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Intrinsic *Alu* affects for RNA splicing in a minigene model

Mina Nakama [a,b,c,*], Bunta Imanaka [a], Yuma Kimoto [b]

[a] Graduate School of Science and Engineering Research, Kindai University, Osaka, Japan
[b] Department of Life Science, Faculty of Science and Engineering, Kindai University, Osaka, Japan
[c] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

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ABSTRACT

Alu elements are commonly located in the introns of primate genomes and, once transcribed, can alter splicing patterns. The insertion of an antisense *Alu* element into intron 9 was shown to enhance exon 10 skipping in a previously developed *ACAT1* minigene model including exon 9–exon 11. This study investigates two intrinsic original *Alus*' role located in the intron in *ACAT1* sequence using the same minigene splicing system. The deletion of intrinsic full *Alu*Sx originally located in intron 10 resulted in intron 10 retention, whereas the partial *Alu*Jb or antisense *Alu*Sx in the same intron was not sufficient for this process. Even normal splicing transcript wasn't shown without intrinsic full *Alu*Sx. Exon skipping was induced under the condition in which the intronic splice out prior to. Also, exon skipping was required with two close *Alu* elements with inverse orientations such as head-to-head and tail-to-tail in our minigene model. Intron retention seems to have been affected by shortening of introns or deletion of *Alu*'s splicing regulatory elements. Either way, *Alus* are associated with human gene expression incorporating themself and adopting in the human genome splicing system.

1. Introduction

Alu elements are among the most highly transposable elements in primate genomes and are categorized as short interspersed elements (SINEs). These elements are non-autonomous and require *trans*-acting factors for their amplification from long interspersed element-1 (LINE-1) [1]. The retrotransposition of *Alu* elements results in insertion mutation, and crossing-over between homologous elements is one of the sources of genetic variations potentially responsible for genetic diseases [2]. Intronic *Alus* can affect the selection of constitutive exons [3]. An *Alu* element can form an intramolecular RNA duplex with a nearby inverted *Alu* repeat sequence, which can serve as a substrate for A-to-I RNA editing [4,5]. This RNA secondary structure may affect pre-mRNA splicing. Our previous research found that the intronic antisense *Alu*Sx affected downstream exon skipping in an *ACAT1* minigene model and demonstrated the occurrence of process by inserting *Alus* into upstream intron 9 when the exon 10 splice acceptor site was suboptimal [6]. In particular, only 16 nucleotides of the partial *Alu* were shown to exert the same effect on exon 10 skipping [7]. *ACAT1* alternative splicing plays critical roles in generating functional diversity and regulating metabolic pathways, directly leading to mitochondrial acetoacetyl-CoA thiolase deficiency. We previously reported that exon 10 skipping of this gene disrupting enzyme activity was induced by two mutations in an exonic splicing enhancer within *ACAT1* exon 10 in patients [8,9]. In intron 10 of *ACAT1*, there are two intrinsic original *Alu* elements that are included in the evolution of humans. Here, the term "intrinsic" is used in the sense of "present" in the current human reference genome, GRCh38. This study focused on the role played by intrinsic *Alus* in intron 10 in alternative splicing in our splicing model.

2. Materials and methods

2.1. Minigene constructs

A basic minigene construct was built based on a previously established minigene containing the truncated introns 9 and 10 of *ACAT1* (Fig. 1A, lane 1) [6,8]. A total of twelve constructs were generated by replacing the inserted fragment or by mutagenesis using the KOD-Plus-Mutagenesis kit (TOYOBO Co., Ltd., Osaka, Japan) or the In-Fusion kit (Takara Bio Inc., Shiga, Japan), respectively, and were subsequently confirmed via direct sequencing.

* Corresponding author. Department of Life Science, Faculty of Science and Engineering, Kindai University, 3-4-1 Kowakae, Higashiosaka, Osaka, 577-8502, Japan.
E-mail address: nakama@life.kindai.ac.jp (M. Nakama).

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2.2. Splicing assay

SV40-transformed fibroblasts obtained from a patient with mitochondrial acetoacetyl-CoA thiolase deficiency (GK03 fibroblasts) [10] were cultured to 70 % confluency. Each expression vector was transfected into GK03 fibroblasts using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). RNA was harvested 48 h after transfection and reverse-transcribed using the strand-specific β-glo2 primer and oligo (dT)12–18 primer. Chimeric cDNAs were amplified using the Ex9 and β-glo3 primers [6,8]. The PCR products were subjected to electrophoresis on a 5 % polyacrylamide gel, and the results showed that normal splicing resulted in a 309-bp fragment, while exon 10 skipping produced a 244-bp fragment. The experimental details have been described in our previous study [6].

