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Research paper

Intronic antisense *Alu* elements have a negative splicing effect on the inclusion of adjacent downstream exons

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ABSTRACT

Alu elements occupy 10% of the human genome. However, although they contribute to genomic and transcriptomic diversity, their function is still not fully understood. We hypothesized that intronic *Alu* elements may contribute to alternative splicing. We therefore examined their effect on splicing using minigene constructs including exon 9–exon 11 inclusive of *ACAT1* with truncated introns 9 and 10. These constructs contained a suboptimal splice acceptor site for intron 9. Insertion of *Alu*Y–partial *Alu*Sz6–*Alu*Sx, originally located in *ACAT1* intron 5, in an antisense direction within intron 9 had a negative effect on exon 10 inclusion. This effect was additive with that of an exonic splicing enhancer mutation in exon 10, and was canceled by the substitution of G for C at the first nucleotide of exon 10 which optimized the splice acceptor site of intron 9. A sense *Alu*Y–partial *Alu*Sz6–*Alu*Sx insertion had no effect on exon 10 inclusion, and one antisense *Alu*Sx insertion had a similar effect to antisense *Alu*Y–partial *Alu*Sz6–*Alu*Sx insertion. The shorter the distance between the antisense *Alu* element and exon 10, the greater the negative effect on exon 10 inclusion. This distance effect was more evident for suboptimal than optimal splice sites. Based on our data, we propose that intronic antisense *Alu* elements contribute to alternative splicing and transcriptomic diversity in some genes, especially when splice acceptor sites are suboptimal.

1. Introduction

Alu elements are primate-specific repeat elements that belong to the group of transposable elements known as Short INterspersed Elements (SINEs). *Alu* elements have been inserted into genomes for 65 million years, and have since expanded their copy number, reaching 1 million in the human genome which comprises > 10% of the genome mass (Batzer and Deininger, 2002). Most *Alu* elements have accumulated in introns, untranslated regions of genes, and intergenic regions (Batzer and Deininger, 2002). Additionally, older *Alu* elements such as *Alu*S and *Alu*J have undergone more substitutions than more recent *Alu* elements such as *Alu*Yb, especially in CG sequences, to show greater variability (Batzer and Deininger, 2002; Xing et al., 2004). *Alu* elements are non-autonomous; hence, their amplification depends on autonomous human retroelements such as Long INterspersed Element (LINE)-1.

Although *Alu* elements contribute to genomic and transcriptomic diversity, their function is still not fully understood. *Alu* element-meditated non-equal homologous recombination has resulted in human genomic diversity but has also caused intragenic deletions and duplications, leading to genetic disorders (Deininger and Batzer, 1999; Fukao et al., 2013, 2007a, 2007b; Zhang et al., 2006). *Alu* elements in introns have served as major substrates for RNA editing, which requires a double-stranded secondary structure of primary nuclear transcripts, in the formation of two *Alu* elements in sense and antisense orientations (Athanasiadis et al., 2004; Kim et al., 2004; Levanon et al., 2004). The accumulation of substitutions has also resulted in intronic *Alu* elements providing new exons to some genes by *Alu* element exonization (Krull, 2005; Lev-Maor et al., 2003; Sorek et al., 2004). Insertions of antisense *Alu* elements into introns close (< 55 bp) to adjacent exons were reported to cause human diseases through aberrant splicing (Gallus et al., 2010; Ganguly et al., 2003; Tighe et al., 2002; Wallace, 1991).

Alternative splicing is an important mechanism for transcriptomic

Abbreviations: ACAT1, acetyl-CoA acetyltransferase 1; ESE, exonic splicing enhancer; LINE, Long INterspersed Element; RT, reverse transcription; SINE, Short INterspersed Element; SV40, simian virus 40

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diversity, which is necessary for tissue specificity and complexity in primates. In more than half of human genes, alternative splicing produces more than one type of transcript. Because of the abundance of intronic *Alu* elements, some may contribute to alternative splicing in their relationship with adjacent exons. Lev-Maor et al. previously showed that *Alu* elements in intron 2 of human *RABL5* influenced the inclusion of exon 3 in alternative splicing (Lev-Maor et al., 2008), but few studies have experimentally examined the effect of intronic *Alu* elements on alternative splicing.

Here, we examined the effect of intronic *Alu* elements on RNA splicing (exon skipping) in a minigene model consisting of exons 9–11 of the acetyl-CoA acetyltransferase 1 gene (*ACAT1*), including a suboptimal splice acceptor site for intron 9.

2. Materials and methods

2.1. Minigene constructs

Our basic minigene construct contained a fragment of wild-type human *ACAT1* (exon 9–exon 11 inclusive) within the pCAGGS vector (Niwa et al., 1991) based on a previously established minigene construct containing truncated intron 9 and truncated intron 10 (Fukao et al., 2010) (Fig. 1A). The truncated intron 9 contained an *Alu*Jo in the sense orientation, while the truncated intron 10 contained a partial *Alu*Jb and an *Alu*Sx in the sense orientation. The constructs wild-type, c.951C > T, c.941C > G, and c.941C > G c.951C > T have been described previously (Fukao et al., 2010). We inserted a fragment consisting of *Alu*Y–partial *Alu*Sz6–*Alu*Sx, originally located in *ACAT1* intron 5 (Supplementary Fig. S1), into a *Hind* III site 226 bp upstream from the beginning of exon 10 (Fig. 1A) in these constructs. Five nucleotide substitutions were identified in a comparison of GenBank accession number NG_009888 for *ACAT1* (underlined in Supplementary Fig. S1). Other minigene constructs were made 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). We also made five constructs with the substitution of c.941C > G for the above single *Alu*Sx-containing constructs which had distances between *Alu*Sx and exon 10 of 226 bp, 120 bp, 60 bp, 40 bp, and 24 bp. The primers used are listed in Supplementary Table S2.

