that most of the biochemical, and hence biological, activities of MATN3 would be preserved in the fusion protein. In other proteins, the vWFA domain functions as a collagen-binding domain.\textsuperscript{17} \textit{MATN1} binds to type II collagen fibrils and interacts with aggrecan and integrin \(\alpha_5\beta_1\).\textsuperscript{18} Thus, MATN could link the collagen fibril network with the proteoglycan network and serve as a connection to chondrocytes.\textsuperscript{19} EGF-like domains are commonly found in ECM proteins, and when arranged in tandem they give rise to flexible rods, as seen in laminin.\textsuperscript{8,20} Coiled-coil domains mediate covalent multimer formation among the MATNs.\textsuperscript{18} However, because the fusion protein localizes to the cytoplasm, its association with these ECM proteins is unlikely. Further studies are necessary to determine the function of the fusion gene.

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