3. Results

3.1. Intrinsic AluSx contributed to intron splicing out

Two intrinsic *Alus* in intron 10 of our *ACAT1* minigene model were identified, a partial *Alu*Jb and a full *Alu*Sx. As previously reported, the minigene construct inserted with the antisense *Alu*Sx showed downstream exon 10 skipping (Fig. 1A, lane 1). To determine whether those two intrinsic *Alus* were required on exon 10 for alternative splicing, deletion constructions were generated for each of them. Removal of the partial *Alu*Jb from the fragment and leaving the full *Alu*Sx had a similar effect on exon 10 skipping (Fig. 1A, lane 2). Thus, the consequences of splicing did not change with or without the partial *Alu*Jb. In contrast, removal of the full *Alu*Sx and leaving only *Alu*Jb resulted in the appearance of two larger transcript bands and the fading of both normal splicing and exon skipping (Fig. 1A, lane 3). The sequencing of larger PCR products revealed the minigene's [exon 9-*Alu*Jb-intron 10-exon 11] and [exon 9-exon 10-*Alu*Jb-intron 10-exon 11]. Moreover, the removal of both *Alu*Jb and *Alu*Sx mainly resulted in the full retention of intron 10, although only a slight normal splicing signal was detected (Fig. 1A, lane 4). The intrinsic full *Alu*Sx in intron 10 was required for intron splicing, while the partial *Alu*Jb in intron 10 was not sufficient for this process.

3.2. Intronic splicing is dominant in normal splicing under the presence of an intrinsic Alu sequence

The orientation of the *Alu* element in intron 10 was subsequently examined. The PCR product-derived signals for both normal splicing and exon skipping faded and induced the failure of splicing out and then ended with the retention of intron 10 (Fig. 2A lanes 2–4 and 6). The constructs of lanes 1 and 5 of Fig. 2, which maintained a sense-full *Alu*Sx in intron 10, showed a clearer normal splicing than those of lanes 2, 3, 4, and 6. As shown in the Supplementary Figure S1, the constructs of lanes 1–4, which there were the part of *Alu* element required for exon 10 skipping [7] and a sense-full *Alu*Sx in intron 10, showed both normal splicing and exon 10 skipping. The 16-bp nucleotides within sense *Alu*Sx, a sequence complementary to antisense *Alu*, is insufficient for intron 10 splicing (lanes 5–7). Without the intrinsic full *Alu*Sx in intron 10, intron retention was also dominant for normal splicing, and exon skipping was detected only slightly.