We confirmed the accuracy of the minigene constructs by direct sequencing. Because the constructs transcribe a human *ACAT1*–rabbit β-globin fusion mRNA, we could amplify minigene-specific mRNA by reverse transcription (RT)-PCR using a combination of a human *ACAT1* sense primer and a rabbit β-globin antisense primer (Fig. 1B).

2.2. Minigene splicing experiment

SV40-transformed fibroblasts from a patient (GK03) with mitochondrial acetoacetyl-CoA thiolase deficiency were established previously (Fukao et al., 1993), and were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Each expression vector (2 μg) was transfected into 5×10^5 SV40-transformed fibroblasts using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). RNA was prepared from the fibroblasts 48 h after transfection, using an Isogen kit (Nippon Gene, Tokyo, Japan). Total RNA (3 μg) was reverse-transcribed with M-MLV reverse transcriptase (LifeTech, Rockville, MD, USA). Strand-specific reverse transcription was performed using the vector-specific β-glo2 primer and oligo(dT)$_{12–18}$ primer (Life Technologies Japan Ltd., Tokyo, Japan). Chimeric cDNAs were amplified with Ex9 and β-glo3 primers (Supplementary Table S3). PCR conditions were: initial denaturation at 94 °C for 1 min, followed by 32 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. We electrophoresed the PCR products on a 5% polyacrylamide gel; normal splicing resulted in a 309 bp fragment, while exon 10 skipping resulted in a 244 bp fragment (Fig. 1B).

cDNAs with normal splicing or with exon 10 skipping were produced by the above minigene splicing experiments (Fig. 2, lane 2), and were purified using the Geneclean II kit (BIO 101, Vista, CA, USA) and subcloned separately into the pGEM-T easy vector (Promega Corp., Madison, WI, USA). The plasmids were mixed in nine serial molar ratios (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1), and the mixtures (about 5×10^{-5} pmol in total) were used as PCR controls for semi-quantitative evaluation.

3. Results

3.1. Alu elements insertion affected exon 10 skipping

We first inserted the *Alu*Y–partial *Alu*Sz6–*Alu*Sx fragment into the minigene vectors wild-type, c.941C > G, c.951C > T, and c.941C > G c.951C > T in the antisense orientation (Fig. 2A).

As previously reported (Fukao et al., 2010), in the absence of *Alu* insertion, normal splicing only was observed for the wild-type minigene (Fig. 2B, lane 1), while the c.951C > T minigene with a disrupted exonic splicing enhancer resulted in exon 10 skipping corresponding approximately to the 7:3 mixture (lane 5); this effect was canceled with an additional c.941C > G substitution which strengthened recognition of the splice acceptor site (lane 7). When the fragment including the *Alu*

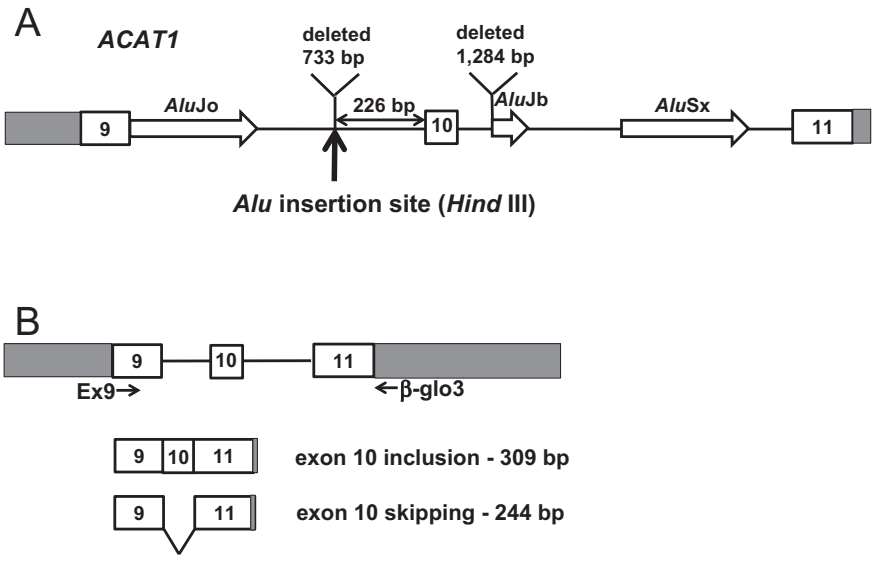

Fig. 1. *ACAT1* minigene construct and minigene-specific RT-PCR. A) Schematic of the *ACAT1* minigene from exon 9 to exon 11. White boxes and white arrows indicate exons and original *Alu* elements, respectively. Gray boxes indicate pCAGGS vector sequence. The *ACAT1* minigene was truncated in intron 9 and in intron 10 using restriction enzyme sites to reduce the length. B) Primer positions for minigene-specific RT-PCR. PCR using *ACAT1* Ex9 and rabbit β-globin gene (β-glo3) primers detected only minigene-specific fused transcripts. Normal splicing (exon 10 inclusion) and aberrant splicing (exon 10 skipping) gave 309 bp and 244 bp fragments, respectively.

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