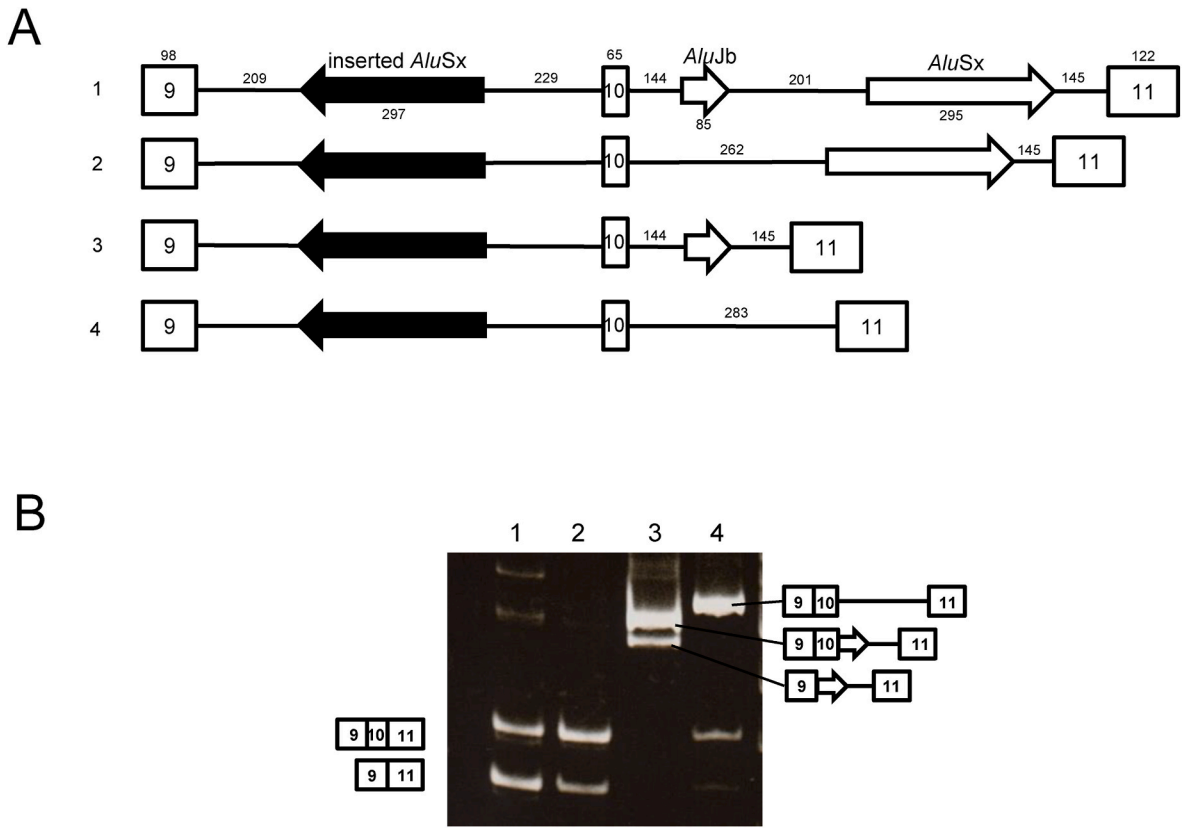

Fig. 1. The intrinsic *Alu*Sx in intron 10 was required for intron 10 splicing
Schematic of the *ACAT1* minigene constructs from exon 9 to exon 11 and the results of minigene splicing experiments. White boxes and white arrows indicate exons and original *Alu* elements, respectively. Newly inserted *Alu*Sx fragment is shown in black arrow. The smaller number represents the length of each element (bp). No.1 construct was generated in the previous report [6]. Other minigene constructs were produced by site-directed mutagenesis methods in this study. Normal splicing (exon 10 inclusion) and exon 10 skipping gave 309 bp and 244 bp fragments, respectively. Aberrant transcripts of intron retention were [exon 9–partial *Alu*Jb–intron 10–exon 11], [exon 9–exon 10– partial *Alu*Jb–intron 10–exon 11], [exon 9–exon 10–intron 10–exon 11] by sequencing.

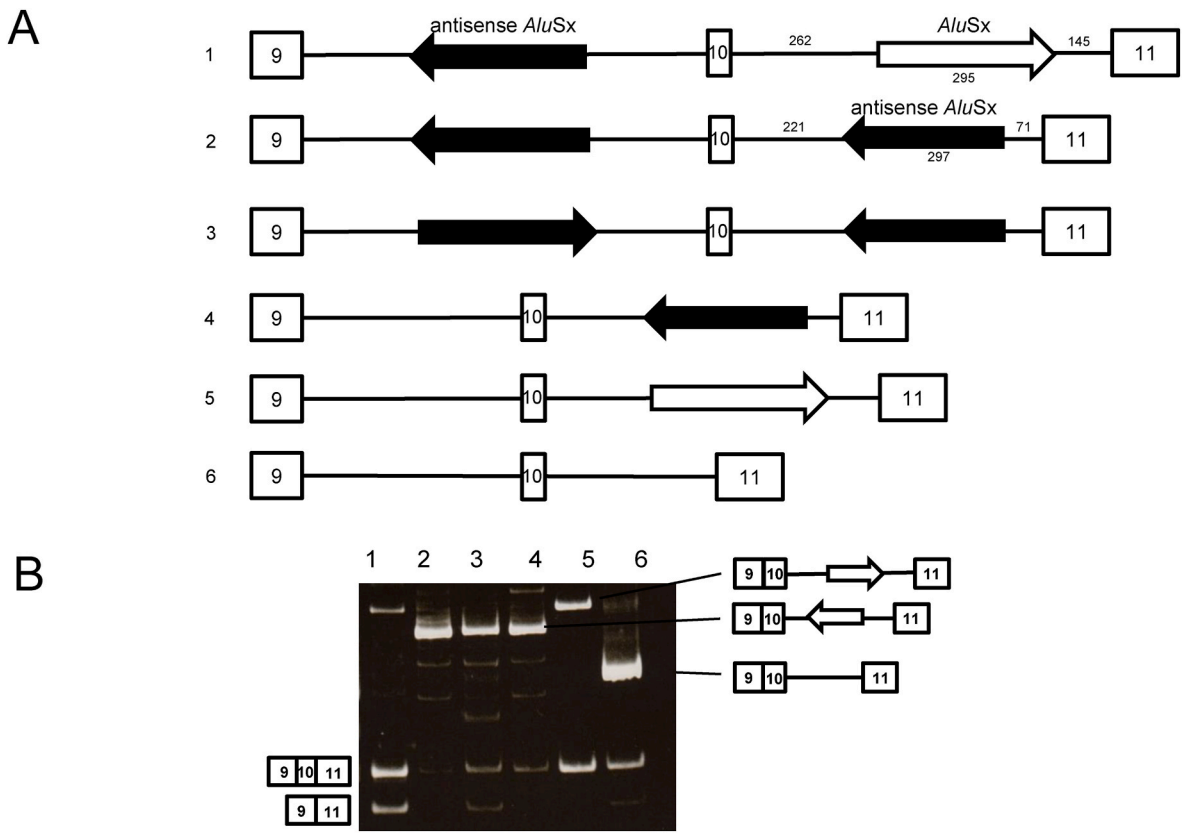

Fig. 2. The exploration of two *Alus'* orientation for exon 10 skipping and intron 10 splicing
Minigene constructs were prepared for one of the different orientations of the intron sequence for splicing experiments. The sample of Fig. 2 lane 1 corresponded to Fig. 1 lane 2. Aberrant transcripts of intron retention were [exon 9–exon 10–intron 10–exon 11], [exon 9–exon 10–intron 10–antisense/sense-*Alu*Sx–exon 11] by sequencing.

4. Discussion

This study revealed a novel role of intrinsic *Alus* in stabilizing RNA splicing. Minigene intron splicing was strongly induced when including the intrinsic sense-*Alu*Sx in intron 10. Without it, intron 10 was not spliced out and remained intact; neither the partial *Alu*Jb nor artificially inserted antisense *Alu*Sx was sufficient. Intron retention is a type of alternative splicing in which introns are retained in mature mRNAs instead of being spliced out. Splicing failure is associated with the sequence of some intronic elements or binding splicing factors [11]. The minigene with partial *Alu*Jb without full *Alu*Sx in intron 10 (lane 3 of Fig. 1) generated the transcripts including partial *Alu*Jb because there was 3' splice site sequence at upstream of it (Supplementary Text S2). It was unclear why intron 10 was not spliced out and remained in pre-mRNA without intrinsic full *Alu*. Among these constructs shown in Fig. 1, the lanes 3 and 4 which lead to strong intron 10 retention had the shorter length of intron 10 than lanes 1 and 2. Moreover, since Fig. 2 lane 2 minigene construct, which has shorter intron 10 length than Fig. 2 lane 1, also lead to intron 10 retention, it is likely that the short intron is a reason for intron retention. Another reason for intron retention of that intrinsic *Alus* was assimilated into human *ACAT1* and contributed as the splicing of conserved sequences like a splicing enhancer. G-rich sequences were reported to enhance splicing through recruiting hnRNPF/H to introns [12]. Since intrinsic sense full *Alu* contains seven G-triplets (GGG), while partial *Alu*Jb of our minigene contains two and antisense *Alu* contains three G triplets (Supplemental Text S3), this difference may have manifested itself in the effect on splicing.

Alus that are located in opposite orientations could form a double-stranded RNA structure, also known as "hairpin structure," which may affect RNA splicing [3]. In this study, exon skipping was also represented by two types of inverse orientations of *Alus* in intron 10 (Fig. 2 lanes 1 and 3). Both tail-to-tail and head-to-head orientations were shown to induce alternative splicing, including exon skipping. *Alus* are associated with human gene expression incorporating themself and adopting in the human genome splicing system.

CRediT authorship contribution statement

Mina Nakama: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Bunta Imanaka:** Writing – review & editing, Validation, Formal analysis. **Yuma Kimoto:** Writing – review & editing, Resources.

Compliance with ethical standards

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University, Japan (Approval number: 29–503) and was carried out in accordance with the principles contained within the Declaration of Helsinki. Informed consent was obtained from the parents of the patients.

Data statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Declaration of competing interest

We declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.102002.

Data availability

Data will be made available on request.

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